TESTING THE APPLICABILITY OF PASSIVE SAMPLING TO CHEMICAL EXPOSURE AND EFFECT SCREENING IN MARINE WILDLIFE

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Introduction

Marine wildlife is encountering multiple threats of natural and anthropogenic origins, among which chemical pollution, particularly persistent bioaccumulative and toxic compounds (PBTs), potentially impacts the health status of exposed populations. Species with long life spans and high lipid content are especially prone to PBT accumulation to elevated levels. In some locations, certain PBT chemical groups in these types of organisms reach concentrations that have caused documented health effects in laboratory animals[1]. However, little is known about the effects of PBT mixtures accumulated over the life span of marine wildlife. Some of the key challenges lay in the traditional extraction and chemical cleanup methods for PBTs in lipid-rich matrices, and their subsequent instrument analysis. These tasks are time and cost intensive and require chemical selectivity, typically resulting in limited sample numbers and contaminant groups assessed.

To address these limitations, we propose to use a polymer sampling device to extract chemical mixtures from lipid-rich tissue based on equilibrium partitioning of chemicals between the polymer and lipid. To estimate the lipid normalised concentration of a chemical in the tissue sampled with such passive sampling devices, its partition coefficient between the polymer and the lipid phase ($K_{polymer\text{-}lipid}$) needs to be known. In addition, for such devices to be applicable to chemical mixtures, the partition coefficient is ideally constant across a wide range of physico-chemical properties. Passive sampling could significantly reduce the time and the vast amount of solvent and clean-up steps involved in conventional wet chemistry approaches and would allow inclusion of less persistent chemicals into bioassays. Among the variety of materials for a passive sampler, polydimethylsiloxane (PDMS) polymer has been extensively explored and applied to sample neutral organic pollutants in abiotic matrices, such as air[2], water[3], soil[4] and sediment[4]. However, there have been so far only few attempts to test and validate such methods in complex biological matrices[5,6]. Pioneering work by Jahnke et al using PDMS based passive samplers for organochlorine pesticides and PCBs in fish oil indicates, however, that the sorptive properties of PDMS for non-polar chemicals appears to be matrix-independent[7]. The present study attempts to further extend the evaluation of PDMS-based passive sampling in biota to very hydrophobic chemicals of toxicological significance in Australian marine wildlife, such as dugongs[8]. The $K_{PDMS\text{-}lipid}$ values for five polychlorinated dibenzo-*p*-dioxin (PCDD) congeners were determined using dugong blubber and compared with those of previously reported compound groups to evaluate the transfer efficiency of PBTs across a range of hydrophobicity. The method was further validated in an *in vitro* bioassay (chemically-activated fluorescence expression or CAFLUX), which is indicative of the activation of the arylhydrocarbon receptor (AhR), and responds specifically to dioxin-like chemicals, to investigate the compatibility of chemical extracts with the biological response and sensitivity of the assay, as well as potential matrix interference due to co-extracted lipid. With these objectives, this study aims to explore the applicability of PDMS-based approaches in exposure and effect assessment of chemical mixtures in biota.

Materials and methods

Chemicals: The selected PCDDs were purchased as a mixture stock (concentration 5 µg/mL per PCDD) from Accustandard. The PCDD congeners included 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 1,2,3,4,7,8-HxCDD, 1,2,3,4,6,7,8-HpCDD and 1,2,3,4,6,7,8,9-OCDD. The 1,2,3,4,7,8,9-heptachlorodibenzofuran (HpCDF) standard solution was purchased from Cambridge Isotope Laboratories. Solvents included acetone (analytical grade; Merck, Darmstadt, Germany), hexane (analytical grade; Sigma Aldrich, St Louis, MO USA), toluene (analytical grade; Merck, Darmstadt, Germany), methanol (analytical grade; Merck, Darmstadt, Germany) and dimethyl sulfoxide (DMSO) (Sigma Aldrich; St Louis, MO USA).

Dugong blubber: Dugong tissue samples were obtained in 2011 during a necropsy at the School of Veterinary Science of the University of Queensland. The dugong was a juvenile male and in good health condition, stranded on the coast of Noosa in Queensland, Australia. Soft blubber tissue, collected from the ventral line beneath the hypodermis was used to test the extraction methods.

Partitioning of PCDDs between PDMS and blubber: Partitioning experiments were conducted bi-directionally (PDMS→Blubber, and Blubber→PDMS) at 20±1°C to ascertain the equilibrium had been reached within the experimental timeframe of 24 hours.

For PDMS→Blubber**,** PDMS disks were first uploaded with dioxins using a method modified from Meyer et al[9]: 16x1 mm PDMS disks (~235 mg) were cut from medical grade PDMS sheet (Specialty Silicone Products, Inc. Ballston, Spa, NY, USA), soxhlet cleaned (300 ml hexane followed by 300 mL methanol, for 2 hours each) and air-dried. 87 µL of PCDD stock solution in toluene (5 µg/mL) was added to each of six 20 mL glass vials and gently blown down under nitrogen at 40°C. 500 µL methanol was then added to each vial. A single clean PDMS disk was placed in each vial and left overnight on a shaker at 100 rpm at 25°C to allow dissolution and partitioning of chemicals. The following day, Milli-Q water was added to each vial, hourly, in order to increase the volume and force the diffusion of dioxins into PDMS disks. The vials were left overnight on the shaker.

For Blubber→PDMS, 620 µL of PCDD stock solution (5 µg/mL) was gently blown down to near dryness under nitrogen at 40°C in a glass insert, and topped up to 71 µL in toluene, resulting in a concentration of 43.5 µg/mL. 16 thin blubber slices (approximately 0.25 g each) were cut and each of 8 pairs was placed in individual wells of a 24-well plate. Three of these pairs were spiked with 10 µL of the concentrated PCDD standard and left overnight for diffusion of the spiked chemicals into the tissue. The remaining pair served as the untreated control. The following day, a PDMS disk was sandwiched between each blubber pairs and left for 24 hours.

Sample extraction: After 24 h partitioning, PDMS disks were removed from the blubber sandwiches and their surfaces wiped thoroughly with acetone to remove adhering lipids. Each PDMS disk was immersed in 5 mL hexane in a glass vial and left overnight on a shaker at 100 rpm at 25°C for extraction. Then the hexane extract was transferred into a glass test tube and the extraction of PDMS was repeated. The combined hexane extract was then concentrated, transferred to a 50 µL glass insert and blown to near dryness, and reconstituted in 75 µL toluene containing 300 pg/µL HpCDF as an internal standard.

Lipid determination: The lipid content of the blubber was determined gravimetrically. Briefly, approximately 8 g of blubber was acid digested using 4 M HCl at 70-80 ˚C for 3-4 hours. The acid digest was liquid-liquid extracted with 100 mL hexane and 150 mL warm water, followed by double extractions with 50 mL of hexane and 100 mL water. The hexane fraction was filtered through sodium sulphate and concentrated, transferred to pre-weighed flasks and further concentrated under a gentle stream of nitrogen until the weight was stable, and the percent lipid was calculated.

Instrumental analysis: PCDD concentrations in the PDMS samples were measured using a Hewlett Packard 5890 Series II gas chromatograph with an electron capture detector (GC-ECD) (Hewlett Packard, Ratingen, Germany) containing a 30 m×0.25 mm DB-5 fused silica capillary column. Five external standard dilution series contained 50 to 800 pg/µL of the same PCDD congeners used to spike samples, and 300 pg/µL HpCDF internal standard was used to calibrate the relative responses of each PCDD congener to HpCDF and to assure the quality of the results.

CAFLUX bioassay: The CAFLUX bioassay was based on rat hepatoma (H4IIE) cells transfected with green fluorescent protein under the control of dioxin response elements. Cells were cultured in Minimum Essential Medium alpha Medium (α-MEM) with L-glutamine and supplemented by 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin and 2% Geneticin at 37˚C in a 5% CO_2 incubator. All tissue culture media were purchased from Invitrogen (Carlsbad, CA USA). Cells were seeded in 96-well, black, clear-bottom microtitre plates with 100 µL of cell suspension at a density of 3 x 10^4 cells per well for 24 hours. Prior to dosing on the

plate, 6 μL of DMSO was added to near-dry PDMS extracts (only extracts from PDMS in contact with spiked blubber were the same as those used for instrumental analysis). The entire samples were then dissolved in 600 μL of medium and serially diluted using a mixing tray. Medium in the cell plate was then removed and 100 μL medium containing either standard TCDD (positive control), DMSO (negative control) or a sample dissolved in DMSO was added to the wells/cells from the mixing tray. The concentrations of the standard dilution series for the positive control ranged from 0.01-100000 nM TCDD, which captured a full dose-response window. After 24 and 48 hours, fluorescence was measured by a fluorometer (FLUostar OPTIMA; BMG LABTECH, Mornington, Australia) with an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Subsequently, cytotoxicity was measured using a MTS assay. Cells were incubated with 120 μL of a mixture of 2.4 mL of the Cell Titer 96 Aqueous Solution (Promega G3581) and 12 mL of fresh medium (DMEM-F12 without phenol red, 5% BFS, 1% non essential amino acids) per well for 2 hours. Absorbance was measured at 490 nm using the Fluostar.

Data analysis: The $K_{PDMS\text{-}lipid}$ for each dioxin congener was calculated by dividing the measured concentration of the compound in PDMS by the spiked concentration in lipid for the lipid→PDMS experiments, or a mass balance based concentration in lipid for the PDMS→lipid experiments. In the latter experiment, we assumed no losses of PCDDs to a third phase. Statistical analyses of $K_{PDMS\text{-}lipid}$ were performed using SigmaStat 3.5. Normality of the data was tested using the Kolmogorov-Smirnov test. The potential difference in $K_{PDMS\text{-}lipid}$ between the bi-directional partitioning experiments was evaluated using a one-way ANOVA. Statistical significance was considered at $p<0.05$.

The EC50 values of samples were derived by sigmoidal dose–response nonlinear regression curve fitting using GraphPadPrism 5.0 and concentration was expressed as fraction of the original sample. The chemical mixture burden in a sample was expressed as toxic equivalent concentrations (TEQ) relative to TCDD. The bioassay-based TEQ (TEQ_{bio}) was compared with the TEQ_{chem} derived from chemical analysis.

Results and discussion

$K_{PDMS\text{-}lipid}$ of dioxin congeners between PDMS and blubber: The $K_{PDMS\text{-}lipid}$ values for each dioxin congener were not significantly ($p>0.05$) different between the two partitioning directions, confirming that equilibrium was attained within 24 hours. The $K_{PDMS\text{-}lipid}$ values of the five dioxin congeners ranged from 0.028-0.05. These values are consistent with $K_{PDMS\text{-}lipid}$ previously determined for PCBs and OC pesticides[5] and demonstrate that $K_{PDMS\text{-}lipid}$-values for these three chemical groups are relatively low and constant across a wide range of hydrophobicity, varying only within a factor of 3. The measured $K_{PDMS\text{-}lipid}$ values were somewhat lower than those predicted as a constant based on the theoretically parallel relationship between $K_{lipid\text{-}water}$ and $K_{PDMS\text{-}water}$. Kinetic issues, instrumental errors and inaccurate measurement of $K_{PDMS\text{-}water}$ could be contributing factors for this discrepancy. Although this warrants more detailed investigations, the PDMS-based approach appears appropriate for the purpose of a screening method for PBT mixtures from biological samples given the uniform sampling across chemicals. Mass balance calculations allow the estimation of total chemical burden based on the fraction of PBT mixture extracted by PDMS from a biological sample for effect assessment.

Evaluation of matrix interference due to lipid in CAFLUX: Weight monitoring revealed an average gain of 1.3 mg of a PDMS disk after exposure to blubber, which suggests 0.3% lipid transfer into PDMS in the current experimental settings. While the residual lipid did not cause significant interferences during instrumental analysis after sample dilution, such an amount of lipid may be of concern to the toxicokinetics of TCDD and thus the biological response in the CAFLUX bioassay. However, a longer exposure time appears to be sufficient to compensate for this kinetic effect. Equally important is to know the response of the endogenous and exogenous AhR-active compounds already present in the blubber and retrieved by PDMS. Therefore, dilution series of (i) extracts from a single, unexposed PDMS disk, (ii) from a PDMS disk with co-extracted lipid (PDMS+lipid) was tested to evaluate their induction effect, and (iii) a dilution series of TCDD spiked into these two extracts was to

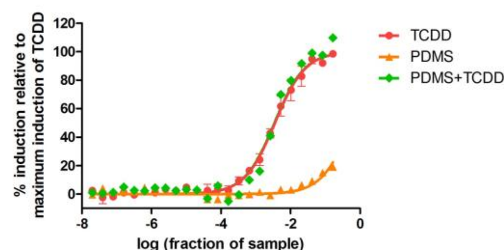

Figure 1. Dose-response evaluation of potential matrix interference due to PDMS

compare their dose-response curves with that of TCDD alone. Results from these tests showed that the induction of the PDMS extract was generally low with the highest induction signal of 20%, where the TCDD equivalent concentration was approximately two orders of magnitude lower than TCDD alone (Figure 1). Furthermore, lipid did not introduce a significant matrix effect (Figure 2). The dose-response curves of TCDD and PDMS+TCDD coincided, further confirming little matrix interference due to PDMS. Similar to PDMS alone, the extracts from PDMS+lipid did not cause any observable induction. The dose-response curves of TCDD, PDMS+TCDD, and PDMS+lipid+TCDD, agreed well, particularly in the linear range and the bottom, including 50% induction effect, but not for the first three dilutions of PDMS+lipid+TCDD, where super induction was observed in an excess of 20-40% compared to the top of TCDD alone. This finding means TEQ derivation based on EC50 is robust even with the current amount of lipid in the bioassay. The super induction of PDMS+lipid+TCDD is more likely to be a result of faster uptake of TCDD by lipid than of the induction caused by compounds contained in the blubber, as the PDMS+lipid samples did not cause significant induction. This hypothesis will be tested by applying a longer exposure time in future experiments.

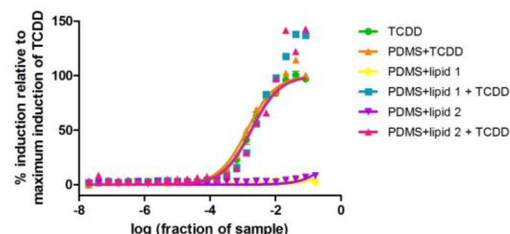

Figure 2. Dose-response evaluation of potential matrix interference due to lipid

CAFLUX responses of PDMS extracts from spiked blubber: We observed relatively small, but consistent variations between TEQ_{chem} and TEQ_{bio}. The bioassay could explain 70-85% of the instrumentally derived chemical burden. The samples did not reach the plateau of 100% induction in their dose-response curves. As with the controls discussed above, there was no significant matrix interference due to co-extracted lipid, and therefore quenching by lipid can be ruled out. Solubility limits and cell viability decline due to high concentrations could account for the observations. The extracts of PDMS after partition experiments with spiked blubber resulted in final concentrations of ~20,000 ng/L for each dioxin congener plus 25,000 ng/L for HpCDF in the very first of the dilution series, as compared to the highest concentration of 318 ng/L for the TCDD standard alone.

The average ratio of TEQ of the PDMS extract to that of the original spike was 0.02, which means approximately 2% of the AhR-mediated toxicity burden could be transferred from blubber to PDMS and dosed into the cells. This transfer efficiency echos the low $K_{PDMS\text{-}lipid}$ values. Ultimately, this means that the method requires upscaling (e.g. increasing the PDMS volume) to meet the sensitivity of the CAFLUX bioassay and beyond when applying PDMS-based passive sampling approaches to effect assessment of chemical mixtures in biological samples.[9]

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