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In vitro biotransformation of surfactants in fish. Part II – Alcohol ethoxylate (C16EO8) and alcohol ethoxylate sulfate (C14EO2S) to estimate bioconcentration potential

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ABSTRACT

Recent regulatory pressures (e.g., REACH, CEPA) requiring bioaccumulation assessments and the need for reduced animal use have increased the necessity for the development of *in vitro*-based methods to estimate bioaccumulation. Our study explored the potential use of subcellular and cellular hepatic systems to determine the biotransformation potential of two surfactants: octaethylene glycol monohexadecyl ether (C16EO8) and diethylene glycol monotetradecyl ether sulfate (C14EO2S). The subcellular systems tested were liver homogenates and microsomes from the common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*). Cellular systems consisted of primary hepatocytes from the common carp (*C. carpio*) and PLHC-1 cells, hepatocarcinoma cells from the desert topminnow (*Poeciliopsis lucida*) cell line. Each *in vitro* system was exposed to radiolabeled test compounds and assayed for biotransformation using liquid scintillation and thin layer chromatographic methods. First-order kinetics were used to estimate rates of biotransformation. Bioconcentration of test materials in fish were predicted using an *in vitro* to *in vivo* metabolic rate extrapolation model linked to a mass-balance model commonly used to predict bioaccumulation in fish. Both subcellular and cellular tests using microsomes, liver homogenates and hepatocytes respectively showed biotransformation of the parent surfactants. Biotransformation rates were fastest for hepatocytes, followed by microsomes and homogenates. Rates were too low from homogenate tests to extrapolate to *in vivo*-based biotransformation rates using the extrapolation model. Trout microsomes metabolized C16EO8 faster than carp microsomes, yet rates were approximately the same for C14EO2S. Predicted BCF values incorporating *in vitro* biotransformation rates from hepatocytes were similar to measured *in vivo* or USEPA's bioconcentration model (BCFWIN) predicted values. Predicted BCF values using microsomal-based rates from trout and carp studies were only slightly less than default BCF values which assumes a linear log Kow to BCF relationship with no biotransformation. However, hepatocyte-based results showed substantially decreased BCFs compared to the default BCF values. These results indicate that BCF estimates based on *in vitro* metabolic rates can provide reasonable estimates of *in vivo* BCF values, therefore, supporting the use of *in vitro* approaches within a tiered approach to assess bioconcentration.

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1. Introduction

Fish are the primary organisms used to assess the bioconcentration factor (BCF) of chemicals. *In vivo* experimental determination of the BCF is expensive and time-consuming (e.g., OECD 305E, 1996), making the potential measurement of thousands

of chemicals via new chemical management procedures such as REACH and CEPA (DGEE, 2003; Government of Canada, 1999) impractical. Additionally, an ethical goal of animal welfare includes the reduction of fish sacrificed during testing, in accordance with societal desires. Within this context, there is a need for biologically-based estimation methods for BCF that can supply the missing data and reduce the numbers of fish tested (Meylan et al., 1999). It is well established that the bioconcentration of a chemical is related to its lipophilicity (Veith et al., 1979), hence

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it is possible to predict the BCF for certain classes of organic chemicals from their octanol/water partition coefficient (Kow; Connell, 1993; Neff, 2002). Even so, some organic chemicals do not fit with this model as some are metabolized relatively rapidly by fish and/or are eliminated faster than would be expected if diffusion was the only process involved (Cravedi, 2002). Fish have similar biotransformation enzymes as those found in mammals, including those catalyzing phase I and phase II reactions, although reactivity is often lower (Cravedi 2002; Han et al., 2007). Meylan et al. (1999) empirically incorporated metabolism into a general estimation model for BCF by defining correction factors for those chemical classes for which it is known to have a significant influence. However, published studies on *in vivo* or *in vitro* fish metabolism are scarce for a large number of chemical classes for which correction factors might be developed. This is especially the situation for surfactants.

Three recent reviews have illustrated the potential for *in vitro* systems to provide biotransformation rates for the estimation of an *in vivo* 'B' (bioconcentration and/or bioaccumulation) assessment (DeWolf et al., 2006; Nichols et al., 2006, 2007). Cravedi (2002) and Nichols et al. (2006, 2007) acknowledge that biotransformation rate is a dominant factor in determining the extent to which chemicals may bioconcentrate. For example, (Nichols et al., 2006, 2007) illustrated that *in vitro* biotransformation rates from hepatic systems (e.g., microsomes, S9 fractions and hepatocytes) can be used as inputs in a fish bioaccumulation model (Arnot and Gobas, 2004, also see <http://www.rem.sfu.ca/toxicology/models/models.htm>) that incorporates the use of metabolism. Recent studies by Han et al. (2007, 2009), Cowan-Ellsberry et al. (2008) and Dyer et al. (2008) have shown how *in vitro* biotransformation rates can be scaled up to *in vivo* rates, thereby enabling the prediction of BCF values that incorporate metabolism.

Research has shown that measured bioconcentration factors (BCFs) of surfactants in fish are lower than predictions via Kow-based models due to the metabolism and rapid depuration (Tolls et al., 1994, 1995, 1997, 1998, 1999; Newsome et al., 1995). Tolls et al., (2000) demonstrated via *in vivo* experiments with fish exposed to the alcohol ethoxylate (AE) C16EO8 and other homologs that measured BCFs were less than predicted, based on Kow-only expectations. AEs have been shown to be extensively metabolized by mammals. For example Drotman (1980) found that rats orally dosed with AEs (e.g., C12EO6, C13EO6) were rapidly eliminated in urine, presumably as polar metabolites. According to Newsome et al. (1995) and Tolls and Sijm (1999), AEs may be biotransformed via a couple of pathways, such as: initial ω -oxidation of the alkyl-terminus and/or ether cleavage leading to a short chain polyethoxylate glycol and a carboxylic acid followed by β -oxidation. AEs have also been shown to be biotransformed in wastewater via ether cleavage (Matthijs et al., 1995).

Since determining biotransformation *in vitro* in fish is a relatively new field, our study investigated the potential use of several *in vitro* systems to assess *in vivo* biotransformation and bioconcentration in fish by exposing hepatic cells and subcellular fractions to C16EO8 and C14EO2S. Furthermore, these *in vitro* biotransformation kinetics were extrapolated up to *in vivo* responses (kinetics, BCF) to demonstrate the validity of the *in vitro* approach.

In the cell systems, we determined cytotoxicity, uptake and biotransformation in two *in vitro* cellular systems: fresh isolated primary hepatocytes from the common carp (*Cyprinus carpio*) and an immortalized cell line (PLHC-1). In subcellular systems, we determined biotransformation rates in liver homogenates and microsomal fractions collected from rainbow trout (*Oncorhynchus mykiss*) and the common carp. This study (as well as Dyer et al., 2008) was conducted under an umbrella of surfactant-based re-

search sponsored by ERASM (Environmental Risk Assessment and Management, see <http://www.erasm.org/study.htm>) with the purpose of developing *in vitro* methods for bioaccumulation.

2. Methods

2.1. Test chemicals and characterization

2.1.1. Octaethylene glycol monohexadecyl ether (C16EO8)

Octaethylene glycol monohexadecyl ether was obtained from Fluka Chemical Corp (Milwaukee, WI; catalogue #74717). An assay by GC area listed on manufacturer's certificate of analysis stated 100% purity. An in-house characterization by super fluid critical mass spectroscopy (SFC/MS) indicated 92.1% purity. Additional in-house characterization by NMR indicated an average chain length of C16EO8.5.

Radiolabeled C16EO8 (^{14}C -C16EO8) was synthesized by Procter and Gamble Company (P&G, Cincinnati, Ohio) synthesis laboratory in 2003. The label was located on the alkyl chain alpha to the ethoxylate. The specific activity was 1030.45 MBq/g. Thin layer chromatography using radioactivity detection (TLC-RAD) using two different solvent systems showed a single peak. Proton NMR indicated a spectrum consistent with the structure.

2.1.2. Diethylene glycol monotetradecyl ether sulfate (C14EO2S)

Non-radiolabeled C14EO2S (purity 94.7%) and ^{14}C -C14EO2S (specific activity 1073.3 MBq/g) were synthesized by the Procter and Gamble Company (P&G), Cincinnati, Ohio. The radiolabel was located on the alkyl chain alpha to the ethoxylate and normal phase TLC-RAD indicated a single peak.

7-Ethoxy[3- ^{14}C]-coumarin (2038.7 MBq/mmol, radiochemical purity > 98%), used to measure cytochrome P450 activity, was from Amersham (Les Ulis, France). Unlabeled ethoxycoumarin was from Sigma (Saint Quentin Fallavier, France).

2.2. Fish culture

2.2.1. Culture I

C. carpio used by P&G were obtained through Aquatic Research Organisms (ARO, Hampton, NH, USA) and were fed Hikari Koi Staple Diet (Hayward, California, USA) twice daily. Fish were acclimated for a minimum of 8 d before use and were not fed approximately 18 h prior to test initiation. Cultures were maintained in large (890 L) flow-through aquaria supplied with blended well water (hardness \sim 180 mg CaCO_3/L ; pH \sim 8; and temperature 22–26 °C). Aquaria water was aerated and filtered through canister filters containing carbon and zeolite. A 16 h light: 8 h dark cycle at light intensities ranging from 50 to 90 LUX was provided. Fish wet weights ranged from 156.6 to 385.5 g at the time of sacrifice. Liver and gonads were then immediately removed and weighed.

2.2.2. Culture II

Carp (*C. carpio*) weighing approximately 250 g were obtained from "Station Experimentale Piscicole de l'Indre" (SEPIB, Le Blanc, France) and were fed a carp intensive diet (SARB, Tours, France) twice daily. The constant water conditions were kept: water temperature (24 ± 2 °C), hardness (150–220 mg CaCO_3/L) and pH \sim 7.2.

2.2.3. Culture III

Rainbow trout (*O. mykiss*) having a mean weight of approximately 220 g were from INRA pisciculture at Donzacq, France, and were fed once a day a pelleted commercial feed (Trouvit, Fontaine Les Vervins, France). The trout culture conditions were: water temperature (17 ± 1 °C), hardness (\sim 120 mg CaCO_3/L) and an average pH of 7.4.

2.3. Cellular and subcellular preparation and characterization

2.3.1. Isolated primary hepatocyte preparation (from Culture I)

Hepatocyte preparation followed the protocol described by Dyer et al. (2008). Briefly, hepatocytes were isolated via a two step process involving an initial digestion with 1 M collagenase (Type 1, Sigma Aldrich, St. Louis, MO, USA) and 2 M calcium chloride in a trypsinizing flask at room temp ($\sim 20^\circ\text{C}$) for 20 min using a stir plate, followed by a 1–2 h trypsinization step (0.05% trypsin and 0.53 mM ethylenediaminetetraacetic acid (EDTA) per 3 g of tissue). Cells in the supernatant were transferred into Leibovitz –15 (L-15, Gibco[®], Invitrogen Corp., Carlsbad, CA, USA) media supplemented with 5% FBS (fetal bovine serum) and gentamicin sulfate. This final step halts the trypsin-based reaction. Cells in media were centrifuged at 100 g for 7 min. The resulting pellet was remixed with 10–15 mL of L-15 media and then filtered through a nylon filter (250 μm , Sefar Filtration, Inc., Kansas City, MO, USA). Mean and standard deviation of cellular yields were 8.4×10^7 and 2.1×10^7 cells/g liver, respectively. Approximately 400 000 primary cells (determined via direct counting using a hemacytometer) were seeded per well in 96-well microtiter plates. Primaria[™] plates (BD Biosciences, Rockville, MD, USA) were used for primary cells to obtain sufficient attachment.

2.3.2. Culture of PLHC-1 cells

The immortalized cell line, PLHC-1, was derived from a hepatocellular carcinoma induced in *Poeciliopsis lucida*, a desert topminnow, using 7,12-dimethyl-benz(a)anthracene (Schultz and Schultz, 1985). Cells were originally obtained from Dr. L. Hightower, Univ. Connecticut, Storrs, CT, USA and cryogenically preserved in liquid nitrogen. Cells were cultured in Leibovitz-15 media, supplemented 5% FBS (Gibco[®]) and gentamicin sulfate. Cultures were used within 20 passages of being removed from cryogenic storage. Cells were grown to confluency in T-75 flasks and then subcultured by dissociating with 0.05% (w/v) trypsin and 0.5 mM EDTA in calcium and magnesium free phosphate buffered saline. Approximately 100 000 PLHC-1 cells (determined via direct counting using a hemacytometer) seeded per well in 96-well microtiter plates.

2.3.3. Subcellular system preparations (from Cultures II and III)

Carp livers were weighed and divided into three pools of 15 livers each. Trout livers were collected from nine fish and pooled in three groups of three samples each. Thawed livers were sliced into small pieces then washed twice with cold homogenizing buffer consisting of 25 mM HEPES, 1.25 mM EDTA, 1 mM DTT and 10% glycerol adjusted to pH 7.4. Liver fragments were then homogenized mechanically using a Potter homogenizer as described by Perdu-Durand and Cravedi (1989). The suspension was filtered through two cotton meshes that were laid upon another and then adjusted to 4 mL buffer per gram of liver. The first centrifugation was performed at 600g for 10 min. Supernatants were carefully removed and 1 mL aliquots of homogenate suspension were frozen in appropriate vials and stored at -80°C . The remaining supernatant was transferred into ultracentrifuge tubes for a second centrifugation at 10 000g (ultracentrifuge model Centrikon T-1045 – Kontron Instruments, Zürich, Switzerland) for 20 min. For microsomal fractions, the supernatant obtained was transferred again into ultracentrifuge tubes and spun at 105 000g for 60 min. The resulting pellets were then resuspended and homogenized mechanically with Potter homogenizer in a buffer $\text{PO}_4 \text{ Na/K}$ 50 mM, pH 7.4 containing 1 mM EDTA, 1 mM DTT and 20% glycerol. The microsomal suspension was brought to a total volume of 1 mL buffer per gram of liver. Aliquots of microsomes (0.5 mL) were frozen and stored at -80°C .

2.3.4. Characterization of the subcellular fractions

Subcellular fractions were characterized for total protein, esterase and ECOD (Ethoxycoumarin O-Deethylase) activities. Total protein was determined via the Lowry method, using bovine serum albumin as a standard and was measured by the reaction with Folin's phenol reagent for each of the pooled samples (homogenate, microsomes). Measurements were performed on an UV spectrophotometer set at 750 nm (Uvikon 810 – Kontron Instruments, Zürich, Switzerland). Samples were analyzed in triplicate and compared to the standard curve with bovine serum albumin concentration ranging from 0.02–0.20 mg/mL. Protein concentration was expressed in mg/mL. Esterase activity was assayed by measuring at 405 nm the production of p-nitrophenol from p-nitrophenyl acetate. The protein subcellular fraction (0.02 mg) was added to 3 mL medium containing 20 mM phosphate buffer pH 7.4, 1 mM EDTA and 0.1% Triton X100. The reaction was performed at 25°C and was started by addition of 10 μL of 0.1 M p-nitrophenol acetate in ice-cold methanol and followed up for 3 min. A blank (without enzyme) was run simultaneous in order to permit correction for spontaneous substrate hydrolysis. A calibration curve was used to calculate the quantity of p-nitrophenol formed. Samples were measured in triplicate and esterase activity was expressed in μmol p-nitrophenol formed/h/mg protein. ECOD activity was determined using ^{14}C -labeled 7-ethoxycoumarin as the substrate (10 nmol, 9167 Bq) and incubated in 1 mL $\text{PO}_4 \text{ Na/K}$ 0.1 M, 5 mM MgCl_2 buffer (pH 7.4) containing the subcellular fraction (10 mg protein homogenate, or 2 mg protein microsomes) and a NADPH-generating system (NADP 1.27 mM, glucose 6-phosphate 5 mM and glucose 6-phosphate dehydrogenase 2 IU).

After 2 h incubations at 25°C , 3 mL methanol was added to stop the reaction process and samples were stored at 4°C until HPLC analysis. Post incubation, samples were centrifuged, protein precipitated and then evaporated and analyzed by HPLC as described by Zalko et al. (1998). The analyses were performed on a C-18 reversed-phase column (ODS2 Spherisorb, $4.6 \times 250 \text{ mm}$; 5 μm ; Interchrom, Montluçon, France) using a Spectra Physics P1000 system (Les Ulis, France). The flow rate was 1 mL/min and the temperature was controlled at 30°C . Mobile phases consisted of ammonium acetate buffer (50 mM, pH 5) and acetonitrile 85:15 (v/v) for A and ammonium acetate buffer (50 mM, pH 5) and acetonitrile 20:80 (v/v) for B. A two-step gradient was developed as follows: 0–5 min, 100% A; 5–25 min, linear gradient from 100% A to 50:50 (v/v) A/B; linear gradient leading to 100% B. The eluent was monitored by ^{14}C detection with a Packard Flo-One A500 (Packard Instrument Co, Downers Grove, IL), using Flo-Scint II as liquid scintillation cocktail (Perkin Elmer Life and Analytical Sciences, Shelton, CT, USA). The chromatographic conditions permitted the separation and the quantification of 7-ethoxycoumarin and its major metabolite, 7-hydroxycoumarin. ECOD activity was expressed as pmol hydroxycoumarin formed/h/mg protein. The effect of surfactant concentrations on ECOD activity were investigated by exposing carp homogenate and microsomes to 10 and 100 μM of C16E08 and C14E02S.

2.4. Cytotoxicity assays

Cytotoxicity tests were conducted to determine the maximum concentration suitable for cellular biotransformation assays (i.e., sans-toxicity, $\leq \text{EC}_{50}$). Cytotoxicity for C16E08 and C14E02S was determined via uptake of neutral red (NRU) dye into lysosomes. Concentration-responses were expressed as percent control compared to micromolar (μM) surfactant concentration. Neutral red uptake serves as an indicator of living cells.

Ninety-six well microtiter plates were seeded with PLHC-1 or primary cells 24 h prior to test initiation and incubated with supplemented L-15 media at 30°C . After 24 h, media was removed

from the plate wells and replaced with 200 μL of dosing dilutions containing the test substance prepared in supplemented L-15 media. Nine dosing solutions were developed for C16E08 ranging from 10 to 90 μM for primary cells and 2 to 34 μM for PLHC-1 cells. Nine dosing solutions for C14E02S ranged from 50 to 250 μM for both cell types.

Cells were incubated with test chemicals for approximately 24 (± 3) h. Test solutions were subsequently removed and replaced with 200 μL media containing neutral red (40 $\mu\text{g}/\text{mL}$) and were incubated at 30 $^{\circ}\text{C}$ for 2–3 h. Cells were then fixed with 100 μL of 10% formalin/10% calcium chloride for approximately 3 min and then media removed. After air drying, 100 μL of extraction solution (50% ethanol and 1% acetic acid in water) was added to microplates, mixed for 10 min, then another 100 μL added and mixed again for 10 min. Absorbance was read on a spectrophotometer at 540 nm (baseline subtract at 630 nm).

Concentration-responses were determined by Table Curve 2D (Systat Software Inc., Richmond, California, USA) using logistic dose response equations. EC50 and 95% confidence limit values were determined from Table Curve predicted evaluation tables.

2.5. Biotransformation studies

2.5.1. Incubations with hepatocytes

Incubations for kinetics studies were conducted at concentrations below the EC0 as indicated by NRU. Due to a slight increased sensitivity of PLHC-1 cells to C16E08 compared to primary hepatocytes, kinetics assays were conducted at 2.5 and 5 μM , respectively. All kinetic assays for AES were conducted at 40 μM .

Several Primaria™ microtiter plates were seeded with both primary cells and PLHC-1 at densities of 400 000 and 100 000 cells per well, respectively, approximately 24 h prior to dosing with surfactant. Stock solutions were prepared in L-15 media and verified via liquid scintillation counting. Media were removed from seeded microplates and then each test well was dosed with 200 μL of dosing solution. Control wells were filled with 200 μL of L-15 media only. Additionally, wells without cells (blanks) were dosed for determination of surfactant absorption to plate well surfaces.

Microtiter plates were sacrificed at several time points: 0.5-, 2-, 5-, 10-, 24- and 48-h. Media overlying the cells was placed into vials for total radioactivity counts (LSC) and to determine the proportion of radiolabel biotransformed (via TLC–RAD, thin layer chromatography–radioactivity detection). Cells were subsequently removed from wells via trypsinization (30 μL of 0.05% (w/v) trypsin/0.5 mM EDTA for 1 min, followed by two rinses of either Dulbecco's phosphate buffered saline (DPBS) or de-ionized water) and counted for radioactivity using LSC and TLC–RAD. Samples for radioactivity analysis used water as the final rinse, whereas wells that were enumerated for cell number used DPBS. The 'well rinse' of cell blank controls was subtracted from the cell fraction to account for well wall adsorption. Biotransformation in media and cells was halted by immediately freezing collected samples in liquid nitrogen, and then stored at -80°C . Triplicate wells were sampled for each time point for the kinetics studies.

2.5.2. Incubations with subcellular fractions

Frozen aliquots of carp liver homogenates or microsomes were thawed and kept on ice. ^{14}C -Labeled surfactants were incubated in a phosphate buffer (1 mL PO_4 Na/K buffer 0.1 M, 5 mM MgCl_2 , pH 7.4) with a NADPH-generating system (NADP 1.27 mM, glucose 6-phosphate 5 mM and glucose 6-phosphate dehydrogenase 2 IU) containing the subcellular fractions (homogenate: 10 mg protein, or microsomes: 2 mg protein). Incubations were conducted at 25 $^{\circ}\text{C}$ for 2 h, while shaking. Various concentrations were tested. Blank controls (inactivated subcellular fraction via immersion in 100 $^{\circ}\text{C}$ water for 3 min) were systematically conducted in parallel

with treatments. Biotransformation reactions were halted by adding 3 mL methanol. Subsequently, samples were placed on ice for 10 min to precipitate the proteins and then were centrifuged at 8000 rpm for 10 min. The supernatant was transferred into 15 mL Corex tubes, and submitted to a second centrifugation (8000 rpm, 10 min). An aliquot of the supernatant (500 μL , corresponding to approximately 500 Bq) was evaporated to dryness with a vacuum concentrator Speed Vac SC 110A (Thermo Savant, USA). Dried samples were resuspended in 20 μL methanol prior to TLC analysis.

2.6. Analytical

2.6.1. Liquid scintillation counting

The radioactivity of each incubation medium was determined by direct counting on a scintillation counter (Tricarb 2200CA, Packard Instruments, Meriden, CT, USA), using Ultima Gold (Perkin Elmer Life and Analytical Sciences, Shelton, CT, USA) as the scintillation cocktail.

2.6.2. Thin layer chromatographic analysis for hepatocytes

Frozen samples were lyophilized to dryness and then stored at 4 $^{\circ}\text{C}$ until extracted with chloroform: methanol (25:75, v/v). Cell samples, due to their low activity, were extracted with 400 μL whereas media samples were extracted with 600 μL by vortex mixing and sonication (~ 20 min at 40 $^{\circ}\text{C}$). Aliquots of extraction solvent were counted by LSC to determine extraction efficiency. Another aliquot was used for TLC analysis. For cells, extracts from multiple vials were used for increased activity.

C16E08 was analyzed using normal phase thin layer chromatography with non-conditioned plates (Whatman, Florham Park, New Jersey, USA, LK5 Silica gel 15 A, 250 μM) and an elution solvent of chloroform/methanol/formic acid (90:10:1, v/v/v). The samples of the C14E02S cell kinetics test were analyzed using normal phase TLC with non-preconditioned silica gel. The elution solvent consisted of chloroform/methanol/formic acid (160:50:2, v/v/v). All plates were scanned on a Bioscan Imaging Scanner for radioactivity. Chromatograms were manually integrated and the percent of total activity for individual peaks was reported.

2.6.3. Thin layer chromatographic analysis for subcellular fractions

Thin Layer Chromatography (TLC) separation of C16E08 and C14E02S and corresponding metabolites produced in incubations with subcellular fractions was performed on silica gel pre-coated plates (Kieselgel 60, without fluorescent indicator, 0.25 mm) from Merck, Darmstadt, Germany. Radioactive areas were located and quantified by an automatic TLC–Linear Analyzer LB 284/285 (Bertold, Wilbad, Germany) equipped with a Chroma 1D software. Two solvent mixtures were used: chloroform/methanol/formic acid 90:10:1 v/v/v for C16E08 and chloroform/methanol/water/formic acid 80:25:3:2 v/v/v/v for C14E02S.

2.7. Kinetics

Uptake and loss of parent molecules and generation of metabolites were determined via three different rate equations, each in Table Curve 2D.

$$[A](t) = [A]_0 e^{-k_1 t} \quad (1)$$

$$[B](t) = \frac{k_1}{k_2 - k_1} (e^{k_1 t}) \quad (2)$$

$$[C](t) = \left(1 - \frac{k_2 e^{k_1 t} - e^{k_2 t}}{k_2 - k_1} \right) \quad (3)$$

Eq. (1), an integrated first-order decay equation, was used to determine the rate of loss per hour (h^{-1}) of parent molecules (A) in the exposure media and test system for primary hepatocytes and

PLHC-1 cells, where $[A_0]$ refers to the initial dosed concentration at time zero and k refers to the first-order rate loss for time t . Eq. (2) is the integrated form of a first-order formation and decay function illustrating the uptake ($\mu\text{mol/g/h}$) of parent molecules into cells (step A to B) and loss (h^{-1}) through time due to biotransformation (step B to C). For this equation to be valid, it needs to be established that biotransformation was indeed measured. Eq. (3) describes the formation of total metabolites (C) in both media and cell fractions ($1/\text{h}$). All three equations together allow a mass balance of radiolabel in the microtiter plate test system to be determined.

2.8. Extrapolation to bioconcentration in fish

Intrinsic clearance rates (V_{max}/K_m) from subcellular tests and parent loss (due to biotransformation) from hepatocytes were used as inputs to an *in vitro* to *in vivo* extrapolation model for bioconcentration developed by Cowan-Ellsberry et al. (2008). Briefly, this model takes *in vitro* loss rates and scales them to *in vivo* liver loss rates and then, by using information on the amount of blood flowing through the liver and the effect of chemical binding to proteins and lipid in blood relative to that in the test system, the *in vivo* hepatic clearance is estimated. The final step is to estimate the whole body biotransformation rate constant, k_{MET} , that takes into account how much of the chemical will be distributed from the blood to other tissues (i.e., volume of distribution). BCFs were determined by the mass-balance model described by Arnot and Gobas (2003, 2004). Default BCFs were calculated, assuming no metabolism ($k_{\text{MET}} = 0$) for a 1 kg trout or carp with 10% lipid content exposed to the parent materials at 12 and 23 °C, respectively.

2.9. Statistics

Values were compared using one-way analysis of variance (ANOVA) and are expressed as means \pm SD. When significant ($p < 0.05$), differences were further tested using a Tukey multiple comparison test.

3. Results

3.1. Cytotoxicity

Cytotoxicity of C16E08 was determined for both primary hepatocytes and PLHC-1 cells. PLHC-1 cells were more sensitive than primary hepatocytes, as the EC50s were 7.9 and 29.2 μM , respectively (Table 1). Similarly, PLHC-1 cells were more sensitive to C14E02S than primary cells. The EC50 values for C14E02S were 125.8 and 105.3 μM for primary and PLHC-1 cells, respectively (Table 1). Based on the concentration response relationships, incubations for kinetics studies with C16E08 were conducted at 2.5 and 5 μM for PLHC-1 and primary cells, whereas for C14E02S, both PLHC-1 and primary cells were incubated at 40 μM .

3.2. Subcellular characterization

Esterase activity and biotransformation of 7-ethoxycoumarin (ECOD) were obtained for carp liver homogenates and microsomes

as well as rainbow trout microsomes. Regarding esterase activity (expressed as $\mu\text{mol/h/mg}$ protein), carp microsomal activity was significantly greater than carp homogenates (data in Dyer et al., 2008). ECOD activities of microsomes from carp and trout were greater than carp homogenates (data in Dyer et al., 2008). The ECOD activity in trout was similar to that reported by Banka et al. (1997).

The effect of the surfactants on ECOD activity in carp liver homogenates and microsomes was investigated. Compared to controls, no significant differences in activities were observed in both subcellular fractions at 100 μM C16E08 and C14E02S.

3.3. Cellular kinetics

3.3.1. C16E08

A mass balance of radiolabeled C16E08 in primary hepatocytes and PLHC-1 cells was determined. The total average recovery for the primary cell system was $94.4 \pm 6.9\%$ and $93.4 \pm 2.8\%$ for PLHC-1 cells, as determined by LSC. The average extraction efficiency of media samples for both cell types via TLC was $108.4 \pm 32.5\%$. For both cell types, the average extraction efficiency was $102.4 \pm 31.7\%$.

A majority of radiolabeled parent C16E08 was found in the overlying media for both cell systems. Mean media fractions in the primary cell experiments throughout all time points ranged from 89.3% to 65.7%, decreasing throughout the 72 h study. The remaining fraction was detected in the cellular fraction where means ranged from 2.1% to 23.8%, increasing throughout the study period. Media blanks (no cells) ranged from 92.1% to 88.4% and well rinses ranged from 0.6% to 1.8%, but with no temporal pattern. Similar results were found for the PLHC-1 cells where media fractions ranged from 94.1% to 78.6%, decreasing through time while the cellular fractions ranged from 1.6% to 6.9%, increasing through time. Media blanks ranged from 84.1% to 93.6% and well rinses from 0.9% to 1.3%, both with no temporal change.

Thin layer chromatograms provided data for the proportion of parent C16E08 in overlying media, cells and metabolites found in both cell and media fractions. Parent C16E08 was rapidly taken up by the primary hepatocyte cell fraction and biotransformed to both polar and non-polar metabolites resulting in a loss over time (Fig. 1). The proportion of metabolites increased throughout the exposure period in both cellular and media fractions. The uptake and loss (attributable to biotransformation) of the parent material in the hepatocytes was modeled using a first-order formation and decay model. The loss of parent material from the overlying media was modeled using a first-order decay model. Biotransformation products in both media and cellular fractions were modeled using a first-order formation model. Coefficients of determination (R^2) for all models were between 0.95 and 0.98. The overall good fit for each of the first-order models indicated that the loss of the parent material from the media was due to the uptake of the parent into the cell and then biotransformed. The resulting metabolites were found in both media and cellular fractions, evidence of metabolite efflux from the cellular fraction as well as partitioning within the cell. Considering these findings and assuming a completely mixed model, the parent loss rate from the entire test sys-

Table 1

Summary of cytotoxicity results for primary hepatocytes and PLHC-1 cells exposed to C16E08 and C14E02S for 24 h. Effect concentrations which were relative to uptake of Neutral Red dye into lysosomes of controls are presented in molar and mass bases. Coefficient of determination refers to the molar model. All models were significant ($p < 0.05$).

| Cell type | Test compound | EC50 (μM) | 95% confidence limits | EC50 (mg/L) | R^2 |
|-----------|---------------|------------------------|-----------------------|-------------|-------|
| Primary | C16E08 | 29.2 | 26.4–31.9 | 49.1 | 0.91 |
| PLHC-1 | C16E08 | 7.9 | 7.4–8.5 | 13.3 | 0.97 |
| Primary | C14E02S | 125.8 | 119.7–132.3 | 311.4 | 0.86 |
| PLHC-1 | C14E02S | 105.3 | 103.3–107.6 | 260.6 | 0.98 |

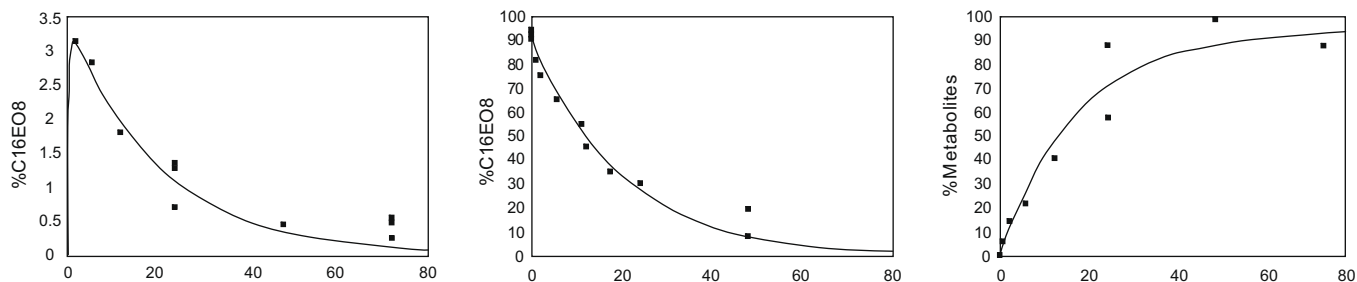


Fig. 1. Kinetics by hour (x-axis). At left, uptake/loss of C16EO8 into primary hepatocytes and loss ($R^2 = 0.95$). Middle plot, loss of C16EO8 from overlying media and cells as result of metabolism ($R^2 = 0.98$). Right figure, percent C16EO8 metabolites from cell and media fractions ($R^2 = 0.95$). Note Y-axis scale per plot is based on percent of initial parent molecule radioactivity.

tem (media and cell fractions) was determined to be 0.069 nmol/cell/h. Considering the test volume and concentration of test material, the *in vitro* intrinsic clearance rate was 2.78E-06 mL/cell/h (Table 2). This value was used in the Cowan-Ellsberry et al. (2008) extrapolation model to predict the *in vivo* metabolism rate (k_{MET}) and bioconcentration factor. The predicted k_{MET} and BCF were 0.768 d^{-1} and 123.4, respectively. Using the Cowan-Ellsberry model, assuming no *in vivo* biotransformation, the default BCF was 2556 for carp.

PLHC-1 cells illustrated uptake of the parent C16EO8 and generation of polar metabolites in cellular and media fractions. However, the proportion of radiolabeled material converted to metabolites was much less, compared to primary cells, suggesting that efflux pumps may play a greater role in parent loss from cells compared to primary hepatocytes (Fig. 2). Normalized on a per protein basis, the rate of uptake C16EO8 in PLHC-1 was similar to that of primary cells, however, the rate of loss was nearly an order of magnitude slower (Table 2).

3.3.2. C14EO2S

For C14EO2S, the total average recovery for primary cell system was 93.6 ± 3.4% as determined by LSC. The average extraction efficiency of all media samples via TLC was 98.1%. Similar results were found with PLHC-1 cells where the total recovery and extraction efficiencies for media and cells were 96.5 ± 1.7%, 98.1 ± 6.1% and 114.6 ± 6.8%, respectively.

A majority of radiolabeled parent C14EO2S was found in the overlying media for both cell systems. Mean media fractions in the primary cell experiments throughout all time points ranged from 86.1% to 75.9%, decreasing throughout the 72 h study. The remaining fraction was detected in the cellular fraction where means ranged from 1.6% to 17.2%, increasing throughout the study period. Media blanks (no cells) ranged from 97.8% to 85.8% and well rinses ranged from 0.8% to 1.6%, but with no temporal pattern. Similar results were observed for PLHC-1 studies where mean media fractions ranged from 91.5% to 85.8%, decreasing throughout the 72 h study. Remaining cellular fractions ranged from 3.8% to 8.5%, increasing throughout the study period. Media blanks (no

cells) ranged from 94.0% to 121% and well rinses ranged from 0.8% to 1.6%, but with no temporal pattern.

TLCs provided data for the proportion of parent C14EO2S in overlying media, cells and metabolites generated in both cell and media fractions. Parent C14EO2S was rapidly taken up by the primary hepatocyte cell fraction and biotransformed to both polar and non-polar metabolites resulting in a loss over time (Fig. 3). The proportion of metabolites increased throughout the exposure period in both cellular and media fractions. The uptake and loss (attributable to biotransformation) of the parent material in the hepatocytes was modeled using a first-order formation and decay model. The loss of parent material from the overlying media was modeled using a first-order decay model. Biotransformation products in both media and cellular fractions were modeled using a first-order formation model. Coefficients of determination (R^2) for all models were between 0.87 and 0.98. The overall good fit for each of the first-order models indicated that the loss of the parent material from the media was due to the uptake of the parent into the cell and then biotransformed. The resulting metabolites were found in both media and cellular fractions, evidence of metabolite efflux from the cellular fraction as well as partitioning within the cell. Considering these findings and assuming a completely mixed model, the parent loss rate from the entire test system (media and cell fractions) was determined to be 0.0079 nmol/cell/h. Considering the test volume and concentration of test material, the *in vitro* intrinsic clearance rate was 3.95E-08 mL/cell/h (Table 2). This value was used in the Cowan-Ellsberry et al. (2008) extrapolation model to predict the *in vivo* metabolism rate (k_{MET}) and bioconcentration factor of 0.157 d^{-1} and 10.3, respectively. Assuming no *in vivo* biotransformation, the default BCF was 13.2 for carp. The kinetics of loss of C14EO2S from media, uptake into PLHC-1 cells and generation of metabolites did not follow first order-rate kinetics.

3.4. Subcellular kinetics

Biotransformation activity of C16EO8 over a 2 h period was investigated in carp microsomes and homogenates as well as in

Table 2
First order *in vitro* clearance rates based on exposures of C16EO8 and C14EO2S to rainbow trout and carp microsomes and primary hepatocytes from carp. Clearance rates were extrapolated to predict *in vivo* metabolism (k_{MET}) rates and BCF using the approach discussed in Cowan-Ellsberry et al. (2008). Default BCFs were based on 1 kg fish with a lipid content of 0.1, a k_{MET} of zero with trout and carp being exposed to 12 and 23 °C, respectively.

| Chemical | Species | <i>In vitro</i> clearance rates | | Predicted fish k_{MET} | | |
|----------|---------|---------------------------------|----------------------|--------------------------|---------------|-------------|
| | | Subcellular (mL/h/g protein) | Cellular (mL/cell/h) | (d^{-1}) | Predicted BCF | Default BCF |
| C16EO8 | Trout | 229.3 | | 0.031 | 1516 | 2881 |
| C16EO8 | Carp | 30.5 | | 0.009 | 2073 | 2556 |
| C14EO2S | Trout | 38.7 | | 0.024 | 13.1 | 13.2 |
| C14EO2S | Carp | 28.6 | | 0.035 | 13.1 | 13.2 |
| C16EO8 | Carp | | 2.78E-06 | 0.768 | 123.4 | 2556 |
| C14EO2S | Carp | | 3.95E-08 | 0.157 | 10.3 | 13.2 |

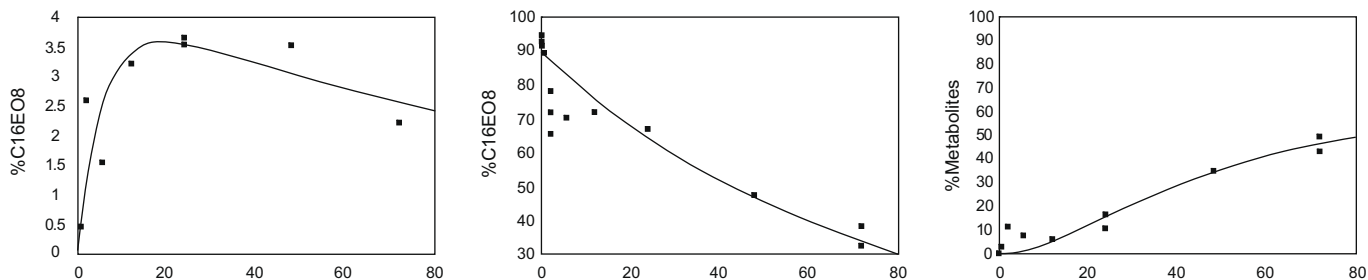


Fig. 2. Kinetics by hour (x-axis). At left, uptake/loss of C16EO8 into PLHC-1 cells and loss ($R^2 = 0.64$). Middle plot, loss of C16EO8 from overlying media and cells as result of metabolism ($R^2 = 0.91$). Right figure, percent C16EO8 metabolites from cell and media fractions ($R^2 = 0.92$). Note Y-axis scale per plot.

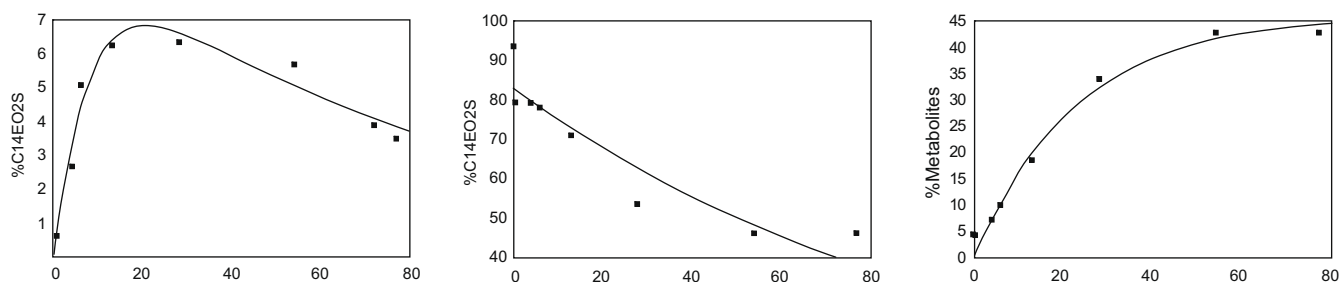


Fig. 3. Kinetics by hour (x-axis). At left, uptake of C14EO2S into primary hepatocytes and loss ($R^2 = 0.94$). Middle plot, loss of C16EO8 from overlying media and cells as result of metabolism ($R^2 = 0.87$). Right figure, percent C16EO8 metabolites from cell and media fractions ($R^2 = 0.98$). Note Y-axis per plot.

rainbow trout microsomes. Four C16EO8 concentrations (10, 50, 100 and 200 μM) were incubated with carp liver subcellular fractions. In microsomes as in homogenates, two metabolites were produced and migrated at Rf 0.80 and 0.87. In homogenates, at the 200 μM concentration, metabolite production was too low to be measured. The production of metabolites followed Michaelis–Menten kinetics increasing with the concentration of C16EO8 and reaching saturation at 100 μM in microsomal fractions (Fig. 4A). However, microsomal biotransformation of C16EO8 in trout was greater than found in carp (Fig. 4B). Michaelis–Menten parameters (V_{max} and K_m) for the production of metabolites were calculated according to the nonlinear regression equation from microsomal incubations. Mean V_{max} , (pmol/min/mg prot), and K_m (μM), parameters for carp microsomes incubated with C16EO8 were 68.94 and 135.68, respectively, whereas they were 289.97 and 75.87 from trout microsomal incubations.

Biotransformation of C14EO2S was also greater in carp microsomes compared to homogenates (Fig. 5A). However, the rate of microsomal biotransformation from carp was very similar to that of rainbow trout (Fig. 5B). Mean V_{max} and K_m parameters from carp microsomes incubated with C14EO2S were 76.86 and 161.46, whereas the same parameters observed via incubations with trout microsomes were 47.87 and 74.17. The ratio of the V_{max} and K_m provided the *in vitro* intrinsic clearance rates presented in Table 2.

3.5. Extrapolations

Predicted *in vivo* metabolism rates (k_{MET}) ranged from 0.009 to 0.035 (d^{-1}) for both surfactants and fish species based on microsomal *in vitro* clearance rates (Table 2). However, much faster k_{MET} values were estimated based on carp hepatocyte tests, ranging from 0.768 (d^{-1}) for C16EO8 to 0.157 (d^{-1}) for C14EO2S. In all cases, the predicted BCFs based on the *in vitro* to *in vivo* extrapolation methods by Cowan-Ellsberry et al. (2008) were less than the default BCF values (assuming no biotransformation) based on a 1 kg fish with a lipid content of 10% with trout and carp being exposed to 12 and 23 $^{\circ}\text{C}$, respectively. The greatest difference from

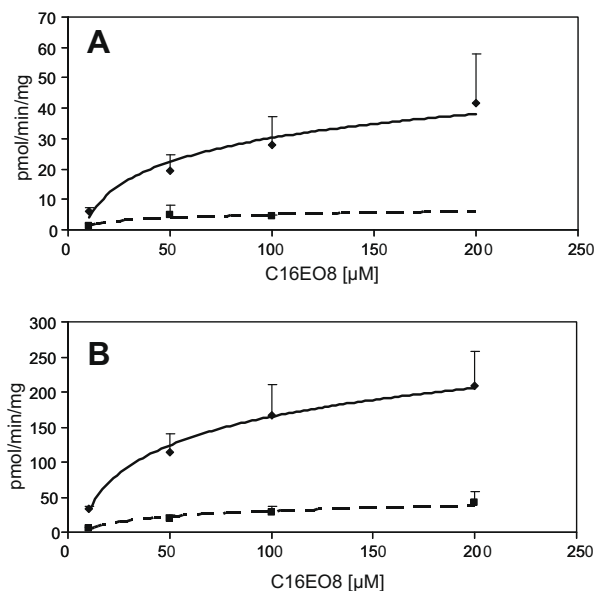


Fig. 4. (A). Michaelis–Menten kinetics for the biotransformation of C16EO8 in carp microsomes (diamonds, solid line) and homogenates (squares, dashed line). (B). Comparison of kinetics for the biotransformation of C16EO8 in microsomes from rainbow trout (diamonds, solid line) and carp (squares, dashed line). Values are means \pm SD from three pools of fish.

default values were found for hepatocyte tests. For example, the default BCF value for C16EO8 at 23 $^{\circ}\text{C}$ was 2556 whereas when incorporating the hepatocyte-derived *in vitro* clearance rate, the predicted BCF was 123.4.

4. Discussion

For both primary hepatocytes and PLHC-1 cells, C16EO8 was found to be more cytotoxic than C14EO2S. Of the two cell types,

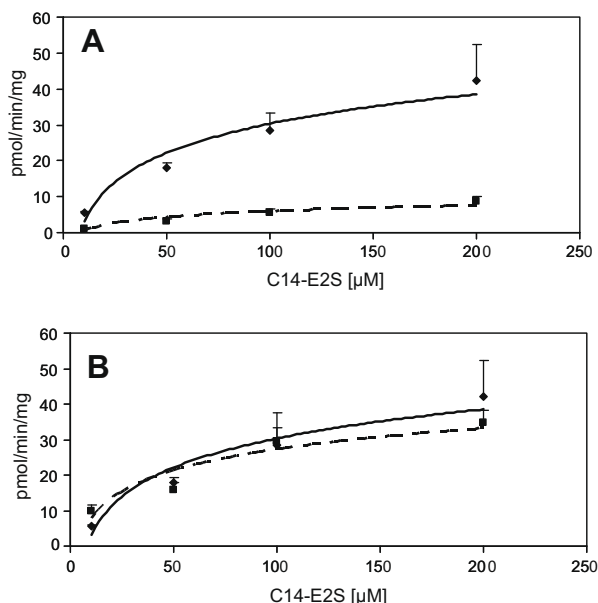


Fig. 5. (A). Michaelis–Menten kinetics for the biotransformation of C14EO2S in carp microsomes (diamonds, solid line) and homogenates (squares, dashed line). (B). Comparison of kinetics for the biotransformation of C14EO2S in microsomes from rainbow trout (diamonds, solid line) and carp (squares, dashed line). Values are means \pm SD from three pools of fish.

PLHC-1 cells were more sensitive to both surfactants. Median effect concentrations reported here were based on nominal concentrations, a typical practice in cytotoxicological studies. Our past studies with surfactants have shown that nominal concentrations are useful to gain a relative understanding of toxicity, whereas determining cell burdens associated with toxicity provides a direct measure of cytotoxicity (Bernhard and Dyer, 2005). In this case we investigated cytotoxicity to determine the maximum test chemical concentration for which biotransformation could be assessed without causing toxicity.

Both PLHC-1 and primary hepatocytes from the carp showed uptake and loss of C16EO8 due to metabolism. Via a first-order formation and decay model, rates of uptake into fish cells and loss of parent compounds from cells were determined (Figs. 1 and 2). First-order uptake and loss due to metabolism was also found in primary hepatocytes exposed to C14EO2S (Fig. 3) however, PLHC-1 cells exposed to C14EO2S did not conform to the first-order rate model. For both test materials the fraction of total radioactivity measured as metabolites in cells and overlying media was greater with the primary hepatocytes than PLHC-1 cells. Thin layer chromatograms for both media and cellular fractions illustrated that the number of potential metabolites were also greatest in primary cells (not shown). It should be noted that our observations of the majority of the parent surfactants partitioning within the overlying media fraction instead of the cells and plastic microtiter plate walls is due to their amphiphilic property (i.e., polar and non-polar ends). It is possible that the microtiter plated-based method may prove difficult with more highly hydrophobic materials (e.g., non-polar with log Kow > 4.5), as these materials will have a propensity to strongly bind to plastic walls.

All the tested fractions (microsomes and liver homogenates) were suitable for measuring ECOD and esterase activities and the comparisons between microsomes and homogenates using the surfactants as substrates indicated similar qualitative results (data in Dyer et al., 2008). Nevertheless, the fastest observed metabolic rates were with microsomes, calculated on a protein basis, therefore microsomes would be preferred for assessing the potential

for *in vivo* biotransformation. The present study showed that C16EO8 was metabolized at a greater extent by trout hepatic microsomes than by those from carp (Table 2 and Fig. 4), whereas C14EO2S was biotransformed similarly in both species (Fig. 5). The comparison of our results with the data reported previously for other surfactants (Dyer et al., 2008) indicates that in carp, C16EO8 and C14EO2S are biotransformed at a lower extent than C12LAS and C13EO8. In trout, the biotransformation rate for C16EO8 was slightly greater than the values measured for C12LAS and C13EO8, whereas the biotransformation of C14EO2S was limited as compared with these compounds (Dyer et al., 2008).

The common pathway for the biotransformation of surfactants is either an enzymatic cleavage of the non-polar and polar surfactant moieties (forming a fatty alcohol/acid and a hydrophilic product) or a terminal oxidation and subsequent stepwise degradation of the alkyl chain (leaving again a more hydrophilic product). The metabolism of the surfactant alkyl chain through a combination of ω - and β -oxidations, with subsequent excretion of a short chain derivative has been demonstrated for several fish species (Newsome et al., 1995; Thibaut et al., 1998; Van Egmond et al., 1999; Tolls and sijn, 1999; Arukwe et al., 2000; Cravedi et al., 2001). The enzyme systems involved in these metabolic steps (ω - and ω -1, and β -oxidations) are mainly present in the liver and are located in microsomes and mitochondria (Thibaut et al., 2002).

In vitro test system clearance rates of parent chemical substrates from both cellular and subcellular test systems were determined and used as inputs into the extrapolation model by Cowan-Ellsberry et al. (2008). This model incorporates exposure temperature and physiological characteristics of fish, including hepatic blood flow, to estimate the whole body biotransformation rate (k_{MET}). The k_{MET} is one of the most important modifying factors in the bioconcentration model by Arnot and Gobas (2003, 2004). Hence, the extrapolation model provides a link between *in vitro* metabolism rate to *in vivo* metabolism rate and then bioconcentration. This approach has also been discussed by Han et al., (2007) and Nichols et al. (2006, 2007). Based on a log Kow of 4.5 for C16EO8, the default BCFs, assuming no metabolism, were 2881 and 2556 for rainbow trout and carp exposed at 12 and 23 °C, respectively. For C14EO2S, the default BCFs for trout and carp were the same, 13.2, due to the relatively low log Kow value of 2.1. Inclusion of *in vitro* clearance in the extrapolation model yielded predicted BCFs for C16EO8 of 1516 and 2073 via microsomal tests with trout and carp, respectively. A BCF of 13.1 was predicted based on carp and trout microsomal tests with C14EO2S. Lower predicted BCFs resulted from tests with primary hepatocytes from carp, where the BCFs of 123.4 and 10.3 were estimated for C16EO8 and C14EO2S, respectively. The relative difference between hepatocytes and microsomes have also been observed by Han et al. (2009), where intrinsic *in vitro* rates and extrapolated BCFs were compared to measured BCFs for a wide array of compounds, such as molinate, Michler's ketone, 4-nonylphenol, 2,4-di-tert-butylphenol and benzo(a)pyrene. In that study, rates from hepatocytes always exceeded that of subcellular fractions, therefore also leading to lower BCFs.

The predicted BCFs for C16EO8 from microsomal and cellular studies bounded the only measured *in vivo* BCF value, 387.5 for fat-head minnow (Tolls et al., 2000). The USEPA program BCFWIN predicted an *in vivo* value of 62. No published *in vivo* BCF data are available for C14EO2S. However, BCFWIN predicted a BCF value of 71 for this material, which was similar to data from an unpublished study by P&G where fish were exposed to a commercial alcohol ethoxylate sulfate containing C14EO2S in an experimental stream study.

It has been proposed that there is a need for *in vitro* systems for investigating biotransformation, toxicity, mechanisms and kinetics of potentially bioaccumulative chemicals (Blaauboer et al., 1994;

DeWolf et al., 2006; Nichols et al., 2006; Nichols et al., 2007; Han et al., 2007; Cowan-Ellsberry et al., 2008). The validity of these systems needs to be urgently evaluated as there are increasing regulatory needs to assess biotransformation and toxicity. For example, under the REACH (Registration, Evaluation and Authorization of Chemicals) regulatory framework for chemicals in the European Union, a chemical is considered 'bioaccumulative' if the BCF is greater than 2000, and 'very bioaccumulative' if the BCF is greater than 5000 (DGEE, 2003). Based on log Kow alone, C16E08 could have been interpreted to have a default BCF exceeding the 'bioaccumulative' criterion, contrary to results from rainbow trout microsomes and carp hepatocytes which clearly show that predicted BCFs that incorporate metabolism resulted in a non-bioaccumulative classification status. Within REACH, *in vitro* systems can potentially play a very important role in future BCF assessments by providing rapid screening for chemical biotransformation while reducing animal usage, time and cost.

Between our data (this study and Dyer et al., 2008) and that of (Han et al., 2007, 2009), it is becoming quite clear that subcellular-based tests provide more conservative (slower) *in vitro* clearance rates than those from hepatocyte-based tests. While these findings fit well within a tiered structure proceeding from subcellular to cellular to modified OECD 305 studies and culminating in official OECD 305 BCF studies, it does beg the question why even conduct subcellular tests and/or how can hepatocyte studies become more accessible? At present, there is a commercial supply for rainbow trout S9, hence any laboratory may be able to conduct preliminary BCF assessments that include S9-based metabolic corrections. Needed, however, are commercial supplies of cryopreserved fish hepatocytes that will enable any laboratory to conduct BCF assessments, but by including more realistic hepatic clearance rates. For future acceptance of *in vitro* methods, it is imperative that development of such cryopreserved cells be created and evaluated, with the hope the metabolically-corrected BCFs can become a routine exercise for bioconcentration assessments.

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