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IN VITRO-IN VIVO EXTRAPOLATION OF HEPATIC AND GASTROINTESTINAL BIOTRANSFORMATION RATES OF HYDROPHOBIC CHEMICALS IN RAINBOW TROUT

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Abstract

Hepatic *in vitro* biotransformation assays, in combination with *in vitro-in vivo* extrapolation (IVIVE) and bioaccumulation modeling, can be used to support regulatory bioaccumulation assessments. In most applications, however, these methods ignore the possibility of extrahepatic metabolism. Here we evaluated intestinal biotransformation in rainbow trout using S9 fractions prepared from the upper intestinal (GIT) epithelium. Measured levels of activity determined using standard substrates for phase I and phase II biotransformation enzymes were within 2-fold of activities measured in hepatic S9 fractions. *In vitro* intrinsic clearance rates for 2-ethylhexyl-4-methoxycinnamate (EHMC; an organic sunscreen agent) and two polycyclic aromatic hydrocarbons (pyrene [PYR] and benzo(a)pyrene [BAP]) were significantly higher in liver S9 fractions than in GIT S9 fractions. For octocrylene (OCT; a second sunscreen agent), however, *in vitro* intrinsic clearance rates were higher in GIT S9 fractions compared to liver S9 fractions. An existing 'liver only' IVIVE model was expanded to consider biotransformation in both the liver and GIT. Relevant IVIVE scaling factors were developed by morphological, histological, and biochemical evaluation of trout intestines. For chemicals biotransformed at higher rates by hepatic S9 fractions (i.e., BAP, PYR, EHMC), the 'liver & GIT' model yielded whole-body biotransformation rate constants (k_{MET}) that were within 1.2 to 1.4-fold of those estimated using the 'liver only' model. In contrast to these findings, the mean k_{MET} for OCT obtained using the 'liver & GIT' model was 3.3 times higher than the mean k_{MET} derived using the 'liver only' model and was in good agreement with empirical k_{MET} estimates determined previously for trout (<20% difference). The results of this study suggest that current 'liver only' IVIVE approaches may underestimate *in vivo* biotransformation rates for chemicals that undergo substantial biotransformation in the GIT.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplemental data

Supplementary material related to this article can be found, in the online version, at <https://doi.org/10.1016/j.aquatox.2020.105629>.

Keywords

bioaccumulation; biotransformation; animal alternatives; in vitro-in vivo extrapolation; intestinal metabolism; rainbow trout

1. Introduction

The assessment of bioaccumulation potential in fish is an integral part of the evaluation of chemical substances for their risk to the environment. A metric that is commonly used to describe a chemical's potential for bioaccumulation is the bioconcentration factor (BCF), which is defined as the steady-state chemical concentration in an organism divided by that in water, resulting from an aqueous (water-only) exposure. A BCF for fish may be determined in standardized laboratory experiments (OECD, 2012). For most chemicals, however, an empirical BCF is unavailable, so bioaccumulation models may be used to predict a BCF based on a chemical's octanol to water partition coefficient (K_{OW}) (Arnot and Gobas, 2003, 2004). For chemicals that are biotransformed, BCFs predicted by K_{OW} -based models (simple regression type or a mechanistic description) may overestimate the true degree of accumulation, resulting in possible misclassification of chemical bioaccumulation potential (Nichols et al., 2009). For this reason, knowledge of biotransformation in fish has been identified as a key requirement in chemical bioaccumulation assessments (Weisbrod et al., 2009).

To improve bioaccumulation assessments for fish, methods are needed to measure or estimate chemical biotransformation rates and incorporate this information into BCF prediction models. One well-known approach involves the measurement of *in vitro* biotransformation rate using hepatocytes or liver S9 fractions (OECD, 2018a, 2018b). The measured rate of intrinsic clearance is scaled to the intact tissue and used as an input to a physiological model of the liver to estimate hepatic clearance. The calculated hepatic clearance rate is then scaled to the whole animal to obtain a whole-body (*in vivo*) biotransformation rate constant (Nichols et al., 2006; Cowan-Ellsberry et al., 2008; Han et al., 2009, Laue et al., 2014). For biotransformed chemicals, the inclusion of extrapolated *in vitro* rate constants into bioaccumulation models improves BCF predictions resulting in modelled BCFs that are much closer to empirical BCFs than those generated assuming no biotransformation (Han et al., 2009; Cowan-Ellsberry et al., 2008; Dyer et al., 2008; Laue et al., 2014; Fay et al., 2016). However, there is a tendency for IVIVE methods to overestimate BCFs relative to measured values (Escher et al., 2011; Nichols et al., 2018a; Saunders et al., 2019).

The true rate of *in vitro* intrinsic clearance may be underestimated if the assay is performed at inappropriately high substrate concentrations (Lo et al., 2015a; Nichols et al., 2018a, Saunders et al., 2019). In such cases, failure to predict *in vivo* levels of activity is a result of the *in vitro* data used to perform the extrapolation. Alternatively, the well-stirred liver model employed as part of the extrapolation procedure may be insufficient to predict hepatic clearance of some highly bound chemicals (Escher et al., 2011). Finally, current IVIVE methods assume that biotransformation occurs primarily in the liver. If substantial

biotransformation occurs in other tissues, this approach will tend to underestimate the true whole-body biotransformation rate constant (Nichols et al., 2013b).

Few attempts have been made to quantitatively relate measured *in vitro* activity for extrahepatic tissues to chemical bioconcentration in fish. Exceptions include studies by Gomez et al. (2010) and Stadnicka-Michalak et al. (2018). Biotransformation rates for ibuprofen, propranolol, and norethindrone were measured in rainbow trout gill and liver S9 fractions (Gomez et al., 2010). These rates were then extrapolated to estimates of whole-body clearance and used as inputs to a one-compartment BCF prediction model. For ibuprofen and propranolol, BCFs predicted in this manner were significantly lower than BCFs estimated using only hepatic biotransformation rate constants (Gomez et al., 2010). Gill, liver, and intestinal cell lines were shown to metabolize benzo(a)pyrene. These data were subsequently incorporated into a physiologically based toxicokinetic (PBTK) model for fish, resulting in improved agreement between predicted and empirical BCFs (Stadnicka-Michalak et al., 2018).

Microsomes isolated from intestinal tissues of several fish species have been shown to exhibit measurable activity toward standard substrates for phase I and phase II biotransformation enzymes (Van Veld et al., 1988; 1990; 1991; James et al., 1997; Lee et al., 2001; Lou et al., 2002), suggesting that the intestine is an important site for biotransformation. Additional work indicates that biotransformation in the gastrointestinal tract may substantially reduce chemical uptake in fish from contaminated food (Van Veld et al., 1988). Presently, however, there are no standardized methods for measuring *in vitro* intrinsic intestinal clearance rates in fish (including all relevant biotransformation pathways), and there is limited knowledge of the scaling factors required to extrapolate this activity to the intact tissue. Although the impact of hepatic and intestinal biotransformation on chemical accumulation in fish may be predicted using a full PBTK model (Stadnicka-Michalak et al., 2018), a simple IVIVE approach that could be used to inform one-compartment BCF prediction models currently does not exist.

In the present study, *in vitro* biotransformation rate constants were measured for two organic sunscreen agents (2-ethylhexyl-4-methoxycinnamate [EHMC] and octocrylene [OCT]) and two polycyclic aromatic hydrocarbons (pyrene [PYR] and benzo(a)pyrene [BAP]), using hepatic and intestinal S9 fractions from rainbow trout. An existing IVIVE model (Nichols et al., 2013b) was expanded to consider biotransformation in both the intestines and liver. Scaling factors required to support this effort were developed by morphological, histological, and biochemical evaluation of trout intestines. The expanded IVIVE model was evaluated by comparing estimated whole-body biotransformation rate constants to rate constants extrapolated using a 'liver only' IVIVE model. Additional comparisons were then made to empirical rate constants generated in *in vivo* studies with trout.

2. Materials and Methods

2.1. Chemicals

Adenosine 3'-phosphate 5'-phosphosulfate (PAPS; 80% pure) was obtained from EMD Millipore (Calbiochem). The reduced form of β -nicotinamide adenine dinucleotide

phosphate (β -NADPH, > 95% pure) was purchased from Oriental Yeast. Solvents were obtained from Fisher Chemical. All other chemicals and cofactors were purchased from Sigma-Aldrich and were reagent-grade or higher in quality.

2.2. Homogenates and S9 fractions

Rainbow trout (*Oncorhynchus mykiss*, Erwin strain) were obtained as eggs from the US Geological Survey Upper Midwest Environmental Sciences Center in LaCrosse, WI, and reared to the desired size (~400–700 g; Table 1) at the USEPA laboratory in Duluth, MN. The gonadosomatic index (GSI; gonad mass/body mass $\times$ 100) was determined for each test animal. Measured values ranged from 0.03 to 0.06 for males and 0.24 to 0.56 for females (Supporting Information, Table S1), demonstrating that fish were sexually immature at the time of use (Nichols et al., 2018a). Fish were fed Silver Cup trout chow (Nelson and Sons) and were maintained on a natural photo-period at 11 ± 1 °C. Water for fish holding was obtained directly from Lake Superior (single-pass, sand-filtered, and ultraviolet treated). All fish were fasted for at least 24 h prior to use.

Three groups of 3 trout were processed individually to obtain paired pools of liver and intestinal S9 fractions (3 sample pairs in total). Details pertaining to the preparation liver S9 fractions are given elsewhere (Johanning et al., 2012). Briefly, fish were euthanized with ethyl-3-aminobenzoate methanesulfonate (MS 222, 300 mg/L) buffered with 900 mg/L NaHCO_3 . The hepatic vein was severed and 10 to 20 mL of ice-cold clearing buffer (Hank's balanced salt solution with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid and 3 mM ethylenediamine tetraacetic acid [EDTA], pH 7.8) was perfused through the hepatic portal vein. Livers were then excised, rinsed with clearing buffer, minced in 2 volumes of homogenization buffer (50 mM Tris, 2 mM EDTA, 1 mM dithiothreitol [DTT], 150 mM KCl, and 250 mM sucrose; pH 7.8), and homogenized using a Potter-Elvehjem mortar and pestle.

Procedures used to prepare intestinal S9 fractions were adapted from methods used to obtain intestinal microsomes (James et al., 1997; Kleinow et al., 1998; McElroy and Kleinow 1992). Briefly, the anterior intestine (upper intestine minus the pyloric ceca; Figure S1) was excised and rinsed with ice-cold 0.9% NaCl solution to remove luminal contents and expose the mucosal (epithelial) cell layer. The tissue was transferred to an ice-cold glass plate with the mucosal side facing upward. Epithelial cells were then removed by gentle scraping with the edge of a glass microscope slide, pooled, and placed in 10 mL of ice-cold homogenization buffer (phenylmethylsulfonyl fluoride (PMSF) free; see Supporting Information, *Preliminary studies with PMSF*, and). In these preparations, the epithelial cell types were not identified but it was assumed that enterocytes provided most of the metabolic activity. The cells were sedimented at 2,000 *g*, weighed, and homogenized in 4 volumes of homogenization buffer using a Potter-Elvehjem mortar and pestle. Portions of the liver and intestinal cell homogenates were retained on ice while the remaining portions were centrifuged at 13,000 *g* for 20 min (4 °C) to obtain S9 fractions. The S9 fractions and crude homogenates were flash frozen in liquid nitrogen and stored at -80 °C.

The protein content of pooled S9 samples was determined via Peterson's modification of the Lowry method (Sigma-Aldrich, 2003) using bovine serum albumin (fraction V) as the

standard. The measured protein content of liver and intestinal S9 fractions averaged 25 and 11 mg/mL, respectively (Table S3). This difference in protein content was largely due to different dilution factors (2-fold and 4-fold, respectively) employed during tissue homogenization. The total CYP content of S9 samples and crude tissue homogenates was determined using a dithionite difference spectroscopy method (Matsubara et al., 1976) modified for use with fish (Nichols et al., 2013b). The number of sample replicates for each assay is given in Table S3.

2.3. Characterization assays

The metabolic activities of liver and intestinal S9 fractions were evaluated by performing a set of assays using model substrates for cytochrome P450 1A (CYP1A), cytochrome P450 3A (CYP3A), uridine 5'-diphospho-glucuronosyltransferase (UGT), and glutathione-S-transferase (GST). CYP1A activity was characterized by measuring the rate of 7-ethoxyresorufin-*O*-dealkylation (EROD assay; Burke and Mayer, 1974). UGT activity was characterized by measuring glucuronidation of *p*-nitrophenol (Ladd et al., 2016). GST activity was assessed by measuring glutathione conjugation of 1-chloro-2,4-dinitrobenzene (Habig et al., 1974). Details pertaining to these assays are given elsewhere (Nichols et al., 2013a).

CYP3A activity was characterized by measuring the hydroxylation of testosterone to 6 β -hydroxy-testosterone (T6BH; Han et al., 2009). Reaction mixtures (700 μ L) were incubated for 30 min and consisted of 1 mg/mL S9 protein, 2 mM NADPH, and 130 μ M testosterone in 100 mM potassium phosphate buffer containing 1 mM MgCl₂. T6BH was quantified by pipetting 200 μ L of the reaction mixture into 800 μ L MeOH containing 250 ng/mL of 11-ketoandrostenedione as the internal standard. The samples were held at 4 °C overnight to precipitate S9 protein, re-mixed, and centrifuged at 3,000 *g* for 10 min (4 °C). Subsamples of the supernatant were then diluted 20-fold with MeOH:MQ water (4:1; v/v) and analyzed by liquid chromatography-mass spectrometry (LC-MS). All characterization assays were performed using saturating substrate concentrations at the physiological temperature (11 °C) and pH (7.8) for trout. Negative controls with matrix blanks and denatured S9 fractions were run simultaneously with each assay and measured activities were blank corrected. The number of sample replicates for each assay is given in Table S3.

2.4. In vitro biotransformation assay

Substrate depletion experiments were conducted at 11 $\pm$ 1 °C in a 1 mL reaction vessel containing 100 mM potassium phosphate buffer (pH 7.8) and the cofactors β -NADPH, uridine 5'-diphosphoglucuronic acid (UDPGA), adenosine 3'-phosphate 5-phosphosulfate (PAPS), and reduced glutathione (GSH) at final concentrations of 2 mM, 2 mM, 0.1 mM, and 5 mM, respectively. Buffer, S9 fractions (1 mg/mL S9 protein), and 25 mg/mL alamethicin were mixed and pre-incubated on ice for 15 min. Alamethicin is a pore-forming peptide that increases the permeability of the ER membrane and is added to enhance UGT activity (Ladd et al. 2016). Reactions were then initiated by adding the test chemicals in acetone carrier (0.5% [v/v] final concentration). Initial concentrations of test chemicals in the incubation media averaged 0.056, 0.013, 0.10, and 0.47 μ M for PYR, BAP, EHMC, and OCT, respectively. These concentrations are well below previously reported apparent

Michaelis-Menten constants (K_M) for each reaction (Nichols et al., 2018a; Saunders et al., 2019) and were at least 30 times greater than instrument detection limits (Nichols et al., 2018a). All depletion experiments were performed in duplicate for each chemical in each S9 pool ($n=3$). Matrix blanks and heat-inactivated S9 controls were run with each assay to evaluate chemical extraction efficiency and characterize potential non-enzymatic chemical losses. Reactions were terminated by transferring 100 μ L aliquots of the incubation media into 300 μ L of ice-cold acetonitrile. Up to 9 aliquots were removed from each reaction vial. Incubation periods were adjusted to account for chemical-specific difference in the rate of biotransformation, and did not exceed 75 min.

Samples containing PYR and BAP were vortexed and centrifuged at 3,000 g for 6 min (4 $^{\circ}$ C). Supernatants were then analyzed by high-performance liquid chromatography (HPLC). For samples containing EHMC and OCT, 5 μ L of 0.5 μ M d^{12} -chrysene (internal standard) was added to each vial and shaken on a vortex mixer for 10 s. A 1.0 mL volume of n -hexane was added to each vial and vials were mixed for 5 min using a vortex mixer to extract EHMC or OCT and d^{12} -chrysene. Sample vials were then centrifuged at 3,000 g for 10 min. The hexane supernatants were then transferred to 2 mL amber glass vials for gas chromatography-mass spectrometry (GC-MS) analysis.

2.5. Instrumental analyses

Descriptions of the conditions for analysis by LC-MS, GC-MS, and HPLC are included in the Supporting Information.

2.6. Calculation of *in vitro* intrinsic clearance

An *in vitro-in vivo* extrapolation (IVIVE) model was developed to estimate whole-body rates of biotransformation from measured rates of *in vitro* intrinsic clearance in liver ($CL_{INT,LIVS9}$) and intestinal ($CL_{INT,GITS9}$) S9 fractions (Figure 1). Measured chemical concentrations from substrate depletion experiments were transformed to their natural logarithms and regressed against time (t). First-order depletion rate constants (k_{DEP} ; 1/h) were determined from the slope of the fitted linear relationship:

$$\ln C_t = \ln C_0 - k_{DEP} \times t \quad (1)$$

where C_0 and C_t are the concentrations of the test chemical (μ M) in the incubation medium at time 0 and time t (h). The k_{DEP} values obtained using liver ($k_{DEP,L}$) and intestinal ($k_{DEP,G}$) S9 fractions were then divided by the measured concentration of S9 protein ($C_{S9,L}$ or $C_{S9,G}$; 1 mg/mL) to calculate $CL_{INT,LIVS9}$ and $CL_{INT,GITS9}$ with units of mL/h/mg protein.

2.7. Calculation of hepatic clearance

Multiplying $CL_{INT,LIVS9}$ by the S9 content of liver tissue (L_{S9} ; mg S9 protein/g liver) and liver weight as a fraction of total body weight (L_{FBW} ; g liver/g fish) yields the *in vivo* hepatic intrinsic clearance ($CL_{INT,LIV}$; mL/h/g fish). Further multiplication by 24 h 1/d provides units of mL/d/g fish (equal to L/d/kg fish). The L_{FBW} (0.0092; Table 1) was determined in a separate pool of rainbow trout ($n=7$; Table S4) and is within the range previously reported values (0.7–2.9%; Cowan-Ellsberry et al., 2008). The L_{S9} was estimated

using a ratio method analogous to that used to estimate the microsomal protein content of liver tissue (Nichols et al., 2013b):

$$L_{S9} = \frac{\text{pmol CYP in liver homogenate g tissue}^{-1}}{\text{pmol CYP in liver S9 fraction mg S9 protein}^{-1}} \quad (2)$$

where pmol CYP refers to the total CYP content of crude homogenates and S9 fractions. Application of this ratio method assumes that any loss of S9 protein during the processing of liver samples is proportional to the loss of microsomal protein.

Hepatic clearance (CL_H ; L/d/kg fish) was calculated using a well-stirred liver model that accounts for potential rate limitations caused by blood flow to the liver (Q_L ; L/d/kg fish) (Wilkinson and Shand, 1975). The model also includes a correction to account for differences in the water content of the S9 system (v_{WS9} ; 0.99 L/L) and blood plasma (v_{WBL} ; 0.84 L/L) (Krause and Goss, 2018):

$$CL_H = \frac{\left(Q_L \times f_U \times CL_{INT, LIV} \times \left[\frac{v_{WS9}}{v_{WBL}} \right] \right)}{\left(Q_L + \left(f_U \times CL_{INT, LIV} \times \left[\frac{v_{WS9}}{v_{WBL}} \right] \right) \right)} \quad (3)$$

The Q_L was set equal to 25.9% of cardiac output (Q_C ; L/d/kg fish; Nichols et al., 1990). The Q_C for trout was estimated using an empirical equation that accounts for differences in fish acclimation temperature (T ; °C) and body weight ($W_{B,E}$; g) (Erickson and McKim, 1990):

$$Q_C = \left[(0.023 T - 0.78) \times \left(\frac{W_{B,E}}{500} \right)^{-0.1} \right] \times 24 \quad (4)$$

The well-stirred model includes a binding parameter (f_U ; unitless) that is calculated as the ratio of free chemical fractions in blood plasma (ϕ_P ; unitless) and the S9 incubation medium (ϕ_{S9} ; unitless) (Nichols et al., 2013a):

$$f_U = \frac{\phi_P}{\phi_{S9}} \quad (5)$$

Implied by this calculation is the assumption that only freely dissolved chemical is available to hepatic biotransformation enzymes *in vitro* and *in vivo*. Henceforth, f_U values calculated in this manner are denoted with the abbreviation $f_{U,calc}$, and the underlying basis for this calculation is referred to as the “full” binding assumption.

The ϕ_P was calculated as:

$$\phi_P = v_{WBL} / P_{BW} \quad (6)$$

where P_{BW} is the blood-water partition coefficient (L/L; Fitzsimmons et al. 2001):

$$P_{BW} = ([10^{0.73 \log K_{OW}} \times 0.16] + 0.84) \quad (7)$$

The ϕ_{S9} was calculated as (Han et al., 2009):

$$\phi_{S9} = v_{WS9} / (C_{S9} \times 10^{0.694 \log K_{OW} - 2.158} + 1.0) \quad (8)$$

These binding algorithms are appropriate for neutral organic chemicals that bind non-specifically to organic molecules such as lipids and proteins. Because the lipid and protein content of trout plasma is considerably higher than that of liver S9 fractions (Escher et al., 2011; Saunders et al., 2019), the calculated ϕ_P for a hydrophobic chemical ($\log K_{OW} > 3$) will be much greater than ϕ_{S9} , yielding an f_U value $\ll 1$. Binding terms ($f_{U,calc}$) calculated for the selected test chemicals ranged from 0.021 to 0.026 (Table S5).

Additional estimates of CL_H were then obtained under the assumption that $\phi_P = \phi_{S9}$ (i.e., $f_U = 1.0$). This assumption implies the possibility that chemical bioavailability to biotransformation enzymes *in vitro* (S9) and *in vivo* is effectively the same, despite differences in ϕ_P and ϕ_{S9} (Nichols et al. 2013a; Laue et al. 2014).

2.8. Calculation of intestinal clearance

In vivo intestinal intrinsic clearance ($CL_{INT,GIT}$; L/d/kg fish) was estimated by multiplying $CL_{INT,GITS9}$ by the fractional mucosal cell weight (MC_{FBW} ; g mucosal cells/g fish), the S9 content of mucosal cells (MC_{S9} ; mg S9/g mucosal cells), and the units conversion factor 24 h 1/d. The MC_{S9} was calculated using the ratio method described in Equation 2. For this exercise, however, we used measured CYP content values for mucosal cell homogenates and S9 fractions. The MC_{FBW} accounts for differences in total mass of epithelial cells that line the pyloric ceca ($\Phi_{MC,PC}$) and anterior intestine ($\Phi_{MC,AG}$), expressed as fractions of tissue weight. Estimates of $\Phi_{MC,PC}$ and $\Phi_{MC,AG}$ (Table 1) were obtained from stained transverse sections of pyloric ceca and anterior intestine (Figure S2, Table S6, and accompanying text in Supporting Information). These values were then multiplied by the weights of the pyloric ceca (W_{PC} ; g) and anterior intestine (W_{AG} ; g) to estimate the total mucosal cell weight (W_{MC} ; g):

$$W_{MC} = (\Phi_{MC,PC} \times W_{PC}) + (\Phi_{MC,AG} \times W_{AG}) \quad (9)$$

The W_{AG} was set equal to the measured weight of the anterior intestine, determined in this study (0.99 g; Table 1). An estimate of W_{PC} was obtained by subtracting W_{AG} from the estimated total weight of the upper intestine (W_{UI} ; g, including both pyloric ceca and anterior intestine), determined using data from a separate pool of rainbow trout ($n=7$; Table S4). Expressed as a fraction of body weight, this total tissue weight (0.99%) is in good agreement with previously reported values for trout (0.8–2.1%; Barron et al., 1987; Nichols et al., 2004). Finally, the W_{MC} was divided by mean fish body weight (Table 1) to generate MC_{FBW} (0.0074 g mucosal cells/g fish).

Calculation of intestinal clearance (CL_G ; L/d/kg fish) was also performed using a well-stirred model:

$$CL_G = \frac{\left(Q_L \times f_U \times CL_{INT, GIT} \times \left[\frac{v_{WS9}}{v_{WBL}} \right] \right)}{\left(Q_L + \left(f_U \times CL_{INT, GIT} \times \left[\frac{v_{WS9}}{v_{WBL}} \right] \right) \right)} \quad (10)$$

In rainbow trout, most of the blood that perfuses the liver originates as venous blood draining the intestines. Blood flow to the intestines was therefore set equal to that flowing to the liver. Expressed as a percent of Q_C (25.9%), this value is within the range of reported intestinal blood flows in fish (10–40% of Q_C ; Seth et al., 2010). The measured amount of protein in both the intestinal and liver S9 assays was the same (1 mg/mL). Therefore, both the binding term f_U and the water content of the S9 system (v_{WS9}) used to calculate CL_G were set equal to those used in the calculation of CL_H .

2.9. Calculation of total clearance

The independently calculated hepatic and intestinal clearance terms were used to calculate total clearances (CL_{TOT} ; L/d/kg fish) for the selected test chemicals. This required that consideration be given to the series arrangement of the tissues. That is, the amount of chemical cleared by the liver depends on that remaining in blood following intestinal biotransformation (Nichols et al., 2007). Assuming as indicated above that most of the blood flowing to the liver originates from the intestines, this total clearance may be calculated as (Gillette, 1982):

$$CL_{TOT} = CL_G + (CL_H \times ([Q_L - CL_G]/Q_L)) \quad (11)$$

2.10. Calculation of whole-body biotransformation rate constants

Whole-body (*in vivo*) biotransformation rate constants (k_{MET} ; 1/d) were estimated by dividing CL_H or CL_{TOT} by each chemical's volume of distribution (V_D ; L/kg) (Nichols et al., 2013b):

$$k_{MET} = \frac{CL_H \text{ or } CL_{TOT}}{V_D} \quad (12)$$

where the V_D is the sorptive capacity of the fish relative to that of blood. The V_D for each chemical was calculated as:

$$V_D = \frac{(v_{LWB} \times K_{OW})/d_L}{P_{BW}} \quad (13)$$

where v_{LWB} represents the lipid content of the organism (kg/kg) and d_L is the density of fish lipid (0.90 kg/L). For each chemical the v_{LWB} was set to equal the mean of measured values determined in *in vivo* biotransformation studies (Table 2). All equations and parameters included in the IVIVE model are provided in the Supporting Information, Table S5.

2.11. Empirical biotransformation rate constants

Empirical *in vivo* biotransformation rate constants (k_{MET} ; Table 2) for EHMC and OCT were reported previously in a single study performed with rainbow trout following exposure to either chemical at three different dietary concentrations (Saunders et al., 2020). No dose-dependent effects on k_{MET} , fish weight, or lipid content was observed. Therefore, in the extrapolation of EHMC and OCT biotransformation rates, modeled fish weights and fractional lipid contents were set to the mean of the three reported values (Table 2; Saunders et al., 2020). Empirical k_{MET} values for BAP and PYR in trout were obtained from multiple studies that used different sized trout (Niimi and Palazzo, 1986; Lo et al., 2015b; 2016; DiMauro, 2018). Therefore, to improve comparisons between the different studies, all rate constants for BAP and PYR were normalized to a 10 g fish at 11°C using the relationship (Arnot et al., 2008):

$$k_{\text{MET},N} = k_{\text{MET},i} \left(\frac{W_{B,N}}{W_{B,i}} \right)^{-0.25} \exp[0.01(T_N - T_i)] \quad (14)$$

where $k_{\text{MET},N}$ is the normalized rate constant, $k_{\text{MET},i}$ is the study-specific rate constant, $W_{B,N}$ is the normalized mass of the organism (10 g), $W_{B,i}$ is the original study-specific mass of the organism (g), T_N is the normalized water temperature (11°C), and T_i is the original study-specific water temperature (°C). For PYR and BAP, the v_{LWB} was set to equal the mean of reported values (0.040 and 0.048, respectively; Table 2). Study-specific information used to calculate the normalized k_{MET} values is provided in Table S7.

2.12. IVIVE model evaluation

In vitro depletion rate constants for each chemical were extrapolated to estimate k_{MET} using an existing ('liver only') IVIVE model (Nichols et al., 2013b) and the expanded ('liver & GIT') IVIVE model presented here (Figure 1). The expanded IVIVE model was evaluated by comparing predicted whole-body biotransformation rate constants to rate constants predicted using the 'liver only' IVIVE model. Additional comparisons were then made to empirical rate constants generated in *in vivo* studies with trout (Table 2). Due to differences in the way that extrapolated and empirical k_{MET} values were derived, a statistical analysis was deemed inappropriate (see Supporting Information, *IVIVE model evaluation*). Hence, we elected instead to evaluate the fold difference between *in vitro*- and *in vivo*-derived rate constants.

Previous studies have evaluated IVIVE models by comparing relative agreement between empirical BCFs and BCFs predicted using *in vitro* biotransformation rates (Escher et al., 2011; Laue et al., 2014; Nichols et al., 2018b; Saunders et al., 2019). We instead evaluated the IVIVE models presented here by directly comparing *in vitro* and *in vivo*-derived biotransformation rate constants. The comparison of biotransformation rates allows for an improved assessment of IVIVE model performance because it eliminates uncertainties in BCF model inputs that are unrelated to biotransformation (e.g., predicted rates of chemical uptake and elimination across the gills).

2.13. Statistical analyses

Linear regression was used to test whether the test chemicals exhibited a significant log-linear decrease in chemical concentration over time in heat-treated, liver, and intestinal S9 fractions (slope = zero). A Welch's *t* test was used to evaluate differences in mean enzyme activities between the liver and intestinal S9 fractions, differences in total CYP content between liver and intestinal S9 fractions and homogenates, and differences in test chemical depletion rate constants between liver and intestinal S9 fractions. All statistical analyses were performed in R (Version 3.3.3) at an α level of 0.05.

3. Results and Discussion

3.1. Characterization of trout liver and intestinal S9 fractions

The mean ($\pm$ SE) total CYP content of liver ($10,721 \pm 1,233$ pmol CYP/g) and intestinal ($11,035 \pm 1,062$ pmol CYP/g) homogenates did not differ significantly ($p=0.8567$; Table 3). However, the CYP content of intestinal S9 fractions (48.09 ± 2.86 pmol CYP/mg S9 protein) was approximately 2-fold lower than that of liver S9 fractions (80.52 ± 4.09 pmol CYP/mg S9 protein; $p=0.0042$). This difference in CYP recovery is accounted for in the IVIVE model by calculating the S9 protein content of liver tissue (L_{S9}) and mucosal epithelial cells (MC_{S9}) (Equation 2). The calculated values for L_{S9} and MC_{S9} were 133.4 ± 14.4 mg S9/g and 228.5 ± 9.34 mg S9/g, respectively (Table 3). To our knowledge, the MC_{S9} has never been measured in fish. The L_{S9} determined here is within the range of values reported previously for trout (79–170 mg S9/g liver; Cowan-Ellsberry et al., 2008; Nichols et al., 2013b).

A possible reason for the lower CYP content of the intestinal S9 fractions is that intestinal mucous impacts the separation of intact cells, cell debris, and microsomal protein during the centrifugation of tissue homogenates. Treatment with dithiothreitol (DTT) was shown to increase yields of isolated epithelial cells from Rhesus Macaques intestinal tissue (Pan et al., 2012). DTT decreases mucous viscosity by reducing disulfide bonds, thereby dissociating mucin fibers into monomeric subunits. In the present study, DTT was added to the homogenization buffers at concentrations comparable to other enterocyte isolation procedures (Goodyear et al., 2014; Pan et al., 2012; Salinas et al., 2007). By adding DTT to intestinal washing buffers it may be possible to 'break down' mucous and improve CYP recovery in intestinal S9 fractions; however, this would have to be determined.

Measured EROD, T6BH, UGT, and GST activities in liver S9 fractions ranged from 3.5–4.4 pmol/min/mg protein, 32–53 pmol/min/mg protein, 711–1281 pmol/min/mg protein and 614–824 nmol/min/mg protein, respectively (Table S3). Similar values have been reported previously for trout liver S9 fractions (Han et al., 2009; Nichols et al., 2018a). Measured EROD and GST activities in intestinal S9 fractions were approximately 2-fold lower than those determined in liver S9 fractions (Figure 2, Panels A and D; $p<0.05$). The UGT activities of intestinal and liver S9 fractions did not differ statistically (Figure 2C; $p=0.5057$). Previous authors have reported that EROD activities in liver microsomes prepared from different species of un-induced fish are between 4- and 27-fold higher than activities in intestinal microsomes (Van Veld et al., 1988; 1990; 1991; James et al., 1997).

Other studies have shown modest (2-fold) differences in GST and UGT activities between liver and intestinal subcellular fractions (Lindström-Seppä et al., 1981; Van Veld et al., 1991).

Measured T6BH activities in intestinal S9 fractions were 1.6-fold higher than those in liver S9 fractions (Figure 2B; $p=0.0352$). This difference is comparable to reported differences in T6BH activity in liver and intestinal microsomes from channel catfish and rainbow trout (Lee et al., 2001; Lou et al., 2002). Immunohistochemical and mRNA analyses of CYP3A subfamily genes have demonstrated strong responses in the intestine and liver of fish (Cok et al., 1998). In particular, the subfamily isoforms CYP3A27 (Lee et al., 2001) and CYP3A45 (Lee and Buhler 2003) are expressed abundantly in the rainbow trout intestine. These findings suggest that CYP3A isoforms are involved in the first-pass clearance of xenobiotics (Hegelund and Celander, 2003). Collectively, the results of this and previous studies indicate that the intestinal epithelium in fish possesses a robust capacity to biotransform chemical contaminants.

Total CYP content, EROD activity, and aryl hydrocarbon hydroxylase activity in the pyloric ceca of spot (*Leiostomus xanthurus*) were approximately 2-fold higher than values measured in the anterior intestine (Van Veld et al., 1988). Similarly, the total CYP content in the pyloric ceca of scup (*Stenotomus versicolor*) was approximately 2-fold higher than in the hindgut (Stegeman et al., 1979). Higher enzymatic activities in the proximal portion of the intestine in spot and scup may reflect the fact that this region is the first line of defense against dietary xenobiotics. In the present study, intestinal S9 fractions were obtained from the anterior portion of the upper intestine, distal to the pyloric ceca (Figure S1). If, as in spot and scup, the concentration of biotransformation enzymes in the trout intestine is higher in the pyloric ceca than in other regions, then *in vivo* intrinsic clearance rates calculated here for the entire upper intestine may underestimate true levels of activity.

3.2. In vitro biotransformation rates

Assays with heat-treated S9 showed no significant depletion of any test chemical ($p > 0.05$; Figure S3). All test chemicals exhibited a significant log-linear decrease in chemical concentration over time in liver and intestinal S9 fractions (negative slope $\neq$ zero, $p < 0.05$; Figure S3). Liver and intestinal *in vitro* clearance rates are provided in Table S8. Mean ($\pm$ SE) intrinsic clearance rates determined for the three liver S9 pools were 18 ± 1.9 mL/h/mg protein, 40 ± 3.6 mL/h/mg protein, 3.5 ± 0.42 mL/h/mg protein, and 0.88 ± 0.030 mL/h/mg protein, for PYR, BAP, EHMC, and OCT, respectively. In the intestinal S9 fractions, mean ($\pm$ SE) intestinal *in vitro* clearance rates determined for PYR, BAP, EHMC, and OCT were 4.2 ± 0.68 mL/h/mg protein, 9.4 ± 0.91 mL/h/mg protein, 1.4 ± 0.19 mL/h/mg protein, and 1.7 ± 0.19 mL/h/mg protein, respectively (Figure 3). For PYR, BAP, and EHMC the mean $CL_{INT,S9}$ measured using liver S9 fractions was significantly higher than that determined using intestinal S9 fractions ($p < 0.05$ for each). For OCT, the mean $CL_{INT,S9}$ measured in intestinal S9 fractions was approximately 1.5-fold higher than that in liver S9 fractions ($p=0.0349$).

The primary pathway for PAH biotransformation in fish involves hydroxylation of one or more aromatic rings, followed by sulfation and glucuronidation of hydroxylated products

(Varanasi et al., 1989). These hydroxylation reactions are catalyzed predominantly by CYP1A, although other CYP enzymes may contribute (Schlenk et al., 2008). Observed differences in biotransformation of PYR and BAP in liver and intestinal S9 fractions (liver > intestine) were similar to tissue-specific differences in EROD activity shown here (Figure 2A) and in previous studies (James et al., 1997; Van Veld et al., 1988; 1990; 1991). This finding is also consistent with relative differences in measured *in vitro* intrinsic clearance of BAP determined in liver and intestinal cells lines (Stadnicka-Michalak et al., 2018).

Previous studies with trout liver S9 fractions have shown that hydrolysis by carboxylesterases and CYP-mediated biotransformation are important metabolic routes for EHMC and OCT (Saunders et al., 2019). It is possible, therefore, that tissue-specific differences in distribution of carboxylesterases and/or CYP enzymes in the intestines and liver could explain the differences in clearance rates observed for EHMC and OCT. To our knowledge, the CYP isoforms involved in biotransformation of EHMC and OCT in fish have not yet been identified. It is interesting to note, however, that relative differences in OCT clearance rates (Figure 3B) and mean T6BH activities (Figure 2B) in liver and intestinal S9 fractions were similar. The existence of higher T6BH activity in the intestinal S9 fraction suggests an enrichment of CYP3A in the intestinal epithelium of rainbow trout, as compared to the liver. It is possible, therefore, that OCT is a substrate for CYP3A while EHMC is a substrate for other CYPs. Previous authors have noted the localization of CYP3A enzymes in the fish intestine (Husøy et al., 1994; Cok et al., 1998; Lee et al., 2001; Lee and Buhler, 2003; Hegelund and Celandier, 2003; McArthur et al., 2003). The prototypical human CYP3A4 substrates diltiazem and carbamazepine were not significantly biotransformed in trout liver S9 fractions (Connors et al., 2013). It would be of interest to determine if these substrates are biotransformed by intestinal S9 fractions.

3.3. Extrapolated whole-body biotransformation rate constants

Prior to evaluating the expanded IVIVE model, hepatic *in vitro* rate constants were extrapolated using the ‘liver only’ model to assess two binding assumptions, $f_U = f_{U,calc}$ (Equation 5) and $f_U = 1.0$. Relatively good agreement between estimated and empirical k_{MET} values was obtained for all test chemicals under the full binding assumption (within 0.4- to 4.4-fold, based on mean values; Figure S4). In contrast, setting $f_U = 1.0$ resulted in k_{MET} estimates that exceeded empirical values (Table 2) by a factor of 2.1 to 8.6. Previous authors have reported that setting $f_U = 1.0$ often results in improved agreement between empirical BCFs and BCFs predicted using measured rates of *in vitro* biotransformation, while use of the full binding assumption tends to yield BCFs that overestimate empirical values (Escher et al., 2011; Laue et al., 2014; Nichols et al., 2018b; Saunders et al., 2019). The use of predicted BCFs to evaluate IVIVE methods is greatly complicated, however, by uncertainties in model inputs unrelated to biotransformation (e.g., predicted rates of chemical uptake and elimination across the gills).

An examination of Equations 13 and 14 indicates that any addition of intestinal biotransformation to the IVIVE model will yield a larger predicted k_{MET} value than that generated by the ‘liver only’ description. In the present study, this could only increase the extent to which k_{MET} estimates exceed empirical values, assuming $f_U = 1.0$. Subsequent

evaluation of the expanded ('liver & GIT') IVIVE model was therefore performed under the full binding assumption ($f_U = f_{U,calc}$).

Whole-body biotransformation rate constants (k_{MET}) predicted for PYR, BAP, EHMC, and OCT by the 'liver only' model were (mean $\pm$ SE) 1.39 ± 0.07 1/d, 0.73 ± 0.03 1/d, 0.24 ± 0.02 1/d, and 0.033 ± 0.001 1/d, respectively (Figure 4). The k_{MET} values predicted by the 'liver & GIT' model for the same four chemicals were (mean $\pm$ SE) 1.73 ± 0.01 1/d, 0.88 ± 0.01 1/d, 0.33 ± 0.01 1/d, and 0.11 ± 0.01 1/d, respectively (Figure 4). For PYR, BAP, and EHMC the k_{MET} values obtained using the 'liver only' model were approximately 1.2- to 1.4-fold lower than those predicted by the 'liver & GIT' model (Figures 4A–C). For each of these chemicals, estimated hepatic clearance rates were significantly higher (2-fold) than estimated intestinal clearance rates. This finding suggests that for chemicals that have significantly higher rates of hepatic clearance relative to intestinal clearance, hepatic *in vitro* biotransformation assays may be sufficient to estimate whole-body biotransformation rate constants.

Empirical k_{MET} values for BAP and PYR were obtained from multiple studies performed with rainbow trout (Niimi and Palazzo, 1986; Lo et al., 2015b; 2016; DiMauro, 2018). Both of the extrapolated k_{MET} values for BAP ('liver only' and 'liver & GIT') fell within the upper range of empirical k_{MET} data (Figure 4B), while the extrapolated k_{MET} values for PYR were 4.4- ('liver only') and 5.5-fold higher ('liver & GIT') than the mean empirical k_{MET} (Figure 4A). A possible reason for the overestimates of the extrapolated biotransformation rates for PYR may be because the available empirical data are not well-matched to the *in vitro* data generated here. For example, interstrain differences in enzyme activities (Koponen et al., 1997) could result in differences in chemical biotransformation rates measured different strains or cultures of trout. Furthermore, some of the available empirical k_{MET} values for BAP and PYR are from studies that involved simultaneous exposure to several aromatic hydrocarbons (Lo et al., 2015b; Lo et al., 2016). Competitive inhibition among these chemicals could have resulted in biotransformation rates that are lower than those expected in single chemical exposures (Lee et al., 2014). Additionally, all empirical rate constants for BAP and PYR were normalized to a 10 g fish at 11 °C using Equation 14. This normalization was required to permit direct comparisons to k_{MET} values predicted by the IVIVE models. However, the parameters in Equation 14 reflect assumptions regarding the weight- and temperature-dependence of biotransformation rates in fish that are largely untested.

Unlike PYR and BAP, *in vitro* and *in vivo* biotransformation rate constants for EHMC and OCT were obtained from the same population of trout held under identical conditions. The k_{MET} for EHMC, obtained by extrapolating measured rates of hepatic *in vitro* clearance to the whole animal ('liver only' model), was approximately 2.3-fold lower than the mean empirical k_{MET} of 0.54 1/d (Figure 4C). When intestinal biotransformation was incorporated into this extrapolation, the k_{MET} for EHMC increased from 0.24 to 0.33 1/d. For OCT, the inclusion of measured intestinal activity into the extrapolation ('liver & GIT' model) increased the estimated k_{MET} by 3.3-fold from 0.033 to 0.11 1/d (Figure 4B). This latter value is in good agreement (i.e., <20% difference) with the empirical k_{MET} (0.093 1/d) determined previously by Saunders et al. (2020). These modeled results suggest that

biotransformation in the intestinal epithelial may have a large impact on the whole-body clearance rate of OCT in rainbow trout. Moreover, these findings indicate that for chemicals that undergo substantial biotransformation in intestinal epithelium, incorporating this activity into current IVIVE approaches could lead to improved estimates of whole-body biotransformation rate constants.

4. Conclusions

The results of the present study show that S9 fractions prepared from the intestinal epithelium in rainbow trout biotransform a variety of chemical substances. Basal-level metabolic activities toward standard substrates for phase I and phase II biotransformation enzymes were within 2-fold of activities measured in hepatic S9 fractions. Measured rates of testosterone hydroxylation were significantly higher in intestinal S9 fractions than in hepatic S9 fractions, suggesting that the intestinal epithelium is an important site for biotransformation of CYP3A substrates in fish.

Observed differences in biotransformation of PYR and BAP by liver and intestinal S9 fractions (liver > intestine) were comparable to measured differences in EROD activity. This finding is consistent with previous work which indicates that hydroxylation of PAHs is catalyzed predominantly by CYP1A. EHMC depletion rate constants were also comparatively higher in liver S9 fractions, whereas OCT depletion rate constants were higher in intestinal S9 fractions. These differences may indicate that biotransformation of EHMC and OCT are mediated by different CYP isoforms. For chemicals biotransformed at higher rates by hepatic S9 fractions (i.e., BAP, PYR, EHMC), the ‘liver only’ IVIVE model may be sufficient to estimate whole-body biotransformation rate constants (k_{MET}), as the extrapolated rate constants do not differ substantially (i.e., < 1.5-fold difference) from those obtained using a ‘liver & GIT’ model. In contrast, the k_{MET} value predicted for OCT using the ‘liver & GIT’ model was 3.3-fold higher than that obtained using the ‘liver only’ model and exhibited much better agreement with empirical values. This finding suggests that current ‘liver only’ IVIVE approaches may underestimate whole-body *in vivo* biotransformation rates for chemicals that are subject to significant intestinal biotransformation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability

Data and associated metadata pertaining to this manuscript may be accessed through the USEPA Environmental Data Gateway at <https://doi.org/10.23719/1519218>.

Abbreviations

ϕ_P	Fraction unbound in blood plasma (unitless)
ϕ_{S9}	Fraction unbound in S9 fractions (unitless)
$\Phi_{MC,AG}$	Fraction of mucosal cells covering anterior intestine (unitless)
$\Phi_{MC,PC}$	Fraction of mucosal cells covering pyloric ceca (unitless)
θ	Proportionality constant to reflect the sorptive capacity of protein relative to octanol (0.05)
BAP	Benzo(a)pyrene
BCF	Bioconcentration factor (L water/kg fish)
C_0	Initial concentration; concentration in the incubation medium at time 0 (μM)
$C_{S9,G}$	S9 protein content in intestinal S9 fractions (mg protein/mL S9)
$C_{S9,L}$	S9 protein content in hepatic S9 fractions (mg protein/mL S9)
C_t	concentration in the incubation medium at time t (μM)
CL_G	Intestinal clearance (L blood/d/kg fish)
CL_H	Hepatic clearance (L blood/d/kg fish)
$CL_{INT,GIT}$	<i>In vivo</i> intestinal intrinsic clearance rate (L S9/d/kg fish)
$CL_{INT,GITS9}$	<i>In vitro</i> intestinal intrinsic clearance rate (mL S9/h/mg protein)
$CL_{INT,LIV}$	<i>In vivo</i> hepatic intrinsic clearance rate (L S9/d/kg fish)
$CL_{INT,LIVS9}$	<i>In vitro</i> hepatic intrinsic clearance rate (mL S9/h/mg protein)
$CL_{INT,S9}$	<i>In vitro</i> intrinsic clearance rate (mL S9/h/mg protein)
CL_{TOT}	Total clearance (L blood/d/kg fish)
CYP	Cytochrome P450
d_L	Density of fish lipid (kg lipid/L water)
DTT	Dithiothreitol
EHMC	2-Ethylhexyl-4-methoxycinnamate

EROD	Ethoxyresorufin- <i>O</i> -deethylase
$f_{U,calc}$	Calculated binding correction factor
$f_U=1.0$	Binding correction factor set equal to 1.0
G_{FBW}	Fractional upper intestine weight (g upper intestine/g fish)
GC-MS	Gas chromatography mass spectrometry
GIT	Gastrointestinal tract
GSI	Gonadosomatic Index
GST	Glutathione S-transferase
HPLC	High performance liquid chromatography
IVIVE	<i>In vitro-in vivo</i> extrapolation
k_{DEP}	First-order <i>in vitro</i> depletion rate constant (1/h)
$k_{DEP,G}$	<i>In vitro</i> depletion rate constant measured in intestinal S9 fractions (1/h)
$k_{DEP,L}$	<i>In vitro</i> depletion rate constant measured in liver S9 fractions (1/h)
K_M	Michaelis-Menten constant
k_{MET}	Whole-body <i>in vivo</i> biotransformation rate constant (1/d)
$k_{MET,i}$	Study-specific whole-body <i>in vivo</i> biotransformation rate constant (1/d)
$k_{MET,N}$	Normalized whole-body <i>in vivo</i> biotransformation rate constant (1/d)
K_{OW}	Octanol-water partition coefficient
LC-MS	Liquid chromatography mass spectrometry
L_{FBW}	Fractional liver weight (g liver/g fish)
L_{S9}	Liver S9 protein content (mg S9 protein/g liver)
MC_{FBW}	Fractional mucosal cell weight (g mucosal cells/g fish)
MC_{S9}	Mucosal cell S9 protein content (mg S9 protein/g mucosal cells)
NADPH	Reduced β -nicotinamide adenine dinucleotide phosphate
OECD	Organisation for Economic Co-operation and Development
OCT	Octocrylene

P_{BW}	Equilibrium blood-water partition coefficient (L water/L blood)
PAH	Polycyclic aromatic hydrocarbon
PAPS	Adenosine 3'-phosphate 5'-phosphosulfate
PBTK	Physiologically based toxicokinetic model
PMSF	Phenylmethylsulfonyl fluoride
PYR	Pyrene
Q_C	Cardiac output (L blood/d/kg fish)
Q_L	Tissue blood flow (L blood/d/kg fish)
S9	Supernatant fraction obtained from liver or intestinal homogenate by centrifuging at 13,000 <i>g</i> for 20 min in phosphate buffer
SD	Standard deviation
SE	Standard error
T	Time
T_i	Study-specific water temperature (°C)
T_N	Normalized water temperature (°C)
T6BH	Testosterone-6 β -hydroxylation
UGT	Glucuronosyltransferase
V_D	Apparent volume of distribution (L blood/kg fish)
v_{LWB}	Fractional whole-body lipid content (kg lipid/kg fish)
v_{WBL}	Fractional water content of blood (L water/L blood)
v_{WS9}	Fractional water content of S9 (L water/L S9)
W_{AG}	Anterior intestine weight (g)
W_B	Fish body weight (g)
$W_{B,i}$	Study-specific fish body weight (g)
$W_{B,E}$	Body weight of extrapolated fish (g)
$W_{B,N}$	Normalized fish body weight (g)
W_{MC}	Total mucosal cell weight (g)
W_{PC}	Estimated pyloric ceca weight (g)

W_{UI}

Estimated upper intestine weight (g)

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Figure 1.
In vitro-in vivo extrapolation (IVIVE) model for estimating whole-body biotransformation rate constants from *in vitro* depletion rate constants measured in hepatic and intestinal S9 fractions.

Figure 2. Mean activities of 7-ethoxyresorufin-*O*-dealkylation (EROD; A), testosterone-6 β -hydroxylation (T6BH; B), glucuronidation of *p*-nitrophenol (UGT; C) and glutathione conjugation of 1-chloro-2,4-dinitrobenzene (GST; D) in liver and intestinal (GIT) S9 fractions. Error bars represent the standard error of the mean ($n=3$). The p -values are the results from a Welch's t test used to compare activities of the standard substrates between liver and GIT S9 fractions ($p<0.05$).

Figure 3.

Mean *in vitro* intrinsic clearance rates ($CL_{INT,S9}$) for (A) 0.056 μM pyrene (PYR), (B) 0.013 μM benzo(a)pyrene (BAP), (C) 0.10 μM 2-ethylhexyl-4-methoxycinnamate (EHMC), and (D) 0.47 μM octocrylene (OCT) measured using liver and intestinal (GIT) S9 fractions.

Error bars represent the standard error of the mean ($n=3$). The p -values are the results from a Welch's t test used to compare activities of the substrates between liver and GIT S9 fractions ($\alpha = 0.05$).

Figure 4.

Boxplots of whole-body biotransformation rate constants (k_{MET}) for PYR (A), BAP (B), EHMC (C), and OCT (D) extrapolated from *in vitro* hepatic activities ('liver only'; triangles) and from *in vitro* hepatic and intestinal activities ('liver & GIT'; squares). *In vitro* rate constants were extrapolated by setting the binding correction factor (f_{U}) equal to $f_{\text{U,calc}}$ (Table S5). Empirical *in vivo* rate constants (Table 2) are also provided (circles). The box boundaries indicate the 1st and 3rd quartiles, the horizontal line within the box is the median, and the whiskers denote the 5th and 95th percentiles. The data points represent the raw data.

Table 1.

Mean (SD) fish and tissue weights, and liver and intestinal (GIT) scaling factors determined in rainbow trout.

Parameter	Value	Equation
Fish and tissue weights^a		
Fish body weight (W_B ; g)	534 (121)	
Anterior intestine weight (W_{AG} ; g)	0.99 (0.23)	
Liver Scaling Factors		
Fractional liver weight (L_{FBW} ; g/g) ^b	0.0092 (0.0017)	
GIT Scaling Factors		
Fractional upper intestine weight (G_{FBW} ; g/g) ^b	0.0099 (0.0013)	
Estimated upper intestine weight (W_{UI} ; g)	5.29	$G_{FBW} \times W_B$
Estimated pyloric ceca weight (W_{PC} ; g)	4.30	$W_{UI} - W_{AG}$
Fraction of mucosal cells covering pyloric ceca ($\Phi_{MC,PC}$) ^c	0.77 (0.03)	
Fraction of mucosal cells covering anterior intestine ($\Phi_{MC,AG}$) ^c	0.67 (0.004)	
Total mucosal cell weight (W_{MC} ; g)	3.90	$\Phi_{MC,PC} \times W_{PC} + \Phi_{MC,AG} \times W_{AG}$
Fractional mucosal cell weight (MC_{FBW} ; g/g)	0.0073	W_{MC} / W_B

^aMean ($n=9$) values determined from the fish selected for S9 preparation (Table S1)

^bMean ($n=7$) values determined from a separate pool of trout (Table S4)

^cValues determined from the quantification of mucosal cell areas of the upper intestinal tract (Equation S1; Table S6).

Table 2.

Fish weight ($W_{B,E}$; g), fractional whole-body lipid content (ν_{LWB}), and whole-body *in vivo* biotransformation rate constants (k_{MET} ; 1/d) of the four test chemicals. The k_{MET} values for EHMC and OCT were obtained at three different dose levels from a single study whereas k_{MET} values for PYR and BAP were obtained from multiple studies.

Chemical	$W_{B,E}$	ν_{LWB}	k_{MET}	Source
EHMC	42	0.042	0.467	Saunders et al., 2020
	39	0.046	0.653	
	39	0.038	0.502	
OCT	32	0.036	0.077	Saunders et al., 2020
	36	0.038	0.104	
	40	0.038	0.097	
BAP ^a	10	0.050 ^b	1.050	Niimi and Palazzo, 1986 ^c
	10	0.067	0.140	Lo et al., 2015b
	10	0.020	0.320	Lo et al., 2016
	10	0.050	0.620	Lo et al., 2016
PYR ^a	10	0.030	0.450	Lo et al., 2016
	10	0.049	0.183	DiMauro, 2018

^aValues for k_{MET} were adjusted to a 10 g fish at 11 °C (Equation 14).

^bAssumed value of 0.05 because measured values were not reported.

^cOriginal data source; derived k_{MET} value is from Arnot et al., 2008

Table 3.

Calculated S9 protein content of trout liver and gastrointestinal tract (GIT) mucosal cells determined from the ratio of mean cytochrome P450 (CYP) content of S9 fractions and crude tissue homogenates^a

CYP Measurements	Liver	GIT
CYP content of homogenates (pmol CYP/g tissue)	10721 ± 1233	11035 ± 1062
Protein normalized CYP content of S9 fractions (pmol CYP/mg S9 protein)	80.52 ± 4.09	48.09 ± 2.86
Calculated S9 protein content (L _{S9} or MC _{S9} ; mg S9 protein/g tissue)	133.4 ± 14.4	228.5 ± 9.34

^a All values are reported as the mean ± SE, *n*=3.