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Mixture effect assessment applying *in vitro* bioassays to in-tissue silicone extracts of traditional foods prepared from beluga whale blubber†

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We complement an earlier study on the nutrient and environmental contaminant levels in Arctic beluga whale traditional foods by mixture effect assessment using *in vitro* bioassays. Mixtures were extracted by in-tissue sampling of raw blubber and several traditional food preparations including Muktuk and Uqsuq using silicone (polydimethylsiloxane, PDMS) as sampler. PDMS extracts persistent and degradable neutral organic chemicals of a wide range of hydrophobicity with defined lipid-PDMS partition ratios. The solvent extracts of PDMS were dosed in various reporter gene assays based on human cell lines. Cytotoxicity was consistent across all cell lines and was a good indicator of overall chemical burden. No hormone-like effects on the estrogen receptor, the progesterone receptor and the glucocorticoid receptor were observed but a few samples activated the androgen receptor, albeit with low potency. The peroxisome-proliferator activated receptor (PPAR γ) was the most sensitive endpoint followed by activation of oxidative stress response and activation of the arylhydrocarbon (AhR) receptor. The detected pollutants only explained a small fraction of the experimental mixture effects, indicating additional bioactive pollutants. The effect levels of the extracted mixtures were higher than those observed in blubber extracts of dugongs living off the shore of Australia. Roasting over an open fire or food preparation near a smokehouse led to increased PAH levels that were reflected in increased oxidative stress response and activation of the AhR. So far *in vitro* assays have only been used to quantify persistent dioxin-like chemicals in food and feed but this pilot study demonstrates a much broader potential for food safety evaluations complementing chemical analytical monitoring.

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Environmental significance

Traditional food from Arctic beluga whale comes with great nutritional and cultural value but is also a source of exposure to environmental pollutants. Individual persistent organic pollutants have been well investigated, also in relation to the food preparation method. We complement this evaluation of dietary exposure risk by assessing the mixture effects of persistent and nonpersistent organic pollutants with *in vitro* bioassays to capture the full picture of possible contamination by persistent and nonpersistent bioactive chemicals. The approach introduced here could potentially play an important role in a wide range of dietary risk assessments, complementing methods based on concentrations measured in extracted lipids.

1. Introduction

The Inuvialuit of the Western Canadian Arctic have been using traditional food (TF) prepared from beluga whales, qilalukkut

(*Delphinapterus leucas*) for many centuries. The beluga whale hunt is not only of tremendous cultural and social value, but also provides an important local source of nutrition.¹

The numerous benefits of such TFs need to be weighed against the health risks posed by the presence of anthropogenic contaminants in the tissues of the whales. As long-lived, fish-eating marine mammals, beluga whales have been exposed to, and have accumulated, persistent organic pollutants (POP) such as organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PDBEs) and other dioxin-like chemicals^{2,3} but might also be affected by more near-field pollution of less persistent chemicals. TFs derived from the lipid-rich blubber, such as Muktuk and Uqsuq are of particular

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concern because of the lipophilicity of many organic contaminants.

In an earlier study, we determined the chemical burden of various types of TFs prepared from outer and inner beluga blubber and compared it to that in the raw blubber.³ Most POPs, including OCPs, PCBs and PDBEs were detected and the concentrations did not change much with food preparation.³ If the food was roasted or prepared near a smokehouse, levels of polycyclic aromatic hydrocarbons (PAHs) increased substantially.³ Human health risk assessment performed in this earlier study³ indicated that several individual POPs exceeded their minimum risk levels. This assessment was clearly incomplete, as it did not account for the occurrence of POPs in mixtures and because it was limited to the chemicals that were part of the analytical target list and had been detected. In reality, (i) chemicals exert toxic effects in concert, (ii) many more chemicals, congeners and transformation products are likely to be present in the samples than were targeted by the analytical techniques and (iii) chemicals below the analytical detection limit can still contribute to mixture effects.

In vitro bioassays may provide more direct and more comprehensive information on the health risks posed by contaminants in foods. Such assays based on the aryl hydrocarbon receptor (AhR) have been applied for decades in the monitoring of food and feed for dioxin-like chemicals.^{4–6}

Here we propose an approach to a more comprehensive assessment of the health risk posed by contaminants in food, that combines a greatly expanded battery of bioassays with in-tissue extraction with a silicone polymer (polydimethylsiloxane, PDMS) and illustrate its application using the beluga TF samples from the study by Binnington *et al.*³ The battery of bioassays we rely upon (Fig. 1)⁷ covers several relevant steps along the cellular toxicity pathway, with representative endpoints from xenobiotic metabolism, hormone receptor binding, and adaptive stress response to apical effects such as cell viability. Thereby this test battery covers a wide range of health-relevant toxicity endpoints.

Given that PCBs, PAHs and OCPs were detected in the beluga TF samples, and they are known to activate the AhR in addition to dioxin-like chemicals, we included a highly specific AhR-CALUX assay⁸ (Table 1). Another biological endpoint relevant

for the development of metabolic disorders is the activation of the peroxisome proliferator-activated receptor γ (PPAR γ), which was quantified with the PPAR γ -bla assay⁷ (Table 1). The AREc32 assay for oxidative stress response has been previously applied to PDMS extracts of blubber samples and found very active.⁹ The oxidative stress response is activated directly by electrophilic chemicals but also indirectly by chemicals that cause imbalance of the redox status of the cell or by carcinogenic chemicals.^{10,11} Chemicals that trigger the oxidative stress response were already previously observed in marine mammals.¹²

Furthermore, the battery includes bioassays for activation of hormone receptors¹³ (estrogen receptor (ER-bla), androgen receptor (AR-bla), glucocorticoid receptor (GR-bla), progesterone receptor (PR-bla), Table 1) to evaluate if endocrine disruption effects could be elicited and also for their connection to the oxidative stress response pathway.¹⁴ In addition, cytotoxicity was assessed in all cell lines. The test battery is not comprehensive with respect to toxicological effects but covers a much wider range of chemicals than group-specific chemical analysis and can therefore be viewed as a bioanalytical test battery that quantifies risk-scaled body burden by mixtures of known and unknown chemicals. Mixture models were used to compare the effects predicted from the detected chemicals with those directly measured with bioassays.¹⁵

2. Methods

The study design with all experiments and how they relate to each other, and the research questions are given in the ESI, Fig. S1.†

2.1 Beluga blubber samples

Samples were from two male beluga whales (HI-14-06 37 years old, HI-14-11 24 years old) caught in 2014 by local Inuvialuit hunters off Hendrickson Island (69° 30'N, 133° 35'W) in the Beaufort Sea.³ From the blubber of each whale, 11 beluga TF types were prepared:³ The outer blubber and skin (called Muktuk) was sampled at various stages of the preparation process. Samples were collected following initial drying on the ground (Muktuk Air Dry) or hang drying (Muktuk Hang Dry), while additional samples were isolated after subjecting dried Muktuk to boiling in a drum (Muktuk Boil Large Drum) or a pot (Muktuk Boil Pot), roasting over an open flame (Muktuk Roast) or ageing for 2d (Muktuk Age 2 Days) or 5d (Muktuk Age 5 Days) in raw Uqsuq. Uqsuq is composed of the inner blubber and the sample “Uqsuq Baseline” represents the raw sample before any food preparation. Uqsuq was then fermented for several days, and samples were taken after 2d (Uqsuq Age 2 Days) and 5d (Uqsuq Age 5 Days). At 5 days an oil had separated that was collected (Uqsuq Oil) and tested separately.

The 22 TF samples were characterised in detail by Binnington *et al.*³ for nutrients and chemicals. Direct sample extraction with dichloromethane using accelerated solvent extraction followed by clean-up with gel permeation chromatography³ yielded the concentrations of 26 OCPs, 11 PAHs, 9 PCBs and 7 PBDEs. For convenience the detected concentrations are

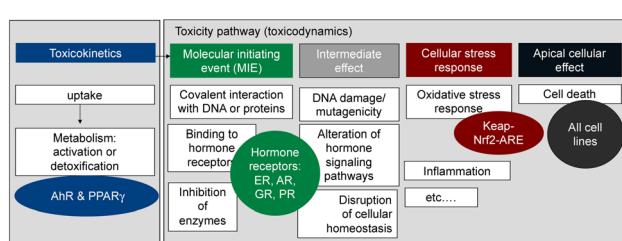


Fig. 1 Battery of *in vitro* assay applied to measure the effects of the extracted mixtures. AhR = aryl hydrocarbon receptor, PPAR γ = peroxisome proliferator-activated receptor gamma, ER = estrogen receptor, AR = androgen receptor, GR = glucocorticoid receptor, PR = progesterone receptor, keap-Nrf2-ARE pathway for adaptive stress response to reactive oxygen species and oxidative stress. Figure adapted from Neale *et al.* 2017.⁷



Table 1 Battery of *in vitro* assay applied to measure the effects of the single chemicals and extracted mixtures. QA/QC reference compound run in parallel on each plate, reference compound for iceberg modelling. AhR = aryl hydrocarbon receptor, PPAR γ = peroxisome proliferator-activated receptor gamma, ER = estrogen receptor, AR = androgen receptor, GR = glucocorticoid receptor, PR = progesterone receptor. The MDL in ng L $^{-1}$ is the concentration of a reference compound that can be resolved in the bioassay at that concentration (detectable response = mean of controls plus 3 \times standard deviation), and the MDL in ng g $_{lip}^{-1}$ accounts for the highest tested REF of 100 g $_{PDMS}$ L $^{-1}$ and the $K_{lipid/PDMS}$ of 37 according to Jin *et al.*⁹

| Mode of toxic action | Endpoint | Cell line/ bioassay | Reference compound (QA/QC) | Method detection limit (MDL) | Reference compound (BEQ $_{bio}$ and iceberg modelling) |
|---|---|--|--|---|--|
| Metabolism | | | | | |
| Induction of the arylhydrocarbon receptor AhR | Induction of reporter gene encoding for luciferase (EC $_{10}$) | AhR-CALUX (H4L1.1c4 rat) ⁸ | 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) EC $_{10}$ = 0.57 ng L $^{-1}$ | 0.19 ng $_{TCDD}$ L $^{-1}$ (TCDD-EQ of 70 ng $_{TCDD}$ g $_{lip}^{-1}$) P) EC $_{10}$ = 1.23 μ g L $^{-1}$ (ref. 33) | Benzo[a]pyrene (B[a]P) |
| Peroxisome proliferator activated receptor γ | Induction of reporter gene for PPAR γ (EC $_{10}$) | PPAR γ -bla (CellSensor PPAR γ -UAS-BLA293-H) ⁷ | Rosiglitazone EC $_{10}$ = 308 \pm 6 ng L $^{-1}$ | 276 ng $_{Rosiglitazone}$ L $^{-1}$ (rosiglitazone-EQ of 102 308 \pm 6 ng L $^{-1}$) | Rosiglitazone EC $_{10}$ = ng $_{Rosiglitazone}$ g $_{lip}^{-1}$ |
| Specific (receptor-mediated) toxicity | | | | | |
| Estrogenicity (ER) | Induction of the estrogen receptor ER α with reporter gene encoding for β -lactamase (EC $_{10}$) | ER α -bla (CellSensor ER α UAS BLA GRIPTITE) ¹³ | 17 β Estradiol (E2) EC $_{10}$ = 6.8 \pm 0.2 ng L $^{-1}$ | 1.6 ng $_{E2}$ L $^{-1}$ (EEQ of 0.6 n/a ng $_{E2}$ g $_{lip}^{-1}$) | n/a |
| Androgenicity (AR) | Induction of androgen receptor (AR) with reporter gene encoding for β -lactamase (EC $_{10}$) | AR-bla (CellSensor AR UAS BLA GRIPTITE) ¹³ | Metribolone (R1881) EC $_{10}$ = 67.3 \pm 2.1 ng L $^{-1}$ | 20 ng $_{R1881}$ L $^{-1}$ (R1881-EQ of 7.4 ng $_{R1881}$ g $_{lip}^{-1}$) | n/a |
| Glucocorticoid receptor (GR) | Induction of GR with reporter gene encoding for β -lactamase (EC $_{10}$) | GR-bla (CellSensor GR UAS-BLA HEK293T) ¹³ | Dexamethasone EC $_{10}$ = 327 \pm 13 ng L $^{-1}$ | 72 ng $_{dexamethasone}$ L $^{-1}$ (dexamethasone-EQ of 27 ng $_{dexamethasone}$ g $_{lip}^{-1}$) | n/a |
| Progesterone receptor (PR) | Induction of PR with reporter gene encoding for β -lactamase (EC $_{10}$) | PR-bla (CellSensor PR UAS-BLA HEK293T) ¹³ | Promegestone EC $_{10}$ = 48.1 \pm 2.9 ng L $^{-1}$ | 6.4 ng $_{promegestone}$ L $^{-1}$ (promegestone-EQ of 2.4 ng $_{promegestone}$ g $_{lip}^{-1}$) | n/a |
| Adaptive stress response | | | | | |
| Oxidative stress | Induction of Nrf2 protein (EC $_{IR1.5}$) | AREc32 gene reporter assay (based on MCF7) ⁴³ | t-Butylhydroquinone (tBHQ) EC $_{IR1.5}$ = 0.48 \pm 0.1 mg L $^{-1}$ | 250 μ g $_{tBHQ}$ L $^{-1}$ (tBHQ-EQ of 93 μ g $_{tBHQ}$ g $_{lip}^{-1}$) P) EC $_{10}$ = 41.6 μ g L $^{-1}$ (ref. 33) | Benzo[a]pyrene (B[a]P) |
| Non-specific toxicity (baseline toxicity) | | | | | |
| Cytotoxicity | Cell viability (IC $_{10}$) with imaging (only for AhR) ¹⁹ and with ToxBLAzer ⁷ for all other assays | All mammalian cell lines above | n/a | | n/a |

reprinted from Binnington *et al.*³ in Table S2.† In addition to this previously published chemical characterization of direct extracts,³ a few selected compounds were also quantified in in-tissue PDMS extracts.¹⁶

2.2 PDMS-extraction

In-tissue PDMS extraction was performed according to previously published methods.¹⁷ In brief, circular 18 mm diameter thin-film discs were cut from PDMS sheets (SSP-M823, 380 μ m thickness, Elasto Proxy Inc, Boisbriand, QC) using a hollow punch and pre-cleaned overnight with acetone *via* Soxhlet extraction. Prior to insertion in blubber samples, discs were briefly air-dried on clean lint-free tissues. A scalpel was used to cut slots into blubber tissues, into which single discs were immersed for 1 day. For blubber (raw) and Muktuk samples,

disc slots were consistently cut in the outer layer of fat, within 1–2 cm of the skin surface, to minimize environmental contaminant concentration variability between blubber layers.¹⁸ Since Uqsuq sampling necessitated randomly collecting inner blubber strips from ageing buckets, this type of approach to limit variability in PDMS sampling sites was not possible. However, Uqsuq samples all originated from the same larger blubber chunk, such that POP concentration variability between body regions was avoided.

To slow down tissue decay during the 24 h equilibration period, disc-containing samples were wrapped in aluminium foil and stored in a refrigerator (4 °C). Discs were then removed from the blubber and thoroughly wiped using lint-free tissues to eliminate any remaining lipid on the disc surface. Following cleaning, discs were stored in pre-cleaned glass test tubes.



For the bioassay work, 11 Beluga TF samples were extracted with 25 PDMS discs each and 11 samples were extracted with 10 PDMS discs each, amounting to approximately 2.2 to 2.4 g and 0.9 to 1 g of PDMS per sample, respectively (Table S1†). 40 Blank PDMS discs were used. This set of discs was sent to Germany for extraction and bioassay testing. Blanks were additionally prepared at UFZ. All blank disks and sample disks were extracted in ethyl acetate and run in the bioassays as described in Section 2.3 below.

For chemical analysis, PDMS extraction was performed in triplicate, such that each blubber sample contained 3 replicates of 5 discs each for a total of 15 discs; except aged Muktuk and Uqsuq samples ($n = 4$) from whale HI-14-11, which contained 3 replicates of 4 discs each. This set of discs was extracted for chemical analysis as described in Section 2.4 below.

The weight gain of the PDMS during the equilibration ranged from 0.89 to 2.89% (Table S1†), which necessitated the correction of the lipid–PDMS partition constant $K_{\text{lipid/PDMS}}$ by the lipid taken up into a PDMS disk with a mass of m_{PDMS} . The true mean $K_{\text{lipid/PDMS}}$ for bioassays was derived by Jin *et al.*⁹ and amounted to $37 \text{ g}_{\text{PDMS}} \text{ g}_{\text{lipid}}^{-1}$. The measured mass gain was assumed to be equal to the mass of co-extracted lipid $m_{\text{coextracted lipid}}$. The lipid corrected distribution ratio $D_{\text{lipid/PDMS+coextracted lipid}}$ was calculated with eqn (1) (Table S1†).

$$D_{\text{lipid/PDMS+coextracted lipid}} = \left(\frac{1}{K_{\text{lipid/PDMS}}} + \frac{m_{\text{coextracted lipid}}}{m_{\text{PDMS}}} \right)^{-1} \quad (1)$$

2.3 In vitro bioassays

The details of the used cell lines are given in Table 1. The AhR CALUX assay used the novel (third generation) more sensitive H4L1.1c4 rat cell line.⁸ The AhR-CALUX was performed as described by Neale *et al.*⁷ with modifications of the cytotoxicity assessment described by Escher *et al.*¹⁹ The AREc32 and PPAR γ -bla assays were performed as described by Neale *et al.*⁷ using the ToxBLAzer as cytotoxicity indicator. The hormone receptor GeneBLAzer assays were described by König *et al.*²⁰ Materials, media and cell cultures were described previously in detail.²¹

2.3.1. Dosing. The dose-metric for experiments with PDMS extracts is the relative extraction factor (REF), which is the product of an extraction factor (EF) and a dilution factor in the bioassay (DF).

$$\text{REF} = \text{EF} \times \text{DF} = \frac{m_{\text{PDMS}}}{V_{\text{extract}}} \times \frac{V_{\text{extract dosed}}}{V_{\text{bioassay}}} \quad (2)$$

The PDMS were extracted with two times 15 mL ethyl acetate (EA), blown down and redissolved in EA yielding an EF of 1 $\text{kg}_{\text{PDMS}} \text{ LEA}^{-1}$. An appropriate aliquot (typically approximately 50 μL) of that extract was then blown down to dryness and resolubilized with 120 μL of bioassay medium (e.g., DF = 2.4 in the dosing vial). This medium stock of the PDMS extract was diluted two-fold 11 times and 10 μL each of the resulting dilution series transferred in duplicate to a 384 well plate that contained 2500–5000 cells in 30 μL medium, resulting in an approximate highest REF in the bioassay of 0.1 kg_{PDMS}

$\text{L}_{\text{bioassay}}^{-1}$. Exposed cells were incubated in 5% CO_2 atmosphere at 37 °C for 24 h before detection.

2.3.2. Detection. For AhR and AREc32, the confluence of the cells before and after 24 h of incubation with and without reference chemicals or samples was determined with an IncuCyte S3 live cell imaging system (Essen BioScience, Ann Arbor, Michigan, USA). For these assays, luciferase is the reporter protein and it was quantified after cell lysis and addition of the substrate luciferin and ATP.²¹

For the detection of the activity of the β -lactamase in the GeneBlazer assays 8 μL of ToxBLAzer™ substrate (ThermoFisher Scientific) were added to each well of the plate and fluorescence was measured immediately for autofluorescence correction and after 2 h incubation using a Tecan Infinite® M1000 plate reader. Fluorescence was excited at 409 nm emission measured at 460 nm (blue) and 530 nm (green) to derive the blue/green ratio as a measure of β -lactamase concentration and thus indirectly reporter gene activation. Cytotoxicity was assessed by fluorescence, excitation 590 nm, emission 665 nm.

2.3.3. Data evaluation. The concentration–response curves (CRC) for the response cytotoxicity were fitted with a log–logistic model, from which the REFs causing 10% cytotoxicity (IC_{10}) were derived. Toxic units ($\text{TU}_{\text{bio, PDMS}}$) were calculated as the reciprocal of IC_{10} .

$$\text{TU}_{\text{bio, PDMS}} = \frac{1}{\text{IC}_{10}(\text{sample})} \quad (3)$$

These $\text{TU}_{\text{bio, PDMS}}$ in units of $\text{L}_{\text{bioassay}} \text{ kg}_{\text{PDMS}}^{-1}$ were converted to lipid-based TU_{bio} ($\text{L}_{\text{bioassay}} \text{ kg}_{\text{lipid}}^{-1}$) using the lipid corrected $D_{\text{lipid/PDMS+coextracted lipid}}$ calculated with eqn (1).

$$\text{TU}_{\text{bio}} = D_{\text{lipid/PDMS+coextracted lipid}} \times \text{TU}_{\text{bio, PDMS}} \quad (4)$$

For activation of reporter genes, a linear, low effect-level model is preferred for complex extracts because cytotoxicity masks activation at high concentrations²² but for the AhR-CALUX also a log–logistic CRC model had to be used for reasons given below. For both types of CRC, only concentrations up to IC_{10} were used and the benchmark concentration derived from the CRC is the EC_{10} for all cell lines with the exception of AREc32, where no maximum effect could be attained and for which the $\text{EC}_{\text{IR1.5}}$ describes the 50% increase in induction ratio (IR) over the control.

The bioanalytical equivalent concentrations (BEQ_{bio}) were calculated by dividing the effect concentration EC_{10} or $\text{EC}_{\text{IR1.5}}$ of a reference chemical by the EC_{10} or $\text{EC}_{\text{IR1.5}}$ of the sample.

$$\text{BEQ}_{\text{bio, PDMS}} = \frac{\text{EC}_{10}(\text{ref})}{\text{EC}_{10}(\text{sample})} \text{ or } \frac{\text{EC}_{\text{IR1.5}}(\text{ref})}{\text{EC}_{\text{IR1.5}}(\text{sample})} \quad (5)$$

The conversion to lipid-based BEQ_{bio} (eqn (6)) was performed analogously to eqn (4).

$$\text{BEQ}_{\text{bio}} \left(\frac{\text{ng}_{\text{ref}}}{\text{kg}_{\text{lipid}}} \right) = D_{\text{lipid/PDMS+coextracted lipid}} \times \text{BEQ}_{\text{bio, PDMS}} \left(\frac{\text{ng}_{\text{ref}}}{\text{kg}_{\text{PDMS}}} \right) \quad (6)$$



The reference chemicals for quality assurance/control (QA/QC), listed in Table 1, were run on each plate for quality control. Most are identical with the reference chemicals for calculating the BEQ_{bio} but for AhR-CALUX and AREc32 we used benzo[a]pyrene (B[a]P) as reference chemical for BEQ_{bio} (Table 1). The BEQ are then defined accordingly as B[a]P-EQ_{bio}, Rosiglitazone-EQ_{bio}, estradiol-EQ_{bio} (EEQ_{bio}), R1881-EQ_{bio} and dexamethasone-EQ_{bio} (Table 1).

2.4 Chemical analysis of PDMS extracts

Pooled PDMS discs destined for chemical analysis were immersed overnight in 10 mL of acetone spiked with internal standard (50 μ L of 200 pg μ L⁻¹ ¹³C₁₂-PCBs, in isoctane). Additionally, 5 discs were extracted as a corresponding blank for each sample [4 for the aged Muktuk/Uqsuq samples from whale HI-14-11]. PDMS extracts were subjected to clean-up prior to analysis by pipetting them onto silica gel columns pre-cleaned with hexane and containing from bottom to top glass wool, Na₂SO₄, SiO₂ and Na₂SO₄. Chemicals were eluted using hexane and DCM, and then concentrated first by rotary evaporation to 2 mL, then exchanged to iso-octane as a keeper and further evaporated under a gentle stream of high-purity nitrogen gas to 1 mL. Note that no such clean-up was performed on PDMS extracts destined for the bioassays to avoid any loss of nonpersistent chemicals. This does not limit the comparison between bioassays and chemical analysis with iceberg modelling (Section 2.5) but some of the missing predicted mixture effect from chemical analysis will be due to removal during clean-up.

Quantification of three indicator OCPs [HCB, *o,p'*-DDT, *p,p'*-DDT, log K_{ow} range 5.64–6.39],⁴³ and seven indicator PCB congeners [PCB-28, -52, -101, -118, -138, -153, and 180, log K_{ow} range 5.66–7.19]²³ was achieved using an Agilent 7890 gas chromatograph (GC) coupled to a 7000 triple-quad MS/MS operated in negative ionization mode using the method described in Binnington *et al.*³ A DB-5 column (60 m, 0.25 mm internal diameter, 0.3 μ m film thickness) was used for separation. Quality control and quality assurance was described by Binnington.¹⁶

The concentration of chemicals in lipid (C_{lipid}) was calculated by multiplying the measured PDMS concentrations (C_{PDMS}) with $K_{lipid/PDMS}$ (eqn (7)).

$$C_{lipid} = C_{PDMS} \times K_{lipid/PDMS} \quad (7)$$

2.5 Iceberg modelling

With iceberg modelling, we refer to the comparison of measured mixture effects BEQ_{bio} with the mixture effects BEQ_{chem} predicted for the detected chemicals.¹⁵ The mixture effects of the detected chemicals BEQ_{chem} can be predicted with eqn (8), provided there are single chemical effect data EC₁₀(*i*) or EC_{IR1.5}(*i*) available for the detected chemicals (*i*) with concentration C_i , from which the relative effect potency (REP_{*i*}) can be

calculated in relation to the reference compound's EC₁₀(ref) or EC_{IR1.5}(ref) (eqn (9)).

$$BEQ_{chem} = \sum_{i=1}^n BEQ_{chem,i} = \sum_{i=1}^n REP_i \times C_i \quad (8)$$

$$REP_i = \frac{EC_{10}(ref)}{EC_{10}(i)} \text{ or } \frac{EC_{IR1.5}(ref)}{EC_{IR1.5}(i)} \quad (9)$$

The summation of BEQ_{chem,i} to BEQ_{chem} (eqn (8)) follows the mixture toxicity concepts of concentration addition and independent action at <10% effect levels,²⁴ which has been demonstrated to be well applicable to environmental mixtures^{25,26} and dugong blubber.⁹

3. Results

3.1 Cytotoxicity of the blubber extracts

As the extracts contained complex mixtures of chemicals, the specific effects were often masked by cytotoxicity. Only non-cytotoxic concentrations of the extracts (<10% cytotoxicity) were evaluated for the specific endpoints but cytotoxicity itself is an indicator of the overall burden of chemicals acting together as mixtures.

The toxic units for 10% cytotoxicity related to the PDMS-based concentration TU_{bio}, PDMS (eqn (3)) varied more between different samples (coefficient of variation 10.6%) than the TU_{bio} (eqn (4)) (coefficient of variation 6.3%) due to the variable amount of co-extracted lipid, that was accounted for by the sample-specific $D_{lipid/PDMS+coextracted\ lipid}$ (Table S1†).

TU_{bio} varied by less than a factor of ten (1.9 to 9.5) between individual samples (Fig. 2 and Table S3†). The TU_{bio}s of each sample type were not paired by food preparation between TF prepared from the two beluga whales HI-14-11 and HI-14-06 (paired *T*-test, *p* = 0.123). TU_{bio} were very similar between the two whales with exception of the samples "Muktuk Roast" and "Muktuk Age 5 days", which had a higher TU_{bio} in whale HI-14-11 (Fig. S2†).

According to sensitivity distributions of log TU_{bio} (Fig. S3†), the most sensitive cell lines were ER-bla, GR-bla and PR-bla, followed by AR-bla, which was equipotent to PPAR γ -bla. AhR-CALUX followed and AREc32 was the least sensitive cell line for cytotoxicity. The GeneBLAzer reporter gene assays are all based on HEK293T and HEK293H cells and the assay is run in

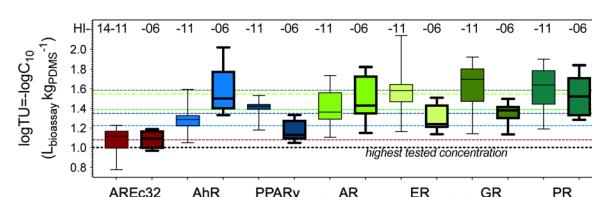


Fig. 2 Comparison of toxic units TU for cytotoxicity between the different cell lines and the two animals – HI-14-11 (thin lines) and HI-14-06 (thick lines), sorted according to increasing mean TU (dotted lines with matching colours, mean of all data from both animals).



medium supplemented with only 2% fetal bovine serum (FBS). These assays were therefore expected to be more sensitive than the cell lines supplemented with 10% FBS (AhR-CALUX derived from H4IIE and AREc32 derived from MCF7 cells (Table 1)) because chemicals bind to serum proteins, which reduces their bioavailability to cells. This was essentially confirmed but the MCF7 cell line additionally seems to be intrinsically more robust than the other cell lines.

Given the consistency of cytotoxicity across cell lines and since the variability of the cytotoxicity measure is relatively high due to the nature of the endpoint, we averaged the $\log \text{TU}_{\text{bio}}$ of all cell lines with exception of AREc32. The coefficient of variation of the mean was 1% to 10% confirming the good comparability of cytotoxicity between cell lines (Table S6†).

All samples varied in the $\log \text{TU}_{\text{bio}}$ by a maximum of 0.5 log units (factor of 3), which indicates that food preparation had no impact on the cytotoxicity of the pollutants and bioactive endogenous compounds extractable with PDMS.

3.2 Specific bioassay responses

3.2.1. Activation of AhR. The six PDMS blanks did not induce any AhR-activating effects apart from two PDMS blanks, which elicited 11% and 15% AhR activation at the highest tested REF_{PDMS} of $0.1 \text{ kg}_{\text{PDMS}} \text{ L}_{\text{bioassay}}^{-1}$. However, the cell viability was also compromised with 77% and 84% of maximum cell viability (Fig. S4a†). Under these conditions the reporter gene can be triggered non-specifically. This so-called cytotoxicity burst refers to an artifact observed close to cell death when all stress responses and consequently also all reporter genes are activated in a final burst before cell death.^{21,27} Therefore, only effects were considered where cell viability was $>90\%$, and hence the blanks were deemed not to activate AhR.

All samples showed activation of AhR with the EC_{10} ranging from $\text{REF} 1.1$ to $4.5 \text{ glipid L}_{\text{bioassay}}^{-1}$ (Table S4†). The % effect levels never reached 100% because cytotoxicity always kicked in before the effect reached a maximum in the CRCs (Fig. S5 and S6†). Because linear evaluation of the CRC unexpectedly did not yield a good fit, the EC_{10} was derived from a log-logistic fit.

The EC_{10} values for the activation of AhR were often very close to IC_{10} for cytotoxicity indicating a low specificity of the assay for the extracted mixtures. The largest specificity ratio ($\text{SR} = \text{IC}_{10}/\text{EC}_{10}$)²¹ of 1.9 was observed for HI-14-11 Muktuk Roast, which contained high concentrations of PAHs, that are specifically acting on the AhR, followed by HI-14-06 Muktuk Roast with a SR of 1.7. Whereas one normally would exclude any sample with a SR < 1 as “not specifically acting”, we reported EC_{10} for all samples since only one sample had a SR of 0.87, and four had a SR between 0.95 and 0.99. At an $\text{SR} \leq 1$, the activation occurs at the same concentration as cytotoxicity, which means that the effect is not specific.

The EC_{10} were converted to benzo[a]pyrene equivalent concentrations ($\text{B}[a]\text{P-EQ}_{\text{bio}}$) because PAHs were analysed in the samples and therefore the direct comparison in iceberg modelling described below is facilitated (Table S7†).

All samples were compared with the baseline Uqsuq sample before food preparation in Fig. 3. Several preparation methods

reduced the $\text{B}[a]\text{P-EQ}_{\text{bio}}$. $\text{B}[a]\text{P-EQ}_{\text{bio}}$ of both specimens were significantly reduced in the Boil Drum preparation (One-way Anova, Dunnett's multiple comparison test $p = 0.0176$), while others showed differences between both specimens. For instance, drying (AirDry) reduced the $\text{B}[a]\text{P-EQ}_{\text{bio}}$, while hanging Muktuk for drying did not change the $\text{B}[a]\text{P-EQ}_{\text{bio}}$ for HI-14-11 but increased it for HI-14-06.

Clearly, direct roasting increased the $\text{B}[a]\text{P-EQ}_{\text{bio}}$ presumably by producing PAHs consistent with the analytical data of PAHs.³ Muktuk Age 5 Day and Uqsuq Oil of HI-14-11 were also increased over baseline which had also been observed for the total concentration of PAHs. The difference between the two specimens was explained by different setups of the food preparation with HI-14-11 aged within the smokehouse and HI-14-06 smoked far away from a smokehouse.

Although the pattern was similar to the chemical analysis of PAHs, one main difference was that the $\text{B}[a]\text{P-EQ}_{\text{bio}}$ were already high in the samples unaffected by smoke, indicating that chemicals other than PAHs contribute to the $\text{B}[a]\text{P-EQ}_{\text{bio}}$. Iceberg modelling below will further investigate those contributions.

In order to facilitate comparison with data reported in the literature, we also calculated the more common $\text{TCDD-EQ}_{\text{bio}}$ which ranged from 126 to $500 \text{ pg}_{\text{TCDD}} \text{ glipid}^{-1}$. The baseline values were very similar in the two beluga whales (257 and $259 \text{ pg}_{\text{TCDD}} \text{ glipid}^{-1}$ for HI-14-06 and HI-14-11, respectively). The experimental $\text{TCDD-EQ}_{\text{bio}}$ were up to five times higher than the median $\text{TCDD-EQ}_{\text{bio}}$ detected in Australian dugongs that ranged from 16 to $230 \text{ pg}_{\text{TCDD}} \text{ glipid}^{-1}$.⁹ The distribution in dugongs was statistically different from the beluga baseline (Wilcoxon signed rank test, $p = 0.001$).

3.2.2. Activation of PPAR γ . No blank effects were detected up to a REF of $0.1 \text{ kg}_{\text{PDMS}} \text{ L}_{\text{bioassay}}^{-1}$ (Fig. S4b†). Unlike for AhR, cytotoxicity kicked in at much higher concentrations than the activation of PPAR γ and the linear CRC model could be used to derive the EC_{10} (Fig. S7 and S8†). The SR were high, ranging from 14 to 50, with a mean of 26.

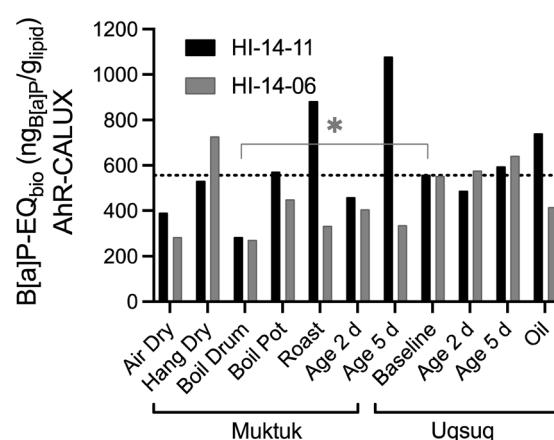


Fig. 3 Comparison of benzo[a]pyrene equivalent concentrations $\text{B}[a]\text{P-EQ}_{\text{bio}}$ in the AhR GeneBLAzer assay between the two animals HI-14-11 (black bars) and HI-14-06 (grey bars). Data in Table S7.† The dotted line refers to the mean of the baseline samples.



The baseline samples were again consistent for both specimens with a mean Rosiglitazone-EQ of $4.85 \text{ ng}_{\text{Rosiglitazone glipid}}^{-1}$ (Fig. 4). Food preparation generally did not change or decrease the effect with the exception of the oil sample, which was higher for HI-14-11 and much lower for HI-14-06.

Since PPAR γ plays a role in lipid metabolism and is also activated by long-chain alkane carboxylic acids,²⁸ it is possible that coextracted lipids would interfere if also fatty acids were coextracted. To evaluate this possibility, the % weight gain was also plotted against the Rosiglitazone-EQ (Fig. S9†) but there was no positive association. Therefore, we deem the contribution of natural fatty acids as negligible, also because they would be fully charged and charged chemicals do not partition to PDMS.²⁹

3.2.3. Activation of the hormone receptors. Most extracts did not activate the investigated hormone receptors up to cytotoxic concentrations. All blanks were clean (Fig. S4†). A few samples slightly activated ER α -bla (Fig. S10 and S11†) but only at concentrations higher than IC₁₀, so this effect is invalid and presumably caused by the cytotoxicity burst. This is a different situation than for AhR CALUX, which already had upwards trending CRCs and IC₁₀ was just around the 10% effect level.

Five samples activated the AR-bla (Fig. S12 and S13†), namely both Muktuk samples dried on the ground (Air Dry) and also Muktuk Roast, Uqsuq Age 5 Days and Uqsuq Oil of HI-14-06. The low androgenic effects with EC₁₀ ranging from 0.7 to 2.7 $\text{ng}_{\text{glipid}} \text{ L}_{\text{bioassay}}^{-1}$ (Table S4†) were presumably not caused by chemicals in the whales, where effects were absent, but introduced by contamination during food preparation. In contrast no activation was observed in GR-bla (Fig. S14 and S15†) and PR-bla (Fig. S16 and S17†).

Environmental pollutants are known to interfere with the hormone systems of marine mammals,³⁰ but the effects might be much more subtle than the direct binding to a hormone receptor.

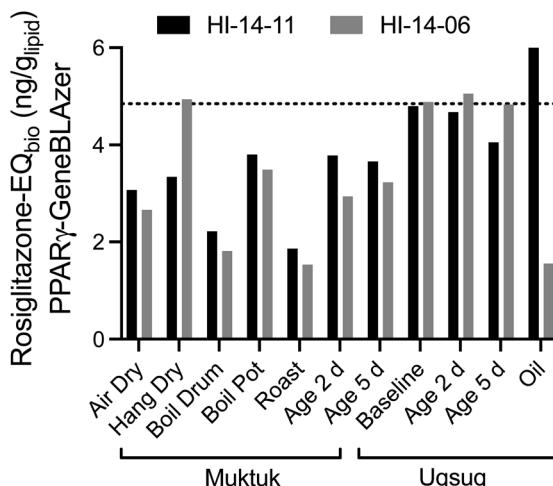


Fig. 4 Comparison of Rosiglitazone-EQ_{bio} in the PPAR γ GeneBLAzer assay between the two animals HI-14-11 (black bars) and HI-14-06 (grey bars). Data in Table S7.† The dotted line refers to the mean of the baseline samples.

3.2.4. Activation of the oxidative stress response. The blanks did not show any effect in AREc32 (Fig. S4†). All samples activated the oxidative stress response (Fig. S18 and S19†). SR ranged from 5 to 25 for the 8 samples where cytotoxicity was measurable. The pattern of B[a]P-EQ_{bio} for AREc32 (Fig. 5) resembled that of AhR-bla (Fig. 3), apart from the Muktuk Roast sample that was much lower in comparison. HI-14-06 had again the lowest B[a]P-EQ_{bio} for the Uqsuq Oil, followed by Roast.

3.2.5. Comparison of the bioassay responses. Comparing all activation data on the EC₁₀ or EC_{IR1.5} level (Fig. S20a†), the PPAR γ -bla was the most sensitive bioassay with lowest effect concentrations, followed by AREc32, AR-bla (for the few active samples) and AhR-CALUX. The high response of PPAR γ -bla was unexpected, and effects from endogenous compounds cannot be ruled out. AREc32 had also been more sensitive than AhR-CALUX in dugong blubber extracted with the same method.⁹

Blubber, liver, kidney and brain-tissues of harbour porpoises, harbour seals, ringed seals and orcas from the North Sea and the Baltic Sea were also evaluated with AhR-CALUX, PPAR γ -bla and AREc32.^{31,32} While effects were not converted to lipid concentrations in these studies,^{31,32} the direct comparison of the effect concentrations based on PDMS indicated that the mixture effect levels were very similar between the Canadian belugas and the marine mammals from European seas (Fig. S20b†).

3.3 Comparison of concentrations of analytes in lipids from direct extraction and via PDMS extraction

Ideally, chemical concentrations used for iceberg modelling should apply to the same type of in-tissue-PDMS extracts (converted into lipid-based concentrations using eqn (7)) as the extracts used in the bioassays. Most chemical analyses on the beluga blubber samples had, however, been done on direct lipid extracts.³ The analysis of a selection of chemicals (exclusively POPs) in in-tissue-PDMS extracts allows for a comparison of the

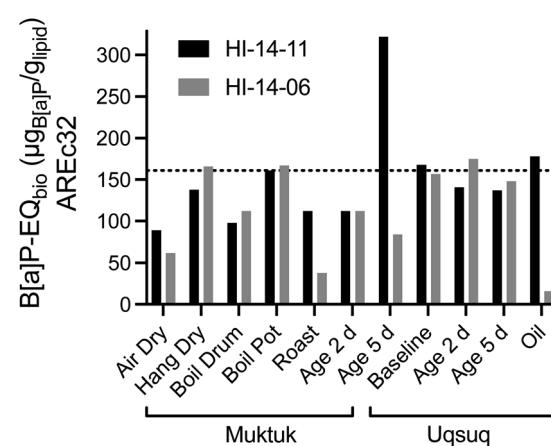


Fig. 5 Comparison of B[a]P-EQ_{bio} in the AREc32 assay between the two animals HI-14-11 (black bars) and HI-14-06 (grey bars). Data in Table S7.† The dotted line refers to the mean of the baseline samples.



final lipid-based concentrations obtained by either direct lipid extraction (Table S2†) or PDMS extraction (Table S5†). Excellent agreement between both methods (shown in Fig. S21†) justifies using the analytical data from direct extraction for the iceberg modelling. We caution that the PDMS extracts used for chemical analysis underwent an acid silica clean-up, while the bioassays were subjected to raw extract to capture persistent and non-persistent chemicals.

3.4 Iceberg modelling

34 Chemicals were quantified in the TF samples but EC₁₀ values for AhR-CALUX were available for only 14 of them (Table S6†). The hexachlorocyclohexanes, naphthalene, acenaphthylene, acenaphthene and fluorene were too volatile to be tested in a plate-based bioassays because their predicted bioassay medium-air partition constants $K_{\text{medium/air}}$ was below the threshold of 10⁴ (Fig. S22†).¹⁹ Hexachlorobenzene was newly characterized for the present study because its concentrations were fairly high but its $K_{\text{medium/air}}$ is also close to the threshold (Fig. S22†), which was also the case for mirex, which had measured effect concentrations that are likely to be rather uncertain due to potential losses of chemical from the assay plate during the 24 h incubation at 37 °C. Of the remaining chemicals 11 were either inactive or cytotoxic before activation started or they precipitated before any activity could be determined. A comparison with the predicted baseline toxicity IC₁₀ (Fig. S23†) showed that many of the active chemicals were close to baseline toxicity.³³ Highly specific in AhR-CALUX were PCB118, chrysene and B[a]P (Fig. S23a†).

These and other PAHs contributed a large fraction to B[a]P-EQ_{chem} in HI-14-11 Muktuk Roast (Fig. 6, and S24a†). In contrast, the consistently high B[a]P-EQ_{chem} in HI-14-06 stemmed rather from PCBs and PBDEs (Fig. 6, and S24b†). If Fig. 3 and 6 are superimposed, it is evident that their patterns are very similar, although the B[a]P-EQ_{chem} is very much lower than the B[a]P-EQ_{bio}.

The B[a]P-EQ_{chem} of the detected chemicals explained >1% of the AhR-activating effect (B[a]P-EQ_{bio}) in all samples of HI-14-

06, with highest percentage effect explained by HI-14-06 Muktuk Air Dry (3.7%), Muktuk Boil Large Drum (3.2%) and Muktuk Roast (2.5%), while HI-14-11 preparations had much lower explained fractions B[a]P-EQ_{chem}/B[a]P-EQ_{bio} with exception of Muktuk Boil Large Drum and Roast of HI-14-11 (Fig. S25a†). This seems a small fraction but is not implausible given that there are thousands of chemicals in complex mixtures.

In PPAR γ -bla only phenanthrene, fluoranthene, PCB28 and PDDE47 were active (Table S6†) and their Rosiglitazone-EQ_{chem} explained <0.00018% of the Rosiglitazone-EQ_{bio} (Fig. S25b and Table S7†).

In AREc32, the B[a]P-EQ_{chem} of the six active chemicals, HCB, endosulfan, anthracene, fluoranthene, chrysene and B[a]P (Table S6†) explained <0.013% (Fig. S25c and Table S7†) and here the PAHs were the largest contributors to B[a]P-EQ_{chem}.

Many different PAHs have been detected in marine mammals.¹ Hence it is conceivable that some of the gap between B[a]P-EQ_{chem} and B[a]P-EQ_{bio} could be closed by additional PAHs, such as alkylated and otherwise substituted PAHs. The accompanying study³ had not included polychlorinated dibenzodioxins (PCDD) and dibenzofurans (PCDF) as target analytes in the analytical methods. If PCDD and PCDFs were included in the analysis, almost 100% of the effect in AhR-CALUX were explained by PCDD/PCDFs in a previous study with extracts from dugong blubber.⁹ However for the activation of oxidative stress response more than 98% of the effect still remained unexplained when PCDD and PCDFs were included in iceberg modelling in dugong blubber.⁹

Iceberg modelling was also performed on PDMS extracts from various tissues of harbour porpoises, harbour seals, ringed seals and orcas from European seas.³² The BEQ_{chem} for the AhR-CALUX were dominated by the PCBs and BEQ_{chem} often explained more of the BEQ_{bio} than in the present study (Fig. S25a†). The picture was very similar for PPAR γ -bla between the present study and ref. 32 with <<0.1% of BEQ_{bio} explained by BEQ_{chem}. As for AhR-CALUX, the BEQ_{chem} of AREc32 were much more variable in ref. 32 but often explained a higher fraction of BEQ_{bio} than in the present study.

4. Discussion

Assessing the risk of contaminant exposure arising from the dietary intake of TFs is exceptionally challenging because it has to be weighed against the enormous social, cultural and nutritional benefits of TFs for indigenous populations. Such risk assessment relies typically on the quantification of a selection of individual contaminants in solvent extracts of TFs and the comparison of their concentrations with threshold values separating acceptable from unacceptable exposures. Limitations of this approach include that only a small fraction of the bioactive compounds present in TFs are being quantified and their ability to exert toxic effect in concert is generally not taken in account.

A bioassay-based quantification of mixture effects using a PDMS-based extraction procedure as introduced here allows for a more comprehensive and unbiased chemical risk

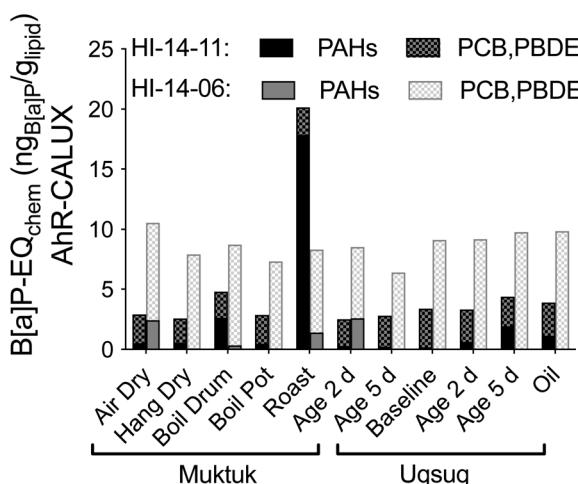


Fig. 6 Contribution of nonpersistent PAHs and persistent PCBs and PBDEs to B[a]P-EQ_{chem} in the AhR-CALUX assay. Data in Table S7.†



assessment of food contaminants, because it does not overlook any chemicals but accounts for the concerted action of both, persistent and nonpersistent bioactive chemicals. The iceberg modelling revealed that the contaminant concentrations in the exhaustive lipid extracts of different beluga TFs reported previously³ can only explain a tiny fraction of the observed mixture effects. The PDMS-extraction method combined with bioassays has previously been applied to tissues from other marine mammals such as dugongs,³⁴ porpoises, whales and seals³¹ and also for human adipose tissue.³⁵ However, it is applied here for the first time in the context of food in general and TFs in particular.

Bioassays have found use in dietary risk assessment previously. Specifically, the AhR-CALUX assay has been widely used to assess dioxin-like residues in food and feed for 20 years,^{36–38} and recently detailed recommendations have been given for the use of AhR-CALUX assays to quantify dioxins and PCBs in EU-regulated foods.^{4,39,40}

These bioassay methods are typically only applied to exhaustive lipid extracts, from which lipids were removed through a clean-up process that is targeted to remove not only lipids but also nonpersistent organics. This is appropriate if one is only interested in dioxin-like chemicals, but bioactivity can also be introduced by nonpersistent chemicals. Our approach is unbiased with respect to the extraction of neutral organic chemicals, although the extraction efficacy of ionizable organic chemicals is admittedly limited.²⁹

The maximum level of mixtures of PCDD/Fs and dioxin-like PCBs accepted in pork for consumption is defined as 1.25 pg_{WHO-PCDD/F-PCB-TEQ} g_{lipid}⁻¹ and the action levels is 0.75 pg_{WHO-PCDD/F-TEQ} g_{lipid}⁻¹.⁶ These values were derived for mixtures of 7 PCDDs, 10 PCDF and 12 planar PCBs from chemical analysis and would be equivalent to TCDD-EQ_{chem} accounting only for these 29 POPs. The TCDD-EQ_{chem} were shown to agree reasonably well with the TCDD-EQ_{bio} of POPs extracted from pork with exhaustive extraction/cleanup using different AhR-CALUX assays, among them also the cell line we used.⁶ Pork meat contained 0.30 to 5.29 pg_{WHO-PCDD/F-TEQ} g_{lipid}⁻¹, which would be 49 to 860 times lower than the TCDD-EQ_{bio} found in the baseline beluga whale samples.

This comparison is an indication that a lot of BEQ_{bio} detected in beluga stemmed from nonpersistent organics but could partially be contributed by a higher POP level in beluga than in pork. As the food consumption thresholds are defined only for POPs, future work should also include the analysis of PCDD/Fs and should apportion the effect contribution from persistent and non-persistent mixture components.

A concern raised when using whole extract testing is that the bioassays also respond to endogenous compounds present in the extracted mixtures, thereby raising the prospect of false positives. For this reason, a certain background level of effect must be considered acceptable, although this acceptable background effect, which will be bioassay-specific, still needs to be defined. Small quantities of coextracted endogenous lipids decrease the sensitivity of the assays by lowering the bioavailability of the dosed chemicals.⁴¹ This phenomenon is well characterized and a model has been developed to account for

this decrease in sensitivity.⁴¹ Applying this model, we could demonstrate that at the concentration of EC₁₀ the fraction of coextracted lipid was too low to cause any artifacts.

Presently, no effect-based trigger (EBT) values are available differentiating between acceptable and unacceptable exposure based on bioassay-based results, that would take the place of Minimum Risk Levels (MRL) for individual chemicals and the action levels related to WHO-PCDD/F-TEQ for animal food. The first experiences with application of effect-based methods for the quantification of mixture effects in the present study will need to be expanded to eventually develop a sufficiently large database to derive EBTs.

An analogy can be drawn to *in vitro* bioassays in water quality assessment, where initially mainly comparative assessments were performed, *e.g.*, to assess the treatment efficacy of a wastewater treatment plant or a drinking water plant and later the tools were also applied for surface water quality monitoring by using EBT values.¹⁵

Such comparative assessment can also be done in the present case, such as the comparison of effects seen in the extracts from Australian dugongs and Canadian Arctic belugas mentioned throughout the results section. The approach also allows for a comparison between mixture effects observed in extracts from different types of TFs and in the tissues of the two different whales. Overall, this work indicated that the observed effects are not strongly influenced by the TF preparation method, although drying near a smokehouse or roasting the Muktuk increases the AhR-activating effect. The findings thereby confirmed the chemical analysis, which noted the similar contamination levels in the different beluga TFs, but also the introduction of PAHs to roasted Muktuk and a sample aged close to a smokehouse. When comparing the two whales, we find that the BEQ_{bio} for the activation of the AhR were similar for corresponding samples from the two specimens but the effects in the older HI-14-06 was driven by classic POPs, while the effects in the younger HI-14-11 was driven by PAHs and unknown contaminants. Therefore, no single experimental approach is superior to the other, only in combination can chemical analysis and *in vitro* bioassays give us the full picture of environmental and food contamination.⁴²

5. Conclusions

This pilot study clearly demonstrated the usefulness of *in vitro* bioassays for obtaining a full picture of contamination with environmental pollutants and for comprehensively assessing the chemical risk of TF preparations. The measurement of mixture effects is useful to complement chemical analysis and can clearly demonstrate differences between sample types and preparation methods, but at this stage the evaluation is more comparative than absolute because acceptable effect levels (EBTs) are still missing.

In future work it is recommended to run the AhR-CALUX assay in duplicate, one without clean-up to evaluate the entire bioactive mixture but also one with clean-up to assess the fraction of persistent organic pollutants in the mixture. We



would expect then that the PAHs are destroyed with clean-up and what remains are persistent pollutants.

Author contributions

Beate Escher: conceptualisation, methodology, formal analysis, visualization, writing – original draft. Matt Binnington: methodology and investigation, writing – review & editing. Maria König: methodology and investigation, writing – review & editing. Ying Duan Lei: methodology, Frank Wania: conceptualisation, writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

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