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Quantifying Processes Determining the Free Concentration of Phenanthrene in Basal Cytotoxicity Assays

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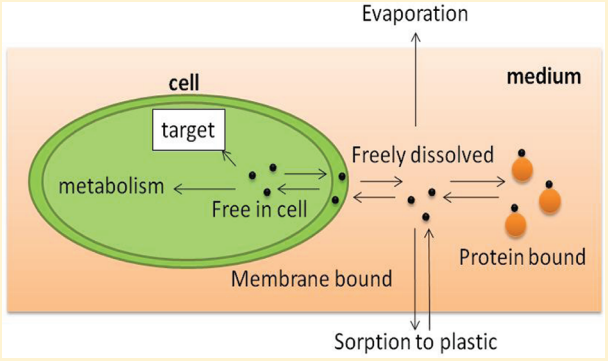
ABSTRACT: Difficulties may arise when extrapolating *in vitro* derived toxicity data to *in vivo* toxicity data because of the high variability and occasional low sensitivity of *in vitro* results. Differences in the free concentration of a test compound between *in vitro* and *in vivo* systems and between different *in vitro* systems may in part explain this variability and sensitivity difference. The aim of this study was to determine what assay components influence the free concentration of phenanthrene in a Balb/c 3T3 and RTgill-W1 MTT assay. Partition coefficients of phenanthrene to serum, well plate plastic, cells, and headspace were measured and subsequently used to model the free concentration of the compound *in vitro*. The estimated free concentration was compared to the free concentration measured in the assays using solid phase microextraction (SPME). Results indicate that the free concentration of phenanthrene, a relatively volatile and hydrophobic compound, is significantly reduced in a typical *in vitro* setup as it binds to matrices such as serum protein and well plate plastic. A reduction in free concentration due to increasing serum protein levels is accompanied by an increase in the median effect concentration (EC_{50}) and can be modeled, with the exception of evaporation, using the partition coefficients of the compound to assay components.



INTRODUCTION

Given the expensive nature of animal tests in both economical and ethical terms, much toxicological research is currently geared toward reducing, refining, and replacing animal tests with, among others, (*in vitro*) cell assays.[1,2] Generally, good correlations have been found between *in vitro* and fish and mammalian derived acute toxic dose data, but there is room for improvement when considering the low absolute sensitivity of *in vitro* assays.[3,4] Assay sensitivity is important in (eco)-toxicological hazard assessment because toxic thresholds determined in toxicity tests are used to define safe concentrations or doses of test chemicals.[5] One explanation for deviating *in vitro* and *in vivo* data is that a single cell culture will generally have a limited number of target sites in comparison to a whole organism and, therefore, cannot detect all possible target sites of the compound. Developing batteries of *in vitro* tests where organ specific modes of action have been conserved and measuring a range of end points will likely improve the prediction power of *in vitro* assays.[4,6,7]

Another explanation for differences in *in vitro* and *in vivo* data is that freely available, unbound concentrations of test compounds between *in vitro* and *in vivo* systems may vary considerably. Generally, only the free concentration of a chemical is considered available to the organism or tissue to cause an effect.[8,9] However, measuring or estimating the free concentrations in *in vitro* systems has been limited. Effect concentrations of chemicals *in vitro* are normally expressed as nominal or total concentrations. Yet, recent studies have found that serum proteins in cell culture medium significantly bind test compounds and that an increase in serum levels increased effect concentrations.[10−13] Studies have also found evaporation and binding of compounds to culture plate plastic to be significant.[14−16] Other studies have found lipid binding and, thus, cell concentrations to be significant determinants of bioavailability of a compound *in vitro*.[17,18]

Several techniques exist to estimate binding affinities to extracellular matrices and free concentrations of compounds. These include equilibrium dialysis, ultrafiltration, and centrifugation. However, these techniques prove to be labor intensive and time-consuming, and are not always compatible with the sample matrix.[19,20] Recent studies have therefore focused on solid-phase microextraction (SPME).[21−24] A number of studies have successfully used the technique to estimate the extent of protein binding of compounds in aqueous phases and to directly measure free concentrations in *in vitro* systems.[11,25−28]

A few studies have modeled free concentrations in *in vitro* systems.[29−31] In these studies, physicochemical properties of test compounds, such as the octanol−water partition coefficient (K_{OW}), a proxy for the hydrophobicity of a compound, and the

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Henry's law constant (H), a measure of volatility, have been associated with the loss of free concentrations *in vitro* due to serum protein binding, lipid binding, and evaporation.[7,13,30,32] These properties, in turn, have been used as model input parameters to estimate free concentrations *in vitro*. The models, however, each describe only one or a few loss pathways and have generally not been validated with measured free concentrations.

Because of the patchwork of knowledge on factors included in current models of the free concentration *in vitro*, the aim of this study was to measure and model the individual effects of different system components on the free concentration of phenanthrene in a mouse fibroblast (Balb/c 3T3) and rainbow trout gill (RTgill W1) basal cytotoxicity assay. Phenanthrene is a hydrophobic and relatively volatile polycyclic aromatic hydrocarbon and is slightly cytotoxic *in vitro*.[16,33] Cytotoxicity of phenanthrene was measured at different serum concentrations in exposure medium and expressed in terms of nominal, measured free, and modeled free concentrations of phenanthrene. Free concentrations were modeled using an equilibrium partition model and compared to measured free concentrations using negligible-depletion SPME (nd-SPME). In modeling the different fate pathways of phenanthrene, a quantitative understanding of the factors affecting the free concentration of test chemicals in *in vitro* basal cytotoxicity assays was developed, which, in turn, could serve as a basis for developing more general models for estimating free concentrations *in vitro* in the future.

MATERIALS AND METHODS

Theory. The model in this study describes how phenanthrene partitions from culture medium to the various elements in a Balb/c 3T3 and RTgill-W1 basal cytotoxicity assay. The compound is considered to partition between five phases in a closed system: bare culture medium, serum protein, well plate plastic, cells, and air (Figure 1). The concentration of phenanthrene in each phase is assumed to be at equilibrium. These concentrations are related to each other by their partition or sorption coefficient, K.[34] Bare culture medium is taken as the reference phase, and phenanthrene in this phase is considered unbound and freely available. Thus, the partition coefficient of phenanthrene between the sorbing phase and bare medium, K, is given by the following equation:

$$K_x = C_x/C_{\text{free}} \tag{1}$$

Under unsaturated conditions, the free fraction of phenanthrene, F, is given by

$$F = 1/(1 + K_x \cdot [X]) \tag{2}$$

The parameters C_x and C_{free} refer to the concentration of phenanthrene found in or at the sorbing phase and free in the medium, respectively. The parameter $[X]$ refers to the concentration of the sorbing phase. In the case of serum protein and cells, this concentration refers to the concentration of serum protein and cells per unit of medium. In the case of well plate plastic, it is assumed that phenanthrene adsorbs to the plastic surface, and therefore, the concentration of plastic is expressed as the surface area of plastic exposed per unit of medium. In the case of evaporation into the headspace, the concentration of the headspace is expressed as the volume ratio of air to medium.

Equation 2 can be further extended to include all sorbing phases and corresponding partition coefficients in a basal cytotoxicity assay. Thus, the free fraction of phenanthrene in a basal cytotoxicity assay is given by

$$F = \frac{1}{1 + K_s[S] + K_p[P] + K_c[C] + K_a \cdot \frac{V_a}{V_m}} \tag{3}$$

where K_s, K_p, K_c, and K_a refer to the partition coefficients of phenanthrene to serum protein, plastic, cells, and air, respectively. The volumes of air and medium are indicated by V_a and V_m, respectively. The concentrations of serum protein, plastic, and cells in medium are indicated by [S], [P], and [C], respectively.

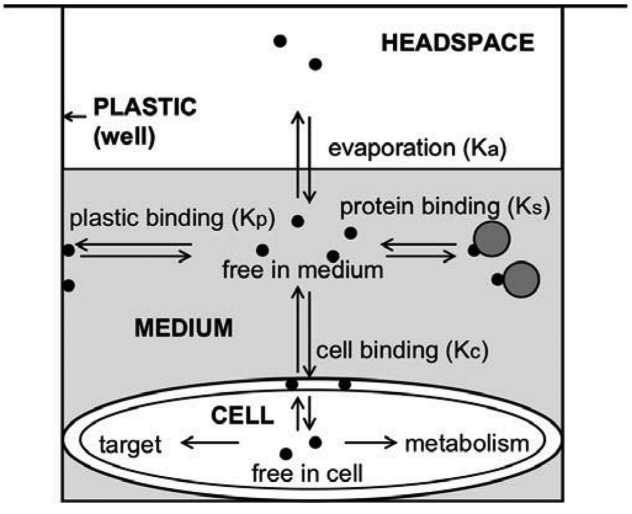

Figure 1. Schematic representation of the various processes that determine the availability of a test compound to a cell in a basal cytotoxicity assay. In this study, metabolism is assumed negligible and phenanthrene toxicity occurs via cell membrane disruption (narcosis). Phenanthrene is thought to partition from bare culture medium into four phases with corresponding partition coefficients: serum constituents (K_s), plastic (K_p), cells (K_c), and headspace (K_a).

Chemicals, Fibers, Media, and Solvents. Phenanthrene (98%) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Bovine serum albumin (BSA, 98% purity, essentially fatty-acid and γ-globulin free) was supplied by Sigma (St. Louis, MO). Sodium azide was purchased from Merck (Amsterdam, The Netherlands). Unless otherwise stated, all cell culture media and buffers were purchased from Gibco BRL (Breda, The Netherlands). Bare cell culture medium for Balb/c 3T3 cells consisted of Dulbecco's modified Eagle's medium (DMEM) with 25 mM glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, 40 μM phenol red, 100 U/L penicillin, and 100 μg/L streptomycin. Bare culture for RTgill-W1 consisted of Leibovitz L-15 with 5 mM galactose, 5 mM sodium pyruvate, 2.05 mM L-glutamine, 100 U/L penicillin, and 100 μg/L streptomycin. Bare culture medium for Balb/c 3T3 and RTgill-W1 was supplemented with 0−10% heat inactivated newborn bovine calf serum (NCS, with estimated 31 g/L BSA as communicated by the supplier) and fetal bovine calf serum (FBS, with an estimated 44 g/L BSA as communicated by the supplier), respectively. It should be noted that the aforementioned BSA concentrations are indicative only, as concentrations vary significantly between batches of serum. Polydimethylsiloxane (PDMS) fibers, with a glass core diameter of 100 μm and PDMS coating thicknesses of 7.0 and 28.5 μm, were purchased from Poly Micro Industries (Phoenix, AZ). Acetonitrile, methanol, *n*-hexane, isopropanol (Labscan, Dublin, Ireland), and dimethyl sulfoxide (DMSO, Fluka, Buchs, Germany) were of analytical grade (99.9, 99.9, 95, 95, and 99% purity, respectively). Pure deionized water was prepared using a Millipore water purification system equipped with an organic-free kit (Millipore Waters, Amsterdam, The Netherlands).

Determining Sorption Coefficients. SPME was used to determine serum protein and cell binding constants. PDMS coated SPME fibers 2.5 cm in length and 28.5 μm in PDMS thickness were exposed for 48 h at 20 °C to 5 mL of bare culture medium supplemented with 0, 0.1 g/L BSA, 0.2% NCS or FCS, or

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approximately 70,000 Balb/c 3T3 or RTgill-W1 cells, and phenanthrene concentrations varying between 0, 12−1000 μg/L (0.5% v/v DMSO/medium) in 5 mL aluminum capped glass vials (Aluglas B.V., Uithoorn, The Netherlands). The vials were shaken on a rock 'n' roller (5 cycles/min., Snijders Scientific, Tilburg, The Netherlands). The experiment was carried out under sterile conditions, in the dark, and in triplicate. After exposure, each fiber was gently blotted dry on a tissue and put into 1.8 mL autosampler vials with 200−1500 μL of acetonitrile for 24 h to extract phenanthrene out of the fiber coating.[35] To determine the mass balance of the system, the medium was extracted by diluting medium aliquots in 20× acetonitrile in autosampler vials. The samples were stored at −20 °C prior to analysis. For an accurate determination of partition coefficients, the amount bound to the binding phases tested (i.e., cells, albumin, and serum) was checked to be at least 30% of the total phenanthrene added. Likewise, for the determination of accurate fiber−medium partition coefficients, the test system was set up such that at least 30% of the total phenanthrene added to the system was extracted by the fiber.[27,35] It should also be noted that cells were dying (but mainly intact, as determined by trypan blue staining) after the 48 h exposure in a serum-free, airtight SPME system. Similar to previous studies,[36] it was assumed that partitioning of phenanthrene into dying cells was similar to that in live cells.

To determine the sorption coefficient of phenanthrene to well plate plastic, individual TC coated polystyrene 35 mm diameter × 10 mm height culture dishes (CELLSTAR, Greiner Bio-one, Alphen a/d Rijn, The Netherlands) were added to 20 mL of bare culture medium supplemented with 50 mg/L sodium azide to inhibit bacterial degradation. The medium was spiked with phenanthrene concentrations varying between 0, 12−1000 μg/L (0.5% v/v DMSO/medium) in 45 mL glass jars (65 × 40 mm, VWR International B.V., Amsterdam). The systems were exposed for 48 h on a horizontal shaker in the dark, at 20 °C, and in triplicate. Pilot studies indicated that 48 h was sufficient for the system to reach equilibrium. Phenanthrene was extracted from the medium before and after exposure as described earlier. In order to estimate the mass balance in the test system, phenanthrene was also extracted from plastic by shaking each dish in 8 mL of acetonitrile overnight on a horizontal shaker, diluting and transferring the extracts to autosampler vials, and storing at −20 °C.

A dimensionless air−medium partition coefficient, K_a, was estimated using the Henry's law constant for phenanthrene (H) at 34.7 and 20 °C, 7.90 and 3.63 Pa m^3/mol,[37] and the following equation:

$$K_a = H/(R \cdot T) \quad (4)$$

where R is the universal gas constant, and T is the system's temperature in Kelvin.[34] In so doing, it is assumed that evaporation of phenanthrene from water and from bare culture medium is the same and that the system is at equilibrium.

Cell Culture and Cytotoxicity Assays. The Balb/c 3T3 cell line clone 31 and RTgill-W1 cell line were purchased from American Type Culture Collection (CCL-163, Manassas, VA). Balb/c 3T3 were cultured in bare culture medium supplemented with 10% NCS and incubated at 37 °C, 90% humidity, and 5% CO_2. RTgill-W1 were cultured in bare culture medium supplemented with 10% FBS and incubated at 20 °C. Experiments were performed with passages 75−93.

To test for the toxicity and concentrations of phenanthrene in each phase of a cell assay, Balb/c 3T3 and RTgill-W1 were seeded at a density of 3 × 10^4 and 15 × 10^4 cells/well in 1 mL of 10% serum-supplemented medium in 24-well plates. After seeding, cells were incubated for 24 h. Seeding medium was subsequently replaced with 2 mL/well medium containing phenanthrene (0, 2.6−100.9 μM, 0.5% v/v DMSO/medium) and 1.25, 2.5, and 5% NCS or 0, 2, and 5% FCS, for Balb/c 3T3 and RTgill-W1, respectively. The experiment was carried out in triplicate and included control cultures exposed to serum-supplemented medium with and without 0.5% DMSO. To minimize and contain the evaporation of phenanthrene, well plates were covered with sterilized aluminum foil, a Viton sheet (Rubber BV, Hilversum, The Netherlands), and the plate lid.

After 48 h of exposure to phenanthrene, cell viability and cell protein content was determined using the MTT assay[38] and the Lowry assay.[39] For the MTT assay, the cells were washed with PBS and incubated for 40 min with 1 mL/well DMEM with 1 mg/L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO). Cells were washed, formazan, produced by mitochondrial succinate dehydrogenase in living cells from MTT, was extracted with 1 mL/well isopropanol for 15 min, and absorbance was measured spectrophotometrically at 595 nm. The Lowry assay was performed on separate plates. The cells were washed with PBS, and 150 μL of Millipore water, 750 μL of freshly prepared Lowry C solution (20 g/L sodium carbonate, 4 g/L sodium hydroxide, 0.2 g/L sodium−potassium-tartrate, and 0.1 g/L copper sulfate in Millipore water), and 40 μL Folin−Ciocalteu's phenol (Merck KGaA, Darmstadt, Germany) were added to each well. Protein concentrations were spectrophotometrically determined after 30-min incubations using an absorbance wavelength of 750 nm and calibration standards of 0.1−1.0 g/L BSA.

Measuring Freely Dissolved, Well Plate Bound, Cell Bound, and Protein Bound Phenanthrene. The cytotoxicity assays and phenanthrene concentration determinations in these cytotoxicity assays were performed in separately exposed well plates. Free concentrations of phenanthrene in the cytotoxicity assays were determined using nd-SPME.[11,19] SPME fibers with a 7 μm thick PDMS coating, cut into 1 cm pieces, were fully submerged in the exposure medium of each well. After 48 h of exposure, each fiber was extracted as aforementioned. To determine whether the depletion of phenanthrene into the fiber was negligible, the amount in the fiber was checked to be <5% of the spiked quantity. Exposure times were deemed long enough for equilibrium to establish based on pilot kinetic studies.

Phenanthrene was also extracted from the exposure medium, cells, and well plate plastic. Phenanthrene in medium and plastic was extracted as aforementioned. Phenanthrene from cells was extracted by incubating cells for 1 min with 50 μL/well 0.5% trypsin and ethylenediaminetetraacetic acid (EDTA) in PBS, suspending cells in an additional 100 μL/well Millipore water, and diluting the cell suspension 20 times in acetonitrile. The experiments were done in triplicate.

Analysis of Phenanthrene Concentrations. Analyses determining phenanthrene concentrations from the various extracted media were performed using HPLC fluorescence. The HPLC system was equipped with a Shimadzu DGU 14A degasser (Den Bosch, The Netherlands), a Varian 9012 pump, a Merck Hitachi F-1050 fluorescence spectrophotometer (Maarssen, The Netherlands), and a 100 mm × 3 mm i.d. × 5 μm PAH ChromSpher 5 C18 column (Varian) that was operated at 30 °C. All analyses were performed at a flow rate of 500 μL/minute and an injection volume of 20 μL. An elution of 85% methanol and 15% Millipore water was used, and the excitation and emission wavelengths of phenanthrene were set at 252/370 nm. Quantification was done using calibration standards prepared for phenanthrene (0.82−200 μg/L acetonitrile). The detection limit was ∼0.15 μg/L. Chromatograms were analyzed using Chromcard v. 2.3.3 (Milan, Italy).

Data Analysis. Free concentrations measured in each well were determined using the measured fiber concentrations, the fiber−bare medium partition coefficient, K_f, and eq 1.[11,19,27] Since the experiments determining partition coefficients of phenanthrene to fiber (K_f), serum (K_s), and cells (K_c) were not done under negligible depletive conditions, they were determined using measured phenanthrene concentrations in fiber, bare medium, medium containing serum, or medium containing cells in a variation of eq 2

$$F_f = \frac{1}{1 + \frac{V_m}{V_f K_f}(1 + K_{c,s}[C, S])} \quad (5)$$

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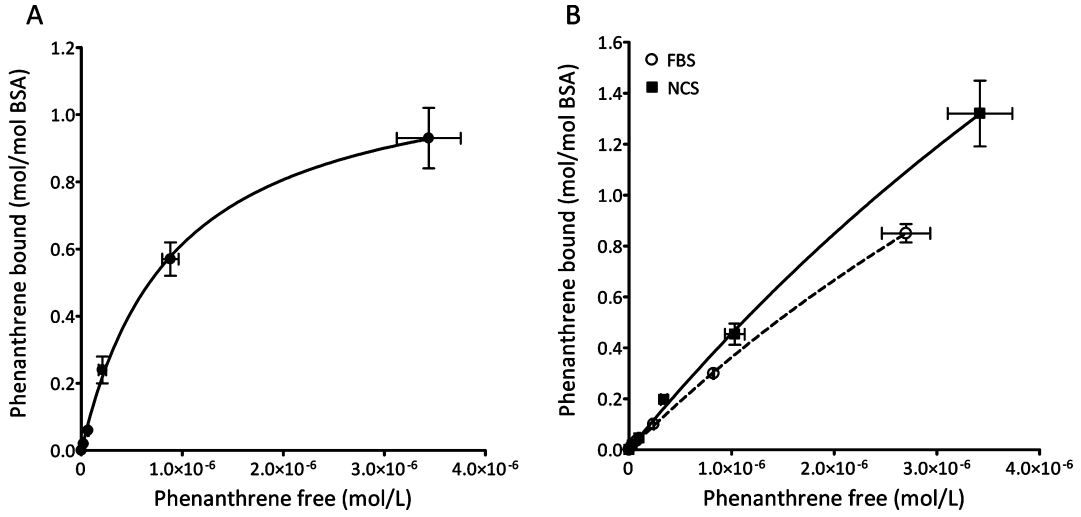

Figure 2. Saturation profile of phenanthrene to BSA (A), NCS (B solid line), and FBS (B dashed line), as a function of BSA concentration (assuming 31 and 44 g/L BSA in 100% NCS and FBS, respectively, eq 6). R^2 for all fits was greater than 0.99.

where F_f refers to the fraction of total spiked phenanthrene in the fiber at equilibrium, and V_f refers to the volume of the fiber coating. To account for saturation in serum protein binding, free and protein bound concentration of phenanthrene, derived using measured fiber concentrations (eqs 1 and 5), were used to construct a sorption curve

$$C_b = \frac{C_{b\max} \cdot C_{\text{free}}}{K_d + C_{\text{free}}} \quad (6)$$

where C_b (mol/mol protein), C_{free} (mol/L), and K_d refer to the bound, unbound concentration of phenanthrene, and the protein dissociation constant, respectively. K_p was calculated using the measured plastic and medium concentrations in a Freundlich fit,[34] where K_p refers to the sorption coefficient at a defined aqueous concentration, and n is the parameter describing the sorption linearity.

$$\log C_p = n \log C_{\text{free}} + \log K_p \quad (7)$$

Using the cytotoxicity assay absorbance measurements, sigmoidal dose–response curves were constructed using the Hill slope function with a variable slope and as a function of log concentrations of phenanthrene against the percentage of absorbance of the DMSO control. Statistical differences among treatments were determined by a two-tailed Student t test, with a significance level set at >95% ($p < 0.05$). All error propagations, statistical analyses, and fits were done using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA).

RESULTS AND DISCUSSION

Partitioning to Serum Constituents. The sorption curves for phenanthrene to BSA, NCS, and FBS are depicted in Figure 2. The log fiber–DMEM and log fiber–L-15 partition coefficients (log K_f) were separately determined to be 3.76 ± 0.05 and 3.80 ± 0.01, respectively. The fiber–DMEM and log fiber–L-15 partition coefficients are in line with fiber–water partition coefficients (3.73 ± 0.05), suggesting that there are no major binding elements in either type of bare culture medium.[40] Average recovery of phenanthrene after exposure to the fiber–medium–serum system was 100 ± 4%.

The sorption curve for BSA suggests that there is one binding site for phenanthrene on each BSA molecule ($C_{b\max}$ is 1.17 ± 0.03 mol/mol BSA, Figure 2). Full saturation occurs near the solubility of phenanthrene in bare medium, which is assumed to be equal to that of water at 25 °C (4.6 μmol/L).[41] As for phenanthrene binding to NCS and FBS, one binding site per protein and saturation below or at water solubility cannot be assumed. Arguably, there are more binding agents in serum than BSA (e.g., lipoproteins), despite BSA being the most dominant binding protein.[42] Saturation of all serum protein is, therefore, less significant than for BSA alone. For simplicity, we therefore assume that binding to serum protein is a linear partition process. The serum–medium partition coefficient of phenanthrene, K_s, can be referred to as an apparent, compounded association constant incorporating the mean number of binding sites on all binding elements available in serum-enriched medium. Using eq 5, log K_s is estimated to be 3.94 ± 0.07 and 3.94 ± 0.01 L/kg for NCS and FBS, respectively. This indicates that in culture medium, containing typically 5% serum, more than 93% of phenanthrene is bound to serum constituents (eq 2).

Partitioning to Well Plate Plastic. The sorption curve of phenanthrene to polystyrene well plate plastic is depicted in Figure 3. The average total recovery of phenanthrene after

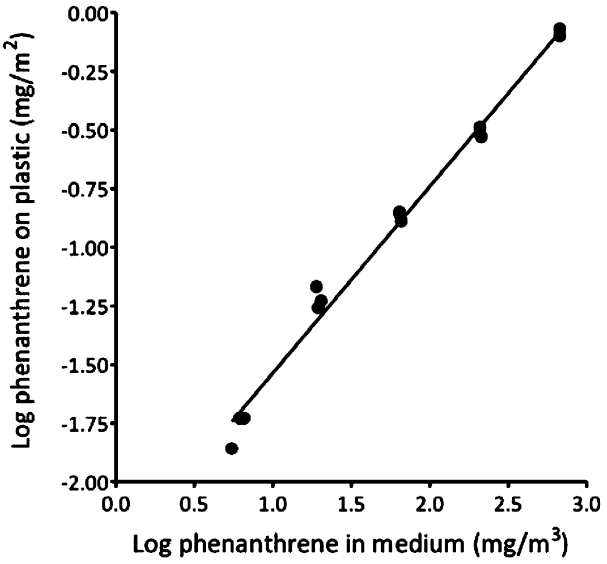

Figure 3. Sorption isotherm of phenanthrene to well plate plastic. Points were fit using the Freundlich equation. Log K_p is −2.33 ± 0.04 (m), and n is 0.80 ± 0.02. R^2 of the fit is 0.99.

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exposure was 98 ± 5% after 48 h of exposure. The average log K_p across the concentration range tested was found to be −2.33 ± 0.16 m, and no significant difference in partitioning to plastic between Balb/c 3T3 and RTgill-W1 bare culture medium was found, as is expected given the similar K_f values of the two media. The sorption linearity parameter, n, was found to be 0.80 ± 0.02, suggesting that saturation of phenanthrene on plastic does occur at high phenanthrene concentrations. When ignoring saturation, a log K_p of −2.33 (K_p in m) suggests that 62% of phenanthrene binds to plastic in a typical 24-well plate cell assay, where an estimated 701 mm^2 plastic is exposed to medium (eq 2). Schirmer et al.[15] found that 60−70% of fluoranthene, a slightly more lipophilic PAH than phenanthrene, was bound to well plastic in a serum-free fish cell assay, thus supporting this study's plastic binding findings.

Partitioning to Air. The ratio of the volume of air to total well volume in a closed well of a 24-well plate with 2 mL of medium is calculated to be 0.41. Using eq 4, 7.90, and 3.63 Pa m^3 mol^{-1} as the Henry's law constants for phenanthrene at 37 and 20 °C, an ideal gas constant of 8.31 m^3 Pa K^{-1} mol^{-1}, and a temperature of 310 and 293 K, the log air−medium partition coefficient of phenanthrene, log K_a, is estimated to be −2.51 and −2.83, respectively. This is in accordance with the log K_a value of −2.58 used by Schreiber et al.[16] to describe the evaporation of phenanthrene from water in a glass 24-well plate at 26.5 °C. This means that if no other sorption processes occur, a negligible (0−1%) amount of phenanthrene partitions into the headspace of a well (eq 2).

Partitioning to Cells. The sorption curve of phenanthrene to Balb/c 3T3 and RTgill-W1 cells in bare culture medium is depicted in Figure 4. Average recovery of phenanthrene for both cell types was 96 ± 4%, which is expected as the loss through metabolism by the metabolically inactive Balb/c 3T3 and RTgill-W1 cells is unlikely. On the basis of Lowry measurements, Balb/c 3T3 and RTgill-W1 cells, respectively, contain 0.46 ± 0.01 and 0.21 ± 0.02 mg protein/10^6 cells. Given an estimated 0.23 mg lipid/mg protein, Balb/c 3T3 and RTgill-W1 also, respectively, contain 0.11 and 0.04 mg lipid/10^6 cells.[30] Log K_c calculated in L/kg lipid is therefore 5.07 ± 0.04 and 5.16 ± 0.02 for Balb/c 3T3 and RTgill-W1, respectively. These partition coefficients coincide with the liposome−water partition coefficient for phenanthrene of 5.07 ± 0.04 L/kg reported in Jonker and Van Der Heijden.[36] This suggests that partitioning of phenanthrene to cells is driven by cell lipid concentrations and that this is a simple linear process. Indeed, linearity is illustrated by the sorption linearity parameter, n, of 1.02 ± 0.02 for Balb/c 3T3, suggesting that no saturation occurs. For RTgill-W1, n is slightly below 1 (0.95 ± 0.01) suggesting some minor saturation of cell binding, possibly by cell protein. Nevertheless, when assuming only linear sorption to cells lipids, the measured log K_c indicates that, if no cell growth and other sorption processes occur, a minimum of 11% of phenanthrene is bound to cells in a cytotoxicity assay using the seeding density and assay setup described in this study. Notably, the percentage of phenanthrene bound to cells would increase when considering cell growth over the exposure time.

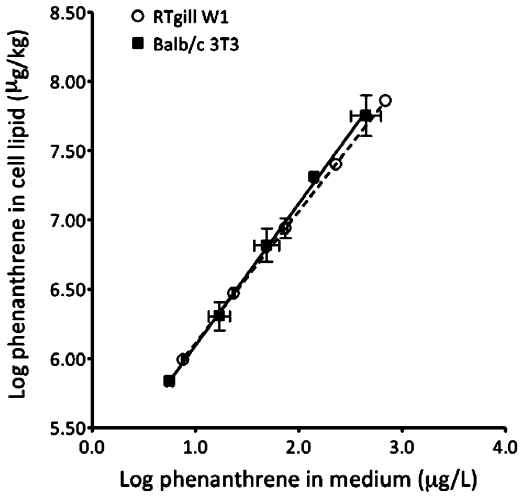

Figure 4. Sorption isotherms of phenanthrene to Balb/c 3T3 fibroblasts (solid line) and RTgill-W1 gill epithelial (dashed line). The slopes of the linear regression lines are 1.02 ± 0.02 and 0.95 ± 0.01, the log partition coefficients of phenanthrene to cells are 5.07 ± 0.04 and 5.16 ± 0.01 (L/kg cell lipid), and the R^2 of the fits are 1.00 and 1.00 for Balb/c 3T3 and RTgill-W1, respectively.

Modeling and Measuring Freely Available Phenanthrene Concentrations *in Vitro*. The partition coefficients of phenanthrene to serum protein, well plate plastic, headspace, and cells were used in eq 3 to estimate the fraction of phenanthrene free in a 48-h Balb/c 3T3 and RTgill-W1 cytotoxicity assay. The modeled free fractions calculated for medium containing 0, 2, and 5% FBS and 1.25, 2.5, and 5% NCS are given in Table 1. Results illustrate how the free fraction is estimated to be less than a third of the nominal concentration of phenanthrene. Moreover, with small changes in serum concentrations, the free fraction is estimated to change significantly as doubling the serum level approximately halves the free concentration.

The measured free concentration of phenanthrene in medium with various serum types and concentrations in a 48-h cytotoxicity assay are also noted in Table 1. The amount of phenanthrene in each SPME fiber used to measure the free fraction was less than 5% of the total amount dosed in medium, fulfilling the requirements of negligible-depletion SPME.[19] There was no significant difference in measured free concentrations at the different phenanthrene concentrations in medium (2-tailed Student's t test, $p > 0.05$), suggesting that no observable saturation of serum, plastic, or cell binding occurred. The free fraction was found to significantly decrease with increasing serum concentrations (2-tailed Student's t test, $p < 0.05$), supporting the model findings. The measured free phenanthrene concentrations, however, were 1−2 times lower than the modeled fractions, with the cell assays with the lowest serum levels in medium having the greatest difference between modeled and measured free concentrations.

The difference in modeled and measured free fractions for assays with low serum levels is likely due to the poorly defined evaporation process of phenanthrene in the partition model. It is likely that in a microtiter plate setup, the air and medium concentrations of phenanthrene are not in equilibrium, unlike the model assumes. Twenty-four well plates covered with aluminum foil are not airtight and thus allow continuous evaporation of phenanthrene to occur. This is supported by Halling-Sorensen et al.,[43] Mayer et al.,[44] Thellen et al.,[45] Schreiber et al.,[16] and Riedl and Altenburger.[14] They found that for chemicals with similar Henry's law constants as those of phenanthrene, effect concentrations were higher in algal test systems conducted in open flasks than with sealed airtight flasks. They argued that this difference was due to the

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Table 1. Modeled and Measured Fractions of Phenanthrene in Medium (Free and Total), Plastic, and Cells[a]

cell type	RTgill-W1			Balb/c 3T3		
serum %, type	0% FBS	2% FBS	5% FBS	1.25% NCS	2.5% NCS	5% NCS
measured % free ± SD	20.4 ± 1.1%	7.8 ± 1.0%	4.8 ± 0.1%	6.9 ± 0.5%	5.0 ± 0.3%	3.2 ± 0.2%
modeled % free	32.5%	9.2%	4.5%	15.2%	9.9%	5.8%
modeled % free[b]	30.2%	8.4%	4.2%	8.9%	6.2%	4.1%
measured % in medium ± SD	30.2 ± 6.3%	73.0 ± 1.4%	84.7 ± 1.9%	51.5 ± 3.2%	65.8 ± 4.7%	82.9 ± 2.7%
modeled % in medium	32.5%	80.0%	90.1%	66.3%	76.7%	83.8%
measured % in plastic ± SD	42.8 ± 4.5%	10.8 ± 3.6%	6.6 ± 1.1%	25.9 ± 1.6%	17.8 ± 3.7%	12.9 ± 3.3%
modeled % in plastic	53.3%	15.2%	7.3%	24.9%	16.4%	9.5%
measured % in cells ± SD	9.9 ± 4.8%	3.9 ± 1.4%	1.9 ± 1.5%	2.5 ± 0.2%	1.9 ± 0.2%	1.9 ± 0.2%
modeled % in cells	14.1%	4.8%	2.6%	8.8%	7.0%	6.7%
measure fraction lost ± SD	17.1 ± 9.1%	12.4 ± 4.3%	6.9 ± 2.7%	20.2 ± 3.6%	14.6 ± 6.0%	2.3 ± 4.3%
modeled fraction lost	0.03%	0.01%	0.00%	0.03%	0.02%	0.01%
log nominal EC_{50} (μM) ± SE	1.78 ± 0.03	2.07 ± 0.07	2.33 ± 0.05	1.86 ± 0.04	2.02 ± 0.02	2.27 ± 0.06
log measured free EC_{50} (μM) ± SE	1.05 ± 0.04	0.96 ± 0.08	1.01 ± 0.06	0.70 ± 0.04	0.72 ± 0.02	0.78 ± 0.06
log modeled free EC_{50} (μM) ± SE	1.30 ± 0.05	1.03 ± 0.09	0.98 ± 0.07	1.04 ± 0.04	1.02 ± 0.02	1.03 ± 0.06
nominal EC_{50} (μM)	60.7	116.1	213.6	72.9	105.3	186.6
measured EC_{50} (μM)	11.2	9.0	10.2	5.0	5.3	6.0
modeled EC_{50} (μM)	19.8	10.7	9.5	11.1	10.4	10.8

[a]Nominal, modeled, and measured free EC_{50} values (cell viability as % of control) of phenanthrene in a Balb/c 3T3 and RTgill-W1MTT assay. The measured values are average values across all concentrations of phenanthrene tested in the cell assays. [b]On the basis of measured 48 h medium concentrations and log K_s only.

evaporation of compounds. Moreover, the problem of evaporation was aggravated in well plates as the surface area to volume ratio is high in these small volume wells, and the evaporated substances may not only dissipate but also evoke effects in adjacent wells.[46]

Mass Balances. Table 1 also shows the model estimates and the measured fractions of phenanthrene in the various compartments of a Balb/c 3T3 and RTgill-W1 cell assay after 48 h of exposure to noncytotoxic concentrations of phenanthrene. In serum containing assays, the majority of phenanthrene was recovered from the medium, supporting modeling results that serum is the most dominant binding element in a Balb/c 3T3 and RTgill-W1 assay. Measured plastic concentrations also show that well plate plastic is also an important sink as 7−35% of phenanthrene is bound to it in the settings tested in this study. The importance of this sink increases with decreasing serum levels, which is supported by model estimates and by the finding that only in the absence of FBS, 60−67% of the fluoranthene was recovered from plastic in Schirmer et al.[15]

Only a small fraction of phenanthrene was recovered from the cells (less than 10%). Using Lowry measurements of cell protein levels in each well after exposure to estimate cell lipid concentrations, the model too predicts minor fractions of phenanthrene in cells, although its predictions are notably higher than that measured in the cells. The higher modeled values are likely due to the evaporation loss process that is greater at 37 °C than 20 °C and is poorly modeled in this study. Nevertheless, measured and modeled fractions together suggest that, despite the relatively high binding affinity of phenanthrene to cell lipids, the amount of cell lipid, in comparison to the volumes of medium, the concentration of serum, and the area of exposed plastic, is minor. Thus, cells can be considered an insignificant sink for phenanthrene *in vitro*. However, like with plastic binding, the importance of cell binding increases with decreasing serum levels. Figure 5 depicts the measured amount of phenanthrene per cell in the Balb/c and RTgill-W1 MTT assay with increasing concentrations of

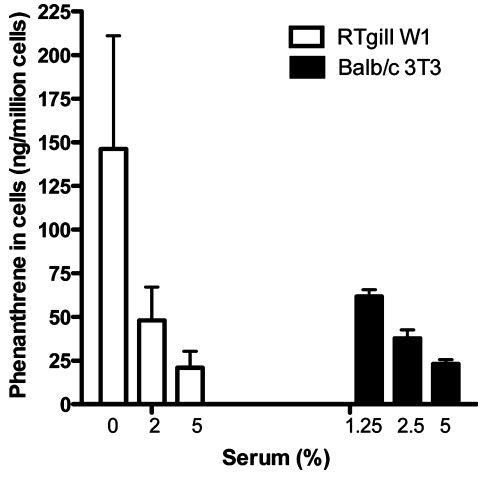

Figure 5. Measured concentrations of phenanthrene per million Balb/c 3T3 (solid bars) and RTgill-W1 (open bars) cells after 48 h exposure to medium spiked with 111 μg/L phenanthrene containing 1.25, 2.5, and 5% NCS and 0, 2, and 5% FBS, respectively.

serum in medium. It supports the notion that the free concentration, as opposed to the nominal concentration, is available for uptake into cells and can cause toxicity. The model in this study may be used to estimate internal concentrations at which toxicity occurs. This allows for a more direct, system-independent comparison of cell sensitivities to phenanthrene than when nominal medium concentrations are used to compare sensitivities.

The remainder of phenanthrene not found in the medium, plastic, and cells and was assumed to be lost via evaporation (Table 1). Increasing loss of phenanthrene with decreasing serum levels indicates that serum retains the compound in solution. As noted earlier, this loss in recovery points to a deficiency in the model in capturing evaporation. The observed losses are not likely to be due to inefficiencies in the extraction procedure as the same extraction procedures were used to determine the aforementioned individual partition coefficients

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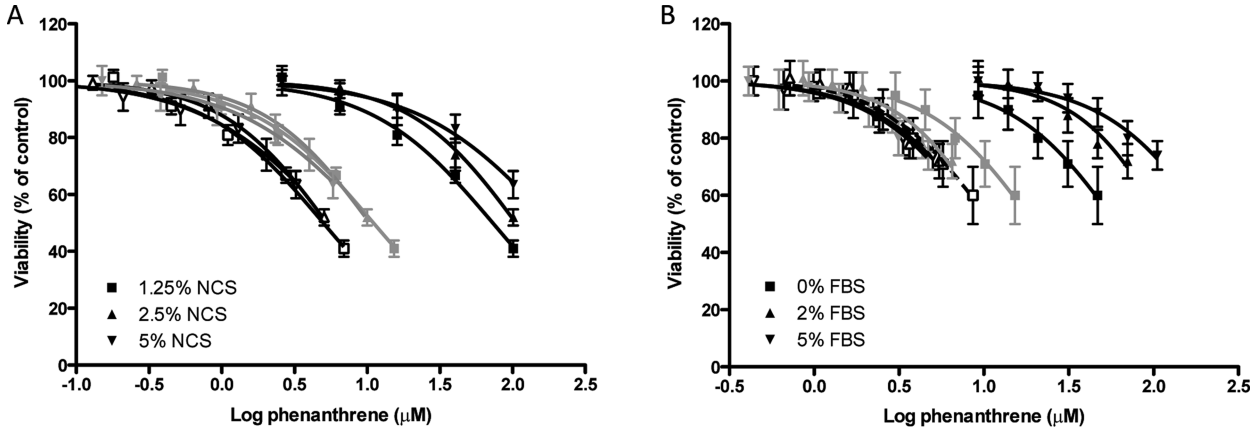

Figure 6. Dose−response curves of Balb/c 3T3 (A) and RTgill-W1 (B) exposed for 48 h to phenanthrene in medium with 1.25% NCS (squares, A) or 0% FBS (squares, B), 2.5% NCS (upward triangle, A) or 2% FBS (upward triangle, B), and 5% NCS (downward-pointing triangle, A) or 5% FBS (downward-pointing triangle, B). The curves are based on nominal (solid black symbols and lines), measured free (open symbols and dashed lines), and modeled free concentrations (gray solid symbols and lines). The top and bottom effects were set at 100 and 0%, respectively. The hill slopes and R^2 for Balb/c 3T3 (A) were −1.05 ± 0.11, −1.19 ± 0.07, −0.95 ± 0.11, and 0.99, 1.00, and 0.98, respectively. The hillslopes and R^2 for RTgill-W1 (B) were −1.41 ± 0.11, −1.66 ± 0.33, −1.39 ± 0.14 and 0.99, 0.94, and 0.98, respectively.

of phenanthrene to the *in vitro* compartments, which were generally around 100%.

The difference between observed and modeled loss of phenanthrene is greater in the Balb/c 3T3 cytotoxicity assay than in the RTgill-W1 cytotoxicity assay. One explanation for this is the higher incubation temperature of the Balb/c 3T3 assay. An increase in temperature increases the log K_a and thus the extent of evaporation, which is only partly captured in the model. Partition coefficients of phenanthrene to SPME fibers and lipid are also known to be negatively related to temperature.[47] Higher temperatures could therefore yield lower partition coefficients and therefore higher free and evaporated fractions. The partition coefficients used in this study's model, however, have been measured at 20 °C only.

A sensitivity analysis was performed to determine the robustness of the model with changing partition coefficients. Muijs and Jonker[47] found a 0.24 log unit difference in K_f for phenanthrene to 30 μm PDMS fiber in water with a 25 °C change in temperature and extrapolated a log K_f of 3.60 at 37 °C. Changes of this magnitude in K_f change the measured free concentration approximately 1.5 times, which explains some of the difference in measured and modeled free fractions in a Balb/c 3T3 cytotoxicity assay. Changes in each K_c and K_p of similar magnitudes results in insignificant changes in modeled free concentrations, as is expected as the free concentration is dominated by serum binding in the Balb/c 3T3 assay in this study. Changes in K_s of 0.2 log units changes the modeled free concentration of phenanthrene in 1.25−5% NCS supplemented medium 1.3−1.5 times or 1−4% percentage points, respectively. Although for the results of this model, changes in individual partition coefficients due to changes in temperature have limited impact on modeled free concentrations, the relationship between temperature and partitioning should be kept in mind when using the model to extrapolate to other setups. Likewise, saturation of plastic and serum protein are not taken into account in this model, but there is saturation occurring, and this should be kept in mind when calculating free concentrations at high phenanthrene concentrations.

To circumvent the issues of estimating loss through difficult processes like evaporation, the free concentration may also be modeled using only the partition coefficient of phenanthrene to serum, K_s, and a measured total concentrations in the medium after 48 h of exposure to the cell assay. When doing so, the modeled free concentrations in both the Balb/c 3T3 and RTgill-W1 cytotoxicity assay with medium containing serum are closer to the measured free concentrations (Table 1). Using measured medium concentrations after exposure, as opposed to initial dosing concentrations in medium, implicitly takes loss through plastic sorption and evaporation into account, allowing for a more robust estimate of free concentrations in this study. This strategy of modeling free concentrations may prove to be a simple reliable strategy to apply to other compounds in serum containing *in vitro* cytotoxicity assays.

Cytotoxicity at Different Serum Levels. To understand the significance of changing free concentrations of phenanthrene *in vitro* with changing serum concentrations, basal cytotoxicity of phenanthrene in medium with different serum levels was tested in a Balb/c 3T3 and RTgill-W1MTT assay. Dose−response curves of phenanthrene tested in different media are depicted in Figure 6. Corresponding median effect concentrations, EC_{50}s, are listed in Table 1. The conventional nominal dose−response curves moved to the left and estimated EC_{50}s decreased with decreasing serum levels, indicating that the toxicity of phenanthrene increased with decreasing serum levels. Gülden et al.,[10] Heringa et al.,[11] and Hestermann et al.[12] found similar increases in *in vitro* toxicity, estrogenicity, and cytochrome P450 induction of organochlorine pesticides, (xeno)estrogens, and halogenated aromatic hydrocarbons, respectively, with decreasing levels of serum in medium. Figure 6 and Table 1 also show that the EC_{50}s based on measured and modeled free concentrations are a tenth of the EC_{50}s based on nominal concentrations and are independent of serum concentrations. Moreover, the EC_{50}s based on measured free concentrations suggest that Balb/c 3T3 cells are more sensitive to phenanthrene than RTgill-W1 cells.

Significance of Free Concentrations *in Vitro*. The cytotoxicity results for phenanthrene in this study support the free concentration concept and strengthen the plea to use free concentrations as opposed to nominal concentrations in cell assays. The free concentration better represents the concentration taken up by the cells. Indeed, the free concentration of a compound as hydrophobic and volatile as phenanthrene is

significantly reduced in a typical Balb/c 3T3 assay. Moreover, it varies significantly when small changes in assay setup are introduced, such as changes in serum levels. It follows that when using *in vitro* assays in quantitative environmental and human risk assessment, strict standardization of the assays is advised. A guideline for good cell culture practice is therefore a welcome development.[48]

The results of this study also support numerous developments within the field of *in vitro* toxicology. A number of techniques have been developed to measure free concentrations directly or indirectly, such as SPME. These techniques can facilitate the development of *in vitro*−*in vivo* concentration extrapolation models.[11,13,20] Moreover, a number of *in vitro* techniques have been developed to minimize the loss of a compound through nonspecific binding or evaporation. These include the use of serum-free cell assays, the use of plate sealers, the use of cell suspension cultures in culture plates of nonbinding material, and the use of solvent-free/continuous dosing techniques.[49−55]

Arguably, however, modeling the free concentration of a test compound *in vitro*, as opposed to measuring it or using techniques to circumvent nonspecific binding or evaporation, will be less cumbersome and will give sufficiently useful results. The model in this study suggests that for similar *in vitro* assay setups and for compounds with similar physicochemical and cell-uptake properties as those of phenanthrene, the free concentration is significantly determined by the serum concentration and can generally be calculated using the serum binding constant. In the absence of serum and in a closed system, the free concentration is determined by the extent of evaporation, the amount of exposed well plate plastic, and the plastic binding constant.

The model in this study can form the basis of a model to estimate the free concentration *in vitro* for organic test compounds in general. Indeed, serum and plastic binding constants of neutral and nonpolar organic contaminants are generally related to log K_{OW} (hydrophobicity). Evaporation is related to the Henry's law constant (H, volatility).[14,30] Gülden and Seibert,[30] for example, proposed a partition model to estimate free fraction of organic chemicals *in vitro* based on log K_{OW}, as a proxy for hydrophobicity, and serum protein binding parameters, which they derived experimentally. Using this algorithm, the authors were able to show that the extrapolation of *in vitro* Balb/c 3T3 cytotoxicity data correlated significantly better, both in relative and absolute terms, with fathead minnow acute toxicity of pesticides.[56] This model, however, still needs to be validated with measured free concentrations and does not take into account evaporation or sorption to plastic of the test compounds, which, as shown in this study, may be particularly important when serum-free cell assays are used.

Riedl and Altenburger,[14] however, developed a model to estimate the ratio between EC_{50}s from algal toxicity assays using microtiter plates and airtight glass containers. They used log K_{OW} and H as proxies for the extent of plastic binding and evaporation in an empirical regression model that calculates the ratio of algal EC_{50}s in a reference (glass and closed) and microtiter plate (plastic and open) system. This model, however, is meant as a simple alert system, which indicates when free concentrations in microtiter plates are likely to differ from nominal concentrations in algal toxicity tests.

All in all, however, the Gülden and Seibert[30] and the Riedl and Altenburger[14] models support the notion that the free concentration *in vitro* can be modeled using physicochemical properties of compounds. The partition coefficients of neutral organic contaminants in the model in this study may in the future be estimated using log K_{OW} and H. For more polar chemicals, differences in hydrogen bonding capacity will have to be included as well, and multiparameter linear free energy relationship models are needed.[57] Moreover, before extrapolating the model in this study to other test compounds, a better understanding of the dynamics and modeling of active transport of test chemicals across cell membranes (causing differences between intra and extracellular free concentrations), metabolism of test chemicals in metabolically active cell cultures (causing a continuous decrease of free concentrations over time), and evaporation of test chemicals in microtiter plates needs to be established. A way around modeling the dynamics of evaporation is to measure exposed medium concentrations and model the free concentration using only association constants to serum protein.

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ABBREVIATIONS

BSA, bovine serum albumin; DMEM, Dulbecco's Modified Eagle's medium; DMSO, dimethyl sulfoxide; EC_{50}, median effect concentration; FBS, fetal bovine serum; H, Henry's law constant; K_{OW}, octanol−water partition coefficient; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; n/a, not applicable; NCS, newborn calf serum; Nd-SPME, negligible-depletion solid phase microextraction; PAH, polycyclic aromatic hydrocarbon; PDMS, polydimethylsiloxane; SPME, solid phase microextraction

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