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TOWARD IMPROVED MODELS FOR PREDICTING BIOCONCENTRATION OF WELL-METABOLIZED COMPOUNDS BY RAINBOW TROUT USING MEASURED RATES OF IN VITRO INTRINSIC CLEARANCE

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Abstract: Models were developed to predict the bioconcentration of well-metabolized chemicals by rainbow trout. The models employ intrinsic clearance data from in vitro studies with liver S9 fractions or isolated hepatocytes to estimate a liver clearance rate, which is extrapolated to a whole-body biotransformation rate constant (k_{MET}). Estimated k_{MET} values are then used as inputs to a mass-balance bioconcentration prediction model. An updated algorithm based on measured binding values in trout is used to predict unbound chemical fractions in blood, while other model parameters are designed to be representative of small fish typically used in whole-animal bioconcentration testing efforts. Overall model behavior was shown to be strongly dependent on the relative hydrophobicity of the test compound and assumed rate of in vitro activity. The results of a restricted sensitivity analysis highlight critical research needs and provide guidance on the use of in vitro biotransformation data in a tiered approach to bioaccumulation assessment. *Environ Toxicol Chem* 2013;32:1611–1622. © 2013 SETAC

Keywords: Bioaccumulation Bioconcentration Biotransformation In vitro–in vivo extrapolation Fish

INTRODUCTION

Biotransformation can strongly impact the extent to which hydrophobic organic chemicals accumulate in fish [1–4]. Until recently, however, methods that could be used to predict this activity were unavailable. Fortunately, this situation is changing. Using a mass-balance modeling approach, Arnot et al. [5,6] evaluated measured bioconcentration factors (BCFs) and dietary bioaccumulation data for fish to estimate whole-body rates of biotransformation for over 600 compounds. This information was then used to develop a screening-level quantitative structure–activity relationship (QSAR) model that predicts biotransformation rates based on chemical structure [7]. The biotransformation rate QSAR has been integrated with a screening-level BCF and bioaccumulation factor (BAF) prediction model [8] and incorporated into the BCFBAF module of the US Environmental Protection Agency's (USEPA's) Estimation Programs Interface Suite software program [9]. Additional QSAR approaches based on documented chemical transformations in rats have been developed to estimate the probability that a chemical will undergo biotransformation in fish and predict likely products of this activity [10].

Despite this progress, however, a need remains for methods to directly assess biotransformation of chemicals not adequately covered by existing databases or for which greater certainty in the prediction is required. Ideally, this need would be met using methods that do not involve additional whole-animal testing. One approach that may satisfy this need involves the use of in vitro metabolizing systems derived from liver tissue. Using procedures developed initially by the pharmaceutical industry, several groups have extrapolated in vitro biotransformation data for fish to the intact animal and used this information to predict impacts on chemical accumulation [11–15]. In each case, incorporating in vitro information into established models of chemical accumulation substantially improved model performance, resulting in predicted BCFs that were in better agreement with measured values than predictions obtained assuming no biotransformation.

Collectively, these studies have provided a "proof of concept" for the use of in vitro biotransformation data in bioaccumulation modeling efforts with fish. However, a closer examination of these efforts indicates a number of differences in assumed extrapolation factors and physiological inputs. Some of these differences can be attributed to the use of different fish species (trout, carp, and catfish), while others reflect differences in reported values for a particular species (see Table 1 in Cowan-Ellsberry et al. [13]). In addition, all of these studies have provided BCF predictions for relatively large fish (trout: 200–1000 g; carp: 375 g; catfish: 160 g) containing a substantial amount of whole-body lipid (10%). While this is understandable, given that these animals were used as the source of in vitro material (S9 fractions, microsomes, or hepatocytes), most of the BCF testing with fish has been performed using much smaller animals. Failure to account for differences in fish mass and lipid content may result in a mismatch between predicted BCFs and the measured BCFs used to evaluate model accuracy and performance.

All Supplemental Data may be found in the online version of this article.
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Table 1. Independent variable inputs to the S9-bioconcentration (S9-BCF) model

Parameter (symbol)	Value	Units	Reference
Octanol–water partition coefficient (K_{OW}) of test compound	Measured or estimated	Unitless	
Log K_{OW} of test compound	From K_{OW}	Unitless	
Body weight of modeled fish (Bwg_M)	Set by investigator; here, assumed to be 10	g	
Body weight of fish used as source of S9 protein (Bwg_{S9})	Measured	g	
S9 protein concentration (C_{S9})	Set by investigator; typically 0.5 to 2.0	mg/mL	
Reaction rate (*Rate*)	Measured; from substrate depletion assay	1/h	
Fish holding temperature (T, for BCF testing)	Set by investigator; here, assumed to be 15	Celsius	
Liver S9 protein content (L_{S9})	163	mg/g liver	The present study
Liver weight as fraction of whole-body weight (L_{FBW})	0.015	Unitless	[38]
Liver blood flow as fraction of cardiac output (Q_{HFRAC})	0.259; includes both arterial and portal blood flows	Unitless	[29]
Fractional water content of blood (ν_{WBL})	0.84	Unitless	[57]
Fractional whole-body lipid content (ν_{LWB})[a]	Set by investigator; here, assumed to be 0.05	Unitless	
Particulate organic carbon content (C_{POC})	4.6×10^{-6}	kg/L	[39]
POC binding constant (α_{POC})	0.35	Unitless	[41]
Dissolved organic carbon content (C_{DOC})	1.0×10^{-6}	kg/L	[39]
DOC affinity constant (α_{DOC})	0.08	Unitless	[40]
Total aqueous chemical concentration ($C_{W,TOT}$)	Set by investigator; does not impact BCF calculation	mg/L	

[a]Although fixed by the investigator, this parameter was varied in the sensitivity analysis.
POC = particulate organic carbon; DOC = dissolved organic carbon

The purpose of this report is to provide updated models for predicting the bioconcentration of well-metabolized chemicals by rainbow trout. The models incorporate recent advances in our knowledge of key extrapolation factors and chemical partitioning relationships and are designed to predict steady-state BCFs for a reference fish, defined as a 10-g rainbow trout held at 15 °C that contains 5% whole-body lipid. This definition recognizes that most of the in vivo testing with fish has been performed using small animals or juveniles of larger species [16] and is consistent with recommendations for testing trout given in current guidance [17,18]. The models are flexible, however, and contain algorithms that adjust for user-specified changes in temperature, lipid content, fish mass, and several extrapolation factors. A limited sensitivity analysis was conducted to evaluate model sensitivity to changes in selected input parameters. The results of this analysis highlight critical research needs and provide guidance for using these models in a tiered approach to bioaccumulation assessment.

MODEL DESCRIPTION

Two computational models were developed to predict the bioconcentration of well-metabolized chemicals by rainbow trout. Both employ a 1-compartment description of the animal and use data obtained from an in vitro test system to estimate a whole-body biotransformation rate constant (k_{MET}). The S9-BCF model uses data derived from liver S9 fractions, while the HEP-BCF model relies on data from isolated hepatocytes. Both models are configured as Microsoft Excel spreadsheets, which are available on request from the corresponding author. Table 1 provides a list of variable inputs for the S9-BCF model. Dependent variables calculated by the model are given in Table 2, along with the equations that perform these calculations. Input parameters for the HEP-BCF model that differ from those used in the S9-BCF model are given in Table 3.

In vitro–in vivo extrapolation of intrinsic hepatic clearance

The approach used to extrapolate in vitro intrinsic clearance to an estimate of the in vivo whole-body biotransformation rate constant has been outlined previously [13,19]. Briefly, substrate depletion experiments are performed under first-order reaction conditions to characterize the rate of chemical disappearance from the system. Measured concentrations of the parent chemical are $\log_{10}$-transformed, and a linear regression is performed on the transformed values to calculate a first-order elimination rate constant (1/h), which is related to the regression slope by the expression $Rate = -2.3\ Slope$.

Rate is divided by the measured concentration of S9 protein (C_{S9}; mg/mL) or hepatocytes (C_{HEP}; 10^6 cells/mL) to calculate in vitro intrinsic clearance ($CL_{IN\ VITRO,INT}$; mL/h/mg protein or 10^6 hepatocytes). For reactions that exhibit classical Michaelis-Menten kinetics, $CL_{IN\ VITRO,INT}$ is equal to the maximum rate of reaction (V_{MAX}; μmole/h/mg protein) divided by the affinity constant (K_M; μmole/mL), where K_M is the substrate concentration resulting in half-maximal activity. Multiplying $CL_{IN\ VITRO,\ INT}$ by the S9 content of liver tissue (L_{S9}; mg/g liver) or hepatocellularity (L_{HEP}; 10^6 cells/g liver) and liver weight as a fraction of total body weight (L_{FBW}; g liver/g fish) yields the in vivo intrinsic clearance ($CL_{IN\ VIVO,INT}$; mL/h/g fish), while further multiplication by 24 (h/d) provides units of mL/d/g fish (equivalent to L/d/kg fish).

A well-stirred liver submodel [20,21] is used to calculate hepatic clearance (CL_H; L/d/kg fish)

$$CL_H = Q_H\, f_U\, CL_{IN\ VIVO,\ INT} / \left(Q_H + f_U\, CL_{IN\ VIVO,\ INT}\right) \quad (1)$$

This description accounts for possible rate limitations imposed by the rate of liver blood flow (Q_H; L/d/kg fish). The model also includes a term (f_U; unitless) that corrects for potential binding effects on clearance. In the default approach taken in the present study, f_U is calculated as the ratio of free chemical fractions in blood plasma ($f_{U,P}$; unitless) and in the in vitro system ($f_{U,S9}$ or $f_{U,HEP}$; unitless) [22–25].

The binding term $f_{U,S9}$ is calculated using an empirical relationship given by Han et al. [12]

$$f_{U,S9} = 1/\left(C_{S9} \times 10^{0.694\ log\ Kow - 2.158} + 1.0\right) \quad (2)$$

Although this relationship was originally developed using binding data from rat liver microsomes [25], predicted levels of

Table 2. Dependent variables calculated by the S9-bioconcentration factor (S9-BCF) and hepatocyte-bioconcentration factor (HEP-BCF) models

Parameter (symbol)	Units	Equation used for calculation
Body weight of modeled fish ($Bwkg_M$)	kg	$Bwg_M/1000$
In vitro intrinsic clearance ($CL_{IN\ VITRO,INT}$)	mL/h/mg protein or mL/h/10^6 cells	$Rate/C_{S9}$ or $Rate/C_{HEP}$
In vivo intrinsic clearance ($CL_{IN\ VIVO,INT}$)	L/d/kg fish	$CL_{IN\ VITRO,INT}\ L_{S9}\ L_{FBW} \times 24$ or $CL_{IN\ VITRO,INT}\ L_{HEP}\ L_{FBW} \times 24$
Unbound fraction in the S9 system ($f_{U,S9}$)	Unitless	$1/(C_{S9} \times 10^{0.694\ log\ Kow\ -\ 2.158} + 1.0)$
Unbound fraction in the hepatocyte system ($f_{U,HEP}$)	Unitless	$1/[(C_{HEP}/2 \times 10^6) \times 10^{0.676\ log\ Kow\ -\ 2.215} + 1.0]$
Blood to water partition coefficient (P_{BW})	Unitless	$(10^{0.73\ log\ Kow} \times 0.16) + 0.84$
Unbound fraction in blood plasma ($f_{U,P}$)	Unitless	ν_{WBL}/P_{BW}
Hepatic clearance binding term (f_U)	Unitless	$f_{U,P}/f_{U,S9}$ or $f_{U,P}/f_{U,HEP}$
Cardiac output, scaled for temperature and body weight (Q_C)	L/d/kg fish	$[(0.023\ T) - 0.78] \times (Bwg_M/500)^{-0.1} \times 24$
Blood flow to the liver (Q_H)	L/d/kg fish	$Q_C\ Q_{HFRAC}$
Hepatic clearance (CL_H)	L/d/kg liver	$Q_H\ f_U\ CL_{IN\ VIVO,\ INT}/(Q_H + f_U\ CL_{IN\ VIVO,INT})$
Partitioning-based BCF (BCF_P)	Unitless	$\nu_{LWB}\ K_{OW}$
Apparent volume of distribution ($V_{D,BL}$)	L/kg	BCF_P/P_{BW}
Whole-body metabolism rate constant (k_{MET})	1/d	$CL_H/V_{D,BL}$
Gill uptake rate constant (k_1)	L/d/kg fish	$1/[(0.01 + 1/K_{OW}) \times Bwkg_M^{0.4}]$
Gill elimination rate constant (k_2)	1/d	k_1/BCF_P
Fecal egestion rate constant (k_E)	1/d	$0.125[0.02\ Bwkg_M^{-0.15} e^{(0.06\ T)}/(5.1 \times 10^{-8} K_{OW} + 2)]$
Freely dissolved chemical fraction in water (FD)	Unitless	$1/(1 + C_{POC}\ \alpha_{POC}\ K_{OW} + C_{DOC}\ \alpha_{DOC}\ K_{OW})$
Freely dissolved chemical concentration in water ($C_{W,FD}$)	mg/L	$C_{W,TOT}\ FD$
Steady-state chemical concentration in fish ($C_{FISH,SS}$)	mg/kg	$k_1\ C_{W,FD}/(k_2 + k_{MET} + k_E)$
BCF expressed on a total chemical basis (BCF_{TOT})	L/kg	$C_{FISH,SS}/C_{W,TOT}$
BCF normalized for lipid content and expressed on a freely dissolved chemical basis ($BCF_{FD,L}$)	L/kg lipid	$C_{FISH,SS}/(C_{W,FD}\ \nu_{LWB})$

K_{OW} = $\log_{10}$ octanol–water partition coefficient; T = temperature.

binding for several test compounds were found to be consistent with measured values in trout liver S9 fractions [26].

The binding term $f_{U,HEP}$ is calculated using a modification of the relationship given by Han et al. [11]

$$f_{U,HEP} = 1/\left[C_{HEP}/\left(2 \times 10^6\right) \times 10^{0.676\ log\ Kow - 2.215} + 1.0\right] \quad (3)$$

Equation 3 differs from that given by Han et al. [11] with respect to inclusion of a new term, $C_{HEP}/(2 \times 10^6)$, which corrects for differences in hepatocyte concentration. Binding values for trout used to develop the original relationship were measured in a solution containing 2×10^6 hepatocytes/mL. By using Equation 3, it is possible to predict binding in solutions containing higher or lower cell concentrations.

Several authors have developed empirically based algorithms that predict chemical binding in fish plasma [11,19,27]. In order to evaluate these relationships, we assembled a data set of measured blood (or blood plasma) binding values for rainbow trout (Supplemental Data, Figure S1). Twenty values are reported for chemicals with octanol-water partition coefficient ($\log K_{OW}$) values ranging from approximately 1.5 to greater than 8. When expressed as the log of the bound/free ratio, these data describe a linear relationship with $\log K_{OW}$.

A description of methods used to assess model fit is provided in the Supplemental Data, along with a brief discussion of modeled results. Based on this analysis, we recommend use of the binding algorithm given by Fitzsimmons et al. [27]

$$P_{BW} = \left(10^{0.73\ log\ Kow} \times 0.16\right) + 0.84 \quad (4)$$

where P_{BW} is the equilibrium blood-water partition coefficient.

Assuming that the chemical present in the aqueous fraction of blood is freely dissolved, P_{BW} is related to $f_{U,P}$ by [19]

$$f_{U,P} = \nu_{WBL}/P_{BW} \quad (5)$$

where ν_{WBL} is the fractional water content of blood. This relationship also assumes that chemical binding in plasma and whole blood is approximately equal.

The Q_H is calculated as the product of total cardiac output (Q_C; L/d/kg fish) and the fraction of Q_C that flows to the liver via the hepatic artery and hepatic portal vein (Q_{HFRAC}; unitless). This fractional amount (0.259) is based on data for trout provided by Barron et al. [28], using assumptions given by Nichols et al. [29]. Total cardiac output (Q_C) as a function of temperature and body weight is calculated using an empirical equation developed previously for rainbow trout [30]

$$Q_C = \left[(0.023\ T - 0.78) \times (Bwg_M/500)^{-0.1}\right] \times 24 \quad (6)$$

where Bwg_M is the weight of the modeled fish in grams and T is temperature (Celsius).

Table 3. Inputs to the hepatocyte-bioconcentration factor (HEP-BCF) model that differ from those used in the S9-bioconcentration factor (S9-BCF) model

Parameter (symbol)	Value	Units	Reference
Assay hepatocyte concentration (C_{HEP})	Controlled by investigator; typically 0.5 to 2.0 $\times$ 10^6	Cells/mL	
Hepatocellularity (L_{HEP})	500 $\times$ 10^6	Cells/g liver	The present study

A whole-body biotransformation rate constant (k_{MET}; 1/d) is calculated by dividing CL_H by the compound's apparent volume of distribution ($V_{D,BL}$; L/kg), referenced to the chemical concentration in blood [19]

$$k_{MET} = CL_H / V_{D,BL} \quad (7)$$

The $V_{D,BL}$ may be thought of as the sorption capacity of the fish relative to that of blood, and its value can be calculated as the equilibrium fish–water partition coefficient (BCF_P; L/kg) divided by P_{BW}. The term BCF_P is equivalent to a steady-state BCF in the absence of biotransformation or organism growth, and its value may be approximated as [9]

$$BCF_P = \nu_{LWB}\ K_{OW} \quad (8)$$

where ν_{LWB} (unitless) is the fractional lipid content of the organism.

Importantly, the approach outlined above assumes that the liver is the principal site of chemical biotransformation. If substantial biotransformation occurs in nonhepatic tissues, this approach will tend to underpredict the actual whole-body biotransformation rate constant.

Prediction of steady-state chemical bioconcentration in fish

The total chemical concentration in fish at steady state ($C_{FISH,SS}$; mg/kg) is predicted using the 1-compartment model given by Arnot and Gobas [9]. Assuming a water-only exposure and no organism growth, the model that describes these conditions is

$$C_{FISH,SS} = k_1\ FD\ C_{W,TOT} / (k_2 + k_E + k_{MET}) \quad (9)$$

where k_1 (L/d/kg) and k_2 (1/d) are rate constants that describe chemical uptake and loss across the gills; $C_{W,TOT}$ is the total chemical concentration in water; *FD* (unitless) is the fraction of this total that is freely dissolved and bioavailable for uptake; and k_E (1/d) is the fecal egestion rate constant.

If necessary, growth can be accounted for by incorporating an additional rate constant (k_G; 1/d) into the denominator (summed with k_2, k_E, and k_{MET}), provided that growth is first-order with respect to fish body weight. Alternatively, Equation 9 can be used to simulate measured chemical concentrations in growing fish if experimental data are growth-corrected to eliminate growth effects. The advantage of this latter approach is that the growth correction can be applied regardless of the actual pattern of growth, provided that this pattern is adequately described. The model constants k_1, k_2, k_E, and *FD* were calculated using the relationships given by Arnot and Gobas [9].

The models calculate steady-state BCFs for trout in 2 ways. Dividing $C_{FISH,SS}$ by $C_{W,TOT}$ results in a BCF value expressed on a total chemical basis (BCF_{TOT}; L/kg). A second "normalized" BCF ($BCF_{FD,L}$; L/kg lipid) is calculated as $C_{FISH,SS}$ divided by the freely dissolved chemical concentration in water ($C_{W,FD}$; mg/L, equal to the product term $FD \times C_{W,TOT}$) and the fish's fractional lipid content.

MODEL PARAMETERIZATION

Use of the S9-BCF and HEP-BCF models requires specification of parameters for the compound of interest, the in vitro metabolizing system, the modeled animal, and the assumed set of exposure conditions. Most of these parameters remain fixed once they are set for a particular experimental design. In practice, the parameters most likely to change for each test substance are K_{OW} and the measured substrate depletion rate constant.

In vitro metabolizing system

In vitro reactions are generally conducted in a small volume (e.g., 0.2 mL) of buffer to conserve biological material and minimize the need for reaction cofactors [31]. Concentrations of S9 protein (C_{S9}) or hepatocytes (C_{HEP}) can be adjusted to a limited extent to achieve measurable levels of activity while minimizing undesired binding effects and possible product inhibition. The S9 concentration typically ranges from 0.1 to 2.0 mg/mL, whereas the hepatocyte concentration generally ranges from 0.5 to 2.0×10^6 cells/mL.

In vitro–in vivo extrapolation factors

The S9 protein content of liver tissue (L_{S9}; mg S9 protein/g liver) refers to the total amount of S9 protein in the tissue and not the amount determined by measuring the protein content of S9 fractions. Differences between these 2 quantities arise if tissue processing results in incomplete recovery of proteins that might otherwise contribute to the S9 fraction due, for example, to incomplete cell lysis or sedimentation of protein with the cell debris pellet. The S9 protein content of trout liver was estimated using a ratio method analogous to that routinely used to estimate microsomal protein content [32–34]. In the present study we used 2 markers of microsomal protein, total cytochrome P450 (CYP) content and glucose-6-phosphatase (G6P) activity, to relate the microsomal content of the S9 fraction to that of the tissue homogenate from which it was obtained. Descriptions of these methods are provided in Supplemental Data along with estimated values for 5 livers, determined using both procedures. The resulting information suggests that the S9 protein content of trout liver tissue ranges from 155 mg/g liver (G6P method) to 171 mg/g liver (CYP method). For this modeling effort, we used an average value of 163 mg S9 protein/g liver.

Hampton et al. [35] employed morphometric methods to calculate hepatocellularity values for spawning male (510×10^6 cells/g liver) and female (860×10^6 cells/g liver) trout. Using a protein ratio method, Han et al. [36] determined the hepatocellularity of livers from sexually immature male trout to be 540×10^6 cells/g liver. We used the protein ratio method as well as a CYP ratio method [37] to evaluate hepatocellularity in sexually immature male and female trout. Measured values were somewhat lower (420 to 540×10^6 cells/g liver) than those given by Hampton et al. [35] or Han et al. [36] but did not differ among the sexes (J. Nichols, USEPA, Duluth, MN). Based on this information, we assumed an average hepatocellularity value of 500×10^6 cells/g liver.

In trout, reported liver weights as a fraction of total body weight range from 0.7% to 2.9% [13]. Factors that contribute to this observed range include animal size, gender, and state of sexual maturation. In the present study, we employed a value of 1.5%, based on data given by Schultz and Hayton [38] for small (3–7 g) animals.

BCF prediction model

The freely dissolved chemical fraction in water (*FD*) was calculated using values for the concentration of dissolved organic carbon (C_{DOC}) and of particulate organic carbon (C_{POC}) given by the USEPA ([39]; Tables 6–10, means for all types of surface water). These mean values, which are based on thousands of individual measurements, are 4.6×10^{-6} kg/L and 1.0×10^{-6} kg/L for C_{DOC} and C_{POC}, respectively. The

binding values α_{DOC} and α_{POC} were set equal to 0.08 [40] and 0.35 [41], respectively.

MODEL EVALUATION

The S9-BCF and HEP-BCF models were evaluated by calculating BCF values for a hypothetical set of organic chemicals with log K_{OW} values ranging from 2 to 8. Chemicals with log K_{OW} values less than 2 were not modeled because these compounds seldom accumulate in fish. Chemicals with log K_{OW} values greater than 8 have high potential to accumulate in fish; however, uptake from water is unlikely to contribute substantially to this accumulation (the diet is the primary route of uptake), and traditional BCF testing methods are difficult or impossible to employ. All compounds were assumed to accumulate in fish as a consequence of nonspecific partitioning to tissue lipids and proteins. The models do not explicitly account for chemical ionization or high-affinity binding to proteins.

To perform these simulations, we assumed a standard temperature of 15 °C and a total chemical concentration in water ($C_{W,TOT}$) of 1.0 mg/L. The modeled fish was assigned a weight of 10 g and a whole-body lipid content of 5%. In vitro concentrations of S9 (C_{S9}) protein and hepatocytes (C_{HEP}) were set equal to 1 mg/mL and 2×10^6 cells/mL, respectively.

Hypothetical rates of substrate depletion were varied from 0.001/h to 10/h. A depletion rate of 10/h results in a solution half-life of just a few minutes. When extrapolated to the standardized test animal using the S9-BCF model, this rate of biotransformation corresponds to a k_{MET} value of 0.65/d. A depletion rate of 0.01/h results in an in vitro half-life of approximately 70 h and corresponds to a k_{MET} value of 0.001/d. Our own experience suggests that depletion rates less than 0.05/h (in vitro $t_{1/2} \approx 13.9$ h) are difficult to measure due to the limited working lifetime of these in vitro preparations (2–3 h for S9 fractions and somewhat longer for hepatocytes). We have modeled lower rates, however, to illustrate the potential effects of biotransformation on chemical accumulation.

SENSITIVITY ANALYSIS

To further evaluate model performance, we conducted a limited analysis of model sensitivity to changes in selected parameters. Previous modeling efforts have shown that BCF predictions are highly sensitive to changes in the value of k_{MET} [1–4]. We therefore focused the sensitivity analysis on parameters used to extrapolate measured levels of in vitro activity to an estimate of k_{MET}.

The sensitivity analysis was conducted by independently changing selected model input parameters (*PAR*) by +1% and examining the effects of these changes on predicted steady-state BCF values (BCF_{TOT} and $BCF_{FD,L}$) for a set of hypothetical compounds with log K_{OW} values ranging from 2 to 8. Normalized sensitivity coefficients (*SENS*) were calculated as the percentage change in the BCF (numeric value without $\log_{10}$ transformation) divided by the percentage change in *PAR*. A sensitivity coefficient of 1.0 indicates that the BCF changes in direct proportion to a change in *PAR*, whereas values less than 1.0 indicate a less-than-proportional relationship. Negative values of *SENS* indicate an inverse relationship between the BCF and *PAR*. Initially, we focused on independent model variables that directly impact calculation of k_{MET}, including *Rate*, L_{FBW}, L_{HEP}, and Q_{HFRAC}. We then evaluated the dependent variables BCF_P, $V_{D,BL}$, P_{BW}, and f_U, all of which are functions of log K_{OW}, as well as Q_C, which is a function of temperature and fish body weight. In each case, model sensitivity to changes in the selected input variable was evaluated at 4 assumed levels of in vitro metabolic activity (0.01/h, 0.1/h, 1/h, and 10/h). Finally, we evaluated the effect of changing fish lipid content (ν_{LWB}). Although this parameter is assigned a fixed value in the current parameterization of the model, our experience with BCF testing indicates that substantial differences among individual test animals can be expected. It was of interest, therefore, to evaluate model sensitivity to this term.

EVALUATION OF CHEMICAL BINDING EFFECTS

Limited data suggest that correcting for chemical binding effects in plasma and in the in vitro system used to assess biotransformation may result in underestimation of true hepatic clearance for hydrophobic environmental contaminants [26]. To further evaluate this possibility, we ran a series of simulations assuming that $f_U = 1.0$. This assignment implies that the chemical concentration available to metabolizing enzymes in vivo and in vitro is effectively the same.

ALLOMETRIC SCALING OF BIOTRANSFORMATION

The models given in the present study are designed to predict chemical bioconcentration in a 10-g fish. Generally, however, in vitro metabolizing systems (S9 fractions or hepatocytes) are obtained from larger animals (typically >200 g), resulting in a large difference in the mass of tested and modeled animals. Presently, the models assume that the specific biotransformation rate (i.e., normalized to body weight) does not change with fish mass. Given current uncertainty in this assumption, however, additional simulations were run assuming that the specific biotransformation rate scales to an exponent of –0.25. To perform these simulations, we derived an allometric expression to calculate the $CL_{IN\ VIVO,\ INT}$ expected of a 10-g animal (see Supplemental Data for derivation and relevant citations)

$$CL_{IN\ VIVO,\ INT,\ 10} = CL_{IN\ VIVO,\ INT}(10/BW)^{-0.25} \qquad (10)$$

In this equation the term BW (body weight) is the measured mass of fish (grams) from which the in vitro material was obtained.

RESULTS

The models were initially run using inputs given in Tables 1 and 2. The resulting model predictions are shown in Figure 1A. For an assumed level of substrate depletion, BCF values predicted by the S9-BCF model are higher than those predicted by the HEP-BCF model. This occurs because different extrapolation factors are used to translate the measured rate into an estimate of $CL_{IN\ VIVO,INT}$. Unless otherwise noted, all simulations shown in the present study were generated using the S9-BCF model. Nearly identical plots were obtained using the HEP-BCF model when rate values were set equal to 0.24 times those used in the S9-BCF model.

Calculated BCFs are expressed in Figure 1A on a total chemical basis (BCF_{TOT}) because this is the value used in most regulatory applications. When the biotransformation rate constant is set equal to a very low value (e.g., 0.001/h), BCF_{TOT} increases in a nearly proportional manner with log K_{OW} for log K_{OW} values ranging from 2 to 5. As log K_{OW} values increase above 5, predicted BCF_{TOT} values increase in a less-than-proportional manner with log K_{OW}, becoming maximal at a log K_{OW} value of approximately 6.3. In effect, the progressive decrease in chemical bioavailability that occurs when log K_{OW}

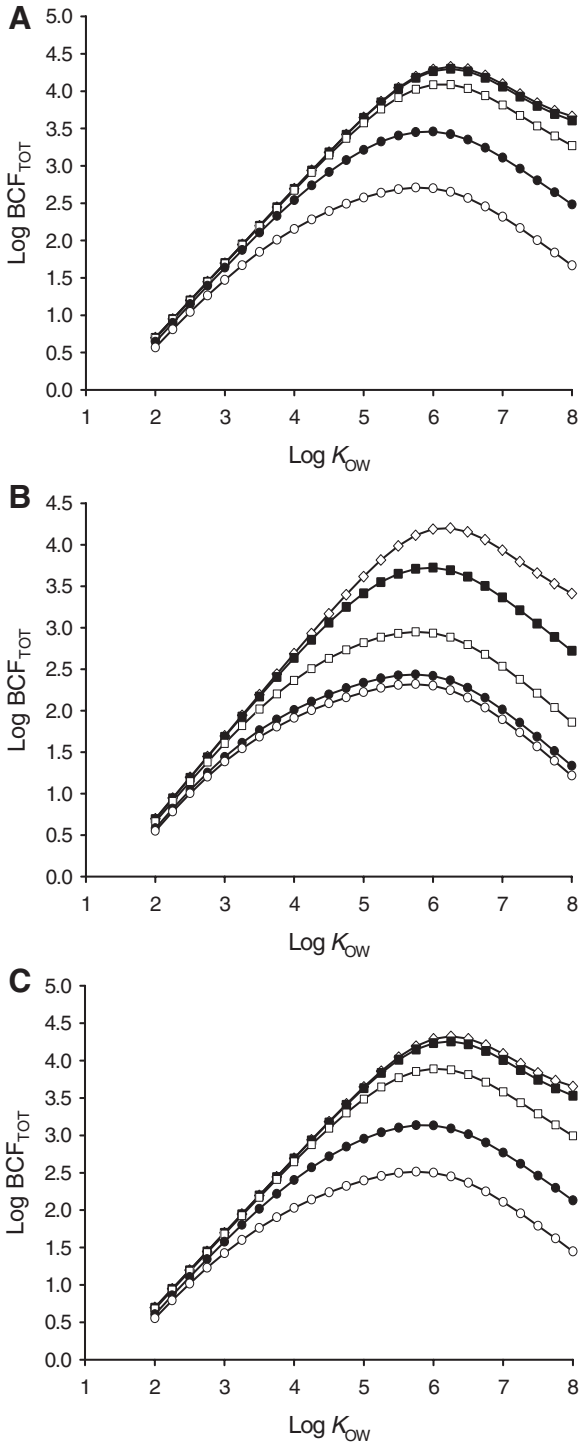

Figure 1. Effect of hepatic biotransformation on chemical bioconcentration in small trout predicted using a 1-compartment steady-state model. The bioconcentration factor (BCF_{TOT}) is defined as the total steady-state chemical concentration in fish divided by that in water, resulting from a water-only exposure. Individual plots correspond to hypothetical rates of in vitro S9 metabolic activity equal to 0.001/h (open diamonds), 0.01/h (solid squares), 0.1/h (open squares), 1/h (solid circles), or 10/h (open circles). (**A**) Bioconcentration factors predicted using model inputs given in Tables 1 and 2. (**B**) Bioconcentration factors predicted by setting the chemical binding term (f_U) used to adjust for free chemical fractions in plasma and in the in vitro metabolizing system equal to 1.0. (**C**) Bioconcentration factors predicted assuming that measured in vitro rates of metabolic activity scale to a body weight exponent of –0.25. Fish used as the source of biological material (liver S9 fraction) were assumed to weigh 400 g. Log K_{OW} = $\log_{10}$ octanol–water partition coefficient.

values exceed 5 (due to DOC and POC binding) reduces the chemical concentration in water with which fish achieve equilibrium. A similar plot of normalized bioconcentration factors ($BCF_{FD,L}$) is given as Figure S2 in Supplemental Data. When the biotransformation rate constant is set equal to a very low value, $BCF_{FD,L}$ increases in a nearly proportional manner with increasing log K_{OW} at all log K_{OW} values.

Figure 1A shows that predicted impacts of biotransformation on chemical accumulation depend strongly on chemical log K_{OW}. For compounds with relatively low log K_{OW} values (<3), the rate of chemical elimination across the gills exceeds modeled rates of whole-body biotransformation. Under these circumstances, biotransformation has a relatively small impact on predicted steady-state BCFs. Biotransformation has a large impact on chemical accumulation at log K_{OW} values >5.

Figure 2A presents the same modeled output, plotted in a manner that shows more clearly how different rates of in vitro

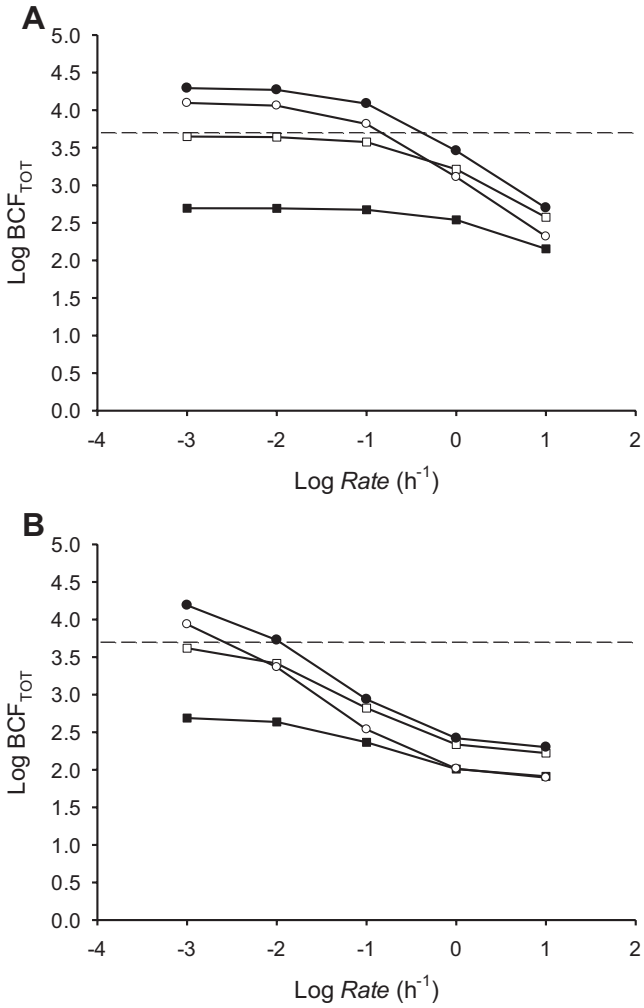

Figure 2. Effect of hepatic biotransformation on chemical bioconcentration in small trout predicted using a 1-compartment steady-state model. Steady-state bioconcentration factor (BCF_{TOT}) values are plotted against the log of the assumed in vitro biotransformation rate for hypothetical compounds with octanol–water partition coefficient (log K_{OW}) values of 4 (solid squares), 5 (open squares), 6 (open circles), or 7 (solid circles). The dashed line corresponds to a BCF_{TOT} of 5000. (**A**) Bioconcentration factors predicted using model inputs given in Tables 1 and 2. (**B**) Bioconcentration factors predicted by setting the chemical binding term (f_U) used to adjust for free chemical fractions in plasma and in the in vitro metabolizing system equal to 1.0.

activity impact predicted chemical accumulation. A similar plot was given previously by Cowan-Ellsberry et al. [13]. For an assumed set of modeled conditions, this plot can be used to identify a hypothetical level of in vitro activity that would be required to reduce accumulation of all compounds below a specified value. In the present study, a depletion rate of 0.56/h measured using the S9 fraction was predicted to reduce the accumulation of all compounds in small trout to a BCF_{TOT} value less than 5000, designated in Figure 2 by a dashed line.

Sensitivity analysis

The sensitivity analysis revealed several characteristic patterns of model response to changes in selected input parameters (Figure 3A–E). Unless otherwise noted, similar plots were obtained regardless of whether normalized sensitivity coefficients (*SENS*) were generated using BCF_{TOT} or $BCF_{FD,L}$ values. Figure 3A shows the sensitivity of the model to a +1% change in the in vitro biotransformation rate constant. Identical plots were obtained by changing liver weight as a fraction of total

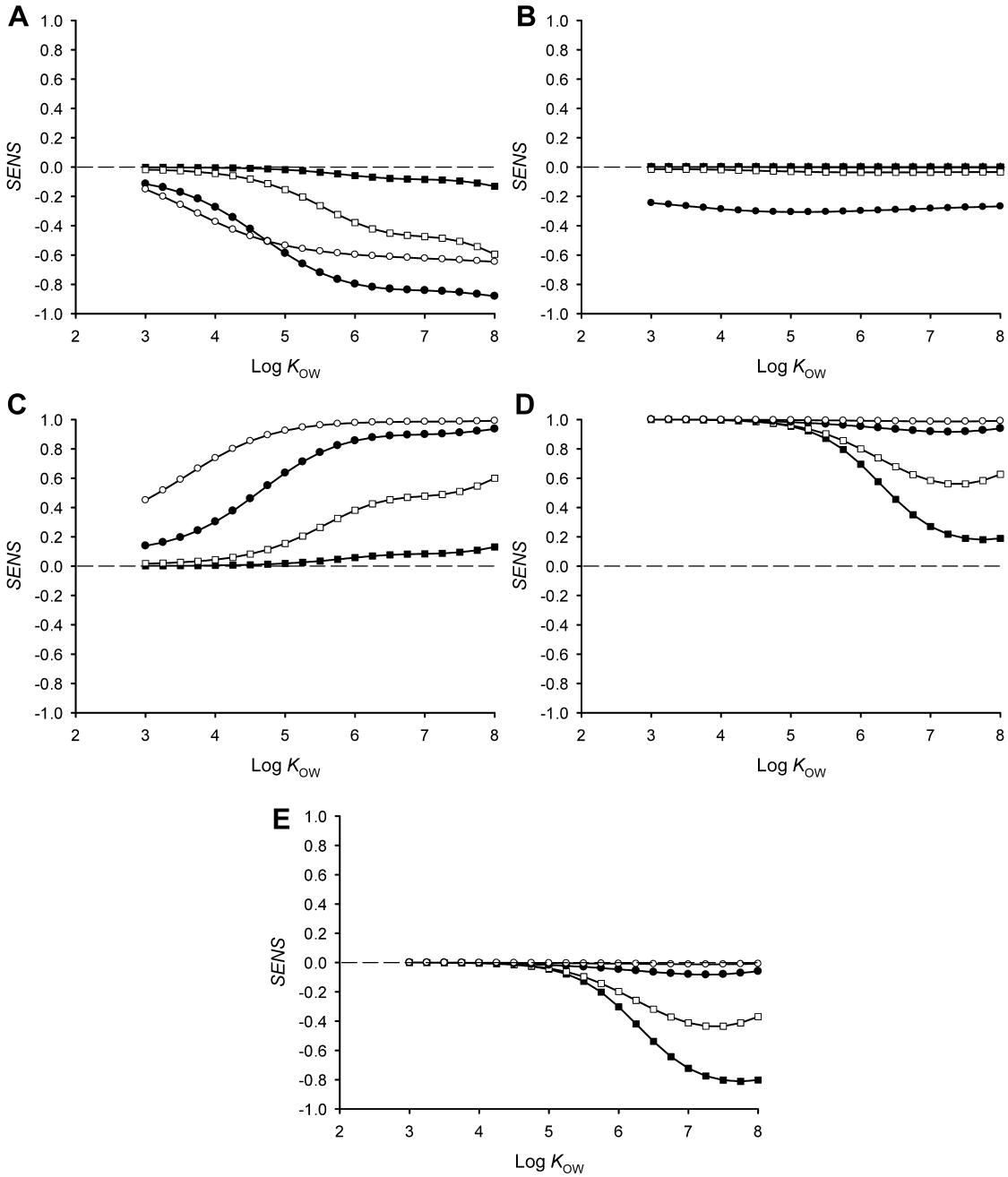

Figure 3. Sensitivity of the model to changes in selected parameter inputs. Normalized sensitivity coefficients (*SENS*) were calculated as the percentage change in the bioconcentration factor expressed on a total concentration basis (BCF_{TOT}) divided by the percentage change (+1%) in the selected input parameter. Individual plots show *SENS* values calculated at 4 assumed levels of in vitro metabolic activity: 0.01/h (solid squares), 0.1/h (open squares), 1/h (solid circles), and 10/h (open circles). (**A**) Sensitivity to changes in the rate of in vitro S9 metabolic activity (*Rate*). (**B**) Sensitivity to changes in liver blood flow as a fraction of cardiac output (Q_{HFRAC}). (**C**) Sensitivity to changes in the apparent volume of distribution ($V_{D,BL}$). (**D**) Sensitivity to changes in the equilibrium fish–water partition coefficient (BCF_P). (**E**) Sensitivity to changes in whole-body lipid content (ν_{LWB}). Log K_{OW} = $\log_{10}$ octanol–water partition coefficient.

body weight (L_{FBW}), the S9 protein content of liver tissue (L_{S9}), and the binding term f_U. This consistent response is explained by the fact that all 3 parameters contribute directly to the product term f_U $CL_{IN\ VIVO,\ INT}$, which appears in both the numerator and the denominator of the well-stirred liver model. All of these plots show that model sensitivity to changes in a particular parameter increases with log K_{OW}. This result is due to the general dependence of model behavior on chemical log K_{OW} noted earlier (Figure 1). Model sensitivity to changes in rate, L_{FBW}, L_{S9}, and f_U decreases at very high rates of biotransformation (Figure 3A, comparing simulations for 1/h and 10/h). Under these circumstances hepatic clearance becomes rate-limited by liver blood flow (the "flow-limited" case of the well-stirred liver model; Equation 1) and is insensitive to changes in the product term f_U $CL_{IN\ VIVO,INT}$.

Figure 3B shows the sensitivity of the model to changes in liver blood flow as a fraction of cardiac output (Q_{HFRAC}). A similar plot was obtained by changing cardiac output (Q_C) by +1%. The model is relatively insensitive to changes in either parameter except when the rate of in vitro activity is set equal to a very high value. This result is expected since both of these parameters factor into the calculation of Q_H. As noted above, Q_H becomes rate-limiting on CL_H only at very high rates of biotransformation.

A plot identical to that shown in Figure 3B was obtained when the blood to water partition coefficient (P_{BW}) was changed by +1%; however, the basis for this result differs from that described for Q_{HFRAC} and Q_C. The term P_{BW} appears twice in the model, first as a factor used to calculate f_U and again as a factor used to calculate the apparent volume of distribution ($V_{D,BL}$). When biotransformation rates are low, effects of P_{BW} on model performance occurring through its impact on f_U and $V_{D,BL}$ tend to cancel out. This is because f_U tends to impact k_{MET} in a proportional manner (through the product term f_U $CL_{IN\ VITRO,\ INT}$; Equation 1), whereas changes in $V_{D,BL}$ impact k_{MET} inversely (Equation 7). At high rates of metabolic activity, changes in P_{BW} operating through its impact on f_U have little effect on k_{MET} because CL_H is rate-limited by Q_H. Under the same conditions, however, changes in P_{BW} continue to impact $V_{D,BL}$. The result is that the model becomes sensitive to changes in P_{BW} in much the same way that it is sensitive to changes in Q_H. This is because CL_H is approximately equal to Q_H when the biotransformation rate is fast, and $V_{D,BL}$ is simply a factor through which Q_H is converted to k_{MET}.

The model exhibits a high level of sensitivity to changes in $V_{D,BL}$ regardless of whether CL_H is rate-limited by biotransformation or Q_H (Figure 3C). This is because $V_{D,BL}$ operates directly on CL_H to determine k_{MET} (Equation 7). The value of $V_{D,BL}$ is itself calculated as the ratio of BCF_P and P_{BW}. As indicated previously, the model is relatively insensitive to changes in P_{BW} except at high rates of biotransformation. In contrast, the model exhibits high sensitivity to changes in BCF_P across the entire range of modeled log K_{OW} values (Figure 3D). The term BCF_P appears twice in the model, impacting both $V_{D,BL}$ (through a proportional relationship) and the gill efflux rate constant k_2 (through an inverse relationship). These effects operate in concert to impact predicted BCFs.

The sensitivity of the model to changes in fractional whole-body lipid content (ν_{LWB}) was found to be dependent on the modeled output used to assess this behavior. Sensitivity coefficients calculated from BCF values expressed on a total concentration basis (i.e., BCF_{TOT}) described the same patterns shown in Figure 3D (for model sensitivity to changes in BCF_P). This is expected since ν_{LWB} is used to calculate BCF_P. When lipid-normalized BCF values (i.e., $BCF_{FD,L}$) are used to evaluate model sensitivity to changes in ν_{LWB}, the entire plot shifts downward (Figure 3E). This shift occurs because the impacts of ν_{LWB} on predicted BCF_{TOT} values are offset by the lipid-normalization procedure itself. Thus, an increase in ν_{LWB} results in increased BCF_{TOT} values (by virtue of its impact on k_{MET} and k_2); however, these changes are accompanied by an increase in the lipid-normalization factor (i.e., ν_{LWB}), which is divided into BCF_{TOT} to obtain $BCF_{FD,L}$.

Evaluation of chemical binding effects

For compounds possessing log K_{OW} values greater than 2, binding algorithms given previously (Equations 4–6) predict that free chemical fractions in plasma ($f_{U,P}$) will be substantially less than those in an S9 fraction ($f_{U,S9}$) or solution of hepatocytes ($f_{U,HEP}$). The result is that f_U values predicted by the full model (where $f_U = f_{U,P}/f_{U,S9}$ or $f_{U,P}/f_{U,HEP}$) are considerably less than 1.0.

When the binding term f_U is set equal to 1.0, predicted BCFs tend to be lower than those obtained with the full model. This can be seen by comparing panels A (full model) and B ($f_U = 1.0$) in Figure 1. A closer examination of this figure suggests, however, that the effect of binding depends on the assumed level of in vitro activity. When the assumed rate is low (0.01/h or 0.1/h), setting $f_U = 1.0$ has a large effect on predicted BCFs. Relatively smaller changes in predicted BCFs are observed when the assumed rate of activity is high. Moreover, Figure 1B suggests that predicted BCFs for all modeled log K_{OW} values tend to converge on a minimum set of values at high (1/h) and very high (10/h) rates of biotransformation. There is no suggestion of this convergent behavior in Figure 1A.

These observations are consistent with the structure of the well-stirred liver model, which predicts that changes in chemical binding will have a large impact on CL_H when the product term f_U $CL_{IN\ VIVO,INT}$ is much smaller than Q_H (the "enzyme-limited" case). When f_U is set equal to 1.0, the product term f_U $CL_{IN\ VIVO,\ INT}$ becomes much larger than values predicted by the full model, driving the well-stirred liver model toward the "flow-limited" case.

The impact of chemical binding on predicted levels of accumulation also can be shown by plotting predicted BCFs against assumed rates of in vitro biotransformation (Figure 2B). This plot, which can be compared directly to Figure 2A, suggests that different binding assumptions strongly impact the extent to which a fixed level of in vitro activity reduces predicted levels of accumulation. In Figure 2B the rate of in vitro activity (measured using S9 fractions) required to reduce accumulation of all compounds to a BCF less than 5000 is approximately 0.01/h, assuming that $f_U = 1.0$.

Allometric scaling of biotransformation

Figure 1C shows the set of model predictions obtained when in vitro biotransformation is scaled to a body weight exponent of –0.25. The resulting BCFs are somewhat lower than those shown in Figure 1A, suggesting that incorporation of allometric scaling increases the predicted impact of this activity. Under these circumstances a biotransformation rate greater than 0.23/h will reduce accumulation of all compounds to a BCF less than 5000. Using the algebraic expression derived in the Supplemental Data (Equation S11), one may calculate the factor used to scale biotransformation as $(10/BW)^{-0.25}$, where 10 is the mass (grams) of the modeled animal and BW is the mass of the animal used to provide in vitro material (S9 fraction or hepatocytes). Assuming a BW of 400 g, this factor is approximately 2.5.

Because this scaling factor is multiplied by the in vitro biotransformation rate constant, model sensitivity to changes in the body weight exponent depends on the assumed level of metabolic activity. Moreover, the effect of allometric scaling also depends on a compound's log K_{OW} value. Model sensitivity to a change in the allometric exponent is greatest when biotransformation rates are low and log K_{OW} values are high (>5). Under these circumstances, adopting an allometric exponent of –0.25 results in a 2.5-fold decrease in predicted BCFs. When biotransformation rates are high (tending toward "flow-limited" hepatic clearance) or log K_{OW} values are low (<3), changing this exponent has little effect on predicted BCFs.

DISCUSSION

In the present study we describe models that employ measured in vitro rates of metabolic activity to predict chemical accumulation in fish resulting from steady-state waterborne exposures. Similar models have been given previously by other authors [11–15]. The goal of this effort was to update these procedures using new information and develop models to serve a specific purpose—that of predicting measured levels of accumulation in small trout commonly used in BCF testing efforts.

Recommended changes to published in vitro–in vivo extrapolation procedures include adoption of a different algorithm to predict free chemical fractions in blood (i.e., the equation given by Fitzsimmons et al. [27]) and a revised estimate of the S9 protein content of liver tissue, corrected for incomplete recovery. Additional changes reflect attributes of small (10 g) trout, including specification of a lower whole-body lipid content (5%). Accurate specification of this latter parameter is critical because it impacts calculation of both the gill elimination rate constant (k_2, equal to k_1/BCF_P) and the whole-body biotransformation constant (k_{MET}; equal to $CL_H/V_{D,BL}$, where $V_{D,BL} = BCF_P/P_{BW}$).

The models given in the present study represent our best judgment regarding current approaches and assumptions. We recognize, however, that they may change as new information becomes available. One potential change involves the question of allometric scaling. Although studies of allometric scaling in fish are complicated by confounding influences of temperature, pH, and other factors, numerous studies have shown that intraspecific metabolic rate (measured as resting oxygen consumption) tends to scale to a fractional exponent of body weight, typically ranging from 0.6 to 0.9 when expressed on a whole-animal basis [42–48]. Allometric relationships containing similar exponents also figure prominently in many published models for chemical accumulation by fish, reflecting assumed or characterized relationships between fish size and biological determinants of chemical flux, including gill ventilation volume, cardiac output, food consumption rate, and absorptive surface area [49–51].

We are not aware of any studies that have investigated allometric scaling of biotransformation in fish. Lacking this information, we adopted the assumption that biotransformation rates do not change with body mass. Nevertheless, model simulations reflecting differences in the size of fish used for in vitro studies and BCF testing illustrate the effect that size-dependent changes in biotransformation could have on BCF predictions (see Figure 1A and C). It is important, therefore, that this question be investigated more thoroughly.

The effect of chemical binding on hepatic clearance represents another source of modeling uncertainty. When the well-stirred liver model was initially formulated, the binding term f_U was interpreted as the free chemical fraction in plasma [20,21]. Later work with a number of pharmaceutical compounds showed that in vitro–in vivo biotransformation extrapolations are often improved when f_U is calculated from the ratio of free chemical fractions in plasma and the in vitro system used to measure this activity [22–25]. A possible exception to this rule exists when hepatocytes are exposed to compounds that are substrates for membrane transporters. In several such cases, accurate predictions of measured in vivo clearance have been obtained by setting $f_U = 1.0$ [52].

In the models given in the present study, the default assumption is that $f_U = f_{U,P}/f_{U,S9}$ or $f_{U,P}/f_{U,HEP}$. However, accumulating evidence suggests that this assumption may be inappropriate for some in vitro–in vivo biotransformation extrapolations with fish. Escher et al. [26] measured binding of several hydrophobic compounds in fish plasma and S9 fractions. These compounds were selected because their rates of in vitro biotransformation and measured BCFs had been given by other authors. When measured binding values were used to predict f_U (as $f_{U,P}/f_{U,S9}$), Escher et al. [26] found that predicted BCFs were substantially higher than measured values. By comparison, setting f_U equal to 1.0 resulted in much better prediction of measured BCFs.

One possible explanation for these findings is that neutral hydrophobic compounds do not bind to plasma proteins in the same manner as many drugs. Drug binding to proteins often saturates at high chemical concentrations, indicating a specific interaction with 1 or more binding sites. In contrast, plasma binding of hydrophobic environmental contaminants tends to be independent of concentration, suggesting nonspecific partitioning behavior. Kinetic considerations also may allow "bound" compounds to rapidly dissociate from molecular binding sites in plasma, making them available to metabolizing enzymes of the liver within the time frame available for this activity [26]. Presently, it seems reasonable to use the 2 different binding assumptions to estimate upper and lower limits on hepatic clearance. Unfortunately, when intrinsic clearance values are low, these 2 competing assumptions result in substantial differences in calculated hepatic clearance rates and resulting BCF estimates. More work is needed to determine which binding assumption best predicts the biotransformation of hydrophobic environmental contaminants by fish.

Additional guidance for future research can be obtained from the results of the sensitivity analysis. Two parameters emerge from this analysis as critical model inputs requiring additional research focus. One, the binding term f_U, has already been discussed. The second key parameter is the apparent volume of distribution ($V_{D,BL}$). As indicated previously, $V_{D,BL}$ can be estimated as the ratio of BCF_P and P_{BW}. However, calculating $V_{D,BL}$ in this manner combines errors associated with prediction of these 2 terms individually. A more direct way to estimate $V_{D,BL}$ is to develop a QSAR prediction algorithm based on measured chemical concentrations in the whole animal and in blood plasma under steady-state conditions. The advantage of this approach is that there is no requirement to measure chemical concentrations in water (as needed for BCF_P and P_{BW}), which is difficult for high log K_{OW} compounds. For some compounds, the true value of $V_{D,BL}$ will differ substantially from that predicted by simple partitioning relationships, particularly if a chemical binds with high affinity to a specific class of proteins in tissues or plasma. Under these circumstances, it may be necessary to measure $V_{D,BL}$ directly. Alternatively, it may be possible to determine $V_{D,BL}$ by using a model-based approach to evaluate

plasma concentration time-course data from appropriately designed kinetic experiments [53].

The sensitivity analysis also provides guidance on the use of these models within a regulatory context. Biotransformation is unlikely to impact accumulation of low (<3) log K_{OW} compounds, regardless of the rate of activity. In contrast, biotransformation may have a large impact on accumulation of higher log K_{OW} compounds; however, confidence in model-based predictions tends to decrease with decreasing levels of metabolic activity. This occurs because model sensitivity to several key input parameters (e.g., f_U, $V_{D,BL}$) increases as the rate of in vitro activity declines.

Confidence in estimated BCF values increases substantially at high measured rates of in vitro activity. Under these conditions, chemical binding effects become unimportant and the models tend to converge on a BCF prediction controlled by the rate of hepatic blood flow. The need to accurately estimate true levels of $CL_{IN\ VIVO,INT}$ also declines as the overall rate of metabolic activity increases. Thus, substantial errors in f_U or $CL_{IN\ VIVO,INT}$ can be tolerated as long as the product term f_U $CL_{IN\ VIVO,INT}$ is considerably greater than Q_H.

Although future improvements to the models presented in the present study are likely, the principles on which they are based are well established. Presently, in vitro systems derived from liver tissue can be used with confidence to identify compounds possessing high, medium, or low levels of metabolic stability. This information has substantial value for prioritizing limited testing resources and may provide important support for bioaccumulation assessments within the context of a weight-of-evidence approach [54]. Eventually, we expect these or similar methods to be endorsed by governmental regulatory authorities as part of a tiered approach to bioaccumulation assessment [54,55]. To achieve this goal it will be necessary to evaluate the models using well-matched (same species, life stage, temperature, etc.) in vitro biotransformation and in vivo BCF data. Unfortunately, all of the proof-of-concept studies performed to date have suffered from a lack of this type of information.

Regulatory acceptance of these procedures also requires a better understanding of their domain for applicability, including identification of the metabolic pathways for which they are most useful. To date, in vitro–in vivo metabolism extrapolations with fish have been performed using a variety of chemical substrates including herbicides (atrazine [11], trifluralin [11], and molinate [11,12]), pesticides (chlorpyrifos and zoxamide [13]), pharmaceuticals (ibuprofen, norethindrone, and propranolol [14]), alcohol ethoxylates (C16EO8 [13] and C13EO8 [14]), a benzophenone derivative (4,4-bis[dimethylamino]-benzophenone [11,12]), a surfactant (4-nonylphenol [11,12]), an alkylphenol (2,4-di-*tert*-butylphenol [11,12]), a polycyclic aromatic hydrocarbon (benzo[*a*]pyrene [11,12]), and an alkylbenzene sulfonate (C12LAS [13]). The biotransformation pathways responsible for metabolic clearance of these compounds in mammals are well known (see, for example, Table 1 in Han et al. [11]). Enzymes (e.g., CYPs) that catalyze these reactions may not exist in fish as similar forms. Nevertheless, fish can perform most, if not all, of these reactions, including hydrolysis, *N*-dealkylation, hydroxylation, sulfoxidation, glucuronidation, glutathione conjugation, and *N*-acetylation [56]. Additional extrapolation efforts with fish are needed utilizing substrates for other biotransformation reactions. Given their importance for metabolism of many environmental contaminants, dehalogenation reactions are of special interest.

A second question of critical importance is whether bioaccumulation prediction models developed for compounds that partition nonspecifically to tissue lipids can be applied to compounds that ionize at environmental pH values and/or bind with high affinity to proteins. Researchers are actively working to develop models that can account for these behaviors. In the interim, it should be noted that procedures given in the present study for extrapolating in vitro metabolism data to the intact animal were initially developed to evaluate pharmaceutical compounds, many of which exhibit high-affinity protein binding and are weak acids or bases. In principle, therefore, it may be possible to apply these methods to a wide range of organic compounds provided that one can measure or estimate critical binding parameters (e.g., $f_{U,P}$, $f_{U,S9}$, $f_{U,HEP}$) and a compound's apparent volume of distribution ($V_{D,BL}$).

As indicated previously, there are practical limitations on the lower limit of metabolic activity that can be measured using current in vitro methods (about 0.05/h using S9 fractions). Future improvements to these methods may provide a means by which to measure lower rates of clearance. Isolated hepatocytes hold particular promise in this regard because they remain viable for an extended period of time.

Over time it will become important to develop similar models for test animals other than rainbow trout. These models can be expected to differ with respect to individual parameter values (e.g., liver S9 protein content, liver weight as a fraction of whole-body weight) but are likely to retain the same basic structure described in the present study. Moreover, the results of the sensitivity analysis are expected to be broadly applicable and can be used to guide research on other species.

SUPPLEMENTAL DATA

Measured blood binding values for trout are used to evaluate competing algorithms for prediction of this behavior, and a ratio method based on measured CYP content or G6P activity is used to estimate the S9 protein content of trout liver. We also derive an expression that can be used to scale specific metabolic rates among fish of different sizes using an assumed allometric scaling constant of –0.25.

Table S1.

Figures S1 and S2 (270 KB PDF).

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