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Metabolism of ^{14}C-octamethylcyclotetrasiloxane ([^{14}C]D_4) or ^{14}C-decamethylcyclopentasiloxane ([^{14}C]D_5) orally gavaged in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Critical factors (uptake, distribution, metabolism and elimination) for understanding the bioaccumulation/biomagnification potential of Octamethylcyclotetrasiloxane (D_4) and Decamethylcyclopentasiloxane (D_5) siloxanes in fish were investigated to address whether these chemicals meet the "B" criteria of the Persistent, Bioaccumulative, and Toxic (PBT) classification. A metabolism study was conducted in rainbow trout whereby a 15 mg [^{14}C]D_4/kg bw or [^{14}C]D_5/kg bw as a single bolus oral dose was administered via gavage. Of the administered dose, 79% (D_4) and 78% (D_5) was recovered by the end of the study (96-h). Eighty-two percent and 25% of the recovered dose was absorbed based on the percentage of recovered dose in carcass (69% and 17%), tissues, bile and blood (12% and 8%) and urine (1%) for D_4 and D_5, respectively. A significant portion of the recovered dose (i.e. 18% for D_4 and 75% for D_5) was eliminated in feces. Maximum blood concentrations were 1.6 and 1.4 µg D_4 or D_5/g blood at 24 h post-dosing, with elimination half-lives of 39 h (D_4) and 70 h (D_5). Modeling of parent and metabolite blood concentrations resulted in estimated metabolism rate constants ($k_{m\,(blood)}$) of 0.15 (D_4) and 0.17 day^{-1}(D_5). Metabolites in tissues, bile, blood, and urine totaled a minimum of 2% (D_4) and 14% (D_5) of the absorbed dose. The highest concentration of ^{14}C-activity in the fish following D_4 administration was in mesenteric fat followed by bile, but the opposite was true for D_5. Metabolites were not detected in fat, only parent chemical. In bile, 94% (D_4) and 99% (D_5) of the ^{14}C-activity was due to metabolites. Metabolites were also detected in the digestive tract, liver and gonads. Approximately 40% of the ^{14}C-activity detected in the liver was due to the presence of metabolites. Urinary elimination represented a minor pathway, but all the ^{14}C-activity in the urine was associated with metabolites. Clearance may occur via enterohepatic circulation of metabolic products in bile with excretion via the digestive tract and urinary clearance of polar metabolites.

1. Introduction

The cyclic volatile methyl siloxanes (cVMS) are a class of silicone compounds that have an unusual combination of physico-chemical properties that results in their wide use in consumer (e.g. shampoos, deodorant, cosmetic) and industrial (e.g. polymer production, dry cleaning solvents, industrial cleaning fluids) applications (Horii and Kannan, 2008; Wang et al., 2009). Octamethylcyclotetrasiloxane (D_4) and decamethylcyclopentasiloxane (D_5) are silicon based materials with octanol-water partitioning coefficients (i.e. log K_{ow}, a surrogate measure of lipophilicity) of 6.98 and 8.07 (Xu and Kropscott, 2012, 2014), respectively, and water solubilities of 56 and 17 µg/L (Varaprath et al., 1996), respectively.

Through use in down the drain consumer products and as a result of manufacturing, these siloxanes may be released into the environment. The release of these chemicals to the environment have raised concerns as to their fate and effects in aquatic ecosystems for governments in Canada (Environment Canada, 2010), the UK (Environment Agency, 2009a; Environment Agency, 2009b), and the Nordic States (Norden, 2005; Lennert et al., 2004). In particular, a judicial review on D_5 has occurred in Canada (Environment Canada, 2011).

Bioconcentration or bioaccumulation potential is a key parameter for chemical classification and labeling, regulatory management, pollution prevention initiatives, and environmental risk assessments.

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For decades, a fundamental premise for assessing chemical bioaccumulation has been the knowledge of a compound's K_{ow} value, which was sufficient for preliminary assessment of bioaccumulation behavior (Barron, 1990; European Chemicals Bureau, 2003). More recently, the preferred metrics for identifying the bioaccumulation potential of a chemical are the compound's bioaccumulation factor (BAF) or bioconcentration factor (BCF). Chemicals with a BAF or BCF equal to or greater than 5000 L/kg-wet weight (ww) are considered to be bioaccumulative in nature (Environment Canada, 1995). In absence of both BAF and BCF data, the log K_{ow} has been identified as a surrogate measure of a chemical's bioaccumulation potential and chemicals with a log $K_{ow} \geq 5$ are considered to have bioaccumulative potential (Environment Canada, 1995).

The process of bioaccumulation may be considered the product of both gill and dietary uptake, attenuated by depuration mechanisms such as gill and gastrointestinal elimination, organism growth, and metabolic transformation.

$$d(C_{fish})/dt = \text{Uptake} - \text{Elimination} - \text{Growth} - \text{Metabolism}$$

Bioaccumulation models for aquatic organisms, validated using carbon-based substances, do exist (Arnot and Gobas, 2003; Arnot and Gobas, 2004) and are commonly used to predict bioaccumulation potential (i.e. “B” in a PBT classification) based on physico-chemical properties such as the compound's log K_{ow} value and the organism's trophic level position. In these models, the aquatic organism metabolism rate (k_m) is usually not known and considered as zero. Metabolic transformation is one of the important attenuation mechanisms and cannot be ignored since it can drastically change whether a substance will magnify or dilute with increasing trophic level (trophic magnification factor, TMF).

Numerous researchers have applied mass balance models and other techniques to in vivo fish bioconcentration/bioaccumulation data to estimate biotransformation rate constants (Arnot and Gobas, 2003; Arnot et al., 2008; van der Linde et al., 2001; Tolls and Sijm, 1999). Mackay et al. (2013) have examined how rapid metabolism or other means of elimination may be an indication of low bioaccumulation (B) potential for a compound. Aquatic food web models (Arnot and Gobas, 2003) have suggested that chemicals with rapid k_m values should not biomagnify in aquatic food webs (i.e., $k_m \geq 0.05\ \text{day}^{-1}$). Several computer models for calculating biotransformation rates are available, but these models still need further refinement (Gobas et al., 2009; Brown et al., 2012; Papa et al., 2014).

In vivo experimental results on metabolism and excretion from toxicokinetic studies in fish represent valuable information in assessing a chemical's ‘B’ potential. The objective of this work is to present experimental data regarding the scope and rate of in vivo fish metabolism for the cyclic siloxanes, D_4 and D_5 and to obtain an estimate of whole-body k_m from blood time course data ($k_{m(\text{blood})}$). Metabolism studies were conducted to determine the extent of metabolism in blood and tissues and to derive an estimate of k_m from blood time course data in rainbow trout following a single oral gavage administration. Without knowledge of the extent of metabolism or the whole-body metabolic rate constant, k_m, bioaccumulation model predictions will continue to use zero as the default k_m value.

2. Materials and methods

2.1. Test substances

The ^{14}C-octamethylcyclotetrasiloxane (D_4) and ^{14}C-decamethylcyclopentasiloxane (D_5) test substances were provided by the Dow Corning Corporation, Auburn, MI. [^{14}C]D_4 and [^{14}C]D_5 had specific activities of 6.883 and 6.090 mCi/g, respectively.

2.2. Test fish

Mature rainbow trout (*Oncorhynchus mykiss*) were purchased from Greenspring Trout Farms, Inc., Newville, PA and were acclimated to laboratory conditions for 2 weeks prior to use. Variations in water temperature did not exceed ± 3° C in any 72-h period while the fish were held. During the pre-test period, the test fish were fed at least once daily. The diet consisted of commercial food (Ziegler Silver Floating Pellets, 5.0 mm, Ziegler Bros., Gardners, PA). Food was withheld for approximately 24 h prior to the test. This fasting period was chosen so that the digestive tract would still contain some food at the time of dosing and to avoid any regurgitation that might result from introducing the dosing solution into an empty stomach.

The trout used for the D_4 study, conducted in August, were mature males weighing between 1.0 and 1.4 kg. Mature females weighing between 0.7 and 1.4 kg were used for the D_5 study conducted in February. The supply of the 1 kg trout used for testing was very limited, and the gender of fish available varied over time. Thus, the gender of fish could not be randomized within tests, and the two compounds were tested using different genders. The fish appeared to be healthy at the time of testing.

2.3. Water supply

Basic water chemistry measurements during the 4 weeks preceding the tests were hardness = 136–140 mg/L as $CaCO_3$ (moderately hard), alkalinity = 180–182 (mg/L as $CaCO_3$), pH = 8.2–8.2, and specific conductance = 300–300 (μmhos/cm). Well water was analyzed for selected organic and inorganic constituents. Heavy metal and pesticide concentrations in the water were below levels that might affect the tests.

2.4. Environmental conditions

Lighting during acclimation and testing was provided by fluorescent tubes emitting wavelengths similar to natural sunlight (e.g., Colortone® 50). The photoperiod was 16 h of light and 8 h of dark. A 30-min transition period of low light intensity was provided when lights were turned on and off to avoid sudden changes in light intensity.

The flow of pre-chilled water into the test chambers was adjusted to maintain constant temperature and oxygen levels and the water in the test chambers was also aerated. Water temperature ranges were 12 ± 2 °C (D_4 study) and 13 ± 2 °C (D_5 study). Dissolved oxygen levels ranged between 8.1–10.8 ppm (D_4 study) and 8.8–10.1 ppm (D_5 study), and were always above 75% saturation during the studies.

2.5. Test fish preparation and chambers

The fish were surgically prepared (i.e. insertion of a urinary tract catheter and a cannula in the dorsal aorta) (Smith and Bell, 1964) and placed in plexiglass flow-through test chambers similar to the respirometer-metabolism chambers of McKim and Goeden (McKim and Goeden, 1982). Following confirmation that the aortic cannulas and urinary catheters were properly placed and unobstructed, the fish were allowed to recover overnight. After the 0-h blood sample was collected the next morning, fish were administered an oral bolus gavage dose of [^{14}C]D_4 or [^{14}C]D_5 in a corn oil vehicle.

2.6. Preparation of dosing solutions

[^{14}C]D_4 and [^{14}C]D_5 dosing solutions were prepared in corn oil acquired from Sigma–Aldrich, St. Louis, MO. Target concentrations in the dosing solutions were 30 mg D_4/mL and 30 mg D_5/mL so that the target dose of 15 mg of D_4 or D_5 per kilogram of fish (~103 μCi per fish and ~75 μCi per fish respectively) could be delivered in a single 0.5 mL/kg body weight bolus oral gavage dose. Three 0.1 mL samples

of each dosing solution were taken for analytical verification of the concentration and homogeneity.

2.7. Dosing

Dosing solutions were administered using a glass syringe and a 14-gauge stainless-steel feeding tube that was inserted directly into the stomach of the fish. Each fish was weighed, and the volume of dosing solution required to achieve a nominal 15 mg D_4/kg body weight or 15 mg D_5/kg body weight dose was calculated. The dose was determined gravimetrically.

Fish were dosed immediately after the 0-h blood samples were taken. Prior to dosing, fish were lightly anesthetized using tricaine methanesulfonate (MS-222). When the fish became quiescent, its head was gently lifted from the water, the gavage tube was inserted, and the dose was delivered. The water flow into the tank allowed the anesthetic to be flushed from the tank so that the fish was allowed to recover. Following dose administration, the empty feeding tube and syringe were weighed to determine the weight of the dosing solution delivered and the fish were monitored for physical condition and signs of regurgitation of the dose delivered.

2.8. Sample collection

Dorsal aortic blood samples (~0.5 mL) were collected from each fish at approximately 0, 2, 4, 8, 12, 24, 48, 72 and 96 h post-dosing.

Urine samples were collected from fish at the following collections intervals; 0–2, 2–4, 4–8, 8–12, 12–24, 24–48, 48–72, and 72–96 h. Collection of urine for the 0 h sample was initiated on the day before dosing, and continued up to the time of dosing. At this time, a clean sample container was positioned in place of the one used for the hour 0 sample, and collection of urine for the 0–2 h sample was initiated. This process was repeated for all subsequent samples. Urine samples were frozen at −80 °C until analysis.

Fecal samples were pooled by collection interval; 0–24, 24–48, 48–72 and 72–96 h post-dosing and stored frozen until analysis.

During the first 2 h following dosing, minute amounts of oil were observed on the surface of the water in some of the metabolism chambers. When observed, this oil was aspirated from the surface water (along with incidentally collected water) and placed in labeled glass vial so that dose loss could be quantified by liquid scintillation counting. The tanks were monitored for the presence of fecal material and extruded eggs at each sampling interval. If these materials were found, they were collected by siphoning the solid materials and incidentally collected water into glass sample bottles, and handled in the same way as other biological samples.

At the end of the experiment (~96 h), the fish were euthanized by anesthetization with MS-222 and all fish were dissected. The abdominal cavity of each was opened, and any fat deposits visible in the abdominal cavity were collected for later analysis. The entire volume of the bile in the gall bladder was collected using a needle and syringe. The entire mass of the liver was collected, and the gastrointestinal tract from the esophagus to the anus was removed. All biological samples, blood, bile, tissues (liver, fat, egg sacs, milt, and digestive tract), remaining carcass, urine, and feces were frozen at −80 °C until analysis.

2.9. Study design

With D_4 there were two trials conducted and only one trial with D_5. A second trial was conducted with D_4 since additional urine samples were needed to confirm a urinary metabolite profile that was observed in the first trial. All samples were collected in the second trial with D_4 and only the urine was analyzed for radioactivity and a second metabolite profile generated.

2.10. Analytical chemistry

All biological tissues were collected and extracted in tetrahydrofuran (THF). A stable-isotope (^{13}C) isomer of the test material served as an internal standard. Extraction solvent was pre-aliquotted in a volume targeting a minimum 2:1 (v/w) ratio of solvent to tissue mass. Digestive tract, egg mass or milt, fat, and liver were subjected to physical mincing after being introduced to extraction solvent. All tissues were vortex-mixed for 5 min, sonicated for 5 min (except blood and bile), centrifuged, and extract removed to a new vial. A second identical extraction was performed using neat THF. The second extract was combined with the first to produce a single combined extract for each sample.

A separate aliquot of each extract was dried using magnesium sulfate and analyzed by Gas Chromatography with Mass Spectrometric detection (GC/MS). Analysis was performed in electron ionization (EI) mode on a Hewlett Packard 6890GC/5973N MSD. The analytical column used was a Hewlett Packard HP-5MS (30 m × 0.25 mm × 0.25 μm). Parent D_4, [^{13}C]D_4, parent D_5, and [^{13}C]D_5 were quantitated from the ion fragments *m/z* 281, 285, 355, and 360, respectively.

Radioactivity was quantitated in the THF extracts of bile, blood, digestive tract, egg sacs, fat, and liver by direct analysis using a Packard Tri-Carb 3100TR liquid scintillation analyzer. In addition, the remaining pellet after THF extraction was solubilized using 35% tetraethyl ammonium hydroxide (TEAH). Aliquots of the solubilized pellet were processed for liquid scintillation analysis to determine the amount of radioactivity remaining after extraction. Remaining carcass and fecal samples were also solubilized in TEAH and analyzed for radioactivity. Any water associated with the collected fecal samples was analyzed by liquid scintillation analysis. Total radioactivity was calculated by summing radioactivity from extracts and solubilized pellets.

Selected urine samples and 96 h extracts of bile, digestive tract, and liver were analyzed by high performance liquid chromatography with radiochemical detection (HPLC/RAD) for a qualitative metabolite profile.

The concentration of parent D_4 or D_5 in fish tissues was reported as μg D_4 or D_5/g sample. The radioactivity concentrations were reported as μg eq per g sample. The calculation of equivalents was based upon the specific activity of the dose solution administered.

2.11. Data analysis

Calculations of mean and standard deviation of sample concentrations were performed using Microsoft Excel™ 2000. Calculations of analytical results were performed using Provantis™ Version 6.5.0.21 and Microsoft Excel™ Version 11. Blood Area-Under-the-Curves (AUC)s for parent and radioactivity including statistical analyses were calculated using SAS/STAT software, Version 9.13 (SAS Institute Inc, 2006).

2.12. Estimation of k_m

A metabolic rate constant ($k_{m(\text{blood})}$) was derived for D_4 and D_5 using the trout blood data. Dietary bioaccumulative processes in fish (i.e., uptake, metabolism, elimination, growth, etc.) may be generically expressed as a first-order kinetic model:

$$dC_{fish}/dt = K_1 * C_{diet} - (k_2 + k_m + k_g) * C_{fish} \quad (1)$$

where C_{fish} is the chemical concentration in the fish (μg/g-wet weight); t is time; K_1 is the chemical dietary uptake rate constant from the food matrix of interest (g-food/g-fish/time); C_{diet} is the chemical concentration (μg/g) in food/bolus dose, etc.; and k_2, k_m, and k_g are rate constants (time^{-1}) representing total chemical elimination from the organism or to storage or waste (i.e., gill ventilation, fat, bile, urine, feces, etc.), overall metabolism, and growth dilution, respectively. In Eq. (1) k_m refers to a whole-body metabolism rate constant. In the current metabolism studies, the in-life period (96 h) is short enough that

growth dilution is assumed to be negligible, i.e., k_g was set to zero. An estimate of the whole-body k_m was estimated from blood time course data and is referred to as $k_{m(\text{blood})}$.

Using first-order expressions similar to Eq. (1), pharmacokinetic compartmental modeling of (1) parent D_4 and total metabolite concentrations and (2) parent D_5 and total metabolite concentrations were conducted in order to determine elimination and metabolism rate constants (i.e., k_2 and $k_{m(\text{blood})}$ values, respectively) for bolus-administered D_4 or D_5 in trout. The fish compartmental model utilized the ^{14}C-radiolabeled measured concentrations of (1) parent D_4 and total metabolites and (2) parent D_5 and total metabolites in fish blood that were collected at six time points during the 96-h study. Researchers have shown previously in work with lipophilic organic compounds in fish that blood concentrations of such compounds are routinely used to reflect both the magnitude and uptake/depuration kinetics of chemical concentrations expressed on a whole-body basis (Nichols et al., 2004; Barron et al., 1991; Barron et al., 1993).

In this approach, the fish is considered to be a single compartment with regard to uptake and excretion/storage of D_4 or D_5, with concomitant D_4 or D_5 metabolism. The bolus dose of D_4 or D_5 is transported from the fish gut into the bloodstream at an uptake rate K_1 (g-food/g-fish/time). In this model, the dynamic relationship between parent D_4 and its metabolites and also parent D_5 and its metabolites in trout may be described with a few simple differential equations (Eqs. (2)–(4)). Equations that follow describe D_4 and the same equations can be used to describe D_5:

Movement of D_4 or D_5 from bolus dose into blood:

$$dC_{D4,Bolus}/dt = -K_1{*}C_{D4,Bolus} \quad (2)$$

where C_{D4_bolus} is the chemical concentration in the bolus dose (μg/g); t is time; and K_1 is the chemical uptake rate constant (g-food/g-fish/time).

The distribution of D_4 or D_5 in blood is modeled as a balance between uptake from bolus and loss via metabolism and elimination/storage:

$$dC_{D4,Blood}/dt = K_1{*}C_{D4,Bolus} - C_{D4,Blood}{*}(k_m + k_2) \quad (3)$$

where C_{D4_Blood} is the chemical concentration of D_4 in fish blood (μg/g), k_m is the D_4 metabolism rate constant (time^{-1}), and k_2 is the D_4 depuration or elimination rate constant (time^{-1}) representing D_4 elimination to storage/waste by the fish.

The distribution of D_4 or D_5 metabolites in fish blood is a balance between production via D_4 or D_5 metabolism and elimination/storage of metabolites by the fish:

$$dC_{Metab,Blood}/dt = k_m{*}C_{D4,Blood} - k_e{*}C_{Metab,Blood} \quad (4)$$

where C_{Metab_Blood} is the chemical concentration of total D_4 metabolites in fish blood (μg D_4 equivalents/g) and k_e is the rate constant (time^{-1}) representing total metabolite elimination to storage/waste by the fish.

2.13. Additional studies

A summary of methods for the following additional studies and data in rainbow trout are provided in Supplementary Data: (1) [^{14}C]D_4 or [^{14}C]D_5 was loaded on fish feed (targeted dose of ~1–2 mg/kg bw) to determine the metabolite profiles in whole fish extracts and (2) [^{14}C]D_4 was administered to rainbow trout loaded on fish feed (targeted dose of 15 mg/kg bw) to determine body burden.

3. Results

The dosing solutions were found to be homogenous for both the [^{14}C]D_4 and [^{14}C]D_5 studies. The measured dose solution concentrations for the D_4 trials were 92% (Trial 1) and 101% (Trial 2) of the targeted dose. For the D_5 study, the dosing solution was 78% of nominal.

In the initial D_4 rainbow trout metabolism study, Trial 1, doses in a corn oil vehicle administered to fish 1, 2, 3, and 4 were determined to be 16,237, 17,413, 12,445 and 9170 μg [^{14}C]D_4 (Table 1), respectively or 12.4, 14.2, 10.9 and 9.5 mg/kg body weight, respectively. An overall average of 79% of the ^{14}C-labeled administered dose was recovered in collected blood, tissues, remaining carcass, bile, urine and feces. An average of 69% of the recovered dose was found in the remaining carcass, 12% was recovered in tissues, bile and blood, 1% in urine and 18% in feces (Table 2). The average percentage of the recovered dose of D_4 absorbed from the bolus dose (percentage recovered in carcass, tissues, bile, blood and urine) was 82%.

In the second trial (2T) with D_4, doses in a corn oil vehicle administered to fish 1 (2T), 2 (2T), 3 (2T), and 4 (2T) were determined to be 16,800, 18,500, 19,200 and 22,300 μg [^{14}C]D_4, respectively or 15.8, 13.5, 15.1 and 17.5 mg/kg body weight, respectively.

The doses in a corn oil vehicle administered to fish 6, 7, and 8, in the rainbow trout D_5 metabolism study, were 13,197, 10,205, and 13,319 μg [^{14}C]D_5, respectively or 9.4, 13, and 12 mg/kg body weight, respectively (Table 1). The overall average percentage of the administered dose (μg eq D_5) recovered in collected blood, tissues, remaining carcass, bile and excreta was 78%. An average of 17% of the recovered dose was found in the remaining carcass and 8% was recovered in tissues, bile and blood (Table 3). The largest percentage of the recovered dose (75%) was excreted in the feces. Small amounts of ^{14}C-activity were recovered in the urine and considered to be near 0%. Approximately 25% of the bolus dose of D_5 administered was absorbed in trout.

[^{14}C]D_4 or [^{14}C]D_5 administered in a corn oil solution at a targeted dose of 15 mg/kg bw was absorbed and distributed in the body, as radioactivity and parent were found in blood, tissues, remaining carcass (analyzed for radioactivity only), bile, and urine (no parent). Parent D_4 or D_5 (μg D_4 or D_5/g blood) and radioactivity (μg eq D_4 or D_5/g blood) were detected in blood throughout the 96 h experimental period (Fig. 1). Parent D_4 was detected at 8 h post-dosing and through 96 h post-dosing and total radioactivity was detected at the first blood collection of 2 h and through 96 h post-dosing. The peak blood radioactivity concentrations were at 24 and 12 h for D_4 and D_5, respectively. The difference between parent and total ^{14}C-radioactivity curves represent the amount of metabolites in the blood. Calculated areas under-the-curves (AUCs) for both total radioactivity and parent chemi-

Table 1
Percentage of the administered dose recovered at 96 h post-dosing in rainbow trout with a targeted dose level of 15 mg D_4 or D_5/kg bw via a single oral gavage in a corn oil vehicle.

[^{14}C]D_4				[^{14}C]D_5			
Fish ID	μg dosed	μg eq recovered	% recovered	Fish ID	μg dosed	μg eq recovered	% recovered
1	16,237	12,996	80				
2	17,413	14,408	83	6	13,197	8851	67
3	12,445	11,164	90	7	10,205	8589	84
4	9170	5955	65	8	13,319	11,032	83
Mean	13,816	11,131	79	Mean	12,240	9491	78
SE	1877	1849	5	SE	1018	774	5

Table 2
Percentage of radioactivity recovered in samples (based on total radioactivity recovered) at 96 h post-dosing in rainbow trout with a targeted dose level of 15 mg [^{14}C]D_4/kg bw via a single oral gavage in a corn oil vehicle.

Fish ID	Carcass		Tissues, bile and blood		Urine		Feces	
	µg eq	% dose recovered	µg eq	% dose recovered	µg eq	% dose recovered	µg eq	% dose recovered
1	7125	55	1096	8	76	1	4694	36
2	8671	60	2170	15	60	0	3502	24
3	8978	80	1154	10	41	0	988	9
4	4897	82	798	13	72	1	218	4
Mean	7418	69	1297	12	62	1	2350	18
SE	933	7	303	1	8	0	1050	7

Table 3
Percentage of radioactivity recovered in samples (based on total radioactivity recovered) at 96 h post-dosing in rainbow trout with a targeted dose level of 15 mg [^{14}C]D_5/kg bw via a single oral gavage in a corn oil vehicle.

Fish ID	Carcass		Tissues, bile and blood		Urine		Feces	
	µg eq	% dose recovered	µg eq	% dose recovered	µg eq	% dose recovered	µg eq	% dose recovered
6	1386	16	438	5	13	0	6976	79
7	1291	15	1052	12	40	0	6182	72
8	2171	20	667	6	9	0	8153	74
Mean	1616	17	719	8	21	0	7104	75
SE	279	1	179	2	10	0.1	572	2

cal indicate a statistically significant difference ($p < 0.05$) between these AUCs for both the D_4 and D_5 fish metabolism studies (Table 4). The calculated elimination half-lifes of total ^{14}C-radioactivity in blood for D_4 and D_5 were 39 h and 70 h, respectively, based on the average blood radioactivity concentrations at each time point.

Metabolism of bolus administered D_4 and D_5 in adult rainbow trout did occur in selected tissues and bile. The concentrations of parent D_4 or D_5 and total ^{14}C-radioactivity were determined in fat, bile, digestive tract without contents, liver, milt (D_4 only) and eggs (D_5 only) (Tables 5 and 6). Parent chemical as the percentage of the total ^{14}C-radioactivity in tissues and bile was calculated. In the D_4 study, ^{14}C-radioactivity in fat and digestive tract contained the first and third highest concentration of radioactivity, respectively. The fat and digestive tract did not contain a significant percentage of metabolites, as the parent percentage (mean) of total radioacitvty was 103 and 98%, respectively. The second highest concentration of radioactivity in samples analyzed was found in the bile, on average, 5% of the total radioactivity was attributed to parent D_4 (i.e. 95% of the total radioactivity as metabolites). In liver and milt samples, an average of 60 and 80% of the ^{14}C-radioactivity found, respectively, was present as parent D_4 (i.e. 40 and 20% of total radioactivity was attributed to metabolites, respectively).

In the D_5 study, the highest concentration of ^{14}C-radioactivity was found in the bile (174 µg eq/g) with < 1% of the recovered dose attributed to parent D_5 (i.e. 99% of total radioactivity was metabolites) (Table 6). Average liver and digestive tract (minus content) samples contained 60 and 76% of the total radioactivity, respectively, as parent D_5 (i.e. 40 and 24% of the total ^{14}C-radioactivity concentration were metabolites). In the egg sacs, 56% of the total ^{14}C-radioactivity concentration was parent D_5 (i.e. 44% was metabolites). Fat was the only tissue that did not contain metabolites (all ^{14}C-radioactivity being parent D_5).

For both D_4 and D_5 a portion of the administered dose was eliminated in the urine (Supplementary Data, Table S1). No parent D_4 or D_5 was found in the urine samples analyzed. The urine which consisted entirely of metabolites, expressed as µg eq/g urine or total µg eq for each collection interval. All urine from a given collection interval was collected. In the D_4 study, the highest metabolite concentration in the urine was found during the 48–72 h collection interval (mean of ~0.38 µg equivalents of D_4/g urine). In the D_5 study, the highest mean level of radioactivity in the urine was found in the 72–96 h collection interval (~0.35 µg equivalents of D_5/g urine).

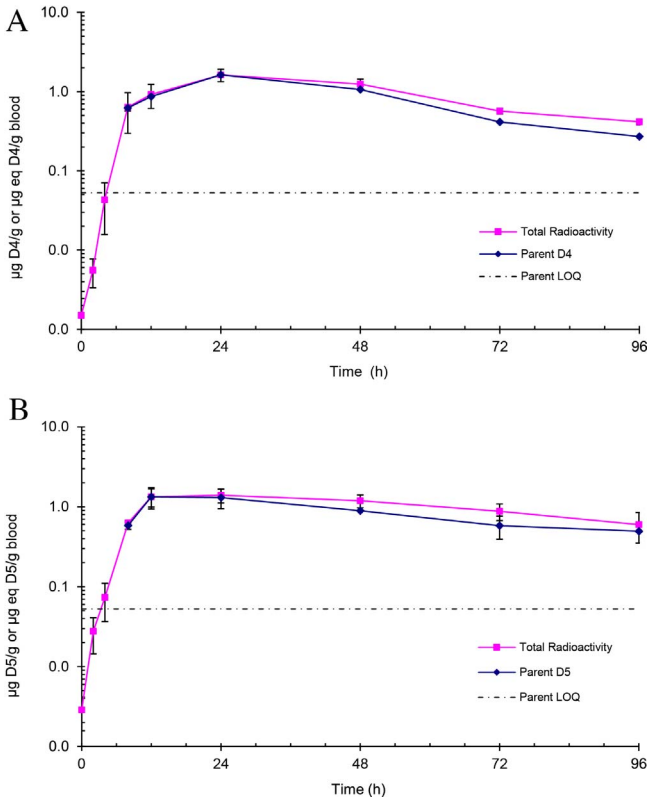

Fig. 1. Blood time-course of parent and radioactivity concentrations (mean ± SE) in rainbow trout through 96 h following oral gavage dosing with (A) [^{14}C]D_4 or (B) [^{14}C]D_5 at 15 mg of test article/kg body weight (targeted).

Urine samples from the 24–48, 48–72, and 72–96 collection intervals from all four D_4 treated fish and 48–72 and 72–96 collection intervals from one D_5 treated fish were analyzed by HPLC/RAD in order

Table 4
Blood radioactivity and parent area-under-the curves (AUC) following a single oral gavage targeted dose level of 15 mg [^{14}C]D_4 or [^{14}C]D_5/kg bw in a corn oil vehicle in rainbow trout.

Fish ID	[^{14}C]D_4		Fish ID	[^{14}C]D_5	
	Radioactivity AUC µg eq-h/g	Parent AUC µg-h/g		Radioactivity AUC µg eq-h/g	Parent AUC µg-h/g
1	80.92	71.19			
2	94.56	82.00	6	69.17	47.85
3	115.62	111.31	7	118.28	97.09
4	74.93	56.70	8	119.94	107.40
Mean	91.51	80.30[a]	Mean	102.46	84.11[a]
SE	9.03	11.56	SE	16.65	18.37

[a] Statistically significant difference, $p < 0.05$, between radioactivity and parent AUCs.

Table 5
Concentration of parent D_4, radioactivity and percentage of parent as total radioactivity at 96 h post-dosing in rainbow trout with a targeted dose level of 15 mg [^{14}C]D_4/kg bw via oral gavage in a corn oil vehicle.

	Bile			Liver			Milt		
Fish ID	Parent mg/g	Metabolite mg eq/g	Parent % of Total	Parent mg/g	Metabolite mg eq/g	Parent % of Total	Parent mg/g	Metabolite mg eq/g	Parent % of Total
1	1.91	47.97	4	2.67	1.31	67	0.81	0.26	76
2	3.38	84.79	4	2.04	2.07	50	0.77	0.19	80
3	3.51	31.56	10	2.36	1.39	63	0.86	0.09	90
4	2.33	52.87	4	1.61	1.08	60	0.63	0.23	73
Mean	2.78	54.30	5	2.17	1.46	60	0.77	0.19	80
SE	0.39	11.14	1.51	0.23	0.21	3.72	0.05	0.04	3.79

	Digestive Tract			Fat		
Fish ID	Parent mg/g	Metabolite mg eq/g	Parent % of Total	Parent mg/g	Metabolite mg eq/g	Parent % of Total
1	14.26	0.00	101	120.45	0.00	103
2	26.61	0.00	102	90.06	0.00	101
3	17.11	0.40	98	81.75	0.00	104
4	12.51	1.05	92	72.71	0.00	103
Mean	17.62	0.36	98	91.24	0.00	103
SE	3.14	0.25	2.11	10.36	0.00	0.52

Parent limit of quantitation for milt, fat, bile, digestive tract and liver; 0.04, 0.10, 0.08, 0.04 and 0.04 µg D_4/g sample, respectively.

Table 6
Concentration of parent D_5, radioactivity and percentage of parent as total radioactivity at 96 h post-dosing in rainbow trout with a targeted dose level of 15 mg [^{14}C]D_5/kg bw via oral gavage in a corn oil vehicle.

	Bile			Eggs			Liver		
Fish ID	Parent mg/g	Metabolite mg eq/g	Parent % of Total	Parent mg/g	Metabolite mg eq/g	Parent % of Total	Parent mg/g	Metabolite mg eq/g	Parent % of Total
6	0.201	66.6	0.301	0.198	0.390	33.7	0.770	0.845	47.7
7	BLQ	285	0.00	1.45	0.442	76.6	2.95	0.988	74.9
8	2.55	171	1.47	1.01	0.704	58.8	1.97	1.45	57.6
Mean	1.38	174	0.592	0.885	0.512	56.4	1.90	1.09	60.1
SE	NA	63.1	0.450	0.366	0.097	12.4	0.630	0.182	7.96

	Digestive Tract			Fat		
Fish ID	Parent mg/g	Metabolite mg eq/g	Parent % of Total	Parent mg/g	Metabolite mg eq/g	Parent % of Total
6	1.50	0.276	84.5	14.6	0.0	111
7	27.6	16.7	62.3	24.3	0.0	114
8	2.54	0.619	80.4	18.4	0.0	116
Mean	10.5	5.86	75.7	19.1	0.0	114
SE	8.53	5.42	6.82	2.82	0.0	1.43

Parent limit of quantitation for eggs, fat, bile, digestive tract and liver; 0.00026, 0.0113, 0.0242, 0.00113, and 0.00170 µg D_5/g sample, respectively.

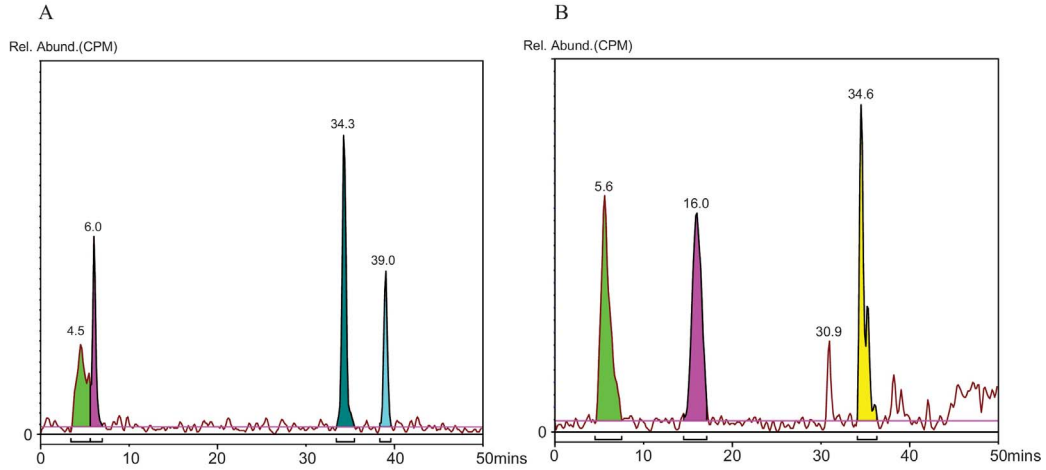

Fig. 2. Urinary metabolite profile analysis of urine collected from fish dosed with D_4 or D_5 at 15 mg/kg body weight (A) Urine collected 24–48 h post-dosing with [^{14}C]D_4, Retention time: parent D_4 = 49.4 min, not observed and methylsilanetriol retention time is 6 min. The absence of dimethylsilanediol in urine was confirmed with fish from the second trial. (B) Urine collected 72–96 h post-dosing with [^{14}C]D_5, retention time of dimethylsilanediol is 16 min, parent D_5 = 51.2 min; not observed. Methylsilanetriol retention time is 5.6 min.

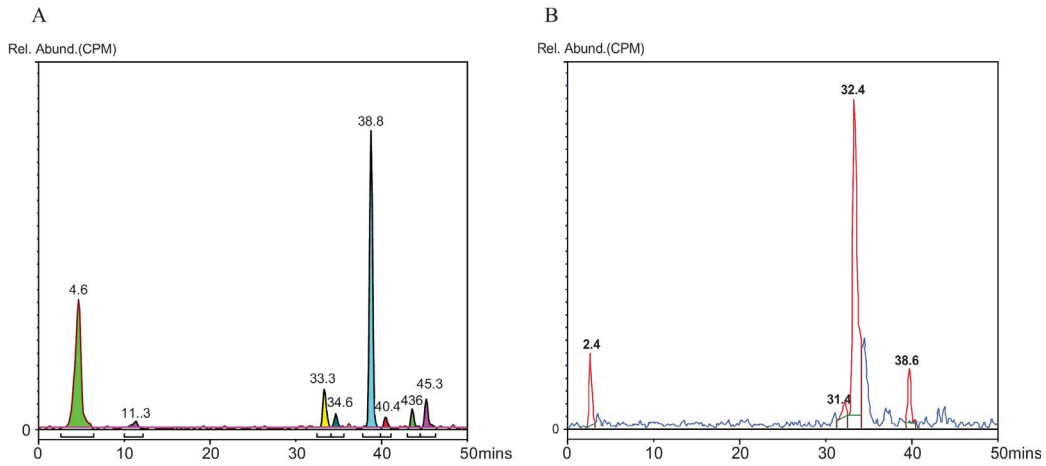

Fig. 3. Biliary metabolite profile analysis from bile collected at 96 h post-dosing with (A) [^{14}C]D_4 or (B) [^{14}C]D_5 at 15 mg/kg body weight. Retention time: parent D_4 = 45.2 min, parent D_5 = 43.8 min.

to determine the metabolite profile of the ^{14}C-radioactivity. Metabolite profiling of the urine shows that the ^{14}C-activity consisted entirely of metabolites more polar than D_4 (Fig. 2A) or D_5 (Fig. 2B). Metabolite identification was not undertaken however retention time match was done for two common metabolites typically seen in mammalian studies. The metabolites methylsilanetriol and dimethylsilandiol, based on an approximated retention time match (Varaprath et al., 1999; Varaprath et al., 2003), were observed in the urine from the D_5 treated fish but only methylsilanetriol was seen in D_4 treated fish.

In both the D_4 and D_5 fish metabolism studies, a percentage of the administered dose was eliminated in the feces (Supplementary Data, Table S2). The amount recovered in the feces was on average 18% of the average total μg eq of D_4 recovered. The μg eq recovered in the water associated with the fecal samples was negligible. Analysis of the fecal samples for parent D_4 indicated the majority of the radioactivity was present as parent D_4 (data not shown).

The average μg eq of D_5 recovered in the feces through 96 h was 75% of the total μg eq D_5 recovered. The μg eq recovered in the water associated with the fecal samples was negligible. Fecal samples were not analyzed for parent chemical, however, it is anticipated that a portion of the radioactivity could be attributed to metabolites since biliary excretion is occurring and metabolites represent the majority of the radioactivity associated with bile.

Extracts of bile were profiled for metabolites of D_4 and D_5 with HPLC radiochromatography. The result of the metabolite profile analysis of bile extracts from representative fish orally gavaged with D_4 or D_5 are presented in Fig. 3. The bile extract profiles indicated the presence of some parent D_4 or D_5, which is consistent with the independent GC–MS analysis results. Bile also contained several metabolites that were more polar than D_4 or D_5.

Extracts of digestive tract and liver were also profiled for metabolites from fish orally gavaged with D_5 (data not shown). All three of the tissue extracts contained some parent D_5, which is consistent with the independent GC–MS analysis results. The high limits of detection (LOD) for the GC–MS precluded the detection of metabolites in the liver.

In the D_4 study, no metabolites were observed in the fat collected from any of the fish. An average of 76, 23, 11, and 6 μg eq of metabolites were detected in bile, liver, digestive tract and milt; respectively (Supplementary Data, Table S3). The average sum total μg eq of metabolites in the tissues was 116 μg eq. In urine, parent D_4 was not observed, with metabolites accounting for a total of 62 μg eq. The average total μg eq of metabolites found in the tissues and urine was 178 μg eq. The total μg eq of D_4 absorbed in the fish from the bolus dose was determined to be 8777 μg eq (7418 μg eq in carcass, 1297 μg eq in tissues and blood, and 62 μg eq in urine). The calculated percent of D_4 metabolism measured in rainbow trout over the 96 h period was estimated to be at least 2%. Parent D_4 was not measured in the remaining carcass; therefore, the estimate of metabolism over the 96-h period may be higher. An additional study measured the percentage of metabolism observed in metabolite profiles from whole fish

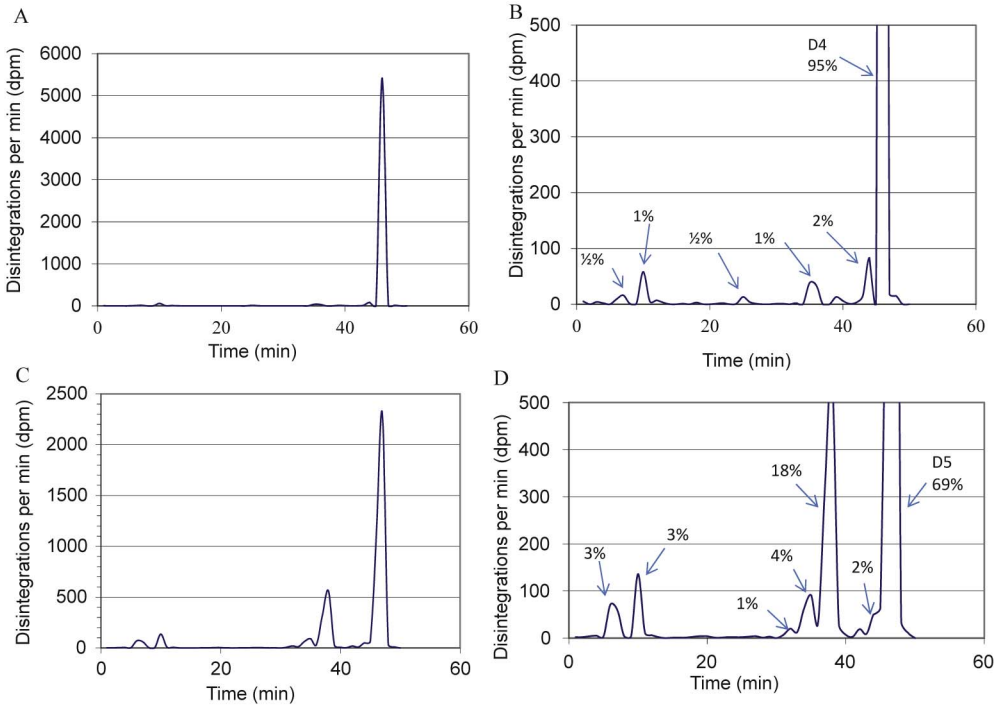

Fig. 4. (A and B) Reconstructed HPLC from tetrahydrofuran extracts of a whole fish fed a nominal concentration of 500 µg [^{14}C]D_4/g of feed, ~1 g/day for 5 days, ~1–2 mg/kg bw/day. Total dpm: 5761. Percentages listed are radioactivity associated with metabolites or parent material for each peak. (A) Full scale and (B) Expanded scale (scaled to 500 dpm) with metabolite and peak area percentages. (C and D) Reconstructed HPLC from tetrahydrofuran extracts of a whole fish fed a nominal concentration of 250 µg [^{14}C]D_5/g of feed, ~1 g/day for 5 days, ~0.5–1.0 mg/kg bw/day. Total dpm: 5037. Percentages listed are radioactivity associated with metabolites or parent material for each peak. (C) Full scale and (D) Expanded scale (scaled to 500 dpm) with metabolite and peak area percentages.

homogenate extracts taken following the fifth day of dietary administration. Metabolite profiling determined that metabolites accounted for 5% of the total radioactivity (Fig. 4A and B; Supplementary Methods: whole fish extracts), which is consistent with metabolism observed in this 96-h metabolism study.

In the D_5 study, the average µg eq of metabolites were 151, 88.8, 71.1, and 17.3 in bile, digestive tract, egg sacs, and liver, respectively (Supplementary Data, Table S4). No metabolites were observed in the fat collected from any of the fish. Parent D_5 was not observed in the urine. The total µg eq of metabolites in urine (average of fish 6, 7, and 8) was 20.9 µg eq. The calculated percent of parent D_5 metabolism in rainbow trout was estimated to be at least 14%. This percentage is an estimate since parent D_5 was not measured in the remaining carcass. If metabolites account for a high level of the total radioactivity remaining in the carcass, then metabolism would be more robust than is apparent from the current data set. An additional study measured the percentage of D_5 metabolism observed in metabolite profiles from whole fish homogenate extracts after 5 days of dietary administration. Metabolite profiling determined that metabolites account for 31% of the total radioactivity (Fig. 4C and D: Supplementary Methods: whole fish extracts), which is consistent with metabolism observed in this 96-h metabolism study.

The k_m for D_4 in trout using mean residue data in this study was determined to be 0.00431 h^{-1} or 0.10 day^{-1} (Fig. 5A). Assuming first-order kinetics, the resulting fish metabolism half-life for D_4 is approximately 6.7 days and the overall D_4 dissipation half-life (metabolism + loss due to elimination/storage) in trout was approximately 1.2 days. This calculated fish dissipation half-life for D_4 is supported by experimental results, as fish blood data indicate that D_4 levels in fish blood declined about 40% from a peak concentration of 1.628 µg/g at 24 h to 1.063 µg/g at 48 h, a cumulative timeframe of 24 h or 1 day; there was a concomitant increase in total metabolites over the same time period.

The k_m for D_5 in trout using mean blood data in this study was determined to be 0.0068 h^{-1} or 0.17 day^{-1} (Fig. 5B). Assuming first-order kinetics, the resulting fish metabolism half-life for D_5 is approximately 4 days and the overall D_5 dissipation half-life (metabolism + loss due to elimination/storage) in trout was approximately 2.3 days. This calculated fish dissipation half-life for D_5 is supported by experimental results, as fish blood data indicate that D_5 levels in fish blood declined about 50% from a peak concentration of 1.33 µg/g at 12 h to 0.583 µg/g at 72 h, a cumulative timeframe of 60 h or 2.5 days; there was a concomitant increase in total metabolites over the same time period.

4. Discussion

When considering fish metabolism of D_4 and D_5, the intravenous route of administration (dorsal aorta) is not appropriate due to their high lipophilicity and low water solubility. These parameters suggest that uptake via food is a more relevant exposure route. Previous studies reported success using a gavage technique to administer an oral bolus dose of a chemical to rainbow trout. Corn oil was found to be a suitable vehicle for dietary administration.

To demonstrate that the absorption of [^{14}C]D_4 was not different if administered as a single corn oil oral bolus dose verse a single dietary administration, [^{14}C]D_4 was loaded on feed and fed to rainbow trout at ~5 mg/kg bw. The percentage of the dose recovered and absorbed in rainbow trout is similar whether the [^{14}C]D_4 was administered in corn oil (fasted fish, not freely swimming and with a dorsal aorta cannula and urinary catheter) or on fish feed (non-fasted fish, freely swimming and no surgical intervention) (Supplementary Data, Tables S5 and S6).

When comparing the trout [^{14}C]D_4 urinary metabolite profile to D_4-treated rats, an important intermediate in the rat metabolic degradation scheme, dimethylsilanediol, is missing (Varaprath et al., 1999). The exact reason why the intermediate is missing in trout is unknown. However, factors that may contribute to this apparent lack of metabolite include saturation of metabolism at the dose of 15 mg [^{14}C]D_4/kg bw and influence of the freezing point (17.5 °C) of D_4 since the rainbow

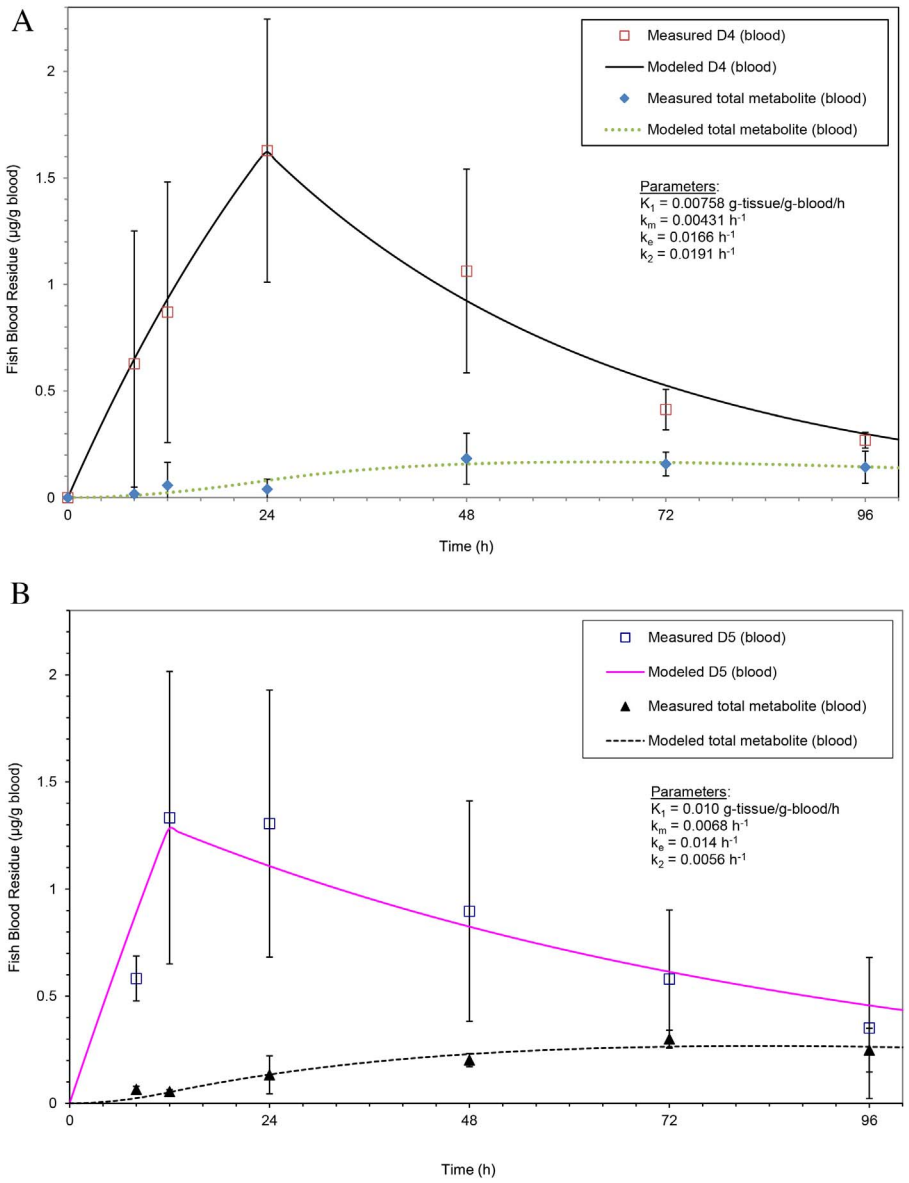

Fig. 5. (A) Time-course of parent D_4 and metabolite(s) concentrations in blood (mean ± SD) of rainbow trout ($n = 4$) following an average oral bolus gavage administration of 11.75 mg [^{14}C]D_4/kg body weight in corn oil. Plot of mean D_4 and total metabolite residue data versus modeled fit of fish compartmental model. (B) Time-course of parent D_5 and metabolite(s) concentrations in blood (mean ± SD) of rainbow trout ($n = 3$) following an average oral bolus gavage administration of 11.6 mg [^{14}C]D_5/kg body weight in corn oil. Plot of mean D_5 and total metabolite residue data versus modeled fit of fish compartmental model.

trout study was conducted at 12 °C.

In these metabolism studies, the percentage of metabolites in the absorbed dose was estimated to be at least 2% and 14% following administration of 15 mg of [^{14}C]D_4 or [^{14}C]D_5/kg bw to rainbow trout, respectively. The proportion of radioactivity in the carcass that represents metabolites is not known since analysis for parent D_4 or D_5 in the remaining carcass was not conducted. If metabolites account for a high percentage of the total radioactivity in the carcass, then overall metabolism would be more robust than is apparent from this 96-h metabolism study. The percentage of metabolism observed in metabolite profiles from whole fish homogenate extracts in a separate 4-day feeding study was determined to be 5% and 31% of the total radioactivity for D_4 and D_5, respectively (Figs. 4B and 4D). In addition to the 96-h metabolism studies and the studies where metabolites were observed in whole fish extracts, metabolism has been observed in separate D_4 and D_5 bioaccumulation studies with trout (Woodburn et al., 2013).

In the bioaccumulation study with [^{14}C]D_4 where rainbow trout were fed 15 mg/kg bw during the 35-day uptake phase, livers were collected in the 42-day depuration phase and analyzed for parent D_4 and total radioactivity. Comparison of total radioactivity to D_4 parent concentrations in liver extracts indicated the presence of one or more metabolites (Woodburn et al., 2013). Whole body autoradiography (WBA) conducted during the depuration phase showed that the highest amounts of radioactivity were present in the digestive tract, liver and adipose tissue (Domoradzki et al., 2015). At day 42 of the depuration phase, there was a significant amount of radioactivity in the gall bladder. Moderate amounts of radioactivity were observed in the liver and contents of the intestinal tract. Comparison of ^{14}C-radioactivity to parent D_4 in the liver and metabolite characterization of liver extracts provide evidence that D_4 can be metabolized in immature rainbow trout (< 10 g). Continued presence of parent D_4 in the intestinal tract contents 42 days post-dosing also demonstrates elimination of parent D_4 via enterohepatic circulation.

Similarly in the [^{14}C]D_5 bioaccumulation study (Woodburn et al., 2013), fish were fed 15 mg/kg bw/day for 35 days and analysis of

radioactivity in whole body fish extracts and WBA (Drottar et al., 2006) provide evidence that D_5 is metabolized and eliminated via the digestive tract. Approximately 23% of the observed depuration was due to factors other than growth. The observation that the concentration of radioactivity remains high in the digestive tract even after dosing was discontinued is suggestive of metabolism with metabolite(s) entering the digestive tract through enterohepatic circulation. This hypothesis is strengthened when combined with the WBA results (Domoradzki et al., 2015). After 42 days of depuration, most of the ^{14}C-radioactivity was found in the liver and digestive tract contents.

The estimated metabolism rate constants ($k_{m(\text{blood})}$) derived from the blood time-course data analysis of the D_4 and D_5 fish metabolism studies will prove useful in parameterizing bioconcentration or bioaccumulation models regarding the fate of D_4 and D_5 in aquatic organisms.

5. Conclusions

Metabolism of D_4 and D_5 has been measured in rainbow trout. Approximately 40% of the total radioactivity detected in the liver for both D_4 and D_5 was due to the presence of metabolites. An important aspect observed in these studies was the biliary excretion of metabolites, (~95% for D_4 and 99% for D_5) which is indicative of hepatic metabolism. The findings of this study are consistent with other findings from an oral feed study where whole-body extract metabolite concentrations were 5% (D_4) and 31% (D_5) of the total radioactivity.

Incorporation of metabolism into bioaccumulation/biomagnification models for "B" assessment of D_4 and D_5 will be valuable in determining the behavior of these chemicals in food webs. For D_5, metabolism may be less critical than for D_4 in bioaccumulation modeling since the chemical's high lipophilicity indicates minimal gut transfer rates. With D_4 and D_5 a conservative estimate of $k_{m(\text{blood})}$, ≥ 0.01 day^{-1}, was derived.

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Transparency document

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2017.03.025.

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