

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

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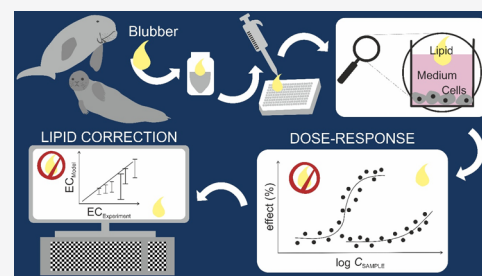


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ABSTRACT: Extraction of chemicals from biota leads to co-extraction of lipids. When dosing such extracts into in vitro bioassays, co-dosed lipids act as an additional phase that can reduce the bioavailability of the chemicals and the apparent sensitivity 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-filtration⁶ 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 0.5⁵ 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.²⁵ 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 model²⁶ 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 S11). 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 $K_{\text{medium/air}}$ of the tested chemicals was above the volatility cut-off of $K_{\text{medium/air}} > 10^4$.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.

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

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

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).

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

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

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

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

Triolein served as a surrogate for co-dosed lipids, and experimental triolein/water partition constants, $K_{\text{triolein/w}}$ were used as a proxy for $K_{\text{lipid/w}}$. 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

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

We assume that EC_{cell} is constant²⁸ and independent of Vf_{lipid} , that is, EC_{cell} with lipid in the system ($EC_{\text{cell},x\% \text{ lipid}}$) is equal to EC_{cell} without lipid in the system ($EC_{\text{cell},0\% \text{ lipid}}$). Therefore, the EC with lipids in the system ($EC_{x\% \text{ lipid}}$) can be predicted from the measured EC without lipids in the system ($EC_{0\% \text{ lipid}}$) for any Vf_{lipid} (eq 7).

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

MATERIALS AND METHODS

Chemicals and Materials. The test chemicals for the AREc32 assay were benzo[*a*]pyrene (Sigma-Aldrich, #50-32-8, $\geq 96\%$) 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, $\geq 98\%$) (chemical structures in Figure S12). 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, $\geq 99.5\%$). Triolein was purchased from Sigma-Aldrich ($\geq 99\%$). 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 line²⁹ obtained by the courtesy of C. Roland Wolf, Cancer Research UK, and the AhR activation with the AhR-CALUX cell line³⁰ 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

(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 cells³¹ are given in Section S11 and Table S11. 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 S11).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 S13.

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 V_{lipid} and the V_{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). V_{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 V_{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, $K_{\text{lipid/PDMS}}$, of 30 $L_{\text{PDMS}} L_{\text{lipid}}^{-1}$ 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

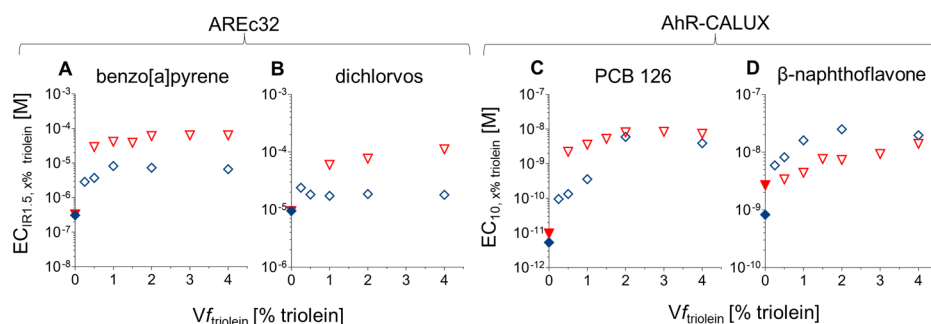


Figure 1. Comparison between “0 h” samples (red triangles) and “24 h” samples (blue diamonds) in dependency of the $V_{f_{\text{triolein}}}$: $EC_{IR1.5}$ in the AREc32 assay for (A) benzo[a]pyrene and (B) dichlorvos and EC_{10} in the AhR assay for (C) PCB 126 and (D) β -naphthoflavone. The filled symbols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

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 S11). 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 $V_{f_{\text{triolein}}}$ (Figures S14–13) were well repeatable, and therefore, a common $EC_{x\% \text{ triolein}}$ was derived from triplicate measurements³⁵ (Table S12).

Dosing-spiked triolein to the cells resulted in a higher $EC_{x\% \text{ triolein}}$ than without triolein ($EC_{0\% \text{ triolein}}$) for all test chemicals, and $EC_{x\% \text{ triolein}}$ increased with the increasing $V_{f_{\text{triolein}}}$ (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 $V_{f_{\text{triolein}}}$. 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 $EC_{10,0\% \text{ triolein}}$ 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 S12). 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 $EC_{0\% \text{ triolein}}$ of the “0 h” experiments was used to predict the $EC_{x\% \text{ triolein}}$ 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 S12 and Table S13.

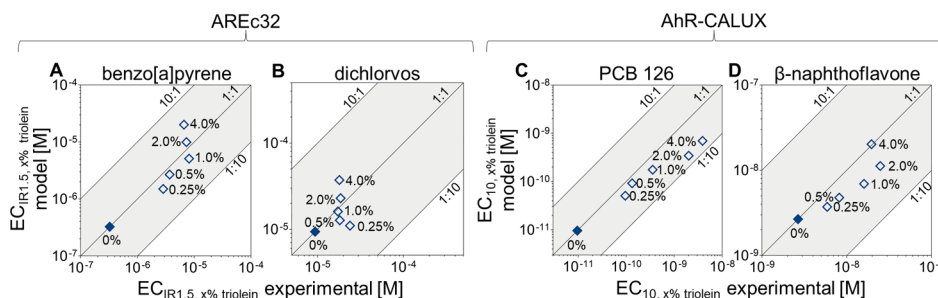


Figure 2. Comparison between experimental EC of the “24 h” experiments and EC predicted with eq 7: $EC_{IR1.5, x\% \text{ triolein}}$ for the AREc32 assay for (A) benzo[*a*]pyrene and (B) dichlorvos and EC_{10} for the AhR-CALUX assay for (C) PCB 126 and (D) β -naphthoflavone. The filled symbols represent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

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 $EC_{IR1.5, x\% \text{ triolein}}$ was increased by a factor of 9–25 compared to $EC_{IR1.5, 0\% \text{ triolein}}$ (Figure 1A) but only by a factor of 2–3 for dichlorvos (Figure 1B). The ratio between modeled and experimental $EC_{IR1.5, x\% \text{ triolein}}$ 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 126³⁸ 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 $EC_{10, x\% \text{ triolein}}$ within a factor between 1.5–5.8 (Figure 2C). $EC_{10, 0\% \text{ triolein}}$ for β -naphthoflavone were 2–9 times lower than $EC_{10, x\% \text{ triolein}}$ (Figure 1D). $EC_{10, x\% \text{ triolein}}$ was predicted within a factor of 1.0–2.3 (Figure 2D).

$EC_{IR1.5, x\% \text{ triolein}}$ for benzo[*a*]pyrene and dichlorvos and $EC_{10, x\% \text{ triolein}}$ 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 $K_{\text{triolein}/w}$ 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

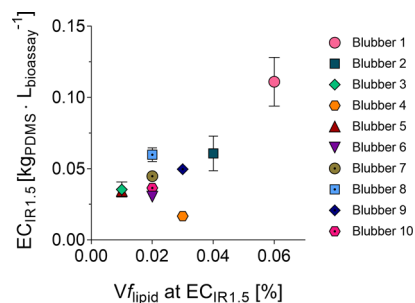


Figure 3. Relationship between $EC_{IR1.5}$ and Vf_{lipid} measured in the AREc32 assay (“24 h” dosing). Bars represent the standard error (in some cases smaller than the symbols). PDMS extracts from blubber tissue are plotted using different symbols: blubber 1–8 from dugong, blubber 9 from ringed seal, 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 $\log K_{\text{lipid}/w}$ is linearly correlated with $\log K_{ow}$, $\log K_{\text{medium}/w}$ and $\log K_{\text{cell}/w}$ are slightly offset from that correlation for less-hydrophobic chemicals because of the contribution of sorption to proteins and water in the

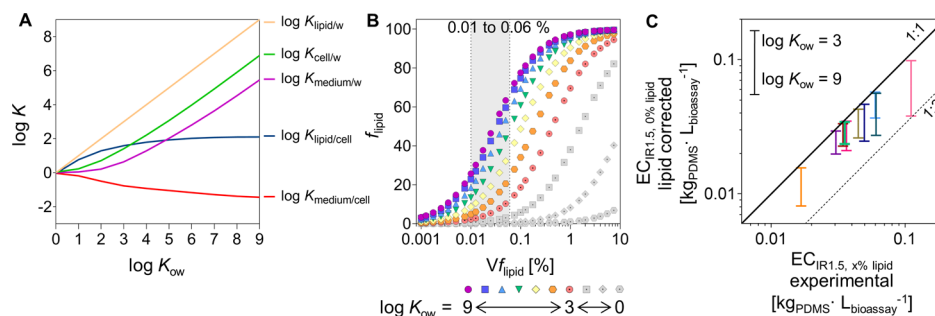


Figure 4. (A) Partition constants $\log K_{\text{lipid/water}}$, $\log K_{\text{cell/water}}$, $\log K_{\text{medium/water}}$, $\log K_{\text{lipid/cell}}$ and $\log K_{\text{medium/cell}}$ as a function of $\log K_{\text{ow}}$. (B) Relationship between f_{lipid} and $V_{f_{\text{lipid}}}$ for $\log K_{\text{ow}}$ between 0 and 9. The shaded area highlights $V_{f_{\text{lipid}}}$ 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 $\log K_{\text{ow}}$ 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, $\log K_{\text{medium/cell}}$ and $\log K_{\text{lipid/cell}}$ are fairly constant at $\log K_{\text{ow}} > 3$ (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 $V_{f_{\text{lipid}}}$. Even for a low $V_{f_{\text{lipid}}}$ of 0.01%, the f_{lipid} for chemicals with $\log K_{\text{ow}}$ between 3 and 9 ranges from 2–26%. For $V_{f_{\text{lipid}}}$ of 0.06%, f_{lipid} increases to 12–68% for the same $\log K_{\text{ow}}$ range. f_{lipid} is only moderately dependent on the hydrophobicity for chemicals, with $\log K_{\text{ow}}$ between 3 and 9 (Figure 4B). On this understanding, we estimated a range of lipid-corrected $\text{EC}_{\text{IR1.5, 0% lipid}}$ in Figure 4C. The lipid correction was performed for the “24 h” PDMS-based blubber extracts for $\log K_{\text{ow}}$ range between 3 and 9. The span of lipid-corrected $\text{EC}_{\text{IR1.5, 0% lipid}}$ and the distance to the experimentally determined value is dependent on $V_{f_{\text{lipid}}}$. Although the lipid correction covered 6 orders of magnitude of K_{ow} , the corrected span of $\text{EC}_{\text{IR1.5, 0% lipid}}$ ranged up only to a factor of 3. Up to $V_{f_{\text{lipid}}}$ of 0.27%, the deviation between non-corrected EC to the lipid-corrected value $\text{EC}_{\text{IR1.5, 0% lipid}}$ is expected to be less than 1 order of magnitude.

Lipid correction to a precise $\text{EC}_{0\% \text{ lipid}}$ 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 $V_{f_{\text{lipid}}}$ 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>.

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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Notes

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