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Modeling Exposure in the Tox21 *in Vitro* Bioassays

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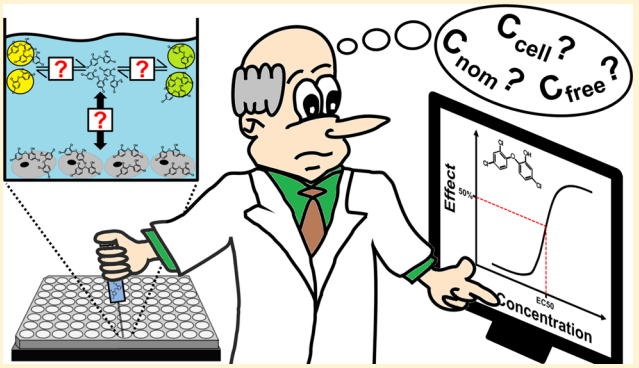
Supporting Information

ABSTRACT: High-throughput *in vitro* bioassays are becoming increasingly important in the risk characterization of anthropogenic chemicals. Large databases gather nominal effect concentrations (C_{nom}) for diverse modes of action. However, the biologically effective concentration can substantially deviate due to differences in chemical partitioning. In this study, we modeled freely dissolved (C_{free}), cellular (C_{cell}), and membrane concentrations (C_{mem}) in the Tox21 GeneBLAzer bioassays for a set of neutral and ionogenic organic chemicals covering a large physicochemical space. Cells and medium constituents were experimentally characterized for their lipid and protein content, and partition constants were either collected from the literature or predicted by mechanistic models. The chemicals exhibited multifaceted partitioning to proteins and lipids with distribution ratios spanning over 8 orders of magnitude. Modeled C_{free} deviated over 5 orders of magnitude from C_{nom} and can be compared to *in vivo* effect data, environmental concentrations, and the unbound fraction in plasma, which is needed for the *in vitro* to *in vivo* extrapolation. C_{cell} was relatively constant for chemicals with membrane lipid−water distribution ratios of 1000 or higher and proportional to C_{nom}. Representing a sum parameter for exposure that integrates the entire dose from intracellular partitioning, C_{cell} is particularly suitable for the effect characterization of chemicals with multiple target sites and the calculation of their relative effect potencies. Effective membrane concentrations indicated that the specific effects of very hydrophobic chemicals in multiple bioassays are occurring at concentrations close to baseline toxicity. The equilibrium partitioning model including all relevant system parameters and a generic bioassay setup is attached as an excel workbook to this paper and can readily be applied to diverse *in vitro* bioassays.



1. INTRODUCTION

In vitro cell bioassays present a simple, fast and cost-efficient tool for the effect characterization of chemicals. Particularly when run in high-throughput screening (HTS) format, *in vitro* bioassays will meet the high demands of effect data for the increasing number and variety of anthropogenic chemicals. The dose metric in *in vitro* testing is typically the nominal concentration (C_{nom}). However, only the freely dissolved concentration (C_{free}) is available for uptake and thus driving effects.[1] C_{free} should be quantified for interassay comparison and interpretation of *in vitro* effect data and for subsequent use in the quantitative *in vitro* to *in vivo* extrapolation (QIVIVE).[2−4] The total cellular concentration (C_{cell}) is directly related to C_{free} by the cell−water partition constant ($K_{cell/w}$) under equilibrium conditions and can serve as a proxy for the biological dose of chemicals.[5,6]

In *in vitro* bioassays, C_{free} can be depleted by sorption to medium constituents, such as the proteins and lipids from the medium.[7,8] Continuous reduction can be caused by evaporation,[9] partitioning into plate materials,[10] and degradation processes.[11] The relative importance of these processes depends on the bioassay setup and the physicochemical properties of the tested chemical.[12,13] These differences may result in a large and nonproportional deviation of C_{free} from C_{nom} and hence to reduced sensitivity of and low comparability between *in vitro* bioassays.

Recent studies demonstrated the variability of *in vitro* bioavailability as a result of chemical equilibrium partitioning between medium constituents and cells in different bioassay formats.[8,13] These studies normalized C_{cell} to the total cellular lipid content leading to underestimated C_{cell} of hydrophilic chemicals and chemicals that exhibit high protein sorption, such as anions.[14] Relevant partition constants were predicted by the chemicals' octanol−water partition constants (K_{ow}). However, the sorption of chemicals to biomolecules such as serum proteins can be very complex and dependent on various molecular interactions between the chemical and the sorbent, in

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particular for ionogenic organic chemicals (IOCs), leading to high errors in the prediction of partition constants via K_{ow}.[14,15]

A large *in vitro* database is available on the iCSS ToxCast Dashboard (https://actor.epa.gov/dashboard) provided by the US EPA, in which data from ToxCast (https://www.epa.gov/chemical-research/toxicity-forecasting) and Tox21 (https://www.epa.gov/chemical-research/toxicology-testing-21st-century-tox21) are collected. While ToxCast included more than 700 bioassays, the number of chemicals was limited to ∼1800. In contrast, in Tox21, ∼10,000 chemicals (Tox21 10K library) were tested in ∼50 *in vitro* bioassays covering various steps of the cellular signaling pathway.[16] Reporter gene assays based on various nuclear receptors and transcription factors are included in Tox21, and experiments were performed in HTS format in 1536 well plates using robotic systems.[16] In the iCSS ToxCast Dashboard, measured toxicity is expressed as 50% activity concentration (AC50), hereinafter referred to as 50% effect concentration (EC50). The use of 1536 well plates and the very small medium volumes of 5−6 μL hampered the analytical quantification of C_{free} and C_{cell}; thus, all reported effect data are based on C_{nom}.

As representative examples of the Tox21 bioassays, we chose 26 bioassays of the GeneBLAzer platform,[17−19] which use various human cell lines with reporter genes for diverse receptors. The cells constitutively coexpress a fusion protein of the ligand binding domain of the human nuclear receptor or transcription factor (DNA-binding domain).[17] Upon ligand binding, the fusion protein translocates to the nucleus and binds to the Upstream Activator Sequence (UAS) which controls the transcription of β-lactamase. The amount of β-lactamase is quantified via the changing fluorescence property of a specific substrate that is transformed by β-lactamase.[17]

This study aimed (i) at modeling 50% effective freely dissolved, cellular and membrane concentrations ($EC50_{free}$, $EC50_{cell}$, $EC50_{mem}$) of 100 neutral, anionic, cationic, and multiprotic (multiple acid and base functional groups) river pollutants with heterogeneous physicochemical properties and multiple modes of action[20] that have been comprehensively assessed with the Tox21 GeneBLAzer assays and (ii) to discuss the utility of the different dose metrics. As the basis for our model, relevant system parameters such as water, protein, and lipid contents in the assay media and cells were determined by experiment. Bovine serum albumin (BSA) and phospholipid liposomes (lip) were used in the modeling approach as surrogates for assay proteins and lipids, respectively. For both sorptive matrices, several databases of experimental partition constants are available in the literature,[14,15,21−23] and validated prediction approaches have been developed.[15,24−26] These were used to derive speciation-corrected distribution ratios at pH 7.4 between both surrogates and water ($D_{BSA/w}$, $D_{lip/w}$). We hypothesized that the chemicals would exhibit multifaceted partitioning between and within the medium and the cells dependent on the physicochemical properties of the chemicals and the assay setup, leading to a large variability in exposure between different chemicals and bioassays. This diversity could have hampered the risk prioritization within the Tox21 program because the differences in the EC50 could be due to both, differences in exposure and differences in the reporter gene construct of the particular cell line. Here, we address the former point, the variability in exposure and the insufficiency of nominal concentrations as a dose metric. Experimental determination of assay parameters as well as the use of state-of-the-art methods for the prediction of partition constants will enhance the precision and accuracy of model outputs while extending their domain of applicability to complex chemicals such as IOCs. Linking effect data to C_{free} and C_{cell} would improve the comparability between different *in vitro* bioassays, the analysis and interpretation of *in vitro* effect data, as well as its suitability for the QIVIVE.[4,27]

2. THEORY

As described by Armitage et al.,[13] the partition properties of *in vitro* assays can be defined by the composition and volumes of their system compartments, such as the medium, cells, headspace, and well plate materials as well as the respective partition constants for the chemicals of interest. Since we assume equilibrium, kinetic processes such as evaporation, diffusion into well plate materials, cellular uptake kinetics, and degradation were neglected for this study.

Quantifying evaporative losses from the well would only be possible in closed systems with a defined headspace volume, which generally hampers *in vitro* toxicity testing of volatile chemicals in unsealed HTS plate format. Therefore, only nonvolatile chemicals (air−water partition constant ≤0.03) were chosen for this study. The binding to plate material may be an important and time-dependent influencing factor in aqueous media with low organic matter content, e.g., in the *Aliivibrio fischeri*,[28] the *Danio rerio* fish embryo,[9] and the algae toxicity test, for which considerable losses are generally expected to occur for neutral chemicals with a log K_{ow} > 3.[10] Availability of partition constants and diffusion coefficients to polystyrene is relatively low, impeding the integration of sorption to plate materials into the model so far.

Preliminary experiments showed that equilibrium partitioning kinetics between the medium and the cells can be slow and different between neutral chemicals and IOCs,[29] which can lead to an underestimation of C_{cell} for a large period of the test duration. Because of this variability, equilibrium might not be achieved within the typical assay duration of up to 24 h, requiring modeling approaches to quantify exposure over time in future studies.

Proteins and lipids are the major sorptive colloids for organic chemicals in *in vitro* bioassays.[12,30] Thus, we define that the medium and the cells are composed of their respective volumes of proteins ($V_{protein}$ in L, density 1.36 kg L^{-1}), lipids (V_{lipid} in L, density 1.0 kg L^{-1}), and water (V_w in L, density 1.0 kg L^{-1}) (eqs 1 and 2) and that components with a low sorptive capacity such as carbohydrates are neglected.

$$V_{medium} = V_{protein,medium} + V_{lipid,medium} + V_{w,medium} \quad (1)$$

$$V_{cell} = V_{protein,cell} + V_{lipid,cell} + V_{w,cell} \quad (2)$$

Assuming equilibrium state and similar physical conditions (pH) between and within the medium and the cells results in equal medium and intracellular C_{free} as well as defined C_{cell}, as illustrated in Figure 1.

BSA and lip were used as surrogates for all medium and cellular proteins and lipids, respectively. However, given that both medium and, foremost, cells are composed of innumerable proteins and lipids of variable structure, this could represent a source of uncertainty. The distribution ratios between medium and water ($D_{medium/w}$, L_w L_{medium}^{-1}) as well as cells and water ($D_{cell/w}$, L_w L_{cell}^{-1}) are given as a function of their respective volume fractions (*VF*) of proteins, lipids, and water and the chemicals $D_{BSA/w}$ and $D_{lip/w}$ (eqs 3 and 4).

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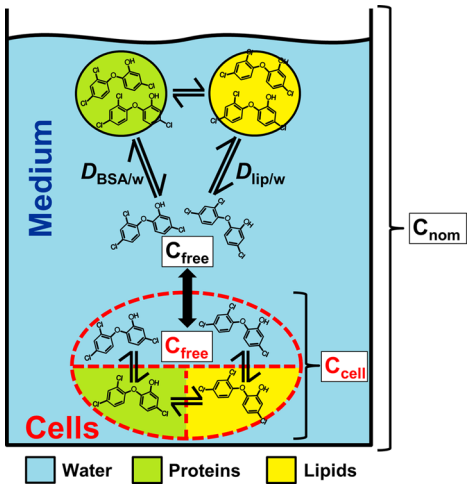

Figure 1. Mass balance model used for this study. The chemical partitioning was calculated from the distribution ratios between medium and cells at a medium pH of 7.4. Both compartments are composed of water, proteins, and lipids. Proteins and lipids are represented by BSA and lip.

$$D_{medium/w}\ (\text{pH } 7.4) = VF_{protein,medium} \times D_{BSA/w} + VF_{lipid,medium} \times D_{lip/w} + VF_{w,medium} \quad (3)$$

$$D_{cell/w}\ (\text{pH } 7.4) = VF_{protein,cell} \times D_{BSA/w} + VF_{lipid,cell} \times D_{lip/w} + VF_{w,cell} \quad (4)$$

$D_{BSA/w}$ ($L_w\ L_{BSA}^{-1}$) and $D_{lip/w}$ ($L_w\ L_{lip}^{-1}$) result from the fraction of neutral and ionized species at medium pH of 7.4 and their associated partition constants (eqs 5 and 6).

$$D_{BSA/w}\ (\text{pH } 7.4) = f_{neutral} \times K_{BSA/w}(\text{neutral}) + f_{ion} \times K_{BSA/w}(\text{ion}) \quad (5)$$

$$D_{lip/w}\ (\text{pH } 7.4) = f_{neutral} \times K_{lip/w}(\text{neutral}) + f_{ion} \times K_{lip/w}(\text{ion}) \quad (6)$$

Chemical partitioning between medium and cells ($D_{medium/cell}$, $L_{cell}\ L_{medium}^{-1}$) was quantified with eq 7.

$$D_{medium/cell}\ (\text{pH } 7.4) = \frac{D_{medium/w}}{D_{cell/w}} \quad (7)$$

The chemical fractions in the cells and the medium were calculated by a mass balance equation (eqs 8 and 9).

$$f_{cell} = \left(1 + D_{medium/cell} \times \frac{V_{medium}}{V_{cell}}\right)^{-1} \quad (8)$$

$$f_{medium} = 1 - f_{cell} \quad (9)$$

Fractions within the two compartments (cells and medium) were calculated by the respective phase volumes (proteins, lipids, and water). For instance, the freely dissolved fraction in the medium ($f_{free,medium}$) and the fraction in cell membranes (f_{mem}) are defined by eqs 10 and 11.

$$f_{free,medium} = \left(1 + D_{BSA/w} \times \frac{V_{protein,medium}}{V_{w,medium}} + D_{lip/w} \times \frac{V_{lipid,medium}}{V_{w,medium}} + D_{cell/w} \times \frac{V_{cell}}{V_{w,medium}}\right)^{-1} \quad (10)$$

Table 1. Assay-Specific Information of the 26 Evaluated Tox21 Reporter Gene Assays of the GeneBLAzer Panel

assay name	biological target	medium information	cell line	cell number
AR BLA agonist ratio	androgen receptor (AR)	6 μL of OptiMEM + 10% d-FBS	HEK293T	2000
AR BLA antagonist ratio	androgen receptor (AR)	5 μL of OptiMEM + 10% d-FBS	HEK293T	2000
AR BLA antagonist viability	cell viability	5 μL of OptiMEM + 10% d-FBS	HEK293T	2000
ER BLA agonist ratio	estrogen receptor (ER)	6 μL of DMEM phenol-red free +2% cs-FBS	HEK293T	5000
ER BLA antagonist ratio	estrogen receptor (ER)	5 μL of DMEM phenol-red free +2% cs-FBS	HEK293T	5000
ER BLA antagonist viability	cell viability	5 μL of DMEM phenol-red free +2% cs-FBS	HEK293T	5000
GR BLA agonist ratio	glucocorticoid receptor (GR)	6 μL of OptiMEM + 1% d-FBS	HEK293T	1500
GR BLA antagonist ratio	glucocorticoid receptor (GR)	5 μL of OptiMEM + 1% d-FBS	HEK293T	1500
GR BLA antagonist viability	cell viability	5 μL of OptiMEM + 1% d-FBS	HEK293T	1500
PPARG BLA agonist ratio	peroxisome proliferator-activated receptor γ (PPARG)	6 μL of DMEM phenol-red free + 1% cs-FBS	HEK293H	3000
PPARG BLA antagonist ratio	peroxisome proliferator-activated receptor γ (PPARG)	6 μL of DMEM phenol-red free + 1% cs-FBS	HEK293H	3000
PPARG BLA antagonist viability	cell viability	6 μL of DMEM phenol-red free + 1% cs-FBS	HEK293H	3000
ARE BLA agonist ratio	nuclear factor: (erythroid-derived 2)-like 2	5 μL of DMEM GlutaMAX + 1% FBS	HepG2	2000
ARE BLA agonist viability	cell viability	5 μL of DMEM GlutaMAX + 1% FBS	HepG2	2000
P53 BLA p1−5 ratio	nuclear factor: tumor protein p53	5 μL of OptiMEM + 0.5% d-FBS	HCT 116	4000
P53 BLA p1−5 viability	cell viability	5 μL of OptiMEM + 0.5% d-FBS	HCT 116	4000
NFKB BLA agonist ratio	nuclear factor: kappa light polypeptide gene	6 μL of OptiMEM + 10% d-FBS	ME-180	2000
NFKB BLA agonist viability	cell viability	6 μL of OptiMEM + 10% d-FBS	ME-180	2000

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$$f_{mem} = \left(1 + \frac{1}{D_{lip/w}} \times \frac{V_{w,cell}}{V_{lipid,cell}} + \frac{D_{BSA/w}}{D_{lip/w}} \times \frac{V_{protein,cell}}{V_{lipid,cell}} + \frac{D_{medium/w}}{D_{lip/w}} \times \frac{V_{medium}}{V_{lipid,cell}}\right)^{-1} \quad (11)$$

$(E)C_{free}$ in the medium (mol $L_{w,medium}^{-1}$) can thereof be calculated by multiplying the free fraction in the medium ($f_{free,medium}$) with $(E)C_{nom}$ (mol L_{total}^{-1}) and the ratio of the system volume (V_{system}, L_{total}) to the water phase volume of the medium ($V_{w,medium}$, $L_{w,medium}$).

$$(E)C_{free} = (E)C_{nom} \times f_{free,medium} \times \frac{V_{system}}{V_{w,medium}} \quad (12)$$

$(E)C_{cell}$ (mol L_{cell}^{-1}) can be derived by multiplying $(E)C_{free}$ (mol $L_{w,medium}^{-1}$) with $D_{cell/w}$ (L_w L_{cell}^{-1}).

$$(E)C_{cell} = (E)C_{free} \times D_{cell/w} \quad (13)$$

$(E)C_{mem}$ (mol L_{mem}^{-1}) is the product of f_{mem}, $(E)C_{nom}$ (mol L_{total}^{-1}), and the quotient of the system volume (V_{system}, L_{total}) with the lipid volume of the cells ($V_{lipid,cell}$, L_{mem}), assuming that all cellular lipids are membrane lipids.

$$(E)C_{mem} = (E)C_{nom} \times f_{mem} \times \frac{V_{system}}{V_{lipid,cell}} \quad (14)$$

3. MATERIALS AND METHODS

3.1. System Parameters. The information on the experimental setup of the selected GeneBLAzer assays was gathered from the iCSS ToxCast Dashboard (https://actor.epa.gov/dashboard/, accessed on June 15, 2016). They were performed in 1536 well plate format with medium volumes of 5−6 μL and a maximum of 1 vol % of dimethyl sulfoxide. All cells were adherent. The end points, either binding to nuclear receptors or transcription factor activity measured as a fluorescence signal produced by GAL4 β-lactamase inducible reporter technology or cell viability based on luciferase-coupled ATP quantification, were quantified after 24 h of chemical exposure.

3.1.1. Determination of Water, Protein, and Lipid Contents of Cells and Media. In the 26 evaluated Tox21 GeneBLAzer assays (Table 1), either Opti Minimum Essential Medium (OptiMEM) or Dulbecco's Modified Eagle Medium (DMEM, phenol-red free or GlutaMAX) were used as basic medium with fetal bovine serum as supplement, either untreated (FBS), charcoal stripped (cs-FBS), or dialyzed (d-FBS). We used the cell lines HEK293T (ER alpha Griptite, CN: K1393), HEK293H (PPAR gamma-UAS-*bla*, CN: K1419), HepG2 (ARE-*bla*, CN: K1208), HCT116 (p53RE-*bla*, CN: K1202), and ME-180 (HRE-*bla*, CN: K1202) for the quantification of cell parameters. All medium constituents and cells were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

To determine the water content of the cell lines, a defined number of cells (3−8 million cells) of each cell line was washed once with phosphate buffered saline (8% NaCl in H_2O, 0.2% KCl in H_2O, 1.442% Na_2HPO_4 in H_2O, and 0.25% KH_2PO_4 in H_2O, v/v 1:1:1:1), gathered in a microtube and centrifuged. The cell number was quantified with the automated cell counter CASY MODEL TT (Roche Innovatis, Reutlingen, Germany). Wet weight of the cell pellet was measured. Subsequently, the cells were freeze-dried for at least 24 h, and their dry weight was measured. The water content of the cells was defined as the difference between wet and dry weights.

The protein contents of cells and media were quantified with the Lowry assay.[31] The cells were homogenized for 1 min by ultrasonic treatment (Sonoplus 2070, Bandelin, Berlin, Germany). Both the homogenized cells and medium constituents were diluted in bidistilled water yielding protein concentrations that fell into the range of the BSA calibration (0.1−1 mg mL^{-1}, Carl Roth GmbH + Co. KG, Karlsruhe, Germany). In a 96-well plate (clear, flat bottom, Corning, Lowell, MA, USA), 200 μL of Lowry reagent (1% $CuSO_4$ in H_2O, 2% K−Na-tartrate in H_2O, 2% Na_2CO_3 in H_2O, v/v 0.5:0.5:25) was added to 20 μL of BSA standard or sample. The well plate was shaken on a vortex mixer (VXR basic, IKA, Staufen, Germany) for 5 min at 900 rpm. Twenty microliters of a 1 M Folin−Ciocalteu reagent (Sigma-Aldrich, Munich, Germany) was added to each well. After 30 min at 900 rpm, the color intensity at 750 nm was measured in an UV/Vis plate reader (F200 Infinite Pro, TECAN, Männedorf, Switzerland), whereby 16 measurements were automatically performed at different positions in the well. The protein content of the samples was then quantified by the resulting linear regression equation of the BSA calibration series.

Lipids in the cells and media constituents were extracted according to a modified Bligh−Dyer method without use of chlorinated solvents.[32,33] Briefly, 2-propanol (99% purity, Merck KGaA, Darmstadt, Deutschland) and cyclohexane (99% purity, Merck) were added to samples of homogenized cells in bidistilled water and single medium constituents yielding a volume ratio of 8:10:11. The tubes were then vortexed for 30 s, sonicated for 5 min in a water bath at room temperature and centrifuged at 2000 rpm for 5 min (Centrifuge 5804, Eppendorf, Hamburg, Germany). The resulting upper cyclohexane layer was removed from the tubes and gathered. The extraction was repeated once with 1 mL of cyclohexane. The lipid contents of the extracts were subsequently determined with three different methods. The total lipid content in the extract was determined gravimetrically. Lipids were further quantified by a modified sulfo-phosphovanillin method via the number of double bonds on the fatty acids.[34] The extracts were transferred to 1.5 mL HPLC vials and dried under nitrogen, followed by the addition of 0.2 mL 95% concentrated sulfuric acid (Merck) and heating at 100 °C for 10 min. After adding 0.8 mL of phosphoric acid-vanillin reagent (0.2 g vanillin (Sigma-Aldrich) in 80 mL of H_2O and 20 mL of 85% phosphoric acid (Merck)) and 10 min of incubation at room temperature, absorbance was measured at 530 nm. Triolein (Sigma-Aldrich), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, Avanti Polar Lipids, Alabaster, AL, USA), and cholesterol (Sigma-Aldrich) were used as calibration standards (0−100 μg). Since the lipid compositions of cells and media are unknown, the mean value of the three calibrations with three different calibration standards was finally used for quantification. The total organic phosphorus content (TOP) of the extracts was determined in addition to estimate the phospholipid content as representative of membrane lipids. For the TOP measurements, the cyclohexane extracts were gathered in 250 mL round flasks and evaporated in a rotary evaporator (RE 121, Büchi, Essen, Germany) to form thin lipid films for improved formation of membrane lipid vesicles. After adding 8 mL of bidistilled water and resuspension in an ultrasonic bath for 5 min, the TOP was determined by inductively coupled plasma atomic emission spectroscopy. The phospholipid concentration in POPC equivalents (C_{POPC}) was calculated based on the molecular weight of phosphorus (M_P = 31 g mol^{-1}) and POPC (M_{POPC} = 760 g mol^{-1}):

$$C_{POPC} = \frac{C_P}{\frac{M_P}{M_{POPC}}} \quad (15)$$

The sulfo-phosphovanillin method was found to be most suitable for the lipid determination of *in vitro* test media and cells due to the small amounts of lipids that rendered the gravimetric method unreliable. The values from the sulfo-phosphovanillin method were used for modeling. A discussion on the different lipid determination methods can be found in the Supporting Information (section S1).

All results from the water, protein and lipid determinations can be found in the Supporting Information (Table S1).

3.2. Chemical Descriptors. The 100 chemicals cover a wide range of physicochemical properties. The hydrophobicity of the neutral species of the chemicals spans almost 9 orders of magnitude with log K_{ow} ranging from 0.07 to 8.48 (Table S3) with water solubility differing from easily soluble (e.g., caffeine with 200 g L^{-1}) to almost insoluble in

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Table 2. System Parameters of Two Reporter Gene Assays of the GeneBLAzer Panel[a]

androgen receptor (AR) assay			antioxidant response element (ARE) assay		
medium			medium		
volume	6 μL		volume	5 μL	
medium	OptiMEM + 10% d-FBS		medium	DMEM GlutaMAX + 1% FBS	
V_{water}	5.97×10^{-03} μL	99.45%	V_{water}	4.99×10^{-03} μL	99.84%
$V_{protein}$	3.12×10^{-05} μL	0.52%	$V_{protein}$	6.90×10^{-06} μL	0.14%
V_{lipid}	1.66×10^{-06} μL	0.03%	V_{lipid}	1.04×10^{-06} μL	0.02%
cells			cells		
cell line	HEK293T		cell line	HepG2	
cell number	2000		cell number	2000	
V_{water}	1.32×10^{-05} μL	90.57%	V_{water}	5.31×10^{-06} μL	87.35%
$V_{protein}$	1.23×10^{-06} μL	8.43%	$V_{protein}$	5.75×10^{-07} μL	9.46%
V_{lipid}	1.46×10^{-07} μL	1.00%	V_{lipid}	1.94×10^{-07} μL	3.19%

[a]The water, protein, and lipid contents of the cells and media were experimentally determined as described in section 3.1. The characteristics of all other assays are described in Table S2.

water (e.g., indeno(1,2,3-c,d)pyrene with 0.01 μg L^{-1}). Four chemicals are permanently charged, and 46 chemicals are ionizable, of which 31 are >99% ionized in water at medium pH of 7.4, 16 are present with a fraction of the neutral species ≤98%, and 6 are multiprotic (forming dicationic, dianionic, and zwitterionic species).

The partition constants that were used to calculate the distribution ratio of the chemicals were preferably experimental values from the literature. If no experimental data were available, partition constants for the neutral and ionic species were modeled as described in the following sections. The complete data set including all relevant physicochemical properties and distribution ratios D for the chemicals at pH 7.4 is provided in the Supporting Information (Table S3).

3.2.1. PP-LFERs to Predict Partition Constants of Neutral Species. For neutral chemicals and the neutral fraction of IOCs, the polyparameter linear free energy relationships (PP-LFERs) shown in eqs 16[15] and 17[23] were used to predict $K_{BSA/w}$ and $K_{lip/w}$.

$$\log K_{BSA/w} = 0.35 + 0.28 \times L - 0.46 \times S + 0.2 \times A - 3.18 \times B + 1.84 \times V \quad (16)$$

$$\log K_{lip/w} = 0.53 + 0.49 \times L - 0.93 \times S - 0.18 \times A - 3.75 \times B + 1.73 \times V \quad (17)$$

The capital letters describe the properties of the chemical, wherein S is the dipolarity/polarizability parameter, A is the solute's H-bond acidity, B is the solute's H-bond basicity, V is the molar volume, and L is the logarithm of the hexadecane-air partition constant. These chemical descriptors are multiplied with system descriptors that depend on the difference in chemical properties between two sorbates and are derived from multiple linear regression analysis against experimental partition constants. Experimental substance and system descriptors were obtained from the UFZ-LSER database,[35] and missing substance descriptors were calculated using the Absolv module available in ACD/Laboratories.[36]

3.2.2. 3D-QSARs. A 3D quantitative structure-activity relationship (3D-QSAR) model was used to predict $K_{BSA/w}$ for those ions that have no reported experimental values on partition constants or fraction bound in plasma.[37] Two experimental data sets of 83 neutral chemicals[15] and 45 anionic chemicals[14] were used to construct a 3D-QSAR model with open3DQSAR.[38] The detailed procedure is reported elsewhere[39] and shall be explained here only briefly. The basis for the calculation is the COSMO-RS theory[40] and the representation of chemicals through their polarized surface (sigma surface). The sigma surface is used for the alignment[41] of the 3D-QSAR model, and the model is calibrated in the same way as it was done in a previous publication for various data sets.[37] After model calibration, $K_{BSA/w}$ could be estimated with a RMSE of 0.63 ± 0.10 and R^2 of 0.52 ± 0.15 for the 128 chemicals of the data set.[39]

3.2.3. COSMOmic. In those cases where no experimental values were available for $K_{lip/w}$ of ions, we used COSMOmic for prediction.[24,42] COSMOmic is a mechanistic, quantum mechanistically based model, which extends the Conductor-like Screening Model for Real Solvents (COSMO-RS)[40] in order to describe anisotropic micelle or lipid bilayer systems.[24] The implementation of the membrane bilayer potential enables the calculation of $K_{lip/w}$ of charged organic chemicals[42] both more reliably and in a more mechanistically based manner using other models.[26] COSMOmic accounts for the anisotropy of a phospholipid bilayer by virtually slicing the bilayer into consecutive layers and successively calculating the Gibbs free energy of the chemicals between a bulk water layer and each phospholipid layer. Thereby, the different contributions of the depth-dependent chemical environments of a phospholipid bilayer can be taken into account.

3.3. Tox21 Data Extraction. We extracted $EC50_{nom}$ from the iCSS ToxCast Dashboard (https://actor.epa.gov/dashboard, accessed on January 27, 2017) for the active chemicals in the selected bioassays. Between 2 and 31 chemicals were active in 25 of the 26 selected assays, and the literature $EC50_{nom}$ values are compiled in Table S4.

4. RESULTS AND DISCUSSION

4.1. System Parameters of the Tox21 GeneBLAzer Reporter Gene Battery. As is common for human cell-based bioassays, the media used in the Tox21 GeneBLAzer battery are consistently composed of a basic medium amended with a defined amount of FBS. The experimental quantification of protein, lipid, and water volumes revealed that protein and lipid contents of the three different basic media (OptiMEM, DMEM GlutaMAX, and phenol-red free DMEM) varied by 46 and 18%, respectively. Variability among FBS, d-FBS, and cs-FBS was 17% in terms of protein and 5% in terms of lipid content. Cellular water, protein, and lipid contents of the five cell lines differed by 52%, 41%, and 20% from the average.

The experimental setup regarding basic medium, FBS content, as well as used cell line and cell number varied considerably between the assays (Table 1). For example, the androgen receptor (AR) assay was performed in OptiMEM with a d-FBS content of 10%, whereas DMEM GlutaMAX with only 1% FBS was used in the antioxidant response element (ARE) assay (Table 2). The medium made up >99% of the total volume in all evaluated assays, and both, the medium and the cells, were predominantly composed of water with minor volume contributions of proteins and lipids (Table S2). Proteins and lipids are considered the main sorptive phases in *in vitro* bioassays. The proportion of proteins and lipids were

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consistently higher in the cells (AR assay: $VF_{protein,cell}$ = 8.43%; $VF_{lipid,cell}$ = 1.00%) compared to the medium (AR assay: $VF_{protein,medium}$ = 0.52%; $VF_{lipid,medium}$ = 0.03%) (Table 2), whereby the volume of proteins was higher than the volume of lipids in the medium (AR assay: 17.3:1) and in the cells (AR assay: 8.4:1).

These differences led to a 4.5 times higher protein and a 1.6 times higher lipid content in the AR medium than in the ARE medium. The bioassays were also performed with different cell lines. In the AR assay, HEK293T cells derived from a human embryonic kidney were used, whereas the human liver cancer cell line HepG2 was used in the ARE assay (Table 2). Although having a 2.4 times higher total volume and being composed of 2.5 times and 2.1 times more water and proteins, respectively, the VF_{lipid} of the HEK293T cells was measured to be 3.2 times lower compared to that of the HepG2 cells. HepG2 cells synthesize fatty acids during cultivation which explains their high lipid content.[43] The system parameters of all evaluated GeneBLAzer assays with associated volumes of water, proteins, and lipids can be found in Table S2.

4.2. Chemical Partitioning. The sorption affinities to BSA and liposomes vary by orders of magnitude between the chemicals, with log $D_{BSA/w}$ and log $D_{lip/w}$ ranging from −0.2 up to 6.5 and −0.4 up to 8 (Figure 2), respectively. Even very

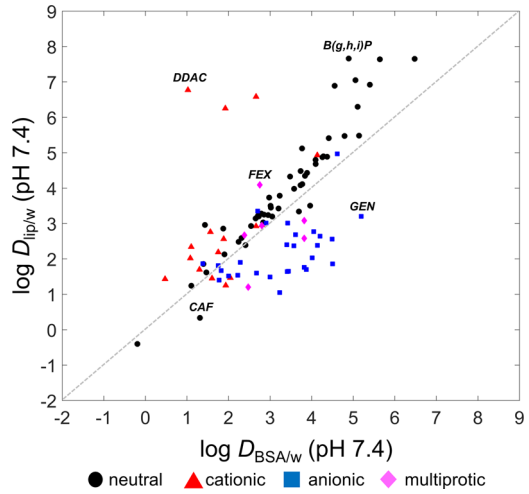

Figure 2. $D_{lip/w}$ and $D_{BSA/w}$ of neutral, cationic, anionic, and multiprotic chemicals at a medium pH of 7.4. Didecyldimethylammonium chloride (DDAC), caffeine (CAF), fexofenadine (FEX), genistein (GEN), and benzo(g,h,i)perylene (B(g,h,i)P) are emphasized as examples.

hydrophilic chemicals such as caffeine (CAF, log $D_{lip/w}$ = 0.33) sorb considerably to BSA (log $D_{BSA/w}$ = 1.31), while polycyclic aromatic hydrocarbons such as benzo(g,h,i)perylene (B(g,h,i)P) show very high sorption affinity to both surrogates (log $D_{BSA/w}$ = 4.89, log $D_{lip/w}$ = 7.66).

For the neutral chemicals ($f_{neutral}$ > 98%), there is an apparent correlation between log $D_{BSA/w}$ and log $D_{lip/w}$ with binding to liposomes a factor of up to 1000 times stronger than that to BSA. Although most of the investigated anionic chemicals (f_{anion} ≥ 2%) show higher affinity to proteins compared to phospholipids, e.g., genistein (GEN) (f_{anion} = 0.61, log $D_{BSA/w}$ = 5.19, log $D_{lip/w}$ = 3.20), there are also exceptions, such as pentobarbital (f_{anion} = 0.16, log $D_{BSA/w}$ = 1.38, log $D_{lip/w}$ = 1.68). Most of the considered cations are predominantly available in the water phase of the medium, but quaternary ammonium cations with long carbon chains, such as didecyldimethylammonium chloride (DDAC) (f_{cation} = 1, log $D_{BSA/w}$ = 1.02, log $D_{lip/w}$ = 6.77), are >99% bound to lipids, probably as a result of structural similarities to phospholipids that make up liposomes.[23]

The distribution ratios for the 100 chemicals illustrate the complexity of chemical partitioning to proteins and lipids, which emphasizes that their derivation by log K_{ow} correlation can lead to a high uncertainty and inaccuracy.[14,23,26] Using prediction methods such as PP-LFERs, COSMOmic, and 3D-QSARs that include several chemical properties and even 3D interactions can substantially improve the quality of predicted partition constants compared to predictions via log K_{ow}.

4.3. Freely Dissolved Concentration as the *in Vitro* Dose Metric. A key objective of *in vitro* effect testing is the comparison and extrapolation of observed effects to realistic exposure scenarios, e.g., in the environment. C_{free} serves as most appropriate dose metric for this purpose since it is safe to assume that the target site concentration is in equilibrium with C_{free} in the vicinity of the target site in most scenarios. C_{free} can thus be used as an indicator of whether an effect is to be expected.[6,8,12,13,44] In other words, if an assay shows an effect at a given C_{free}, then we can expect to measure the same effects at equal C_{free} in any other exposure scenario.

As shown for the AR assay for a constant C_{nom} of 10 μM (Figure 3A), modeled C_{free} can differ considerably and

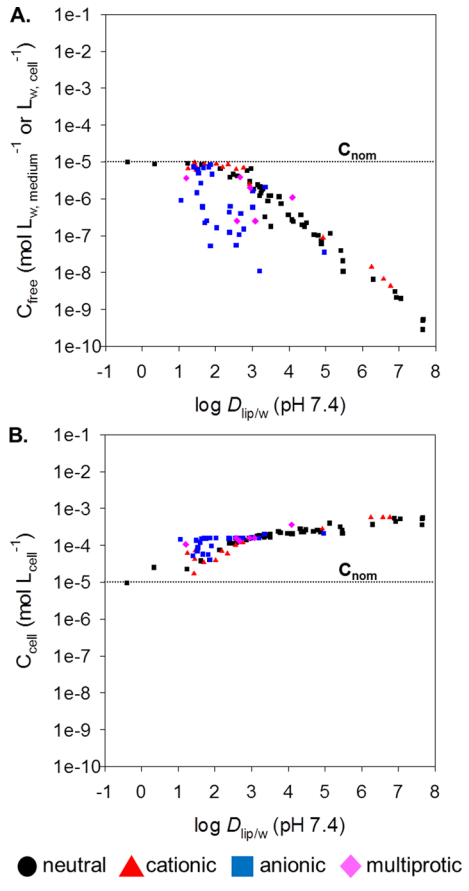

Figure 3. Modeled freely dissolved (A) and cellular (B) concentrations of neutral, cationic, anionic, and multiprotic chemicals in the AR assay based on a nominal concentration of 10 μM, sorted by chemicals' $D_{lip/w}$ at medium pH 7.4. Note that chemicals could have been sorted just as well by $D_{BSA/w}$.

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nonproportionally from C_{nom} with a deviation of up to 5 orders of magnitude for very hydrophobic chemicals. There are also large deviations of C_{free} from C_{nom} for anionic chemicals with log $D_{lip/w}$ < 3, confirming that C_{nom} is not suitable as an approximation for C_{free} in the Tox21 GeneBLAzer assays.

Modeling C_{free} by our approach can substantially increase the comparability within the Tox21 GeneBLAzer battery, to other *in vitro* constructs, and to *in vivo* systems, for which controlling and measuring C_{free} is progressively becoming the norm.[7,45−47] Since environmental monitoring of chemicals is increasingly based on C_{free} measured by, e.g., *in situ* passive sampling devices,[48,49] modeled C_{free} can directly be compared to environmental concentrations. C_{free} represents the most appropriate input parameter for the QIVIVE since it can directly be related to the unbound fraction in plasma.[4,27]

4.4. Cellular Concentration as the *in Vitro* Dose Metric. The toxic effects of a chemical most probably result from complex combinations of specific (e.g., hormone receptor binding), reactive, and baseline effects. As discussed in earlier studies, internal effect concentrations (IEC) can serve as proxy for the biologically effective dose in whole organisms.[5,50,51] Analogous to this concept, C_{cell} represents a sum parameter that integrates the entire intracellular dose from partitioning into different biological targets, e.g., suitable to describe exposure to electrophilic chemicals that can react with biological nucleophiles located in different cell organelles[52] or to evaluate baseline toxicity based on membrane concentrations.[53]

At a constant C_{nom} in the AR assay, C_{cell} is fairly constant for chemicals with log $D_{lip/w}$ > 3, and there is only a narrow gap of 1 order of magnitude between C_{nom} and C_{cell} (Figure 3B). This finding is relevant considering the approach to characterize the *in vitro* toxicity of chemicals and mixtures based on relative effect potencies (REP), which relate the effect of a chemical to a potent reference chemical.[54,55] Because of the systematic difference between C_{nom} and C_{cell}, the REP values in *in vitro* assays when derived from C_{nom} and C_{cell} are very similar for a given chemical across a large chemical space. Thus, the approach remains valid for *in vitro* systems even though bioavailability can vary greatly. The WHO toxic equivalency factors (TEF) are consensus values for comparison of the potency of dioxin-like chemicals,[56,57] and they were derived from measured *in vivo* REPs mainly from mammalian tests using a dose metric of mass per kg body weight, hence a nominal dose. For the derivation of TEF for fish,[57] *in vivo* biomarkers and fish cell lines were used and appear to also have been based on nominal concentrations in most cases. The REP derived from C_{nom} and C_{cell} are thus good proxies for *in vivo* REP and can be used in the future for TEF revisions. The proportionality between C_{nom} and C_{cell} over a wide range of hydrophobicity is given for this particular *in vitro* battery as a result of the colloid-rich medium. C_{cell} deviates nonproportionally from C_{nom} in a colloid-free cell-based system (Figure S3). The same would hold for the cell-free assays that are among the ToxCast assays. For cell-free assays, a simpler model solely based on receptor binding could be invoked, which lies beyond the scope of this study.

4.5. Chemical Partitioning in the Tox21 GeneBLAzer Assays. The dependency of chemical partitioning on the composition of the medium and cells is illustrated in Figure 4. B(g,h,i)P is mainly bound to lipids in medium and cells. Although being >90% available in the water phase of the medium of the AR assay (Figure 4A), within the cells CAF is mainly bound to proteins (Figure 4B). The multimedia

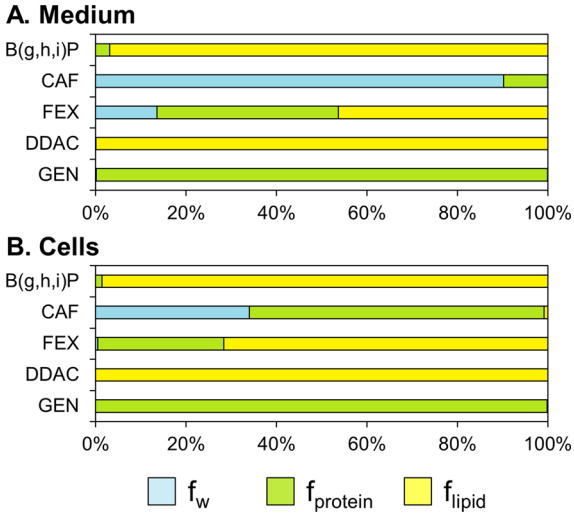

Figure 4. Mass balance of exemplary chemicals in water, proteins, and lipids of (A) the medium and (B) the cells of the AR assay. Note that the mass balance differs considerably between the compartments, as a result of their respective water, lipid, and protein volume fractions (Table 2).

chemical fexofenadine (FEX) is distributed between all phases in the medium but mainly resides in the lipids within the cells.

In contrast, for DDAC and GEN, the distribution does not differ much within the cells and within the medium, caused by the higher sorption affinity of DDAC to lipids compared to proteins (f_{cation} = 1, log $D_{BSA/w}$ = 1.02, log $D_{lip/w}$ = 6.77), and contrarily of GEN to proteins compared to lipids (f_{anion} = 0.61, log $D_{BSA/w}$ = 5.19, log $D_{lip/w}$ = 3.20).

The partitioning of all evaluated chemicals and the resulting large variability in exposure are illustrated for the AR and ARE assays (Figure 5). The medium generally represents the dominant sink for the chemicals in all Tox21 GeneBLAzer assays (>84%, Figure 5A and D). This phenomenon results from the large volume fraction of the medium (>99%) and the fact that the medium makes up a large proportion of total system proteins and lipids, for instance, 96.2% and 91.9% in the AR assay, respectively. This leads to high reductions in the C_{free} of chemicals with high $D_{lip/w}$ and $D_{BSA/w}$ and a large deviation from C_{nom} (Figure 3A).

In the AR medium (Figure 5B), 72 of the 100 chemicals were modeled to partition >70% to proteins and lipids, mainly as a result of the 10% FBS content of the medium leading to a $VF_{protein}$ of 0.52%, whereas only four chemicals are ≥90% freely dissolved in the water phase. In the medium of the ARE assay with 1% FBS and a consequently lower $VF_{protein}$ of 0.14% (Figure 5E), 52 chemicals were bound >70%, whereas 12 were >90% freely available. These findings indicate that C_{free} and thus the measured effects can considerably be reduced in *in vitro* bioassays by the colloid-rich media,[1,30] wherein the degree of reduction highly depends on the FBS content in the medium.

Because of the lower VF_w in the cells (90.6% for AR and 87.4% for ARE) compared to the medium (>99%), the proteins and lipids are the dominant sink for most chemicals in the cells. However, due to the higher volume fraction of lipids (VF_{lipid} = 3.2% in ARE compared to 1.0% in AR), chemical partitioning to the lipid phase of the ARE cells is considerably higher compared to the AR cells (Figure 5C and F).

In the ARE assay with a medium amended with only 1% FBS, the partitioning into the cells becomes more relevant in the

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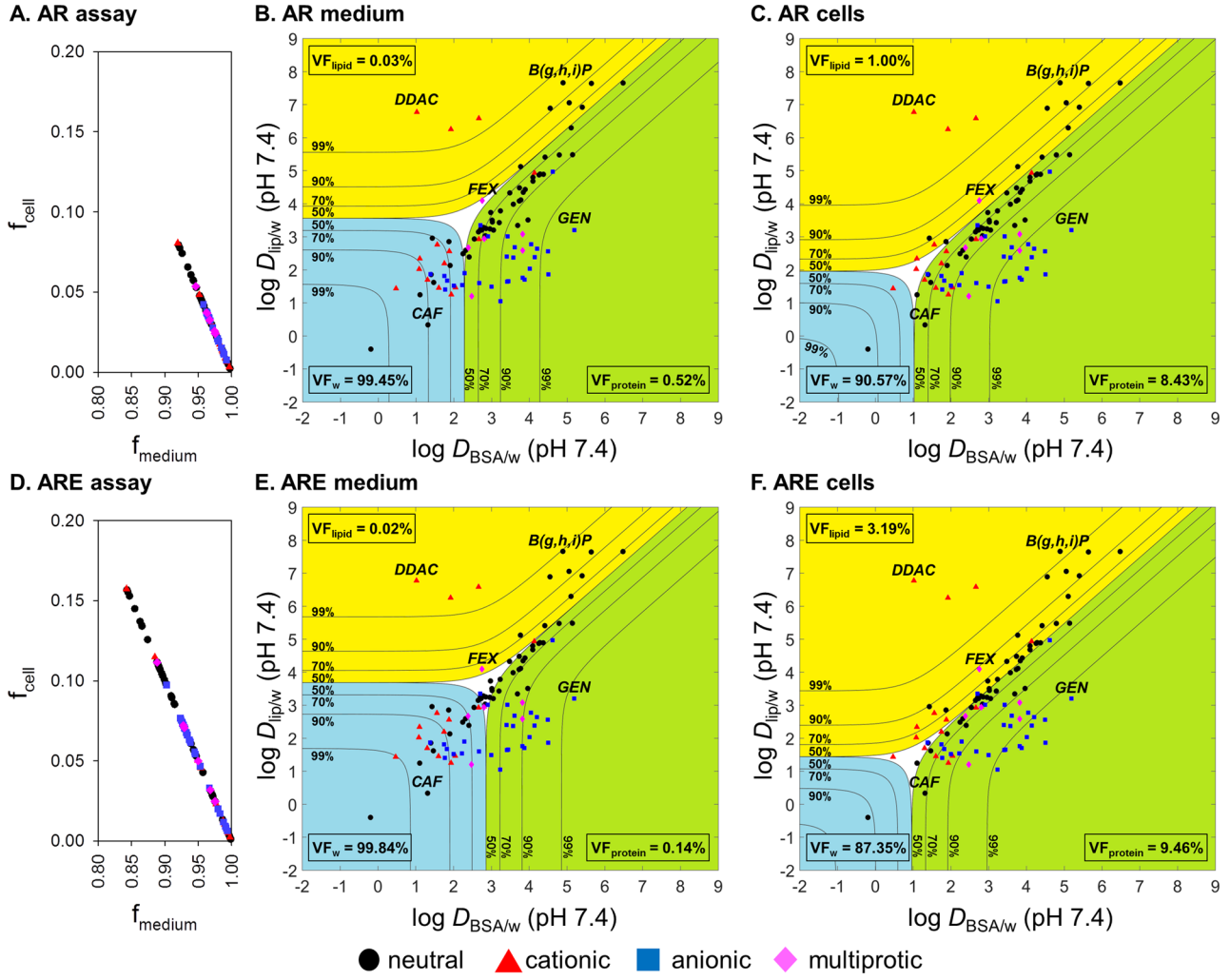

Figure 5. Modeled chemical partitioning of neutral, cationic, anionic, and multiprotic chemicals between cells and medium as well as among lipids (yellow), proteins (green), and water (blue) within the medium and the cells in the AR (A,B,C) and the ARE (D,E,F) assays, based on chemicals' $D_{lip/w}$ and $D_{BSA/w}$ at a medium pH of 7.4. The marked lines indicate the 50%, 70%, 90%, and 99% thresholds that result from the respective phase volume fractions (*VF*) in cells and medium.

overall mass balance (Figure 5D). Although accounting for only 0.12% of the total system volume, the cells make up 8.0% and 15.8% of total system proteins and lipids (opposed to only 3.8% and 8.1% in the AR assay); thus, particularly chemicals with high $D_{lip/w}$ and $D_{BSA/w}$ partition to a larger proportion into the cells (up to 16%). In contrast, a medium such as the AR medium with 10% FBS is generally less depleted by cellular uptake (<8%, Figure 5A); however, at the same C_{nom}, C_{cell} in the AR assay are lower compared to that of the ARE assay.

4.6. Application of the Model to GeneBLAzer Effect Data. 48 of the 100 evaluated chemicals were active in at least one of the evaluated GeneBLAzer assays, of which 28 are neutral, 13 partially anionic, 1 partially cationic, 1 permanently charged anionic, 2 permanently charged cationic, and 3 multiprotic at medium pH of 7.4. Note that 13 of the chemicals were not tested within the Tox21 GeneBLAzer battery, and 3 were only tested in a subset of the assays. The very hydrophobic neutral polycyclic aromatic hydrocarbons benzo(a)pyrene (B(a)P) and benzo(*b*)fluoranthene as well as the cationic long chain quaternary ammonium compounds DDAC and hexadecyltrimethylammonium were active in various assays, while other chemicals were only active in a single assay, such as CAF (ARE) and indometacin (PPARG). The literature $EC50_{nom}$ and corresponding modeled effect concentrations of all active chemicals in all evaluated bioassays can be found in Table S4.

Figure 6 shows the REP of 6 of the 31 active chemicals in the Tox 21 ARE agonist assay in relation to the highly potent and very hydrophobic reference chemical 2,3,7,8-tetrachlorodibenzodioxin (2,3,7,8-TCDD) (Figure 6A) and the REP with the very hydrophilic chemical CAF as reference (Figure 6B). The REP_{free} based on the $EC50_{free}$ may underestimate the potency of a more hydrophilic chemical if the reference chemical is hydrophobic, whereas the REP_{free} is overestimated for hydrophobic chemicals if the reference chemical is hydrophilic. These differences are caused by the large deviations in chemical partitioning and thus large variety in C_{free} (Figure 3A). Representing a sum parameter for internal exposure, C_{cell} integrates differences in intracellular chemical partitioning between the test chemical and the reference chemical which result from differences in the physicochemical properties, which may span several orders of magnitude, as exemplified by 2,3,7,8-TCDD (log $D_{lip/w}$ = 6.89, Figure 6A) and CAF (log $D_{lip/w}$ = 0.33, Figure 6B). $EC50_{cell}$ thus represents a better input than $EC50_{free}$ for the REP calculations in the same *in vitro* test system. Considering the proportionality of C_{nom} and C_{cell} for

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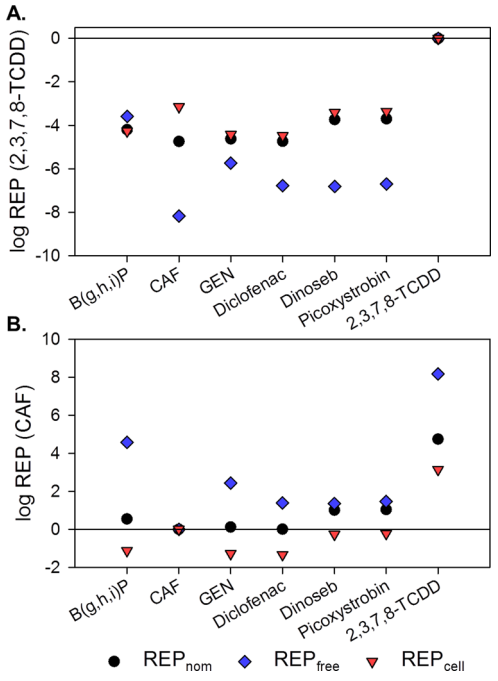

Figure 6. REP of six active chemicals in the ARE agonist assay with (A) 2,3,7,8-tetrachlorodibenzodioxin (2,3,7,8-TCDD) and (B) caffeine (CAF) as reference chemicals on basis of $EC50_{nom}$, $EC50_{free}$, and $EC50_{cell}$.

most of the chemicals (Figure 3B), C_{nom} may thereby serve as proxy for C_{cell} because REP_{nom} is very close to the REP_{cell} independent of the physicochemical properties of the reference chemical (Figure 6).

Baseline toxicity of organic chemicals occurs above a defined membrane concentration of approximately 300 mmol L_{mem}^{-1} in isolated cell membranes[58] and is considered to be related to membrane concentrations of 0.1 to 1 mol L_{lipid}^{-1} in aquatic species.[59,60] Figure 7 shows the EC50 values of agonist chemicals in the ARE assay based on C_{mem}.

Although most of the $EC50_{mem}$ are below the threshold for baseline toxicity, which constitutes the minimum toxicity a chemical can have, particularly neutral hydrophobic chemicals with high log $D_{lip/w}$ are close to the critical membrane concentration for baseline toxicity (prazepam and B(a)P).

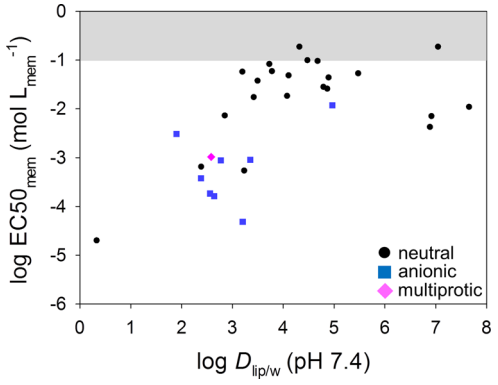

Figure 7. Modeled $EC50_{mem}$ of active neutral, anionic, and multiprotic chemicals in the ARE agonist assay plotted against their $D_{lip/w}$ at pH 7.4. The membrane concentration range of 0.1−1 mol L_{mem}^{-1} is given as a threshold for baseline toxicity (gray shading).[59,60] No cationic study chemicals were active in this bioassay.

Given the fact that the biological target site of the ARE assay is located in the cytosol, measured activities of hydrophobic chemicals might be less specific since concentration−response testing was performed up to cytotoxic concentrations (i.e., critical C_{mem}), also explaining their broad activity in assays indicative of different toxic mechanisms (e.g., B(a)P is active in 10 of the 26 evaluated assays). These results agree with the findings of a recent study by Judson et al.,[61] who revealed that chemicals that tend to cause cytotoxicity within a nominal concentration range of up to 100 μM activated 12% of the evaluated specific end points, whereas chemicals that did not show cytotoxicity within the concentration range were active in only 1.3% of the 815 assays. This phenomenon probably resulted from high C_{mem} since most of the broadly active chemicals are hydrophobic and thus exhibit high sorption to biological membranes, such as 7,12-dimethylbenz(*a*)anthracene (log $K_{lip/w}$ = 6.99). Modeling $EC50_{mem}$ can help to differentiate between chemicals showing specific effects and chemicals for which reported EC50 values might be partly influenced by underlying cytotoxicity. The reported differences in cell sensitivity to chemical-induced cytotoxicity between different cell lines[62] could result from differences in cellular composition resulting in different C_{mem} for similar C_{nom} of the chemical (Figure 5C and F). However, it could also be explained by differences in the medium composition of the considered *in vitro* bioassays.

Most of the target sites in *in vitro* bioassays are located either in the cytosol or in cell compartments such as the nucleus. More detailed modeling of chemical partitioning between different cell compartments could further enhance the interpretability of a cellular response. Further parametrization of compartment-specific proteins and lipids is therefore needed considering the complexity of chemical sorption to biomolecules.[14,15]

4.7. Implication for Chemical Risk Assessment and Future Steps. The protein- and lipid-rich nutrient medium used in cellular assays with mammalian cell lines could have led to a decreased sensitivity of the Tox21 *in vitro* bioassays due to reduced C_{free}. The model output is consistent with the conclusion of earlier studies that C_{nom} is generally not an adequate metric to describe the *in vitro* exposure of most of the evaluated chemicals since they are bound to a large extent even in media with low FBS content.[6,12,13] Given the fact that measured effects were exclusively linked to C_{nom} within the Tox21 program, chemical risk classification and prioritization hence suffers from low comparability between assays at a constant C_{nom} (Figure S4) and particularly to other exposure scenarios, such as *in vivo* toxicity data. Taking into account chemical partitioning by equilibrium model approaches such as those presented here considerably enhances the interpretability of *in vitro* toxicity data, but further refinement of the model, system, and chemical parameters as well as moving from an equilibrium to a kinetically resolved model will further improve the accuracy of the dose descriptors. However, the presented model is already a major leap as compared to purely K_{ow}-based equilibrium partitioning models.

The relative distribution of the chemicals in the Tox21 GeneBLAzer assays shows that chemical partitioning in *in vitro* assays can vary greatly between and within the cells and medium. C_{free} is strongly dependent on (i) the physicochemical properties of all species of the chemicals as well as (ii) the composition of the medium and the cells in terms of volume fractions of lipids, proteins, and water. Inconsistency in basic

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medium and FBS content as well as the natural variability of the FBS can thereby lead to highly variable C_{free} between bioassays of a single experimental platform but even more so across different platforms. One should be aware of this variability when comparing effect data of bioassays carried out with different setups or when changing the experimental setup of an assay.

Modeling C_{free}, C_{cell}, and even target site concentrations such as C_{mem} allows evaluation of whether differences in measured toxicity between *in vitro* platforms are the result of intrinsic sensitivity differences of the cell lines, the receptor expression, and/or the reporter gene design or whether they are simply the result of differences in bioavailability. However, in most databases, system parameters are either poorly reported or not available. Clear and traceable information on the system parameters (e.g., medium composition) should always be part of published *in vitro* effect data, in order to allow retrospective bioavailability calculations. Extending the equilibrium model to kinetic processes such as sorption to plate materials, degradation, cellular growth, and uptake kinetics can further improve the accuracy and correctness of the model output.

BSA and liposomes were used as surrogates for all medium and cellular proteins and lipids in order to improve the model over previous K_{ow}-based estimates. Sorption to proteins can be very complex,[63,64] in particular for IOCs for which strong steric and 3D structure effects on BSA sorption were measured for isomeric chemicals.[14] Studies are therefore needed that test the suitability of BSA and liposomes as surrogates for medium and cellular proteins and lipids when testing IOCs. The sorption of a chemical might be considerably different between proteins due to differences in the structure and conformation of binding sites, in particular when comparing serum proteins with structural proteins.[65] In contrast to neutral chemicals, ions are not expected to partition into storage lipids, but some have higher affinity to membrane bilayers. A more detailed parametrization of the medium and cell composition is warranted to find out if a more complex model is required or if the present model is sufficient for routine applications.

The model can readily be used and is included as Supporting Information. We provide experimental data on water, protein, and lipid contents of different media and different cell lines in Table S1, and there is also an average generic set of system parameters that can be applied for cells and media, for which protein and lipid contents have not been characterized. The modified Lowry assay and sulfo-phosphovanillin method are both rapid, robust photometric tests performed in multiwell plate format and can be implemented with low equipment and personnel costs. Considering that FBS is produced from natural blood, meaning its composition can vary considerably, we recommend quantifying proteins and lipids when using a new lot. Cellular composition may differ for similar cell lines of different culture. The partition constants of the study chemicals (log $K_{BSA/w}$ and log $K_{lip/w}$) are gathered in Table S3. Input values needed to perform predictions for neutral organic chemicals via PP-LFERs are moreover freely available for >8,000 chemicals.[35] Although state-of-the-art prediction approaches for ions such as COSMOmic and 3D-QSARs are not that easily accessible so far, there are ongoing efforts to develop user-friendly models for the prediction of IOCs. Considering the complexity of partitioning of ions into complex matrices such as BSA,[14,63,64] we need more experimental data for the mass balance model but also to improve the prediction methods for the binding constants. Methods are needed to robustly measure partition constants of IOCs for the relevant binding matrices, such as third-phase partitioning approaches[66] based on novel sampling devices for IOCs.[67]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.7b00023.

Model calculations (XLSX)

Experimental data on the system parameters, the relevant partition constants and modeled effect data for the study chemicals were gathered (XLSX)

Utility of the experimental methods used for lipid determination and additional plots (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

C_{nom}, nominal concentration; C_{free}, freely dissolved concentration; C_{cell}, cellular concentration; C_{mem}, membrane concentration; IOC, ionogenic organic chemicals; K_{ow}, octanol−water partition constant; $D_{lip/w}$, liposome−water distribution ratio; $D_{BSA/w}$, bovine serum albumin−water distribution ratio

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Supporting Information

Modeling exposure in the Tox21 *in vitro* bioassays

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Content overview: 5 Pages, 4 Figures, 2 Excel Workbooks

S1. Discussion on lipid determination methods

We used three different methods to determine the lipid content of the extracts obtained by the Smedes extraction (Figure S1).[1]

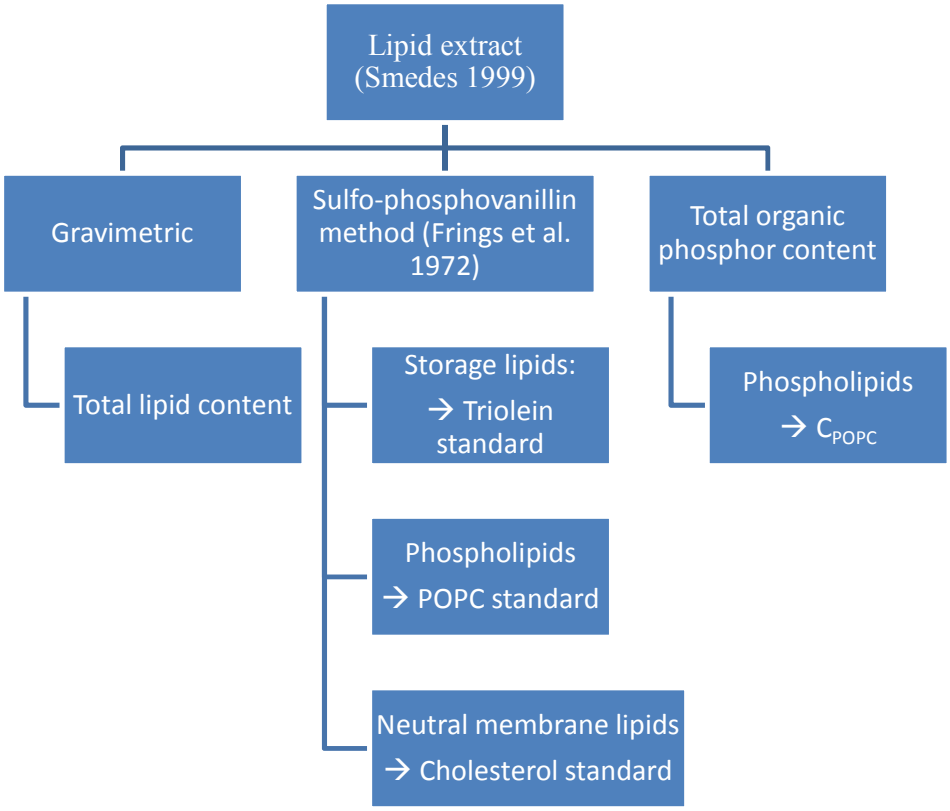

Figure S1: Methods that were used for determining the lipid content in the Smedes extracts including the lipid species that are accounted for by the determination method.

Determining the lipid content gravimetrically carries the advantage that the sum of all lipids is being captured. However, proteins, carbohydrates, and salts may be included in the dried lipid extracts.[2] Using the gravimetric method may thus lead to an overestimation of the total lipid content.[3] The lipid content determination of small samples such as cell with lipid extracts with dry weights of <1 mg can furthermore lead to large random errors while weighing and is thus not recommended.

The sulfo-phosphovanillin method (SPV method) is a rapid and simple spectrophotometric based test to determine the lipid content of samples via reference calibration standards. The method is based on the reaction of sulfuric acid with the carbon-carbon double bonds of the lipids and the subsequent reaction of a phosphovanillin reagent with the formed carbonium ion which leads to the formation of colored molecule.[4] We used triolein, POPC and cholesterol as calibration standards which serve as surrogates for storage lipids, phospholipids, and neutral membrane lipids, respectively. The strength of the colorimetric reaction should correlate with the number of double bonds contained in the sample, which satisfactory holds for the calibration standards used for the study (Figure S2).

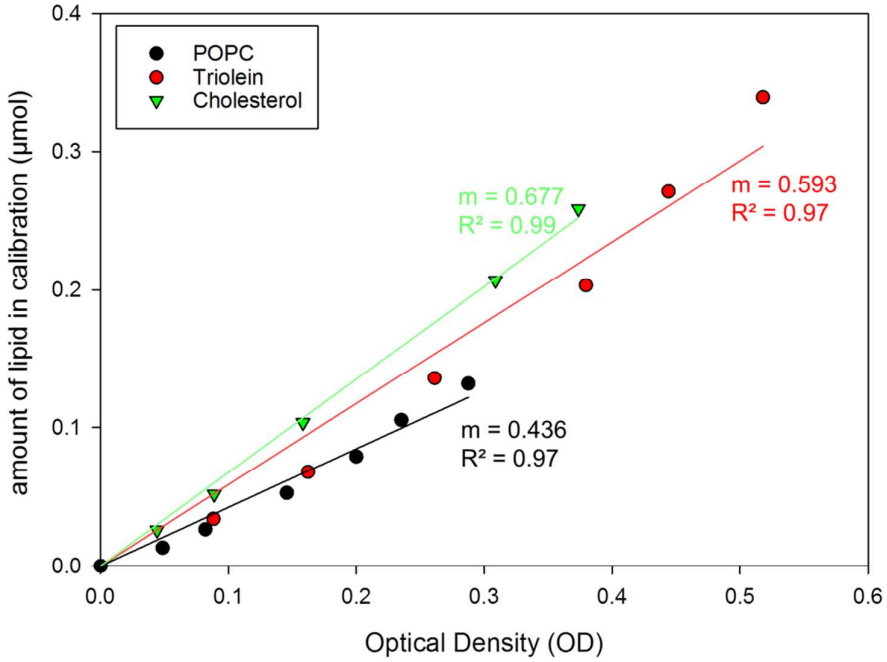

Figure S2: Linear regression of the optical density measured after the sulfo-phosphovanillin reaction against the amount of lipid standard in the sample (POPC, triolein, and cholesterol).

Most recently, the SPV-method was modified to be used in microplate format,[5,6] which was adapted for this study. This modification enables the analysis of lipids in very small samples, which simplifies the determination of cellular lipids since only a small number of cells has to be cultured (< 1 million cells are sufficient for multiple measurements) and enables high-throughput analysis. Requiring a small number of reagents and low minimum hardware, the method can easily be implemented in everyday laboratory practice.

The analysis of the total phosphor content (TOP) and subsequent conversion in POPC equivalents (Eq. 16) might be a good method to determine the phospholipids content of samples, which is particularly interesting for cell samples. However, the method will underestimate the lipid content of, e.g., the test medium, since we expect a large number of non-phosphoric lipids. Furthermore, the current method suffers from high variability between the replicates, low sensitivity (large amounts are needed to reach detection limits) and high laboratory efforts, impeding its application as rapid test procedure.

Our results confirm that the gravimetric method leads to higher lipid contents in the medium constituents and particularly, the cells than using the SPV-method (Table S1). Lipid contents of the medium constituents were partly higher when using the TOP method, however, standard deviations were unsatisfactory high. In summary, the SPV-method proved to be most robust, and using the mean value of the three standards should be the most reliable method used. However, provided that enough sample material is available, the gravimetric method can be performed in addition, covering also lipids without carbon-carbon double bonds.

S2. C_{cell} in colloid-free medium

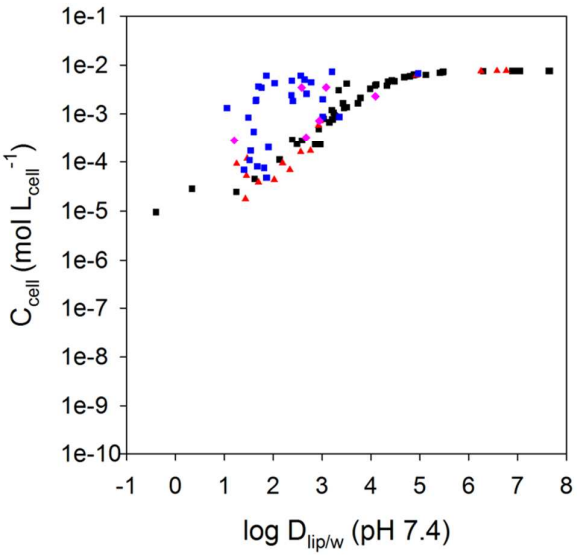

Figure S3: Modeled cellular (B.) concentrations of neutral, cationic, anionic and multiprotic test chemicals in a medium without sorptive colloids and 2000 cells, based on a nominal concentration of 10 µM and sorted by the chemicals' $D_{lip/w}$ at medium pH 7.4. Note that chemicals could as well be sorted by $D_{BSA/w}$.

S3. Inter-assay exposure variability

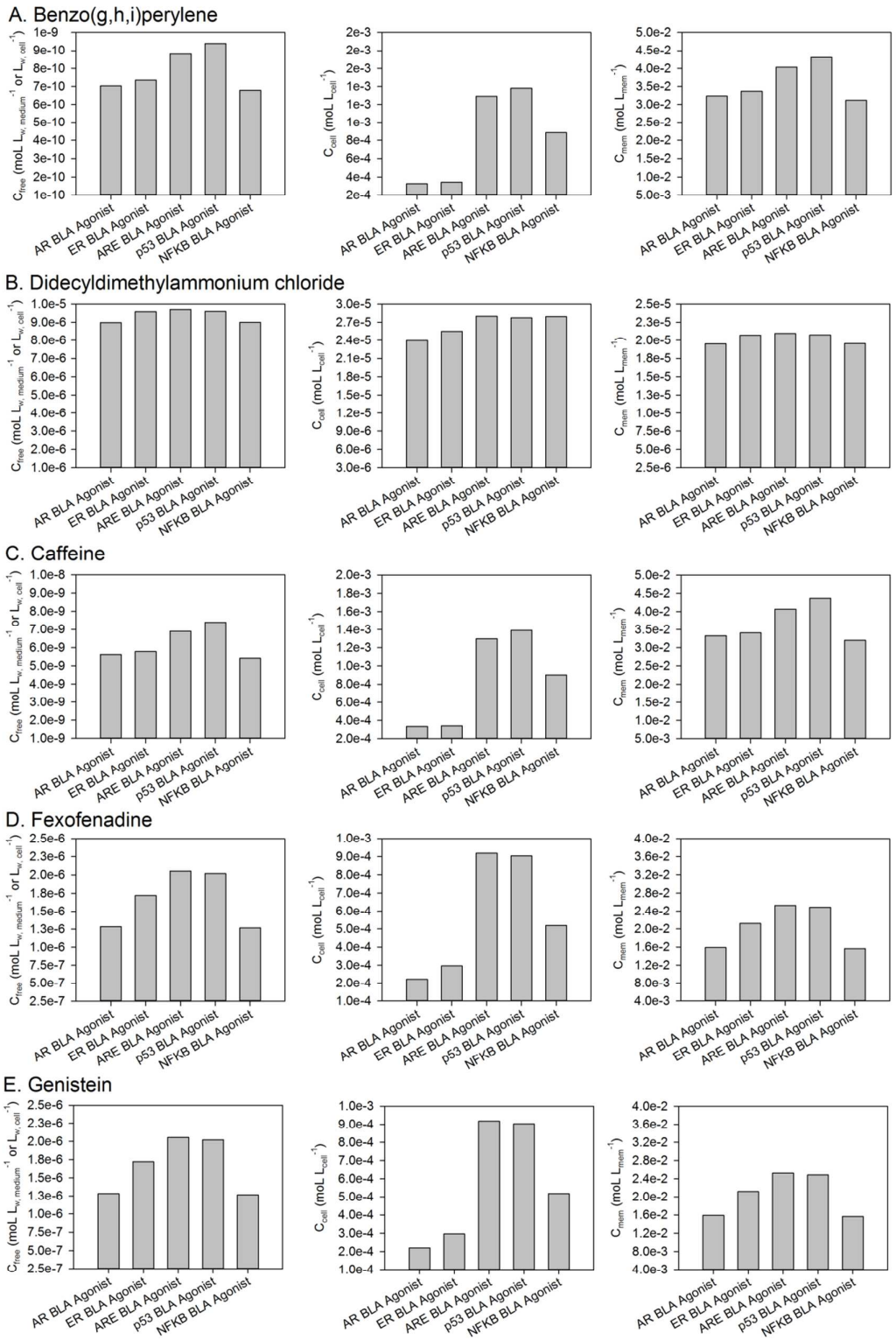

Figure S4: Modeled freely dissolved, cellular, and membrane concentrations of benzo(g,h,i)perylene (A.), didecyldimethylammonium chloride (B.), caffeine (C.), fexofenadine (D.), and genistein (E.) in different *in vitro* bioassays which were carried out with different experimental setups (see Table S2).

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