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IN VITRO BIOTRANSFORMATION RATES IN FISH LIVER S9: EFFECT OF DOSING TECHNIQUES

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Abstract: In vitro biotransformation assays are currently being explored to improve estimates of bioconcentration factors of potentially bioaccumulative organic chemicals in fish. The present study compares thin-film and solvent-delivery dosing techniques as well as single versus multiple chemical dosing for measuring biotransformation rates of selected polycyclic aromatic hydrocarbons in rainbow trout (*Oncorhynchus mykiss*) liver S9. The findings show that biotransformation rates of very hydrophobic substances can be accurately measured in thin-film sorbent-dosing assays from concentration–time profiles in the incubation medium but not from those in the sorbent phase because of low chemical film-to-incubation-medium mass-transfer rates at the incubation temperature of 13.5 °C required for trout liver assays. Biotransformation rates determined by thin-film dosing were greater than those determined by solvent-delivery dosing for chrysene (octanol–water partition coefficient [K_{OW}] = $10^{5.60}$) and benzo[*a*]pyrene ($K_{OW} = 10^{6.04}$), whereas there were no statistical differences in pyrene ($K_{OW} = 10^{5.18}$) biotransformation rates between the 2 methods. In sorbent delivery–based assays, simultaneous multiple-chemical dosing produced biotransformation rates that were not statistically different from those measured in single-chemical dosing experiments for pyrene and benzo[*a*]pyrene but not for chrysene. In solvent-delivery experiments, multiple-chemical dosing produced biotransformation rates that were much smaller than those in single-chemical dosing experiments for all test chemicals. While thin-film sorbent-phase and solvent delivery–based dosing methods are both suitable methods for measuring biotransformation rates of substances of intermediate hydrophobicity, thin-film sorbent-phase dosing may be more suitable for superhydrophobic chemicals. *Environ Toxicol Chem* 2014;33:1885–1893. © 2014 SETAC

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INTRODUCTION

Biotransformation may play an important role in the elimination and bioaccumulation of xenobiotic chemicals. However, the lack of a priori consideration of biotransformation rates is a major limitation in current bioaccumulation assessments. The bioconcentration factor (BCF), bioaccumulation factor (BAF), and the octanol–water partition coefficient (K_{OW}) are routinely used to assess the bioaccumulative behavior of chemicals according to national and international regulations such as the Canadian Environmental Protection Act; the US Toxic Substances Control Act; the European Union's Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH); the Japanese Chemical Substances Control Law; and the Stockholm Convention on Persistent Organic Pollutants [1]. Empirical BCFs and BAFs do not exist for the vast majority of commercial chemicals. Therefore, to date, bioaccumulation assessments have often relied on the K_{OW} or on bioaccumulation models, which in most cases did not consider biotransformation because of a lack of information on biotransformation rates. This approach can lead to misidentification of the bioaccumulation potential of chemicals in screening and risk assessments. Recently, 2 approaches have emerged to include biotransformation in bioaccumulation assessments. One approach is the development of quantitative structure–activity relationships for calculating the BCF and BAF that take into account biotransformation [2]. The other approach is the application of experimental in vitro biotransformation tests [3–8].

In vitro analysis of chemical biotransformation rates in liver preparations has been advocated for predicting biotransformation rates in whole organisms in a manner that is cost-effective and reduces animal use [9]. Developing protocols for in vitro biotransformation tests using fish liver is of particular interest because existing bioaccumulation models for fish can use the bioassay data to estimate BCF and BAF values [3–6,10]. Various fish liver preparations include 9000-*g* supernatants of liver homogenate (S9) [4,7,8,11], microsomes [6,8], freshly isolated hepatocytes [4,7,12,13], and cultured hepatocytes [13]. Ex vivo assays using isolated perfused fish livers have also been proposed to measure biotransformation rates that can be extrapolated to the whole body [14]. However, in vitro assays for measuring biotransformation rates of highly hydrophobic chemicals ($\log K_{OW} > 5$) with high bioaccumulation potential can be challenging when using conventional solvent-delivery dosing methods where chemicals are introduced into the incubation medium dissolved in a small volume of an organic solvent [15]. These challenges include incomplete dissolution of the hydrophobic chemical in the largely aqueous assay medium and inhibition of enzyme activity by the spiking solvent. A solvent-free dosing approach has the potential to overcome the above-mentioned problems and may reduce error in measurements of the in vitro biotransformation rates of very hydrophobic chemicals (i.e., those with high bioaccumulation potential) [15]. In addition, sorbent-phase dosing allows for direct measurement of the unbound chemical fraction in the incubation and reduces

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substrate saturation of enzyme by using incubations with an initial substrate concentration of 0.

Solvent-free passive dosing techniques have been developed and applied to improve toxicity testing of highly hydrophobic chemicals by loading the test chemical into a sorbent phase such as poly(dimethylsiloxane), silicone O-rings, or ethylene vinyl acetate (EVA), and then delivering the chemical into the assay medium by passive diffusion [11,16–22]. Passive dosing using silicone O-rings as the dosing polymer has also been used to measure biodegradation kinetics of phenanthrene and fluoranthene in a bacterial strain at defined dissolved chemical concentrations ranging over 4 orders of magnitude [23]. An EVA thin-film sorbent-phase dosing approach has been developed and applied to measure the in vitro biotransformation rates of superhydrophobic chemicals by rat liver S9 fractions, and it was found that the in vitro biotransformation rates measured using the sorbent-phase dosing method were greater than those measured using a solvent-delivery dosing method under the same conditions [15].

The objective of the present study was to develop and evaluate the sorbent-phase dosing technique to measure in vitro biotransformation rates of hydrophobic chemicals by liver S9 fractions from rainbow trout (*Oncorhynchus mykiss*). In vitro biotransformation rates obtained from sorbent-phase dosing were compared with biotransformation rates measured using conventional solvent-delivery dosing to evaluate method performance. To date, solvent-free passive dosing techniques have not been applied to fish liver preparations, yet fish remain the predominant species used in bioaccumulation assessment. In addition, we investigated multiple chemical dosing using both sorbent-phase and solvent-delivery dosing approaches; measuring the biotransformation rates of multiple chemicals in the same incubation would reduce time, costs, and animal use. The ultimate goal of the present studies was to improve current bioaccumulation assessments.

THEORY

In thin-film sorbent-phase dosing systems, the test chemical is transferred by passive diffusion from the sorbent phase (EVA thin films) to the incubation medium containing liver S9 with active metabolic capacity (test) or inactivated enzymes (control). The theory and inherent assumptions of the thin-film sorbent-phase dosing approach are presented in Lee et al. [15]. A 2-compartment mass-transfer model is used to describe the exchange of the test chemical between the sorbent phase and the incubation medium considering simultaneous diffusive transfer and biotransformation, as described by the Equations 1 and 2

$$\frac{dC_e}{dt} = k_2 \frac{V_m}{V_e} C_m - k_1 C_e \quad (1)$$

$$\frac{dC_m}{dt} = k_1 \frac{V_e}{V_m} C_e - (k_2 + k_r) C_m \quad (2)$$

where C_e and C_m are concentrations (mol/m^3) of the chemical in the EVA sorbent phase and the incubation medium, respectively; V_e and V_m are volumes (m^3) of the EVA thin film and the incubation medium, respectively; k_1 and k_2 are mass-transfer rate constants (min^{-1}) describing the transfer of the chemical from the sorbent phase to the incubation medium (k_1) and from the medium back to the sorbent phase (k_2); and k_r is the in vitro biotransformation rate constant (min^{-1}). It is required that the mass-transfer rate constants (k_1 and k_2) of the chemical in the test are the same in the test and control and that k_r is 0 in the control.

One of the features of the thin-film sorbent-phase dosing technique is that biotransformation rates can be determined by measuring chemical concentrations in the EVA films over time, which is analytically less difficult than measuring concentrations in highly complex liver preparations. However, this requires the diffusive mass-transfer rate of the chemical from the sorbent to the incubation mixture to be greater than the rate of biotransformation (i.e., $k_1 > k_r$) to ensure that the mass transfer is not the rate-limiting step in the biotransformation process [15]. If this requirement is not met and $k_1 \leq k_r$, then the biotransformation rate can be determined from the chemical concentration in the incubation medium over time but not from the concentrations in the films.

MATERIALS AND METHODS

Chemicals

Pyrene, chrysene, benzo[*a*]pyrene, and chrysene-d^{12} were purchased from Sigma-Aldrich with purities of 98% or higher. Ethylene vinyl acetate, Elvax 40W, was obtained from DuPont. Potassium dihydrogen phosphate and high-performance liquid chromatography (HPLC)–grade acetonitrile were obtained from Caledon Laboratories. Potassium phosphate dibasic was obtained from Anachemia Canada. Potassium chloride and HPLC-grade hexane were obtained from EMD Millipore. All other chemicals, if not specified, were purchased from Sigma-Aldrich.

Animals

Nine male rainbow trout (*O. mykiss*, approximately 1000 g body wt) were obtained from Miracle Springs. The fish were held in tanks equipped with a dechlorinated tap water flow-through system ($13.5 \pm 2\,^{\circ}$C) under a 16:8-h light:dark cycle for at least 2 wk and fed EWOS Pacific 3.0-mm pellets once daily.

Preparation of trout liver S9 fraction

The trout were euthanized by overdose exposure to 0.3 g L^{-1} tricaine methanesulfonate (MS222, buffered with 0.3 g L^{-1} sodium bicarbonate). Exposure to this concentration of MS222 for 5 min has no effect on microsomal P450 activities [24]. Livers were immediately excised and rinsed in ice-cold 1.15% (w/v) KCl. Each liver was weighed, minced on ice with a razor blade, and homogenized on ice using a Potter-Elvehjem glass tissue grinder with a Teflon pestle (Kontes) in 1 volume (g mL^{-1}) of ice-cold 0.20 M phosphate buffer (pH 7.4) containing 1.15% (w/v) KCl. The liver homogenates were pooled into 3 groups (each group was comprised of 3 trout livers) and centrifuged at 9000 *g* for 20 min at 4 °C (Hermle Z360K centrifuge). The 9000-*g* supernatant fraction (S9) was collected and stored at −80 °C until use (held for <3 mo). The protein concentration of the S9 fraction was determined by the method of Bradford [25] using bovine serum albumin (Fraction V; Sigma-Aldrich) as the standard.

Thin-film preparation

The EVA thin film was prepared according to Lee et al. [15]. Briefly, a 0.135-g L^{-1} EVA solution was prepared by dissolving EVA beads in dichloromethane. The test chemicals were pyrene, chrysene, and benzo[*a*]pyrene, which have log K_{OW} values of 5.18, 5.60, and 6.04, respectively [26]. These chemicals were added to the EVA solution individually or as a mixture producing nominal concentrations of 4.05 μg mL^{-1},

4.57 μg mL^{-1}, and 12.62 μg mL^{-1}, respectively. The maximum possible concentration in the incubation medium (assuming all chemical in the sorbent is instantaneously released into the incubation medium) was 1.0 μM for pyrene and chrysene and 2.5 μM for benzo[*a*]pyrene. These concentrations are less than reported Michaelis-Menten constants (K_m) of 15.1 μM for pyrene hydroxylation by isolated hepatocytes from rainbow trout [27] and 33 μM to 125 μM for benzo[*a*]pyrene hydroxylation by liver microsomes of rainbow trout [28]. Thin films of EVA containing the test chemicals were formed on the interior surface of 2-mL silanized amber glass vials (Agilent) by adding 25 μL of the spiked EVA solution and rolling the vials slowly to evaporate the solvent. The thin films were 4 nm thick and contained 0.0035 μL (3.4 μg) EVA. Thicker films (20 nm containing 17 μg EVA) were also studied. Film thickness was estimated by dividing the volume of EVA film by the interior surface area of the vial.

Incubation conditions of sorbent-phase dosing system

The reactions were started by adding 0.50 mL of the incubation mixture containing trout liver S9 (preincubated at 13.5 °C for 5 min) to the EVA–coated vials. The incubation mixture consisted of 0.38 mL phosphate buffer (0.20 M, pH 7.4) containing 1.15% (w/v) KCl, 0.10 mL reduced nicotinamide adenine dinucleotide phosphate (NADPH)–generating system (0.8 μmol nicotinamide adenine dinucleotide phosphate, 8 μmol glucose-6-phosphate, 1.6 units glucose-6-phosphate dehydrogenase, and 4 μmol $MgCl_2$ prepared in phosphate buffer), and 0.020 mL trout liver S9 (containing ~3 mg S9 protein in the incubation mixture). Incubations were conducted at 13.5 ± 1 °C in a water bath equipped with an immersion cooler (Grant CS 200G). The vials were capped with polytetrafluoroethylene-lined screw caps and rolled horizontally at 80 rpm in the water bath with a roller designed in-house to optimize contact between the incubation medium and the EVA thin film. The reactions were stopped at various times (10 min, 20 min, 30 min, 40 min, 60 min, 80 min, 100 min, and 140 min in single-chemical dosing experiments for pyrene and 5 min, 10 min, 15 min, 30 min, 45 min, 60 min, 90 min, 120 min, 150 min, and 180 min in all other experiments) by quickly transferring 0.40 mL of incubation medium to 1.0 mL ice-cold hexane and removing the remaining medium from the EVA-coated vials. The EVA thin films were then rinsed twice with 0.20 mL of deionized water, and 1.0 mL hexane was added to the vials to extract chemicals from the films. Chemical concentrations were measured in both the EVA and medium phases.

A no-cofactor control system using inactive liver S9 (incubated at 13.5 °C overnight and no NADPH-generating system included in the incubation mixture) was run in parallel with each test system using the incubation conditions described above. Two vials without incubation medium were included in the incubation bath and their films extracted to determine the initial concentration of the test chemicals in the EVA thin films (C_e at $t = 0$). Test chemicals were dosed individually or in a mixture under the same experimental conditions to examine mixture effects. Triplicate incubations using different pools of liver S9 (3 fish per pool, 9 fish used in total) were conducted to determine the mean in vitro biotransformation rates of the test chemicals.

Incubation conditions of the solvent-delivery dosing system

The same trout liver S9 preparations and incubation conditions were used in both sorbent-phase dosing and solvent-delivery dosing experiments. Briefly, pyrene, chrysene, and benzo[*a*]pyrene were dissolved individually or in a mixture in acetonitrile. To initiate the reactions, 2.4 μL of the spiked solvent was added to 0.50 mL incubation mixture in 2-mL vials preincubated at 13.5 °C for 5 min. Final concentrations in the incubation medium were 0.50 μM for pyrene and chrysene and 1.0 μM for benzo[*a*]pyrene. The final acetonitrile concentration was <0.5% (v/v). Incubations were conducted at 13.5 ± 1 °C, and reactions were terminated at various times (0 min, 10 min, 20 min, 30 min, 40 min, 60 min, 80 min, 100 min, and 140 min in single-chemical dosing experiments for pyrene and 0 min, 5 min, 10 min, 15 min, 30 min, 45 min, 60 min, 90 min, 120 min, 150 min, and 180 min in other experiments) by adding 1.0 mL ice-cold hexane to the incubation medium. A no-cofactor control system in which the trout liver S9 had been incubated at 13.5 °C overnight for enzyme inactivation and the NADPH-generating system was omitted from the incubation mixture was run in parallel with a test system using the incubation conditions described above. Test chemicals were dosed individually or in a mixture under the same experimental conditions to examine mixture effects. Triplicate experiments using the same S9 preparations used in the sorbent-phase dosing (3 fish per pool, 9 fish used in total) were conducted to determine the mean in vitro biotransformation rates of the test chemicals.

Chemical extraction

Chemical extraction procedures were according to Lee et al. [15]. Briefly, prior to chemical extraction from the films, an nternal standard (0.21 nmol chrysene-d^{12}) was added to the hexane extraction solvent. Test chemicals remaining in the film after the termination of incubation were extracted into the hexane by shaking the vials on a vortex mixer for 1 min. Extracts were transferred to 2-mL autosampler vials for gas chromatography/mass spectrometry (GC/MS) analysis. Internal standard (0.21 nmol chrysene-d^{12}) was added to the vials containing S9 plus hexane, and the vials were shaken on a vortex mixer for 2 min. The vials were then centrifuged at 800 *g* for 10 min (IEC Centra-CL2; Thermo Scientific). The upper organic layer was transferred to a 2-mL autosampler vial for GC/MS analysis.

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). 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 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. The dynamic range and relative response factor (obtained by dividing the ratio of peak area by the concentration of the test chemical to that of the internal standard) for each test chemical were determined using an 8-point calibration curve (concentration range, 1–500 ng mL^{-1}). Strong linearity ($r^2 > 0.99$) was

shown in the calibration curves, and constant relative response factor values were obtained over the concentration range.

Data analysis

In the sorbent-phase dosing experiments, the mass-transfer rate constants (k_1, k_2) and in vitro biotransformation rate constants (k_r) and their 95% confidence intervals were estimated as described by Lee et al. [15] by fitting the measured chemical concentrations in either the sorbent phase or the incubation medium using a nonlinear regression and a Runge-Kutta numerical differential equation solver using MATLAB R2009a (Mathworks).

To derive the biotransformation rate constants in solvent-delivery dosing experiments, the declining concentrations in the incubation medium over time were fitted by a first-order kinetic model

$$\frac{dC_m}{dt} = -k_r \times C_m \quad (3)$$

where C_m is the control-corrected chemical concentration in the incubation medium (μM) and k_r is the apparent first-order biotransformation rate constant (min^{-1}). The k_r value was estimated using a linear regression from the slope of ln ($C_m/C_{m,t=0}$) versus time (i.e., ln $[C_m/C_{m,t=0}] = -k_r \times t$), where $C_{m,t=0}$ is the initial dosing concentration of the chemical in the incubation medium (μM). Only data showing apparent first-order depletion were used for data analysis.

Determination of unbound fraction

The unbound fractions of the test chemicals in the incubation medium were determined as C_e/(EVA–water partition coefficient $[K_{EW}] \times C_m$) as described by Lee et al. [15]. Briefly, $C_e/(K_{EW} \times C_m)$ was obtained from the control data in the sorbent-phase dosing experiments, where C_e/C_m is the ratio of chemical concentration in the sorbent phase to chemical concentration in the incubation medium at steady state in the control, calculated as $(k_2 \times V_m)/(k_1 \times V_e)$ obtained from Equation 1 when $dC_e/dt = 0$ or from Equation 2 when $dC_m/dt = 0$ and $k_r = 0$; K_{EW} is $10^{5.84}$, $10^{6.40}$, and $10^{6.52}$ for pyrene, chrysene and benzo[*a*]pyrene, respectively [29].

RESULTS AND DISCUSSION

Thin-film sorbent-phase dosing of trout liver S9

Extraction efficiencies of all test chemicals from the sorbent phase were high at 102 ± 1%, and sorbent concentrations were therefore not corrected for extraction efficiency. Extraction efficiencies from the trout liver incubation mixture were 79 ± 2%, 70 ± 4%, and 61 ± 4% (mean ± standard deviation [SD]) for pyrene, chrysene, and benzo[*a*]pyrene, respectively. Concentrations of the test chemicals in the incubation medium were corrected for extraction efficiencies to ensure that mass balance was obtained. The lower extraction efficiencies from the incubation medium compared with the sorbent phase illustrate the greater ease and lower error of sorbent-phase extractions.

When test chemicals were dosed individually via the thin-film sorbent phase, concentrations of pyrene, chrysene, and benzo[*a*]pyrene in the sorbent phase declined over time as the test chemical was transferred from the sorbent phase to the incubation medium (Figure 1). The magnitude of concentration decline over time was greatest for pyrene, whereas chrysene and benzo[*a*]pyrene exhibited similar concentration declines over time (Figure 1). For all test chemicals, the rates of concentration decline over time in the test (thin films exposed to active trout liver S9) and control (thin films exposed to inactive trout liver S9) were similar and did not show statistically significant ($p < 0.05$) differences (Figure 1). Corresponding concentrations in incubation media containing inactive S9 increased over time and reached a plateau (Figure 1). Concentrations in the active liver incubations increased over time, reached a maximum, and then decreased over time for all test chemicals (Figure 1). Concentration profiles in the incubation mixture showed highly statistically significant differences between test and control ($p < 0.05$). The findings show that the biotransformation rate constant, k_r, of the chemicals in the present study can be derived from the concentration profiles in the incubation phase but not from those in the sorbent phase. Figure 2 illustrates the reasons for these findings. It shows that the similarity between chemical concentration profiles in the sorbent phase of the test and control incubations is a result of the slow chemical delivery rate of the test chemicals from the sorbent phase to the incubation medium (k_1) relative to the biotransformation rate in the incubation medium (k_r). The measured in vitro biotransformation rate constants for chrysene and benzo[*a*]pyrene obtained from concentrations measured in the incubation medium were $0.17 \pm 0.03\ min^{-1}$ and $0.037 \pm 0.006\ min^{-1}$ (mean ± SD), respectively. These rate constants are significantly higher ($p < 0.05$) than the corresponding k_1 values (Figure 2B and C). For pyrene, k_r was $0.012 \pm 0.005\ min^{-1}$ (mean ± SD) and significantly ($p = 0.04$) smaller than k_1, but k_1 did not exceed k_r by a large enough margin to measure k_r using the sorbent-phase concentration time course.

In similar experiments of the same test chemicals in rat liver S9 [15], delivery rates of chrysene ($0.28\ min^{-1}$) and benzo[*a*]pyrene ($0.13\ min^{-1}$) from the sorbent phase to the liver incubation medium were much greater than those measured in the present experiment using fish liver S9 (i.e., $0.012 \pm 0.005\ min^{-1}$ and $0.012 \pm 0.002\ min^{-1}$, mean ± SD), despite the thinner EVA films (4 nm) used in the fish study compared with the rat study (20 nm). In trout liver incubation experiments at 13.5 °C using thin films of 20-nm thickness, equal to that used in the rat liver incubation studies at 37 °C, k_1 values were $0.0038 \pm 0.006\ min^{-1}$ and $0.0013 \pm 0.002\ min^{-1}$ for chrysene and benzo[*a*]pyrene, respectively, 73 and 96 times, respectively, lower than those measured in the rat study (Supplemental Data, Figure S1). While the rat and trout liver S9 incubation mixture did vary in composition (12 mg protein mL^{-1} and 3 mg protein mL^{-1} in rat and fish, respectively), we expect that the lower incubation temperature in the trout study (13.5 °C) compared with the rat study (37 °C) is the main factor causing the lower sorbent delivery rates in the trout liver S9 incubations. Diffusion coefficients are recognized to decline with decreasing temperature [30]. The lower temperature required in bioassays with rainbow trout liver (13.5 °C in the present study) compared with 37 °C in mammalian liver bioassays can therefore limit the ability of measuring biotransformation rates from concentration measurements in the thin films. Sorbent delivery rates can be increased by employing thinner films. However, there are practical limits to the use of very thin films because of test chemical evaporation from the sorbent phase during film preparation and handling processes, which contributes error. The decline in diffusion rates with decreasing temperature does not affect the ability to measure biotransformation rates from concentration measurements in the incubation medium.

Figure 2 shows that for all test chemicals there were no statistically significant differences ($p > 0.05$) between sorbent-to-liver medium mass-transfer rate constants (k_1) obtained from

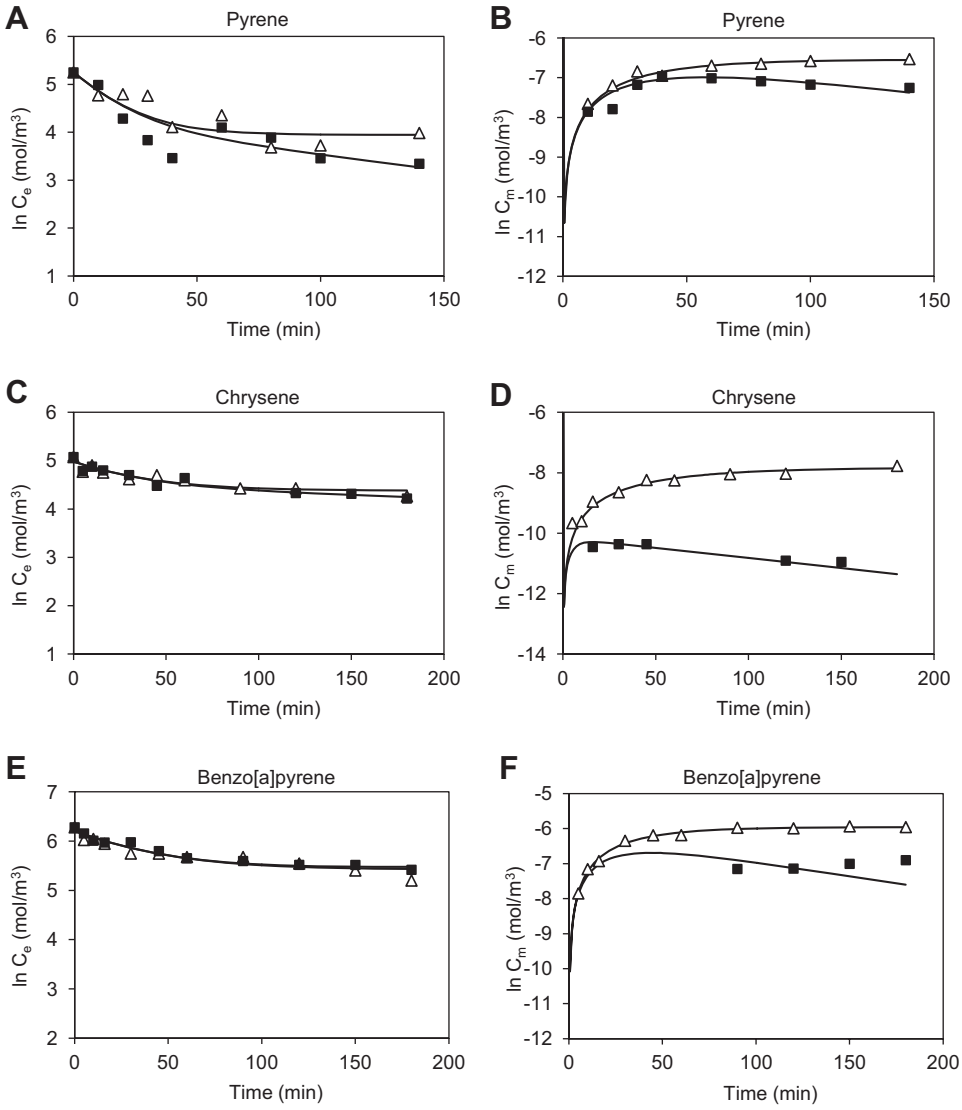

Figure 1. Natural logarithm of concentration–time profiles in the ethylene vinyl acetate thin-film sorbent phase ($\ln C_e$; **A**, **C**, **E**) and in the incubation medium ($\ln C_m$; **B**, **D**, **F**) containing active (solid squares) or inactive (open triangles) rainbow trout liver S9 (control) using the sorbent-phase single-chemical dosing approach for pyrene (**A**, **B**), chrysene (**C**, **D**), and benzo[*a*]pyrene (**E**, **F**). Solid lines represent nonlinear regressions. Data from 1 of 3 experiments are shown.

concentrations in the sorbent phase and those obtained from concentrations in the incubation medium. Similarly, medium-to-sorbent mass-transfer rate constants (k_2) determined from concentrations measured in the sorbent phase were not significantly different from those determined using the concentrations in the incubation medium ($p > 0.05$). This illustrates that mass-transfer rate constants can be determined using concentrations in either the sorbent or the medium phase. However, there were differences in the magnitude of error in the determination of the mass-transfer rate constants. For pyrene, the error in the k_1 and k_2 measurements obtained from concentrations in the sorbent phase was greater than that obtained from concentrations in the medium, but this was not observed for chrysene and benzo[*a*]pyrene. This may be a result of the greater volatility of pyrene, which introduces variability among replicates because of loss of chemical from the sorbent phase during preparation and handling of the thin films. Pyrene has a lower octanol–air partition coefficient (K_{OA}; log K_{OA} = 8.60) compared with chrysene (log K_{OA} = 10.40) and benzo[*a*]pyrene (log K_{OA} = 10.80) [26]. For benzo[*a*]pyrene, the error in the k_1 and k_2 measurements obtained from concentrations in the incubation medium was greater than that obtained from concentrations in the sorbent phase. The lower extraction efficiency and fewer detectable measured concentrations for benzo[*a*]pyrene in the incubation medium (Figure 1) compared with those from the sorbent phase are likely the main causes of the difference in error. Therefore, to obtain accurate sorbent-to-medium and medium-to-sorbent mass-transfer rate constants, concentration measurements in the liver medium may be preferred over measurements in the sorbent phase for relatively low-K_{OA} chemicals, whereas concentrations measured in the sorbent phase may be more suitable for high-K_{OW} and high-K_{OA} chemicals.

Concentrations of pyrene and benzo[*a*]pyrene measured in the incubation mixture were well below (by 1–3 orders of magnitude) reported Michaelis-Menten constants of 15 μM for pyrene measured using isolated hepatocytes from rainbow trout [27] and 33 μM to 125 μM for benzo[*a*]pyrene measured using liver microsomes from rainbow trout [28]. This suggests that enzyme saturation likely did not occur and that first-order enzyme kinetics were maintained. Substrate concentrations below the Michaelis-Menten constant are necessary in substrate

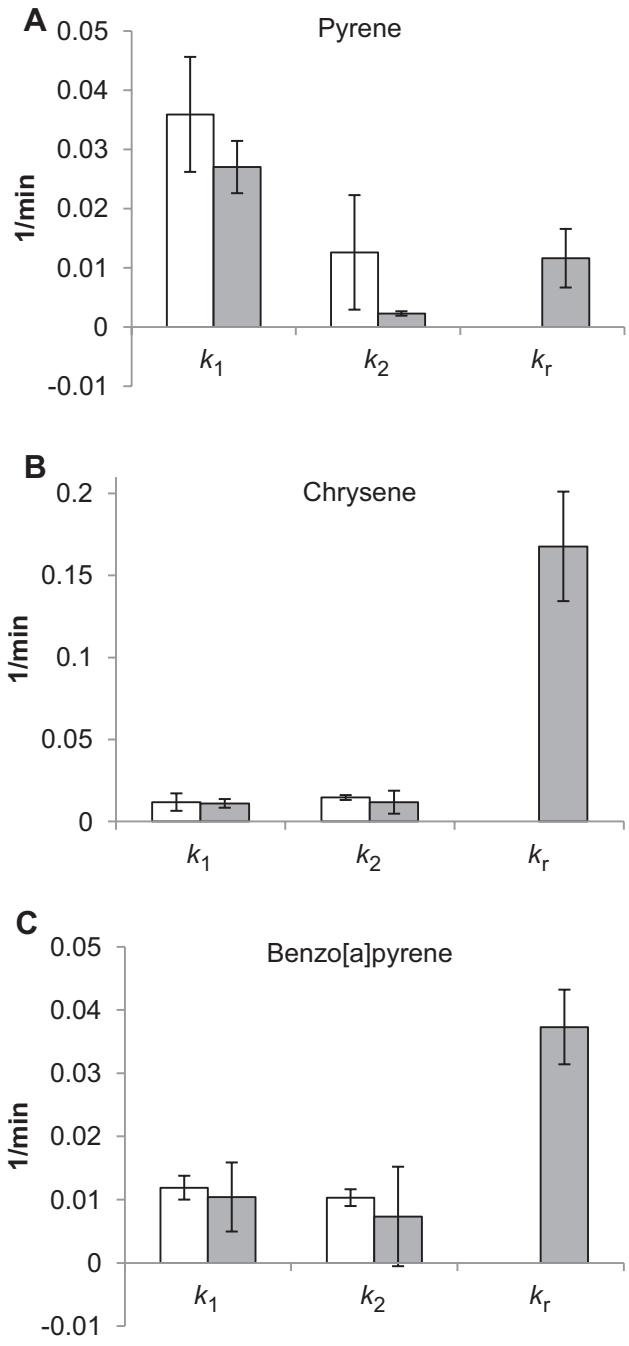

Figure 2. Measured rate constants for mass-transfer (k_1 and k_2) and in vitro biotransformation rate (k_r) in sorbent-phase single-chemical dosing experiments ($n = 3$) for pyrene (**A**), chrysene (**B**), and benzo[*a*]pyrene (**C**). Results obtained from 3 independent experiments using concentrations in the sorbent phase (empty bars) or the liver incubation mixture (filled bars). Error bars represent the standard deviation.

depletion experiments to avoid enzyme saturation and subsequent underestimation of the depletion rate.

The extrapolation of in vitro biotransformation rates to in vivo rates requires information about the fraction of unbound substrate in incubation mixtures [31–33]. In a sorbent-dosing approach, the unbound substrate can be measured from the concentration profiles in the control (inactive S9) incubations [15]. In the present study, the unbound fractions of pyrene, chrysene, and benzo[*a*]pyrene in the incubation medium were 0.033 ± 0.001, 0.087 ± 0.053, and 0.04 ± 0.01 (mean $\pm$ SD), respectively. The mean measured unbound fractions of pyrene, chrysene, and benzo[*a*]pyrene were approximately 3-fold, 14-fold, and 13-fold higher than those calculated using a K_{OW}-dependent empirical relationship used by others [6,11] for fish liver S9. One of the factors contributing to the observed differences between measured and calculated unbound fractions may be that the empirical relationship was obtained using heat-denatured liver S9 or microsomes. The S9 preparations used in the present experiments were not heat-treated. Using the measured unbound fractions and the measured S9 protein content of 2.83 ± 0.42 mg S9 protein/mL (mean $\pm$ SD), unbound intrinsic clearance rates of 0.13 ± 0.06 mL min^{-1} mg^{-1} S9 protein, 0.69 ± 0.45 mL min^{-1} mg^{-1} S9 protein, and 0.33 ± 0.11 mL min^{-1} mg^{-1} S9 protein (mean $\pm$ SD) for, respectively, pyrene, chrysene, and benzo[*a*]pyrene can be derived. The unbound intrinsic clearance rates for chrysene and benzo[*a*]pyrene in rainbow trout liver are much lower than those measured using the same technique in rat liver [15]: 10.9 ± 1.5 mL min^{-1} mg^{-1} S9 protein (mean $\pm$ SD) for chrysene and 15.3 ± 4.1 mL min^{-1} mg^{-1} S9 protein (mean $\pm$ SD) for benzo[*a*]pyrene. This supports the general assumption that biotransformation rates in fish are lower than those in mammals.

Solvent-delivery dosing using trout liver S9

Figure 3 illustrates that when dosed individually using the solvent delivery method, the concentrations of pyrene, chrysene, and benzo[*a*]pyrene in the active liver S9 showed a statistically significant log-linear decline with incubation time ($p < 0.05$ for the slopes), indicating apparent first-order kinetics of substrate depletion within the first hour of incubation (later time points flattening the depletion curves were omitted from data analysis). The estimated in vitro biotransformation rate constants (k_r) for pyrene, chrysene, and benzo[*a*]pyrene were 0.021 ± 0.005 min^{-1}, 0.008 ± 0.002 min^{-1}, and 0.019 ± 0.006 min^{-1} (mean $\pm$ SD), respectively. The k_r values obtained for pyrene and benzo[*a*]pyrene were similar ($p > 0.05$), and both were significantly higher than the measured k_r value for chrysene ($p < 0.05$). Benzo[*a*]pyrene was also biotransformed faster than chrysene by liver microsomes from brown bullheads [34]. The k_r value measured in the present study for pyrene was in good agreement with the value of 0.015 ± 0.002 min^{-1} (mean $\pm$ SD) found earlier in solvent-delivery experiments in this laboratory [35]. For benzo[*a*]pyrene, the measured in vitro biotransformation rate normalized to protein concentration in the incubation medium was 0.37 ± 0.12 mL h^{-1} mg^{-1} S9 protein, which is approximately 5 times higher than that reported by Han et al. [8] in rainbow trout liver S9. The apparent difference in biotransformation rate may be a result of the higher substrate concentration of 2 μM and the lower protein concentration of 2 mg protein mL^{-1} in Han et al. compared with those of the present study, 1.0 μM and 2.8 mg protein mL^{-1}. Both factors can contribute to lower biotransformation rates according to Michaelis-Menten theory.

The in vitro biotransformation rate of pyrene obtained from the solvent-delivery dosing experiments was not significantly different ($p > 0.05$) from that obtained in the sorbent-phase dosing experiments using the same trout liver S9 preparation (Figure 4A and 4B). For chrysene and benzo[*a*]pyrene, however, the in vitro biotransformation rates measured using the thin-film sorbent-phase dosing system were significantly higher (20 times and 2 times, respectively) than those measured using the solvent-delivery dosing system ($p < 0.05$). This agrees with experiments using rat liver S9 in which k_r values were also greater than those obtained from solvent-delivery dosing experiments for both chemicals [15]. The higher biotransformation rates obtained in

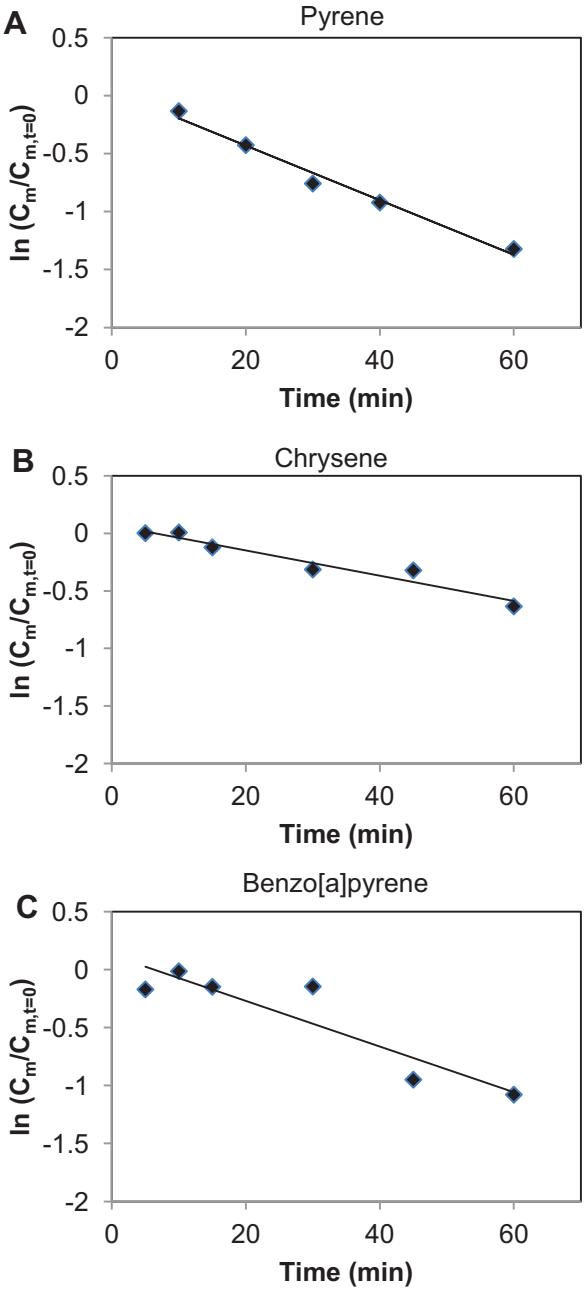

Figure 3. Natural logarithm of concentration–time profiles in the trout liver S9 in the solvent-delivery single-chemical dosing experiments for pyrene (**A**), chrysene (**B**), and benzo[*a*]pyrene (**C**). Concentrations expressed as the ratio of chemical concentraion in the incubation medium in the test (C_m control adjusted) to initial chemical concentrations in the incubation medium ($C_{m,t=0}$). Data from 1 of 3 experiments are shown.

thin-film sorbent-phase dosing experiments may result from lower initial incubation substrate concentrations in the sorbent delivery–based experiments, a lack of solvent inhibition of enzyme activities, better chemical dissolution in the incubation medium, and reduced biotransformation product inhibition by product diffusion into the sorbent phase. The greatest difference between biotransformation rates measured in sorbent and solvent delivery–based systems was for chrysene. Chrysene has the lowest aqueous solubility of the chemicals tested—2 μg/L at 25 °C compared with 4 μg/L and 130 μg/L for benzo[*a*]pyrene and pyrene, respectively [26]. It has been suggested that when superhydrophobic chemicals are spiked using a carrier solvent and delivered into a largely aqueous liver preparation, their concentrations may locally exceed their aqueous solubility, causing the formation of microcrystals [20] that may limit substrate access to enzymes during the relatively short incubation phase, causing underestimations of in vitro biotransformation rates in the solvent-delivery dosing experiments. For chemicals with greater water solubility, such as pyrene, incomplete dissolution in the incubation medium may not be an important issue, and in vitro biotransformation rates in sorbent-phase dosing and solvent-delivery dosing systems are similar. The results suggest that the solvent-delivery dosing approach is appropriate for measuring in vitro biotransformation rates of less hydrophobic chemicals (e.g., log $K_{OW} < 5$) with relatively high water solubility. The sorbent-phase dosing approach may be more useful for measuring in vitro biotransformation rates for very hydrophobic chemicals (e.g., log $K_{OW} > 5$) with very low water solubility.

Dosing with chemical mixtures

Thin-film sorbent-phase dosing. Supplemental Data, Figure S2 illustrates the time course of chemical concentrations in the EVA film and in the incubation medium in the multichemical dosing experiment. Sorbent-to-medium mass-transfer rate constants (k_1) obtained from multichemical dosing experiments were not significantly different from those obtained from single-chemical sorbent-phase dosing experiments for all 3 test chemicals ($p > 0.05$; Figure 4C). Also, the medium-to-sorbent mass-transfer rate constants (k_2) were similar in single-chemical and mixture incubations (Figure 4D). For chrysene, the difference in k_2 values between single-chemical and chemical mixture incubations was very small but statistically significant ($p = 0.022$) because of very low variability among replicates. The observation that film-incubation medium mass-transfer rate constants were not affected by the presence of other chemicals spiked simultaneously in the sorbent phase is consistent with diffusive mass-transfer of chemicals being controlled by the molecular diffusion coefficients and thickness of the diffusion layers [36], neither of which are affected by multichemical dosing conditions. There was also no significant mixture effect on in vitro biotransformation rates (k_r) measured for pyrene and benzo[*a*]pyrene in the sorbent-phase dosing experiments (Figure 4A). This suggests that codelivery of approximately equimolar concentrations of the 3 test chemicals to the incubation medium does not affect the dissolution in the incubation medium or enzyme–substrate interactions of pyrene and benzo[*a*]pyrene. However, the k_r measured for chrysene in the single-chemical sorbent-phase dosing experiments was significantly higher ($p < 0.05$) than that in the multiple-chemical dosing experiments. This may be a result of competitive inhibition of chrysene oxidation by benzo[*a*]pyrene as both chemicals are catalyzed by CYP1A [37]. Constitutive levels of CYP1A in fish are low [34,38,39], and benzo[*a*]pyrene concentrations in the incubation medium are greater than those of chrysene (Supplemental Data, Figure S2), making competitive inhibition more likely.

Solvent-delivery dosing. When test chemicals were dosed together as a mixture, no statistically significant declines ($p > 0.05$) in the pyrene and chrysene concentrations with incubation time were observed (Supplemental Data, Figure S3). For benzo[*a*]pyrene there was a statistically significant decline ($p < 0.05$) in concentrations in the incubation medium over time (Supplemental Data, Figure S3), but the in vitro biotransformation rate in the mixture incubation was much lower than that in single-chemical incubations. In general, in

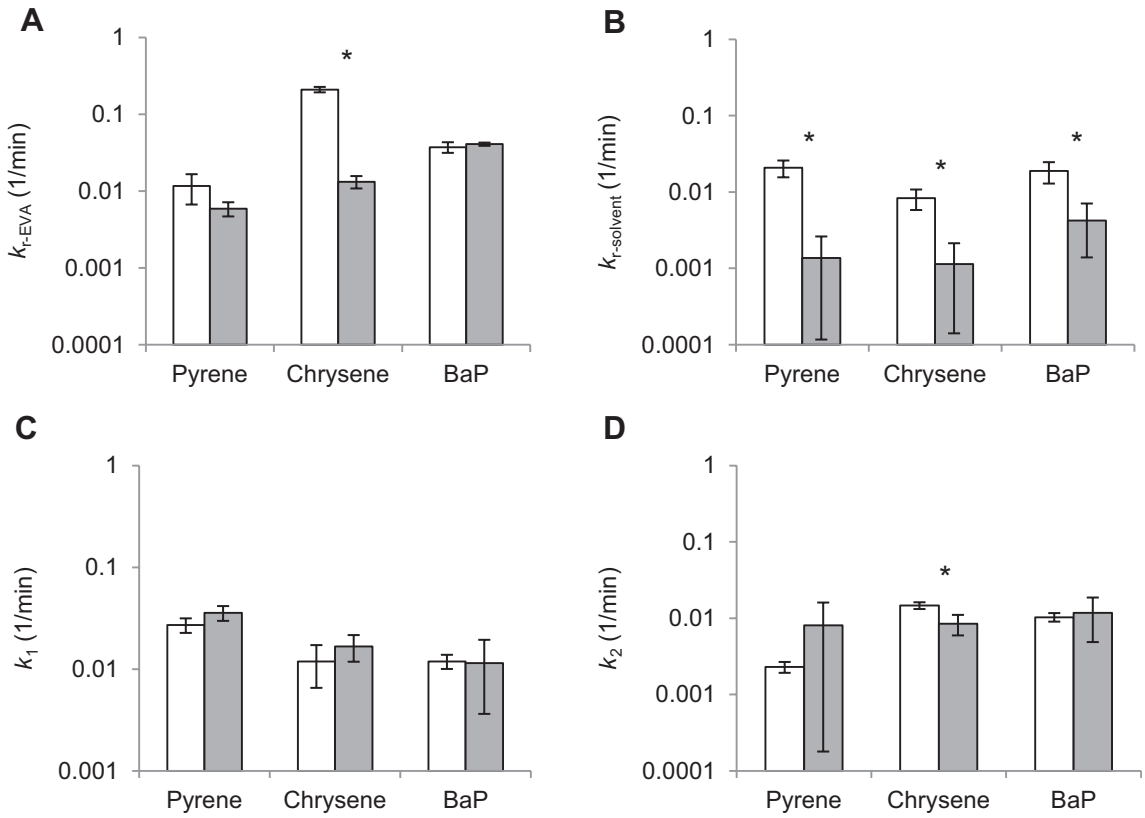

Figure 4. Measured in vitro biotransformation rates determined in the sorbent-phase dosing experiments ($k_{r\text{-}EVA}$; **A**) or in the solvent-delivery dosing experiments ($k_{r\text{-}solvent}$; **B**), sorbent-to-medium mass transfer rate constants (k_1; **C**), and medium-to-sorbent mass-transfer rate constants (k_2; **D**) obtained from single-chemical dosing experiments (empty bars) or multichemical dosing experiments (filled bars). Mass-transfer rate constants for pyrene were obtained from concentrations in the liver incubation medium; mass transfer rate constants for chrysene and benzo[*a*]pyrene (BaP) were obtained from concentrations in the sorbent phase. In vitro biotransformation rates were obtained from concentrations in the liver medium for all test chemicals. Error bars represent the standard deviation ($n = 3$). *Significant difference ($p < 0.05$). EVA = ethylene vinyl acetate.

vitro biotransformation rate constants (k_r) obtained from single-chemical dosing experiments were significantly greater than those obtained from multiple-chemical dosing experiments (Figure 4B). Similar results were reported for chrysene and benzo[*a*]pyrene biotransformation in a solvent delivery dosing experiment with rainbow trout hepatocytes (J. Trowell, Simon Fraser University, Burnaby, BC, Canada, unpublished data). The results suggest that the biotransformation of the test chemicals is inhibited by the presence of other substrates in the incubation mixture. In solvent-delivery experiments, therefore, chemicals should be dosed individually to measure the full metabolic capacity of the liver preparation. However, the observation that the mixture effect was much smaller in the sorbent phase dosing experiments than that in the solvent delivery–based dosing experiments (and apparently absent for pyrene and benzo[*a*]pyrene) suggests that the dosing method also has an effect on the apparent biotransformation rates of chemicals when dosed together. The lower initial substrate concentrations in the incubation medium in the thin-film dosing experiments compared with those in the solvent-delivery dosing experiments may reduce competitive inhibition and be partly responsible for this observation. A sorbent-delivery system may therefore have greater potential for measuring biotransformation rates of multiple chemicals, although further work on this issue appears necessary.

The results of the present study suggest that thin-film sorbent-phase dosing is a particularly useful method for measuring in vitro biotransformation rates of substances that are highly hydrophobic (e.g., log $K_{OW} > 5$), and hence very difficult to fully dissolve in aqueous media, and that have a high log K_{OA} (e.g., log $K_{OA} > 5$), which reduces measurement error among replicate thin-film preparations concentration because of reduction of evaporative losses of the test chemical from the films. An important advantage of thin-film sorbent dosing over solvent delivery–based dosing is the direct measurement of the fraction of unbound test chemical in the incubation. The unbound fraction of very hydrophobic chemicals can be very low and play an important role in the in vitro to in vivo extrapolation of biotransformation rates and may be difficult to estimate by other means. A disadvantage of the thin-film sorbent phase dosing method is that the release rate of the chemical from the film to the incubation medium falls with decreasing temperature and with increasing K_{OW}. Although slow thin-film release rates do not pose a fundamental problem to the application of the thin-film sorbent technique to measure biotransformation rates, they do require the adoption of more sensitive techniques for chemical detection and lower detection limits. The sorbent-phase dosing technique has shown potential for simultaneous measurement of biotransformation rates of multiple chemicals, whereas the solvent delivery–based experiments have indicated that biotransformation rates are best measured when dosing chemicals individually. Further investigations are needed to refine the sorbent-phase dosing system, to explore its application to compounds other than polycyclic aromatic hydrocarbons, and to investigate its use for simultaneous measurement of biotransformation rates of multiple

chemicals, with the ultimate goal of improving bioaccumulation assessments.

SUPPLEMENTAL DATA

Figures S1–S3. (209 KB DOCX).

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REFERENCES

1. Arnot JA, Gobas FAPC. 2006. A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. *Environ Rev* 14:257–297.
2. Arnot JA, Meylan W, Tunkel J, Howard PH, Mackay D, Bonnell M, Boethling RS. 2009. A quantitative structure–activity relationship for predicting metabolic biotransformation rates for organic chemicals in fish. *Environ Toxicol Chem* 28:1168–1177.
3. Nichols JW, Schultz IR, Fitzsimmons PN. 2006. In vitro–in vivo extrapolation of quantitative hepatic biotransformation data for fish I. A review of methods and strategies for incorporating intrinsic clearance estimates into chemical kinetic models. *Aquat Toxicol* 78:74–90.
4. Cowan-Ellsberry CE, Dyer SD, Erhardt S, Bernhard MJ, Roe AL, Dowty ME, Weisbrod AV. 2008. Approach for extrapolating in vitro metabolism data to refine bioconcentration factor estimates. *Chemosphere* 70:1804–1817.
5. Nichols JW, Bonnell M, Dimitrov SD, Escher BI, Han X, Kramer NI. 2009. Bioaccumulation assessment using predictive approaches. *Integr Environ Assess Manag* 5:577–597.
6. 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.
7. Dyer SD, Bernhard MJ, Cowan-Ellsberry C, Perdu-Durand E, Demmerle S, Cravedi JP. 2008. In vitro biotransformation of surfactants in fish. Part I: Linear alkylbenzene sulfonate (C12-LAS) and alcohol ethoxylate (C13EO8). *Chemosphere* 72:850–862.
8. 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.
9. Weisbrod AV, Sahi J, Segner H, James MO, Nichols J, Schultz I, Erhardt S, Cowan-Ellsberry C, Bonnell M, Hoeger B. 2009. The state of in vitro science for use in bioaccumulation assessments for fish. *Environ Toxicol Chem* 28:86–96.
10. Arnot JA, Gobas FAPC. 2004. A food web bioaccumulation model for organic chemicals in aquatic ecosystems. *Environ Toxicol Chem* 23:2343–2355.
11. Escher BI, Cowan-Ellsberry CE, Dyer S, Embry MR, Erhardt S, Halder M, Kwon JH, Johanning K, Oosterwijk MTT, Rutishauser S, Segner H, Nichols J. 2011. Protein and lipid binding parameters in rainbow trout (Oncorhynchus mykiss) blood and liver fractions to extrapolate from an in vitro metabolic degradation assay to in vivo bioaccumulation potential of hydrophobic organic chemicals. *Chem Res Toxicol* 24:1134–1143.
12. 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.
13. Uchea C, Sarda S, Schulz-Utermoehl T, Owen S, Chipman KJ. 2013. In vitro models of xenobiotic metabolism in trout for use in environmental bioaccumulation studies. *Xenobiotica* 43:421–431.
14. Nichols JW, Hoffman AD, Fitzsimmons PN. 2009. Optimization of an isolated perfused rainbow trout liver model: Clearance studies with 7-ethoxycoumarin. *Aquat Toxicol* 95:182–194.
15. 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.
16. Mayer P, Wernsing J, Tolls J, de Maagd PGJ, Sijm DTHM. 1999. Establishing and controlling dissolved concentrations of hydrophobic organics by partitioning from a solid phase. *Environ Sci Technol* 33: 2284–2290.
17. Brown RS, Akhtar P, Akerman J, Hampel L, Kozin IS, Villerius LA, Klamer HJC. 2001. Partition controlled delivery of hydrophobic substances in toxicity tests using poly(dimethylsiloxane) (PDMS) films. *Environ Sci Technol* 35:4097–4102.
18. Kiparissis Y, Akhtar P, Hodson PV, Brown RS. 2003. Partition-controlled delivery of toxicants: A novel in vivo approach for embryo toxicity testing. *Environ Sci Technol* 37:2262–2266.
19. Mayer P, Holmstrup M. 2008. Passive dosing of soil invertebrates with polycyclic aromatic hydrocarbons: Limited chemical activity explains toxicity cutoff. *Environ Sci Technol* 42:7516–7521.
20. Kwon JH, Wuethrich T, Mayer P, Escher BI. 2009. Development of a dynamic delivery method for in vitro bioassays. *Chemosphere* 76:83–90.
21. Smith KEC, Oostingh GJ, Mayer P. 2010. Passive dosing for producing defined and constant exposure of hydrophobic organic compounds during in vitro toxicity tests. *Chem Res Toxicol* 23:55–65.
22. Kramer NI, Busser FJM, Oosterwijk MTT, Schirmer K, Escher BI, Hermens JLM. 2010. Development of a partition-controlled dosing system for cell assays. *Chem Res Toxicol* 23:1806–1814.
23. Smith KEC, Rein A, Trapp S, Mayer P, Karlson UG. 2012. Dynamic passive dosing for studying the biotransformation of hydrophobic organic chemicals: Microbial degradation as an example. *Environ Sci Technol* 46:4852–4860.
24. Kolanczyk RC, Fitzsimmons PN, McKim JM, Ericson RJ, Schmeider PK. 2003. Effects of anesthesia (tricaine methanesulfonate, MS222) on liver biotransformation in rainbow trout (Oncorhynchus mykiss). *Aquat Toxicol* 64:177–184.
25. Bradford MM. 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Anal Biochem* 72:248–254.
26. 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 Press, Boca Raton, FL, USA.
27. Law FCP, Abedini S, Kennedy CJ. 1991. A biologically based toxicokinetic model for pyrene in rainbow trout. *Toxicol Appl Pharmacol* 110:390–402.
28. 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 (Oncorhynchus mykiss). *Comp Biochem Physiol Part C* 97:127–132.
29. Golding CJ, Gobas FAPC, Birch GE. 2008. A fugacity approach for assessing the bioaccumulation of hydrophobic organic compounds from estuarine sediment. *Environ Toxicol Chem* 27:1047–1054.
30. Niesner R, Heintz A. 2000. Diffusion coefficients of aromatics in aqueous solution. *J Chem Eng Data* 45:1121–1124.
31. Obach RS. 1997. Nonspecific binding to microsomes: Impact on scale-up of in vitro intrinsic clearance to hepatic clearance as assessed through examination of warfarin, imipramine, and propranolol. *Drug Metab Dispos* 25:1359–1369.
32. Obach RS. 1999. Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: An examination of in vitro half-life approach and nonspecific binding to microsomes. *Drug Metab Dispos* 27:1350–1359.
33. Poulin P, Kenny JR, Hop CECA, Haddad S. 2012. In vitro–in vivo extrapolation of clearance: Modeling hepatic metabolic clearance of highly bound drugs and comparative assessment with existing calculation methods. *J Pharm Sci* 101:838–851.
34. Pangrekar J, Kandaswami C, Kole P, Kumar S, Sikka HC. 1995. Comparative metabolism of benzo[a]pyrene, chrysene and phenanthrene by brown bullhead liver microsomes. *Mar Environ Res* 39:51–55.
35. Johanning K, Hancock G, Escher BI, Adekola A, Bernhard M, Cowan-Ellsberry C, Domoradzki J, Dyer S, Eickhoff C, Erhardt S, Fitzsimmons P, Halder M, Nichols J, Rutishauser S, Sharpe A, Segner H, Schultz I, Embry M. 2012. In vitro metabolism using rainbow trout liver S9. Summary report of the HESI Bioaccumulation Committee. [cited 2014 January 22]. Available from: http://www.hesiglobal.org/files/public/Committees/Bioaccumulation/Presentations%20and%20Data%20Resources/S9_report_FINAL_20Nov2012.pdf.
36. Kwon JH, Wuethrich T, Mayer P, Escher BI. 2007. Dynamic permeation method to determine partition coefficients of highly hydrophobic chemicals between poly(dimethylsiloxane) and water. *Anal Chem* 79: 6816–6822.
37. Stegeman JJ, Soballa V, Morrison HG, Jacob J, Doehmer J. 1998. Catalytic properties of scup cytochrome P4501A expressed in Chinese hamster V79 cells. *Mar Environ Res* 46:395.
38. Schlenk D, Celander M, Gallagher EP, George S, James M, Kullman SW, van den Hurk P, Willett K. 2008. Biotransformation in fishes. In DiGiulio RT, Hinton DE, eds, *The Toxicology of Fishes*. CRC Press, Boca Raton, FL, USA, pp 9–54.
39. Uno T, Ishizuka M, Itakura T. 2012. Cytochrome P450 (CYP) in fish. *Environ Toxicol Pharmacol* 34:1–34.