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In vitro to *in vivo* extrapolation of effective dosimetry in developmental toxicity testing: Application of a generic PBK modelling approach

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ABSTRACT

Incorporation of kinetics to quantitative *in vitro* to *in vivo* extrapolations (QIVIVE) is a key step for the realization of a non-animal testing paradigm, in the sphere of regulatory toxicology. The use of Physiologically-Based Kinetic (PBK) modelling for determining systemic doses of chemicals at the target site is accepted to be an indispensable element for such purposes. Nonetheless, PBK models are usually designed for a single or a group of compounds and are considered demanding, with respect to experimental data needed for model parameterization. Alternatively, we evaluate here the use of a more generic approach, *i.e.* the so-called IndusChemFate model, which is based on incorporated QSAR model parametrization. The model was used to simulate the *in vivo* kinetics of three diverse classes of developmental toxicants: triazoles, glycol ethers' alkoxyacetic acid metabolites and phthalate primary metabolites. The model required specific input per each class of compounds. These compounds were previously tested in three alternative assays: the whole-embryo culture (WEC), the zebrafish embryo test (ZET), and the mouse embryonic stem cell test (EST). Thereafter, the PBK-simulated blood levels at toxic *in vivo* doses were compared to the respective *in vitro* effective concentrations. Comparisons pertaining to relative potency and potency ranking with integration of kinetics were similar to previously obtained comparisons. Additionally, all three *in vitro* systems produced quite comparable results, and hence, a combination of alternative tests is still preferable for predicting the endpoint of developmental toxicity *in vivo*. This approach is put forward as biologically more plausible since plasma concentrations, rather than external administered doses, constitute the most direct *in vivo* dose metric.

1. Introduction

The transition from animal experiments to alternative mechanism-based *in vitro* assays or assays with lower organisms, as the main information source for chemical risk assessment, meets with significant challenges. To begin with, the toxicity endpoints examined *in vitro* diverge from those assessed *in vivo*, and hence, their relevance for the prediction of adversity at intact organism level needs extrapolation of the underlying toxicodynamics and toxicokinetics (Blaauboer, 2008; Blaauboer et al., 2012; Gülden and Seibert, 2005). This problem can partially be overcome by using a combination of *in vitro* and other alternative tests, which can measure several different endpoints and various mechanisms of toxicity, rather than one single assay (Gülden and Seibert, 2005; Kroese et al., 2015; Schenk et al., 2010; Piersma, 2006).

Another critical issue is the quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) of effective concentrations (Blaauboer, 2010; Gülden and Seibert, 2005) corresponding with the points of departure for the risk assessment. *In vitro* and *in vivo* exposure situations differ fundamentally, making such extrapolations complex. In the *in vitro* assays, the compound of interest is directly added to the assay medium, thereby allowing an apparently simple exposure situation when compared to the *in vivo* situation. However, even *in vitro* the exposure situation is not that obvious, as free (active) *versus* bound (inactive) compound fraction needs to be considered, as well as possible time-dependent decomposition and/or evaporation of the test substance from the culture medium (Kramer et al., 2012; Groothuis et al., 2015). Similarly, *in vivo*, binding of a substance into plasma or serum proteins will make it unavailable for diffusion/transport across cell membranes (Alder et al., 2011; Banker and Clark, 2008). In addition, in an intact organism the route from external exposure to the target organ is confounded with absorption, distribution, metabolism and excretion (ADME) characteristics, determining actual target organ exposure levels both in terms of concentration and in terms of time-dependency. Such processes are lacking in *in vitro* systems.

Clearly, linking the toxic dose metric measured *in vitro* and the *in vivo* relevant effective dose, requires the integration of kinetics of both systems (Alder et al., 2011; NRC, 2007; Blaauboer, 2010). Here the use of Physiologically Based Kinetic (PBK) modelling is deemed to be a key element (Alder et al., 2011; Bessems et al., 2014; Bouvier d'Yvoire

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et al., 2007; Hartung et al., 2011; NRC, 2007; Punt et al., 2011; Yoon et al., 2015). PBK models can estimate the systemic effective doses of substances at a specific target site and *vice versa*, whereas with reverse dosimetry, they can be used for the prediction of external effective doses *in vivo* starting from the *in vitro* toxic concentrations, *i.e.* the presumed target doses (Alder et al., 2011; Blaauboer, 2008). Several PBK-reverse dosimetry approaches have been hitherto performed for different toxicity endpoints, as for example: neurotoxicity (Forsby and Blaauboer, 2007), nephrotoxicity (Abdullah et al., 2016) and hepatotoxicity (Klein et al., 2016). Furthermore reverse-dosimetry PBK modelling has been applied on high-throughput chemicals on the basis of *in vitro* assays on metabolism and protein binding and QSAR physical-chemical properties (Wambaugh et al., 2015; Wetmore et al., 2012, 2013). Here we will focus on the *in vitro*/*in vivo* extrapolation of the endpoint of developmental toxicity.

We previously correlated directly *in vitro* benchmark concentrations, occurring specifically from three alternative developmental toxicity models, with *in vivo* benchmark doses from existing animal experiments, for a series of embryotoxic compounds (de Jong et al., 2009, 2011; Hermsen et al., 2011; Piersma et al., 2008). The assays employed were the rodent post-implantation Whole-Embryo Culture method (WEC), the zebrafish embryo test (ZET), and the mouse embryonic stem cell test (EST). Amongst these the EST is the only test not requiring the sacrifice of animals, by utilizing a permanent murine cell line. The test uses the capacity of embryonic stem cells to differentiate *in vitro* to contracting cardiac myoblasts. Inhibition of this differentiation process, in the absence of cytotoxicity, is taken as predicting embryotoxicity (Scholz et al., 1999; Seiler and Spielmann, 2011). In contrast to EST, the WEC and ZET involve the development of whole embryos, either after explantation from a pregnant rat or using zebra fish eggs, respectively. In the WEC experimental model, the effects of substances given during a narrow exposure window (early organogenesis, gestation day (GD) 10-12) are examined in culture, after isolation of the embryos from pregnant animals (Chapin et al., 2008; Piersma et al., 2004). On the other hand, the ZET assesses chemical toxicity during up to 120 h of embryogenesis including hatching of the larvae (Brannen et al., 2010; Hill et al., 2005). The advantage of both tests is that they mirror general morphogenesis, at least within a given developmental time window, due to their use of the whole embryo, rather than a plain cell-line (Chapin et al., 2008).

In that research, it was demonstrated that for a more meaningful extrapolation of such alternative methods *in vivo*, integration of kinetics is necessary. Inevitably, this presupposes that the alternative systems sufficiently represent the *in vivo* situation. Regarding the EST assay some examples have been published, where *in vitro* concentration-response data were translated into *in vivo* dose-response data with the use of PBK modelling (glycol ethers and retinoic acid: Louisse et al., 2010, 2015; phenols: Strikwold et al., 2013, 2016; glycol ethers: Verwei et al., 2006). Those studies aimed at deriving a predicted human *in vivo* point of departure to be used for risk assessment, and illustrate the potential of combining *in vitro* results and PBK modelling in deriving human toxicity standards. The PBK model parameters were derived either from combined *in vivo*/*in vitro* data (Verwei et al., 2006; Louisse et al., 2010), or solely from *in vitro* and *in silico* data (Strikwold et al., 2013, 2016).

Notwithstanding the fact that these studies proved the concept of reverse PBK dosimetry in deriving human toxicity standards, they all needed chemical specific PBK models, whereas the ever increasing number of chemicals would favour a more generic PBK modelling approach (Basketter et al., 2012; Bessems et al., 2014). Proving the suitability of a single PBK model concept, which only requires a minimum input of information, for different groups of chemicals, would facilitate the PBK application for extrapolation purposes, thereby facilitating an animal-free toxicity testing paradigm. Furthermore, the *in vitro* - *in vivo* extrapolation rests on the assumption that the *in vitro* concentrations have equal potency in inducing toxicity in the developing embryo. Finally, the applied reverse dosimetry was limited to the EST assay.

The objectives of the current study were therefore (1) to assess the feasibility of a generic PBK model in order to predict *in vivo* kinetics, (2) to correlate PBK predicted *in vivo* dosimetry, *i.e.* venous blood plasma concentrations corresponding to toxic *in vivo* effect levels, with the respective *in vitro* effect levels and (3) to extend the foregoing analysis beyond the EST assay, by including other relevant alternative developmental toxicity assays.

The PBK model IndusChemFate (Jongeneelen and Ten Berge, 2011) was used, a cross-chemical predictive model, readily accessible, and in a form of an MS EXCEL Spreadsheet. The features of IndusChemFate are in line with what has been previously suggested to form the basis for the build-up of generic PBK-platforms: relatively simple, open access, with inclusion of a physiological data base, multiple exposure routes (oral, inhalatory, dermal) (Basketter et al., 2012; Leist et al., 2014) and species applicability (human, rat, mouse).

Next to the EST, the WEC and the ZET assays were chosen as alternative embryotoxicity assays for comparison. Finally, three different classes of developmentally toxic chemicals were chosen as model compounds, *i.e.* six 1,2,4-triazole compounds, four glycol ether alkoxyacetic acid metabolites, and two monophthalates. These compounds represent three different classes in terms of challenges for PBK modelling, the complexity of the modelling moving from toxicity induced by the parent compound (triazoles), by hepatic formation of a primary metabolite (glycol ethers) or by metabolite formation in the gastrointestinal tract (phthalates), thereby allowing us to evaluate the extent to which the IndusChemFate model can generically be employed.

2. Materials and methods

2.1. In vivo toxicity data

2.1.1. 1,2,4-Triazoles derivatives. 1,2,4-Triazoles derivatives, referred to herein as triazoles, are fungicides some of which are known to induce developmental effects in laboratory animals (EFSA, 2009). The parent compound is known to be more potent than the metabolite free triazole (EFSA, 2009; FAO/WHO, 2008). Six members of the group were used: hexaconazole (HEX), flusilazole (FLU), cyproconazole (CYP), triadimefon (TDI), myclobutanyl (MYC), triticonazole (TTC). *In vivo* developmental toxicity studies were used as selected previously by de Jong et al. (2011). Study information was collected as presented in that article. In all studies the animal model was the rat, exposed orally (mostly by gavage) during gestation days 6 to 15 or 7 to 16. Other routes of administration and other species were not considered. Benchmark doses at a 10% effect size (BMD10) for skeletal variations[1] were determined by de Jong et al. (2011), as this was the most sensitive endpoint for most of the substances (with the exception of flusilazole and myclobutanyl). The order followed in Table 1 is based on the BMD10 values (most potent to least potent).

2.1.2. Glycol ethers. Likewise, existing *in vivo* data were collected for the glycol ethers. *In vivo* BMD10 values for critical embryo toxic endpoints (malformations, fetal viability, skeletal variations) were taken for the glycol ethers ethylene glycol methyl ether (EGME) and ethylene glycol ethyl ether (EGEE) from Hermsen et al. (2011), and for ethylene glycol butyl ether (EGBE) from Louisse et al. (2010). The corresponding developmental toxicity studies were found either in the published literature or in international evaluations of each compound by US EPA or the EU Risk Assessment Committee (RAC). A standard developmental toxicity test with the parent substance ethylene glycol phenyl ether (EGPE) was identified in the available REACH dossier for this substance (study performed by BASF found at ECHA's website). In this study the tested

[1] Skeletal variation (supernumerary ribs, extra ossification centers in ribs, unossified sternebrae) was chosen as a sensitive developmental endpoint, previously by de Jong et al. (2011). The BMR was defined as a 10% additional incidence of skeletal variations (de Jong et al., 2011).

Table 1
Developmental toxicity of the triazoles in rats.

Substance	Rat strain	Route	Exposure period	Dose (mg/kg bw/day)	dLEL (mg/kg bw/day)[a]	BMD10 skeletal variations (mg/kg bw/day)[a]
HEX	Wistar	Oral gavage	GD 7–16	0, 2.5, 25, 250	2.5	2.5
FLU	CRL:CD (SD)	Oral in diet	GD 7–16	0, 0.4, 2, 10, 50, 250	0.4[b]	2.9
CYP	Wistar	Oral gavage	GD 6–15	0, 6, 12, 24, 48	12	15.6
TDI	CRL:CD (SD)	Oral gavage	GD 6–15	0, 10, 25, 50, 100	50	26.9
MYC	SD	Oral gavage	GD 6–15	0, 31.3, 93.8, 312.6, 468.9	312.6[c]	314.8
TTC	CRL:CD (SD)	Oral gavage	GD 6–15	0, 40, 200, 1000	1000	1182.3[d]

[a] dLEL and BMD10 values taken from de Jong et al. (2011) (presented in the paper in μmol/kg, transformed here into mg/kg bw/day). The dLEL represents the Low Effect Level for the most sensitive endpoint, *i.e.* skeletal variations, except for the case of flusilazole and myclobutanyl (see below).
[b] This is the dLEL for flusilazole on urogenital malformations, while for skeletal development the dLEL was 10 mg/kg bw/day.
[c] dLEL for skeletal variations was not the most sensitive for myclobutanyl; decreased viability index was recorded at 93.8 mg/kg bw/day.
[d] BMD10 value is above the highest dose tested.

parent substance EGPE, did not exert any fetotoxic effect. In all four toxicity studies the animal species was the rat, exposed only *via* the oral route during specific days of the gestation period (see Table 2).

2.1.3. Phthalates. The *in vivo* information for two representatives of the phthalates, was taken from two studies (Table 2), as selected previously (Janer et al., 2008a). The two compounds were di(2-ethylhexyl) phthalate (DEHP) and di(n-butyl) phthalate (DBP), known to be developmentally toxic (Ema et al., 1993; Ema, 2002; ECB, 2008). As with the two previous sets of chemicals, priority was given to rat studies. The animals were treated orally by gavage during gestation days 7 to 15. BMD50 for malformations and resorptions/implantation loss and BMD05 for fetal body weights were determined (Janer et al., 2008a). For all endpoints the compounds had similar potencies, but the BMD05 for fetal body weight was used here, as the lowest BMD.

2.2. Physiologically based kinetic model

In order to simulate organ exposure, the PBK model IndusChemFate, developed by Jongeneelen and Ten Berge (Jongeneelen and Ten Berge, 2011), was applied to all the selected substances. This model comprises, next to the blood, twelve body compartments,[2] and it can be applied for different routes of exposure (dermal, inhalation or oral), for different species (man, rat, mice) and for different exposure durations (single peak *versus* repeated chronic exposure). In this study, we applied the model for the rat and for single and repeated, daily oral exposure. In order to mimic the fetal exposure, the chemical's average and average-peak concentration in the maternal blood were used as a proxy, in accordance with the developmental toxicity exposure windows. The model thus assumes that the maternal blood is an effective measure for fetal exposure to either of the investigated chemicals.

As input the model requires physiological/anatomical parameters (organ volumes, blood flows, cardiac output and alveolar ventilation), biochemical parameters (hepatic Michaelis-Menten kinetics, *i.e.*, maximal metabolic rate (V_{max}), affinity constant for the parent compound and metabolites (K_M) and physicochemical parameters (octanol-water partition coefficient, vapour pressure, molecular weight, water solubility and density). In the model the latter parameters are used for calculating blood concentration: organ partition coefficients and renal clearance. In IndusChemFate chemical metabolism is implemented in a sequential way, *i.e.*, the parent compound is only metabolized into its primary metabolite followed by metabolism of the primary metabolite into a secondary metabolite, *etc.* In the case of triazoles this results in the following principal metabolic pathway: biologically parent compound > less active metabolite. In the case of glycol ethers and phthalates the following pathway was modelled: parent compound → toxicologically active acidic metabolite.

[2] Twelve body compartments: lung, heart, brain, skin, adipose tissue, muscles, bone, bone marrow, stomach and intestines, liver and kidney.

Physiological/anatomical parameters for the rat were as described in the IndusChemFate user manual (version 2.00). Physicochemical parameters (organ:blood partition coefficients; renal clearance) were obtained from QSAR models (see Table 3). Three model software packages (KOWWIN, MPBPWIN and WSKOW) automatically give the experimental value used to derive the predictive model, whenever a substance is present in the PhysProp database which is bundled with the EpiSuite software containing the model software packages (US EPA, 2016). If an experimental value was available this was preferred over the estimated value. For this particular set of substances the differences between the experimental and estimated values were negligible. All models give a direct estimate of the needed PBK parameter, except for the estimate for the biochemical parameters, *i.e.* hepatic V_{max} and K_M, which is due to lack of general (freely available) QSAR estimation models for these parameters.

The biochemical parameters were obtained as follows. In the case of the triazoles the parent compound itself is known to be developmentally toxic (EFSA, 2009) and much more potent than the metabolite (EFSA, 2009; FAO/WHO, 2008). Hence, the kinetics of the parent fungicides are of major importance. For the modelling of the concentration of parent triazole compounds in maternal blood, the parent triazoles metabolic parameters, *i.e.* V_{max} and K_M values, are needed. For the triazoles used in this study no PBK models or *in vitro* metabolic data from which V_{max} or K_m may be obtained have yet been developed. However, for these compounds whole body half-lives ranging from 22 to 53 h are available (for details see Supplementary Data). Though the whole body half-life *per se* does not provide a PBK metabolism parameter, it allows for the setting of the ratio of the hepatic V_{max}/K_M values as a first-order metabolic rate constant in concordance with the whole body half-life.

For the glycol ethers not the parent compound, but a primary metabolite is responsible for the induced developmental toxicity (Brown et al., 1984; Cheever et al., 1984; Foster et al., 1984; Giavini et al., 1993), *i.e.* methoxyacetic acid (MAA), ethoxyacetic acid (EAA), butoxyacetic acid (BAA) and phenoxyacetic acid (PAA). Therefore, the kinetics of both the respective parent substance and its alkoxy acetic metabolite have to be modelled. Here existing glycol ether PBK models provided the necessary metabolism parameter information: Hays et al. (2000) on EGME metabolism to MAA and urinary excretion of MAA (observed plasma half-life: 20 h), Gargas et al. (2000) on the metabolism of EGEE to EAA and urinary excretion of EAA (observed plasma half-life: 8 h), Corley et al. (1994) on the metabolism of EGBE to BAA and urinary excretion of BAA (observed plasma half-life 1.5 h) and Troutman et al. (2015) on the metabolism of EGPE to PAA, the metabolism of PAA and the urinary excretion of PAA (observed plasma half-life: 0.7 h). These studies were also used for PBK model verification.

For the phthalates DEHP and DBP, the embryo toxic derivatives are their monoesters, mono(2-ethylhexyl)phthalate (MEHP) and mono(*n*-butyl) phthalate (MBP), respectively, rather than the parent di-ester (Janer et al., 2008a,b). The metabolites are formed in the GI tract by

Table 2
Developmental toxicity of the glycol ethers and two phthalates in rats

Substance	Rat strain	Route	Exposure period	Dose (mg/kg bw/day)	Critical endpoint	dLOAEL (mg/kg bw/day)	BMD10 (mg/kg bw/day)[a]	Reference
EGME (MAA)	SD	Oral, in diet	GD 7–18	0, 16, 31, 73, 140, 198, 290, 620	Fetal malformations: cardiovascular	31	38[b]	Nelson et al., 1989
EGEE (EAA)	Wistar	Oral gavage	GD 1–21	0, 11.5, 23, 46.5, 93, 186, 372	Skeletal variations & retardation	46.5	83[b]	Stenger et al., 1971[d]
EGBE (BAA)	F344	Oral gavage	GD 9–11	0, 30, 100, 200	Fetal viability (resorptions)	200	185[c]	Sleet et al., 1989[e]
EGPE (PAA)	Wistar	Oral gavage	GD 6–19	0, 100, 300, 1000	No effects on the fetus	NOAEL: 1000	–	ECHA disseminated REACH dossier[f]

Substance	Rat strain	Route	Exposure period	Dose (mg/kg bw/day)	Critical endpoint	dLOAEL (mg/kg bw/day)	BMD05 (mg/kg bw/day)	Reference
DEHP (MEHP)	Wistar	Oral gavage	GD 7–15	0, 40, 200, 1000	Growth	1000	507.7	Hellwig et al., 1997
DBP (MBP)	Wistar	Oral gavage	GD 7–15	0, 500, 630, 750, 1000	Growth	500 (lowest dose tested)	528.8	Ema et al., 1993

[a] BMD10 values were taken from the respective publications (see below) in μmol/kg bw/day and calculated back to mg/kg bw/day based on the molecular weight of the parent compound.
[b] Hermsen et al., 2011.
[c] Louisse et al., 2010.
[d] This is the original reference; however, the information presented was taken from the report of the EU RAC Committee on EGEE (2011).
[e] This is the original reference; however, data were collected from US EPA evaluation (US EPA, 2009) on EGBE.
[f] ECHA disseminated dossier on EGPE.

hydrolysis of the di-phthalates (Keys et al., 1999, 2000). Existing PBK models for DEHP (Keys et al., 1999) and DBP (Keys et al., 2000) indicate that this conversion occurs relative fast and that the formed metabolites are absorbed much faster that their respective parents. For this reason the exposure to DEHP and DBP was modelled as instantaneous conversion of an orally administered dose of these compounds to their monoalkyl metabolites, followed by absorption of the formed metabolites. As indicated by Keys et al. (1999) the DEHP → MEHP conversion amounted to 6.5%, *i.e.* an oral dose of 100 mg DEHP/kg bw/day resulting in the same systemic exposure to MEHP as an oral dose of 6.5 mg mono(2-ethylhexyl)phthalate/kg bw/day. Similarly, Keys et al. (2000) indicate 27% conversion of an oral DBP dose to MBP.

2.3. In vitro toxicity data

2.3.1. Triazoles. Existing *in vitro* data for the six triazoles were collected from earlier work, as published by de Jong et al. (2011) and Hermsen et al. (2011). The substances were evaluated in three developmental toxicity alternative assays, the WEC, the ZET and the EST. Critical concentration levels representing thresholds of adverse effects in each of the assays were compiled. The results of all three tests were previously analyzed with a Benchmark Dose (BMD) approach, with the use of the PROAST software, and are presented in the aforesaid publications. The following benchmark responses were used as dose metrics for the *in vitro* assays: in the WEC the concentration associated with a 5% decrease in the Total Morphological Score (TMS), *i.e.* the $BMC05_{TMS}$ (de Jong et al., 2011), in the ZET the 5% decrease on the General Morphology Score (GMS), *i.e.* the $BMC05_{GMS}$ (Hermsen et al., 2011), and in the EST the concentration corresponding with a 50% decrease in the number of culture wells with beating embryoid bodies, *i.e.* BMCd50 (de Jong et al., 2011) (see Table 4).

Table 3
Models used to estimate input parameters required for the generic PBK model.

Phys chem parameter	QSAR models used for prediction
Log D at pH 5.4 (intestines) and pH 7.4 (blood serum)	KOWWIN v1.68 (US EPA, 2016), with JChem estimated pKa/pKb for dissociating substances, (Szegezdi and Csizmadia, 2007)
Density (g/cm^3)	ChemSketch v.11 (ACD/ChemSketch, 2011)
Molecular weight (g/mol)	Calculated from structural formula
Vapour pressure (Pa)	MPBPWIN v1.43 (US EPA, 2016)
Water solubility (mg/L)	WSKOW v1.42 (US EPA, 2016)

2.3.2. Glycol ethers. Likewise, *in vitro* data were collected, as previously performed, for the glycol ethers, but in this case not for the parents, but for their embryotoxic alkoxyacetic acid metabolites. The *in vitro* data (Table 5) were taken from the published studies of de Jong et al. (2009) and Hermsen et al. (2011), for the EST and ZET, respectively. The tests were conducted directly with the toxic metabolites because the systems essentially lack metabolizing capacity (Piersma et al., 2004; Verwei et al., 2006). For the WEC, only data for three of the metabolites (excluding PAA) were available (Giavini et al., 1993).

As for the fungicides, the Benchmark Concentrations (BMCs) were obtained for ZET and EST from the published results, $BMC05_{GMS}$ (Hermsen et al., 2011) and BMCd50 (de Jong et al., 2009), respectively. BAA and PAA did not induce any embryotoxic effects in the ZET. For the WEC only LOAECs were available (Giavini et al., 1993), while no data were found for PAA in this assay.

2.3.3. Phthalates. For the two phthalates DEHP and DBP, *in vitro* data were collected for the respective embryotoxic monoesters (MEHP and MBP), for the WEC and EST (Table 5). Unfortunately, benchmark responses were not available for the EST assay, and hence the ID50 concentrations were used, as presented in the relevant paper (Schulpen et al., 2013). No information on the embryotoxic potential of the two monophthalates in the ZET assay could be identified in the public domain.

2.4. Correlation analysis

In order to determine correlations between the calculated PBK blood concentrations at the respective *in vivo* BMD10s and the corresponding *in vitro* BMC or LOAEC values, the triazole data were plotted against each

Table 4
Effect of the triazoles on embryonic development in the WEC and the ZET and on the differentiation of ES cells into beating cardiomyocytes (EST), as collected from published literature.[a]

Substance	WEC BMC05TMS (μM)[a]	ZET BMC05GMS (μM)[a]	EST BMCd50 (μM)[a]
FLU	19	4.8	5.7
CYP	335.9	27.7	31.8
TDI	178.6	29.2	32.2
HEX	149.9	7	16.6
MYC	138.6	30.2	30.5
TTC	272.1	80.5	35.8

[a] *In vitro* BMC values taken from de Jong et al., 2011.

Table 5
Effect of the glycol ethers alkoxyacetic acid metabolites on embryonic development in the WEC and the ZET and on the differentiation of ES cells into beating cardiomyocytes (EST).

Substance	WEC LOAEC (mM)[a]	ZET BMC05GMS (mM)[b]	EST BMCd50 (mM)[c]
MAA	0.1	2.7	2.4
EAA	0.2	3.1	3.4
BAA	0.4	No effect	5.2
PAA	Not tested	No effect	6.2
Substance	WEC BMC05 TMS (μM)[d]	No data on ZET	EST ID50 (μM)[e]
MEHP	600		410
MBP	2900		1440

[a] Giavini et al., 1993.
[b] Hermsen et al., 2011.
[c] de Jong et al., 2009; Presented here is the average between the two lab results (given separately in the publication).
[d] Janer et al., 2008.
[e] Schulpen et al., 2013.

other and analyzed with a power function in Microsoft Excel. This procedure is in concordance with previous work of our group (de Jong et al., 2011; Hermsen et al., 2011; Piersma et al., 2008).

3. Results

3.1. Verification of the PBK model

3.1.1. Triazoles. In the case of triazoles the available kinetic information was used for the calculation of the whole body half-life, which ranged from 22 h for triadimefon to 53 h for cyproconazole. Without exception, physicochemical QSARs incorporated in the PBK model indicated the lipophilic character for all of these compounds. As expected the PBK model showed highest triazole levels in the maternal adipose tissue. Note the relative small difference between the average blood concentration and the peak concentration (see Fig. 1 for flusilazole).

In a similar way, respective blood concentrations of the fungicide were calculated, for the dose range applied in the selected *in vivo* developmental toxicity study, *i.e.* orally 0.4–250 mg/kg bw/day during the whole exposure period, *i.e.* gestation days 7 to 16 (study data shown in Table 1). As the appropriate *in vivo* dose metric for fetal exposure, either the average or the peak-average 10-day maternal blood concentrations could be taken. The predicted blood levels were plotted against the external administered doses (Fig. 2). The relationship shown in Fig. 2 then was used to calculate the flusilazole average and peak-average concentrations at the BMD10 level of the *in vivo* toxicity experiment (in the case of fluzilazole 2.9 mg/kg bw/day). The results indicated that there is no substantial difference between the two blood concentrations (average and average-peak), and potentially they could both represent a suitable *proxy* for fetal exposure. The same procedure was repeated for each of the triazole compounds (data not shown).

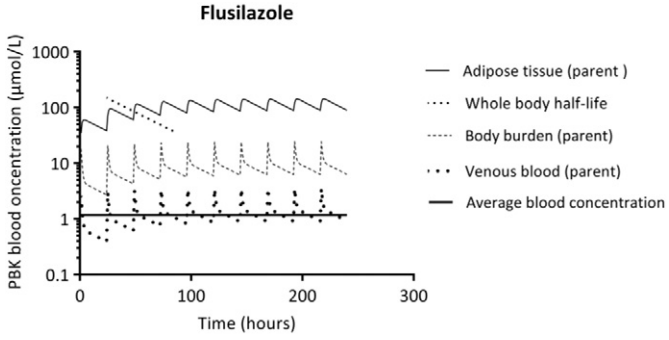

Fig. 1. Time-course PBK model simulation of flusilazole exposure for the whole body, adipose tissue, venous blood, after oral administration of the substance for 10 consecutive days (external dose used in the simulations is the 2 mg/kg bw/day, chosen from the doses tested in the developmental toxicity study).

3.1.2. Glycol ethers. In the case of glycol ethers existing PBK models provided suitable model verification data, *i.e.* time-course curves of both the parent compounds and its primary toxic acetic acid metabolite after gavage administration. Initial simulations revealed that the default IndusChemFate, predicting negligible urinary reabsorption of glycol ether metabolites, grossly overpredicted the clearance of such metabolites from the blood (data not shown). This overprediction could be avoided by incorporating substantial urinary reabsorption of glycol ether metabolites from tubular urine, the latter being in concordance with the modelling of the formation of glycolic acid from glycol (Corley et al., 2005). Fig. 3 illustrates the PBK simulations for EGME/MAA in venous blood as made with the modified IndusChemFate model, after oral exposure to the parent substance. The produced PBK results were close to the *in vivo* measured concentrations from the experimental study of Hays et al. (2000), albeit with slight underprediction (Fig. 3).

These results suggest that IndusChemFate can satisfactory estimate the *in vivo* blood levels of the primary metabolite MAA, after dosing of the parent compound. Similar results were found with the other three glycol ethers and their metabolites (see Supplementary Data).

As for the triazoles, the PBK model was used to predict both the average and peak-average venous blood concentrations of the glycol ethers' primary metabolites in the rat, after consecutive daily oral exposure to the parent substance, for the whole dose range given in the selected developmental toxicity study (Fig. 4).

The average and peak blood levels significantly differed for EAA, BAA and PAA, but not so much for MAA. The differences were more pronounced with the least potent compound BAA and with the non-developmentally toxic PAA (Fig. 4).

As with the triazoles, the PBK simulated relationship between the metabolite blood concentrations and the respective oral doses of the parent substances was determined and used to translate the *in vivo* external effect doses of the parent substance, as defined by the benchmark approach (BMD10), into blood concentrations of the corresponding alkoxyacetic acid metabolite, during the respective exposure period for each substance. As expected the difference between corresponding average and peak levels was remarkable (data not shown).

3.1.3. Phthalates. As a default the IndusChemFate PBK model describes chemical kinetics as perfusion limited, *i.e.* kinetics being limited by the blood flowing to the organs. As shown by chemical specific PBK models this concept is unable to describe phthalate kinetics (Keys et al., 1999, 2000). In concordance with the findings of Keys et al. indeed it was found that the default IndusChemfate PBK model leads to a gross overestimation of the concentration of mono-phthalate metabolites after gavage exposure to the parent diphthalate (data not shown). However, as also shown by Keys et al. the incorporation of enterohepatic circulation leads to a satisfactory description of phthalate kinetics. In the case of phthalates inherent enterohepatic circulation of the IndusChemFate model was taken into account. This led to a satisfactory description of the time-course of the blood concentrations of toxic monoester phthalates (Fig. 5), the PBK simulations are comparable to the verification data, *i.e. in vivo* measured concentrations from the experimental studies of Keys et al., 1999, 2000). As with the glycol ethers the produced simulations were close to measured data (Keys et al., 1999, 2000).

The PBK model was used to predict both the average and peak-average venous blood concentrations of monoesters in the rat, after daily gavage exposure to the di-esters, for the whole dose range given in the selected developmental toxicity study (Fig. 6). The two metrics differed between them clearly for both compounds.

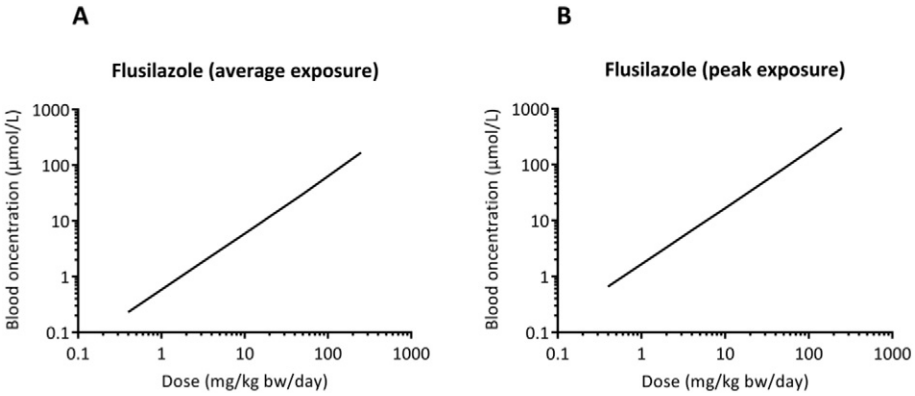

Fig. 2. Flusilazole average venous blood (A) and peak- average venous blood (B) concentration (μmol/L) in relation to the orally administered substance (mg/kg bw/day), for 10 consecutive days, as calculated by PBK-modelling.

As with the triazoles and glycol ethers the PBK simulated relationship between the metabolite blood concentrations and the respective oral doses of the parent substances was calculated, the latter being calculated as a % conversion of the parent phthalate. The resulting relationships were used to predict the plasma levels of the metabolite at the corresponding BMD05 on fetal growth (data not shown).

3.2. In vitro-in vivo comparisons

3.2.1. Triazoles. The fetotoxicity potency ranking of the triazoles resulting from the PBK model-predicted blood concentrations (average and peak), at the BMD10 level for skeletal variations, as well as from each alternative developmental toxicity test, is presented in Fig. 7. As a dose metric for the *in vitro* assays the corresponding BMC values are used (Table 1). The results show that the triazoles' effective internal concentration (average or average-peak) after oral exposure to each individual substance, produces the same potency ranking as that based on the BMD10 values. Hexaconazole seems to be the most potent compound of all six. The overall ranking is as follows: hexaconazole > flusilazole > cyproconazole > triadimefon > myclobutanyl > triticonazole. On the other hand, none of the three alternative assays could rank 100% correctly all six compounds, in agreement with their *in vivo* potency.

For the *in vivo-in vitro* correlations the average blood concentration was chosen as dose metric. Fig. 8 illustrates the triazoles predicted average blood concentrations at the *in vivo* BMD10 levels (skeletal variations), in correlation with the BMC values for the triazole compounds, as found in the three alternative developmental toxicity assays. The highest correlation with a coefficient R^2 of 0.85 was produced with the results from the ZET. The EST showed a moderate correlation (R^2: 0.54), and the WEC a low correlation (R^2: 0.29).

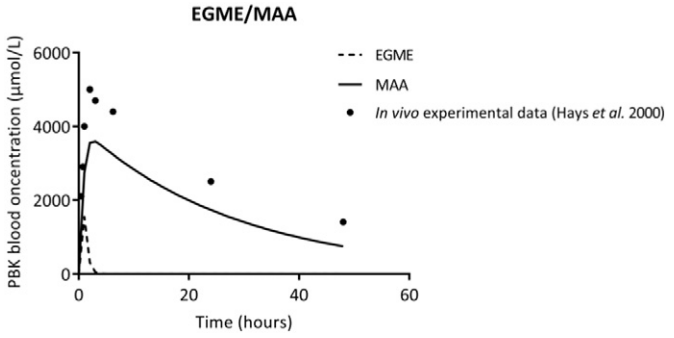

Fig. 3. Time-course PBK model simulation of the parent substance EGME and its metabolite MAA concentrations in the venous blood, after a single oral administration of EGME (3.3 mmol/kg bw) in the rat. PBK model incorporating reabsorption of MAA from tubular urine.

The line of identity (slope equal to 1) was drawn, in order to depict the absolute differences between effect levels recorded *in vitro* and *in vivo* estimated blood levels (Fig. 8); if the data points are precisely on the line of identity the *in vitro* and PBK effect levels are exactly the same. Results within the same order of magnitude are considered to be comparable, since such differences can stem solely from biological variation (Janer et al., 2008a). The compounds TDI and CYP were (almost) on the line of identity for both the ZET and the EST assays. MYC effective concentrations (*in vitro vs. in vivo*) differed more than one order of magnitude in these two tests (13.9-fold and 13.7-fold, respectively), with a lower potency *in vivo*. The same was seen for TTC in the ZET (14.9-fold), but a higher difference (33.6-fold) was detected with the EST. Again here, as for MYC, the compound was shown to be more toxic *in vitro* than *in vivo*. In the WEC the main outliers are HEX and CYP, for which the potency is under-predicted (differences with blood effect levels: 110-fold, 15-fold, respectively). The fungicide FLU was borderline in respect to the 10-fold scale (11-fold), which is considered to be a normal variation in *in vivo* toxicity studies (Janer et al., 2008b).

3.2.2. Glycol ethers. In contrast to the result with the triazoles, the embryotoxicity potency ranking of the glycol ethers' primary metabolites, *i.e.* the alkoxy acetic acids, as resulting from the PBK model-predicted blood concentrations, appeared different for the average and average-peak blood concentrations (Fig. 9). The ranking for the internal estimated concentrations corresponding to the peak exposures was in agreement with the order as sorted with the *in vivo* BMD10 values: MAA > EAA > BAA. This outcome suggests that in the case of glycol ethers the developmental effect might be primarily driven by the peak exposures rather than the average exposures of the embryo. The *in vitro* potency ranking of the glycol ether metabolites was the same as the *in vivo* BMD10 ranking, confirming the *in vitro in vivo* extrapolation. BAA and PAA did not induce any embryotoxic effects in the ZET. This is in fact in agreement with the *in vivo* data. BAA is embryotoxic at doses at which maternal toxicity is also observed (Sleet et al., 1989). In the oral developmental toxicity selected for EGPE, the substance, and consequently its metabolite, did not exert any adverse effects on the fetal development. Hence, comparison with these two compounds was not possible.

For the *in vivo-in vitro* correlations both the average and average-peak blood concentrations were used. Fig. 10 (A and B) illustrates the metabolites' predicted blood concentrations at the *in vivo* BMD10 levels, in correlation with the BMC values for the metabolites, as found in the two alternative developmental toxicity assays. The line of identity in the graphs demonstrates that in the WEC test the effective concentrations are slightly overestimated, as compared to the PBK simulated *in vivo* situation. On the other hand, in the EST the BMC values are slightly higher in relation to the predicted effect blood concentrations at the BMD levels. This outcome indicates that the alternative assays might

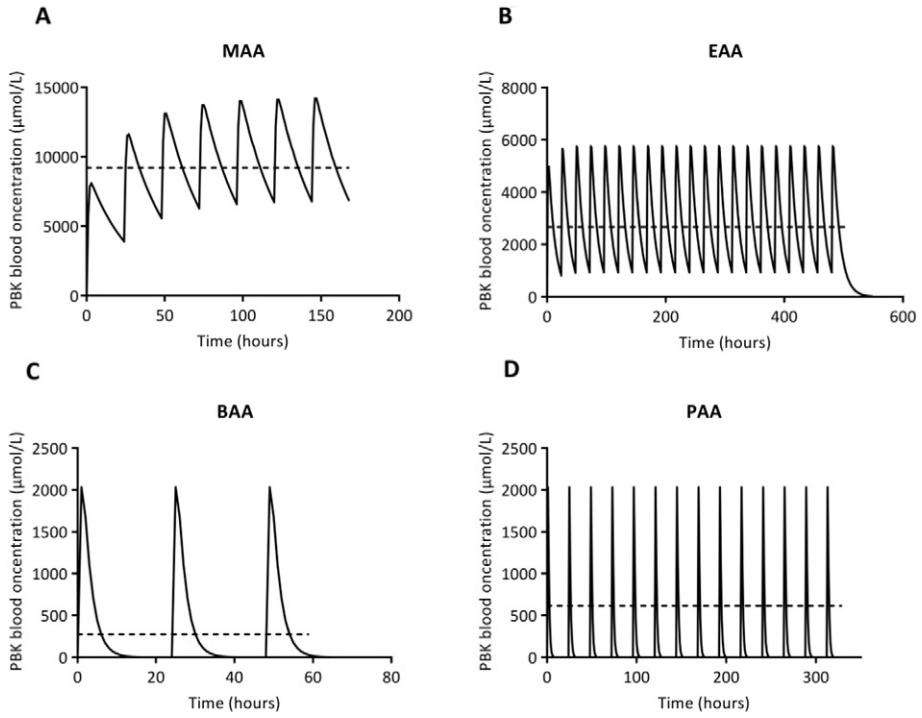

Fig. 4. (A) Time-course PBK model simulation of MAA in venous blood, after oral administration of eight consecutive daily doses of the parent EGME (dose: 620 mg/kg bw/day). The average venous blood terminal half-life of MAA was 20 h. (B) Time-course model simulation of EAA in venous blood, after oral administration of 21 consecutive daily doses of the parent EGEE (dose: 372 mg/kg bw/day). The venous blood terminal half-life of EAA was 8 h. (C) Time-course model simulation of BAA in venous blood, after oral administration of three consecutive daily doses of the parent EGBE (dose: 200 mg/kg bw/day). The venous blood terminal half-life of BAA was 1.5 h. (D) Time-course model simulation of PAA in venous blood, after oral administration of 14 consecutive daily doses of the parent EGPE (dose: 300 mg/kg bw/day). The venous blood terminal half-life of PAA was 0.7 h.

be over-sensitive or under-sensitive with respect to predicting the observed *in vivo* toxic effects. Nonetheless, with respect to the calculated peak blood concentrations, equivalent to the *in vivo* BMD10s (critical endpoints), the predicted effect levels by both tests did not differ more than 4.5-fold (2- to 4.5-fold).

3.2.3. Phthalates. *In vivo*, DEHP and DBP have comparable potency based on the two selected studies, with similar BMD05 values on fetal growth and BMD50s on malformations (Janer et al., 2008a,b). Nevertheless, in the *in vitro* assays WEC and EST, the presumed toxic monophthalate MEHP appears to be more potent than MBP (3.5- to almost 5-fold), which could perhaps be a result of kinetics differences.

Indeed, the estimated blood concentrations of the two metabolites, corresponding to the BMD05 of fetal growth (or the BMD50s on malformations), have larger relative differences than the external BMD05 values. In particular, the peak plasma concentration of MBP was almost 10-fold higher than the respective blood level for the MEHP monoester, despite the similarity in the BMD05 doses. This outcome demonstrates a difference in potency between the two metabolites, in agreement with the observations of the two alternative tests. The PBK calculated peak blood concentrations at the BMD05 and the effect levels measured in the *in vitro* assays differ an order of magnitude or less, *i.e.* they are within the expected variation observed also in animal experiments *in vivo* (Janer et al., 2008b). However, this is not the case for the average blood concentrations (Fig. 11).

4. Discussion

A pivotal step towards QIVIVE concerns the correlation of the toxic potency of compounds in *in vitro* systems with that observed *in vivo*. As a first tier this may be obtained by direct comparison of *in vitro* biologically active concentrations with *in vivo* effective doses. This comparison may be improved by comparing biologically active *in vitro* and *in vivo* concentration at the cellular level, *i.e.* the concentration delivered at the relevant target site. *In vitro* this is relatively easy, initially being the nominal concentrations added to the cells. However, *in vivo* the delivery of an administered dose to the cells is subsequently affected by the ADME processes. In other words, linking *in vitro* to *in vivo* dosimetry needs the integration of the kinetics of both systems. Though *in vivo* kinetic PBK models have been shown to be an indispensable tool here, they are mostly chemical- (Li et al., 2017; Louisse et al., 2015; Strikwold et al., 2013) or chemical class- (Louisse et al., 2010; Strikwold et al., 2016) specific. The purpose of the present work was, as a proof of principle, to examine whether a PBK model with features which are in line with generic PBK modelling can be used for the extrapolation of *in vitro* observed developmental toxicity to the *in vivo* situation for three different classes of chemicals known to be developmentally toxic in the rat, *i.e.* the triazoles, the glycol ethers and the phthalates. By employing the model for these diverse classes, we survey the generic nature and applicability domain of the model used.

4.1. Feasibility of IndusChemFate as a generic PBK model

The results of this study show that PBK modelling is able to extrapolate *in vitro* reproductive toxicity to systemic exposure in the intact organism, thereby refining the extrapolation paradigm previously applied by our group (de Jong et al., 2011; Hermsen et al., 2011). Based on input parameters taken either from previously reported PBK models (metabolism parameters for glycol ethers and phthalates), physicochemical QSARs (all compounds) and from published regulatory literature (whole body half-life of triazoles) our results demonstrate that IndusChemFate can simulate the *in vivo* kinetics in the rat, for widely different chemical compounds. IndusChemFate incorporates several key features of a generic modelling approach: open access, inclusion of a physiological data base, multiple exposure routes (oral, inhalatory, dermal) (Basketter et al., 2012; Leist et al., 2014) and species (human, rat, mouse). The model avoids the disadvantage common to the development of PBK models which is generally considered quite complex

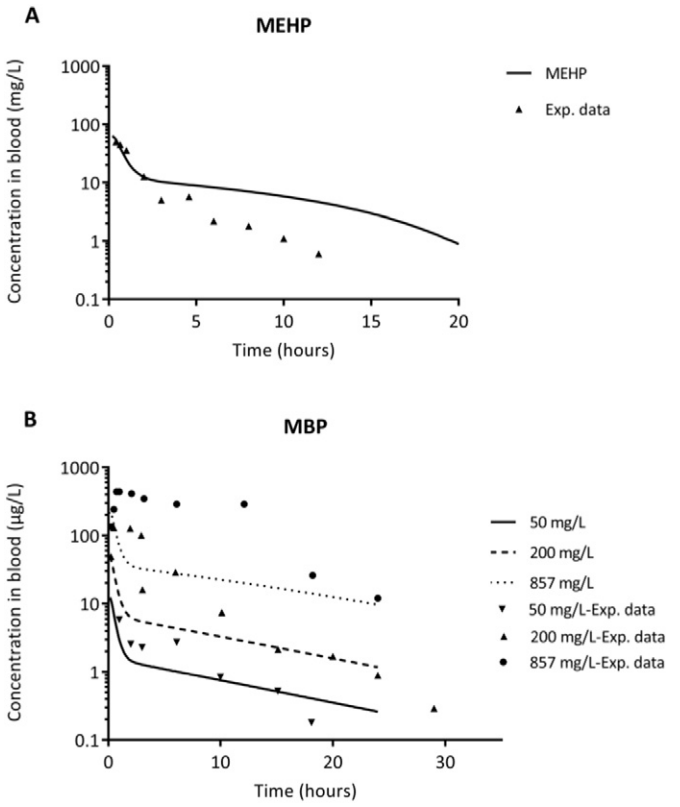

Fig. 5. (A) Time-course PBK model simulation of MEHP in venous blood, after a single oral administration of the metabolite MEHP (100 mg/kg bw), with the PBK model incorporating enterohepatic circulation of the formed metabolite. The experimental data were taken from (Keys et al., 1999, Fig. 5). (B) Time-course model simulation of MBP in venous blood, after oral administration of single doses of the parent DBP (doses: 50, 200, 857 mg/kg bw). PBK model incorporating enterohepatic circulation of the formed metabolite. The experimental data were taken from (Keys et al., 2000, Fig. 4A).

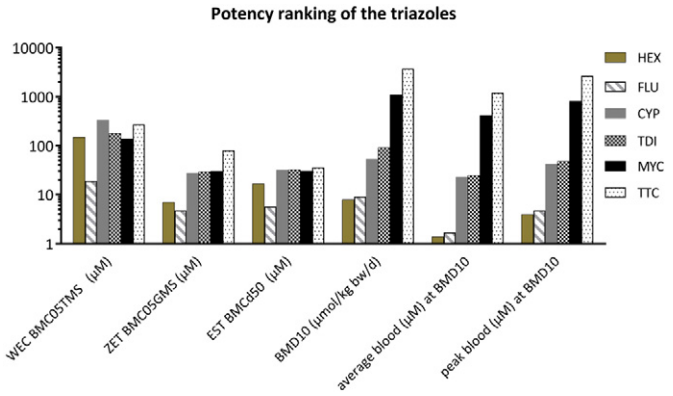

Fig. 7. Potency ranking of the triazoles as resulting from each developmental toxicity alternative test, *in vivo* experiments and the PBK model-simulated venous blood concentrations. The potency is demonstrated either by the *in vitro* respective BMCs (µM) for the WEC, ZET and EST, the *in vivo* BMD10 skeletal variations, or the PBK simulated average and peak blood concentrations (µM) at the BMD10 level. Note that the higher the graph bar the lower the potency.

and needs mathematical and programming expertise (Bessems et al., 2014). Furthermore, it overcomes the problem pertaining to availability of all partitioning model parameters of a substance, by incorporating QSARs, developed to predict such parameters solely from physicochemical characteristics (Jongeneelen and Ten Berge, 2011). However, in addition, the model needs substantial non-QSAR input. Firstly, metabolism parameters should be available. Though such parameters may be obtained from available PBK models or by fitting the model to experimental *in vivo* kinetics they preferably should be obtained from *in vitro* experimental measurements using cellular or subcellular organ fraction. In this context the *in vitro* measurements of these parameters by Green et al. (1996) for glycol ethers still are exemplary. Secondly, essential chemical characteristics such as reabsorption from tubular urine (glycol ether metabolites) and stability in the gastrointestinal tract (phthalate parent compounds) *a priori* should be known in order to lead to successful modelling. In this context the Kow based QSAR for tubular reabsorption clearly was found at variance with the *in vivo* kinetics of glycol ether metabolites.

4.2. IndusChemFate: fine tuning

In IndusChemFate, blood:organ partitioning is based on the distribution of the non-ionised compound, between the blood and the organs. In the case of triazoles, parent glycol ethers and phthalates this is a valid approach for the parent compounds. However for primary glycol ether and phthalate metabolites it may not, because these metabolites contain an acetic acid moiety, which at the pH of the blood or the organs is highly ionised. Though the present study and specific PBK models (Keys et al., 1999, 2000) indicates that the current PBK model concept gives a satisfactory description of glycol ether and phthalate kinetics without incorporation of a partitioning mechanism, which takes ionization explicitly into account the extension of the current PBK model concept with pH dependent ionization partitioning may improve the modelling of ionised compounds. As, in combination with perfusion-diffusion limitation, the non-ionised/ionised partitioning is the most

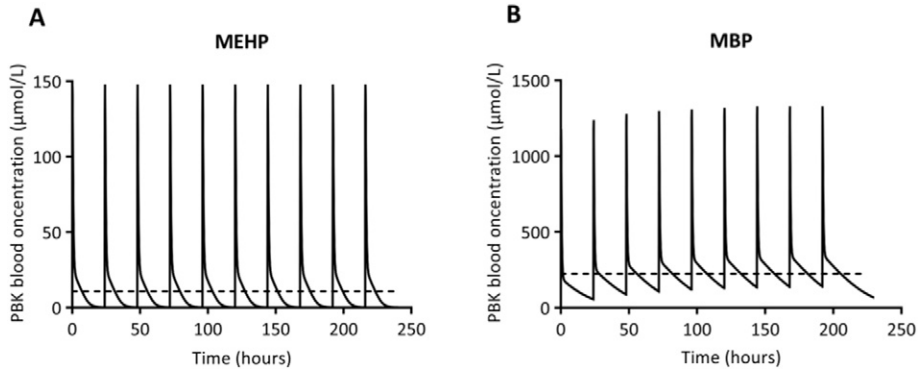

Fig. 6. (A) Time-course PBK model simulation of the monophthalate MEHP concentrations in the venous blood, after repeated oral administration of the parent (1 g DEHP/kg bw/day → 65 mg MEHP/kg bw/day, *i.e.* 6.5% conversion in the GI tract) in the rat, with activated enterohepatic circulation. (B) Time-course PBK model simulation of the monophthalate DBP concentrations in the venous blood, after repeated oral administration of the parent (1 g DBP/kg bw/d → 270 mg MBP/kg bw/day, *i.e.* 27% conversion in the GI tract) in the rat, with activated enterohepatic circulation.

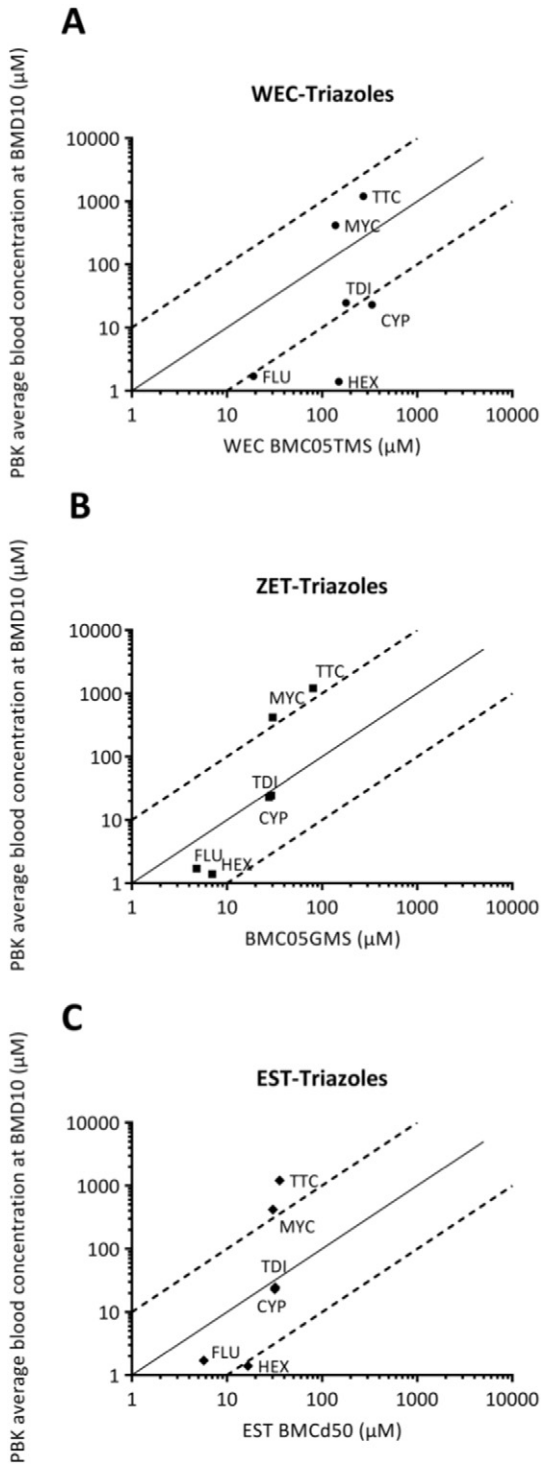

Fig. 8. PBK-predicted average venous blood concentrations of the triazoles, corresponding to the *in vivo* BMD10 values for developmental toxicity endpoints (BMD10 values taken from de Jong et al., 2011) plotted against (A) *in vitro* WEC BMC values (de Jong et al., 2011), (B) ZET BMC values (Hermsen et al., 2011), and (C) EST BMC values (de Jong et al., 2011).

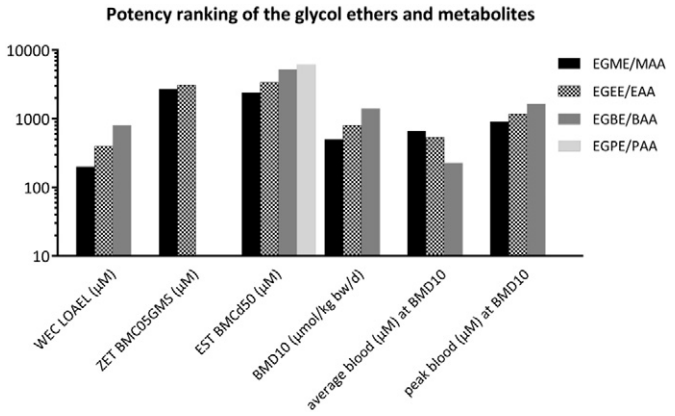

Fig. 9. Potency ranking of the alkoxy acetic acid metabolites or the respective parents glycol ethers as resulting from each developmental toxicity alternative test, *in vivo* experiments and the PBK model-simulated venous blood concentrations. The potency is demonstrated either by the *in vitro* WEC LOAEL (μM), the *in vitro* respective BMCs (μM) for the ZET and EST, the *in vivo* BMD10 skeletal variations, or the PBK simulated average and peak blood concentrations (μM) at the BMD10 level. Note that the higher the graph bar the lower the potency.

generic chemical PBK distribution mechanism, we currently are extending the IndusChemFate model according to this mechanism.

A potential limitation is the use of maternal blood as a proxy for fetal exposure. Here it should be realised that combining *in vitro* developmental toxicity results with a quantitative measure of placental diffusion (as revealed by the BeWo transport system) was found to increase the predictive power of the *in vitro* reproductive assays with respect to *in vivo* developmental toxicity (Li et al., 2015, 2016, 2017). Clearly, for such compounds *in vitro* toxicity testing should be combined with PBK modelling and the BeWo placental transport system in order to predict *in vivo* reproductive toxicity. As indicated by Li et al. (2017) only in the case of a high placental transfer rate a combination of PBK modelling an *in vitro* toxicity testing suffices to predict *in vivo* developmental toxicity. In that case, maternal blood is the ideal surrogate for fetal exposure, whereas in the case of chemicals with a low placental transfer rate the PBK model should be extended with a separate fetal sub-compartment. In that case, alterations in chemical kinetics due to physiological changes occurring during pregnancy, or changes in kinetics due to placenta formation have to be taken into account In this study it was assumed that for all substances the placental barrier is negligible. Nonetheless, as shown for triazoles (Li et al., 2016) it cannot be excluded that the compounds have different transfer rates through the placenta and hence, this can influence the effect levels *in vivo*.

4.3. Peak versus average exposure

Developmental toxicity is thought to result from a relatively short exposure period, *i.e.* a peak, even single, exposure during a well-defined critical time period within organogenesis. The sensitive window of specific morphogenetic processes may amount to less than 2-days. In contrast, toxicity may also be related to more sustained exposure, *i.e.* a substantial part or even the total duration of pregnancy. Kinetically both exposure situations relate to simulating the maximal (C_{max} approach) or the average (*AUC*-area under the curve approach) blood concentration. In this study we considered these exposure metrics and the results indicate that, depending on the chemical's kinetic profile, both approaches may lead to different results. For example, triazoles display relatively slow kinetics. As a consequence, after repeated exposure, these chemicals are expected to reach a so-called quasi steady state situation in the body and the blood relatively quickly (see Fig. 1). In such a situation additional dosing will lead to relatively low peak concentrations. Hence, the extrapolation of *in vitro* to *in vivo* does not differ much, whether based on a C_{max} or an *AUC* approach. This contrasts sharply with glycol ethers and phthalates which show much faster kinetics and, consequently, more variable blood kinetics after repeated exposure. As expected, the *in vitro* to *in vivo* extrapolation then may substantially differ when based on the C_{max} or the *AUC* approach.

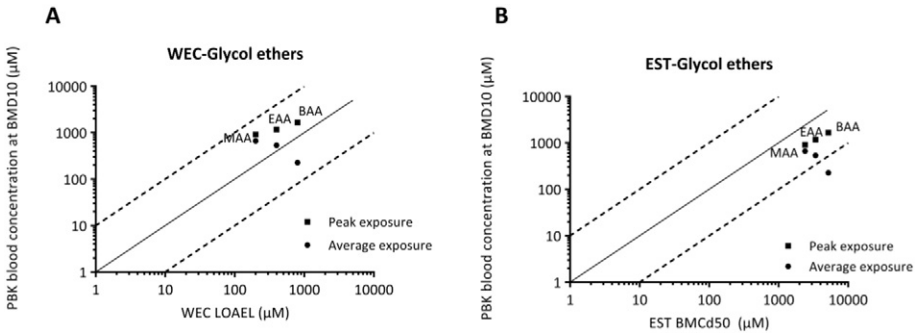

Fig. 10. PBK-predicted average and peak venous blood concentrations of the glycol ethers alkoxyacetic metabolites (MAA, EAA and BAA), corresponding to the *in vivo* BMD10 values for developmental toxicity endpoints (BMD10 values taken from Hermsen et al., 2011 and Louisse et al., 2010) plotted against (A) *in vitro* WEC LOAEL values (Giavini et al., 1993) and (B) the *in vitro* EST BMCd50 values (de Jong et al., 2009).

4.4. In vitro/in vivo comparison

Previous work directly correlated *in vivo* reference values of external dose *versus in vitro* effective concentrations, for the triazoles and the glycol ethers (de Jong et al., 2009, 2011; Hermsen et al., 2011). The current study used a more refined approach, *i.e.* a comparison of the nominal *in vitro* effective concentration with the PBK simulated concentration in the blood after gavage exposure at the level of *in vivo* reproductive toxicity. This comparison showed that for the triazoles, correlations pertaining to relative potency and potency ranking with integration of kinetics, remain comparable with previous correlations (de Jong et al., 2011). For both the ZET and EST assays the fungicides MYC and TTC, were outside the 10-fold scale, which is considered to be a normal variation in *in vivo* toxicity studies (Janer et al., 2008b). Their predicted potencies differed at least one order of magnitude from the PBK estimated plasma concentrations (ZET: 13.9-fold and 14.9-fold, EST: 13.7-fold and 33.6-fold, respectively), while *in vivo* the two compounds appeared less potent. In the WEC the main outliers are HEX, which exerted a 110-fold lower potency in the *in vitro* assay, compared to its *in vivo* effect level, followed by CYP with a 15-fold difference.

Within the class of glycol ethers, the toxic potency and ranking obtained from all three alternative assays was already in agreement with the ranking based on *in vivo* BMD10 values (embryotoxicity) of the parent substances. The *in vitro* effective concentrations were within the range (one order of magnitude) of estimated blood concentrations, corresponding to external effective doses from animal experiments.

The two chosen phthalates, DEHP and DBP, were shown to have comparable potency in animal experimental studies, with similar BMD05 values on fetal growth and BMD50s on malformations (Janer et al., 2008b). This was not seen in the alternative tests, where MEHP is 3.5- to almost 5-fold more toxic than MBP. Even so, such differences are within the allowable 10-fold scale. The calculated PBK peak blood concentrations showed an analogous pattern to the findings recorded *in vitro*, indicating that integration of kinetics in such extrapolations can quantitatively refine the comparisons. The *in vitro* effective concentrations differed less than an order of magnitude (or in one case an order of magnitude) from the plasma concentrations, corresponding to the *in vivo* BMC05s.

Previously the straightforward assumption was made that equal concentrations at the target site *in vitro* and *in vivo* will induce similar toxic effects (Louisse et al., 2010, 2015; Strikwold et al., 2013, 2016; Li et al., 2017). Nonetheless, the interpretation of reproductive alternative assays as to what effect constitutes adversity *versus* non-toxic physiological changes and *in vitro versus in vivo* toxic potency warrants further elucidation. In the WEC, the ID20 on the total morphological score (TMS) is taken as the standard. This effect size does constitute clear adversity as a 20% reduction of TMS indicates significant retardation of embryo development. For the ZET, the ID20 on the general morphology score (GMS) is defined as the reference value, again based on a significant retardation of development at that effect size. In the EST, the ID50 on cardiomyocyte differentiation has been classically used as the easiest measure to derive on the sigmoid dose-response curves, which this method provides. This standard approach was also applied in the current comparison. The three assays used showed very comparable patterns as to *in vitro* to *in vivo* extrapolation of individual chemicals studied.

In conclusion, the IndusChemFate model was found to be capable of describing the *in vivo* kinetics of the three classes of developmental toxicants employed, though at the expense of several chemical specific adaptations. However, future modelling will still need fine-tuning, in terms of including for instance a placental-fetal compartment, alternative partitioning mechanisms such as ionization/non-ionization, diffusion-limitation, the fate of chemicals in the GI tract and renal clearance.

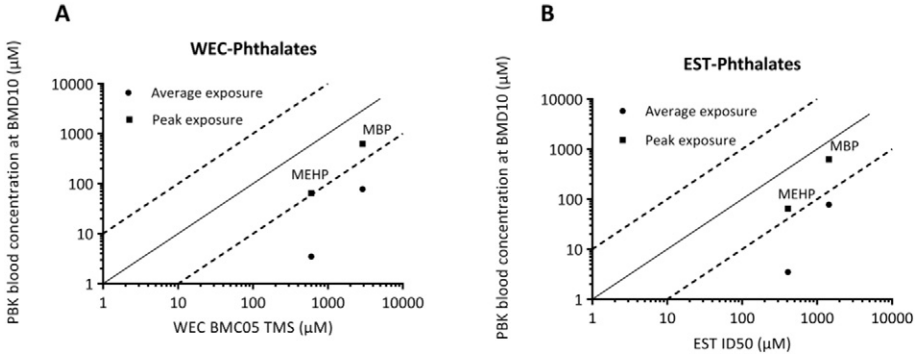

Fig. 11. PBK-predicted average and peak venous blood concentrations of the monophthalates MEHP and MBP, corresponding to the *in vivo* BMD05 values for developmental toxicity endpoints (BMD05 on fetal growth, taken from Janer et al., 2008b) plotted against (A) *in vitro* WEC BMC05 TMS values (Janer et al., 2008b) and (B) the *in vitro* EST ID50 values (Schulpen et al., 2013).

Furthermore, we performed comparisons with three different developmental toxicity alternative assays. The current results indicate that for the time being it is not possible to discriminate which of the three assays outweighs the others in predicting *in vivo* toxicity. Hence, a combination of tests is preferable for predicting the endpoint of developmental toxicity (Genschow et al., 2004; Piersma et al., 2013).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2017.07.021.

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