Toxicology 332 (2015) 30–40

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Dose metric considerations in *in vitro* assays to improve quantitative *in vitro–in vivo* dose extrapolations

Floris A. Groothuis[a,*], Minne B. Heringa[b], Beate Nicol[c], Joop L.M. Hermens[a], Bas. J. Blaauboer[a], Nynke I. Kramer[a]

[a] *Institute for Risk Assessment Sciences, Utrecht University, PO Box 80177, 3508 TD Utrecht, The Netherlands*
[b] *National Institute of Public Health and the Environment (RIVM), PO Box 1, 3720 BA Bilthoven, The Netherlands*
[c] *Unilever U.K., Safety & Environmental Assurance Centre, Colworth Science Park, Sharnbrook, Bedford MK44 1LQ, United Kingdom*

ARTICLE INFO

Article history:
Received 5 November 2012
Received in revised form 17 July 2013
Accepted 14 August 2013
Available online 23 August 2013

Keywords:
In vitro assay
Dose
Quantitative *in vitro–in vivo* extrapolation (QIVIVE)
Mechanism of action
Free concentration

ABSTRACT

Challenges to improve toxicological risk assessment to meet the demands of the EU chemical's legislation, REACH, and the EU 7th Amendment of the Cosmetics Directive have accelerated the development of non-animal based methods. Unfortunately, uncertainties remain surrounding the power of alternative methods such as *in vitro* assays to predict *in vivo* dose–response relationships, which impedes their use in regulatory toxicology. One issue reviewed here, is the lack of a well-defined dose metric for use in concentration-effect relationships obtained from *in vitro* cell assays. Traditionally, the nominal concentration has been used to define *in vitro* concentration–effect relationships. However, chemicals may differentially and non-specifically bind to medium constituents, well plate plastic and cells. They may also evaporate, degrade or be metabolized over the exposure period at different rates. Studies have shown that these processes may reduce the bioavailable and biologically effective dose of test chemicals in *in vitro* assays to levels far below their nominal concentration. This subsequently hampers the interpretation of *in vitro* data to predict and compare the true toxic potency of test chemicals. Therefore, this review discusses a number of dose metrics and their dependency on *in vitro* assay setup. Recommendations are given on when to consider alternative dose metrics instead of nominal concentrations, in order to reduce effect concentration variability between *in vitro* assays and between *in vitro* and *in vivo* assays in toxicology.

1. Introduction

It is estimated that the European Union's (EU) new chemicals legislation, REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), will significantly increase the number of laboratory animals used for toxicity testing (Hofer et al., 2004; Breithaupt, 2006; EU Parliament and Council, 2006; ECHA, 2009; Hartung and Rovida, 2009). REACH, as well as other regulations like the EU 7th Amendment to the Cosmetics Directive, banning testing of cosmetic ingredients on animals, and the proposed revision of the Toxic Substance Control Act (TSCA) by the US Environmental Protection Agency (EPA), have strengthened the call for non-animal based methods in toxicological risk assessment (EU Parliament and Council, 2003; Hartung, 2011). Promising methods include (quantitative) structure activity relationships ((Q)SARs), (human cell-based) *in vitro* assays and physiologically based biokinetic (PBBK) modelling, which may be combined in integrated testing strategies (ITS) (Health Council of the Netherlands, 2001; Blaauboer, 2002; Cronin et al., 2003a,b; Gubbels-van Hal et al., 2005; Lipscomb et al., 2012; Coecke et al., 2013). The implementation of such strategies for toxicological risk assessment purposes

Abbreviations: AUC, Area under the curve; TWA, Time weighted average; BED, Biologically effective dose; EC_{50}, Median effect concentration; MeOA, Mechanism of action; PBBK, Physiological based biokinetic modelling, also referred to in literature as physiological based pharmacokinetic modelling (PBPK) or physiological based toxicokinetic modelling (PBTK); (Q)IVIVE, (Quantitative) *in vitro–in vivo* extrapolation; REACH, Registration, evaluation, authorisation and restriction of chemicals; NRC, US National Research Council; OECD, Organisation of Economic Cooperation and Development; SPME, Solid-phase microextraction; PDMS, Polydimethylsiloxane; PAH, Polycyclic aromatic hydrocarbons; HAH, Halogenated aromatic hydrocarbons; DMSO, Dimethylsulfoxide; K_{OW}, octanol–water partition coefficient, Log form also referred to as LogP; BK/TD, Biokinetic/toxicodynamic modelling also referred to in literature as toxicokinetic/toxicodynamic (TK/TD) or pharmacokinetic pharmacodynamic (PKPD) modelling.

* Corresponding author. Tel.: +31 30 253 5328; fax: +31 30 253 5077.
E-mail addresses: f.a.groothuis@uu.nl (F.A. Groothuis), minne.heringa@rivm.nl (M.B. Heringa), Beate.Nicol@unilever.com (B. Nicol), j.hermens@uu.nl (J.L.M. Hermens), b.blaauboer@uu.nl (Bas.J. Blaauboer), N.I.Kramer@uu.nl (N.I. Kramer).

0300-483X/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tox.2013.08.012

was recognised in the report by the US National Research Council (NRC) entitled "Toxicity Testing in the 21st Century: A Vision and a Strategy" (NRC, 2007).

In vitro assays form the backbone of integrated testing strategies as they may provide both the initial concentration–response relationship and the ADME (absorption, distribution, metabolism and excretion) parameters needed for *in silico* modelling to estimate toxic doses to humans and the environment (Blaauboer et al., 2012). Note that a dose here refers to a specified quantity of a chemical agent to which organisms or cells are exposed, while concentration refers to a dose per volume. In this paper, considerations of dose encompass both an amount and a concentration. Typical *in vitro* systems consist of subcellular fractions, primary cells, cell lines, or tissue slices in either glass vessels or plastic well plates (*i.e.* microtitre and tissue culture plates) (Bhogal et al., 2005). These are exposed to varying concentrations of a test chemical in exposure medium and assessed for molecular and cellular changes. Their applicability as alternatives to animal models in toxicology is evident from the idea that chemicals generally initiate effects at a cellular level (Ekwall, 1983; Schirmer, 2006). A major advantage of using *in vitro* assays include the fact that numerous test chemicals can be analysed in high throughput systems, thus reducing the animals used and toxic waste produced (Schirmer, 2006).

Several studies have successfully predicted systemic toxicological effects *in vivo* from *in vitro* assays, with and without making use of additional *in silico* methods. Castaño et al. (2003) and Schirmer (2006), in their respective reviews of literature comparing *in vitro* cytotoxicity data with that from the acute fish toxicity test, report good correlations between the *in vitro* derived median effect concentrations (EC_{50}) and median lethal concentrations to fish (LC_{50}). Likewise, the Multicenter Evaluation of *In vitro* Cytotoxicity (MEIC) programme revealed considerable correlations between cytotoxicity data from a battery of *in vitro* toxicity assays with human cell lines and acutely lethal peak concentrations in human blood (Clemedson et al., 1996; Clemedson and Ekwall, 1999; Ekwall, 1999). Moreover, a limited number of studies using PBBK models to predict the *in vivo* exposure conditions that produce chemical concentration in the target tissue equivalent to the concentrations at which effects were observed *in vitro*, resulted in predicted effect concentrations that corresponded well with animal toxicity data (de Jongh et al., 1999; Verwei et al., 2006; Louisse et al., 2010; Punt et al., 2013). Other *in vitro–in vivo* extrapolation (IVIVE) successes have been achieved predicting body clearance, rather than toxic effects (Andersson et al., 2001; Blanchard et al., 2006; Baker and Parton, 2007).

Despite the promising correlations between *in vitro* and *in vivo* dose–response relationships, there is room for improvement when considering the large inter-assay variation and the occasionally low absolute sensitivity of *in vitro* assays to predict *in vivo* toxicity (Clemedson et al., 1996; Ekwall, 1999; Castaño et al., 2003; Schirmer, 2006). One explanation for deviating *in vitro* and *in vivo* data, is the fact that single cell cultures will have a limited number of target sites and can only reveal a limited number of the perturbations in toxicity pathways that occur on a multi-organ level (Schirmer, 2006; Hartung, 2010; Astashkina et al., 2012; Blaauboer et al., 2012). A related issue is the limited organ specific functionality of cells in culture compared to their *in situ* counterparts, including differences in types and levels of transport proteins, receptors and biotransformation enzymes (Lin and Will, 2012). Specifically, differences in metabolic clearance and toxic metabolite formation between *in vitro* assays and between *in vitro* and *in vivo* assays are considered to be problematic (Guillouzo, 1998; Wilkening et al., 2003; Gubbels-van Hal et al., 2005; Coecke et al., 2006). The problems regarding *in vivo* resemblance can be partially countered by making use of batteries of *in vitro* assays or sophisticated systems like co-cultures, 3D cell models, tissue slices and engineered tissue (Griffith and Swartz, 2006; Astashkina et al., 2012). Such *in vitro* assays potentially represent *in vivo* effects to a greater extent, but are technically more difficult to use and generally have a lower throughput.

Another reason for the variation and occasionally low sensitivity of *in vitro* assays predicting systemic toxicity is the difference in the 'bioavailability' of test chemicals between *in vitro* assays and between *in vitro* and *in vivo* test systems. Bioavailability, in this context, refers to the fraction of test chemical in a system that is available for uptake into cells or tissue. There may be a difference in intracellular concentrations despite total extracellular concentrations being equal between two *in vitro* assays or between *in vitro* and *in vivo* assays. This difference, in turn, may in some degree be attributable to differences in the concentration of the test chemical available for uptake into cells (Kramer et al., 2012). Generally, nominal concentrations, *i.e.* the amount of added compound divided by the volume of the exposure medium, are used to construct concentration–effect relationships *in vitro*. Such a dose metric may greatly differ from the concentration at the target site in cells because no corrections are made for non-specific binding to extracellular matrices (such as serum proteins and the plastic of well plates or other lab equipment used in sample handling), evaporation or degradation of chemicals (Fig. 1). Moreover, the choice of dose metric of the *in vivo* assay to be predicted by the *in vitro* assay may also affect the potential success of the IVIVE. Correlations between *in vitro* and *in vivo* effect concentrations are likely to be higher when trying to predict *in vivo* concentrations in blood or aquarium water of fish toxicity tests because the *in vitro* cell assay generally resembles the blood/water-cell interface better than for example an external exposure *via* food or a bolus injection.

To quantitatively predict *in vivo* effects from *in vitro* toxicity tests, the choice of a particular dose metric for *in vitro* test assays needs to be carefully considered. Therefore, the aim of this review is to discuss the scientific literature considering the bioavailability of test chemicals in *in vitro* assays, as well as to provide guidance in choosing appropriate dose metrics for different *in vitro* setups. To meet this aim, major dose metrics available for both animal and *in vitro* toxicity assays, 'loss' pathways of chemicals in *in vitro* assays, as well as physicochemical properties of chemicals affecting their bioavailability *in vitro* are considered. Moreover, techniques to determine and model the various dose metrics in *in vitro* assays are discussed. Finally, a flow chart is presented to recommend appropriate dose metrics for *in vitro* assays based on the mechanism of action of the chemical tested, *in vitro* assay setup and physicochemical properties of test chemicals.

2. Dose metrics in toxicology

The reliability and accuracy of an *in vitro*-derived prediction of *in vivo* outcomes depends on the quality of the *in vitro* model. The prediction may be unreliable if the dose metric is poorly chosen. A dose metric (*i.e.* exposure metric) is defined as a measure of dose, a specified quantity of a test chemical in an entity like an *in vitro* assay or (part of an) organism. In toxicology, a variety of different dose metrics exist. The most commonly used metrics are defined in Table 1 and Fig. 2. The list of dose metrics is non-comprehensive, as it does not define dose metrics for a particular chemical entity in a defined medium such as the surface area per volume for nanoparticles (Han et al., 2012).

In theory, the most relevant dose metric explaining an *in vitro* response would be the concentration at the site of action in or on cells, such as the concentration at specific receptors, because this dose is most closely related to the initial molecular changes caused by the chemical in the cell (Escher and Hermens, 2002). Paustenbach (2000) defines this dose as the biologically effective

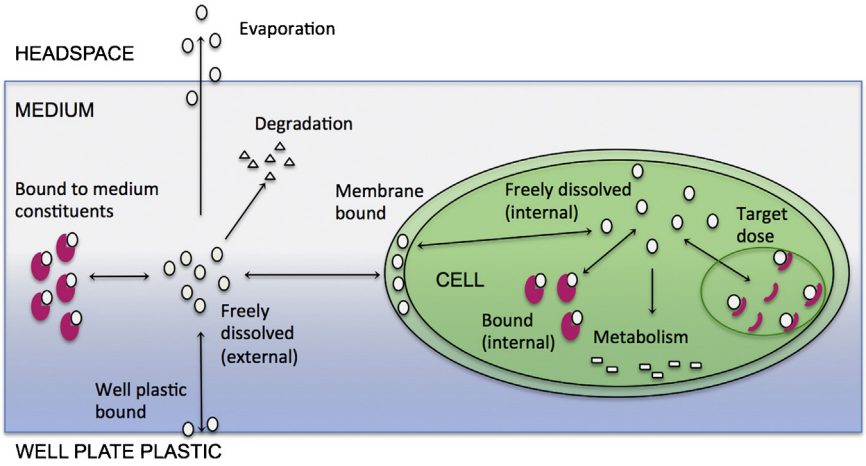

Fig. 1. An illustration of the different processes influencing the bioavailability of a chemical in a typical *in vitro* cell-based assay. The concentration of the test chemical at the target in a cell is determined by the extent of evaporation, metabolism and degradation over time. It is also determined by the extent to which the chemical binds to plastic, medium constituents, cell membranes and constituents in the cytoplasm (which may or may not be the target site). The open circles represent the chemical and the filled circles represent serum protein and lipids. Similar illustrations can also be found in Heringa et al. (2004) and Kramer et al. (2012).

dose (BED), "the amount that actually reaches cells, sites, or membranes where adverse effects occur, [this] may represent only a fraction of the delivered dose, but it is obviously the best one for predicting adverse effects." Unfortunately, such a dose is not practically measurable in most cases. Thus, surrogates are used including (1) the internal cell, organ or organism concentration, *i.e.* the total concentration inside cells, tissues or organisms at a specified time, (2) the freely available concentration, *i.e.* the concentration of the chemical not bound to matrices in the exposure medium surrounding the cells, tissues or organism, and (3) the nominal or total concentration in the medium to which a cell or organism is exposed (Table 1). The advantages and disadvantages on using each of these surrogate dose metrics in *in vitro* assays are discussed below.

Table 1
Glossary of common dose metrics used in toxicology.

Dose metric	Definition
Nominal concentration	Total amount of chemical divided by the volume of exposure medium to which the chemical is added (*e.g.* μmol/L medium)
Total concentration	Analytically measured concentrations in exposure medium (*e.g.* μmol/L medium) Includes chemicals freely available in medium and bound to medium constituents.
Freely available/free concentration	The unbound concentration of a test chemical in exposure medium (*e.g.* μmol/L medium)
Total internal concentration	The concentration of the test chemical associated with cells (*e.g.* μmol/10^6 cells)
Cytoplasm concentration and membrane concentration	The concentration in the cytoplasm of cells, either freely available or bound to constituents in the cytoplasm (*e.g.* μmol/L cytoplasm). Similar, the membrane concentration refers to the concentration associated with the membrane fraction of cells
Target concentration/biologically effective dose (BED)	The dose at the target site (*e.g.* DNA, cytoplasm or membrane receptors) in cells or tissues that causes a (toxicological) effect (*e.g.* μmol/μmol receptor)
Area under the curve (AUC)	Any of the above-mentioned dose metrics integrated over time (*e.g.* μmol/L × min)
Time weighted average (TWA)	Any of the above-mentioned dose metrics averaged over time (*e.g.* average μmol/L)
Biokinetic/toxicodynamic modelling (BK/TD)	Any of the above-mentioned dose metrics modelled over time

3. Nominal and total concentrations: Assay setup determining effect concentrations

As aforementioned, the nominal or total concentration is most commonly used to construct concentration–effect relationships in *in vitro* toxicology. Whereas the nominal concentration refers to the added dose divided by the volume of exposure medium, the total concentration refers to the analytically measured concentration of test chemicals in the exposure medium (excluding cells). Nominal and total concentrations are simple measures for quantifying concentration–effect relationships, which is why they are so widely used. However, both concentrations may differ significantly from the actual concentration available for uptake into cells, as the chemical may evaporate or degrade over time and bind to extracellular matrices such as plastic, serum protein and lipids (Fig. 1). Both the physiochemical properties of the test chemical as well as the specific assay conditions determine the fraction of a chemical available for uptake into cells *in vitro*. Notably, by referring to these processes in the context of what dose metric would be most appropriate to use, it is assumed they cause unintentional changes in the bioavailability of the tested chemicals as opposed to studies that may deliberately test for metabolic rates, degrading rates or binding affinities to *e.g.* serum proteins.

Only the freely dissolved, unbound concentration of a chemical (*i.e.* the free concentration) is considered available for uptake into organisms, tissue, or cells, to cause toxicity (Hervé et al., 1994; Escher and Hermens, 2004; Howard et al., 2010; Vaes et al., 1996, 1997; Gulden and Seibert, 1997). In *in vitro* assays, one of the most important bioavailability reducing factors is the serum commonly present in cell culture medium. Serum significantly decreases the unbound concentration of a chemical *in vitro* as many chemicals bind to its constituents, predominantly albumin (Schirmer et al., 1997; Seibert et al., 2002; Heringa et al., 2004; Kramer et al., 2012). Kramer et al. (2007, 2010, 2012) found that polycyclic aromatic hydrocarbons (PAHs, *i.e.* phenanthrene, pyrene and benzo(*a*)pyrene) were for 93–99.9% bound to serum constituents in the medium of basal cytotoxicity assays supplemented with a standard serum concentration of 5%. The authors also found that

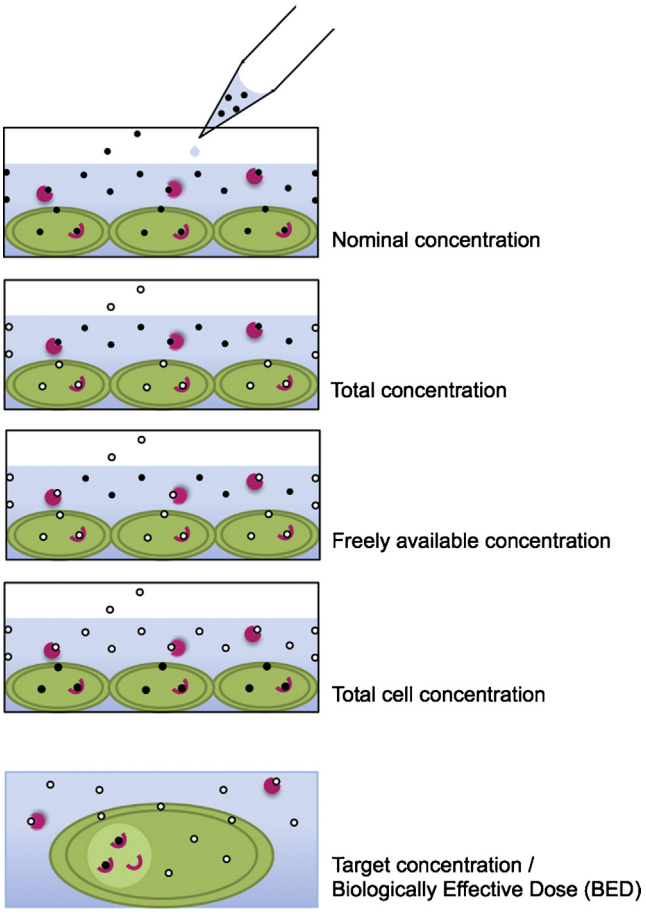

Fig. 2. Illustrations of different (surrogate) dose metrics in a typical *in vitro* assay. At the bottom, the theoretical target dose, also termed biologically effective dose (BED) is shown. Towards the top, surrogate dose metrics further away from the target site are shown. Solid black circles represent the chemical fraction included in the depicted measure of dose, while the open black circles represent the fraction not determined by the depicted dose metric.

the greater the concentration of serum, the greater the bound fraction of phenanthrene in medium, the lower the freely available concentration of phenanthrene, the lower the concentration of phenanthrene in cells, and subsequently the lower the observed cytotoxicity was. Similarly, Hestermann et al. (2000) found that halogenated aromatic hydrocarbons (HAHs) were highly bound to serum constituents and they measured significant increases of EC_{50} values at higher serum levels in medium in EROD (ethoxyresorufin *O*-deethylase activity) assays using PHLC-1 cells. These PAHs and HAHs are very lipophilic and likely to bind to serum lipids and albumin, coinciding with the evidence that the log affinity constant, K_a, or equivalently, the log albumin-water partition coefficient ($K_{albumin/water}$) of neutral organic chemicals, is positively correlated with the log octanol–water partition coefficient (Log K_{OW} or Log*P*, a proxy for lipophilicity) (deBruyn and Gobas, 2007; Endo and Goss, 2011). However, specific binding of polar, charged and more hydrophilic chemicals to serum protein may also occur to a significant extent, suggesting more specific interactions between the chemical and albumin binding sites (Kratochwil et al., 2004; Gülden and Seibert, 2005). Moreover, the size and three-dimensional structure may also influence sorption to protein (Endo and Goss, 2011).

Test chemicals may also differentially bind to the plastic of well plates and other lab equipment used during the preparation and transfer of the dosing solution. This reduces the unbound concentration and the observed toxicity of the test chemical (Schirmer et al., 1997; Gellert and Stommel, 1999; Riedl and Altenburger, 2007; Schreiber et al., 2008; Kramer et al., 2012). Kramer et al. (2012) and Schirmer et al. (1997) found that 43% and 60% of phenanthrene and fluoranthene, respectively, bound to the well plate plastic in cytotoxicity assays when no serum was present in the medium. Interestingly, the extent of binding to plastic was insignificant in the presence of serum. Serum, therefore, was found to be a greater determinant of the free fraction of test chemicals than plastic. Moreover, like with binding to serum constituents, studies by Gellert and Stommel (1999) and Riedl and Altenburger (2007) showed that the extent of binding to well plate plastic is correlated with the lipophilicity of the chemical.

Gülden et al. (2001, 2010) measured EC_{50} values for several compounds in cytotoxicity assays and found that the cell density had a significant impact on the free concentration. They observed that the greater the amount of cells used, the lower the amount of chemical per cell and the lower the observed toxicity of the chemical was. Cell binding and accumulation in cells may be affected by specific transport mechanisms, accumulation in lipids and membranes, cell protein binding and trapping in lysosomes. Again, lipophilic chemicals tend to bind more strongly to cells and this binding may be approximated by the linear relationship between log K_{OW} and log liposome–water partition coefficients (Gobas et al., 1988; Vaes et al., 1998; Jonker and van der Heijden, 2007). Notably, positively charged chemicals (cations, basic at pH 7.4) and negatively charged compounds (anions, acidic at pH 7.4) show different cell-partitioning behaviour in medium. The partitioning of positively charged chemicals to membranes is similar or even enhanced compared to that of neutral compounds with equal hydrophobicity towards cell membranes or microsomes (Austin et al., 1995, 2002; Escher and Schwarzenbach, 1996; Kramer et al., 1998; Schmitt, 2008). An explanation is the attraction of these chemicals to the negative charges of the phospholipids present in membranes (Katagi, 2001). In contrast, negatively charged chemicals are repelled by the anionic surface of membranes. Instead, they have been found to bind more strongly to bovine serum albumin (BSA) compared to positively charged compounds (Austin et al., 2005).

Evaporation of a chemical from an *in vitro* system over the exposure period significantly reduces the total concentration over time, where the greater the loss of a chemical due to evaporation, the lower the observed toxicity of a chemical (Gülden et al., 2010; Tanneberger et al., 2010; Knöbel et al., 2012). Kramer et al. (2012) found that the concentrations of highly volatile chemicals like di- and trichlorobenzenes were less than a tenth of the original nominal concentration after 24 h of exposure in a basal cytotoxicity assay with fish gill cells. When the loss of evaporation was compensated for using a partition-controlled dosing system, measured EC_{50} values were at least four times lower than when evaporation was not compensated for. Evaporation in well plates may additionally introduce a bias by causing the chemical to enter into adjacent wells and eliciting effects there (Eisentraeger et al., 2003). Riedl and Altenburger (2007) found that evaporation significantly reduced the concentration of 16 industrial organic test chemicals present in an algal growth inhibition test using well plates. EC_{50} values of the volatile chemicals were lower in airtight glass vessels than in well plates. The effect was considered substantial when the log Henry constant (Log *H*, where *H* is in Pa m^3/mol) of the chemical, a proxy for volatility, was higher than −5.6 or when the log air–water partition coefficient, K_{AW} was more than −3.

New developments in the field of *in vitro* toxicology may help overcome some of the problems associated with evaporation and binding to medium constituents, cell membranes and well plate plastic. These include the standardisation of assay protocols with fixed cell concentrations and, where feasible, the establishment of serum-free cell assays to avoid serum protein binding, the use of plate sealers to minimize evaporation out of well plates and the use

of cell suspension cultures in well plates of non-binding material to avoid sorption of the test chemical to plastic (Ackermann and Fent, 1998; Mori and Wakabayashi, 2000; Coecke et al., 2005). Studies investigating the effect of co-solvents on the solubility, bioavailability and toxicity of test chemicals *in vitro*, allow researchers to carefully choose co-solvents for their *in vitro* assays. Indeed, it is common practice to prepare highly concentrated stock solutions of test chemicals in dimethylsulfoxide (DMSO), which are then used to spike culture medium. This can potentially lead to supersaturated solutions and a free concentration change as the chemical drops out of solution (Schnell et al., 2009; Tanneberger et al., 2010). Another useful development is the use of partition-controlled dosing systems for *in vitro* assays to compensate for the loss of test chemicals (Mayer et al., 1999; Brown et al., 2001; Kiparissis et al., 2003; Kramer et al., 2010; Smith et al., 2010; Bougeard et al., 2011; Smith et al., 2013). Kramer et al. (2010), for example, loaded polydimethylsiloxane (PDMS) sheets with the test chemical and placed them in the wells of a 24-well plate with culture medium and fish cells on inserts. In so doing, losses of the test chemical out of the medium due to evaporation were compensated by the release of the chemical from PDMS in order to maintain the partition equilibrium between PDMS and medium.

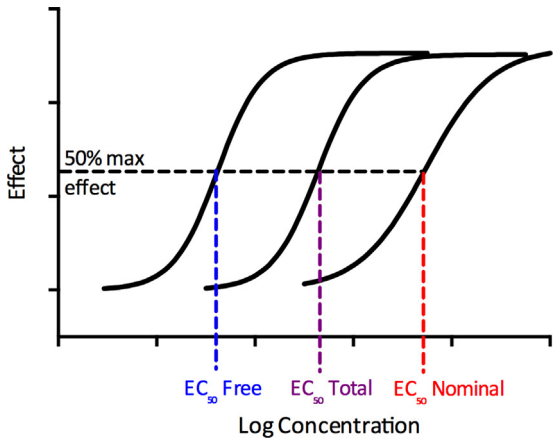

Fig. 3. Theoretical concentration–effect relationships of a test chemical in an *in vitro* assay based on nominal, total and free concentrations, (adapted from Escher and Hermens (2004)). The EC_{50} based on nominal and total concentrations may be different when the chemical significantly binds to plastic and cells, evaporates and degrades. The EC_{50} based on total, nominal and free concentrations may be different when the chemical significantly binds to serum constituents.

4. Free concentration as dose metric: Measuring and modelling techniques

Unintentional reductions of test chemical concentration through evaporation, metabolism, degradation and binding to plastic or other equipment may be detected by measuring total concentrations in medium over time. However, binding to medium constituents and cells in suspension is not distinguished using this method. In such cases, the freely available, unbound concentration in medium may be a better estimate of the BED (biologically effective dose) than the total or nominal concentration. Heringa et al. (2004) and Kramer et al. (2012) measured effect concentrations of test chemicals based on nominal and free concentrations in exposure medium with varying serum concentrations in oestrogen reporter gene assays and basal cytotoxicity assays, respectively. The authors found that the free concentration was a more consistent dose metric to use than the nominal concentration, as the free concentration was not dependent on the *in vitro* assay setup (*i.e.* serum levels). In contrast, the nominal concentration was shown to underestimate the toxic or *in vitro* potency *i.e.* the extent of toxicity caused by similar chemical concentrations, because a significant fraction of the tested chemical was not available to cause toxicity (Fig. 3).

The importance of using free instead of nominal concentrations for IVIVE is evident from the study by Gülden and Seibert (2005). The authors calculated the nominal cytotoxic concentration (EC_{50}) and the free cytotoxic concentration (EC_{50u}) for a number of organic chemicals ranging widely in cytotoxic potency. They correlated the *in vitro* derived EC_{50} and EC_{50u} data with LC_{50}-values from acute fish toxicity assays and found that EC_{50u} and LC_{50} corresponded better than EC_{50} and LC_{50} values, indicating that at least part of the variation could be explained by differences in bioavailability. Moreover, the difference in nominal and free concentrations, and thus the difference between the EC_{50} and EC_{50u}, was greater for the more cytotoxic chemicals than for the less cytotoxic chemicals. The authors suggest that for chemicals with a low cytotoxic potency, the reduction in bioavailability due to serum protein binding is not significant because protein binding will be saturated at the chemical concentrations needed to elicit toxicity.

Several techniques exist to measure the free concentrations and binding affinities of test chemicals to (extra)cellular matrices. These include equilibrium dialysis, ultrafiltration and centrifugation (Oravcová et al., 1996; Heringa and Hermens, 2003). Recent studies have also focused on solid-phase microextraction (SPME) as a relatively simple technique to measure the binding affinities of test chemicals to serum constituents (Vaes et al., 1996; Yuan et al., 1999; Musteata et al., 2006). This technology uses glass or metal fibres, which are coated with a polymer, to only extract the freely available chemical from the test solution (Arthur and Pawliszyn, 1990; Lord and Pawliszyn, 2000; Ulrich, 2000). Heringa et al. (2004) and Kramer et al. (2012) successfully used a variation of this technique, termed negligible-depletion (nd-)SPME, to directly measure free concentrations in an oestrogen-receptor reporter gene assay and a basal cytotoxicity assay, respectively. Another variation of SPME for determining free concentrations *in vitro* is partition-controlled dosing. Kramer et al. (2010) simultaneously maintained constant concentrations in medium over the exposure period and measured the free fraction of volatile and lipophilic chemicals in the exposure medium. The latter was achieved using the partition-coefficient of the test chemicals to PDMS in bare culture medium. A number of other studies have successfully used similar partition controlled dosing systems to maintain free chemical concentrations *in vitro* (Smith et al., 2010, 2013; Bougeard et al., 2011).

Arguably, however, modelling the free concentration of a test compound *in vitro* would be less cumbersome than measuring it. Indeed, equilibrium dialysis, ultrafiltration and centrifugation are difficult to use for hydrophobic test chemicals and are not always compatible with *in vitro* matrices (Oravcová et al., 1996; Heringa and Hermens, 2003). Likewise, the use of SPME directly in the cell assay is limited by the compatibility of the test chemical with the fibre coating and the kinetics of chemical uptake into the fibre (Vuckovic et al., 2009). Very hydrophobic chemicals partition only slowly into the fibre. Thus, equilibrium between the fibre and culture medium may not be reached within the exposure period of the cell assay. One would then have to resort to the more complicated non-equilibrium SPME techniques (Musteata et al., 2008). Since the SPME fibres in *in vitro* systems are small and take up minute amounts of the test chemical, the detection limit of the test chemical using currently available analytical techniques can be limiting (Heringa et al., 2004).

A number of mathematical models to estimate the free concentrations in *in vitro* systems have been described in the literature (Austin et al., 2002, 2005; Gülden and Seibert, 2003; Riedl and Altenburger, 2007; Kilford et al.,

2008; Kramer et al., 2012). Gülden and Seibert (2003), for example, proposed an extrapolation model for estimating the EC_{50} of chemicals in human serum equivalent to *in vitro* derived EC_{50}, taking into account the possible difference in the bioavailability of chemicals between *in vitro* assays and human serum. Their model, based on the equilibrium partitioning theory (EPT), estimates free concentrations of organic chemicals *in vitro* using the log K_{OW}, the amount of lipid in cells and medium, and the amount of protein in the medium. The association constant to protein K_a was derived from EC_{50} measurements at different serum levels *in vitro*. Based on their model, a rule system for four classes of chemicals was derived. Chemicals with a log $K_{OW} < 2$ or a low *in vitro* potency ($EC_{50} > 1000\,\mu M$) will have human serum effect concentrations that are equal to the nominal *in vitro* effect concentrations (rule 1). Compounds with a log $K_{OW} > 2$ and low *in vitro* potency should be corrected for the lipid fractions in culture medium and plasma (rule 2). Compounds with a log $K_{OW} < 2$ and a high potency ($EC_{50} < 1000\,\mu M$) should be corrected for the fraction bound to plasma proteins (rule 3). Chemicals with a log $K_{OW} > 2$ and a high potency should be corrected for lipid fractions and plasma proteins (rule 4). Verwei et al. (2006) successfully applied this rule-system to estimate *in vivo* developmentally toxic doses of embryotoxic chemicals using *in vitro* effect concentrations in the embryonic stem cell test.

Riedl and Altenburger (2007) developed an empirical model to estimate the ratio between EC_{50} values from algal toxicity assays using well plates and airtight glass containers. They used K_{OW} to account for plastic sorption and the Henry's law constant to account for evaporation. The model by Kramer et al. (2012) attempts to describe all major 'loss' pathways in a basal cytotoxicity assay (*i.e.* serum protein binding, cell partitioning, sorption to well plate plastic and evaporation). Like the Gülden and Seibert (2003) model, this model uses an equilibrium partitioning approach to estimate free fractions based on the K_a, the amount of serum protein, lipid–water partition coefficient, cell lipid content, plastic–water partition coefficient, the area of plastic exposed, the Henry's law constant of the chemical and the amount of headspace in each well of a well plate (assuming the well plate is sealed and airtight). The partition coefficients to serum, plastic and cell lipid may be estimated using the chemical's K_{OW}. This model was successfully applied to predict the sorption of phenanthrene to cells, plastic and serum protein. However, the model assumes a closed well system, where the concentration ratio of the chemical in the air and water is constant. This is unrealistic as evaporation is usually a continuous process, which is not captured by the model. Alternatively, the time course of the concentration versus the effect could be estimated using a biokinetic and biodynamic (BK/BD) model that describes the amount of chemical in a certain part of the system (*e.g.* external or internal cell concentration) over time. Such models are potentially more suitable to account for evaporation in open systems and are able to provide more information on the effects of a chemical over time (also referred to as the dynamics of chemicals in a test system). More information on this topic is provided in Section 6 where time-dependent changes in concentration and effects are described in more detail.

Additionally it should be noted that the models described above need to be validated for their chemical applicability domain. The equilibrium partitioning models for example have been developed for neutral organic chemicals. More experimental work is needed to establish models to estimate the free concentrations of less hydrophobic, more polar and charged chemicals in *in vitro* assays. Recent work by Endo et al. (2011) and Endo and Goss (2011) on polyparameter linear free energy relationships are useful in filling in this knowledge gap.

5. Concentration in cells

As aforementioned, the free concentration is considered a better measure to use in quantitative IVIVE compared to nominal and total medium concentrations. However, the free concentration itself is not always a good estimate of the BED. For example, the free concentration in the culture medium may decrease over time through evaporation, degradation, membrane transporter action or metabolism of a chemical *in vitro*. This in turn reduces the concentration that is available for uptake into the cells and the amount reaching the target site. For a number of organic industrial chemicals, Knöbel et al. (2012) and Kramer et al. (2010, 2012) found the (free) concentration *in vitro* to change over time and debated the use of either the free concentration in exposure medium at the end of the exposure period, or the geometric mean of the initial concentration and the concentration at the end of the exposure period. Notably, the partition-controlled dosing system developed by, amongst others Kramer et al. (2010) maintains constant free medium concentrations over the exposure period, but this method may contribute to the accumulation of the chemical in cells, hampering the interpretation of the true potency of the chemical.

In another study, DelRaso et al. (2003) found cadmium toxicity, based on free concentrations in medium, to increase with increasing serum concentrations in rat hepatocyte cultures. The authors attributed this to the enhanced rate of uptake of cadmium into the cells in the presence of serum proteins (*i.e.* facilitated transport by serum protein). Thus, the study illustrates that the free concentration, although closer to the BED, is not necessarily the most appropriate concentration to express *in vitro* toxicity. Binding matrices, such as serum protein, may enhance the uptake of toxicants into cells and if toxicity is dependent on the uptake rate of a chemical, the free concentration at equilibrium may not be sufficient to express toxicity.

Other than facilitated transport of chemicals by serum protein, the uptake rate into cells may also be affected by the presence of uptake and efflux transporters. *In vivo*, blood–brain barrier epithelia, gut epithelia and hepatocytes, for example, contain uptake and efflux transporters that actively transport specific chemicals across the cell membrane (Mizuno et al., 2003; Berezowski et al., 2004; Hayeshi et al., 2008). Lin and Lin (1990) found that increased serum protein binding significantly decreased the uptake of drugs by the brain, but to a lesser extent than predicted from the free concentration *in vitro*, suggesting that drug binding to serum protein did not limit the transport of drugs through the blood–brain barrier, likely because of the presence of transporters. Cells lacking specific transporters, as a number of cell lines do, will be poor surrogates for *in vivo* toxicity regulated by these transporters (Webborn et al., 2007).

Thus, given that effect concentrations expressed as free concentrations may also be dependent on *in vitro* assay setup, it could be argued that *in vitro* toxicity data should be based on the concentration of a test chemical in the cells (or at a target within the cell) over an exposure period. The internal dose is defined for *in vivo* systems as the concentration in a tissue (Escher and Hermens, 2002; McCarty et al., 2011). This is a dose surrogate closer to the site of toxic action and should therefore be preferred over external concentrations in *e.g.* blood or food. Thus far, concentrations per cell over time have been largely ignored in (*in vitro*) toxicology. To assess internal concentrations, one could measure critical cell burdens, analogous to critical body residues (CBR), the concentration of a test chemical in or on cells at a point in time that causes a perturbation to a toxicity pathway (McCarty et al., 2011). Additionally, internal concentrations could be modelled by using, if available, data about the partitioning and uptake rate into cells. Admittedly, the extraction of tissues or cells for concentration measurements remains delicate work, which is probably why very few

have tried it (Escher et al., 2011). However, Bopp et al. (2006) did investigate internal concentrations *in vitro* and illustrated that, like freely available concentrations, internal concentrations are better to use for dose–response relationships compared to nominal concentrations. The authors estimated internal concentrations by using cell-environment partitioning characteristics. Similar to the studies comparing free and nominal concentrations, the authors found that nominal concentrations used in the dose–effect relationship greatly overestimated median effect concentrations compared to internal concentrations.

6. Time-dependent dose metrics and mechanisms of action

The effect of exposure time on the concentration taken up by cells and causing toxicity *in vitro* has been little explored. One study considered time-dependent dose metrics in *in vitro* assays and found that cytotoxic medium concentrations of hydrogen peroxide in C6 glioma cells decreased from 500 to 30 μM with increasing incubation time from 1 to 24 h (Gülden et al., 2010). Twenty-four hours proved to be sufficient to determine an incipient cytotoxic concentration. This concentration resembles the cytotoxicity after an infinite time of exposure. These were linearly related to the amount of cells and thus the incipient effect concentration could be expressed as a dose per cell, *i.e.* in this case 430 nmol/mg cell protein. Moreover, with decreasing cell concentrations, the hydrogen peroxide elimination decelerates and thus exposure to the chemical applied as a bolus approached a continuous exposure to a steady concentration, indicating that the area under the concentration versus time curve (AUC) better characterizes the potency of hydrogen peroxide *in vitro*.

The above example suggests that, in cases of long exposure, the use of a cumulative dose, the area under the curve (AUC) or time-weighted average (TWA) may be appropriate. Time-weighted averages are expressed as doses divided by the time period of dosing and are often used in carcinogenic risk assessment, as well as dose–response relationships to estimate lifetime risks (Paustenbach, 2000). The AUC, in contrast, is the integrated dose over time (Paustenbach, 2000). AUC and TWA measures are generally based on nominal or total concentrations, but may also be based on free and internal concentrations. The AUC measure in a study by Gülden et al. (2010) was effective because the hydrogen peroxide toxicity can be viewed as mostly irreversible, resulting in an increase in effect over time, leading to equal AUC–effect relationships while the nominal concentration–effect relationships varied. The case of hydrogen peroxide indicates the possible influence of the mechanisms of action (MeOA) on the choice of the most appropriate dose metric *in vitro*.

A toxic mechanism of action (MeOA) refers to the biochemical process or interaction resulting in (adverse) effects (Escher et al., 2011). These adverse effects caused by a chemical are commonly referred to as the chemical's mode of action (MoOAP) (Escher et al., 2011). According to the US National Research Council, knowledge about the mechanisms by which a chemical affects the cells will be critical for accurate, quantitative prediction of hazardous exposure levels *in vivo* (NRC, 2007). The mechanism will give information about the initial location of the adverse effect and the type of chemical reaction.

The range of mechanisms through which a compound can act is broad. Chemicals can simply partition and accumulate in membranes, causing non-specific baseline toxicity, referred to as narcosis in ecotoxicology (Könemann, 1981; McCarty et al., 1992; Ekwall, 1995; Escher et al., 2011). Baseline toxicity can be relatively easily predicted with QSARs (quantitative structure activity relationships) for neutral organic chemicals by using their solubility (Mackay et al., 2009). The effect is generally considered a reversible effect, thus a peak concentration in the membrane, or in cells, could be calculated to express the toxic potency of these chemicals *in vitro*. The quantification of membrane specific toxicity such as narcosis, could potentially benefit from doses based on membrane concentrations, not on nominal, free or cytoplasmic concentrations. Indeed, the molar concentration of different narcotic chemicals in aquatic organisms at death in acute toxicity assays was found to be approximately constant, amounting to 40–160 mmol/kg lipid. *In vitro* cell assays may similarly react to narcotic chemicals and differences in sensitivity between cell types in basal cytotoxicity assays may be minimized when expressing effect concentrations per cell lipid content.

If the chemical exerts a stronger effect than predicted for baseline toxicity, the chemical also has a more specific or reactive MeOA through, for example, receptor binding or the formation of covalent bonds with macromolecules. Furthermore, reactive and specific acting chemicals may act through both reversible or irreversible mechanisms. The effects of reactive chemicals are mostly irreversible, meaning the damage is cumulative and requires the additional use of a time-dependent exposure metric for adequate quantification (Reinert et al., 2002). Legierse et al. (1999) and Verhaar et al. (1999) for example, have successfully predicted aquatic toxicity of chemicals with irreversible mechanisms by using the AUC instead of peak exposure. Indeed, the peak concentration would not account for damage accumulation over time. Chlorpyrifos, as another example, has been shown to cause death in *Daphnia pulex* well beyond the exposure period (van der Hoeven and Gerritsen, 1997). Chlorpyrifos, like other organophophates, irreversibly binds to the acetylcholineesterase. The latency effect may thus be caused by further action of remaining organophosphates in the body, even though external exposure has stopped, because the previous damage cannot be repaired (Jager and Kooijman, 2005).

Sometimes animals, but also cells are exposed in pulses (as in repeated dose studies) in an attempt to better reflect the exposure conditions occurring in the environment (Ashauer et al., 2007). Reinert et al. (2002) described different studies that repeatedly exposed fish for a certain amount of time to a test chemical. When the fish could recover quickly enough between pulses, indicating a more reversible mechanism, then a time-dependent exposure would become less important compared to simple peak exposure. However, the term reversibility depends on whether the cells or animals are capable of recovering before a new exposure occurs. Indeed, the rate of diffusion of very hydrophobic chemicals out of membranes may be too slow for the cell or organism to recover (Escher et al., 2011). Thus, the threshold between reversible or irreversible, as well as the accumulative potential of a chemical should be accounted for in the decision whether to use time-dependent exposure metrics.

Another way to incorporate time effects such as pulsed exposure in *in vitro* assays is to make use of a biokinetic and toxicodynamic (BK/TD) model that describes concentration–effect relationships over time. More specifically, biokinetics refers to the distribution of the test chemical in the studied system over time (*e.g.* bound/unbound, external/internal), while the toxicodynamics is a description of the (adverse) changes occurring in a biological system or organism caused by the chemical entity over time (Jager et al., 2011). As opposed to the static AUC or TWA measures, BK/TD models may provide more information about the dynamics as the effects over the whole time range is incorporated based on the distribution of the chemical. In the past, such models have often been applied in ecotoxicology and *in vivo* (McCarty and Mackay, 1993; Ashauer et al., 2007; Nyman et al., 2012; Boxall et al., 2013). Interestingly, a few studies have used BK/TD modelling approaches to describe *in vitro* systems as well (Kedderis et al., 1993; Nielsen et al., 2007; Poirier et al., 2008). Nielsen et al. (2007) for

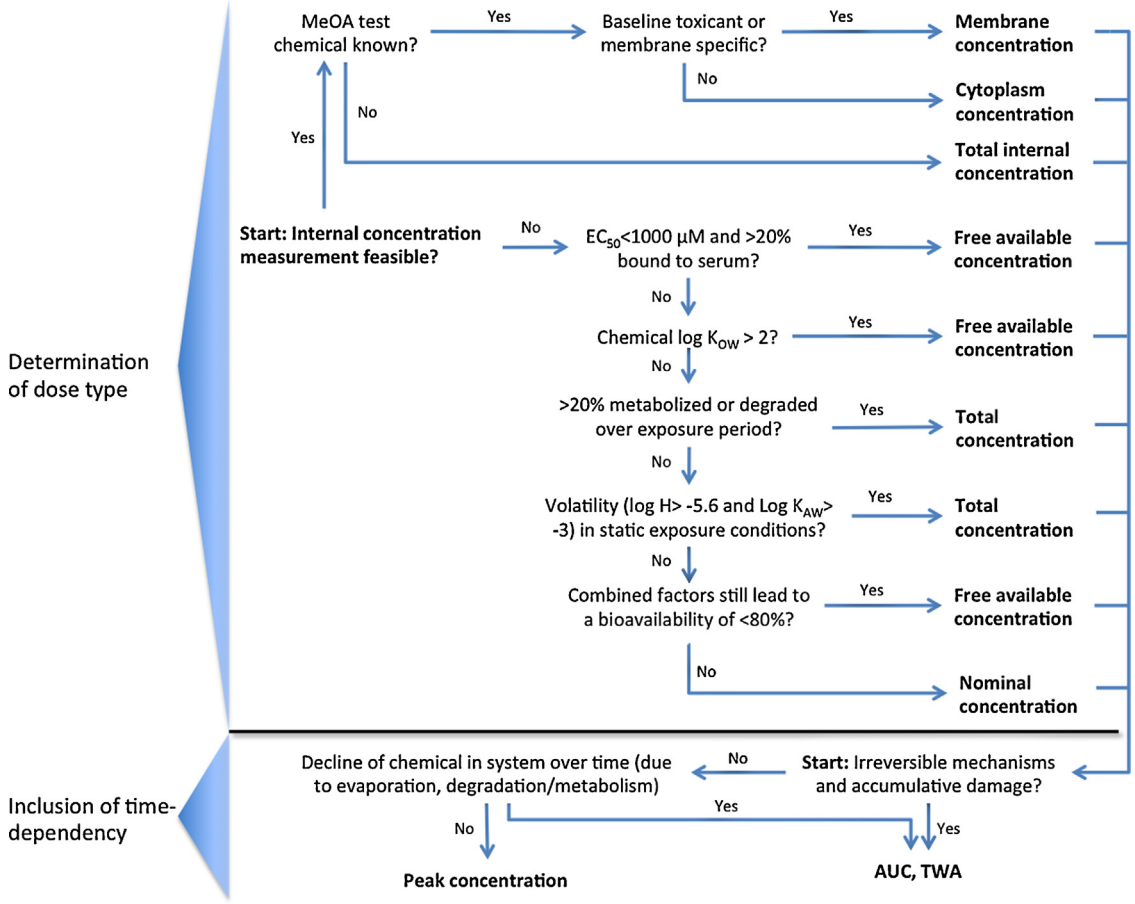

Fig. 4. Flow chart to aid in choosing an appropriate dose metric for a specific *in vitro* toxicity test. First, a choice should be made for dose type based on the characteristics of the chemical and available knowledge. Then, the metric can be integrated or averaged in case of time-dependent exposure and irreversible mechanisms, or steady reduction over time. Peak concentration is defined here as the maximum concentration reached during the exposure period. BK/TD may be applied to model partitioning and assess concentration changes over time. The chart has been compiled using literature data (Austin et al., 2002; Reinert et al., 2002; Gülden and Seibert, 2003; OECD, 2006a; OECD, 2006b; 2011; Riedl and Altenburger, 2007; Gülden et al., 2010; Knöbel et al., 2012).

example dosed antibiotics to bacteria (*Streptococcus pyogenes*) and the viability together with the chemical concentration was measured at different time points. They developed a BK/TD model that fitted these time-kill curve experiments while accounting for degradation of the chemicals and persistence of bacterial subgroups. This way the authors were able to study the time course of toxicity, including an initial rapid killing rate and an effect decline with time. These findings may not have been obtainable when part of the kinetics of the compounds, in this case degradation, would not have been accounted for.

This review focuses mainly on the kinetics of chemicals *in vitro*, but it is just as important to consider in more detail how the kinetics can influence the changes in adverse effects. By combining the knowledge about *in vitro* kinetics and dynamics in an *in vitro* BK/TD model with other *in silico* methods needed for *IVIVE* (*e.g.* PBBK), it could improve toxicological risk assessments while also reducing animal based toxicity testing.

7. Conclusion: The most appropriate dose metric *in vitro*

In this review, a number of dose metrics in *in vitro* toxicology have been discussed specifically with regards to their dependency on assay setup. From the literature it appears that three main factors determine the choice for the right dose metric in an *in vitro* assay: (1) the physicochemical properties and toxicity of the chemical (*e.g.* lipophilicity, volatility, *in vitro* potency, reactivity, stability), as they determine whether losses of chemical in the assay can affect the toxic effect, (2) the assay setup (*e.g.* the exposure regime and time, metabolic potential of cells, the cell number, the presence of serum etc.) and (3) the MeOA measured in the particular assay. For more irreversible mechanisms, peak exposure concentrations may not suffice as perturbations to cell functioning accumulate over time. In such cases, toxicity may be better captured using time-dependent dose metrics such as AUC and TWA, or BK/TD models. The flow chart presented in Fig. 4 provides a two-step guide to the reader to consider an appropriate and feasible dose metric.

The chart is divided into two sections. Initially a choice can be made between an internal dose metric including total internal, cytoplasm and membrane concentrations, or an external dose metric including nominal, total and free concentrations. The choice for an internal or external dose metric depends on both the knowledge about the MeOA of the compound and the feasibility to measure internal concentrations. For example, if the chemical is a baseline toxicant, acting through accumulation in the cell membrane causing narcosis or basal cytotoxicity, the amount of chemical per cell or cell lipid may be considered. The choice for an internal concentration will also depend on whether the increased accuracy of the obtained dose–response is worth the investment. When external concentrations are used, the choice for free, nominal or total concentrations is based on the information available on the physicochemical properties and *in vitro* potency of the test compound. The chart lists roughly defined criteria the chemical needs to meet for the free or total concentration to be significantly lower than the nominal concentration. Finally, the dose metrics can be combined

with a time-dependent metric such as a peak concentration (the maximum concentration reached over the exposure period), AUC or TWA. Time-dependent metrics are justified when testing reactive or specifically acting chemicals causing (irreversible) cumulative damage or if the chemical concentration in the test system changes significantly over time. It may also be possible to apply BK/TD models to assess concentration changes over time and relate this to the effects of a chemical.

The chart criteria are indicative only because they are obtained from the limited amount of literature available. For example, the threshold reduction in bioavailability (20%) that is used in Fig. 4 is derived from a number of OECD guidelines (OECD, 2006a,b, 2011). Further research is needed to specify the cut-offs in physicochemical properties and assay parameters. Indeed, the cut-off value for the free fraction, below which *in vitro* data based on nominal concentrations becomes problematic for IVIVE, is unclear. As a theoretical example, a free fraction lower than 50% in the *in vitro* test may still insignificantly impact an *in vivo* dose estimate. Thus, further research is welcome on this topic and on how the relevant parameters to determine the appropriate dose metric, such as protein binding and volatility, may be estimated by the more basic properties of the chemical, such as the K_{ow} and molecular weight.

Research assessing the effect of mechanistic processes on the target concentration and the effects of using these alternative dose metrics in *in vitro* assays for *in vitro-in vivo* dose extrapolations is still in its infancy. Yet this research may be highly valuable for toxicological risk assessment. Such research on dose metrics in *in vitro* assays falls within two high profile paradigm shifts in toxicology. The first shift is towards integrated testing strategies as an alternative to traditional animal tests in toxicological risk assessment. Illustrating this shift is the vision described in the influential U.S. National Research Council's report, Toxicity Testing in the Twenty-first Century (NRC, 2007). In this vision, high-throughput *in vitro* (human) cell assay batteries identify perturbations to critical toxicity pathways across a wide dose range of a test chemical. In turn, systems biology approaches and PBBK reverse dosimetry modelling use these *in vitro* dose–response curves to identify exposure situations likely to cause toxicity in humans. The second shift concerns the shift towards identifying the exposome as opposed to estimating concentrations of regulated contaminants in air, water and food by exposure scientists. The exposome refers to defining and monitoring exposures as fluctuating levels of biologically active chemicals in the human body's internal environment over a lifetime (Rappaport and Smith, 2010; Rappaport, 2011). Data on internal concentrations over time of chemicals disturbing critical toxicological pathways *in vitro* may be compared with monitored internal tissue concentrations to discover key exposures responsible for chronic disease.

Brief

To improve quantitative *in vitro–in vivo* dose extrapolations in toxicological risk assessment, care should be taken when defining a dose metric for *in vitro* concentration–effect relationships.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

Ackermann, G.E., Fent, K., 1998. The adaptation of the permanent fish cell lines PLHC-1 and RTG-2 to FCS-free media results in similar growth rates compared to FCS-containing conditions. Mar. Environ. Res. 46, 363–367.

Andersson, T.B., Sjoberg, H., Hoffmann, K.J., Boobis, A.R., Watts, P., Edwards, R.J., Lake, B.G., Price, R.J., Renwick, A.B., Gomez-Lechon, M.J., Castell, J.V., Ingelman-Sundberg, M., Hidestrand, M., Goldfarb, P.S., Lewis, D.F.V., Corcos, L., Guillouzo, A., Taavitsainen, P., Pelkonen, O., 2001. An assessment of human liver-derived *in vitro* systems to predict the *in vivo* metabolism and clearance of almokalant. Drug Metab. Dispos. 29, 712–720.

Arthur, C.L., Pawliszyn, J., 1990. Solid-phase microextraction with thermal-desorption using fused-silica optical fibers. Anal. Chem. 62, 2145–2148.

Ashauer, R., Boxall, A.B.A., Brown, C.D., 2007. New ecotoxicological model to simulate survival of aquatic invertebrates after exposure to fluctuating and sequential pulses of pesticides. Environ. Sci. Technol. 41, 1480–1486.

Astashkina, A., Mann, B., Grainger, D.W., 2012. A critical evaluation of *in vitro* cell culture models for high-throughput drug screening and toxicity. Pharmacol. Therapeut. 134, 82–106.

Austin, R.P., Barton, P., Cockroft, S.L., Wenlock, M.C., Riley, R.J., 2002. The influence of nonspecific microsomal binding on apparent intrinsic clearance, and its prediction from physicochemical properties. Drug Metab. Dispos. 30, 1497–1503.

Austin, R.P., Barton, P., Mohmed, S., Riley, R.J., 2005. The binding of drugs to hepatocytes and its relationship to physicochemical properties. Drug Metab. Dispos. 33, 419–425.

Austin, R.P., Davis, A.M., Manners, C.N., 1995. Partitioning of ionizing molecules between aqueous buffers and phospholipid-vesicles. J. Pharmacol. Sci. 84, 1180–1183.

Baker, M., Parton, T., 2007. Kinetic determinants of hepatic clearance: plasma protein binding and hepatic uptake. Xenobiotica 37, 1110–1134.

Berezowski, V., Landry, C., Dehouck, M.P., Cecchelli, R., Fenart, L., 2004. Contribution of glial cells and pericytes to the mRNA profiles of *p*-glycoprotein and multidrug resistance-associated proteins in an *in vitro* model of the blood–brain barrier. Brain Res. 1018, 1–9.

Bhogal, N., Grindon, C., Combes, R., Balls, M., 2005. Toxicity testing: creating a revolution based on new technologies. Trends Biotechnol. 23, 299–307.

Blaauboer, B.J., 2002. The applicability of *in vitro*-derived data in hazard identification and characterisation of chemicals. Environ. Toxicol. Pharmacol. 11, 213–225.

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. T4 Workshop Report ALTEX 29, 411–425.

Blanchard, N., Hewitt, N.J., Silber, P., Jones, H., Coassolo, P., Lave, T., 2006. Prediction of hepatic clearance using cryopreserved human hepatocytes: a comparison of serum and serum-free incubations. J. Pharm. Pharmacol. 58, 633–641.

Bopp, S.K., Bols, N.C., Schirmer, K., 2006. Development of a solvent-free, solid-phase *in vitro* bioassay using vertebrate cells. Environ. Toxicol. Chem. 25, 1390–1398.

Bougeard, C., Gallampois, C., Brack, W., 2011. Passive dosing: an approach to control mutagen exposure in the Ames fluctuation test. Chemosphere 83, 409–414.

Boxall, A.B.A., Fogg, L.A., Ashauer, R., Bowles, T., Sinclair, C.J., Colyer, A., Brain, R.A., 2013. Effects of repeated pulsed herbicide exposures on the growth of aquatic macrophytes. Environ. Toxicol. Chem. 32, 193–200.

Breithaupt, H., 2006. The costs of REACH. REACH is largely welcomed, but the requirement to test existing chemicals for adverse effects is not good news for all. EMBO Rep. 7, 968–971.

Brown, R.S., Akhtar, P., Akerman, J., Hampel, L., Kozin, I.S., Villerius, L.A., Klamer, H.J.C., 2001. Partition controlled delivery of hydrophobic substances in toxicity tests using poly(dimethylsiloxane) (PDMS) films. Environ. Sci. Technol. 35, 4097–4102.

Castaño, A., Bols, N., Braunbeck, T., Dierickx, P., Halder, M., Isomaa, B., Kawahara, K., Lee, L.E.J., Mothersill, C., Part, P., Repetto, G., Sintes, J.R., Rufli, H., Smith, R., Wood, C., Segner, H., 2003. The use of fish cells in ecotoxicology—the report and recommendations of ECVAM workshop 47. ATLA—Altern. Lab. Anim. 31, 317–351.

Clemedson, C., Ekwall, B., 1999. Overview of the final MEIC results: I. The *in vitro–in vitro* evaluation. Toxicol. In Vitro 13, 657–663.

Clemedson, C., McFarlaneAbdulla, E., Andersson, M., Barile, F.A., Calleja, M.C., Chesne, C., Clothier, R., Cottin, M., Curren, R., Dierickx, P., Ferro, M., Fiskesjo, G., GarzaOcanas, L., GomezLechon, M.J., Gülden, M., Isomaa, B., Janus, J., Judge, P., Kahru, A., Kemp, R.B., Kerszman, G., Kristen, U., Kunimoto, M., Karenlampi, S., Lavrijsen, K., Lewan, L., Lilius, H., Malmsten, A., Ohno, T., Persoone, G., Pettersson, R., Roguet, R., Romert, L., Sandberg, M., Sawyer, T.W., Seibert, H., Shrivastava, R., Sjostrom, M., Stammati, A., Tanaka, N., TorresAlanis, O., Voss, J.U., Wakuri, S., Walum, E., Wang, X.H., Zucco, F., Ekwall, B., 1996. MEIC evaluation of acute systemic toxicity. 2. *In vitro* results from 68 toxicity assays used to test the first 30 reference chemicals and a comparative cytotoxicity analysis. ATLA—Altern. Lab. Anim. 24, 273–311.

Coecke, S., Ahr, H., Blaauboer, B.J., Bremer, S., Casati, S., Castell, J., Combes, R., Corvi, R., Crespi, C.L., Cunningham, M.L., Elaut, G., Eletti, B., Freidig, A., Gennari, A., Ghersi-Egea, J.-., Guillouzo, A., Hartung, T., Hoet, P., Ingelman-Sundberg, M., Munn, S., Janssens, W., Ladstetter, B., Leahy, D., Long, A., Meneguz, A., Monshouwer, M., Morath, S., Nagelkerke, F., Pelkonen, O., Ponti, J., Prieto, P., Richert, L., Sabbioni, E., Schaack, B., Steiling, W., Testai, E., Vericat, J.-., Worth, A., 2006. Metabolism: a bottleneck in *in vitro* toxicological test development. ATLA—Altern. Lab. Anim. 34, 49–84.

Coecke, S., Balls, M., Bowe, G., Davis, J., Gstraunthaler, G., Hartung, T., Hay, R., Merten, O.W., Price, A., Schechtman, L., Stacey, G., Stokes, W., 2005. Guidance on good cell culture practice: a report of the second ECVAM Task Force on good cell culture practice. ATLA—Alternatives Lab. Anim. 33, 261–287.

Coecke, S., Pelkonen, O., Leite, S.B., Bernauer, U., Bessems, J.G., Bois, F.Y., Gundert-Remy, U., Loizou, G., Testai, E., Zaldivar, J., 2013. Toxicokinetics as a key to the

integrated toxicity risk assessment based primarily on non-animal approaches. Toxicol. In Vitro 27, 1570–1577.
Cronin, M.T.D., Jaworska, J.S., Walker, J.D., Comber, M.H.I., Watts, C.D., Worth, A.P., 2003a. Use of QSARs in international decision-making frameworks to predict health effects of chemical substances. Environ. Health Perspect. 111, 1391–1401.
Cronin, M.T.D., Walker, J.D., Jaworska, J.S., Comber, M.H.I., Watts, C.D., Worth, A.P., 2003b. Use of QSARs in international decision-making frameworks to predict ecologic effects and environmental fate of chemical substances. Environ. Health Perspect. 111, 1376–1390.
de Jongh, J., Forsby, A., Houston, J.B., Beckman, M., Combes, R., Blaauboer, B.J., 1999. An integrated approach to the prediction of systemic toxicity using computer-based biokinetic models and biological *in vitro* test methods: overview of a prevalidation study based on the ECITTS project. Toxicol. In Vitro 13, 549–554.
deBruyn, A.M.H., Gobas, F.A.P.C., 2007. The sorptive capacity of animal protein. Environ. Toxicol. Chem. 26, 1803–1808.
DelRaso, N.J., Foy, B.D., Gearhart, J.M., Frazier, J.M., 2003. Cadmium uptake kinetics in rat hepatocytes: correction for albumin binding. Toxicol. Sci. 72, 19–30.
ECHA2009, Press Release ECHA/PR/09/11 2009. New study on the number of test animals for REACH.
Eisentraeger, A., Dott, W., Klein, J., Hahn, S., 2003. Comparative studies on algal toxicity testing using fluorometric microplate and Erlenmeyer flask growth-inhibition assays. Ecotoxicol. Environ. Saf. 54, 346–354.
Ekwall, B., 1999. Overview of the final MEIC results: II. The *in vitro–in vivo* evaluation: including the selection of a practical battery of cell tests for prediction of acute lethal blood concentrations in humans. Toxicol. In Vitro 13, 665–673.
Ekwall, B., 1995. The basal cytotoxicity concept. In: Goldberg, A.M., van Zupthen, L.F.M. (Eds.), The World Congress on Alternatives and Animal Use in the Life Sciences: Education, Research, Testing. Mary Ann Liebert, New York, pp. 721–725.
Ekwall, B., 1983. Screening of toxic compounds in mammalian cell cultures. Ann. N.Y. Acad. Sci. 407, 64–77.
Endo, S., Escher, B.I., Goss, K.U., 2011. Capacities of membrane lipids to accumulate neutral organic chemicals. Environ. Sci. Technol. 45, 5912–5921.
Endo, S., Goss, K.U., 2011. Serum albumin binding of structurally diverse neutral organic compounds: data and models. Chem. Res. Toxicol. 24, 2293–2301.
Escher, B.I., Hermens, J.L.M., 2004. Internal exposure: linking bioavailability to effects. Environ. Sci. Technol. 38, 455A–462A.
Escher, B.I., Hermens, J.L.M., 2002. Modes of action in ecotoxicology: their role in body burdens, species sensitivity, QSARs, and mixture effects. Environ. Sci. Technol. 36, 4201–4217.
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.
Escher, B.I., Ashauer, R., Dyer, S., Hermens, J.L.M., Lee, J., Leslie, H.A., Mayer, P., Meador, J.P., Warne, 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. Manage. 7, 28–49.
EU Parliament and Council, 2006. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006. Concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. Off. J. Eur. Union L136, 3–280.
EU Parliament and Council, 2003. Directive 2003/15/EC of the European Parliament and of the Council of 27 February 2003 amending Council Directive 76/768/EEC on the Approximation of the Laws of the Member States Relating to Cosmetic Products. http://ec.europa.eu.proxy.library.uu.nl/consumers/sectors/cosmetics/documents/directive/
Gellert, G., Stommel, A., 1999. Influence of microplate material on the sensitivity of growth inhibition tests with bacteria assessing toxic organic substances in water and waste water. Environ. Toxicol. 14, 424–428.
Gobas, F.A.P.C., Lahittete, J.M., Garofalo, G., Wan, Y.S., Mackay, D., 1988. A novel method for measuring membrane-water partition-coefficients of hydrophobic organic-chemicals - comparison with 1-octanol-water partitioning. J. Pharmacol. Sci. 77, 265–272.
Griffith, L.G., Swartz, M.A., 2006. Capturing complex 3D tissue physiology *in vitro*. Nat. Rev. Mol. Cell Biol. 7, 211–224.
Gubbels-van Hal, W.M.L.G., Blaauboer, B.J., Barentsen, H.M., Hoitink, M.A., Meerts, I.A.T.M., van der Hoeven, J.C.M., 2005. An alternative approach for the safety evaluation of new and existing chemicals, an exercise in integrated testing. Regul. Toxicol. Pharm. 42, 284–295.
Guillouzo, A., 1998. Liver cell models in *in vitro* toxicology. Environ. Health Perspect. 106, 511–532.
Gülden, M., Morchel, S., Seibert, H., 2001. Factors influencing nominal effective concentrations of chemical compounds *in vitro*: cell concentration. Toxicol. In Vitro 15, 233–243.
Gulden, M., Seibert, H., 1997. Influence of protein binding and lipophilicity on the distribution of chemical compounds in *in vitro* systems. Toxicol. In Vitro 11, 479–483.
Gülden, M., Seibert, H., 2005. Impact of bioavailability on the correlation between *in vitro* cytotoxic and *in vivo* acute fish toxic concentrations of chemicals. Aquat. Toxicol. 72, 327–337.
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.
Gülden, M., Jess, A., Kammann, J., Maser, E., Seibert, H., 2010. Cytotoxic potency of H_2O_2 in cell cultures: impact of cell concentration and exposure time. Free Radic. Biol. Med. 49, 1298–1305.
Han, X., Corson, N., Wade-Mercer, P., Gelein, R., Jiang, J., Sahu, M., Biswas, P., Finkelstein, J.N., Elder, A., Oberdorster, G., 2012. Assessing the relevance of in vitro studies in nanotoxicology by examining correlations between in vitro and in vivo data. Toxicology 297, 1–9.
Hartung, T., 2010. Food for thought. . .on alternative methods for chemical safety testing. ALTEX 27, 3–14.
Hartung, T., 2011. From alternative methods to a new toxicology. Eur. J. Pharm. Biopharm. 77, 338–349.
Hartung, T., Rovida, C., 2009. Chemical regulators have overreached. Nature 460, 1080–1081.
Hayeshi, R., Masimirembwa, C., Mukanganyama, S., Ungell, A.L.B., 2008. Lysosomal trapping of amodiaquine: impact on transport across intestinal epithelia models. Biopharm. Drug Dispos. 29, 324–334.
Health Council of the Netherlands, 2001. Toxicity Testing: A More Efficient Approach. The Hague, Publication no. 2001/24E.
Heringa, M.B., Hermens, J.L.M., 2003. Measurement of free concentrations using negligible depletion-solid phase microextraction (nd-SPME). TrAC, Trends Anal. Chem. 22, 575–587.
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.
Hervé, F., Urien, S., Albengres, E., Duche, J.C., Tillement, J.P., 1994. Drug-binding in plasma—a summary of recent trends in the study of drug and hormone-binding. Clin. Pharmacokinet. 26, 44–58.
Hestermann, E.V., Stegeman, J.J., Hahn, M.E., 2000. Serum alters the uptake and relative potencies of halogenated aromatic hydrocarbons in cell culture bioassays. Toxicol. Sci. 53, 316–325.
Hofer, T., Gerner, I., Gundert-Remy, U., Liebsch, M., Schulte, A., Spielmann, H., Vogel, R., Wettig, K., 2004. Animal testing and alternative approaches for the human health risk assessment under the proposed new European chemicals regulation. Arch. Toxicol. 78, 549–564.
Howard, M.L., Hill, J.J., Galluppi, G.R., McLean, M.A., 2010. Plasma protein binding in drug discovery and development. Comb. Chem. High Throughput Screening 13, 170–187.
Jager, T., Kooijman, S.A.L.M., 2005. Modeling receptor kinetics in the analysis of survival data for organophosphorus pesticides. Environ. Sci. Technol. 39, 8307–8314.
Jager, T., Albert, C., Preuss, T.G., Ashauer, R., 2011. General unified threshold model of survival—a toxicokinetic–toxicodynamic framework for ecotoxicology. Environ. Sci. Technol. 45, 2529–2540.
Jonker, M.T.O., van der Heijden, S.A., 2007. Bioconcentration factor hydrophobicity cutoff: an artificial phenomenon reconstructed. Environ. Sci. Technol. 41, 7363–7369.
Katagi, T., 2001. Partition of 2,4-dichlorophenoxyacetic acid derivatives in phosphatidylcholine multi-lamellar vesicles. J. Pestic. Sci. 26, 354–360.
Kedderis, G.L., Carfagna, M.A., Held, S.D., Batra, R., Murphy, J.E., Gargas, M.L., 1993. Kinetic-analysis of furan biotransformation by F344 rats *in-vivo* and *in-vitro*. Toxicol. Appl. Pharm. 123, 274–282.
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.
Kiparissis, Y., Akhtar, P., Hodson, P.V., Brown, R.S., 2003. Partition-controlled delivery of toxicants: a novel *in vivo* approach for embryo toxicity testing. Environ. Sci. Technol. 37, 2262–2266.
Knöbel, M., Busser, F.J.M., Rico-Rico, A., Kramer, N.I., Hermens, J.L.M., Hafner, C., Tanneberger, K., Schirmer, K., Scholz, S., 2012. Predicting adult fish acute lethality with the zebrafish embryo: relevance of test duration, endpoints, compound properties, and exposure concentration analysis. Environ. Sci. Technol. 46, 9690–9700.
Könemann, H., 1981. Quantitative structure–activity-relationships in fish toxicity studies. 1. Relationship for 50 industrial pollutants. Toxicology 19, 209–221.
Kramer, N.I., Busser, F.J.M., Oosterwijk, M.T.T., Schirmer, K., Escher, B.I., Hermens, J.L.M., 2010. Development of a partition-controlled dosing system for cell assays. Chem. Res. Toxicol. 23, 1806–1814.
Kramer, N.I., Krismartina, M., Rico-Rico, A., Blaauboer, B.J., Hermens, J.L.M., 2012. Quantifying processes determining the free concentration of phenanthrene in basal cytotoxicity assays. Chem. Res. Toxicol. 25, 436–445.
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.
Kramer, S.D., Braun, A., Jakits-Deiser, C., Wunderli-Allenspach, H., 1998. Towards the predictability of drug–lipid membrane interactions: the pH-dependent affinity of propranolol to phosphatidylinositol containing liposomes. Pharmacol. Res. 15, 739–744.
Kratochwil, N.A., Huber, W., Müller, F., Kansy, M., Gerber, P.R., 2004. Predicting plasma protein binding of drugs—revisited. Curr. Opin. Drug Discovery Dev. 7, 507–512.
Legierse, K.C.H.M., Verhaar, H.J.M., Vaes, W.H.J., De Bruijn, J.H.M., Hermens, J.L.M., 1999. Analysis of the time-dependent acute aquatic toxicity of organophosphorus pesticides: the critical target occupation model. Environ. Sci. Technol. 33, 917–925.

Lin, T.H., Lin, J.H., 1990. Effects of protein binding and experimental disease states on brain uptake of benzodiazepines in rats. J. Pharmacol. Exp. Ther. 253, 45–50.
Lin, Z., Will, Y., 2012. Evaluation of drugs with specific organ toxicities in organ-specific cell lines. Toxicol. Sci. 126, 114–127.
Lipscomb, J.C., Haddad, S., Poet, T., Krishnan, K., 2012. Physiologically-based pharmacokinetic (PBPK) models in toxicity testing and risk assessment. Adv. Exp. Med. Biol. 745, 76–95.
Lord, H., Pawliszyn, J., 2000. Evolution of solid-phase microextraction technology. J. Chromatogr. A 885, 153–193.
Louisse, 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.
Mackay, D., Arnot, J.A., Petkova, E.P., Wallace, K.B., Call, D.J., Brooke, L.T., Veith, G.D., 2009. The physicochemical basis of QSARs for baseline toxicity. SAR QSAR Environ. Res. 20, 393–414.
Mayer, P., Wernsing, J., Tolls, J., De Maagd, P.G.J., Sijm, D.T.H.M., 1999. Establishing and controlling dissolved concentrations of hydrophobic organics by partitioning from a solid phase. Environ. Sci. Technol. 33, 2284–2290.
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. Manage. 7, 7–27.
McCarty, L.S., Mackay, D., Smith, A.D., Ozburn, G.W., Dixon, D.G., 1992. Residue-based interpretation of toxicity and bioconcentration QSARs from aquatic bioassays—neutral narcotic organics. Environ. Toxicol. Chem. 11, 917–930.
McCarty, L., Mackay, D., 1993. Enhancing ecotoxicological modeling and assessment. Environ. Sci. Technol. 27, 1719–1728.
Mizuno, N., Niwa, T., Yotsumoto, Y., Sugiyama, Y., 2003. Impact of drug transporter studies on drug discovery and development. Pharmacol. Rev. 55, 425–461.
Mori, M., Wakabayashi, M., 2000. Cytotoxicity evaluation of synthesized chemicals using suspension-cultured fish cells. Fish. Sci. 66, 871–875.
Musteata, F.M., Pawliszyn, J., Qian, M.G., Wu, J.-., Miwa, G.T., 2006. Determination of drug plasma protein binding by solid phase microextraction. J. Pharmacol. Sci. 95, 1712–1722.
Musteata, F.M., de Lannoy, I., Gien, B., Pawliszyn, J., 2008. Blood sampling without blood draws for *in vivo* pharmacokinetic studies in rats. J. Pharmaceut. Biomed. 47, 907–912.
Nielsen, E.I., Viberg, A., Lowdin, E., Cars, O., Karlsson, M.O., Sandstrom, M., 2007. Semimechanistic pharmacokinetic/pharmacodynamic model for assessment of activity of antibacterial agents from time-kill curve experiments. Antimicrob. Agents Chemother. 51, 128–136.
NRC2007, 2007. National research council, committee on toxicity testing and assessment of environmental agents. In: Toxicity Testing in the 21st Century: A Vision and a Strategy. The National Academies Press, pp. 1–216.
Nyman, A., Schirmer, K., Ashauer, R., 2012. Toxicokinetic-toxicodynamic modelling of survival of *Gammarus pulex* in multiple pulse exposures to propiconazole: model assumptions, calibration data requirements and predictive power. Ecotoxicology 21, 1828–1840.
OECD, 2011. OECD guideline for testing of chemicals. Section 2: effects on biotic systems. In: Guideline 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test. Organisation for Economic Cooperation and Development, Paris, France.
OECD, 2006a. OECD Guideline for testing of chemicals. Draft proposal for a new guideline. In: Fish Embryo Toxicity (FET) Test. Organisation for Economic Cooperation and Development, Paris, France.
OECD, 2006b. OECD guideline for testing of chemicals. Section 2: Effects on biotic systems. In: Guideline 221: Lemna sp. Growth Inhibition Test. Organisation for Economic Cooperation and Development, Paris, France.
Oravcová, J., Böhs, B., Lindner, W., 1996. Drug-protein binding studies new trends in analytical and experimental methodology. J. Chromatogr. B 677, 1–28.
Paustenbach, D., 2000. The practice of exposure assessment: a state-of-the-art review (Reprinted from Principles and Methods of Toxicology, 4th ed., 2001). J. Toxicol. Environ. Health 3, 179–291.
Poirier, A., Lave, T., Portmann, R., Brun, M., Senner, F., Kansy, M., Grimm, H., Funk, C., 2008. Design, data analysis, and simulation of *in vitro* drug transport kinetic experiments using a mechanistic *in vitro* model. Drug Metab. Dispos. 36, 2434–2444.
Punt, A., Brand, W., Murk, A.J., van Wezel, A.P., Schriks, M., Heringa, M.B., 2013. Effect of combining *in vitro* estrogenicity data with kinetic characteristics of estrogenic compounds on the *in vivo* predictive value. Toxicol. In Vitro 27, 44–51.
Rappaport, S.M., 2011. Implications of the exposome for exposure science. J. Exposure Anal. Environ. Epidemiol. 21, 5–9.
Rappaport, S.M., Smith, M.T., 2010. Environment and disease risks. Science 330, 460–461.
Reinert, K.H., Giddings, J.A., Judd, L., 2002. Effects analysis of time-varying or repeated exposures in aquatic ecological risk assessment of agrochemicals. Environ. Toxicol. Chem. 21, 1977–1992.
Riedl, J., Altenburger, R., 2007. Physicochemical substance properties as indicators for unreliable exposure in microplate-based bioassays. Chemosphere 67, 2210–2220.
Schirmer, K., Chan, A.G.J., Greenberg, B.M., Dixon, D.G., Bols, N.C., 1997. Methodology for demonstrating and measuring the photocytotoxicity of fluoranthene to fish cells in culture. Toxicol. In Vitro 11, 107–119.
Schirmer, K., 2006. Proposal to improve vertebrate cell cultures to establish them as substitutes for the regulatory testing of chemicals and effluents using fish. Toxicology 224, 163–183.
Schmitt, W., 2008. General approach for the calculation of tissue to plasma partition coefficients. Toxicol. Vitro 22, 457–467.
Schnell, S., Kawano, A., Porte, C., Lee, L.E.J., Bols, N.C., 2009. Effects of ibuprofen on the viability and proliferation of rainbow trout liver cell lines and potential problems and interactions in effects assessment. Environ. Toxicol. 24, 157–165.
Schreiber, R., Altenburger, R., Paschke, A., Kuester, E., 2008. How to deal with lipophilic and volatile organic substances in microtiter plate assays. Environ. Toxicol. Chem. 27, 1676–1682.
Seibert, H., Morchel, S., Gülden, M., 2002. Factors influencing nominal effective concentrations of chemical compounds *in vitro*: medium protein concentration. Toxicol. In Vitro 16, 289–297.
Smith, K.E.C., Dom, N., Blust, R., Mayer, P., 2010. Controlling and maintaining exposure of hydrophobic organic compounds in aquatic toxicity tests by passive dosing. Aquat. Toxicol. 98, 15–24.
Smith, K.E.C., Heringa, M.B., Uytewaal, M., Mayer, P., 2013. The dosing determines mutagenicity of hydrophobic compounds in the Ames II assay with metabolic transformation: passive dosing versus solvent spiking. Mutat. Res.: Genet. Toxicol. Environ. Mutagen. 750, 12–18.
Tanneberger, K., Rico-Rico, A., Kramer, N.I., Busser, F.J.M., Hermens, J.L.M., Schirmer, K., 2010. Effects of solvents and dosing procedure on chemical toxicity in cell-based *in vitro* assays. Environ. Sci. Technol. 44, 4775–4781.
Ulrich, S., 2000. Solid-phase microextraction in biomedical analysis. J. Chromatogr. A 902, 167–194.
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.
Vaes, W.H.J., Ramos, E.U., Verhaar, H.J.M., Seinen, W., Hermens, J.L.M., 1996. Measurement of the free concentration using solid-phase microextraction: binding to protein. Anal. Chem. 68, 4463–4467.
Vaes, W., Ramos, E., Hamwijk, C., vanHolsteijn, I., Blaauboer, B., Seinen, W., Verhaar, H., Hermens, J., 1997. Solid phase microextraction as a tool to determine membrane/water partition coefficients and bioavailable concentrations in *in vitro* systems. Chem. Res. Toxicol. 10, 1067–1072.
van der Hoeven, N., Gerritsen, A.A.M., 1997. Effects of chlorpyrifos on individuals and populations of *Daphnia pulex* in the laboratory and field. Environ. Toxicol. Chem. 16, 2438–2447.
Verhaar, H.J.M., De Wolf, W., Dyer, S., Legierse, K.C.H.M., Seinen, W., Hermens, J.L.M., 1999. An LC50 vs. time model for the aquatic toxicity of reactive and receptor-mediated compounds. Consequences for bioconcentration kinetics and risk assessment. Environ. Sci. Technol. 33, 758–763.
Verwei, M., van Burgsteden, J.A., Krul, C.A.M., van de Sandt, J.J.M., Freidig, A.P., 2006. Prediction of *in vivo* embryotoxic effect levels with a combination of *in vitro* studies and PBPK modelling. Toxicol. Lett. 165, 79–87.
Vuckovic, D., Shirey, R., Chen, Y., Sidisky, L., Aurand, C., Stenerson, K., Pawliszyn, J., 2009. *In vitro* evaluation of new biocompatible coatings for solid-phase microextraction: implications for drug analysis and *in vivo* sampling applications. Anal. Chim. Acta 638, 175–185.
Webborn, P.J.H., Parker, A.J., Denton, R.L., Riley, R.J., 2007. *In vitro–in vivo* extrapolation of hepatic clearance involving active uptake: theoretical and experimental aspects. Xenobiotica 37, 1090–1109.
Wilkening, S., Stahl, F., Bader, A., 2003. Comparison of primary human hepatocytes and hepatoma cell line HEPG2 with regard to their biotransformation properties. Drug Metab. Dispos. 31, 1035–1042.
Yuan, H., Ranatunga, R., Carr, P.W., Pawliszyn, J., 1999. Determination of equilibrium constant of alkylbenzenes binding to bovine serum albumin by solid phase microextraction. Analyst 124, 1443–1448.