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Application of Mass Balance Models and the Chemical Activity Concept To Facilitate the Use of in Vitro Toxicity Data for Risk Assessment

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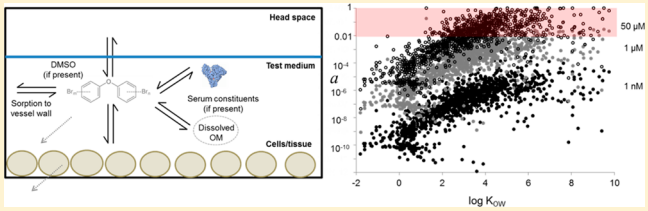
Supporting Information

ABSTRACT: Practical, financial, and ethical considerations related to conducting extensive animal testing have resulted in various initiatives to promote and expand the use of in vitro testing data for chemical evaluations. Nominal concentrations in the aqueous phase corresponding to an effect (or biological activity) are commonly reported and used to characterize toxicity (or biological response). However, the true concentration in the aqueous phase can be substantially different from the nominal. To support in vitro test design and aid the interpretation of in vitro toxicity data, we developed a mass balance model that can be parametrized and applied to represent typical in vitro test systems. The model calculates the mass distribution, freely dissolved concentrations, and cell/tissue concentrations corresponding to the initial nominal concentration and experimental conditions specified by the user. Chemical activity, a metric which can be used to assess the potential for baseline toxicity to occur, is also calculated. The model is first applied to a set of hypothetical chemicals to illustrate the degree to which test conditions (e.g., presence or absence of serum) influence the distribution of the chemical in the test system. The model is then applied to set of 1194 real substances (predominantly from the ToxCast chemical database) to calculate the potential range of concentrations and chemical activities under assumed test conditions. The model demonstrates how both concentrations and chemical activities can vary by orders of magnitude for the same nominal concentration.



INTRODUCTION

Reliable and representative toxicity and effects data are a prerequisite for conducting chemical risk assessment (RA). However, only a small proportion of the many thousands of chemicals in use can be considered well characterized with respect to toxicity and potential to cause adverse effects.[1] For ecological risk assessment (ERA), wildlife exposure and effects data are often compared using external concentration metrics (e.g., LC50s derived from aquatic toxicity tests; LD50s derived from dietary exposure studies) and in some cases on a whole body or tissue-specific basis.[2,3] For human health risk assessment (HHRA), estimates of total daily intake rates (exposures) are compared with thresholds of toxicological concern (TTC) or reference doses based on extrapolations of appropriate dose-based end points (e.g., LD50s derived from dietary exposure studies).[4−7] Because of the practical, financial, and ethical considerations related to conducting extensive animal testing, initiatives to promote and expand the use of in vitro toxicity testing are gaining prominence (e.g., the US EPA's Tox21 (http://epa.gov/ncct/Tox21) and ToxCast (www.epa.gov/ncct/toxcast) programs; the EU sixth Framework Programme project OSIRIS; www.ufz.de/osiris/; the CellSens project www.cefic-lri.org/projects).[8−16] For example, in vitro concentration metrics termed Activity Concentrations (AC50, concentration at which biological activity related to a response following exposure is 50% of its maximum) are being used to derive exposure metrics applicable to the whole organism (e.g., oral equivalent doses) through extrapolation techniques.[12,14,16] Complementary methods to translate other in vitro toxicity data (e.g., EC50s) into metrics amenable to the needs of RA are required, as reliance on such data will increase in the future.

In vitro test systems are multicomponent environments, and differences in chemical behavior in these systems have long been recognized as important factors influencing responses.[17−30] In particular, the dependence of cytotoxicity on the serum content of the bulk in vitro medium is well-established. This dependency follows from the reduction in freely dissolved concentration of chemical due to sorption to plasma proteins and other biological macromolecules (e.g., lipids) present in the medium. In other words, the total assumed (i.e., nominal, unmeasured) concentration in the medium is not an adequate indicator of exposure when interpreting and comparing in vitro toxicity data for different

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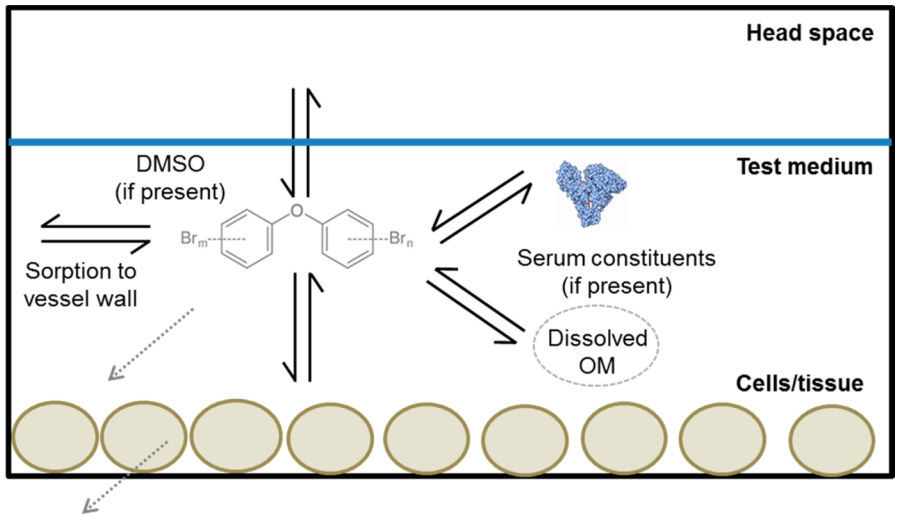

Figure 1. Conceptual representation of an in vitro test system. DMSO: dimethyl sulfoxide, an example of a cosolvent. OM: organic matter.

chemicals and between different in vitro systems. As succinctly stated by Gülden and Seibert,[19] "concentration-effect relationships determined in vitro depend not only on the activity of the chemical compounds or the sensitivity of the targets but also on the distribution of compounds in the in vitro systems". Various factors influencing chemical distribution in an in vitro test system have been explored by these authors and others. Based on this research, a partitioning-based approach for extrapolating in vitro concentration data to equivalent human serum concentrations has been developed.[31,32] Partitioning-based approaches have also been applied to characterize the behavior of chemicals in microsomal incubation systems and their sorption to hepatocytes suspended in culture medium.[33,34] The ability to confidently relate nominal (assumed) concentrations to cell/tissue concentrations would clearly be valuable for high-throughput screening (HTS) as well.

Chemical activity, its relationship to chemical concentration, and its use to characterize baseline toxicity (narcosis) were first described by Ferguson over 70 years ago.[35] More recently, the utility of chemical activity for distinguishing between chemicals exerting baseline toxicity and more specific modes of action and for applications in RA has been explored.[36−40] Just as with in vivo toxicity data, the ability to categorize in vitro toxicity test results in terms of mode of action would be useful. Accordingly, the main objective of this paper is to develop a generic mass balance modeling approach to estimate the distribution of neutral organic chemicals in in vitro test systems and the corresponding chemical activity. Note that chemical activity is a well-defined metric rooted in thermodynamic considerations, whereas biological activity and Activity Concentrations are generic terms for characterizing any sort of biological response to chemical exposure. The model is first applied to a set of hypothetical chemicals to illustrate the significance of chemical distribution in a typical in vitro test system across a range of partitioning properties. The model is then applied to chemicals being used in high-throughput in vitro bioassay and toxicity screening to illustrate how both mass balance models and the chemical activity concept can potentially be used to help interpret and apply such results for HTS and prioritization.

MATERIALS AND METHODS

An in vitro test system is represented conceptually in Figure 1. The exposure scenario considered here is a single addition of chemical to the test system (e.g., via "solvent spiking"), which yields a nominal test concentration based on the amount of chemical added (mol) and the volume of bulk medium (L). It does not refer to passive dosing approaches which use sorbent materials (e.g., silicone O-rings[41,42]) loaded with chemical to supply the bulk medium with test compounds. As illustrated, the mass of chemical added to the bulk medium is expected to distribute between the key phases in the system (i.e., aqueous phase, head space, cells/tissue) according to the partitioning properties of the chemical. Figure 1 also illustrates that serum constituents (i.e., plasma proteins and lipids) and dissolved organic matter (DOM) may be present in the bulk medium, both of which can influence the distribution of the chemical in the test system. DOM could be present unintentionally (e.g., cell exudates) or intentionally (e.g., other types of proteins present in the cell culture medium). Besides partitioning processes, degradation in the aqueous phase or within the cells/tissues may also occur. Sorption to the vessel walls has also been observed but is dependent on the type of material used and experimental conditions.[29,43,44] The approaches applied to characterize the distribution of neutral organic chemicals in in vitro test systems are presented in the following sections.

Equilibrium Distribution Model. The model assumes instantaneous equilibrium partitioning between the various phases present in the in vitro test system. The nominal test concentration is converted to total amount (M_T, mol) and then used along with phase volumes and partition coefficients to calculate the freely dissolved aqueous concentration in the test system (C_W, in mol L^{-1}, M) as

$$C_W = \frac{M_T}{K_{AW}V_A + V_W + K_{SaW}V_{Sa} + K_{SlW}V_{Sl} + K_{DW}V_D + K_{CW}V_C} \quad (1)$$

where K_{AW} is the air−water partition coefficient, V_A is the volume of head space, V_W is the volume of medium (aqueous phase only), K_{SaW} is the serum albumin−water partition coefficient, V_{Sa} is the volume of serum albumin, K_{SlW} is the serum lipid−water partition coefficient, V_{Sl} is the volume of serum lipids, K_{DW} is the dissolved organic matter (DOM)−water partition coefficient, V_D is the volume of DOM, K_{CW} is

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the cell/tissue-water partition coefficient, and V_C is the volume of cells/tissue. All partition coefficients are dimensionless (i.e., L L^{-1}), and all phase volumes are expressed in liters (L) for the calculations. In the absence of user-specified values, K_{CW} is estimated as K_{OW} multiplied by the volume fraction of lipid equivalents (i.e., pseudo-octanol content) (f_L),[45] and serum albumin−water partitioning (K_{SaW}) is based on the data analysis of Endo and Goss.[46] It is assumed that interactions with serum albumin occur according to a linear sorption isotherm (i.e., the potential for saturation at high substrate concentrations is not considered). Methods for estimating other partition coefficients are described in the Supporting Information (section S1). If a phase is not present in the system, its volume can be set to zero. The model is intended for neutral organic chemicals and should only be applied to ionogenic organic chemicals (IOCs) if the compound is predominantly neutral at the test pH.

Once calculated, C_W is then compared to the water solubility limit of the chemical (S_W). If the water solubility limit is exceeded (i.e., $C_W > S_W$), the excess is assumed to precipitate out of solution (i.e., be unavailable to partition into any other phase) and S_W is used to recalculate the mass of chemical freely dissolved in the test system (M_W) along with the concentrations of chemical associated with all other phases. If the water solubility is not exceeded, C_W is used for all calculations as

$$M_W = (C_W \text{ or } S_W)V_W, \quad C_X = (C_W \text{ or } S_W)K_{XW}, \quad M_X = C_X V_X \tag{2}$$

where C_X, K_{XW}, M_X, and V_X are chemical concentrations, partition coefficients with water, masses of chemicals, and volumes for other phases in the system. As justified in the Supporting Information (section S2), the potential influence of cosolvents (e.g., dimethyl sulfoxide, DMSO) on S_W is ignored. Adsorption to the vessel walls is also ignored in the current calculations. The potential influence of adsorption to the vessel walls is likely to be most important in bulk media with very low serum volume fractions (VFs), particularly when concentrations exceed the water solubility limit. For example, the estimated fraction of brominated diphenyl ether (BDE-47) associated with vessel walls was approximately 25−40% in experiments with no serum added, approximately 15% with a serum VF of 0.01 and negligible with a serum VF of 0.10.[43] Available data suggests that polymer−water partition coefficients (log K_{PW}, volumetric) are substantially lower than log K_{OW} (and hence lipid−water partition coefficients).[47−51] These data are consistent with the relationship between serum VF and mass fraction sorbed to vessel wall described above (i.e., chemicals have greater affinity for serum lipids than the vessel walls). Additional studies are required to validate a generic approach to quantify this phenomenon under realistic in vitro test conditions. Exposure duration, contact surface area, and the relative sorption capacities of the vessel material and bulk medium are key variables. The implications of ignoring sorption to the vessel walls are discussed following presentation of the model output.

The modeling approach implicitly assumes no degradation in all phases, and accordingly, will overestimate masses and concentrations of chemicals highly prone to degradation in the test system. The potential bias is particularly relevant for chemicals that degrade quickly (e.g., via hydrolysis) and for tests using cells/tissues with a high capacity to biotransform chemicals (e.g., hepatocytes). Another assumption of the modeling approach is that the cells/tissues exhibit no growth during the exposure period. As the experimental scenario considered here is static (i.e., fixed amount of chemical added to system, not constant input), steady-state modeling approaches cannot be applied. The treatment of the in vitro test system is thus analogous to a Level I multimedia environmental fate model.[45]

Chemical Activity Concept and Baseline Toxicity. For a chemical dissolved in water, the chemical activity (a; unitless) is calculated as[38]

$$a = \frac{C_W}{S_W^L} \tag{3}$$

where S_W^L is the subcooled liquid water solubility limit if the chemical is a solid at the test system temperature or the water solubility limit if the chemical is a liquid. If the chemical is a solid at the test system temperature, S_W^L is calculated using the water solubility of the solid (S_W^S) and the fugacity ratio (F).[37,45] Under this approach, the chemical activity of a solid will always be less than 1 even at the water solubility limit because $S_W^L > S_W^S$, as determined by F. At equilibrium, the chemical activity in the cell/tissue (and all other phases) is equivalent to the chemical activity in the aqueous phase. The main uncertainties in chemical activity calculations stem from potential errors in water solubility limits, melting point, and entropies of fusion. See the Supporting Information (section S3) for details. All chemicals are expected to exert baseline toxicity regardless of whether or not other modes of action are exhibited. For the purposes of this exercise, it is assumed that a chemical activity of 0.01 or higher indicates the potential for baseline toxicity to occur.[36−40] In an in vitro cell assay, baseline toxicity is assumed to reflect disruption of membrane integrity and functioning (e.g., loss of insular capacity).[52]

Model Parametrization and Application. The model is implemented in Microsoft Excel 2010 using the Visual Basic for Applications (VBA) programming language and is provided as Supporting Information. The tool can be parametrized for any in vitro test system as long as estimated volumes and compositions of the various components are available. For the current study, the model was parametrized to represent a 25 cm^2 cell culture flask with a total volume of 50 mL. All calculations assume a temperature of 37 °C and an ionic strength of 0.15 M. The total volume of the bulk medium (i.e., aqueous phase, serum albumin/lipids, DOM) excluding cells/tissues was set to 5 mL. The mass of cells/tissue was assumed to be 3 mg, corresponding to a volume of 3 μL given a density of 1 kg L^{-1}. The volume of head space (V_A) is then the remainder (i.e., ∼45 mL). The model can be reparameterized to simulate in vitro tests conducted in well plates commonly used for HTS but would require the user to estimate an appropriate volume of head space above the medium.

The volumes of serum albumin and serum lipids are calculated from the user-defined serum VF in the bulk medium, concentrations of albumin and lipids in the serum (g L^{-1}), and densities of 1.36 and 1 kg L^{-1} for serum albumin and lipids, respectively.[46,53] Calculations presented here assume serum albumin and lipid concentrations of 24 and 1.9 g L^{-1} respectively, representative values for fetal bovine serum (FBS).[32] For reference, serum albumin and lipid concentrations in human serum were reported as 38 and 7.2 g L^{-1}, respectively, in the same publication. The bulk medium was assumed to contain negligible amounts of DOM (i.e., V_{DOM} = 0 mL).

Assuming f_L = 0.05 and the VF of FBS in the bulk medium is 0.10, the model parametrization described above corresponds to a test system with 3.0×10^{-5} mL cell/tissue lipid per mL bulk medium and 1.8×10^{-3} mL serum albumin and 1.8×10^{-4} mL serum lipid per mL bulk medium, respectively. Assuming f_L = 0.01, the test system has 6.0×10^{-6} mL cell/tissue lipid per mL bulk medium. These values are broadly consistent with the phase VFs reported in an empirical study by Gülden et al.[32]

Two illustrative applications of the model were conducted. The model was first applied to a set of hypothetical chemicals with log K_{OW} ranging from 1 to 10 and log K_{AW} ranging from −4 to 5. Water solubility limits were estimated using K_{OW} and an assumed solubility in octanol (S_O), as detailed in the Supporting Information (section S4). The VF of serum in the bulk medium was varied from 0−0.20, and f_L was assumed to range between 0.01−0.05. The main purpose of this model application is to highlight the relationship between partitioning properties and the behavior of neutral organic chemicals in the test system under different conditions. The behavior is characterized by the mass distribution (MD), the cell/tissue enrichment factor (EF), and the nominal concentration depletion factor (DF). EF and DF are calculated as

$$\mathrm{EF} = \frac{C_C}{C_{N,I}} \quad \text{and} \quad \mathrm{DF} = \left(\frac{C_W}{C_{N,I}}\right)^{-1} \tag{4}$$

where C_C is the concentration in the cell/tissue (wet weight) and $C_{N,I}$ is the nominal concentration in the test system (0.1 nM for the generic application). The EF metric indicates the extent to which the nominal concentration reflects the exposure-relevant concentration in the cells/tissue at equilibrium, whereas the DF metric indicates the extent to which the nominal concentration reflects the bioavailable (i.e., freely dissolved) concentration in the bulk medium at equilibrium.

The second illustrative application of the model used the physical-chemical properties of 1194 real chemicals; 1181 from the ToxCast chemical database. The procedures for compiling required property values and screening for ionization are described in the Supporting Information (section S5). Only neutral organic chemicals and IOCs that are predominantly neutral at pH 7.4 were selected for this application. Note that unlike for the application to the hypothetical chemicals (see above), the user is expected to enter measured or estimated water solubility data. The reactivity of these chemicals in the test system was not considered (i.e., we assume there is no chemical degradation or transformation during the test period). Model output was generated for nominal test concentrations ranging from 1 nM−50 μM, f_L from 0.01−0.05, and FBS VFs in serum ranging from 0−0.20. This range of nominal test concentrations is consistent with data reported for some persistent organic pollutants (POPs)[43,54−57] and the ToxCast in vitro assays.[11] Molar concentrations in cells/tissue are expressed in units more commonly reported for risk assessment (e.g., ng g^{-1}). The main objective of this application is to demonstrate the potential utility of the model for interpreting in vitro toxicity test data and, in particular, the value of relating nominal test concentrations to cell/tissue concentrations and chemical activity. As such, the model is not parametrized to the various bioassay-specific test conditions. The purpose of using chemicals from the ToxCast database is to explore the potential range of partitioning behavior in a generic in vitro test system environment for substances already established to be of scientific and regulatory interest. Key assumptions and limitations for the proposed model are summarized in the Supporting Information (section S6).

RESULTS AND DISCUSSION

Generic Model Application. Figure 2 presents the MDs, EFs, and DFs for the set of hypothetical chemicals as a function of log K_{OW} and log K_{AW} assuming there is no serum present in the bulk medium and FBS is present at a VF of 0.10, i.e., common cell/tissue in vitro test conditions. Note that f_L of the cells/tissue was assumed to be 0.05 for the results displayed in Figure 2. The mass distribution plots identify partitioning property combinations which result in two-thirds of the total mass in the head space, bulk medium (i.e., dissolved phase of medium plus serum albumin and lipids, if present), and cells/tissues, respectively. To help put this application into context, partitioning properties for real chemicals are superimposed in Figure 2.

As illustrated in Figure 2, the head space in the 50 mL culture flask is the dominant phase for chemicals with log K_{AW} > −0.5 and log K_{OW} < 5 under the current model assumptions. As K_{AW} decreases and/or hydrophobicity increases (i.e., log $K_{OW}\uparrow$), the cells/tissues or bulk medium become the main reservoirs. A key result shown in the MD panels of Figure 2 is the dominance of serum albumin and lipids over cell/tissue lipids as a mass reservoir when FBS is present. This model output is driven by the VFs of serum albumin and lipids in the test system being much greater than the VF of cell/tissue lipids (see the Materials and Methods) and is generated across the typical range of serum VFs in cell culture medium (0.02−0.20).

The presence of serum albumin and lipids also drives the differences in EF displayed in Figure 2. For example, the maximum EF in the absence of serum (1670) approximates the ratio between the volume of bulk medium (5 mL) and volume of cell/tissue (3 μL or 0.003 mL). However, once serum is present in the aqueous phase of the test system, the maximum EF is sensitive to the assumed concentrations (g L^{-1}) and partitioning properties of serum albumin and lipids. The value assumed for f_L is also a key determinant. For example, the maximum EF when f_L = 0.05 is 1000, 390, 220, 150, and 120 with FBS VFs of 0.01, 0.05, 0.1, 0.15, and 0.20 respectively. If f_L is assumed to be 0.01, the maximum EFs are 390, 95, 50, 35, and 25 for the same FBS volume fractions. If FBS is replaced with human serum (see characteristics reported above), the maximum EFs when f_L = 0.05 fall to 485, 125, 65, 45, and 35, respectively, for the same serum VFs. Both the VF and characteristics of serum present in bulk medium are key parameters for determining the distribution of neutral organic chemicals in in vitro test systems, consistent with empirical data.[19,24,43] Note that whereas the fractional distributions displayed in Figure 2 will not be influenced by sorption to vessel walls, the absolute values of EF will be sensitive to this phenomenon. For example, if 50% of the total mass is sorbed to the vessel walls, the EFs would be 2-fold lower. However, as losses due to this process under typical in vitro test conditions appear to be lower (e.g., 10−20%[43,44]), other factors (e.g., differences in serum VF) are likely to be more important considerations when interpreting in vitro toxicity data. More systematic data characterizing the relationship between chemical properties, test conditions, and propensity to sorb to vessel walls are required to explicitly include this process in the model calculations.

The main conclusion following from the generic model exercise is that in most cases nominal concentrations in the test

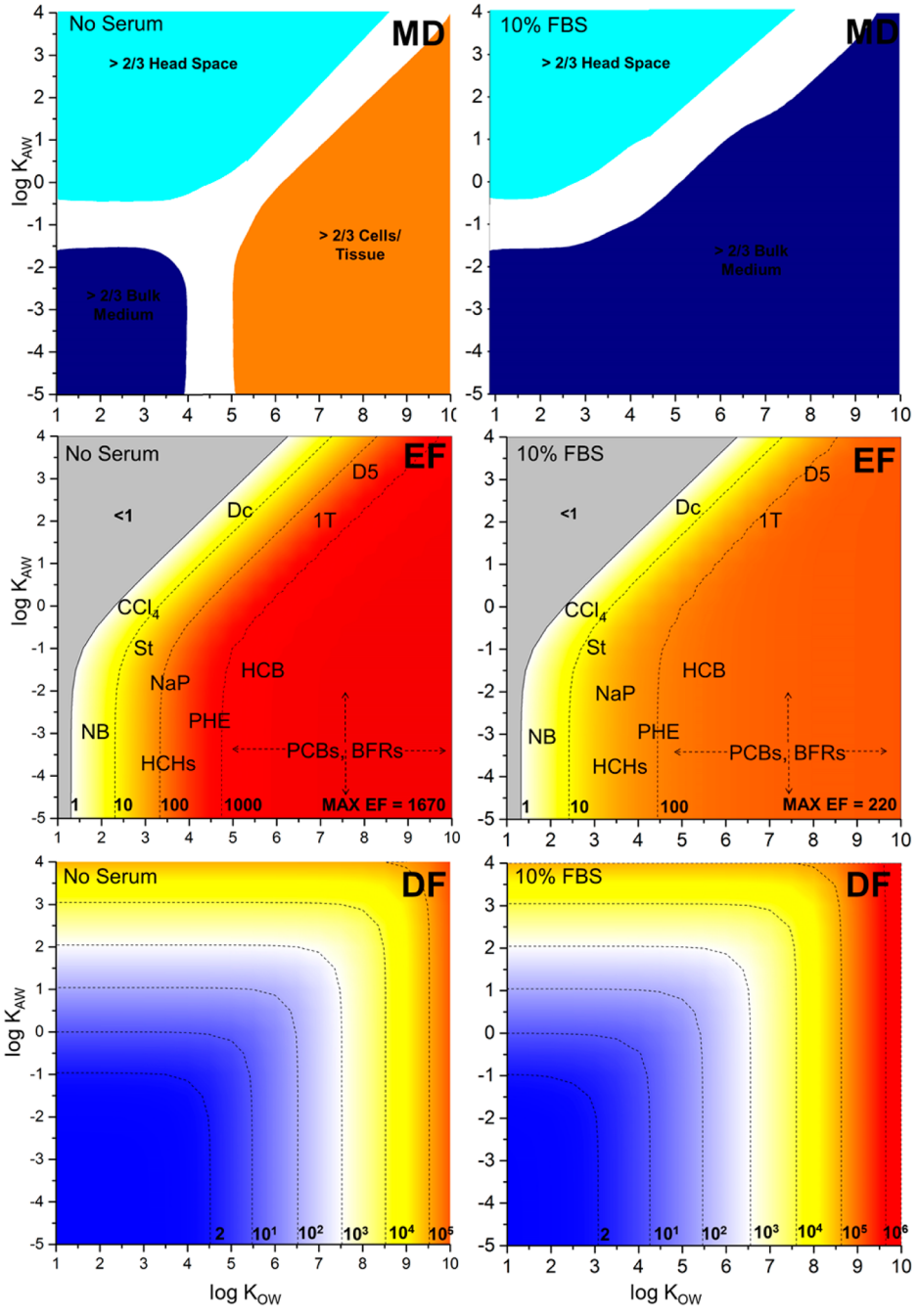

Figure 2. Mass distribution (MD), enrichment factors (EF), and depletion factors (DFs) as a function of partitioning properties in bulk medium with no serum and a serum (fetal bovine serum, FBS) volume fraction of 0.10. The approximate partitioning properties of nitrobenzene (NB), carbon tetrachloride (CCl_4), styrene (St), naphthalene (NaP), phenanthrene (PHE), hexachlorocyclohexanes (HCHs), decane (Dc), hexachlorobenzne (HCB), 1-tetradecene (1T), polychlorinated biphenyls (PCBs), brominated flame retardants (BFRs), and decamethylcyclopentasiloxane (D5) are indicated on the EF panels.

system cannot be used as a proxy for either cell/tissue concentration or freely dissolved concentrations. Screening and prioritizing chemicals with respect to "potency" or potential hazard using nominal concentrations is also problematic. For example, chemicals may cause an "effect" in the in vitro test system at (freely dissolved) concentrations much lower than the nominal concentrations (i.e., they are more "potent" than they may appear). While this conclusion is not novel, the generic application demonstrates that it is true across a wider range of partitioning properties than may be appreciated based on the available empirical data for individual chemicals. The presence and characteristics of serum in the bulk medium is confirmed as a key confounding factor limiting the comparability of in vitro test data reported using nominal concentrations.

Modeled versus Measured EFs: Persistent Organic Pollutants (POPs). Evaluation of model performance is hindered by a general lack of appropriate measurement data. In typical in vitro toxicity and bioassay studies, only the nominal test concentrations are reported along with some details on the cells (e.g., type and initial seeding density) and medium used (e.g., commercial name, serum type, and VF).

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Where EFs or cell/tissue concentrations are measured, parametrization of the model to precisely match the experimental conditions is difficult because some of the required details (e.g., mass of cells/tissue, cell/tissue lipid content, lipid and serum albumin levels in serum) are unavailable. These issues limit the number of opportunities to assess model performance; however, some comparisons are still possible.

The generic model output indicates that EFs in the range of 50−1000 can be expected for typical hydrophobic POPs (i.e., log K_{OW} > 5), depending on serum type, serum VF, and cell/tissue lipid content. EFs of this magnitude tend to exceed expectations based on empirical data for BDE-47 (EF = 20−125, serum VF = 0−0.10)[43] and Aroclor 1254 (mixture of PCBs) (EF = 30−150, serum VF = 0).[54] However, the BDE-47 data cited above were reported after 60 and 120 min exposures, which may be insufficient for equilibrium conditions to be approached. Additionally, the nominal test concentration in the study (1 μM) is approximately 20-fold higher than the estimated solubility limit of BDE-47 (50 nM).[58] The nominal test concentration in the Aroclor 1254 study (~2 μM) also implies supersaturated conditions in the test system, given that the commonly assumed "average" water solubility of Aroclor 1254 is approximately 0.01 μM (3.4 μg/L at 25 °C).[59] In other words, the actual medium concentration corresponding to the observations is lower than the nominal concentration. Generic model output using a 1 μM nominal test concentration is presented in the Supporting Information (section S7). Supersaturation occurs for chemicals with log K_{OW} > 5.5 (serum VF = 0) or log K_{OW} > 6.5 (serum VF = 0.10), reducing modeled *EF*s approximately 2−500-fold compared to output at a nominal test concentration of 1 nM. Modeled EFs assuming 1 and 2 μM nominal concentrations (serum VF = 0) are consistent with expectations based on the empirical data for chemicals with partitioning properties in the range of brominated flame retardants (BFRs) and PCBs but are still elevated compared to the BDE-47 data at serum VF = 0.10. Exposure duration and serum characteristics are plausible factors underlying these differences. Additional empirical data characterizing the relationship between nominal test concentrations and cell/tissue concentration for BDE-47 in three cell lines (MCF-7, HepG2, PC12) are summarized in the Supporting Information (section S8).[56] The exposure duration (3 days) and use of nominal test concentrations below the solubility limit make these data more appropriate for evaluating model performance (although cell and serum characteristics were not adequately reported). The measured wet weight concentrations correspond to empirical EFs ranging from approximately 37−62 in MCF-7 cells, 57−84 in HepG2 cells, and 130−320 in PC12 cells. The modeled EFs tend to overestimate empirical EFs for MCF-7 and underestimate the empirical EFs for the PC12 cell line, whereas the modeled *EF*s and empirical EFs for the HepG2 cells span a similar range of values. As shown in the Supporting Information (section S8), the modeled EFs are within 1 order of magnitude, and hence, performance is deemed acceptable for screening level purposes, especially given the uncertainties in the model parametrization and variability in the empirical data.

Illustrative Model Application Using Partitioning Properties of Real Chemicals. Cell/tissue concentrations and chemical activities calculated for the set of real chemicals assuming f_L = 0.05 and a serum VF of 0.10 (FBS) with nominal concentrations of 1 nM, 1 μM, and 50 μM are presented as a function of log K_{OW} in Figure 3. Cell/tissue concentrations and chemical activities calculated for the same scenarios but

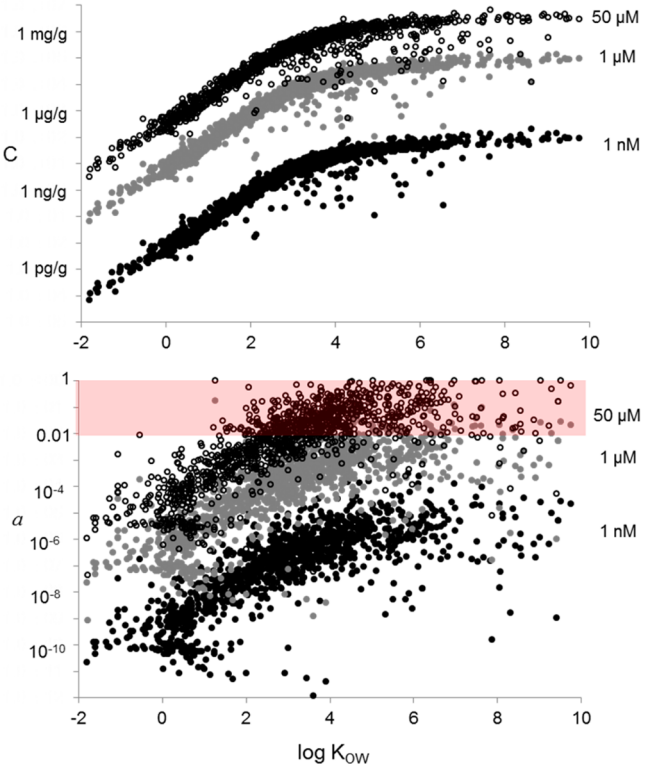

Figure 3. Estimated concentration in cell/tissue (*C*, wet weight) (top panel) and chemical activity (a) (bottom panel) at equilibrium as a function of log K_{OW} and initial nominal concentration in the test system assuming assuming f_L = 0.05 and initial nominal concentrations of 50 μM (open circles), 1 μM (gray circles), and 1 nM (black circles). The volume fraction of fetal bovine serum (FBS) in the bulk medium was set to 0.10 for these calculations. The red transparent bar in the bottom panel indicates approximate range of chemical activities associated with baseline toxicity ($a \geq 0.01$).

assuming f_L = 0.01 are presented in the Supporting Information (section S9). Evaporative losses (i.e., extensive partitioning into head space; see Figure 2) for this model application are also characterized in the Supporting Information (section S9).

Figure 3 illustrates how equilibrium cell/tissue concentrations can vary over orders of magnitude for a given nominal test concentration solely due to differences in either hydrophobicity (K_{OW}) or volatility (K_{AW}). For example, a nominal test concentration of 1 nM corresponds to cell/tissue concentrations in the fg g^{-1} to ng g^{-1} wet weight range. Modeled wet weight concentrations scale with the assumed value of f_L, (i.e., are proportional to the lipid equivalent content and, hence, 5-fold lower assuming f_L = 0.01 vs 0.05) (Supporting Information, section S9). The large range in cell/tissue concentrations corresponding to the same nominal concentration does not stem from an unrepresentative selection of compounds but simply reflects the diversity of estimated property values exhibited by chemicals selected for high-throughput screening (Supporting Information, Table S2). The ng g^{-1} concentration range is achieved for chemicals with log $K_{OW} \geq$ ~2, as long as the K_{AW} is low enough so that partitioning into the head space is minimal. Model output scales proportionally with the amount of chemical added and mg g^{-1} wet weight concentrations are possible for hydrophobic

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chemicals when nominal test concentrations are greater than 1 μM. A figure where modeled cell/tissue concentrations are expressed on a molar basis is presented in the Supporting Information (section S9). Toxicity thresholds based on the critical body residue (CBR) approach for baseline toxicity (e.g., 1−10 mmol kg^{-1})[60,61] are also indicated and can be reached by more hydrophobic chemicals once nominal test concentrations are greater than 1 μM. The modeled cell/tissue concentrations are sensitive to the serum VF, particularly for the more hydrophobic chemicals, which can exhibit differences of up to 20-fold over the range of serum VFs considered.

The relationship between nominal test concentration and chemical activity presented in Figure 3 is also highly variable. Cell/tissue concentrations and chemical activities span multiple orders of magnitude for the same nominal concentration but are more scattered as a function of log K_{OW}. At a nominal test concentration of 50 μM and serum VF of 0.10, 47.6% of the test chemicals exhibit modeled chemical activities corresponding to levels typically associated with baseline toxicity (membrane disruption) (i.e., $a \geq 0.01$), whereas at 1 μM, the proportion falls to 5.9%. Model output is similar assuming f_L = 0.01 as opposed to f_L = 0.05 (Supporting Information, section S9). Chemicals with $a \geq 0.01$ at nominal test concentrations of 1 μM are generally more hydrophobic (log K_{OW} > 5) and include several brominated flame retardants (e.g., γ-hexabromocyclododecane, BDE-99, BDE-153) and pyrethroids (e.g., esfenvalerate, permethrin, etofenprox). Note that 0.5% and 12.8% of the chemicals simulated were first corrected for supersaturation following the initial equilibrium distribution calculation (i.e., exhibited $C_W > S_W$ after the first iteration of the calculation) at nominal test concentrations of 1 and 50 μM, respectively. With no serum present in the bulk medium, corrections for supersaturation are required for 3.5% and 25.9% of the compounds at nominal test concentrations of 1 and 50 μM, respectively. Many of the chemicals included in this case study are also being tested in high-throughput cell-free assays. Supporting Information (section S9, Table S6) summarizes the screening results when the cell/tissue volume (and hence lipids) are assumed to be zero. While the proportion of chemicals exhibiting supersaturation and $a \geq 0.01$ are similar, the baseline narcosis activity level is not directly applicable because the test system does not include the target site, i.e., no cell membranes. Nevertheless, it is still useful to consider cell-free assays in terms of concentrations, water solubility limits, and chemical activity.

The sensitivity of the model output to the parametrization of the bulk medium highlights the importance of detailed characterization and reporting of the composition of these mixtures. Additionally, the model demonstrates how in vitro testing requires careful consideration of chemical properties to ensure that tests are conducted below saturation (solubility) limits. The general error when concentrations are above solubility limits is an overestimation of the dissolved (bioavailable) concentration corresponding to the observed biological response, i.e., underestimation of toxicity. A complicating factor is that the observed biological response may be a function of the supersaturated condition (i.e., presence of precipitates) and not a function of a chemical−biological interaction that would occur under standard (not supersaturated) conditions.[62] Overall, Figure 3 demonstrates how the proposed modeling approach is valuable as a screening tool to be applied prior to conducting in vitro toxicity testing. Specifically, models such as this can be used to guide the selection of appropriate nominal test concentrations expected to result in cell/tissue concentrations bracketing the range of observed or modeled tissue concentrations in different organisms. Experimental conditions with the potential to cause supersaturation of the aqueous phase can also be identified and avoided.

Relevance for Risk Assessment. The most important question to address in risk assessment is whether observed (or predicted) concentrations are likely to result in significant adverse effects being experienced by the organism. This modeling study reaffirms that in vitro toxicity test data expressed in terms of nominal test concentration are of limited value for quantifying risk associated with chemical exposure because nominal concentrations are not the bioavailable/freely dissolved concentrations in the aqueous phase or the cell/tissue concentrations associated with toxicity.[17,19,22,27] The distribution of the chemical in the test system needs to be quantified.

In addition to prospective applications (i.e., study design), the modeling tool can be applied retrospectively if sufficient data characterizing the in vitro test system are available. If this is the case, empirical EC50s for the true chemical concentration corresponding to the observed effect or biological response derived from nominal test concentrations can be related to corresponding chemical activities. As illustrated in the Supporting Information (section S9), model output generated by the tool can also be interpreted using the critical body or critical cell residue (CBR, CCR) approach.[52,60,61,63−65] Either way, chemical toxicity can be ranked in a more robust manner. For example, if two chemicals cause the same (biological) response at the same nominal concentration (e.g., EC50 = 1 nM for both), the perception may be that the chemicals are "equi-toxic" for that particular end point. However, the model output in Figure 3 shows that the less hydrophobic chemical of the two can be the "more toxic" chemical because it elicits the response at a lower chemical activity (and cell/tissue concentration). Given the scatter in the model output for chemical activity, this pattern should not be assumed to be universal. Exceptions are possible, and discrepancies can arise due to errors in physical−chemical property data (e.g., lack of internal consistency[66]).

It may also be possible to use in vitro testing data in combination with the modeling tool to hypothesize whether or not a chemical exerts baseline toxicity or a more specific mode of action. For example, nominal EC50s (growth inhibition) reported for γ-HCH, dieldrin, and 4-nonylphenol under different experimental conditions are screened in the Supporting Information (section S10).[32] The modeling results implicate baseline toxicity as the mode of action in all experimental scenarios evaluated (a > 0.01), but this conclusion is subject to uncertainty given that growth dilution is not accounted for in the model calculations.

Limitations and Future Considerations. The main concern when applying the modeling tool described here is the dependence on the equilibrium distribution assumption. Because of this assumption, the model cannot be applied to simulate time-dependent accumulation of chemicals in cells/tissues nor can it explicitly account for degradation or cell/tissue growth during the exposure period.[43,44,67] The bias in the equilibrium distribution calculations related to ignoring growth will depend on the relative rates of uptake into the cells/tissue versus cell doubling time. Rapid uptake kinetics may be sufficient to largely counteract growth dilution during cell division. Degradation is not expected to be a key factor for

typical POPs but can be anticipated to substantially widen the range of cell/tissue concentrations and chemical activities corresponding to a given nominal test concentration for more labile chemicals. Future modeling efforts should be dedicated to developing and evaluating a dynamic version of the model (e.g., a Level IV fugacity-based approach). Diffusivity in water and membrane permeability could potentially be used to estimate mass-transfer coefficients characterizing exchange kinetics for the cells/tissues (assuming surface area for exchange can be estimated reliably). Changes in cell mass (volume) can be simulated, and degradation in all compartments can be accounted for in the calculations using first-order rate constants (or Michaelis–Menten kinetics for biotransformation).

While the equilibrium distribution modeling approach is subject to limitations, uncertainties, and potential bias, there is clearly great value in the application of such tools to (i) plan appropriate in vitro exposure scenarios based on physical–chemical properties, (ii) avoid the confounding factor of supersaturating the aqueous phase, and (iii) facilitate the interpretation and use of in vitro toxicity and bioassay data for risk assessment and prioritization. Further development, application, and evaluation of modeling approaches for in vitro test systems is warranted. Modeling efforts to address uncertainty in quantifying the concentrations and chemical activities corresponding with in vitro biological responses would be greatly assisted by more detailed characterization of cell/tissue properties and other experimental conditions used for in vitro toxicity and bioassay testing. Test-specific parameters required by the model (and other similar ones, e.g., ref 32) should be measured and reported to the greatest extent possible. More systematic data relating physical–chemical properties to the propensity to sorb to vessel walls under different test conditions (e.g., presence/absence of serum) are required. An additional task is to expand the approach for neutral organic chemicals to ionogenic organic chemicals that are predominantly charged in the test system.

■ ASSOCIATED CONTENT

Supporting Information

Additional details on the modeling approach, property values, model inputs, and empirical data. A version of the model is also included. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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