



Review

Effective exposure of chemicals in *in vitro* cell systems: A review of chemical distribution models

Susana Proença^{a,*}, Beate I. Escher^{b,c}, Fabian C. Fischer^{b,d}, Ciarán Fisher^e, Sébastien Grégoire^f, Nicky J. Hewitt^g, Beate Nicol^h, Alicia Painiⁱ, Nynke I. Kramer^a

^a Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands

^b Department of Cell Toxicology, Helmholtz Centre for Environmental Research – UFZ, Permoserstr. 15, DE-04318 Leipzig, Germany

^c Environmental Toxicology, Center for Applied Geoscience, Eberhard Karls University Tübingen, Schnarrenbergstr. 94-96, DE-72076 Tübingen, Germany

^d National Institute for Environmental Studies (NIES), Center for Health and Environmental Risk Research, Onogawa 16-2, 305-8506 Tsukuba, Ibaraki, Japan

^e Certara UK Limited, Simcyp Division, Acero, 1 Concourse Way, Sheffield S1 2BJ, UK

^f L'Oréal Research & Innovation, Aulnay-Sous-Bois, France

^g ADME Task Force, Cosmetic Europe, Brussels, Belgium

^h Unilever U.K., Safety & Environmental Assurance Centre, Colworth Science Park, Sharnbrook, Bedford MK44 1LQ, United Kingdom

ⁱ European Commission, Joint Research Centre (JRC), Ispra, Italy



ARTICLE INFO

Keywords:

in vitro assays
mass balance
pharmacokinetics
partitioning
QIVIVE
free concentration

ABSTRACT

Nominal effect concentrations from *in vitro* toxicity assays may lead to inaccurate estimations of *in vivo* toxic doses because the nominal concentration poorly reflects the concentration at the molecular target in cells *in vitro*, which is responsible for initiating effects and can be referred to as the biologically effective dose. Chemicals can differentially distribute between *in vitro* assay compartments, including serum constituents in exposure medium, microtitre plate plastic, headspace and extracellular matrices. The partitioning of test chemicals to these extracellular compartments reduces the concentration at the molecular target. Free concentrations in medium and cell-associated concentrations are considered better proxies of the biologically effective dose. This paper reviews the mechanisms by which test chemicals distribute between *in vitro* assay compartments, and also lists the physicochemical properties driving the extent of this distribution. The mechanisms and physicochemical properties driving the distribution of test chemical *in vitro* help explain the makeup of mass balance models that estimate free concentrations and cell-associated concentrations in *in vitro* toxicity assays. A thorough understanding of the distribution processes and assumptions underlying these mass balance models helps define chemical and biological applicability domains of individual models, as well as provide a perspective on how to improve model predictivity and quantitative *in vitro-in vivo* extrapolations.

1. Introducing exposure conditions in *in vitro* systems

Toxicity testing in the 21st century aims to make use of the advances in cellular and molecular technologies to circumvent animal testing and

develop a more mechanistic approach to characterise the hazards of chemical exposure in humans (Leist et al., 2008; NRC, 2007). High-throughput cooperative screening programs, such as the US Tox21 programme, not only increased the availability of *in vitro* bioassay data,

Abbreviations: 3Rs, Replace, Reduce and Refine animal testing; ADME, Absorption, Distribution, Metabolism and Excretion; AOP, Adverse Outcome Pathway; BED, Biologically effective dose; BSA, Bovine Serum Albumin; ECM, Extracellular matrix; EC₅₀, Median effect concentration; HSA, Human Serum Albumin; $K_{\text{albumin/w}}$, Albumin-water partition coefficient; $K_{\text{medium/air}}$, Medium-air partition coefficient; K_{aw} , Air-water partition coefficient; $K_{\text{liposomes/w}}$, Liposomes-water partition coefficient; K_{H} , Henry Law Constant; K_{ow} , Octanol-water partition coefficient; $K_{\text{triolein/w}}$, Triolein-water partition coefficient; $K_{\text{PS/medium}}$, Polystyrene-medium partition coefficient; LPDE, Low density polyethylene; MIE, Molecular Initiating Event; PAH, Polycyclic Aromatic Hydrocarbon; PBK, Physiologically-based kinetic modelling, also referred to as physiologically-based pharmacokinetic (PBPK), physiologically-based toxicokinetic (PBTk) and physiologically-based biokinetic (PBBK) modelling; PCB, Polychlorinated biphenyls; QIVIVE, Quantitative *in vitro-in vivo* extrapolation; QSPR, Quantitative structure-property relationship; SPME, Solid phase microextraction; TD, Toxicodynamics; TK, Toxicokinetics; VCBA, Virtual cell-based assay; VIVD, Virtual *in vitro* distribution model.

* Corresponding author at: Institute for Risk Assessment Sciences, Utrecht University, P.O. Box 80177, 3508 TD Utrecht, The Netherlands.

E-mail address: s.proenca@uu.nl (S. Proença).

<https://doi.org/10.1016/j.tiv.2021.105133>

Received 22 October 2020; Received in revised form 11 February 2021; Accepted 25 February 2021

Available online 1 March 2021

0887-2333/© 2021 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

but also improved the credibility of *in vitro* assay data by undergoing rigid quality control and showing transparent outcomes (Krewski et al., 2020; Tice et al., 2013). However, the extrapolation of effective doses *in vitro* to *in vivo* exposure remains a bottleneck in the interpretation of *in vitro* effect data. Reverse-dosimetry using physiologically-based kinetic (PBK) models simulates the absorption, distribution, metabolism, and excretion (ADME) processes acting on chemicals in the body, to derive external exposures from target tissue concentrations (Coecke et al., 2013). These models are increasingly used to perform quantitative *in vitro-in vivo* extrapolations (QIVIVE) for chemical hazard characterisation. (Blaauboer et al., 2012; Louisse et al., 2010; Wetmore et al., 2015). However, QIVIVE studies usually relate *in vitro* nominal concentrations (C_{nominal}), which is defined in Groothuis et al. (2015) as the mass of added chemical per volume exposure medium¹, to the free concentration (C_{free}) in blood plasma in the target organ. The C_{nominal} *in vitro* and C_{free} *in vivo* that cause the same effect are not necessarily similar. The discrepancies found in *in vitro* chemical hazard characterisation can be partly explained with the lack of consideration for differences in fraction of chemical available to interact with the molecular target (Groothuis et al., 2015; Honda et al., 2019; Kramer et al., 2009).

Toxicity is a function of both the exposure at the molecular target site and the chemical's intrinsic potency to activate the molecular initiating event (MIE), which initiates a cascade of key events and resulting in an adverse outcome *in vivo* (Ankley et al., 2010; Escher et al., 2005). Ideally, the concentration at the molecular target *in vitro* and *in vivo* (referred to in Paustenbach (2000) as the biologically effective dose, BED²) should be linked for accurate QIVIVE. Since the experimental determination of BED is challenging, experimentally accessible dose metrics closely related to the BED should be compared instead. *In vivo*, the dose metric approximating the BED is the tissue concentration (C_{tissue}) or free plasma concentration ($C_{\text{free plasma}}$), which can be derived from externally exposed dose or C_{blood} using PBK models (Derendorf and Schmidt, 2019). *In vitro*, the cell-associated concentration (C_{cell} , i.e. the concentration of a chemical accumulated in cells) or freely available concentration in the medium (C_{free}) are considered appropriate proxies for the BED (Groothuis et al., 2015; Heringa et al., 2004; McCarty et al., 2011). The relationship between C_{free} , C_{cell} and C_{nominal} depends on chemical distribution processes in the *in vitro* system, including evaporation and sorption to microtitre plastic and medium constituents.

Typical high-throughput *in vitro* systems consist of a plastic microtitre plate allowing for gas exchange in which primary or immortalized cells are exposed to chemicals in cell culture medium. Chemicals distribute in the *in vitro* system as schematized in Fig. 1. The distribution depends on their physicochemical properties (e.g. binding affinity for medium constituents and volatility) and the experimental setup (e.g. volume of medium, presence of medium supplements and exposure temperatures). A fraction of the chemical mass can be irreversibly lost through evaporation and degradation or reversibly sorb/bind to well plastic, extracellular matrices and medium constituents like serum protein. This decreases C_{free} relative to the nominal concentration (Groothuis et al., 2015; Kramer et al., 2015). It is often assumed that only the freely dissolved molecules in medium can be distribute into cells, and therefore in steady state conditions, C_{free} is equal to the free concentration in cell ($C_{\text{cell free}}$). The assumption does not hold for ionised chemicals as the pH differs between the extracellular and intracellular environment, which results in different fraction of neutral chemicals in and outside the cell. Moreover, due to ionic molecules low passive permeability, transporters activity might play a greater role in their influx and efflux in cells (Varma et al., 2015). It should be noted that the

dose metric that is measurable, C_{cell} , also includes the amount bound to cell components like membrane lipids and proteins. This non-specific sorption to cellular components can cause cells themselves to appreciably reduce the C_{free} , especially if used in high numbers with low concentrations of highly accumulating xenobiotics (Gülden and Seibert, 2003). As an example, the same nominal concentration of the same chemical is added to two different assays. If one assay has higher concentrations of cells or serum, it may have lower C_{free} and, consequently, lower C_{cell} , $C_{\text{cell free}}$ and BED, and higher observed nominal effect concentrations (Gülden et al., 2001; Heringa et al., 2004; Seibert et al., 2002).

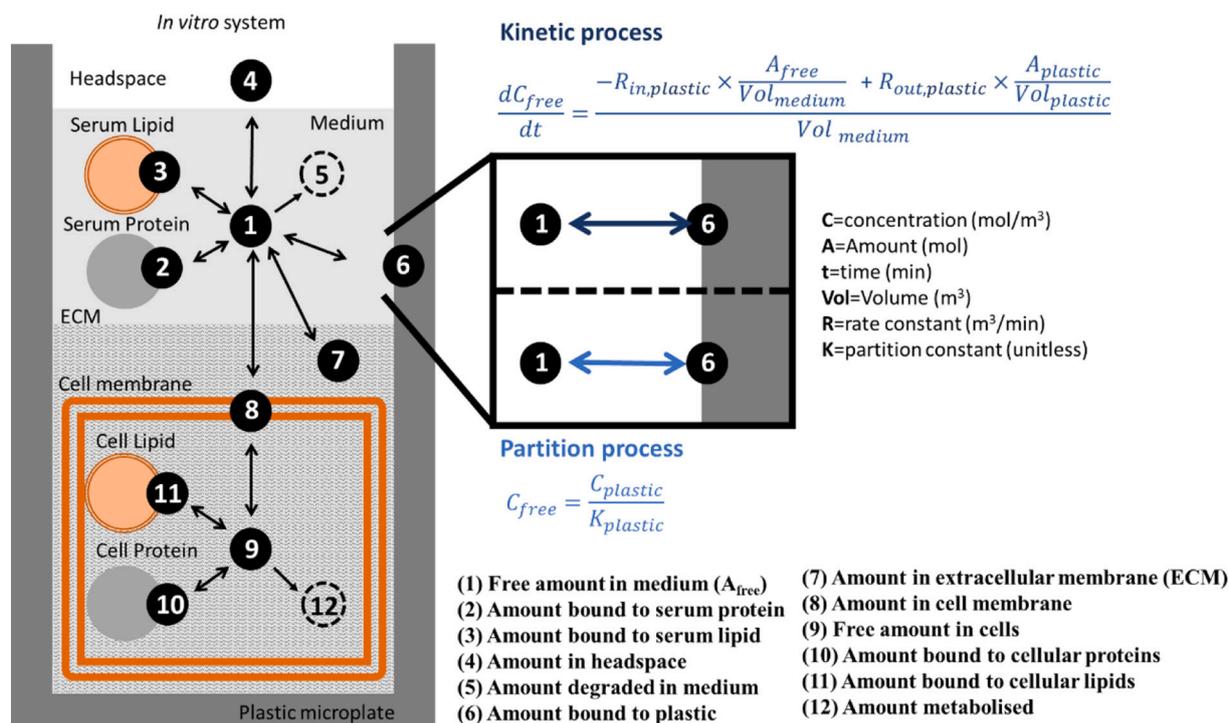
To avoid extensive analytical measurements, but also better understand the distribution of chemicals in *in vitro* systems, several mathematical models have been developed to predict *in vitro* C_{free} and C_{cell} from C_{nominal} . These mass balance models simulate the distribution of the chemicals through a set of equations that describe distribution processes, defining the movement of the chemical between two different *in vitro* compartments using partition coefficients or rate constants. The general structure of this type of models is depicted in Fig. 1. Static models make use of a partition coefficient and assume that the *in vitro* system is at steady-state equilibrium, meaning that there are no changes in chemical concentrations between compartments over time. This assumption reduces the complexity of the models and make them less data intensive than kinetic models. However, this simplified view of chemical distribution insufficiently captures cases where a thermodynamic equilibrium takes significant time compared to the overall duration of the assay. Kinetic models consider the evolution of the chemical distribution in the different compartments in time using differential equations parameterized with rate constants. Some of the models in literature integrate both approaches assuming thermodynamic equilibria for fast distribution processes like serum constituent binding and rate equations for slower processes like uptake into cells. Partition constants and distribution rate constants used in these *in vitro* distribution models can be derived from experimental data (e.g. analytically determined chemical concentrations in medium and cells) or using Quantitative Structure-Property Relationships (QSPRs) that predict these parameters based on the chemical physicochemical properties.

The choice of model compartments, equations, and parameterisation differs per model, and depends on the aim of the model (e.g. intrinsic clearance assays require the modelling of the rate of metabolism), the assumptions made (e.g. a steady-state between cell and medium may be assumed for exposures of stable and non-volatile chemicals over 24 hours), the test chemicals (e.g. volatile chemicals require the modelling of evaporation) and experimental setup (e.g. the distribution to extracellular matrix is modelled when estimating the distribution in hepatic sandwich cultures). Hence, each model simulating the chemical distribution in *in vitro* systems has a specific chemical as well as system setup applicability domains (AD). Models should generally be used to only simulate conditions within their AD, since extrapolations to outside the AD can be very inaccurate. However, understanding the ADs of these type of models in literature is not an easy task, given that these are not explicitly reported, and the model structures are complex and largely overlap. Moreover, the impact of using the models outside their AD, on the prediction's accuracy, should be determined to understand where model improvements are required.

To allow researchers to make an informed decision on what *in vitro* mass balance (i.e. chemical distribution) model to select for a given *in vitro* experiment, this review aims to clarify AD's of common models and describe the uncertainties associated with the different model assumptions. Since the distribution process of the chemical into each compartment is dependent on the matrix characteristics of the compartment, in a first step, we focus on the processes decreasing the C_{free} . We discuss our current knowledge of matrix characteristics and how these characteristics determine the extent to which chemicals associate with it (see 'Distribution processes in *in vitro* systems' section).

¹ Groothuis et al. (2015) distinguish between C_{nominal} and C_{total} , where the latter refers to analytically determined test chemical concentrations in exposure medium before exposure.

² In this paper, 'dose' is considered an umbrella term for measures of chemical exposure.



Frequently used dose metrics:

$$C_{nominal} = \frac{(A_1 + A_2 + A_3 + A_4 + A_5 + A_6 + A_7 + A_8 + A_9 + A_{10} + A_{11})}{Vol_{medium}}$$

$$C_{medium} = \frac{(A_1 + A_2 + A_3)}{Vol_{medium}}$$

$$C_{cell} = \frac{(A_7 + A_8 + A_9)}{Vol_{cells}}$$

Fig. 1. Scheme illustrating how test chemicals distribute between different *in vitro* assay compartments, which are numbered and explained in the legend on the right of the figure. Two strategies for modelling the chemical distribution *in vitro* are depicted: modelling the distribution as a series of kinetic processes (dark blue) or as partition processes where a thermodynamic equilibrium is assumed (light blue). Examples of equations estimating the distribution into plastic as a kinetic or partition process are presented as examples. Besides A_{free} or C_{free} , most of the models in literature also predict the dose metrics C_{cell} and A_{cell} .

With the theories behind distribution processes clarified, it is easier to discuss different model structures reported in literature: naming the compartments, the reason for including partition and/or kinetic equations, and the strategies for parameterisation (see ‘*In silico models of in vitro system chemical mass balance*’ section). The ADs of the different models are then discussed. This review ends with an advice how to direct future research efforts based on the ADs of currently available models. Overall, we aim to highlight the relevance of understanding test chemical distributions in *in vitro* systems to accurately compare chemical potencies between *in vitro* assays and between *in vitro* and *in vivo* assays.

2. Distribution processes in *in vitro* systems

2.1. Binding to serum constituents

In vitro exposure media are often supplemented with 2-20% foetal bovine serum (FBS), which means that the concentrations of serum protein and lipids in *in vitro* exposure medium and *in vivo* plasma differ. The impact of chemical binding to serum lipids and proteins in *in vitro* exposure medium on C_{free} , has been recognized in *in vivo* studies (Smith et al., 2010; Trainor, 2007), *in vitro* intrinsic clearance (Austin et al., 2005; Bowman and Benet, 2018; Kilford et al., 2008; Poulin et al., 2016) and drug potency assays (Smith et al., 2010; Wienkers and Heath, 2005). It has not been as well recognized and considered in chemical safety assessment and development of new *in vitro* models. Ignoring the binding of test chemicals to serum proteins and lipids might cause a mischaracterisation of intrinsic chemical toxic potencies. Chemicals with a high affinity for serum protein and/or serum lipids will have a

lower C_{free} compared to $C_{nominal}$ than chemicals with a low binding affinity in *in vitro* assays with high serum levels in exposure medium. Chemicals with a high affinity for serum protein and/or lipids will have a lower C_{free} in *in vitro* assays with high serum levels in exposure medium than in *in vitro* assays with low serum levels in exposure medium.

Serum lipids are mostly organised as lipoproteins aggregates, which consist in a core of hydrophobic lipids surrounded by one layer of phospholipids and apoproteins (Fielding and Fielding, 2008). Chemical binding to serum lipids can be described in a similar manner as the binding to cellular lipids. Details on this binding process are discussed in Section 2.4, which covers the binding of chemicals to cell components, focussing on different types of lipids.

The globular protein bovine serum albumin (BSA) accounts for 60% (ranging 51-84% for different lots) of the total protein in FBS (Price and Gregory, 1982) and is considered the dominant chemical sorptive protein in cell culture media. The two main chemical-binding sites for albumin are size-constrained cavities that mediate hydrophobic interactions. These cavities also contain a small cluster of positively charged amino acids that can interact with electronegative groups of chemicals such as organic acids (Balaz, 2009; Ghuman et al., 2005). Besides these two cavities, other binding sites that mostly mediate hydrophobic and nonspecific interaction have been found in the albumin protein (Colmenarejo, 2003; Ghuman et al., 2005).

It should be noted that albumin binding can saturate, particularly when there are low levels of serum added to the exposure medium. Under these conditions, the impact of albumin binding on C_{free} and C_{cell} decreases with increasing test chemical concentrations. Also, despite the presence of multiple chemical binding sites on albumin, chemicals such

as organic acids (Henneberger et al., 2020) and polycyclic aromatic hydrocarbons (PAHs) (Kramer, 2010) appear to only have one binding site, which results in the saturation at relatively low concentrations of test chemical concentrations in culture medium (Gülden and Seibert, 2003; Henneberger et al., 2020).

Where binding affinities for BSA are not available, binding affinities to human serum albumin (HSA) may be used. These proteins are highly conserved (Majorek et al., 2012) and since the unspecific interactions of many chemicals with albumin is somewhat independent of the albumin's tertiary structure, it is assumed that the small amino acid differences between the two species do not change association constants ($K_{\text{albumin/w}}$) significantly (Kwon et al., 2020). Thus, the differences between BSA and HSA are frequently ignored in *in vivo* and *in vitro* distribution models. However, it should be noted that for some specifically interacting chemicals (e.g. warfarin), significantly different association constants due to structural differences between BSA and HSA have been reported (Kosa et al., 1997).

As illustrated in Fig. 1, the albumin concentration and association constant are needed for the calculation of C_{free} *in vitro*. Some *in vitro* distribution models consider saturation of serum protein binding, requiring knowledge of the number of binding sites on albumin for a given chemical (Henneberger et al., 2020; Kramer, 2010; Seibert et al., 2002). Often the albumin concentration used to calculate the bound concentration and C_{free} is the concentration measured or reported to be in serum. However, it should be noted that some cells in *in vitro* assays, such as hepatocytes, also produce albumin, increasing the total concentration of albumin in *in vitro* system. However, the excreted levels of albumin in these systems are calculated to be equivalent to a supplementation of 0.0003–0.0009%³ of FBS and are therefore considered to be an insignificant determinant of C_{free} . To analytically measure $K_{\text{albumin/w}}$, tools using matrices that extract unbound chemicals are used, like polymer coatings in solid phase microextraction (SPME) or dialysis membranes. Variations in experimental protocols (e.g. pH, salt concentration and temperature) can impact the $K_{\text{albumin/w}}$ (Bowman and Benet, 2018; Henneberger et al., 2016a). Additionally, lipophilic chemicals bind to plastic and dialysis membranes, which decreases the C_{free} , leading to overpredictions of $K_{\text{albumin/w}}$ if appropriate controls are not used (Kramer et al., 2007). Nevertheless, the robustness and standardisation of these techniques has improved in recent years, making the experimental determinations of $K_{\text{albumin/w}}$ more reliable and more applicable to high-throughput screening (Azizi et al., 2019; Boyacı et al., 2018).

Since $K_{\text{albumin/w}}$ is such an important determinant of C_{free} *in vitro* and *in vivo*, a number of Quantitative Structure Property Relationships (QSPRs) have been developed to estimate $K_{\text{albumin/w}}$ using physicochemical properties, circumventing the need to perform analytical measurements. Due to the nonspecific interactions between albumin and most chemicals, the octanol/water partition coefficient (K_{ow}) is the most common chemical descriptor used for $K_{\text{albumin/w}}$ QSPRs. Using different chemical classes in their training sets, several authors found that linear regressions of $\log K_{\text{ow}}$ could reasonably predict $\log K_{\text{albumin/w}}$ for lipophilic neutral chemicals (de Bruyn and Gobas, 2007; Endo and Goss, 2011; Kramer, 2010; Vandenberg et al., 1972). On the other hand, since polar chemicals can interact with albumin protein through hydrogen-bonds (Zsila et al., 2011), lipophilicity and thus $\log K_{\text{ow}}$, does not correlate as strongly to the binding affinity to albumin of these chemicals, which highlights the need for more descriptors. Endo and Goss (2011) explored other descriptors to predict $\log K_{\text{albumin/w}}$ of polar and apolar neutral chemicals by applying polyparameter linear free relationships (PP-LFERs) (Endo and Goss, 2014). Despite including more varied descriptors, the PP-LFER did not improve the $\log K_{\text{albumin/w}}$

predictions significantly relative to $\log K_{\text{ow}}$ -based QSPRs for neutral chemicals. For ionic chemicals, a PP-LFER that included two ionic descriptors (Abraham and Zhao, 2004) was found to predict $\log K_{\text{albumin/w}}$ significantly better than $\log K_{\text{ow}}$ -based QSPRs (Henneberger et al., 2016a).

There are QSPRs that use more complex descriptors (e.g. electrotopological descriptors (Hall et al., 2003)), or instead of linear regressions, use machine learning algorithms (e.g. genetic algorithms (Colmenarejo, 2003)) and random forest (Toma et al., 2019)). Besides electrotopological descriptors derived from 2D structures of the chemical molecule, descriptors can also be obtained from the 3D conformation that the chemical adopts when bound to albumin binding sites (Linden et al., 2017). This method allows for more accurate predictions of $K_{\text{albumin/w}}$ for ionised chemicals. However, more complex descriptors frequently require software that may not be sufficiently maintained and updated. Ideally, for the QSPR to be used by a wider public, they should be easily integrated into different software platforms that derive the descriptors and run the QSPR (e.g. Dragon molecular descriptors extension in KNIME⁴). Moreover, due to the complexity and variety of possible interactions between chemicals and albumin, generating a sufficiently reliable generic QSPR is unlikely. Therefore, specific QSPRs that take into account the different classes of chemicals are recommended for accurate determination of $K_{\text{albumin/w}}$ (Colmenarejo, 2003; Henneberger et al., 2016a), even when these QSPRs consider only $\log K_{\text{ow}}$ as descriptor. Ideally the QSPRs are based on enough descriptors that the physicochemical properties driving the distribution process are represented but are also simple enough to allow for mechanistic interpretations.

Binding to serum is usually considered a partition process that decreases C_{free} and consequently C_{cell} . In reality, there are several rate processes (e.g. sorption and desorption from albumin, diffusion of free chemical across aqueous medium, etc.) which are generally quick enough to be negligible and therefore these assumptions hold true for most *in vitro* test scenarios. A number of studies investigated whether the 'immediate partitioning to albumin' assumption holds in intrinsic clearance assays, since any rate process that is slower than the intrinsic clearance can lead to underpredictions of clearance (Krause and Goss, 2018; Kwon et al., 2020; Weisiger, 1985). Predictions using experimental desorption rates from albumin revealed that a slow desorption rate of rapidly metabolised chemicals with a high affinity to albumin may indeed hamper clearance calculations *in vivo* (Krause and Goss, 2018). In these simulations, the slow albumin desorption kinetics together with the relatively quick perfusion of plasma were shown to limit the amount of chemical released from albumin into liver sinusoids. Given that most *in vitro* systems are not perfused, a similar hindrance in clearance calculations is not expected. However, with the advent of microfluidic systems, it will be worth investigating and comparing perfusion, desorption and diffusion rates *in vitro*.

Likewise, the assumption that a decrease of C_{free} in medium leads to a proportional decrease of C_{cell} may not always hold. Several authors report that *in vitro* clearance did not decrease in the presence of serum as would be expected given the decrease in C_{free} (Andersson et al., 2004; Fukuchi et al., 2017; Obach, 1999; Riley et al., 2005). The proposed mechanism is that albumin binding to some chemicals enhances their rate of diffusion and/or cellular uptake (Bowman and Benet, 2018; Poulin et al., 2016). Similarly, lipoproteins vesicles have been found to facilitate uptake of chemical into cells (Shireman and Wei, 1986). In fact, this increase in diffusion and/or serum constituent-mediated cellular uptake, has also been observed in cells with low metabolic activity (Fischer et al., 2018a; Hjelmberg et al., 2008), meaning that cells in assays with supplemented serum achieve steady state C_{cell} sooner than in conditions without serum.

³ Calculations based on an albumin production rate of 7.5 pg/day/cell (Gunnness et al., 2013) and 2.5 $\mu\text{g/day}/10^6\text{cells}$ (Lübberstedt et al., 2011) and a monolayer of 125,000 cells in a 24-well microplate with 0.5 mL medium.

⁴ http://www.taletе.mi.it/help/dragon_help/index.html?knime_extension.html (23/04/2020)

2.2. Distribution to and through the extracellular matrix (ECM)

While the use of extracellular matrices (ECM) in *in vitro* systems has been studied to improve organ-specific functions of cells (LeCluyse et al., 1999; Sellaro and Ranade, 2009; Streuli, 1999), its impact on the distribution of chemicals and uptake kinetics into cells has been less studied. As is the case with serum proteins in *in vitro* assays, ECM may sorb chemicals, decreasing the overall C_{free} of test chemicals. Some of the chemicals tested in EU FP7 Predict-IV project were found to be associated with ECM in cell assays (e.g. 15% of the dose of ibuprofen was found sorbed to collagen after acute exposure while 5-10% of a dose of amiodarone was found sorbed to Geltrex™ after repeated exposure) (Kramer et al., 2015). However, an overview of partitioning of neutral organic chemicals to animal protein indicated that, in general, chemicals have lower binding affinities to collagen and gelatin (hydrolysed collagen which is also used in *in vitro* assays) than muscle protein and BSA (Endo et al., 2012). These low binding coefficients to ECM together with higher binding coefficient to other compartments (e.g. plastic and serum protein) results in a negligible fraction of compounds associated to ECM (e.g. cyclosporin A and chlorpromazine sorption to Geltrex™ (Bellwon et al., 2015; Broeders et al., 2015)).

Despite the relatively low binding affinity of chemicals to ECM compared to other assay components, the ECM may hamper quantification of observed effects in kinetic assays as it may slow the uptake rate of chemicals into cells (Broeders et al., 2015). Since the ECM is a mesh of fibres, its tortuosity increases the diffusion length of both free and bound chemicals, which decelerates overall diffusion. Collagen matrices were reported to significantly restrict diffusion of large molecules (e.g. proteins and dextran) depending on the collagen concentration, hydration, extent of cross-linking, as well as macromolecules size (Gilbert et al., 2006; Ramanujan et al., 2002). The diffusion of cationic chemicals was found to be hindered in ECM-containing assays more than other chemical classes due to collagen being slightly positively charged. On the other hand, selective filtration of anionic macromolecules has been observed in glomerular basement membrane, which is constituted by collagen, but also contains many negatively charged components, such as laminin and heparan sulphate proteoglycans. Therefore, besides steric and hydrodynamic restrictions, electrostatic repulsion can also decelerate the overall mass transport of chemicals (Stylianopoulos et al., 2010). It should be noted that there are conflicting reports for ECM selective filtration, possibly due to differences in ECM gel constitution, hydration, extension of polymerization and the presence of cells (Bolton et al., 1998; Ferrell et al., 2011).

The impact of ECM slowing the rate of diffusion of chemical on C_{cell} *in vitro* can be especially high in static *in vitro* intrinsic clearance assays where diffusion through the ECM is the rate-limiting step of the observed clearance. However, since ECM-hindered diffusion was so far only observed for large macromolecules, diffusion of smaller organic chemicals may not be significantly hampered. For warfarin (slow clearance) and 7-hydroxycoumarin (fast clearance) in serum-free intrinsic clearance assays containing hepatocytes within an ECM gel, it has been shown that intrinsic clearance rate was rate limiting the overall reaction rather than the diffusion in the ECM (Treijtel et al., 2005). Future experiments with well-designed controls will provide greater clarity on the exact *in vitro* conditions (e.g. level and type of ECM, exposure time, physicochemical properties and intrinsic clearance of the chemical) needed for the presence of ECM to significantly affect *in vitro* readout due to reduced chemical diffusion rates and sorption to ECM.

2.3. Plastic sorption

Current *in vitro* assays rely on plastic materials. The most common material used for microtitre plates is polystyrene. This hydrophobic polymer is treated to make its surface more hydrophilic and negatively charged, which facilitates cell attachment (Curtis et al., 1983; Lerman et al., 2018; Wilson et al., 2005). Several studies investigated the impact

of sorption to microtitre plate plastic on the C_{free} in *in vitro* assays. For neutral chemicals with a $\log K_{\text{ow}}$ higher than 5.5 and under serum-free conditions, 25-75% of the added mass of chemical sorbed to plastic (Bourez et al., 2013; Hestermann et al., 2000; Mundy et al., 2004).

The amount of chemical associated to microplate (A_{plastic}) was found to be time-dependent (Bourez et al., 2013; Mundy et al., 2004). In fact, a two-phase kinetic profile has been proposed, where the rate of sorption occurs rapidly in the first hours of exposure and then continues more slowly afterwards (Fischer et al., 2018b). The existence of these two kinetic steps in plastic sorption is possibly because the sorption to plastic consists of adsorption (binding of molecules to the surface of the polymer) as well as absorption (diffusion within the polymer). Slower absorption kinetics demand measurement of samples spaced in time to accurately measure the sorption equilibrium. Equilibrium between the chemical in medium and sorbed to plastic was found to take several hours or even days for high $\log K_{\text{ow}}$ chemicals (Stadnicka-Michalak et al., 2014). Gavara et al. (1996) derived an equation to estimate the length of time to reach equilibrium in polystyrene films given a chemical's diffusion rate in polystyrene (D_{PS}). The authors then calculated that 90% chemical equilibrium is reached *in vitro* after 50 days of exposure to limonene ($D_{\text{PS}} = 3 \times 10^{-18} \text{ m}^2/\text{s}$). Experimentally it was found that 4-31 days were needed for several neutral chemicals to reach equilibrium between C_{free} and the concentration sorbed in 25 μm thick polystyrene sheets (Fischer et al., 2018b). Since chemical sorption to plastic is a reversible process and diffusion in polystyrene is a slow process, desorption from plastic can potentially be a rate limiting step in intrinsic clearance assays.

Since the absorption rate is significantly slower than adsorption rate to plastic, adsorption dominates the mass transfer of chemical into plastic in *in vitro* assays with standard exposures times (Fischer et al., 2018b; Schreiber et al., 2008). Therefore, some authors assume an instantaneous chemical equilibrium between plastic and medium described by an apparent partition coefficient ($K_{\text{PS}/\text{medium}}$) to determine the fraction associated to well plate plastic. Following the finding that sorption to plastic affected especially highly lipophilic chemicals (Riedl and Altenburger, 2007; Schreiber et al., 2008), Kramer (2010) studied the $K_{\text{PS}/\text{medium}}$ of seven PAHs ($\log K_{\text{ow}}$ ranging from 3.3 to 6.1) to the tissue-culture treated polystyrene. Freundlich isotherms were used to derive $\log K_{\text{PS}/\text{medium}}$. It was found that saturation of plastic binding of PAHs occurs at concentrations higher than the solubility in water and could therefore be ignored when estimating the extent of plastic binding in the *in vitro* assay examined in the study. The $\log K_{\text{PS}/\text{medium}}$ for these PAHs linearly correlated with $\log K_{\text{ow}}$. It should be noted that by fitting $\log K_{\text{PS}/\text{medium}}$ to experimental data, even if assuming adsorption only, the resulting partition coefficient will reflect both adsorption and absorption. Hence, *in vitro* distribution kinetics models that use these calculated partition coefficients may still predict accurately plastic concentrations, as long as it is for the same length of exposure as used experimentally.

Stadnicka-Michalak et al. (2014) studied how the amount associated to plastic changed over 48 hours for eight chemicals. While for most chemicals an apparent equilibrium in the plastic was achieved within one day, three chemicals took more than one day to achieve it. For each chemical, rates of influx and efflux from plastic were fitted to experimental data. The rate of influx was shown to also correlate positively with $\log K_{\text{ow}}$ while the rate of efflux had an inverse second order polynomial correlation with $\log K_{\text{ow}}$. Fischer et al. (2018b) studied adsorption and absorption for 22 chemicals, using thin 25 μm polystyrene disks to achieve equilibrium in reasonable experimental time. Absorption was modelled through Fick's second law of diffusion which describes the change of chemical concentration in plastic over time and depends on a diffusion coefficient (D_{PS}). The model for plastic sorption was used to fit $K_{\text{PS}/\text{medium}}$ and D_{PS} from experimental data. The model and resulting parameters successfully simulated the decrease of C_{free} after 24 and 96 hours in polystyrene plates with and without serum. The model was published in Excel and is available to calculate the impact of plastic

sorption in different well plate formats. In the same study by Fischer et al. (2018b), while the logarithm of $K_{PS/medium}$ correlated linearly with $\log K_{ow}$, $\log D_{PS}$ did not correlate with $\log K_{ow}$ and contrary to some literature (Berens and Hopfenberg, 1982; Dole et al., 2011; Kim et al., 1993; Rusina et al., 2010) nor with molecular weight.

While the study of sorption to microplates has been focused on polystyrene, the use of other plastics for culture plates is increasing. Microfluidic systems, for example, often use polydimethylsiloxane (PDMS) which has markedly different chemical sorption characteristics to polystyrene (Nianzhen Li et al., 2009). The diffusion of chemicals through polymeric systems depends on the free volume of the polymer, segmental mobility of the side-chains and overall “stiffness” of the polymer (George and Thomas, 2001). For the latter parameter, the lower the glass transition temperature relative to the experimental temperature, the softer and more flexible the polymer is, thus allowing chemicals to diffuse more quickly and partition better (in)to the polymer. Schreiber et al. (2008) compared the diffusion of phenanthrene in different polymers and showed that while 2% of the chemical sorbed to glass and 28% to plexiglass, 94 % sorbed to polystyrene. However, higher percentages of the total chemical mass were observed sorbed to polyethylene, illustrating how chemicals can differentially sorb to different polymers.

2.4. C_{cell} : accumulation and permeability

The cell-associated concentration (C_{cell}) is the result of chemical uptake into the cell, accumulation inside the cell by binding to cellular components, and metabolic degradation of the chemical. While binding to the cellular components is usually considered an instantaneous process and thus can be calculated using partition coefficients, permeability and metabolism are rates and therefore need to be modelled through kinetic models. It should be noted that cellular uptake for some chemicals also depend on xenobiotic transporters (i.e. proteins that mediate facilitated and active membrane transport) (Clerbaux et al., 2018). The expression and activity of these transporters depend highly on the cell model and experimental conditions used (De Bruyn et al., 2013; Tiong et al., 2014). Moreover, predicting the facilitated or active transport rate is considerably challenging due to the complexity of membrane protein-substrate interactions. Also, the microenvironment (e.g. membrane composition) affects membrane transporter expression and activity (Lagares et al., 2020; Montanari and Ecker, 2015).

Despite the existence of facilitated or active transport of chemicals across cell membranes, studies frequently assume that for most chemicals *in vitro*, passive permeability dominates the overall transport in and out of cells. For these reasons, published *in vitro* distribution models do not include the process of active transport of chemicals into cells. Similarly, metabolic clearance depends on enzymes differentially expressed across *in vitro* assays. Predicting clearance rates of chemicals is equally challenging and generally ignored in *in vitro* distribution models. In this section, we therefore focus on the different partitioning processes that drive accumulation and list physicochemical properties correlating with partition coefficients and passive permeability.

As mentioned in the introduction, C_{cell} incorporates $C_{cell,free}$, $C_{cell,membrane}$ and $C_{cell,bound}$ (Fig. 1). Only free chemical molecules (C_{free} or $C_{cell,free}$) are considered available to interact with a molecular target (McCarty et al., 2011; Smith et al., 2010). For chemicals with high sorption affinities to cells in assays with high cell numbers, the intracellular accumulation of a chemical can deplete the medium C_{free} , and consequently $C_{cell,free}$ and the concentration at the molecular target. Indeed Glden et al. (2001) found a correlation between the cytotoxic effective concentration and the number of bovine sperm cells in suspension. A similar effect was observed with zinc oxide particles in a human cell line (Heng et al., 2011) and with copper in unicellular algae (Franklin et al., 2002). Unspecific binding to cell components also decreases $C_{cell,free}$ in intrinsic clearance assays; the number of microsomes in an experimental condition was found to inversely correlate to the

derived intrinsic clearance rate (Margolis and Obach, 2003). Although the measured effective dose or intrinsic clearance can be affected by other factors such as cell confluency, pH differences and saturable intrinsic clearance, the decrease of $C_{cell,free}$ due to unspecific binding to cell components is the only process that can occur in all the aforementioned *in vitro* systems.

Cell lipids and proteins drive the accumulation of chemicals in cells *in vitro* and *in vivo*. Carbohydrates have not been pointed out as significant sinks for chemicals, although literature on partitioning into carbohydrates is scarce (Schwarzenbach et al., 2016). To estimate partitioning to cellular proteins, some *in vitro* chemical mass balance models use $K_{albumin/w}$ (Fischer et al., 2017; Zaldivar Comenges et al., 2016). While $K_{albumin/w}$ does reasonably predict C_{cell} for neutral and cationic chemicals, it generally overestimates C_{cell} of organic acids (Henneberger et al., 2020). Predictions improved for these chemicals when partition coefficients to structural proteins (chicken muscle), instead of albumin, are used as surrogates for cell proteins (Henneberger et al., 2020). While albumin can have more specific interactions with acidic chemicals, resulting in higher $K_{albumin/w}$, non-specific interactions are expected for structural proteins, lowering the partition coefficient (Henneberger et al., 2016b). Moreover, partitioning to structural proteins does not exhibit saturation and is less influenced by steric effects of chemicals than $K_{albumin/w}$ (Henneberger et al., 2016b). A PP-LFER has been created for predicting partition coefficients of neutral chemicals to chicken muscle (Endo et al., 2012) and has meanwhile been extended to ionised chemicals (Henneberger et al., 2016b). It should be noted the test set for the latter is not rich in cationic chemicals and therefore its accuracy for this class of chemicals is uncertain.

There is a strong correlation between bioaccumulation and lipid content of organisms in ecotoxicity studies (Barron, 1990; Jonker and Van Der Heijden, 2007; Van Der Heijden and Jonker, 2011). Lipids tend to be the dominant determinant of distribution to *in vivo* tissues. Neutral chemicals tend to have significantly higher partition coefficients to lipids than proteins, especially structural proteins (Endo et al., 2013; Henneberger et al., 2020). Yet, for several organs and cell lines, the protein content was measured to be 3-8 times higher than the lipid content and thus proteins can have a major role in driving partitioning (Bertelsen et al., 1998; Fischer et al., 2017; Henneberger et al., 2019; Zaldivar Comenges et al., 2016).

Partition coefficients to cell lipids have an obvious direct correlation with the general lipophilicity proxy, K_{ow} (Austin et al., 2005; Kramer, 2010; Quinn et al., 2014). It should be noted, however, that cellular lipids include membrane lipids⁵ (e.g. phospholipids, cholesterol, sphingomyelin) and storage lipids (mostly triglycerides and derivatives). Especially polar and ionised chemicals have different binding affinities for different lipids types.

For more accurate C_{cell} predictions, solvents or sorbent matrices that better represent each type of lipids have been studied. Partition coefficients of neutral lipophilic chemicals to triolein ($\log K_{triolein/water}$), a specific triglyceride, strongly correlate with partition coefficients to abdominal fat and MCF-7 cells (Quinn et al., 2014). In turn, $\log K_{triolein/water}$ had a linear correlation with $\log K_{ow}$, although there is a trend for K_{ow} to be lower than $\log K_{triolein/water}$. References in literature report opposing results: $\log K_{ow}$ either over- or underpredicts $\log K_{triolein/water}$ (Chiou, 1985; Jabusch and Swackhamer, 2005; Quinn et al., 2014). These discrepancies may be due to experimental artifacts and/or differences in chemicals tested. Since octanol is a more favourable solvent for polar chemicals than triolein (Abraham and Acree, 2016), test sets containing chemicals of different polarity may show different correlations between $\log K_{ow}$ and $K_{triolein/water}$. Besides $K_{triolein/water}$, using partition coefficients to oils (e.g. olive oil, fish oil) other than octanol may help improve the prediction of the partition coefficients to storage

⁵ Cell membranes, myelin membranes, the nucleus membrane, endoplasmic reticulum and mitochondrial membranes differ in their lipid composition.

lipids (Geisler et al., 2012; Poulin and Krishnan, 1995).

Liposomes, bilayers of organised phospholipids, strongly resemble cell membranes and, hence, have been used extensively to study how chemicals partition to cell membranes *in vitro* (Escher and Schwarzenbach, 1996). Unlike triolein and octanol, phospholipids have either zwitterionic or negatively charged headgroups, with the latter having good hydrogen bond acceptors capabilities (Fahy et al., 2005). These phospholipids self-organize in membranes, with the highly polar surfaces surrounding the densely packed lipophilic hydrocarbon core. K_{ow} and $K_{triolein/w}$ are considered ill-suited to act as direct proxies for liposome/water partition coefficients ($K_{liposomes/w}$). Two different PP-LFERS were fit to $\log K_{liposomes/w}$ (Endo et al., 2011; Vaes et al., 1998). The lipophilicity related parameter showed to have a positive correlation to $\log K_{liposomes/w}$. Both PP-LFERS also captured the negative influence of hydrogen-bond acceptor capability in the value of the predicted $K_{liposomes/w}$, reflecting the repulsion of hydrogen-bond acceptors from the phospholipids headgroups. The impact of hydrogen bond donors on $\log K_{liposomes/w}$ was overall low. A comparison of one of the $\log K_{liposomes/w}$ PP-LFER (Endo et al., 2011) with a $\log K_{ow}$ PP-LFER using the same descriptors, showed that, although polarity has a negative impact on both partition coefficients, it has a greater impact on $\log K_{ow}$ (Abraham and Acree, 2016; Schwarzenbach et al., 2016).

$\log K_{liposomes/w}$ and $\log K_{ow}$ differ more for ionised chemicals than for neutral chemicals. The $\log K_{liposomes/w}$ and $\log K_{ow}$ of neutral molecules of phenols are similar, and with increased pH and consequently increased number of negatively ionised molecules, both partition coefficients decrease. However, for $\log K_{ow}$, the decrease is much more pronounced and depends more on the presence of cationic salts (Escher and Schwarzenbach, 1996). The liposomes' membrane structure promotes more efficient counterion-ionic molecules "neutralization", possibly due to its higher surface area to volume ratio than octanol (Escher and Schwarzenbach, 1996).

To further explore the importance of membrane structure and anisotropy on partitioning of chemical, Bittermann et al. (2014) calculated $K_{liposomes/w}$ for a set of neutral, cationic and anionic chemicals to membranes with different levels of complexity. The authors used COSMOtherm, a software that calculates solvation mixture thermodynamics. $K_{liposomes/w}$ values for neutral chemicals, predicted using COSMOtherm

and assuming disorganised phospholipids, were comparable to experimental $K_{liposomes/w}$ values and K_{ow} -predicted $K_{liposomes/w}$ values. However, when compared to experimental $K_{liposomes/w}$ values, $K_{liposomes/w}$ was overpredicted for cations and underpredicted for anions. Phospholipids were also modelled in COSMOtherm as organised membranes, and $K_{liposomes/w}$ values were computed from the chemical's solubility in the different sections of the membrane. Although $K_{liposomes/w}$ was better predicted when COSMOtherm assumed organised membranes as opposed to disorganised phospholipids, the tendency to over- and underpredict $K_{liposomes/w}$ of cationic and anionic chemicals persisted. As aforementioned, phospholipids are amphiphilic molecules and its organisation in membranes causes an electronic heterogeneity and dislocation of charges (dipole), which generates a positive electronic potential. This potential was optimised for $K_{liposomes/w}$ of neutral and ionised chemicals. Partitioning simulations including the optimized potential resulted in improved predictions of $\log K_{liposomes/w}$ for ionised chemicals; the membrane positive potential lightly repels cations, reducing their overprediction, and slightly attracts anions, reducing their underprediction. COSMOtherm remains the most accurate tool to predict the partition behaviour of ions, without requiring more computationally laborious methods (e.g. software that includes molecular dynamics (Venable et al., 2019)).

In summary, cell partitioning can be calculated with individual partition coefficients to cell protein, storage lipids and membrane lipids. Depending on whether the chemical is neutral, polar or ionic, different methods are available to estimate these partition coefficients. Fig. 2 presents an overview of methods estimating partition coefficients to cell proteins, storage lipids and membrane lipids. The methods differentially balance prediction accuracy and throughput. Measurements of the different lipids in cells can be a hurdle in modelling partitioning to cells as distinguishing lipid types is analytically challenging. There is little need to distinguish between storage and membrane lipids when estimating K_{cell} and C_{cell} for neutral chemicals. For polar and especially ionic chemicals, however, assuming bulk lipid partitioning can lead to over- and underestimations. Future studies should investigate the extent and significance of this over- and underestimation. Moreover, since the other components of membranes (e.g. glycolipids, sterols and transmembrane proteins) are not included in liposomes, also it should be

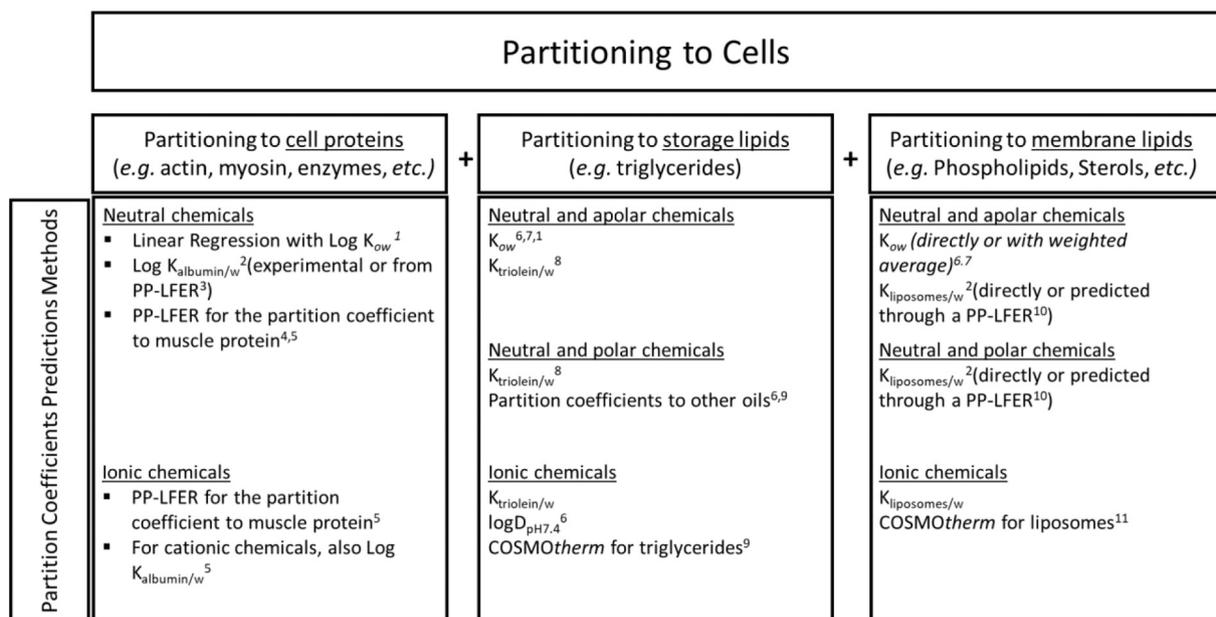


Fig. 2. An overview of surrogate parameters and QSPRs for predicting a chemical's affinity to the different components of cells *in vitro*. The correct surrogate or QSPR to use is dependent on the chemical class of the test chemical. References: 1- (Zaldivar Comenges et al., 2016), 2-(Fischer et al., 2017), 3- (Endo and Goss, 2011), 4-(Endo et al., 2012), 5-(Henneberger et al., 2016a), 6-(Poulin and Krishnan, 1995), 7-(Rodgers et al., 2005; Rodgers and Rowland, 2006), 8-(Quinn et al., 2014), 9-(Geisler et al., 2015), 10-(Endo et al., 2011), 11-(Bittermann et al., 2014)

studied if these components can significantly affect the partition coefficient to cell membranes.

Whereas partition coefficients to cells describe the distribution of a chemical between medium and cells at equilibrium, permeability describes the flux of molecules across the membrane. Hence, permeability defines how quickly the chemical accumulates in the cell before it reaches this chemical equilibrium. Depending on the chemical properties and other processes affecting the C_{cell} (e.g. transport and metabolism), it may take hours to days for cells to reach a steady state concentration in cells (Fischer et al., 2018a; Stadnicka-Michalak et al., 2014). Therefore, for *in vitro* assays of short duration (i.e. shorter than 8 hours), a chemical's permeability can significantly influence the observed *in vitro* readout.

Most literature studying permeability test the passage of chemical across phospholipid membranes (e.g. parallel artificial membrane permeability assays and black lipid membrane assays) or cell barriers (e.g. Caco-2 or brain-blood barrier endothelial cells apparent permeability P_{app} assays) in transwell systems (Youdim et al., 2003). Permeability measured with phospholipid membranes cannot be directly compared to P_{app} . Besides assessing the permeability across phospholipid membranes, P_{app} also includes influx and efflux transporter activity, lateral membrane diffusion, transcellular diffusion, and diffusion across the cytosol and transwell membrane (Bittermann and Goss, 2017). Therefore, mechanistic modelling that discriminates between individual diffusive processes driving membrane permeability is needed for comparing membrane permeability to P_{app} .

Because permeability describes a flux of molecules it can be described with Fick's law of diffusion (Eq. (1)), which states that the transfer of a chemical mass from a donor compartment to a receiver compartment in time (dM/dt) depends on the chemical's diffusion coefficient (D), the distance the chemical needs to cross (l), the concentration gradient between the receiver and donor compartment (ΔC) and the surface area of the membrane (SA). For permeability studies it is common practice to lump D and l in a permeability coefficient (P_{coef} , in cm^2/min), assuming the membrane thickness is identical in all membranes.

$$\frac{dM}{dt} = \frac{D (\text{cm}^2/\text{min})}{l(\text{cm})} \times \Delta C (\mu\text{mol}/\text{cm}^3) \times SA (\text{cm}^2) \quad (1)$$

It is assumed that only the unbound molecules can permeate, hence ΔC is about the C_{free} and the $C_{\text{cell, free}}$. Once chemicals reach the cytosol, they can quickly partition to cell lipids and proteins, hence decreasing $C_{\text{cell, free}}$. Therefore, partitioning of chemicals to cells lipids and protein, generates a higher ΔC than if cellular partitioning was not considered. This results in a higher flux of molecules in cells with higher lipid and protein content. Bourez et al. (2013) found that uptake of PCBs is faster in cells with more triglycerides. As an important driver of the flux of molecules across membranes, ΔC must be correctly incorporated in experimental derivations of P_{coef} and models of the distributions of chemicals in *in vitro* systems.

Several processes determine P_{coef} : diffusion of molecules in the unstirred aqueous layer around the (cell) membrane, partitioning between medium and the membrane and diffusion through the membrane, across sections differing in lipophilicity and polarity. Therefore, several physicochemical properties of a substance influence permeability, summarized in the Lipinski rule of 5 (Lipinski et al., 1997): lipophilicity, molecular weight, and polarity (H-bond-donor and acceptors). Accordingly, there are several membrane permeability QSPRs that take these properties into account. Recently COSMOtherm added a feature that predicts a "true" passive membrane permeability based on the free energy of chemicals into individual sections of the membrane (Ebert et al., 2020).

Although lipophilicity generally increases permeability because lipophilic chemicals diffuse readily through membranes and have high partition coefficients to membranes, extremely lipophilic chemicals (log

K_{ow} higher than 5) have low cell membrane permeabilities. This is because diffusion through the unstirred aqueous boundary layer and across phospholipids with polar headgroups becomes prohibitively slow (Bittermann and Goss, 2017). Similarly, chemicals with several H-bond donor or acceptor groups diffuse more easily across the aqueous and polar environments, but not within the membrane lipophilic core. Due to their optimal affinity to membranes, amphiphilic molecules, such as digitonin, prefer accumulating in membrane instead of diffusing across it (Balaz, 2009; Glden et al., 2001).

The molecular volume negatively affects P_{coef} because every diffusion process has a resistance created by the "viscosity drag", which depends on the surrounding matrix viscosity and the molecule size (Edward, 1970). Therefore, high volume chemicals have lower diffusion constants than low volume chemicals. With the membrane being a more viscous environment than medium or cytosol, the decreased diffusion of these large and less compact molecules is more pronounced. It is noteworthy that unfavourable interactions between the chemical and the surrounding matrix also increase the diffusion drag. In fact, for a set of ten fluorescent chemicals, the anionic and zwitterion chemicals had lower uptake in cells relatively to neutral chemicals (Fischer et al., 2018a).

The difference between membrane partitioning and permeability explains the controversy surrounding the question to what extent molecular volume and high lipophilicity have a negative impact on bioaccumulation (Jonker and Van Der Heijden, 2007). As aforementioned, molecular volume and high lipophilicity have deleterious effect on permeability. This is not true for partitioning to membrane. If a deleterious effect of these characteristics on partitioning to cell is observed, the chemical equilibrium between the aqueous medium and the membrane surrogate was likely not achieved during the experiment. In fact, it was calculated that for some extremely lipophilic chemicals, extraordinary time lengths (e.g. 0.5 years for log K_{ow} of 6) are needed to achieve equilibrium (Hawker and Connell, 1985). Therefore, especially in assays with short exposure durations, it should be evaluated whether partitioning processes reach equilibrium and consequently whether partition coefficients predict C_{cell} sufficiently.

2.5. Loss processes to gas phase

Problematic evaporation of the test chemicals has been extensively reported in aquatic toxicity assays and is therefore a large focus point in the OECD guidance document on aqueous-phase aquatic toxicity testing of difficult chemicals (OECD, 2019). This OECD guideline indicates that significant fractions of chemicals with a Henry Law constant (K_{H}) of 1-10 $\text{Pa} \times \text{m}^3/\text{mol}$ are lost to evaporation, which hampers the calculation of effect concentrations. With the advent of *in vitro* high-throughput screening using smaller medium volumes in microtitre plates, gas surface area to medium ratios are becoming higher and consequently the problems associated with the evaporation of test chemicals will become more apparent. (Vaal and Forkerts, 1998).

Modelling evaporation in *in vitro* assays is challenging. Most *in vitro* chemical mass balance models taking evaporation of a chemical into account, assume the *in vitro* assay is closed system. In a closed system, the volatile chemical quickly reaches an equilibrium between air and media, which can be described by the chemicals air-water partition coefficient, K_{aw} . K_{aw} can be derived from K_{H} . However, most *in vitro* assays are semi-open systems. The mass flow of air in and out of the well continually dilutes the chemical in the headspace, making it virtually impossible to attain a chemical equilibrium between medium. Eventually, all chemical mass will have evaporated out of the system. The *in vitro* distribution model in Comenges et al (2016) includes equations simulating this mass flow of air. It should be noted, however, that the air mass flow is a chaotic and highly variable process, and consequently, models of this flow are fraught with uncertainties.

Besides contributing to the loss of chemical mass from the system, evaporation can also cause the cross-contamination of wells with

chemical. In 96-well microtitre plates, phenol with a K_H of 0.155 Pa \times m³/mol evaporated and migrated from the phenol-exposed wells to the control wells (100% viability), slowing cell growth in these controls (Thellen et al., 1989). Therefore, the cell growth in phenol-exposed wells was not as low relative to controls as it would be expected if there was no cross-contamination, reducing the observed toxic potency of the chemical. This cross contamination was dependent on the orientation of test wells relative to control wells, buffer zones between conditions, and whether the plates were sealed, emphasizing the importance of test setup standardisation.

Another pitfall of modelling evaporation lies in the need to extrapolate K_H for different temperatures. While common chemical databases list K_H at 25 °C, *in vitro* toxicity assays with mammalian cells are routinely performed at 37°C. Since K_H changes with temperature (Ten Hulscher et al., 1992), predicting the volatilization at 37°C with the constant for 25°C can lead to great underestimations of evaporation. K_H can be extrapolated from 25°C to 37°C through calculations using the chemical vapor pressure or enthalpy or a PP-LFERs for K_{aw} in different temperatures (Smith and Harvey, 2007).

Several strategies have been studied to adapt *in vitro* assays to a closed system format to reliably test volatile chemicals and model C_{free} . Plastic adhesives were used to cover wells in microtitre plates used for cytotoxicity assays with phenanthrene (K_H of 4.28 \times Pa \cdot m³/mol). Yet, after 24 hours exposure, a loss of 98 % of phenanthrene to evaporation was still observed. When microplates were covered with aluminium instead of plastic, losses decreased to 75%, suggesting the plastic adhesive worked as a sink for evaporated compound, enhancing evaporation (Schreiber et al., 2008). The potential for aluminium covers to minimize evaporation was further studied in Birch et al. (2019). In general, the aluminium covers did not decrease evaporation significantly. However, cross-contamination of wells was prevented for 8 volatile chemicals by using the aluminium cover. Thus, the loss of chemical mass in closed wells is most probably due to the glue of the aluminium cover sorbing the chemical than to the presence of gas transfer from out of the well to inside.

To accurately predict C_{free} using K_{aw} , the *in vitro* system needs to be closed, which is easier to do with cell cultures that do not require attachment to a surface, such as suspension cultures and spheroids (Liu et al., 2013), since these cultures can be done in tightly capped glass vials. Still, monolayers of SH-SY5Y (McDermott et al., 2007) and A549 (Liu et al., 2013) cell lines were able to attach to glass vials, and also HepG2, after coating the bottom of the vial with Poly-Lysine (Mochalski et al., 2013). Instead of ensuring a chemical equilibrium between the medium and gaseous phase in *in vitro* systems, the gaseous headspace may be eliminated altogether. Stalter et al. (2013) used tightly capped glass vials without headspace and found higher recoveries of test chemicals than in sealed wells with a gaseous headspace. Although these closed-system methods have their advantages, many cells used in *in vitro* assays require high oxygen levels in medium (Wenger et al., 2015) and gas exchanges with the CO₂ atmosphere inside the cell incubator, which these systems cannot deliver.

As aforementioned, usually loss of mass of the chemicals by evaporation can be predicted through K_H . However, binding to *in vitro* set up components, such as serum, decreases C_{medium} and C_{free} and consequently the amount of chemical available for evaporation. Taking this in account, a volatile cut-off was proposed that was not based on the K_H , but rather on the medium-air partition constant $K_{medium/air}$ of 10000 (Escher et al., 2019). This $K_{medium/air}$ represent both the K_{aw} , but also how much chemical is bound to lipid and protein components in medium.

2.6. Abiotic degradation

Abiotic degradation of test chemicals may occur in *in vitro* assays, thus decreasing C_{medium} and C_{free} . Some chemicals, such as bupropion and coumarin (Dragojević et al., 2011; O'Byrne et al., 2010), degrade

relatively quickly (e.g. half-life of 72 h and 2.5-16h, respectively) in aqueous solutions at pH 7.4. Assuming similarities to environment abiotic degradation (Nolte and Ragas, 2017), degradation *in vitro* is likely to occur by reacting with radicals (mainly oxygen radicals) or photolysis in the medium and headspace. This means degradation will occur at different rates in medium and headspace due to different concentration of radicals and light penetration in each phase. Radicals occur naturally (often through photolysis of water and atmospheric gases), but may also get into medium through metabolism reactions in cells (Phaniendra et al., 2015). Components of cell culture serum can also increase radicals concentration (Wang et al., 2018). Moreover, partial oxygen pressure in *in vitro* is often higher than tissues and blood in *in vivo*, which could result in a more oxidative environment (Halliwell, 2003). However, it should be also noted that oxygen concentration in medium drops quickly throughout the static culture medium depth (Wenger et al., 2015). Most media often do contain antioxidants such as glutathione that reduce the reactivity of radicals. Altogether it is difficult to conclude how similar abiotic degradation rates in cell culture media and in *in vivo* are.

It is assumed that only unbound molecules in medium are available for degradation, since bound molecules are shielded from light and degradation by radicals (Schwarzenbach et al., 2016). Similarly, to intrinsic clearance assays, if the rate of albumin or plastic desorption of the chemical is slow enough it potentially affects the degradation of the chemical. Thus, future studies are needed to elucidate the similarities of mass loss in *in vitro* and *in vivo* due to abiotic degradation and the best strategy for QIVIVE of unstable chemicals.

While models for abiotic degradation exist, they have been developed for environmental compartments and rely on complex chemical descriptors (e.g. electron orbitals of the atoms in the molecules) (Nolte and Ragas, 2017). The U.S. EPA EPISuite toolbox includes models predicting aqueous hydrolysis rates (HYDROWIN) and atmospheric oxidation (AopWIN), which consist of hydroxy radical reactions. Besides, experimental data for atmospheric hydroxylation rate of a number of chemicals is available in US and EU chemical databases (Comptox Dashboard and REACH dossiers, respectively). There is a need for more relevant *in silico* tools to predict abiotic degradation under *in vitro* test conditions.

3. In silico models to estimate the distribution kinetics of chemicals *in vitro*

The use of $C_{nominal}$ for comparing chemical potencies, assay sensitivity or deriving a Point of Departure (PoD) for QIVIVE is often inappropriate because $C_{nominal}$ is not necessarily similarly proportional to the BED across chemicals and bioassays. The distribution processes described above explain why, when and by how much $C_{nominal}$ and BED differ. The C_{free} and C_{cell} that cause a certain effect are dose metrics that are more independent of distribution processes in an *in vitro* system and are therefore more comparable across systems.

Although individual distribution processes have their individual contribution to the decrease of available chemical, often several processes occur in the same *in vitro* system, with plastic, serum, cells, etc. competing to sorb the same unbound molecules. There are a few experimental examples of the interplay between *in vitro* distribution processes. Since serum constituent-bound test chemical are unavailable for sorption to other compartments, the presence of serum minimizes the fraction sorbed to plastic and cells (Hestermann et al., 2000; Mundy et al., 2004). Testing the same volatile chemical in glass vessels instead of polystyrene ones resulted in more evaporation since the chemical molecules that binds to the polystyrene vessel do not evaporate, effect that does not happen in glass vessels (Riedl and Altenburger, 2007; Schreiber et al., 2008). In conclusion, the same chemical can have different distribution profiles in different experimental set ups (Bellwon et al., 2015a; Broeders et al., 2015; Pomponio et al., 2015; Truisci et al., 2015). Hence, to predict *in vitro* C_{free} and other alternative dose metrics

to C_{nominal} , *in silico* models describing the rate and partitioning of test chemicals to the different compartments in *in vitro* systems, have been developed (Fig. 3 and Table 1).

Compartmental models of chemical distribution in *in vitro* set up (Fig. 1) describe the process of chemicals moving between compartments by means of uptake and elimination rate constants and concentration gradients. Assuming steady-state in the compartment, the ratio of uptake and efflux can be described as a partition coefficient. Whether to include a specific process (e.g. abiotic degradation) and compartment (e.g. serum) in an *in vitro* chemical distribution model depends on the physicochemical properties of the chemical, assay setup and dose metric needed for a specific study. For a detailed analysis of these models, a selection has been made to have a diverse representation of the different compartments and methods to simulate the distribution processes. This selection allows to evaluate the domains of applicability of different models.

3.1. *In vitro* compartments

Earlier models focus on serum protein binding to estimate C_{free} *in vitro*. Based on previous studies indicating that specific serum proteins (e.g. sex hormone binding globulin, alpha-fetoprotein and albumin) strongly bind estrogenic chemicals, Heringa et al. (2004) and Teeguarden and Barton (2004) divided serum into a number of separate model compartments (Fig. 3A and B). The addition of these serum proteins in the models allowed the evaluation of availability of estrogenic chemicals in different *in vitro* assays. Teeguarden et al. (2004) extended this work further to understand how xenobiotics could compete with oestrogen for its receptor, by simulating a mixture of the two chemicals.

Several authors noted the dependency of intrinsic clearance and effect concentrations on cell number. They proposed to include cells as an additional model compartment (with cell lipids as a surrogate for the cell biomass) to model C_{free} (Fig. 3D-G) (Armitage et al., 2014; Glden and Seibert, 2003; Kramer, 2010; Kwon et al., 2020). Following the discovery that highly lipophilic chemicals in low serum conditions significantly sorbed to the plastic of microtitre plates, plastic was also added as a compartment (Fig. 3F) (Kramer, 2010).

After studies reporting that while organic chemicals generally had higher partition coefficients to lipids, tissues contained more protein than lipids, the models also started to add both a serum lipid compartment but also a cell protein compartment (Fig. 3H,I and N). ECM was used in experiments in the Predict-IV (Bellwon et al., 2015b; Broeders et al., 2015; Pomponio et al., 2015; Truisi et al., 2015). The amount of drug detected in medium, plastic, cells and ECM were measured. Since results showed that little concentration of the chemical was found associated with the ECM, it was not included as a compartment in the *in silico* model (Fig. 3H). However the model of Treijtel et al. (2005, 2004) (Fig. 3L) included ECM as a compartment, for the aim of the study was to evaluate the impact of ECM on intrinsic clearance assays.

Some chemicals toxic mode of action requires their uptake into subcellular compartments such as mitochondria and lysosomes. Since the distribution into these compartments can also be predicted based on chemical physicochemical properties (Horobin et al., 2007; Trapp et al., 2008), the compartments were added to two generic models (Fig. 3I and N). Thus, these models have a semi-integration of chemical distribution and toxicodynamics.

The inclusion of headspace as a compartment introduces a great deal of uncertainty. While some general models have included it to account for evaporation of volatile compounds (Fig. 3F,G, I and N) (Armitage et al., 2014; Fisher et al., 2019; Kramer, 2010; Zaldivar Comenges et al., 2016), testing volatile chemicals in standard *in vitro* systems leads to very unreliable results. Moreover, the current *in silico* distribution models consider the wells of microtitre plates as closed system, ignoring the flow of air in and out of the well. Ideally, testing of suspected volatile chemicals should be performed in a more suitable closed *in vitro* system

such as a capped glass vial (Liu et al., 2013; McDermott et al., 2007; Mochalski et al., 2013), provided that cells can be cultured under such conditions. Then an *in silico* model of distribution can be useful to predict in equilibrium situations how much chemical is in the headspace (Liu et al., 2013). For setups in glass vials, there is no need for polystyrene as a compartment.

3.2. Partitioning or kinetic processes

Each distribution process in an *in vitro* system can be modelled using partition coefficients, thus assuming the system is instantaneously in a steady-state equilibrium. The distribution process can also be modelled as a kinetic process, using differential equations (Fig. 1). Except for unidirectional reactions (e.g. degradation), kinetic processes must be modelled through either a rate in and out or as a diffusion, both strategies taking in account concentration gradients between compartments. The majority of the *in silico* models reported in the literature (Fig. 3) are based solely on partitioning. Based on the knowledge of the different distribution processes, despite this assumption being reasonable for most chemicals and *in vitro* systems, some cases justify resolving the kinetics of distribution processes.

The assumption that the system will quickly reach equilibrium or steady state can be incorrect for experiments where metabolism or evaporation of the chemical into an open system occurs. To have general steady-state in these *in vitro* assays, the rates of sorption/desorption from serum, plastic and cells and diffusion across ECM need to be significantly faster than the rate of depletion of the chemical. If the rates are not faster than the depletion, then the amount of chemical found in serum, plastic and cells will be lower than the partition coefficient would predict, leading to overestimations of the chemical sorbed into these compartments. Moreover, the measured rate of depletion will then be influenced by the speed of the rates of those distribution processes. Kwon et al. (2020) studied this issue (Fig. 3D), by using different *in silico* models to simulate *in vitro* PAHs clearance assays with varied concentrations of microsomal protein in glass vials, so sorption to polystyrene would not play a role. The model with most accurate simulations included a rate of delivery of the chemical bound to cell components to the enzymatic complex. Diffusion in polystyrene can be quite slow (Fischer et al., 2018b), typically slower than the clearance rate of quickly metabolized chemicals. Most clearance assays are completed within minutes to hours. Therefore, plastic sorption as a kinetic process for clearance assays or any other assays where there will be depletion of chemical will need to be considered if the clearance has slow kinetics.

While most of the *in vitro* models for intrinsic hepatic clearance assays do incorporate the distribution to protein and cells as instantaneous partition (Austin et al., 2005; Kilford et al., 2008), often they underpredict the intrinsic clearance of highly lipophilic chemicals in the presence of serum. This underprediction is possibly because they do not account for the facilitated diffusion or cellular uptake of chemical albumin-bound chemical (Bowman and Benet, 2018; Poulin et al., 2016). Since diffusion through ECM could also be a rate-limiting step in clearance assays, Treijtel et al. (2005, 2004) added this kinetic process to a *in silico* model of chemical distribution in a hepatocytes sandwich culture (Fig. 3L). In fact, diffusion through collagen seemed to impact minimally the measured clearance. Still, more knowledge and experimental data is needed to properly assess the extension of delayed diffusion by the ECM, since it also depends on presence of serum, cellular uptake rate and clearance rate of the specific drug.

A specific case where $C_{\text{cell, free}}$ and C_{free} do not reach equilibrium is short exposures (e.g. 4-6 hours exposure) of chemicals with slow membrane permeability. For example, PCBs accumulation in adipocytes did not reach equilibrium in 4 hours (Bourez et al., 2013), cypermethrin took more than 8 hours to reach equilibrium (Stadnicka-Michalak et al., 2014) and the cell accumulation of five out of the ten chemicals tested by Fischer et al. (2018a), did not reach equilibrium after five hours. Most importantly, temporal C_{cell} followed a first order kinetic curve and

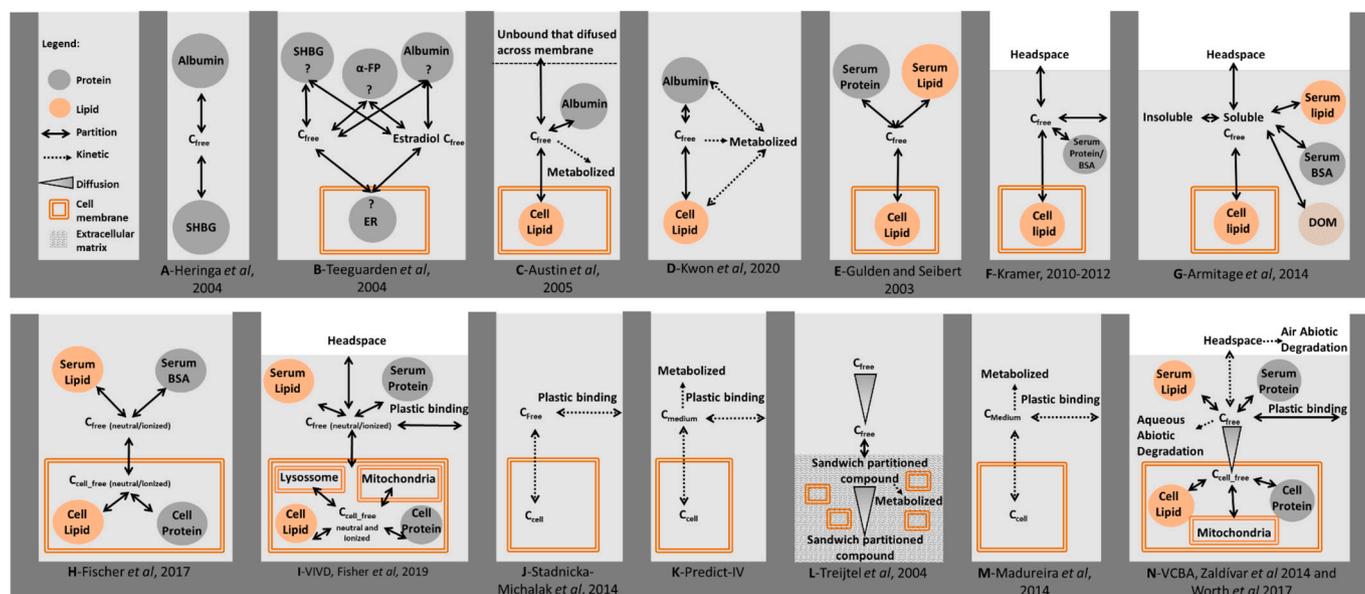


Fig. 3. Schematic representation of *in silico* models that describe the *in vitro* distribution of test chemicals. Grey and orange circles denote proteins and lipids, respectively, and the orange double line represents the cell membrane. Black arrows indicate whether the distribution process is modelled using partition coefficients (solid line), rate constants (dashed line) or diffusion constants (triangles). Question marks represent competition for binding sites. Some models estimate C_{medium} instead of C_{free} in medium because serum constituent binding is not accounted for. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

therefore, most of the accumulation occurs within a short duration. This means that predictions using partition coefficients might still be sufficiently accurate for C_{cell} and $C_{\text{cell, free}}$. The models in Fig. 3C, D, J, K-N do include distribution processes as kinetics (Austin et al., 2006; Bellwon et al., 2015b; Kwon et al., 2020; Madureira et al., 2014; Stadnicka-Michalak et al., 2014; Treijtel et al., 2005; Wilmes et al., 2013; Zaldivar Comenges et al., 2016); Zaldivar et al (2016) includes permeability as a rate which can potentially better simulate short exposures scenarios or slowly permeable chemicals, but also intrinsic clearance (Paini et al., 2017). The model in the Predict-IV project includes distribution into all compartments as rates.

3.3. Parameterisation of models

Model structure depends on the available data to parameterize it. Knowledge of the *in vitro* setup, such as test temperature, medium volume and composition and surface area of medium-exposed plastic, is generally readily available from the suppliers of the assay and assay components. It is more challenging to obtain detailed information on the cells used in the *in vitro* assay, e.g. the number over time, volume, lipid and protein content, and expression levels of xenobiotic transporters and biotransformation enzymes in the cells of interest. Complex measurements are needed for cell volume, intracellular lipid and protein. In many long-lived *in vitro* assays, cell numbers and therefore concentration per cell change over the exposure period. However, these changing cell numbers over the exposure time is rarely determined. Of the models depicted in Fig. 3, only the VCBA model includes cell growth, but it does not reflect contact inhibition of cell proliferation and the effect of serum on cell growth (Proença et al., 2019). In exposure conditions where cell death occurs, the number of cells and therefore total volume of cells will decrease. Dead cells release their intracellular content, including cell proteins, lipids and associated test chemicals, into the exposure medium. This process is represented in the model in Zaldivar et al. (2016) although it is not shown in Fig. 3. The model simulates that the amount of cell lipid and protein in the system remains the same, and thus, C_{free} should not change. However, this way of modelling assumes that intracellular lipids and proteins retain the same partitioning characteristics inside and out of the cells and that once the cell die these

components do not degrade extensively. Again, specific experiments could clarify the best strategy to model the contents of dead cells, although it would probably only be relevant for serum-free or low serum conditions.

Although all models in Fig. 3 used cell line specific parameters (e.g. cell volume, lipid and protein fraction in cells), measuring these parameters is hardly high throughput or standardized. Moreover, phenotypic characteristics of the same cell line vary significantly between experiments and labs (Liu et al., 2019; Zucco et al., 2005). Henneberger et al. (2020) measured C_{cell} for the same 21 chemicals in four different cell lines. Except for few chemicals (warfarin, torsemide, naproxen, coumarin, 6-gingerol) the steady-state partition ratios between cells and water varied by less than one log-unit, and besides, no systematic differences between the cells were observed. Therefore, to simulate C_{free} and C_{cell} in *in vitro* experiments with several cell lines, the authors used cell parameters calculated from the average of different cell lines. They recommended that differences in cell volume, lipids and protein content between cell lines and within cell lines cultured under different conditions (e.g. passages, serum supplementation, growth stage, etc.) should be better assessed in the future to understand which strategy offers the best balance between being high throughput and being accurate.

To obtain the partition coefficients and rate constants that are used in these *in silico* models that predict C_{free} or C_{cell} in *in vitro* assay, three strategies can be identified from the models in Fig. 3. The models are categorized according to these strategies in Table 1.

1. Collecting the parameters (e.g. partitioning coefficients for estrogenic chemicals and different proteins in serum) from literature (Fig. 3A, B and E) (Heringa et al., 2004; Teeguarden and Barton, 2004). This strategy is easy but relies on very specific data which is not frequently available in literature, especially for new chemicals. Thus, the number of chemicals these models can simulate are limited.
2. Deriving the parameters by fitting the model to experimental data (e.g. concentrations of chemical in the different compartments in time) (Fig. 3C, D, J, K, L and M). These types of models are generally more explanatory than predictive for different scenarios (Bellwon et al., 2015b; Broeders et al., 2015; Madureira et al., 2014; Pomponio et al.,

Table 1

Summary of in silico models that describe in vitro distribution of test chemicals and their applicability domains (ADs). The chemical applicability domain distinguishes between specific (for specific chemicals) and generic, meaning it is for chemicals within the range of physicochemical properties indicated.

Reference model	<i>In vitro</i> system applicability domain	Chemical applicability domain	Partition to serum	Partition to Plastic	Partition to Headspace	Partition to cells or cell components	Other Partitions	Kinetic and Dynamic processes
Fig. 2 -A (Heringa et al., 2004)	Culture media with serum	<u>Specific</u> estradiol and octylphenol	experimental					No
Fig. 2B (Teeguarden and Barton, 2004)	Culture media with serum and monolayer of ER expressing cells	<u>Specific</u> estradiol, genistein, bisphenol A and octylphenol	experimental			experimental		No
Fig. 2C (Austin et al., 2005)	Static culture media with and without BSA, with rat primary hepatocytes in suspension in glass vials	<u>Generic</u> Neutral and Ionised Non-volatile				$\text{Log}D_{\text{ow}(\text{pH } 7.4)}$		Intrinsic Clearance
Fig. 2D (Kwon et al., 2020)	Agitating culture media with and without BSA, with S9 fractions in suspension in glass vials	<u>Specific</u> Phenanthrene Anthracene Pyrene Benzo(a)pyrene						Intrinsic Clearance, Desorption from cell components and albumin
Fig. 2E (Gülden and Seibert, 2003)	Culture media with and without serum and general cell suspension in glass Erlenmeyers	<u>Specific</u> 33 chemicals, inorganic and ionised organic and organic	experimental			$\text{Log}K_{\text{ow}}$		No
Fig. 2F (Kramer, 2010)	Culture media with and without BSA, with cell monolayer in plastic microplates	<u>Generic (PAHs)</u> Neutral and Apolar Non-volatile and volatile	$\text{Log}K_{\text{ow}}$ QSPR	$\text{Log}K_{\text{ow}}$ QSPR	K_{H}	$\text{Log}K_{\text{ow}}$ QSPR		No
Fig. 2G (Armitage et al., 2014)	Culture media with serum and cells monolayer	<u>Generic</u> Neutral Non-volatile and volatile	$\text{Log}K_{\text{ow}}$ extrapolation equation		K_{H}	$\text{Log}K_{\text{ow}}$	Solubility and chemical activity	No
Fig. 2H (Fischer et al., 2017)	Generic	<u>Generic</u> Neutral and Ionised Non-volatile	PP-LFER and a 3D-QSPR			PP-LFER or COSMOtherm software		No
Fig. 2I (VIVD (Fisher et al., 2019)	Generic	<u>Generic</u> Neutral and Ionised Non-volatile and volatile	$\text{Log}K_{\text{ow}}$	$\text{Log}K_{\text{ow}}$ extrapolation equation	K_{H}	$\text{Log}K_{\text{ow}}$ olive oil/water partition for neutral lipids, $\text{Log}K_{\text{ow}}$ partition for neutral phospholipids	Lysosome and mitochondria partitioning	No
Fig. 2J (Stadnicka-Michalak et al., 2014)	Static culture media without serum, with cells monolayer in a 24-well plastic microplate	<u>Generic</u> Neutral and Ionised Non volatile		$\text{Log}K_{\text{ow}}$ extrapolation equation		$\text{Log}K_{\text{ow}}$ extrapolation equation		Sorption to plastic and cellular uptake
Fig. 2K (Predict-IV ^a)	The specific <i>in vitro</i> system of each paper	<u>Specific</u> cyclosporin A, chlorpromazine, ibuprofen, amiodarone	Experimental	Experimental		experimental	Extracellular matrix	Intrinsic clearance and plastic binding
Fig. 2L (Treijtel et al., 2004, Treijtel et al., 2005)	Static culture media without serum and rat primary hepatocytes in a sandwich culture	<u>Specific</u> tolbutamide, warfarin and 7-ethoxycoumarin				$\text{Log}K_{\text{ow}}$ based extrapolation and fitted parameters		Clearance and diffusion in collagen
Fig. 2M (Madureira et al., 2014)	Static culture media with 7% serum and Hepa1c1c7 monolayer in a 75 cm ² flasks	<u>Specific</u> benzo(a)pyrene		experimental		experimental		Uptake, metabolism, and formation of DNA adducts
Fig. 2N (VCBA (Zaldivar Comenges et al., 2016)	Generic	<u>Generic</u> Neutral Non-volatile and volatile	$\text{Log}K_{\text{ow}}$ extrapolation equation	$\text{Log}K_{\text{ow}}$ extrapolation equation	K_{H}	$\text{Log}K_{\text{ow}}$ extrapolation equation	Mitochondria partition	Evaporation, abiotic degradation, cell permeability and cell growth/ death

^a Refers to the general presentation of model used in EU FP7 Predict-IV project. The project included several *in vitro* systems and the model was fit and adapted to each type of system.

2015; Treijtel et al., 2004). These models have the potential to accurately predict chemical distributions in specific experiments but cannot be applied to other experimental setups or chemicals.

- Using QSPRs (Fig. 3F, G- J and N). This strategy is based on the knowledge of how physicochemical properties drive the accumulation of chemical in a certain compartment. Here, authors of the model measure partition coefficients or rate constants for a group of chemicals and derive QSPRs to estimate partition coefficients and rate constants of similar chemicals (Kramer, 2010). Other authors use existing QSPRs from literature (Armitage et al., 2014; Fischer et al., 2017; Fisher et al., 2019; Zaldivar Comenges et al., 2016). Therefore, these types of models can be used to predict distribution of new chemicals from which there is not a lot of information.

To exemplify these *in silico* models, simulations were run with five generic models to predict the distribution of six test chemicals in *in vitro* assays with HepaRG cells at a nominal concentration of 1 μ M. Two *in vitro* assay setups were tested: 96-well plastic microplates with 0 or 10% (v/v) FBS in exposure medium (Fig. 3). Steady-state is assumed for all models with the exception of the dynamic VCBA model. The VCBA model was run for 24 hours of simulated time and the distribution of compound predicted at 24 hours assumed comparable to steady-state. The input chemical parameters needed for each model are given in Fig. 4 except for the model described by Fischer et al. This model requires specific descriptors which can be obtained in UFZ-LSER database and, specifically for ionized molecules, requires parameters from CosmoTherm and from 3D-QSAR (Table 1). For the purpose of these simulations, we only found the ionic molecule descriptors for triclosan. For all other ionic chemicals (diphenhydramine and ibuprofen), neutrality was assumed ⁶.

Fig. 4 illustrates how predictions of chemical partitioning are dependent on the physicochemical properties of the chemical: lipophilic chemicals (*i.e.* diphenhydramine, naphthalene, triclosan and ibuprofen) are predicted by some of the models to sorb to plastic (approximately 10%, 10%, 25% and 70%, respectively) when no serum is present in the exposure medium. However from these four chemicals, only naphthalene is entirely neutral. While triclosan is partially anionic and therefore there are still neutral molecules that might still sorb to plastic, ibuprofen and diphenhydramine are fully ionized and therefore, significant sorption to plastic is unlikely. Naphthalene, an example of a volatile chemical, only shows $\leq 5\%$ of loss into the headspace in the models where evaporation is considered (*i.e.* Kramer, 2010, VIVD, Armitage and VCBA). In reality evaporation of naphthalene in an *in vitro* system is likely to be more extensive as standard *in vitro* test systems are not closed systems with a fixed headspace, which the models assume.

For Fig. 4B, the C_{cell} for the selected chemicals was calculated taking in account the number of cells (2500) and the volume of an HepaRG cell ($1.67 \times 10^{-15} \text{ m}^3$ (Paini et al., 2017)). It should be noted that Kramer's model and Armitage's model only take in account partitioning to cell lipids, ignoring the distribution into the cytosol and binding to cell protein. Ignoring the cytosol and cell protein compartment may lead to lower simulated cell associated concentrations, especially for less lipophilic chemicals. However, as observed in Fig. 4B C_{cell} simulations of Kramer and Armitage's models do not clearly stand apart from other

model's predictions. Fig. 4B also shows how in the presence of serum, the C_{cell} decreases substantially for the more lipophilic chemicals (diphenhydramine, naphthalene, ibuprofen and triclosan). It also clearly illustrates the variation in predicted cell-associated concentrations between models, explained by both their variation in model compartments and QSARs used to predict partitioning. For all the simulated chemicals in conditions without serum, the model by Fischer et al. (2017) simulates the highest C_{cell} . The models from Fischer et al. (2017) and Armitage et al. (2014) do not consider plastic sorption, and therefore in the absence of serum there is not a compartment, besides cells, to which the chemical can distribute into. This would explain a higher C_{cell} of chemicals in these two models, in the absence of serum. However, C_{cell} for the several chemicals resulting from the Armitage *et al* model are substantially lower than Fischer's *et al* model which is probably related to both the cellular compartments added (*i.e.* just lipid or protein and lipid) and QSPRs used ($\log K_{OW}$ -based QSPRs or PP-LFERS based on other descriptors).

It should be noted that Fig. 4A and 4B are merely meant to illustrate the output and variations therein from *in vitro* distribution kinetics models. A comparison of model predictive performance is only possible with adequate experimental data, and is beyond the scope of this review.

3.4. Applicability domain (AD) of the models

Each one of the *in vitro* mass balance models was developed for a specific aim, usually for a specific group of chemical and/or a certain experimental setup, and is based on certain assumptions, which result in the model having an applicability domain (AD). While the accuracy of a model in predicting scenarios within its AD (interpolation) depends on the quality of the model, the accuracy in predicting outside its domain of applicability depends how much of the assumptions and chemical-property relations are still valid. Therefore, to clarify where certain models can be more accurate and identify possible gaps and improvements, here (Table 1) we briefly evaluate the chemical and experimental set up AD of each of the models in Fig. 3.

3.4.1. Chemical AD

The most discussed AD in literature is the chemical space. For these *in vitro* mass balance *in silico* models, the chemical AD, summarized in Table 1, results from either the specific test chemicals used (in case of models parameterized with values from literature or fitted to experimental data) or the training sets used in the QSPRs, but also on the structure of the model. Since the distribution processes depend on the physicochemical properties of chemicals, by structuring a model with certain compartments and processes as partitioning or kinetic, the authors of the models have some assumptions about the chemical distribution. For example, most of the models in Fig. 3 do not include headspace as a compartment because it is assumed the chemicals to be predicted are not volatile. The same is true for both abiotic and biotic degradation. Some of the generic models assume that chemical binding to serum protein can be explained solely by the partition coefficient to BSA, which is not necessarily true for chemicals with high binding to α -acid glycoprotein (Qin et al., 1994).

In the cases where QSPRs were used, there is an intention of predicting the parameters for different chemicals within the AD. There are several methods to measure QSAR/QSPRs applicability domain and similarity of chemicals. The small size of the training sets of the QSPRs and the fact that one model uses several QSPRs makes it difficult to present the chemical space in a more detailed way. Therefore, for ease of interpretation, each model's chemical space is summarized merely as neutral apolar, neutral (including both polar and apolar) and ionised chemicals, and volatile/non-volatile chemicals. A few of the models use

⁶ Experimental values for ibuprofen and diphenhydramine exist (Henneberger et al., 2020) and there are some differences between these experimental values that account ionization and the PP-LFER predictions, which do not: for ibuprofen experimental $\log K_{albumin/w}$ was 4.02 and $\log K_{liposomes/w}$ was 1.81 while PP/LFERS predictions were 3.55 and 3.41, respectively. For diphenhydramine experimental $K_{albumin/w}$ was 1.99 and $\log K_{liposomes/w}$ was 2.17 while PP-LFERS predictions were 3.61 and 3.27, respectively.

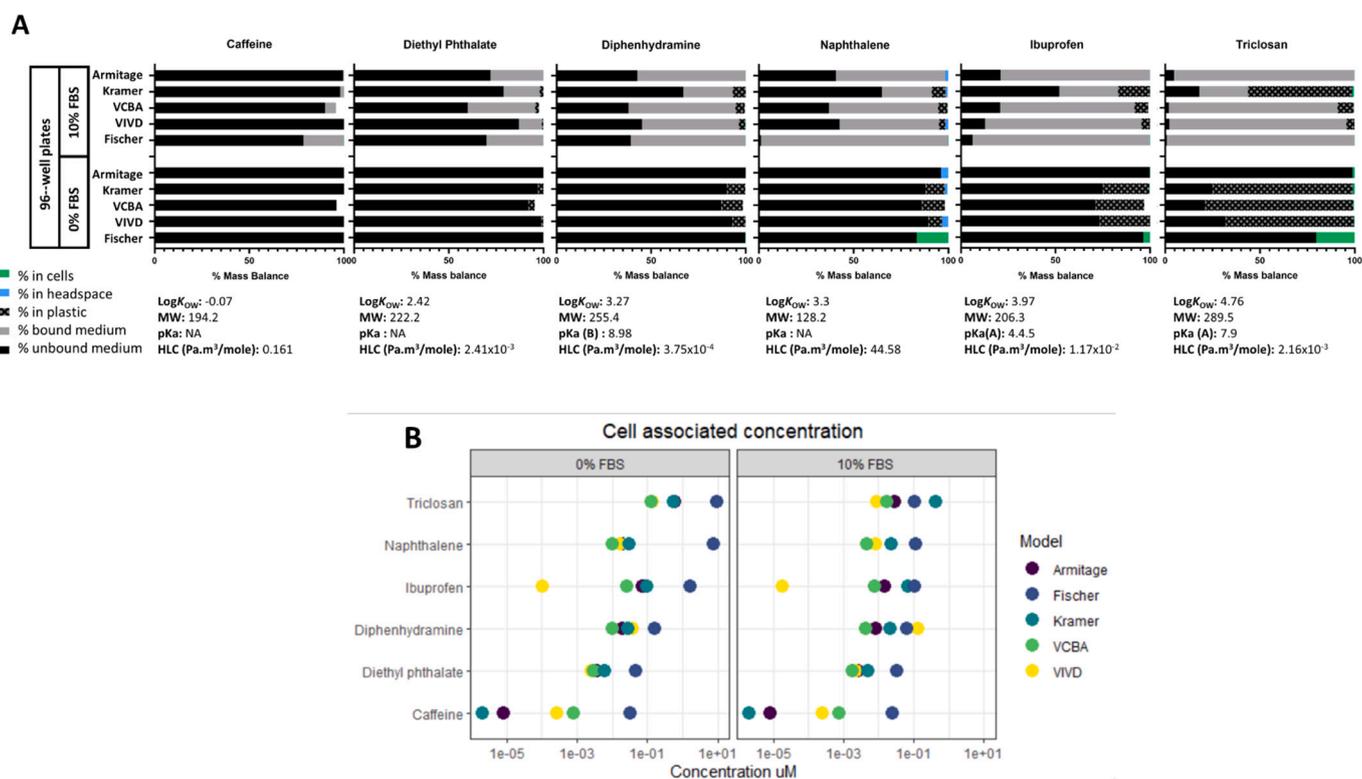


Fig. 4. Simulations of several chemicals distribution in an in vitro experiment set up with HepaRG monolayer in a 96-well plate, exposed for 24 hours to 1 μ M of nominal concentration, with and without FBS supplementation. Simulations were made with several generic in silico models reviewed here. A-Distribution of the chemical mass into the different in vitro system compartments depending on model, chemical and presence or absence of serum. B-Different chemical intracellular accumulation depending on model, chemical and presence or absence of serum. NOTE: the VCBA model accounts for possible abiotic degradation over time, but the mass of degraded compound is not included in the plots above, so apparent mass-balance can be less than 100%.

log K_{ow} as a proxy for partition to cell components (Armitage et al., 2014; Gülden and Seibert, 2003), which is not a full QSPR since is based on general knowledge, thus making it difficult to define the AD. Several generic models use the QSPR derived from PAHs for plastic partitioning which can limit the overall mass balance model chemical space to neutral apolar chemicals. Ionogenic chemicals are not expected to have extensive sorption to plastic, but this is only correctly modelled if the fraction of neutral/ionized molecules for a given chemical is calculated. The VCBA model does not make this distinction but some of the QSPRs are based also on polar and even ionised chemicals and thus, while here its chemical space is defined as neutral chemicals, we acknowledge it is not completely clear. Currently there is a QSPR for plastic sorption and absorption, which covers a larger range of neutral chemicals (Fischer et al., 2018b).

Ionised molecules will often behave differently from corresponding neutral molecules. For example, partitioning to plastic is generally negligible for ionised chemicals (Seidensticker et al., 2018). Local QSPRs for neutral and charged AD are most often more accurate (e.g. for albumin binding) (Henneberger et al., 2020), where binding mechanisms differ for neutral and charged species. In contrast, membrane partitioning (Escher and Sigg, 2004) is more similar between neutral and charged species than K_{ow} based predictions will show. A bulk solvent such as octanol is not a good surrogate for anisotropic lipid bilayer membranes, especially for partitioning of ionised chemicals. Of the generic models in Table 1 only Fischer et al. (2017) and Fisher et al. (2019) make distinction of neutral and ionised molecules.

3.4.2. Experimental set up AD

The *in silico* model's structure depends on the *in vitro* system it describes. Therefore, we aimed to extend the model's ADs according to the corresponding *in vitro* system. Since defining these ADs is currently

subjective and novel, some of the rationale behind the model structure are described below:

- The polystyrene walls are included as a compartment to simulate experiments in plastic microplates. However, since sorption to plastic is most significant in the absence of serum (Fischer et al., 2017; Proença et al., 2019), some models for simulating experiments in microplates do not include the compartment, therefore making their AD limited to experiments with serum (Armitage et al., 2014).
- The distinction between static and agitating medium only makes sense for models that include distribution processes as a rate. This is relevant for the slow diffusion of some very lipophilic chemicals. It should be noted that used agitating medium is only possible in well plates with lower well numbers (e.g. 24-well and lower wells).
- Defining the *in vitro* system AD with the type of cells depends on whether the model considers the cell culture characteristics (number cells, lipid and protein content), but also whether any of the distribution processes depends on the type of cells, such as intrinsic clearance (Fig. 3C, D, K, L and M). The same is true for the type of microtitre plate, since some models do not normalise the chemical sorbed to plastic per surface area of exposed polystyrene.
- It is more challenging to define the *in vitro* experiment AD for models based on QSPRs derived from different studies, since they aim to simulate diverse *in vitro* systems by taking in account volumes of medium, surface, etc. However, these models generally focus on *in vitro* systems used on high-throughput platforms (e.g. Fischer et al. (2017) with ToxCast⁷ and Tox21⁸ assays, Armitage et al. (2014)

⁷ <https://www.epa.gov/chemical-research/toxicity-forecasting>

⁸ <https://www.epa.gov/chemical-research/toxicology-testing-21st-century-tox21>

focused on ToxCast data, the VCBA model (Proença et al., 2019) has been applied with the ACuteTox project (Clemedson et al., 2007) and finally the VIVD model is currently being applied in the context of EU-TOXRISK project⁹.

4. Discussion

The ultimate goal of QIVIVE and extrapolation between *in vitro* assays is to predict the exposure (dose) for which no (adverse) effect is expected in a system different from the one in which the data has been generated in. The accuracy of these extrapolations is dependent on how the point-of-departure (POD) or no-observed adverse effect levels (NOAEL) are determined. The concentration available in the target site and thus the level of bioactivity observed in *in vitro* assays depend on the distribution of the chemical. *In silico* mass balance models that simulate these distribution processes can derive the *in vitro* C_{free} and C_{cell} which are considered equivalent to the $C_{free\ plasma}$ and C_{tissue} , respectively. Some of the models discussed in this review propose to use the predicted EC_{free} or EC_{cell} to make QIVIVE extrapolations (Gülden and Seibert, 2003; Riedl and Altenburger, 2007; Stadnicka-Michalak et al., 2014). However, since the EC_{50} values after a certain exposure duration (e.g. 24 hours) only represent a snapshot of the exposure these extrapolations may only be accurate for exposures with the same duration. Some authors have suggested alternatives to the use of just one concentration for QIVIVE, such as the geometric mean of the free concentration in the beginning and end of exposure, the area under the curve or the time-weighted average of concentrations (Groothuis et al., 2015). Extrapolating different exposures requires acknowledging the time dimension of toxicodynamics (Ashauer and Escher, 2010; Zhang et al., 2015): usually once the chemical molecule (BED) interacts with its molecular target it takes time to initiate each of the following key events, with cell death usually being a later event. In most key events there are recovery mechanisms, which prevent further progression of the AOP. Therefore, the observed toxicity depends not only on BED in time but also on these “rates of damage” and “recovery rates”. Some of the models in Fig. 3 incorporated these toxicodynamics rates; Stadnicka et al integrated the *in vitro* kinetics with toxicodynamic rates of toxicity by using the General Unified Threshold Model of Survival (Stadnicka-Michalak et al., 2015). The VCBA model (Zaldivar Comenges et al., 2016) has a similar strategy by including a general non-effect concentration and killing rate. Madureira et al. (2014) calculated the rate of DNA adduct formation by benzo[a]pyrene, but unfortunately did not integrate with distribution of the chemical in the *in vitro* system.

Modelling efforts also must put careful consideration on the dose metrics used (Groothuis et al., 2015). While most of the *in silico* models reviewed here derive C_{free} , C_{cell} and $C_{cell\ free}$, these dose metrics are not necessarily always proportional to each other or to BED. C_{cell} depends not only on C_{free} , but also the intracellular bioaccumulation of the chemical. Although theoretically C_{free} and $C_{cell\ free}$ should be the same, slow permeability, cellular metabolism, or other processes that disrupt steady state, can result in relatively lower $C_{cell\ free}$. The proportionality to BED is dependent on the toxic mode of action (Escher et al., 2011). If it is an intracellular receptor (e.g. Ahr) $C_{cell\ free}$ is closest proxy since the potency of the effect will depend on the ratio between the affinity to the target molecule but also affinity to other non-target cellular matrices. If toxicity occurs in a subcellular compartment into which chemicals highly partition due to their physicochemical properties, the concentration of chemical in that compartment can be a better representative of BED (e.g. concentration in membranes (Escher et al., 2020; Escher and Schwarzenbach, 1996), concentration in lysosomes (Fisher et al., 2019) and concentration mitochondria (Fisher et al., 2019; Worth et al., 2017)). It is more challenging to define a BED proxy for reactive chemicals since they do not have a specific target, but for the time being

C_{cell} perhaps be considered acceptable (Escher et al., 2011). Therefore, the dose metric most suitable as a Point of Departure for extrapolation to *in vivo* or to other *in vitro* assays, depends on the toxic mode of action and functional differences (transporters and metabolic activity but also recovery mechanism) between the system to be compared (*in vitro-in vivo* or *in vitro-in vitro*).

As discussed in this review, several *in silico* models of chemical distribution in *in vitro* assays are already available. Despite these models having slightly different applicability domains they mostly have similar structure (compartments and processes). Specially for the generic models more extensive experimental validation is long overdue. The experimental validation allows assessment of in which experiments the assumptions of the models are correct or not and therefore evaluate the model's structure. Recently, Henneberger et al. (2020) observed that for anionic chemicals sorption to albumin cannot be predicted with a simple partition since saturation of albumin binding sites for these chemicals occurs at concentrations relevant for toxicity testing. Hopefully, this review highlighted *in vitro* exposure scenarios that might be more challenging to simulate under the common assumptions of the models, such as short acute exposure and exposure of rapidly metabolized chemicals or slowly permeable chemicals. In addition, also some repeated exposure scenarios might require more complex models (e.g. simulating kinetic processes which requires more experimental data).

The chemical AD of models has been expanded in more recent studies (Fischer et al., 2017; Fisher et al., 2019) that focused on the differences between neutral and ionised chemicals. The main limitation remains accurate QSPRs to predict partitioning of heavily charged chemicals. Moreover, there is still a gap for inorganic and nanoparticles distribution in *in vitro* systems. Although the distribution of these chemicals in *in vitro* systems has been broadly discussed in literature (e.g. protein corona effect of cellular uptake) (Drasler et al., 2017), there are currently no models to predict the distribution of these chemicals. Models for inorganic and nanoparticles need to include different physicochemical properties but also may also be required to include different processes (McCarty et al., 2011; Teeguarden et al., 2007).

As seen in Table 1, the models have an AD mostly focused on high-throughput screening platforms: monolayers of cells in plastic microplates and static medium. Conversely, the development of new models such as spheroids, organs-on-a-chip (OOC) and microfluidics systems is among the strategies to improve *in vitro* cells phenotype and better reflect the *in vivo* microenvironment. These *in vitro* systems have very particular distribution processes that should be addressed for better translatability of the models to *in vivo* but also to compare functionality with other *in vitro* systems:

- OOC and microfluidics often use materials other than polystyrene and glass as a surface for cells to attach and a capillary system for the flow of culture medium (Shen et al., 2010; Sung et al., 2019; Wu et al., 2006). Chemicals can have even higher partition to these materials than to polystyrene (Auner et al., 2019; Schreiber et al., 2008).
- OOC and microfluidics tend to use a smaller volume of medium relative to the number of cells compared to traditional static monolayer incubations. In cases where the chemical is dosed in this static medium in a closed system, the ratio of medium volume: number of cells, and consequently amount of chemical/number of cells, is decreased. This means that the resulting C_{free} in these systems is possibly less than for the same $C_{nominal}$ in a standard microplate.
- OOC and microfluidics often do not have headspace into which the chemical can evaporate.
- While chemical distribution into cell suspension or monolayers can be described as a partition for most exposure scenarios, spheroids are characterized by several layers of cells that can delay the chemical uptake (Minchinton and Tannock, 2006). There are some models which focus only on the step-wise permeability and diffusion across the spheroids; however, they still ignore the intracellular

⁹ <https://www.eu-toxrisk.eu/>

accumulation explained by sorption into intracellular lipids and proteins (Leedale et al., 2020; Leite et al., 2012).

Of note, OOC and microfluidic systems tend to employ perfused media, although there is lack of standardisation on this perfusion (e.g. whether the media is an open or closed system). Integration of the *in vitro* distribution models described here with models simulating medium flow (Maass et al., 2018) can greatly improve the assessment of cell exposure to chemicals in these new *in vitro* systems.

In conclusion, simulations of the chemical distribution should always be performed in *in vitro* chemical hazard characterisation. These simulations are also important for the validation of new *in vitro* models since it often requires testing the toxicity of specific chemicals (e.g. testing acetaminophen and valproic acid in hepatic models) (Vinken and Hengstler, 2018). But to be feasible for a more general scientific public to use these models, the *in silico* models have to accommodate the set ups of new *in vitro* models, but also of more diverse group of chemicals. Often the mass balance simulations to derive the actual available concentrations after performing the assay will be enough, but for certain chemicals, simulations should be performed *a priori* of experiments so that a better dosing system can be applied (Fischer et al., 2019).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This review was commissioned by Cosmetics Europe as part of the IV-Kin project within its LRSS programme. We gratefully acknowledge the financial support given to the individual authors to draft the manuscript, including the Marie Skłodowska-Curie Action-Innovative Training Network-project in3, under grant no. 721975, the CEFIC LRI ECO36 project funded by CEFIC Long-Range Research Initiative (LRI), and the EU Horizon 2020 EUToxRisk project No 681002.

References

Abraham, M.H., Acree, W.E., 2016. Equations for water-triolein partition coefficients for neutral species; comparison with other water-solvent partitions, and environmental and toxicological processes. *Chemosphere* 154, 48–54. <https://doi.org/10.1016/j.chemosphere.2016.03.086>.

Abraham, M.H., Zhao, Y.H., 2004. Determination of solvation descriptors for ionic species: Hydrogen bond acidity and basicity. *J. Organomet. Chem.* 69, 4677–4685. <https://doi.org/10.1021/jo049766y>.

Andersson, T.B., Bredberg, E., Ericsson, H., Sjöberg, H., 2004. An evaluation of the *in vitro* metabolism data for predicting the clearance and drug-drug interaction potential of CYP2C9 substrates. *Drug Metab. Dispos.* 32, 715–721. <https://doi.org/10.1124/dmd.32.7.715>.

Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R.D., Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., Serrano, J.A., Tietge, J.E., Villeneuve, D.L., 2010. Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. *Environ. Toxicol. Chem.* 29, 730–741. <https://doi.org/10.1002/etc.34>.

Armitage, J.M., Wania, F., Arnot, J.A., 2014. Application of mass balance models and the chemical activity concept to facilitate the use of *in vitro* toxicity data for risk assessment. *Environ. Sci. Technol.* 48, 9770–9779. <https://doi.org/10.1021/es501955g>.

Ashauer, R., Escher, B.I., 2010. Advantages of toxicokinetic and toxicodynamic modelling in aquatic ecotoxicology and risk assessment. *J. Environ. Monit.* 12, 2056–2061. <https://doi.org/10.1039/c0em00234h>.

Auner, A.W., Tasneem, K.M., Markov, D.A., 2019. Lab on a chip chemical-PDMS binding kinetics and implications. *Lab Chip* 19, 864–874. <https://doi.org/10.1039/C8LC00796A>.

Austin, R.P., Barton, P., Mohamed, S., Riley, R.J., 2005. The binding of drugs to hepatocytes and its relationship to physicochemical properties. *Pharmacology* 33, 419–425. <https://doi.org/10.1124/dmd.104.002436.where>.

Austin, R.P., Barton, P., Riley, R.J., 2006. binding of drugs to hepatic microsomes: Comment and assessment of current prediction methodology with recommendation for improvement. *Drug Metab. Dispos.* 34, 727. <https://doi.org/10.1124/dmd.105.009142>.

Azizi, A., Shahhoseini, F., Modir-Rousta, A., Bottaro, C.S., 2019. High throughput direct analysis of water using solvothermal headspace desorption with porous thin films. *Anal. Chim. Acta* 1087, 51–61. <https://doi.org/10.1016/j.aca.2019.08.022>.

Balaz, S., 2009. Modeling kinetics of subcellular disposition of chemicals. *Chem. Rev.* 109, 1793–1899. <https://doi.org/10.1021/cr030440j>.

Barron, M.G., 1990. Bioconcentration, p. 24. <https://doi.org/10.1021/es00081a001>.

Bellwon, P., Culot, M., Wilmes, A., Schmidt, T., Zurich, M.G., Schultz, L., Schmal, O., Gramowski-Voss, A., Weiss, D.G., Jennings, P., Bal-Price, A., Testai, E., Dekant, W., 2015a. Cyclosporine A kinetics in brain cell cultures and its potential of crossing the blood-brain barrier. *Toxicol. in Vitro* 30, 166–175. <https://doi.org/10.1016/j.tiv.2015.01.003>.

Bellwon, P., Truiss, G.L., Bois, F.Y., Wilmes, A., Schmidt, T., Savary, C.C., Parmentier, C., Hewitt, P.G., Schmal, O., Josse, R., Richert, L., Guillouzo, A., Mueller, S.O., Jennings, P., Testai, E., Dekant, W., 2015b. Kinetics and dynamics of cyclosporine A in three hepatic cell culture systems. *Toxicol. in Vitro* 30, 62–78. <https://doi.org/10.1016/j.tiv.2015.07.016>.

Berens, A.R., Hopfenberg, H.B., 1982. Diffusion of organic vapors at low concentrations in glassy PVC, polystyrene, and PMMA. *J. Membr. Sci.* 10, 283–303. [https://doi.org/10.1016/S0376-7388\(00\)81415-5](https://doi.org/10.1016/S0376-7388(00)81415-5).

Bertelsen, S.L., Hoffman, A.D., Gallinat, C.A., Elonen, C.M., Nichols, J.W., 1998. Evaluation of log K(OW) and tissue lipid content as predictors of chemical partitioning to fish tissues. *Environ. Toxicol. Chem.* 17, 1447–1455. [https://doi.org/10.1897/1551-5028\(1998\)017<1447:EOLKOA>2.3.CO;2](https://doi.org/10.1897/1551-5028(1998)017<1447:EOLKOA>2.3.CO;2).

Birch, H., Kramer, N.I., Mayer, P., 2019. Time-resolved freely dissolved concentrations of semivolatiles and hydrophobic test chemicals in *in vitro* assays measuring high losses and crossover by headspace solid-phase microextraction. *Chem. Res. Toxicol.* 32, 1780–1790. <https://doi.org/10.1021/acs.chemrestox.9b00133>.

Bittermann, K., Goss, K.U., 2017. Predicting apparent passive permeability of Caco-2 and MDCK cell-monolayers: a mechanistic model. *PLoS One* 12, 1–20. <https://doi.org/10.1371/journal.pone.0190319>.

Bittermann, K., Spycher, S., Endo, S., Pohler, L., Humiar, U., Goss, K.U., Klamt, A., 2014. Prediction of phospholipid-water partition coefficients of ionic organic chemicals using the mechanistic model COSMOmic. *J. Phys. Chem. B* 118, 14833–14842. <https://doi.org/10.1021/jp509348a>.

Blaauboer, B.J., Boekelheide, K., Clewell, H.J., Daneshian, M., Dingemans, M.M.L., Goldberg, A.M., Heneweer, M., Jaworska, J., Kramer, N.I., Leist, M., Seibert, H., Testai, E., Vandebriel, R.J., Yager, J.D., Zurlo, J., 2012. The use of biomarkers of toxicity for integrating *in vitro* hazard estimates into risk assessment for humans. *ALTEX* 29, 411–425. <https://doi.org/10.14573/altex.2012.4.411>.

Bolton, G.R., Deen, W.M., Daniels, B.S., 1998. Assessment of the charge selectivity of glomerular basement membrane using Ficoll sulfate. *Am. J. Phys.* 274, F889–F896.

Bourez, S., Van den Daelen, C., Le Lay, S., Poupaert, J., Larondelle, Y., Thomé, J.P., Schneider, Y.J., Dugail, I., Debier, C., 2013. The dynamics of accumulation of PCBs in cultured adipocytes vary with the cell lipid content and the lipophilicity of the congener. *Toxicol. Lett.* 216, 40–46. <https://doi.org/10.1016/j.toxlet.2012.09.027>.

Bowman, C.M., Benet, L.Z., 2018. An examination of protein binding and protein-facilitated uptake relating to *in vitro-in vivo* extrapolation. *Eur. J. Pharm. Sci.* 123, 502–514. <https://doi.org/10.1016/j.ejps.2018.08.008>.

Boyaçlı, E., Bojko, B., Reyes-Garcés, N., Poole, J.J., Gómez-Ríos, G.A., Teixeira, A., Nicol, B., Pawliszyn, J., 2018. High-throughput analysis using non-depletive SPME: Challenges and applications to the determination of free and total concentrations in small sample volumes. *Sci. Rep.* 8, 1–10. <https://doi.org/10.1038/s41598-018-19313-1>.

Broeders, J.J.W., Parmentier, C., Truiss, G.L., Jossé, R., Alexandre, E., Savary, C.C., Hewitt, P.G., Mueller, S.O., Guillouzo, A., Richert, L., van Eijkeren, J.C.H., Hermens, J.L.M., Blaauboer, B.J., 2015. Biokinetics of chlorpromazine in primary rat and human hepatocytes and human HepaRG cells after repeated exposure. *Toxicol. in Vitro* 30, 52–61. <https://doi.org/10.1016/j.tiv.2014.08.012>.

Chiou, C.T., 1985. Partition Coefficients of Organic Compounds in Lipid-Water Systems and Correlations with Fish Bioconcentration Factors. *Environ. Sci. Technol.* 19, 57–62. <https://doi.org/10.1021/es00131a005>.

Clemmedson, Cecilia, Ada, Kolman, Anna, Forsby, 2007. The Integrated Acute Systemic Toxicity Project (ACuteTox) for the Optimisation and Validation of Alternative *In Vitro* Tests. *ATLA* 35, 33–38, 2005.

Clerbaux, L.A., Coecke, S., Lumen, A., Kliment, T., Worth, A.P., Paini, A., 2018. Capturing the applicability of *in vitro-in silico* membrane transporter data in chemical risk assessment and biomedical research. *Sci. Total Environ.* 645, 97–108. <https://doi.org/10.1016/j.scitotenv.2018.07.122>.

Coecke, S., Pelkonen, O., Leite, S.B., Bernauer, U., Bessems, J.G.M., Bois, F.Y., Gundert-Remy, U., Loizou, G., Testai, E., Zaldívar, J.M., 2013. Toxicokinetics as a key to the integrated toxicity risk assessment based primarily on non-animal approaches. *Toxicol. in Vitro* 27, 1570–1577. <https://doi.org/10.1016/j.tiv.2012.06.012>.

Colmenarejo, G., 2003. *In silico* prediction of drug-binding strengths to human serum albumin. *Med. Res. Rev.* 23, 275–301. <https://doi.org/10.1002/med.10039>.

Comenges, J.M.Z., 2016. Theoretical and mathematical foundation of the Virtual Cell Based Assay – A review. *Toxicology in Vitro*. <https://doi.org/10.1016/j.tiv.2016.07.013>.

Curtis, A.S.G., Forrester, J.V., McInnes, C., Lawrie, F., 1983. Adhesion of cells to polystyrene surfaces. *J. Cell Biol.* 97, 1500–1506. <https://doi.org/10.1083/jcb.97.5.1500>.

de Bruyn, A.M.H., Gobas, F.A.P.C., 2007. The sorptive capacity of animal protein. *Environ. Toxicol. Chem.* 26, 1803. <https://doi.org/10.1897/07-016R.1>.

De Bruyn, T., Chatterjee, S., Fattah, S., Keemink, J., Nicolai, J., Augustijns, P., Annaert, P., 2013. Sandwich-cultured hepatocytes: utility for *in vitro* exploration of hepatobiliary drug disposition and drug-induced hepatotoxicity. *Expert Opin. Drug Metab. Toxicol.* 9, 589–616. <https://doi.org/10.1517/17425255.2013.773973>.

- Derendorf, H., Schmidt, S., 2019. Rowland and Tozer's Clinical Pharmacokinetics and Pharmacodynamics: Concepts and Applications, 5th edition.
- Dole, P., Feigenbaum, A.E., La Cruz, C.D., Pastorelli, S., Paseiro, P., Hankemeier, T., Voulzatis, Y., Aucejo, S., Saillard, P., Pappaspyrides, C., Dole, P., Feigenbaum, A.E., La Cruz, C.D., Paseiro, P., Hankemeier, T., Voulzatis, Y., Aucejo, S., 2011. Typical diffusion behaviour in packaging polymers – application to functional barriers. *Food Addit. Contam.* 23, 202–211. <https://doi.org/10.1080/02652030500373661>.
- Dragojević, S., Šunjić, V., Bencetić-Mihaljević, V., Ralić, J., Mesić, M., Elenkov, I.J., Sucić, A.F., Klonekay, A.C., Lerman, L., Ilijaš, M., Gabelica-Marković, V., Malnar, I., 2011. Determination of aqueous stability and degradation products of series of coumarin dimers. *J. Pharm. Biomed. Anal.* 54, 37–47. <https://doi.org/10.1016/j.jpba.2010.08.002>.
- Drasler, B., Sayre, P., Steinhäuser, K.G., Petri-Fink, A., Rothen-Rutishauser, B., 2017. In vitro approaches to assess the hazard of nanomaterials. *NanoImpact* 8, 99–116. <https://doi.org/10.1016/j.impact.2017.08.002>.
- Ebert, A., Bittermann, K., Huniar, U., Goss, K., Klamt, A., Accepted, J., 2020. COSMO perm: Mechanistic Prediction of Passive Membrane Permeability for Neutral Compounds and Ions, and its pH Dependence. <https://doi.org/10.1021/acs.jpcc.9b11728>.
- Edward, J.T., 1970. Molecular volumes and the Stokes-Einstein equation. *J. Chem. Educ.* 47, 261. <https://doi.org/10.1021/ed047p261>.
- Endo, S., Goss, K.-U., 2011. Serum albumin binding of structurally diverse neutral organic compounds: data and models. *Chem. Res. Toxicol.* 24, 2293–2301. <https://doi.org/10.1021/tx200431b>.
- Endo, S., Goss, K.U., 2014. Applications of polyparameter linear free energy relationships in environmental chemistry. *Environ. Sci. Technol.* 48, 12477–12491. <https://doi.org/10.1021/es503369t>.
- Endo, S., Escher, B.I., Goss, K.U., 2011. Capacities of membrane lipids to accumulate neutral organic chemicals. *Environ. Sci. Technol.* 45, 5912–5921. <https://doi.org/10.1021/es200855w>.
- Endo, S., Bauerfeind, J., Goss, K.U., 2012. Partitioning of neutral organic compounds to structural proteins. *Environ. Sci. Technol.* 46, 12697–12703. <https://doi.org/10.1021/es303379y>.
- Endo, S., Brown, T.N., Goss, K.U., 2013. General model for estimating partition coefficients to organisms and their tissues using the biological compositions and polyparameter linear free energy relationships. *Environ. Sci. Technol.* 47, 6630–6639. <https://doi.org/10.1021/es401772m>.
- Escher, B.I., Schwarzenbach, R.P., 1996. Partitioning of substituted phenols in liposome - water, biomembrane - water, and octanol - water systems. *Environ. Sci. Technol.* 30, 260–270. <https://doi.org/10.1021/es9503084>.
- Escher, B.I., Sigg, L., 2004. Chemical speciation of organics and of metals at biological interfaces. In: Sons, J.W. (Ed.), *Physicochemical Kinetics and Transport at Biointerfaces*.
- Escher, B., Hermens, J., Schwarzenbach, R., 2005. International workshop: Internal exposure - Linking bioavailability to effects. *Environ. Sci. Pollut. Res.* 12, 57–60. <https://doi.org/10.1065/espr2005.01.004>.
- Escher, B.I., Ashauer, R., Dyer, S., Hermens, J.L.M., Lee, J.H., Leslie, H.A., Mayer, P., Meador, J.P., Warnekk, M.S.J., 2011. Crucial role of mechanisms and modes of toxic action for understanding tissue residue toxicity and internal effect concentrations of organic chemicals. *Integr. Environ. Assess. Manag.* <https://doi.org/10.1002/ieam.100>.
- Escher, B.I., Glauch, L., Mayer, P., Schlichting, R., 2019. Baseline toxicity and volatility cutoff in reporter gene assays used for high-throughput screening. *Chem. Res. Toxicol.* <https://doi.org/10.1021/acs.chemrestox.9b00182>.
- Escher, B.I., Henneberger, L., König, M., Schlichting, R., Fischer, F.C., 2020. Cytotoxicity burst? Differentiating specific from nonspecific effects in tox21 in vitro reporter gene assays. *Environ. Health Perspect.* 128, 1–10. <https://doi.org/10.1289/EHP6664>.
- Fahy, E., Subramaniam, S., Brown, H.A., Glass, C.K., Merrill, A.H., Murphy, R.C., Raetz, C.R.H., Russell, D.W., Seyama, Y., Shaw, W., Shimizu, T., Spener, F., van Meer, G., VanNieuwenhze, M.S., White, S.H., Witztum, J.L., Dennis, E.A., 2005. A comprehensive classification system for lipids. *Eur. J. Lipid Sci. Technol.* 107, 337–364. <https://doi.org/10.1002/ejlt.2004005001>.
- Ferrell, N., Groszek, J., Li, L., Smith, R., Butler, R.S., Zorman, C.A., Roy, S., Fissell, W.H., 2011. Basal lamina secreted by MDCK cells has size- and charge-selective properties. *Am. J. Physiol. Ren. Physiol.* 300, F86–F90. <https://doi.org/10.1152/ajprenal.00484.2010>.
- Fielding, C.J., Fielding, P.E., 2008. Dynamics of lipoprotein transport in the circulatory system. *Biochem. Lipids Lipoproteins Membr.* 533–553. <https://doi.org/10.1016/B978-044453219-0.50021-0>.
- Fischer, F.C., Henneberger, L., König, M., Bittermann, K., Linden, L., Goss, K.U., Escher, B.I., 2017. Modeling exposure in the Tox21 in vitro bioassays. *Chem. Res. Toxicol.* 30, 1197–1208. <https://doi.org/10.1021/acs.chemrestox.7b00023>.
- Fischer, A.C., Droge, S.T.J., Henneberger, L., König, M., Schlichting, R., Scholz, S., Escher, B.I., 2018a. Cellular uptake kinetics of neutral and charged chemicals in in vitro assays measured by fluorescence microscopy. *Chem. Res. Toxicol.* <https://doi.org/10.1021/acs.chemrestox.8b00019>.
- Fischer, C., Cirpka O.A., Goss, K.-U., Henneberger, L., Escher, B.I., 2018b. Application of experimental polystyrene partition constants and diffusion coefficients to predict the sorption of neutral organic chemicals to multiwell plates in in vivo and in vitro bioassays. *Environ. Sci. Technol.* 52, 13511–13522. <https://doi.org/10.1021/acs.est.8b04246>.
- Fischer, F.C., Henneberger, L., Schlichting, R., Escher, B.I., 2019. How to improve the dosing of chemicals in high-throughput in vitro mammalian cell assays. *Chem. Res. Toxicol.* 32, 1462–1468. <https://doi.org/10.1021/acs.chemrestox.9b00167>.
- Fisher, C., Siméon, S., Jamei, M., Gardner, I., Bois, Y.F., 2019. Toxicology in Vitro VIVD: Virtual in vitro distribution model for the mechanistic prediction of intracellular concentrations of chemicals in in vitro toxicity assays. *Toxicol. in Vitro* 58, 42–50. <https://doi.org/10.1016/j.tiv.2018.12.017>.
- Franklin, N.M., Stauber, J.L., Apte, S.C., Lim, R.P., 2002. Effect of initial cell density on the bioavailability and toxicity of copper in microalgal bioassays. *Environ. Toxicol. Chem.* 21, 742–751. [https://doi.org/10.1897/1551-5028\(2002\)021<0742:EOICDO>2.0.CO;2](https://doi.org/10.1897/1551-5028(2002)021<0742:EOICDO>2.0.CO;2).
- Fukuchi, Y., Tshimoto, K., Mori, T., Kakimoto, K., Tobe, Y., Sawada, T., Asaumi, R., Iwata, T., Hashimoto, Y., Nunoya, K. Ichi, Imawaka, H., Miyachi, S., Sugiyam, Y., 2017. Analysis of nonlinear pharmacokinetics of a highly albumin-bound compound: contribution of albumin-mediated hepatic uptake mechanism. *J. Pharm. Sci.* 106, 2704–2714. <https://doi.org/10.1016/j.xphs.2017.04.052>.
- Gavara, R., Hernandez, R., Giann, J., 1996. Methods to determine partition coefficient of organic compounds in water/polystyrene systems. *J. Food Sci.* 61, 947–952. <https://doi.org/10.1111/j.1365-2621.1996.tb10908.x>.
- Geisler, A., Endo, S., Goss, K.U., 2012. Partitioning of organic chemicals to storage lipids: elucidating the dependence on fatty acid composition and temperature. *Environ. Sci. Technol.* 46, 9519–9524. <https://doi.org/10.1021/es301921w>.
- Geisler, A., Oemisch, L., Endo, S., Goss, K.U., 2015. Predicting storage-lipid water partitioning of organic solutes from molecular structure. *Environ. Sci. Technol.* 49, 5538–5545. <https://doi.org/10.1021/es506336m>.
- George, S.C., Thomas, S., 2001. Transport phenomena through polymeric systems. *Prog. Polym. Sci.* 26, 985–1017. [https://doi.org/10.1016/S0079-6700\(00\)00036-8](https://doi.org/10.1016/S0079-6700(00)00036-8).
- Ghuman, J., Zunsain, P.A., Petipras, I., Bhattacharya, A.A., Otagiri, M., Curry, S., 2005. Structural basis of the drug-binding specificity of human serum albumin. *J. Mol. Biol.* 353, 38–52. <https://doi.org/10.1016/j.jmb.2005.07.075>.
- Gilbert, T.W., Sellaro, T.L., Badylak, S.F., 2006. Decellularization of tissues and organs. *Biomaterials* 27, 3675–3683. <https://doi.org/10.1016/j.biomaterials.2006.02.014>.
- Groothuis, F.A., Heringa, M.B., Nicol, B., Hermens, J.L.M., Blaauwboer, B.J., Kramer, N.I., 2015. Dose metric considerations in in vitro assays to improve quantitative in vitro-in vivo dose extrapolations. *Toxicology* 332, 30–40. <https://doi.org/10.1016/j.tox.2013.08.012>.
- Gülden, M., Seibert, H., 2003. In vitro-in vivo extrapolation: estimation of human serum concentrations of chemicals equivalent to cytotoxic concentrations in vitro. *Toxicology* 189, 211–222. [https://doi.org/10.1016/S0300-483X\(03\)00146-X](https://doi.org/10.1016/S0300-483X(03)00146-X).
- Gülden, M., Mörchel, S., Seibert, H., 2001. Factors influencing nominal effective concentrations of chemical compounds in vitro: cell concentration. *Toxicol. in Vitro* 15, 233–243.
- Gunness, P., Mueller, D., Shevchenko, V., Heinze, E., Ingelman-Sundberg, M., Noor, F., 2013. 3D organotypic cultures of human HepaRG cells: a tool for in vitro toxicity studies. *Toxicol. Sci.* 133, 67–78. <https://doi.org/10.1093/toxsci/ktf021>.
- Hall, L.M., Hall, L.H., Kier, L.B., 2003. Modeling drug albumin binding affinity with e-state topological structure representation. *J. Chem. Inf. Comput. Sci.* 43, 2120–2128. <https://doi.org/10.1021/ci030019w>.
- Halliwell, B., 2003. Oxidative stress in cell culture: an under-appreciated problem? *FEBS Lett.* 540, 3–6. [https://doi.org/10.1016/S0014-5793\(03\)00235-7](https://doi.org/10.1016/S0014-5793(03)00235-7).
- Hawker, D.W., Connell, D.W., 1985. Relationships between partition coefficient, uptake rate constant, clearance rate constant and time to equilibrium for bioaccumulation. *Chemosphere* 14, 1205–1219.
- Heng, B.C., Zhao, X., Xiong, S., Ng, K.W., Boey, F.Y.C., Loo, J.S.C., 2011. Cytotoxicity of zinc oxide (ZnO) nanoparticles is influenced by cell density and culture format. *Arch. Toxicol.* 85, 695–704. <https://doi.org/10.1007/s00204-010-0608-7>.
- Henneberger, L., Goss, K.U., Endo, S., 2016a. Equilibrium sorption of structurally diverse organic ions to bovine serum albumin. *Environ. Sci. Technol.* 50, 5119–5126. <https://doi.org/10.1021/acs.est.5b06176>.
- Henneberger, L., Goss, K.U., Endo, S., 2016b. Partitioning of organic ions to muscle protein: experimental data, modeling, and implications for in vivo distribution of organic ions. *Environ. Sci. Technol.* 50, 7029–7036. <https://doi.org/10.1021/acs.est.6b01417>.
- Henneberger, L., Mühlenbrink, M., Fischer, F.C., Escher, B.I., 2019. C18-coated solid-phase microextraction fibers for the quantification of partitioning of organic acids to proteins, lipids, and cells. *Chem. Res. Toxicol.* 32, 168–178. <https://doi.org/10.1021/acs.chemrestox.8b00249>.
- Henneberger, L., Mu, M., Heinrich, D.J., Teixeira, A., Nicol, B., Escher, B.I., 2020. Experimental validation of mass balance models for in vitro cell-based bioassays. *Environ. Sci. Technol.* 54 <https://doi.org/10.1021/acs.est.9b06144>.
- Heringa, M.B., Schreurs, R.H.M.M., Busser, F., Van Der Saag, P.T., Van Der Burg, B., Hermens, J.L.M., 2004. Toward more useful in vitro toxicity data with measured free concentrations. *Environ. Sci. Technol.* 38, 6263–6270. <https://doi.org/10.1021/es049285w>.
- Hestermann, E.V., Stegeman, J.J., Hahn, M.E., 2000. Cell culture serum alters the uptake and relative potencies of halogenated aromatic hydrocarbons in PLHC-1 cells. *Mar. Environ. Res.* 50, 545–546. [https://doi.org/10.1016/S0141-1136\(00\)00233-6](https://doi.org/10.1016/S0141-1136(00)00233-6).
- Hjelmberg, P.S., Andreassen, T.K., Bonfeld-Jørgensen, E.C., 2008. Cellular uptake of lipoproteins and persistent organic compounds—An update and new data. *Environ. Res.* 108, 192–198. <https://doi.org/10.1016/j.envres.2008.07.019>.
- Honda, G.S., Pearce, R.G., Pham, L.L., Setzer, R.W., Wetmore, B.A., Sipes, N.S., Gilbert, J., Franz, B., Thomas, R.S., Wambaugh, J.F., 2019. Using the concordance of in vitro and in vivo data to evaluate extrapolation assumptions. *PLoS One.* <https://doi.org/10.1371/journal.pone.0217564>.
- Horobin, R.W., Trapp, S., Weissig, V., 2007. Mitochondriotropics: A review of their mode of action, and their applications for drug and DNA delivery to mammalian mitochondria. *J. Control. Release* 121, 125–136. <https://doi.org/10.1016/j.jconrel.2007.05.040>.
- Jabusch, T.W., Swackhamer, D.L., 2005. Partitioning of polychlorinated biphenyls in octanol/water, triolein/water, and membrane/water systems. *Chemosphere* 60, 1270–1278. <https://doi.org/10.1016/j.chemosphere.2005.01.076>.

- Jonker, M.T.O., Van Der Heijden, S.A., 2007. Bioconcentration factor hydrophobicity cutoff: an artificial phenomenon reconstructed. *Environ. Sci. Technol.* 41, 7363–7369. <https://doi.org/10.1021/es0709977>.
- Kilford, P.J., Gertz, M., Houston, J.B., Galetin, A., 2008. Hepatocellular binding of drugs: correction for unbound fraction in hepatocyte incubations using microsomal binding or drug lipophilicity data. *Drug Metab. Dispos.* 36, 1194–1197. <https://doi.org/10.1124/dmd.108.020834>.
- Kim, D., Caruthers, J.M., Peppas, N.A., 1993. Penetrant transport in cross-linked polystyrene. *Macromolecules* 26, 1841–1847. <https://doi.org/10.1021/ma00060a008>.
- Kosa, T., Maruyama, T., Otagiri, M., 1997. Species differences of serum albumins: I. Drug binding sites. *Pharm. Res.* <https://doi.org/10.1023/A:1012138604016>.
- Kramer, 2010. *Measuring, Modeling, and Increasing the Free Concentration of Test Chemicals in Cell Assays*. Utrecht University.
- Kramer, N.I., Van Eijkeren, J.C.H., Hermens, J.L.M., 2007. Influence of albumin on sorption kinetics in solid-phase microextraction: consequences for chemical analyses and uptake processes. *Anal. Chem.* 79, 6941–6948. <https://doi.org/10.1021/ac070574n>.
- Kramer, N.I., Hermens, J.L.M., Schirmer, K., 2009. The influence of modes of action and physicochemical properties of chemicals on the correlation between in vitro and acute fish toxicity data. *Toxicol. in Vitro* 23, 1372–1379. <https://doi.org/10.1016/j.tiv.2009.07.029>.
- Kramer, N.I., Di Consiglio, E., Blaauboer, B.J., Testai, E., 2015. Biokinetics in repeated-dosing in vitro drug toxicity studies. *Toxicol. in Vitro* 30, 217–224. <https://doi.org/10.1016/j.tiv.2015.09.005>.
- Krause, S., Goss, K.U., 2018. The impact of desorption kinetics from albumin on hepatic extraction efficiency and hepatic clearance: a model study. *Arch. Toxicol.* 92, 2175–2182. <https://doi.org/10.1007/s00204-018-2224-x>.
- Krewski, D., Andersen, M.E., Tyshenko, M.G., Krishnan, K., Hartung, T., Boekelheide, K., Wambaugh, J.F., Jones, D., Whelan, M., Thomas, R., Yauk, C., Barton-Maclaren, T., Cote, I., 2020. Toxicity testing in the 21st century: progress in the past decade and future perspectives. *Arch. Toxicol. Springer Berlin Heidelberg*. <https://doi.org/10.1007/s00204-019-02613-4>.
- Kwon, J.-H., Lee, H.-J., Escher, B.I., 2020. Bioavailability of hydrophobic organic chemicals on an in vitro metabolic transformation using rat liver S9 fraction. *Toxicol. in Vitro* 66. <https://doi.org/10.1016/j.tiv.2020.104835>.
- Lagares, L.M., Minovski, N., Alfonso, A.Y.C., Benfenati, E., Wellens, S., Culot, M., Gosselet, F., Novič, M., 2020. Homology modeling of the human p-glycoprotein (Abcb1) and insights into ligand binding through molecular docking studies. *Int. J. Mol. Sci.* 21, 1–37. <https://doi.org/10.3390/ijms21114058>.
- LeCluyse, E., Bullock, P., Madan, A., 1999. Influence of extracellular matrix overlay and medium formulation on the induction of cytochrome P-450 2B enzymes in primary cultures of rat hepatocytes. *Drug Metab.* 27, 909–915.
- Leedale, J.A., Kyffin, J.A., Harding, A.L., Colley, H.E., Murdoch, C., Sharma, P., Williams, D.P., Webb, S.D., Bearon, R.N., 2020. Multiscale modelling of drug transport and metabolism in liver spheroids. *Interface Focus* 10. <https://doi.org/10.1098/rsfs.2019.0041>.
- Leist, M., Hartung, T., Nicotera, P., 2008. The dawning of a new age of toxicology Doerenkamp-Zbinden chair for alternative in vitro methods. *ALTEX* 103–114.
- Leite, S.B., Wilk-Zasadna, I., Zaldivar, J.M., Airola, E., Reis-Fernandes, M.A., Mennecozi, M., Guguen-Guillouzo, C., Chesne, C., Guillou, C., Alves, P.M., Coecke, S., 2012. Three-dimensional HepaRG model as an attractive tool for toxicity testing. *Toxicol. Sci.* 130, 106–116. <https://doi.org/10.1093/toxsci/kfs232>.
- Lerman, M.J., Lomborg, J., Muramoto, S., Gillen, G., Fisher, J.P., 2018. The evolution of polystyrene as a cell culture material. *Tissue Eng. Part B* 24, 359–372. <https://doi.org/10.1089/ten.teb.2018.0056>.
- Li, Nianzhen, Schwartz, M., Ionescu-Zanetti, C., 2009. PDMS compound adsorption in context. *J. Biomol. Screen.* 14, 194–202. <https://doi.org/10.1177/1087057108327326>.
- Linden, L., Goss, K.U., Endo, S., 2017. 3D-QSAR predictions for bovine serum albumin-water partition coefficients of organic anions using quantum mechanically based descriptors. *Environ Sci Process Impacts* 19, 261–269. <https://doi.org/10.1039/c6em00555a>.
- Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J., 1997. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* 23, 3–25.
- Liu, F.F., Peng, C., Escher, B.I., Fantino, E., Giles, C., Were, S., Duffy, L., Ng, J.C., 2013. Hanging drop: an in vitro air toxic exposure model using human lung cells in 2D and 3D structures. *J. Hazard. Mater.* 261, 701–710. <https://doi.org/10.1016/j.jhazmat.2013.01.027>.
- Liu, Y., Mi, Y., Mueller, T., Kreibich, S., Williams, E.G., Van Drogen, A., Borel, C., Frank, M., Germain, P.L., Bludau, I., Mehnert, M., Seifert, M., Emmenlauer, M., Sorg, I., Bezrukov, F., Bena, F.S., Zhou, H., Dehio, C., Testa, G., Saez-Rodriguez, J., Antonarakis, S.E., Hardt, W.D., Aebbersold, R., 2019. Multi-omic measurements of heterogeneity in HeLa cells across laboratories. *Nat. Biotechnol.* 37, 314–322. <https://doi.org/10.1038/s41587-019-0037-y>.
- Louisie, J., de Jong, E., van de Sandt, J.J.M., Blaauboer, B.J., Woutersen, R.A., Piersma, A.H., Rietjens, I.M.C.M., Verwei, M., 2010. The use of in vitro toxicity data and physiologically based kinetic modeling to predict dose-response curves for in vivo developmental toxicity of glycol ethers in rat and man. *Toxicol. Sci.* 118, 470–484. <https://doi.org/10.1093/toxsci/kfq270>.
- Lübbertstedt, M., Müller-Vieira, U., Mayer, M., Biemel, K.M., Knöspel, F., Knobeloch, D., Nüssler, A.K., Gerlach, J.C., Zeilinger, K., 2011. HepaRG human hepatic cell line utility as a surrogate for primary human hepatocytes in drug metabolism assessment in vitro. *J. Pharmacol. Toxicol. Methods* 63, 59–68. <https://doi.org/10.1016/j.vascn.2010.04.013>.
- Maass, C., Dallas, M., Labarge, M.E., Shockley, M., Valdez, J., Geishecker, E., Stokes, C.L., Griffith, L.G., Cirit, M., 2018. Establishing quasi-steady state operations of microphysiological systems (MPS) using tissue-specific metabolic dependencies. *Sci. Rep.* 8, 1–13. <https://doi.org/10.1038/s41598-018-25971-y>.
- Madureira, D.J., Weiss, F.T., Van Midwoud, P., Helbling, D.E., Sturla, S.J., Schirmer, K., 2014. Systems toxicology approach to understand the kinetics of benzo(a)pyrene uptake, biotransformation, and dna adduct formation in a liver cell model. *Chem. Res. Toxicol.* 27, 443–453. <https://doi.org/10.1021/tx400446q>.
- Majorek, K.A., Porebski, P.J., Dayal, A., Zimmerman, M.D., Jablonska, K., Stewart, A.J., Chruszcz, M., Minor, W., 2012. Structural and immunologic characterization of bovine, horse and rabbit serum albumins. *Mol. Immunol.* 52, 174–182.
- Margolis, J.M., Obach, R.S., 2003. Impact of nonspecific binding to microsomes and phospholipid on the inhibition of cytochrome P4502D6: Implications for relating in vitro inhibition data to in vivo drug interactions. *Drug Metab. Dispos.* 31, 606–611. <https://doi.org/10.1124/dmd.31.5.606>.
- McCarty, L.S., Landrum, P.F., Luoma, S.N., Meador, J.P., Merten, A.A., Shephard, B.K., van Wezel, A.P., 2011. Advancing environmental toxicology through chemical dosimetry: external exposures versus tissue residues. *Integr. Environ. Assess. Manag.* 7, 7–27. <https://doi.org/10.1002/ieam.98>.
- McDermott, C., Allshire, A., van Pelt, F.N.A.M., Heffron, J.J.A., 2007. Validation of a method for acute and subchronic exposure of cells in vitro to volatile organic solvents. *Toxicol. in Vitro* 21, 116–124. <https://doi.org/10.1016/j.tiv.2006.08.002>.
- Minchinton, A.L., Tannock, I.F., 2006. Drug penetration in solid tumours. *Nat. Rev. Cancer* 6, 583–592. <https://doi.org/10.1038/nrc1893>.
- Mochalski, P., Sponring, A., King, J., Unterkofler, K., Troppmair, J., Amann, A., 2013. Release and uptake of volatile organic compounds by human hepatocellular carcinoma cells (HepG2) in vitro. *Cancer Cell Int.* 13. <https://doi-org.proxy.library.uu.nl/10.1186/1475-2867-13-72>.
- Montanari, F., Ecker, G.F., 2015. Prediction of drug-ABC-transporter interaction - Recent advances and future challenges. *Adv. Drug Deliv. Rev.* <https://doi.org/10.1016/j.addr.2015.03.001>.
- Mundy, W.R., Freudenrich, T.M., Crofton, K.M., DeVito, M.K., 2004. Accumulation of PBDE-47 in primary cultures of rat neocortical cells. *Toxicol. Sci.* 82, 164–169. <https://doi.org/10.1093/toxsci/kfh239>.
- Nolte, T.M., Ragas, A.M.J., 2017. A review of quantitative structure-property relationships for the fate of ionizable organic chemicals in water matrices and identification of knowledge gaps. *Environ Sci Process Impacts* 19, 221–246. <https://doi.org/10.1039/c7em00034k>.
- NRC, 2007. *Toxicity Testing in the 21st Century: A Vision and a Strategy*. Natl. Acad. Test.
- O'Byrne, P.M., Williams, R., Walsh, J.J., Gilmer, J.F., 2010. The aqueous stability of bupropion. *J. Pharm. Biomed. Anal.* 53, 376–381. <https://doi.org/10.1016/j.jpba.2010.04.024>.
- Obach, R.S., 1999. Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: an examination of in vitro half-life approach and nonspecific binding to microsomes. *Drug Metab. Dispos.* 27, 1350–1359.
- OECD, 2019. *Guidance Document on Aqueous-phase Aquatic Toxicity Testing of Difficult Test Chemicals* 23, pp. 1–81.
- Paini, A., Mennecozi, M., Horvat, T., Gerloff, K., Palosaari, T., Sala Benito, J.V., Worth, A., 2017. Practical use of the virtual cell based assay: simulation of repeated exposure experiments in liver cell lines. *Toxicol. in Vitro* 45, 233–240. <https://doi.org/10.1016/j.tiv.2016.10.007>.
- Paustenbach, Dennis J., 2000. The practice of exposure assessment: a state-of-the-art review. *Journal of Toxicology and Environmental Health Part B: Critical Reviews* 33, 179–291. <https://doi.org/10.1080/10937400050045264>.
- Phaniendra, A., Jestadi, D.B., Periyasamy, L., 2015. Free radicals: properties, sources, targets, and their implication in various diseases. *Indian J. Clin. Biochem.* 30, 11–26. <https://doi.org/10.1007/s12291-014-0446-0>.
- Pomponio, G., Savary, C.C., Parmentier, C., Bois, F., Guillouzo, A., Romanelli, L., Richert, L., Di Consiglio, E., Testai, E., 2015. In vitro kinetics of amiodolone and its major metabolite in two human liver cell models after acute and repeated treatments. *Toxicol. in Vitro* 30, 36–51. <https://doi.org/10.1016/j.tiv.2014.12.012>.
- Poulin, P., Krishnan, K., 1995. An algorithm for predicting tissue: Blood partition coefficients of organic chemicals from n-octanol: water partition coefficient data. *J. Toxicol. Environ. Health* 46, 117–129. <https://doi.org/10.1080/15287399509532021>.
- Poulin, P., Burczynski, F.J., Haddad, S., 2016. The role of extracellular binding proteins in the cellular uptake of drugs: impact on quantitative in vitro-to-in vivo extrapolations of toxicity and efficacy in physiologically based pharmacokinetic-pharmacodynamic research. *J. Pharm. Sci.* 105, 497–508. <https://doi.org/10.1002/jps.24571>.
- Price, P.J., Gregory, E.A., 1982. Relationship between in vitro growth promotion and biophysical and biochemical properties of the serum supplement. *In Vitro* 18, 576–584. <https://doi.org/10.1007/BF02810081>.
- Proença, S., Paini, A., Joossens, E., Vicente, J., Benito, S., Berggren, E., 2019. Insights into in vitro biokinetics using virtual cell based assay simulations. *ALTEX* 1, 447–461. <https://doi.org/10.14573/altex.1812101>.
- Qin, M., Nilsson, M., Oie, S., 1994. Decreased elimination of drug in the presence of alpha-1-acid glycoprotein is related to a reduced hepatocyte uptake. *J. Pharmacol. Exp. Ther.* 269, 1176–1181.
- Quinn, C.L., Van Der Heijden, S.A., Wania, F., Jonker, M.T.O., 2014. Partitioning of polychlorinated biphenyls into human cells and adipose tissues: evaluation of octanol, triolein, and liposomes as surrogates. *Environ. Sci. Technol.* 48, 5920–5928. <https://doi.org/10.1021/es500090x>.
- Ramanujan, S., Pluen, A., McKee, T.D., Brown, E.B., Boucher, Y., Jain, R.K., 2002. Diffusion and convection in collagen gels: Implications for transport in the tumor

- interstitium. *Biophys. J.* 83, 1650–1660. [https://doi.org/10.1016/S0006-3495\(02\)73933-7](https://doi.org/10.1016/S0006-3495(02)73933-7).
- Riedel, J., Altenburger, R., 2007. Physicochemical substance properties as indicators for unreliable exposure in microplate-based bioassays. *Chemosphere* 67, 2210–2220. <https://doi.org/10.1016/j.chemosphere.2006.12.022>.
- Riley, R.J., McGinness, D.F., Austin, R.P., 2005. A unified model for predicting human hepatic, metabolic clearance from in vitro intrinsic clearance data in hepatocytes and microsomes. *Pharmacology* 33, 1304–1311. <https://doi.org/10.1124/dmd.105.004259.lenged>.
- Rodgers, T., Rowland, M., 2006. Physiologically based pharmacokinetic modelling 2: predicting the tissue distribution of acids, very weak bases, neutrals and zwitterions. *J. Pharm. Sci.* 95, 1238–1257. <https://doi.org/10.1002/jps.20502>.
- Rodgers, T., Leahy, D., Rowland, M., 2005. Physiologically based pharmacokinetic modeling 1: predicting the tissue distribution of moderate-to-strong bases. *J. Pharm. Sci.* 94, 1259–1276. <https://doi.org/10.1002/jps.20322>.
- Rusina, T., Smedes, F., Klanova, J., 2010. Diffusion coefficients of polychlorinated biphenyls and polycyclic aromatic hydrocarbons in polydimethylsiloxane and low-density polyethylene polymers. *J. Appl. Polym. Sci.* 116, 1803–1810.
- Schreiber, R., Altenburger, R., Paschke, A., Kuster, E., 2008. How to deal with lipophilic and volatile organic substances in. *Environ. Toxicol.* 27, 1676–1682. <https://doi.org/10.1897/07-504.1>.
- Schwarzenbach, R., Gschwend, P.M., Imboden, D.M., 2016. Sorption II: Partitioning to living media-bioaccumulation and baseline toxicity. In: *Environmental Organic Chemistry*. Wiley.
- Seibert, H., Mörchel, S., Gülden, M., 2002. Factors influencing nominal effective concentrations of chemical compounds in vitro: medium protein concentration. *Toxicol. in Vitro* 16, 289–297. [https://doi.org/10.1016/S0887-2333\(02\)00014-0](https://doi.org/10.1016/S0887-2333(02)00014-0).
- Seidensticker, S., Grathwohl, P., Lamprecht, J., Zarfl, C., 2018. A combined experimental and modeling study to evaluate pH-dependent sorption of polar and non-polar compounds to polyethylene and polystyrene microplastics. *Environ. Sci. Eur.* 30, 1–12. <https://doi.org/10.1186/s12302-018-0155-z>.
- Sellaro, T., Ranade, A., 2009. Maintenance of human hepatocyte function in vitro by liver-derived extracellular matrix gels. *Eng. Part A* 16.
- Shen, C., Zhang, G., Meng, Q., 2010. Enhancement of the predicted drug hepatotoxicity in gel entrapped hepatocytes within polysulfone-g-poly (ethylene glycol) modified hollow fiber. *Toxicol. Appl. Pharmacol.* 249, 140–147. <https://doi.org/10.1016/j.taap.2010.08.028>.
- Shireman, R.B., Wei, C.I., 1986. Uptake of 2,3,7,8-tetrachlorodibenzo-p-dioxin from plasma lipoproteins by cultured human fibroblasts. *Chem. Biol. Interact.* 58, 1–12. [https://doi.org/10.1016/S0009-2797\(86\)80082-5](https://doi.org/10.1016/S0009-2797(86)80082-5).
- Smith, F.L., Harvey, A.H., 2007. Avoid common pitfalls when using Henry's law. *Chem. Eng. Prog.* 103, 33–39.
- Smith, D.A., Di, L., Kerns, E.H., 2010. The effect of plasma protein binding on in vivo efficacy: misconceptions in drug discovery. *Nat. Rev. Drug Discov.* 9, 929–939. <https://doi.org/10.1038/nrd3287>.
- Stadnicka-Michalak, J., Tanneberger, K., Schirmer, K., Ashauer, R., 2014. Measured and modeled toxicokinetics in cultured fish cells and application to in vitro - In vivo toxicity extrapolation. *PLoS One* 9. <https://doi.org/10.1371/journal.pone.0092303>.
- Stadnicka-Michalak, J., Schirmer, K., Ashauer, R., 2015. Toxicology across scales: cell population growth in vitro predicts reduced fish growth. *Sci. Adv.* 1–8. <https://doi.org/10.1126/sciadv.1500302>.
- Stalter, D., Dutt, M., Escher, B.I., 2013. Headspace-free setup of in vitro bioassays for the evaluation of volatile disinfection by-products. *Chem. Res. Toxicol.* 26, 1605–1614. <https://doi.org/10.1021/tx400263h>.
- Streuli, C., 1999. Extracellular matrix remodelling and cellular differentiation. *Curr. Opin. Cell Biol.* 11, 634–640.
- Stylianopoulos, T., Poh, M.Z., Insin, N., Bawendi, M.G., Fukumura, D., Munn, L.L., Jain, R.K., 2010. Diffusion of particles in the extracellular matrix: the effect of repulsive electrostatic interactions. *Biophys. J.* 99, 1342–1349. <https://doi.org/10.1016/j.bpj.2010.06.016>.
- Sung, J.H., Koo, J., Shuler, M.L., 2019. Mimicking the human physiology with microphysiological systems (MPS). *BioChip J.* 13, 115–126. <https://doi.org/10.1007/s13206-019-3201-z>.
- Teeguarden, J.G., Barton, H.A., 2004. Computational modeling of serum-binding proteins and clearance in extrapolations across life stages and species for endocrine active compounds. *Risk Anal.* 24, 751–770. <https://doi.org/10.1111/j.0272-4332.2004.00473.x>.
- Teeguarden, J.G., Hinderliter, P.M., Orr, G., Thrall, B.D., Pounds, J.G., 2007. Particle kinetics in vitro: dosimetry considerations for in vitro nanoparticle toxicity assessments. *Toxicol. Sci.* 95, 300–312. <https://doi.org/10.1093/toxsci/kfl165>.
- Ten Hulscher, T.E.M., Van Der Velde, L.E., Bruggeman, W.A., 1992. Temperature dependence of Henry's Law constants for selected chlorobenzenes, polychlorinated biphenyls and polycyclic aromatic hydrocarbons. *Environ. Toxicol. Chem.* 11, 1595–1603. <https://doi.org/10.1002/etc.5620111109>.
- Thellen, C., Blaise, C., Roy, Y., Hickey, C., 1989. Round Robin testing with the *Selenastrum capricornutum* microplate toxicity assay. *Hydrobiologia* 188–189, 259–268. <https://doi.org/10.1007/BF00027791>.
- Tice, R.R., Austin, C.P., Kavlock, R.J., Bucher, J.R., 2013. Improving the human hazard characterization of chemicals: a Tox21 update. *Environ. Health Perspect.* 121, 756–765. <https://doi.org/10.1289/ehp.1205784>.
- Tiong, H.Y., Huang, P., Xiong, S., Li, Y., Vathsala, A., Zink, D., 2014. Drug-induced nephrotoxicity: clinical impact and preclinical in vitro models. *Mol. Pharm.* 11, 1933–1948. <https://doi.org/10.1021/mp400720w>.
- Toma, C., Gadaleta, D., Roncaglioni, A., Toropov, A., Toropova, A., Marzo, M., Benfenati, E., 2019. QSAR development for plasma protein binding: influence of the ionization state. *Pharm. Res.* 36. <https://doi.org/10.1007/s11095-018-2561-8>.
- Trainor, G.L., 2007. The importance of plasma protein binding in drug discovery. *Expert Opin. Drug Discovery* 2, 51–64. <https://doi.org/10.1517/17460441.2.1.51>.
- Trapp, S., Rosania, G.R., Horobin, R.W., Kornhuber, J., 2008. Quantitative modeling of selective lysosomal targeting for drug design. *Eur. Biophys. J.* 37, 1317–1328. <https://doi.org/10.1007/s00249-008-0338-4>.
- Treijtel, N., Barendregt, A., Freidig, A.P., Blaauboer, B.J., Van Eijkeren, J.C.H., 2004. Modeling the in vitro intrinsic clearance of the slowly metabolized compound tobutamide determined in sandwich-cultured rat hepatocytes. *Drug Metab. Dispos.* 32, 884–891. <https://doi.org/10.1124/dmd.32.8.884>.
- Treijtel, N., Van Helvoort, H., Barendregt, A., Blaauboer, B.J., Van Eijkeren, J.C.H., 2005. The use of sandwich-cultured rat hepatocytes to determine the intrinsic clearance of compounds with different extraction ratios: 7-Ethoxycoumarin and warfarin. *Drug Metab. Dispos.* 33, 1325–1332. <https://doi.org/10.1124/dmd.105.004390>.
- Truisi, G.L., Di Consiglio, E., Parmentier, C., Savary, C.C., Pomponio, G., Bois, F., Lauer, B., Jossé, R., Hewitt, P.G., Mueller, S.O., Richert, L., Guillouzo, A., Testai, E., 2015. Understanding the biokinetics of ibuprofen after single and repeated treatments in rat and human in vitro liver cell systems. *Toxicol. Lett.* 233, 172–186. <https://doi.org/10.1016/j.toxlet.2015.01.006>.
- Vaal, M.A., Forkerts, A.J., 1998. Sensitivity of Microscale Ecotoxicity Tests and Their Suitability to Measure Toxicity of Environmental Samples.
- Vaes, W.H.J., Ramos, E.U., Verhaar, H.J.M., Cramer, C.J., Hermens, J.L.M., 1998. Understanding and estimating membrane/water partition coefficients: approaches to derive quantitative structure property relationships. *Chem. Res. Toxicol.* 11, 847–854. <https://doi.org/10.1021/tx970210y>.
- Van Der Heijden, S.A., Jonker, M.T.O., 2011. Intra- and interspecies variation in bioconcentration potential of polychlorinated biphenyls: are all lipids equal? *Environ. Sci. Technol.* 45, 10408–10414. <https://doi.org/10.1021/es2022158>.
- Vandenbelt, J.M., Hansen, C., Church, C., 1972. Binding of apolar molecules by serum albumin. *J. Med. Chem.* 15, 787–789. <https://doi.org/10.1021/jm00278a001>.
- Varma, M.V., Steyn, S.J., Allerton, C., El-Kattan, A.F., 2015. Predicting clearance mechanism in drug discovery: extended clearance classification system (ECCS). *Pharm. Res.* 32, 3785–3802. <https://doi.org/10.1007/s11095-015-1749-4>.
- Venable, R.M., Krämer, A., Pastor, R.W., 2019. Molecular dynamics simulations of membrane permeability. *Chem. Rev.* 119, 5954–5997. <https://doi.org/10.1021/acs.chemrev.8b00486>.
- Vinken, M., Hengstler, J.G., 2018. Characterization of hepatocyte-based in vitro systems for reliable toxicity testing. *Arch. Toxicol.* 92, 2981–2986. <https://doi.org/10.1007/s00204-018-2297-6>.
- Wang, L., Liu, Y., Qi, C., Shen, L., Wang, J., Liu, X., Zhang, N., Bing, T., Shanguan, D., 2018. Oxidative degradation of polyamines by serum supplement causes cytotoxicity on cultured cells. *Nat. Sci. Rep.* 8, 1–9. <https://doi.org/10.1038/s41598-018-28648-8>.
- Weisiger, R.A., 1985. Dissociation from albumin: a potentially rate-limiting step in the clearance of substances by the liver. *Proc. Natl. Acad. Sci. U. S. A.* 82, 1563–1567. <https://doi.org/10.1073/pnas.82.5.1563>.
- Wenger, R., Kurtcuoglu, V., Scholz, C., Marti, H., Hoogewijs, D., 2015. Frequently asked questions in hypoxia research. *Hypoxia* 35. <https://doi.org/10.2147/hp.s92198>.
- Wetmore, B.A., Wambaugh, J.F., Allen, B., Ferguson, S.S., Sochaski, M.A., Setzer, R.W., Houck, K.A., Strobe, C.L., Cantwell, K., Judson, R.S., LeCluyse, E., Clewell, H.J., Thomas, R.S., Andersen, M.E., 2015. Incorporating high-throughput exposure predictions with dosimetry-adjusted in vitro bioactivity to inform chemical toxicity testing. *Toxicol. Sci.* 148, 121–136. <https://doi.org/10.1093/toxsci/kfv171>.
- Wienkers, L.C., Heath, T.G., 2005. Predicting in vivo drug interactions from in vitro drug discovery data. *Nat. Rev. Drug Discov.* 4, 825–833. <https://doi.org/10.1038/nrd1851>.
- Wilmes, A., Limonciel, A., Aschauer, L., Moenks, K., Bielow, C., Leonard, M.O., Hamon, J., Carpi, D., Ruzek, S., Handler, A., Schmal, O., Herrgen, G., Bellwon, P., Burek, C., Truisi, G.L., Hewitt, P., Di Consiglio, E., Testai, E., Blaauboer, B.J., Guillou, C., Huber, C.G., Lukas, A., Pfaller, W., Mueller, S.O., Bois, F.Y., Dekant, W., Jennings, P., 2013. Application of integrated transcriptomic, proteomic and metabolomic profiling for the delineation of mechanisms of drug induced cell stress. *J. Proteome* 79, 180–194. <https://doi.org/10.1016/j.jpro.2012.11.022>.
- Wilson, C.J., Clegg, R.E., Leavesley, D.L., Pearcy, M.J., 2005. Mediation of biomaterial-cell interactions by adsorbed proteins: a review. *Tissue Eng.* 11, 1–18. <https://doi.org/10.1089/ten.2005.11.1>.
- Worth, A.P., Louise, J., Macko, P., Sala Benito, J.V., Paini, A., 2017. Virtual cell based assay simulations of intra-mitochondrial concentrations in hepatocytes and cardiomyocytes. *Toxicol. in Vitro* 45, 222–232. <https://doi.org/10.1016/j.tiv.2017.09.009>.
- Wu, M., Urban, J., Cui, Z., Cui, Z.F., 2006. Development of PDMS microreactor with well-defined and homogenous culture environment for chondrocyte 3-D culture. *Biomed. Microdevices* 331–340. <https://doi.org/10.1007/s10544-006-9597-y>.
- Youdim, K.A., Avdeef, A., Abbott, N.J., 2003. In vitro trans-monomer permeability calculations: often forgotten assumptions. *Drug Discov. Today* 8, 997–1003. [https://doi.org/10.1016/S1359-6446\(03\)02873-3](https://doi.org/10.1016/S1359-6446(03)02873-3).
- Zaldivar Comenges, J.M., Joossens, E., Benito, J.V.S., Worth, A., Paini, A., 2016. Theoretical and mathematical foundation of the virtual cell based assay – A review. *Toxicol. in Vitro*. <https://doi.org/10.1016/j.tiv.2016.07.013>.
- Zhang, Q., Bhattacharya, S., Pi, J., Clewell, R.A., Carmichael, P.L., Andersen, M.E., 2015. Adaptive posttranslational control in cellular stress response pathways and its

- relationship to toxicity testing and safety assessment. *Toxicol. Sci.* 147, 302–316. <https://doi.org/10.1093/toxsci/kfv130>.
- Zsila, F., Bikadi, Z., Malik, D., Hari, P., Pechan, I., Berces, A., Hazai, E., 2011. Evaluation of drug-human serum albumin binding interactions with support vector machine aided online automated docking. *Bioinformatics* 27, 1806–1813. <https://doi.org/10.1093/bioinformatics/btr284>.
- Zucco, F., Batto, A.F., Bises, G., Chambaz, J., Chiusolo, A., Consalvo, R., Cross, H., Dal Negro, G., De Angelis, I., Fabre, G., Guillou, F., Hoffman, S., Laplanche, L., Morel, E., Pinçon-Raymond, M., Prieto, P., Turco, L., Ranaldi, G., Rousset, M., Sambuy, Y., Scarino, M.L., Torreilles, F., Stamatii, A., 2005. An inter-laboratory study to evaluate the effects of medium composition on the differentiation and barrier function of Caco-2 cell lines. *ATLA Altern. Lab. Anim.* 33, 603–618. <https://doi.org/10.1177/026119290503300618>.