SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 34, No. 12, pp. 2782–2790, 2015

Printed in the USA

CONCENTRATION DEPENDENCE OF BIOTRANSFORMATION IN FISH LIVER S9: OPTIMIZING SUBSTRATE CONCENTRATIONS TO ESTIMATE HEPATIC CLEARANCE FOR BIOACCUMULATION ASSESSMENT

JUSTIN C. LO,† GAYATRI N. ALLARD,§ S.VICTORIA OTTON,§ DAVID A. CAMPBELL,‡ and FRANK A.P.C. GOBAS*†§
†Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada
‡Department of Statistics and Actuarial Science, Simon Fraser University, Surrey, British Columbia, Canada
§School of Resource and Environmental Management, Simon Fraser University, Burnaby, British Columbia, Canada

(Submitted 17 December 2014; Returned for Revision 8 February 2015; Accepted 9 June 2015)

Abstract: In vitro bioassays to estimate biotransformation rate constants of contaminants in fish are currently being investigated to improve bioaccumulation assessments of hydrophobic contaminants. The present study investigates the relationship between chemical substrate concentration and in vitro biotransformation rate of 4 environmental contaminants (9-methylanthracene, pyrene, chrysene, and benzo[*a*]pyrene) in rainbow trout (*Oncorhynchus mykiss*) liver S9 fractions and methods to determine maximum first-order biotransformation rate constants. Substrate depletion experiments using a series of initial substrate concentrations showed that in vitro biotransformation rates exhibit strong concentration dependence, consistent with a Michaelis–Menten kinetic model. The results indicate that depletion rate constants measured at initial substrate concentrations of 1 μM (a current convention) could underestimate the in vitro biotransformation potential and may cause bioconcentration factors to be overestimated if in vitro biotransformation rates are used to assess bioconcentration factors in fish. Depletion rate constants measured using thin-film sorbent dosing experiments were not statistically different from the maximum depletion rate constants derived using a series of solvent delivery–based depletion experiments for 3 of the 4 test chemicals. Multiple solvent delivery–based depletion experiments at a range of initial concentrations are recommended for determining the concentration dependence of in vitro biotransformation rates in fish liver fractions, whereas a single sorbent phase dosing experiment may be able to provide reasonable approximations of maximum depletion rates of very hydrophobic substances. *Environ Toxicol Chem* 2015;34:2782–2790. © 2015 SETAC

Keywords: Biotransformation Bioaccumulation In vitro Hydrophobic chemicals Fish

INTRODUCTION

Bioaccumulation of organic contaminants is usually assessed using the octanol–water partition coefficient (K_{OW}) or the bioconcentration factor (BCF) in fish [1]. Because experimentally determined BCFs of most chemicals requiring evaluation are unknown, the bioaccumulation potential of chemicals is often assessed by K_{OW} or K_{OW}-based bioaccumulation models [1]. However, K_{OW} and K_{OW}-based bioaccumulation models cannot provide a priori estimates of biotransformation rates. There is interest in developing methods for assessing chemical biotransformation rates to avoid incorrectly classifying chemicals as bioaccumulative based solely on a high K_{OW} value. One approach is the development of quantitative structure–activity relationship (QSAR) models to predict biotransformation rates and corresponding BCFs based on chemical structure [2,3]. Another approach involves measuring biotransformation rates in vitro using subcellular liver preparations [4,5] or hepatocytes [6], followed by scaling and extrapolating the results to whole fish [7]. Compared with in vivo testing, in vitro testing is time- and cost-effective and reduces animal use [8].

In vitro biotransformation assays were first developed to estimate hepatic clearance rates of prospective drugs during the early stages of drug development [9–11]. In these assays, the initial increase in metabolite concentration, C_M (μM) (i.e., $\frac{dC_M}{dt}$ in units of μM min^{-1}), at various substrate concentrations in the incubation medium, C_I (μM), is measured, and kinetic parameters are determined by fitting the concentration data to the classical Michaelis–Menten equation

$$\frac{dC_M}{dt} = \frac{V_{MAX} \times C_I}{K_M + C_I} \quad (1)$$

where V_{MAX} (μM min^{-1}) is the maximum formation rate of the metabolite, and K_M is the Michaelis constant (μM) for the reaction (i.e., the substrate concentration at $0.5\,V_{MAX}$). This method requires knowledge of the major metabolite formed and the analytical tools (including authentic standards) for their quantification. However, the metabolic pathways of the vast majority of environmental chemicals requiring bioaccumulation assessment are unknown. Furthermore, the formation of more than 1 metabolite may need to be characterized to obtain complete information on the chemical's metabolic stability.

An alternative method measures the rate of loss (or depletion) of the chemical in the incubation medium as a result of biotransformation [4–8,12]. Here, the first-order depletion rate constant, k_{dep} (min^{-1}), is measured as the slope of the relationship between the natural logarithm of the remaining substrate concentration in the incubation medium (C_I) at incubation time (t)

$$k_{dep} = \frac{\ln\left(\frac{C_{I,t=0}}{C_I}\right)}{t} \quad (2)$$

where $C_{I,t=0}$ is the initial substrate concentration in the incubation medium. The substrate depletion method has been

All Supplemental Data may be found in the online version of this article.
* Address correspondence to gobas@sfu.ca
Published online 15 June 2015 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.3117

proposed for measuring hepatic clearance rates of environmental contaminants whose metabolic pathways are unknown [4–8,12]. These studies were generally conducted using a starting substrate concentration of 1 μM. Johanning et al. [12] recommended that starting concentrations of test chemical ($C_{I,t=0}$) should be 1 μM or less. Such concentrations are assumed to be sufficiently below the K_M of the major biotransformation reaction(s) to provide a k_{dep} that reflects the initial reaction rate (i.e., the biotransformation rate at substrate concentrations that are well below enzyme saturation). To date, there has been no investigation of the potential dependence of a contaminant's in vitro biotransformation rate (as measured by k_{dep}) using a range of initial chemical concentrations.

Instead of relying on the assumption that $C_{I,t=0} << K_M$, in vitro depletion rates (k_{dep}) at multiple initial chemical concentrations can be determined and fitted to a rewritten form of the Michaelis–Menten equation as described by Obach and Reed-Hagen [13]

$$k_{dep} = k_{dep,C\rightarrow 0}\left(1 - \frac{C_{I,t=0}}{C_{I,t=0} + K_M}\right) \quad (3)$$

where $k_{dep,C\rightarrow 0}$ is the theoretical k_{dep} at an infinitesimally low substrate concentration. Equation 3 describes a decrease of k_{dep} as $C_{I,t=0}$ increases through the range of the K_M value and approaches 0 when the enzymes are presumably saturated. The inflection point in the fitted curve represents K_M. This is the substrate concentration that corresponds with half of the maximum depletion rate constant ($k_{dep,C\rightarrow 0}$). Equation 3 can be solved to yield both K_M and $k_{dep,C\rightarrow 0}$. If the depletion rate represents a simple 1-enzyme, 1-product type of metabolic reaction, then the K_M value is theoretically identical to the K_M determined in classical product formation assays [14]. This has been observed to be the case for drugs oxidized by predominantly 1 cytochrome P450 enzyme to form 1 product [13,15,16]. However, for a chemical metabolized by multiple enzymes present in a liver preparation, the K_M determined from substrate depletion experiments represents a composite of the K_M values of all the major metabolic reactions of the test chemical.

A method developed in our laboratory for measuring in vitro biotransformation rates of highly hydrophobic chemicals employs sorbent-phase dosing [17,18]. In this method, the test chemical is dissolved in a thin-film ethylene vinyl acetate (EVA) sorbent phase and exposed to the enzyme preparation. The chemical is delivered solvent-free via passive diffusion into the incubation medium over the course of the incubation. The thin-film configuration intends to provide a high surface to volume ratio to accelerate the release of the substrate in the incubation medium. A high surface area to volume relationship is of particular importance for very hydrophobic substrates, because they diffuse very slowly from the sorbent phase into the largely aqueous incubation medium. One relevant feature of this method is that the initial substrate concentration in the incubation medium is essentially 0, whereas in a conventional solvent delivery–based system the initial concentration is at its maximum value. Because of the low substrate concentrations in the incubation medium, the initial biotransformation rate can be expected to represent the maximum depletion rate constant, $k_{dep,C\rightarrow 0}$. This feature may provide a method for measuring biotransformation rates without the need to determine K_M and the associated concentration dependence of the biotransformation rate. In a sorbent delivery–based test, the transfer of the test chemical between the sorbent (EVA in the present study) and the incubation medium can be described by a 2-compartment mass-transfer model

$$\frac{dC_E}{dt} = \left(k_2 \times \frac{V_I}{V_E} \times C_I\right) - (k_1 \times C_E) \quad (4)$$

$$\frac{dC_I}{dt} = \left(k_1 \times \frac{V_E}{V_I} \times C_E\right) - (k_2 + k_{dep,EVA})C_I \quad (5)$$

where C_E and C_I are the chemical substrate concentrations (mol/m^3) in the EVA and the incubation medium, respectively; V_E and V_I are the volumes (m^3) of the EVA and the incubation medium; k_1 and k_2 are the mass transfer rate constants (min^{-1}) between the EVA and incubation medium; and $k_{dep,EVA}$ is the in vitro biotransformation rate constant (min^{-1}) derived in EVA thin-film sorbent delivery experiments. The value of $k_{dep,EVA}$ is derived from the mass transfer constants between the 2 phases as described by Lee et al. [17]. By adding the appropriate amount of test chemical to the sorbent phase, the maximum possible concentration in the incubation medium can be controlled to avoid exceeding a specific target value (e.g., 1 μM). Hence, if K_M is known, experiments can be conducted where C_I does not exceed K_M.

The main objective of the present study is to investigate the dependence on substrate concentration of in vitro biotransformation rates of several very hydrophobic test chemicals. To investigate this, depletion rate constants are measured at multiple substrate concentrations through multiple conventional solvent delivery–based tests and fitted to a nonlinear model (Equation 3) to determine kinetic parameters for the hydrophobic test chemicals. Using the same S9, depletion rate constants are measured using a sorbent-phase dosing system [17,18] and compared with the kinetic parameters estimated from the solvent delivery–based tests. The second objective of the present study is to explore how a sorbent-phase dosing method may avoid the scientific challenges presented by concentration dependence compared with a solvent delivery dosing system. The ultimate goal of this research is to improve bioaccumulation assessments of environmental contaminants.

MATERIALS AND METHODS

Chemicals

The test chemicals 9-methylathracene (log $K_{OW} = 5.07$) [19], pyrene (log $K_{OW} = 5.18$) [20], chrysene (log $K_{OW} = 5.81$) [21], benzo[*a*]pyrene (log $K_{OW} = 6.13$) [21] and the internal standard chrysene-d^{12}, methanol, high-performance liquid chromatography (HPLC)-grade n-hexane, and β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt hydrate (β-NADPH; ≥97% purity) were purchased from Sigma-Aldrich. Ethylene vinyl acetate (Elvax 40W) was obtained from DuPont. Potassium dihydrogen phosphate and HPLC-grade acetonitrile were from Caledon Laboratories. Potassium phosphate dibasic was obtained from Anachemia Canada. Potassium chloride was purchased from EMD Millipore. Unless specified, all other materials were purchased from Sigma-Aldrich.

Animals

Eight rainbow trout (*Oncorhynchus mykiss*) were obtained from Miracle Springs fish hatchery. The fish were held in a flow-through tank supplied with dechlorinated water.

Water temperatures were kept at 12.5 °C to 14.5 °C (mean temperature = 13.5 °C). The holding environment followed a 16:8-h light:dark cycle. Fish were acclimatized for more than 2 wk and fed EWOS Pacific 3.0-mm pellets at a rate of 1% body weight d^{-1}. The average body weight was 386 ± 68 g (standard deviation [SD], $n = 8$) at the time of euthanasia.

Liver S9 preparation

Fish were euthanized by a 5-min exposure to 0.3 g L^{-1} tricaine methanesulfonate (MS 222) buffered with 0.3 g L^{-1} sodium bicarbonate. Exposure to this concentration of MS222 for 5 min has been shown to have no significant effect on microsomal cytochrome P450 activities in rainbow trout [22]. The fish livers were excised and immediately rinsed in ice-cold 1.15% (w/v) KCl. The liver weights were 5.3 ± 1.0 g ($n = 8$). The livers were minced on ice with a razor blade and homogenized using a Potter–Elvehjem glass tissue grinder with a Teflon pestle (Kontes) in 7 volumes (g mL^{-1}) of ice-cold 0.2 M phosphate buffer (pH 7.4) containing 1.15% (w/v) KCl. The liver homogenates were centrifuged at 10 000 *g* for 20 min at 4 °C (Hermle Z360K centrifuge). Following centrifugation, the top lipid layer was carefully removed and discarded, and the postmitochondrial supernatant fraction (S9) was collected and stored in aliquots at –80 °C until use (held for <3 mo). The protein concentration of the S9 was determined by the method of Bradford [23] using bovine serum albumin (fraction V) as the standard.

Solvent delivery dosing preparation

Stock solutions of 9-methylanthracene, pyrene, chrysene, and benzo[*a*]pyrene were prepared in acetonitrile and further diluted in acetonitrile, resulting in substrate concentrations in the incubation medium of 0.056 μM, 0.01 μM, 0.18 μM, 0.32 μM, 0.56 μM, 1.0 μM, 1.78 μM, 3.16 μM, 5.62 μM, 10 μM, and 18 μM. The final volume of acetonitrile in the incubation mixture was <0.5% (v/v).

Sorbent-phase dosing preparation

An EVA solution of 0.06735 g L^{-1} in dichloromethane was prepared. The test substrates 9-methylanthracene, pyrene, chrysene, or benzo[*a*]pyrene were added to the EVA solution at 10 μM, 5 μM, 10 μM, and 25 μM, respectively. Each substrate was incubated individually. A 50-μL volume of the spiked EVA solution was transferred to a 2-mL silanized amber glass vial (Agilent). Uncapped, the vial was rolled to evaporate the solvent. This resulted in 0.0035 μL (3.4 μg) of EVA in the vial and an estimated EVA film thickness of 4 nm (calculated by dividing the volume of EVA film by the interior surface of the test vial). Assuming that the entire chemical mass in the sorbent phase was released into the incubation medium (0.5 mL), this corresponded to maximum possible concentrations in the incubation medium of 1.0 μM for 9-methylanthracene and chrysene, 0.5 μM for pyrene, and 2.5 μM for benzo[*a*]pyrene.

Selection of sampling time points

In preliminary depletion experiments at an initial substrate concentration of 0.5 μM, substrate concentrations declined in a log-linear fashion for a period of 80 min. Therefore, incubations were conducted for 80 min or less if the concentration fell below the detection limit. In preliminary sorbent-phase delivery experiments with inactive S9, k_1 and k_2 rate constants for the 4 test chemicals were measured. These values were then used in simulations to select 11 sampling time points that produced the smallest variance in $k_{dep,EVA}$ estimates for a range of possible $k_{dep,EVA}$ values. The remaining 9 incubation time points were selected at regular intervals at 0 min, 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 70 min, and 80 min, for a total of 20 incubation times ($n = 20$) per sorbent-phase delivery experiment.

Incubations

All in vitro assays in the present study used a single S9 homogenate, pooled from 8 fish. Multiple ($n = 20$) independent incubations were carried out to measure the decline of the test chemical concentration for each chemical over time. A single S9 homogenate was used to remove confounding variables resulting from differences in enzymatic activities among S9 liver homogenates used for sorbent and solvent delivery–based experiments. Each test chemical was incubated individually (not as mixture). Incubations were not subsampled. The incubations were done in a cold room maintained at 12.9 °C to 14.2 °C. In solvent delivery experiments, the test substrate dissolved in acetonitrile was transferred to a 2-mL amber glass vial (Agilent) containing 300 μL of incubation buffer (0.2 M, pH 7.4 phosphate buffer containing 1.15% [w/v] KCl and 1.5 mM β-NADPH). The reaction was started by adding 200 μL of pooled S9. In sorbent-phase dosing experiments, 300 μL of incubation buffer (containing 1.5 mM β-NADPH) and 200 μL of pooled S9 were added to the EVA-coated vials to start the reaction. The vials were capped with polytetrafluoroethylene-lined screw caps (Agilent) and rolled horizontally at 60 rpm on a rocker/roller (Stuart SRT9D). Incubations where the test chemical in acetonitrile was added to the liver homogenate produced depletion rates that were not significantly different from those where the liver homogenate was added to the test chemical solution (Supplemental Data, Figure S1). In solvent delivery experiments, the incubation reactions were stopped by adding 200 μL of ice-cold methanol to the reaction vial, followed by shaking on a vortex mixer for 20 s. In sorbent-phase dosing experiments, the incubation reactions were stopped by transferring 400 μL of the incubation medium to a 2-mL amber glass vial (Agilent) containing 200 μL ice-cold methanol and shaken on a vortex mixer for 20 s. The remaining incubation medium (100 μL) was removed from the EVA-coated vial. The EVA film was then rinsed 4 times with 1.0 mL of deionized water. A no-cofactor control experiment using inactive liver S9 (left at room temperature overnight and with no β-NADPH included in the incubation mixture) was conducted in parallel with each test system. Two EVA-coated vials with no added incubation medium were included to determine the presence of initial concentrations of the test substrate in the EVA thin films.

Chemical extraction

Following the termination of the incubation in solvent delivery experiments, the internal standard (0.5 nmol chrysene-d^{12} dissolved in 10 μL of methanol) was added to the test vial and mixed using a vortex mixer for 30 s. Then 1.0 mL of n-hexane was transferred to the vial and shaken on a vortex mixer for 1 min to extract the test chemical and internal standard. The same procedure was carried out for the vials containing the incubation media from the sorbent-phase dosing experiments. For the EVA-coated test vials, 1.0 mL of n-hexane containing the internal standard (0.5 nmol chrysene-d^{12}) was transferred to the vial. Following the hexane extraction, the vials were centrifuged at 1560 *g* for 10 min (IEC Centra-CL2; Thermo Scientific). The upper organic layer was transferred to a 2-mL amber glass vial (Agilent) for gas chromatography/

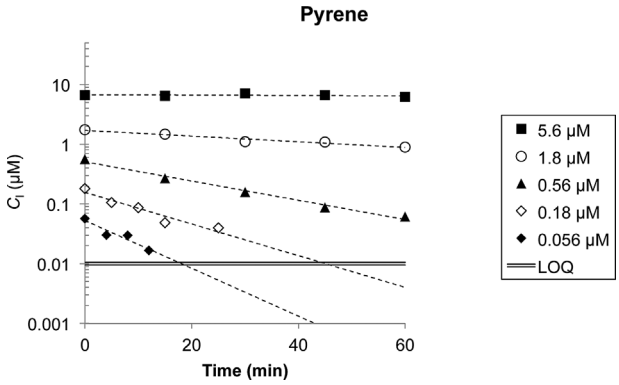

Figure 1. Decline of the pyrene concentration in the incubation medium for different initial substrate concentrations in the incubation medium (C_I) in solvent delivery dosing experiments. The limit of quantification (LOQ) is illustrated by the double solid line.

mass spectrometry (GC/MS) analysis. In the sorbent dosing experiments, chemical concentrations were measured in both the EVA films and the incubation media.

GC/MS analysis

Test chemicals were analyzed using an Agilent 6890 GC coupled to an Agilent 5973 MS and an Agilent 7683 autosampler. The GC was fitted with a cool-on-column capillary inlet, and the injection volume was 1 μL. Chemicals were separated on an HP-5MS 5% phenyl methylpolysiloxane-coated column (30 m × 0.25 mm inner diameter, 0.25 μm film thickness) connected to a fused-silica deactivated guard column (5 m × 0.53 mm inner diameter). The oven was held at an initial temperature of 60 °C for 0.5 min, then increased at 25 °C min^{-1} to 200 °C (held for 0.5 min), followed by an increase at 20 °C min^{-1} to a final temperature of 300 °C (held for 4 min for 9-methylathracene, pyrene, and chrysene, and for 8 min for benzo[*a*]pyrene analysis). Helium was used as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. Conditions for MS measurements were as follows: electron impact ionization at 70 eV; ion source temperature at 230 °C; and selected ions at mass-to-charge ratios of 192 (9-methylanthracene), 202 (pyrene), 228 (chrysene), 240 (chrysene-d^{12}), and 252 (benzo[*a*]pyrene). Agilent MSD ChemStation software (G1701CA) was used for instrument control and data processing. An 8-point calibration curve (concentration range 0.005–5 μM) was constructed and run for each chemical. Strong linearity ($r^2 > 0.99$) was shown in the calibration curves, with constant relative response factor values obtained over the range. The limits of quantification for 9-methylanthracene, pyrene, chrysene, and benzo[*a*]pyrene were approximately 0.02 μM, 0.01 μM, 0.01 μM, and 0.03 μM, during the solvent delivery dosing experiments and 0.1 μM, 0.5 μM, 0.03 μM, and 0.05 μM during the EVA sorbent-phase dosing experiments, respectively.

Kinetic analyses

First-order depletion rate constants (k_{dep}) were determined as the slope of the regression lines relating the natural logarithm of the remaining substrate concentration to incubation time according to Equation 2. Maximum depletion rate constants at infinitesimally low substrate concentrations ($k_{dep,C\to 0}$) and the apparent K_M were determined by fitting Equation 3 to k_{dep} estimates measured over a range of substrate concentrations. Because the k_{dep} values estimated at low and high initial substrate concentrations do not carry the same precision, each k_{dep} estimate was weighted by the reciprocal of the standard error of the estimate. A weighted nonlinear least squares regression was used to get the best fit of the k_{dep} data to its corresponding starting substrate concentrations. This weighted nonlinear least squares analysis followed the Analytic Gauss–Newton algorithm in JMP 10.

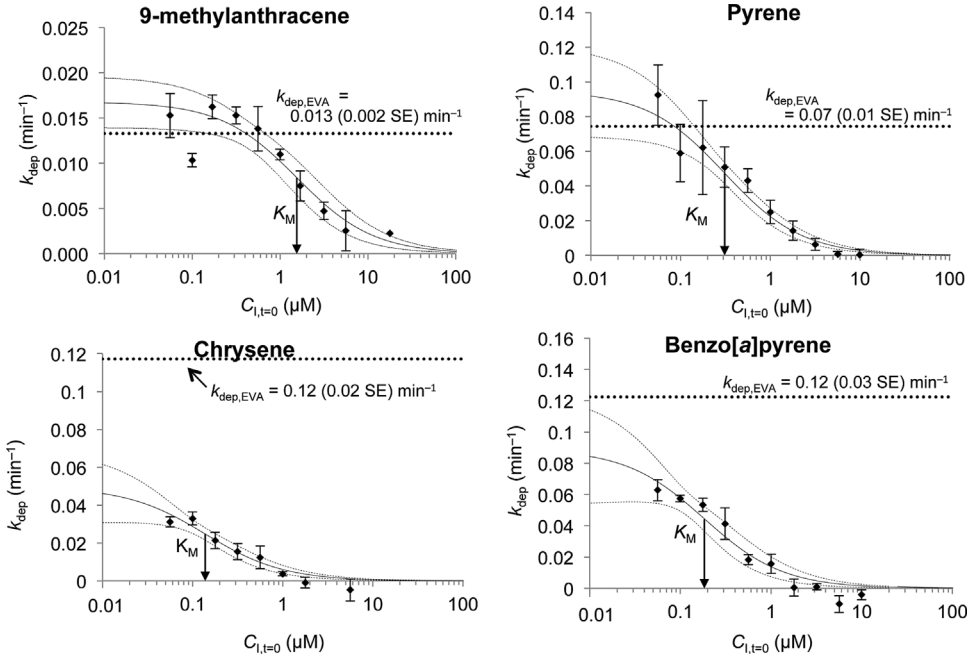

Figure 2. Depletion rate constants (k_{dep}) versus the initial substrate concentrations ($C_{I,t=0}$) in solvent delivery dosing experiments for 9-methylanthracene, pyrene, chrysene, and benzo[*a*]pyrene. Error bars represent the standard errors of the mean. Dashed lines represent the 95% confidence interval of the mean k_{dep} estimate. The arrow presents the apparent Michaelis constant (K_M). The dotted line represents the depletion rate constant estimated from the ethylene vinyl acetate (EVA) sorbent-phase delivery dosing method results ($k_{dep,EVA}$).

Table 1. Comparison of the depletion rate constants (min^{-1}) of 4 hydrophobic test chemicals at 1 μM substrate concentration (k_{dep}), the maximum depletion rate constants ($k_{dep,C\to0}$), the apparent Michaelis constants (K_M), and the depletion rate constants measured in thin-film ethylene vinyl acetate sorbent-phase delivery dosing experiments ($k_{dep,EVA}$)[a]

Substrate	k_{dep} (1 μM)	$k_{dep,C\to0}$ (min^{-1})	K_M (μM)	$k_{dep,EVA}$ (min^{-1})
9-Methylanthracene	0.011 (0.002)	0.017 (0.001)	1.6 (0.4)	0.013 (0.002)
Pyrene	0.021 (0.007)	0.09 (0.01)	0.31 (0.08)	0.07 (0.01)
Chrysene	0.004 (0.001)	0.049 (0.008)	0.14 (0.05)	0.12 (0.02)
Benzo[*a*]pyrene	0.016 (0.006)	0.09 (0.02)	0.18 (0.08)	0.12 (0.03)

[a]Error is represented by the standard error (SE) of the mean, in parentheses.

In sorbent-phase dosing experiments, the observed substrate concentrations in the EVA (C_E) and in the incubation media (C_I) for the inactive (control) and active S9 (test) incubations were plotted versus time. To estimate the mass-transfer rate constants (k_1, k_2) and the in vitro depletion rate constant ($k_{dep,EVA}$), the data were fitted by the model described in Equations 4 and 5 through nonlinear least squares. Because the control and test for both the EVA and the incubation medium phases share the same parameters, all 4 data sources (i.e., EVA-control, EVA-test, media-control, media-test) were combined to obtain the least squares parameter estimates. This nonlinear least squares analysis was solved using MATLAB R2014a (Mathworks). To avoid suboptimal parameter estimates as a result of a potentially multimodal likelihood, multiple random starting points for the parameters were selected for initializing the least squares algorithm, with the best overall least squares minima returned. Time 0 incubation media concentrations were fixed at 0 μM.

RESULTS AND DISCUSSION

S9 liver preparation

The protein concentration of the pooled S9 from 8 fish livers was 8.8 mg mL^{-1} (0.2 mg mL^{-1} standard error [SE], $n = 3$ measurements). Each incubation contained a final protein concentration of 3.5 mg mL^{-1}. Because the same S9 was used in all incubations, reported depletion rates are expressed in units of min^{-1} and are not normalized for protein. For all test chemicals, no biotransformation was observed in the control incubations (containing inactive S9 and no added β-NADPH). Extraction efficiencies of the 4 test chemicals from the incubation mixture and from the EVA thin films were not significantly different from 100%. Therefore, chemical concentrations were not corrected for extraction efficiency.

Solvent delivery dosing experiments

Figure 1 illustrates substrate depletion over time for pyrene. Similar depletion curves were observed for the other test chemicals (Supplemental Data, Figure S2). The concentration of the test chemicals declined in a log-linear fashion with time for all test chemicals and at all initial concentrations except for incubations with initial pyrene concentrations greater than 2 μM. At concentrations in excess of 2 μM, the enzyme(s) involved in the biotransformation reaction(s) were presumed to be saturated, and biotransformation had a negligible and undetectable effect on substrate concentrations. At initial pyrene concentrations less than 2 μM, the apparent first order substrate depletion rate, k_{dep}, increased with decreasing initial pyrene concentrations. A similar inverse relationship between the apparent first-order substrate depletion rate constant and the initial substrate concentration, consistent with Michaelis–Menten reaction kinetics, was observed for the other test chemicals.

Figure 2 shows the relationship between k_{dep} and $C_{I,t=0}$, and the good fit of the Michaelis–Menten kinetic model as formulated in Equation 3 to the biotransformation rate data. The apparent K_M and $k_{dep,C\to0}$ values were estimated through nonlinear regression of the data to the model. The $k_{dep,C\to0}$ values for 9-methylanthracene, pyrene, chrysene, and benzo[*a*]pyrene were estimated at 0.017 min^{-1} (0.001 min^{-1} SE), 0.09 min^{-1} (0.01 min^{-1}SE), 0.049 min^{-1} (0.008 min^{-1}SE), and 0.09 min^{-1} (0.02 min^{-1} SE), respectively. The apparent K_M values were 1.6 μM (0.4 μM SE) μM for 9-methylanthracene, 0.31 μM (0.08 μM SE) for pyrene, 0.14 μM (0.05 μM SE) for chrysene, and 0.18 μM (0.08 μM SE) for benzo[*a*]pyrene (Table 1). Despite a reasonable fit of the depletion rate constants for chrysene to the Michaelis–Menten model (Figure 2), $k_{dep,C\to0}$ estimates for chrysene should be interpreted with caution, as measurements of the depletion rate constants at the lowest concentrations could not be completed because concentrations were below the detection limit.

Figure 3 provides a comparison of the K_M of benzo[*a*]pyrene observed in the present study with values reported in other studies using fish liver. It shows that for a single chemical (benzo[*a*]pyrene), literature K_M values can vary by more than 3 orders of magnitude. Fitzsimmons et al. [24] made a similar observation. The large differences in reported K_M values can be the result of differences in the biotransformation study design (i.e., product formation vs substrate depletion), fish species, life

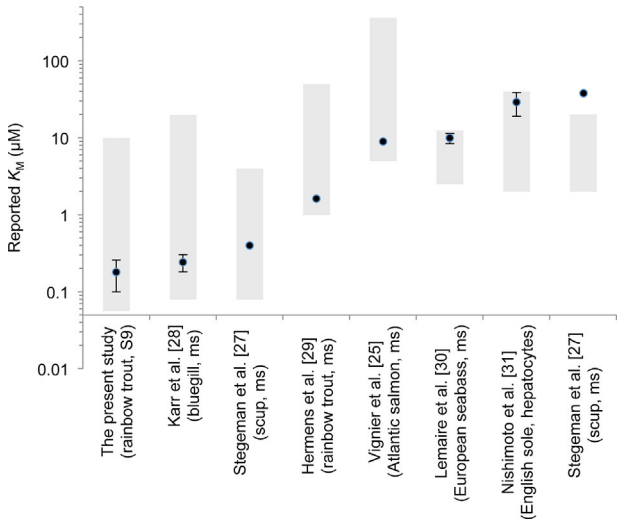

Figure 3. A comparison of the concentration range (shaded box) and apparent Michaelis constant (K_M) estimates of benzo[*a*]pyrene (circles) in various fish liver S9 and microsomal (ms) preparations. Error bars are standard errors of the mean. Results from the present study monitored the substrate depletion. All other studies measured the metabolite formation. Details of each study can be found in the Supplemental Data, Table S1.

Table 2. The thin film to incubation medium transfer rate constant (k_1; min^{-1}), the incubation medium to thin film transfer rate constant (k_2; min^{-1}), the initial chemical concentration in the ethylene vinyl acetate thin film ($C_{EVA,t=0}$, mM), and the fraction unbound in the incubation medium in the sorbent-phase delivery–based experiments

Substrate	k_1 (min^{-1})	k_2 (min^{-1})	$C_{EVA,t=0}$ (mM)	Fraction unbound[a]
9-Methylanthracene	0.29 (0.03)	0.009 (0.004)	117.5 (3.5)	0.038
Pyrene	0.16 (0.01)	0.017 (0.005)	47.0 (1.4)	0.014
Chrysene	0.065 (0.004)	0.020 (0.004)	108.5 (2.9)	0.025
Benzo[*a*]pyrene	0.0113 (0.0007)	0.0003 (0.0001)	495.1 (5.5)	0.0009

[a]The fraction unbound was calculated as K_{EI}/K_{EW}, where K_{EI} is the partition coefficient between the ethylene vinyl acetate (EVA) sorbent phase and the incubation medium and K_{EW} is the partition coefficient between EVA sorbent phase and water. K_{EI} was estimated as $k_2\ V_I/k_1\ V_{EVA}$, where $V_I = 0.5$ mL and $V_{EVA} = 3.5\ 10^{-6}$ mL. K_{EW} was calculated from K_{OW} using $\log K_{EW} = 1.04\ (\log K_{OW}) \pm 0.22$ in George et al. [32].

stage [25], thermal acclimation [26], the type of in vitro liver preparation (i.e., S9, microsomes, isolated hepatocytes), and the range of substrate concentrations tested. Interestingly, in the 3 studies (including the present study) that have investigated benzo[*a*]pyrene biotransformation at concentrations less than 1 μM, the apparent K_M measurements vary only 2.2-fold, despite the different fish species and liver preparations used. The other reports examined benzo[*a*]pyrene biotransformation at concentrations from 1 μM to 360 μM and would be unable to detect a K_M value of 0.18 μM (Table 1). The study by Stegeman et al. [27] is notable because the authors observed an apparent K_M of 0.4 μM when a range of low benzo[*a*]pyrene concentrations was examined and a K_M of 38 μM when incubations were done using a higher range of benzo[*a*]pyrene concentrations. Because of the many factors that may affect concentration dependence of the in vitro biotransformation rate, it is important to be cautious when relying on literature K_M measurements as a benchmark for selecting substrate concentrations for biotransformation assays. Conducting multiple depletion experiments using a range of initial substrate concentrations may be the most appropriate approach to obtain meaningful in vitro biotransformation rates for in vitro to in vivo extrapolation (Table 2).

Figure 4 shows that maximum, first-order depletion rate constants of pyrene, chrysene, and benzo[*a*]pyrene ($k_{dep,C\to0}$) were 4 to 12 times higher than k_{dep} measured at 1 μM initial substrate concentrations (values given in Table 1). An initial concentration of 1 μM or less has been suggested as a possible test concentration for measuring first-order depletion rate constants [12]. Only for 9-methylanthracene was the k_{dep}, determined at a 1 μM initial concentration ($0.017\ min^{-1}$), not significantly different from $k_{dep,C\to0}$ ($0.011\ min^{-1}$). This is because of the relatively high K_M of 9-methylanthracene (1.6 [0.4 SE] μM), which exceeds 1 μM. Figure 4 suggests that k_{dep} of these substrates measured at initial concentrations of 1 μM can substantially underestimate the chemical's in vitro biotransformation rate, which in turn can cause hepatic clearance rates to be underestimated and BCFs to be overestimated in in vitro to in vivo extrapolations.

Figure 5 illustrates the measured concentrations of the test chemicals in EVA thin films and in the incubation medium. Figure 5A, C, E, and G shows that concentrations of the test chemicals in the EVA declined over time as the chemicals were transferred to the incubation medium. There were no statistically significant differences between the EVA depletion curves with active and inactivated liver S9. This behavior has been observed before and is attributable to biotransformation rates exceeding the sorbent-phase delivery rates [17]. Figure 5B, D, F, and H shows that concentrations of the chemicals in the incubation medium initially increased with time (as test chemicals were transferred from the EVA into the incubation medium), then reached a maximum (as delivery rates matched biotransformation rates), and finally declined over time in active S9 (but not in inactive S9) as biotransformation rates exceeded thin-film delivery rates. For all test chemicals, there were distinct differences in the concentration–time curves for the active and inactivated S9 incubation media, indicating clear evidence of biotransformation. Figure 5B and D shows that pyrene and 9-methylanthracene concentrations in the active S9 liver media were well below the apparent K_M measured from the multiple depletion curve experiments. The sorbent dosing–derived depletion rate constants ($k_{dep,EVA}$) of $0.07\ min^{-1}$ ($0.01\ min^{-1}$SE) for pyrene and $0.013\ min^{-1}$ ($0.002\ min^{-1}$SE) for 9-methylanthracene from the EVA dosing experiments (Table 2) are not statistically different (Student's *t* test) from the $k_{dep,C\to0}$ of $0.09\ min^{-1}$ ($0.01\ min^{-1}$SE) and $0.017\ min^{-1}$ ($0.001\ min^{-1}$SE) discussed earlier (Table 1). Figures 5F and H show that concentrations of chrysene and benzo[*a*]pyrene in the active S9 liver media were at or below the apparent K_M value throughout most, but not all, of the incubation period. The benzo[*a*]pyrene depletion rate constant estimated from the EVA dosing experiment ($k_{dep,EVA}$) of $0.12\ min^{-1}$ ($0.03\ min^{-1}$SE) (Table 2) is also not statistically significantly different (Student's *t* test) from the maximum depletion rate ($k_{dep,C\to0}$) of $0.09\ min^{-1}$ ($0.02\ min^{-1}$SE). The depletion rate constant of chrysene of $0.12\ min^{-1}$ ($0.07\ min^{-1}$SE) derived from the EVA dosing experiment (Table 2) is greater and statistically significantly different from the $k_{dep,C\to0}$ of $0.049\ min^{-1}$ ($0.008\ min^{-1}$SE; Figure 5). This, we expect, may be attributable

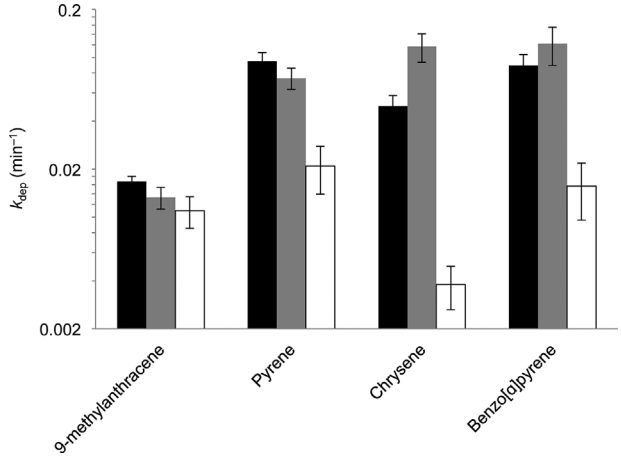

Figure 4. Comparison of the maximum depletion rate constant ($k_{dep,C\to0}$) estimated from the series of solvent delivery dosing experiments (black), the depletion rate constant ($k_{dep,EVA}$) estimated from the ethylene vinyl acetate (EVA) dosing experiments (gray), and the depletion rate constant (k_{dep}) estimated from a solvent delivery experiment at the initial substrate concentration of 1 μM (white). Error bars are standard errors of the mean.

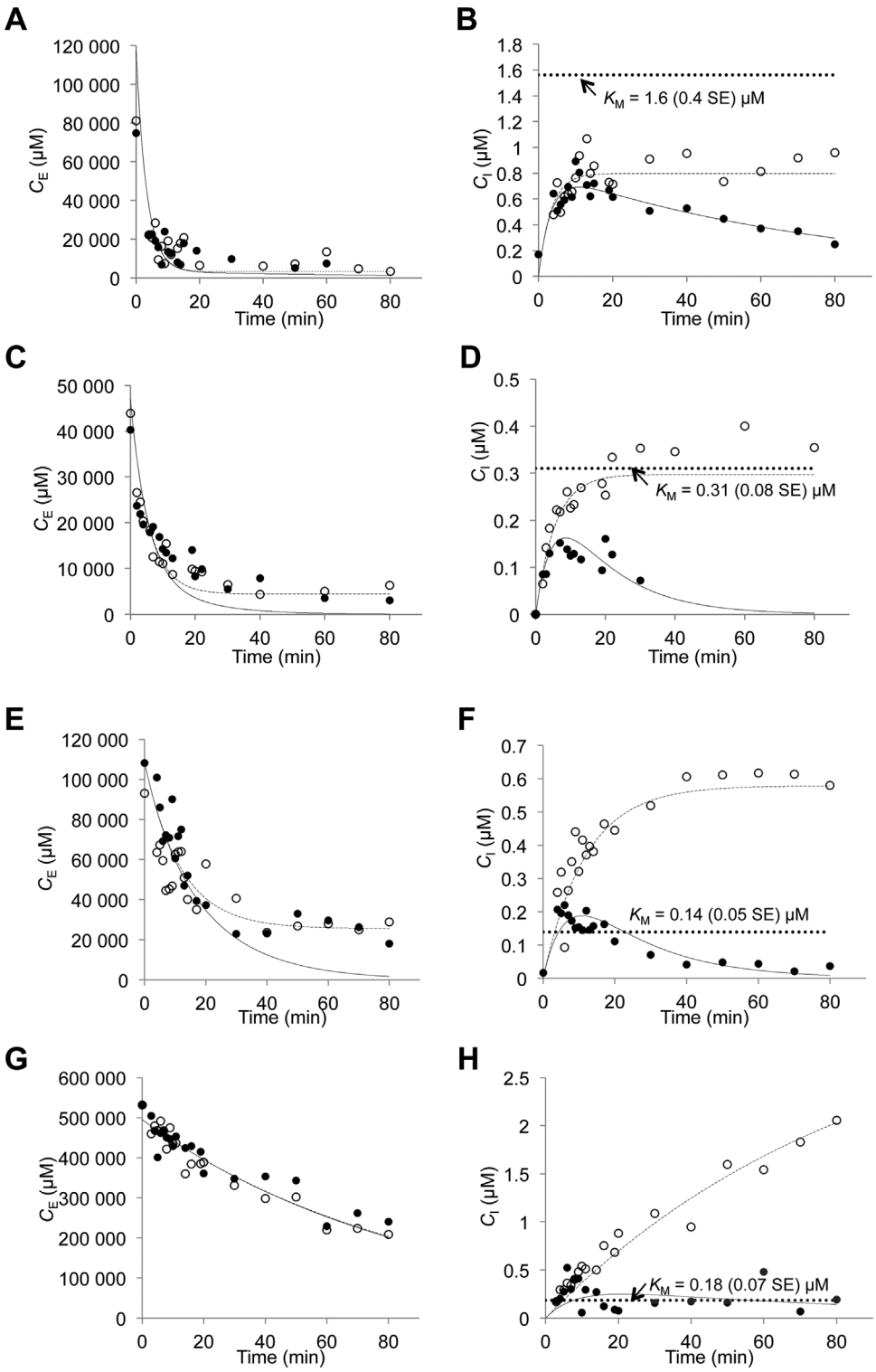

Figure 5. Concentration–time profiles in the sorbent-phase delivery dosing experiments for 9-methylanthracene (**A**,**B**), pyrene (**C**,**D**), chrysene (**E**,**F**), and benzo[*a*]pyrene (**G**,**H**) in both the ethylene vinyl acetate (EVA) sorbent-phase (C_E; **A**, **C**, **E**, **G**) and the incubation medium (C_I; **B**, **D**, **F**, **G**). Solid circles and solid lines represent the active trout liver S9 data and nonlinear regression estimates. Open circles and dashed lines represent the inactive S9 data and nonlinear regression estimates. The dotted line represents the apparent Michaelis constant (K_M) estimated from the series of solvent delivery dosing experiments.

to analytical detection limits for chrysene preventing the determination of the full relationship between k_{dep} and log $C_{I,t=0}$ at low substrate concentrations in the incubation medium, possibly causing the $k_{dep,C\to 0}$ of chrysene to be misidentified. The good agreement between $k_{dep,EVA}$ and $k_{dep,C\to 0}$ estimates from multiple depletion curves method for 3 of the 4 test chemicals indicates the potential of sorbent-phase methods to approximate $k_{dep,C\to 0}$ without the need to conduct experiments at multiple concentrations and characterize K_M. However, it should be stressed that sorbent-phase dosing methods can produce incubation concentrations above K_M. Also, it is advantageous that, in sorbent delivery–based experiment, the initial concentration of the test chemical in the incubation medium is at its lowest possible value (i.e., near 0) rather than at

its maximum concentration. Furthermore, chemicals that require bioaccumulation assessments (i.e., those having a log $K_{OW} > 5$) are very hydrophobic and are therefore released slowly into the incubation medium, hence producing low concentrations in the incubation medium. Measured in vitro biotransformation rates from sorbent-phase dosing experiments can therefore in many cases be expected to be closer to the theoretical maximum depletion rate constant at an infinitesimally low-substrate concentration ($k_{dep,C\rightarrow 0}$) than comparable measurements in solvent delivery–based dosing experiments. This low initial substrate concentration in sorbent delivery–based dosing methods may explain that in previous studies in both rat [17] and fish [18] liver S9 preparations depletion rate constants measured in sorbent delivery–based dosing methods were higher than those measured in solvent delivery–based experiments.

The results of the present study indicate that conducting multiple solvent delivery–based experiments within an appropriate concentration range provides the most accurate method for determining biotransformation potential, as the experiments reveal the full Michaelis–Menten relationship between substrate concentration and biotransformation rate. The sorbent-phase delivery–based dosing method appears to provide a reasonable alternative to the multiple solvent delivery–based experiments, as it provided good estimates of the maximum first-order depletion rate constant for the test chemicals in the present study. The main advantage of the EVA dosing method is that it does not require multiple dosing experiments and hence may be less costly and less time-consuming and may require fewer animals. In addition, the sorbent-phase delivery–based dosing method provides an estimate of the fraction unbound (a valuable extrapolation parameter), requires no solvents, and prevents incomplete dissolution of the test chemical in the incubation medium. However, in contrast to the multiple solvent delivery method where $k_{dep,C\rightarrow 0}$ is determined, the sorbent-phase delivery–based dosing method does not determine K_M and cannot reveal the full relationship between the in vitro biotransformation rate and the substrate concentration. The relationship between chemical concentration and biotransformation rate likely plays an important role in the extrapolation of in vitro to in vivo biotransformation rates. Models for extrapolation of in vitro to in vivo biotransformation rates may benefit from incorporating the concentration dependence of the biotransformation rate.

SUPPLEMENTAL DATA

Tables S1–S2.
Figures S1–S2. (72 KB DOC).

Acknowledgment—The authors acknowledge the National Science and Engineering Research Council of Canada for support.

Data availability—All original data are included in the Supplemental Data and in the body of the text.

REFERENCES

1. Arnot JA, Gobas FAPC. 2006. A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in fish. *Environ Rev* 14:257–297.
2. Arnot JA, Meylan W, Tunkel J, Howard PH, Mackay D, Bonnell M. 2009. A qualitative structure-activity relationship for predicting metabolic biotransformation rates for organic chemicals in fish. *Environ Toxicol Chem* 28:1168–1177.
3. Papa E, van der Wall, Arnot JA, Gramatica P. 2014. Metabolic biotransformation half-lives in fish: QSAR modeling and consensus analysis. *Sci Total Environ* 470–471:1040–1046.
4. Han X, Nabb DL, Yang CH, Snajdr SI, Mingoia RT. 2009. Liver microsomes and S9 from rainbow trout (*Oncorhynchus mykiss*): Comparison of basal-level enzyme activities with rat and determination of xenobiotic intrinsic clearance in support of bioaccumulation assessment. *Environ Toxicol Chem* 28:481–488.
5. Laue H, Gfeller H, Jenner KJ, Nichols JW, Kern S, Natsch A. 2014. Predicting the bioconcentration of fragrance ingredients by rainbow trout using measured rates of in-vitro intrinsic clearance. *Environ Sci Technol* 48:9486–9495.
6. Han X, Nabb DL, Mingoia RT, Yang CH. 2007. Determination of xenobiotic intrinsic clearance in freshly isolated hepatocytes from rainbow trout (*Oncorhynchus mykiss*) and rat and its application in bioaccumulation assessment. *Environ Sci Technol* 41:3269–3276.
7. Nichols JW, Huggett DB, Arnot JA, Fitzsimmons PN, Cowan-Ellsberry CE. 2013. Towards improved models for predicting bioconcentration of well-metabolized compounds by rainbow trout using measured rates of in-vitro intrinsic clearance. *Environ Toxicol Chem* 32:1611–1622.
8. Weisbrod AV, Sahi J, Segner H, James MO, Nichols J, Schultz I, Erhardt S, Cowan-Ellsberry C, Bonnell M, Goeger B. 2009. The state of in-vitro science for use in bioaccumulation assessments for fish. *Environ Toxicol Chem* 28:86–96.
9. Rane A, Wilkinson GR, Shand DG. 1977. Prediction of hepatic extraction ratio from in-vitro measurement of intrinsic clearance. *J Pharmacol Exp Ther* 200:420–424.
10. Houston JB. 1994. Utility of in-vitro drug metabolism data in predicting in vivo metabolic clearance. *Biochem Pharmacol* 47:1469–1479.
11. Carlile DJ, Hakooz N, Bayliss MK, Houston JB. 1999. Microsomal prediction of in vivo clearance of CYP2C9 substrates in humans. *Br J Clin Pharmacol* 47:625–635.
12. Johanning K, Hancock G, Escher B, Adekola A, Bernhard MJ, Cowan-Ellsberry C, Domoradzki J, Dyer S, Eickhoff C, Embry M, Erhardt S, Fitzsimmons P, Halder M, Hill J, Holden D, Johnson R, Rutishauser S, Segner H, Schultz I, Nichols J. 2012. Assessment of metabolic stability using the rainbow trout (*Oncorhynchus mykiss*) liver S9 fraction. *Curr Protoc Toxicol* 2012:14.10.1–14.10.28.
13. Obach RS, Reed-Hagen AE. 2002. Measurement of Michaelis constants for cytochrome P450-mediated biotransformation reactions using a substrate depletion approach. *Drug Metab Dispos* 30:831–837.
14. Nath A, Atkins WM. 2006. Short communication: A theoretical validation of the substrate depletion approach to determining kinetic parameters. *Drug Metab Dispos* 34:1433–1435.
15. Mohutsky MA, Chien JY, Ring BJ, Wrighton SA. 2006. Predictions of the in vivo clearance of drugs from rate of loss using human liver microsomes for phase I and phase II biotransformations. *Pharm Res* 23:654–662.
16. Sjögren E, Lennernäs H, Andersson TB, Gråsjö J, Bredberg U. 2009. The multiple depletion curves method provides accurate estimates of intrinsic clearance (CL_{int}), maximum velocity of the metabolic reaction (V_{max}), and Michaelis constant (K_m): Accuracy and robustness evaluated through experimental data and Monte Carlo simulations. *Drug Metab Dispos* 37:47–58.
17. Lee YS, Otton SV, Campbell DA, Moore MM, Kennedy CJ, Gobas FAPC. 2012. Measuring in-vitro biotransformation rates of super hydrophobic chemicals in rat liver S9 fractions using thin-film sorbent-phase dosing. *Environ Sci Technol* 46:410–418.
18. Lee YS, Lee DH, Delafoulhouze M, Otton SV, Moore MM, Kennedy CJ, Gobas FAPC. 2014. In-vitro biotransformation rates in fish liver S9: Effect of dosing techniques. *Environ Toxicol Chem* 33:1885–1893.
19. Sangster J. 1994. *LOGKOW Databank*. Sangster Research Laboratories, Montreal, Quebec, Canada.
20. Mackay D, Shiu WY, Ma K-C, Lee SC. 2006. *Handbook of Physical-Chemical Properties and Environmental Fate for Organic Chemicals*, Vol 1—Introduction to Hydrocarbons. CRC, Boca Raton, FL, USA.
21. De Maagd PGJ, ten Hulscher TEM, Huevel HVD, Opperhuizen A, Sijm DTHM. 1998. Physicochemical properties of polycyclic aromatic hydrocarbons: Aqueous solubilities, n-octanol/water partition coefficients, and Henry's law constants. *Environ Toxicol Chem* 17:251–257.
22. Rolanczyk RC, Fitzsimmons PN, McKim JM, Erickson RJ, Schmieder PK. 2003. Effects of anesthesia (tricaine methanesulfonate, MS222) on liver biotransformation in rainbow trout (*Oncorhynchus mykiss*). *Aquat Toxicol* 64:177–184.
23. Bradford MM. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Anal Biochem* 72:248–254.
24. Fitzsimmons PN, Lien GJ, Nichols JW. 2007. A compilation of in-vitro rate and affinity values for xenobiotic biotransformation in fish, measured under physiological conditions. *Comp Biochem Physiol C* 145:485–506.

25. Vignier V, Vandermeulen HJ, Mossman D. 1996. Assay conditions and basal activity of CYP1A-dependent mixed function oxidase in parr and smolt of Atlantic salmon (*Salmo salar*). *Comp Biochem Physiol C* 113:325–330
26. Carpenter HM, Fredrickson LS, Williams DE, Buhler DR, Curtis LR. 1990. The effect of thermal acclimation on the activity of arylhydrocarbon hydroxylase in rainbow trout, *Salmo gairdneri*. *Comp Biochem Physiol C* 97:127–132.
27. Stegeman JJ, Binder RL, Orren A. 1979. Hepatic and extrahepatic microsomal electron transport components and mixed-function oxygenases in the marine fish *Stenotomus versicolor*. *Biochem Pharmacol* 28:3431–3439.
28. Karr SW, Reinert RE, Wade AE. 1985. The effects of temperature on the cytochrome P-450 system of thermally acclimated bluegill. *Comp Biochem Physiol C* 80:135–139.
29. Hermens JLM, Bradbury SP, Broderius SJ. 1990. Influence of cytochrome P450 mixed-function oxidase induction on the acute toxicity to rainbow trout (*Salmo gairdneri*) of primary aromatic amines. *Ecotox Environ Safe* 20:156–166.
30. Lemaire P, Mathieu A, Carrière S, Narbonne J-F, Lafaurie M, Giudicelli J. 1992. Hepatic biotransformation enzymes in aquaculture European sea bass (*Dicentrarchus labrax*): Kinetic parameters and induction with benzo(A) pyrene. *Comp Biochem Physiol B* 103:847–853.
31. Nishimoto M, Yanagida GK, Stein JE, Baird WM, Varanasi U. 1992. The metabolism of benzo(a) pyrene in English sole (*Parophrys vetulus*): Comparison between isolated hepatocytes in-vitro and liver in vivo. *Xenobiotica* 22:949–961.
32. George TS, Vlahos P, Harner T, Helm P, Wilfort B. 2011. A rapidly equilibrating, thin film, passive water sampler for organic contaminants; characterization and field testing. *Environ Pollut* 159:481–486.