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View Article Online
View Journal | View Issue



Cite this: *Environ. Sci.: Processes Impacts*, 2023, 25, 1802

Characterizing the marine mammal exposome by iceberg modeling, linking chemical analysis and *in vitro* bioassays†

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The present study complements work on mixture effects measured with *in vitro* bioassays of passive equilibrium sampling extracts using the silicone polydimethylsiloxane (PDMS) in organs from marine mammals with chemical profiling. Blubber, liver, kidney and brain tissues of harbor porpoise (*Phocoena phocoena*), harbor seal (*Phoca vitulina*), ringed seal (*Phoca hispida*) and orca (*Orcinus orca*) from the North and Baltic Seas were investigated. We analyzed 117 chemicals including legacy and emerging contaminants using gas chromatography-high resolution mass spectrometry and quantified 70 of those chemicals in at least one sample. No systematic differences between the organs were found. Only for single compounds a clear distribution pattern was observed. For example, 4,4'-dichlorodiphenyltrichloroethane, enzacamene and etofenprox were mainly detected in blubber, whereas tonalide and the hexachlorocyclohexanes were more often found in liver. Furthermore, we compared the chemical profiling with the bioanalytical results using an iceberg mixture model, evaluating how much of the biological effect could be explained by the analyzed chemicals. The mixture effect predicted from the quantified chemical concentrations explained 0.014–83% of the aryl hydrocarbon receptor activating effect (AhR-CALUX), but less than 0.13% for the activation of the oxidative stress response (AREC32) and peroxisome-proliferator activated receptor (PPAR γ). The quantified chemicals also explained between 0.044–45% of the cytotoxic effect measured with the AhR-CALUX. The largest fraction of the observed effect was explained for the orca, which was the individual with the highest chemical burden. This study underlines that chemical analysis and bioassays are complementary to comprehensively characterize the mixture exposome of marine mammals.

Received 27th January 2023
Accepted 2nd April 2023

DOI: 10.1039/d3em00033h

rsc.li/espi

Environmental significance

In their natural habitat marine mammals are exposed to a large number and variety of environmental pollutants. Studying the chemical burden of biota tissues and identifying the mixture effects can help in characterizing the internal exposure of chemicals. By using passive equilibrium sampling with silicone with so-called “chemometers” it is possible to transfer environmental mixtures of chemicals into an extract without changing their composition, allowing one to study the mixtures of environmental pollutants extracted from biota and their effects without bias. The combination of *in vitro* bioassays and chemical profiling of legacy and emerging contaminants helps to understand the mixture effects causing the activation of cellular toxicity pathways.

1 Introduction

Numerous studies showed that marine mammals are globally exposed to different anthropogenic influences, including encounters with fishery activities (e.g. bycatch), noise pollution and exposure to chemicals such as Hydrophobic Organic Compounds (HOCs). HOCs like Polychlorinated Biphenyls (PCBs), Organochlorine Pesticides (OCPs), Polybrominated Diphenyl Ethers (PBDEs), Chlorinated Hydrocarbons (CHCs), and Polycyclic Aromatic Hydrocarbons (PAHs) are known legacy chemicals often found in marine ecosystems.^{1–12} Most HOCs are persistent in the environment, resist biodegradation and can cause adverse effects on aquatic organisms. Due to their natural

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† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d3em00033h>



habitat, long life span, large blubber fraction and elevated trophic level, marine mammals may accumulate a wide variety of HOCs in their tissues, and in some cases they experience adverse health effects.^{5,11,13,14} Already young marine mammals possess a high chemical burden, as lipophilic chemicals are transferred from mother to their offspring during pregnancy and lactation.^{11,15–19} HOCs have a negative impact on the health and survival of marine mammals,^{11,20,21} and can cause, amongst others, immunodeficient effects,^{22–25} reproduction dysfunction,^{26–28} and endocrine disruption.^{3,29–31} The production and use of some HOCs are regulated; they have partly been banned almost two decades ago to prevent adverse environmental and health effects, *e.g.* in the Stockholm Convention on Persistent Organic Pollutants (POPs), including PCBs, hexachlorobenzene (HCB), chemicals from the OCP-group and others.³² Besides these regulated legacy POPs, other emerging organic contaminants with similar properties are of concern, especially as many of them are not regulated, *e.g.* personal care products, chemicals with unknown structures, transformation products, and others. Furthermore, mixtures of different chemicals (even at low dose) should be considered as they can jointly elicit effects which can be additive, cumulative or interactive.³³

To identify and quantify the relevant compounds in tissues of marine mammals, the chemicals need to be extracted from the tissues. A useful tool to transfer environmental mixtures of nonpolar HOCs to different profiling tools, such as chemical analysis and bioanalysis, without changing the chemical composition, is passive equilibrium sampling with a chemometer, *e.g.* the silicone polydimethylsiloxane (PDMS). Chemometers are understood as a common, universal and well-defined polymer reference phase for passive sampling, reaching thermodynamic equilibrium partitioning of a large range of nonpolar organic pollutants in different matrices like biota, sediment and potentially water.³⁴ Using chemometers, organic chemicals with a broad range of physicochemical properties are transferred into the extract largely without changing the chemical composition.^{35–37} By using this approach, extracts from biota tissue can conveniently be submitted to instrumental analysis and/or bioanalytical profiling, to analyze the composition or effects of the contained mixture of chemicals. The resulting concentrations in the silicone reference phase can be directly compared across different tissues, individuals and species, circumventing potential bias due to normalization to lipid mass, as would be required with conventional approaches such as traditional exhaustive extraction.^{38,39} Using a chemometer additionally reduces the amount of matrix transferred to the extract and thus a non-destructive cleanup is sufficient to maintain the broad chemical composition in the extract for further analyses.^{37,40}

In a previous study,⁴¹ we examined the chemical mixture effects of silicone chemometers equilibrated with different organs (*i.e.* liver, kidney, brain and blubber) of seven marine mammals from the North and Baltic Seas. Chemometer extracts were tested in three cell-based *in vitro* bioassays to investigate different modes of action: activation of the xenobiotic metabolism, including the activation of (I) the peroxisome

proliferator-activated receptor gamma (PPAR γ) with the PPAR γ -bla GeneBLAzer assay⁴² and (II) the aryl hydrocarbon receptor (AhR) with the AhR-CALUX assay⁴³ as well as (III) the adaptive Nrf2-dependent oxidative stress response with the AREc32 assay.⁴⁴ The results indicated that the extracts from liver caused higher bio activation than the corresponding blubber extracts, more precisely 11 ± 0.26 ($n = 7$) times higher activation for PPAR γ -bla and 1.9 ± 0.32 ($n = 4$) times higher activation of the AREc32 assay. In the AhR-CALUX the blubber extracts did not activate the AhR up to concentrations where cytotoxicity occurred, whereas for all seven liver extracts an activation for AhR was measured.⁴¹

The main objective in this study was to submit liver, kidney, brain and blubber tissues from marine mammals to chemometer sampling, cleanup and chemical profiling to determine chemical patterns across different tissues for a broad range of legacy and emerging HOCs. For this purpose, we analyzed the above-mentioned seven marine mammals, sampled the chemicals from organs of five additional marine mammals and compared our results to literature data. Additionally, we assessed the relationship of the mixture effects measured previously with the bioanalytical assays⁴¹ and the predicted mixture effects of the detected, targeted chemicals of the same sample. To characterize the exposome of the animals, we applied two complementary approaches: broad chemical screening in combination with bioanalytical assessment⁴¹ of mixture effects. Targeted chemical analysis provides quantitative data regarding a defined number of compounds and can give an impression of the total exposure. By means of bioanalytical testing, mixture effects can be captured and thus the totality of chemicals extracted from a sample can be characterized. Combining both approaches by so-called iceberg modeling allows to estimate which fraction of the observed effect can be explained by the known and quantified chemicals, opposed to which fraction remains unexplained.⁴⁵

2 Methods

2.1 Biota samples

In this study tissues from seven harbor porpoises (*Phocoena phocoena*, P.p.), three harbor seals (*Phoca vitulina*, P.v.), one ringed seal (*Pusa hispida*, P.h.) and one orca (*Orcinus orca*, O.o.) were analyzed. The samples were obtained from deceased animals with different causes of death, *e.g.* stranding, bycatch or pneumonia and final sepsis so that they had to be euthanized, on the German coasts of the North and Baltic Sea between 2016 and 2019. All tissues were collected in moderate to good condition. For eight animals a full set of liver, blubber, brain and kidney, and for four animals a core set of liver and blubber were available. For details on the animals' available organs, sex, age group and stranding location, see Fig. 1 and Table S1.† Samples were abbreviated with their species name (P.p., P.v., P.h. and O.o.) plus a running number. For coherent sample labeling and to facilitate direct comparison, samples analyzed in Reiter *et al.*⁴¹ were termed with the identical name (P.p.1–5, P.v.1, O.o.1) as before. The additional samples received a running number. The order of the samples was



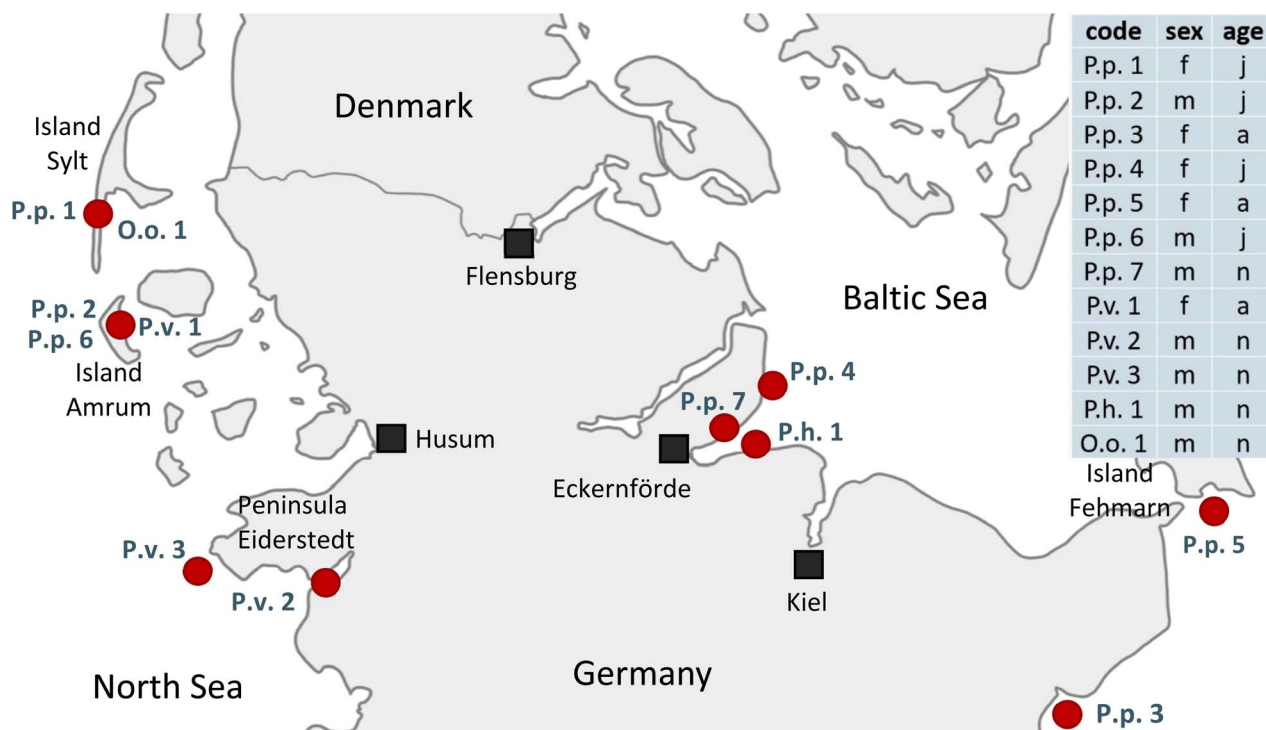


Fig. 1 Stranding locations of the twelve examined animals on the shores of Schleswig-Holstein, Germany, and core information of the individuals analyzed: code (P.p.: harbor porpoise, P.v.: harbor seal, P.h.: ringed seal, O.o.: orca, plus running number), sex (f: female, m: male) and age group (a: adult, j: juvenile, n: neonate). For more information, e.g. stranding year, available organs and references to previous studies, see Table S1.†

random. The tissues were processed as described in detail by Reiter *et al.*⁴¹ Briefly, the samples were homogenized with a blender (see Table S2†) and stored at -20°C . For all samples the lipid content was determined gravimetrically following extraction with various mixtures of solvents;⁴⁶ for the detailed method, see Text S1.†

2.2 Solvents and standards

If not otherwise specified, ethyl acetate (EtAc), acetonitrile, *n*-hexane and isopropanol of gas chromatography grade were purchased from Merck, Darmstadt, Germany and used. Diethyl ether (PESTINORM®) was purchased from VWR International, Darmstadt, Germany. Analytical standards of at least 98% purity were purchased from Dr Ehrenstorfer (Augsburg, Germany) and from Sigma-Aldrich (Germany), stable isotope-labeled standards were purchased from Wellington Laboratories (Guelph, ON, Canada) and Campro Scientific (Berlin, Germany). All standards are listed in Table S3.†

2.3 Extraction of chemicals

2.3.1 Chemometer sampling. Chemometer sampling and extraction was performed with the silicone PDMS according to previously published studies.^{41,47,48} Briefly, PDMS sheets (SSP-M823, Special Silicone Products, Ballston, USA) with thicknesses of 125 μm , 250 μm and 350 μm were cut (see Table S2† for dimensions), solvent-cleaned using Soxhlet extraction with EtAc (Honeywell, HPLC grade) for at least 20 hours and stored in

fresh EtAc (Honeywell, HPLC grade) at room temperature until usage. Before chemometer sampling, the sheets were air-dried under the fume hood for approximately 2 h and the PDMS weight was determined with a micro-analytical balance (Mettler Toledo, Gießen, Germany). The equilibration of the chemometers with the oil-like blubber homogenate was performed statically. The PDMS was immersed in a vial with homogenized blubber and equilibrated for 48 h at 4°C ; except for the blubber tissue from O.o.1 that was equilibrated for 72 h, due to lower lipid content (Table S2†), to make sure equilibrium partitioning between the sample and the chemometer was approached.⁴¹ To equilibrate the PDMS with tissue from liver, brain and kidney, dynamic sampling with manual relocations was carried out.^{41,48} The PDMS sheets were layered between the homogenized tissue, regularly relocated and the tissue mixed, *i.e.* eight to ten relocations per day (every 90 to 110 minutes) were performed and samples left static overnight. During the experiment, the samples were kept at 4°C for seven to nine days. Up to 70 relocations were carried out unless the texture of the tissue homogenate did not allow any more for continued sampling. The maximum mass of PDMS to be used in each single sample met the negligible depletion conditions (<5% depletion of pollutants by partitioning to PDMS).⁴⁹ For selected tissues of sufficient mass, we sampled duplicates of 125 μm , 250 μm and 350 μm thick PDMS sheets each; for the other tissues, duplicates of 350 μm sheets were taken. See Table S2† for detailed PDMS sheet replicate numbers, dimensions and weights, as well as the number of relocations and the total exposure time.



All samples were analyzed by gas chromatography-high resolution mass spectrometry (GC-HRMS, QExactive, Thermo Fisher Scientific, Germany), as described elsewhere.^{41,52} In total, 117

experimentally determined $K_{\text{lipid/PDMS}}$, with our measured c_{lipid} values from total exhaustive extraction (Table S6†). The results are described in detail in Text S5.†

$$c_{\text{lipid, eq.}} = K_{\text{lipid/PDMS}} \times c_{\text{PDMS}}^{-1} \quad (1)$$

2.5 Iceberg modeling

In our previous study,⁴¹ PDMS extracts from the organs of P.p.1–5, P.v.1 and O.o.1 were measured in three cell-based *in vitro* bioassays: the PPAR γ -bla GeneBLAzer assay,⁴² AhR-CALUX assay⁴³ and AREc32 assay.⁴⁴ With the earlier generated data⁴¹ and the data from the chemical analysis, iceberg modeling was carried out. The detailed description of the iceberg modeling is given in Text S6.† Briefly, the bioanalytical equivalent concentrations (BEQ) measured from the results of the cell-based *in vitro* bioassays (BEQ_{bio}, eqn S4†), reported in our earlier study,⁴¹ were compared with the predicted effect, calculated by the sum of detected compounds in the extract (BEQ_{chem}, eqn S5†). The ratio of BEQ_{chem}/BEQ_{bio} indicates which fraction of the measured effect in the bioassay can be explained by the detected compounds. In addition, iceberg modeling was applied to the cytotoxic effects, detected in the AhR-CALUX, by the ratio of the predicted Toxic Unit (TU) of all detected compounds, TU_{chem}, and the bioanalytically measured TU_{bio} in the AhR-CALUX (TU_{chem}/TU_{bio}, eqn S7†).

2.6 Statistical evaluations

To calculate differences between variables of two different organs, a ratio paired *t*-test was used. Gaussian normal distribution of the results was confirmed. Statistical significance was defined with a *p*-value <0.05 (*). The *t*-test was calculated with the Software GraphPad Prism V 9.5.0.

3 Results and discussion

3.1 Characterization of tissue and lipid weight gain

The lipid content varied greatly between and within tissues, *i.e.* 37 to 101 g_{lipid} g_{blubber}⁻¹, 1.9 to 23 g_{lipid} g_{liver}⁻¹, 6.8 to 12 g_{lipid} g_{brain}⁻¹, 2.0 to 3.9 g_{lipid} g_{kidney}⁻¹ (Table S2†). *In tissue* passive sampling comes along with a weight gain of the PDMS due to lipid uptake into the polymer.³⁶ Although the lipid content of the investigated tissues ranged from 1.9 to 101%, the lipid uptake into the PDMS was rather uniform, *i.e.* $0.88 \pm 0.62\%$ (mean $\pm$ SD, $n = 40$). The lipid uptake into the PDMS thus was independent of the lipid content of the tissue but tissue-specific, *e.g.* lower lipid uptake was observed for brain tissue ($0.30 \pm 0.26\%$ ($n = 8$)) than for kidney tissue ($0.64 \pm 0.24\%$ ($n = 8$)), although the lipid content of the brain was higher than that of the kidney (Fig. S1†).

3.2 Chemical burden of marine mammals

The chemical concentrations in the PDMS sheets, c_{PDMS} in $\text{pg}_{\text{analyte}} \text{mg}_{\text{PDMS}}^{-1}$, were calculated and are summarized in

Table S5.† 70 out of 117 target compounds were detected in at least one out of 40 samples and thus 47 targeted chemicals were not found in any sample. Fig. 2 shows a heatmap with the detected targeted chemicals and the samples in which they were found. Additionally, the total chemical burden of the different compound groups is shown in Fig. S2.† Eight compounds were found in 90% or more of the samples (*i.e.*, 36–40 samples), six of which were PCBs (PCB 101, 138, 149, 153, 170, 180), one PAH (fluorene) and one OCP (4,4'-DDE). Compounds that were found in particularly high concentrations in most samples were PCB 153 (geometric mean $\pm$ geometric SD: 157 ± 3.98 pg mg_{PDMS}⁻¹, $n = 40$), PCB 138 (90.2 ± 3.99 pg mg_{PDMS}⁻¹, $n = 39$), 4,4'-DDE (142 ± 5.16 pg mg_{PDMS}⁻¹, $n = 40$) and dieldrin (115 ± 3.64 pg mg_{PDMS}⁻¹, $n = 15$). The total burden of the samples varied greatly between animals and organs. Exceptionally high concentrations were found in O.o.1, a neonate orca stranded and found in the North Sea (especially PCBs, DDX (4,4'-DDE, 4,4'-DDD and 4,4'-DDT) and BDEs), see Fig. 2. High concentrations of PCBs in this individuum were also observed in an earlier study by Schnitzler *et al.*⁵⁹ The lowest chemical burden was found in the two adult female harbor porpoises, stranded and found in the Baltic Sea, P.p.3 and P.p.5, and in the neonate male ringed seal P.h.1 from the Baltic Sea. The concentrations of selected chemicals found in some extracts were above the maximal concentration of the calibration which is discussed in detail in Text S7.† Consequently, the extract concentrations of these compounds were extrapolated from the linear concentration curve; the single extracts that exceeded the maximal calibration of a compound are highlighted in Table S5.†

Comparing the tissues within each animal revealed that some of the compounds tended to accumulate in the four organs to different extents. The compound 4,4'-DDT was found in every blubber sample from all analyzed individuals ($n = 12$) and in five (out of eight) brain samples, whereas it was only detected in two (out of eight) kidney samples and one liver sample (out of twelve). As blubber and brain are less metabolically active than liver and kidney, this was an expected observation. Another compound that was frequently found in blubber samples was the UV filter enzacamene (4-methylbenzylidene camphor, $n = 11$), which was also found in five kidney samples, whereas it was rarely found in liver and brain (each $n = 2$). Enzacamene is used in personal care products and was detected before in dolphins from the Brazilian coast.^{60,61} Furthermore, the pyrethroid etofenprox (often used as insecticide) and the PAH benzo[*b*]fluorene were frequently found in blubber ($n = 9$), whereas both compounds were less frequently detected in the other organs ($n = 1$ –3). Etofenprox and pyrethroids in general can be metabolized by mammals, explaining the higher detection frequency in the less metabolically active blubber tissue.⁶²

The other way around, the polycyclic musk tonalide was found in more than half of the liver samples ($n = 7$) but it was detected less frequently in the other organs ($n = 2-3$). Hexachlorocyclohexanes (HCHs, α -, β -, γ - and δ -isomers), were not found in blubber, whereas the α -isomer was found in four liver samples, the β -isomer in three liver samples, one kidney and one brain samples, the γ -isomer in three liver samples, two

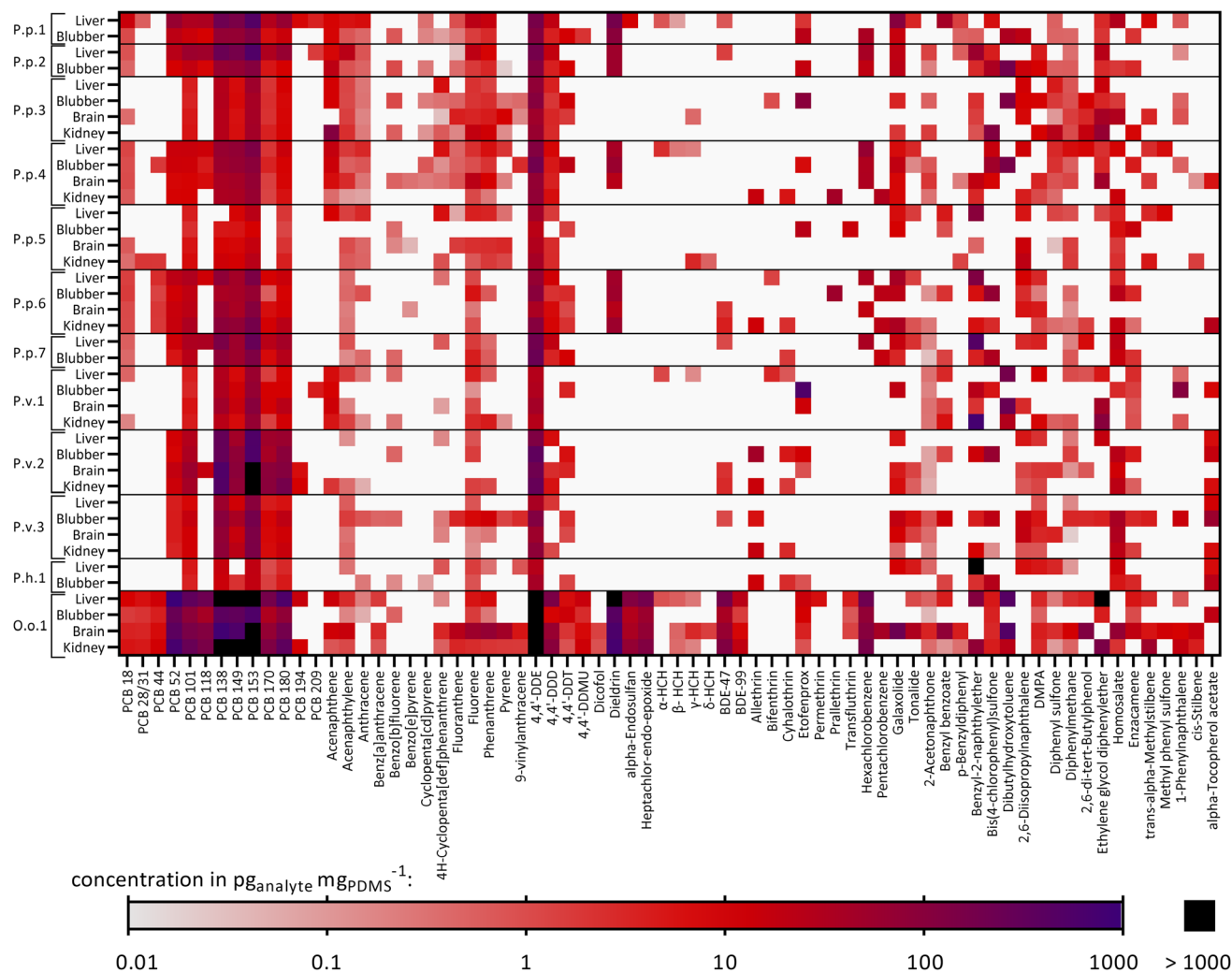


Fig. 2 Heatmap showing the detected targeted compounds found in each animal and their corresponding organs: P.p.: harbor porpoise, P.v.: harbor seal, P.h.: ringed seal, O.o.: orca, plus running number. White: the chemical was not found in the sample. Concentrations in $\text{pg}_{\text{analyte}} \text{mg}_{\text{PDMS}}^{-1}$. Concentrations above $1000 \text{ pg}_{\text{analyte}} \text{mg}_{\text{PDMS}}^{-1}$ are colored in black. Furthermore, some extracts exceeded the maximal concentration of the highest concentrated calibration solutions and hence, have a semi-quantitative character (discussed in detail in Text S6†). Affected samples for which every replicate exceeded the calibration are P.p.1 liver: PCB153; P.p.7 liver: benzyl-2-naphthylether; P.v.2 all organs: PCB153; P.v.2 brain, kidney: PCB138; P.h.1 liver: benzyl-2-naphthylether; O.o.1 liver: PCB52, dieldrin, ethylene glycol diphenylether; O.o.1 liver, brain, kidney: PCBs 138, 149, 153; O.o.1 all organs: 4,4'-DDE. Detailed data are given in Table S5.†

kidney and brain samples and the δ -isomer in one kidney and brain sample. Still, HCHs were detected in earlier studies in blubber samples from harbor porpoises from the Baltic and North Seas.^{63,64}

Fig. S3† illustrates the specific contaminant patterns of each organ and animal. Overall, a high fraction of PCBs (mean $\pm$ SD: $44 \pm 22\%$), OCPs ($24 \pm 16\%$) and chemicals from the “Others” category (incl. antioxidants, UV filters and other compounds) ($22 \pm 22\%$) was detected in all samples. This pattern was similar for all organs, except for blubber samples, where a higher pyrethroid fraction was additionally identified ($12 \pm 19\%$); contrarily, pyrethroids were found in less than 1% of the liver and brain samples and on average in $4.9 \pm 7.2\%$ in the kidney samples.

3.3 Chemical distribution between liver and blubber

Paired liver and blubber samples were available for all twelve animals. In our previous work,⁴¹ the bioanalytical results of paired liver and blubber samples from P.p.1–5, P.v.1 and O.o.1 indicated a higher activation of the cell assays from liver extracts, compared to the corresponding blubber samples; more specifically, for PPAR γ -bla by a factor of 11 ± 0.26 ($n = 7$), for AREc32 by a factor of 1.9 ± 0.32 ($n = 4$) and in the AhR-CALUX activation was only measured for liver extracts ($n = 7$), whereas the blubber extracts did not activate the AhR up to concentrations where cytotoxicity occurred. In this work, a ratio paired t -test was used to compare the concentrations, measured with GC-HRMS, in the chemometers equilibrated with the liver ($c_{\text{PDMS, liver}}$), to the corresponding equilibrated blubber sample

Most compounds were close to equilibrium partitioning within the organism (chemical activity ratio = 1), indicating that the tissues within the organism are at equilibrium. For

DDXs often mark the largest share of the OCP-burden,^{63,67,68} and the lipid-normalized sum concentrations of 4,4'-DDE, 4,4'-

Concentration range of the compound (group) reported in mg kg _{lipid} ⁻¹	Sample details	Reference
Polychlorinated biphenyls (PCBs)		
∑PCB ₁₇ : 0.21–90	<i>n</i> = 112, Danish waters, 2003–2019, different age groups, male and female	11
∑PCB ₃₅ : 1.1–82	<i>n</i> = 28, German North Sea, 1990–2008, different age groups, male and female	69
Organochlorine pesticides (OCPs)		
∑DDX ₆ : 0.4–22.9	<i>n</i> = 28, German North Sea, 1990–2008, different age groups, male and female	69
4,4'-DDE: 0.59–12	<i>n</i> = 29, North and Baltic Sea, 1994–1995, mostly immature animals, male and female	63
Hexachlorobenzene (HCB)		
HCB: 0.10–0.92	<i>n</i> = 29, North and Baltic Sea, 1994–1995, mostly immature animals, male and female	63
HCB: 0.013–0.42	<i>n</i> = 34, Danish waters, 2003–2019, different age groups, male and female	11

DDD and 4,4'-DDT ($\sum \text{DDX}_3$) in blubber tissues in this study ranged from 0.45 mg kg_{lipid}⁻¹ in P.p.5 to 6.3 mg kg_{lipid}⁻¹ in P.v.2. Only O.o.1 exceeded this range by a factor of 11 at 74.4 mg kg_{lipid}⁻¹. Among the DDXs, 4,4'-DDE was the most abundant representative, which is a common pattern in marine mammals,^{67,68} and ranged from 0.40 to 4.3 mg kg_{lipid}⁻¹. Except for O.o.1, the DDX concentrations determined in this study correspond with previously reported results, listed in Table 1.

HCB was only found in five out of the seven harbor porpoise samples as well as in the orca, but not in the harbor seal or ringed seal samples. Concentrations in the respective blubber tissues ranged from 0.22 to 0.88 mg kg_{lipid}⁻¹. This range is consistent with the scale of observations made in several older and recent studies, summarized in Table 1.

It is important to put the different concentrations into context with known thresholds of physiological impacts in marine mammals, which are based on mixture effects. At present, there are three commonly used $\sum \text{PCB}$ thresholds that provide guidance for assessing PCB contamination of marine mammal tissues: (I) 9.0 mg kg_{lipid}⁻¹ ($\sum \text{PCB}_{23}$), which marks the general onset of physiological impacts in marine mammals,^{70,71} (II) 11.0 mg kg_{lipid}⁻¹ ($\sum \text{PCB}_{25}$), from which on infertility and reproductive failure can be expected in female sexually mature harbor porpoises^{41,72} and (III) 41.0 mg kg_{lipid}⁻¹ ($\sum \text{PCB}_{23}$), for the onset of profound reproductive impairment of Baltic ringed seals.^{71,73}

The lowest threshold (I) was transgressed by 14 samples and in at least one organ from P.p.1, P.p.2, P.p.3, P.p.6, P.p.7, P.v.2, O.o.1. The second lowest threshold (II) was transgressed by 12 samples by the same individuals mentioned above. The highest threshold (III) was transgressed by all examined organs from O.o.1 and in the brain and kidney of P.v.2. It is important to notice that in this study, the $\sum \text{PCB}$ trespassing some thresholds is corresponding to $\sum \text{PCB}_{13}$ and not to $\sum \text{PCB}_{23}$ or $\sum \text{PCB}_{25}$, respectively. A summary is shown in Fig. S6.† In the past decades, numerous studies have found PCB levels in marine mammals that are believed to trigger various adverse effects.^{3,29,74,75} For instance, Das *et al.*³ hypothesized that concentrations as low as 7.66 ± 5.08 mg kg_{lipid}⁻¹ ($\sum \text{PCB}_6$) interfere with the harbor porpoise thyroid functions leading to severe interfollicular fibrosis. This threshold was transgressed in at least one organ of eight animals, namely P.p.1, P.p.2, P.p.4, P.p.6, P.p.7, P.v.2, P.v.3 and O.o.1. Recently, it has been specified that the risk of death from infectious diseases is raised by 5% for every 1 mg kg_{lipid}⁻¹ increase of the $\sum \text{PCB}_{25}$ burden, which poses risk to all marine mammals.⁷⁵

As PAHs are metabolized in higher organisms, elevated concentrations of PAHs might primarily reflect on recent exposure.⁷⁶ In this study the contamination levels of PAHs relative to the total chemical burden in blubber were between 0.041–4.2% and hence might not indicate a major recent exposure. However, carcinogenic properties of PAHs have been identified in mammals, but to the best of our knowledge, no effect thresholds have been established for marine mammals.^{67,77}

Limited thresholds for adverse effects on marine mammals have been established for OCPs, however, several studies

indicated that OCPs adversely affect marine mammals, *e.g.* by impairing thyroid function or by acting as endocrine disrupting chemicals potentially affecting the reproduction.^{3,68} Furthermore, 4,4'-DDT levels of 2.44 ± 0.37 mg kg_{lipid}⁻¹ found in Baltic harbor seals are suspected to trigger immunotoxic effects,⁷⁸ but no exceedance was observed in the blubber samples analyzed in this study. Furthermore, organohalogen compounds, such as DDX, are considered toxic from concentrations of 1 mg kg_{wet weight}⁻¹.⁷⁹ Considering the lipid content of each blubber sample (Table S1†), nine (out of twelve) blubber samples (P.p.1, P.p.2, P.p.3, P.p.4, P.p.6, P.p.7, P.v.2, P.p.3, O.o.1) exceeded this threshold for $\sum \text{DDX}_3$, suggesting that 75% of the analyzed individuals could have suffered from adverse effects caused by their $\sum \text{DDX}_3$ burden.

3.5 Iceberg modeling

To determine how much of the bioanalytically measured mixture effect (BEQ_{bio})⁴¹ can be explained by analytically determined chemicals and their predicted mixture effect (BEQ_{chem}), iceberg modeling was applied. In addition, for the AhR-CALUX assay, cytotoxic effects from bioanalysis (TU_{bio}) and the predicted cytotoxic effects from chemical analysis (TU_{chem}) were compared. The bioanalytical results for PPAR γ -bla, AhR and AREc32 were reported and discussed in detail in an earlier study.⁴¹ Values used for the iceberg modeling are summarized in Table S8.†

From the 70 detected compounds, 9 chemicals cannot be captured in bioassays because they are too volatile ($K_{\text{medium/air}}$ below a threshold of 10^4 , Fig. S7 and Table S4†).⁸⁰ From the 59 remaining chemicals, effect data for 37 compounds were available (Table S4†), of which 11 chemicals did not activate one of the three bioassays, and no cytotoxic effects were measurable for the AhR-CALUX. A fraction between 27–95% (geometric mean $\pm$ geometric SD: $77 \pm 1.3\%$), normalized to the total concentration, could be captured with the 37 compounds with known effect data. Consequently between 2.2–72% ($11 \pm 2.3\%$) remained unknown, due to unavailable compounds (24 chemicals) and between 0.58–28% ($3.9 \pm 2.7\%$) stayed unexplained due to chemicals with explicit volatility, as shown in Fig. S8.† For most extracts the major compound burden could be captured with the effect data, although especially for the extracts of P.p.5 liver and P.v.1 kidney, more than 50% could not be explained.

Twelve compounds of the detected chemicals are known to activate the AhR-CALUX, *i.e.*, 4 PCBs, 5 PAHs, 2 BDEs, and ethylene glycol diphenyl ether. The PCB congeners 118, 138 and 180 explained most of the effect, whereas the other compounds in the extracts played a minor role (Fig. 3A). Overall, BEQ_{chem} explained between 0.014–83% of the AhR-activating effect (BEQ_{bio}) by the 12 compounds (Fig. 4A). Most of the effect was explained for the liver extract of O.o.1 (83%), but also for the corresponding brain and kidney samples, a substantial fraction was explained (22% and 26%). Between 1.2 and 12% of the effect was explained for the liver extracts of P.p.1 and P.p.2 and the brain extract of P.p.3. Less than 1% AhR-activation was determined in liver extracts of P.p.3, P.p.4, P.p.5 and all extracts



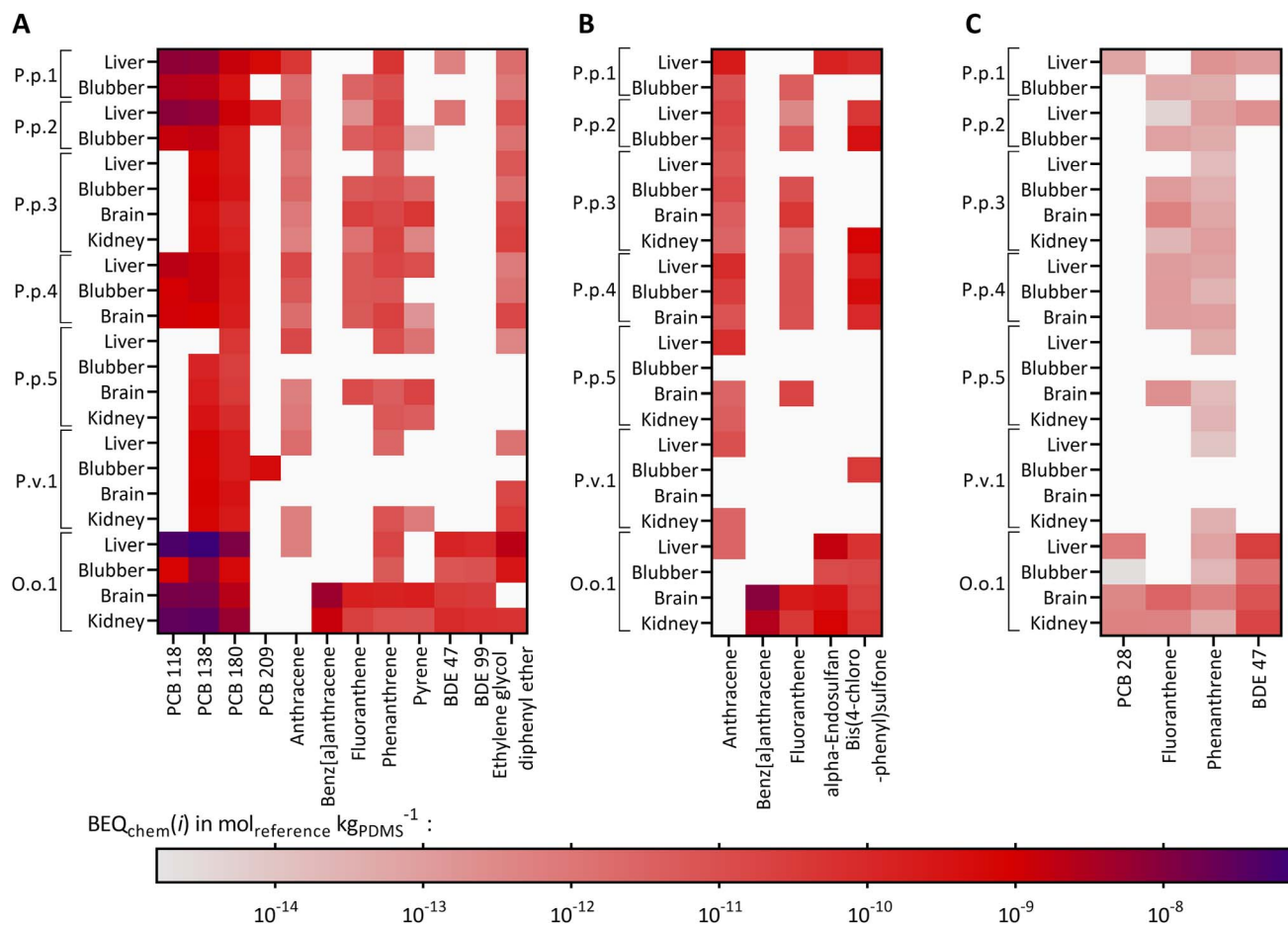


Fig. 3 Heatmap of compounds activating the (A) AhR-CALUX bioassay, (B) oxidative stress response in the AREc32 bioassay and (C) the PPAR γ -bla bioassay, as $BEQ_{chem}(i)$ of the compound's activation of the assay in $mol_{reference} kg_{PDMS}^{-1}$ for the analyzed animals and their corresponding organs: P.p.: harbor porpoise, P.v.: harbor seal, O.o.: orca, plus running number. White: the chemical was not found in the sample. Reference chemicals for AhR-CALUX and AREc32 are benzo[a]pyrene and for PPAR γ -bla Rosiglitazone.

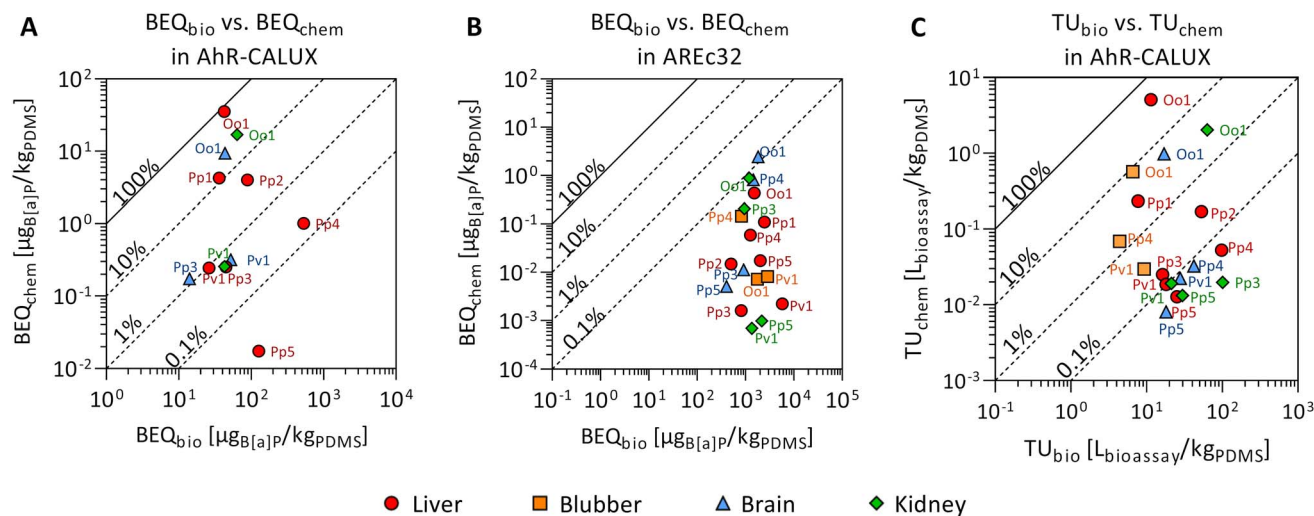
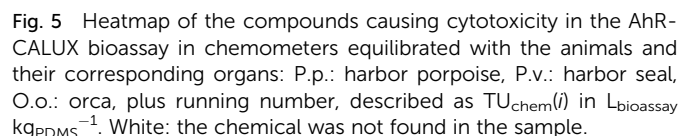


Fig. 4 Mixture effects from liver (red circle), blubber (orange square), brain (blue triangle) and kidney (green diamond) samples from seven marine mammals (P.p.: harbor porpoise, P.v.: harbor seal, P.h.: ringed seal, O.o.: orca, plus running number). Comparison of the predicted mixture effect from the detected compounds, described as bioanalytical equivalent concentration, BEQ_{chem} , to the bioanalytically derived mixture effects, BEQ_{bio} , in the (A) AhR-CALUX and (B) AREc32 assays. Reference chemical for AhR-CALUX and AREc32 is benzo[a]pyrene (B[a]P). (C) Comparison of the cytotoxic effects measured in the AhR-CALUX assay (TU_{bio}) with the predicted effect (TU_{chem}), data in Tables S4 and S8.† The symbols are labeled with the corresponding code from the different animals.

The animal with the highest chemical burden in this study was the orca O.o.1 and thus a larger fraction of the observed effects was explained for the tissues of this animal than for the remaining individuals. Contrarily, for P.p.3 and P.p.5, as the animals with the lowest overall concentrations of the targeted compounds, the smallest fractions of the observed effects were explained. In contrast to the chemical analysis, the measured effects in the bioassays (Effect and Toxic Units) for O.o.1 were



5 Conclusion

The results from chemical analysis have shown that compounds banned years to decades ago (*e.g.* PCBs) can still be highly

relevant in the environment since they continue to be found in high concentrations in marine mammals, partly exceeding known threshold values of physiological impacts. Nonetheless, including these contaminants into iceberg modeling to explain measured cellular effects, in most cases only small fractions could be accounted for. This discrepancy indicates that there are still a lot of unknown (or untargeted) chemicals, activating toxicity pathways, supporting and clarifying previous findings.³³

Besides the high contaminant burden of marine mammals demonstrated in this study, other reports from the literature show that these animals are under severe health stress and at high risk of dying from infectious diseases.^{3,11,29,86–89} In addition to adult males, which do not transfer part of their HOCs body burden to their offspring, especially young marine mammals (most of the individuals analyzed in this study fall into this category) are exposed to high concentrations of HOCs due to maternal transfer of HOCs during pregnancy and lactation, potentially causing physiological effects such as immunosuppression.¹¹ The measurement and monitoring of the chemical burden by quantification of legacy compounds as well as emerging compounds with similar properties helps to strengthen the link between exposure and adverse health outcomes for the organisms and the ecosystem.

Author contributions

EBR: conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing – original draft; BIE: formal analysis, methodology, writing – review & editing; ERN: formal analysis, investigation, validation, writing – review & editing; HN: investigation, validation, writing – review & editing; US: resources, writing – review & editing; AJ: conceptualization, investigation, methodology, project administration, supervision, validation, funding acquisition, writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement no. 715173, CHEMO-RISK), and the Helmholtz Association, HGF Project EXPOSOMETER 2020–2025, funding of first-time appointments of excellent women scientists, W2/W3-126. We gratefully appreciate access to the large infrastructure platform CITEPro (Chemicals in the Environment Profiler) funded by the Helmholtz Association. The marine mammals were collected through the stranding network in Schleswig-Holstein and necropsied partly with funding of the Landesbetrieb für Küstenschutz, Nationalpark und Meeresschutz Schleswig-Holstein (LKN.SH) and Ministry of Energy Transition, Agriculture, Environment, Nature and Digitalisation of Schleswig-Holstein (MEKUN SH), Germany. The authors acknowledge the work of ITAW

colleagues, for preparing and collecting the samples from the marine mammals in frame of projects funded partly by LKN.SH and MEKUN SH. We are grateful for the technical help of Jörg Watzke, Fanny Rosello and Njoud Alsabbagh, especially during the handling of the tissues. We also thank Aleksandra Piotrowska and Sandra Jäsch for support during the GC-HRMS measurements and maintaining the high quality. And we thank Nadin Ulrich for the assistance with the LSER-Database.

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