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SOMATIC AND GASTROINTESTINAL IN VIVO BIOTRANSFORMATION RATES OF HYDROPHOBIC CHEMICALS IN FISH

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Abstract: To improve current bioaccumulation assessment methods, a methodology is developed, applied, and investigated for measuring in vivo biotransformation rates of hydrophobic organic substances in the body (soma) and gastrointestinal tract of the fish. The method resembles the Organisation for Economic Co-operation and Development (OECD) 305 dietary bioaccumulation test but includes reference chemicals to determine both somatic and gastrointestinal biotransformation rates of test chemicals. Somatic biotransformation rate constants for the test chemicals ranged between 0 d^{-1} and 0.38 (standard error [SE] 0.03)/d^{-1}. Gastrointestinal biotransformation rate constants varied from 0 d^{-1} to 46 (SE 7) d^{-1}. Gastrointestinal biotransformation contributed more to the overall biotransformation in fish than somatic biotransformation for all test substances but 1. Results suggest that biomagnification tests can reveal the full extent of biotransformation in fish. The common presumption that the liver is the main site of biotransformation may not apply to many substances exposed through the diet. The results suggest that the application of quantitative structure-activity relationships (QSARs) for somatic biotransformation rates and hepatic in vitro models to assess the effect of biotransformation on bioaccumulation can underestimate biotransformation rates and overestimate the biomagnification potential of chemicals that are biotransformed in the gastrointestinal tract. With some modifications, the OECD 305 test can generate somatic and gastrointestinal biotransformation data to develop biotransformation QSARs and test in vitro–in vivo biotransformation extrapolation methods. *Environ Toxicol Chem* 2015;34:2282–2294. © 2015 SETAC

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INTRODUCTION

Bioaccumulation is the process that involves the absorption, internal distribution, biotransformation, and excretion of chemical substances. Bioaccumulation can lead to high chemical concentrations in organisms that may make organisms more susceptible to toxic effects. Quantitative estimates of the degree of bioaccumulation in biota can be helpful in identifying substances that are bioaccumulative in nature and in estimating internal concentrations in organisms and associated risks. Currently, regulatory programs such as the Canadian Environmental Protection Act, the US Toxic Substances Control Act, and the European Union's (EU) Registration, Evaluation, Authorization and Restriction of Chemicals include the assessment of the bioaccumulative behavior of commercial chemicals using laboratory derived bioconcentration factors (BCFs; $C_{Organism}/C_{Water}$), the octanol–water partition coefficients (K_{OW}; $C_{Octanol}/C_{Water}$), and, in Canada, field-derived bioaccumulation factors (BAFs; $C_{Organism}/C_{Water}$) [1]. Recently, the EU included provisions for considering other bioaccumulation metrics such as the biomagnification factor (BMF) and trophic magnification factor (TMF) to increase the weight of evidence in bioaccumulation assessments [2,3]. However, regulatory programs often largely rely on BCF determinations. A challenge of this approach is that empirical BCFs and BAFs are typically available for only a small fraction of the many commercial chemicals that require assessment [4]. As a result, the octanol–water partition coefficient is often used to evaluate a chemical's bioaccumulation potential. However, the octanol–water partition coefficient represents a chemical partitioning process between a lipid surrogate (i.e., 1-octanol) and water, which oversimplifies the bioaccumulation process of many chemicals, including those with a very high octanol–water partition coefficient (e.g., $K_{OW} > 10^5$) and those that are biotransformed. Mechanistic bioaccumulation models, which can represent details of uptake and excretion of chemicals, have proven to be useful alternatives to the K_{OW} for the bioaccumulation assessment of many commercial chemicals in fish and other organisms. However, the inability of bioaccumulation models to a priori estimate biotransformation rates of absorbed chemicals has remained a key challenge in conducting realistic bioaccumulation assessments. If the rate of chemical excretion is very slow (e.g., for high K_{OW}, potentially bioaccumulative substances), biotransformation can be an important elimination process. Absence of data on biotransformation rates may cause many hydrophobic chemicals to be evaluated as bioaccumulative when they are not and hence result in false positives and unnecessary prioritization in chemical management programs.

To develop methods to include biotransformation in bioaccumulation assessment, quantitative structure-activity relationship (QSAR) models have been developed to predict biotransformation rates and corresponding BCFs of hydrophobic organic chemicals in fish based on chemical structure [5]. These biotransformation rate models can be useful for screening-level assessments and have been incorporated into regulatory software programs such as the US Environmental Protection Agency's (US EPA) Estimation Programs Interface Suite [6]. Developing QSARs requires good quality data.

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However, few data on in vivo biotransformation rates of chemicals in fish and other organisms exist to date, and there are no established methods to make direct measurements of in vivo biotransformation rates in fish.

Another initiative aimed at including biotransformation in bioaccumulation assessment involves developing in vitro biotransformation rate assays using fish hepatocytes and liver S9 and microsomal fractions [7,8]. The success of this initiative depends on the ability to extrapolate in vitro biotransformation rates to in vivo biotransformation rates. The lack of reliable in vivo biotransformation rate data or methods to measure in vivo biotransformation rates provides a barrier to successfully validate and eventually implement in vitro bioassays for measuring biotransformation rates.

The lack of reliable methods to measure in vivo biotransformation rates has precluded the development of a biotransformation rate database for QSAR model development. Previously, biotransformation rates have been estimated from BCF data and bioaccumulation models [9,10]. In the present study, we propose and apply a new method that uses biotransformation-resistant reference chemicals to measure in vivo biotransformation rates of hydrophobic organic chemicals that are useful in bioaccumulation assessment. This method may be included relatively easily in the current OECD 305 test guideline [11] for measuring bioconcentration and biomagnification factors. The present study illustrates the application and underlying theory of the method. The aim of this method is to provide a methodology for generating high quality in vivo biotransformation rate data that can be used to further develop methods for bioaccumulation assessment in fish.

THEORY

Bioaccumulation of contaminants in fish is often described by a fish–water–diet, 3 compartment, first order kinetic model [12] as

$$dC_F/dt = k_1\, C_W + k_D\, C_D - (k_2 + k_E + k_G + k_M)\, C_F \quad (1)$$

where C_F is the chemical concentration in the fish (g chemical kg fish^{-1}); C_W is the chemical concentration in the water (g chemical L^{-1}); k_1 is the uptake clearance rate for respiratory uptake (L water kg fish^{-1} d^{-1}); k_D (kg food kg fish^{-1} d^{-1}) is the rate constant for chemical uptake through the diet, C_D (g chemical kg food^{-1}) is the concentration of the chemical in the diet, k_2 (d^{-1}) is the rate constant for respiratory elimination, k_E (d^{-1}) is the rate constant for elimination via fecal egestion, k_G (d^{-1}) is the rate constant for pseudo elimination via growth dilution, and k_M (d^{-1}) is the rate constant for biotransformation of the chemical in the fish and t is time (d; Figure 1A). This model can represent chemical bioconcentration (i.e., $C_D = 0$) as represented in the OECD 305 test guideline [11], by the steady-state BCF (L water kg fish^{-1})

$$BCF = C_F/C_W = k_1/(k_2{+}k_E{+}k_G{+}k_M) = k_1/k_T \quad (2)$$

where k_T (d^{-1}) represents the sum of k_2, k_E, k_M and k_G. The model can also represent dietary bioaccumulation (i.e., $C_W = 0$) in the form of the steady-state BMF (kg food kg fish^{-1}), as measured in dietary bioaccumulation tests such as the new OECD 305 test guideline [11].

$$BCF = C_F/C_D = k_D/(k_2{+}k_E{+}k_G{+}k_M) = k_D/k_T \quad (3)$$

It is important to stress that in this modeling approach, the fish is viewed as a single compartment and includes the gastrointestinal contents (Figure 1A). Standard BCF assays are consistent with this modeling approach as measurements of C_F are usually determined by homogenizing the entire fish including the intestinal contents. The OECD 305 testing protocol [11] also states that the BMF is normally determined using test substance analysis of whole fish, even though the mass of chemical in the intestines can contribute considerably to the total mass of chemical in the fish, especially for substances that biotransformed rapidly in the body of the fish.

To better represent biotransformation and in particular biotransformation in the gastrointestinal tract, we can refine this model by distinguishing between the intestinal tract and the body (soma) of the fish as is shown in Figure 1B. This model can be implemented experimentally by removing the intestinal content from the fish before analysis. The intestinal tract is viewed as consisting of the gut lumen. The lumen contains the intestinal content or digesta and includes the intestinal flora and gastric enzymes secreted by enterocytes. The fish's body includes all parts of the fish but not the content of the intestinal tract. Chemicals enter the lumen as a result of food ingestion and chemicals transfer from the body of the fish into the intestinal content (including bile excretion). Chemicals are removed from the lumen through chemical transfer into the body of the fish, fecal egestion, and transformation in the intestines. Chemicals in the body of the fish are the result of uptake from the intestinal lumen and from water via the gills and the skin. Chemicals are removed from the body of the fish via chemical transfer from the fish into the intestinal content (including bile excretion), respiratory elimination via the gills and skin, biotransformation in the body of the fish (somatic biotransformation), and pseudo removed through growth dilution. Enterohepatic recirculation of contaminants in this model is represented by the chemical exchange between the intestinal content and the fish (i.e., k_{BG} and k_{GB}). The mass balance equations for the body of the fish (B) and the gastrointestinal contents (G) are

$$dM_B/dt = k^*_{B1} M_W + k_{GB} M_G - (k_{B2}{+}k_{BG}{+}k_{GD}{+}k_{BM}) M_B \quad (4)$$

$$dM_G/dt = G_I C_D + k_{BG} M_B - (k_{GB}{+}k_{GE}{+}k_{GM}) M_G \quad (5)$$

where M_B and M_G are the chemical masses (g) in the body of the fish and the digesta; G_I is the food ingestion rate (kg food d^{-1}); C_D is the concentration of the chemical in ingested diet (g chemical kg food^{-1}); and k^*_{B1} k_{B2}, k_{GB}, k_{BG}, k_{GD}, k_{BM}, k_{GE}, and k_{GM} are the rate constants (d^{-1}) for respiratory uptake, respiratory elimination, chemical transfer from the gastrointestinal content to the fish body, chemical transfer from the fish body to the gastrointestinal content, growth dilution, biotransformation of the chemical in the body of the fish, that is, somatic biotransformation, fecal egestion from the gastro-intestinal tract, and biotransformation of the chemical in the gastro-intestinal content, respectively (Figure 1B). The combined depuration rate constant from the fish's body ($k_{B2} + k_{BG} + k_{GD} + k_{BM}$) is k_{BT}.

Assuming a steady-state in the gastrointestinal contents, that is, $dM_G/dt = 0$, Equation 5 can be rewritten as

$$M_G = (G_I C_D + k_{BG} M_B)/(k_{GB}{+}k_{GE}{+}k_{GM}) \quad (6)$$

which after substitution in Equation 4 and recognizing that the chemical concentration in the body of the fish C_B

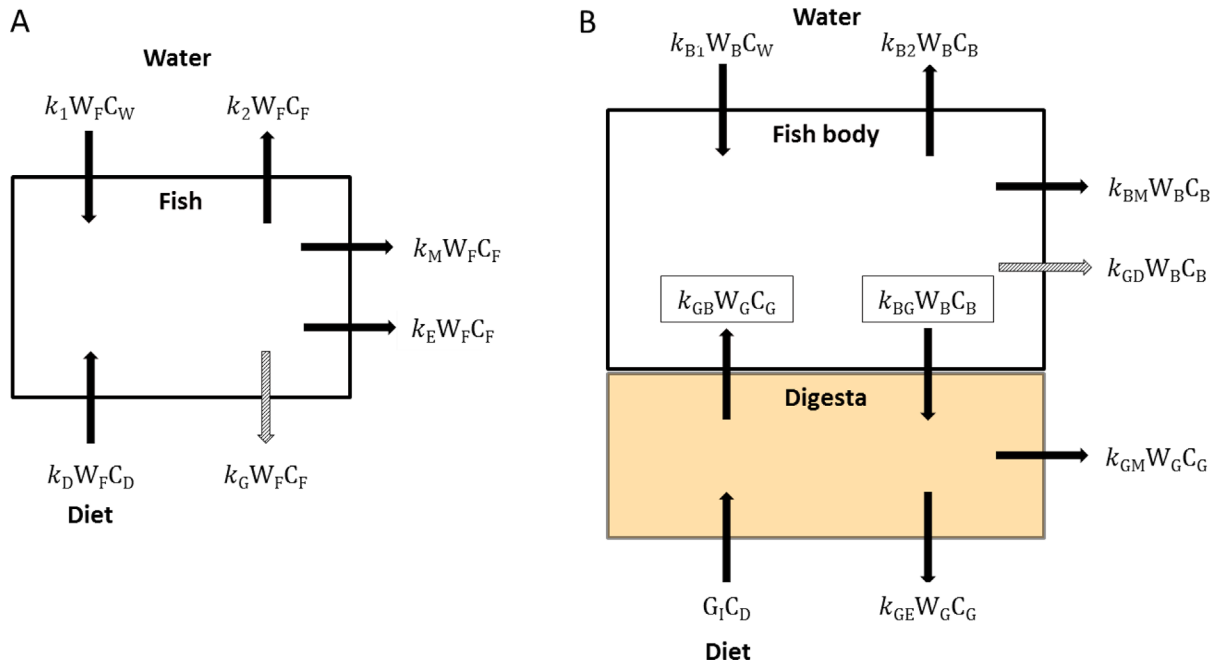

Figure 1. Conceptual diagram of the transport and transformation kinetics of hydrophobic organic chemicals in a 1 compartment fish model (**A**) illustrating the role of biotransformation in the whole fish (k_M), and in a 2 compartment model separating the fish body from the contents of the digestive tract (**B**) illustrating the role of somatic biotransformation (k_{BM}) and gastro intestinal biotransformation (k_{GM}).

(g kg body wt^{-1}) is the ratio of M_B (g) and the fish's body weight W_B (kg), that is, $C_B = M_B/W_B$ becomes

$$dC_B/dt = k_{B1}C_W + (k_{GB}/(k_{GB}+k_{GE}+k_{GM}))(G_I/W_B)C_D - (k_{B2}+k_{BG}\cdot(k_{GE}+k_{GM})/(k_{GB}+k_{GE}+k_{GM})+k_{GD}+k_{BM})C_B \quad (7)$$

where k_{B1} is the uptake clearance rate for respiratory uptake (L water kg body wt^{-1} d^{-1}) and (G_I / W_B) is the proportional feeding rate of the fish expressed as a percentage of the fish's body weight per day. A comparison of Equation 1 and Equation 7 shows a similarity in both expressions. Equation 7, however, applies to the body of the fish, whereas Equation 1 applies to the body of the fish and the intestinal content. Hence, the rate constants k_1 and k_{B1} as well as k_2 and k_{B2} and k_M and k_{BM} are not the same. If the mass of the chemical in the body of the fish is large compared with that in the digesta, then k_{B1} approaches k_1, k_{B2} approaches k_2, k_{BM} approaches k_M, and k_{BT} approaches k_T. However, if the mass of the chemical in the fish body is comparable to or smaller than that in the digesta, for example, due to rapid biotransformation of the chemical in the body of the fish, then $k_1 < k_{B1}$, $k_2 < k_{B2}$, $k_M < k_{BM}$, and $k_T < k_{BT}$.

Figure 2 illustrates that in this model, the ingested chemical flux (in units of g chemical d^{-1}), that is, $G_I C_D$ is fractionated in the intestinal tract in 1) the flux (g d^{-1}) that is absorbed by the fish body, that is, $(k_{GB} / (k_{GB} + k_{GE} + k_{GM})) \times G_I \times C_D$; 2) the flux (g d^{-1}) that is egested from the intestinal tract, that is, $(k_{GE} / (k_{GB} + k_{GE} + k_{GM})) \times G_I \times C_D$; and 3) the flux (g d^{-1}) that is transformed in the intestinal tract, that is, $(k_{GM} / (k_{GB} + k_{GE} + k_{GM})) \times G_I \times C_D$. The chemical flux from the body of the fish to the intestines, that is, $k_{BG} \times W_B \times C_B$, is also fractionated in the intestinal tract into 1) the flux (g d^{-1}) that is recirculated back into the fish body, that is, $(k_{BG} \times k_{GB} / (k_{GB} + k_{GE} + k_{GM})) \times W_B \times C_B$; 2) the flux (g d^{-1}) that is egested from the intestinal tract, that is, $(k_{BG} \times k_{GE} / (k_{GB} + k_{GE} + k_{GM})) \times W_B \times C_B$; and 3) the flux (g d^{-1}) that is transformed in the intestinal tract, that is, $(k_{BG} \times k_{GM} / (k_{GB} + k_{GE} + k_{GM})) \times W_B \times C_B$. Figure 2 illustrates that intestinal biotransformation is made up of 2 contributions: chemical transformation on ingestion and chemical transformation after absorption and subsequent elimination from the body of the fish. Both contributions express the gastrointestinal biotransformation rate constant k_{GM}. At the start of a dietary bioaccumulation study ($t = 0$), there is only contribution to the gastro-intestinal biotransformation rate, that is, $(k_{GM} / (k_{GB} + k_{GE} + k_{GM})) \times G_I \times C_D$ as $C_B = 0$. This provides an opportunity to determine k_{GM} from information typically collected in a dietary bioaccumulation study.

If the fish is viewed as the body of the fish, it is possible to redefine the dietary uptake rate constant as k_{BD} (kg food kg bodywt^{-1} d^{-1})

$$k_{BD} = (k_{GB}/(k_{GB}+k_{GE}+k_{GM}))(G_I/W_B) \quad (8)$$

In Equation 8, the dietary uptake efficiency for a substance that is biotransformed in the gastrointestinal tract ($E_{D,M}$) is

$$E_{D,M} = k_{GB}/(k_{GB}+k_{GE}+k_{GM}) \quad (9)$$

which equates k_{BD} to its more recognizable form of $E_{D,M} \times G_I/W_B$. In Equation 9, k_{GB}, k_{GE}, and k_{GM} represent the relative rates of chemical uptake from the intestines into the body of the fish, egestion in fecal matter, and biotransformation in the lumen of the digestive tract. The dietary uptake efficiency for a dietary contaminant that is not biotransformed in the gastrointestinal tract ($E_{D,N}$), that is, $k_{GM} = 0$, is

$$E_{D,N} = k_{GB}/(k_{GB}+k_{GE}) \quad (10)$$

It has been shown that $E_{D,N}$ for nonbiotransforming chemicals follows a nonlinear relationship with K_{OW}, which can be used to estimate $E_{D,N}$ from K_{OW} for neutral hydrophobic chemicals, that is,

$$E_{D,N}{}^{-1} = \alpha K_{OW} + \beta \quad (11)$$

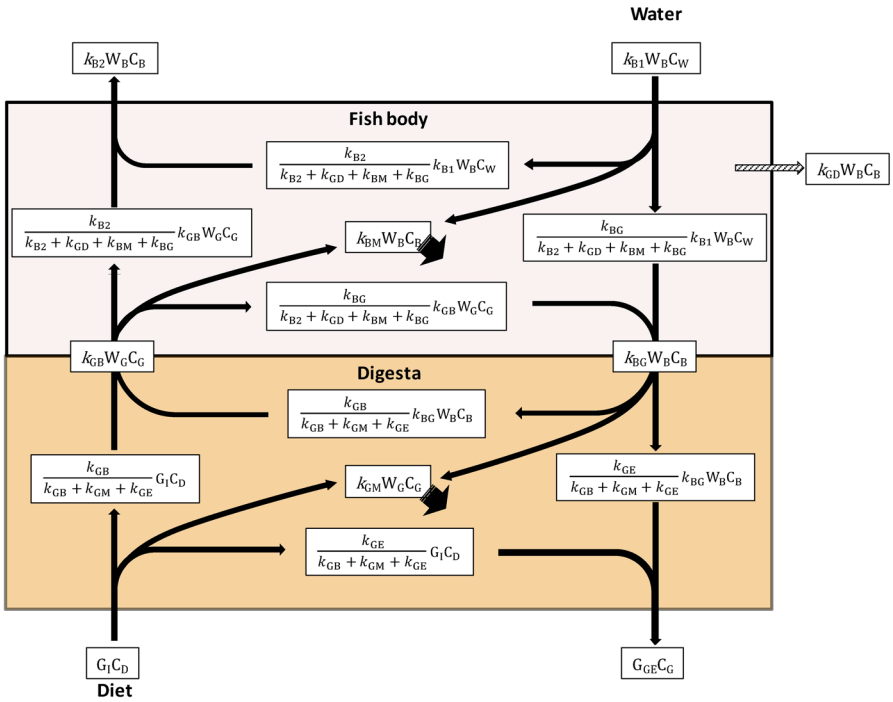

Figure 2. A more detailed conceptual diagram of the transport and transformation kinetics of hydrophobic organic chemicals in a 2 compartment model separating the fish body from the contents of the digestive tract illustrating the role of biotransformation (represented by the arrow) in the body (k_{BM}) and the gastrointestinal tract (k_{GM}) of the fish.

where α and β are coefficients that can be determined via regression of empirical $E_{D,N}$ observations [13]. If the fish is viewed as the body of the fish, it is also possible to redefine the fecal egestion rate constant k_E in Equation 1 in terms of the fecal egestion rate constant from the fish body (k_{BE} in d^{-1}) as

$$\begin{aligned} k_{BE} &= k_{BG}((k_{GE}+k_{GM})/(k_{GB}+k_{GE}+k_{GM})) \\ &= k_{BG}(k_{GE}/(k_{GB}+k_{GE}+k_{GM})) \\ &\quad + k_{BG}(k_{GM}/(k_{GB}+k_{GE}+k_{GM})) \end{aligned} \tag{12}$$

where k_{GE} / (k_{GB} + k_{GE} + k_{GM}) is the fraction of the ingested chemical that is egested from the intestinal tract in fecal matter; k_{BG} (k_{GE} / (k_{GB} + k_{GE} + k_{GM})) is the fraction of the fish absorbed chemical that is eliminated from the fish body untransformed (i.e., as parent chemical) in fecal matter; k_{GM} / (k_{GB} + k_{GE} + k_{GM}) is the fraction of the ingested chemical that is biotransformed in the intestinal tract; and k_{BG} (k_{GM} / (k_{GB} + k_{GE} + k_{GM})) is the fraction of the chemical mass eliminated by the fish that is biotransformed in the intestinal tract.

The model illustrates that biotransformation rates in the body of the fish (i.e., somatic biotransformation) and in the gastrointestinal tract (i.e., gastrointestinal biotransformation) are represented in bioaccumulation metrics in distinctly different fashions. Somatic (including hepatic) biotransformation is represented as k_{BM} in the combined depuration rate constant (k_{B2} + k_{BG} + k_{GD} + k_{BM}) or k_{BT}. The k_{BT} can be estimated from the concentrations in the body of the fish (C_B) during the depuration phase of a bioconcentration or biomagnification test in the same fashion as k_T is derived from C_F through log linear regression. Gastrointestinal biotransformation is reflected in the dietary uptake rate constant (k_{BD}) or the dietary uptake efficiency ($E_{D,M}$). $E_{D,M}$ can be determined from the initial ($t = 0$) increase in chemical concentration in the fish over time when there is no chemical in the fish.

Equation 4 and Equation 5 show that k_{BM} can be determined from the total depuration rate constant k_{BT} provided the elimination rate constants through nonmetabolic pathways, that is, (k_{B2} + k_{BG} + k_{GD}) or k_{SE} are known. One way to determine k_{SE} is to stop biotransformation of the chemical in the fish such that k_{BT} equals k_{SE}. The biotransformation rate constant k_{BM} can then be found in the experiment by subtracting k_{SE} from k_{BT} determined in the experiment where biotransformation is allowed to occur. This approach has been applied by Sijm et al. [14] and Myamoto et al. [15], who used inhibitors of the cytochrome P-450 system to stop or reduce the biotransformation of their test chemicals. The application of this method requires prior knowledge of the metabolic pathway of the test chemical, may not capture all applicable biotransformation pathways, and can involve treatment of test animals that may interfere with animal welfare.

Another approach, explored in the present study, is to determine k_{SE} by exposing test animals with nonbiotransformable reference chemicals together with a biotransformable test chemical. Given that elimination rates are known to be related to K_{OW}, it would be ideal to use a reference chemical of the same log K_{OW} values as the test chemical. However, such a reference chemical may be difficult to find. Alternatively, a range of nonbiotransformable reference chemicals with varying K_{OW} can be used to develop an empirical relationship between k_{SE} and K_{OW} for nonbiotransformable chemicals that can be used to derive the k_{SE} for test chemicals of varying K_{OW}. In theory, a range of numerical relationships (e.g., regression, polynomial equations) can be used to fit the relationship between k_{SE} and K_{OW} of the reference chemicals. However, we prefer to use a previously developed mechanistic model [16] to fit the empirical depuration rate constant data for the nonbiotransformable reference chemicals to derive the k_{SE} and K_{OW}. This model may provide a better description of the functional relationship between k_{SE} and K_{OW} than regression models,

which are not based on the same mechanistic considerations. The fitted model allows k_{SE} of the test chemical to be derived from the K_{OW} of the test chemical and k_{BM} then follows from the measurement of the total depuration rate constant k_{BT} of the biotransformable test chemical as

$$k_{BM} = k_{BT} - k_{SE} \quad (13)$$

Equation 9 and Equation 10 illustrate how the biotransformation rate constant in the gastrointestinal tract (k_{GM}) can be determined from the dietary uptake efficiency of the test and the reference chemicals as:

$$k_{GM} = (E_{D,M}{}^{-1} - E_{D,N}{}^{-1})k_{GB} \quad (14)$$

where k_{GB} can be derived by rearranging Equation 10 and considering that k_{GE} is the ratio of the fecal egestion rate G_{GE} (kg digesta d^{-1}) and the amount of digesta W_G (kg) in the gastro–intestinal tract, that is, $k_{GE} = G_{GE}/W_G$, as

$$\begin{aligned} k_{GB} &= (E_{D,N}/(1 - E_{D,N}))k_{GE} \\ &= (E_{D,N}/(1 - E_{D,N}))(G_{GE}/\ W_G) \end{aligned} \quad (15)$$

G_{GE} can be determined experimentally from fecal collection measurements or by adding inabsorbable chromic oxide to the diet and measuring the increase in chromic oxide concentrations in the fecal matter over that in administered food that occurs as a result of food absorption by the fish [17,18]. For example, in previous work in rainbow trout (*Oncorhynchus mykiss*) in our laboratory [18], the ratio of chromic oxide concentrations in the digesta (g kg digesta^{-1}) and in the diet (g kg food^{-1}) was measured as 2.1 (SE 0.4; kg food dry wt kg digesta dry wt^{-1}), indicating a G_{GE}/G_I ratio or γ_{GI} of 0.48 kg digesta dry weight kg food dry weight^{-1} and a corresponding dietary assimilation efficiency ε_f (unitless) of approximately 52% on a dry weight basis. Also, G_{GE} can be estimated from the dietary ingestion rate, the composition of the diet, and the assimilation efficiencies of the diet constituents [16] using values for the assimilation efficiencies of the various food constituents. Hence, G_{GE} can be determined as

$$\begin{aligned} G_{GE} = \{&(1 - \varepsilon_L)\phi_{DL} + (1 - \varepsilon_P)\phi_{DP} + (1 - \varepsilon_N)\phi_{DN} \\ &+ (1 - \varepsilon_W)\phi_{DW}\}G_I = \gamma_{GI}G_I \end{aligned} \quad (16)$$

where ϕ_{DL}, ϕ_{DP}, ϕ_{DN}, and ϕ_{DW} are the fractions of lipid (kg lipid kg diet^{-1}), protein (kg protein kg diet^{-1}), nondigestible organic matter (kg nondigestible organic matter kg diet^{-1}), and water (kg water kg diet^{-1}) of the fish's diet, and ε_L, ε_P, ε_N and ε_W are the dietary assimilation efficiencies of lipids, protein, nondigestible organic matters, and water, and where γ_{GI} is the ratio of the fecal egestion and dietary ingestion rates. The amount of digesta W_G (kg digesta dry wt) in the intestinal tract can be estimated from the feeding rate and the instantaneous evacuation rate of digesta (i.e., through food absorption and fecal egestion) from the gastrointestinal tract of the fish

$$dW_G/dt = G_I - \delta W_G \quad (17)$$

where δ is the digesta evacuation rate constant expressed as a fraction of digesta W_G that is evacuated (d^{-1}). Equation 17 is consistent with observations indicating that the decrease in gastrointestinal content follows an exponential relationship with time, suggesting that the rate of emptying the gastrointestinal tract in units of g d^{-1} is proportional to the amount of food in the intestinal tract [19]. A mean steady-state amount of digesta can then be estimated as a result of a constant feeding rate (G_I) and a constant digesta evacuation rate (δ), because if dW_G/dt is 0 (i.e., at steady-state), then Equation 17 shows that $W_G = G_I/\delta$, where G_I is known from experimental conditions, and δ can be estimated from digestive tract emptying times. For example, 100% emptying times ($t_{E,100}$) have been compiled by Fänge and Grove [19] and may reasonably approximate 95% emptying times ($t_{E,95}$), which are related to δ as $3/t_{E,95}$. The rate constant for chemical excretion from the gastrointestinal tract can then be derived as

$$k_{GE} = G_{GE}/\ W_G \quad (18)$$

Equations 14 through 18 provide a method to derive the gastrointestinal biotransformation rate constant k_{GM} for a test chemical from the dietary uptake efficiencies of the test chemical and a nonbiotransfomable reference chemical. For example, a 100 g fish that is fed 1% of its body weight per day (i.e., 1 g food d^{-1}) produces a fecal egestion rate of approximately 0.5 g digesta d^{-1}. If the fish's 95% gastrointestinal evacuation time ($t_{E,95}$) for a meal is 1.5 d, then δ is 3/1.5 d^{-1} or 2 d^{-1}, and the steady-state amount of digesta W_G in the gastrointestinal tract is 1 g food d^{-1} /2 d^{-1} or 0.5 g. This means that k_{GE} or G_{GE}/W_G of 0.5 g digesta d^{-1}/0.5 g digesta = 1 d^{-1}. If, in our example, the $E_{D,N}$ for the nonbiotransformable reference chemical is 0.50, then following Equation 15, $k_{GB} = k_{GE} = G_{GE}/W_G = 1\ d^{-1}$. If $E_{D,M}$ for the test biotransformable chemical is, for example, 0.25, then k_{GM} can be determined as $(0.25^{-1} - 0.50^{-1}) \times 1\ d^{-1} = 2\ d^{-1}$. It should be emphasized that k_{GM} applies to the mass of chemical in the gastrointestinal tract (M_G), whereas k_{BM} applies to the chemical mass in the fish's body (M_B). To compare the relative importance of gastrointestinal and somatic biotransformation, the rate constants need to be multiplied by the corresponding masses of the parent substance in the intestinal tract (M_G) and the fish's body (M_B). The mass of parent test chemical in the fish body can be determined at steady-state ($dM_B/dt = 0$) as

$$M_B = C_B W_B = C_D W_B k_{BD}/k_{BT} \quad (19)$$

where C_D, W_B, k_{BD}, and k_{BT} are parameters determined in a dietary bioaccumulation study.

The mass of the parent test chemical in the fish's intestinal tract can be determined from Equation 5 at steady-state ($dM_G/dt = 0$) as

$$M_G = C_G W_G = (C_D G_I + k_{BG}\, M_B)/(k_{GB} + k_{GE} + k_{GM}) \quad (20)$$

where the chemical concentration in the diet C_D and the feeding rate G_I are known from the experimental conditions; k_{GM}, k_{GE}, and k_{GB} can be determined from Equations 14 through 18 and k_{BG}, that is, the rate constant for chemical from the fish into the gastrointestinal tract, can be estimated as

$$k_{BG} = K_{GB} k_{GB} \quad (21)$$

where K_{GB} is the unitless chemical partition coefficient between the gastrointestinal content and the fish, which can be estimated

from the composition of the digesta and the body of the fish as [16]:

$$K_{GB} = (\phi_{GL}K_{OW}+\phi_{GP}\chi K_{OW}+\phi_{GN}\theta K_{OW}+\phi_{GW})\,d_G/ (\phi_{BL}K_{OW}+\phi_{BP}\chi K_{OW}+\phi_{BN}\theta K_{OW}+\phi_{BW})d_B \quad (22)$$

where ϕ_{GL}, ϕ_{GP}, ϕ_{GN}, and ϕ_{GW} are the fractions of lipid (kg lipid kg digesta^{-1}), protein (kg protein kg digesta^{-1}), nondigestible organic matter (kg nondigestible organic matter kg digesta^{-1}), and water (kg water kg digesta^{-1}) in the gastrointestinal contents of the fish; ϕ_{BL}, ϕ_{BP}, ϕ_{BN}, and ϕ_{BW} are the fractions of lipid (kg lipid kg fish body^{-1}), protein (kg protein kg fish body^{-1}), nondigestible organic matter (kg nondigestible organic matter kg fish body^{-1}), and water (kg water kg fish body^{-1}) of the body of the fish; d_G and d_B are the densities of the digesta and body of the fish (kg L^{-1}); χ and θ are proportionality constants comparing the absorptive capacity of proteins and nondigestible organic matter, respectively, to that of lipids (assumed to be equal to that of octanol) and can be assumed to be 0.05 for certain classes of hydrophobic organic chemicals [20]. Whereas the lipid and protein contents of the fish body and fish food are often known or easily measurable, the composition of the digesta is usually not known and difficult to measure. It can be approximated, however, from the dietary composition using estimates of the dietary assimilation efficiencies of lipids (ε_L), protein (ε_P), nondigestible organic matter (ε_N), and water (ε_W) following Arnot and Gobas [16]

$$\phi_{GL} = (1-\varepsilon_L)\phi_{DL}/\{(1-\varepsilon_L)\phi_{DL}+(1-\varepsilon_P)\phi_{DP} +(1-\varepsilon_N)\phi_{DN}+(1-\varepsilon_W)\phi_{DW}\} \quad (23)$$

$$\phi_{GP} = (1-\varepsilon_P)\phi_{DP}/\{(1-\varepsilon_L)\phi_{DL}+(1-\varepsilon_P)\phi_{DP} +(1-\varepsilon_N)\phi_{DN}+(1-\varepsilon_W)\phi_{DW}\} \quad (24)$$

$$\phi_{GN} = (1-\varepsilon_N)\phi_{DN}/\{(1-\varepsilon_L)\phi_{DL}+(1-\varepsilon_P)\phi_{DP} +(1-\varepsilon_N)\phi_{DN}+(1-\varepsilon_W)\phi_{DW}\} \quad (25)$$

$$\phi_{GW} = (1-\varepsilon_W)\phi_{DW}/\{(1-\varepsilon_L)\phi_{DL}+(1-\varepsilon_P)\phi_{DP} +(1-\varepsilon_N)\phi_{DN}+(1-\varepsilon_W)\phi_{DW}\} \quad (26)$$

where ϕ_{DL}, $\phi_{DP,}$ ϕ_{DN}, and ϕ_{DW} are the fractions of lipid (kg lipid kg food^{-1}), protein (kg protein kg food^{-1}), nondigestible organic matter (kg nondigestible organic matter kg food^{-1}), and water (kg water kg food^{-1}) of the fish's diet. The dietary lipid assimilation efficiency is approximately 92% in rainbow trout [18], and protein and water assimilation efficiencies in fish are approximately 75% and 50%. The dietary assimilation efficiency of nondigestible organic matter can be assumed to be 0%.

There are several areas of uncertainty in deriving gastrointestinal biotransformation rate constants from dietary absorption efficiencies. One of these originates from the practice of using dried fish foods in dietary bioaccumulation experiments and the lack of measuring the amount of water absorbed with the food. Fortunately, as demonstrated in detail in the Supplemental Data, the absorption of water with the food by fish does not have a significant effect on the determination of intestinal biotransformation rates for very hydrophobic neutral organic substances with a very high K_{OW} ($\log K_{OW} > 5$). As a result, calculations based on a dry weight basis will produce estimates of biotransformation rates that are not significantly different from those conducted on a wet weight basis. The main reason for the insignificant role of water on the dietary uptake dynamics of very hydrophobic substances in fish is that water has a negligible capacity to solubilize very hydrophobic chemicals compared with lipids, proteins, and other organic materials. As a result, the mass balance equations for the uptake of very hydrophobic chemicals in fish can be described on a wet weight or dry weight basis without introducing a significant error due to ignoring the chemical in aqueous parts of the diet and digesta in the dry weight-based calculations. This is advantageous in dietary bioaccumulation experiments because often, as is the case in the present study, the chemical is administered in the form of dry food that is applied to water. Because the water content of the actual diet and digesta of the fish are, in most cases, not characterized in dietary bioaccumulation experiments, wet weight-based calculations involving the feeding and fecal egestion rates are difficult to perform. Hence, we recommend that the calculation of gastrointestinal biotransformation rates for very hydrophobic organic chemicals be conducted on a dry weight basis.

Another area of uncertainty originates from the inherent assumption of the bioaccumulation model that food consumption is a continuous process. Observations by Fänge and Grove [19] suggest that this assumption may be reasonable for fish in controlled feeding experiments, because the dynamics of intestinal evacuation in fish are consistent with generating a relatively constant amount of digesta. However, the assumption of continuity and the recognized effect of temperature, meal size, food type, fish size, method of feeding, and feeding history on gastric evacuation times [19] contribute uncertainty in characterizing the amount of digesta W_G in the intestinal tract of the fish, which contributes uncertainty to determining k_{GE}, k_{GB}, and k_{GM}. This uncertainty may further increase when extending the applicability of domain of the presented approach (i.e., dietary bioaccumulation experiments) to field applications, where fish may not feed for extended periods of time. Fortunately, the uncertainty in W_G is removed, to a large extent, from the determination of the gastrointestinal biotransformation rate (expressed in g chemical d^{-1}), that is, $k_{GM} \times M_G$ or $k_{GM} \times W_G \times C_G$, because k_{GM} follows an inverse relationship with W_G, whereas the chemical mass in the digesta is proportional to W_G. Hence, errors in determining W_G have a tendency to partially cancel out when determining gastrointestinal biotransformation rates.

The model description shown in the present study illustrates that the contribution of gastrointestinal and somatic in vivo biotransformation can be derived from measurements typically made in a dietary bioaccumulation study if nonbiotransformable reference chemicals are added to the experimental protocol of the test, and the chemical mass in the intestinal content of the fish is not included in measuring the chemical concentration in the fish. The contribution of somatic biotransformation as a proportion of the total mass of chemical biotransformed (Φ_{BM}) in the fish can be estimated as:

$$\Phi_{BM} = k_{BM}M_B/(k_{BM}M_B+k_{GM}M_G) \quad (27)$$

One of consequences of gastrointestinal biotransformation is that it counteracts the gastrointestinal magnification effect in the gastrointestinal tract. Gastrointestinal magnification is the increase of the chemical fugacity (or thermodynamic) activity in the intestinal content over that in the diet, occurring as a result of food absorption and food digestion [18]. Gastrointestinal magnification (which is defined as an increase in the chemical fugacity in the digesta over that in the diet [21]) is generally viewed as the underlying mechanism why hydrophobic organic

chemicals biomagnify (defined as an increase in the chemical fugacity in the body of the fish over that in the diet) in fish [18]. As illustrated in the Supplemental Data, it is possible to determine the magnitude of the gastro-intestinal biotransformation rate constant k^{*}_{GM} (d^{-1}) that prevents gastrointestinal magnification and hence biomagnification in fish as

$$k^{*}_{GM} = (G_I / W_G)(K_{DG} - \gamma_{GI}) \quad (28)$$

where K_{DG} (kg digesta dry wt kg food dry wt^{-1}) is the diet–digesta partition coefficient, which can be estimated from the composition of the diet as

$$K_{DG} = C_D / C_G = (\phi_{DL}K_{OW} + \phi_{DP}\,\chi K_{OW} + \phi_{DN}\,\theta K_{OW} + \phi_{DW}) / (\phi_{GL}K_{OW} + \phi_{GP}\,\chi K_{OW} + \phi_{GN}\,\theta K_{OW} + \phi_{GW}) \quad (29)$$

As explained in more detail in the Supplemental Data, substitution of Equation 28 into Equation 14 provides a threshold dietary uptake efficiency $E^{*}_{D,M}$ which, if not exceeded, indicates that the chemical cannot be subject to gastrointestinal magnification and hence is not expected to biomagnify in fish:

$$\frac{1}{E^{*}_{D,M}} = \frac{K_{DG}}{\gamma_{GI}} \times \frac{(1 - E_{D,N})}{E_{D,N}} + \frac{1}{E_{D,N}} \quad (30)$$

If substances exhibit a dietary uptake efficiency greater than $E^{*}_{D,M}$, then it is still possible that the substance cannot biomagnify provided the somatic biotransformation rate is sufficiently high.

MATERIALS AND METHODS

Fish

Rainbow trout (*O. mykiss*, ~30 g body wt) were purchased from Miracle Springs Hatchery and Trout Farm and acclimatized for 4 wk before initiating the experiments. Fish were held in 4 (3 test and 1 control) flow-through glass aquaria supplied with dechlorinated water. The water was oxygenated with air stones, resulting in dissolved oxygen concentrations in water that were 90% of saturation. The aquaria were housed in a cold room at Simon Fraser University with a 14:10-h light:dark schedule. Water temperatures were kept at 11.4 °C to 13.3 °C (mean temperature = 12.6 °C) throughout the acclimation period and the experiment with a thermostatted water chiller. Fish were fed commercial fish chow at a daily rate of 1.5% of the pre-experiment mean fish body weight. On administration, fish food (1.5 mm EWOS Pacific Complete Feed for Salmonids) contained 18.6% lipids, 46.6% protein, 32.4% of nondigestible organic materials, and 2.4% water.

Chemicals

Fish were administered a control or a contaminated diet containing 15 chemicals at concentrations listed in Table 1 along with the K_{OW} of the chemicals. The reference chemicals were selected because of their resistance to biotransformation in fish and microbial degradation and to represent a range in K_{OW}. Although the reference chemicals are recognized for their persistence, some biotransformation of polychlorinated biphenyl (PCB) 52 has been observed in fish [22]. To prepare the diet, test chemicals were dissolved in 3 mL of corn oil and 15 mL of toluene. This spiking solution was then added slowly to 400 g of fish feed, mechanically stirring the fish feed in an open system overnight. The spiked diet was stored at 2 °C in a sealed container. Fish feed for the control diet was prepared in a similar manner but without the test chemicals.

Dosing design

Fish in the exposure group ($n = 51$) were housed in 3, 40 gallon glass flow-through tanks and exposed to the test chemicals in the diet for 14 d, followed by a 114 d depuration period when fish were fed a noncontaminated control diet. Three fish were collected, 1 from each of the 3 exposure tanks, on day 0, day 2, day 5, day 9, day 14, day 14.17, day 14.5, day 14.75, day 15, day 17, day 19, day 23, day 31.4, day 48.2, day 86, and day 128, and analyzed independently. Unexposed control fish ($n = 12$, housed in a single 40 gallon glass flow-through aquarium) were fed an uncontaminated control diet throughout the entire 128 d experiment and shared the same dechlorinated freshwater source as the exposed fish to monitor for any potential chemical uptake from water. Three fish were collected on day 0, day 5, day 23, and day 128, and analyzed independently to test for potential uptake of test and reference chemicals from the water due to chemical leaching from administered fish food and exposed fish to the water. Fish were

Table 1. Concentrations of reference and test chemicals in the diet of the fish, the log K_{OW}, the combined depuration rate constant from the fish body k_{BT} (day^{-1}) with its standard error, and the dietary uptake efficiency E_D (unitless) and its standard error for the reference and test chemicals in the present study

	Nominal food concentrations (mg kg wet wt^{-1})	log K_{OW}	$k_{BT} \pm$ SE (day^{-1})	$E_D \pm$ SE (%)
Reference chemicals				
1,2,4,5-tetrachlorobenzene	18.1	4.64 [36]	0.045 ± 0.016	63 ± 13
Pentachlorobenzene (PCBz)	52.9	5.17 [36]	0.024 ± 0.002	55 ± 8
Hexachlorobenzene (HCBz)	8.8	5.73 [37]	0.0088 ± 0.0019	53 ± 10
2,2′,5,5′-PCB (PCB 52)	12.5	6.09 [36]	0.0028 ± 0.0020	45 ± 8
2,2′,4,4′,6,6′-PCB (PCB 155)	12.5	7.55 [36]	0.0012 ± 0.0022	46 ± 6
2,2′,4,4′,5,5′-PCB (PCB 153)	12.5	7.75 [36]	0.00069 ± 0.0021	46 ± 7
2,2′,3,3′,4,4′,5’,5′,6,6′-PCB (PCB 209)	12.5	8.27 [37]	0.0011 ± 0.0021	34 ± 3
Test chemicals				
1,2,3,4-tetramethyl benzene	120	4.00 [38]	0.36 (±0.10)	44 ± 12
β-hexachlorocyclohexane (β -HCH)	8.5	4.14 [36]	0.23 (±0.079)	55 ± 5
Trans-decalin	499	4.79 [39]	0.038 (±0.0025)	19 ± 5
9-methylanthracene	129	5.07 [38]	0.41 (±0.10)	13 ± 9
Chrysene	28.2	5.81 [40]	0.39 (±0.12)	4.9 ± 1.4
Hexylcyclohexane	488	6.05 [6]	0.043 (±0.020)	14 ± 4
2,6-dimethyldecane	476	6.09 [6]	0.18 (±0.043)	33 ± 14
Benzo[*a*]pyrene	27.8	6.13 [40]	0.094 (±0.031)	2.3 ± 1.2

sacrificed using an overdose of Finquel MS-222 (Argent Laboratories) and split into liver, carcass, and intestines. Each compartment was analyzed individually.

Sample extraction

The whole liver was used for sample extraction. For carcass samples, a homogenized fraction of the whole carcass (minus the liver) was used. These samples were homogenized with an Oster 18-speed blender/blade (Sunbeam Products) used with glass canning jars (Benardin). All experimental equipment were washed with detergent and rinsed with hexane and dichloromethane (DCM) before use. Liver (ranging in weight from 0.15–1.22 g) and carcass samples (4.17–5.27 g) were weighed and homogenized with 20 g (for liver samples) or 40 g (carcass samples) of sodium sulphate (Caleon laboratory Chemicals). To prevent volatilization of chemicals in the extraction process, 0.2 mL (for liver) and 0.4 mL (for carcass) of corn oil were added. Internal standards were also added, including d^8-naphthalene (for trans-decalin, 2,6-dimethyl decane, 1,2,3,4-tetramethylbenzene, hexylcyclohexane, 1,2,4,5-tetrachlorobenzene), d^{10}-acenaphthene (for pentachlorobenzene), ^{13}C-hexachlorobenzene (for hexachlorobenzene, beta-hexachlorocyclohexane), PCB 115 (for PCB 52, 9-methylanthracene, PCB 155, PCB 153), and d^{12}-chrysene (for chrysene, benzo[*a*]pyrene), PCB 207 (for PCB 209). Samples were extracted 3 times with 1:1 DCM–Hexane (40 mL, 20 mL, 20 mL for liver samples; 60 mL, 30 mL, 30 mL for carcass samples) under sonication for 20 min. Under a stream of nitrogen, extracts were concentrated to approximately 5 mL. The concentrated samples were then eluted through a column packed with 10 g of de-activated florisil and eluted with 40 mL hexanes. The samples were then eluted with 90 mL of 1:1 DCM–Hexane, which was concentrated down to 0.3 mL to 0.5 mL under a steady nitrogen stream and diluted with n-hexane to a total volume of 1 mL. For carcass samples, the samples were diluted 20-fold. No dilution was necessary for the analysis of liver samples.

Gas chromatography mass spectrometry analysis

Extracts were analyzed for the test chemicals using an Agilent 6890 gas chromatograph (GC) attached to an Agilent 5973N mass spectrometer (MS), with a programmable cool on-column injection port, a 30 m × 250 μm × 0.25 μm HP –5MS 5% phenyl methyl siloxane-coated column (Agilent), and a 5 m × 530 μm × 0.25 μm fused-silica deactivated guard column (Agilent). The oven temperature was 45 °C for 1.5 min, increasing to 150 °C at 15 °C min^{-1}, and finally increasing 10 °C min^{-1} to 285 °C, and held for 5 min. The injection port and ion source temperatures were 45 °C and 230 °C respectively. The carrier gas was helium at 1 mL min^{-1} flow rate. The MS data was acquired in the selected ion monitoring mode (*m/z* 138 for trans-decalin; 85 for 2,6-dimethyldecane; 119 for 1,2,3,4-tetramethylbenzene; 136 for d^8-naphthalene; 82 for hexylcyclohexane; 216 for 1,2,4,5-tetrachlorobenzene; 164 for d^{10}-acenaphthene; 250 for pentachlorobenzene; 290 for ^{13}C-hexachlorobenzene; 284 for hexachlorobenzene; 219 for beta-hexachlorocyclohexane; 292 for PCB 52; 192 for 9-methylanthracene; 360 for PCB 155; 326 for PCB 115; 360 for PCB 153; 240 for d^{12}-chrysene, 228 for chrysene; 464 for PCB 207; 252 for benzo[*a*]pyrene; and 498 for PCB 209). These ions were selected based on the properties of high intensity with low interference. A 1.00 μL sample of the extract was injected into the column by a 5 μL gas-tight glass syringe (Agilent). Peak areas were integrated and used to quantify the test chemicals using Chemstation software (Hewlett Packard). Chemical concentrations were calculated using the relative response factor approach.

Fish body concentrations

Chemical concentrations in the body of the fish (C_B) were determined by adding the chemical masses in the liver and carcass of each fish and dividing by the combined wet weights of the 2 compartments.

Somatic uptake and depuration rate constants

Somatic depuration rate constants (k_{BT}) were derived from the test chemical concentrations in the body of the fish during the depuration phase of the experiment by a weighted linear regression of the natural logarithm of the concentrations in the fish body measured after day 14.5 (i.e., 12 h after the last feeding period) versus time. The dietary uptake rate constant (k_{BD}) for each chemical was derived using nonlinear regression of

$$C_B = (k_{BD}/k_{BT})C_D(1 - \exp(-k_{BT}t)) \quad (31)$$

which is the analytical solution of Equation 7 if C_D is constant over time. The dietary uptake efficiency for test and reference chemicals was determined from k_{BD} following Equation 8 using a daily feeding rate of 1.2% of the mean fish body weight during the uptake period.

Somatic k_{SE} rate constant

To determine k_{SE}, the fish bioaccumulation model described in Arnot and Gobas [16], was parameterized (Supplemental Data, Table S1) to represent the experimental fish under the experimental conditions to produce a nonlinear relationship between k_{SE} (i.e., k_{BT} with $k_{BM} = 0$) and K_{OW}. This model was fitted to the experimental depuration rate constant data for the reference chemicals using a weighted nonlinear least squares Gauss-Newton algorithm under JMP® 9.0.2. The reciprocal of the standard errors of the depuration rate constants for the reference chemicals were used for weighting. The fitting involved determining the fish body lipid content and growth rate constant that best fitted the empirical $k_{SE} - K_{OW}$ relationship.

Somatic biotransformation rate constant (k_{BM})

The rate constant of somatic biotransformation (k_{BM}) of the test chemicals was determined by subtracting k_{SE} from k_{BT} following Equation 13.

$E_{D,N}$

To determine the relationship between $E_{D,N}$ and K_{OW} for the reference chemicals, dietary absorption efficiencies for the reference chemicals were fitted to Equation 11 using the nonlinear weighted least squares Gauss-Newton algorithm under JMP 9.0.2 with the reciprocal of the standard errors of the $E_{D,N}$ estimates as the weight.

Gastrointestinal biotransformation rate constant (k_{GM})

The rate constant for gastrointestinal biotransformation (k_{GM}) of the test chemicals was determined from the dietary uptake efficiency $E_{D,M}$ and $E_{D,N}$ following Equations 14 through 18.

RESULTS AND DISCUSSION

Fish

No fish mortalities were observed throughout the experiment in either the exposure or control groups. Behavior and appearance of fish in the exposure and control groups were

similar. Concentrations of the test and reference chemicals in the control fish were below their limit of quantitation (0.4–6 μmol g wet wt^{-1}). Growth rate constants (k_{GD}) were calculated using the standard OECD 305 method [11] as the slope of the natural logarithm of 1/weight (g) versus time (day). There was no evidence of a difference in the growth rate constant for the test fish (0.0066 [SE 0.0012] d^{-1}) and control fish (0.0066 [SE 0.0015] d^{-1}; Supplemental Data, Figure S1). Fish exhibited an initial body weight of 32 g (standard deviation [SD] 2, $n = 3$), which increased over time to 42 g (SD 4, $n = 3$) at the end of the 14 d uptake period, and to 81 g (SD 39, $n = 7$) at the end of the 128 d experiment. The average body weight and lipid content of the fish during the uptake period was 37 g (SE 4) and 6.7% (SE 1.0) respectively, and the average daily feeding rate was 0.012 g (SE 0.002) food wet weight/g fish wet weight or 0.0116 g (SE 0.002) food dry weight/g fish wet weight during the exposure period. Using a dietary assimilation efficiencies for lipids, protein, and nondigestible organic matter of 92%, 75%, and 0%, respectively, the dry weight based dietary assimilation efficiency was calculated to be $(0.92 \times 0.186 + 0.75 \times 0.466 + 0 \times 0.324) \times (0.012/0.0116)$ or 53%, similar to the 52% (SD 4.0) measured previously using chromic oxide concentrations in Gobas et al. [18]. Assuming that the 100% digestive emptying time of 35 h or 1.45 d reported for 60 g to 80 g rainbow trout at 13.5 °C [19] is a reasonable estimate for the 95% digestive emptying time in the fish in this experiment, δ can be estimated as 3/1.45 d^{-1} or 2.1 d^{-1}, and the steady-state amount of digesta in the fish can be estimated as $(0.0116 \times 37)/2.1 =$ 0.20 g dry weight (Equation 17).

Somatic biotransformation

Supplemental Data, Figure S2, illustrates that throughout the uptake phase, the mean chemical concentration in fish (of 3 replicates) increased over time and reached a steady-state concentration for certain substances, after which the concentration remained constant throughout the remainder of the uptake phase. For other substances, the chemical concentration in the fish increased throughout the uptake period and steady state was never achieved. After fish were fed noncontaminated diet (i.e., depuration phase), mean concentrations declined over time. Whole fish body total depuration rate constants (k_{BT}) for the reference and test chemicals were derived through log linear regression version of concentrations (C_B) versus time (t) and are listed in Table 1.

Figure 3 shows that the total depuration rate constant (k_{SE}) for the reference chemicals decreased with increasing log K_{OW} to a minimum value of approximately 0.001 d^{-1} for chemicals with a log K_{OW} greater than approximately 7.5. This minimum k_{SE} value may represent growth dilution, as with increasing log K_{OW}, k_{B2}, and k_{BE} become increasingly smaller causing k_{SE} to approach k_{GD}. However, it should be emphasized that the error in the depuration rate constants (k_{BT}) of PCB 52, PCB 155, PCB 153, and PCB 209 is large due to the small decline in concentration achieved over the duration of the depuration phase. This error has several consequences. First, it means that a small rate of biotransformation of a chemical (i.e., a rate within the margins of error), such as may occur for PCB 52, is not necessarily an impediment to using that chemical as a reference chemical. Second, because for very hydrophobic chemicals, the error is large enough for the depuration rate constant to not be statistically different from 0, it is sometimes reasonable to assume that k_{SE} is essentially 0 for very hydrophobic reference chemicals. Third, although the relative error in determining the depuration rate constant of the reference chemical can be large,

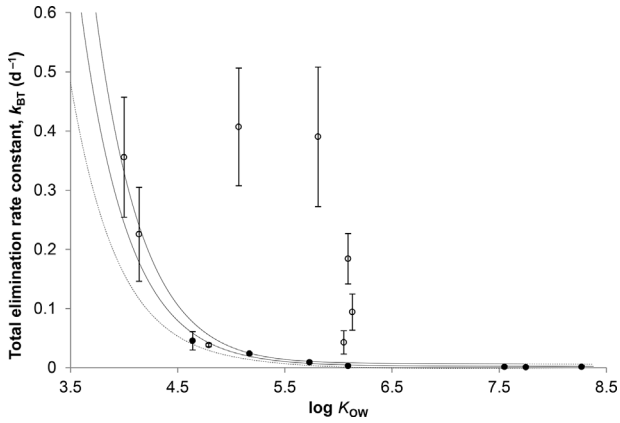

Figure 3. Rate constants for total elimination from the fish body k_{BT} (day^{-1}) of reference chemicals (filled round circles) and test chemicals (open round circles) versus log K_{OW} (standard errors reported in error bars). Test chemicals from left to right: 1,2,3,4-tetramethylbenzene, β-hexachlorocyclohexane, trans-decalin, 9-methylanthracene, chrysene, hexylcyclohexane, 2,6-dimethyldecane, and benzo[*a*]pyrene. The solid line represents the model used to fit the depuration rate constant data for the nonbiotransformable reference chemicals. The dotted lines represent the 95% confidence intervals for the predicted model values.

it may contribute little error in determining the somatic biotransformation rate constant (k_{BM}) according to Equation 13, provided the somatic biotransformation rate is sufficiently high. Fourth, the error also means that BMFs of very hydrophobic nonbiotransformable substances derived using a kinetic approach (e.g., as the ratio of k_{BD} and k_{BT}) can contain large errors.

Figure 3 illustrates that the bioaccumulation model provides a good fit of the relationship between the depuration rate constants (k_{BT}) and log K_{OW} for the reference chemicals, hence providing a method for determining k_{SE} of nonionized hydrophobic test chemicals. Figure 3 shows that the depuration rate constants (k_{BT}) of the test chemicals 1,2,3,4-tetramethylbenzene and β-hexachlorocyclohexane (β-HCH) were close to their corresponding k_{SE} values, indicating a small somatic biotransformation rate constant (k_{BM}), not different from 0. These findings do not necessarily indicate that these substances do not biotransform in the body of the fish, but that the biotransformation rate constant k_{BM} is small compared to k_{SE} for these substances. Both 1,2,3,4-tetramethylbenzene and β-HCH have a relatively low log K_{OW} and eliminate from fish to the water (via the gills) relatively quickly. For substances with a relatively low log K_{OW} (e.g., less than 3.5), which are eliminated quickly in fish by nonmetabolic pathways, the reference chemical method applied in the present study may not be a suitable method for measuring somatic biotransformation rates.

Figure 3 illustrates that the depuration rate constants (k_{BT}) of 2,6-dimethyldecane, 9-methylanthracene, chrysene, and benzo-[*a*]pyrene are greater than their corresponding k_{SE} values. For these substances, values for the somatic biotransformation rate constant (k_{BM}) can be determined with confidence (Table 2) because the difference between k_{BT} and k_{SE} is large compared to the error in the measurements of k_{BT} and k_{SE}. The biotransformation of some of these test chemicals in the present study, as well as related substances has been reported in prior studies [23,24]. Quantitative structure-activity relationships predictions of biotransformation rate constants obtained from EpiSuite 4.11 [6] for a 62 g fish (i.e., mean weight of the experimental fish during the depuration phase) at 12.6 °C (Table 2) also indicate that these chemicals can be expected to

Table 2. Somatic biotransformation rate constants (k_{BM}), modeled somatic k_{SE} rate constants, intestinal biotransformation rate constants (k_{GM}), BCFBAF (Ver 3.00) [6] predicted fish biotransformation rate constants (k_M), proportion of total mass biotransformed in the gut (ϕ_{GM}), and proportion of total mass biotransformed in the fish body (ϕ_{BM})[a]

Chemical	$k_{BM} \pm$ SE (1/day)	$k_{SE} \pm$ SE (1/day)	$k_{GM} \pm$ SE (1/day)	$k_{M,BCFBAF}$ (1/day)	ϕ_{GM}	ϕ_{BM}
1,2,3,4-tetramethylbenzene	0.11 ± 0.03	0.24 ± 0.04	0.45 ± 0.15	0.66	0.63	0.37
β-HCH	0.047 ± 0.025	0.18 ± 0.03	–0.09 ± 0.03	0.025	0	1
Trans-decalin	–0.004 ± 0.003	0.042 ± 0.007	3.7 ± 1.0	0.06	1.0	0
9-methylanthracene	0.38 ± 0.03	0.023 ± 0.003	6.6 ± 2.0	0.22	0.87	0.13
Chrysene	0.38 ± 0.04	0.006 ± 0.002	20 ± 5	0.14	0.95	0.05
Hexylcyclohexane	0.0378 ± 0.005	0.005 ± 0.002	5.7 ± 1.4	0.07	0.92	0.08
2,6-dimethyldecane	0.18 ± 0.01	0.005 ± 0.002	1.2 ± 0.4	0.09	0.64	0.36
Benzo[*a*]pyrene	0.09 ± 0.01	0.004 ± 0.002	46 ± 10	0.48	0.98	0.02

[a]BCFBAF predicted k_M values were adjusted to 62 g fish in water at a temperature of 12.6 °C
SE = standard error; β-HCH = β-hexachlorocyclohexane.

be biotransformed by fish. The k_{BM} values derived for the test chemicals in the present study can provide a preliminary test of the QSAR-based biotransformation rate predictions by EpiSuite 4.11. Supplemental Data, Figure S5, illustrates that the QSAR predicted k_M values of 2,6-dimethyldecane, 9-methylanthracene, chrysene, transdecalin, β-HCH, and hexylcyclohexane are in reasonable agreement with the observed k_{BM} values. However, the EpiSuite predictions of the biotransformation rate constant of 1,2,3,4-tetramethylbenzene and benzo[*a*]pyrene are approximately 10 times greater than the observed values in the present study. The lack of good agreement between QSAR predicted and observed biotransformation rate constants for 1,2,3,4-tetramethylbenzene may be due to the relatively low K_{OW} of 1,2,3,4-tetramethylbenzene, which produces a relatively high k_{SE} and hence makes it difficult to detect and accurately quantify the contribution of biotransformation to the overall depuration rate constant. This limitation affects both the determination of the biotransformation rate constant in the present study, as well as the training set of chemicals used in the development of the biotransformation QSAR. The lack of good agreement between QSAR predicted and observed k_{BM} values for benzo[*a*]pyrene is more difficult to explain. It is possible, however, that in a multiple chemical dosing design (as conducted in the present study), involving several aromatic hydrocarbons, competitive inhibition among the various test chemicals may cause biotransformation rates to be lower than in single chemical exposure studies. Competitive inhibition of biotransformation rates of benzo[*a*]pyrene, chrysene, and 9-methylanthracene has been observed in in vitro biotransformation studies involving S9 rainbow trout liver homogenates [25]. It may also be possible that due to the common practice of analyzing whole fish (fish body and intestines), the biotransformation rates used to develop the QSARs may have been influenced by biotransformation in the gastrointestinal tract.

Gastrointestinal biotransformation

Nonlinear regression of the reciprocal of observed dietary uptake efficiencies of nonbiotransformable reference chemicals ($E_{D,N}$) as a function K_{OW} using Equation 11 produced the following relationship (Figure 4):

$$E_{DN}{}^{-1} = 5.6.10^{-9}(\text{SE } 1.8.10^{-9})K_{OW} + 1.9(\text{SE } 0.1) \quad (32)$$

This relationship is similar to the relationship between the dietary uptake efficiency and K_{OW} observed in previous studies for similar chemical substances [13,18] administered in the diet over a prolonged period of time. The basic relationship is also apparent in a recent study by Xiao et al. [26], who reported benchmarked dietary uptake efficiencies and applied a different methodology to determine dietary uptake efficiencies than that used in the present study. Figure 4 shows that the mean dietary uptake efficiency for nonbiotransformable chemicals is approximately constant at 52% (SE 4) for substances with a log K_{OW} up to approximately 7 and then declines with increasing log K_{OW}. Figure 4 shows that with the exception of 1,2,3,4-tetramethyl benzene and β-HCH, all test chemicals exhibit dietary uptake efficiencies ($E_{D,M}$) that are significantly smaller than those derived by Equation 32 for the same chemical in absence of gastrointestinal biotransformation. The highest dietary uptake efficiency (55% [SE 8]) was observed for β-HCH and was not significantly different ($p > 0.05$) from that derived by Equation 32 for the predicted reference compound at the same log K_{OW}. The smallest dietary uptake efficiencies were observed for benzo[*a*]pyrene (2.3% [SE 1.2]) and chrysene (4.9% [SE 1.4]). These findings are in good agreement with several studies observing low dietary uptake efficiencies of benzo[*a*]pyrene [25,27,28], 9-methyl anthracene [29], and related substances [29] in fish and trophic dilution in field studies [30,31,32]. The

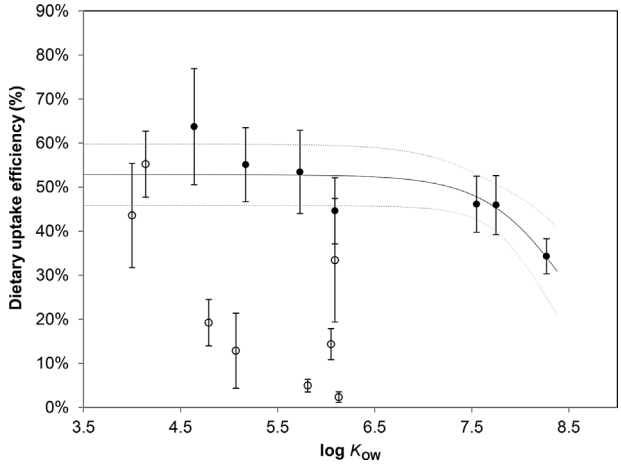

Figure 4. Dietary uptake efficiencies of the reference chemicals ($E_{D,N}$, filled round circles) and test chemicals ($E_{D,M}$, open round circles) versus log K_{OW} (standard errors reported in error bars). Test chemicals from left to right: 1,2,3,4-tetramethylbenzene, β-hexachlorocyclohexane, trans-decalin, 9-methylanthracene, chrysene, hexylcyclohexane, 2,6-dimethyldecane, and benzo[*a*]pyrene. The line represents nonlinear regression fit to the dietary uptake efficiency data of the reference chemicals (Equation 32). The dotted lines represent the 95% confidence intervals for the predicted mean.

low dietary uptake efficiencies of 6 of the 8 test chemicals relative to those of reference chemicals indicate significant gastrointestinal biotransformation of these test chemicals. Gastrointestinal biotransformation rate constants (k_{GM}) for these substances can be derived from the dietary uptake efficiencies of the test and reference chemicals according to Equation 14 and Equation 15, if the fecal egestion rate G_{GE} and W_G are known. Equation 16 indicates that throughout the uptake phase of the experiment in which fish exhibit an average weight of 37 g, G_{GE} was $(1-0.53)\times 0.0116\times 37 = 0.20$ g digesta dry weight/d^{-1} and W_G was approximately 0.20 g. Intestinal biotransformation rate constants ranged between 0 d^{-1} (for β-HCH) to 46 d^{-1} (Table 2) and were, with the exception of k_{GM} for β-HCH, greater than their corresponding somatic biotransformation rate constants (k_{BM}). A direct comparison between k_{GM} and k_{BM}, however, is not meaningful because the rate constants apply to different compartments, that is, the digesta in the intestinal tract for k_{GM} and the fish's body weight for k_{BM}. To estimate the relative importance of somatic and gastrointestinal biotransformation, k_{GM} and k_{BM} need to be multiplied by the mass of test chemical in the gastrointestinal tract M_G and in the fish body weight M_B, respectively, which can be derived at steady state from the empirical observations according to Equations 19 and 20. Figure 5, which shows the relative importance of somatic and intestinal biotransformation at steady state as ϕ_{BM}, suggests that for all test chemicals except β-HCH, gastrointestinal biotransformation is the main contributor to biotransformation within the fish. In the gastrointestinal tract of the fish, β-HCH did not appear to be biotransformed. In certain terrestrial organisms, β-HCH is a substance that is known to biomagnify, but not in fish due to a high degree of elimination through respiratory ventilation of water [33]. Gastrointestinal biotransformation accounted for 63% of the total biotransformation for 1,2,3,4-tetramethylbenzene; 64% for 2,6-dimethyldecane; 87% for 9-methylantharcene; 92% for hexylcyclohexane; 95% for chrysene; and 98% for benzo[*a*]pyrene. Supplemental Data, Figure S3, shows that for substances that were biotransformed in both the gastrointestinal tract and in the body of the fish, there is a weak and statistically insignificant ($p = 0.059$, $n = 6$) correlation between somatic and gastrointestinal biotransformation rates (g parent test chemical biotransformed.d^{-1}), but this correlation does not apply to trans-decalin and β-HCH. Supplemental Data, Figure S3, suggests that somatic biotransformation rates may, in some cases, be indicative of gastrointestinal biotransformation rates, but that there can also be distinct differences in the capacity for biotransformation between the fish's body and the gastrointestinal tract. Supplemental Data, Figure S4, shows that there was no correlation between k_{BM} and K_{OW} or between k_{GM} and K_{OW}.

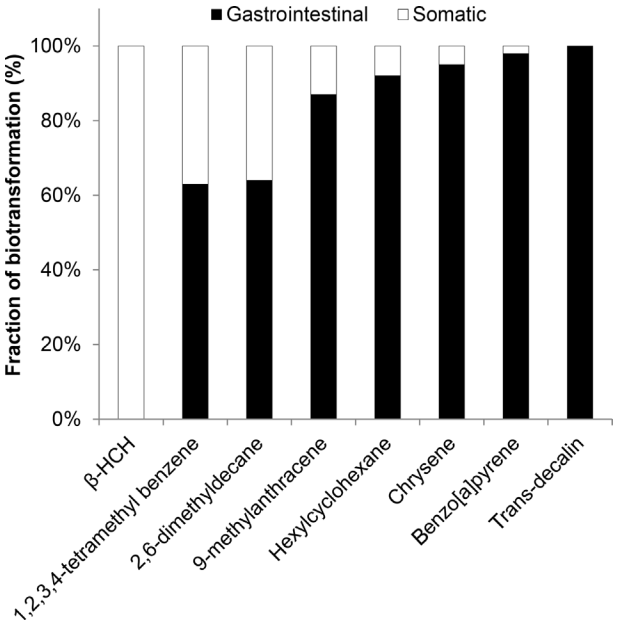

Figure 5. Contribution of somatic (black) and gastrointestinal (white) biotransformation to the overall mass of chemical biotransformed. β-HCH = β-hexachlorocyclohexane.

The observation that gastrointestinal biotransformation exceeds somatic (including hepatic) biotransformation challenges the common presumption that the liver is the main site of biotransformation in fish. Although the liver is the main site for biotransformation of many pharmaceutical drugs dosed via the blood or respiratory route (e.g., gills in fish), this may not be the case for many bioaccumulative substances that are primarily absorbed via the diet.

The apparent dominant role of gastrointestinal biotransformation in the biotransformation of most of the hydrophobic test substances in the present study may point to the different roles that lipids play in the gastrointestinal tract and in the body of the fish. In the body of the fish, lipids function as storage compartments of very hydrophobic substances that reduce the bioavailability of very hydrophobic compounds to biotransforming enzymes. In the gastrointestinal tract, lipids increase the availability of compounds dissolved in the lipids to biotransforming enzymes and microorganisms due to their high degree of digestion and absorption [18], which makes hydrophobic chemicals present in dietary lipids available to gastrointestinal microflora and digestive enzymes.

Biotransformation in the gastrointestinal tract lowers the gastrointestinal magnification that can occur. Equation 28 illustrates that under the experimental conditions of the test, gastrointestinal magnification can be prevented if k^*_{GM} exceeds a value of approximately $(0.0116\times 37/0.20)(2.85-0.47) = 5.1\ d^{-1}$, which in the present study corresponds with a dietary uptake efficiency lower than approximately 13% for substances with a log K_{OW} up to 7. The measurement of the dietary uptake efficiency can be used to identify substances that lack the ability to biomagnify in fish due to biotransformation in the gastrointestinal tract. The measurement of k_{BT} identifies substances that lack the ability to biomagnify in fish due to their ability to be biotransformed in the body of the fish. Substances that are resistant to both gastrointestinal and somatic biotransformation and also eliminate and excrete slowly (e.g., substances with a log $K_{OW} > 5$) can be expected to have biomagnification potential.

Biomagnification factors

Figure 6 illustrates that the BMFs of the reference chemicals increase with increasing log K_{OW} from approximately 0.47 for 1,2,4,5-tetrachlorobenzene to values as high as 22 for PCB 153. The BMFs for the highest K_{OW} chemicals, in particular PCB 52, PCB 153, and PCB 155 are subject to large errors due to the large error in the measurement of the depuration rate constant in fish body resulting from the very slow depuration rate. The lipid normalized BMFs (kg lipid/kg lipid) of the test chemicals are all far below 1, illustrating that both somatic and intestinal biotransformation can prevent biomagnification of the parent substance.

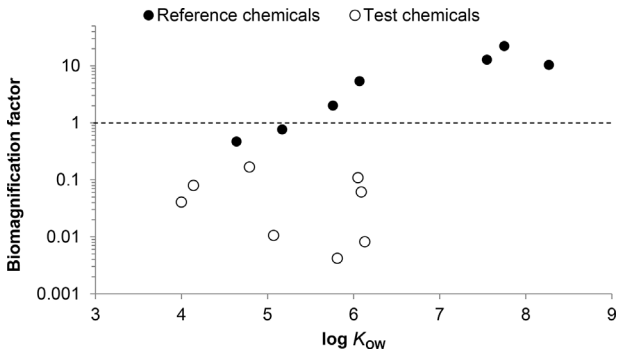

Figure 6. Lipid normalized log biomagnification factor (BMF) versus log K_{OW} for reference and test chemicals. The BMF was calculated as $(0.0116 \times E_D \times \varphi_{LD}) / (k_{BT} \times \varphi_{LB})$.

Regulatory implications

The large contribution of gastrointestinal biotransformation to the overall biotransformation of the majority of the test chemicals in the present study has implications for regulatory bioaccumulation screening of commercial chemicals.

First, the results show that current bioconcentration tests cannot account for the full degree of biotransformation that chemical substances experience in fish. This is because gastrointestinal biotransformation does not occur in bioconcentration tests to the same degree as in biomagnification tests. In the environment, however, many hydrophobic, potentially bioaccumulative substances are predominantly absorbed via the diet [34,35]. Bioconcentration tests may provide inaccurate estimates of the biomagnification potential of a chemical if the chemical is subject to significant biotransformation in the gastrointestinal tract. For example, in the present study, trans-decalin appeared not to be biotransformed in the fish's body, whereas a high degree of gastrointestinal biotransformation was observed. The gastrointestinal biotransformation rate constant of trans-decalin was not significantly different from the rate constant required to prevent gastrointestinal magnification in the fish and hence avoid biomagnification. High K_{OW} chemicals (e.g., log $K_{OW} > 5$), which eliminate and excrete slowly from the fish's body but that are rapidly biotransformed in the gastrointestinal tract but not in the body of the fish may therefore produce high BCFs in bioconcentration tests but cannot biomagnify. The application of a dietary bioaccumulation test using reference chemicals as described in the present study may provide the additional insights to distinguish between chemicals that can and cannot biomagnify. The recently revamped OECD 305 protocol for a bioconcentration test already includes a dietary study protocol that is similar in design as the present study and also recommends the use of reference substances. In the OECD 305 protocol, reference substances are primarily used to confirm that the method used for spiking food with test chemicals is adequate to achieve homogeneity and bioavailability of the test substances. As the present study illustrates, reference substances can also aid in measuring in vivo biotransformation rates of the test chemicals. The broader application of reference chemicals may provide a feasible extension of the existing protocol, which can yield in vivo biotransformation rate data that are invaluable for developing QSARs for biotransformation and the testing of in vitro-in vivo biotransformation rate extrapolation methods.

Second, the methodology used to derive biotransformation rate QSARs from bioconcentration factors derived from bioconcentration tests (which do not involve dietary exposure) may be appropriate for estimating BCFs, but may underestimate the contribution of biotransformation to the biomagnification process. Perhaps, QSARs for gastrointestinal biotransformation can be developed based on measured dietary absorption efficiencies. These QSARs can be useful for identifying potentially biomagnifying substances.

Third, the application of in vitro bioassays using hepatic media such as liver S9 homogenates and hepatocytes may be appropriate for the estimation of BCFs, but they are likely inadequate for estimating the BMFs for many chemicals, especially those that are biotransformed in the gastrointestinal tract. Developing methods to measure in vitro gastrointestinal biotransformation rates can be suggested as an important area of research to further strengthen bioaccumulation screening.

SUPPLEMENTAL DATA

Table S1.
Figures S1–S5.
Equations S1–S19. (570 KB DOC).

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Data availability—Data, associated metadata, and calculation tools are available in the Supplemental Data.

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