Experimental exposure assessment of ionizable organic chemicals in *in vitro* cell-based bioassays

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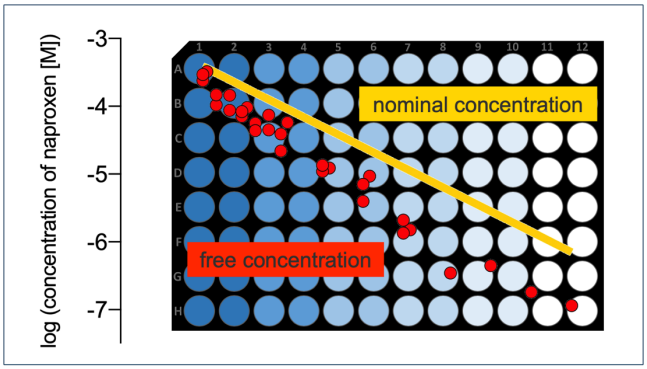
KEYWORDS

QIVIVE, protein binding, solid-phase microextraction, mass balance models

Table of Contents Graphic



ABSTRACT

Exposure assessment in *in vitro* cell-based bioassays is challenging for ionizable organic chemicals (IOCs), because they are present as more than one chemical species in the bioassay medium. Furthermore, compared to neutral organic chemicals, their binding to medium proteins and lipids is driven by more complex molecular interactions. Total medium concentrations ($C_{total,medium}$) and/or freely dissolved medium concentrations ($C_{free,medium}$) were determined for one neutral chemical and 14 IOCs (acids, bases, multifunctional) at concentrations relevant for determination of cytotoxicity and effect. $C_{free,medium}$ were measured in two *in vitro* bioassays at the time of dosing and after 24 h of incubation using solid-phase microextraction (SPME). $C_{free,medium}$ were maximally 1.7 times lower than the nominal concentrations (C_{nom}) for the hydrophilic chemicals (caffeine and lamotrigine). For the organic acids (naproxen, ibuprofen, warfarin and diclofenac), $C_{free,medium}$ was by a factor of 4 lower than C_{nom} at high concentrations but the ratio was much higher at low concentrations, indicating a non-linear binding behavior. The experimental $C_{free,medium}$ were also compared with $C_{free,medium}$ predicted with a mass balance model accounting for binding to medium proteins and lipids. The mass balance model performed well for five of the test chemicals (within a factor of 10) but it underestimated $C_{free,medium}$ by up to a factor of 1200 for chemicals that showed

non-linear binding to medium components. These findings emphasize that experimental exposure assessment is required for improved understanding of *in vitro* toxicity data.

Introduction

The program "Toxicity Testing in the 21st Century" (Tox21) of the US National Research Council was an important step towards toxicity testing with the focus on *in vitro* methods.[1] Compared to *in vivo* test systems, *in vitro* cell-based bioassays are cost-effective, time-saving and can be automated. However, they do not yield information of systemic effects or toxicokinetics. This complicates the association of *in vitro* effects with effects *in vivo*. Quantitative *in vitro*-to-*in vivo* extrapolation (QIVIVE) may solve this problem. To generate meaningful data with QIVIVE the chemical exposure in *in vitro* assays must be better understood.[2] The nominal concentration (C_{nom}) is predominantly used in the field of *in vitro* toxicity, but can differ widely from the actual exposure. Binding to medium proteins and lipids, cellular uptake and metabolism, diffusion into well plate material and volatilization can lead to a reduction of the bioavailable concentration.[3-5] To overcome this problem, the use of the freely dissolved concentration in the assay medium ($C_{free,medium}$) instead of the nominal concentration (C_{nom}) has been suggested, but depending on the application, other concentration metrics can be relevant as well.

The following concentration metrics were used in this study (Fig. 1). The total concentration of the chemicals added to the test system is C_{nom}, and $C_{total,medium}$ and $C_{total,cell}$ are the total concentration in the assay medium and in the cells, respectively. In both compartments, medium and cells, the chemicals are either freely dissolved in the water phase ($C_{free,medium}$ and $C_{free,cell}$) or bound to proteins and lipids ($C_{bound,medium}$ and $C_{bound,cell}$). Within the cells, a fraction of the bound molecules is partitioning to the cell membranes ($C_{membrane,cell}$), eventually causing cytotoxicity, if a certain $C_{membrane,cell}$ is exceeded (baseline toxicity). While $C_{total,medium}$, $C_{free,medium}$ and $C_{bound,medium}$

can be easily determined experimentally from aliquots of assay medium without disturbing the detection of the specific endpoint of interest,[3] the cells have to be extracted with solvents to determine $C_{total,cell}$. If chemical equilibrium is attained in the bioassay, $C_{free,cell}$ equals $C_{free,medium}$ and does not need to be determined by experiment. Both, $C_{free,cell}$ and $C_{bound,cell}$ cannot be determined by experiment and need to be calculated using computational tools. The same applies to $C_{membrane,cell}$.

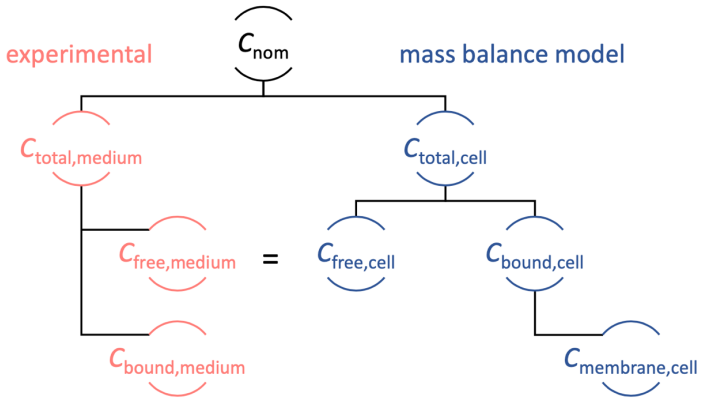


Fig. 1 Concentration metrics for *in vitro* cell-based bioassays that can either be determined experimentally (red icons) or calculated using mass balance models (blue icons).

Various mass balance models have been developed to predict $C_{free,medium}$ and other concentration metrics in *in vitro* bioassays from the partition constants or distribution ratios of the test chemicals between the different compartments of the assay system[4] or by using surrogate phases, e.g., octanol[6] or liposomes and serum albumin.[5] However, only few studies have focused on the experimental determination of $C_{free,medium}$.[3, 4, 7] Conventional methods for the measurement of $C_{free,medium}$, such as equilibrium dialysis, ultrafiltration and centrifugation are difficult to integrate in a routine assay workflow.[4] Solid-phase microextraction (SPME) was found to be suitable for an integration into bioassay workflows[3] and is also compatible with small sample volumes and protein and lipid-rich biological sample matrix.[8]

Many toxicologically and environmentally relevant chemicals like surfactants, pharmaceuticals and pesticides are ionic and ionizable organic chemicals (IOCs).[9-11] Exposure assessment is especially challenging for IOCs, because these chemicals can be present in different forms (e. g., neutral, cationic, anionic or with multiple charges) depending on the pH value of the medium. Prediction models for the partitioning of ionized molecules are still widely missing.[12] The development of such models is also complicated by the fact that ions undergo more complex sorption processes like ion exchange or ion pair partitioning compared to neutral molecules and often show non-linear sorption isotherms.[12, 13]

The aim of this work was to experimentally determine the exposure of 15 chemicals, 14 IOCs and one neutral chemical, in two *in vitro* cell-based bioassays, testing for oxidative stress response and activation of the peroxisome proliferator-activated receptor gamma (PPARγ), respectively. The selected test chemicals were chosen to represent different exposure scenarios from nearly completely freely dissolved (e.g., caffeine) to highly protein-bound chemicals (e.g., naproxen and ibuprofen), explicitly including chemicals that showed non-linear binding isotherms to medium proteins[14] and consequently concentration-variable exposure in previous studies (e.g., diclofenac).[3] A SPME technique[3] was used for the quantification of $C_{free,medium}$ in medium aliquots sampled at the time point of dosing and after 24 h of incubation of the cells with the test chemicals. Since this method for the determination of $C_{free,medium}$ was based on a mass balance calculation (see eq. 8, section "Data evaluation"), the stability of the test chemicals was checked by additionally measuring $C_{total,medium}$ in the medium aliquots. To detect non-linear binding to medium components, $C_{free,medium}$ was determined for the entire concentration range tested in the bioassay. From the obtained assay data and measured concentrations, the nominal and freely dissolved effect concentrations were calculated and compared. The freely dissolved effect concentrations obtained

experimentally were also compared with the calculated values from a mass balance model[5] to assess the reliability of this prediction model for exposure assessment of IOCs.

Materials and methods

Test chemicals

For this study, 15 chemicals were tested, one neutral hydrophilic chemical (caffeine), five monoprotic bases, six monoprotic acids, and three chemicals with multiple functional groups (Table 1). Caffeine, metoprolol and propranolol showed weak binding to the media components and high free fractions in the medium.[3] However, there were problems measuring the concentration of the bases using SPME. Five bases were selected for this work to investigate whether these chemicals are stable for the duration of the assay, as metabolic activity was recently detected in one of the cell lines used.[15] Some of the organic acids (e.g., diclofenac, 2,4-D, ibuprofen, naproxen and warfarin) showed non-linear binding to FBS at high concentrations of the chemicals in a previous study.[14] This observation should be verified for the concentrations used in the bioassays. Little is known about the exposure of multifunctional chemicals. For this reason, a few complex substances of different speciation were selected to test whether the SPME method also works for this type of chemicals and whether the mass balance model can predict their exposure. Further information on the test chemicals (Cas-No., purity and supplier) can be found in the Supporting Information (SI) Table S1.

Table 1. Test chemicals of this study with their respective speciation and logarithmic bovine serum albumin-water (log $D_{BSA/w}$) and liposome-water distribution ratios (log $D_{lip/w}$) at pH 7.4.

Chemical	Chemical class	Acidity constant pK_a	Speciation at pH 7.4	log $D_{BSA/w}$ [L_w/L_{BSA}] pH 7.4, 37°C	log $D_{lip/w}$ [L_w/L_{lip}] pH 7.4, 37°C

Caffeine	Neutral	-	100 % Neutral	1.66 [k]	0.08 [m]
Lamotrigine	Base	5.34 [a]	>99 % Neutral	2.16 [k]	2.06 [k]
Venlafaxine	Base	8.4 [b]	91 % Cationic, 9 % neutral	1.58 [k]	1.64 [k]
Metoprolol	Base	9.68 [c]	>99 % Cationic	1.51 [k]	1.42 [k]
Diphenhydramine	Base	8.98 [d]	97 % Cationic, 3 % neutral	1.99 [k]	2.17 [k]
Propranolol	Base	9.42 [d]	>99 % Cationic	1.78 [k]	2.73 [k]
Diclofenac	Acid	4.15 [d]	>99 % Anionic	4.40 [l]	2.64 [f]
2,4-Dichlorophenoxy-acetic acid (2,4-D)	Acid	2.9 [e]	>99 % Anionic	3.56 [l]	2.02 [l]
Naproxen	Acid	4.15 [d]	>99 % Anionic	5.21 [l]	2.17 [l]
Ibuprofen	Acid	4.45 [f]	>99 % Anionic	4.02 [l]	1.81 [f]
Torasemide	Acid	6.68 [g]	84 % Anionic, 16 % neutral	3.81 [l]	2.05 [l]
Warfarin	Acid	4.9 [h]	>99 % Anionic	3.46 [l]	1.62 [l]
Genistein	Multifunctional	7.2, 10, 13.1 [i]	61 % Anionic, 39 % neutral	2.83 [l]	3.32 [l]
Telmisartan	Multifunctional	3.3, 4.0, 6.2 [b]	94 % Anionic, 6 % zwitterionic	3.39 [n]	4.73 [n]
Labetalol	Multifunctional	7.35, 9.11 [j]	53 % Zwitterionic, 47 % cationic	1.24 [k]	3.26 [k]

[a] ref [16], [b] Predicted using pKa GALAS tool of ACD/Labs 2015 release (Build 2726.), [c] ref [17], [d] PhysProp database accessed via EPISuite version 4.1, [e] ref [18], [f] ref [19], [g] ref [20], [h] ref [21], [i] ref [22], [j] ref [23], [k] ref [24], [l] ref [14], [m] Predicted using the UFZ-LSER database[25], [n] data of this study (see SI section S2 for more details)

Experimental procedure

In vitro cell-based bioassays

All chemicals were tested in two reporter gene bioassays. The AREc32 assay was developed by Wang et al.[26] for the detection of oxidative stress response and is based on the human MCF7 cell line containing a luciferase gene construct under the control of eight copies of the antioxidant response element (ARE). The PPARγ assay uses commercially available GeneBLAzer PPARγ-UAS-*bla* HEK293H cells developed by Invitrogen (Thermo Fisher Scientific) and detects chemicals that activate the peroxisome proliferator-activated receptor gamma (PPARγ). The AREc32 medium contained 10% untreated FBS and 90% DMEM and the PPARγ medium was composed of 2% charcoal-stripped FBS and 98% Opti-MEM. 50 mM (AREc32) or 10 mM

(PPARγ) HEPES were added to the media to keep the pH stable over the 24 h incubation time. The layout of the assay plates is depicted in Fig. S2. 96-Well plates made of polystyrene (AREc32 assay) and polystyrene coated with poly-d-lysine coating (PPARγ assay) from Corning were used. The cells were exposed to different concentrations of the test chemicals. Dilutions were either prepared manually (serial dilution) or with a digital dispenser (linear dilution) in a dosing plate. All chemicals were tested with a serial dilution series first and only chemicals that were classified as being active were repeated in duplicate with a linear dilution series. The serial dosing was conducted as described previously.[3] For the linear dosing, a glass-coated 96-deep well plate was filled with 500 µL of either PPARγ assay medium or AREc32 assay medium per well. For the dilution, a defined volume of a DMSO stock of the chemicals was pipetted into the dispense head cassette of a digital dispenser (D300e, Tecan). The device automatically added the necessary volume to each well to obtain the desired dilution. The plates were sealed and shaken at 1000 rpm for 30-45 minutes (BioShake iQ, Q Instruments). The DMSO content was kept <1% in the dosing plates.

The test chemicals were dosed to the cells in duplicates by manually transferring 170 µL from each well of the dosing plate to the cell plates containing the seeded cells in 50 µL assay medium. The total volume in the cell plate after dosing was 220 µL per well. The cells were incubated at 37°C, 5 % CO_2 and 100 % humidity for 24 h. The confluency of the cells was measured non-invasively with the IncuCyte S3 Automated Live-Cell Imager (Essen BioScience) and the related software (IncuCyte S3 v2019A) before the dosing and after 24 h exposure. The cell viability and cytotoxicity were determined by measuring and comparing the confluency of the exposed and unexposed cells. Before the detection of the specific endpoints, 200 µL of the medium were removed from each well, transferred to a glass-coated 96-well plate and stored for the chemical

analysis. The protocol for the detection of the activation of the ARE elements can be found in Escher et al.[27] The detailed protocol for the PPARγ assay is described in Neale et al.[28]

Aliquots were taken at two different time points of the assay for the determination of $C_{free,medium}$ and $C_{total,medium}$ of the chemicals in the assay medium. The medium from the dosing vial (serial dilution) or the dosing plate (linear dilution) was sampled to measure $C_{free,medium}$ and $C_{total,medium}$ before the incubation with the cells at t_{0h}. After 24 h incubation, the medium removed from the assay plates was used to determine $C_{free,medium}$ and $C_{total,medium}$ at t_{24h}.

Total concentration measurements

Total medium concentrations were measured from the dosing vials (serial dilutions) or from the four highest concentrations in the dosing plates (linear dilutions) at the beginning of the test (t_{0h}) and from the four highest concentrations of the assay plate after 24 h of incubation (t_{24h}). Proteins and phospholipids were removed from the samples using Phree Phospholipid Removal Plates from Phenomenex. Phree plates were placed onto glass-coated deep-well collection plates and each well was filled with 400 µL acetonitrile with 1% formic acid. 100 µL of the medium sample were added and the plate was shaken for 10 min at 1500 rpm (High-Speed Microplate Shaker DMS-2500, VWR International or BioShake iQ, Q Instruments). For the elution, the plate was centrifuged at 500 × g for 5 min (Megafuge 40, Heraeus). 500 µL of acetonitrile with 1% formic acid were added to each well of the Phree plate again and the centrifugation was repeated. The eluates were combined, transferred to 1.5 mL glass vials and evaporated using an XcelVap evaporation system (Horizon) until the samples were dry (6 PSIG - 12 PSIG for 180 minutes). Samples were re-dissolved in 10/ 90 (v/v) acetonitrile/ H_2O (caffeine), 50/ 50 acetonitrile/ H_2O (lamotrigine, venlafaxine, metoprolol, diphenhydramine, propranolol, diclofenac, genistein, telmisartan, labetalol) or 50/ 50 methanol/ H_2O (2,4-D, naproxen, ibuprofen, torasemide, warfarin) for the subsequent LC-MS/MS analysis.

Freely dissolved concentration measurements

Solid-phase microextraction (SPME) was used to determine $C_{free,medium}$ of the chemicals in the AREc32 and the PPARγ assay medium as described previously.[3] The SPME fibers were obtained from Sigma Aldrich (Supelco). The metal alloy fibers had a coating of C18 particles (5 µm) embedded in the biocompatible polymer polyacrylonitrile (coating thickness 45 µm). Fibers with three different coating lengths were used (see Table S3 for which coating length was used for which chemical). Blunt cannulas (Braun, 0.8 × 22 mm) were used to insert the fibers into the samples. Fibers were stored in methanol and equilibrated in MilliQ before each experiment for at least 20 minutes, according to the literature[8] and the manufacturer of the fibers. Aliquots of 180 µL of the respective assay medium from the dosing vial or the cell plate were transferred to glass vials with insert. The fibers were quickly transferred to the sample vials and the samples were incubated at 37°C and 250 or 1200 rpm for 24 h (Orbital Shaker MaxQ 2000 SHKE, Thermo Scientific or High-Speed Microplate Shaker DMS-2500, VWR International). For the desorption, the fibers were moved to another vial containing 180 µL of the desorption solution and incubated on the orbital shaker/high-speed shaker at 37 °C and 250/1200 rpm for 2 h. After desorption, the fibers were removed, and the samples were analyzed using LC-MS/MS. Table S3 shows the SPME conditions for the different chemicals. Control samples in PBS were run in parallel for all chemicals to obtain the fiber-water distribution ratios ($D_{f/w}$) and to check the mass balance. $C_{free,medium}$ was not determined with SPME for the bases (venlafaxine, metoprolol, diphenhydramine and propranolol) and labetalol, because experimental artifacts were observed in previous work.[3] These chemicals bind to a very low extend (calculated fraction bound <50 %) to the proteins and lipids of the assay medium.[29, 30] Hence, $C_{free,medium}$ was expected to be similar to $C_{total,medium}$ for these chemicals and the measurement of $C_{total,medium}$ was assumed to be sufficient to

monitor their exposure. To confirm this assumption, $C_{free,medium}$ of propranolol and labetalol was measured in both assay media via equilibrium dialysis (see SI section S5).

Instrumental analysis

Quantification of chemical concentrations for all samples was performed using a liquid chromatograph (Agilent 1260 Infinity II) coupled to a mass spectrometer (Agilent 6420 Triple Quad) equipped either with a Kinetex 1.7 µm, C18, 100 Å, LC column (50 × 2.1 mm), a BioZen 1.6 µm, Peptide PS-C18 LC column (50 × 2.1 mm) or a Luna Omega 1.6 µm, Polar C18, 100 Å, LC column (50 × 2.1 mm) depending on the analyte. The LC- and MS-parameters are listed in the SI (Tables S4+S5). Data evaluation was performed using the Agilent MassHunter Software and Microsoft Excel. Standard solutions of different concentrations (1 ng/mL to 10,000 ng/mL), prepared in the same solvents as the respective samples, were measured before and after the samples to quantify the chemical concentrations. To check the quality of the measurement and to exclude the carry-over between samples, blanks consisting of 100% acetonitrile and standard solutions were measured after every tenth sample.

Data evaluation

To derive the concentration response curves (CRCs), the effects (cytotoxicity, activation of oxidative stress response or activation of PPARγ) were plotted against C_{nom} or the experimentally determined $C_{free,medium}$. The concentration (nominal or free) which led to a reduction of the cell viability of 10 % (IC_{10}) was calculated using eq. 1 from the slope of the linear range of the CRC.[31]

$$IC_{10} = \frac{10\%}{slope} \qquad \text{eq. 1}$$

The reference compound in the AREc32 assay was *tert*-butylhydroquinone (tBHQ).[32] The induction ratio (IR) was calculated. The concentration which lead to an IR of 1.5 ($EC_{IR1.5}$) was

used as activity benchmark.[27] The $EC_{IR1.5}$ was calculated from the linear range of the CRCs at concentrations below cytotoxicity using eq. 2.

$$EC_{IR1.5} = \frac{0.5}{\text{slope}} \quad \text{eq. 2}$$

The reference compound in the PPARγ assay was the antidiabetic drug rosiglitazone and was used to determine the maximum effect. The concentration which leads to an effect of 10% of the maximum is the EC_{10}. It was calculated from the slope of the linear range of the CRCs at concentrations below cytotoxicity using eq. 3.

$$EC_{10} = \frac{10\%}{\text{slope}} \quad \text{eq. 3}$$

To compare the measured IC_{10} with baseline toxicity, the toxic ratios (TR) of the chemicals were calculated using eq. 4. Baseline toxicity is the minimum toxicity a substance can cause and is elicited by the integration of the chemical into biological membranes.[33]

$$\text{Toxic ratio (TR)} = \frac{IC_{10,\text{ baseline (nom or free)}}}{IC_{10,\text{ experimental (nom or free)}}} \quad \text{eq. 4}$$

The nominal $IC_{10,baseline}$ was calculated using the baseline toxicity QSARS for the respective assays from Escher et al.[34] The freely dissolved $IC_{10,baseline}$ was derived by dividing the critical membrane concentration (i.e., $C_{membrane,cell}$) of 69 mmol/L[34] by the $D_{lip/w}$ of the chemical. The specificity ratio (SR) (eq. 5) describes how much lower the effect concentration is than the corresponding inhibitory concentration of cytotoxicity. Only if SR > 1, an EC_{10} is valid and only if SR >10 the effect is specific.

$$\text{Specificity ratio (SR)} = \frac{IC_{10,nom(free)}}{EC_{10,nom(free)} \text{ or } EC_{IR1.5,nom(free)}} \quad \text{eq. 5}$$

The absolute recovery of the chemicals from assay medium was calculated using eq. 6. Additionally, recoveries were normalized by the recovery at t_{0h} of the respective experiment to obtain the relative recoveries at both time points (t_{0h}/t_{0h} and t_{24h}/t_{0h}). The concentration of chemical

in the solvent after clean-up and the volume of solvent used are indicated by $C_{solvent}$ and $V_{solvent}$, $V_{aliquot}$ is the volume of the assay medium aliquot.

$$\text{Absolute recovery [\%]} = \frac{C_{solvent} \times V_{solvent}}{C_{nom} \times V_{aliquot}} \quad \text{eq. 6}$$

The concentration of the test chemicals in the SPME fiber (C_f) was calculated from the concentration measured in the desorption solution (C_{des}) and the volumes of desorption solution (V_{des}) and fiber (V_f) using (eq. 7).

$$C_f = \frac{C_{des} \times V_{des}}{V_f} \quad \text{eq. 7}$$

For the calculation of $C_{free,medium}$, eq. 8 was used.[3] The total amount of test chemical in the sample is n_{total}, which was calculated from C_{nom}.

$$C_{free,medium} = \frac{n_{total}}{D_{f/w} \times \left(\frac{n_{total}}{C_f} - V_f\right)} \quad \text{eq. 8}$$

A mass balance model was used for the prediction of $C_{free,medium}$ (eq. 9).[5]

$$C_{free,medium} = \frac{n_{total}}{D_{BSA/w} \times V_{prot,medium} + D_{lip/w} \times V_{lip,medium} + D_{cell/w} \times V_{cell} + V_{w,medium}} \quad \text{eq. 9}$$

The system parameters required for the calculation were the volumes of proteins, lipids and water of the medium ($V_{prot,medium}$, $V_{lip,medium}$, $V_{w,medium}$), the total volume of cells in the well (V_{cell}) and the cell-water distribution ratios of the chemicals ($D_{cell/w}$) and were taken from Henneberger et al.[3] Serum-albumin water ($D_{BSA/w}$) and liposome-water distribution ratios ($D_{lip/w}$) from different sources were used (see Table 1). All test chemicals of this study were non-volatile (medium-air partition constant >10,000)[34] ionizable or neutral hydrophilic chemicals and no losses to plastic well plates and air were expected.

Results

Total concentration measurements

For the majority of the test chemicals, absolute recoveries were high (>75 %) after extraction with the Phree Phospholipid Removal Plates (calculated using eq. 6, see Table S6, left columns). For some chemicals, low but still reproducible absolute recoveries were found, e.g., for lamotrigine from the AREc32 assay medium (45 %) and for ibuprofen from the PPARγ assay medium (48 %). For better comparison, the absolute recoveries were normalized to the recoveries at the beginning of the assay (see Table S6, right columns). These relative recoveries were used to assess whether $C_{total,medium}$ of the test chemicals was stable. For both bioassays, the relative recoveries determined at t_{0h} and t_{24h} did not differ significantly for the majority of the test chemicals (unpaired t test, see recoveries in Table S6 and Fig S3), indicating stable exposure conditions. For diphenhydramine in the AREc32 assay and warfarin and telmisartan in the PPARγ assay, the relative recovery from assay medium was significantly increasing over time (up to 43 %). Increasing recovery can be caused by a loss of water from the wells during incubation of the assay plate (chemical-independent) or can be an experimental artifact of the used clean-up method (chemical-specific). For genistein and propranolol in the AREc32 assay, $C_{total,medium}$ was decreasing over time by 27 and 17 % after 24 h, respectively. Two previous studies suggest that the genetically modified MCF7 cells used in the AREc32 assay can metabolize xenobiotic chemicals like benzo[a]pyrene[3, 35] and cellular metabolism might have caused the loss of chemical over time.

Nominal and freely dissolved effect concentrations

The experimentally determined nominal and freely dissolved effect concentrations for cytotoxicity ($IC_{10,nom}$ and $IC_{10,free}$), activation of oxidative stress response ($EC_{IR1.5,nom}$ and $EC_{IR1.5,free}$) and activation of PPARγ ($EC_{10,nom}$ and $EC_{10,free}$) are summarized in Table 2. The nominal and freely dissolved concentration-response curves of all test chemicals for both assays

can be found in the SI section S8. All chemicals were cytotoxic in the AREc32 assay and twelve of the 15 test chemicals were cytotoxic in the PPARγ assay. Three chemicals activated the oxidative stress response in the AREc32 assay (propranolol, genistein and labetalol) and nine chemicals (caffeine, lamotrigine, diclofenac, 2,4-D, naproxen, ibuprofen, torasemide, warfarin, telmisartan), including all monoprotic organic acids, activated PPARγ. The lowest $EC_{10,nom}$ and $EC_{10,free}$ were determined for telmisartan, a known activator of PPARγ.[36, 37]

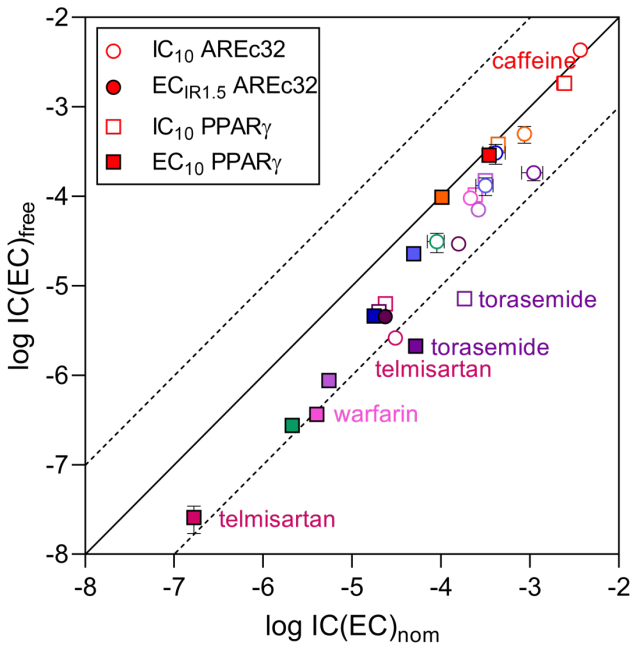
For caffeine and lamotrigine, $IC_{10,nom}$ and $IC_{10,free}$ from both assays, as well as $EC_{10,nom}$ and $EC_{10,free}$ in the PPARγ assay, were very similar due to the high free fractions in assay medium (Fig. 2). For caffeine, this finding is in line with the results of a study previously published.[3] The organic acids diclofenac, naproxen, ibuprofen and warfarin showed similar $IC_{10,nom}$ and $IC_{10,free}$ (within a factor of 4). In contrast, $EC_{10,nom}$ and $EC_{10,free}$ of the organic acids in the PPARγ assay differed by up to a factor of 11, because of the lower free fractions at low concentrations. Ratios between $IC_{10,nom}$ and $IC_{10,free}$ were also less than a factor of ten for all other chemicals in both assays (Fig. 2), except for torasemide in the PPARγ assay ($IC_{10,free}$ 26× lower than $IC_{10,nom}$) and for telmisartan in AREc32 assay ($IC_{10,free}$ 12× lower than $IC_{10,nom}$). Genistein was the only chemical with experimentally determined $C_{free,medium}$ that was active in AREc32, and both $IC_{10,free}$ and $EC_{IR1.5,free}$ were five times lower than the respective nominal effect concentrations. Lower free fractions were expected for the AREc32 medium, because it contained more lipids and proteins than the medium of the PPARγ assay.[3] For the acid torasemide, $C_{free,medium}$ was increasing linearly with C_{nom} in the PPARγ assay and both, $IC_{10,free}$ and $EC_{10,free}$, were about a factor of 25 lower than the respective nominal effect concentrations. Interestingly, $IC_{10,free}$ of torasemide in the AREc32 assay was only 5 times lower than $IC_{10,nom.}$ However, the freely dissolved concentrations of both media are hardly comparable for most of the chemicals due to non-linear binding to medium components.

Table 2 Nominal and freely dissolved effect concentrations of the test chemicals.

AREc32 assay								
	Cytotoxicity				*Activation of oxidative stress response*			
Chemical	$IC_{10,nom}$ [M]	CV [%]	$IC_{10,free}$ [M]	CV [%]	$EC_{IR1.5,nom}$ [M]	CV [%]	$EC_{IR1.5,free}$ [M]	CV [%]
Caffeine	3.68×10^{-3}	7.7	4.33×10^{-3}	9.4	Not active up to IC_{10}			
Lamotrigine	8.63×10^{-4}	9.0	5.00×10^{-4}	21.1	Not active up to IC_{10}			
Venlafaxine	4.51×10^{-4}	4.7	4.51×10^{-4} [a]	4.7[a]	Not active up to IC_{10}			
Metoprolol	7.89×10^{-4}	13.8	7.89×10^{-4} [a]	13.8[a]	Not active up to IC_{10}			
Diphenhydramine	1.69×10^{-4}	6.0	1.69×10^{-4} [a]	6.0[a]	Not active up to IC_{10}			
Propranolol	1.47×10^{-4}	11.3	1.47×10^{-4} [a]	11.3[a]	5.84×10^{-5}	6.6	5.84×10^{-5} [a]	6.6[a]
Diclofenac	8.94×10^{-5}	21.6	3.12×10^{-5}	24.4	Not active up to IC_{10}			
2,4-D	3.16×10^{-4}	21.9	1.32×10^{-4}	22.5	Not active up to IC_{10}			
Naproxen	4.11×10^{-4}	28.3	3.06×10^{-4}	25.2	Not active up to IC_{10}			
Ibuprofen	2.63×10^{-4}	6.3	7.09×10^{-5}	5.5	Not active up to IC_{10}			
Torasemide	1.10×10^{-3}	27.1	1.84×10^{-4}	18.7	Not active up to IC_{10}			
Warfarin	2.15×10^{-4}	8.0	9.56×10^{-5}	6.4	Not active up to IC_{10}			
Genistein	1.58×10^{-4}	7.9	2.96×10^{-5}	10.1	2.36×10^{-5}	5.3	4.55×10^{-6}	4.9
Telmisartan	3.07×10^{-5}	5.2	2.62×10^{-6}	8.1	Not active up to IC_{10}			
Labetalol	8.90×10^{-5}	7.2	8.90×10^{-5} [a]	7.2[a]	3.36×10^{-5}	7.0	3.36×10^{-5} [a]	7.0[a]
PPARγ assay								
	Cytotoxicity				*Activation of PPARγ*			
Chemical	$IC_{10,nom}$ [M]	CV [%]	$IC_{10,free}$ [M]	CV [%]	$EC_{10,nom}$ [M]	CV [%]	$EC_{10,free}$ [M]	CV [%]
Caffeine	2.44×10^{-3}	18.2	1.84×10^{-3}	13.7	3.48×10^{-4}	6.6	2.89×10^{-4}	8.9
Lamotrigine	4.39×10^{-4}	13.8	3.82×10^{-4}	15.6	1.02×10^{-4}	10.5	9.81×10^{-5}	10.7
Venlafaxine	2.11×10^{-4}	23.7	2.11×10^{-4} [a]	23.7[a]	Not active up to IC_{10}			
Metoprolol	2.31×10^{-4}	20.8	2.31×10^{-4} [a]	20.8[a]	Not active up to IC_{10}			
Diphenhydramine	3.60×10^{-5}	3.0	3.60×10^{-5} [a]	3.0[a]	Not active up to IC_{10}			
Propranolol	1.84×10^{-5}	4.4	1.84×10^{-5} [a]	4.4[a]	Not active up to IC_{10}			
Diclofenac	[b]				2.12×10^{-6}	11.0	2.75×10^{-7}	10.4
2,4-D	Not active up to 4.64×10^{-4} M		Not active up to 2.19×10^{-4} M		4.92×10^{-5}	3.1	2.28×10^{-5}	4.3
Naproxen	Not active up to 3.86×10^{-4} M		Not active up to 3.27×10^{-4} M		1.77×10^{-5}	4.2	4.62×10^{-6}	5.3
Ibuprofen	3.16×10^{-4}	15.7	1.50×10^{-4}	11.1	5.46×10^{-6}	9.1	8.78×10^{-7}	11.1
Torasemide	1.84×10^{-4}	7.7	7.19×10^{-6}	6.7	5.19×10^{-5}	8.1	2.12×10^{-6}	8.3
Warfarin	2.42×10^{-4}	10.5	1.04×10^{-4}	15.2	4.01×10^{-6}	5.2	3.67×10^{-7}	10.6
Genistein	2.01×10^{-5}	5.2	5.14×10^{-6}	4.6	Not active up to IC_{10}			
Telmisartan	2.37×10^{-5}	12.4	6.32×10^{-6}	12.5	1.67×10^{-7}	9.6	2.58×10^{-8}	33.4
Labetalol	2.31×10^{-5}	3.4	2.31×10^{-5} [a]	3.4[a]	Not active up to IC_{10}			

[a] Freely dissolved effect concentrations were not determined experimentally, but assumed to be equal to nominal effect concentrations; [b] Cytotoxicity observed, but <10%

Fig. 2 Comparison of freely dissolved and nominal effect concentrations. Data for venlafaxine, metoprolol, diphenhydramine, propranolol and labetalol are not shown, because freely dissolved effect concentrations were assumed to be equal to nominal effect concentrations.



For the bases venlafaxine, metoprolol, diphenhydramine, propranolol and the multifunctional chemical labetalol, $C_{free,medium}$ was expected to be similar to C_{nom}. The equilibrium dialysis experiments conducted for propranolol and labetalol with both assay media confirmed this hypothesis. The free fractions of propranolol and labetalol were about 100 % in the PPARγ assay medium and 65 and 93 %, respectively in the AREc32 assay medium. Consequently, the freely dissolved effect concentrations were assumed to be equal to the nominal effect concentrations (Table 2).

We compared the activity data of this study with the results from the US EPA Tox21 program (downloaded from https://comptox.epa.gov/dashboard/chemical_lists/toxcast on 28 April 2020, see Table S7). In Tox21 the same cell line was used for the PPARγ assay, but a different cell line (ARE_BLA) was used for the detection of oxidative stress response. The comparison showed an

agreement with the Tox21 data for the activation of the oxidative stress response for ten of the 15 test chemicals. Caffeine, diclofenac and telmisartan did not activate the oxidative stress response in this study, but were classified as active chemicals in the Tox21 program. Propranolol and labetalol, on the contrary, were classified as active in this study, but showed no activation of the oxidative stress response in Tox21. The SRs determined for the activation of the oxidative stress response in this study were all below 10, which shows that the oxidative stress response is a rather unspecific effect that is difficult to distinguish from cytotoxicity (see also discussion in next section). For the PPARγ assay, the comparison with the Tox21 data also showed agreement for ten of the 15 test chemicals. Caffeine, lamotrigine, torasemide, diclofenac and ibuprofen activated the PPARγ in this work but were classified as non-active in Tox21. However, in this study, caffeine, lamotrigine and torasemide were classified as weak activators of the PPARγ with SRs <10.

Nominal and freely dissolved effect concentrations of five chemicals tested in this study (caffeine, metoprolol, propranolol, diclofenac, labetalol) were also in good agreement with results from an earlier work.[3] The majority of the data agreed within a factor of 2.5. For diclofenac, the $EC_{10,nom}$ in the PPARγ assay differed by a factor of 5.3 and for caffeine, the $IC_{10,free}$ in the AREc32 assay differed by a factor of 3.8. Labetalol, previously classified as not active, showed activation of oxidative stress response in this work, but with a low specificity (see also next section).

Toxic ratio (TR) and specificity ratio (SR) analysis

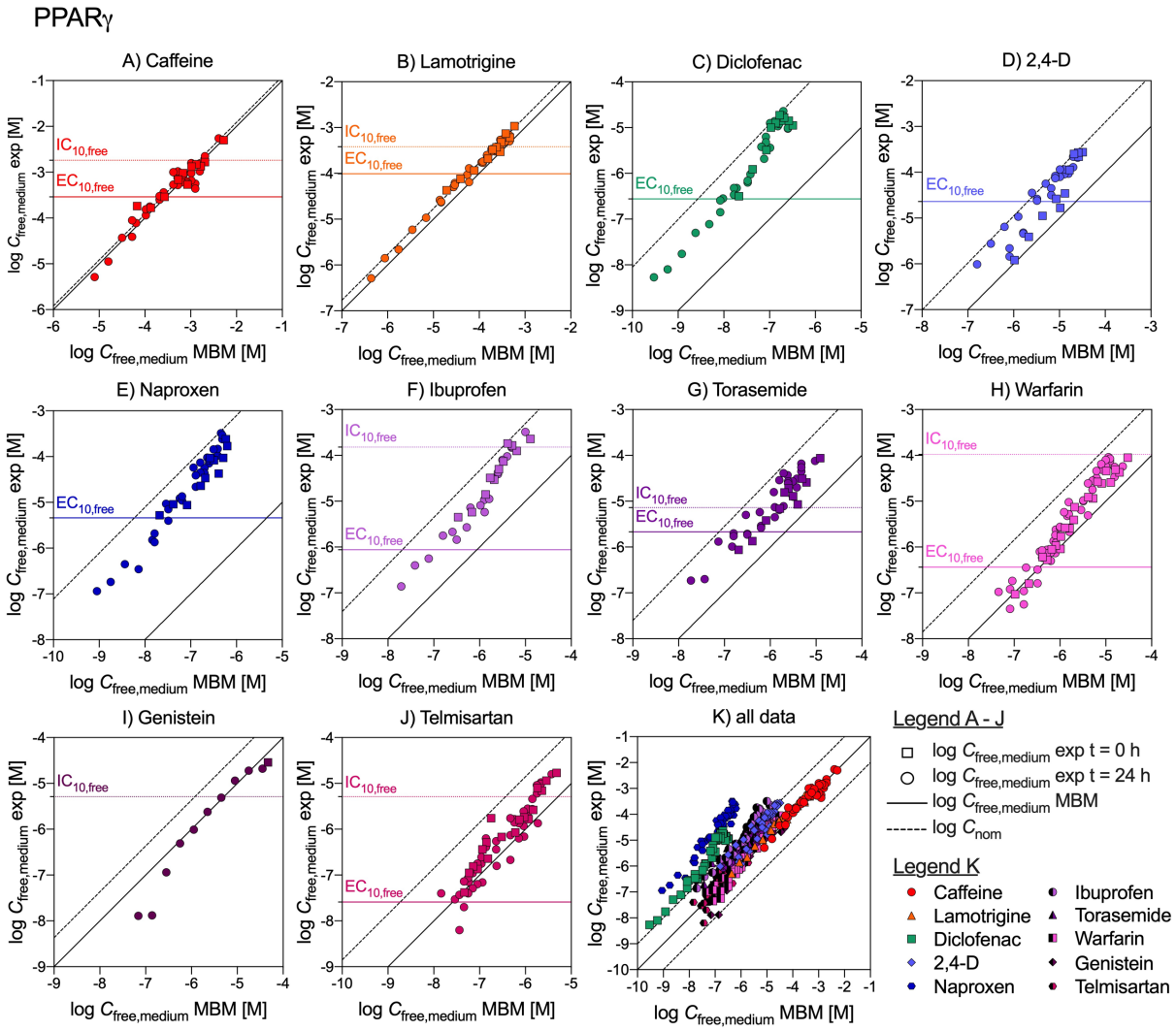
The TRs and SRs calculated from the nominal and freely dissolved effect concentrations were often very similar (Fig. S6). Nearly all nominal TRs were between 1 and 10 (except for diphenhydramine and propranolol in PPARγ), indicating that the majority of the tested chemicals was baseline toxic. If the TRs were calculated from $IC_{10,free}$, more chemicals exceeded the threshold of 10, indicating specific modes of toxic action. The TRs were slightly above 10 for

caffeine, ibuprofen and warfarin in the AREc32 assay and for caffeine, diphenhydramine, propranolol and warfarin in the PPARγ assay. The highest TR calculated based on $IC_{10,free}$ was 85 for torasemide in the PPARγ assay. The specificity ratio analysis revealed that activation of oxidative stress response in the AREc32 assay was probably caused by the cytotoxicity burst phenomenon[38, 39] for all chemicals, as SRs were below 10. Even genistein, a phytoestrogen that is known to induce oxidative stress response,[40] showed an SR below 10 in this study. The same applied to caffeine, lamotrigine and torasemide for the activation of PPARγ. For ibuprofen, warfarin and telmisartan the SRs for the PPARγ assay based on the freely dissolved concentrations were above 100 indicating highly specific activation of PPARγ. For diclofenac, 2,4-D and naproxen, no SR could be calculated for PPARγ activation, because the chemicals were not cytotoxic. Hence, the activation was likely also specific for these chemicals.

Comparison of experimentally determined and predicted $C_{free,medium}$

For ten of the 15 test chemicals, $C_{free,medium}$ was determined for all tested concentration levels in both bioassays (see Fig. 3 for PPARγ assay and Fig. S8 for AREc32 assay) at two different time points of the assay. No difference between $C_{free,medium}$ at the beginning of the assay (t_{0h}, squares in Fig. 3A-J) and after 24 h of exposure (circles in Fig. 3A-J) was found, which is line with the results from the total concentration measurements and confirms again stable exposure conditions. The free fractions of the test chemicals were found to be generally smaller in the AREc32 assay compared to the PPARγ assay. The AREc32 assay medium contained five times more FBS than the PPARγ medium[3] leading to a larger depletion of $C_{free,medium}$ by binding of the chemicals to medium proteins and lipids.

Fig. 3 Comparison of experimentally determined freely dissolved concentrations in the medium sampled from the PPARγ assay ($C_{free,medium}$ exp) with freely dissolved medium concentrations ($C_{free,medium}$ MBM) predicted using the mass balance model[5] (MBM).



The distribution of the test chemicals between the different phases of the assay medium (water, lipids and proteins) was predicted by the mass balance model and visualized by a chemical space plot (Fig. S7). For caffeine, lamotrigine, venlafaxine, metoprolol, diphenhydramine, propranolol, and labetalol, a high unbound fraction (>50 %) was calculated in both assay media, while for all other chemicals more than 70 % of the molecules were predicted to be bound to the medium

proteins. For diclofenac and naproxen, the fraction bound to proteins was even above 99 % in both assay media.

To assess the performance of the mass balance model, the RMSE was calculated (Table S7). For the hydrophilic chemicals caffeine and lamotrigine and the acid genistein, predicted and experimentally determined $C_{free,medium}$ agreed well for both bioassays, indicated by an RMSE of <0.3 log-units (Fig. 3A, B and I and Fig. S8A, B and I). For the acid warfarin and the complex chemical telmisartan, a reasonable agreement of modelled and measured $C_{free,medium}$ was found (RMSE <0.6 log-units, Fig. 3H, J and Fig. S8H, J). The organic acids diclofenac, naproxen, ibuprofen and warfarin showed a non-linear increase of $C_{free,medium}$ with increasing C_{nom} in both assays (see Fig. 3C, E, F & H for PPARγ assay and Fig S8C, E, F & H for AREc32 assay). Therefore, $C_{free,medium}$ was close to C_{nom} at high concentrations for these chemicals and the mass balance model underestimated $C_{free,medium}$ for diclofenac, 2,4-D, naproxen, ibuprofen and torasemide (RMSE >0.8 log-units, Fig. 3C-G, Fig. S8C-G). The highest discrepancies between predicted and experimentally determined $C_{free,medium}$ were found for naproxen (RMSE >2.3 log-units, Fig. 3E, Fig. S8E). Because the free fraction increased non-linearly with the nominal concentration (e.g., diclofenac, naproxen, ibuprofen and warfarin), the differences between modelled and measured $C_{free,medium}$ increased as well. Interestingly, for some of the organic acids (e.g., diclofenac and naproxen) the mass balance model underestimated $C_{free,medium}$ even at low concentrations.

Discussion

For caffeine and lamotrigine, the measurement of $C_{free,medium}$ showed only little deviation from C_{nom}. For the bases, $C_{free,\ medium}$ was not determined with SPME, but the results of equilibrium dialysis of propranolol and labetalol showed very low binding to media components for these

chemicals. Hence, the determination of $C_{total,medium}$ at two different time points was found to be sufficient to quantify *in vitro* exposure for these chemicals. Furthermore, if $C_{total,medium}$ is stable for the duration of the assay, which was the case for all chemicals of this study, nominal effect concentrations can be used for QIVIVE. However, we recommend to use freely dissolved effect concentrations if $C_{free,medium}$ is reduced by >20 % to account for binding to medium components. This is especially important for organic acids, because $C_{free,medium}$ was not only reduced by the medium, but no linear relationship between $C_{free,medium}$ and C_{nom} was found in this study for diclofenac, naproxen, ibuprofen, warfarin, torasemide and telmisartan in at least one assay medium (see Figures 3 and S8), which is in line with previously published data.[3, 14] This phenomenon can be explained by the limited number of high affinity binding sites on serum albumin and is especially relevant for the calculation of $IC_{10,free}$, because cytotoxicity occurs at high concentrations of the chemicals. The saturation phenomenon is probably less relevant for specific (i.e., receptor-mediated effects) that are observed at lower concentrations of the chemicals.

Mass balance models have been proposed as simple tools to predict $C_{free,medium}$. However, only for three chemicals that showed significant binding to medium components (warfarin, genistein, telmisartan), the mass balance model provided reliable estimates of $C_{free,medium}$. For organic acids, freely dissolved effect concentrations could not be derived by a simple mass balance model. Experimental determination of $C_{free,medium}$ not only at two different time points, but also at different concentrations was required to derive the freely dissolved effect concentrations for these chemicals. For some of the organic acids tested (e.g., diclofenac and naproxen), the model was found to underpredict $C_{free,medium}$ also at low concentrations. This can be explained by the fact that the protein binding data used as input parameters for the mass balance model were generated using

purified, essentially fatty acid free albumin,[14] while the assay medium contained fatty acids as nutrients that can compete with the test chemicals for binding sites.[41, 42]

The results of this study emphasize again the need for experimental determination of chemical concentrations in *in vitro* cell-based bioassays as many processes such as saturation of and competition for specific protein binding sites cannot be covered by currently available prediction models. The extension of the models by non-linear or competitive binding is theoretically possible, but challenging owing to the complexity of these sorption processes.

Experimental exposure assessment can also help to exclude carry-over, mistakes during the assay workflow and loss processes like metabolization or abiotic degradation of the chemical over the duration of the bioassay. Exposure assessment, at least determination of $C_{\text{total,medium}}$, is also recommended for chemicals with high free fractions, as they can be degraded abiotically or metabolized during the assay leading to unstable exposure conditions. If the free fraction in the medium is reduced by >20 %, $C_{\text{free,medium}}$ should be measured additionally to account for binding to medium components. Experimental determination of $C_{\text{free,medium}}$ may be conducted using the SPME approach of this study or other methods like equilibrium dialysis. Future studies should also focus on the further investigation of medium saturation, not only for IOCs (e.g., multiprotic acids, multifunctional chemicals), but also for neutral chemicals of different hydrophobicity. This study and previous work from our group have provided a convenient workflow for exposure assessment of single chemicals in 96-well plates. The next challenges will be the exposure assessment for assays with longer incubation times, as well as the application of the SPME method to higher-tier well plate formats (i.e., 384-well plates), to neutral hydrophobic chemicals and to chemical mixtures.

ASSOCIATED CONTENT

Supporting Information.

The following files are available free of charge.

Additional information on the test chemicals, SPME method, assay plate layout, equilibrium dialysis, instrumental analysis and total concentration measurements, nominal and freely dissolved concentration-effect curves, figures showing the toxic ratio and specificity ratios, chemical space plots and comparison of experimental and predicted $C_{free,medium}$ in the AREc32 assay (PDF)

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