Combining Chemical Analysis and *In Vitro* Bioassays to Characterize the Exposome of Organic Contaminants in Marine Mammals

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Summary

Marine mammals are exposed to a wide range of environmental pollutants. Considering the complex mixture of chemicals to which marine mammals are exposed to, monitoring the chemical burden is necessary to evaluate the health risk posed by the exposure to mixtures. Within the framework of this thesis, passive equilibrium sampling (PES) with silicone was used to study the mixtures of chemicals in four organs of marine mammals, including liver, kidney, brain, and blubber. Tissues from seven harbor porpoises (*Phocoena phocoena*), three harbor seals (*Phoca vitulina*), one ringed seal (*Pusa hispida*) and one orca (*Orcinus orca*) were analyzed.

The PES method with silicone was established for marine mammal organs to capture a broad variety of hydrophobic organic compounds (HOCs). For lipid-rich tissue (i.e., tissue with a lipid content >10 %, such as blubber), HOCs reach a thermodynamic equilibrium between silicone and tissue with static equilibration within 24 h. In order to achieve thermodynamic equilibrium between the silicone and tissues of liver, kidney, and brain (with lipid contents <10 %), a dynamic PES method needed to be performed. It was demonstrated that a manual relocation of the silicone in the tissue up to 70 times (within 9 days) was sufficient for the HOCs to reach equilibrium between silicone and liver, kidney, and brain. At equilibrium, the HOCs were extracted from the silicone using solvent, allowing the investigation of the exposome (i.e., the total chemical burden) of marine mammals by using two analytical techniques: *in vitro* cell-based reporter gene assays and targeted chemical analysis with gas chromatography-high resolution mass spectrometry (GC-HRMS).

Usually, the extraction of biota samples leads to a co-extraction of lipid matrix constituents. When dosing these residual co-extracted lipids to *in vitro* cell-based reporter bioassays, the partitioning of the dosed chemicals is influenced. Co-dosed lipids reduce the apparent sensitivity of the assay, as the bioavailability of the chemicals is reduced. Hence, the equilibrium partitioning between the assay constituents (i.e., medium, cells and co-dosed lipids) was described with a mass balance model (MBM) and experimentally verified. The MBM allowed the interpretation of *in vitro* bioassay results for lipid-containing biota extracts of known lipid volume fraction. Furthermore, a lipid correction of the bioassay effect data, i.e., prediction of the bioanalytical effect without lipid influence, was possible for compounds with known partitioning properties and hydrophobicity. In order to omit a lipid correction for biota extracts of unknown chemical mixture, a lipid volume fraction of 0.27 % should not be exceeded.

With the method developments and the MBM, the mixture effects from marine mammal organ PES extracts were quantified with three *in vitro* assays, using mammalian cell lines. Bioassays targeting the activation of the aryl hydrocarbon receptor (AhR-CALUX), the peroxisome proliferator-activated receptor gamma (PPARγ-bla) and the Nrf2-dependent oxidative stress response (AREc32) were tested for liver (*n*=7), kidney (*n*=4), brain (*n*=5), and blubber (*n*=7) extracts. The activation of the PPARγ-bla indicated elevated effects elicited by extracts from liver, followed by extracts from kidney, brain, and blubber tissues. More specifically, the activation of PPARγ-bla and the AREc32 was 11±0.26 (mean±SD, *n*=7) and 1.9±0.32 (*n*=4), respectively, times higher for liver extracts relative to the corresponding blubber extracts. Minor differences were detected between the other organs tested with the AREc32. The tested blubber extracts did not activate the AhR-CALUX up to concentrations where cytotoxicity occurred, whereas an activation was measured for liver extracts (*n*=7).

The analytical results highlight the complexity of chemical mixtures that can be found within the marine mammals: 70 of 117 targeted HOCs were detected with GC-HRMS, such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), and polycyclic aromatic hydrocarbons (PAHs). A distribution pattern between different organs of the same animal was observed for single compounds. For example, metabolizable compounds like 4,4'-dichlorodiphenyltrichloroethane (4,4'-DDT) or etofenprox were mainly detected in the extracts of the less metabolically active blubber. Compounds that cannot be (easily) metabolized by mammals, were detected in all organs, such as higher chlorinated PCBs. Overall, the analytical investigation indicated a uniform chemical distribution within the organs of the same animal.

The results of targeted chemical analysis and bioanalytical assessment were combined using 'iceberg modeling'. While the detected effects measured in the AhR-CALUX was explained between 0.014–83 % by the quantified chemical mixture, less than 0.13 % and less than 0.034 % was explained for the bioanalytically measured activation of the AREc32 and PPARγ-bla, respectively. This demonstrated that in most cases a large fraction of undetected (and/or unknown) chemicals were responsible for the activation of the cell-based *in vitro* bioassays.

Monitoring the mixtures of environmental pollutants in marine mammals by a combination of chemical analysis and bioassays is efficient in assessing trends and to derive potential effects of these contaminants in the marine environment. The characterization of the exposome is useful for the determination of pathological effect thresholds and large-scale monitoring and conservation programs.

Zusammenfassung

Marine Säugetiere sind einer Vielzahl von Umweltschadstoffen ausgesetzt. Unter Berücksichtigung der komplexen Mischungen von Chemikalien, denen die Meeressäuger ausgesetzt sind, ist es notwenig die chemische Belastung zu aufzuzeichnen, um das Gesundheitsrisiko durch die Exposition gegenüber dieser Mischungen abzuschätzen. Im Rahmen dieser Dissertation wurden die Mischungseffekte von Umweltchemikalien in vier Organen - Leber, Niere, Gehirn und Blubber - mariner Säugetiere mittels passiver Gleichgewichtsprobennahme mit Silikon untersucht. Hierbei wurden die Organe von sieben Schweinswalen (*Phocoena phocoena*), drei Seehunden (*Phoca vitulina*), einer Ringelrobbe (*Phoca hispida*) und eines Schwertwales (*Orcinus orca*) untersucht.

Um eine Vielzahl hydrophober organischer Chemikalien (HOCs) zu erfassen, wurde die Methode der passiven Gleichgewichtsprobennahme mit Silikon für marine Säugetierorgane etabliert. In lipidreichem Gewebe (d.h. Gewebe mit einem Lipidgehalt >10 %, wie z.B. Blubber) erreichen HOCs ein thermodynamisches Gleichgewicht zwischen Silikon und Gewebe innerhalb von 24 h unter statischer Beprobung. Um ein thermodynamisches Gleichgewicht zwischen Silikon und Gewebe mit einem Lipidgehalt <10 % (d.h. Leber, Niere und Gehirn) zu erreichen, muss die Probennahme dynamisch durchgeführt werden. Es wurde gezeigt, dass eine bis zu 70-malige Umlagerung des Silikons im Gewebe (innerhalb von 9 Tagen) ausreichte, um ein Gleichgewicht der HOCs zwischen Silikon und Leber, Niere und Gehirn zu erreichen. Im Gleichgewicht wurden die HOCs unter Verwendung von Lösungsmittel aus dem Silikon extrahiert. Dies ermöglichte die Untersuchung des Exposoms (d.h. die Gesamtheit der chemischen Belastung) von Meeressäugern mittels zweier Analysetechniken: zellbasierte *in vitro* Reportergen-Assays und chemische Analyse durch Gaschromatographie gekoppelt mit hochauflösender Massenspektrometrie (GC-HRMS).

Die Extraktion an Biota-Proben führt in der Regel zu einer Co-Extraktion von Lipidmatrixbestandteilen. Diese co-extrahierten Lipide beeinflussen die Verteilung der Chemikalien im System der zellbasierten *in vitro* Reportergen-Assays. Dabei verringern die co-dosierten Lipide die scheinbare Empfindlichkeit des Assays, da die Bioverfügbarkeit der Chemikalien verringert wird. Um die Gleichgewichtsverteilung der Chemikalien zwischen den Assay-Bestandteilen (d.h. Medium, Zellen und co-dosierten Lipiden) zu beschreiben, wurde ein Massenbilanzmodell (MBM) berechnet und experimentell verifiziert. Bei bekannter Lipid-Volumenfraktion ermöglichte das beschriebene MBM die Interpretation von *in vitro* Bioassay-Ergebnissen für lipidhaltige Extrakte aus Biota-Proben. Darüber hinaus war eine Lipidkorrektur der Bioassay-Effektdaten (d. h. die Vorhersage des bioanalytischen Effekts ohne Lipideinfluss) für

Chemikalien mit bekannten Verteilungseigenschaften und Hydrophobizität möglich. Bei Biota-Extrakten mit unbekannter chemischer Mischung und einer Lipid-Volumenfraktion <0,27 % kann eine Lipidkorrektur vermieden werden.

Mit der entwickelten und verifizierten passiven Probennahmemethode und dem MBM konnten die Mischungseffekte aus den Organ-Extrakten der marinen Säugetiere mit drei *in vitro* Reportergen-Assays quantifiziert werden. Die Aktivierung des Aryl-Hydrocarbon-Rezeptors (AhR-CALUX), des Peroxisom-Proliferator-aktivierten Rezeptors Gamma (PPARγ-bla) und der Nrf2-abhängigen oxidativen Stressantwort (AREc32) wurden gemessen. Alle Zellassays basieren auf Säugetierzelllinien. Getestet wurden Leber- (*n*=7), Niere- (*n*=4), Gehirn- (*n*=5) und Blubber- (*n*=7) Extrakte. Die Messung des PPARγ-bla deutete auf eine erhöhte Aktivierung durch die Leberextrakte hin, gefolgt von den Extrakten aus Niere, Gehirn und zuletzt Blubber. Dabei war die Aktivierung für die Leberextrakte im PPARγ-bla und AREc32 11±0,26 (Mittelwert±Standardabweichung, *n*=7) bzw. 1,9±0,32 (*n*=4) mal höher, im Vergleich zu den Extrakten aus Blubber desselben Individuums. Geringfügige Unterschiede wurden zwischen den weiteren getesteten Organen in AREc32 festgestellt. Für die Blubberextrakte wurde keine Aktivierung des AhR-CALUX, für Konzentrationen unterhalb der Zytotoxizität, gemessen, während für alle Leberextrate (*n*=7) eine Aktivierung gemessen wurde.

Die Ergebnisse der chemischen Analytik unterstreicht die Komplexität chemischer Mischungen in den Meeressäugern: 70 von 117 untersuchten HOCs wurden mittels GC-HRMS nachgewiesen, darunter polychlorierte Biphenyle (PCBs), chlororganische Pestizide (OCPs) und polyzyklische aromatische Kohlenwasserstoffe (PAHs). Für einzelne Chemikalien konnte ein Verteilungsmuster zwischen den Organen desselben Tieres festgestellt werden. Beispielsweise wurden metabolisierbare Verbindungen wie 4,4'-Dichlordiphenyltrichlorethan (4,4'-DDT) oder Etofenprox hauptsächlich in den Extrakten des weniger stoffwechselaktiven Blubbers nachgewiesen. Schadstoffe, die von Säugetieren nicht (schnell) metabolisiert werden können, wie z.B. höher-chlorierte PCBs, wurden in allen Organen nachgewiesen. Insgesamt deutete die analytische Untersuchung auf eine gleichmäßige Verteilung der Schadstoffe zwischen den Organen innerhalb der Tiere hin.

Die Ergebnisse der chemischen Analyse und der bioanalytischen Messung wurden mittels „Eisberg-Modellierung" miteinander kombiniert. Die mittels GC-HRMS quantifizierte Chemikalienmischung erklärte zwischen 0,014–83 % der im AhR-CALUX gemessenen Effekte, während für AREc32 und PPARγ-bla weniger als 0,13 % bzw. weniger als 0,034 % erklärt wurden. Dies verdeutlichte, dass in den meisten

Fällen ein großer Teil unentdeckter (und/oder unbekannter) Chemikalien für die Aktivierung der zellbasierten *in vitro* Bioassays verantwortlich war.

Die Bewertung von organischen Schadstoffmischungen in der Umwelt durch eine Kombination aus chemischer Analyse und Bioassays ist eine aufschlussreiche Methode um die potenziellen Auswirkungen der Chemikalienmischungen zu beurteilen. Zudem ist die Charakterisierung des Exposoms nützlich, um pathologische Wirkungsschwellen zu bestimmen. Überdies können die Daten von groß angelegten Überwachungs- und Konservierungsprogrammen verwendet werden.

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“So long, and thanks for all the fish!”

- Douglas Adams

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List of Abbreviations

AhR	aryl hydrocarbon receptor
AMAP	Arctic Monitoring and Assessment Programme
B[a]P	benzo[a]pyrene
BDE	brominated diphenyl ether
BEQ	bioanalytical equivalent concentrations
BFR	brominated flame retardant
CHC	chlorinated hydrocarbon
CIS	cold injection system
DDD	dichlorodiphenyldichloroethylene
DDE	dichlorodiphenyldichloroethane
DDT	dichlorodiphenyltrichloroethane
DDX	sum of DDE, DDD and DDT
EC	effect concentration
EI	electron ionization
EU	effect unit
GC	gas chromatography
GC-HRMS	gas chromatography-high resolution mass spectrometry
HCB	hexachlorobenzene
HCH	hexachlorocyclohexanes
HELCOM	Helsinki Commission
HOC	hydrophobic organic compound
HRMS	high resolution mass spectrometry
IC	inhibitory concentration
MBM	mass balance model
MS	mass spectrometry
O.o.	*Orcinus orca* (orca)
OCP	organochlorine pesticide
OSPAR	Oslo and Paris Commission
P.h.	*Pusa hispida* (ringed seal)
P.p.	*Phocoena phocoena* (harbor porpoise)

P.v.	*Phoca vitulina* (harbor seal)
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
PDMS	polydimethylsiloxane
PES	passive equilibrium sampling
POPs	persistent organic pollutants
PPARγ	peroxisome proliferator-activated receptor gamma
PSA	primary secondary amine
Pyr	pyrethroid
R^2	coefficient of determination
SD	standard deviation
tBHQ	tert-butylhydroquinone
TCDD	2,3,7,8-tetrachloro-dibenzodioxin
TDU	thermal desorption unit
TU	toxic unit

1 Introduction

1.1 Marine Mammals under Anthropogenic Stress

Our modern lifestyle benefits from the usage of diverse chemicals. At the same time the use of chemicals also triggers the release of countless compounds into the environment. Wang et al. (2020) concluded that over 350.000 chemicals and mixtures of chemicals were registered in national or regional inventories. The purposes of these chemicals are diverse and cover the use as pharmaceuticals, personal care products, pesticides, and industrial chemicals (Escher and Fenner 2011). Beside the advantages that chemicals bring for humans, the increasing number of released chemicals that can be found in drinking water, food and the atmosphere can adversely affect human and wildlife health (Rappaport and Smith 2010; Escher et al. 2020b).

As long-lived organisms on a high trophic level, marine mammals accumulate complex mixtures of different chemicals. Eventually, marine mammals accumulate the highest levels of environmental contaminants in the overall wildlife. Due to their natural habitat and the number of anthropogenic compounds that can be found in water, fish, or sediment, marine mammals are particularly exposed to hazardous chemicals (Ross 2000; Letcher et al. 2010; Siebert et al. 2012; Jepson and Law 2016a). Especially pollutants that are hydrophobic and resistant to metabolic degradation persist in the environment. These can be biomagnified through the food web towards higher trophic level organisms (Mössner et al. 1994; Ross 2000; Jepson and Law 2016a). Chemicals of this category are persistent organic pollutants (POPs). The countless number of various chemicals within individual organisms increase the risk of adverse effects with a negative impact on the health status of marine animals (Desforges et al. 2016; Slobodnik et al. 2022).

1.1.1 Hydrophobic Organic Chemicals (HOCs)

Pollutants of great concern in the environment, including marine wildlife, are hydrophobic organic chemicals (HOCs). HOCs of concern are, among others, POPs, which include polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs), brominated flame retardants (BFRs) as well as degradable but continuously produced polycyclic aromatic hydrocarbons (PAHs). Due to the high hydrophobicity of HOCs, these chemicals tend to partition into lipid-rich compartments in the organism (Mössner et al. 1994; Ross 2000; Kelly et al. 2007). Exposure to HOCs has been associated with several health effects, such as

endocrine disruption, reproductive disorders, cancer, and cardiovascular diseases (Desforges et al. 2016; Guo et al. 2019; Slobodnik et al. 2022). The toxic effects of POPs in the environment are recognized and addressed in the Stockholm Convention on POPs by the United Nations Environment Programme, which resulted in a limitation of the manufacturing and use of these compounds (UNEP 2019). In 2004, Stockholm Convention entered into force after having been ratified by 90 countries, phrasing out the initial 12 POPs: aldrin, chlordane, dichlorodiphenyltrichloroethane (DDT), dieldrin, endrin, heptachlor, hexachlorobenzene (HCB), mirex, toxaphene, PCBs, polychlorinated dibenzo-p-dioxins (dioxins), and polychlorinated dibenzofurans (furans). Since 2004, 18 additional POPs and POP groups have been added to the listed substances (UNEP 2019).

The worldwide distribution and fate of HOCs is determined by their persistent properties in combination with their semi-volatile and lipophilic nature which makes them prone to bioaccumulation. HOCs can be found in biotic and abiotic phases, e.g. they occur in the vapor phase or bind to particulate matter. Released from point and diffusive sources (e.g. industry, agriculture, households, traffic emissions), HOCs enter the environment and are distributed via the atmosphere. The capability of HOCs for long-range transport via atmospheric circulation led to a worldwide distribution (Wania and Mackay 1996). Eventually this led to the accumulation of HOCs in remote regions of the world, such as the Arctic (Muir et al. 1992; Desforges et al. 2018; Dietz et al. 2019).

1.1.2 Physiological Impact of HOCs on Marine Mammals

The effects of elevated HOC concentrations in mammals are diverse and can increase the risk of endocrine disruption (Beineke et al. 2005; Das et al. 2006; Schnitzler et al. 2008; Imazaki et al. 2015), reproductive disorders (Schwacke et al. 2002; Sonne et al. 2009; Jepson and Law 2016a), and impairments of the immune system (Ross et al. 1995; Lehnert et al. 2016; Desforges et al. 2017; Hall et al. 2018).Thus, the health and survival of marine mammals may be negatively affected (Sonne et al. 2020; van den Heuvel-Greve et al. 2021; Boyi et al. 2022). During gestation and lactation HOCs are transferred from the mother to their offspring (Debier et al. 2003; Sørmo et al. 2003; Vanden Berghe et al. 2012; Williams et al. 2020b; van den Heuvel-Greve et al. 2021; Hayes et al. 2022). Compared to adult animals, neonate and juvenile animals have an elevated metabolic activity and higher caloric consumption per kg of body weight (Innes et al. 1987). In consequence, already young mammals are at high risk to accumulate large amounts of HOCs. The chemical pattern that can be found in an organism is influenced by many factors, such as age, reproductive status, sex, diet, geographical location (habitat) and is known to change over the lifetime.

The entirety of exposure within an organism can be captured by the concept of the exposome (Wild 2005). The exposome includes exogenous compounds, such as environmental pollutants, and endogenous chemicals, such as compounds formed by metabolic processes in the organism (Rappaport and Smith 2010). Consequently, the overall exposome of all chemicals within an organism is represented by a complex mixture of different environmental pollutants, their transformation products and endogenous chemicals. Moreover, the exposome is changing throughout the lifespan, due to diet, infections, diseases and exposure events (Rappaport and Smith 2010; Rappaport 2011; Miller and Jones 2014). Furthermore, the factor of the environmental health risks is included in the term of "eco-exposome", which was framed by Scholz et al. (2022): "The eco-exposome represents the totality of internal exposure over a lifetime to individuals of a given species. This includes exposure to anthropogenic chemicals, their biotransformation products, and/or adducts. Endogenous signaling chemicals, changed in response to exposure of anthropogenic chemicals, could contribute to the totality of an internal exposure and the translation of this exposure to biological responses."

The physiological impact of the chemical mixture is difficult to predict as molecular interactions and mechanisms can be additive, synergistic and antagonistic. Individual chemicals might not cause any toxic effects at a low concentration, however, the sum of all chemicals within an organism may lead to adverse effects (Walter et al. 2002; Boobis et al. 2011; Altenburger et al. 2018; Escher et al. 2021). The comprehensive assessment of the chemical exposome of marine mammals is important to determine potential adverse health effects and pathophysiological effect thresholds.

1.2 Assessment of Organic Compounds in Biota

Different approaches can be used to comprehensively determine and identify the chemical burden. Due to the high diversity of chemical compounds and potentially low concentrations, highly sensitive and reliable analytical techniques are of utmost importance. For instance, extracts from environmental samples can be characterized by their chemical composition, i.e., the identification and quantification of single compounds. A complementary approach is to account the whole mixture of chemicals by the identification of the potential effects or endpoints in biological systems. To address both characterization approaches, dedicated analytical techniques need to be used, e.g. chemical analysis and bioanalytical assessment.

1.2.1 Sampling Approach: Passive Equilibrium Sampling with Silicone Chemometer

In order to enable the analysis of a chemical mixture with instrumental or bioanalytical measurements, the analytes need to be transferred from the tissue into an extract. Passive equilibrium sampling (PES) with a chemometer provides a suitable method for the analysis of HOCs. Chemometers are a well-defined polymer reference phase for the assessment of diverse organic pollutants in different matrices (Rojo-Nieto and Jahnke 2023). In this thesis, the silicone polydimethylsiloxane (PDMS, Figure 1), was used as chemometer, due to its apolar and inert properties. PDMS can capture nonpolar HOCs with a wide range of physicochemical properties. As a commonly used chemometer, PDMS is well characterized and suitable to transfer complex environmental mixtures of chemicals into an extract without changing their mixture composition (Mayer et al. 2003; Jahnke et al. 2008; Ossiander et al. 2008; Jahnke et al. 2014; Smedes et al. 2017; Rojo-Nieto and Jahnke 2023). Based on a thermodynamic equilibrium between the chemometer and the sample, chemometers can be applied to examine the exposure and fate of HOCs in the environment, e.g. biota tissue.

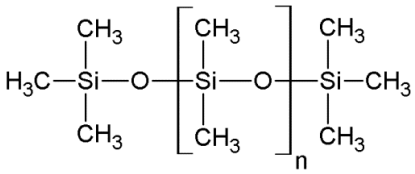

Figure 1. Structural formula of the polymer polydimethylsiloxane (PDMS), that was used as a chemometer in this thesis.

For PES, the chemometer is brought into direct contact with the homogenized tissue to allow the chemicals to partition between the tissue and the chemometer, until a thermodynamic equilibrium is established. At equilibrium, the HOCs are extracted from the silicone and the chemical concentrations in the chemometer, c_{PDMS}, can be quantified. The traditional approach to analyze and report chemical concentrations in biota is to carry out an exhaustive extraction of the tissues and to normalize the measured concentration to the lipid weight (c_{lipid}), which represents the main sorption phase of HOCs. Hence, the comparison of the observed exposure in different organs with varying lipid contents and the following normalization to the lipid weight could introduce errors and lead to biased results. These errors can be introduced due to different extraction protocols and varying sorptive capacities, e.g. of storage lipids and membrane lipids, opposed to proteins (Jahnke et al. 2015; Mäenpää et al. 2015; Rojo-Nieto et al. 2019).

The uptake of the compounds into the chemometer until equilibrium, is a time-dependent process and can be described by a first-order one-compartment model (Mayer et al. 2003), simplified in Figure 2. To apply the chemometer approach in biological matrices, three defined criteria must be met: (i) in order to not alter the equilibrium concentration, the chemometer must not deplete the sample by more than 5 % of the total mass of the chemical, i.e., negligible depletion conditions must be met; (ii) the equilibrium must be reached within an appropriate time (e.g., before the tissue starts to decay); (iii) the sorptive capacity of the chemometer must not be altered by the sampled tissue and matrix (Mayer et al. 2003; Ossiander et al. 2008).

The thermodynamic equilibrium between silicone and lipid-rich tissue, e.g. blubber, is achieved within few hours with static equilibration, as the lipids enhance the diffusion of HOCs throughout the tissue (Jahnke et al. 2008; Ossiander et al. 2008). Conversely, for tissues with low lipid content (<10 %) the diffusion of the HOCs within the tissue might be too slow to establish equilibrium partitioning. Consequently, applying static equilibration on lean tissue could lead to local depletion of the tissue close to the surface of the chemometer; as a consequence, the uptake rate of the compounds into the silicone is reduced and a thermodynamic equilibrium between silicone and lean tissue may not be achieved before tissue decay (Jahnke et al. 2009). Rusina et al. (2017) showed that local depletion can be avoided by using a dynamic sampling approach, ensuring multiple relocations of the chemometer in the tissue over time. Various dynamic sampling approaches for PES with lean tissue have been developed and proven to be successful: (i) manual relocations of silicone sheets in homogenized fish tissue (Rojo-Nieto et al. 2019), (ii) rolling silicone-coated vials to sample animal feces and animal food (e.g., horse meat, chicken, fruits, vegetables, etc.) (Chen et al. 2020; Chen et al. 2022) and (III) using an automatic device that constantly stirs silicone sheets in fish fillet cubes (Rusina et al. 2017; Smedes et al. 2020) and homogenized mammalian tissue (Baumer et al. 2021a; Baumer et al. 2021b).

The chemometer technique circumvents normalizations, as the silicone is introduced as a common sorptive reference phase. The resulting c_{PDMS} can be directly compared across different tissues and the chemical activity can be estimated (Mayer et al. 2003; Jahnke et al. 2009). It is possible to convert the concentrations in the silicone, c_{PDMS}, to lipid-based equilibrium concentrations in biota, $c_{lipid,\ eq.}$. This can be realized by using a polymer-specific lipid-silicone partition coefficient $K_{lipid/silicone}$ (Jahnke et al. 2008; Smedes et al. 2017). Eventually, the conversion allows the comparison of silicone-based data with measurements using exhaustive extraction.

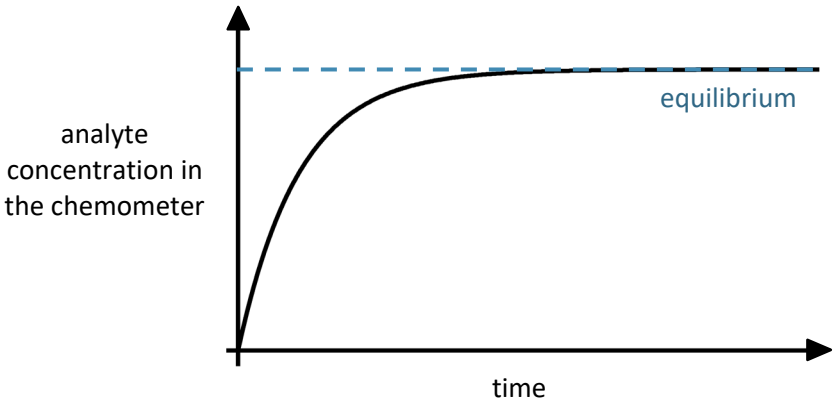

Figure 2. Simplified uptake kinetic of a chemical into the chemometer. The analyte concentration in the chemometer is plotted over the time. The uptake curve reaches the plateau when the equilibrium is approached.

Chemometer extracts can directly be dosed to bioassays to characterize the exposome, as the chemicals and proportions present in the mixture from the tissue are maintained. Furthermore, the extract can be introduced to chemical analysis for quantification of compounds and hence, providing additional information on the chemical burden. With this, chemometers are an adequate tool to characterize chemical exposure in marine mammals.

1.2.2 Chemical Analysis

The traditional approach to report the chemical burden is to analyze specific targeted compounds with instrumental analysis. Typically, the identification of chemicals in a sample involves the isolation of the compounds from the sample, a cleanup step, followed by the separation, and determination of the target compounds. Gas chromatography (GC) is a commonly used and well-established separation technique for non-polar and (semi-)volatile organic compounds. The separation is achieved by an analytical column through which a carrier gas transports the (vaporized) mixture of chemicals. The chromatographic separation of the compounds depends on the interaction with the column. By coupling the GC with mass spectrometry (MS) measurement, information for the identification of compounds is provided. The molecules elute from the analytical column and pass an ion source, e.g., an electron ionization (EI) source. The neutral molecules are ionized and fragmented and accelerated through the mass analyzer, e.g., an ion trap. Finally, the ions are separated based on their mass-to-charge ratio (*m/z*). With the monitored information (i.e., retention time, the major characteristic fragment (*m/z* ion) and a confirmation

fragment), the compound can be identified. The addition of a calibration (i.e., a mixture with known substances and varying concentrations) and internal standard (i.e., compound(s) with a constant concentration in every sample and not originally present in the environment) enables the reliable identification, qualification and quantification of the analytes. To precisely determine targeted compounds in an environmental sample, the analytical instrument needs to be sensitive and selective. Environmental samples comprise a large number of compounds which can be available at low concentrations and, consequently, missed by the instruments' detection (and quantification) limit. For highly sensitive detection of (semi-)volatile analytes in complex environmental samples, high resolution mass spectrometry (HRMS) can provide sensitive and reliable results (Peterson et al. 2014).

Monitoring programs for environmental pollutants in marine mammals with chemical analysis put a large focus on POPs, such as PCBs, OCPs or dioxins. However, constant development of analytical tools permits the identification of new contaminants, e.g. chemicals of emerging concern, such as organotin compounds (Berge et al. 2004; Ciesielski et al. 2004; Sham et al. 2020), personal care products (Kannan et al. 2005; Hart et al. 2018; Combi et al. 2022) and other relevant chemicals that may induce adverse health effects.

1.2.3 Bioanalytical Assessment

While chemical analysis can identify and quantify single chemicals, bioanalytical tools provide information about the activation of important cellular toxicity pathways triggered by the overall chemical mixture present in an extract. A commonly used method to characterize an environmental extract is the usage of *in vitro* cell-based reporter gene bioassays. The underlying principle is the usage of recombinant cells, i.e., genetically modified cells. These reporter gene cells encode a detectable and quantifiable product (i.e., a reporter protein) when activated to a specific stimulus, e.g., elicited by xenobiotic chemicals. With these recombinant cells an activation of a specific mode of action by a chemical mixture can be identified and quantified. In addition, cytotoxic effects can be measured by monitoring the cell growth and cell viability.

An important cellular toxicity pathway is the toxicokinetic pathway of the xenobiotic metabolism. A xenobiotic chemical is a compound that is foreign to the organism and thus does not occur naturally. Xenobiotic chemicals can interfere with different cellular mechanisms and can activate xenobiotic receptors. Commonly investigated xenobiotic receptors are the aryl hydrocarbon receptor (AhR) and peroxisome proliferator-activated receptors (PPAR) (Omiecinski et al. 2011; Escher et al. 2021). Examples for environmental pollutants that act as AhR ligands are dioxins, furans, PCBs and some PAHs, such as

benzo[a]pyrene (B[a]P) (Piskorska-Pliszczynska et al. 1986; Safe 1990). PPARγ ligands include phthalates, perfluorinated compounds, halogenated derivates of bisphenol A, and organophosphorus compounds (Riu et al. 2011; Grimaldi et al. 2015; Garoche et al. 2021).

Another prominent cellular toxicity pathway is the activation of the cellular oxidative stress response. The Nrf2-mediated oxidative stress response activates metabolic enzymes to counteract oxidative stress. However, excessive activation of the oxidative stress response will lead to adverse outcomes. Elevated oxidative stress can cause cell damage, such as DNA damage or apoptosis, resulting in the pathogenesis of several adverse health effects, e.g., cancer, inflammation, and neurodegenerative disorders (Canli et al. 2017; Habtemariam 2019). The oxidative stress response can be induced by numerous environmental chemicals, such as PAHs, quinones, and dioxins (Escher et al. 2012; Shukla et al. 2012; Jin et al. 2015; Neale et al. 2017a). Oxidative stress response is a valuable early indicator of the presence of stressors within a biological system (Escher et al. 2021).

The information that can be gained from bioanalytical measurements (i.e., the activation of a molecular pathway) can be linked to the chemical composition of an environmental sample, such as marine mammals. As such, PES extracts from marine mammals (i.e., dugongs from the east coast of Australia) activated the AhR and the Nrf2-mediated oxidative stress response (Jin et al. 2013; Jin et al. 2015). The characterization by cell-based bioassays captures all compounds that can activate a specific cellular, and hence, provide substantive information for biomonitoring.

1.2.4 Combination of Chemical Analysis with *In Vitro* Bioassays: Iceberg Modeling

Targeted chemical analysis determines selected known chemicals at quantifiable levels within an environmental sample. Apart from that, *in vitro* reporter gene assays provide information on a specific effect caused by biologically active chemicals in the sample. The results from both analyses can be linked together by the concept of the so-called ‘iceberg modeling’ approach, illustrated in Figure 3 (Escher et al. 2021). With this analogy, the biological effect measured with the bioassay is represented by the entire iceberg, whereas the identified chemicals, that are known to elicit the measured effect, are symbolized as the visible tip of the iceberg. In consequence, the submerged part of the iceberg represents all chemicals, that were not detected (e.g., unknown compounds, or chemicals below the detection limit of the instrument) or chemicals with unknown effects.

In other words, by iceberg modeling the mixture effect measured with the bioanalytical tool is compared with the predicted effect of the detected chemicals from instrumental analysis. This approach helps to

understand how much of the measured endpoint effect can be explained by the detected chemicals and how much remains unknown (Weijs and Zaccaroni 2016; König et al. 2017; Escher et al. 2020b; Neale and Escher 2020).

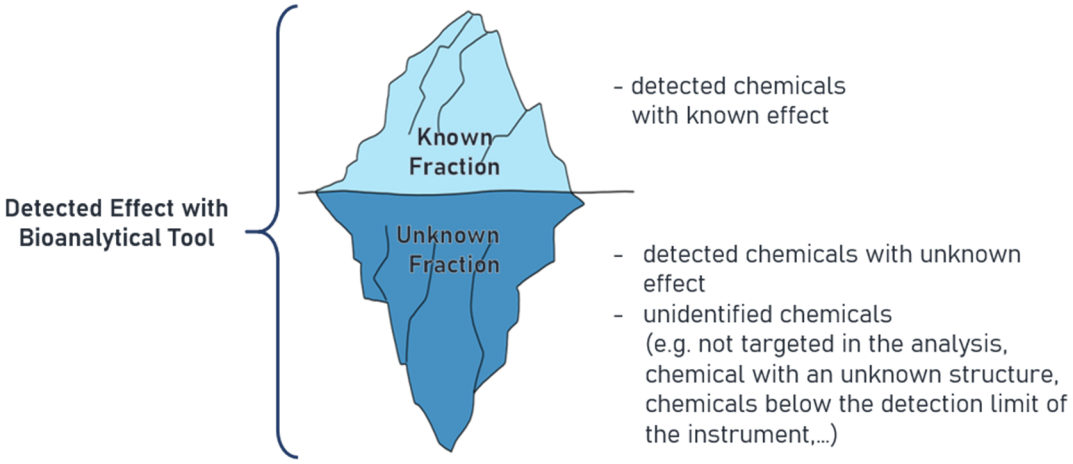

Figure 3. The iceberg modeling approach combines bioanalytical measurements and chemical analysis. The detected chemicals with known effect represent the visible tip of the iceberg on the surface, whereas the unexplained effect is represented by the hidden part of the iceberg underwater. The bioanalytical tool can characterize the size of the entire iceberg. (Figure adapted from Escher et al. (2021)).

1.3 Aim of this Doctoral Thesis

The goal of this thesis was to examine the chemical burden of HOCs in different organs of marine mammals. For the assessment of chemical exposure in marine mammals, various tissues from the same individual were chosen: lipid-storage blubber, liver, kidney and brain. Consequently, tissue-specific toxicological information is provided. The combination of chemical analysis and bioanalytical measurements allows the comprehensive characterization of the mixture exposome.

The core structure of this thesis is outlined in Figure 4. To monitor the chemical exposure in marine mammals, the chemical mixture in the biota tissue needed to be transferred into an extract that is suitable for analytical and bioanalytical measurements. For that reason, PES with the silicone PDMS was carried out. The development and validation of a suitable and successful sampling method with the chemometer for lean tissues of marine mammals was performed (*Publication II*).

Usually, processing biota material includes matrix transfer into the extract. Consequently, extracts containing matrix need to be introduced to a cleanup method to remove all disturbing matrix constituents, such as lipids and proteins. PES with a silicone minimizes co-extraction of biological matrix. Minor amounts of lipid residues are still transferred to the extract. The distribution of chemicals dosed to an *in vitro* cell-based bioassay system can be simplified with the major partitioning compartments of medium and cells. Furthermore, medium and cells are each composed of lipid, protein, and water. Dosing extracts containing co-extracted lipids into the bioassay system, will add an additional third partitioning phase to this system. The influence of the co-dosed lipids on the partitioning of the dosed chemicals in the cell-based bioassay system was thoroughly investigated. Moreover, the dosing strategy of biota extracts to *in vitro* bioassays was improved (*Publication I*).

To reduce the amount of lipid matrix constituents in the extract while maintaining the composition of its diverse chemical mixture, the influence of a non-destructive cleanup method with reliable recoveries was tested with the *in vitro* bioassays (*Publication II*).

With an optimized sampling and dosing method, bioanalytical measurements with three cell-based reporter gene assays (*Publication II*) and chemical analysis with gas chromatography-high resolution mass spectrometry (GC-HRMS) (*Publication III*) were carried out. Finally, iceberg modeling was applied to identify the known and unknown fractions in the biological extracts (*Publication III*).

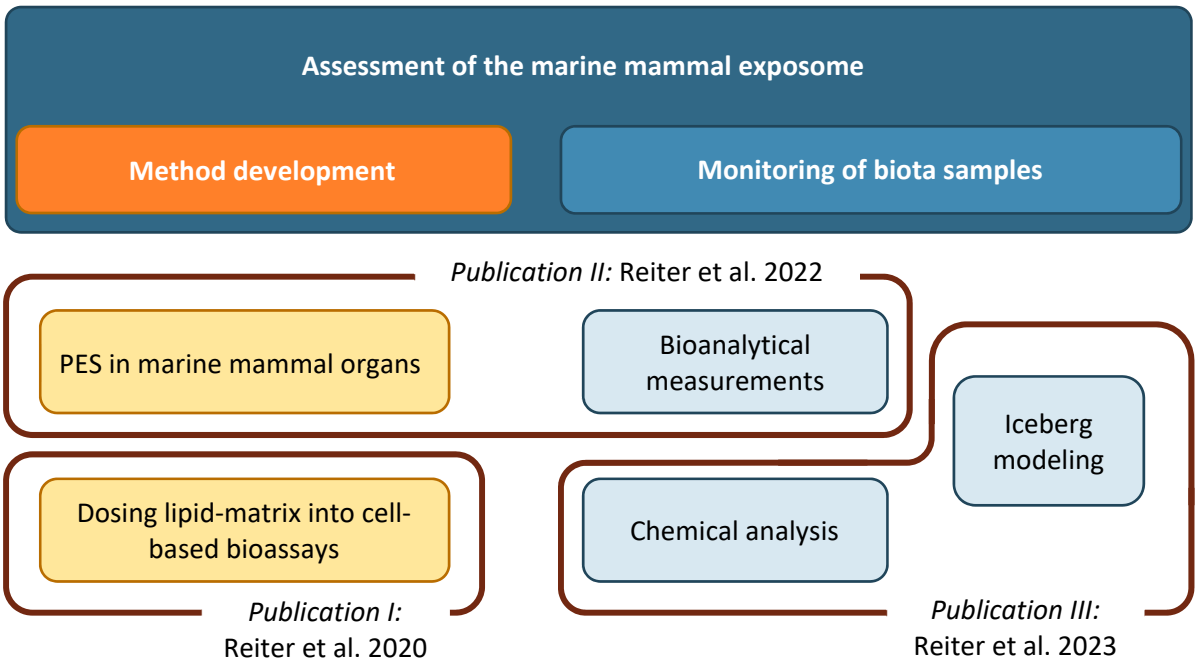

Figure 4. Outline of this thesis and the associated publications. The assessment of the marine mammal exposome required method development steps: Passive equilibrium sampling (PES) with silicone chemometers in marine mammal organs (*Publication II*), and dosing procedure of biota extracts containing lipid matrix constituents (*Publication I*). With the developed and evaluated method, the biota extracts were characterized using bioanalytical measurements (*Publication II*) and instrumental chemical analysis (*Publication III*). The (bio)analytical results were combined with iceberg modeling (*Publication III*).

2 Materials and Methods

All material and methods are described in detail in the referred publications. For reasons of clarity and comprehensibility, some contents are summarized in the following.

2.1 Sample Material

The characterization of the exposome was carried out by analyzing 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.). The samples were provided by the Institute for Terrestrial and Aquatic Wildlife Research in Büsum, Germany, and were obtained from animals deceased and stranded between 2016 - 2019. The stranding locations along the shores of Schleswig-Holstein, Germany, are shown in Figure 5. Additional information on sex, age and stranding year are given in Table 1. For eight animals a complete set of liver, blubber, brain and kidney were provided and for four animals a core set of liver and blubber were available (Table 1). All tissues were homogenized with a blender and stored at -20 °C.

Table 1. Information on the twelve individuals analyzed in this thesis: sample code, analyzed organs, sex, age group, stranding year and waters found. The sample code includes the species name plus a running number: P.p.: harbor porpoise, P.v.: common seal, P.h.: ringed seal, O.o.: orca. Either a core set of two organs were available (liver and blubber) or the full set of four organs (liver, blubber, brain and kidney). The table was taken from *Publication III*.

SAMPLE CODE	ORGANS AVAILABLE	SEX	AGE GROUP	STRANDING YEAR	WATERS
P.p. 1	2	female	juvenile	2018	North Sea
P.p. 2	2	male	juvenile	2019	North Sea
P.p. 3	4	female	adult	2019	Baltic Sea
P.p. 4	4	female	juvenile	2019	Baltic Sea
P.p. 5	4	female	adult	2018	Baltic Sea
P.p. 6	4	male	juvenile	2019	North Sea
P.p. 7	2	male	neonate	2019	Baltic Sea
P.v. 1	4	female	adult	2018	North Sea
P.v. 2	4	male	neonate	2019	North Sea
P.v. 3	4	male	neonate	2019	North Sea
P.h. 1	2	male	neonate	2018	Baltic Sea
O.o. 1	4	male	neonate	2016	North Sea

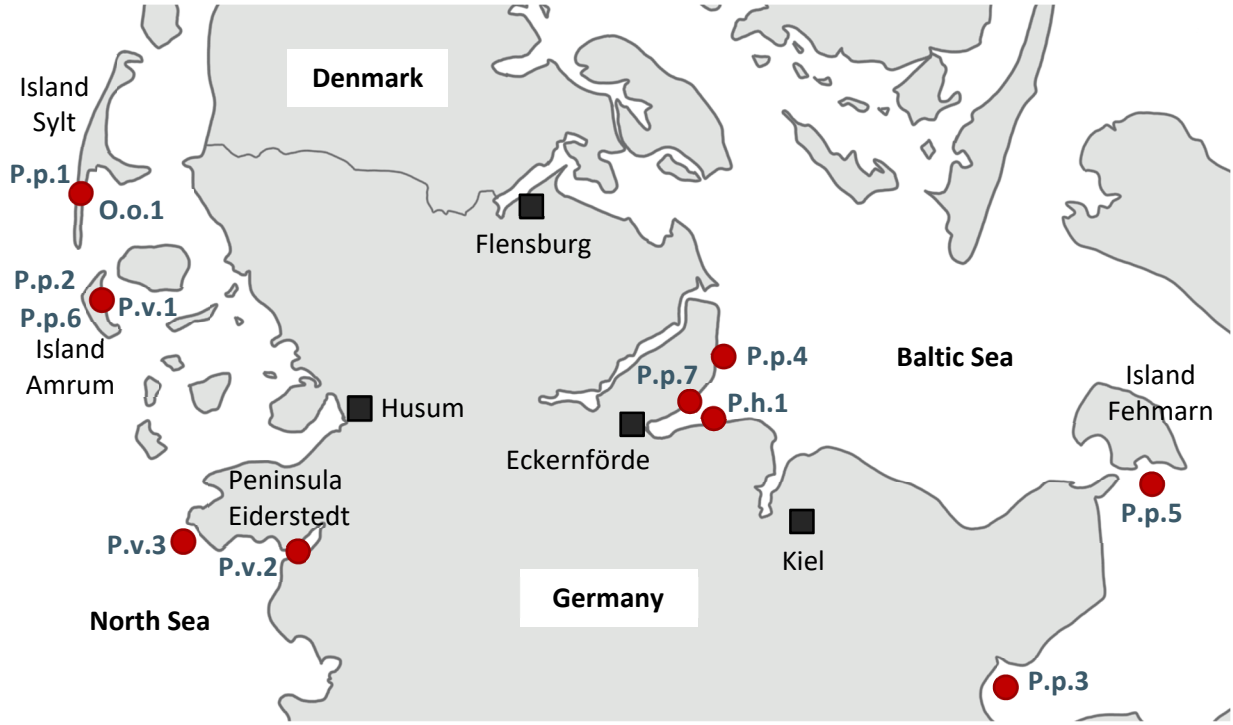

Figure 5. Stranding locations on the shores of Germany of the twelve marine mammals analyzed in this thesis (red dots). More detailed information on the sex, age and stranding year is provided in Table 1. The sample code includes the species name plus a running number: P.p.: harbor porpoise, P.v.: common seal, P.h.: ringed seal, O.o.: orca. The figure was modified from *Publication III.*

2.2 Passive Equilibrium Sampling

PDMS sheets (SSP-M823, Special Silicone Products, Ballston, USA) with a density of 1.17 g·cm^{-3}, and thicknesses of either 1000 µm, 350 µm, 250 µm or 125 µm were used in the studies of this thesis. The PDMS sheets were solvent-cleaned using Soxhlet extraction with ethyl acetate for at least 20 h and stored in fresh aliquots of ethyl acetate in the dark at room temperature until usage. The air-dried PDMS sheets were weight before the experiments with a micro-analytical balance (Mettler Toledo, Gießen, Germany). The criterium of negligible depletion conditions (<5 % depletion of the pollutants from the tissue by partitioning to the silicone) was met in all experiments. The minimum mass of the tissue required to meet the 5 % negligible depletion criterion was calculated based on lipid fraction of the tissues and with an average partition constant between lipid and PDMS, $K_{lipid/PDMS}$, of 30 L_{PDMS} L_{lipid}^{-1} (Jin et al. 2013). Additionally, at least 30 % more tissue homogenate and at least 5 g of additional tissue, was used.

Static equilibrium with PDMS sheets in blubber:

The PES procedure with PDMS sheets and blubber is described in detail in *Publication I*, *II* and *III*. Briefly, PDMS sheets were sandwiched or immersed in the homogenized blubber tissue, as illustrated in Figure 6A. PDMS and blubber were equilibrated for at least 24 h and up to 72 h at 4 °C.

Manual relocations with PDMS sheets in liver, kidney and brain:

The manual relocation process is described in detail in *Publication II* and *III*. Briefly, homogenized tissue (liver, kidney, or brain) was filled into clean glass vials and PDMS sheets were immersed in the tissue, as illustrated in Figure 6A. The silicone sheets were relocated 8 times per day (i.e., every 1.5 h), with static sampling at night, over nine days. To assess the uptake kinetics (*Publication II*), 350 µm tick silicone samplers were collected at 10 time points between 5 and 64-70 relocations (corresponding to 5 to 216 h of exposure of the sampler in the tissue). As an additional measure to confirm equilibrium partitioning (*Publication II* and *III*), silicone sheets with thicknesses of 350 µm, 250 µm and 125 µm were used and collected after the last relocation (64-70 relocations). For bioanalytical measurements, 350 µm thick PDMS sheets were collected after the last relocation. During the equilibration, the samples were kept at 4 °C.

Automatized relocation in a silicone-coated vial:

To test the applicability of an automatized approach, pre-weighed 20 mL-vials were silicone-coated (DC1–2577, Dow Chemical Company, Midland, Michigan, USA) aiming for three thicknesses (10, 20 and 30 µm) (Reichenberg et al. 2008). Following to casting, curing and cleaning, the coated vials were weighed with a micro-analytical balance, to assess the silicone weight. The vials were filled with the viscous tissue homogenate (liver, kidney, brain, or blubber). As for the PDMS sheets, the criterium of negligible depletion conditions were met. Additionally, metal beads were added to support the mixing and relocation of the homogenate near the silicone surface. The homogenate-filled vials were placed in constant motion on a horizontal roller mixer, as illustrated in Figure 6B. Vials were continuously rolled during the sampling period and kept at 4 °C. To assess the uptake kinetics, vials with a coating thickness of 30 µm were collected at 10 time points (between 5 - 216 h). In addition, vials with coating thicknesses of 30 µm, 20 µm and 10 µm were used for the last exposure time point of 216 h (i.e., nine days), to confirm equilibrium partitioning.

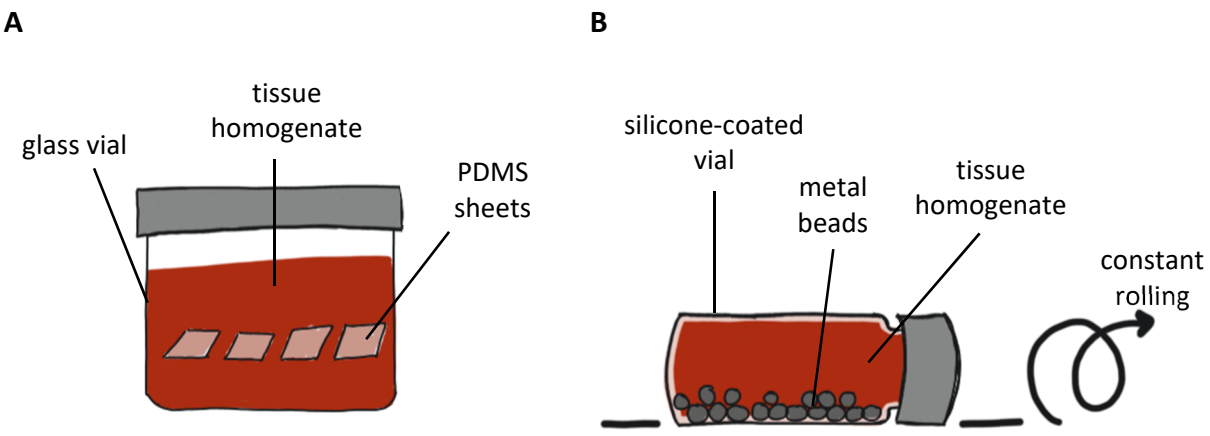

Figure 6. Illustration of the passive sampling approaches used in this thesis. (A) Glass vial filled with tissue homogenate. The PDMS sheets were immersed in the tissue. (B) Silicone-coated vials, filled with tissue homogenate and metal beads, were placed on a roller mixer to keep the tissue in motion (and thus being relocated) on the silicone surface. The metal beads assisted to relocate the viscous tissue.

2.3 Solvent Extraction of the Analytes from the Silicone and Cleanup

After equilibrating the silicone with the tissue homogenates, the PDMS sheets were taken out of the tissue. In case of the silicone-coated vials, the tissue was removed from the vials. The silicone surface was thoroughly cleaned with lint-free tissues, rinsed two times with bidistilled water and dried. The weight of the PDMS sheets and silicone-coated vials were determined with a micro-analytical balance. The analytes were extracted with 1 mL ethyl acetate per 0.1 g silicone, and with a minimum of 1.5 mL ethyl acetate, to ensure full coverage of the silicone with the solvent. The silicone was extracted for at least 2 h at room temperature on a horizontal roller mixer. The solvent was collected and the extraction repeated once with a fresh aliquot of ethyl acetate.

A non-destructive cleanup was performed to reduce the amount of co-extracted lipids in the PES extracts. The procedures are described in detail in *Publication II* and *III*. In a first step, the ethyl acetate extract was evaporated and the solvent was changed to acetonitrile. In a second step, the cleanup was conducted. In a last step, the solvent was changed to ethyl acetate again.

The extracts for bioanalytical measurements were either not submitted to a cleanup, or submitted to a freeze-out cleanup at -20 °C (Muz et al. 2021) in combination with a primary secondary amine (PSA)

sorbent (Agilent Technologies, USA) extraction (Wernicke et al. 2022). After cleanup the extract solvent was blown down to dryness and re-dissolved in ethyl acetate.

For the extracts that were submitted to chemical analysis, a cleanup with Captiva EMR-Lipid cartridges (3 mL, Agilent Technologies, USA) (Muz et al. 2021) in combination with PSA extraction was preferred. The extracts were transferred to a GC vial with insert and evaporated until dryness. The residues were dissolved in ethyl acetate, containing 21 isotope-labeled internal standards: 4,2'-DDT-D_8, Acenaphthen-D_{10}, B[a]P-D_{12}, Beta HCH-$^{13}C_6$, Chrysene-D_{12}, Decachlorobiphenyl-$^{13}C_{12}$, Etofenprox-D_5, Hexachlorobenzene-$^{13}C_6$, Naphthalene-D_8, Pentabromdiphenylether-$^{13}C_{12}$, PCB101-$^{13}C_{12}$, PCB138-$^{13}C_{12}$, PCB153-$^{13}C_{12}$, PCB180-$^{13}C_{12}$, PCB28-$^{13}C_{12}$, PCB52-$^{13}C_{12}$, Perylene-D_{12}, Phenanthrene-D_{10}, Pyrene-D_{10}, Tetrabromodiphenylther-$^{13}C_{12}$, Tonalide-D_3.

2.4 Chemical Analysis with GC-HRMS and Data Analysis

Chemical analysis was performed by gas chromatography-high resolution mass spectrometry (GC-HRMS, QExactive, Thermo Fisher Scientific, Germany). The 117 targeted compounds covered legacy and emerging HOCs: PCBs (*n*=13), PAHs (*n*=26), OCPs (*n*=23), brominated diphenyl ethers (BDEs) (*n*=6), pyrethroids (*n*=13), chlorinated hydrocarbons (CHCs) (*n*=3), musk fragrances (*n*=9) and other industrial compounds, such as antioxidants, UV filters and long-chain chemicals (*n*=23). The compounds are listed in Table 2. The targeted compounds were addressed in earlier relevant environmental studies using PES in marine sediment (Muz et al. 2020).

The instrumental setup is described in detail in *Publication II* and *III*. To summarize, for quantification a 13-point calibration (0.1 – 1000 ng mL^{-1}) was used, and solvent blanks, procedural silicone blanks and quality controls added to every measured batch. The GC system was equipped with a Trace 1310 GC coupled with a thermal desorption unit (TDU, TDU-2, Gerstel, Mülheim/Ruhr, Germany), a cooled injections system (CIS, Gerstel, Mülheim/Ruhr, Germany) and a DB-5ms ultra inert GC column (30 m x 0.25 mm ID, 0.25 µm film thickness). The injection volume was set to 2 µL in splitless mode. The carrier gas was helium with constant flow rate of 1.2 mL/min. The thermal desorption in the TDU was set with a temperature program from 30 °C to 300 °C (rate 300 °C/min) and holding for 5 minutes. The transfer temperature from the TDU to the CIS was set to 320 °C. The cryo-focusing was executed at -25 °C in a glass liner containing deactivated glass wool (CIS-4 TDU, Gerstel, Mülheim, Germany) for 0.2 minutes. The analytes were desorbed at a temperature of up to 300 °C at a rate of 12 °C/s, holding for 10 minutes. The analytes were injected in a splitless mode (2 minutes). The chromatographic separation was performed

with following temperature program: 60 °C (1 minute), heating up to 150 °C at a rate of 30 °C/min, further up to 186 °C at a rate of 6 °C/min rate and finally up to 300 °C at a rate of 4 °C/min (holding 11.5 min). The transfer line temperature was 280 °C and was connected with the QExactive instrument (Thermo Fisher Scientific, Germany). Temperature of the ion source was 250 °C. The mass spectrometric analysis was conducted using electron ionization (EI) at 70 eV (positive polarity), with fullscan mode (scan range: 70-810 *m/z*; resolution: 60,000; full width at half maximum: *m/z* 200). Perfluorotributylamine was used as mass calibrant for the internal calibration and tuning of the instrument.

Table 2. List of target chemicals, their abbreviation, the associated compound category and CAS number. Following compound categories were used: polychlorinated biphenyls (PCBs) (*n*=13), polyaromatic hydrocarbons (PAHs) (*n*=26), organochlorine pesticides (OCPs) (*n*=23), brominated diphenyl ethers (BDEs) (*n*=6), pyrethroids (Pyr) (*n*=13), chlorinated hydrocarbons (CHCs) (*n*=3), musk fragrances (musks) (*n*=9) and other industrial compounds (Others) (*n*=23). Additionally, an "X" indicates if the compound was detected in at least one sample. MDLs are reported in *Publication III*.

COMPOUND NAME	ABBREVIATION	COMPOUND CATEGORY	CAS	"X" IF DETECTED
2,2',5-Trichlorobiphenyl	PCB 18	PCB	37680-65-2	X
2,4,4'-Trichlorobiphenyl	PCB 28/31	PCB	7012-37-5	X
2,2',3,5'-tetrachlorobiphenyl	PCB 44	PCB	41464-39-5	X
2,2',5,5'-Tetrachlorobiphenyl	PCB 52	PCB	35693-99-3	X
2,2',4,5,5'-Pentachlorobiphenyl	PCB 101	PCB	37680-73-2	X
2,3',4,4',5-Pentachlorobiphenyl	PCB 118	PCB	31508-00-6	X
2,2',3,4,4',5'-Hexachlorobiphenyl	PCB 138	PCB	35065-28-2	X
2,2',3,4',5',6-Hexachlorobiphenyl	PCB 149	PCB	38380-04-0	X
2,2',4,4',5,5'-Hexachlorobiphenyl	PCB 153	PCB	35065-27-1	X
2,2',3,3',4,4',5-Heptachlorobiphenyl	PCB 170	PCB	35065-30-6	X
2,2',3,4,4',5,5'-Heptachlorobiphenyl	PCB 180	PCB	35065-29-3	X
2,2',3,3',4,4',5,5'-Octachlorobiphenyl	PCB 194	PCB	35694-08-7	X
Decachlorobiphenyl	PCB 209	PCB	2051-24-3	X
Acenaphthene		PAH	83-32-9	X
Acenaphthylene		PAH	208-96-8	X
Anthracene		PAH	120-12-7	X
Benz[a]anthracene		PAH	56-55-3	X
Benzo[a]pyrene	B[a]P	PAH	50-32-8	
Benzo[b]fluoranthene		PAH	205-99-2	
Benzo[b]fluorene		PAH	243-17-4	X
Benzo[e]pyrene		PAH	192-97-2	X
Benzo[ghi]perylene		PAH	191-24-2	
Benzo[k]fluoranthene		PAH	207-08-9	
Chrysene		PAH	218-01-9	
Cyclopenta[cd]pyrene		PAH	27208-37-3	X
4H-Cyclopenta[def]phenanthrene		PAH	203-64-5	X
Dibenz[a,h]anthracene		PAH	53-70-3	
Dibenzo[a,e]pyrene		PAH	192-65-4	
Fluoranthene		PAH	206-44-0	X

COMPOUND NAME	ABBREVIATION	COMPOUND CATEGORY	CAS	"X" IF DETECTED
Fluorene		PAH	86-73-7	X
Indeno[1,2,3cd]fluoranthene		PAH	193-43-1	
Indeno[1,2,3-cd]pyrene		PAH	193-39-5	
Perylene		PAH	198-55-0	
Phenanthrene		PAH	85-01-8	X
Pyrene		PAH	129-00-0	X
m-Terphenyl		PAH	92-06-8	
o-Terphenyl		PAH	84-15-1	
p-Terphenyl		PAH	92-94-4	
9-vinylanthracene		PAH	2444-68-0	X
Aldrin		OCP	309-00-2	
p,p'-Dichlorodiphenyldichloroethane	4,4'-DDE	OCP	72-55-9	X
p,p'-Dichlorodiphenyldichloroethylene	4,4'-DDD	OCP	72-54-8	X
p,p'-Dichlorodiphenyltrichloroethane	4,4'-DDT	OCP	50-29-3	X
1,1-Bis(p-chlorophenyl)-2-chloroethene	4,4'-DDMU	OCP	1022-22-6	X
1,1-Dichloro-2,2-bis(4-methoxyphenyl)ethan		OCP	7388-31-0	
Dicofol		OCP	115-32-2	X
Dieldrin		OCP	60-57-1	X
alpha-Endosulfan		OCP	959-98-8	X
beta-Endosulfan		OCP	33213-65-9	
Endosulfan sulfate		OCP	1031-07-8	
Endrin		OCP	72-20-8	
Endrin aldehyde		OCP	7421-93-4	
Endrin ketone		OCP	53494-70-5	
Heptachlor		OCP	76-44-8	
Heptachlor-endo-epoxide		OCP	28044-83-9	X
α-hexachlorocyclohexane	α-HCH	OCP	319-84-6	X
β-hexachlorocyclohexane	β- HCH	OCP	319-85-7	X
γ-hexachlorocyclohexane	γ-HCH	OCP	58-89-9	X
δ- hexachlorocyclohexane	δ-HCH	OCP	319-86-8	X
Methoxychlor		OCP	72-43-5	
Pentachloronitrobenzene		OCP	82-68-8	
Triclosan-methyl		OCP	4640-01-1	
3-Bromocarbazole		BDE	1592-95-6	
Hexabromobenzene		BDE	87-82-1	
2,2',4,4'-Tetrabromdiphenyl ether	BDE-47	BDE	5436-43-1	X
2,2',4,4',5-Pentabromodiphenyl ether	BDE-99	BDE	60348-60-9	X
2,2',4,4',5,5'-Hexabromodiphenyl ether	BDE-153	BDE	68631-49-2	
2,2',4,4',5,6'-Hexabromodiphenyl ether	BDE-154	BDE	207122-15-4	
Acrinathrin		Pyr	101007-06-1	
Allethrin		Pyr	584-79-2	X
Bifenthrin		Pyr	82657-04-3	X
Cyfluthrin		Pyr	68359-37-5	
Cyhalothrin		Pyr	91465-08-6	X
Cypermethrin		Pyr	52315-07-8	
Deltamethrin		Pyr	52918-63-5	
Esfenvalerate		Pyr	66230-04-4	
Etofenprox		Pyr	80844-07-1	X
Fluvalinate		Pyr	69409-94-5	

COMPOUND NAME	ABBREVIATION	COMPOUND CATEGORY	CAS	"X" IF DETECTED
Permethrin		Pyr	52645-53-1	X
Prallethrin		Pyr	23031-36-9	X
Tefluthrin		Pyr	79538-32-2	
Transfluthrin		Pyr	118712-89-3	X
1-Chloro-2-nitrobenzene		CHC	88-73-3	
Hexachlorobenzene	HCB	CHC	118-74-1	X
Pentachlorobenzene		CHC	608-93-5	X
Ambrettolide		musks	123-69-3	
Celestolide		musks	13171-00-1	
Galaxolide		musks	1222-05-5	X
Musk ambrette		musks	83-66-9	
Musk ketone		musks	81-14-1	
Musk xylene		musks	81-15-2	
Phantolide		musks	15323-35-0	
Tonalide		musks	21145-77-7	X
Velvione		musks	37609-25-9	
2-Acetonaphthone		Others	93-08-3	X
Benzyl benzoate		Others	120-51-4	X
p-Benzyldiphenyl		Others	613-42-3	X
Benzyl-2-naphthylether		Others	613-62-7	X
Bis(4-chlorophenyl)sulfone		Others	80-07-9	X
Chlorpyrifos		Others	2921-88-2	
Dibutylhydroxytoluene		Others	128-37-0	X
2,6-Diisopropylnaphthalene		Others	24157-81-1	X
2,2-Dimethoxy-2-phenylacetophenone	DMPA	Others	24650-42-8	X
Diphenyl sulfone		Others	127-63-9	X
Diphenylmethane		Others	101-81-5	X
2,6-di-tert-Butylphenol		Others	128-39-2	X
Ethylene glycol diphenylether		Others	104-66-5	X
Homosalate		Others	118-56-9	X
4-Methylbenzylidene camphor	Enzacamene	Others	36861-47-9	X
trans-alpha-Methylstilbene		Others	103-30-0/ 833-81-8	X
Methyl phenyl sulfone		Others	3112-85-4	X
Methyl tolyl sulfone		Others	3185-99-7	
1-Phenylnaphthalene		Others	605-02-7	X
cis-Stilbene		Others	645-49-8	X
Dioctyldiphenylamine		Others	101-67-7	
Piperonyl butoxide		Others	51-03-6	
alpha-Tocopherol acetate		Others	7695-91-2	X

The evaluation of the GC-HRMS data and the integration of peak areas was performed using the software Tracefinder General Quan 5.1 (Thermo Fisher Scientific).

During the extraction of biota with silicone, lipids were taken up into the silicone and thus being co-extracted. Consequently, the analytes concentration in the extract, $n_{extract}$ in $mass_{analyte}/volume_{extract}$, is described as the analyte concentration in the extract in the co-extracted lipid, $n_{co\text{-}extracted\ lipid}$, plus the analyte concentration in the silicone, n_{PDMS} (Equation 1). It was assumed, that the analyte concentration in the co-extracted lipids is the same as in the lipids in the tissue (Baumer et al. 2021a, Equation 2).

$$n_{extract} = n_{co\text{-}extracted\ lipid} + n_{PDMS} \quad \text{(Equation 1)}$$

$$n_{co\text{-}extracted\ lipid} = n_{lipid} \quad \text{(Equation 2)}$$

A correction of the co-extracted lipids in the silicone sheets was performed by subtracting the fraction of co-extracted lipids, by using the partition constant between lipid and PDMS, $K_{lipid/PDMS}$ (Equation 3). The $K_{lipid/PDMS}$ are given in the supplementary information of *Publication III*.

$$n_{PDMS} = n_{extract} * (K_{lipid/PDMS} * m_{lipid} * m_{PDMS}^{-1})^{-1} \quad \text{(Equation 3)}$$

The concentrations discussed in this thesis are given in silicone-based concentrations c_{PDMS} (or $c_{silicone}$) in $mass_{analyte}/mass_{PDMS\ (or\ silicone)}$. In order to compare the concentrations found in literature data, c_{PDMS} needed to be converted to lipid-based equilibrium concentrations $c_{lipid,\ eq.}$, in $mass_{analyte}/mass_{lipid}$. Using the $K_{lipid/PDMS}$ and Equation 4 the concentrations were converted.

$$c_{lipid,\ eq.} = K_{lipid/PDMS} * c_{PDMS}^{-1} \quad \text{(Equation 4)}$$

2.5 Bioanalytical Measurements and Data Evaluation

In this work, three *in vitro* reporter gene bioassays were used to analyze the PES extracts. The measurement of the activation of the AhR was performed with the AhR-CALUX cell line (Brennan et al. 2015; Neale et al. 2017b), obtained by the courtesy of Michael Denison, University of California, Davis, USA. The activation of the PPARγ was measured with the PPARγ-bla GeneBLAzer assay (Thermo Fisher Scientific) (Invitrogen 2010; Neale et al. 2017b). The measurement of the Nrf2-mediated oxidative stress response was done with the AREc32 cell line (Wang et al. 2006; Escher et al. 2012), obtained by the courtesy of C. Roland Wolf, Cancer Research UK. In previous studies, Jin et al. (2013) and Jin et al. (2015) found that bioassays indicative for the activation of the AhR and oxidative stress response can be activated by PES extracts from marine mammals. Furthermore, extracts from marine sediment were activating the

AhR-CALUX, PPARγ-bla and AREc32 assays (Jahnke et al. 2018). Therefore, the three assays were found to be relevant for testing the activation potential of PES extract from marine mammals.

Cell culturing and cell assay dosing is described in detail in *Publication I* and *II*. In brief, the cells were incubated at 37 °C and 5 % CO_2 and were seeded in a defined cell number to 384-well plates, 24 h prior to dosing. The cell confluency was monitored with an IncuCyte S3 live cell imaging system (Essen BioScience, Ann Arbor, Michigan, USA) to measure the cell viability. For dosing, the extract in ethyl acetate was evaporated until dryness and the residue dissolved in cell assay medium (PPARγ-bla: 98% Opti-MEM, 2% cs-FBS; AhR-CALUX and AREc32: 90 % DMEM Glutamax, 10% FBS). The extracts were further diluted to different concentrations with cell assay medium and dosed into the 384-well plates containing the seeded cells.

After 24 h of exposure the cell confluency was measured again. Furthermore, the fluorescence (PPARγ-bla) or luminescence (AhR-CALUX and AREc32) activity of the cells were measured with a microplate reader (Tecan, Männedorf, Switzerland). Samples with a positive reference compound (PPARγ-bla: rosiglitazone; AhR-CALUX: 2,3,7,8-tetrachloro-dibenzodioxin (TCDD); AREc32: tert-butylhydroquinone (tBHQ)), procedural blanks and medium blanks (i.e., untreated cells) were measured in parallel with the samples.

The cell viability was assessed with the monitored cell confluency. With this, a nominal inhibitory concentration for 10 % reduction of the cell viability (IC_{10}) was derived from linear concentration–response curves (up to 40 % inhibition), relative to the untreated cells (Escher et al. 2020a).

From the fluorescence and luminescence data, the effect concentration causing 10 % effect (EC_{10}) relative to the maximum effect of the positive reference compound, was derived from linear concentration–response curves for the PPARγ-bla and AhR-CALUX (up to 40 % effect). With the AREc32 an effect concentration triggering an induction ratio (IR) of 1.5 ($EC_{IR1.5}$), was derived from linear concentration–response curves (up to an IR of 4).

Specific activation of the reporter-gene assays were determined by excluding values at concentrations that caused more than 10% reduction of the cell viability relative to untreated cells (Escher et al. 2020a).

A high IC and EC corresponds to an apparent lower potency of the extract to cause an effect. In order to obtain more intuitive values, i.e. high values that correspond to higher effects and low values correspond to lower effects, the reciprocal values of IC and EC were calculated. The reciprocal value of IC_{10} (i.e., $1/IC_{10}$)

is defined as the bioanalytical toxic unit (TU_{bio}). The reciprocal value of EC_{10} (i.e., $1/EC_{10}$) and $EC_{IR1.5}$ (i.e., $1/EC_{IR1.5}$) is defined as the bioanalytical effect unit (EU_{bio}). The TU_{bio} and EU_{bio} are reported in $L_{bioassay}/g_{PDMS}$.

2.6 Iceberg Modeling

The bioanalytical data from the bioassays and the chemical data from the GC-HRMS measurements was combined using iceberg modeling. The iceberg modeling evaluation is described in *Publication III*. In brief, the bioanalytical equivalent concentrations measured with the bioassays (BEQ_{bio}) were defined as the EC values of a reference compound (Rosiglitazone for PPARγ-bla, and B[a]P for AhR-CALUX and AREc32) divided by the EC of the sample, see Equation 5. With the results from the GC-HRMS measurements and the resulting concentrations of the detected compounds *i*, the corresponding BEQ_{chem} can be predicted. For this, the sum of the single $BEQ_{chem}(i)$ of all detected compounds *i*, defined as their relative effect potencies REP(*i*), were multiplied by the detected concentration c(*i*), see Equation 6 and 7.

$$BEQ_{bio} = EC_{10}(\text{reference}) * EC_{10}(\text{sample})^{-1} \quad \text{or} \quad EC_{IR1.5}(\text{reference}) * EC_{IR1.5}(\text{sample})^{-1} \qquad \text{(Equation 5)}$$

$$BEQ_{chem} = \sum_{i=1}^{n} BEQ_{chem}(i) = \sum_{i=1}^{n} (REP(i) * c(i) \qquad \text{(Equation 6)}$$

$$REP(i) = EC_{10}(\text{reference}) * EC_{10}(i)^{-1} \quad \text{or} \quad EC_{IR1.5}(\text{reference}) * EC_{IR1.5}(i)^{-1} \qquad \text{(Equation 7)}$$

With the ratio of BEQ_{chem}/BEQ_{bio} the fraction of how much effect was explained by the detected chemicals was determined. A schematic illustration of the conjunction of the results generated by bioanalytical measurements and GC-HRMS analysis is outlined in Figure 7.

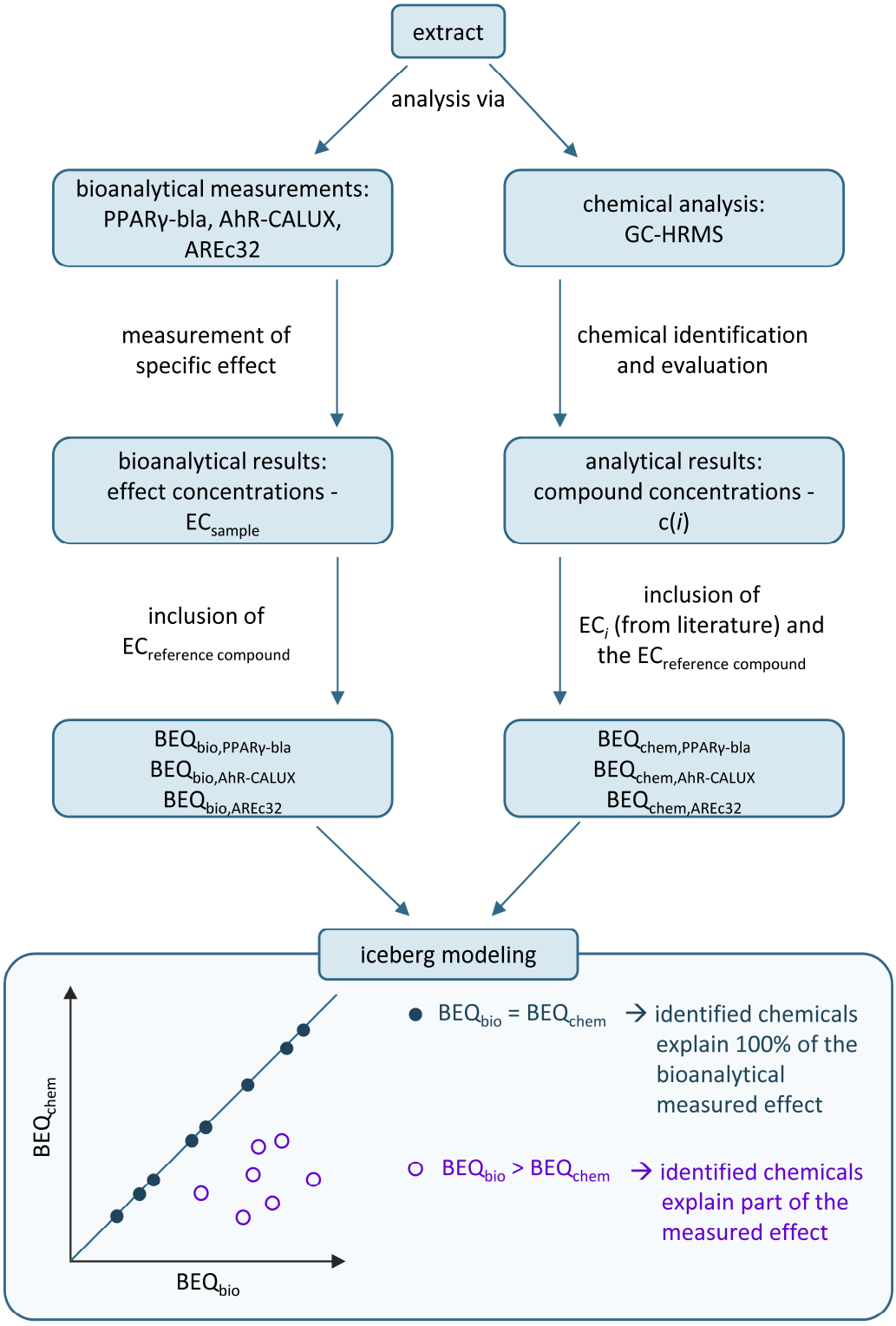

Figure 7. Schematic illustration of the data analysis and the conjunction of the results for iceberg modeling.

3 Passive Equilibrium Sampling in Diverse Biota Tissues

PDMS is a commonly used chemometer to perform PES of HOCs in biota tissue (Jahnke et al. 2008; Jahnke et al. 2011; Jin et al. 2013; Jin et al. 2015; Rusina et al. 2017; Rojo-Nieto et al. 2019; Smedes et al. 2020; Baumer et al. 2021a; Baumer et al. 2021b). An important criterion to carry out PES in biota tissue is to achieve thermodynamic equilibrium between the chemometer and the biota tissue within an appropriate time interval, i.e., before the tissue starts to decay. In addition, for a routine and feasible application, the PES method should be robust, practicable and replicable.

The thermodynamic equilibrium between PDMS and blubber, is achieved within 24 h with static equilibration (Jin et al. 2013).

3.1 Dynamic Sampling in Lean Tissue

The average (± standard deviation (SD)) lipid content of the evaluated marine mammal organs (e.g., from harbor porpoises) were: liver with 4.8±1.2 % (*n*=7), kidney with 3.0±0.70 % (*n*=4), brain with 9.1±2.2 % (*n*=4) and blubber 90±7.1 % (*n*=7) (*Publication III*). Consequently, liver, kidney and brain are considered as lean tissues in the context of the silicone chemometer extraction. To compare the adequacy and capability for PES in lean marine mammal organs, two dynamic sampling methods were tested and compared: (i) manual relocations of silicone sheets in homogenized tissues and (ii) constantly rolling silicone-coated vials filled with the tissue homogenate.

To confirm thermodynamic equilibrium between the chemometer and the tissue, the analyte mass sampled with the chemometer can be compared for different surface area-to-volume ratios of the silicone (i.e., different thicknesses of the silicone with a uniform geometry) at constant sampling time or by performing a time series experiment to evaluate the uptake kinetics (Ossiander et al. 2008; Mäenpää et al. 2011). Both methods were conducted to confirm equilibrium or to identify the time to reach equilibrium for the different sampling approaches. The experiments are described in chapter 2.2. Large-volume samples of liver, kidney and brain from harbor porpoises were processed for this method development; the evaluation was conducted using instrumental analysis with GC-HRMS (Q-Exactive, Thermo Fisher Scientific, Germany) for representative PCBs (see chapter 2.4).

3.1.1 Manual Relocations of Chemometer Sheets and Tissue

The silicone sheets were relocated 8 times per day (i.e., every 1.5 h), over nine days resulting in up to 64-70 relocations. For the uptake kinetics, 350 µm tick silicone samplers were collected at 10 time points between 5 and 64-70 relocations. Additionally, silicone sheets with thicknesses of 350 µm, 250 µm and 125 µm were used and collected after the last relocation (64-70 relocations), after nine days. In general, the time and number of relocations can be varied and planned independently, e.g. more, or less numbers of relocations can be processed per day.

In Figure 8 the results for liver tissue of harbor porpoise P.p.2 for four PCB congeners (PCB 52, 118, 153, 180) are plotted. The linear regressions shown in Figure 8A represent the relationship between the mass of the quantified PCB congeners in the silicone sheets of different thicknesses and the silicone mass. The linear regression forced through the origin resulted in a coefficient of determination (R^2) > 0.98, consequently, indicating a successful establishment of an equilibrium between the silicone and the compounds in the tissue after nine days and corresponding to 64-70 relocations. The uptake kinetics were additionally investigated to determine how long it takes to achieve equilibrium in the present setting. For this purpose, a one-phase exponential curve was fitted ($y = y_{max}*(1-e^{-k*x})$, evaluated with the Software Graph-Pad Prism 9.3.1), where y is defined as the concentration at a certain time and y_{max} is defined as the concentration at equilibrium (i.e., concentration in the plateau, see Figure 2). In this context, the uptake kinetics for the liver tissue of P.p.2 are plotted in Figure 8B. For the depicted PCBs the time to reach 95% of equilibrium, t_{95}, was between 109 and 221 h, suggesting that the full time interval of nine days (or 70 relocations) was needed to achieve equilibrium. The uptake kinetics for kidney and brain were similar; the comprehensive discussion can be found in *Publication II*, as well as the results for other PCB congeners.

Though the manual relocation process is time-consuming and impedes high sample throughput, the approach is highly controlled during the whole sampling process and can be easily modified or adjusted in terms of silicone dimensions, tissue textures and number of relocations per day.

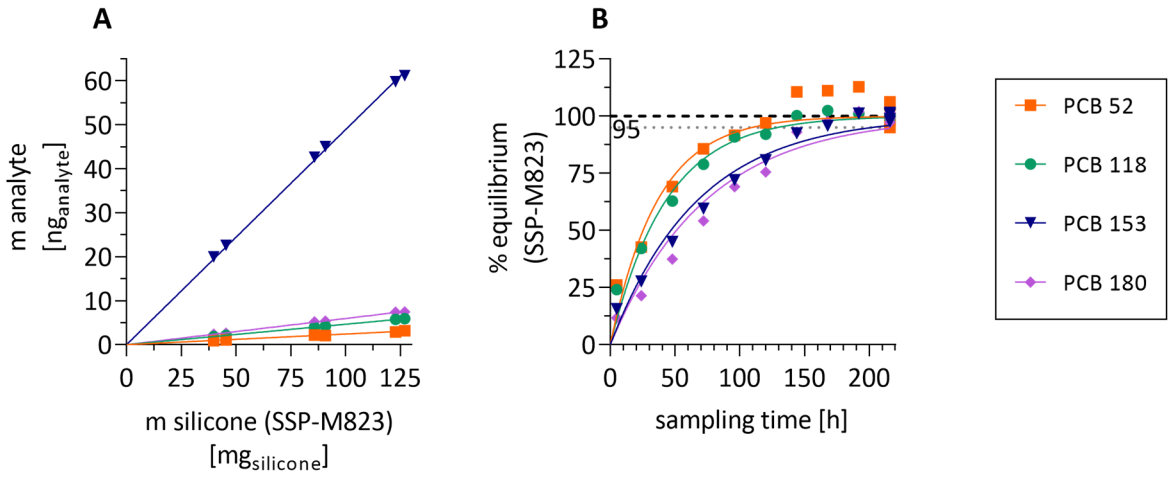

Figure 8. Evaluation of four selected PCBs after manual relocations of silicone sheets (SSP-M823) in the liver tissue of harbor porpoise P.p.2. (A) Plots of the mass of selected PCBs in the silicone sheets of multiple silicone thicknesses (125, 250, 350 µm) after the maximal sampling period of nine days vs. the silicone mass. The solid lines represent the linear regressions through the origin with a $R^2 > 0.98$. (B) Uptake kinetics of the selected PCBs in the silicone sheets in dependency of the sampling time in h. The curves were fitted with an exponential one-phase association with a $R^2 > 0.90$. Additionally, a black broken line indicates 100% and a grey dotted line 95% of equilibrium. The figures were taken and modified from *Publication II.*

3.1.2 Automatized Relocation of Tissue in a Silicone-Coated Vial

Silicone-coated glass vials are a well-established PES device for soil, sediment and particulate suspended matter (Reichenberg et al. 2008; Muz et al. 2020; Wernicke et al. 2022). Furthermore, silicone-coated vials have been used to determine the chemical activity of animal feces and animal food (Chen et al. 2020; Chen et al. 2022).

In accordance with the manual relocation process, the analyte mass was determined and compared for different surface-to-volume ratios (i.e., different thicknesses of the silicone coatings: 10 µm, 20µm and 30 µm) after the last exposure time point of 216 h (i.e., nine days). To assess the uptake kinetics, vials with a coating thickness of 30 µm were collected at 10 time points (between 5 - 216 h).

Results for the liver tissue of harbor porpoise P.p.2, analyzed for four selected PCBs (PCB 52, 118, 153, 180), are displayed in Figure 9. The mass of the quantified PCB congeners in the silicone-coated vials with different thicknesses was plotted against the silicone mass. The linear regression shown in Figure 9A resulted in a $R^2 > 0.85$ and indicated successful equilibrium after the full interval of nine days. The uptake kinetics, shown in Figure 9B, were used to determine the time point when 95% of equilibrium was

reached. For the selected PCBs a t_{95} between 44 to 294 h was identified. Consequently, for the PCB congeners 52, 118 and 153 a time interval of nine days proved to be sufficient to reach equilibrium, however, for the higher chlorinated PCB 180 and a coating thickness of 30 µm more time is needed (i.e., 294 h). In contrast, with the alternative approach (using different surface-to-volume ratios) thermodynamic equilibrium was confirmed. The results for kidney and brain were similar to the described experiment with liver (data not shown).

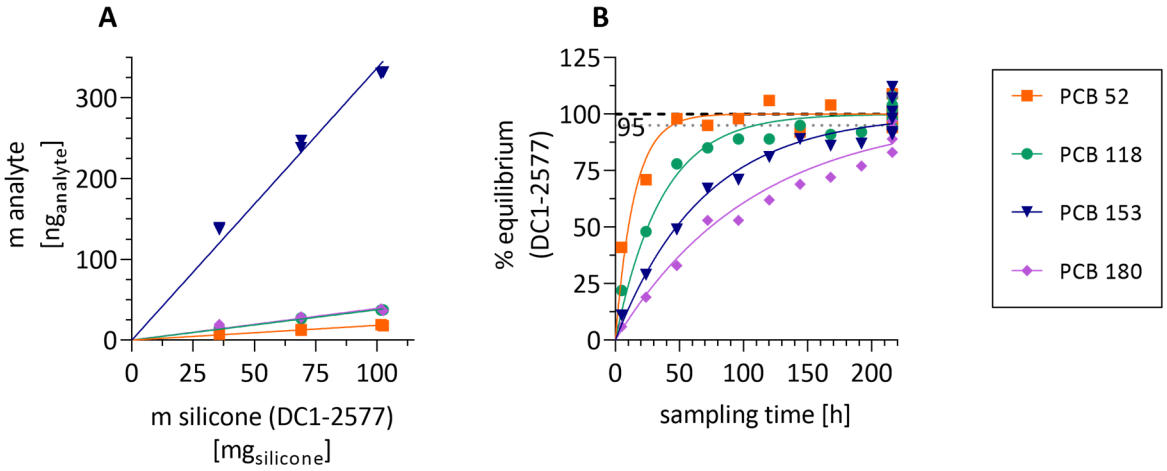

Figure 9. Evaluation of four selected PCBs after automatized relocations in silicone-coated vials (DC1-2577) for the liver tissue of harbor porpoise P.p.2. (A) Plots of the mass of the selected PCBs in the silicone with different coating thicknesses (10, 20, 30µm) vs. silicone mass taken after the maximal sampling period of nine days. The solid lines represent the linear regressions through the origin with a $R^2 > 0.85$. (B) Uptake kinetics of the selected PCBs in dependency of the sampling time in h in the silicone-coated vials. The curves were fitted with an exponential one-phase association with a $R^2 > 0.86$. Additionally, a black broken line indicates 100% and a grey dotted line 95% of equilibrium.

3.1.3 Comparison of Manual Relocations with Automatized Relocations

The coating silicone (DC1-2577) differs in its partitioning properties from silicone sheets (SSP-M823), e.g., in terms of distinct partition constants. The SSP-M823 silicone sheets are well characterized and lipid-silicone partitioning constants $K_{lipid/silicone(SSP-M823)}$ are available for several compounds (Jahnke et al. 2008; Smedes et al. 2017), whereas no explicit $K_{lipid/silicone(DC1-2577)}$ for the coating silicone DC1-2577 are available. However, the partition constants between the two polymers $K_{SSP-M823/DC1-2577}$ are given in Gilbert et al. (2016). With this information, it was possible to unify the concentration of the different silicones ($c_{SSP-M823}$ and $c_{DC1-2577}$) to a comparable value ($c_{silicone}$) with Equations 8 and 9.

$K_{SSP\text{-}M823/DC1\text{-}2577} = c_{SSP\text{-}M823} * c_{DC1\text{-}2577}^{-1}$ (Equation 8)

$c_{SSP\text{-}M823} = c_{silicone} = c_{DC1\text{-}2577} * K_{SSP\text{-}M823/DC1\text{-}2577}$ (Equation 9)

Although our results confirmed that with both PES approaches the thermodynamic equilibrium between the silicone and the tissue was achieved for the last sampling point (i.e., after nine days, Figure 8A and Figure 9A), the PCB concentrations, $c_{silicone}$, were 2-3 times lower in the silicone sheets (SSP-M823) than in the silicone-coated vials (DC1-2577) (Figure 10A-D).

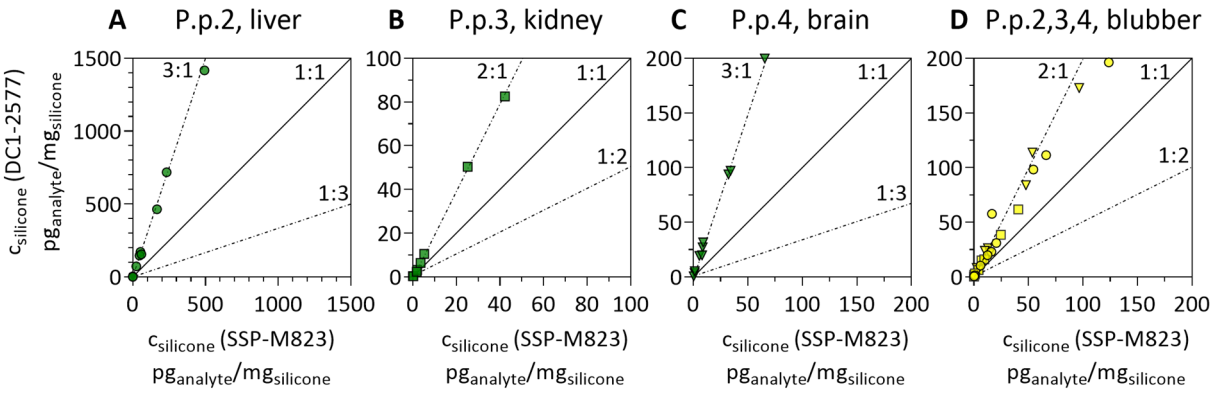

Figure 10. Comparison of the concentrations in the silicone, $c_{silicone}$, of the dynamic sampling approaches (rolling of silicone-coated vials (DC1-2577) and manual relocation of silicone sheets (SSP-M823)). (A, B and C) Concentrations for the liver, kidney and brain tissues of harbor porpoises P.p.2, 3 and 4 and (D) the corresponding blubber samples of all three harbor porpoises. The solid line indicates an 1:1 agreement, whereas dotted lines indicate a factor of 2 or 3 (as described in the graphs) above or below.

To further investigate this systematic deviation, a total (exhaustive solvent) extraction was performed to compare the concentrations in the lipids, c_{lipid}, from total extraction with the lipid-based equilibrium partitioning concentrations from the silicones $c_{lipid, eq.}$. For this, $K_{lipid/silicone(SSP\text{-}M823)}$ from the literature were used (Jahnke et al. 2008; Smedes et al. 2017, Equation 10).

$K_{lipid/silicone} = c_{lipid, eq.} * c_{silicone}^{-1}$ (Equation 10)

The comparison of $c_{lipid, eq.}$, measured with the PES, indicated an overestimation of the chemometer data towards c_{lipid}, measured with total extraction for liver, kidney and brain tissue (Figure 11). More precisely, for silicone sheets (SSP-M823) and silicone-coated vials (DC1-2577) on average 4 and 10 times, respectively, higher concentrations were evaluated compared to the extracts from total extraction. The comparison of $c_{lipid, eq.}$ with c_{lipid} for the blubber tissue indicated similar concentrations: the $c_{lipid, eq.}$ from

silicone sheets (SSP-M823) agreed within an average factor of 1.1, which had been observed for PES (with SSP-M823) in lean fish tissues before (Rojo-Nieto et al. 2019). The $c_{lipid,\ eq.}$ of silicone coated vials (DC1-2577) agreed within an average factor of 2.0. The $K_{lipid/silicone}$ values were determined with pure lipids, e.g. oils. Consequently, the above discussed results might indicate that the $K_{lipid/silicone}$ values are not transferrable to complex mammalian tissues of low lipid content. However, the $K_{lipid/silicone}$ values seem to be applicable for high lipid content tissues (i.e., fat tissue). Especially for lean tissues other sorptive phases besides lipids can play an important role (e.g., proteins) and thus further influence the partitioning process (deBruyn and Gobas 2007; Baumer et al. 2021a).

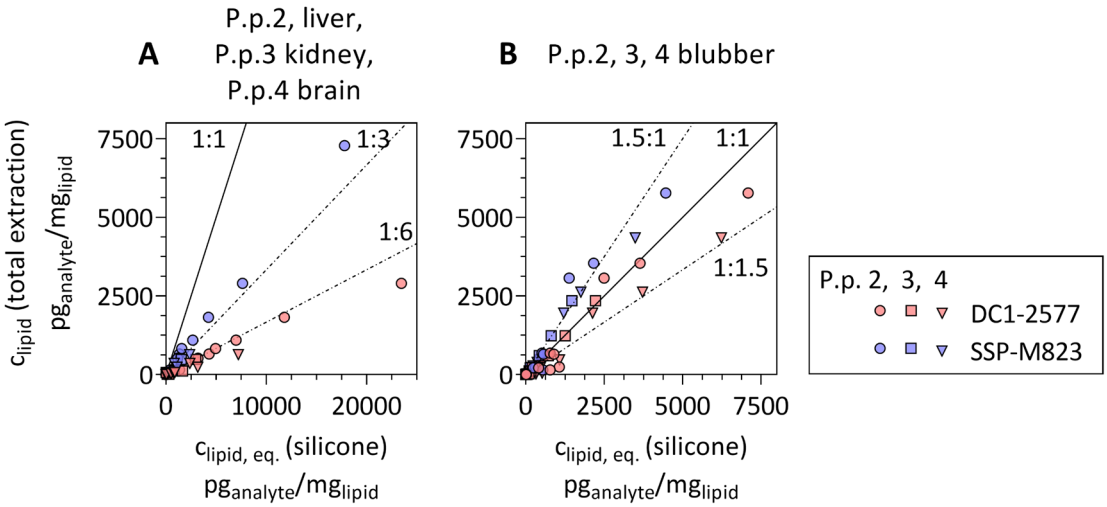

Figure 11. Comparison of the lipid concentrations measured with total extraction, c_{lipid}, vs. the lipid-based equilibrium concentrations, $c_{lipid,\ eq.}$ (i.e., converted concentrations in the silicone, $c_{silicone}$, measured with silicone sheets (SSP-M823) and silicone-coated vials (DC1-2577)). (A) Concentrations in the liver, kidney and brain tissues of harbor porpoises P.p.2, 3 and 4 and (B) the corresponding blubber samples of the three harbor porpoises. The solid line indicates an 1:1 agreement, whereas dotted lines indicate a factor of 1.5, 3 or 6 (as described in the graphs) above or below.

Since the manual relocation approach has been calibrated against traditional exhaustive extraction in a previous study (Rojo-Nieto et al. 2019), the following data presented in this thesis rely on the manual relocation approach. The sampling approach with silicone-coated vials for the evaluation of HOC concentrations in biota tissues was inapplicable. A plausible explanation for this phenomenon has to date not been found. Therefore, the manual relocation approach, for which some SSP-M823 silicone-lipid partition constants are available from literature, was selected as a standardized sampling technique in this thesis.

3.2 Lipid Uptake into the Silicone during PES

The sampling of biota tissues with PDMS led to a rather uniform weight gain of the PDMS during sampling, in comparison to the large lipid content difference for the different tissues (e.g., kidney with approximately 3% and blubber with approximately 87 %). The weight gain is explained by absorption of the lipids into the silicone, rather than an adsorption process onto the silicone surface (Smedes et al. 2017). It usually ranges between 0.5 % to 1.2 % (Jahnke et al. 2009; Jin et al. 2013). Consequently, silicone-based PES extracts contain co-extracted lipids. As an example, the PES blubber extracts from marine mammals, described in *Publication II*, contained between 6.8 to 30.4 $mg_{co\text{-}extracted\ lipid}/g_{PDMS}$.

3.2.1 Influence of Co-Dosed Lipids in the Bioassay System

In vitro cell-based bioassays have been used to measure the effects of diverse extracts, e.g., of mixtures of chemicals from environmental samples such as water (e.g., Neale et al. 2015; König et al. 2017), sediment (e.g., Jahnke et al. 2018; Niu et al. 2020) but also biota samples (e.g., Jin et al. 2013; Jin et al. 2015; Baumer et al. 2021b). The bioassay system can be described with a mass balance model (MBM) between the dosed chemicals (from the extract) and the lipid, protein and water compartments of cells and the medium, using the specific partition constants of the constituents (Fischer et al. 2017). Additional factors can influence the bioavailability of chemicals dosed into the cell assay, for example, the solvent of a dosed extract or even the polymer well plate which the cells are seeded in (Gülden et al. 2002; Clemedson et al. 2003; Tanneberger et al. 2010; Kramer et al. 2015; Fischer et al. 2018).

Consequently, when dosing biota extracts, co-extracted lipids will interfere with the partitioning of the mixtures in the bioassay system. Several studies observed a reduction of the sensitivity when dosing biological samples into cell-based *in vitro* bioassays (Bayen et al. 2004; Simon et al. 2010; Jin et al. 2013). Lipids do not dissolve in cell medium and are not likely to be taken up by cells. As a consequence, co-dosed lipids from biota extracts act as an additional partitioning phase in the bioassay system. The bioavailable fractions of the chemicals in the bioassay system can be described by the partitioning of the dosed chemicals between co-extracted lipids, medium and cells by a three-phase MBM. The derived three-phase MBM constitutes of: (a) the partition constants between lipid and water, medium and water as well as cells and water, (b) the constituents of medium and cells in terms of volume fractions of lipids, proteins and water and (c) the volume fraction of co-extracted lipids (Figure 12).

The theory and application of the three-phase-model is described in detail in *Publication I*. In brief, the model included the partitioning of chemicals between medium, cells and lipid. Furthermore, medium and cells are composed of lipid, protein and water. The fraction of the dosed chemicals in the lipid (f_{lipid}) is described by the mass balance Equation 11, and in the cell (f_{cell}) with Equation 12, including the partition constants K between the compartments and the different volumes V of each compartment (V_{cell}, V_{lipid} and V_{medium}). For the calculation of the cellular effect concentration EC_{cell}, the nominal effect concentration EC, the f_{cell}, the V_{cell} and the total volume (V_{total}, the sum of V_{cell}, V_{lipid} and V_{medium}) is used (see Equation 13). The EC_{cell} is independent of the lipid fraction in the system, i.e., EC_{cell} is constant (Escher et al. 2019). With this, the EC with x % lipids in the system, $EC_{x\%\ lipid}$, can be predicted from an EC without lipids in the system, $EC_{0\%\ lipid}$, using the f_{cell} with and without lipids in the system. The calculation is shown in Equation 14.

$$f_{lipid} = (1 + K_{cell/lipid} * V_{cell} * V_{lipid}^{-1} + K_{medium/lipid} * V_{medium} * V_{lipid}^{-1})^{-1} \quad \text{(Equation 11)}$$

$$f_{cell} = (1 + K_{lipid/cell} * V_{lipid} * V_{cell}^{-1} + K_{medium/cell} * V_{medium} * V_{cell}^{-1})^{-1} \quad \text{(Equation 12)}$$

$$EC_{cell} = EC * f_{cell} * V_{total} * V_{cell}^{-1} \quad \text{(Equation 13)}$$

$$EC_{x\%\ lipid} = EC_{0\%\ lipid} * f_{cell\ (0\%\ lipid)} * f_{cell\ (x\%\ lipid)}^{-1} \quad \text{(Equation 14)}$$

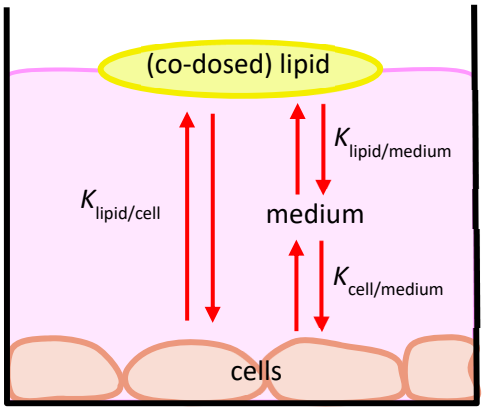

Figure 12. Schematic illustration of the three-phase system used in *Publication I*. After dosing extracts that contain lipids into the bioassay system, chemical partitioning within the bioassay system occurs. Partitioning is characterized by the partition constants K between the lipid droplet, the medium and medium components and the cells. Furthermore, cells and medium are constituted of water, proteins and lipids and are simplified in this model into one system parameter referred to as “cell” and “medium”, respectively. This figure was adapted from *Publication I*.

To verify the derived three-phase MBM, triolein, a synthetic triglyceride, was used to simulate the co-dosed lipid matrix. Furthermore, the influence of different volumes of spiked triolein was investigated and compared with the concentrations predicted by the MBM. For this investigation, the MBM was applied to the cell lines AhR-CALUX and AREc32. For each cell line two bioactive reference compounds with different octanol-water partition coefficients, log K_{ow}, were mixed with triolein and dosed directly into the cell assays, covering triolein volumes between 0.5 - 4 % of the total volume in each well. With a dose-response relationship, the EC_{10} in the AhR-CALUX, relative to the maximum effect caused by the reference agonist TCDD, and the $EC_{IR1.5}$ was derived for the AREc32. The developed and improved dosing technique and the following bioanalytical evaluation are described in detail in *Publication I*.

The bioanalytical results of the extracts containing different volumes of triolein showed that the EC_{10} and $EC_{IR1.5}$ increased with increasing triolein fractions for both assays. The results for the AREc32 assay for the references B[a]P and dichlorvos are summarized in Figure 13A and B; the results for the AhR-CALUX assay are shown in *Figure 1* in *Publication I*. As a higher EC corresponds to an apparent lower potency of effect activation of the tested extract, it was proven that triolein reduced the bioavailability, and hence the apparent sensitivity of the bioassay. Furthermore, it was demonstrated that the three-phase model could adequately predict the nominal EC_{10} and $EC_{IR1.5}$ in the bioassays. The results for the AREc32 assay are shown in Figure 13C for the references B[a]P and Figure 13D for dichlorvos; the results for AhR-CALUX assay are shown in *Figure 2* in *Publication I*.

The derived MBM was applied to bioanalytical data of lipid-containing PES extracts of marine mammal blubber. The extracts composed of an unknown mixture of chemicals. However, it is expected that HOCs between a log K_{ow} of 3 to 9 are most likely to be sampled by the silicone PDMS. The lipid correction (using Equation 14 to calculate $EC_{0\%\ lipid}$) of the PES extract showed that the deviation between the $EC_{x\%\ lipid}$ and the $EC_{0\%\ lipid}$ was less than 1 order of magnitude for samples with a lipid volume fraction below 0.27 % (Figure 14). Hence, testing extracts below a lipid volume fraction of 0.27 %, appeared acceptable to omit a lipid correction for an undefined chemical mixture in PES samples.

The method development, described in *Publication I* and the related validation of the three-phase model hence allowed for the applicability and interpretation of dosing extracts containing lipid-matrix residues up to a lipid volume fraction of 0.27 %.

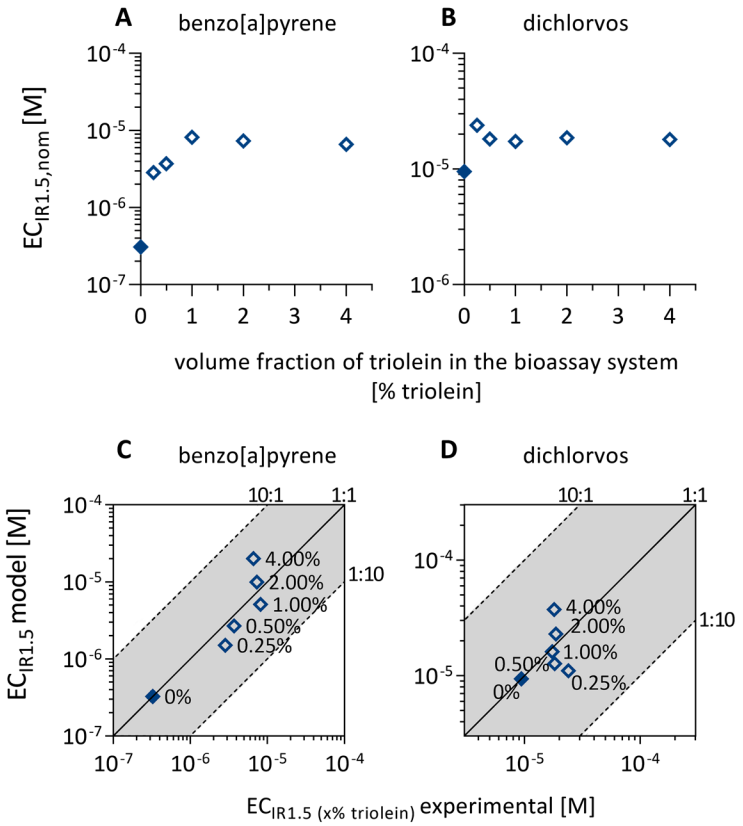

Figure 13. Results for the $EC_{IR1.5}$ measured in the AREc32 assay for (A) and (C) benzo[a]pyrene and for (B) and (D) dichlorvos and different volume fractions (in %) of triolein. The filled diamonds represent the measurements without triolein (0 %), the empty diamonds the measurements with x % triolein. The figures were taken and modified from *Publication I*.

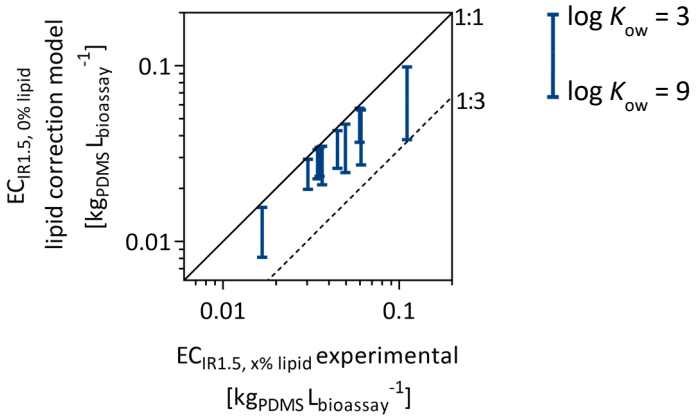

Figure 14. Estimated lipid correction range for chemicals with log K_{ow} of 3–9 for 10 marine mammal blubber PES extracts measured with the AREc32 assay. The solid line indicates a 1:1 line (perfect fit between the lipid correction model for 0 % lipid at the $EC_{IR1.5,\ 0\%\ lipid}$ and the experimentally evaluated $EC_{IR1.5,\ x\%\ lipid}$ with x % lipid). The dotted line indicates a deviation by a factor of 3 from the $EC_{IR1.5,\ 0\%\ lipid}$ to $EC_{IR1.5,\ x\%\ lipid}$ perfect fit. The figure was taken and modified from *Publication I*.

3.2.2 Influence of the Cleanup Procedures on the Chemical Mixture

The measurement of chemical extracts using GC-HRMS is a useful tool for enhanced selectivity and sensitivity for analysis of environmental samples. However, co-extracted matrix residues, such as lipids, can disturb the GC-HRMS system and lead to difficulties regarding the quality of analyses and sensitivity. Furthermore, lipid residues can interfere with the instruments' life time and performance. Consequently, removing the lipid matrix before introducing the extract to GC-HRMS, while maintaining the mixture composition and levels, is essential.

Muz et al. (2021) investigated different non-destructive cleanup methods to remove disturbing matrix constituents without substantially changing the chemical mixture. Best results (i.e., efficient lipid removal at high recovery of a mixture of chemicals) were generated when using Captiva EMR-Lipid cartridges (Agilent Technologies, USA), in comparison to a freeze-out method at -20 °C in Acetonitrile, or using Oasis PRiME HLB cartridges. However, when applying a cleanup with EMR-Lipid cartridges on PES extracts from marine mammals it became apparent that yet a second cleanup was necessary to remove a sufficient amount of co-extracted lipid matrix, in order to maintain a stable performance with the GC-HRMS instrument. Consequently, for PES extracts from marine mammals, the cleanup with EMR-Lipid cartridges was extended by a second cleanup - an extraction with PSA sorbent (Agilent Technologies, USA) (Wernicke et al. 2022). Extracts that were processed with both cleanup methods ensured a sufficiently stable instrument performance for the analysis of larger batches.

In an ideal case, when comparing data from bioanalytical measurements with results from chemical analysis, the analyzed extracts introduced to both analyses should have followed the same preparation steps (sampling, extraction and cleanup). Unfortunately, this procedure was impeded as (i) the extract blanks that have been prepared with an EMR-Lipid cartridge cleanup were found to induce cytotoxic effects and, in addition, (ii) caused an activation of the assay. More explicitly, PDMS silicone blanks submitted to an EMR-cleanup caused detectable effects in the AhR-CALUX assay up to a measurable EC_{10} and IC_{10} value. For PDMS blanks without EMR-cleanup, no effects (i.e., EC_{10} and IC_{10}) in the AhR-CALUX, for the same concentration range, was assessed (Figure 15). Therefore, the freeze-out cleanup procedure followed by a PSA extraction was applied for extracts introduced to bioanalytical measurements (Figure 15). Vice versa, for instrumental GC-HRMS measurement, the reduction of disturbing matrix with freeze-out cleanup in combination with PSA extraction was not sufficient to avoid interferences with the instrument performance under routine measurements. However, according to the study of Muz et al. (2021) the chemical recoveries after EMR- and freeze-out-cleanup were similar (EMR: 80-99 %, *n*=25;

freeze-out: 75-112 %, *n*=26). Thus, the extracts prepared for bioanalytical measurements with freeze-out and PSA cleanup can be expected to be comparable with extracts for instrumental analysis, prepared with EMR and PSA cleanup.

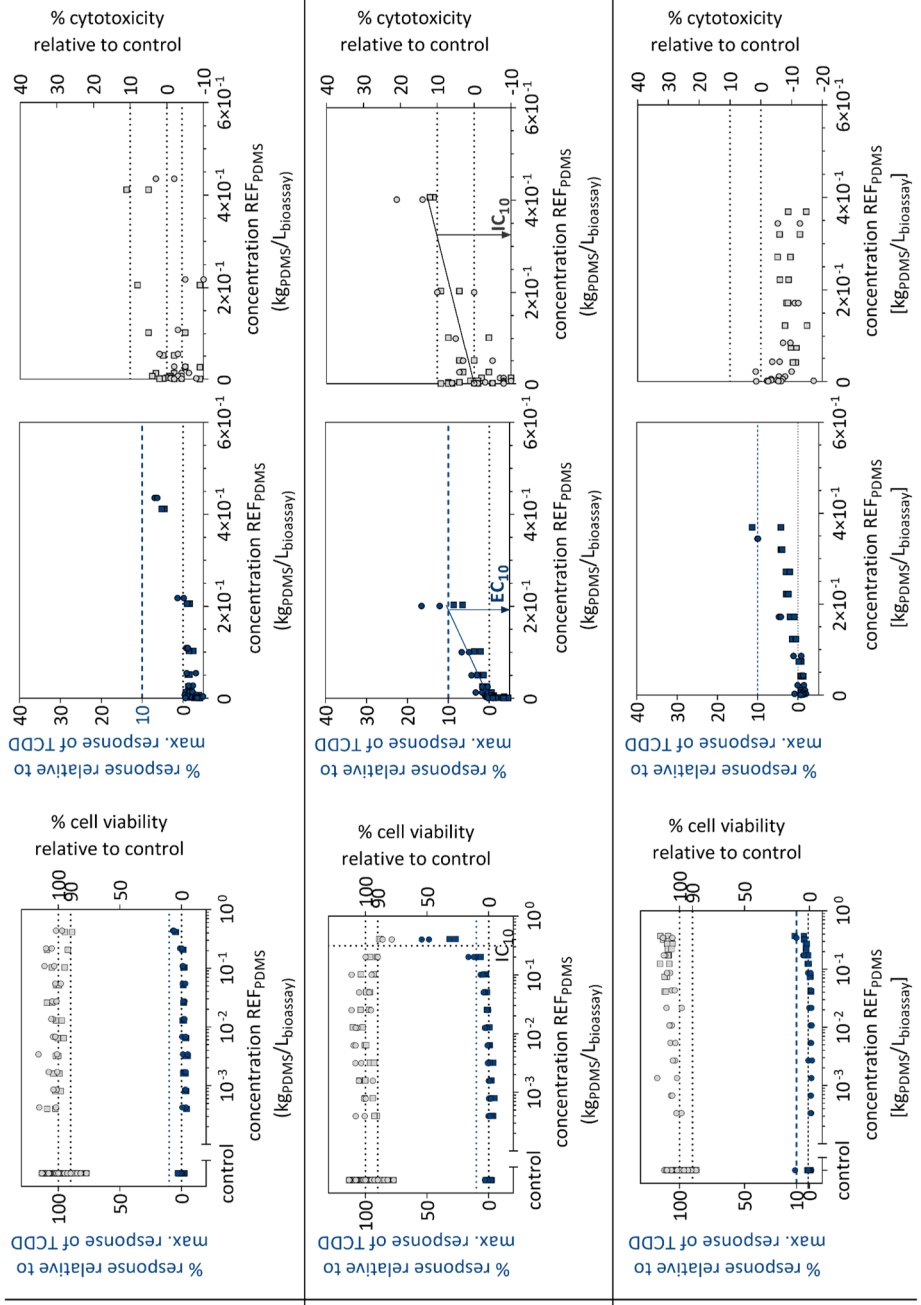

Figure 15. Concentration-effect curves of PDMS blanks without cleanup (top), cleanup via EMR-Lipid-cartridges (middle) and cleanup via freeze-out and PSA-extraction (bottom), measured in the AhR-CALUX assay. The left plots show all experimental data on a logarithmic axis. Blue symbols on the left y-axis refer to % response relative to the maximum triggered by the reference compound TCDD. Grey symbols on the right y-axis refer to the corresponding % cell viability. The middle plots show the % response data for concentrations < IC_{10}. The dotted line indicates an activation of the AhR of 10 % relative to the maximal response of TCDD. A linear regression line through the origin was used to calculate the effect concentration of 10 % (EC_{10}). For the right plots, the % cell viability was converted to % cytotoxicity (% cytotoxicity = 100% - % cell viability). The dotted line indicates a cytotoxic effect of 10 % relative to control cells. A linear regression line through the was used to calculate the inhibitory concentration of 10 %.

3.2.3 Influence of the Cleanup on Bioanalytical Measurements

Dosing extracts with lipid residues into *in vitro* cell-based bioassays, will reduce the apparent sensitivity of the bioavailable chemicals and thus could introduce a bias (see chapter 3.2.1). To reduce the lipid amount dosed into the bioassay system, a non-destructive cleanup can be performed. However, cleanup procedures may alter the chemical mixture composition of the extract and could introduce blank contamination or effects (e.g. cleanup with EMR-Lipid-cartridges, Figure 15).

To examine the influence of a non-destructive cleanup (freeze-out cleanup in combination with PSA extraction) in bioanalytical assays, PES sample extracts from liver, kidney, brain and blubber from marine mammals were analyzed. The activation in the reporter gene assays of AhR-CALUX and PPARγ-bla (activation of the xenobiotic metabolism) and AREc32 (activation of the Nrf2-dependent oxidative stress response) were examined for extracts with and without cleanup. Sample preparation and cleanup procedures are described in detail in *Publication II*.

With the conducted cleanup the lipid residue was significantly ($p<0.05$) reduced by 11.0-92.6 % (geometric mean±SD: 54.4±1.65 %, n=22). When dosing the PES extracts into the bioassays, a reduced activation of PPARγ and oxidative stress response were observed for the extracts with cleanup in the respective assays. The cleanup procedures did not only reduce the lipid amount but also altered the chemical composition, and/or reduced the chemical concentration. The activation measured in the PPARγ-bla was significantly ($p<0.05$) reduced by a factor of 4.3±1.5 (geometric mean±SD, n=22), whereas in the AREc32 a significant reduction by a factor of 2.5±0.23 (n=18) was measured, as shown in Figure 16A (and *Figure 1* in *Publication II*). In contrast, in the AhR-CALUX assay the cleanup did not change the activation potential of the extracts significantly (factor of 1.1±0.075, n=6), whereas the cytotoxic effect was significantly reduced in the extracts with cleanup by a factor of 1.8±0.32 (n=17), as shown in Figure 16B (and *Figure 3* in *Publication II*). For AREc32 a cytotoxic effect was only observed for three sample pairs (i.e., with and without cleanup) for which a lower TU_{bio} was measured for extracts with cleanup (Figure 16B).

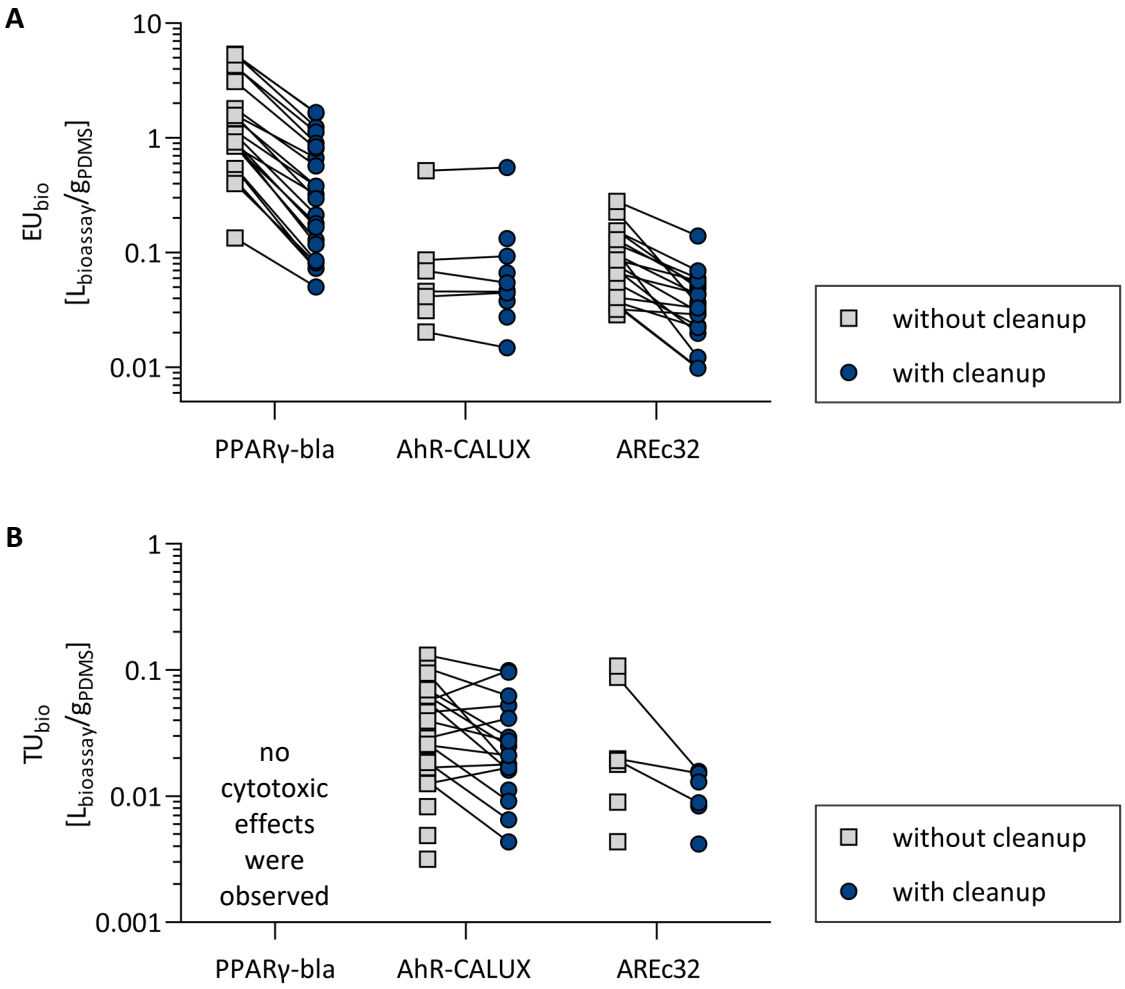

Figure 16. Comparison of effects measured in the reporter gene assays for activation of the PPARγ with the PPARγ-bla assay, the AhR with the AhR-CALUX assay and Nrf2-mediated oxidative stress response with the AREc32 assay, for extracts without cleanup (grey squares) and with cleanup (blue circles). The sample pairs are visualized by connection with a line; some extracts are not connected, because no activation was measured for the corresponding pair. (A) The activation of the effect is expressed as effect units EU_{bio}. Number of pairs: PPARγ-bla: 22; AhR-CALUX: 6 (7 values with no paired analogue); AREc32: 18 (3 values without paired analogue) (B) The cytotoxic effects for the different assays expressed as toxic units TU_{bio}. No cytotoxic effects were measured with the PPARγ-bla assay. Number of pairs: AhR-CALUX: 17 (4 values with no paired analogue); AREc32: 3 (7 values without paired analogue). The figures were taken and modified from *Publication II*.

The lower effect (EU_{bio} and/or TU_{bio}) of extracts with cleanup indicated a loss of chemicals due to the cleanup procedure, i.e., the chemical concentration was reduced or the chemical composition was altered. The altered concentration or composition outweighed the enhanced bioavailability of the dosed chemicals in the bioassays. With the lipid removal the bioavailability of the compounds is enhanced (see chapter 3.2.1), because the lipid partitioning phase in the bioassay system is reduced. To the contrary, extracts submitted to a cleanup showed reduced bioanalytical effects in comparison to the corresponding extract without cleanup, though these extracts contained the larger lipid fraction. A possible activation of the assays endpoint through the lipids itself was investigated by comparing the EU_{bio} with the amount of lipids at the EU_{bio}. Extracts that were highly potent to activate the reporter gene assays needed to be dosed at a low volume (and hence a low volume fraction of lipid). Vice versa, for extracts that were less potent, a higher volume needed to be dosed to the assays (and hence dosed with a higher volume fraction). The amount of co-extracted lipids throughout the samples in the extracts was rather similar. Consequently, the volume fraction of lipids at the EU_{bio} was dependent on the extracts' potency (i.e., the chemicals in the extract). With this observation it was proven that the lipids themselves did not activate the assays endpoints (see *Figure 2* in *Publication II*).

To summarize, both extracts (with and without cleanup) activated the bioassays and the cleanup protocols were not disadvantageous for the measurements. Moreover, due to the reduction of the cytotoxic effect in the AhR-CALUX assay and at the same time the relatively unchanged activation of the AhR, in some cases an activation of the AhR was measurable before cytotoxic effects interfered. With this it was possible to measure additional effects, that were masked due to the cytotoxicity in the extracts without cleanup. Consequently, the extracts with cleanup were more likely to elicit a measurable activation of the AhR at concentrations below cytotoxicity. This observation is a beneficial result for effect measurement with cell-based bioassays. Hence, a non-destructive cleanup is recommended for extracts that contain lipid matrix.

The bioanalytical effects of the individual extracts are described in chapter 4.2.

4 Assessment of the Chemical Burden in Marine Mammals

4.1 Chemical Screening of Targeted Compounds

To assess the chemical burden in marine mammals, tissues from 12 animals were screened for 117 chemicals including legacy and emerging contaminants. More specifically 13 PCBs, 26 PAHs, 23 OCPs, 6 BDEs, 13 pyrethroids, 3 CHCs, 9 musk fragrances and 23 other industrial compounds (e.g. personal care products) were analyzed. Information on the animals can be found in Table 1. All compounds are listed in Table 2. Chemometer extracts from liver, kidney, brain and blubber tissues were prepared and introduced to GC-HRMS. Sampling, extract preparation, analytical screening method and detected concentrations are described in detail in *Publication III*.

From 117 target compounds, 70 compounds were detected in at least one of 40 prepared extracts, see Table 2. Figure 17 shows a summarized heatmap of detected compounds in liver and blubber from the marine mammals for the compound groups of PCBs, PAHs, OCPs, BDEs, and CHCs. The complete heatmap with the additional organs of kidney and brain and the remaining compound groups (pyrethroids, musk fragrances and other industrial compounds) are shown in *Figure 2* in *Publication III*. Prominent compound groups found in all of the samples were PCBs (44±22 %, mean±SD, *n*=40) and OCPs (24±16 %, *n*=40); especially PCB 153 with 20±12 % (*n*=40) and DDX (sum of 4,4'-DDE, 4,4'-DDD and 4,4'-DDT) with 21±15 % (*n*=40).

Differences in chemical contamination patterns across organs within the same animal were found for single chemicals. For example, 4,4'-DDT was found in all examined blubber extracts, whereas it was only found in one liver sample. Liver is a highly metabolically active organ; hence, 4,4'-DDT is metabolized faster to its metabolites 4,4'-DDD and 4,4'-DDE in the liver than in the less metabolically active blubber tissue. Vice versa, the hexachlorocyclohexanes (HCHs, α-, β-, γ- and δ-isomers) were more often found in liver (*n*=10), kidney (*n*=4) or brain (*n*=4), whereas they were not detected in blubber. Comparing the concentration of several compounds with different hydrophobicity (i.e., different log K_{ow}), most compounds did not show a clear accumulation tendency towards a certain organ (see Figure 18). The liver/blubber chemical activity ratio is described by the concentration ratio of an analyte between liver and blubber ($c_{PDMS,liver}/c_{PDMS,blubber}$). Consequently, a concentration ratio close to 1 indicates no accumulation preference towards an organ and implies that the compounds are in equilibrium partition between the organs within the organism. A liver/blubber chemical activity ratio > 1 (i.e., a higher

concentration in the liver compared to the corresponding blubber tissue), could indicate a more recent exposure event, as partitioning to blubber (as lipid storage organ) will take more time. This situation was measured for O.o.1, which was a newborn individuum, stranded on the North Sea coast (Reckendorf et al. 2018).

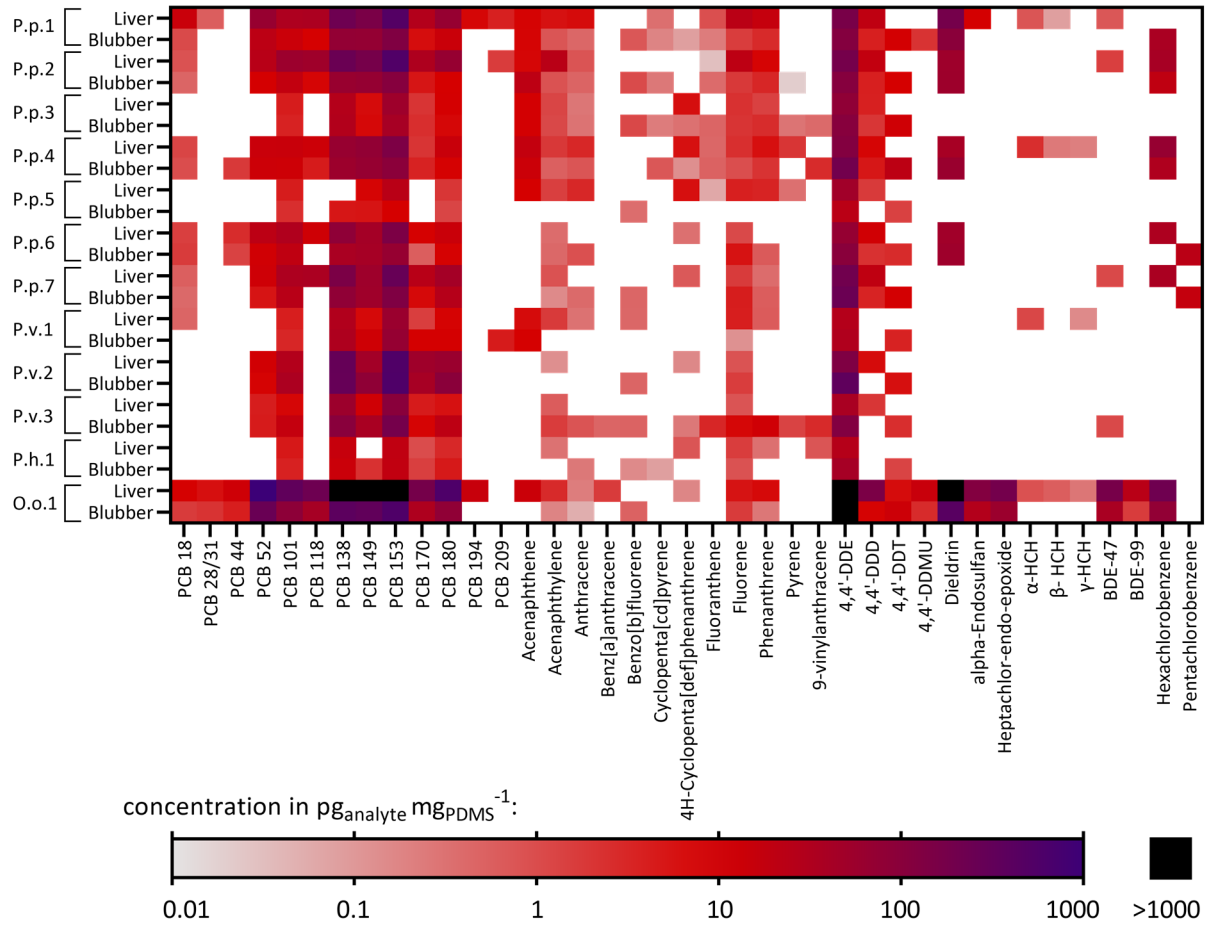

Figure 17. Heatmap showing detected chemicals from the compound group of PCBs (*n*=13), PAHs (*n*=12), OCPs (*n*=10), BDEs (*n*=2), and CHCs (*n*=2) found in the liver and blubber from the twelve animals: P.p.: harbor porpoise, P.v.: harbor seal, P.h.: ringed seal, O.o.: orca, plus running number. The concentration is given in $pg_{analyte}\ mg_{PDMS}^{-1}$. White area means that the chemical was not found in the sample. Concentrations detected above 1000 $pg_{analyte}\ mg_{PDMS}^{-1}$ are shown in black. The figure was taken and modified from *Publication III*.

Comparing the individuals, the highest concentrations were found in the neonate male orca individuum O.o.1 (see Figure 17), especially for PCBs, DDX and BDEs. Lower overall concentrations of the different compounds were found in P.p.3 and P.p.5, both adult female harbor porpoises, and in P.h.1, a neonate male ringed seal.

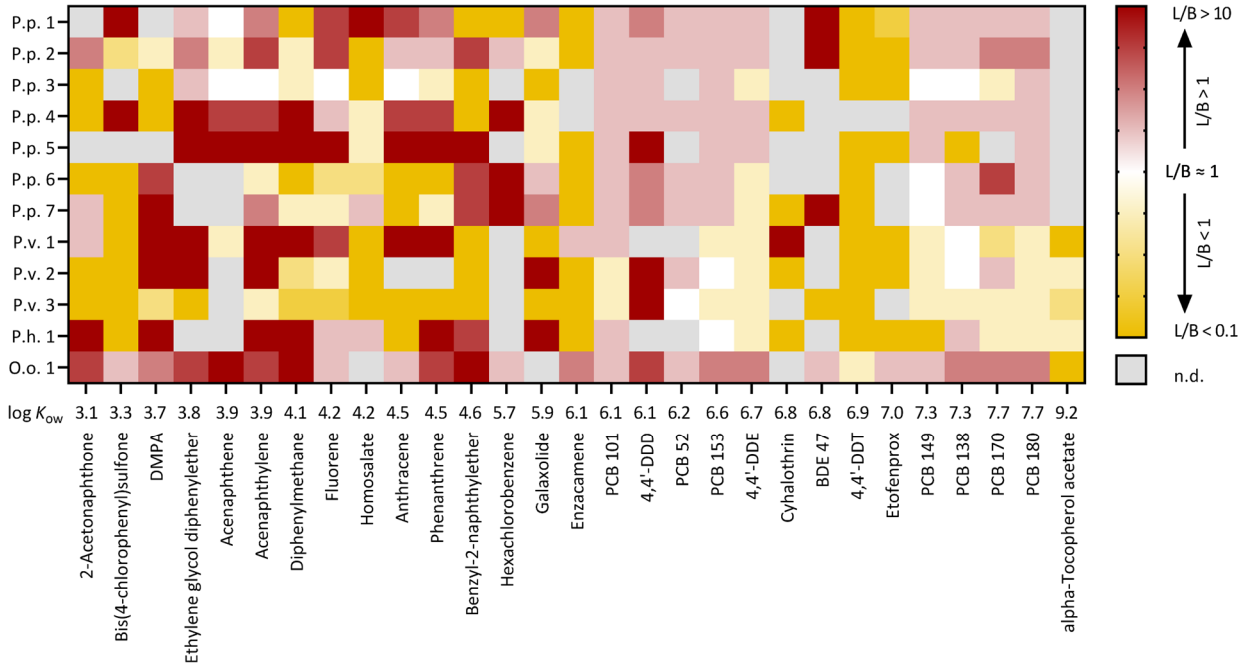

Figure 18. Heatmap showing the chemical activity ratios between liver and blubber (L/B), i.e., the preferred accumulation of selected compounds into liver (red if L/B>1) or blubber (yellow, if L/B<1). For a chemical activity ratio between liver and blubber of around 1 (i.e., 0.9-1.1), no accumulation preference was determined (white color). Grey color marks compounds neither detected in liver nor in blubber. P.p.: harbor porpoise, P.v.: harbor seal, P.h.: ringed seal, O.o.: orca, plus running number. The figure was taken and modified from *Publication III*.

4.1.1 Conversion of Silicone-Normalized Values to Lipid-Based Values

The chemical burden in biota is commonly reported as lipid-normalized concentrations. In order to compare the data generated in this thesis to literature data, the silicone-based concentrations, c_{PDMS}, needed to be converted to lipid-based equilibrium concentrations, $c_{lipid,\ eq.}$. This conversion can be realized by using compound-specific lipid-PDMS partition coefficients, $K_{lipid/PDMS}$ (Equation 4). From the 70 detected chemicals, experimentally determined $K_{lipid/PDMS}$ had been reported for 31 compounds in the literature, including 13 PCBs, 8 PAHs, 8 OCPs and 2 CHCs (Jahnke et al. 2008; Smedes et al. 2017). Alternatively, $K_{lipid/PDMS}$ can be approximated using modeled data, for example using the UFZ-LSER database (Ulrich et al. 2017). The comparison between the literature data and the modeled values with the LSER database resulted in on average 1.4 times higher values from literature, and ranged within a factor between 0.35 and 2.4 (n=27). The 4 HCHs, (α-, β-, γ- and δ-isomers) were excluded for the calculations, as they showed different partitioning behavior in comparison to the other measured compounds (Smedes 2019). To

minimize the error by using possibly uncertain modeled values, an average factor of experimentally determined $K_{lipid/PDMS}$ values can be used. For this, the reported $K_{lipid/PDMS}$ values from Smedes et al. (2017) for the chemicals detected in this study (*Publication III*, excluding the HCH isomers (Smedes 2019)) were considered. As a result, a mean $K_{lipid/PDMS}$ of 23 was determined.

4.1.2 Comparison to Marine Mammal PCB Burden from Literature

The $c_{lipid,eq.}$ for the sum of the 13 analyzed PCB congeners ($\sum PCB_{13}$) in blubber tissues ranged from 0.642 mg/kg$_{lipid}$ (P.p.5) to 44.5 mg/kg$_{lipid}$ (O.o.1). The $c_{lipid, eq.}$ values are reported in the Electronic Supplementary Information of *Publication III*. The geometric mean (±SD) for all analyzed individuals resulted in 5.85 (±3.36) mg/kg$_{lipid}$. Exemplarily, the PCB burden for the harbor porpoises was compared to a selection of previously published studies, listed in Table 3. For all harbor porpoise blubber samples analyzed in this thesis the $\sum PCB_{13}$ concentration ranged between 0.642 to 8.90 mg/kg$_{lipid}$ (n=7). In general, the documented concentrations in this study, corresponded well with the data found in the literature (Figure 19).

Table 3. Data from selected published studies on the PCB burden in harbor porpoise blubber. The number of congeners used to calculate ∑PCB is specified, as well as the sample number. If available, the ∑PCB range (from min – max) is given. For study #3 and #6 a mean (±SD) ∑PCB concentration is given. Sample set details imply the sample collection location and year, as well as the age and sex of the animals. A graphical representation is shown in Figure 19.

#	∑PCB BURDEN	SAMPLE DETAILS	REFERENCE
1	$\sum PCB_{13}$ range: 0.642-8.90 mg/kg$_{lipig}$ (n = 7)	German North Sea, 2018-2019, different age groups, male and female	this study (*Publication III*)
2	$\sum PCB_{17}$ range: 0.21-90 mg/kg$_{lipid}$ (n = 112)	Danish waters, 2003-2019, different age groups, male and female	van den Heuvel-Greve et al. (2021)
3	$\sum PCB_{32}$ average (±SD): 21 (±20) mg/kg$_{lipig}$ (n = 12)	Northeast Atlantic Coast (Spain and Portugal), 2004-2008, different age groups, male and female	Méndez-Fernandez et al. (2014)
4	$\sum PCB_{25}$ range: 1.0-160 mg/kg$_{lipid}$ (n = 440)	UK waters, 1991-2005, different age groups, male and female	Law et al. (2010)
5	$\sum PCB_{35}$ range: 1.1-82 mg/kg$_{lipid}$ (n = 28)	German North Sea, 1990-2008, different age groups, male and female	Weijs et al. (2010)

#	∑PCB BURDEN	SAMPLE DETAILS	REFERENCE
6	$\sum PCB_{16}$ average (±SD): 11 (±11) mg/kg_{lipid} (*n* = 67)	Western European Atlantic Coast (Scotland, Ireland, Netherlands, Belgium, France and Spain), 2001-2003, different age groups, male and female	Pierce et al. (2008)
7	$\sum PCB_{46}$ range: 4.5-39 mg/kg_{lipid} (*n* = 29)	North and Baltic Sea, 1994-1995, mostly immature animals, male and female	Bruhn et al. (1999)
8	$\sum PCB_{46}$ range: 0.88-1.5 mg/kg_{lipid} (*n* = 4)	Arctic waters, 1995, different age groups, male and female	Bruhn et al. (1999)
9	$\sum PCB_{68}$ range: 5.7-13 mg/kg_{lipid} (*n* = 4)	Polish Baltic Sea, 1991-1993, approximately 2-4 years old, male and female	Strandberg et al. (1998)
10	$\sum PCB_{22}$ range: 3.7-65 mg/kg_{lipid} (*n* = 34)	Danish and Norwegian waters, 1987-1991, different age groups, all male	Kleivane et al. (1995)

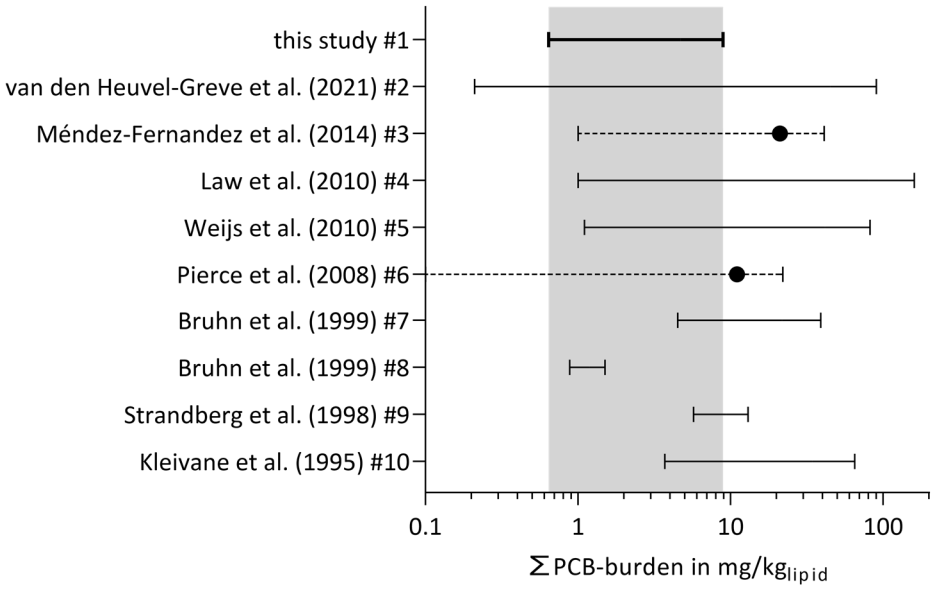

Figure 19. Graphical representation of the comparison of the PCB burden found in harbor porpoise blubber from selected published studies, listed in Table 3. The number of congeners used to calculate ∑PCB in mg/kg_{lipid}, sample number and collection location, year, age and sex of the animals are specified in Table 3. The grey shaded area highlights the concentration range (min-max) of the $\sum PCB_{13}$ burden from the harbor porpoise blubber samples analyzed in this study. If available, the ∑PCB range (from min – max) shown is as solid bar. For study #3 and #6 the mean ∑PCB concentration is shown as a black circle and the SD as broken bars.

4.2 Bioanalytical Evaluation with *In Vitro* Cell-Based Bioassays

Chemometer extracts from liver, kidney, brain and blubber tissue from five harbor porpoises, one common seal and one orca from the North Sea and Baltic Sea were prepared as described in *Publication II*. The extracts were bioanalytically characterized with three *in vitro* cell-based reporter gene bioassays: AhR-CALUX, PPARγ-bla and AREc32 assay.

The detected EU_{bio} values in the different assays and tissues are shown in Figure 20. As described in chapter 3.2.3, a non-destructive cleanup after silicone extraction was beneficial for evaluating more specific effects in the AhR. Consequently, the extracts that were prepared with a cleanup are discussed in the following.

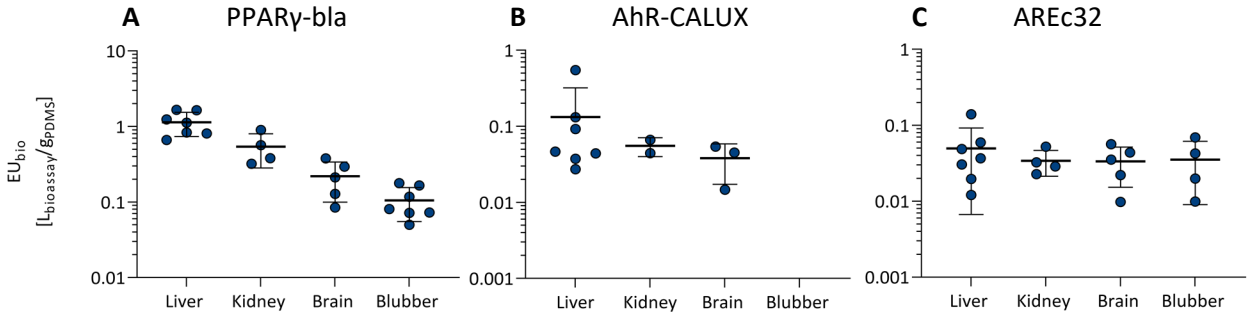

Figure 20. Detected EU_{bio} values for (A) PPARγ-bla, (B) AhR-CALUX and (C) AREc32 for PES extracts from marine mammal organs: liver (*n*=7), kidney (*n*=4), brain (*n*=5) and blubber (*n*=7). The extracts were prepared with a cleanup. The figures were taken and modified from *Publication II*.

The determination of the activation of the AhR showed that 12 samples (out of 23) specifically activated the assay with EU_{bio} values ranging from 0.015-0.55 $L_{bioassay}/g_{PDMS}$ (*n*=12), see Figure 20B. Additionally, 18 samples caused cytotoxic effects in the AhR-CALUX with a TU_{bio} ranging from 0.0044-0.10 $L_{bioassay}/g_{PDMS}$ (*n*=18). For the analysis of the oxidative stress response assay with AREc32, 20 samples activated the assay with an EU_{bio} between 0.010-0.14 $L_{bioassay}/g_{PDMS}$, see Figure 20C. Furthermore, for 6 samples a TU_{bio} between 0.0042-0.016 $L_{bioassay}/g_{PDMS}$ was determined. An activation of the PPARγ was detected for all 23 samples between 0.050-1.7 $L_{bioassay}/g_{PDMS}$, shown in Figure 20A. No cytotoxic effects were measured in the PPARγ-bla up to the maximal dosed concentrations; the maximal concentration dosed to PPARγ-bla ranged between 0.16 to 0.0064 $L_{bioassay}/g_{PDMS}$.

In the PPARγ-bla and AREc32 assays, the observed mixture effects (i.e., EU_{bio}) caused by the liver extracts were higher compared to the blubber extracts for all individual-paired samples. Activation of PPARγ was

significantly ($p<0.05$) higher by a factor of 11±0.26 (geometric mean±SD, n=7) for liver extracts relative to the corresponding blubber extracts. Moreover, a significantly higher EU_{bio} was detected in liver compared to the corresponding kidney and brain extracts (factor of 2.1±0.16 (n=4) and 5.7±0.17 (n=5), respectively). The activation of the oxidative stress response was 1.9±0.32 (n=4) times higher in liver extracts compared to the corresponding blubber extract and as well compared to the corresponding kidney and brain samples (1.5±0.32 (n=4) and 1.5±0.35 (n=5), respectively).

For the examined blubber extracts, no activation of the AhR-CALUX assay was detected (up to concentrations where cytotoxicity occurred or up to an acceptable lipid volume fraction of less than 0.27 % (see chapter 3.2.1)). However, all seven liver extracts activated the AhR-CALUX assay. Due to the limited number of kidney and/or brain extracts that activated the AhR (n=2 for kidney, n=3 for brain), an explicit comparison between organs was not possible.

4.3 Iceberg Modeling: Combining Chemical Analysis with Bioanalytical Tools

While chemical analysis can identify and quantify individual targeted chemicals, bioanalytical measurements capture the integrated effect of complex chemical mixtures. Bringing both analyses together with iceberg modeling, the bioanalytically measured mixture effect (BEQ_{bio}) was compared with the predicted analytical mixture effect from the known and quantified chemicals (BEQ_{chem}) (Neale and Escher 2020). This approach allows the estimation of the fraction of the observed biological effect that can be explained by the targeted chemicals, as opposed to the fraction that remains unknown (see Figure 3). The detailed description of the iceberg modeling approach is given in *Publication III*.

From the 70 detected chemicals (see Table 2), effect data from the literature were available for 37 compounds (Escher et al. 2020a; Baumer et al. 2021b; Lee et al. 2021). Eleven of the detected compounds did not elicit any of the biological effects evaluated in the three bioassays up to the highest tested dose. For iceberg modeling of the effect in AhR-CALUX, twelve compounds were available to estimate the explained fraction (*Figure 3A*, in *Publication III*). With these twelve chemicals between 0.014-83 % of the detected AhR-activating effect was explained (*Figure 4A* in *Publication III*). Consistent with the high chemical burden (see chapter 4.1), most AhR-activating effects were explained for the organs of the orca O.o.1 (22-83 %), the individuum with the highest chemical burden. The lowest fraction of the explained effect was associated with the liver tissue of P.p.5, which also had an overall lower chemical burden. The highly AhR-activating compounds, such as polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans, were not targeted in the instrumental chemical analysis, which most likely explains part

of the gap in the explained fraction. Previous chemical analysis studies on marine mammals originating from the same study site as in this thesis (i.e., North and Baltic Sea) have detected these AhR-activating compounds (Bruhn et al. 1999; Bjurlid et al. 2018).

For AREc32, five detected compounds were found to activate the oxidative stress response. For all samples, an effect of less than 0.13 % was explained (see *Figure 3B* and *Figure 4B* in *Publication III*). Moreover, less than 0.034 % was explained for the PPARγ-activating effect, for which four compounds were available for iceberg modeling (*Figure 3C* in *Publication III*). Neither highly activating compounds for the Nrf2-activating oxidative stress response, such as different PAHs, e.g. B[a]P, and quinones (Escher et al. 2012; Jin et al. 2015), nor strongly PPARγ-activating compounds such as phthalates or perfluorinated compounds (Garoche et al. 2021), were targeted or detected in this study. This discrepancy may explain the large unknown fraction compared to the minor explained effects.

The animal with the highest chemical burden was O.o.1, e.g. the $\sum PCB_{13}$ concentration in the liver was 255 times higher than for the animal with the lowest $\sum PCB_{13}$ burden (P.p.5) and still 8 times higher than the animal with the second highest $\sum PCB_{13}$ burden (P.p.2). Remarkably, the comparison with the results from the bioanalytical measurements showed that the animal O.o.1 did not show the highest effects measured in the *in vitro* reporter gene assays. This relationship is highlighted in Figure 21, showing the BEQ_{bio} and BEQ_{chem} values for the liver extracts of all individuals measured with the AhR-CALUX. The same observation was made for the animals with a comparably lower chemical burden, for example P.p.3 and P.p.5: their BEQ_{bio} were within the range of the extracts from the other animals, and even being higher than the BEQ_{bio} for O.o.1. This observation demonstrates that a large fraction of untargeted and/or unknown chemicals are responsible for further activation of toxicodynamic and cytotoxic pathways that could be captured with different bioanalytical endpoints. The results of the iceberg modeling highlighted the complementarity relationship of bioanalysis and instrumental analysis to comprehensively describe the mixture exposome of marine mammals.

The iceberg modeling of the TU, describing the cytotoxic potential of an extract, was applied to the AhR-CALUX assay. The results and dicussion can be found in *Publication III*.

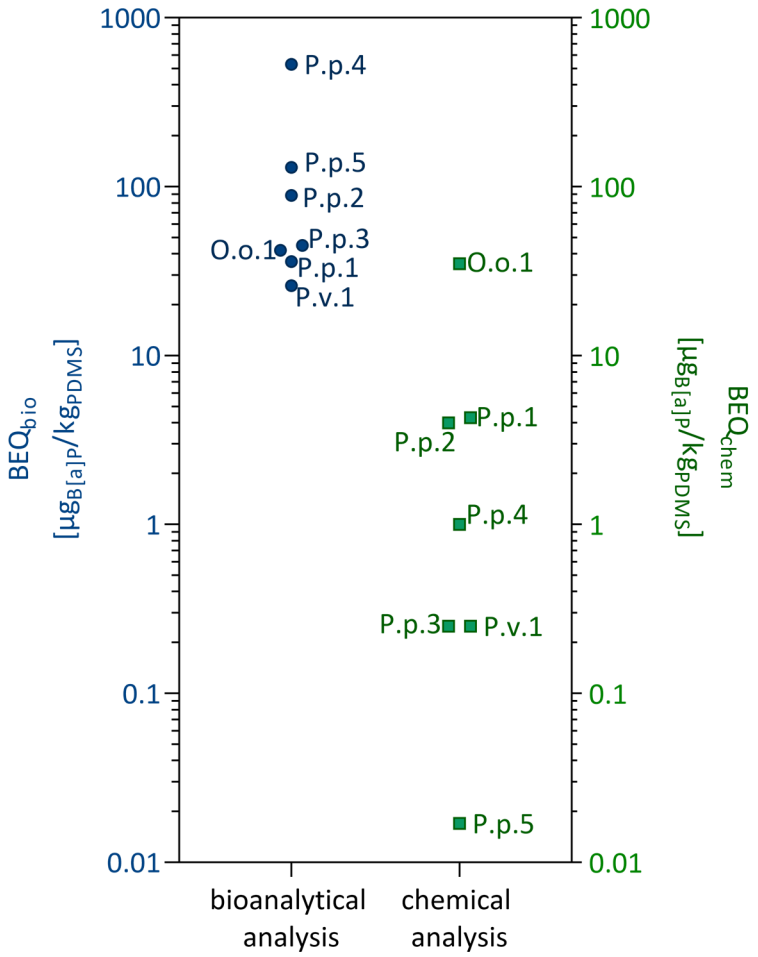

Figure 21. Comparison of bioanalytically measured mixture effects (BEQ_{bio}) (left axis, blue circles) and the predicted analytical mixture effects from the known and quantified chemicals (BEQ_{chem}) (right axis, green squares) in $\mu g_{B[a]P}/kg_{PDMS}$ for the PES extracts from liver measured and calculated for the AhR-CALUX assay. The reference chemical for AhR-CALUX is benzo[a]pyrene (B[a]P). Each point is associated with the corresponding code from the different animals: P.p.: harbor porpoise, P.v.: harbor seal, P.h.: ringed seal, O.o.: orca, plus running number.

5 Implications

5.1 Key Findings

As the analysis of biota is often associated with co-sampling of matrix constituents (e.g., lipid residues), the influence of co-dosed lipids into cell-based bioassays was studied, and an adjusted dosing technique along with a related MBM was established for cell-based bioassays (*Publication I*). Furthermore, in this thesis, a suitable method for PES with silicone chemometers of lean marine mammal organs was developed and proven to be successful (*Publication II*). In addition, the influence on a non-destructive cleanup in bioassays, and thus the reduction of co-dosed lipids, was thoroughly explored (*Publication II*). Based on the developed functional and reliable method, it became possible to examine marine mammalian organs using bioanalytical methods (*Publication II*) and instrumental analysis (*Publication III*). The analysis of the exposome of marine mammals is essential to understand their chemical burden, which can be assessed by iceberg modeling. The contribution of single chemicals with known effect potencies can unravel the total effects and thus the contribution of unidentified compounds or compounds with unknown effect potencies (Figure 3).

It should be recognized that in this thesis a limited number of individuals was analyzed. Due to the diverse method developments, sampling refinements and investigation of cleanup methods, a large volume of the sample tissue was required. Furthermore, only dead stranded or animals in a poor condition that needed to be euthanized were investigated in this study, indicating that these individuals had a poor health status. The limited number of studied organisms in combination with a general inherent variability between individuals did not allow one to make extrapolations towards other individuals or species – the results rather invite for further and deeper investigations of the mixture exposome of marine mammals.

5.2 Internal Exposure and Chemical Distribution within an Organism

The chemical and/or bioanalytical characterization of HOCs extracted from biota is fundamental for understanding and capturing the environmental exposure of long-lived animals, such as marine mammals. The partitioning of chemicals between organs within an organism is defined by their compound-specific physicochemical properties, persistence of the contaminant, and the sorptive capacity of the organ (Levitt 2010; Endo et al. 2013). Comparing different organs within the same organism provides a meaningful approach to investigate internal exposure. Using extracts from silicone chemometers offers the unique

advantage to minimize potential bias that could be introduced when normalizing concentrations to the lipid content or other sorptive phases.

The bioanalytical study of liver, kidney, brain, and blubber extracts showed different potencies in activating the assays' endpoint of organ pairs from the same animal (*Publication II*), e.g., the liver extracts activated the assays with a generally higher potency than the associated blubber extracts. This observation indicated that the chemical activity in the liver tissue was higher than in the corresponding blubber tissue.

For example, the total PCB concentrations varied greatly between the organs within one animal, i.e., the ratio between liver and blubber varied by a factor between 0.5 to 5.5. In Table 4, the descriptive statistics regarding $\sum PCB_{13}$ concentrations, including geometric mean and SD, minimum, median and maximum values, are listed for the four organs. The lowest concentrations (minimum values) were found in P.p.5, whereas the highest concentrations (maximum values) were detected in O.o.1. In the case of $\sum PCB_{13}$, significantly ($p<0.05$) higher concentrations (1.6 times, 95% confidence interval: 1.0-2.5, n=12) were detected in the liver tissue compared to the corresponding blubber sample. The differences among the other organs were not statistically significant.

Apart from the PCB concentration, chemical analysis did not show a clear accumulation tendency of a compound towards any specific organ (*Publication III*). Compared to the bioanalytical measurements, this highlighted the knowledge gap of unidentified compounds (for example due to concentrations below the detection limit, not-targeted substances, unknown chemicals and metabolites as well as endogenous compounds, such as hormones). Single compounds, for which no effect data in the different bioassays had been reported in the literature, lowered the capability to explain the entire effect within iceberg modeling. However, this does not limit the assessment of the chemical burden but rather emphasizes that it is essential not to underestimate the unknown fraction within the exposome.

Table 4. Descriptive statistics of the total PCB concentrations ($\sum PCB_{13}$) in c_{PDMS} in $pg_{analyte}$ mg_{PDMS}^{-1} in the analyzed organs of the 12 marine mammal individuals. Descriptive statistics include the number of samples above detection limit (*n*), the geometric mean and SD, minimum, median und maximum values.

		LIVER	BLUBBER	BRAIN	KIDNEY
c_{PDMS} [$pg_{analyte}$ mg_{PDMS}^{-1}]	geometric mean (±geometric SD)	348 (± 4.90)	221 (± 3.35)	299 (± 4.42)	335 (± 4.95)
	minimum	37.4	24.1	41.8	61.8
	median	346	271	228	223
	maximum	9540	1750	3480	6270
	n	12	12	8	8

5.3 Relevance of Biomonitoring

Monitoring chemical exposure within marine mammals can provide an indication of the animals' health status. Environmental contaminants cause several health dysfunctions (Sonne et al. 2020; van den Heuvel-Greve et al. 2021; Boyi et al. 2022), including suppression of the immune system (Ross et al. 1996; Lehnert et al. 2016; Desforges et al. 2017; Hall et al. 2018), disturbance of the reproductive system (Vos et al. 2000; Schwacke et al. 2002; Sonne et al. 2009; Jepson and Law 2016a), and disruption of the endocrine system (Beineke et al. 2005; Das et al. 2006; Schnitzler et al. 2008; Imazaki et al. 2015).

The population of marine mammals is threatened by anthropogenic influences. A study from Kesselring et al. (2017) reported that the mean age (±SD) at death of female harbor porpoises in the North Sea and Baltic Sea was 5.70 (±0.3) years and 3.67 (±0.3) years, respectively; although, these animals can live over 20 years. However, the sexual maturity of female harbor porpoise is reached only at an age of 4.95 (±0.6) years, highlighting the vulnerable reproduction capacity (Kesselring et al. 2017).

Marine mammals are at a high trophic level and accumulate high concentrations of organic compounds in their tissues as a result of biomagnification (Weijs et al. 2009; Siebert et al. 2012). This thesis shows that even chemicals that have been banned for decades (e.g., PCBs) are still highly relevant. Biomonitoring of marine mammals revealed that PCBs can still be found at high concentrations. PCBs are known to cause adverse effects in marine mammals; hence, the PCB burden is an indicator for the assessment of the health status. A PCB-related immunological impairment, e.g., the depletion of lymphocytes, followed by immunosuppression, and consequently the risk of death from infectious diseases in marine mammals, has been addressed in several studies (Beineke et al. 2005; Das et al. 2006; Yap et al. 2012; Desforges et al.

2016; Jepson et al. 2016b; Williams et al. 2020a). In general, Williams et al. (2020a) expected an increased risk of death due to infectious diseases rising with 5 % for each $\sum PCB_{25}$ concentration of 1 mg/kg_{lipid}. Furthermore, a high PCB exposure was associated with a reduction in thyroid hormone levels and blubber glucose uptake of grey seal pups, influencing their mass gain rate (Bennett et al. 2021). Other PCB-related effects were the alteration of bone mineral density in ringed seals, possibly caused by hormonal disturbances (Routti et al. 2008; Schmidt et al. 2020), increased stillbirth and calf mortality in bottlenose dolphins (Schwacke et al. 2002) and reduced testes weight in male harbor porpoises, potentially reducing fertility (Williams et al. 2021).

Several studies have identified ∑PCB thresholds for the onset of various adverse effects, thereby providing diagnostically conclusive guidance for assessing PCB contamination in marine mammals, listed in Table 5.

Table 5. The ∑PCB effect thresholds determined in the tissues responsible for the onset of various adverse effects in marine mammals.

#	∑PCB THRESHOLD	ADVERSE EFFECT IN MARINE MAMMALS	REFERENCE
(i)	$\sum PCB_{23}$ > 9.0 mg/kg_{lipid}	general onset of physiological impacts in marine mammals	Kannan et al. (2000), Jepson et al. (2016b)
(ii)	$\sum PCB_{25}$ > 11.0 mg/kg_{lipid}	reproductive failure and infertility in female harbor porpoises	Murphy et al. (2015), van den Heuvel-Greve et al. (2021)
(iii)	$\sum PCB_{25}$ > 17.0 mg/kg_{lipid}	thymic involution in harbor porpoises	Kannan et al. (2000), Yap et al. (2012)
(iv)	$\sum PCB_{23}$ > 41.0 mg/kg_{lipid}	profound reproductive impairment of Baltic ringed seals	Helle et al. (1976)

In *Publication III* $\sum PCB_{13}$ was evaluated and the threshold (i) was exceeded by seven individuals in at least one tissue, indicating that general physiological impacts could have been occurred in these animals. Threshold (ii) was exceeded by six animals, from which one was a female harbor porpoise. Thresholds (iii) and (iv) were exceeded by four animals and two animals, respectively. A summary is illustrated in Figure 22. These observations highlight the risks posed by environmental chemicals in marine mammals. In addition to the contamination with PCBs, many other contaminants were detected in the animals examined in this thesis (Table 2). Even at low doses, these complex mixtures of chemicals can interact and

jointly elicit effects (i.e., mixture effects) that can be additive, cumulative, or synergistic (Boobis et al. 2011; Escher et al. 2021).

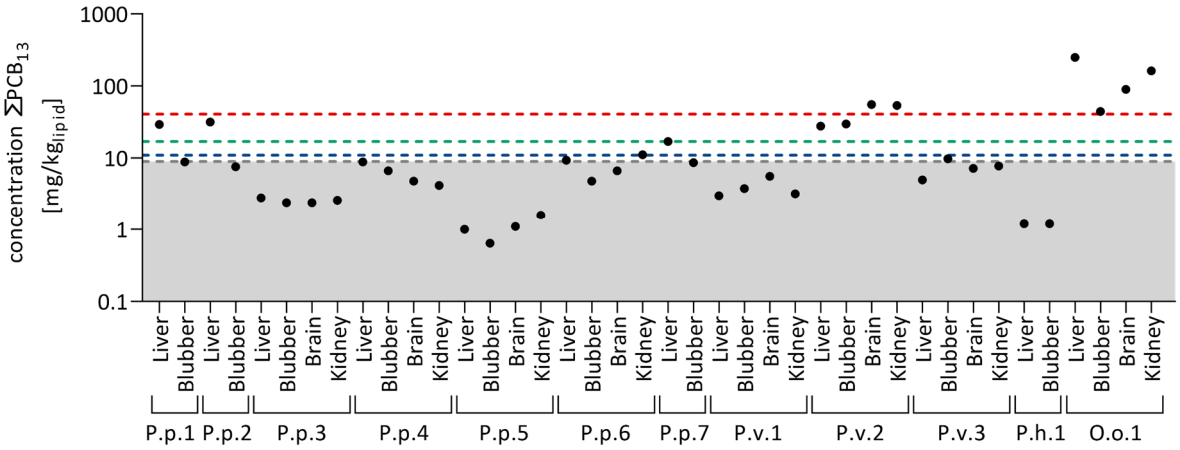

Figure 22. Sum concentrations of 13 PCBs ($\sum PCB_{13}$) for the evaluated animals and their corresponding organs: P.p.: harbor porpoise, P.v.: harbor seal, P.h.: ringed seal, O.o.: orca, plus running number. The grey broken line marks the ∑PCB threshold at (I) $\sum PCB_{23}$: 9.0 mg/kg_{lipid} (Kannan et al. 2000; Jepson et al. 2016b), the blue broken line at (II) $\sum PCB_{25}$: 11.0 mg/kg_{lipid} (Murphy et al. 2015; van den Heuvel-Greve et al. 2021), the green broken line at (III) $\sum PCB_{25}$: 17.0 mg/kg_{lipid} (Kannan et al. 2000; Yap et al. 2012) and the red broken line at (IV) $\sum PCB_{23}$: 41.0 mg/kg_{lipid} (Helle et al. 1976; Jepson et al. 2016b). The grey area indicates the concentration below the general onset of physiological impacts in marine mammals. The figure was taken and modified from *Publication III*.

As described in this thesis, marine mammals are exposed to a diverse cocktail of various chemicals in addition to further hazards. Noise pollution in combination to chemical exposure poses a risk for the health and survival of marine mammals. Substantial noise pollution is caused by marine offshore wind farms (Tougaard et al. 2009; Teilmann and Carstensen 2012), and the detonation of military ammunition (Siebert et al. 2022), additionally affecting the marine mammals. Hearing is essential for the survival of marine mammals, as it is needed for orientation, communication, and sensing fish- and gillnets (Verfuß et al. 2005; Sørensen et al. 2018; Siebert et al. 2022). Hearing loss can be caused by noise pollution, but can also be induced by pollutants, such as PCBs, or by health dysfunctions (Crofton et al. 2000; Rohner et al. 2022). It is assumed that hearing loss could be one of the general physiological impacts in marine mammals that can be detected at a $\sum PCB_{23}$ threshold > 9.0 mg/kg_{lipid} (Kannan et al. 2000; Jepson et al. 2016b). By linking pollutant data with pathological endpoints, new effect thresholds for marine mammals could be established and documented. For this, marine mammals need to be thoroughly investigated by

including necropsy and morphological examination, histopathological analysis, molecular screening for pathogens, in combination to chemical analysis, as carried out by Rohner et al. (2022). The measurement and extensive monitoring of chemical pollution using different analytical approaches, such as quantification of legacy and emerging compounds in chemical analytical methods and bioassays, helps to understand the link between chemical burden and adverse health outcomes. Thus, connecting analytical and bioanalytical data with pathological endpoints has the potential to improve the prediction of physiological effects in marine mammals.

6 Recommendations for Future Work

6.1 Studying Marine Mammals

In this thesis, a sample set of twelve selected individuals was analyzed. Future studies with a larger set of individuals could investigate specific research questions regarding the impact on different species, sex, age, nutrition or health conditions, geographical locations, time trends, or other factors. Stranded animals can introduce a bias into a dataset, due to a potential overrepresentation of animals with bad health conditions or poor nutritional status. Studying marine mammal samples that originate from biopsies of living or hunted animals could avoid this bias, but also need to be in accordance with animal ethics.

Different monitoring programs are active in the investigation of organic pollutants in marine mammals, such as the Helsinki Commission (HELCOM), Oslo and Paris Commission (OSPAR), or the Arctic Monitoring and Assessment Programme (AMAP), continuously improving screening strategies to monitor and document anthropogenic influences on (marine) ecosystems with larger data sets (Gray 1998; Backer et al. 2010; Letcher et al. 2010).

Although certain legacy compounds have been banned over three decades ago (e.g., PCBs) (UNEP 2019), these contaminants continue to remain relevant in long-lived top predators, and in the (marine) environment. In addition to known legacy compounds, new and/or unknown substances, with unknown effects, continuously enter the environment. The determination of these compounds is important for assessing ecosystem risks. For this purpose, other analytical screening approaches, such as non-target screening of environmental samples, can help to evaluate these risks. The data generated by the GC-HRMS in this thesis, provides information for suspect and/or non-target screening methods and consequently allows for further evaluation of the marine mammal exposome (Rebryk et al. 2022; Rebryk and Haglund 2022; Slobodnik et al. 2022).

In addition to the contamination with HOCs, inorganic contaminants, such as metals, are also of concern regarding the health of marine mammals. Besides the essential metals, such as zinc, selenium, copper, and iron, nonessential metals, such as mercury, cadmium, lead, and arsenic, can interfere with cellular mechanisms and threaten the health of mammals (Miyazaki 1994; Ciesielski et al. 2006; Mahfouz et al. 2014; Desforges et al. 2016; Weijs et al. 2016). Heavy metals are released via several sources into the environment, e.g., from industrial wastes during mining, smelting, metal processing in refineries, coal burning, and others. Heavy metals can cause several health issues, such as cardiovascular disorders,

respiratory impairments, neuronal damage, cancer development, mental disturbance, and other disorders (Wang and Shi 2001; Tchounwou et al. 2012; Pratush et al. 2018). To fully capture the chemical burden, monitoring of inorganic contaminants (e.g., heavy metals) in the environment is of importance to comprehensively evaluate and fully understand health risks for marine mammals.

To address differences across geographical locations, marine mammals were sampled and processed in the EXPOSO-METER Project (Helmholtz Association, HGF Project EXPOSO-METER 2020-2025, W2/W3-126). In this project, blubber samples of circa 200 marine mammals, originating from the Arctic, North, Baltic and Mediterranean Sea are planned to be investigated. A comparison of different Arctic toothed whales collected at the southeast coast of Greenland, i.e., pilot whales (*Globicephala melas*), white-beaked dolphins (*Lagenorhynchus albirostris*) and white-sided dolphins (*Lagenorhynchus acutus*), indicated, that the smaller species, white-beaked and white-sided dolphins, were exposed to elevated HOC concentrations compared to the larger pilot whales. This could be a consequence of the higher metabolic rates and a higher requirement of energy intake per kilogram body weight (Williams and Maresh 2015). Further examination of marine top predators from different regions of European Waters, will contribute to the assessment of environmental pollutant exposure data.

6.2 Refining the Data: Determination of Further Variables and Coefficients

The concentrations evaluated in chemometers made of the silicone PDMS (c_{PDMS}) can likely not be used by other researchers, as concentrations in the lipids (c_{lipid}) are commonly reported. Though a conversion from c_{PDMS} to c_{lipid} is possible, reliable partitioning coefficients $K_{lipid/PDMS}$ are necessary (see chapter 4.1.1). In this study, experimentally determined values were not available for all detected compounds, for example UV filters, fragrances, plasticizers, and pyrethroids. Although the modeled or approximated $K_{lipid/PDMS}$ values can be used, the lipid-converted $c_{lipid,\,eq.}$ is more precise with accurately (i.e., experimentally) determined $K_{lipid/PDMS}$ values. Thus, for future work, it is recommended to enhance the range of compounds that can be accurately translated for comparison with data from traditional monitoring.

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8 Publications during Candidature

Peer-reviewed papers: (attached to this thesis)

Publication I:

E. B. Reiter, A. Jahnke, M. König, U. Siebert and B. I. Escher, Influence of Co-Dosed Lipids from Biota Extracts on the Availability of Chemicals in In Vitro Cell-Based Bioassays. *Environ Sci Technol*, 2020, 54, 4240-4247.

Publication II:

E. B. Reiter, B. I. Escher, U. Siebert and A. Jahnke, Activation of the xenobiotic metabolism and oxidative stress response by mixtures of organic pollutants extracted with in-tissue passive sampling from liver, kidney, brain and blubber of marine mammals. *Environ Int*, 2022, 165, 107337.

Publication III:

E. B. Reiter, B. I. Escher, E. Rojo-Nieto, H. Nolte, U. Siebert and A. Jahnke, Characterizing the marine mammal exposome by iceberg modeling, linking chemical analysis and in vitro bioassays. *Environ Sci Process Impacts,* 2023.

Additional study as peer-reviewed paper: (not included to this thesis)

S. Rohner, M. Morell, P. Wohlsein, J. Stürznickel, **E. B. Reiter**, A. Jahnke, E. Prenger-Berninghoff, C. Ewers, G. Walther, L. C. Striewe, A. V. Failla and U. Siebert, Fatal aspergillosis and evidence of unrelated hearing loss in a harbor porpoise (Phocoena phocoena) from the German Baltic Sea. *Front Mar Sci*, 2022, 9.

Conference contributions: (not included to this thesis)

E. B. Reiter, A. Jahnke, A. Jäger, C. Gaus, B. I. Escher, Mixture effects of environmental pollutants in passive sampling extracts of blubber tissue of marine mammals. *International Passive Sampling Symphosium and Workshop*. Dublin, Ireland, May 9-11, 2018, oral presentation.

E. B. Reiter, A. Jahnke, B. I. Escher, Influence of co-dosed lipids from biota extracts on the availability of chemicals in in vitro cell-based bioassays. *SETAC Europe 2019, One Environment. One Health. Sustainable Societies.* Helsinki, Finland, May 26-30, 2019, poster presentation.

E. B. Reiter, A. Jahnke, B. I. Escher, Influence of co-dosed lipids from biota extracts on the availability of chemicals in in vitro cell-based bioassays. *SETAC GLB (German Language Branch)*. Landau, Germany September 4-6, 2019, oral presentation.

E. B. Reiter, B. I. Escher, E. Rojo-Nieto, U. Siebert, A. Jahnke, Bioanalytical Measurement of Passive Sampling Extracts of Different Organs from Marine Mammals. *International Passive Sampling Symphosium and Workshop*. Online, November 4-5, 2021, oral presentation.

E. B. Reiter, B. I. Escher, E. Rojo-Nieto, U. Siebert, A. Jahnke, Bioanalytical Measurement of Passive Sampling Extracts of Different Organs from Marine Mammals. *SETAC Europe 2022, Towards a reduced pollution society*. Copenhagen, Denmark, May 15-19, 2022, oral presentation.

E. Rojo-Nieto, **E. B. Reiter**, A. Jahnke, EXPOSO-METER: Characterizing the environmental and human exposome of mixtures of pollutants at high trophic levels. *SETAC Europe 2022, Towards a reduced pollution society*. Copenhagen, Denmark, May 15-19, 2022, poster presentation.

E. B. Reiter, A. Jahnke, E. Rojo-Nieto. Passive Equilibrium Sampling of Biota with Low Lipid Content: Comparing Silicone Sheets and Silicone-Coated Vials. *International Passive Sampling Symphosium and Workshop*. Utrecht, Netherlands, September 18-20, 2022, poster presentation.

E. Rojo-Nieto, **E. B. Reiter**, T. Wernicke, A. Jahnke, Chemometers: an integrative tool for studying chemicals in biota and surrounding abiotic media. *International Passive Sampling Symphosium and Workshop*. Utrecht, Netherlands, September 18-20, 2022, oral presentation.

A. Miller, **E. B. Reiter**, E. Rojo-Nieto, C. Sonne, R. Dietz, A. Jahnke, Organic pollutants in the Arctic: characterization of contaminant patterns in lipid tissues of toothed whales using passive equilibrium sampling. *International Passive Sampling Symphosium and Workshop*. Utrecht, Netherlands, September 18-20, 2022, poster presentation.

Annex

Publication I

Influence of Co-Dosed Lipids from Biota Extracts on the Availability of Chemicals in *In Vitro* Cell-Based Bioassays

Eva B. Reiter[1]*, Annika Jahnke[1], Maria König[1], Ursula Siebert[2], Beate I. Escher[1,3]

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Influence of Co-Dosed Lipids from Biota Extracts on the Availability of Chemicals in In Vitro Cell-Based Bioassays

Eva B. Reiter,* Annika Jahnke, Maria König, Ursula Siebert, and Beate I. Escher

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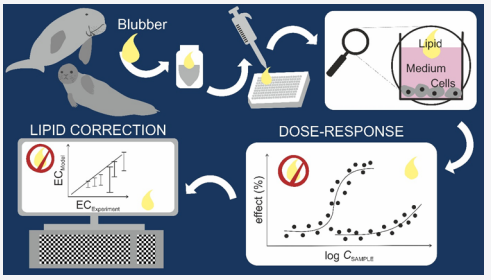
ABSTRACT: Extraction of chemicals from biota leads to co-extraction of lipids. When dosing such extracts into in vitro bioassays, co-dosed lipids act as an additional phase that can reduce the bioavailability of the chemicals and the apparent sensitivity of the assay. Equilibrium partitioning between medium, cells, and co-dosed lipids was described with an existing equilibrium partitioning model for cell-based bioassays extended by an additional lipid phase. We experimentally investigated the influence of co-dosed lipids on the effects elicited by four test chemicals of different hydrophobicity in two bioassays, indicative of the aryl hydrocarbon receptor and oxidative stress response (AREc32). The partitioning model explained the effect of the test chemicals in the presence of spiked triolein within a factor of 0.33−5.83 between the measured and predicted effect concentration (EC). We applied the model to marine mammal blubber extracted with silicone. Extracts dosed in the AREc32 bioassay showed a linear increase of apparent EC with increasing lipid fraction. The partitioning model was used to interpret the role of the co-extracted lipid. A quantitative lipid correction of bioassay results in the presence of co-dosed lipids was possible for known compounds and defined mixtures, while we could only estimate a range for mixtures of unknown chemicals.



INTRODUCTION

Bioanalytical tools are widely used to assess the burden of chemical mixtures, among others in biota extracts, including mussel,[1,2] fish,[3,4] and marine mammals.[5,6] In vitro cell-based reporter gene bioassays are highly sensitive, target specific modes of actions, allow high-throughput applications, and can be used for the effect characterization of the chemical mixture present in an environmental sample.[7] However, the bioavailability of chemicals in the bioassay system can be influenced by various processes, such as binding to medium constituents (lipids and proteins),[8,9] sorption to the well plate,[10,11] and, in the case of biota samples, binding to co-extracted lipids[2,12] that form droplets on the medium surface. Co-dosed lipids add an additional partitioning phase to the bioassay system and can thus reduce the bioavailability of chemicals.

The preparation of biota samples for bioanalytical testing is commonly done using either exhaustive solvent extraction[3,6,12] or passive equilibrium sampling with polymers, like the silicone polydimethylsiloxane (PDMS).[5,13] In the case of solvent extraction, all the lipids present in the sample are extracted along with the chemicals. Hence, extensive clean-up procedures to eliminate the lipid residues, for example, treatment with sulfuric acid,[1,14,15] gel permeation chromatography,[12] sorbent gels,[12,14,16] and acetonitrile extraction, followed by freeze-filtration[6] are required. However, clean-up procedures may alter the chemical mixture composition of the extract and could introduce blank contamination. This can be largely circumvented using extraction with PDMS, which reduces the amount of co-extracted lipids substantially while conserving the chemical composition of the mixture.[17−19] PDMS is suitable for nonpolar, hydrophobic organic chemicals with virtually constant partition constants between PDMS and different matrices,[20] including biota,[5,21−24] for a wide range of chemicals. Sampling the tissue of varying lipid fractions with PDMS led to a rather uniform uptake of between 0.5[5] and 1.2%[22] lipid into the PDMS, which presumably is an absorption process.[25] Therefore, the lipid fraction in PDMS may be considered independent of the sampled tissue and will be transferred into the solvent extract. The lipid fraction that is then co-dosed with the chemical mixture into a bioassay will vary depending on the potency of the sample, which determines how much extract needs to be dosed to elicit the desired response. Former studies observed a reduction of the sensitivity[2,12] in in vitro cell-based bioassays when extracts of biological materials containing lipids were dosed. Jin et al.[5] investigated the kinetic effect of blubber extracts in the AhR-CAFLUX assay (chemically activated fluorescence expression) by comparing the time course of the receptor activation of the reference compound 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) with and without the presence of lipids. The authors

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observed similar effects of TCDD with and without blubber extracts after an exposure time of 72 h but measured an enhanced activation of the receptor after 24 h in the presence of co-extracted lipids, hypothesizing that colloidal lipids can facilitate the uptake into cells.

The main objectives of this study were to develop a model to describe the partitioning of chemicals in in vitro bioassays and to systematically assess the influence of co-dosed lipids in these systems. We hypothesized that lipids act as an additional partitioning phase that reduces the bioavailability of the dosed chemicals and thus the apparent sensitivity of the bioassay. We screened the influence of different dosed volumes of the spiked model lipid triolein, a synthetic triglyceride, in two cell-based in vitro bioassays: AREc32 for the Nrf2-mediated activation of the antioxidant response element (ARE) and AhR-CALUX (chemically activated luciferase expression) for the activation of the aryl hydrocarbon receptor (AhR), which previously have been applied for testing blubber extracts.[5,13] Triolein was spiked with two bioactive test chemicals for each assay that differ in hydrophobicity. Hence, a difference in partitioning from the lipid phase to the bioassay medium and cells was expected. We extended an existing mass balance model[26] by the lipid phase to describe the bioassay system. As both cell lines AREc32 and AhR are cultured in the same assay medium, the test conditions were the same. We validated the model with our experimental data and applied it to bioanalytical data of lipid-containing extracts of the marine mammal blubber from the PDMS-based passive equilibrium sampling. We identified conditions when a correction of bioassay results in the presence of co-dosed lipids is necessary and provide recommendations on how to apply this correction in practice.

THEORY

Partitioning Model for Lipid-Containing Cell-Based In Vitro Bioassays. The equilibrium distribution of chemicals in in vitro cell-based systems has been described as the partitioning between water, medium components, and cells.[26,27] Here, we simplified the bioassay partitioning system and accounted only for two compartments, cells and medium. Each of these compartments is composed of lipid, protein, and water, with water representing all nonsorptive materials. Co-dosed lipids from biota extracts constitute a third compartment (Figure SI1). Binding to the plastic of the well plates is expected to be negligible under the test conditions.[10] Losses to the air were not expected because the medium/air partition constant $K_{medium/air}$ of the tested chemicals was above the volatility cut-off of $K_{medium/air} > 10^4$.[28] The fractions of chemicals in the co-dosed lipid, f_{lipid}, and in the cells, f_{cells}, were calculated with the mass balance eqs 1 and 2.

$$f_{lipid} = \left(1 + K_{cell/lipid} \cdot \frac{V_{cell}}{V_{lipid}} + K_{medium/lipid} \cdot \frac{V_{medium}}{V_{lipid}}\right)^{-1} \quad (1)$$

$$f_{cell} = \left(1 + K_{lipid/cell} \cdot \frac{V_{lipid}}{V_{cell}} + K_{medium/cell} \cdot \frac{V_{medium}}{V_{cell}}\right)^{-1} \quad (2)$$

The volumes of co-dosed lipids, medium, and cells, V_{lipid}, V_{medium}, and V_{cells}, sum up to the total volume in the cell assay system, V_{total}. The fraction of V_{lipid} per V_{total} was termed "volume fraction", Vf_{lipid} (eq 3).

$$Vf_{lipid} = \frac{V_{lipid}}{V_{total}} \quad (3)$$

Partition constants K between the lipid and cells, $K_{lipid/cell}$, and between the medium and cells, $K_{medium/cell}$, were derived from partition constants K between lipid/water, $K_{lipid/w}$, medium/water, $K_{medium/w}$, and cell/water, $K_{cell/w}$ (eqs 4 and 5).

$$K_{lipid/cell} = \frac{K_{lipid/w}}{K_{cell/w}} \quad (4)$$

$$K_{medium/cell} = \frac{K_{medium/w}}{K_{cell/w}} \quad (5)$$

Triolein served as a surrogate for co-dosed lipids, and experimental triolein/water partition constants, $K_{triolein/w}$, were used as a proxy for $K_{lipid/w}$. Triolein is a synthetic triglyceride with known physicochemical properties. Natural oils would pose the disadvantage of unknown purity and chemical partition constants.

The cellular effect concentration, EC_{cell}, can be calculated from the nominal effect concentration, EC, the fraction of chemical in the cell f_{cell}, V_{cell}, and V_{total} (eq 6).[26]

$$EC_{cell} = EC \cdot \frac{f_{cell} \cdot V_{total}}{V_{cell}} \quad (6)$$

We assume that EC_{cell} is constant[28] and independent of Vf_{lipid}, that is, EC_{cell} with lipid in the system ($EC_{cell,x\%\ lipid}$) is equal to EC_{cell} without lipid in the system ($EC_{cell,0\%\ lipid}$). Therefore, the EC with lipids in the system ($EC_{x\%\ lipid}$) can be predicted from the measured EC without lipids in the system ($EC_{0\%\ lipid}$) for any Vf_{lipid} (eq 7).

$$EC_{x\%\ lipid} = EC_{0\%\ lipid} \cdot \frac{f_{cell,0\%\ lipid}}{f_{cell,x\%\ lipid}} \quad (7)$$

MATERIALS AND METHODS

Chemicals and Materials. The test chemicals for the AREc32 assay were benzo[*a*]pyrene (Sigma-Aldrich, #50-32-8, ≥96%) and dichlorvos (Dr. Ehrenstorfer, #62-73-7, 97.6%), and for the AhR-CALUX, they were 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126, Dr. Ehrenstorfer, #57465-28-8, 94.5%) and *β*-naphthoflavone (Sigma-Aldrich, #6051-87-2, ≥98%) (chemical structures in Figure SI2). The reference compound for the AhR-CALUX assay was 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, Dr. Ehrenstorfer). The solvents used were ethyl acetate (Merck, SupraSolv, GC−MS), methanol (Merck, SupraSolv, for GC ECD FID), and dimethyl sulfoxide (DMSO, Applichem, ≥99.5%). Triolein was purchased from Sigma-Aldrich (≥99%). PDMS sheets (SSP-M823) with a thickness of 1 mm, supplied by Specialty Silicone Products (Ballston Spa, USA), were Soxhlet-extracted with ethyl acetate for at least 16 h prior to use.

Cell Lines. In this study, we used two in vitro reporter gene bioassays measuring the Nrf2-dependent oxidative stress response with the AREc32 cell line[29] obtained by the courtesy of C. Roland Wolf, Cancer Research UK, and the AhR activation with the AhR-CALUX cell line[30] obtained by the courtesy of Michael Denison, University of California, Davis, USA. For both cell lines, the same assay medium was used (90% Dulbecco's modified Eagle's medium with Glutamax and 10% fetal bovine serum (FBS), supplemented with penicillin

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(100 U mL^{-1}) and streptomycin (100 μg mL^{-1}), Thermo Fisher Scientific #31966021, #10099-141, #15140-035). The protein and lipid contents of the medium and the cells[31] are given in Section SI1 and Table SI1. Culturing conditions and methods are described in previous studies.[28,32] Two bioactive chemicals were tested per bioassay. Briefly, in preparation of the bioassay experiments, 24 h prior to the dosing of the chemicals, 30 μL of cell suspension with a defined number of cells (AREc32: 2500/well, AhR-CALUX: 3250/well) were plated with a multi-mode Dispenser (Biotek) in a 384-well polystyrene microtiter plate with a clear bottom (AREc32 #3764, AhR-CALUX BioCoat #356663, Corning), termed "cell assay plate". As experience had shown that the cell number stays constant for 24 h since the cells need to adhere and adapt to the new conditions,[33] the seeded cell number was considered as the starting cell number for the experiment. To calculate the mean cell number, the average of the difference between the seeded cell number and the cell number after 24 h exposure to the test chemicals was used, which means 48 h after seeding (Table SI1).[28]

Sample Preparation and Dosing Procedure for Spiked Triolein. Triolein was spiked with each of the test chemicals (benzo[*a*]pyrene, dichlorvos, PCB 126, β-naphthoflavone) individually in a serial dilution of the single chemical. It was then dosed into the wells of the bioassay at a constant triolein volume but with decreasing concentration of the test chemical. Two main experimental set-ups were tested to show the importance of the pre-equilibration of the spiked chemical with triolein and the medium components. In this context, a non-equilibrated set-up (termed "0 h") and a 24 h-equilibrated set-up (termed "24 h") were tested. An overview of the dosing procedures is shown in Figure SI3.

For the "0 h" experiments, spiked triolein was pipetted directly into 40 μL of the medium with cells in a 384-well cell assay plate with a digital analytical syringe (eVol, SGE Analytical Science). The volume of triolein was constant in each well for a given Vf_{lipid}, and the Vf_{lipid} ranged between 0.5 and 4% in the "0 h" experiments. For the "24 h" experiments, spiked triolein was pipetted with the digital analytical syringe to the 70 μL cell assay medium in glass vials. The triolein−medium mix was pre-equilibrated in closed vials for 24 h at room temperature in the dark on an orbital shaker (IKA) at 140 rpm. After 24 h of pre-equilibration, the complete volume in the glass vials was transferred to a glass-coated 384-well plate (Thermo Fischer) for dosing. In preparation of dosing, the medium in the 384-well cell assay plate was removed with a microplate washer (BioTek Instruments), leaving 10 μL assay medium, including cells. 70 μL of each well in the glass-coated 384-well dosing plate was then transferred to the cell assay plate with a multichannel pipette (Eppendorf). Vf_{lipid} in the "24 h" experiments ranged between 0.25 and 4%.

Each experiment was performed in three independent replicates. The first experiment was done with a serial 1:2 dilution of the test chemical in a constant $Vf_{triolein}$ to identify the concentration range of interest. For the second and third experiments, a linear dilution series of the chemical was dosed.

Sample Preparation and Dosing Procedure for Environmental Samples. Biota samples were processed using silicone-based equilibrium sampling of homogenized blubber tissue from marine mammals with PDMS sheets.[5,22] The samples originated from eight individuals of dugongs (Dugong dugon, obtained from Caroline Gaus, the University of Queensland, Queensland, Australia), two harbor porpoises (*Phocoena phocoena*), one ringed seal (*Phoca hispida*), and one gray seal (*Halichoerus grypus*) originating from the waters of Schleswig-Holstein, Germany. All marine mammals were stranded and found dead. The dugongs were found near the beaches of Queensland in Australia. The harbor porpoises and the ringed seal were found in the area of the North Sea in Germany and the gray seal in the Baltic Sea in Germany. Blubber from dugong was homogenized with a cryogenic grinding mill (Retsch), and the other blubber samples were homogenized with a blender (Büchi). The homogenized tissue samples were stored at −80 °C.

2.5−8.7 g homogenized dugong blubber tissue and 1.6−2.3 g PDMS were brought in contact by sandwiching PDMS sheets and blubber and wrapping in an aluminum foil. The homogenized tissues of the harbor porpoises and ringed and gray seals were an oil-like liquid and the PDMS sheets (5.0−5.6 g) were placed vertically in a 125 mL jar in approximately 100 g homogenized blubber, allowing the sample to fill the room between the PDMS sheets. The mass-to-volume ratio satisfied negligible depletion conditions of the blubber tissue (<5% depletion by partitioning to PDMS).[5,22] For this calculation, an average K between the lipid and PDMS, $K_{lipid/PDMS}$, of 30 L_{PDMS} L_{lipid}^{-1}[5] was used. The thermodynamic equilibrium between the blubber and PDMS was typically achieved in less than 24 h with 1 mm PDMS sheets according to previous work,[5,22] and hence, PDMS and blubber were equilibrated for 24 h at 4 °C. After cleaning the PDMS surface thoroughly with lint-free tissues, the sheets were extracted in 10 mL ethyl acetate per 1 g of PDMS for at least 2 h. The extraction was repeated once. The combined solvent was blown down to dryness, and the extract was re-suspended in 2 mL methanol (dugong samples) and 2 mL ethyl acetate (all other samples).

In preparation of the dosing into the bioassay, a defined volume of the sample extract was blown down to dryness and re-suspended in the medium in a dosing vial. The mass of co-dosed lipids in the bioassay system was determined gravimetrically by weighing the preweighed dosing vial with a micro-analytical balance (Mettler Toledo). It was assumed that the weight gain after the blow-down of the solvent is exclusively from lipids, neglecting other co-extracted matrix and the chemicals themselves. As for single chemicals, dosing was performed in two set-ups. The blubber extracts (i) were dosed in a direct set-up without any pre-equilibration (termed "0 h") and (ii) were pre-equilibrated with the medium for 24 h at room temperature in the dark on an orbital shaker (IKA) at 140 rpm in dosing vials prior to the dosing procedure (termed "24 h"). The dosing procedure was described previously by Neale et al.[34] Briefly, two 96-well dilution plates were prepared with a Hamilton robot (Hamilton Microlab Star) by diluting the sample with the medium either in a serial 1:2 dilution or in a linear dilution. For dosing, 10 μL of the diluted sample in the dilution plate was transferred into the 384-well cell plate containing 30 μL of the medium and the cells.

This experiment was performed two times in independent replicates. The first dosing was done with a serial 1:2 dilution of the extracts to identify the concentration range of interest. For the second dosing, a linear dilution series of the extracts was dosed.

Detection of Cytotoxicity and Reporter Gene Activation. The cell viability was measured before dosing and again after 24 h exposure to the dosing extract with an IncuCyte S3 live cell-imaging system (Essen BioScience). The

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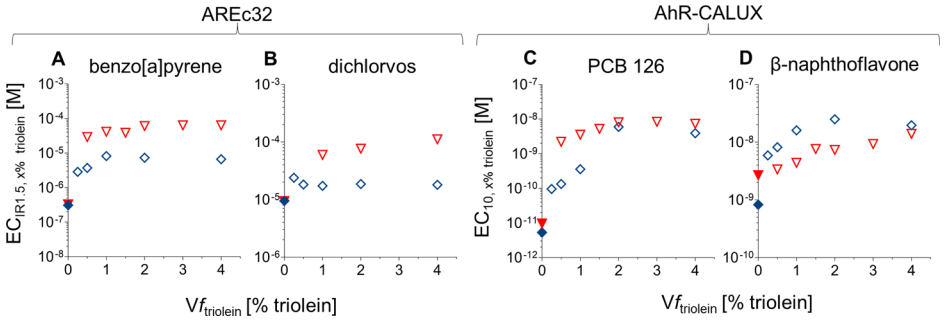

Figure 1. Comparison between "0 h" samples (red triangles) and "24 h" samples (blue diamonds) in dependency of the $Vf_{triolein}$: $EC_{IR1.5}$ in the AREc32 assay for (A) benzo[*a*]pyrene and (B) dichlorvos and EC_{10} in the AhR assay for (C) PCB 126 and (D) *β*-naphthoflavone. The filled symbols represent the measurements without triolein ($EC_{0\%\ triolein}$).

growth of the cells and the cytotoxicity of the extracts were derived from the measured cell confluency in the 384-well cell plates (number of cells given in Table SI1). The nominal inhibitory concentration for 10% reduction of cell viability (IC_{10}) was derived from the concentration−inhibition curves, which were linear up to 40% inhibition.[35]

After monitoring the confluency, the cell plates were prepared for the detection of the reporter gene activation. The AREc32 and AhR-CALUX cell lines have a luciferase reporter gene that was detected after cell lysis by the quantification of the produced luciferase with the substrate luciferin complemented by adenosine triphosphate.[32] The effect concentration, $EC_{IR1.5}$, triggering an induction ratio (IR) of 1.5 in the AREc32 assay was derived from the linear concentration−induction curves up to IR 4, under the condition that these concentrations were below the IC_{10}. The EC_{10} in the AhR-CALUX assay was derived analogously for 10% response relative to the maximum effect elicited by the potent agonist TCDD below 40% and below IC_{10}.[35] The linear regressions were performed with GraphPad Prism 8.2.1.

RESULTS AND DISCUSSION

Effect of the Test Chemicals in the Presence of Constant Triolein Volume Fractions. Linear concentration−response curves of all chemicals in the presence of a certain $Vf_{triolein}$ (Figures SI4−13) were well repeatable, and therefore, a common $EC_{x\%\ triolein}$ was derived from triplicate measurements[35] (Table SI2).

Dosing-spiked triolein to the cells resulted in a higher $EC_{x\%\ triolein}$ than without triolein ($EC_{0\%\ triolein}$) for all test chemicals, and $EC_{x\%\ triolein}$ increased with the increasing $Vf_{triolein}$ (Figure 1) for both assays. A higher EC corresponds to an apparently lower potency of the tested chemical, likely resulting from a reduced bioavailability of the test chemical in the bioassay system.

The dosing of the extract without pre-equilibration ("0 h") led to a 5.1−9.5 times higher $EC_{IR1.5}$ in comparison to the 24 h pre-equilibrated samples with benzo[*a*]pyrene (Figure 1A), 3.4−6.2 times higher $EC_{IR1.5}$ with dichlorvos (Figure 1B), and 1.9−16.5 times higher EC_{10} with PCB 126 (Figure 1C). However, the higher EC was not proportional to the increasing $Vf_{triolein}$. The higher EC at the "0 h" experiment could have resulted from the time needed to reach equilibrium partitioning between triolein and the medium, which would have delayed cellular uptake, which is typically established in standard set-ups within a few hours.[33] In contrast, dosing *β*-naphthoflavone with the "0 h" approach led to a 1.4−3.7 times lower EC_{10} in comparison with the "24 h" samples (Figure 1D). The uptake into the cells took 1.4 h (time to reach 95% of equilibrium) for benzo[*a*]pyrene in a previous study using the medium supplemented with 10% FBS.[33] In the current set-up with the chemicals in triolein dosed to the medium and cells for the "0 h" experiment, the chemicals need to partition from triolein to the medium before cellular uptake is possible. Though it is important to mix the dosing solution with the assay medium in the cell plate properly during dosing,[36] the time to reach the equilibrium between all compartments in the well cannot be accelerated. If this partitioning is slow and is happening during the 24 h of the cell-exposure experiment, the biologically effective dose in the cells is likely to be much smaller than in the absence of the additional triolein phase. In the "24 h" experiment, triolein was pre-equilibrated with the medium for 24 h while shaking to establish an equilibrium between triolein and the medium prior to dosing of the cells, so that the biologically effective dose in the cells can be reached as fast as in the absence of triolein.

If the tested chemicals are not stable under the test conditions, the additional 24 h equilibration with the medium might have led to degradation or reactions with medium components, which could have occurred for *β*-naphthoflavone (Figure 1D). Since the differences were small and since chemicals present in the blubber and other lipid-rich tissues are typically rather persistent, we did not further pursue this issue. The three other test chemicals appeared to be stable in the triolein-containing medium, as supported by very similar $EC_{10,0\%\ triolein}$ for the "0 h" and "24 h" experiments.

We also noticed that dichlorvos spiked to triolein showed higher cytotoxic effects in the "0 h" experiment than in the "24 h" experiment. Since the IC_{10} was at concentrations where the IR had often not yet reached the threshold of IR 1.5, this cytotoxic effect masked the induction (Table SI2). As the spiked triolein was not mixed, the inhomogeneous distribution of dichlorvos could have triggered the higher cytotoxicity of the cells.[36]

Validation of the Three-Phase Partitioning Model for the Reduction of Bioavailability. The experimentally determined ECs from the "24 h" experiments were compared to the predicted values for all test chemicals in Figure 2. The mean $EC_{0\%\ triolein}$ of the "0 h" experiments was used to predict the $EC_{x\%\ triolein}$ of the "24 h" samples that had attained equilibrium to assure that stability issues as encountered with *β*-naphthoflavone did not impact the model. More information on the partition constants used as the model input is given in Section SI2 and Table SI3.

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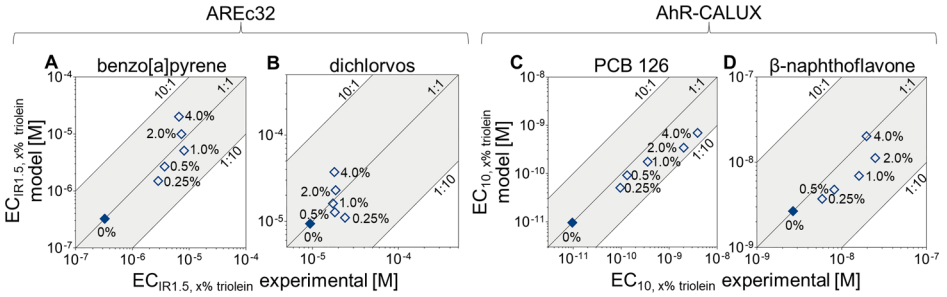

Figure 2. Comparison between experimental EC of the "24 h" experiments and EC predicted with eq 7: $EC_{IR1.5}$ for the AREc32 assay for (A) benzo[*a*]pyrene and (B) dichlorvos and EC_{10} for the AhR-CALUX assay for (C) PCB 126 and (D) *β*-naphthoflavone. The filled symbols represent the measurements without triolein ($EC_{0\%\ triolein}$).

Benzo[*a*]pyrene and dichlorvos were tested in the AREc32 assay because they are known to activate the Nrf2-dependent oxidative stress response.[13,37] $Vf_{triolein}$ ranged from 0.25 to 4% in both experiments, resulting in 89−98% for the more hydrophobic benzo[*a*]pyrene and 15−75% for the less hydrophobic dichlorvos. For this range of $Vf_{triolein}$, the $EC_{IR1.5,x\%\ triolein}$ was increased by a factor of 9−25 compared to $EC_{IR1.5,0\%\ triolein}$ (Figure 1A) but only by a factor of 2−3 for dichlorvos (Figure 1B). The ratio between modeled and experimental $EC_{IR1.5,x\%\ triolein}$ ranged from 0.33−1.9 for benzo[*a*]pyrene (Figure 2A) and from 0.48−2.2 for dichlorvos (Figure 2B).

The two AhR-activating chemicals, PCB 126[38] and *β*-naphthoflavone,[39] were tested in the AhR-CALUX assay at the same $Vf_{triolein}$ in the range 0.25−4%. The $f_{triolein}$ ranged between 81 and 99% for the more hydrophobic PCB 126 and from 28 to 87% for the less hydrophobic *β*-naphthoflavone. Testing spiked triolein with PCB 126 in AhR-CALUX showed a reduction by a factor of 10−400 of the apparent sensitivity in the bioassay with the tested range of $Vf_{triolein}$ (Figure 1C). The model underestimated the effect of triolein but could predict the $EC_{10,x\%\ triolein}$ within a factor between 1.5−5.8 (Figure 2C). $EC_{10,0\%\ triolein}$ for *β*-naphthoflavone were 2−9 times lower than $EC_{10,x\%\ triolein}$ (Figure 1D). $EC_{10,x\%\ triolein}$ was predicted within a factor of 1.0−2.3 (Figure 2D).

$EC_{IR1.5,x\%\ triolein}$ for benzo[*a*]pyrene and dichlorvos and $EC_{10,x\%\ triolein}$ for PCB 126 and *β*-naphthoflavone could be fairly precisely predicted with the three-phase model, indicating that the expansion of the previously published multimedia partition models for cell-based bioassays[27,40] by the additional lipid compartment appears valid. The model was highly sensitive to the partition constants used for the calculations, especially to $K_{BSA/w}$ and $K_{triolein/w}$, as demonstrated by the sensitivity analysis described in Section SI3 and Figure SI14.

Blubber Extracts of Marine Mammals Sampled with PDMS. To test the three-phase partitioning model with environmental samples, PDMS extracts of marine mammal blubber were examined in the AREc32 bioassay. As described above, the PDMS-sampled extracts were either dosed without pre-equilibration ("0 h") or pre-equilibrated for 24 h prior to dosing ("24 h") into the AREc32 assay. The comparison between "0 h" and "24 h" dosing showed that for extracts with a lower Vf_{lipid} the apparent sensitivity of the bioassay can be improved with a 24 h pre-equilibration, as these extracts showed on average a 1.4 lower $EC_{IR1.5}$ (Figures SI15−18 and Table SI5). Since the experiments with single chemicals described above also showed that the apparent sensitivity of the assay was increased by 24 h pre-equilibration, we discuss only the "24 h"-dosing.

Vf_{lipid} in the "24 h" extracts ranged between 0.01 and 0.06% at the $EC_{IR1.5}$ (Table SI5 and Figure 3). For highly potent

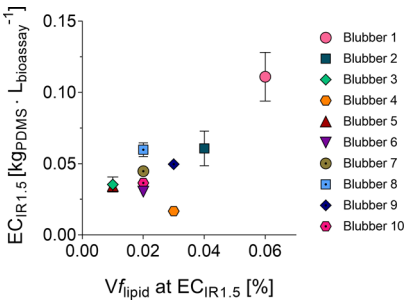

Figure 3. Relationship between $EC_{IR1.5}$ and Vf_{lipid} measured in the AREc32 assay ("24 h" dosing). Bars represent the standard error (in some cases smaller than the symbols). PDMS extracts from blubber tissue are plotted using different symbols: blubber 1−8 from dugong, blubber 9 from ringed seal, and blubber 10 from gray seal.

samples with a low $EC_{IR1.5}$, a low volume of the total extract was dosed, resulting in a low Vf_{lipid} in the cell assay well. For less-potent samples with higher $EC_{IR1.5}$, more extract had to be dosed to achieve an IR of 1.5, and consequently, the Vf_{lipid} at the $EC_{IR1.5}$ was higher. The correlation of Vf_{lipid} to $EC_{IR1.5}$ in Figure 3 had r^2 = 0.61 and root-mean-square error (RMSE) = 0.016 but was driven by one high value at Vf_{lipid} of 0.06%. Therefore, we added the $EC_{IR1.5}$ from the "0 h" experiments and additional samples from previous experiments (unpublished) with 24 additional dugong blubber samples with Vf_{lipid} from 0.007 to 0.1% in Figure SI 19, resulting in a more robust linear correlation of Vf_{lipid} to $EC_{IR1.5}$ with r^2 = 0.71 and RMSE = 0.026. The regression lines are not shown in Figures 3 and SI19 because the database is too weak to derive a model. This correlation can be explained by the same amount of lipid extracted in each experiment but a dosing of higher extract volumes (and hence Vf_{lipid}) of low-potency samples. The effect of lipids appeared independent of the source of the blubber, which was derived from dugong, ringed seal, or gray seal, but species-specific effects remain to be investigated in future studies.

The chemical mixtures present in the PDMS-based blubber extracts are of unknown composition and mainly consist of hydrophobic chemicals. While log $K_{lipid/w}$ is linearly correlated with log K_{ow}, log $K_{medium/w}$ and log $K_{cell/w}$ are slightly offset from that correlation for less-hydrophobic chemicals because of the contribution of sorption to proteins and water in the

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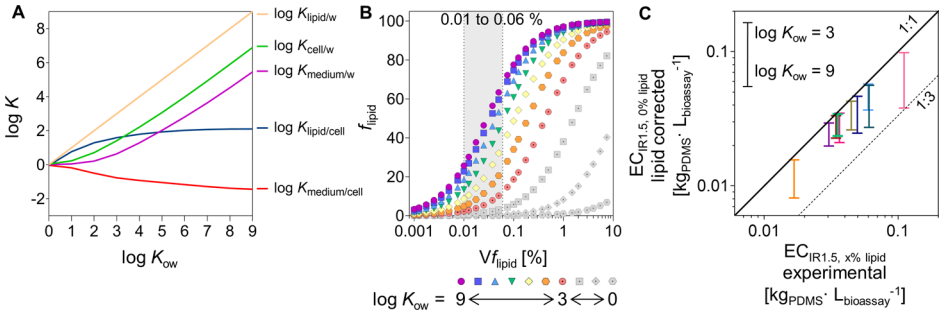

Figure 4. (A) Partition constants log $K_{lipid/water}$, log $K_{cell/water}$, log $K_{medium/water}$, log $K_{lipid/cell}$, and log $K_{medium/cell}$ as a function of log K_{ow}. (B) Relationship between f_{lipid} and Vf_{lipid} for log K_{ow} between 0 and 9. The shaded area highlights Vf_{lipid} at the EC of lipid-containing "24 h" extracts observed in the experiments in this study (0.01−0.06% in the AREc32 assay). (C) Lipid correction for the "24 h" blubber extracts (eq 7). Color code according to Figure 3. The span of the bars shows the range of the lipid correction for chemicals with log K_{ow} of 3−9. The dotted line indicates the deviation by a factor of 3 from the 1:1 line.

medium and cells (Figure 4A). In contrast, log $K_{medium/cell}$ and log $K_{lipid/cell}$ are fairly constant at log K_{ow} > 3 (Figure 4A). Hence, over the hydrophobicity range of chemicals likely to occur in blubber,[41,42] the concentration in the lipid is proportional to the concentration in the cells. Correspondingly, the composition of the mixtures remains intact from the lipid to cell as it is the case for mixtures dosed into the medium without an additional lipid phase. This specification is important because each component of the mixture contributes to the effect approximately in proportion to its fraction in the mixture independent of the presence of the lipid phase.

The additional lipid phase reduces the fraction of chemicals available for cellular uptake and hence the apparent sensitivity of the assay. As shown in Figure 4B, the lipid phase usually is the main sorptive phase, especially for hydrophobic chemicals and higher Vf_{lipid}. Even for a low Vf_{lipid} of 0.01%, the f_{lipid} for chemicals with log K_{ow} between 3 and 9 ranges from 2−26%. For Vf_{lipid} of 0.06%, f_{lipid} increases to 12−68% for the same log K_{ow} range. f_{lipid} is only moderately dependent on the hydrophobicity for chemicals, with log K_{ow} between 3 and 9 (Figure 4B). On this understanding, we estimated a range of lipid-corrected $EC_{x\%\ lipid}$ in Figure 4C. The lipid correction was performed for the "24 h" PDMS-based blubber extracts for log K_{ow} range between 3 and 9. The span of lipid-corrected $EC_{IR1.5,0\%\ lipid}$ and the distance to the experimentally determined value is dependent on Vf_{lipid}. Although the lipid correction covered 6 orders of magnitude of K_{ow}, the corrected span of $EC_{IR1.5,0\%\ lipid}$ ranged up only to a factor of 3. Up to Vf_{lipid} of 0.27%, the deviation between non-corrected EC to the lipid-corrected value $EC_{IR1.5,0\%\ lipid}$ is expected to be less than 1 order of magnitude.

Lipid correction to a precise $EC_{0\%\ lipid}$ for samples with an unknown chemical composition has limitations because the reduction of the bioavailability is dependent on the individual partitioning behavior of each chemical of the mixture. In contrast, precise lipid correction is possible for single compounds with a known partitioning behavior. It is essential to consider experimental differences in the lipid content when linking bioanalytical results with analytical results by the so-called "iceberg" mixture modeling.[37] With the iceberg model, the combination of chemical analysis and mixture effect profiling can explain how much of the total effect can be explained by the fraction of known chemicals.[37] With the application of the lipid correction, the accuracy of the iceberg modeling can be improved.

Recommendations. When dosing samples with co-extracted lipids to cell-based bioassays, the lipid content should be determined before dosing to be able to correct for the reduced bioavailability in the presence of co-dosed lipids. If working with PDMS extracts from passive equilibrium sampling, the amount of co-extracted lipid can be derived from the weight gain of the PDMS during sampling. The lipid-containing extract should be transferred into the dosing vial in a solvent that can dissolve lipids, such as ethyl acetate, to ensure quantitative dosing, but the solvent should be blown down prior to adding the medium to avoid solvent effects.[36]

The model was developed for AhR CALUX and AREc32 because previous studies showed the activity of blubber samples in these assays.[5,13] Provided there is enough information on the lipid and protein content of cells and the medium, the partitioning model can be adapted to any other cell line or bioassay set-up.

To increase the apparent sensitivity of the assay and to facilitate the application of the three-phase partitioning model, we suggest a 24 h pre-equilibration of the extract, ideally blown down to dryness to avoid disturbances by solvents, with assay medium prior to dosing to assure the equilibration of extracted chemicals between the co-extracted lipids and the medium. This approach is possible only for chemicals that are stable under bioassay test conditions, but most chemicals in the blubber are rather persistent and hence likely to be stable during the 48 h of pre-equilibration and incubation in the bioassay.

It is possible to quantitatively correct for the lipid effect on the activity of single chemicals. However, if unresolved complex mixtures are extracted from the tissue, the correction yields only a range of lipid-corrected EC values due to the dependence of the partitioning of the hydrophobicity of the individual components, which are not known. We recommend to test only up to Vf_{lipid} 0.27%, where the range of the lipid-corrected EC is within a factor of 10 for the undefined mixtures in blubber extracts. In such cases, it seems acceptable to omit the lipid correction, especially if similar tissues are compared and no comparison with chemical analysis is attempted.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.9b07850.

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Schematic illustration of the three-phase system, water, protein, and lipid contents of the used medium and the different cell lines; workflow of the dosing procedure; concentration-effect curves for all test chemicals and tested blubber extracts in all assays and supplementary analyses; additional information on the partition constants for the three-phase partitioning model; and sensitivity analysis of the model (PDF)

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Author Contributions

The article was written through contributions of all authors. All authors have given approval to the final version of the article.

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Notes

The authors declare no competing financial interest.

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Publication II

Activation of the xenobiotic metabolism and oxidative stress response by mixtures of organic pollutants extracted with in-tissue passive sampling from liver, kidney, brain and blubber of marine mammals

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Activation of the xenobiotic metabolism and oxidative stress response by mixtures of organic pollutants extracted with *in-tissue* passive sampling from liver, kidney, brain and blubber of marine mammals

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ABSTRACT

We used *in-tissue* passive equilibrium sampling using the silicone polydimethylsiloxane (PDMS) to transfer chemical mixtures present in organs from marine mammals with lipid contents between 2.3 and 99 % into *in vitro* bioassays. Tissues from five harbor porpoises (*Phocoena phocoena*), one harbor seal (*Phoca vitulina*) and one orca (*Orcinus orca*) from the North and Baltic Seas were sampled until thermodynamic equilibrium was reached. Mixture effects were quantified with cellular reporter gene bioassays targeting the activation of the aryl hydrocarbon receptor (AhR-CALUX), the peroxisome proliferator-activated receptor gamma (PPARγ-bla) and the oxidative stress response (AREc32), with parallel cytotoxicity measurements in all assays. After removing co-extracted lipids and other matrix residues with a non-destructive cleanup method (freeze-out of acetonitrile extract followed by a primary secondary amine sorbent extraction), the activation of the PPARγ and AREc32 were reduced by factors of on average 4.3 ± 0.15 (n = 22) and 2.5 ± 0.23 (n = 18), respectively, whereas the activation of the AhR remained largely unaltered: 1.1 ± 0.075 (n = 6). The liver extracts showed the highest activation, followed by the corresponding kidney and brain extracts, and the blubber extracts of the animals were the least active ones. The activation of the PPARγ by the liver extracts was reduced after cleanup by a factor of 11 ± 0.26 (n = 7) and the AREc32 activity by a factor of 1.9 ± 0.32 (n = 4). The blubber extracts did not activate the AhR up to concentrations where cytotoxicity occurred or up to an acceptable lipid volume fraction of 0.27 % as derived from earlier work, whereas all liver extracts that had undergone cleanup activated the AhR. The developed *in-tissue* passive sampling approach allows a direct comparison of the bioassay responses between different tissues without further normalization and serves as a quantitative method suitable for biomonitoring of environmental biota samples.

1. Introduction

Marine mammals are exposed to a wide variety of environmental pollutants due to anthropogenic impacts on their natural habitat, including chemicals like polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons and other legacy and emerging compounds (Desforges et al., 2017a; Sonne et al., 2020). Due to the physicochemical properties of persistent hydrophobic organic contaminants, these chemicals are prone to be enriched in biota and resistant to metabolic degradation, which increases their biomagnification potential towards higher trophic levels (Mössner et al., 1994; Ross, 2000; Kelly et al., 2007). The explicit hydrophobicity of these chemicals drives their

Abbreviations: ACN, Acetonitrile; AhR, Aryl hydrocarbon receptor; CRC, Concentration-response curves; EC, Effect concentration; EtAc, Ethyl acetate; EU, Effect Unit; IC, Inhibitory concentration; IR, Induction ratio; K_{ow}, Octanol/water partition coefficients; O.o., *Orcinus orca*; P.p., *Phocoena phocoena*; P.v., *Phoca vitulina*; PCB, Polychlorinated biphenyl; PDMS, Polydimethylsiloxane; PES, Passive equilibrium sampling; PPARγ, Peroxisome proliferator-activated receptor gamma; PSA, Primary secondary amine; R^2, Coefficient of determination; REF, Relative extraction factor; SD, Standard deviation; SE, Standard error; TU, Toxic Unit.

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accumulation in tissues with high lipid content like blubber (Debier et al., 2003; Vanden Berghe et al., 2012) but they can be also found in liver, kidney and brain tissues of marine mammals (Mössner et al., 1994; Covaci et al., 2002; Weijs et al., 2016). Taken together, and as part of other stressors, this situation implies heavy impacts on the health, development and survival of marine mammals (Sonne et al., 2020).

The isolation of chemicals from biota samples in preparation for their analysis is usually done by conventional exhaustive solvent extraction that implies co-extraction of complex matrix constituents, like lipids. This matrix can impair the quality of the analysis and instrument lifetime; hence it should be removed with a suitable cleanup method (Simon et al., 2010; Desforges et al., 2017b; Muz et al., 2021) that avoids changing their mixture composition. Passive equilibrium sampling (PES) with the silicone polydimethylsiloxane (PDMS) can be used as an alternative approach to extract environmental pollutants from biota tissues (Jin et al., 2013; Jin et al., 2015a; Rojo-Nieto et al., 2019; Smedes et al., 2020; Baumer et al., 2021a; Baumer et al., 2021b).

Two major advantages of using PES over traditional exhaustive extraction approaches are: (i) environmental mixtures of nonpolar, hydrophobic organic chemicals with a broad range of physicochemical properties are transferred into the extract largely without changing the chemical composition (Mayer et al., 2003; Jahnke et al., 2008) and without substantial matrix transfer, avoiding the need to apply destructive cleanup procedures; and (ii) the silicone can be used as a "chemometer", i.e., the concentration in the silicone can be directly compared across different tissues without normalization to lipid mass or other sorption phases (Jahnke et al., 2014).

The thermodynamic equilibrium between PDMS and lipid-rich samples can be attained rapidly with a static equilibration within a few hours, as the lipids accelerate the diffusion of the compounds throughout the tissue (Ossiander et al., 2008; Jahnke et al., 2009; Jin et al., 2013). A static equilibration in tissues with low lipid content (<10 %) does not achieve thermodynamic equilibrium before the tissue starts to decay, as local depletion of chemicals from the tissue in proximity of the PDMS slows down the uptake rates of the compounds into the silicone (Jahnke et al., 2009). To avoid local depletion, tissue with low lipid content needs to be dynamically sampled, i.e., the silicone needs to be moved within the tissue, to ensure achievement of equilibrium partitioning between silicone and tissue (Rusina et al., 2017). Several approaches have been published, including manual relocations of the silicone in homogenized fish tissue (Rojo-Nieto et al., 2019) or automatic relocations with a device that moves the silicone within fish fillet cubes (Rusina et al., 2017; Smedes et al., 2020) and homogenized mammalian tissues (Baumer et al., 2021a; Baumer et al., 2021b).

Bioanalytical tools are suitable to characterize the effects of complex chemical mixtures found within mammals (Jin et al., 2013; Desforges et al., 2017b; Baumer et al., 2021b) as valuable information about the activation of important toxicokinetic pathways is provided. In the present study the bioassays were used as bioanalytical tools, i.e., to quantify the mixture effects of the extracted chemicals (Jin et al., 2015b). In the next step, these measured mixture effects can be compared to the predicted mixture effects elicited by known chemicals in an approach referred to as "iceberg modeling". Despite the reduced co-extraction of complex matrix constituents with PES in comparison with exhaustive solvent extraction, previous studies showed that sampling of biota tissues with PDMS leads to co-extraction of lipids, with a lipid uptake into the PDMS between 0.5 (Jin et al., 2013) and 1.2 % (Jahnke et al., 2009). Dosing these co-extracted lipids into bioanalytical assays could lead to biased results, as co-dosed lipids act as an additional sorption phase in the bioassay system, and hence the bioavailability of chemicals may be reduced (Reiter et al., 2020). Performing a non-destructive lipid cleanup reduces the amount of co-extracted lipids but also alters the composition of the chemical mixture of the extract (Muz et al., 2021), requiring a detailed assessment of these competing effects.

The main objective of this study was to characterize the internal exposure in different organs of marine mammals by characterizing effects of the chemical mixtures of PES extracts with selected key cell-based *in vitro* bioassays. Since blubber and liver are the most frequently sampled, studied and archived tissue types, we focused specifically on these organs and added kidney and brain tissue whenever available for the selected animals. We investigated different modes of toxic action like the activation of the xenobiotic metabolism with the activation of the peroxisome proliferator-activated receptor gamma (PPARγ) and the aryl hydrocarbon receptor (AhR) and activation of the adaptive Nrf2-dependent oxidative stress response. Our expectations were to generate a gradient of exposure: (i) from high effects elicited by extracts from blubber tissue, as storage lipids are expected to be the main reservoir for hydrophobic organic chemicals during the lifespan of the mammals (Debier et al., 2003; Vanden Berghe et al., 2012); (ii) via intermediate effects elicited by the metabolically active organs liver and kidney; (iii) to low effects for the brain tissue, as some chemicals are thought to be held back by the blood–brain-barrier.

To achieve equilibrium between the tissues and the PDMS we manually relocated the PDMS in the tissue as described by Rojo-Nieto et al. (2019), which needed to be optimized for mammalian organ tissues with different lipid contents, and appropriate modifications of the method design needed to be implemented, as their natural texture differs from homogenized fish tissue. Though this method is considered more time-consuming than automated approaches (Rusina et al., 2017; Baumer et al., 2021a), for the manual relocation method no additional devices are needed making it an easy-to-apply and straightforward method that allows full control. Furthermore, we investigated the advantages and disadvantages of reducing the co-extracted lipid fraction in the biota PES extracts concurrent with alterations of the chemical mixtures by performing a non-destructive cleanup. The effects of extracts with and without cleanup were analyzed in parallel using the three cell-based assays, studying both cytotoxicity and the specific modes of action. This study aimed to demonstrate the applicability of the experimental design and to highlight the environmental importance to screen different organs of marine mammals for mixtures of pollutants.

2. Methods

2.1. Biota sample preparation

In this study seven liver, four kidney, five brain and seven blubber tissues from five harbor porpoises (*Phocoena phocoena*, P.p.), one harbor seal (*Phoca vitulina*, P.v.) and one orca (*Orcinus orca*, O.o.) were analyzed. The orca had been analyzed before by pathohistology by Reckendorf et al. (2018) and for PCB concentrations in the blubber tissue by Schnitzler et al. (2019); major findings are summarized in Text A1. The marine mammals had stranded and were found dead on the shores of Schleswig-Holstein, Germany. All carcasses were necropsied within 24 h after the death occurred; hence, the samples were in well-preserved condition. All four organs were available from two harbor porpoises, the harbor seal and the orca. Liver, brain and blubber tissue were available from one additional harbor porpoise and liver and blubber from two harbor porpoises. For details on animal sex, age group and stranding location, see Table B1. All tissues were homogenized with a blender (for harbor porpoises and seal: Mixer B-400, Büchi, Essen, Germany; for the orca, due to small sample size: 8011E, Waring lab, Torrington, USA). No buffer was used for homogenization to avoid altering the tissue composition, and the homogenized tissues were stored at −20 °C. The lipid content of the samples was determined gravimetrically after solvent extraction with cyclohexane and 2-propanol as described by Smedes (1999) and with modifications for smaller amounts of tissue, as described in Baumer et al. (2020). For a detailed method description see Text A2.

3. Passive equilibrium sampling (PES)

PDMS sheets (SSP-M823, Special Silicone Products, Ballston, USA)

with a density of 1.17 g·cm^{-3}, and thicknesses of 350 μm, 250 μm and 125 μm were used for method development with chemical analysis, whereas the 350 μm thick sheets were used to generate enough extract to allow for comprehensive bioanalytical testing. The PDMS sheets were cut (Tables B1 and B2 for dimensions) and solvent-cleaned using Soxhlet extraction with ethyl acetate (EtAc, Honeywell, HPLC grade) for at least 20 h. The clean PDMS sheets were stored in fresh EtAc at room temperature and in the dark until usage. Before sampling the PDMS sheets were air-dried and the exact mass of each single PDMS sheet was determined on a micro-analytical balance (Mettler Toledo, Gießen, Germany). The maximum mass of PDMS to be used in each single sample was calculated based on its lipid fraction, in order to ensure negligible depletion conditions (<5 % depletion of pollutants by partitioning to PDMS) (Jahnke et al., 2009). As an additional countermeasure against depletion, considering a certain unavoidable tissue loss due to handling during the experiment, we always used at least 30 % more of tissue homogenate than the minimum mass.

PES using PDMS in the liver, kidney and brain tissues with different lipid contents was performed by manually relocating the PDMS and mixing the tissue repeatedly during the equilibration as described in detail by Rojo-Nieto et al. (2019) with slight modifications adapted to mammalian tissue, as the natural texture of the organs is more viscous than that of homogenized fish tissue. Briefly, the PDMS sheets were fully immersed in the homogenized tissue in a glass jar, making sure that no air remained between the PDMS sheets and the tissue. For optimal contact, tissue and PDMS sheets were arranged in alternating layers, with tissue at the bottom and top of the glass jar, and were pressed gently together after each relocation event. During the experiment, the samples were kept at 4 °C for nine days. Eight relocations per day were performed, every 90 to 110 min, and the samples were left static overnight. For optimized mixing and relocation, the PDMS sheets were removed from the tissue, the homogenate was thoroughly mixed with a spoon and spatula and the PDMS sheets were fully immersed again in alternating layers with the mixed tissue. To slow down tissue oxidation, the glass jar was gas-flushed with argon prior to the static equilibration overnight. We decided not to use any substance such as sodium azide to inhibit tissue decay for safety reasons as it was necessary to handle the tissues multiple times every day. After maximally 64 to 74 relocations within nine days, the PDMS was removed. See Table B1 and B2 for details of the number of relocation events per tissue.

To determine how many relocations were needed to reach thermodynamic equilibrium between each type of tissue and the PDMS, a time series experiment was performed for liver from P.p.1 and P.p.2, kidney from P.p.3 and brain from P.p.4. For that purpose, additional PDMS sheets were sampled: every day during the nine days one 350 μm thick PDMS sheet was removed for analysis, resulting in a time series of time points collected at between 5 and 70 relocations, corresponding to 5 to 216 h of exposure time. To additionally confirm equilibrium partitioning between the tissue and the PDMS, the concentrations in the PDMS sheets with different mass to volume ratios were compared upon concluding the mixing experiment (Mäenpää et al., 2011). For that reason, the 350 μm PDMS sheets were complemented with sheets with the thickness of 125 and 250 μm for 216 h of exposure time and the maximal number of relocation events.

PDMS-based PES for blubber tissues was performed as described in Reiter et al. (2020). Summarizing, as the homogenized blubber was an oil-like liquid, the PDMS sheets were immersed in a 125 mL jar with 50 to 100 mL of homogenized blubber. Previous work showed that thermodynamic equilibrium partitioning between blubber and PDMS is achieved within 24 h with 1 mm thick PDMS sheets under static conditions (Jahnke et al., 2009; Jin et al., 2013). In this study, 350 μm thick PDMS sheets and blubber from harbor porpoises and seal were equilibrated for 48 h at 4 °C. The orca blubber showed a relatively low lipid content of 37 %, hence the PDMS sheets were equilibrated for 72 h at 4 °C as an additional safety measure.

4. Extraction of the PDMS and cleanup

The PDMS sheets were extracted individually. After removal of the PDMS from the tissue, its surface was cleaned thoroughly with lint-free tissues. Furthermore, the PDMS sheets were briefly dipped into bidistilled water and dried with lint-free tissues. The weight of each PDMS sheet was recorded and set into context with its weight preceding sampling to monitor lipid co-extraction, followed by solvent extraction with 1 mL EtAc (Merck, GCMS-grade) per 100 mg of PDMS for at least 2 h on a horizontal roller mixer. The solvent was transferred to a separate vial, and the extraction was repeated once with fresh EtAc. The extracts were combined and stored at 4 °C before further preparation steps for analysis.

For the bioanalysis, two different preparation methods were compared: dosing the solvent extracts of PDMS without and with cleanup. To prepare the dosing without cleanup, the collected EtAc extract was blown down to dryness in a preweighed vial, the weight of the co-extracted lipid residue was documented to determine the co-extracted lipid mass, m_{lipid}, and the extract was redissolved in 1 mL of EtAc for the final extract. For the corresponding sample submitted to non-destructive cleanup (Muz et al., 2021), we conducted a freeze-out in combination with a primary secondary amine (PSA) sorbent (Agilent Technologies, USA) extraction (Wernicke et al., 2022). The cleanup was performed for lipid removal, with average recoveries of a diverse set of model chemicals using the freeze-out method for high and low amounts of spiked salmon lipids of 97.4 ± 7.6% and 103.1 ± 6.4%, respectively. Further details are given by Muz et al. (2021). For the freeze-out the EtAc extract was blown down to dryness and redissolved in 1.5 mL acetonitrile (ACN, Merck, GC-MS grade) in a vial with conical bottom. The vial was stored at –20 °C for at least 2 h. The lipid residues formed a visible lump that settled to the bottom of the conical vial, and the supernatant was transferred to new vial with a glass pipette. The freeze-out was repeated with another 1.5 mL fresh ACN aliquot and the supernatant transferred to the same vial after a minimum of 2 h. The final 3 mL extract was further submitted to a cleanup with PSA. For this purpose, 35 mg of PSA and 200 mg of dried magnesium sulfate were weighed in a glass centrifuge tube. The 3 mL extract was transferred to the centrifuge tube and washed with 1 mL fresh ACN. After 1 min of mixing with a vortex mixer, the extract was centrifuged for 5 min at 5000 rpm. The supernatant was collected and the mixing and centrifuging repeated with another aliquot of 4 mL fresh ACN. The final 8 mL extract was blown down to ca. 300 μL and, as for the extracts without cleanup, transferred to a preweighed vial and then blown down to dryness. The weight of remaining matrix residues (m_{lipid}) was recorded and the dried extract dissolved in 1 mL EtAc for dosing.

For chemical analysis and the evaluation of the uptake kinetics it was necessary to perform a cleanup and reduce the amount of co-extracted matrix to avoid interferences with the instrument performance and lifetime. The samples were analyzed with a gas chromatography-high resolution Orbitrap mass spectrometry system (GC-HRMS QExactive, Thermo Fisher Scientific, Germany). The GC-HRMS measurement has been described earlier by Muz et al. (2020). The same procedure and cleanup as described above for the bioanalytical extracts with cleanup was also performed for the liver extracts from P.p. 1 and 2 and brain extracts from P.p. 4. Following the freeze-out and PSA cleanup the ACN extract was blown down to dryness and the residue dissolved in EtAc, containing 50–100 ng mL^{-1} of the following internal standards: $^{13}C_{12}$-labelled PCBs 28, 52, 101, 118, 153, 138 and 180 (Wellington, Canada). The kidney extracts from P.p. 3 required an additional cleanup, as remaining matrix still interfered with the instrument's performance. Hence, an additional cleanup with EMR-Lipid-cartridges (3 mL, Agilent Technologies, USA) as described in Muz et al. (2021) was performed: the kidney extract in 1.5 mL ACN was loaded on an EMR cartridge in a vacuum manifold. After the extract passed the cartridge, the extraction vial was washed with 1.5 mL fresh ACN and loaded to the cartridge. Pressure of<0.2 bar was applied with a vacuum pump to recover the

extract. For additional details about the instrumental analysis see Text A3.

5. Cell-based *in vitro* bioassays

Extracts from marine mammal organs were bioanalytically characterized for non-specific cytotoxic and specific effects in three *in vitro* cell-based reporter gene bioassays. The activation of the xenobiotic metabolism was tested with the AhR-CALUX (Neale et al., 2017) and PPARγ-bla GeneBLAzer assays (Invitrogen, 2010), whereas the oxidative stress response was determined with the AREc32 assay (Escher et al., 2012). In addition, cell viability was monitored in each assay to determine non-specific cytotoxic effects. The AhR-CALUX cell line (Brennan et al., 2015) was obtained by courtesy of Michael Denison, University of California, Davis, USA, the GeneBLAzer® cell line was purchased from ThermoFischer® and the AREc32 cell line (Wang et al., 2006) was obtained by courtesy of C. Roland Wolf, Cancer Research UK. Cell culture conditions and dosing methods were described in previous studies (Escher et al., 2012; König et al., 2017; Neale et al., 2017) and are summarized Text A4. An adaptation of the previous studies for optimized dosing of biota samples was followed according to Reiter et al. (2020). Briefly, in preparation for the dosing of the biota extracts into the bioassays, a defined volume of sample extract was blown down to dryness, redissolved in the bioassay medium and pre-equilibrated for 24 h at room temperature in the dark on an orbital shaker at 150 rpm, to ensure equilibrium partitioning between the compartments in the bioassay system, i.e. cells, medium and co-extracted lipids.

The concentration unit of the extracts is defined as the relative extraction factor (REF) in $kg_{PDMS}\ L^{-1}_{bioassay}$, which takes the enrichment of the PDMS extract relative to the sample and the dilution of the extract in the bioassay into account (Escher et al., 2021). The cytotoxicity is defined by the inhibitory concentration (IC_{10}) of 10 % decrease of cell viability compared to unexposed control cells. The IC_{10} and the standard error (SE) were derived from the concentration–response curves (CRC), which showed linearity up to 40 % inhibition (Escher et al., 2018), according to equations (1) and (2).

$$IC_{10} = 10/\text{slope} \tag{1}$$

$$SE(IC_{10}) = (10/\text{slope}^2) \cdot SE(\text{slope}) \tag{2}$$

Analogously to the IC_{10}, the effect concentration causing 10 % effect (EC_{10}) in the PPARγ-bla and AhR-CALUX, relative to the maximum effect caused by the reference agonists Rosiglitazone and 2,3,7,8-tetrachlorodibenzodioxin, respectively, was derived from the linear CRC up to 40 % effect, supposed that these specific effects were measured below the IC_{10}. For the AREc32 assay, the effect concentration triggering an induction ratio (IR) of 1.5 ($EC_{IR1.5}$) was derived from the linear CRC up to an IR of 4, below the IC_{10}. EC_{10} and $EC_{IR1.5}$ and their SE were calculated with equations (3) and (4).

$$EC_{10} = 10/\text{slope} \quad \text{or} \quad EC_{IR1.5} = 0.5/\text{slope} \tag{3}$$

$$SE(EC_{10}) = (10/\text{slope}^2) \cdot SE(\text{slope}) \quad \text{or} \quad SE(EC_{IR1.5}) = (0.5/\text{slope}^2) \cdot SE(\text{slope}) \tag{4}$$

Additionally, specific effects that occurred above the acceptable lipid volume fraction (Vf_{lipid}) of 0.27% (Reiter et al., 2020) were excluded for the evaluation of the EC. Vf_{lipid} is defined by the fraction of V_{lipid} in the extract per the total volume in the bioassay V_{total} (equation (5)), whereas the V_{lipid} was calculated from the co-extracted lipid mass m_{lipid} and the average density of triglycerides (i.e., triolein) of 0.91 g cm^{-3}.

$$Vf_{lipid} = V_{lipid}/V_{total} \tag{5}$$

A high IC_{10}, EC_{10} and $EC_{IR1.5}$ corresponds to an apparent lower potency of the dosed extract. Hence the IC and EC values were inverted to generate more intuitive results. The reciprocal value of the IC_{10} is defined as the Toxic Unit (TU_{bio}, equation (6)) and the reciprocal EC is defined as the Effect Unit (EU_{bio}, equation (7)), both given in $L_{bioassay}\ g^{-1}_{PDMS}$.

$$TU_{bio} = 1/IC_{10} \tag{6}$$

$$EU_{bio} = 1/EC_{10} \quad \text{or} \quad 1/EC_{IR1.5} \tag{7}$$

5.1. Statistical evaluations

To assess relationships between variables we interpreted the coefficient of determination (R^2) of linear regressions and exponential one-phase association curves. Significance between two groups with different cleanup methods or different tissues was calculated using a ratio paired *t*-test. Gaussian distribution of the results was assumed. Definition of statistical significance was a p-value < 0.05. The *t*-test was calculated with the Software GraphPad Prism V 9.3.1. Differences within these groups were not tested because of the small sample size and individuals with different characteristics, i.e., different sex, life stages, species and stranding locations.

6. Results and discussion

6.1. PES with marine mammal organs of different lipid contents

To achieve equilibrium partitioning between the different tissues with lower lipid content than blubber (liver: 8.1 ± 6.1%; kidney: 3.0 ± 0.9%; brain: 9.6 ± 1.4%) and the PDMS, manual relocations as described by Rojo-Nieto et al. (2019) were performed. To demonstrate that equilibrium had been reached, we analyzed selected PCBs with broad range of hydrophobicity (octanol/water partition coefficients, log K_{ow} between 5.60 and 7.76) (Table A2), and compared the results with two different methods to indicate a thermodynamic equilibrium between the tissue and PDMS: (i) the analyte mass was compared to different surface-to-volume ratios of PDMS, i.e. in different thicknesses of PDMS, and (ii) by conducting a time series experiment and evaluating the uptake kinetics.

The PCB concentrations determined in the PDMS are shown in Table B2 in the Supporting Information Excel file SI-B. A linear regression was used to compare the analyte mass found in the different PDMS sheets with varying thicknesses that underwent the maximum number of relocations (Figure A1). For the majority of compounds, the linear regression of PDMS mass vs. analyte mass had an acceptable R^2 between 0.75 and 1.0 (Table A3). Ambiguous regressions resulted mainly due to concentrations close to the limit of quantification resulting in too few points to fit a linear regression.

The uptake kinetics of the time series are shown in Figure A2 and the calculated times to reach 95% of equilibrium (t_{95}) and the R^2 (coefficient of determination) of the exponential one-phase association curves are given in Table A4. For the liver of P.p.1, between 13 and 51 relocations (corresponding to 19 and 152 h of exposure time) were sufficient to achieve 95 % equilibrium for the analyzed PCBs, whereas the liver of P. p.2 required between 32 and 79 relocations (76 to 221 h). For the kidney of P.p.3 and brain of P.p.4 the t_{95} ranged between 8 and 123 relocations (7–349 h) and 54–98 relocations (157–293 h), respectively. In some cases, the calculation of the t_{95} with the exponential one-phase association curve indicated that a higher number of relocations would be required than those maximally performed. Nevertheless, the comparison of different PDMS thicknesses showed that the equilibrium was established after 64–74 relocations for all compounds in all tissues, corresponding to 192–216 h. Performing even more manual relocations was not possible as the tissue texture got more and more solid, impeding further mixing of the tissue. The experiment showed that it was possible to reach equilibrium within a reasonable time under the applied stabilization measures (storage at 4 °C and argon atmosphere for overnight sampling).

6.2. Influence of the lipid matrix reduction

Lipid uptake into the PDMS was assessed by the difference in weight of the PDMS sheets before and after PES. In this experiment lipid weight gains of 0.18 to 3.2 % were observed (Table B1). Although the lipid contents of the same organ of different individuals was similar (Table B1), the lipid co-extraction into the PDMS during passive sampling differed and ranged for liver from 0.67 to 2.0 %, for kidney from 0.41 to 0.97 %, for brain from 0.18 to 0.81 % and for blubber from 0.71 to 3.2 % (Figure A3). For liver and kidney, a tissue-specific tendency is vaguely perceptible (the higher the lipid content of the tissue, the higher the observed weight gain of the PDMS), but this trend cannot be confirmed due to the small sample size. For brain and blubber samples no tendency was observed. The inconsistent lipid uptake could indicate a unique composition of lipids in each tissue type, with differing ratios of triglycerides, phospholipids and steroids.

With the cleanup procedure in this study we reduced the lipid mass in the extracts from 2.3 to 61 to 0.73–35 $mg_{lipid}\ g_{PDMS}^{-1}$ (Figure A4), which is beneficial because co-extracted lipids were shown to lower the sensitivity of the bioassays (Reiter et al., 2020). Although the lipid residues were not fully removed, we reduced them significantly by a factor of 2.8 ± 0.23 (n = 22, geometric mean ± standard deviation (SD); range: 1.1–14), with the applied combination of freeze-out and PSA extraction. The most efficient weight reduction, considered as a proxy of lipid removal, was observed for kidney extracts with a factor of 5.0 ± 0.31 (n = 4, range: 3.3–14), whereas co-extracted lipids in liver and brain extracts were reduced by a factor of 3.3 ± 0.12 (n = 6, range: 2.0–4.2) and 2.5 ± 0.18 (n = 5, range: 1.3–3.8), respectively. The lowest reduction of lipid residues was observed for blubber extracts with a factor of 1.8 ± 0.14 (n = 7, range: 1.1–3.1). Muz et al. (2021) reported a more efficient lipid removal of approximately 83 % (n = 6) with freeze-out only, tested with 10 mg of salmon oil. Although we applied the additional cleanup of PSA-extraction, we still observed a lower overall lipid removal efficiency of 59 % ± 19 % (n = 22). The cleanup efficiency could be reduced due to other lipid matrix constituents, since Muz et al. (2021) tested pure commercially available salmon oil that differs in its composition from complex mammalian organs.

6.3. Reduced EU_{bio} for activation of the PPARγ and AREc32 of extracts with cleanup

EU_{bio} and TU_{bio} values (Table B3) were derived from the linear CRCs (Figures A5-A31). The cleanup-related reduction of the lipid residues at the same time altered the chemical composition in the extract to a certain extent. The samples with cleanup showed a reduced activation in the PPARγ-bla and the AREc32 relative to the extracts without cleanup, shown in Fig. 1. The EU_{bio} measured in the PPARγ-bla was significantly reduced by a factor of 4.3 ± 0.15 for samples with cleanup (n = 22, geometric mean ± SD; range: 2.4–7.8), whereas in the AREc32 a significant reduction of EU_{bio} by factor 2.5 ± 0.23 for samples with cleanup (n = 18, geometric mean ± SD; range: 1.1–8.3) was observed. Contrarily, for the AhR-CALUX, the samples with cleanup were only reduced by a factor 1.1 ± 0.075 (n = 6, range: 0.9–1.4) for samples without cleanup and therefore the cleanup did not change the EU_{bio} significantly (Fig. 1).

The Vf_{lipid} at EU_{bio} in the extracts in the PPARγ-bla, AhR-CALUX and AREc32 ranged from $3.5x10^{-5}$ to 0.024 %, $5.4x10^{-5}$ to 0.62 % and 0.26 to 0.46 %, respectively. Lipids reduce the bioavailability of the chemicals in the bioassay system since they represent an additional sorption phase that competes with the cells. Reiter et al. (2020) recommended that Vf_{lipid} should be<0.27% in the final bioassay system to avoid sensitivity loss, and any specific effect measured with Vf_{lipid} > 0.27% was therefore omitted from further data evaluation. Highly potent extracts activating the assay at lower concentrations (and thus resulting in a high EU_{bio}) showed a low volume of Vf_{lipid}, corresponding to low volumes of the extract (and hence co-extracted lipids) dosed into the assay. Vice versa, low-activity extracts that showed a low EU_{bio} needed to be dosed at higher concentrations, resulting in a higher Vf_{lipid} (see Fig. 2). This observation is consistent for all bioassays and confirms that the assays were not activated by the lipids themselves but by the chemicals in the extracts and their corresponding potency to activate the mode of action (Reiter et al., 2020). It is especially important for the PPARγ-bla, as PPARγ is targeted, amongst others, by fatty acids (Garoche et al., 2021). As with an increasing Vf_{lipid} the EU_{bio} decreased, the receptor was dominantly activated by the extracts' mixtures of pollutants and reduced chemical bioavailability is not expected to play a dominant role. Studies showed that in addition to fatty acids the PPARγ can also be activated by environmental chemicals such as phthalates, perfluorinated compounds, halogenated derivates of bisphenol A and organophosphorus compounds (Riu et al., 2011; Grimaldi et al., 2015; Garoche et al., 2021).

6.4. Cleanup leads to reduction of the TU_{bio} in AhR-CALUX and AREc32

In parallel to the effect activation, the cell viability was evaluated for all three cell lines (see Fig. 3). No reduction of the cell viability of more than 10 % was observed for any of the extracts in the PPARγ-bla since all extracts activated the PPARγ at low concentrations. In the AhR-CALUX, cytotoxicity was most explicit with 17 sample pairs (with and without cleanup) showing cytotoxic effects. In five sample pairs the TU_{bio} was

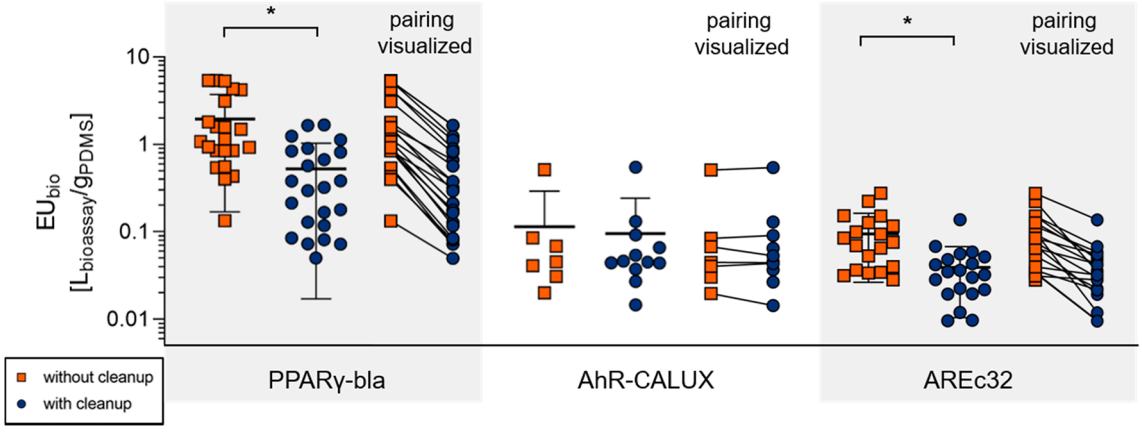

Fig. 1. Activation of specific modes of action expressed in effect units EU_{bio}. Orange squares. without cleanup, blue circles. with cleanup. Line and bar show mean and SD. PPARγ-bla number of pairs. 22. AhR-CALUX number of pairs. 6 (7 values with no paired analogue). AREc32, number of pairs. 18 (3 values without paired analogue). The asterisk indicates significant differences (ratio paired t-test, p-value < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

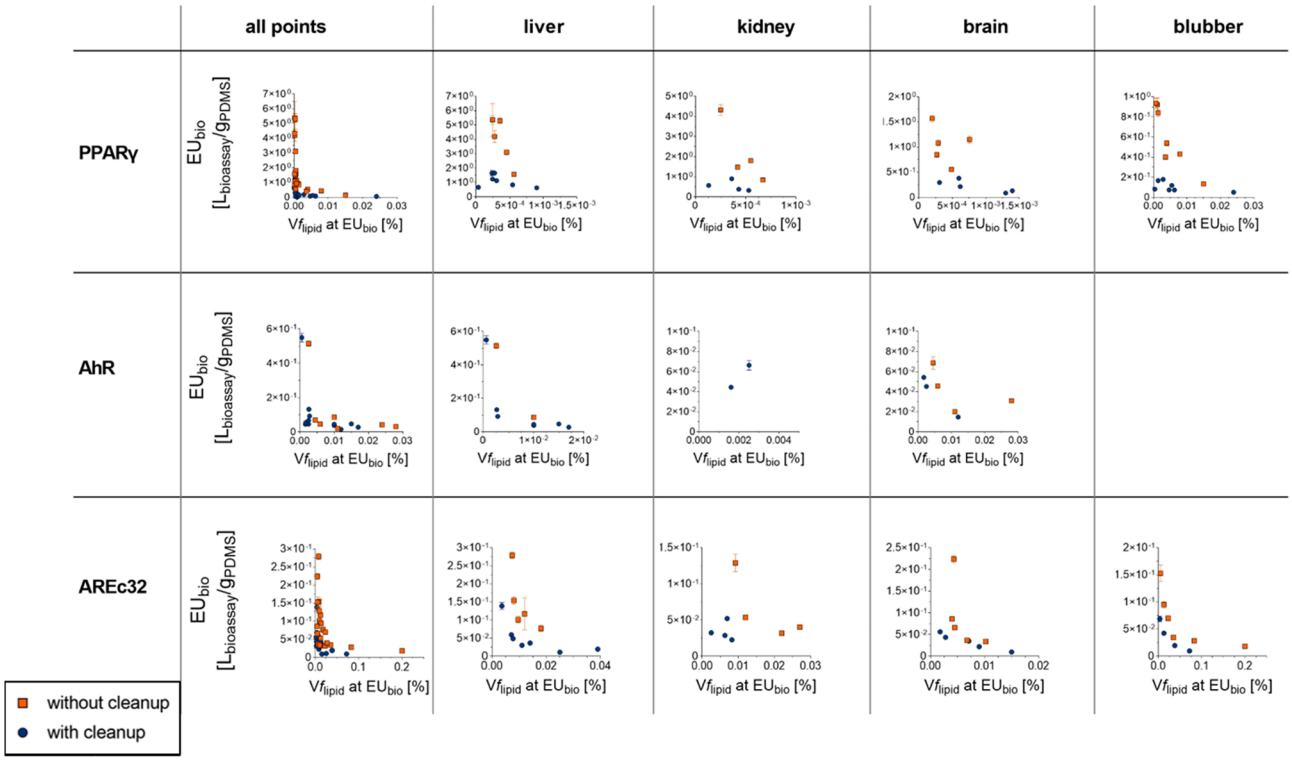

Fig. 2. Volume fraction Vf_{lipid} at EU_{bio} for the three bioassays for all measured extracts and additionally grouped for the single organs. No EU_{bio} was measured for blubber extracts with the AhR-CALUX assay. Orange squares. without cleanup, blue circles. with cleanup. Bars represent the standard error (in most cases smaller than the symbols). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

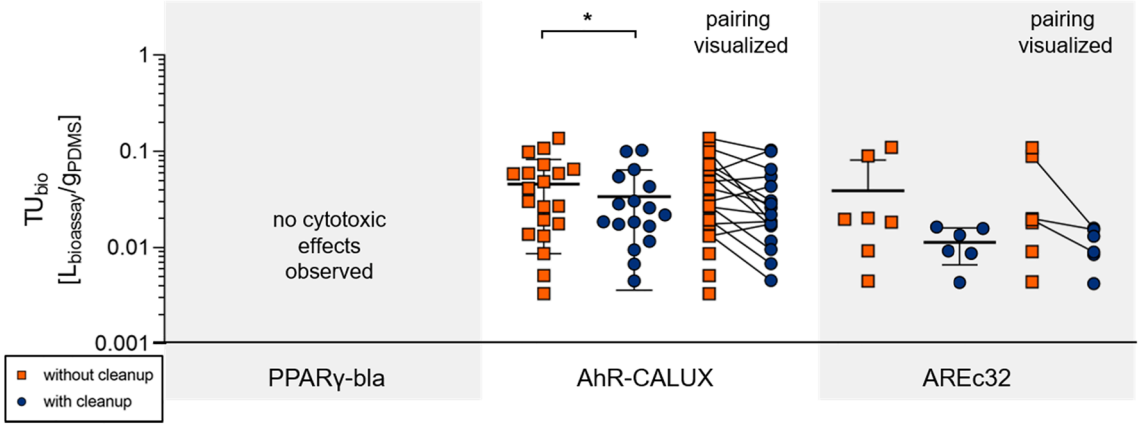

Fig. 3. Cytotoxicity displayed as TU_{bio}. Orange squares. without cleanup, blue circles. with cleanup. Line and bar show mean and SD. AhR-CALUX, number of pairs. 17 (4 single values without pair). AREc32, number of pairs. 3 (7 single values without pair). No cytotoxic effects were observed for PPARγ-bla, as the extracts were more potent and dosing concentrations to reach effect levels hence were lower than for AhR-CALUX and AREc32. The asterisk indicates significant differences (ratio paired *t*-test, p-value < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

higher in samples with cleanup, elevated by a factor of 1.3 ± 0.09 ($n = 5$, geometric mean $\pm$ SD, range: 1.1–1.7). For the other 12 sample pairs the TU_{bio} was 2.6 ± 0.24 times lower ($n = 12$, range: 1.2–7.4) in the samples with cleanup. Taken together, a significant reduction of the TU_{bio} by a factor of 1.8 ± 0.32 for samples with cleanup ($n = 17$) was observed. Only three sample pairs showed cytotoxic effects in the AREc32 assay, for which the TU_{bio} in the samples with cleanup was 2.5 ± 0.32 times lower ($n = 3$).

6.5. *Cleanup allowed additional quantification of specific effects in AhR-CALUX*

For most samples with cleanup the cytotoxic and specific effects were lower in comparison to the samples without cleanup, as discussed above, indicating a certain removal of chemicals concurrent with lipid removal. With the reduction of lipid matrix in the cleanup, and therefore the reduction of the lipid partitioning phase following dosing into the bioassay, the bioavailability of compounds is enhanced, which is expected to increase the sensitivity of the assay (Reiter et al., 2020). When comparing with literature, for the freeze-out, 103.1 ± 6.4 % (mean $\pm$ relative standard deviation) of chemical recovery was observed for a

broad range of chemicals with log K_{ow} ranging from 0.56 to 12.26 (Muz et al., 2021). However, the measured bioanalytical effects of samples submitted to cleanup were reduced in comparison to samples without cleanup with a correspondingly larger fraction of lipid matrix. Nevertheless, the effects were measurable in both types of extracts and therefore the cleanup does not interfere with the bioanalytical results.

An important observation was made when comparing the EU_{bio} and TU_{bio} values in AhR-CALUX. In this assay, five samples without cleanup did not elicit an activation of the AhR until cytotoxicity interfered, whereas for all corresponding samples with cleanup specific effects were determined. As in this assay the TU_{bio} values were reduced in the extracts with cleanup whereas the EU_{bio} values were not (see Fig. 1), we could include valid effects of additional samples in the evaluation.

In Fig. 4 the relationship between TU_{bio} and EU_{bio} is shown. The EU_{bio} values for the data points in the shaded area were extrapolated for the sake of this assessment only because the 10% effect was measured for concentrations that exceeded a 10% reduction of cell viability, hence they are not valid and were excluded for the final evaluation. Fig. 4 shows that eight samples without cleanup (squares in the shaded area) showed a high cytotoxicity and therefore the corresponding EU_{bio}, calculated by extrapolation, was invalid. This situation only occurred for three samples with cleanup (circles in the shaded area), demonstrating the extended data base thanks to the sample cleanup.

6.6. Effect patterns of liver, kidney, brain and blubber

Fig. 5 shows the effect patterns elicited by extracts from the four investigated tissues. For all sample pairs with and without cleanup in the three bioassays, the EU_{bio} values obtained from liver extracts were higher than the corresponding values from blubber extracts. The EU_{bio} for the samples with and without cleanup (geometric mean ± SD) measured with the PPARγ-bla were significantly higher for liver extracts by a mean factor of 11 ± 0.26 (n = 7) and 8.2 ± 0.40 (n = 6), respectively, relative to the corresponding blubber extracts. Furthermore, a significantly higher EU_{bio} was observed in liver compared to kidney by a factor of 2.1 ± 0.16 (n = 4) for samples with cleanup and 2.2 ± 0.31 (n = 3) for samples without cleanup, as well as liver to brain with a

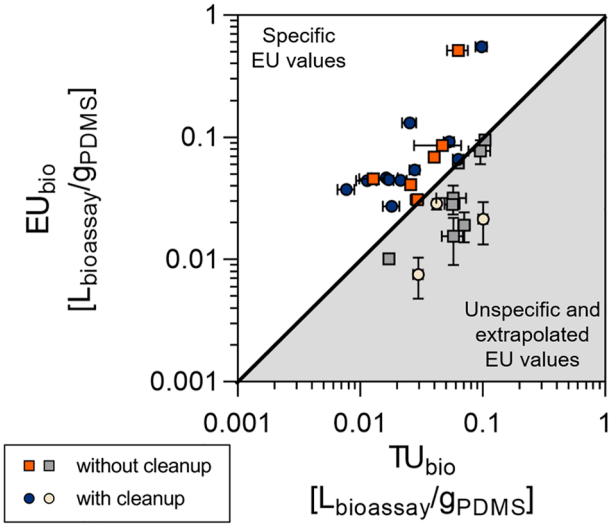

Fig. 4. Specificity plot of EU_{bio} vs. TU_{bio} in the AhR-CALUX assay for assessment reasons. Orange and dark grey squares. without cleanup, blue and light grey circles. with cleanup. Mean ± SE. The black line represents the 1:1 fit. Shaded area below the 1:1 fit line shows unspecific and extrapolated EU_{bio} values, that were invalid and hence were excluded for further evaluations. The further away the values are from the 1:1 line to the upper left, the more specific is the effect. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly higher EU_{bio} of 5.7 ± 0.17 (n = 5) (with cleanup) and 3.9 ± 0.061 (n = 4) (without cleanup), respectively.

The blubber extracts did not activate the AhR up to concentrations where cytotoxicity occurred or up to an acceptable dosed lipid volume fraction, i.e., <0.27 % (Reiter et al., 2020). But all seven liver extracts with cleanup (and two extracts without cleanup) did activate the AhR, which indicates a stronger activation of the liver extracts in comparison to the corresponding blubber extracts. A comparison of extracts between liver and kidney or brain is not possible, as the number of pairs that activated the AhR was limited (n = 2 and 0 for kidney, n = 3 and 1 for brain).

The activation of the AREc32 was also higher in liver extracts compared to the blubber extracts, as the measured EU_{bio} values extracts were 1.9 ± 0.32 times higher (n = 4) for samples with cleanup and 2.4 ± 0.28 (n = 4) (significant) for extracts without cleanup. Similar but not as explicit as for PPARγ-bla, EU_{bio} values for samples with cleanup for kidney and brain were a factor of 1.5 ± 0.32 (n = 4) and 1.5 ± 0.35 (n = 5), respectively, lower in comparison to the EU_{bio} values of liver and for samples without cleanup reduced by a factor of 2.9 ± 0.54, (n = 2) and 1.7 ± 0.61 (n = 3).

6.7. Implications regarding in-tissue sampling using PDMS-based chemometers

The extraction of diverse hydrophobic organic chemicals from biota and their chemical and/or bioanalytical characterization are essential to understand the chemical burden of biota, and in particular mixture effects at different sites of toxic action. The compound-specific physicochemical properties and persistence of each contaminant determines its partitioning between different body compartments and defines the uptake and accumulation potential (Levitt, 2010; Endo et al., 2013). Importantly, diverse compounds show distinct distribution patterns according to their partitioning behavior (Weijs et al., 2016).

The traditional approach to report the chemical burden within an organism is to normalize the compound level to the lipid content of the analyzed tissue. Hence, comparing different organs within one organism with different lipid, protein and water contents, and normalizing to the lipid content can lead to a bias of results, especially when using diverse extraction and cleanup methods for the different sample types (Porta et al., 2009; La Merrill et al., 2013). Using PDMS-based passive equilibrium sampling as a chemometer allows to compare the concentrations in the silicone directly across different tissues without normalization to the lipid mass or other sorptive phases of differing relevance depending on the type and composition of tissue.

PES in tissues with low lipid contents requires manual relocations to avoid local depletion and substantially prolonged sampling kinetics; on the one hand this procedure is time-consuming and impedes high sample throughputs, but on the other hand the experiment is highly controlled. For large sample numbers of similar composition, an automatized dynamic sampling with permanent mixing of silicone and tissue (Rusina et al., 2017; Baumer et al., 2021a) may be preferable to the manual relocation process. However, the manual relocation method is beneficial to allow for adjustments to diverse samples (e.g., different organs), as it is versatile in terms of silicone mass for diverse samples in terms of lipid fraction, texture etc.

The generally elevated potency of liver extracts to activate the assays' modes of action opposed to blubber extracts indicates that the chemical activity in the liver is higher than in blubber. The functionality of the liver as organ with key functions in metabolism and detoxification is central for the organism's health status. A high burden of organic pollutants within marine mammals can cause several health dysfunctionalities (Sonne et al., 2020; van den Heuvel-Greve et al., 2021; Boyi et al., 2022), e.g. disturbance of the immune system (Ross et al., 1996; Lehnert et al., 2016; Desforges et al., 2017b; Hall et al., 2018), reproductive success (Sonne et al., 2009) or endocrine function (Beineke et al., 2005; Das et al., 2006; Schnitzler et al., 2008; Imazaki et al.,

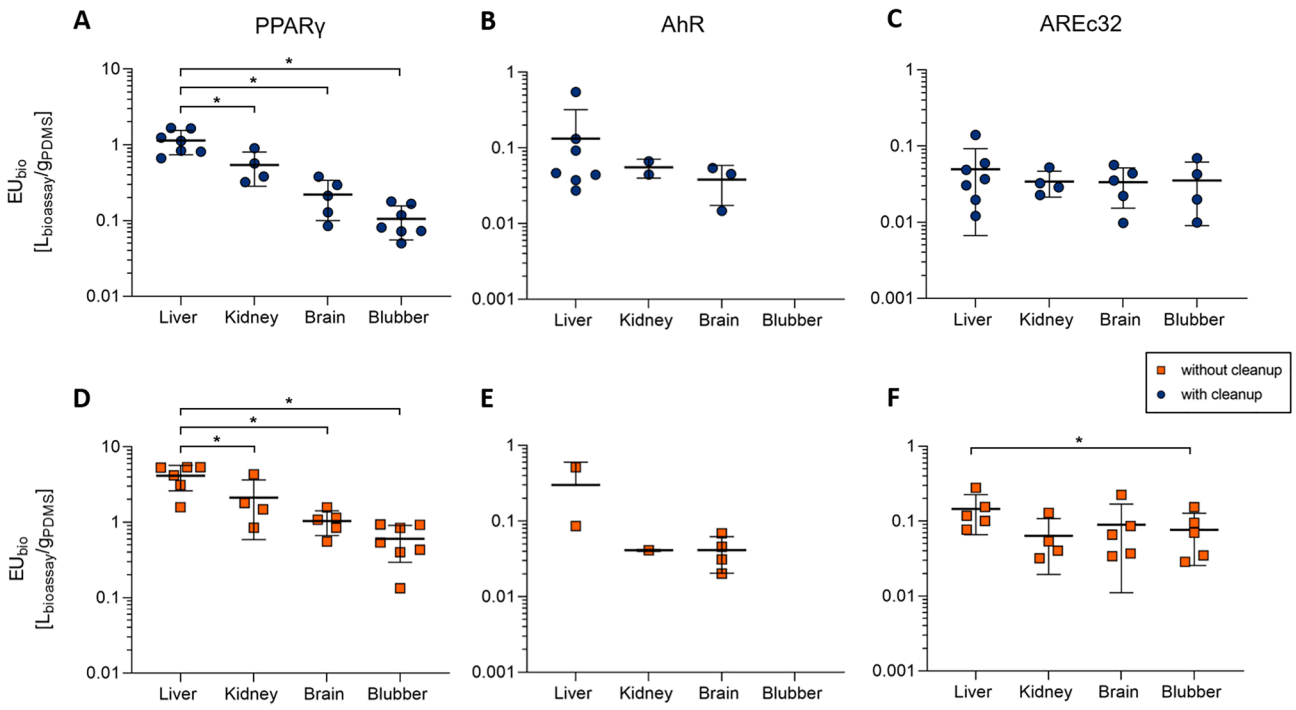

Fig. 5. EU_{bio} of extracts with (A, B, C) and without (D, E, F) cleanup of organs from marine mammals in the PPARγ-bla (A, D), AhR-CALUX (B, E) and AREc32 (C, F) bioassays. Symbols represent the mean EU_{bio} for samples without (orange squares) and with (blue circles) cleanup. No activation of the AhR for blubber extracts was detected. Line and bar show mean and SD of the sample group. The asterisk indicates significant differences (ratio paired *t*-test, p-value < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2015). Besides impacts on the liver, a high exposure of hydrophobic organic contaminants in lipid storage tissue, like blubber, can also lead to lipid metabolic disorders which could affect all organs, like the brain, heart, liver and kidney, resulting from a disorder of the homeostatic regulation (Shan et al., 2020). The potential long-term effects due to chronic internal exposure may be aggravated by explicit biomagnification along food webs and can hence have particularly large impacts on the health of marine mammals at high trophic levels (Barouki, 2010; Shan et al., 2020). Hence, *in-tissue* PES with PDMS, allowing for comprehensive effect assessment with directly comparable results between tissues and individuals, provides a valuable setup for investigating such internal exposure and effects in an unbiased way.

7. Conclusions

Former studies regarding the distribution pattern of organic contaminants within various organs of marine mammals reported either higher concentrations (lipid or wet weight-normalized) in blubber tissue than in liver or kidney (Martineau et al., 1987; McKenzie et al., 1999) or showed the opposite (Orós et al., 2009; Weltmeyer et al., 2021). With the chemometer PES approach described here, a potential bias due to normalization to the lipid fraction can be circumvented and tissues with different lipid contents and additional relevant sorption phases can be directly compared. In our data set, the mixture effects of the blubber extracts were lower than for extracts from the other tissues. The generally elevated mixture effects in liver compared to lipid-rich blubber tissue is indicative of a higher chemical activity of pollutants in liver tissues which appears conceivable as a result of to the metabolically active environment.

Characterizing PES extracts with bioanalytical tools allows for capturing the cellular toxicity and metabolic pathways of chemical mixtures extracted from biota. In this application, bioassays are used as bioanalytical tools, i.e., to quantify the mixture effects of the extracted chemicals, for comparison with chemical analytical data. With target chemical analysis one can only determine the known chemicals that occur at quantifiable levels, whereas new and emerging compounds or contaminants at very low concentrations may be overlooked. An additional step could be to combine the bioanalytical results with comprehensive chemical analysis to broadly characterize the chemical burden and discern the contribution of known compounds to the total effects vs. the contribution of unidentified compounds (Weijs and Zaccaroni, 2016; Escher et al., 2020; Neale and Escher, 2020; Baumer et al., 2021b). For future studies, using one sampling time point (i.e., after 70 manual relocations) is sufficient, opening up for samples from tissue banks, usually limited in available mass. In addition, working with one sampling time point allows for studying larger sample sizes to investigate the impact of age, sex, species, general condition etc. on pollutant levels as well as geographical or time trends, also opening up for statistical evaluation.

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CRediT authorship contribution statement

Eva B. Reite: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Visualization, Writing – original draft. **Beate I. Escher:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing. **Ursula Siebert:** Resources, Writing – review & editing. **Annika Jahnke:** Supervision, Conceptualization, Methodology, Validation, Project administration, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2022.107337.

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Publication III

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

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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 (PPARγ). 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 individuum with the highest chemical burden. This study underlines that chemical analysis and bioassays are complementary to comprehensively characterize the mixture exposome of marine mammals.

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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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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]

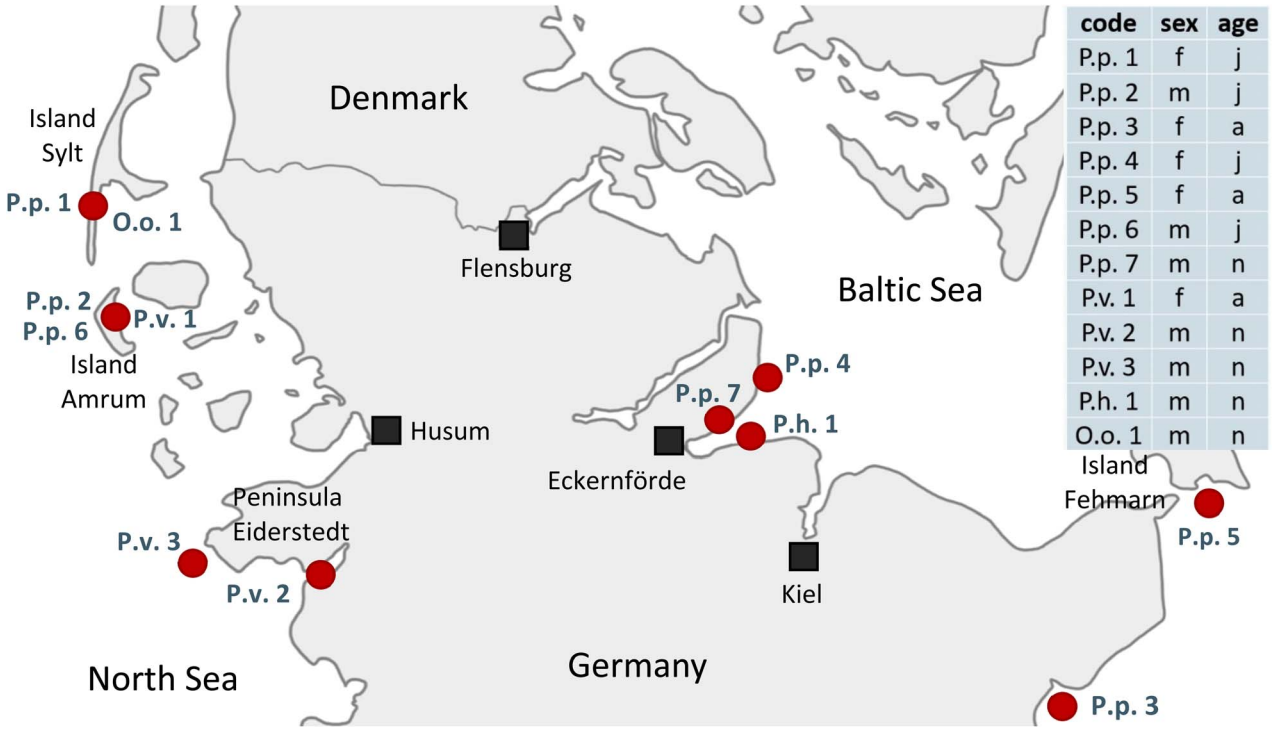
In a previous study,[41] we examined the chemical mixture effects of silicone chemometers equilibrated with different organs (*i.e.* liver, kidney, brain and blubber) of seven marine mammals from the North and Baltic Seas. Chemometer extracts were tested in three cell-based *in vitro* bioassays to investigate different modes of action: activation of the xenobiotic metabolism, including the activation of (I) the peroxisome proliferator-activated receptor gamma (PPARγ) with the PPARγ-bla GeneBLAzer assay[42] and (II) the aryl hydrocarbon receptor (AhR) with the AhR-CALUX assay[43] 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 PPARγ-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 assays[41] 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 assessment[41] 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



code	sex	age
P.p. 1	f	j
P.p. 2	m	j
P.p. 3	f	a
P.p. 4	f	j
P.p. 5	f	a
P.p. 6	m	j
P.p. 7	m	n
P.v. 1	f	a
P.v. 2	m	n
P.v. 3	m	n
P.h. 1	m	n
O.o. 1	m	n

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.

After equilibrating the chemometers with the tissue, the sheets were removed from the tissue and their surface was thoroughly cleaned with lint-free tissues. The PDMS sheets were briefly cleaned with bidistilled water twice and dried with lint-free tissues. The weights of the PDMS sheets were recorded to individually document lipid uptake into the PDMS. For extraction, 1 mL EtAc per 100 mg of PDMS was used; or a minimum volume of 1.5 mL of EtAc for the thinner PDMS sheets to ensure that the sheet was fully immersed in the solvent. Solvent extraction was performed twice for at least 2 h on a horizontal roller mixer and each extract was collected and stored at 4 °C for further processing.

To reduce the amount of co-extracted lipids in the extracts (due to lipid uptake into the PDMS), the samples were submitted to a non-destructive cleanup.[40] As described before,[41] due to matrix interferences in either the bioanalytical test system or the instrumental analysis, different cleanup procedures were required. For bioanalytical measurements a freeze-out cleanup[40] in combination with a primary secondary amine (PSA) sorbent (Agilent Technologies, USA) extraction,[50] was used; for chemical analysis, a combination of Captiva EMR-Lipid cartridges (3 mL, Agilent Technologies, USA)[40] and PSA extraction was preferred. The cleanup procedures are described in detail in Text S2.† In an ideal case, the extracts dosed to the bioassays would have been treated in the identical way as the extracts for chemical analysis. Due to cytotoxic effects that occurred after the EMR cleanup, freeze-out cleanup needed to be applied for the bioanalytical measurements.[41] The other way around, for instrumental GC-HRMS measurement, an EMR cleanup was necessary to avoid interferences with the instrument performance and lifetime. In spite of different applied cleanup procedures, the recoveries of the analyzed chemicals between EMR and freeze-out were similar, as described in Muz *et al.*[40] and Text S2.†

After the cleanup the extracts were blown down to dryness and the residue dissolved in EtAc, spiked with a mixture of 21 isotope-labeled internal standards (50–100 ng mL^{-1}, see Table S3†) and stored at −20 °C until analysis.

2.3.2 Exhaustive solvent extraction. In addition to passive equilibrium sampling, homogenized blubber tissues of the animals P.p.1–5, P.v.1 and O.o.1 were solvent-extracted with the "modified II method" described by Jensen *et al.*[51] Briefly, approximately 10 mg of blubber tissue was extracted in three processing steps with different mixtures of 2-propanol, diethyl ether and *n*-hexane. The collected extract was dried and weighed to determine the extracted lipid weight (micro-analytical balance). As for the chemometers extracts, the extracts were submitted to a non-destructive cleanup (EMR-Lipid cartridges and PSA extraction, see Text S2†) and were finally spiked with isotope-labeled internal standards (100 ng mL^{-1}, Table S3†) and stored at −20 °C until analysis.

2.4 Chemical analysis

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 target compounds, covering a broad range of physicochemical properties and targeting different substance classes of both legacy and emerging hydrophobic organic pollutants, were investigated in our study. The chemical analysis covered legacy and emerging HOCs such as PCBs, PAHs, OCPs, BDEs, Pyrethroids, CHCs, Musks, and other compounds, including antioxidants, UV filters and long-chain chemicals, categorized as "Others" (listed in Table S3†). Detailed instrumental conditions are provided in Text S3.† The target list addressed the compounds found to be relevant in an earlier environmental study using chemometers, analyzing these contaminants in marine sediment[52] as well as in other studies currently ongoing in fresh water biota. Therefore, the detected chemicals in these studies are likely to occur in marine mammals.

Method Detection Limits (MDLs, Table S4†), were determined using a two-tailed *t*-distribution test with 99% intervals, based on the US EPA guidelines, described in detail in Text S4.†[53] Extract concentration of target compounds below the MDL were considered as not detected (n.d.) for further data evaluation. Furthermore, blank subtraction and correction for lipid uptake into the PDMS sheets were carried out. Details on the data evaluation are described in Text S4.† Quality assurance/quality control procedures were in place for the GC-HRMS instrument and the analysis method, including standard operating procedures, trained technicians dedicated to the equipment and traceability (incl. analytical standards and reference materials whenever available), and are described in detail in Text S4.†

2.4.1 Data evaluation: conversion of silicone-based concentrations c_{PDMS} to lipid-based concentrations at equilibrium with the tissue $c_{lipid,\ eq.}$. In this study, the concentration are reported as silicone-based concentrations c_{PDMS} in $mass_{analyte}$ per $mass_{PDMS}$. The PDMS chemometers were used as a common reference phase, circumventing potential bias due to normalization to lipid mass, given that concentrations between the equilibrated chemometers are directly compared. However, the traditional way to report chemical burden in biota is *via* lipid-based concentrations c_{lipid} in $mass_{analyte}$ per $mass_{lipid}$. Thus, in order to compare the concentrations found in this study with those from literature and to make the results accessible for other researchers, the c_{PDMS} was converted to $c_{lipid,\ eq.}$ using compound-specific lipid/PDMS partition coefficients $K_{lipid/PDMS}$ (eqn (1)). For conversion, experimentally determined $K_{lipid/PDMS}$ values are available for 31 compounds (13 PCBs, 8 PAHs, 8 OCPs and 2 CHCs).[36,54] To translate the concentrations for those compounds that were without an experimentally determined partition coefficient, a common method is to use an average value.[52,55,56] For this study, we calculated the average from the experimentally determined $K_{lipid/PDMS}$ values from Smedes *et al.*,[54] for the compounds that were detected in this study (excluding the HCH isomers),[57] resulting in a mean $K_{lipid/PDMS}$ of 23 ($n = 25$). This approximation to an average value agrees with using the theoretical average $K_{lipid/PDMS}$ for all the compounds using modeled values from the UFZ-LSER database[58] (Table S4†). To prove that the converted values using the partition coefficients were appropriate, we compared the converted $c_{lipid,\ eq.}$ values, using

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

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

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 PPARγ-bla GeneBLAzer assay,[42] AhR-CALUX assay[43] and AREc32 assay.[44] With the earlier generated data[41] 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 ± 0.62% (mean ± 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 ± 0.26% ($n = 8$)) than for kidney tissue (0.64 ± 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 $pg_{analyte}\ {mg_{PDMS}}^{-1}$, 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 ± 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

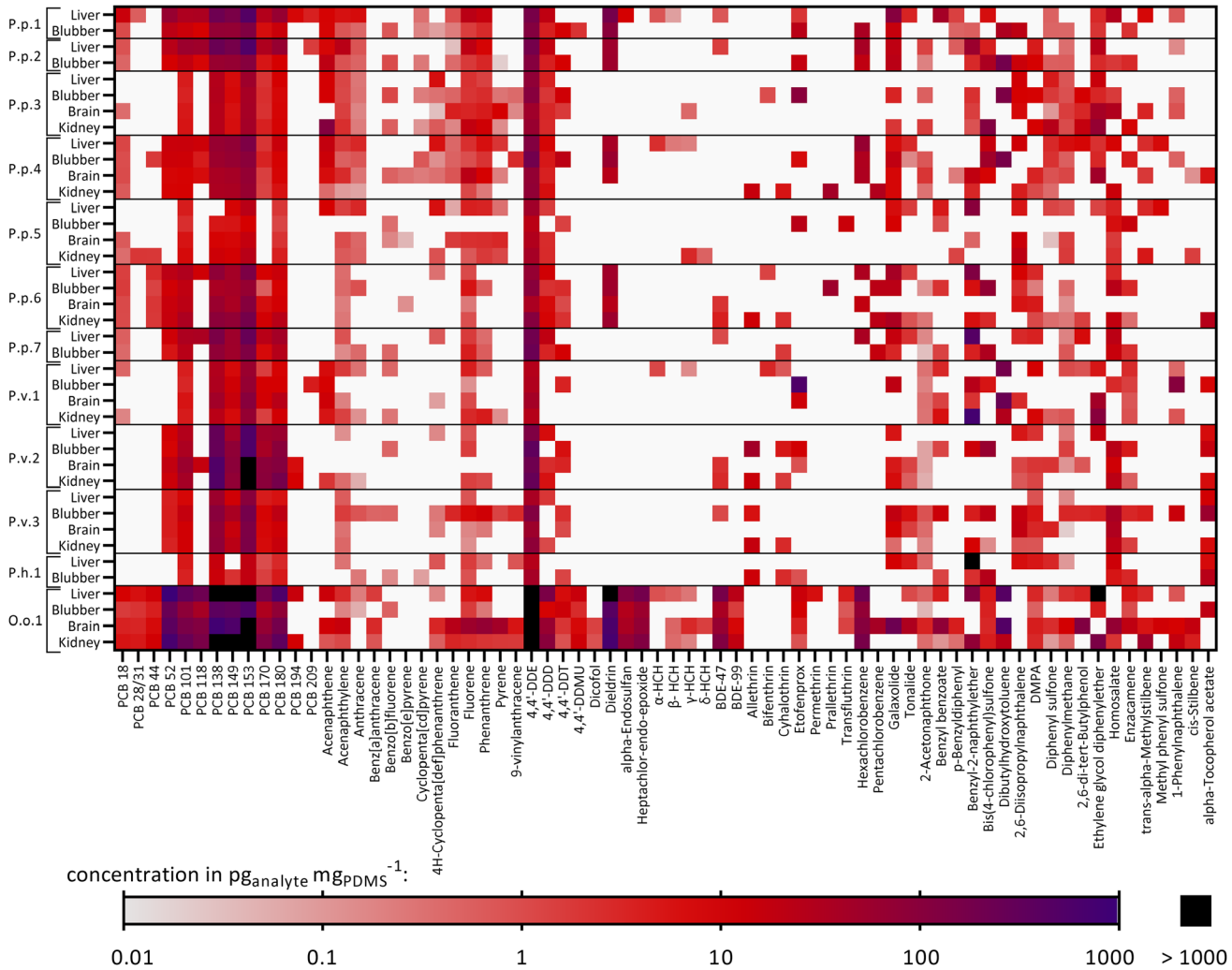


Fig. 2 Heatmap showing the detected targeted compounds found in each animal and their corresponding organs: P.p.: harbor porpoise, P.v.: harbor seal, P.h.: ringed seal, O.o.: orca, plus running number. White: the chemical was not found in the sample. Concentrations in $pg_{analyte}$ mg_{PDMS}^{-1}. Concentrations above 1000 $pg_{analyte}$ mg_{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 ± SD: 44 ± 22%), OCPs (24 ± 16%) and chemicals from the "Others" category (incl. antioxidants, UV filters and other compounds) (22 ± 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 ± 19%); contrarily, pyrethroids were found in less than 1% of the liver and brain samples and on average in 4.9 ± 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 PPARγ-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 ($c_{PDMS,\ liver}$), to the corresponding equilibrated blubber sample

($c_{PDMS,\ blubber}$), describing the liver/blubber chemical activity ratio, $c_{PDMS,\ liver}/c_{PDMS,\ blubber}$. For $\sum PCB_{13}$, a significantly (*, $p < 0.05$) higher concentration of 1.6 times (95% CI: 1.0 to 2.5, *, $n = 12$) was found in $c_{PDMS,\ liver}$, compared to the corresponding $c_{PDMS,\ blubber}$, *e.g.* PCB153 as the PCB congener found in the highest concentration in all samples, was quantified at 1.8 times (1.1 to 2.8, *, $n = 12$) higher concentrations compared to $c_{PDMS,\ blubber}$. Also, for HCB, which was only found in three pairs of liver and blubber, a liver/blubber chemical activity ratio of 2.3 (1.5 to 3.6, *, $n = 3$) was determined. As for the PAHs, $c_{PDMS,\ liver}$ was on average 1.6 (0.58 to 5.2, $n = 12$) times higher, *e.g.* for fluorene 1.7 times (0.50 to 5.9, $n = 11$) higher, compared to $c_{PDMS,\ blubber}$. Regarding the musk compounds, the liver/blubber chemical activity ratio was 1.8 (0.57 to 5.5, $n = 6$), *e.g.*, for galaxolide 1.6 (0.5 to 5.2, $n = 6$). The other way around considering the blubber/liver chemical activity ratio ($c_{PDMS,\ blubber}/c_{PDMS,\ liver}$), for pyrethroids a 13 times (0.25 to 666, $n = 4$) higher $c_{PDMS,\ blubber}$ was found compared to the corresponding $c_{PDMS,\ liver}$. Liver/blubber chemical activity ratios close to unity were found for OCPs (0.95 (0.58 to 1.5, $n = 12$)). The importance to measure the chemical burden in different organs of animals is emphasized by the fact that different patterns are observed in different organs.

Liver/blubber chemical activity ratios are shown for selected compounds and for all animals in Fig. S4.† Compounds like the UV filter homosalate, enzacamene, 4,4′-DDT, the insecticide etofenprox and alpha-tocopherol acetate, a synthetic form of vitamin E, were found in higher concentrations in blubber than in liver. Contrarily, compounds like HCB, 4,4′-DDD, PCB52 and PCB101 tended to be found at higher concentrations in liver. Overall, most compounds showed no clear tendency to accumulate in any organ. Furthermore, there seemed not to be any systematic differences between the organs or a correlation to the chemicals' octanol/water partition coefficients, K_{ow} (see Fig. S4†).

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 O.o.1 most compounds were found in higher concentration in liver than in blubber (Fig. S4†), indicating a more recent exposure of the bioaccumulative HOCs, as partitioning to blubber was not yet in equilibrium.[65] This individuum was only a few days old when it stranded,[66] which could be the reason for consistently lower concentrations of HOCs in blubber.

3.4 Integration of chemical data to previously measured data from literature

The lipid-normalized sum concentrations for all 13 PCBs ($\sum PCB_{13}$) in blubber tissues ranged from 0.64 mg kg_{lipid}^{-1} in P.p.5 to 44.5 mg kg_{lipid}^{-1} in O.o.1. For all individuals, a geometric mean (± geometric SD) of 5.85 ± 3.36 mg kg_{lipid}^{-1} was determined. The concentrations found in the present study correspond well with results from previous studies, see Table 1. In contrast, Schnitzler *et al.*[59] reported 225 mg kg_{lipid}^{-1} ($\sum PCB_{28}$) or 176 mg kg_{lipid}^{-1} ($\sum PCB_{12}$, matching with the PCBs in this study, without PCB 18) for the neonate orca also analyzed here (O.o.1). When comparing the c_{lipid} from the exhaustive solvent extraction in this study (Table S6†), a $\sum PCB_{12}$ of 63.3 mg kg_{lipid}^{-1} was determined, which is 3 times lower than the values reported earlier. Interestingly, lipid contents of the blubber samples analyzed by Schnitzler *et al.* (16–22%, $n = 3$) were approximately 2 times lower than of the blubber samples analyzed in this study (35–39%, $n = 2$), which could indicate that necropsy samples from different layers of the blubber were studied, providing an explanation for (part of) the discrepancy. According to Sørmo *et al.*,[17] the concentration of pollutants in the different layers of blubber tissue from marine mammals can differ, due to distinct lipid compositions and metabolic activities. Although the full blubber layer between skin and muscle fascia was sampled during the necropsy, the distributed subsamples could still differ in composition (as is indicated in the different lipid contents) that might yield in mismatched results.

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′-

Table 1 Data from selected published studies on the chemical burden in harbor porpoise blubber. Concentrations are reported as normalized to the lipid weight (mg kg_{lipid}^{-1}) and in a range from minimum to maximum concentrations. Sample details imply numbers of individuals (*n*), sampling location, years and the age and sex of the animals. ΣPCB_x: sum concentrations for *x* PCB congeners, ΣDDX_6: sum concentrations for 2,4′-DDD, 2,4′-DDT, 2,4′-DDE, 4,4′-DDD, 4,4′-DDE, 4,4′-DDT

Concentration range of the compound (group) reported in mg kg_{lipid}^{-1}	Sample details	Reference
Polychlorinated biphenyls (PCBs)		
$\sum PCB_{17}$: 0.21–90	$n = 112$, Danish waters, 2003–2019, different age groups, male and female	11
$\sum PCB_{35}$: 1.1–82	$n = 28$, German North Sea, 1990–2008, different age groups, male and female	69
Organochlorine pesticides (OCPs)		
$\sum DDX_6$: 0.4–22.9	$n = 28$, German North Sea, 1990–2008, different age groups, male and female	69
4,4′-DDE: 0.59–12	$n = 29$, North and Baltic Sea, 1994–1995, mostly immature animals, male and female	63
Hexachlorobenzene (HCB)		
HCB: 0.10–0.92	$n = 29$, North and Baltic Sea, 1994–1995, mostly immature animals, male and female	63
HCB: 0.013–0.42	$n = 34$, Danish waters, 2003–2019, different age groups, male and female	11

DDD and 4,4′-DDT ($\sum DDX_3$) 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 $\sum PCB$ thresholds that provide guidance for assessing PCB contamination of marine mammal tissues: (I) 9.0 mg kg_{lipid}^{-1} ($\sum PCB_{23}$), which marks the general onset of physiological impacts in marine mammals,[70,71] (II) 11.0 mg kg_{lipid}^{-1} ($\sum PCB_{25}$), from which on infertility and reproductive failure can be expected in female sexually mature harbor porpoises[11,72] and (III) 41.0 mg kg_{lipid}^{-1} ($\sum PCB_{23}$), 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 $\sum PCB$ trespassing some thresholds is corresponding to $\sum PCB_{13}$ and not to $\sum PCB_{23}$ or $\sum PCB_{25}$, 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} ($\sum PCB_6$) 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 $\sum PCB_{25}$ 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 $\sum DDX_3$, suggesting that 75% of the analyzed individuals could have suffered from adverse effects caused by their $\sum DDX_3$ 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 PPARγ-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 ($K_{medium/air}$ 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 ± geometric SD: 77 ± 1.3%), normalized to the total concentration, could be captured with the 37 compounds with known effect data. Consequently between 2.2–72% (11 ± 2.3%) remained unknown, due to unavailable compounds (24 chemicals) and between 0.58–28% (3.9 ± 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

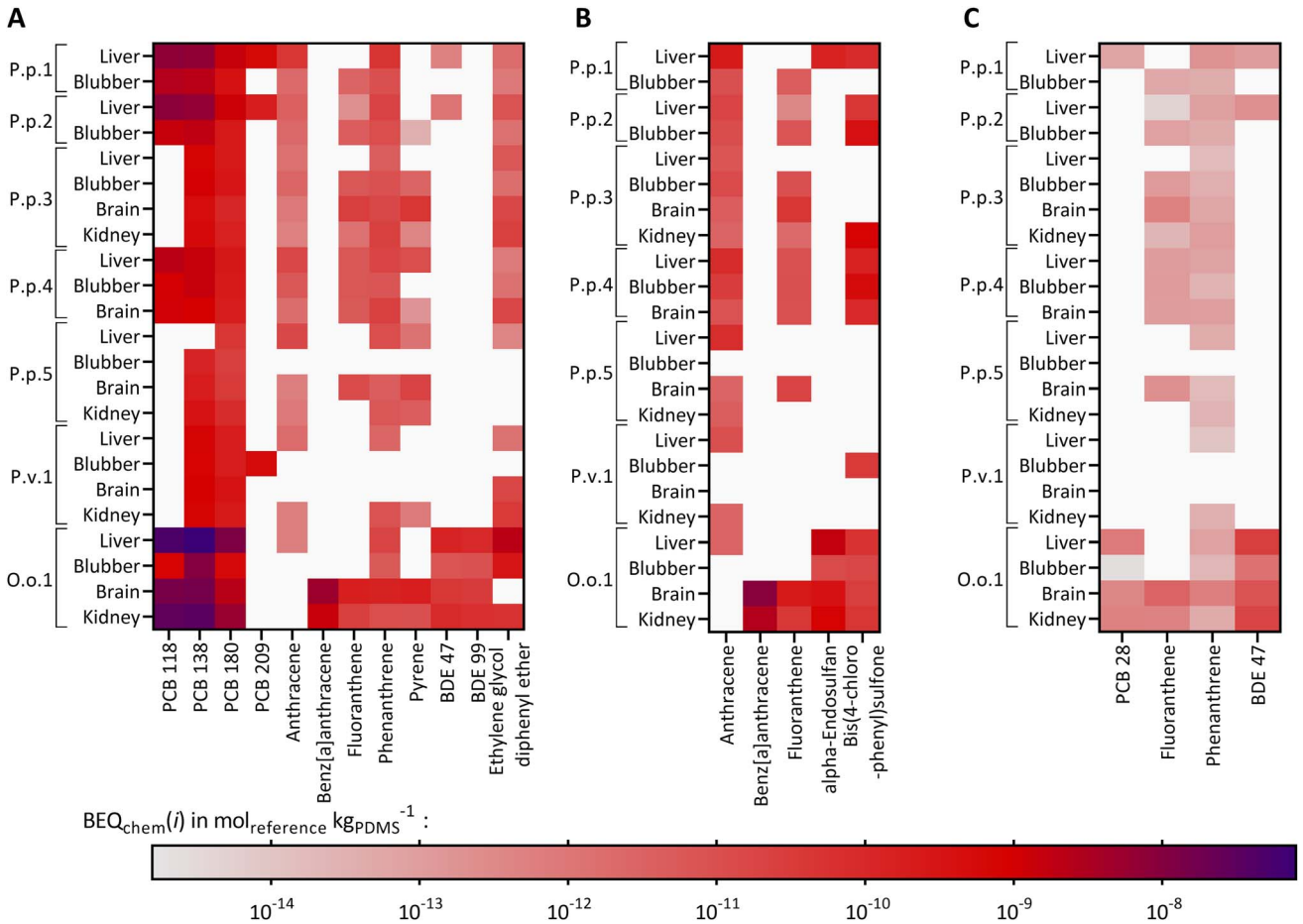


Fig. 3 Heatmap of compounds activating the (A) AhR-CALUX bioassay, (B) oxidative stress response in the AREc32 bioassay and (C) the PPARγ-bla bioassay, as $BEQ_{chem}(i)$ of the compound's activation of the assay in $mol_{reference}\ kg_{PDMS}^{-1}$ for the analyzed animals and their corresponding organs: 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 PPARγ-bla Rosiglitazone.

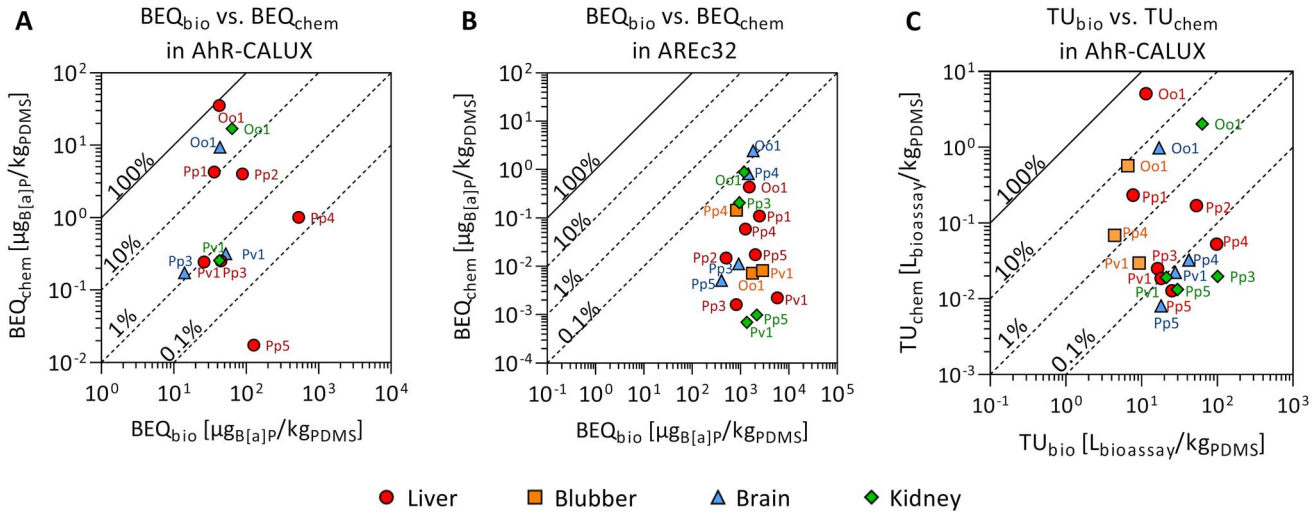


Fig. 4 Mixture effects from liver (red circle), blubber (orange square), brain (blue triangle) and kidney (green diamond) samples from seven marine mammals (P.p.: harbor porpoise, P.v.: harbor seal, P.h.: ringed seal, O.o.: orca, plus running number). Comparison of the predicted mixture effect from the 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.

of P.v.1. The smallest fraction was explained in the liver extract of P.p.5 with 0.014%. This observation correlates with the overall chemical burden, as the highest compound concentrations were found in O.o.1, whereas low concentrations were detected in the extracts from P.p.5. Chemicals that are known to activate the AhR to a great extent are dioxin-like compounds like polychlorinated dibenzodioxins and dibenzofurans, which were not targeted in this study. However, these compounds were detected in marine mammals from the Baltic and North Sea before and thus could play a role in the measured activation of the AhR.[63,81]

For iceberg modeling of the induction of oxidative stress, measured with the AREc32 assay, effect data from five compounds were available: anthracene, benz[*a*]anthracene, fluoranthene, alpha-endosulfan and bis(4-chlorophenyl)sulfone (Fig. 3B). As none of these compounds were detected in the extracts of P.p.5 blubber and P.v.1 brain, no fraction of the observed effect could be explained for these samples. Less than 0.13% was explained for all other samples (Fig. 4B). The Nrf2-dependent oxidative stress response can be activated through various pathways and thus many different chemicals may activate the AREc32 assay. Environmental pollutants such as some PAHs or quinones are known to activate the oxidative stress response pathway, and, as for the AhR, polychlorinated dibenzodioxins.[44,82]

PPARγ is typically activated by long-chain carboxylic acids such as perfluorinated alkane acids and endogenous lipids.[83] Therefore, iceberg modeling is not meaningful for the detected chemicals, only few of which were active in PPARg (PCB28, fluoranthene, phenanthrene and BDE47, shown in Fig. 3C) and consequently negligible fractions (<0.1%) of PPARγ-activation could be explained by the detected chemicals (Fig. S9†). Former studies showed that marine mammals from German Seas were contaminated with polyfluorinated compounds, which activate the PPARγ and could explain part of the observed effect;[8,84,85] however, these compounds were not targeted in this study.

Cytotoxic effects (TU_{bio}) were mainly measured in the AhR-CALUX assay. Amongst the detected chemicals, 22 were characterized to induce cytotoxic effects for the AhR-CALUX cell line; more specifically: 8 PCBs, 4 PAHs, 3 DDXs, 2 BDEs, 3 pyrethroids and 2 musks. The compounds dominating the TU_{chem} were PCB congeners 101, 118, 138, 135, 180 and 4,4′-DDD (Fig. 5). As a result, between 0.044–45% of the cytotoxic effects were explained when comparing TU_{chem} and the measured TU_{bio} (Fig. 4C). Similar to the specific mode of action, a larger fraction of the effect was explained for the tissue samples of O.o.1 with the high chemical burden (3.2–45%), whereas the smallest fractions were explained for the tissue extracts of the low contaminated P.p.5 (0.044–0.050%).

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

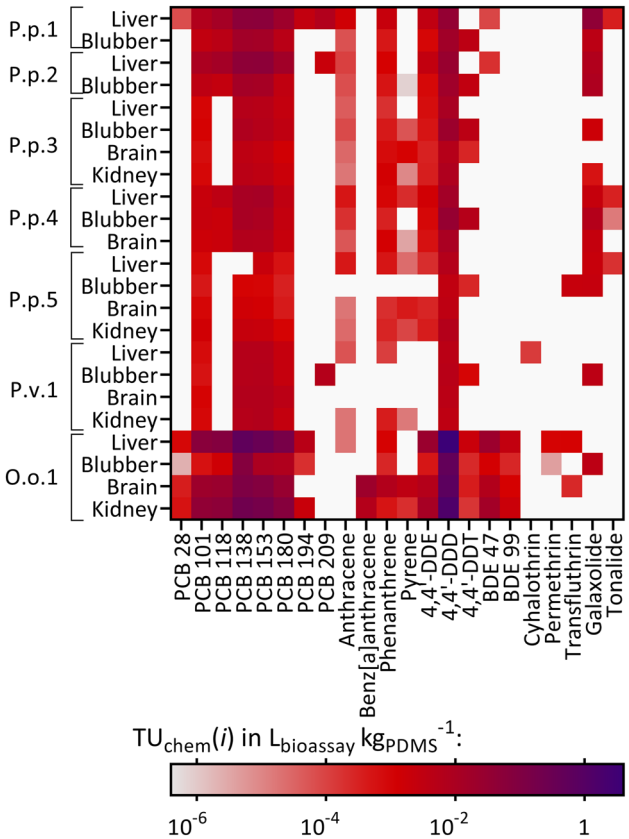


Fig. 5 Heatmap of the compounds causing cytotoxicity in the AhR-CALUX bioassay in chemometers equilibrated with the animals and their corresponding organs: P.p.: harbor porpoise, P.v.: harbor seal, O.o.: orca, plus running number, described as $TU_{chem}(i)$ in $L_{bioassay}$ kg_{PDMS}^{-1}. White: the chemical was not found in the sample.

not higher than for the other animals.[41] *Vice versa*, for animals with lower compound concentrations, like P.p.3 and P.p.5, the specific and non-specific effects measured in the three bioassays were similar to the extracts from other animals. This is an unexpected observation, as especially the cytotoxic effects were rather non-specific and could be caused by numerous chemicals. Our results demonstrate that a large fraction of unknown chemicals (and/or compounds that were not targeted or were below the limit of detection of chemical analysis) contributed substantially to the activation of AhR and PPARγ, receptors that are important for xenobiotic metabolism, the Nrf2-dependent oxidative stress response, as well as cytotoxicity, which integrates all cellular toxicity pathways into one apical outcome. The bioanalytical approach allows the inclusion of unknown and/or unexplained chemicals causing adverse effects in the risk assessment. Combining both data sets using the iceberg model underlines that chemical analysis and bioassays are complementary to comprehensively characterize the mixture exposome.

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.

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