Toxicology 457 (2021) 152819

Contents lists available at ScienceDirect

Toxicology

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

Evaluation and comparison of in vitro intrinsic clearance rates measured using cryopreserved hepatocytes from humans, rats, and rainbow trout

Sherry R. Black [a], John W. Nichols [b], Kellie A. Fay [c], Sharlene R. Matten [d,1], Scott G. Lynn [e,2,*]

[a] RTI International, Discovery Sciences, 3040 East Cornwallis Road, Durham, NC 27709 USA
[b] US Environmental Protection Agency, Office of Research and Development, Great Lakes Toxicology and Ecology Division (GLTED), 6201 Congdon Blvd, Duluth, MN 55804 USA
[c] US Environmental Protection Agency, Office of Pollution Prevention and Toxics (OPPT), William Jefferson Clinton Building, 1200 Pennsylvania Avenue NW, Washington, DC 20460 USA
[d] US Environmental Protection Agency, Office of Science Coordination and Policy (OSCP), William Jefferson Clinton Building, 1200 Pennsylvania Avenue NW, Washington, DC 20460 USA
[e] US Environmental Protection Agency, Office of Science Coordination and Policy (OSCP), William Jefferson Clinton Building, 1200 Pennsylvania Avenue NW, Washington, DC 20460 USA

ARTICLE INFO

Keywords:
Metabolism
Biotransformation
Intrinsic clearance
In vitro-in vivo extrapolation
Cryopreserved hepatocyte

ABSTRACT

In vitro and in silico methods that can reduce the need for animal testing are being used with increasing frequency to assess chemical risks to human health and the environment. The rate of hepatic biotransformation is an important species-specific parameter for determining bioaccumulation potential and extrapolating in vitro bioactivity to in vivo effects. One approach to estimating hepatic biotransformation is to employ in vitro systems derived from liver tissue to measure chemical (substrate) depletion over time which can then be translated to a rate of intrinsic clearance (CL_{int}). In the present study, cryopreserved hepatocytes from humans, rats, and rainbow trout were used to measure CL_{int} values for 54 industrial and pesticidal chemicals at starting test concentrations of 0.1 and 1 μM. A data evaluation framework that emphasizes the behavior of Heat-Treated Controls (HTC) was developed to identify datasets suitable for rate reporting. Measured or estimated ("greater than" or "less than") CL_{int} values were determined for 124 of 226 (55 %) species-chemical-substrate concentration datasets with acceptable analytical chemistry. A large percentage of tested chemicals exhibited low HTC recovery values, indicating a substantial abiotic loss of test chemical over time. An evaluation of K_{OW} values for individual chemicals suggested that in vitro test performance declined with increasing chemical hydrophobicity, although differences in testing devices for mammals and fish also likely played a role. The current findings emphasize the value of negative controls as part of a rigorous approach to data quality assessment for in vitro substrate depletion studies. Changes in current testing protocols can be expected to result in the collection of higher quality data. However, poorly soluble chemicals are likely to remain a challenge for CL_{int} determination.

1. Introduction

Despite a growing demand to reduce the need for animal testing in chemical risk assessments (USEPA, 2019), there is a large inventory of chemicals for which data to support a risk assessment is required. For example, the U.S. Environmental Protection Agency's (EPA) Endocrine Disruptor Screening Program (EDSP) must assess potential endocrine disrupting effects of pesticides and possibly other environmental contaminants in both humans and wildlife (21 U.S.C. § 346a(p) and 42 U.S.C. § 300j-17). The chemical substances covered under the EDSP include pesticide active ingredients, pesticide inert ingredients, and drinking water contaminants, with some overlap between these lists. While

* Corresponding author.
E-mail addresses: sherryb@rti.org (S.R. Black), nichols.john@epa.gov (J.W. Nichols), fay.kellie@epa.gov (K.A. Fay), matten.sharlene@epa.gov (S.R. Matten), lynn.scott@epa.gov (S.G. Lynn).
[1] Present address: US Environmental Protection Agency, Office of Program Support (OPS), William Jefferson Clinton Building, 1200 Pennsylvania Avenue NW, Washington, DC 20460 USA.
[2] Present address: US Environmental Protection Agency, Office of Pesticide Programs (OPP), William Jefferson Clinton Building, 1200 Pennsylvania Avenue NW, Washington, DC 20460 USA.

https://doi.org/10.1016/j.tox.2021.152819
Received 15 February 2021; Received in revised form 17 April 2021; Accepted 5 May 2021
Available online 10 May 2021
0300-483X/Published by Elsevier B.V.

screening for pesticides is mandatory, screening of drinking water contaminants is discretionary. The number of chemicals is likely in the thousands, while only 52 chemicals to date have completed Tier 1 screening (USEPA, 2015). Similarly, the Toxic Substances Control Act (TSCA), as amended by the Frank R. Lautenberg Chemical Safety for the 21st Century Act, requires that EPA prioritize industrial chemicals for assessing their risk to human health and the environment (U.S.C. § 2601 et seq, 2016); more than 40,000 industrial chemicals are legally authorized for use in the stream of commerce and may be considered for prioritization (USEPA, 2020a).

Quantitative in vitro-in vivo extrapolation (QIVIVE) is increasingly being utilized as a non-animal alternative to prioritize chemicals for further evaluation (Basketter et al., 2012; Yoon et al., 2012; Wetmore, 2015; Scholze et al., 2020). The QIVIVE approach involves extrapolation of an adverse in vitro effect concentration to a comparable in vivo concentration in plasma or a target tissue. A chemical mass-balance model is used to relate this in vivo concentration to an equivalent organismal exposure (Liao et al., 2007; Clewell et al., 2008; Blaauboer, 2010; Louisse et al., 2017; Punt et al., 2021). This modeled exposure, typically expressed as a continuous daily dosing rate (e.g., mg/kg body weight/day oral exposure), is then compared to a known or hypothesized exposure to assess the potential for adverse effects. To date, QIVIVE has been used to evaluate potential effects on humans for nearly 60 % of EPA's Toxcast Phase I and II libraries (Rotroff et al., 2010; Wetmore et al., 2012, 2015; Wambaugh et al., 2019). The same approach has been used to predict adverse effects in rats, permitting direct comparisons to existing rodent toxicity data (Honda et al., 2019; Wetmore et al., 2013).

Chemical prioritization and evaluation efforts may also require that chemicals be assessed to determine their potential to bioaccumulate. Generally, the focus of bioaccumulation assessments is on chemical bioaccumulation in fish. Hydrophobic organic compounds are of special interest, as they tend to partition out of the water column and into fish tissues, posing a potential hazard to fish and the animals that consume them. Measured bioaccumulation data for fish are available for >800 chemicals (Arnot and Gobas, 2006), but this is a small fraction of the chemicals in the stream of commerce. Consequently, most of these evaluations are performed using screening-level computational models like those in the BCFBAF module of the U.S. EPA's Estimation Program Interface Suite (EpiSuite™) software package (U.S. EPA, 2012a).

A critical component of both QIVIVE and bioaccumulation assessment is the need to estimate parameters required to run predictive toxicokinetic models. Some model parameters may be estimated using quantitative structure-activity relationships (QSARs) such as those that describe chemical partitioning between blood and tissues (DeJongh et al., 1997; Poulin and Krishnan, 1995; Schmitt, 2008; Peyret et al., 2010) and chemical uptake across fish gills (Arnot and Gobas, 2003). Despite considerable effort, however, several key parameters, including those describing plasma protein binding, distribution mediated by specific membrane transporters, and biotransformation, remain difficult to predict with acceptable accuracy using QSAR methods.

For many chemicals, the rate of hepatic biotransformation is of special interest, as this activity may substantially impact the rate and extent of chemical accumulation over time. Although QSARs have been developed to predict biotransformation rates in both humans (Hansch et al., 2004; Long and Walker, 2003; Peyret et al., 2012; Kirman et al., 2015) and fish (Arnot et al., 2009; Brown et al., 2012; Kuo and Di Toro, 2013; Papa et al., 2014), these tools have limited utility beyond the datasets employed for their development. As an alternative, therefore, QIVIVE of in vitro toxicity data is commonly informed by a parallel activity that involves in vitro-in vivo extrapolation of measured in vitro intrinsic clearance (Yoon et al., 2012; Wilk-Zasadna et al., 2015). The goal of the extrapolation is to estimate in vivo intrinsic clearance from in vitro data. This intrinsic clearance estimate is then used as an input to a physiological model of the liver to predict hepatic clearance due to biotransformation. Cryopreserved hepatocytes are often preferred for this application due to broad coverage of potential metabolic pathways, the presence of enzyme co-factors at physiological levels, and amenability for use in medium-throughput testing efforts (Li et al., 1999; Gebhardt et al., 2003). Similar extrapolation methods employing cryopreserved trout hepatocytes (Mingoia et al., 2010; Fay et al., 2014a, 2015) have been used to inform modeled bioaccumulation assessments for fish (Nichols et al., 2006, 2009; OECD, 2018a).

The procedures used to perform in vitro-in vivo extrapolation of hepatocyte clearance data derive from methods developed by the pharmaceutical industry for pre-clinical screening of drug candidates (Rodrigues, 1997). Because the goal of the extrapolation is to estimate hepatic clearance occurring by all possible biotransformation pathways, these assays are generally performed using a substrate depletion approach (Itwatsubo et al., 1997; Obach et al., 1997; Carlile et al., 1998; Obach, 1999; Obach and Reed-Hagen, 2002). Most drugs are well-suited for application of these methods. In particular, the drug development process favors chemicals that possess low volatility and a moderate-to-high degree of aqueous solubility. Compounds possessing these traits are easily spiked into an in vitro test system and tend to remain in solution unless acted upon. Unlike drugs, many industrial and some pesticidal chemicals possess properties (e.g., high volatility, extreme hydrophobicity) that make them difficult to work with in an in vitro context. In the present study, in vitro depletion assays were performed for 54 industrial and pesticidal chemicals using cryopreserved hepatocytes from humans, rats, and rainbow trout. All assays were conducted using standardized methods employed previously to support QIVIVE efforts (humans/rats) and fish bioaccumulation assessments (trout). The same sampling design and total assay run time were employed throughput, with the goal of evaluating how well these methods perform in a screening-level approach to chemical assessment. The resulting datasets were then analyzed using a data quality evaluation framework to identify data suitable for rate reporting and provide guidance on future use of these methods.

2. Materials and methods

2.1. Test and reference chemicals

Test chemicals were selected to provide broad coverage across the industrial and pesticidal chemical space while also prioritizing chemicals within the published EDSP Universe of Chemicals (USEPA 2012b). Specific chemical-species combinations were chosen to avoid duplication of existing data which precluded testing all chemicals across all three species. In addition, an effort was made to avoid chemicals that could volatilize from the selected in vitro assay systems. In previous depletion studies with rainbow trout hepatocytes, performed at 11 °C, naphthalene was shown to slowly volatilize from inactivated control vials (Fay et al., 2017). Reported Henry's law constants (K_H) for naphthalene average approximately 5×10^{-4} m^3 atm/mol (Sander, 2015). By comparison to the trout assay, chemical volatilization from an assay performed using rat or human hepatocytes may present an even greater challenge due to the higher temperature (37 °C) at which they are run. A Henry's law constant cutoff of 5×10^{-5} m^3 atm/mol was therefore employed to exclude potentially problematic chemicals.

Sources, lot numbers, reported purities, and predicted physicochemical properties ([K_H] and log octanol-water partition coefficient [log K_{OW}]) of test and reference chemicals are provided in Table S1. The identity of each chemical was confirmed by ^{1}H NMR. Predicted K_H and log K_{OW} values were obtained using the OPERA HL and OPERA LogP QSAR models (Mansouri et al., 2018), which were accessed through US EPA's CompTox dashboard (USEPA, 2020b) Reference chemicals were run in parallel with test chemicals as a check on individual assay performance and to evaluate the variability in performance with repeated analysis of the same substrate. The reference chemicals verapamil (human), fenbuconazole (rat), and pyrene (trout) were selected based on ease of analysis and the availability of published in vitro intrinsic

clearance values for these species. Stock solutions of test and reference chemicals were prepared in dimethyl sulfoxide (DMSO: 1 and 10 mM) and stored at −70 °C. Working stocks (10 and 100 μM) were prepared by dilution of primary stock solutions in acetonitrile (for human and rat) or acetone (for trout), as detailed in publications on these assays (Wetmore et al., 2012, 2013; Fay et al., 2015).

2.2. Hepatocyte culture reagents

William's E Medium (WEM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 % GlutaMAX, 0.5x penicillin-streptomycin, Leibovitz's L-15 Medium (no phenol red, with L-glutamate, L-15), and trypan blue (0.4 %) were purchased from Gibco (Grand Island, NY). ITS + Premix was supplied by Corning (Bedford, MA). Hepatic Bio Cryo Recovery Medium (HB-CRM) and dexamethasone (100 nM) were obtained from PhoenixSongs Biologicals (Branford, CT).

2.3. Cryopreserved hepatocytes: sources, preparation and viability

Human and rat hepatocytes were supplied by the QPS lab in Research Triangle Park, NC (QPS, LLC, Newark, DE). Human hepatocytes were obtained as a pool derived from 5 male and 5 female adults. Rat hepatocytes were derived from 8–12-week-old Sprague Dawley rats as sex-specific pooled samples (3 females, 4 males). These sex-specific samples were combined just prior to use to provide a mixed-sex pool. Trout hepatocytes were isolated from sexually immature animals of mixed sex at the USEPA Great Lakes Toxicology and Ecology Division, Duluth, MN, using methods described by Fay et al. (2015).

Cryopreserved human and rat hepatocytes were thawed and resuspended using procedures given by the supplier. Briefly, the cells were thawed (in about 90 s) in a 37 °C water bath, diluted in pre-warmed (37 °C) HB-CRM, and centrifuged at room temperature at 100 × *g* for 8 min (human) or 55 × *g* for 3 min (rat). Media was removed by aspiration and cells were resuspended in pre-warmed (37 °C) incubation medium consisting of serum-free WEM (pH 7.4 at 37 °C) supplemented with 1.5 % HEPES, 1 % ITS+Premix, 1 % GlutaMAX, 0.5X penicillin-streptomycin, and 100 nM dexamethasone. Cryopreserved trout hepatocytes were thawed in a room temperature water bath and suspended in ice-cold L-15 medium (pH 7.8 at 12 °C) using the procedure described by Fay et al. (2015). Viability and hepatocyte counts were determined by trypan blue exclusion. The cell suspensions were then diluted to 0.5 × 10^6 viable hepatocytes/mL (human and rat) or 1.0 × 10^6 viable hepatocytes/mL (trout) in their respective media and stored on ice until use. Heat-inactivated hepatocytes were prepared by boiling hepatocyte suspensions (0.5 × 10^6 hepatocytes/mL for human and rat; 1 × 10^6 hepatocytes/mL for trout) for 15 min. These were prepared as species-specific batches in advance, aliquoted and stored frozen (−20 °C) until needed.

2.4. Hepatocyte characterization assays

The metabolic activity of each lot of hepatocytes was evaluated using prototypical substrates for Phase I and Phase II enzyme activity. For the human and rat hepatocytes, 7-ethoxycoumarin-*O*-deethylase (ECOD) was employed as a marker for Phase I enzymatic activity while Phase II UDP-glucuronosyltransferase (UGT) and sulfotransferase (SULT) activities were measured using 7-hydroxycoumarin as substrate. Human and rat hepatocytes were incubated (0.5 × 10^6 hepatocytes/mL) in triplicate with 100 μM 7-ethoxycoumarin or 7-hydroxycoumarin for 30 min at 37 °C following methods described by Ekins et al. (1995). Trout hepatocyte lysates were assayed for CYP1A activity in incubations with 0.5 μM 7-ethoxyresorufin for 30 min at 12 °C (EROD assay) while glutathione-*S*-transferase (GST) activity was evaluated in incubations with 1000 μM 1-chloro-2,4-dinitrobenzene for 20 min at 12 °C. The procedures used to characterize trout hepatocytes are described in detail by Fay et al. (2014b).

2.5. Substrate depletion assays

Test chemicals were incubated in triplicate with prepared hepatocyte suspensions at nominal concentrations of 0.1 and 1 μM. The human and rat assays were conducted as described by Wetmore et al. (2012, 2013), with minor modifications, while the trout assay was based on Basic Protocol 4 in Fay et al. (2015). Hepatocyte cell densities were 0.5 × 10^6 hepatocytes/mL (human and rat assays) or 1 × 10^6 hepatocytes/mL (trout assays), and the total incubation volume was 1 mL. Assays performed using human and rat hepatocytes were conducted in 48-well deep-well polypropylene plates while those performed using trout hepatocytes were conducted in glass vials. Hepatocyte suspensions were pre-incubated for 10 min at their respective incubation temperatures (37 °C for human/rat; 12 °C for trout). Incubations of human and rat hepatocytes were conducted with gentle orbital shaking in a humidified incubator filled with 5 % CO_2. Assays with trout hepatocytes were performed with gentle shaking in a temperature-controlled chamber equilibrated with room air. All assays were initiated by addition of test chemicals in solvent (working stocks). The final concentration of solvent was ≤1 % (v/v) in all cases. Sub-samples (100 μL) of the total incubation volume were removed at $T = 0$ (immediately after chemical addition), 15, 30, 60, 120, and 240 min, and quenched by addition to 300 μL (for LC–MS/MS) or 100 μL (for GC–MS/MS) acetonitrile. The quenched reaction mixtures were then vortexed and stored at ≤−70 °C prior to analysis.

Species-specific reference chemical controls (0.1 and 1 μM, in triplicate) were included with each assay and sampled as described above. Test chemicals were also incubated in duplicate with heat-inactivated hepatocytes at 0.1 and 1 μM; these Heat-Treated Controls (HTC) were sampled at $T = 0$ and 240 min.

2.6. Sample analysis

Test and reference chemical concentrations in incubation samples were determined using LC–MS/MS, HPLC/UV/Fluoresence or GC–MS/MS methods. Descriptions of analytical methods are provided as Supplementary Data (Analytical methods). Additional analytical details are presented in Tables S2-S7. Standard curves (≥5 points, 1–1000 ng/mL) were used to relate analyte/internal standard (IS) peak area response and analyte concentration in incubation samples. All standard curves met acceptance criteria of a correlation coefficient ≥0.98 and a relative error (% RE) ≤15 % for each concentration (≤ ± 20 % at the limit of quantitation [LOQ]). The experimental LOQ was defined as the lowest concentration of calibration standards that met the acceptance criteria. Quality control (QC) standards (triplicate, three concentrations) were interspersed in each sample set. Precision and accuracy criteria for QC standards were a % RE of ≤ ± 15 % of the nominal value for each concentration and a relative standard deviation ≤ ± 15 %.

2.7. Calculation of in vitro clearance

Data from a single chemical/species/substrate concentration combination were considered a dataset. Measured test chemical concentrations were natural log (Ln)-transformed and plotted against time (three replicates per time point). Datasets with measured chemical concentrations at three or more time points were evaluated by linear regression in Prism8 (GraphPad Software, San Diego, California) to obtain a fitted slope. In those cases where substrate concentrations could be quantified out to 4 h, measured values generally declined in a log-linear manner indicating that the hepatocytes had retained a large fraction of their initial activity over time. In approximately 10 % of cases, however, measured substrate concentrations at later time points suggested a decrease in enzyme activity. In these latter instances, a linear regression was fitted to the initial, log-linear portion of the dataset. All regressions fitted to these truncated datasets were based on at least four data points. An *F*-test (α = 0.05) was used to determine whether fitted slopes was

significantly different from zero. First-order elimination rate constants (k_{dep}; 1/min) were then calculated from significant, negative slopes as: $k_{dep} = -1 \times$ slope. In vitro intrinsic clearance rates (CL_{int}; μL/min/10^6 hepatocytes) were calculated by dividing estimated k_{dep} values by the concentration of viable hepatocytes (10^6 hepatocytes/mL).

$$CL_{int} = \frac{k_{dep}}{hepatocyteconcentration} x1000\mu L \Big/ mL \quad (1)$$

If measured chemical concentrations were obtained at only one or two time points (0 min, or 0 and 15 min), Eq. 2 was used to calculate the minimum slope of the depletion curve that would have resulted in the concentration dropping below the LOQ before the third sampling time point (30 min).

$$-k_{dep} = \frac{\mathrm{Ln}(\mathrm{Concentration}_{T=O} - \mathrm{Concentration}_{LOQ})}{30\ min} \quad (2)$$

The CL_{int} corresponding to this slope was then calculated using Eq. 1. CL_{int} values estimated in this manner are referred to here as "greater than" values and denoted with the symbol ">", since the true rate of depletion may have been faster than that calculated using Eq. 2 but is unlikely to have been slower.

Depletion curves with slopes that did not differ significantly from zero may indicate an absence of biotransformation. Alternatively, biotransformation may have occurred but could not be detected due to the limited time over which the assays were run and/or the variance in repeated measurements. It was of interest, therefore, to estimate the lowest reasonably detectable rate of in vitro activity for each species and substrate concentration category (human, 1 μM; human, 0.1 μM, etc.). This was achieved by calculating the smallest detectable slope ($k_{dep,\ low}$), assuming a power of 0.90 and α-level of 0.05, using the standard experimental design and its "typical" standard error. The standard error of the slope [SE $(\widehat{\beta})$] for a "typical" experiment was estimated as

$$SE\ (\widehat{\beta}) = \sqrt{\frac{\sigma^2}{\sum(x - \bar{x})^2}} \quad (3)$$

where σ^2 is the average within time period variance of the Ln-transformed concentration for all datasets within the category, estimated from the root mean squared error (RMSE), and $\sum(x - \bar{x})^2$ is the sum of squares for x, calculated assuming 3 samples taken at times 0, 0.25, 0.5, 1, 2, and 4 h (summing to 33.91). For this analysis, we used only RMSE values calculated from linear regressions that yielded measurable rates of activity. Additional information pertaining to the power calculation is provided as Supplementary Data (Power calculation).

The $k_{dep,low}$ values calculated for each species and starting substrate concentration were divided by hepatocyte concentration to yield an estimated "less than" CL_{int}, denoted with the symbol "<" (Table 1). For chemicals in this category, the true CL_{int} may have been slower than the estimated lowest detectable rate but is unlikely to have been faster. The estimated lowest detectable CL_{int} values for each species and starting substrate concentration were similar, ranging from 2.57 to 5.07 μL/min/10^6 hepatocytes. There were no obvious relationships between these values and starting substrate concentration.

Heat-Treated Control (HTC) recovery (%) was calculated from the concentration at T =240 min divided by the concentration at T = 0 min × 100 for each of two replicate control wells, and the mean recovery value was reported.

Table 1
Lowest detectable depletion rate constant ($k_{dep,\ low}$) and corresponding lowest detectable in vitro intrinsic clearance rate (CL_{int}), determined by power analysis for each species and starting substrate concentration category.

Species	Substrate concentration (μM)	Average RMSE[a] of datasets	$k_{dep,\ low}$ (1/h)	Lowest detectable CL_{int} (μL/min/10^6 hepatocytes)[b]
Human	0.1	0.13	0.10	3.43
Human	1	0.09	0.08	2.57
Rat	0.1	0.11	0.09	2.93
Rat	1	0.19	0.15	5.07
Trout	0.1	0.21	0.17	2.80
Trout	1	0.25	0.20	3.38

[a] RMSE = root mean square error.
[b] Calculated assuming 0.5 × 10^6 hepatocytes/mL for human and rat, 1 × 10^6 hepatocytes/mL for trout.

3. Results

3.1. Hepatocyte metabolic capacity and viability

Measured ECOD activities determined for the two lots of human hepatocytes were somewhat lower than those given by the supplier (10.6–13.1 vs. 15–18 pmol/min/10^6 hepatocytes), while measured UGT and SULT activities were comparable to supplier-reported values (503–745 pmol/min/10^6 hepatocytes vs. 591–1200 pmol/min/10^6 hepatocytes and 49–76 pmol/min/10^6 hepatocytes vs. 49–82 pmol/min/10^6 hepatocytes activity, respectively). ECOD activities measured for the rat hepatocyte lots were lower than supplier-reported values (3.9–7.8 pmol/min/10^6 hepatocytes vs. 14.9–20.8 pmol/min/10^6 hepatocytes), while UGT and SULT activities were similar to those reported by the supplier (226–404 vs. 200–491 pmol/min/10^6 hepatocytes and 57–286 vs. 49–143 pmol/min/10^6 hepatocytes, respectively). Measured EROD activities for trout hepatocytes (1.3–1.8 pmol/min/mg) were lower than those determined by the supplier (3.7–5.1 pmol/min/mg protein), while measured GST activities (356–378 nmol/min/mg protein) were within the range of supplier-reported values (295–412 nmol/min/mg protein). It is not clear why measured ECOD (humans, rats) and EROD (trout) activities were lower than those given by suppliers. Although some loss of activity associated with shipping and storage may be anticipated, differences between measured and previously determined values were greater than expected. We note, however, that measured phase II activities for all species were similar to or greater than values given by suppliers, while measured CL_{int} values for reference chemicals were comparable to or greater than published values (see Section 3.2, Reference chemical clearance results). It was determined, therefore, that all cell lots possessed acceptable levels of activity. Hepatocyte viability was assessed on each day of use (Table S8) and ranged from 90 to 96 % (human), 85–98 % (rat), and 88–98 % (trout).

3.2. Reference chemical clearance results

Species-specific reference chemicals were run concurrently with test chemicals on each assay day (Tables 2 and S9). The CL_{int} of verapamil by human hepatocytes averaged 24.1 (0.1 μM) and 16.4 (1 μM) μL/min/10^6 hepatocytes, while the CL_{int} of fenbuconazole by rat hepatocytes averaged 26.9 (0.1 μM) and 17.6 (1 μM) μL/min/10^6 hepatocytes, and the CL_{int} of pyrene by trout hepatocytes averaged 27.9 (0.1 μM) and 7.3 (1 μM) μL/min/10^6 hepatocytes. The activity of individual cell lots varied somewhat. For example, the mean CL_{int} of verapamil determined using human cell lot 1 at the 0.1 μM starting concentration was 36.0 μL/min/10^6 hepatocytes, while that determined using human cell lot 2 was 17.8 μL/min/10^6 hepatocytes. For each cell lot, however, there was good replication among repeated measurements, resulting in percent relative standard deviations (% RSD) that averaged 26.2 % (range: 5.8–70.2 %). The CL_{int} values determined here for verapamil and pyrene are within the range of reported values for these species (Tables 2 and S10). The CL_{int} determined for fenbuconazole is approximately 5-fold higher than a previously published value; however, this higher rate was observed consistently throughout the study.

Table 2
Species-specific reference chemical clearance (CL_{int}) and Heat-Treated Control (HTC) recovery.

Species / Reference chemical (CAS No.)	CL_{int} (μL/min/10^6 hepatocytes)[a]	Concentration (μM)[b]	n[c]	HTC recovery % ± SD	n[d]	Source
Human / Verapamil (52-53-9)	24.1 (11.1–45.8)	0.1	10	101 ± 10	7	This study
	16.4 (7.37–28.8)	1	10	100 ± 9	7	
	21.8 (4.65–49.0)	0.25–3	29	–	–	Published literature[e]
Rat / Fenbuconazole (114369-43-6)	26.9 (10.6–45.5)	0.1	9	102 ± 12	7	This study
	17.6 (6.79–37.6)	1	9	112 ± 43	7	
	3.89	1	1	–	–	Wetmore et al., 2013
Trout / Pyrene (129-00-0)	27.9 (12.7–46.9)	0.1	7	115 ± 41	6	This study
	7.33 (2.32–13.2)	1	7	103 ± 34	6	
	45.7 (19.3–66.7)	0.025–0.25	3	–	–	Published literature[e]

[a] Values shown for the present study represent means (low–high) for *n* runs for each starting chemical concentration measured using two lots of human and rat, and four lots of trout hepatocytes. Values given for published literature are reported as means (low–high) for *n* references which utilized a starting chemical concentration within 5-fold of those employed in the present study.
[b] Values shown for the present study represent starting chemical concentrations. Values given for published literature represent the range of reported starting chemical concentrations (low–high).
[c] Sample sizes are given as the number of CL_{int} values determined in the present study (**Table S9**) or the number of values obtained from published literature.
[d] Sample sizes are given for the number of HTC recovery values determined for each species and starting chemical concentration.
[e] References for published literature on verapamil and pyrene are given in **Table S10**.

3.3. Test chemical metabolic clearance results

A total of 54 test chemicals were evaluated at nominal concentrations of 0.1 and 1 μM to determine CL_{int} by human, rat, and trout hepatocytes (Table 3). For 21 of the test chemicals, CL_{int} was evaluated across all three species. For the other chemicals, assays with one or two species were performed, resulting in 238 chemical-species-substrate concentration combinations (i.e., datasets). The complete set of depletion curves for all experiments is shown in Figs. S1-S3. Tabulated clearance data for all tested chemicals are provided in Table S11.

3.4. Framework for data evaluation

Twelve datasets were eliminated from further consideration due to quality control failures including high variance in measured chemical concentrations and/or measured concentrations at $T = 0$ min that were substantially different from target values. For the remaining 226 datasets, a framework for data evaluation was developed to identify those of acceptable quality for rate reporting (Fig. 1). Because it is essential to distinguish between biologically-mediated and abiotic losses of test chemicals, the first tier of the data evaluation framework focused on the HTC recovery results. HTC recovery data for the 226 datasets are shown in Fig. 2. An examination of these values suggested several categories of assay performance, and the datasets were binned accordingly. Approximately 46 % (103) of the datasets had HTC recoveries between 80 and 120 %, indicating little or no abiotic loss of test chemical. Fifty-six datasets (25 %) had HTC recoveries <50 %, suggesting a large abiotic loss of test chemical. Thirty-four datasets (15 %) had HTC recoveries between 50 and 80 %. A sharp break in the data was observed at a recovery value of 130 %. Below this cut-off were 14 datasets (6 %) with values between 120 % and 130 %. The last bin contained 19 datasets (8 %) with HTC recoveries >130 %.

3.5. HTC recovery of 80–120 %

Consistent with guidance provided in OECD (2018a), HTC recoveries between 80 and 120 % were judged to be ideal for CL_{int} determination (e.g., Fig. 3A). Of the 103 datasets in this category, 94 had at least three time points with measured concentrations above the LOQ, permitting the calculation of an CL_{int} value (Table 3). For the remaining 9 datasets, the CL_{int} is reported as a "greater than" rate.

3.6. HTC recovery of 50–80 %

A HTC recovery of 50–80 % suggests a substantial level of abiotic test chemical loss. However, these datasets may still yield acceptable clearance estimates if the rate of loss in the live incubation is sufficiently greater than the rate of loss in the corresponding negative control (Nichols et al., 2013; OECD, 2018b). For example, in the depletion plot for picoxystrobin in rat hepatocyte incubations (Fig. 3B), the slopes of the live hepatocyte depletion curves ($-k_{dep}$, solid symbols) are much greater than that of the corresponding HTC curve, defined by measured concentrations at $T = 0$ and 240 min (open symbols). For datasets with HTC recovery values between 50 and 80 %, the ratio of $-k_{dep}$ to the slope of the HTC curve ($-k_{HTC}$) was calculated as:

$$\text{Ratio} = -k_{dep}/-k_{HTC} \quad (4)$$

For the 34 datasets with HTC recoveries between 50 and 80 %, these calculated ratios ranged from 0 to >160. There was no apparent relationship between the magnitude of this ratio and test species. Calculated values for all species were therefore plotted together to visualize these findings (Fig. 4). When the ratio is >10, the error introduced into calculation of CL_{int} by the unspecified loss of chemical in the HTC is ≤10 %. Therefore, a ratio >10 was set as an acceptance criterion. This criterion was met by 22 datasets. For the 12 datasets that did not meet this criterion, no clearance rate is reported (e.g., Fig. 3C). Datasets that were advanced based on an acceptable ratio were further evaluated with respect to the number of data points in the depletion curve. Clearance rates are reported for the 13 datasets that met the ratio criterion and had ≥3 points in the depletion curve. Nine datasets met the ratio criterion but had <3 points in the depletion curve. For these datasets an estimated "greater than" rate is reported (Table 3).

3.7. HTC recovery of 120–130 %

Fourteen datasets had HTC recoveries between 120 and 130 % (e.g., Fig. 3D). In nearly all these instances, the high recovery value was due primarily to a low concentration measurement in one of the $T = 0$ replicates. All datasets in this category were advanced to the next evaluation consideration: number of data points with measured concentrations above the LOQ. Clearance rates are reported for the ten datasets that had ≥3 acceptable data points while "greater than" rates were estimated for the other four datasets (Table 3).

3.8. HTC recovery <50 % or >130 %

Example depletion curves for chemicals with HTC recoveries <50 % or >130 % are presented in Fig. 3E and F, respectively. Large abiotic chemical losses (HTC recovery <50 %) may be due to volatilization,

Table 3
Measured in vitro clearance rates (μL/min/10^6 hepatocytes) for test chemicals by species and starting substrate concentration.

		Human		Rat		Trout	
Test chemical	CAS #	0.1 μM	1 μM	0.1 μM	1 μM	0.1 μM	1 μM
1,3-Benzenedicarboxylic acid	121-91-5	c	c	c	1.44[a]		
2,2',4,4'-Tetrahydroxybenzophenone	131-55-5			>103[d]	>232[d]	>89.8[d]	29.0[a]
2,5-Di-tert-butylbenzene-1,4-diol	88-58-4	2.75[a]	8.91[a]	e	c		
2-Ethylhexyl paraben	5153-25-3	>128[d]	178[a]			62.0[a]	29.5[a]
3,3',5,5'-Tetrabromobisphenol A	79-94-7	3.22[a]	5.84[a]	15.2[a]	23.0[a]	25.3[a]	22.3[a]
4-(1,1,3,3-Tetramethylbutyl) phenol	140-66-9					55.9[a]	60.9[b]
4,4'-Sulfonylbis[2-(prop-2-en-1-yl) phenol]	41481-66-7	84.7[d]	89.4[a]	80.4[a]	77.6[a]	f	f
4-Hexylresorcinol	136-77-6	f	f	>227[b]	214[a]		
4-Nitroaniline	100-01-6	3.68[a]	<2.57[a]	f	<5.07[a]	<2.80[a]	<3.38[a]
4-n-Nonylphenol	104-40-5	62.6[b]	76.4[b]	>110[b]	121[b]	e	e
4-Nonylphenol (branched)	84852-15-3	>104[b]	141[b]			g	g
4-Octylphenol	1806-26-4					g	11.4[a]
6:2 Fluorotelomer alcohol	647-42-7	e	e	e	e	e	e
Atrazine	1912-24-9			14.1[a]	12.4[a]	<2.80[a]	<3.38[a]
Benzyl butyl phthalate	85-68-7	e	e			g	g
Benzylparaben	94-18-8	>192[d]	>356[a]	e	e		
Bisphenol A	80-05-7					>103[a]	37.4[a]
Bisphenol AF	1478-61-1			165[b]	194[a]	>81.1[a]	37.1[a]
Celecoxib	169590-42-5	24.6[a]	22.9[a]	<2.93[a]	2.97[a]		
Cymoxanil	57966-95-7	e	e	e	e		
Dapsone	80-08-0	1.15[a]	0.92[a]	1.39[a]	1.46[a]		
Deltamethrin	52918-63-5	e	c	e	e	2.43[d]	2.96[a]
Di(2-ethylhexyl) phthalate	117-81-7	g	c			f	4.49[a]
Dibutyl phthalate	84-74-2			>79.3[b]	e	f	>104[b]
Dichlobenil	1194-65-6					f	f
Dicumyl peroxide	80-43-3	e	e	e	e	c	9.18[a]
Ethylparaben	120-47-8					53.9[a]	11.2[a]
Fenthion	55-38-9	e	e	>244[b]	e	e	f
Heptylparaben	1085-12-7	f	f	f	150[d]	33.0[b]	39.9[a]
Hexaflumuron	86479-06-3	f	5.20[d]	f	f	c	<3.38[d]
Hydroxyflutamide	52806-53-8	<3.43[d]	<2.57[a]	<2.93[a]	<5.07[a]	<2.80[d]	<3.38[a]
Ipconazole	125225-28-7	>242[a]	106[a]			1.94[a]	2.06[a]
Isofenphos	25311-71-1	e	e	e	e	e	e
Kaempferol	520-18-3	>95.7[a]	181[b]				
Mestranol	72-33-3	10.2[a]	c	>154[b]	243[b]		
Metconazole	125116-23-6	16.7[a]	4.19[a]			1.14[a]	0.96[a]
Methoxychlor	72-43-5			e	e	3.55[a]	3.40[a]
Myclobutanil	88671-89-0			91.1[a]	68.1[a]	0.67[a]	0.46[a]
Octyl gallate	1034-01-1	g	g	g	g	g	g
p,p'-DDE	72-55-9	c	e	e	e	1.49[a]	2.11[a]
Phenthoate	2597-03-7	>168[a]	>343[b]	e	e	26.6[a]	18.1[d]
Phoxim	14816-18-3	e	e	e	e	f	1.80[a]
Picoxystrobin	117428-22-5	38.2[a]	19.4[a]	88.4[b]	62.3[b]		
Propargite	2312-35-8	e	e	e	e		
Propiconazole	60207-90-1			>232[a]	164[d]	1.12[a]	0.88[a]
Simazine	122-34-9					<2.80[a]	<3.38[a]
Terbuthylazine	5915-41-3	4.10[a]	2.59[a]	12.9[a]	8.57[a]		
Triclocarban	101-20-2	9.45[a]	6.36[a]	38.4[a]	20.5[a]	c	2.99[a]
Triclosan	3380-34-5					64.1[a]	39.4[b]
Trimethyl phosphate	512-56-1	<3.43[a]	<2.57[a]	<2.93[a]	<5.07[a]		
Triphenyl phosphate	115-86-6			>126[b]	140[a]	5.01[a]	c
Triphenyl phosphite	101-02-0	e	e	e	e		
Triphenylethylene	58-72-0	e	e	e	e	14.0[a]	5.00[a]
Zearalenone	17924-92-4	>138[a]	211[a]	>146[a]	213[a]	28.4[b]	14.4[a]

[a]HTC 80–120 %.
[b]HTC between 50 and 80 %, and ratio >10.
[c]HTC between 50 and 80 %, and ratio <10, no rate reported.
[d]HTC between 120 and 130 %.
[e]HTC < 50 %, no rate reported.
[f]HTC > 130 %, no rate reported.
[g]Analytical or technical issues, no rate reported.
Grey = not run.

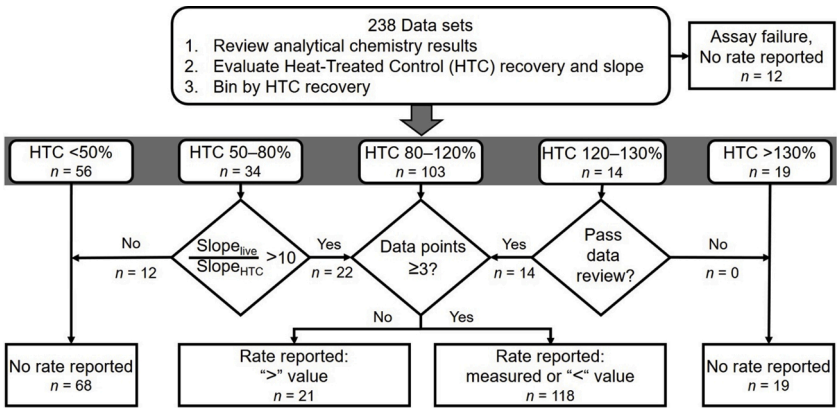

Fig. 1. Data evaluation framework for clearance rate reporting. Bins defined by Heat-Treated Control (HTC) recovery values are arrayed within the shaded rectangle while diamonds indicate decision criteria for determination of rate reporting. The symbols ">" and "< "indicate rates greater than and less than estimated limit values (see text for details).

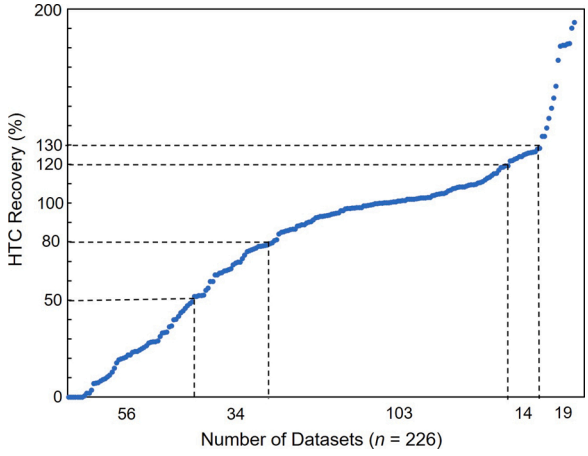

Fig. 2. Distribution of Heat-Treated Control (HTC) recovery values. HTC recovery values for datasets that passed the analytical chemistry review ($n = 226$) are plotted in ascending order. Bins used to group datasets according to their HTC recovery are defined by dashed lines. Numbers shown on the X-axis indicate the number of datasets in each bin.

adsorption to the walls of the reaction vessel, chemical instability, or non-enzymatic reactions with components of the assay system. Under these circumstances, it is impossible to determine the biological contribution to test chemical depletion in the live hepatocyte assays. This behavior was often observed across all tested species and concentrations for a given chemical, as demonstrated by 6:2 fluorotelomer alcohol, cymoxanil, triphenyl phosphite, and others (Table 3). All datasets ($n = 56$) in the <50 % category were determined to be unsuitable for rate reporting.

A HTC recovery >130 % may indicate insufficient mixing of test chemical prior to the $T = 0$ min measurement, especially for those cases where the T =240 min measurement is at the nominal test concentration. This behavior has been reported previously for very hydrophobic chemicals (Fay et al., 2015). If the $T = 0$ min measurement is on target but the T =240 min value is high, an analytical error associated with second measurement is the most likely cause. Datasets in this category ($n = 19$) were also eliminated from rate reporting.

3.9. Relationship of log K_{OW} to assay performance

To evaluate potential effects of adsorption to the reaction vessel wall, individual test chemicals were flagged as "hydrophobic" if they possessed a predicted log octanol-water partition coefficient (log K_{OW}) value greater than 5.0, as determined using US EPA's CompTox dashboard (Table S1). In all, 10 chemicals satisfied this criterion (20 possible test results). Each chemical was then evaluated to determine whether the HTC recovery at 4 h was <80 % or between 80 and 130 %. Ignored in the analysis were any test results that suggested analytical problems, including HTC recovery values >130 %. A similar analysis was performed on the remaining unflagged chemicals. The results for each species were then expressed as a percentage "success" rate [(number of assays with mean HTC recovery between 80 % and 130 % / total number of assays) × 100].

Success rates for hydrophobic chemicals tested using the human and rat hepatocyte assays were quite low (21 % and 14 %, respectively; Table S12). By comparison, the success rate for hydrophobic chemicals tested in the trout hepatocyte assay was much higher (78 %). Non-hydrophobic chemicals performed reasonably well in both the human and rat hepatocyte assays (59 % and 50 % success, respectively). The success rate for non-hydrophobic chemicals in the trout hepatocyte assay was 74 %.

For the hydrophobic chemicals, there was perfect agreement between test results (successful or unsuccessful) obtained using human and rat hepatocytes. Examples include 4-*n*-nonylphenol, deltamethrin, dicumyl peroxide, p,p'-DDE, and triphenylethylene, all of which exhibited HTC recoveries <80 %. There was relatively poor agreement when results from the human and/or rat assays were compared to those obtained using trout hepatocytes. For example, deltamethrin, p,p'-DDE, triphenylethylene, and methoxychlor were lost from the human and/or rat assays (HTC < 80 %) but not from the trout assay. One hydrophobic chemical (3,3′,5,5′-tetrabromobisphenol A; log $K_{OW} = 6.66$) performed successfully in human, rat, and trout assays, while a second (4-*n*-nonylphenol; log $K_{OW} = 5.76$) exhibited unsuccessful performance in all three assays.

3.10. Concentration dependence of clearance rates

The concentration-dependence of CL_{int} was assessed for all datasets yielding reportable depletion rates at both the 0.1 and 1 μM test concentrations. For the 35 datasets that fulfilled this criterion, a second analysis was performed to determine whether fitted slopes for the two depletion curves were significantly different. Finally, the CL_{int} determined at 0.1 μM was divided by that measured at 1 μM to develop a simple activity ratio (Fig. 5).

Thirty-one of 35 (89 %) calculated activity ratios fell between 0.5 and 2.0, indicating good agreement between the two tested substrate concentrations. For six chemicals (3,3′,5,5′-tetrabromobisphenol A, 4,4′-sulfonylbis[2-(prop-2-en-1-yl) phenol, dapsone, picoxystrobin, terbuthylazine, triclocarban), calculated activity ratios were available for

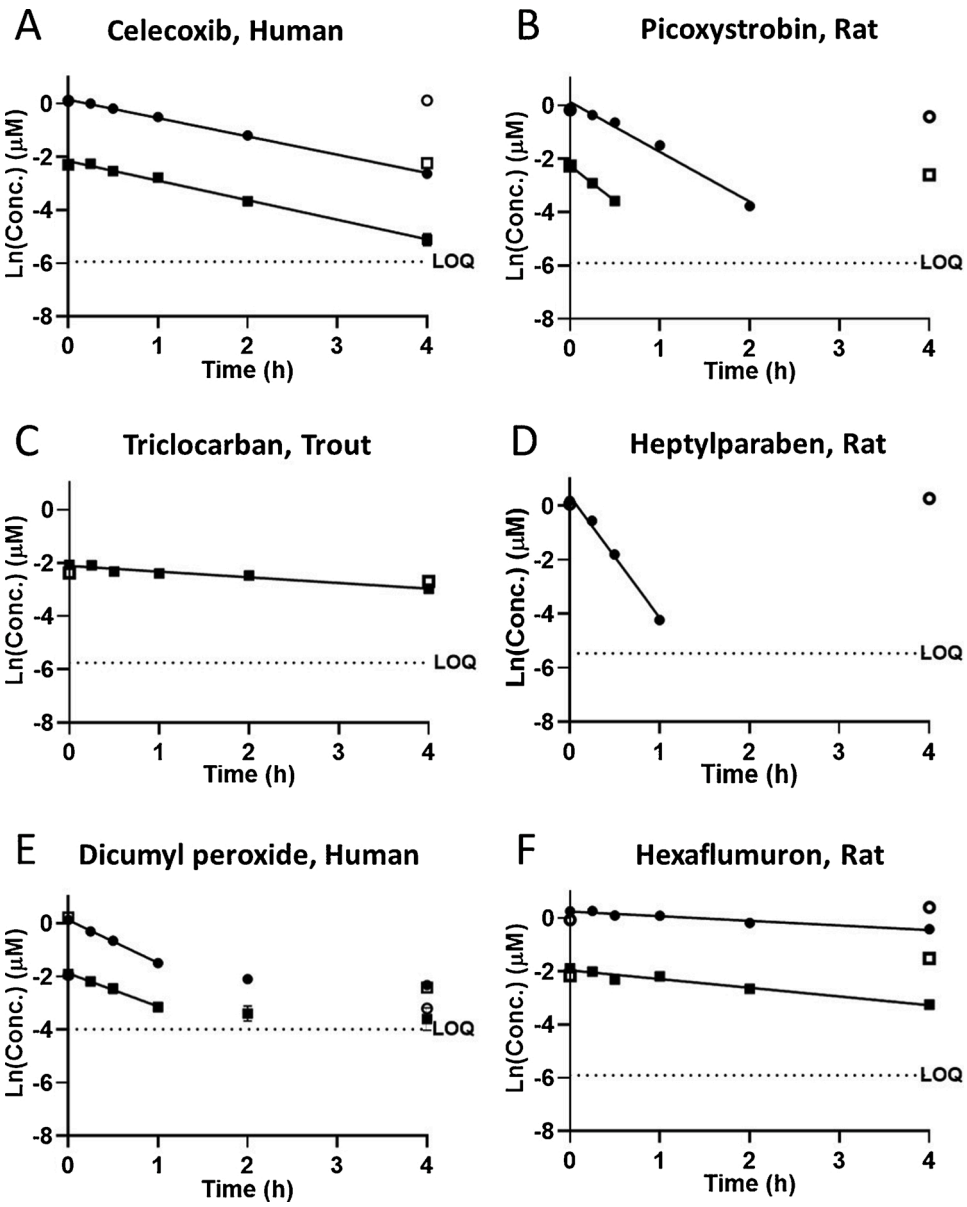

Fig. 3. Depletion plots for datasets in Heat-Treated Control (HTC) recovery bins identified in Figs. 1 and 2. A. HTC recoveries between 80 and 120 %; rates reported. B. HTC recoveries between 50 and 80 %, and ratio k_{dep}/k_{HTC} >10; rates reported. C. HTC recoveries between 50 and 80 %, and ratio k_{dep}/k_{HTC} <10; rates not reported. D. HTC recoveries between 120 and 130 %; data were subjected to additional review and rates reported. E. HTC recoveries <50 %; rates not reported. F. HTC recoveries >130 %; rates not reported. Solid symbols indicate data for active hepatocytes while open symbols show data for HTCs. Each point represents the mean ± SD of two (HTCs) or three measured values (active hepatocytes). Solid lines represent linear regression equations fitted to each active dataset while dotted lines show the analytical limit of quantitation (LOQ).

more than one species. In each of these cases, the ratio fell between 0.5 and 2.0, indicating good agreement among species. Four of 35 (11 %) activity ratios exceeded 2.0. The largest value (4.76) was determined for ethylparaben, when tested using trout hepatocytes. There was no apparent relationship, however, between the magnitude of this ratio and test species.

3.11. *Comparison of extrapolated in vivo intrinsic clearance rates across species*

In vitro intrinsic clearance rates (CL_{int}; μL/min/10^6 hepatocytes, Table 3) were extrapolated to in vivo intrinsic clearance rates ($CL_{int,\ in\ vivo}$; μL/min/g liver) using the following hepatocellularity values (hepatocytes/g liver): human, 110 (Wetmore et al., 2015); rat, 117 (Wetmore et al., 2013); trout, 510 (Fay et al., 2014b) (Fig. 6). The $CL_{int,\ in\ vivo}$ rates for all species demonstrate that these assays have a dynamic range of at least 600-fold (100 to 60,000 μL/min/g liver). For both starting substrate concentrations, the "less than" values for trout liver are higher than those for human or rat liver. This is primarily due to the markedly higher hepatocellularity value for trout.

At the 0.1 μM test concentration, $CL_{int,\ in\ vivo}$ rates were determined for more than one species for 23 chemicals (Fig. 6A). In general, the highest in vivo clearance values were calculated for rat liver tissue while the lowest values were obtained for trout. The most notable exception is 3,3′,5,5′-tetrabromobisphenol A; the calculated $CL_{int,\ in\ vivo}$ of 3,3′,5,5′-tetrabromobisphenol A by trout liver was 7-fold higher than that determined for rat and 36-fold higher than that for human. For several other chemicals (e.g., 2-ethylhexylparaben, 4-nitroaniline, bisphenol AF, and 2,2′,4,4′-tetrahydroxybenzophenone), the $CL_{int,\ in\ vivo}$ calculated for trout liver is comparable to that (within approximately 3-fold) determined for rats or humans. The comparative clearance of propiconazole at 0.1 μM in rat versus trout tissue represents the greatest difference between two species (rat: >27,193 μL/min/g liver; trout: 571 μL/min/g liver).

$CL_{int,\ in\ vivo}$ rates for more than one species at the 1 μM test concentration were determined for 23 chemicals (Fig. 6B). As at the 0.1μM starting concentration, the highest $CL_{int,\ in\ vivo}$ values were usually calculated for rat liver while the lowest values were generally determined for trout. Nevertheless, the calculated $CL_{int,\ in\ vivo}$ of 3,3′,5,5′-tetrabromobisphenol A by trout liver was again greater than that for rats

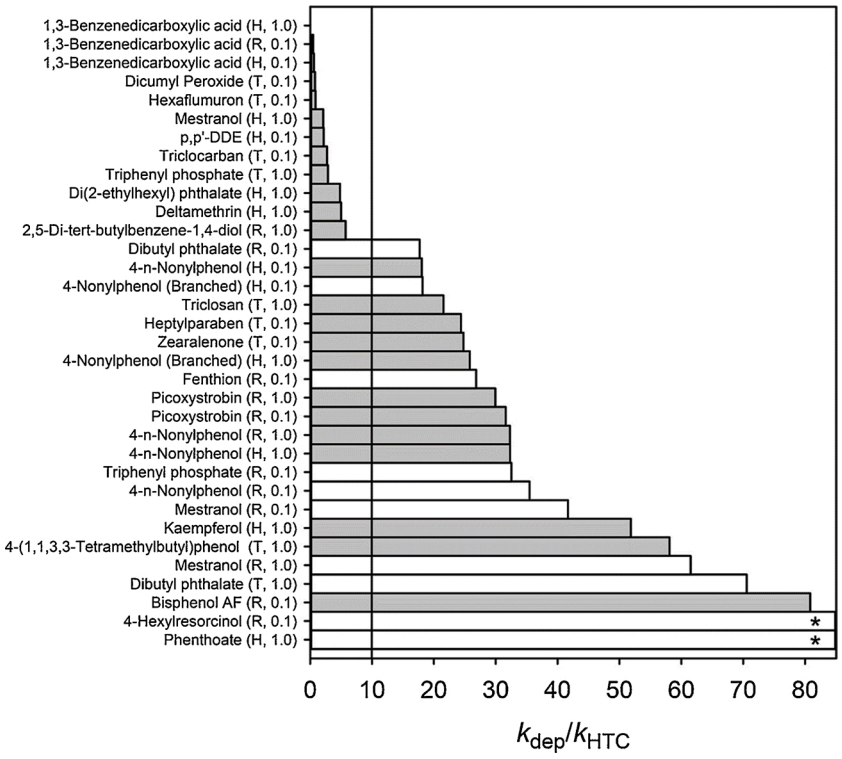

Fig. 4. Ratio of the measured depletion rate constant (k_{dep}) to the slope of the Heat-Treated Control (HTC) recovery curve (k_{HTC}) for datasets with HTC recovery values between 50 and 80 %. Solid bars indicate datasets with ≥3 values above the analytical limit of quantification (LOQ); white bars indicate datasets with <3 values above the LOQ. The vertical line represents a ratio (k_{dep}/k_{HTC}) of 10. *The ratios for 4-hexylresorcinol and phenthoate were 107.6 and 160.5, respectively. Species codes: H = human; R = rat; T = trout.

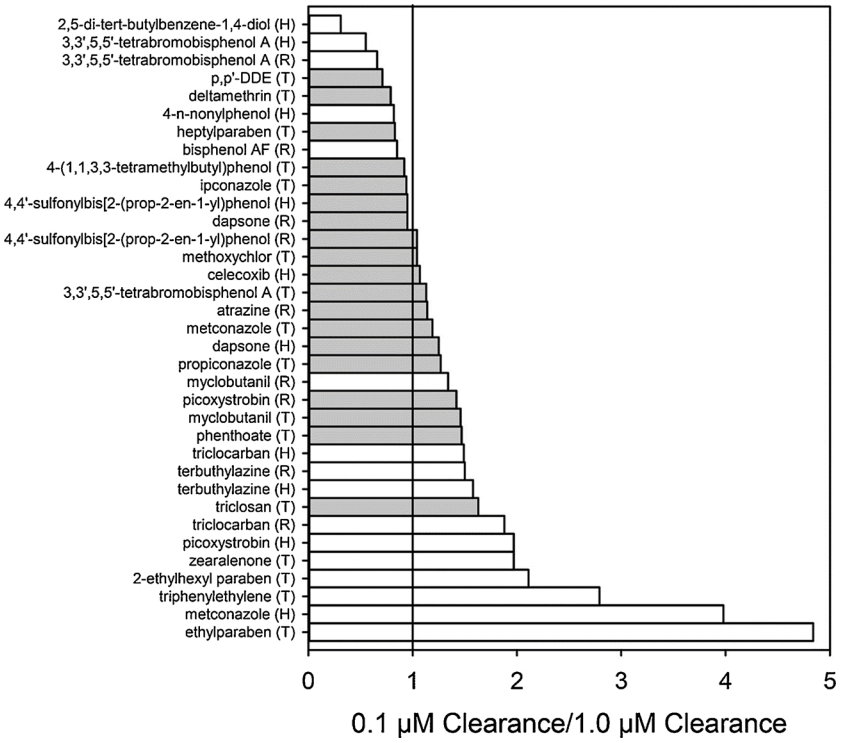

Fig. 5. Ratio of in vitro intrinsic clearance rates (CL_{int}) determined for individual test chemicals at the 0.1 and 1 µM starting substrate concentrations. Grey bars indicate chemicals for which depletion rate constants at 0.1 and 1 µM did not differ significantly (*F*-test, α = 0.05). White bars indicate chemicals for which depletion rate constants at 0.1 and 1 µM were significantly different. The vertical line represents a ratio (0.1 µM/1 µM) of 1. Species codes: H = human; R = rat; T = trout.

(4-fold higher) or humans (18-fold higher). For 4-nitroaniline and hydroxyflutamide, reported rates for all three species are given as "less than" values indicating very low levels of biotransformation. A similar finding for hydroxyflutamide was obtained at the 0.1 µM test concentration. Within the 1 µM dataset, the clearance of propiconazole again represented the greatest difference between two species (rat: 19,241 µL/min/g liver; trout: 449 µL/min/g liver).

Importantly, these species comparisons do not consider potential

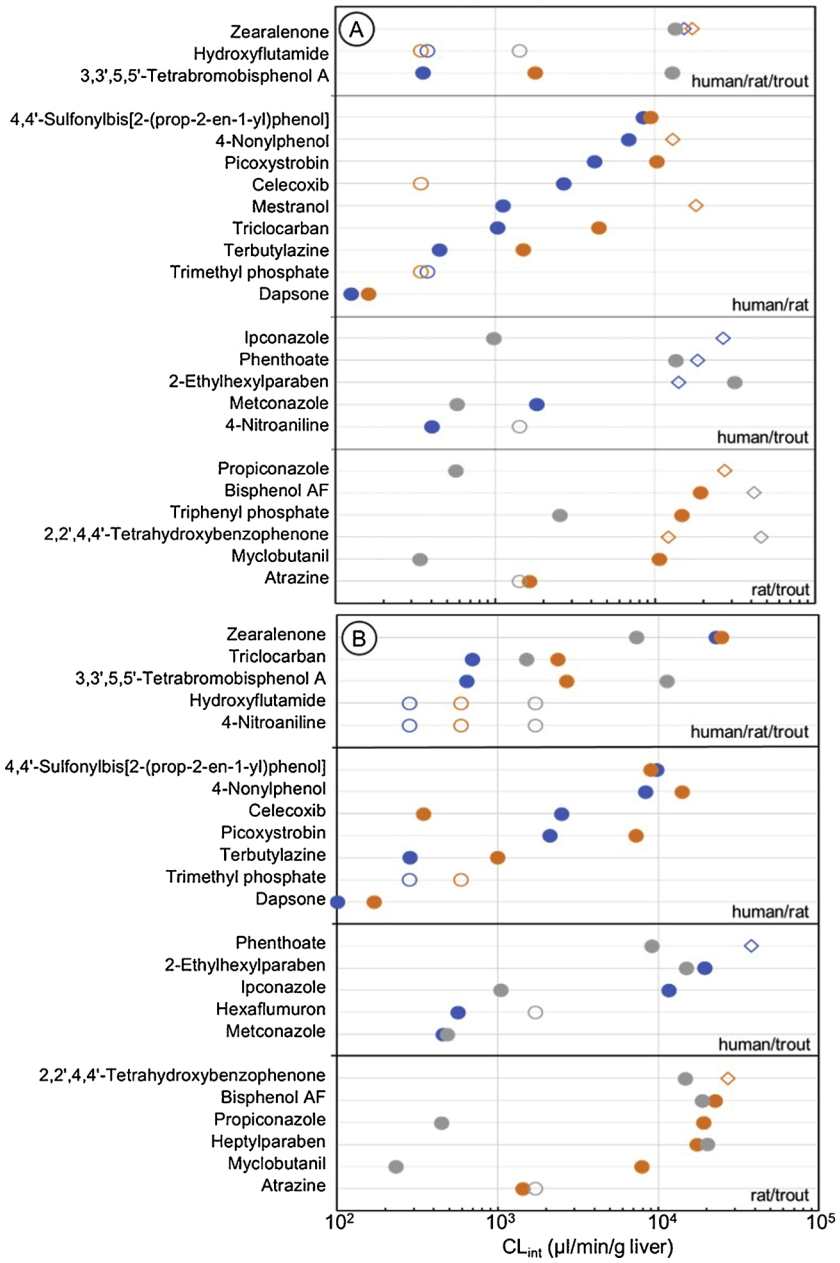

Fig. 6. Cross species comparison of calculated in vivo intrinsic clearance rates ($CL_{int,\ in\ vivo}$; µl/min/g liver). $CL_{int,\ in\ vivo}$ values were calculated from in vitro intrinsic clearance rates measured at starting substrate concentrations of 0.1 µM (A) and 1 µM (B). Dots indicate measured rates, open circles show calculated "less than" rates, and open diamonds indicate "greater than" rates. Color legend: human = blue; rat = orange; trout = grey. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

differences in chemical binding within the different in vitro assays. These differences in binding will be negligible for hydrophilic chemicals that remain freely dissolved in solution. Larger differences can be expected for hydrophobic chemicals that partition into hepatocytes. For such chemicals, binding is inversely related to the fractional volume of the assay occupied by cells (Brown et al., 2007; Hallifax and Houston, 2007). Trout hepatocytes from sexually immature animals are approximately 1/5th the size of human or rat hepatocytes. Thus, binding in a solution that contains human or rat hepatocytes should be greater than that in a solution containing the same number of trout cells. In the present study, this effect of cell size would have been counteracted by the fact that human and rat assays were run using 0.5×10^6 hepatocytes/mL while trout assays employed 1×10^6 hepatocytes/mL. Nevertheless, we can reasonably expect free chemical fractions (f_U) in the trout assay to be slightly higher than those in the human or rat assays, especially for highly bound chemicals. Empirically based algorithms for prediction of f_U in human, rat, and trout hepatocyte assays have been given by several authors (Austin et al., 2005; Han et al., 2007; Kilford et al., 2008); however, the prediction accuracy of these algorithms is relatively low, especially for very hydrophobic chemicals (Kilford et al., 2008). We elected, therefore, to compare intrinsic clearance values without correction for in vitro binding, since relative differences in binding among the three assays are likely to be small (2 to 3-fold or less) while errors in predicted binding for any given test chemical could be substantial.

4. Discussion

In vitro depletion data for 54 industrial and pesticidal chemicals were obtained using cryopreserved hepatocytes from humans, rats, and rainbow trout. The selected test chemicals represent a wide range of structures and physicochemical properties. Given the diverse nature of chemicals that comprise the industrial and pesticidal chemical universe, it is of interest to better understand the domain of applicability of in vitro methods used to measure intrinsic clearance. Additionally, as in vitro methods move toward greater regulatory acceptance, there is a

need to evaluate standardized test methods and develop guidance for predicting which chemicals are unlikely to perform well in such systems. A recent study examined experimental conditions across several studies utilizing human hepatocytes and found that CL_{int} values obtained for the same chemical with divergent methods varied greatly, spanning more than an order of magnitude (Louisse et al., 2020). These findings highlight the need for specific guidance on how to perform and interpret in vitro metabolism studies conducted to support regulatory risk assessments (EFSA, 2019), preferably following principles outlined in the OECD Guidance Document *Good In Vitro Method Practices (GIVIMP)* (OECD, 2018c).

The results of the present study were assessed using a data evaluation framework to identify datasets suitable for rate reporting. The primary emphasis of this framework was on HTC recovery. An initial evaluation showed a surprisingly wide range of values (Fig. 2). Most (~75 %) of the recovery values outside the acceptable range (80–120 %) were lower than 80 %. Plausible explanations for a low HTC recovery value include volatilization, adsorption to the reaction vessel wall, and abiotic chemical reactions including decomposition (e.g., hydrolysis or photolysis) and covalent binding with components of the incubation mixture. Volatilization and decomposition are true loss processes, while adsorption results in removal of chemical from the incubation medium, reducing the amount that is available to be acted on by metabolizing enzymes. Volatilization from a stirred system that is open to the air is likely to follow first-order kinetics. In such cases, it may be possible to subtract the volatilization rate constant from the depletion rate constant for an active sample to calculate a corrected rate of intrinsic clearance (OECD, 2018b). This correction would require several measurements of the HTC over the course of the experiment, as opposed to only the starting and ending times as was done in this study. Application of these methods to volatile substrates may also be facilitated by use of appropriate sealing films. Chemical adsorption to a surface involves diffusion in the unstirred layer at the surface, as well as adsorption to and desorption from the surface itself (Rudzinski and Plazinski, 2006). If the kinetics of adsorption are fast, rapid establishment of an internal binding equilibrium could occur. Alternatively, if the rate of adsorption is slow, the time to equilibrium may be long relative to the kinetics of substrate depletion. Given this complexity, it may or may not be possible to correct depletion rates observed in active samples, given additional HTC data. Abiotic chemical reactions may or may not follow first-order kinetics, depending on the nature of the reaction and the relative concentrations of reactants.

Some low HTC recovery values were seen across all species within a chemical (e.g., 6:2 fluorotelomer alcohol, propargite, triphenyl phosphite) suggesting that this behavior was compound-specific. In other cases, HTC recoveries were low in human and rat assays but the trout recovery value was acceptable (e.g., p,p-DDE, triphenylethylene). To examine the potential impact of chemical hydrophobicity on assay performance, chemicals were flagged as "hydrophobic" if they had a predicted log K_{OW} value >5. One hydrophobic chemical (3,3′,5,5′-tetrabromobisphenol A; log $K_{OW} = 6.66$) exhibited excellent performance in all three assays (with HTC recoveries between 80 and 120 %). Generally, however, hydrophobic chemicals performed poorly in both the human and rat assays, resulting in HTC recoveries <80 %. In contrast, there was no apparent relationship between chemical hydrophobicity and the behavior of HTCs when using the trout hepatocyte assay. These differences in assay performance may have been due to differences in methodology, which are based on protocols taken from the literature. Human and rat hepatocytes were tested in polypropylene plates, while trout hepatocytes were tested in glass vials. Although polypropylene is generally regarded as a low-binding material, numerous studies have shown that adsorption can be impacted by the composition of a test vessel. OECD guidance for using the trout hepatocyte assay recommends the use of glass vials to minimize the effect of chemical adsorption (OECD, 2018a). Importantly, this guidance was developed in anticipation that the assay would be employed for hydrophobic chemicals that possess high bioaccumulation potential. As more information becomes available, it may be possible to develop useful hydrophobicity thresholds for specific assays and/or provide guidance on selection of an appropriate test system (e.g., polypropylene plates or glass vials) for a chemical of interest. Unfortunately, it is difficult to determine how the present findings relate to earlier studies because HTC data are seldom presented. The present study therefore underscores the importance of HTC data and suggests the need for researchers to report this data and discuss how the observed values were used.

In a substantial number of cases ($n = 21$), the chemical concentration dropped below the LOQ within 30 min indicating rapid biotransformation. Had the six sampling time points for these assays been compressed towards the start of the assay, it is likely that more accurate rates could have been reported. Instead, the data were used to calculate "greater than" rates which establish a lower bound on intrinsic clearance. Despite the uncertainty associated with these "greater than" values, the derived rates may still be useful for modeling purposes, since hepatic clearance becomes blood flow-limited at high rates of intrinsic clearance (Rowland et al., 1973; Wilkinson and Shand, 1975). Thus, if the calculated "greater than" rate is sufficiently high, uncertainties regarding its true value may have little impact on predicted rates of hepatic clearance.

The power analysis used to estimate a lowest detectable depletion rate constant was informed by the experimental design as well as typical levels of variance in resulting datasets. If the SE ($\widehat{\beta}$) for a given dataset is smaller than the average value estimated here using Eq. 3, the probability of detecting a significant negative slope will exceed the probability (90 %) used to run the analysis. Within a given experiment, this would translate to an increased likelihood of detecting a significant slope smaller than the "less than" values given in Table 1. There are two examples of this in the current study findings, both involving dapsone. Because of low variance in the data, depletion rate constants lower than the $k_{dep,\ low}$ values calculated for the rat and human assays were found to be significantly different from 0. The result is that CL_{int} values for dapsone measured at both starting substrate concentrations are lower than the "less than" values given for both rat and human (Fig. 6A and B). In principal, it is possible to improve detection of low depletion rate constants by increasing the length of time over which the assay is run, the number of sampling times, and the number of measurements per sampling time; however, current experience suggests that it would be difficult to routinely measure $k_{dep,\ low}$ values much lower than those given in Table 1, using the described methods. Nevertheless, "less than" CL_{int} values developed as described in this study may be useful in the context of performing modeled chemical risk assessments by establishing an upper limit on $CL_{int,\ in\ vivo}$, and by extension hepatic clearance; the true $CL_{int,\ in\ vivo}$ may be lower than this value, but it is unlikely to be higher.

Measured CL_{int} values were used to evaluate the starting concentration-dependence of in vitro activity. The issue of concentration-dependence is important because procedures used to extrapolate in vitro clearance rates to the intact animal generally assume that this activity has been measured under first-order reaction conditions (i.e., starting substrate concentration $<<K_M$; Obach, 1999; Naritomi et al., 2001; Nichols et al., 2006). If this assumption is met, CL_{int} values determined at different starting substrate concentrations will be approximately equal. If not, rates determined at a lower test concentration should exceed those determined at a higher concentration. In the present study, most (31 of 35) of the calculated activity ratios (0.1 μM/1 μM) fell between 0.5 and 2.0, indicating near first-order reaction conditions. Metabolic stability assays conducted by the pharmaceutical industry are commonly performed using a 1 μM starting test concentration, based on the observation that K_M values for most drugs exceed this value (Obach, 1999; Naritomi et al., 2001). Thus, it appears that guidance on starting substrate concentrations provided by the drug-testing experience is adequate for many industrial and pesticidal

chemicals. For several chemicals, however, the calculated activity ratio was greater than 2. An examination of reference chemical clearance data (Table 2) also shows that the activity ratio for pyrene (tested using trout hepatocytes) was greater than 2. For a small number of chemicals, therefore, the 1 μM starting test concentration may be insufficient to achieve first-order reaction conditions.

Calculated $CL_{int,\ in\ vivo}$ rates for several test chemicals were similar for all three test species suggesting qualitative similarities in the activities of specific metabolic pathways (i.e., consistently fast or slow). In most cases, however, clearance rates determined for humans and/or rats were faster than those calculated for trout. Previously, Han et al. (2007) used freshly isolated rat and trout hepatocytes to measure the in vitro intrinsic clearance of six chemicals at starting substrate concentrations ranging from 2 to 15 μM. When these measured CL_{int} values were converted to $CL_{int,\ in\ vivo}$ values (expressed per gram liver), the rates for rats were 1.2 to 20-fold faster than those for trout. Similarly, Han et al. (2009) measured the basal activities of CYP1A, CYP3A, GST and UGT in liver microsomes and S9 fractions from rats and rainbow trout. When normalized to protein content and expressed as a simple ratio, measured S9 activities for rats were 3.0 (UGT) to 10.5 (CYP3A) times higher than those for trout. The $CL_{int,\ in\ vivo}$ rates determined here for rat liver tissue were usually higher than those for humans. Similar results have been reported for some pharmaceuticals (Nishimuta et al., 2013; Hallifax and Houston, 2019). There is some question, however, whether this pattern can be generalized to other chemicals. The datasets published by Wetmore et al. (2012, 2013), Honda et al. (2019) and Wambaugh et al. (2019) provide a unique opportunity to compare $CL_{int,\ in\ vivo}$ rates for rats and humans within the industrial and pesticidal chemical space. These studies used consistent methods and starting substrate concentrations (1 μM). For 6 of the 58 chemicals with data generated in both species, there are two measured values for human hepatocytes yielding 64 total data pairs (rat vs. human). In exactly half of these cases ($n = 32$) the $CL_{int,\ in\ vivo}$ determined for human hepatocytes was faster than that measured using rat hepatocytes, while in the remaining instances the reverse was true.

Published CL_{int} rates that could be compared to the results of this study were identified for 23 unique chemicals (Table 4). The greatest overlap was for chemicals tested using human hepatocytes ($n = 16$). Datasets generated in the present study for 8 of these chemicals did not pass the data quality assessment. For the remaining 8, CL_{int} rates determined for 5 chemicals showed good agreement with values measured previously at a 1 μM starting substrate concentration. For example, clearance rates given here for hydroxyflutamide are reported as "less than" values (indicating no detectable clearance), while previous studies indicated no measurable clearance. Conversely, rapid rates of clearance (>100 μL/min/10^6 hepatocytes) were determined in this and a previous study for benzylparaben and ipconazole. Clearance rates for celecoxib measured in the present study are essentially identical to a value determined earlier. A lower level of agreement between the present work and earlier studies was observed for 2,5-di-tert-butylbenzene-1,4-diol, 3,3′,5,5′-tetrabromobisphenol A, and 4-nitroaniline. In each case, clearance rates determined in the present effort were substantially lower than those measured previously at a 1 μM starting concentration. Relatively good agreement (<4-fold difference) was observed between current results and rates measured earlier at a 10 μM starting test concentration. It is difficult, however, to interpret this finding as clearance rates determined in earlier studies at a 1 μM test concentration were consistently faster than those measured at 10 μM (average of 4.9-fold difference, excepting chemicals for which there was no measurable activity; Table 4). This observation suggests that starting substrate concentrations greater than 1 μM tend to result in non-first-order reaction conditions.

Published CL_{int} rates were available for 7 of 40 chemicals tested in this study using rat hepatocytes. Unacceptable performance of HTCs was observed for 2 of these 7 chemicals (benzylparaben and deltamethrin). For 3 of the remaining 5 chemicals (atrazine, heptylparaben, zearalenone), clearance rates determined in the present effort are in reasonable agreement with reported values (5.2-fold difference or less for rates measured at 1 μM). Clearance of a fourth chemical (trimethyl phosphate) was reported as a "less than" value (indicating no detectable clearance) in this study, while previous studies indicated no measurable clearance. Clearance rates determined in this effort for 4-*n*-nonylphenol are approximately 10 times faster than that measured in a previous study, which was performed using a 5 μM starting test concentration. Additional published data were available for 5 chemicals tested in the present study using trout hepatocytes. Of these, 4-*n*-nonlylphenol and fenthion failed to pass the data quality assessment. For methoxychlor and deltamethrin, clearance rates measured here are in good agreement with published information (<2-fold difference from at least one published value). For atrazine, there was no detectable clearance in this or a previous study.

The extrapolated $CL_{int,\ in\ vivo}$ rates from this study may also be compared to values predicted by established biotransformation QSARs. For example, Fig. 7 compares calculated human and rat $CL_{int,\ in\ vivo}$ values from this study to values generated by ADMET Predictor software v9.5 (Simulations Plus, Lancaster, CA). The rates given by ADMET are reported in units of μL/min/mg microsomal protein. Conversion to units of μL/min/g liver was accomplished using scaling factors of 32 and 61 mg of microsomal protein/g liver for human and rat, respectively (Barter et al., 2007; Smith et al., 2008). For these comparisons, we ignored the role of binding as a determinant of measured (this study) or predicted (by ADMET) in vitro clearance. Existing information indicates, however, that non-specific binding in a solution containing 0.5×10^6 rat or human hepatocytes is comparable to that which occurs in the microsomal assays used to develop the ADMET QSARs (Supplementary Data, Chemical binding to isolated hepatocytes and liver microsomes).

Two-thirds (25/38 = 66 %) of $CL_{int,\ in\ vivo}$ values determined in this study using human hepatocytes were within 10-fold of those predicted by ADMET (Fig. 7A). Where there was substantial (>10-fold) disagreement between extrapolated and predicted values for humans, the extrapolated values were usually higher (10/13 cases). The concordance between extrapolated and predicted $CL_{int,\ in\ vivo}$ values for rats was generally higher than that observed for humans (Fig. 7B). For rats, 30 of 36 (83 %) extrapolated rates were within 10-fold of ADMET-predicted values.

Importantly, $CL_{int,\ in\ vivo}$ values determined in the present study reflect the activity of intact hepatocytes while the QSARs in ADMET predict only microsomal activity. A microsomal preparation contains membrane-bound enzymes (e.g., CYPs and UGTs) responsible for most phase I biotransformation reactions and phase II glucuronidation. It does not contain cytosolic phase II conjugation enzymes, including SULTs and GSTs. If cytosolic enzymes contribute substantially to the in vitro clearance of a tested substrate, an $CL_{int,\ in\ vivo}$ value calculated using hepatocyte data might be expected to exceed that predicted by ADMET. This would tend to shift the plotted point for such a chemical upward in Fig. 7. The cluster of 10 points in the upper left-hand portion of Fig. 7A (for humans) may represent this type of substrate, but this would require confirmation based on detailed knowledge of metabolic products. There is no indication of a similar cluster in the comparison plot for rats (Fig. 7B); however, only one of the chemicals represented by the cluster of points in the human plot (zearalenone) produced an acceptable CL_{int} using rat hepatocytes. Additional research is needed to determine whether in vivo intrinsic clearance rates predicted using biotransformation QSARs or by in vitro-in vivo extrapolation of measured CL_{int} are predictive of true, in vivo values. Absent this information, however, good agreement between rates predicted by both methods may contribute to a higher level of confidence in risk assessments that employ these predictions.

In summary, standardized test methods employing cryopreserved hepatocytes from humans, rats, and trout were used to measure intrinsic clearance rates for a diverse set of industrial and pesticidal chemicals. Reportable rates were obtained for approximately half of the datasets

Table 4
Comparison of measured in vitro clearance rates (μL/min/10^6 hepatocytes) with other published values.

Test chemical	CAS #	Measured value	Test conc. (μM)	Published value[h]	Test conc. (μM)	Reference
Human						
2,5-Di-tert-butylbenzene-1,4-diol	88-58-4	8.91[a]	1	9.86	10	Wambaugh et al., 2019
		2.75[a]	0.1	117	1	
3,3′,5,5′-Tetrabromobisphenol A	79-94-7	5.84[a]	1	13.6	10	Wambaugh et al., 2019
		3.22[a]	0.1	50.5	1	
4-Nitroaniline	100-01-6	<2.57[a] 3.68[a]	1	8.62	10	Wambaugh et al., 2019
			0.1	23.6	1	
Benzyl butyl phthalate	85-68-7	e	1	309	10	Wambaugh et al., 2019
		e	0.1	465	1	
Benzylparaben	94-18-8	>356[a] >192[d]	1	312	10	Wambaugh et al., 2019
			0.1	633	1	
Celecoxib	169590-42-5	22.9[a]	1	25.7	1	Temesi et al., 2010
		24.6[a]	0.1			
Cymoxanil	57966-95-7	e	1	5.05	10	Wambaugh et al., 2019
		e	0.1	32.8	1	
Dapsone	80-08-0	0.92[a]	1	0.00	10	Wambaugh et al., 2019
		1.15[a]	0.1	0.00	1	
Di(2-ethylhexyl) phthalate	117-81-7	c	1	0.00	10	Wetmore et al., 2012
		g	0.1	0.00	1	
Fenthion	55-38-9	e	1	21.6	10	Wetmore et al., 2012
		e	0.1	87.2	1	
Hydroxyflutamide	52806-53-8	<2.57[a] <3.43[d]	1	0.00	10	Wambaugh et al., 2019
			0.1	0.00	1	
Ipconazole	125225-28-7	106[a]	1	17.8	10	Wambaugh et al., 2019
		>242[a]	0.1	101	1	
Isofenphos	25311-71-1	e	1	25.4	10	Wambaugh et al., 2019
		e	0.1	158	1	
Octyl gallate	1034-01-1	g	1	158	10	Wambaugh et al., 2019
		g	0.1	230	1	
p,p'-DDE	72-55-9	e	1	87.0	10	Wambaugh et al., 2019
		c	0.1	199	1	
Propargite	2312-35-8	e	1	22.1	10	Wambaugh et al., 2019
		e	0.1	24.0	1	
Rat						
4-n-Nonylphenol	104-40-5	121[b] >110[b]	1	11.7	2	Han et al., 2007
			0.1			
Atrazine	1912-24-9	12.3[a] 14.1[a]	1	63.5	1	Han et al., 2007
			0.1			
Benzylparaben	94-18-8	e	1	639	5	Honda et al., 2019
		e	0.1			
Deltamethrin	52918-63-5	e	1	7	5	Honda et al., 2019
		e	0.1			
Heptylparaben	1085-12-7	150[d]	1	311	5	Honda et al., 2019
		f	0.1			
Trimethyl phosphate	512-56-1	<5.07[a] <2.93[a]	1	0	5	Honda et al., 2019
			0.1			
Zearalenone	17924-92-4	213[a] >146[a]	1	59.5	5	Honda et al., 2019
			0.1			
Trout						
4-n-Nonylphenol	104-40-5	e	1	1	2	Nichols et al., 2018
		e	0.1	15.3	10	Mingoia et al., 2010
Atrazine	1912-24-9	<3.38[a] <2.80[a]	1	0.033	2	Han et al., 2007
			0.1			
Deltamethrin	52918-63-5	2.96[a]	1	2.55	1	Nichols et al., 2018
		2.43[d]	0.1	9.06	1	
Fenthion	55-38-9	f	1	3.4	0.2	Nichols et al., 2018
		e	0.1	43	2	Fay et al., 2014a
Methoxychlor	72-43-5	3.40[a]	1	0.37	0.32	Fay et al., 2014a
		3.55[a]	0.1	2.35	2	Nichols et al., 2018

[a]HTC 80–120 %.
[b]HTC between 50 and 80 %, and ratio >10.
[c]HTC between 50 and 80 %, and ratio <10, no rate reported.
[d]HTC between 120 and 130 %.
[e]HTC < 50 %, no rate reported.
[f]HTC > 130 %, no rate reported.
[g]Analytical or technical issues, no rate reported.
[h]Lowest and highest intrinsic clearance values found in the literature with respective starting substrate concentration (Test conc.; μM) and reference.

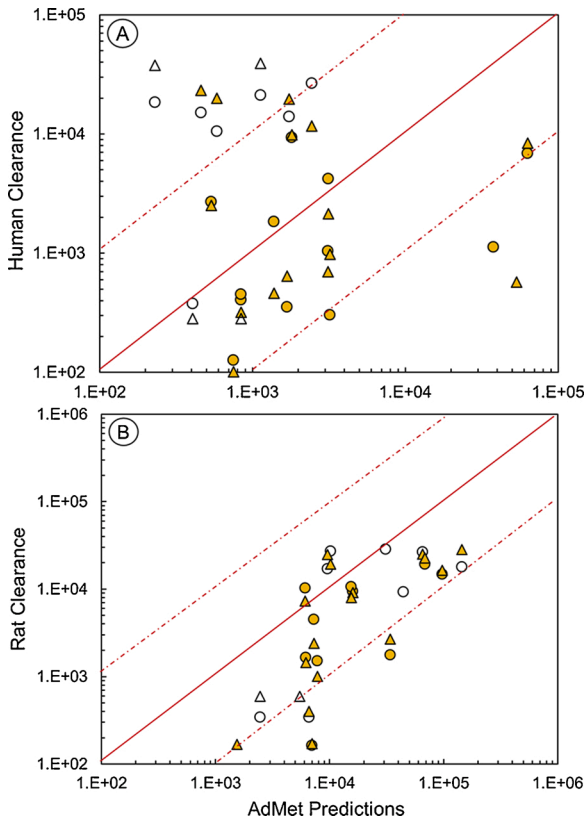

Fig. 7. Comparison of calculated in vivo intrinsic clearance rates ($CL_{int,in\ vivo}$) to $CL_{int,in\ vivo}$ values predicted by ADMET Predictor software v9.5 for human (A) and rat (B). Units for both axes are in μL/min/g of liver. Solid shapes indicate measured rates, while open shapes denote calculated "less than" or "greater than" values. Solid diagonal lines indicate unity, dashed diagonal lines indicate one order of magnitude from unity. Starting substrate concentrations: 0.1 μM = circles; 1 μM = triangles.

(species/chemical combinations). This information is suitable for computational modeling efforts, including QIVIVE and bioaccumulation prediction, and may be used to address questions regarding species differences in biotransformation and the quality of predictions generated by available biotransformation QSARs. Overall, however, the obtained datasets exhibited a wide range of behaviors, emphasizing the need for a robust approach to data quality assessment. While a revised sampling time course would have been likely to result in reportable rates for several of the rapidly metabolized chemicals, low recoveries from negative (HTC) controls cannot be as easily corrected. When in vitro depletion assays are performed to support large scale chemical screening and prioritization efforts, a balance must be struck between the competing needs for reliable data and high throughput. The results of this study suggest that a modest investment in preliminary work to tailor sampling schedules and identify chemicals unlikely to perform well could result in a substantial savings of time and analytical resources. By incorporating more negative control samples, it may also be possible to correct measured rates of clearance for abiotic chemical losses, provided that these abiotic loss processes exhibit first-order kinetics.

Disclaimer

This manuscript does not necessarily represent US Environmental Protection Agency (US EPA) policy. This manuscript has been reviewed in accordance with requirements of the US EPA's Office of Chemical Safety and Pollution Prevention and US EPA's Office of Research and Development. Mention of trade names or commercial products does not indicate endorsement by the US EPA.

Data availability

Data and associated metadata pertaining to this manuscript may be accessed through the USEPA Environmental Dataset Gateway (https://doi.org/10.23719/1520744).

Declaration of Competing Interest

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

Acknowledgements

This work was funded under contract EP-W-11-065, Task Order 12 by U.S. EPA to RTI International. We thank Barbara Wetmore and John Wambaugh for their input on chemical selection, and Joe Swintek for providing statistical support. Critical support for the performance of in vitro depletion assays and analysis of test chemical concentrations was provided by Troy Banks, Cornelia Smith, Scott Watson and Yun Lan Yueh. We also thank Jon Arnot and Alessandro Sangion for providing access to citations in their biotransformation database. Finally, we thank those who reviewed and provided comment on various draft versions of the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.tox.2021.152819.

References

Arnot, J.A., Gobas, F.A.P.C., 2003. A generic QSAR for assessing the bioaccumulation potential of organic chemicals in aquatic food webs. QSAR Comb. Sci. 22, 337–345. https://doi.org/10.1002/qsar.200390023, 2003.

Arnot, J.A., Gobas, F.A.P.C., 2006. A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. Environ. Rev. 14, 257–297. https://doi.org/10.1139/a06-005, 2006.

Arnot, J.A., Meylan, W., Tunkel, J., Howard, P.H., Mackay, D., Bonnell, M., Boethling, R. S., 2009. A quantitative structure-activity relationship for predicting metabolic biotransformation rates for organic chemicals in fish. Environ. Toxicol. Chem. 28, 1168–1177. https://doi.org/10.1897/08-289.1, 2009.

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. https://doi.org/10.1124/dmd.104.002436.

Barter, Z.E., Bayliss, M.K., Beaune, P.H., Boobis, A.R., Carlile, D.J., Edwards, R.J., Houston, J.B., Lake, B.G., Lipscomb, J.C., Pelkonen, O.R., Tucker, G.T., Rostami-Hodjegan, A., 2007. Scaling factors for the extrapolation of in vivo metabolic drug clearance from in vitro data: reaching a consensus on values of human microsomal protein and hepatocellularity per gram of liver. Curr. Drug Metab. 8, 33–45. https://doi.org/10.2174/138920007779315053, 2007.

Basketter, D.A., Clewell, H., Kimber, I., Rossi, A., Blaauboer, B., Burrier, R., Daneshian, M., Eskes, C., Goldberg, A., Hasiwa, N., Hoffmann, S., Jaworska, J., Knudsen, T.B., Landsiedel, R., Leist, M., Locke, P., Maxwell, G., McKim, J., McVey, E. A., Ouedraogo, G., Patlewicz, G., Pelkonen, O., Roggen, E., Rovida, C., Ruhdel, I., Schwarz, M., Schepky, A., Schoeters, G., Skinner, N., Trentz, K., Turner, M., Vanparys, P., Yager, J., Zurlo, J., Hartung, T., 2012. A roadmap for the development of alternative (non-animal) methods for systemic toxicity testing. Altex 29, 3–91. https://doi.org/10.14573/altex.2012.1.003, 2012.

Blaauboer, B.J., 2010. Biokinetic modeling and in vitro-in vivo extrapolations. J. Toxicol. Environ. Health B Crit. Rev. 13, 242–252. https://doi.org/10.1080/10937404.2010.483940, 2010.

Brown, H.S., Griffin, M., Houston, J.B., 2007. Evaluation of cryopreserved hepatocytes as an alternative in vitro system to microsomes for the prediction of metabolic clearance. Drug Metab. Dispos. 35, 293–301. https://doi.org/10.1124/dmd.106.011569.

Brown, T.N., Arnot, J.A., Wania, F., 2012. Iterative fragment selection: a group contribution approach to predicting fish biotransformation half-lives. Environ. Sci. Technol. 46, 8253–8260. https://doi.org/10.1021/es301182a, 2012.

Carlile, D.J., Stevens, A.J., Ashforth, E.I., Waghela, D., Houston, J.B., 1998. In vivo clearance of ethoxycoumarin and its prediction from in vitro systems. Use of drug depletion and metabolite formation methods in hepatic microsomes and isolated hepatocytes. Drug Metab. Dispos. 26, 216–221, 1998.

Clewell, H.J., Tan, Y.M., Campbell, J.L., Andersen, M.E., 2008. Quantitative interpretation of human biomonitoring data. Toxicol. Appl. Pharmacol. 231, 122–133. https://doi.org/10.1016/j.taap.2008.04.021, 2008.

DeJongh, J., Verhaar, H.J., Hermens, J.L., 1997. A quantitative property-property relationship (QPPR) approach to estimate in vitro tissue-blood partition coefficients of organic chemicals in rats and humans. Arch. Toxicol. 72, 17–25. https://doi.org/10.1007/s002040050463, 1997.

EFSA, 2019. EFSA Workshop on in Vitro Comparative Metabolism Studies in Regulatory Pesticide Risk Assessment. https://efsa.onlinelibrary.wiley.com/doi/abs/10.2903/sp.efsa.2019.EN-161810.2903/sp.efsa.2019.EN-1618.

Ekins, S., Murray, G.I., Burke, M.D., Williams, J.A., Marchant, N.C., Hawksworth, G.M., 1995. Quantitative differences in phase I and II metabolism between rat precision-cut liver slices and isolated hepatocytes. Drug Metab. Dispos. 23, 1274–1279, 1995.

Fay, K.A., Fitzsimmons, P.N., Hoffman, A.D., Nichols, J.W., 2017. Comparison of trout hepatocytes and liver S9 fractions as in vitro models for predicting hepatic clearance in fish. Environ. Toxicol. Chem. 36 (2), 463–471. https://doi.org/10.1002/etc.3572.

Fay, K.A., Mingoia, R.T., Goeritz, I., Nabb, D.L., Hoffman, A.D., Ferrell, B.D., Peterson, H. M., Nichols, J.W., Segner, H., Han, X., 2014a. Intra- and interlaboratory reliability of a cryopreserved trout hepatocyte assay for the prediction of chemical bioaccumulation potential. Environ. Sci. Technol. 48, 8170–81782014. https://doi.org/10.1021/es500952a.

Fay, K.A., Fitzsimmons, P.N., Hoffman, A.D., Nichols, J.W., 2014b. Optimizing the use of rainbow trout hepatocytes for bioaccumulation assessments with fish. Xenobiotica 44. https://doi.org/10.3109/00498254.2013.845704, 345-3512014.

Fay, K.A., Nabb, D.L., Mingoia, R.T., Bischof, I., Nichols, J.W., Segner, H., Johanning, K., Han, X., 2015. Determination of metabolic stability using cryopreserved hepatocytes from rainbow trout (Oncorhynchus mykiss). Curr. Protoc. Toxicol. 65 (4.42), 41–44. https://doi.org/10.1002/0471140856.tx0442s65, 42.292015.

Gebhardt, R., Hengstler, J.G., Muller, D., Glockner, R., Buenning, P., Laube, B., Schmelzer, E., Ullrich, M., Utesch, D., Hewitt, N., Ringel, M., Hilz, B.R., Bader, A., Langsch, A., Koose, T., Burger, H.J., Maas, J., Oesch, F., 2003. New hepatocyte in vitro systems for drug metabolism: metabolic capacity and recommendations for application in basic research and drug development, standard operation procedures. Drug Metab. Rev. 35, 145–213. https://doi.org/10.1081/dmr-120023684, 2003.

Hallifax, D., Houston, J.B., 2007. Saturable uptake of lipophilic amine drugs into isolated hepatocytes: mechanisms and consequences for quantitative clearance prediction. Drug Metab. Dispos. 35, 1325–1332. https://doi.org/10.1124/dmd.107.015131.

Hallifax, D., Houston, J.B., 2019. Use of segregated hepatocyte scaling factors and cross-species relationships to resolve clearance dependence in the prediction of human hepatic clearance. Drug Metab. Dispos. 47, 320–327. https://doi.org/10.1124/dmd.118.085191, 2019.

Han, X., Nabb, D.L., Mingoia, R.T., Yang, C.H., 2007. Determination of xenobiotic intrinsic clearance in freshly isolated hepatocytes from rainbow trout (Oncorhynchus mykiss) and rat and its application in bioaccumulation assessment. Environ. Sci. Technol. 41, 3269–3276. https://doi.org/10.1021/es0626279, 2007.

Han, X., Nabb, D.L., Yang, C.H., Snajdr, S.I., Mingoia, R.T., 2009. Liver microsomes and S9 from rainbow trout (Oncorhynchus mykiss): comparison of basal-level enzyme activities with rat and determination of xenobiotic intrinsic clearance in support of bioaccumulation assessment. Environ. Toxicol. Chem. 28, 481–488. https://doi.org/10.1897/08-269.1, 2009.

Hansch, C., Mekapati, S.B., Kurup, A., Verma, R.P., 2004. QSAR of cytochrome P450. Drug Metab. Rev. 36, 105–156. https://doi.org/10.1081/dmr-120028428, 2004.

Honda, G.S., Pearce, R.G., Pham, L.L., Setzer, R.W., Wetmore, B.A., Sipes, N.S., Gilbert, J., Franz, B., Thomas, R.S., Wambaugh, J.F., 2019. Using the concordance of in vitro and in vivo data to evaluate extrapolation assumptions. PLoS One 14. https://doi.org/10.1371/journal.pone.0217564 e0217564 2019.

Itwatsubo, T., Hirota, N., Ooie, T., Suzuki, H., Shimada, N., Chiba, K., Ishizaki, T., Green, C.E., Tyson, C.A., Sugiyama, Y., 1997. Prediction of in vivo drug metabolism in the human liver from in vitro metabolism data. Pharmacol. Ther. 73 (2), 147–171. https://doi.org/10.1016/S0163-7258(96)00184-2.

Kilford, P.J., Gertz, M., Houston, J.B., Galetin, A., 2008. Hepatocellular binding of drug: correction for unbound fraction in hepatocyte incubations using microsomal binding or drug lipophilicity data. Drug Metab. Dispos. 37, 1194–1197. https://doi.org/10.1124/dmd.108.020834.

Kirman, C.R., Aylward, L.L., Wetmore, B.A., Thomas, R.S., Sochaski, M.A., Ferguson, S.S., Csiszar, S.A., Jolliet, O., 2015. Quantitative property–property relationship for screening-level prediction of intrinsic clearance: a tool for exposure modeling for high-throughput toxicity screening data. Appl. In Vitro Toxicol. 1, 140–146. https://doi.org/10.1089/aivt.2014.0008, 2015.

Kuo, D.T., Di Toro, D.M., 2013. Biotransformation model of neutral and weakly polar organic compounds in fish incorporating internal partitioning. Environ. Toxicol. Chem. 32, 1873–1881. https://doi.org/10.1002/etc.2259, 2013.

Li, A.P., Lu, C., Brent, J.A., Pham, C., Fackett, A., Ruegg, C.E., Silber, P.M., 1999. Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential. Chem. Biol. Interact. 121, 17–35. https://doi.org/10.1002/etc.2259, 1999.

Liao, K.H., Tan, Y.M., Clewell 3rd, H.J., 2007. Development of a screening approach to interpret human biomonitoring data on volatile organic compounds: reverse dosimetry on biomonitoring data for trichloroethylene. Risk Anal. 27, 1223–1236. https://doi.org/10.1111/j.1539-6924.2007.00964.x, 2007.

Long, A., Walker, J.D., 2003. Quantitative structure-activity relationships for predicting metabolism and modeling cytochrome p450 enzyme activities. Environ. Toxicol. Chem. 22, 1894–1899. https://doi.org/10.1897/01-480, 2003.

Louisse, J., Beekmann, K., Rietjens, I.M.C.M., 2017. Use of physiologically based kinetic modeling-based reverse dosimetry to predict in vivo toxicity from in vitro data. Chem. Res. Toxicol. 30, 114–125. https://doi.org/10.1021/acs.chemrestox.6b00302.

Louisse, J., Alewijn, M., Peijnenburg, A., Cnubben, N.H.P., Heringa, M.B., Coecke, S., Punt, A., 2020. Towards harmonization of test methods for in vitro hepatic clearance studies. Toxicol. In Vitro 63. https://doi.org/10.1016/j.tiv.2019.104722, 1047222020.

Mansouri, K., Grulke, C.M., Judson, R.S., Williams, A.J., 2018. OPERA models for predicting physicochemical properties and environmental fate endpoints. J. Cheminform. 10, 102018. https://doi.org/10.1186/s13321-018-0263-1.

Mingoia, R.T., Glover, K.P., Nabb, D.L., Yang, C.H., Snajdr, S.I., Han, X., 2010. Cryopreserved hepatocytes from rainbow trout (Oncorhynchus mykiss): a validation study to support their application in bioaccumulation assessment. Environ. Sci. Technol. 44, 3052–3058. https://doi.org/10.1021/es903909g, 2010.

Naritomi, Y., Terashita, S., Kimura, S., Suzuki, A., Kagayama, A., Sugiyama, Y., 2001. Prediction of human hepatic clearance from in vivo animal experiments and in vitro metabolic studies with liver microsomes from animals and humans. Drug Metab. Dispos. 29, 1316–1324, 2001.

Nichols, J.W., Schultz, I.R., Fitzsimmons, P.N., 2006. In vitro-in vivo extrapolation of quantitative hepatic biotransformation data for fish. I. A review of methods, and strategies for incorporating intrinsic clearance estimates into chemical kinetic models. Aquat. Toxicol. 78, 74–90. https://doi.org/10.1016/j.aquatox.2006.01.017, 2006.

Nichols, J.W., Bonnell, M., Dimitrov, S.D., Escher, B.I., Han, X., Kramer, N.I., 2009. Bioaccumulation assessment using predictive approaches. Integr. Environ. Assess. Manag. 5, 577–597. https://doi.org/10.1897/ieam-2008-088.1, 2009.

Nichols, J.W., Hoffman, A.D., ter Laak, T.L., Fitzsimmons, P.N., 2013. Hepatic clearance of 6 polycyclic aromatic hydrocarbons by isolated perfused trout livers: prediction from in vitro clearance by liver S9 fractions. Toxicol. Sci. 136, 359–372. https://doi.org/10.1093/toxsci/kft219, 2013.

Nichols, J., Fay, K., Bernhard, M.J., Bischof, I., Davis, J., Halder, M., Hu, J., Johanning, K., Laue, H., Nabb, D., Schlechtriem, C., Segner, H., Swintek, J., Weeks, J., Embry, M., 2018. Reliability of in vitro methods used to measure intrinsic clearance of hydrophobic organic chemicals by rainbow trout: results of an international ring trial. Toxicol. Sci. 164, 563–575. https://doi.org/10.1093/toxsci/kfy113, 2018.

Nishimuta, H., Nakagawa, T., Nomura, N., Yabuki, M., 2013. Species differences in hepatic and intestinal metabolic activities for 43 human cytochrome P450 substrates between humans and rats or dogs. Xenobiotica 43, 948–955. https://doi.org/10.3109/00498254.2013.787155, 2013.

Obach, R.S., 1999. Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: an examination of in vitro half-life approach and nonspecific binding to microsomes. Drug Metab. Dispos. 27, 1350–1359, 1999.

Obach, R.S., Reed-Hagen, A.E., 2002. Measurement of Michaelis constants for cytochrome P450-mediated biotransformation reactions using a substrate depletion approach. Drug Metab. Dispos. 30, 831–837. https://doi.org/10.1124/dmd.30.7.831, 2002.

Obach, R.S., Baxter, J.G., Liston, T.E., Silber, B.M., Jones, B.C., MacIntyre, F., Rance, D. J., Wastall, P., 1997. The prediction of human pharmacokinetic parameters from preclinical and in vitro metabolism data. J. Pharmacol. Exp. Ther. 283, 46–58, 1997.

OECD, 2018a. Test No. 319A: determination of in vitro intrinsic clearance using cryopreserved rainbow trout hepatocytes (RT-HEP). OECD Guidelines for the Testing of Chemicals. OECD Publishing, Paris France. https://doi.org/10.1787/9789264303218-en. https://www.oecd-ilibrary.org/content/publication/9789264303218-en.

OECD, 2018c. TG 286: guidance document on good in vitro method practices (GIVIMP). OECD Series on Testing and Assessment. OECD Publishing, Paris, France. https://doi.org/10.1787/9789264304796-en. https://www.oecd-ilibrary.org/content/publication/9789264304796-en.

OECD, 2018. No. 280: guidance document on the determination of in vitro intrinsic clearance using cryopreserved hepatocytes (RTHEP) or liver S9 sub-cellular fractions (RT-S9) from rainbow trout and extrapolation to in vivo intrinsic clearance. OECD Series on Testing and Assessment. OECD Publishing, Paris, France. http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2018)12&doclanguage=en.

Papa, E., van der Wal, L., Arnot, J.A., Gramatica, P., 2014. Metabolic biotransformation half-lives in fish: QSAR modeling and consensus analysis. Sci. Total Environ. 470-471, 1040–1046. https://doi.org/10.1016/j.scitotenv.2013.10.068, 2014.

Peyret, T., Krishnan, K., 2012. Quantitative property-property relationship for screening-level prediction of intrinsic clearance of volatile organic chemicals in rats and its integration within PBPK models to predict inhalation pharmacokinetics in humans. J. Toxicol. 2012, 286079. https://doi.org/10.1155/2012/286079, 2012.

Peyret, T., Poulin, P., Krishnan, K., 2010. A unified algorithm for predicting partition coefficients for PBPK modeling of drugs and environmental chemicals. Toxicol. Appl. Pharmacol. 249, 197–207. https://doi.org/10.1016/j.taap.2010.09.010, 2010.

Poulin, P., Krishnan, K., 1995. An algorithm for predicting tissue: blood partition coefficients of organic chemicals from n-octanol: water partition coefficient data. J. Toxicol. Environ. Health 46, 117–129. https://doi.org/10.1080/15287399509532021, 1995.

Punt, A., Pinckaers, N., Peijnenburg, A., Louisse, J., 2021. Development of a web-based toolbox to support quantitative in-vitro-to-in-vivo extrapolations (QIVIVE) within nonanimal testing strategies. Chem. Res. Toxicol. 34, 460–472. https://doi.org/10.1021/acs.chemrestox.0c00307.

Rodrigues, A.D., 1997. Preclinical drug metabolism in the age of high-throughput screening: an industrial perspective. Pharm. Res. 14, 1504–1510. https://doi.org/10.1023/a:1012105713585, 1997.

Rotroff, D.M., Wetmore, B.A., Dix, D.J., Ferguson, S.S., Clewell, H.J., Houck, K.A., Lecluyse, E.L., Andersen, M.E., Judson, R.S., Smith, C.M., Sochaski, M.A., Kavlock, R. J., Boellmann, F., Martin, M.T., Reif, D.M., Wambaugh, J.F., Thomas, R.S., 2010.

Incorporating human dosimetry and exposure into high-throughput in vitro toxicity screening. Toxicol. Sci. 117, 348–358. https://doi.org/10.1093/toxsci/kfq220, 2010.

Rowland, M., Benet, L.Z., Graham, G.G., 1973. Clearance concepts in pharmacokinetics. J. Pharmacokinet. Biopharm. 1, 123–136. https://doi.org/10.1007/BF01059626, 1973.

Rudzinski, W., Plazinski, W., 2006. Kinetics of solute adsorption at solid/solution interfaces: a theoretical development of the empirical pseudo-first and pseudo-second order kinetic rate equations, based on applying the statistical rate theory of interfacial transport. J. Phys. Chem. B 110, 16514–16525. https://doi.org/10.1021/jp061779n, 2006.

Sander, R., 2015. Compilation of Henry's law constants (version 4.0) for water as solvent. Atmos. Chem. Phys. 15, 4399–4981. https://doi.org/10.5194/acp-15-4399-2015.

Schmitt, W., 2008. General approach for the calculation of tissue to plasma partition coefficients. Toxicol. In Vitro 22, 457–467. https://doi.org/10.1016/j.tiv.2007.09.010, 2008.

Scholze, M., Taxvig, C., Kortenkamp, A., Boberg, J., Christiansen, S., Svingen, T., Lauschke, K., Frandsen, H., Ermler, S., Hermann, S.S., Pedersen, M., Lykkeberg, A.K., Axelstad, M., Vinggaard, A.M., 2020. Quantitative in vitro to in vivo extrapolation (QIVIVE) for predicting reduced anogenital distance produced by anti-androgenic pesticides in a rodent model for male reproductive disorders. Environ. Health Perspect. 128, 117005. https://doi.org/10.1289/EHP6774.

Smith, R., Jones, R.D., Ballard, P.G., Griffiths, H.H., 2008. Determination of microsome and hepatocyte scaling factors for in vitro/in vivo extrapolation in the rat and dog. Xenobiotica 38, 1386–1398. https://doi.org/10.1080/00498250802491662, 2008.

Temesi, D.G., Martin, S., Smith, R., Jones, C., Middleton, B., 2010. High-throughput metabolic stability studies in drug discovery by orthogonal acceleration time-of-flight (OATOF) with analogue-to-digital signal capture (ADC). Rapid Commun. Mass Spectrom. 24, 1730–1736. https://doi.org/10.1002/rcm.4546, 2010.

U.S.C. § 2601 et seq, 2016. The Frank R. Lautenberg Chemical Safety for the 21st Century Act. U.S.C.. https://uscode.house.gov/view.xhtml?path=/prelim@title15/chapter53&edition=prelim

U.S.C. § 300j–17, 1996. Estrogenic Substances Screening Program. Public Health and Welfare, Public Health Service, Safety of Public Water Systems, General Provisions. http://www.gpo.gov/fdsys/pkg/USCODE-2010-title42/pdf/USCODE-2010-title42-chap6A-subchapXII-partE-sec300j-17.pdf.

U.S.C. § 346a, 1996. Tolerances and Exemptions for Pesticide Chemical Residues. Food and Drugs, Federal Food, Drug, and Cosmetic Act (FFDCA). http://www.gpo.gov/fdsys/pkg/USCODE-2009-title21/pdf/USCODE-2009-title21-chap9-subchapIV-sec346a.pdf.

USEPA, 2012a. EPI Suite – Estimation Program Interface Suite for Windows, v4.11. Accessed on: November 20, 2017. https://www.epa.gov/tsca-screening-tools/epi-suitetm-estimation-program-interface.

USEPA, 2012b. Endocrine Disruptor Screening Program (EDSP) Universe of Chemicals and General Validation Principles. Accessed on: October 15, 2020. https://www.epa.gov/endocrine-disruption/endocrine-disruptor-screening-program-edsp-universe-chemicals-and-general.

USEPA, 2015. Endocrine Disruptor Screening Program (EDSP) Tier 1 Assessments. Accessed on: February 29, 2020. https://www.epa.gov/endocrine-disruption/endocrine-disruptor-screening-program-tier-1-screening-determinations-and.

USEPA, 2019. Administrator Announcement to Reduce Animal Testing in Risk Assessment. https://www.epa.gov/research/administrator-memo-prioritizing-efforts-reduce-animal-testing-september-10-2019.

USEPA, 2020a. How to Access the TSCA Inventory. Accessed on: April 9, 2020. https://www.epa.gov/tsca-inventory/how-access-tsca-inventory.

USEPA, 2020b. U.S. EPA CompTox Chemicals Dashboard. Accessed on: September 24, 2020. https://comptox.epa.gov/dashboard.

Wambaugh, J.F., Wetmore, B.A., Ring, C.L., Nicolas, C.I., Pearce, R.G., Honda, G.S., Dinallo, R., Angus, D., Gilbert, J., Sierra, T., Badrinarayanan, A., Snodgrass, B., Brockman, A., Strock, C., Setzer, R.W., Thomas, R.S., 2019. Assessing toxicokinetic uncertainty and variability in risk prioritization. Toxicol. Sci. 172, 235–251. https://doi.org/10.1093/toxsci/kfz205, 2019.

Wetmore, B.A., 2015. Quantitative in vitro-to-in vivo extrapolation in a high-throughput environment. Toxicology. 332, 94–101. https://doi.org/10.1016/j.tox.2014.05.012, 2015.

Wetmore, B.A., Wambaugh, J.F., Ferguson, S.S., Sochaski, M.A., Rotroff, D.M., Freeman, K., Clewell 3rd, H.J., Dix, D.J., Andersen, M.E., Houck, K.A., Allen, B., Judson, R.S., Singh, R., Kavlock, R.J., Richard, A.M., Thomas, R.S., 2012. Integration of dosimetry, exposure, and high-throughput screening data in chemical toxicity assessment. Toxicol. Sci. 125, 157–174. https://doi.org/10.1093/toxsci/kfr254, 2012.

Wetmore, B.A., Wambaugh, J.F., Ferguson, S.S., Li, L., Clewell 3rd, H.J., Judson, R.S., Freeman, K., Bao, W., Sochaski, M.A., Chu, T.M., Black, M.B., Healy, E., Allen, B., Andersen, M.E., Wolfinger, R.D., Thomas, R.S., 2013. Relative impact of incorporating pharmacokinetics on predicting in vivo hazard and mode of action from high-throughput in vitro toxicity assays. Toxicol. Sci. 132, 327–346. https://doi.org/10.1093/toxsci/kft012, 2013.

Wetmore, B.A., Wambaugh, J.F., Allen, B., Ferguson, S.S., Sochaski, M.A., Setzer, R.W., Houck, K.A., Strope, C.L., Cantwell, K., Judson, R.S., LeCluyse, E., Clewell, H.J., Thomas, R.S., Andersen, M.E., 2015. Incorporating high-throughput exposure predictions with dosimetry- adjusted in vitro bioactivity to inform chemical toxicity testing. Toxicol. Sci. 148, 121–136. https://doi.org/10.1093/toxsci/kfv171, 2015.

Wilkinson, G.R., Shand, D.G., 1975. Commentary: a physiological approach to hepatic drug clearance. Clin. Pharmacol. Ther. 18, 377–390. https://doi.org/10.1002/cpt1975184377, 1975.

Wilk-Zasadna, I., Bernasconi, C., Pelkonen, O., Coecke, S., 2015. Biotransformation in vitro: an essential consideration in the quantitative in vitro-to-in vivo extrapolation (QIVIVE) of toxicity data. Toxicology 332, 8–19. https://doi.org/10.1016/j.tox.2014.10.006, 2015.

Yoon, M., Campbell, J.L., Andersen, M.E., Clewell, H.J., 2012. Quantitative in vitro to in vivo extrapolation of cell-based toxicity assay results. Crit. Rev. Toxicol. 42, 633–652. https://doi.org/10.3109/10408444.2012.692115, 2012.