Validation of methods for *in vitro-in vivo* extrapolation using hepatic clearance measurements in isolated perfused fish livers

Matthew Schultz[1], Sophia Krause[2], Markus Brinkmann[1,3,4,5*]

[1]Toxicology Center, University of Saskatchewan, 44 Campus Dr, Saskatoon, SK S7N 5B3, Canada

[2]Department of Analytical Environmental Chemistry, Helmholtz Centre for Environmental Research UFZ, Permoserstr. 15, 04318 Leipzig, Germany

[3]School of Environment and Sustainability, University of Saskatchewan, 117 Science Pl, Saskatoon, SK S7N 5C8, Canada

[4]Global Institute for Water Security, University of Saskatchewan, 11 Innovation Blvd, Saskatoon, SK S7N 3H5, Canada

[5]Centre for Hydrology, University of Saskatchewan, 121 Research Dr, Saskatoon, SK S7N 1K2, Canada

*** Corresponding author:**

Markus Brinkmann, PhD

Toxicology Centre, University of Saskatchewan

44 Campus Drive

Saskatoon SK S7N 5B3 Canada

Email: markus.brinkmann@usask.ca

Abstract

In vitro biotransformation assays using hepatocytes or liver sub-cellular fractions, combined with *in vitro-in vivo* extrapolation (IVIVE) models, have been proposed as an alternative to live fish bioconcentration studies. The uncertainty associated with IVIVE approaches to date has been attributed to assay protocols, model assumptions, or variability of *in vivo* data. An isolated perfused trout liver model that measures hepatic clearance has been proposed for validating IVIVE predictions in the absence of other confounding factors. Here, we investigated the hepatic clearances of five chemicals (pyrene, phenanthrene, 4-*n*-nonlyphenol, deltamethrin, and methoxychlor) in this model and compared measured rates to values predicted from published *in vitro* intrinsic clearances for validation of IVIVE models. Additionally, we varied protein concentrations in perfusates to test binding assumptions of these models. We found that measured and predicted hepatic clearances were in very good agreement (root mean squared error 16.8 mL h^{-1} g^{-1}) across three levels of protein binding and across a more diverse chemical space than previously studied within this system. Our results show that current IVIVE methods can reliably predict *in vivo* clearance rates and indicate that discrepancies from measured bioconcentration factors might be driven by other processes, such as extrahepatic biotransformation, etc., and help streamline optimization efforts to the processes that truly matter.

Keywords: Biotransformation, bioconcentration, *in vivo, in vitro, in vivo-in vitro* extrapolation

Synopsis: Hepatic clearance measurements in isolated perfused fish livers were compared to values predicted from *in vitro* assay results to validate existing *in vitro-in vivo* extrapolation models, demonstrating very good predictive performance across five chemicals and three protein concentrations.

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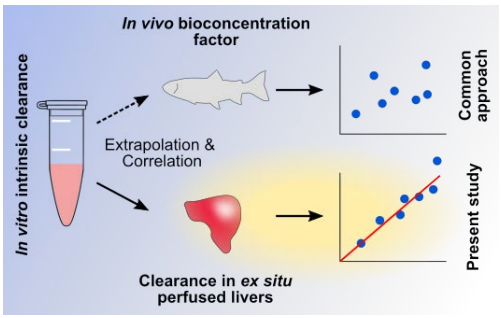

1. Introduction

Chemical risk assessment seeks to characterize environmental contaminants based on standardized methods that can be applied by regulators, stakeholders, and researchers. Contaminants in the aquatic environment are often characterized by three information requirements related to their persistence, bioaccumulation potential, and toxicity (PBT).[1] Of these, studying bioaccumulation potential presents significant challenges due to the costs associated with generating empirical data from whole animal testing, e.g., according to the test guidelines 305 of the Organisation for Economic Co-operation and Development.[2] This guideline outlines methods for determination of the bioconcentration factor (BCF), which is defined as the ratio between a chemical's accumulation within an organism through non-dietary routes of exposure compared to its ambient environment. These studies are experimentally challenging, and require large numbers of animals, resulting in low throughput.[3] Despite these issues, BCF is still recognized as a standard endpoint for bioaccumulation studies within risk assessment.[4] In consequence, results of BCF studies are not always conducted, and simple models and quantitative structure-activity relationships that estimate BCF based on physiochemical characteristics of chemicals, e.g., the *n*-octanol-water partitioning coefficient (*log* K_{OW}) are frequently used in chemical risk assessment.[5,6] These models often agree well with empirical models for chemicals that do not undergo biotransformation. For chemicals that undergo biotransformation, however, these estimates are often inaccurate, leading to sometimes dramatic overestimations of a chemical's bioaccumulation potential.[7,8]

Newer modelling approaches and prediction methods seek to eliminate the use of whole fish on both ethical and economic grounds, while enabling the incorporation of experimental data from alternative test methods, include those from recently standardized *in vitro* assays using isolated rainbow trout (*Oncorhynchus mykiss*) hepatocytes (RT-HEP) or liver sub-cellular fractions (RT-S9).[9–11] Such alternative approaches require extrapolation from a low level of biological organization (*in vitro*) to that of the whole organism (*in vivo*); this process has been termed *in vitro*-*in vivo* extrapolation (IVIVE).[12,13] The goal of these methods is to create data of sufficient quality for use in environmental risk assessment. However, previous studies have focused on a relatively narrow and well-characterized chemical space (specifically polycyclic aromatic hydrocarbons, PAHs), that is not entirely representative of current risk assessment needs.[14–16]Recent studies have sought to expand this understanding using *in vitro* assays to

investigate pesticides, fragrance chemicals, and other emerging contaminants.[17–19]. Thus, there is a need to expand validation of IVIVE approaches to a broader chemical space, potentially by using assays of intermediate biological organization to reduce uncertainty..

Incorporating *in vitro* biotransformation data into predictive models through comprehensive IVIVE methods has been demonstrated to enhance predictive performance markedly but is still limited by several factors. One such factor is the influence of chemical binding to lipids and plasma proteins on freely dissolved chemical concentrations and consequently on *in vitro* biotransformation rates. Many researchers in this space found that incorporating the assumption that no difference in binding exists between *in vitro* assays and blood plasma *in vivo*, which is neither supported by theory nor empirical evidence, improved the apparent accuracy of models.[20,21] More recently, however, consensus has been reached that this practice was misleading and should be avoided, while instead more research is needed to identify the potential reasons for these discrepancies.[5,22] One such reason has been hypothesized to be the potential contribution of extrahepatic biotransformation in organs such as gill and gastrointestinal tract.[23,24] Additionally, the substantial variability of BCF measurements questions the robustness of its use as a comparator for IVIVE predictions.[3,25]

To overcome the current limitations pertaining to the ability to validate current IVIVE approaches, an isolated perfused rainbow trout liver model has been developed that can be used to determine hepatic clearance of chemicals in the absence of these confounding factors.[26] Based on studies with six PAHs, Nichols et al. showed that hepatic clearances measured in isolated perfused trout livers and those predicted using IVIVE were generally in good agreement.[14]

In the present study, we used the isolated perfused liver model to expand the covered chemical space beyond PAHs by measuring the hepatic clearances of pyrene and phenanthrene (both PAHs, to demonstrate comparability with previous studies), as well as 4-*n*-nonylphenol, deltamethrin, and methoxychlor. These chemicals were chosen based on both their relative chemical diversity, and their previous analysis using standardized *in vitro* biotransformation assays. [11] Furthermore, we conducted these experiments at three different concentrations of bovine serum albumin (BSA) to investigate the impact of protein binding on free chemical concentrations and thus hepatic clearance.[14]

2. Materials and Methods

2.1 Organisms

Rainbow trout were acquired as eyed embyros (Troutlodge, Bonney Lake, WA, United States) and raised at the Aquatic Toxicology Research Facility (University of Saskatchewan, Saskatoon, Canada) to suitable size. Fish were fed commercially available size #3 Floating Salmonid Feed (Corey Aquafeeds, Fredericton, Canada) once daily at 1% body weight, and maintained at a photoperiod of 16 h light: 8 h dark at 12 ± 1°C. Water chemistry was consistent at pH 7.8, dissolved oxygen > 80%, with ammonium, chlorine, nitrate, and nitrite all measuring < 0.1 mg L^{-1}. The masses of fish used in this study were between 210 and 440 grams, with livers ranging from 1.4 to 4.0 g in mass. Approval from the University Animal Care Committee was obtained (Animal Use Protocol number 20070049) and all animal use was performed in accordance with Canadian Council on Animal Care (CCAC) regulations.

2.2 Chemicals

The preparation of perfusion and clearing buffers was adapted from that described in Nichols et al.[14,26] This procedure had been specifically optimized to prolong the perfusion time available for data collection. All chemicals were purchased from Sigma-Alrich, unless noted otherwise. Clearing buffer consisted of a solution of 9.5 g L^{-1} Hank's balanced salts, modified without phenol red, magnesium sulfate, or calcium chloride (HBSS), 2.38 g L^{-1} 4-2(-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.55 g L^{-1} sodium pyruvate, 0.73 g L^{-1} ethylenediaminetetraacetic acid (EDTA), and 17.5 mL 1 M aqueous sodium hydroxide solution (NaOH) in 1 L of ultrapure water. Perfusion buffer consisted of 90 g Hank's balanced salts, modified without phenol red, with magnesium sulfate and calcium chloride (HBS), 23.83 g HEPES, 3.5 g sodium bicarbonate, 10.0 g glucose, and 147.0 mL 1 M sodium hydroxide solution in 10 L of ultrapure water. Control perfusate was gassed for 24 hours with carbogen gas (0.5% CO_2/99.5% O_2 mixture), followed by the introduction of bovine serum albumin (BSA) at desired concentrations (1, 2.5, or 10 g L^{-1}) until thoroughly dissolved. Sodium hydroxide and hydrochloric acid were used to adjust and maintain a pH of 7.8 for both clearing and perfusion buffers throughout preparation prior to perfusions.

Pyrene, phenanthrene, 4-*n*-nonylphenol, deltamethrin, and methoxychlor all had purities ≥98%. Stock solutions of test chemicals were prepared in acetone, spiked into perfusion buffers

at nominal concentrations of 0.1, 0.1, 2.0, 1.0, and 0.32 µM, respectively, and given 24 hours to equilibrate prior to perfusions. Acetone concentrations never exceeded 0.05%. Buffers were maintained at 12ºC in a temperature-controlled water bath throughout the experiments.

2.3 Liver isolation and perfusion

Trout livers were isolated and perfused following methods adapted from Nichols et al.[14,26] Fish were fasted for approximately 24 h, then euthanized with an overdose of buffered ethyl 3-aminobenzoate methanesulfonate (MS 222, 250 mg L^{-1}). Fish were weighed prior to initiating the liver isolation procedure. An initial incision along the ventral midline from the anus to the gill isthmus was made, followed by an incision along the lateral line to the operculum and removal of the muscle flap to expose the body cavity. The intestine was severed at the anus, and the gastrointestinal tract was manipulated to fully expose the liver and esophagus, which was severed to permit the careful removal of the intact organ and viscera for transfer to the perfusion equipment. Any damage to the liver, gallbladder, or hepatic portal vein during this process resulted in rejection from the experiment, but was generally rare.

Isolated livers and viscera were transferred in their entirety to a specifically built stainless steel apparatus. The method of full removal and transfer of both liver and viscera and letting perfusate buffer drain freely from the liver was adopted as a change compared to the procedure outlined by Nichols et al., who also cannulated efferent blood vessels and collected efferent perfusates from the cannula. This change was employed to reduce exposure of the liver to non-physiological conditions and thus prevent deterioration, as well as to increase the success rate of the procedure as compared to excising the liver and hepatic vasculature alone. The liver and viscera were manipulated to expose the hepatic portal vein within the apparatus, and 5-0 silk suture was placed around the vein and surrounding vasculature as required to secure the vein in place for cannulation. The portal vein was cannulated directly using appropriately sized intravenous catheters secured by the sutures connected to a syringe pump using Tygon tubing, and the apparatus was placed within a humidified refrigerator maintained at an average internal temperature of 12ºC as monitored by a Type K thermocouple throughout the experiment.

A UP-100 Universal Perfusion System (Harvard Apparatus, Holliston, MA, United States) equipped with a water-jacketed bubble trap was maintained at 12ºC using a recirculating temperature-controlled water bath and was fed by a syringe infusion pump (KD Scientific, KDS

200 Series) using 60 mL BD Luer-Lok syringes. Initially, clearing buffer solution was pumped through at a flow rate of 8.9 mL kg^{-1} body weight min^{-1} to clear the liver of blood. If after 10 minutes the liver was less than ~95% cleared of blood by visual determination, it was rejected from the experiment and discarded. Once cleared of blood, the perfusate was switched to perfusion buffer spiked with the test chemicals. Afferent and efferent samples were collected in 15-minute intervals for the duration of the experiment. Afferent samples were collected by a T-junction sampling port immediately prior to the apparatus chamber. Efferent samples were collected from below the liver in a glass beaker, as the perfusate drained from the liver. Samples of perfusate were analyzed concurrently for pH and glucose over the duration of each experiment using a benchtop pH meter and a handheld glucose test meter (Contour™ Next meter, Ascensia, Basel, Switzerland). Glucose efflux and decrease in pH were calculated as indicators of physiological performance of each liver.

Experiments were designed with *n*=3-4 individual livers per treatment, differing in test chemical, BSA concentration, and duration of experiment. Perfusions followed one of three conditions: (1) perfusion for 2-5 hours at 1 g L^{-1} BSA, (2) perfusion for 3 hours at 2.5 g L^{-1} BSA, (3) perfusion for 3 hours at 10 g L^{-1} BSA. Concentrations of test chemicals and BSA remained constant throughout a single perfusion experiment.

2.4 Sample extraction and analysis

For perfusates containing pyrene, phenanthrene, and 4-*n*-nonylphenol, a 333 μL aliquot was taken from both afferent and efferent samples, mixed with 1 mL of chilled acetonitrile (mass-spectrometry grade, Fisher Scientific) in 1.5 mL centrifuge tubes, and stored on ice for the duration of the experiment. Samples were vortex mixed for 30 s, then centrifuged for 10 min at 10,000 × g. A 1 mL aliquot of the supernatant was removed and transferred to a 2 mL LC vial and stored at 4°C until analysis using liquid chromatography.

For deltamethrin and methoxychlor perfusates, 500 μL aliquots were taken and mixed with 500 μL of chilled acetonitrile, followed by identical storage and centrifugation compared to prior samples. A 5 μL spike of internal standard (isotopically labelled deltamethrin-(phenoxy-d_5) and hexachlorobenzene-$^{13}C_6$, respectively) was added to each sample. A 500 μL aliquot of supernatant was removed and transferred to a 2 mL LC vial and liquid-liquid extracted using 500 μL of hexanes (ACS grade, Fisher Scientific) by vortex-mixing for 30 s and allowing for phase

separation. Finally, a 200 µL aliquot of the supernatant was removed and transferred to a 2 mL LC vial containing a 300 µL glass micro insert for analysis. Blank samples were taken after the addition of BSA, prior to spike chemicals.

Pyrene, phenanthrene, and 4-*n*-nonylphenol were analyzed using an HP Agilent 1100 Series High Performance Liquid Chromatography (HPLC) system with HP 1046A Programmable Fluorescence Detector. A gradient program ranging from 90% A/10%B to 0%A/100%B at a fixed 0.500 mL minute $^{-1}$ over a period of 35 minutes was used. Solvent A consisted of HPLC grade water; solvent B consisted of HPLC purity acetonitrile. The following fluorescence excitation/emission wavelengths (nm) were used: pyrene – 237/385, phenanthrene – 250/390, 4-*n*-nonylphenol – 225/315. Data acquisition and peak integration were performed using Agilent Chemstation software. Quantification of pyrene, phenanthrene, and 4-*n*-nonylphenol was performed based on a seven-point matrix-matched external standard calibration, that is calibration curves were prepared in perfusate buffer containing the same BSA concentrations. Measured concentrations were generally within ± 20% of nominal concentrations.

Deltamethrin and methoxychlor were analyzed using a Thermo Scientific Trace 1300 gas chromatograph equipped with an ISQ 7000 quadrupole mass detector and programmable-temperature vaporizing injector operating in splitless mode. Samples were separated on an Agilent DB-5MS (30 m length, 0.25 mm diameter, 0.25 µm film thickness) capillary column with high purity helium at a flow rate of 1.0 mL min $^{-1}$ as a carrier gas. Data acquisition and processing was performed using Thermo Scientific Chromeleon software in single ion monitoring (SIM) mode.

For analysis of deltamethrin, the injector temperature, transfer line, and ion source temperatures were 280°C, 280°C, and 230°C, respectively. The gas chromatograph followed a temperature gradient program of 70°C held for 1 minute, ramped at 25°C min^{-1} to 250°C, ramped at 5°C min^{-1} to 280°C and held for 8 minutes. Ions with *m/z* 181 and 186 (retention time, RT, 16.9 min for both analytes) were used for quantification of native and mass-labelled deltamethrin, respectively.

For analysis of methoxychlor, the injector temperature, transfer line, and ion source temperatures were 270°C, 300°C, and 250°C, respectively. The gas chromatograph followed a temperature gradient program of 90°C held for 1 minute, ramped at 12°C min^{-1} to 150°C, ramped

at 2°C min^{-1} to 230°C, then ramped at 20°C min^{-1} to 275°C and held for 45 s. Ions with *m/z* 227 (RT 43.1 min) and 290 (RT 14.1 min) were used for quantification of methoxychlor and hexachlorobenzene-$^{13}C_6$ as internal standard, respectively.

Quantification was performed based on a seven-point calibration standard and recovery-corrected based on internal standard recoveries. Across all analyses for both deltamethrin and methoxychlor, internal standard recovery ranged from 80 to 120% and measured concentrations were generally within ± 30% of nominal concentrations.

2.5 Data analysis

Quantification of hepatic clearance was derived from the total concentration of target chemicals within afferent (C_{AFF}, dimensionless) and efferent (C_{EFF}, dimensionless) samples, yielding hepatic extraction efficiency (E_H, dimensionless; Equation 1). Calculation of hepatic clearance was performed according to Nichols et al.[14], using a body weight normalized perfusion rate (*perfusion rate*, mL h^{-1} g $liver^{-1}$; Equation 2) to calculate hepatic clearance of each chemical per sample.

$$E_H = \frac{(C_{AFF} - C_{EFF})}{C_{AFF}} \qquad \text{(Equation 1)}$$

$$CL_H = E_H \times \text{perfusion rate} \qquad \text{(Equation 2)}$$

The average hepatic extraction fraction and thus clearance was calculated for each liver, ignoring the initial 60 minutes of sampling, during which the observed rate of clearance is driven by chemical partitioning rather than biotransformation and therefore not indicative of steady-state clearance. Consequently, the average hepatic clearance values reported were determined based on varying numbers of samples (between 4 and 16) depending on the experimental design. Measurements of glucose efflux as well as pH were used on a qualitative basis to ensure the observed hepatic clearance was the result of physiological activity within each liver, as together these represent an observation of cellular respiration.[14,26]

2.6 In vitro-in vivo extrapolation

To compare the hepatic clearance values obtained using the isolated perfused trout liver model with values predicted from *in vitro* data, we applied an IVIVE approach previously published by Krause & Goss.[27] To this end, a well-stirred liver model that explicitly accounts for the blood flow limitation of the perfused livers was implemented in a Microsoft Excel spreadsheet (Equation 3; attached to this publication in the Supplementary Materials).

$$CL_H = \frac{Q_H \times \frac{f_{blood}^{unbound}}{f_{assay}^{unbound}} \times CL_{in\ vitro,int} \times \frac{w_{assay}}{w_{blood}}}{Q_H + \frac{f_{blood}^{unbound}}{f_{assay}^{unbound}} \times CL_{in\ vitro,int} \times \frac{w_{assay}}{w_{blood}}} \qquad \text{(Equation 3)}$$

Where CL_H is the body weight-normalized hepatic clearance (mL h^{-1} g^{-1} liver), Q_H is the hepatic blood flow or perfusion rate (mL h^{-1} g^{-1} liver), $f_{blood}^{unbound}$ and $f_{assay}^{unbound}$ are the unbound chemical fractions in blood and assay media, respectively, $CL_{in\ vitro,int}$ is the *in vitro* intrinsic clearance determined using *in vitro* test procedures, and w_{assay} and w_{blood} are the water fractions ($mL_{water}/mL_{assay/blood}$) in both assay media and blood, respectively. The term $\frac{f_{blood}^{unbound}}{f_{assay}^{unbound}}$ is often referred to as f_u in the literature.[7] $f_{blood}^{unbound}$ and $f_{assay}^{unbound}$ were calculated based on partition coefficients between assay media and water, as well as liver and water, that were predicted based on combining the contributing sorption to proteins, lipids, and water of the phase of interest as estimated by poly-parameter linear free energy relationships (pp-LFER). This approach allowed us to explicitly account for the differing freely dissolved fractions of the various chemicals between the three different BSA levels that were applied. Using this model, we extrapolated previously published *in vitro* intrinsic clearance values for the chemicals of interest[5,11,28] that were generated using either rainbow trout hepatocytes or RT-S9. For pyrene, an *in vitro* intrinsic clearance value of 1.03 ± 0.12 mL h^{-1} 10^6 $cells^{-1}$ was generated according to OECD 319A using hepatocytes from the same source of fish as the isolated perfused trout livers (Figure S1).

For each chemical-BSA combination, we plotted the arithmetic mean and standard deviation of measured hepatic clearance over time (Figures S2-S6) and against those predicted using the IVIVE.[27] The root mean squared error (RMSE) was calculated as a measure of the

goodness-of-fit. Additionally, we created Bland-Altman plots using Prism 9 software (GraphPad, LaJolla, FL, USA) to estimate potential systematic biases of one method compared to the other.

3. Results and Discussion

3.1 Physiological performance of isolated perfused livers

To obtain reliable and robust clearance measurements in isolated perfused trout livers, it is imperative to ensure proper physiological functioning of the organ during the entire experiment.[14,26] A net glucose efflux was measured across all experiments and chemical treatment (Figure 1A). Values ranged from 2.4 to 79 μmol h^{-1} g^{-1} liver, indicating continuous glycogen mobilization.[24,29] These values are comparable to previously published values by Nichols et al.[14], which ranged from 8.2 to 72 μmol h^{-1} g^{-1} liver. Furthermore, a continuous decrease in pH was detected between afferent and efferent perfusate samples (Figure 1B), indicating active respiration of the organ.[29] The drop in pH ranged from -0.20 to -0.01 and was generally more pronounced than previously reported by Nichols et al.[14], who reported pH decreases ranging from -0.034 to -0.014. These differences might be due to our use of a simple benchtop pH meter, while Nichols et al. used a more sophisticated total blood gas analyzer. Regardless of the absolute magnitude, the determined decrease in pH and concurrent net glucose efflux together are sufficient to demonstrate physiological performance.

3.2 Hepatic clearance in isolated perfused livers

Two sets of chemicals were analyzed in our study: (1) the PAHs pyrene and phenanthrene were studied here to demonstrate proficiency and compare the performance of our experimental setup to the only other published dataset by Nichols et al.[14], while (2) our measurements for 4-*n*-nonylphenol, deltamethrin, and methoxychlor represent the first measurements of hepatic clearance within an isolated perfused trout liver model for these chemicals (Table 1, Figures S1-S5).

The concentration of BSA changes the amount of free chemical fraction available, which had a significant impact on measured hepatic extraction fractions and clearances of all five chemicals, with the 10 g L^{-1} treatment consistently showing the lowest, and the 1.0 g L^{-1}

treatment consistently showing the greatest values (Table 1). Except for deltamethrin, the 2.5 g L^{-1} treatment consistently fell in between the values measured at 1.0 and 10 g L^{-1}, respectively.

Nichols et al.[14] reported hepatic extraction fractions of 85% and 85% for pyrene and 79% and 54% for phenanthrene at 1.0 and 10 g L^{-1} BSA, respectively, while in our study, we measured hepatic extraction fractions of 80.9 and 50.1 for pyrene and 39.5 and 18.6 for phenanthrene at 1.0 and 10 g L^{-1} BSA, respectively (Table 1). While the overall trends agree well between both studies, Nichols et al. measured systematically greater values for both pyrene and phenanthrene. In addition, the authors did not observe the same clear trend in binding-dependent clearance, which they speculate could be attributed to saturation under all measured conditions. Indeed, measured concentrations of pyrene and phenanthrene in perfusates were approx. 0.25 and 1.1 µM, respectively, while those targeted in the present study were somewhat smaller, with nominal concentrations of 0.1 µM for both PAHs. In a different study, Nichols et al.[30] measured the saturable hepatic biotransformation of pyrene and phenanthrene by fitting initial rates of substrate depletion to the Michaelis-Menten model. The Michaelis-Menten constants (K_M; µM) determined in this way were 0.075 and 0.84 µM, respectively, suggesting that slight concentration increases beyond these levels could have marked impacts on the measured clearance rates, thereby explaining the systematic discrepancies between both studies. Indeed, the *in vitro* intrinsic clearance (1.03 ± 0.12 mL h^{-1} 10^6 $cells^{-1}$) of pyrene (Figure S1) generated using hepatocytes from the same source of fish as the isolated perfused trout livers reported here is three-fold lower compared to the average clearance reported by Nichols et al. (3.48 mL h^{-1} 10^6 $cells^{-1}$).[11] Values reported by Nichols et al.[14,26] for S9 in their liver perfusion studies were more in line with those reported in the ring trial.[11] This indicates that the lower overall clearance may be attributed to metabolic variability in the strain of trout used. This might be the result of inter-strain variability, as has been previously reported.[31]

In addition to the two PAHs, one goal of this study was to expand upon and validate the isolated perfused fish liver model based on a broader chemical space compared to prior studies. Here, we generated additional hepatic clearance measurements for 4-*n*-Nonylphenol, deltamethrin, and methoxychlor respectively (Table 1).[11] Measured hepatic extractions fractions for these chemicals ranged from 27.2 to 99.3% at 1.0 g L^{-1} BSA and 21.8 to 67.5% at 10 g L^{-1} BSA, respectively, generally following the trend 4-*n*-Nonylphenol > methoxychlor > deltamethrin. Interestingly, the same trend was not observed when evaluating *in vitro* intrinsic

clearance values generated using S9 and hepatocytes, which followed the trend deltamethrin > 4-*n*-nonylphenol > methoxychlor and 4-*n*-Nonylphenol > deltamethrin > methoxychlor, respectively. These apparent discrepancies are the result of differences in protein binding and partitioning, and flow limitations in case of the perfused livers, between the three experimental systems, thereby further underlining the necessity to extrapolate results of *in vitro* assays to the *in vivo* level (or *ex situ*, in the case of isolated perfused livers) using adequate IVIVE models that explicitly represent these aforementioned differences.

3.4 Comparison of measured and predicted hepatic clearance

Previously published *in vitro* intrinsic clearance measurements from the ring trial reports associated with the standardization of OECD guidelines 319A and B were used as inputs for an IVIVE model that was refined from prior studies[7] and described in detail in Krause and Goss.[27] Hepatic clearance values extrapolated from extrapolated from published *in vitro* RT-S9 or RT-HEP intrinsic clearance values were compared with those directly measured in the isolated perfused liver model for both S9 and hepatocyte *in vitro* data separately (Figure 2).

As was observed for the impacts of protein binding on directly measured hepatic clearances in isolated perfused livers, hepatic clearance was predicted based on the *in vitro* data and partitioning taking into account the predicted binding in presence of the different BSA concentrations, and followed the same trend. The obvious impacts of protein binding on clearances of all five chemicals, with the 10 g L^{-1} treatment consistently showing the lowest, and the 1.0 g L^{-1} treatment consistently showing the greatest values measured values, are another clear indication that setting the term $f_u = \frac{f_{blood}^{unbound}}{f_{assay}^{unbound}}$ to 1 is mechanistically inappropriate as it fails to correct for systemic bioavailability.[5,20–22,32] Generally, the agreement between directly measured and extrapolated hepatic clearances was good for both *in vitro* input data based on hepatocytes (Figure 2A) and S9 (Figure 2C), with RMSEs of 16.8 and 22.7 mL h^{-1} g^{-1} liver, respectively. Deviations of predicted from measured BCFs based on current IVIVE exercises may span several orders of magnitude, especially if the assumption of fu=1.0 is not entertained.[11] Our dataset, on the contrary, indicates that current IVIVE models can be used to extrapolate confidently and quantitatively from *in vitro* measurements to the organ level, with a coefficient of variation of approximately 20% that falls within the observed variability of standardized *in*

391 *vitro* assays.[11,15]Additionally, Bland-Altman analyses indicate that there was very little, if any,
392 systematic bias of 4.1 and 9.8 mL h^{-1} g^{-1} liver between extrapolated and measured hepatic
393 clearances for hepatocytes and S9, respectively (Figure 2B and D).
394 In this light, our dataset provides important insights into the potential reasons for the
395 previously observed difference between measured and modeled BCFs. Our study has added data
396 for the three non-PAH chemicals to the breadth of data available for validation of IVIVE models.
397 It has been discussed previously whether uncertainties arose from *in vitro* assays, IVIVE models,
398 or *in vivo* BCFs used for validation, or all of these combined.[5,25] We believe that the present
399 dataset provides additional confidence that IVIVE based on current models and *in vitro* assay
400 protocols can yield reliable extrapolations from sub-cellular preparations or hepatocytes to the
401 organ level, with a level of variation that does not exceed levels of inter-individual variability
402 observed in standardized biological test systems. Mismatches between predicted and measured
403 BCFs can therefore be assumed to be rooted in factors beyond the organ level and which have
404 been discussed before,[5,11,33] including neglect of extrahepatic biotransformation, variability in
405 BCFs test designs[25], enzyme induction during live fish BCF studies, inaccuracies in partitioning
406 estimates and other kinetic processes (e.g., gill uptake rate constant k_1).
407
408 *3.5 Future applications and research needs*
409 This study has demonstrated that the isolated perfused fish liver model can be reliably
410 used as a tool to quantify hepatic clearance of chemicals. Therefore, this model can be
411 considered valuable for generating high-quality biotransformation data. Without further
412 extrapolation, the obtained hepatic clearance measurements may be used directly as input
413 parameters to physiologically-based toxicokinetic models, such as the ones developed in
414 Brinkmann et al.[34]
415 Furthermore, we show that this quantitative information is useful for validating current
416 approaches for IVIVE. In this way, we demonstrate that current IVIVE models, such that of
417 Krause & Goss[27] applied here, yield quantitatively accurate predictions of hepatic clearance.
418 That is, if differences in binding between *in vitro* assays and perfused livers are accurately
419 accounted for by means of pp-LFERs predictors, and if flow limitations are explicitly
420 incorporated. In this way, we believe that the dataset presented here has the potential to
421 demonstrate whether uncertainties in *in vitro* assay protocols, IVIVE models, BCFs used for

validation, or all these factors combined were the source of commonly observed incongruities in predicted and measured BCFs.

Isolated perfused livers could also be used to further refine IVIVE models, e.g., by systematically studying the dependence of hepatic clearance on perfusion rates. An influence seems plausible based on the assumption that limitations occur through slow desorption of chemicals from plasma proteins, here albumin, or rate-limiting permeation of chemicals through cell membranes. These factors are not currently considered in most IVIVE models, including the one applied here. Krause and Goss[35] show in their recent publication that both factors could impact the results of IVIVE predictions under some circumstances, and it would therefore be useful to study their impact in the isolated perfused trout liver model in greater detail.

Last, our study has also shown that this method can be expanded to a more diverse chemical space than previously studied. It should thus be the goal of subsequent studies to expand the covered chemical space even further. However, the throughput of the isolated perfused liver model is still limited, and our group has thus begun concurrent work investigating mixtures of chemicals in the isolated perfused liver model, representing a move towards substantially higher throughput screening of environmental contaminants.

Supplementary Information

The Supporting Information is available free of charge at https://pubs.acs.org/

- Detailed time-resolved plots of hepatic clearance and extraction fraction over time, broken down according to each chemical and BSA treatment.
- *In vitro-in vivo* extrapolation toolbox including individual measured and modelled hepatic clearance and hepatic extraction fraction values for each liver (XLSX).

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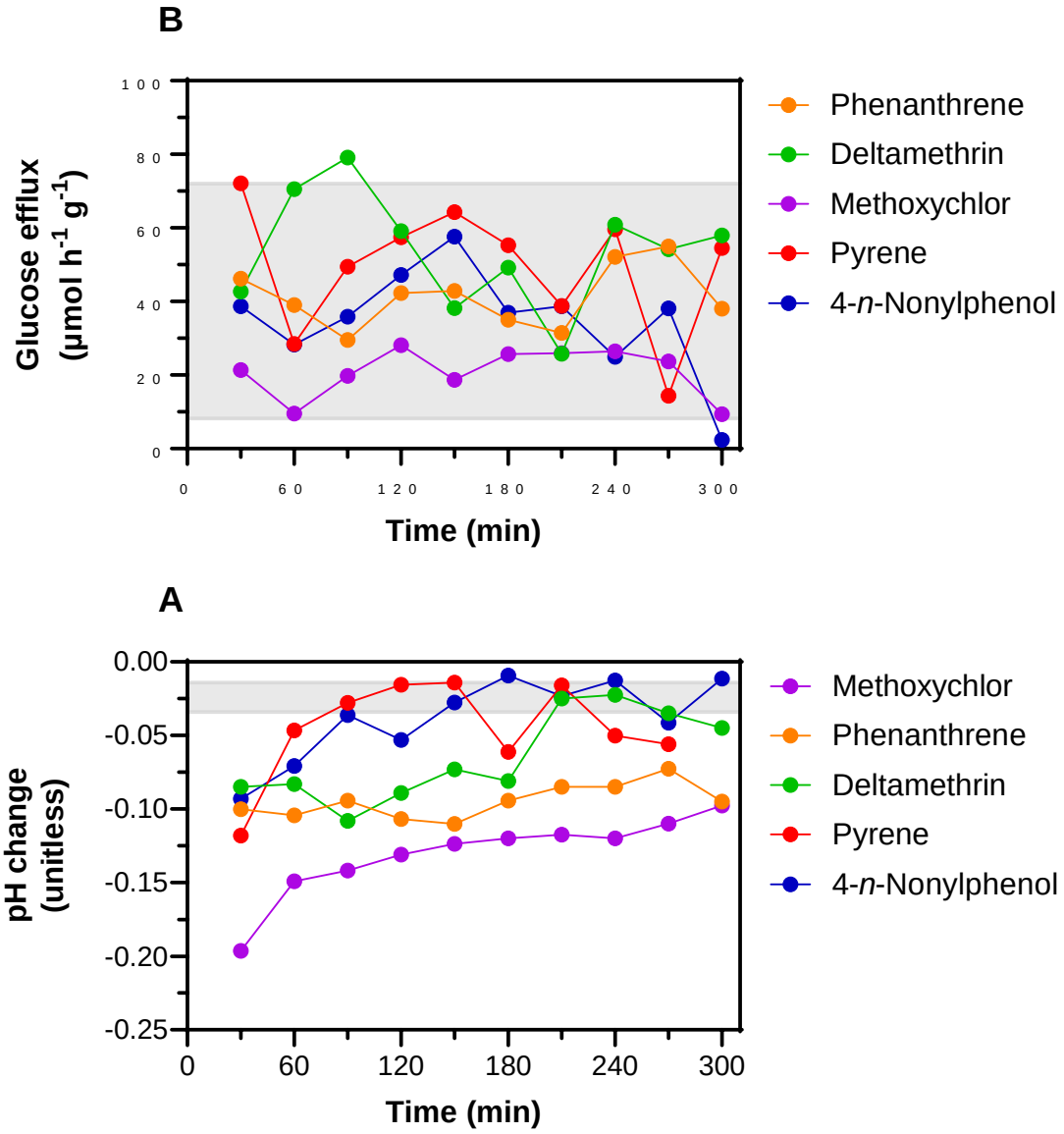
Figures and Tables

Table 1. Experimentally measured hepatic clearance (CL_H, mL h^{-1} g^{-1}) and hepatic extraction fractions (E_H, dimensionless) of the five tested chemicals in isolated perfused trout livers in the presence of 1.0, 2.5, and 10 g L^{-1} BSA. All values are expressed as arithmetic means ± standard deviation of *n*=3-4 replicate livers per condition.

	CL_H (mL h^{-1} g^{-1})			**E_H (%)**		
BSA (g L^{-1})	1.0	2.5	10	1.0	2.5	10
Pyrene	46.4±7.44	37.5±3.52	30.2±18.7	80.9±12.5	49.1±8.81	50.1±14.8
Phenanthrene	24.1±5.64	17.1±2.40	9.81±3.63	39.5±13.8	29.6±7.69	18.6±5.00
4-*n*-Nonylphenol	76.0±12.7	55.4±17.0	47.1±7.01	99.3±1.47	73.8±18.9	67.5±14.4
Deltamethrin	19.0±8.87	7.54±2.46	12.9±8.87	27.2±10.3	12.4±1.44	21.8±16.2
Methoxychlor	39.4±18.7	33.5±16.9	14.9±6.34	64.1±30.5	60.8±30.6	27.0±11.5



593

594 **Figure 1.** Physiological performance of isolated perfused livers was evaluated through
595 measurement of glucose efflux (**A**) and pH change between afferent and efferent samples (**B**). Data
596 shown summarizes all perfusions performed across BSA concentrations. Symbols indicate the
597 arithmetic means of all livers across all conditions per chemical. Gray shaded areas indicate the
598 range of reference values from previous studies conducted by Nichols et al.[14].

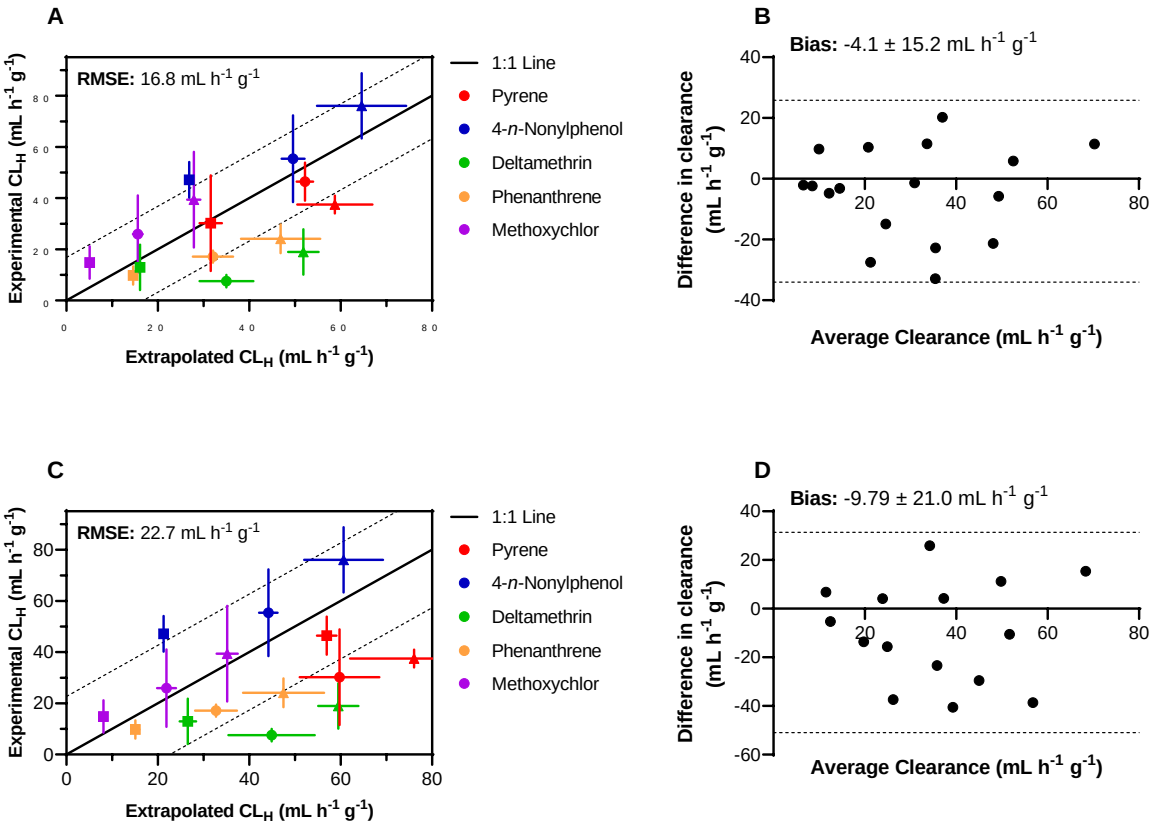


Figure 2. Experimental clearance of assayed chemicals in isolated perfused livers compared with extrapolated clearance in isolated hepatocytes (**A**) or S9 (**C**). Each chemical is represented by a unique color (see legend) in the presence of 1.0, 2.5, and 10 g L^{-1} BSA through squares, circles, and triangles, respectively. The solid line indicates the 1:1 line, dotted lines correspond to ± the root means squared error (RMSE). Symbols indicate the arithmetic means, error bars the standard deviations of *n*=3-4 replicate livers per condition. Horizontal error bars represent the variability in extrapolated hepatic clearance values from RT-HEP and RT-S9, vertical error bars indicate variability of clearance measurements across replicate perfused livers. Bland-Altman plots for clearances predicted from isolated hepatocytes (**B**) or S9 (**D**) versus measured hepatic clearances were generated to test for systematic differences (biases).