Title: 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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PII: S0378-4274(17)30127-3
DOI: http://dx.doi.org/doi:10.1016/j.toxlet.2017.03.025
Reference: TOXLET 9736

To appear in: *Toxicology Letters*

Received date: 28-9-2016
Revised date: 23-3-2017
Accepted date: 26-3-2017

Please cite this article as: Domoradzki, J.Y., Sushynski, J.M., Thackery, L.M., Springer, T.A., Ross, T.L., Woodburn, K.B., Durham, J.A., McNett, D.A.,METABOLISM OF $_{14}$C-OCTAMETHYLCYCLOTETRASILOXANE ([$_{14}$C]D_4) OR $_{14}$C-DECAMETHYLCYCLOPENTASILOXANE ([$_{14}$C]D_5) ORALLY GAVAGED IN RAINBOW TROUT (*Oncorhynchus mykiss*), *Toxicology Letters* (2017), http://dx.doi.org/10.1016/j.toxlet.2017.03.025

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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- Metabolism of octamethylcyclotetrasiloxane (D_4) and decamethylcyclopentasiloxane (D_5) occurs in rainbow trout orally (relevant route for hydrophobic chemicals) gavaged with these materials in a corn oil vehicle.
- Blood elimination half-lives are 39 and 70 h for D_4 and D_5, respectively.
- D4 and D5 are metabolized in rainbow trout with similar estimated biotransformation half-lives ($t_{1/2}$) of less than 7 days.
- Clearance may occur via enterohepatic circulation of metabolic products in bile with excretion via the digestive tract and urinary clearance of metabolites.

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.

Keywords – Metabolism, Elimination, Rainbow Trout, Octamethylcyclotetrasiloxane, Decamethylcyclopentasiloxane.

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 [1, 2]. 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 6.49 and 8.07, respectively, and water solubilities of 56 and 17 μg/L, 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 [3], the UK [4, 5], and the Nordic States [6, 7]. In particular, a judicial review on D_5 has occurred in Canada [8].

Bioconcentration or bioaccumulation potential is a key parameter for chemical classification and labeling, regulatory management, pollution prevention initiatives, and environmental risk assessments. 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 [9, 10]. 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

89 both BAF and BCF data, the log K_{ow} has been identified as a surrogate measure of a
90 chemical's bioaccumulation potential and chemicals with a log $K_{ow} \geq 5$ are considered to
91 have bioaccumulative potential (Environment Canada, 1995).

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

95 $$d(C_{fish})/dt = Uptake - Elimination - Growth - Metabolism$$

96 Bioaccumulation models for aquatic organisms, validated using carbon-based
97 substances, do exist [11, 12] and are commonly used to predict bioaccumulation potential
98 (i.e. "B" in a PBT classification) based on physico-chemical properties such as the
99 compound's log K_{ow} value and the organism's trophic level position. In these models,
100 the aquatic organism metabolism rate (k_m) is usually not known and considered as zero.
101 Metabolic transformation is one of the important attenuation mechanisms and cannot be
102 ignored since it can drastically change whether a substance will magnify or dilute with
103 increasing trophic level (trophic magnification factor, TMF).

104 Numerous researchers have applied mass balance models and other techniques to
105 *in vivo* fish bioconcentration/bioaccumulation data to estimate biotransformation rate
106 constants [11, 13-15]. Mackay [16] et al. have examined how rapid metabolism or other
107 means of elimination may be an indication of low bioaccumulation (B) potential for a
108 compound. Aquatic food web models [11] have suggested that chemicals with rapid k_m
109 values should not biomagnify in aquatic food webs (i.e., $k_m \geq 0.05\ day^{-1}$). Several
110 computer models for calculating biotransformation rates are available, but these models
111 still need further refinement [17-19].

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 ($k_{m\ (blood)}$). 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 two weeks prior to use. Variations in water temperature did not exceed ±°3C 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 females weighing between 0.70 and 1.4 kg. Mature males weighing between 1.0 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 four 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-minute 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 [20] and placed in plexiglass flow-through test chambers similar to the respirometer-metabolism chambers of McKim and Goeden [21]. 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 two hours 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 five minutes, sonicated for 5 minutes (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 x 0.25 mm x 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

247 collected fecal samples was analyzed by liquid scintillation analysis. Total radioactivity
248 was calculated by summing radioactivity from extracts and solubilized pellets.

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

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

256 *2.11. Data analysis*

257 Calculations of mean and standard deviation of sample concentrations were
258 performed using Microsoft Excel™ 2000. Calculations of analytical results were
259 performed using Provantis™ Version 6.5.0.21 and Microsoft Excel™ Version 11. Blood
260 Area-Under-the–Curves (AUC)s for parent and radioactivity including statistical analyses
261 were calculated using SAS/STAT software, Version 9.13 [22].

262 *2.12. Estimation of km*

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

266 $$dC_{fish} / dt = K_1 * C_{diet} - (k_2 + k_m + k_g) * C_{fish} \quad (1)$$

267 where C_{fish} is the chemical concentration in the fish (μg/g-wet weight); t is time; K_1 is the
268 chemical dietary uptake rate constant from the food matrix of interest (g-food/g-
269 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 the Equation (1) k_m refers to a whole-body metabolism rate constant. In the current metabolism studies, the in-life period (96 hour) 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 $k_{m(blood)}$.

Using first-order expressions similar to Equation (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 $k_{m(blood)}$ 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-hour 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 [23-25].

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 (Equations 2 to 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 the Supplemental 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

315 extracts and 2) [^{14}C]D_4 was administered to rainbow trout loaded on fish feed (targeted
316 dose of 15 mg/kg bw) to determine body burden.

317 **3. RESULTS**

318 The dosing solutions were found to be homogenous for both the [^{14}C]D_4 and
319 [^{14}C]D_5 studies. The measured dose solution concentrations for the D_4 trials were 92%
320 (Trial 1) and 101% (Trial 2) of the targeted dose. For the D_5 study, the dosing solution
321 was 78% of nominal.

322 In the initial D_4 rainbow trout metabolism study, Trial 1, doses in a corn oil
323 vehicle administered to fish 1, 2, 3, and 4 were determined to be 16237, 17413, 12445
324 and 9170 μg [^{14}C]D_4 (Table 1), respectively or 12.4, 14.2, 10.9 and 9.5 mg/kg body
325 weight, respectively. An overall average of 79% of the ^{14}C-labeled administered dose
326 was recovered in collected blood, tissues, remaining carcass, bile, urine and feces. An
327 average of 69% of the recovered dose was found in the remaining carcass, 12% was
328 recovered in tissues, bile and blood, 1% in urine and 18% in feces (Table 2). The average
329 percentage of the recovered dose of D_4 absorbed from the bolus dose (percentage
330 recovered in carcass, tissues, bile, blood and urine) was 82%.

331 In the second trial (2T) with D_4, doses in a corn oil vehicle administered to fish 1
332 (2T), 2 (2T), 3 (2T), and 4 (2T) were determined to be 16800, 18500, 19200 and 22300
333 μg [^{14}C]D_4, respectively or 15.8, 13.5, 15.1 and 17.5 mg/kg body weight, respectively.

334 The doses in a corn oil vehicle administered to fish 6, 7, and 8, in the rainbow
335 trout D_5 metabolism study, were 13197, 10205, and 13319 μg [^{14}C]D_5, respectively or
336 9.4, 13, and 12 mg/kg body weight, respectively (Table 1). The overall average
337 percentage of the administered dose (μg eq D_5) recovered in collected blood, tissues,

338 remaining carcass, bile and excreta was 78%. An average of 17% of the recovered dose
339 was found in the remaining carcass and 8% was recovered in tissues, bile and blood
340 (Table 3). The largest percentage of the recovered dose (75%) was excreted in the feces.
341 Small amounts of ^{14}C-activity were recovered in the urine and considered to be near 0%.
342 Approximately 25% of the bolus dose of D_5 administered was absorbed in trout.
343 [^{14}C]D_4 or [^{14}C]D_5 administered in a corn oil solution at a targeted dose of 15
344 mg/kg bw was absorbed and distributed in the body, as radioactivity and parent were
345 found in blood, tissues, remaining carcass (analyzed for radioactivity only), bile, and
346 urine (no parent). Parent D_4 or D_5 (µg D_4 or D_5/g blood) and radioactivity (µg eq D_4 or
347 D_5/g blood) were detected in blood throughout the 96 h experimental period (Figure 1).
348 Parent D_4 was detected at 8 h post-dosing and through 96 h post-dosing and total
349 radioactivity was detected at the first blood collection of 2 h and through 96 h post-
350 dosing. The peak blood radioactivity concentrations were at 24 and 12 h for D_4 and D_5,
351 respectively. The difference between parent and total ^{14}C-radioactivity curves represent
352 the amount of metabolites in the blood. Calculated areas under-the-curves (AUCs) for
353 both total radioactivity and parent chemical indicate a statistically significant difference
354 ($p<0.05$) between these AUCs for both the D_4 and D_5 fish metabolism studies (Table 4).
355 The calculated elimination half-lifes of total ^{14}C-radioactivity in blood for D_4 and D_5
356 were 39 h and 70 h, respectively, based on the average blood radioactivity concentrations
357 at each time point.
358 Metabolism of bolus administered D_4 and D_5 in adult rainbow trout did occur in
359 selected tissues and bile. The concentrations of parent D_4 or D_5 and total ^{14}C-
360 radioactivity were determined in fat, bile, digestive tract without contents, liver, milt (D_4

361 only) and eggs (D_5 only) (Tables 5-6). Parent chemical as the percentage of the total ^{14}C-
362 radioactivity in tissues and bile was calculated. In the D_4 study,^{14}C- radioactivity in fat
363 and digestive tract contained the first and third highest concentration of radioactivity,
364 respectively. The fat and digestive tract did not contain a significant percentage of
365 metabolites, as the parent percentage (mean) of total radioacitvity was 103 and 98%,
366 respectively. The second highest concentration of radioactivity in samples analyzed was
367 found in the bile. On average, 5% of the total radioactivity was attributed to parent D_4
368 (i.e. 95% of the total radioactivity as metabolites). In liver and milt samples, an average
369 of 60 and 80% of the ^{14}C-radioactivity found, respectively, was present as parent D_4 (i.e.
370 40 and 20% of total radioactivity was attributed to metabolites, respectively).
371 In the D_5 study, the highest concentration of ^{14}C-radioactivity was found in the
372 bile (175 µg eq/g) with <1% of the recovered dose attributed to parent D_5 (i.e. 99% of
373 total radioactivity was metabolites) (Table 6). Average liver and digestive tract (minus
374 content) samples contained 60 and 76% of the total radioactivity, respectively, as parent
375 D_5 (i.e. 40 and 24% of the total ^{14}C-radioactivity concentration were metabolites). In the
376 egg sacs, 56% of the total ^{14}C-radioactivity concentration was parent D_5 (i.e. 44% was
377 metabolites). Fat was the only tissue that did not contain metabolites (all ^{14}C-
378 radioactivity being parent D_5).
379 For both D_4 and D_5 a portion of the administered dose was eliminated in the urine
380 (Supplemental Data, Table S1). No parent D_4 or D_5 was found in the urine samples
381 analyzed. The urine which consisted entirely of metabolites, expressed as µg eq/g urine
382 or total µg eq for each collection interval. All urine from a given collection interval was
383 collected. In the D_4 study, the highest metabolite concentration in the urine was found

384 during the 48 - 72 h collection interval (mean of ~0.38 μg equivalents of D_4/g urine). In
385 the D_5 study, the highest mean level of radioactivity in the urine was found in the 72-96 h
386 collection interval (~0.35 μg equivalents of D_5/g urine).

387 Urine samples from the 24-48, 48-72, and 72-96 collection intervals from all four
388 D_4 treated fish and 48-72 and 72-96 collection intervals from one D5 treated fish were
389 analyzed by HPLC/RAD in order to determine the metabolite profile of the ^{14}C-
390 radioactivity. Metabolite profiling of the urine shows that the ^{14}C-activity consisted
391 entirely of metabolites more polar than D_4 (Figure 2A) or D5 (Figure 2B). Metabolite
392 identification was not undertaken however retention time match was done for two
393 common metabolites typically seen in mammalian studies. The metabolites
394 methylsilanetriol and dimethylsilandiol, based on an approximated retention time match
395 [26, 27], were observed in the urine from the D_5 treated fish but only methylsilanetriol
396 was seen in D_4 treated fish.

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

403 The average μg eq of D_5 recovered in the feces through 96 h was 75% of the total
404 μg eq D_5 recovered. The μg eq recovered in the water associated with the fecal samples
405 was negligible. Fecal samples were not analyzed for parent chemical, however, it is
406 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 Figure 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. 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 homogenate extracts taken following the fifth day of dietary administration. Metabolite profiling determined that metabolites accounted for 5% of the total radioactivity (Figures 5A-5B; Supplemental 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. 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% after 96 h. 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 five days of dietary administration. Metabolite profiling determined that metabolites account for 31% of the total radioactivity (Figures 5C-5D: Supplemental 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} (Figure 6A). 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 one 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} (Figure 6B). 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 hours or 2.5 days; there was a concomitant increase in total metabolites over the same time period.

4. DISCUSSION

When considering the 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

476 and absorbed in rainbow trout is similar whether the [^{14}C]D_4 was administered in corn oil
477 (fasted fish, not freely swimming and with a dorsal aorta cannula and urinary catheter) or
478 on fish feed (non-fasted fish, freely swimming and no surgical intervention)
479 (Supplemental Data, Tables S3-S4).

480 When comparing the trout [^{14}C]D_4 urinary metabolite profile to D_4-treated rats, an
481 important intermediate in the rat metabolic degradation scheme, dimethylsilanediol, is
482 missing [26]. The exact reason why the intermediate is missing in trout is unknown.
483 However, factors that may contribute to this apparent lack of metabolite include
484 saturation of metabolism at the dose of 15 mg [^{14}C]D_4/kg bw and influence of the
485 freezing point (17.5^{o}C) of D_4 since the rainbow trout study was conducted at 12^{o}C.

486 In these metabolism studies, the percentage of metabolites in the absorbed dose
487 was estimated to be at least 2% and 14% following administration of 15 mg of [^{14}C]D_4 or
488 [^{14}C]D_5/kg bw to rainbow trout, respectively. The proportion of radioactivity in the
489 carcass that represents metabolites is not known since analysis for parent D_4 or D_5 in the
490 remaining carcass was not conducted. If metabolites account for a high percentage of the
491 total radioactivity in the carcass, then overall metabolism would be more robust than is
492 apparent from this 96-h metabolism study. The percentage of metabolism observed in
493 metabolite profiles from whole fish homogenate extracts in a separate 4-day feeding
494 study was determined to be 4% and 31% of the total radioactivity for D_4 and D_5,
495 respectively (Figures 5-6). In addition to the 96-h metabolism studies and the studies
496 where metabolites were observed in whole fish extracts, metabolism has been observed in
497 separate D_4 and D_5 bioaccumulation studies with trout [28].

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 [28]. 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 [29]. 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 [28], fish were fed 15 mg/kg bw/day for 35 days and analysis of radioactivity in whole body fish extracts and WBA [30] 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 [29]. 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 ($k_{m(blood)}$) 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% (D4) and 31% (D5) 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 $k_{m(blood)}$, ≥ 0.01 day^{-1}, was derived.

Appendix A. Supplementary data

Appendix A. Supplementary material: Methods, Results and Discussion, Tables S1-S4, References.

FIGURE LEGENDS

Figure 1. Blood time-course of parent and radioactivity 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).

Figure 2. Urinary metabolite profile analysis from 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. 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 6 min.

Figure 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.

Figure 4A and 4B. 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 B) Expanded scale (scaled to 500 dpm) with metabolite and peak area percentages.

Figure 4C and 4D. 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 D) Expanded scale (scaled to 500 dpm) with metabolite and peak area percentages.

566 **Figure 5A.** Time-course of parent D_4 and metabolite(s) concentrations in blood (mean ±
567 SD) of rainbow trout (n=4) following an average oral bolus gavage administration of
568 11.75 mg [^{14}C]D_4/kg body weight in corn oil. Plot of mean D_4 and total metabolite
569 residue data versus modeled fit of fish compartmental model.
570 **Figure 5B.** Time-course of parent D_5 and metabolite(s) concentrations in blood (mean ±
571 SD) of rainbow trout (n=3) following an average oral bolus gavage administration of 11.6
572 mg [^{14}C]D_5/kg body weight in corn oil. Plot of mean D_5 and total metabolite residue data
573 versus modeled fit of fish compartmental model.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. John Nichols at the U.S. Environmental Protection Agency Research Facilities in Duluth, MN for help and advice with developing the in-life procedures for metabolism studies in rainbow trout. The authors thank Dr. Duane Huggett for his review and advice during the writing of this manuscript.

FUNDING

Studies were supported in part by the Global Silicone Industry.

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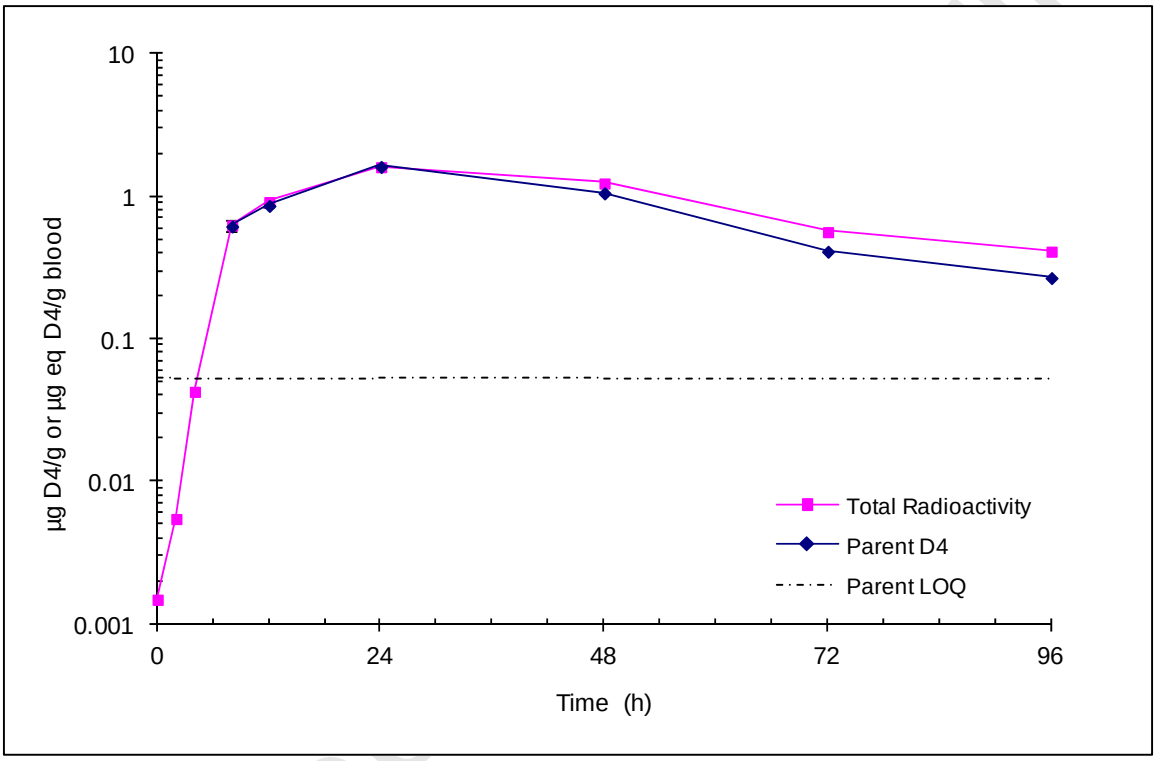
Figure 1A.



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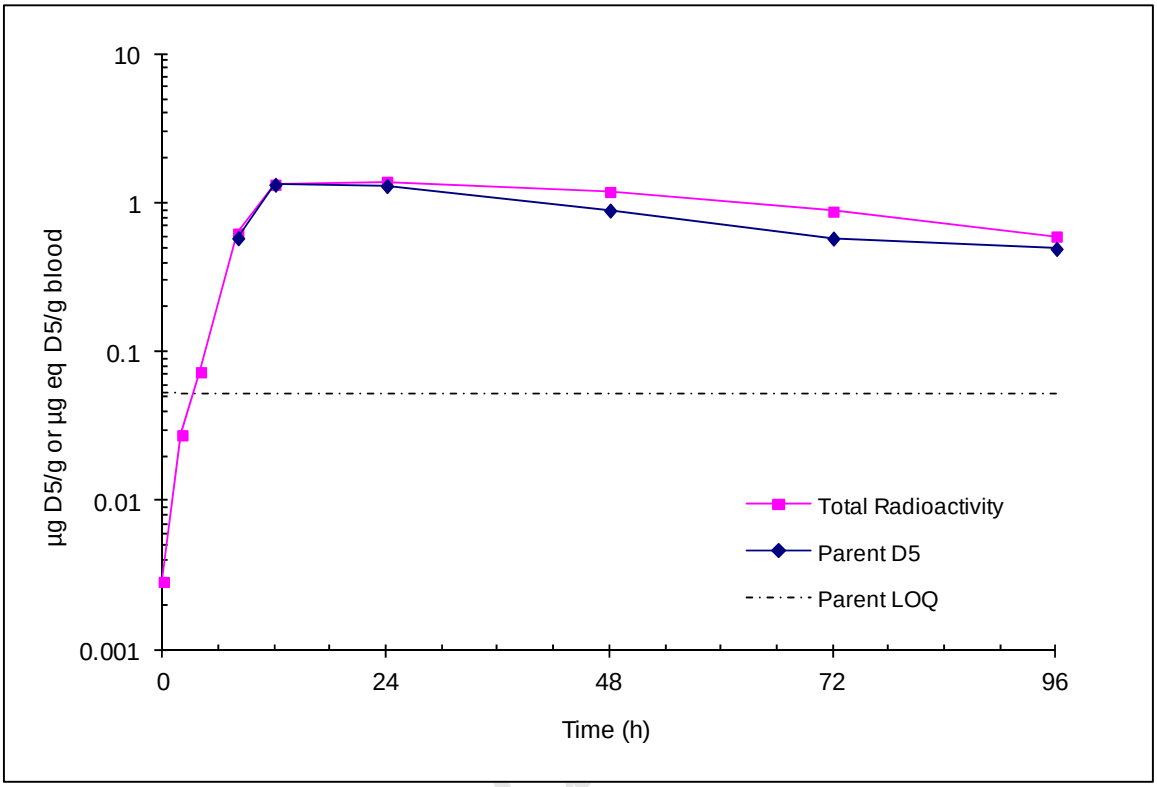

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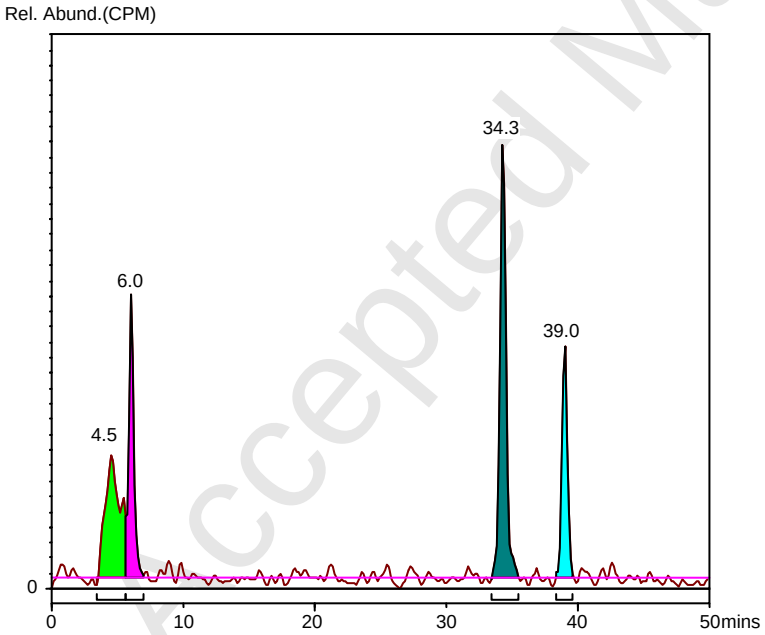

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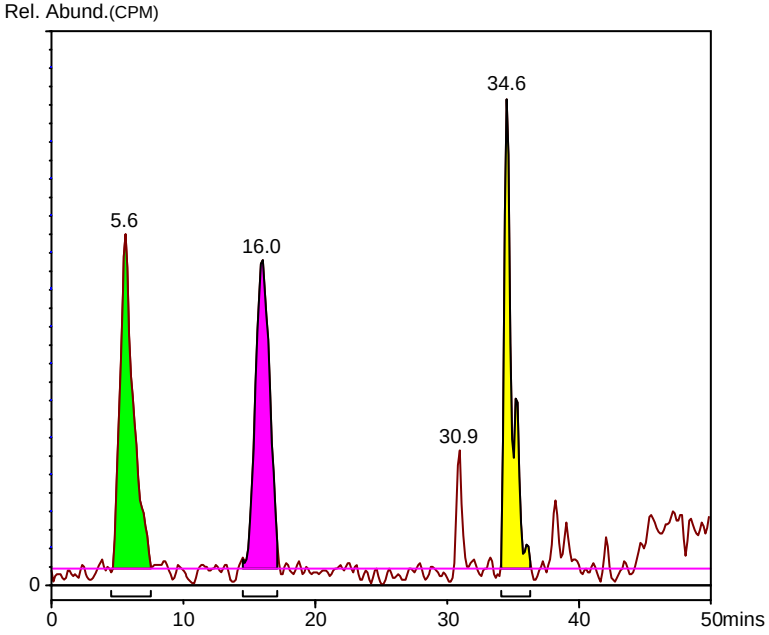

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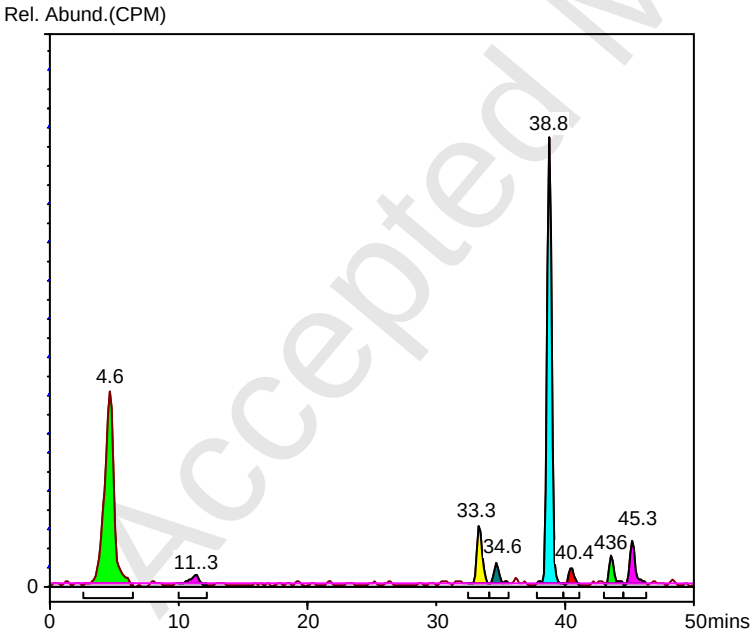

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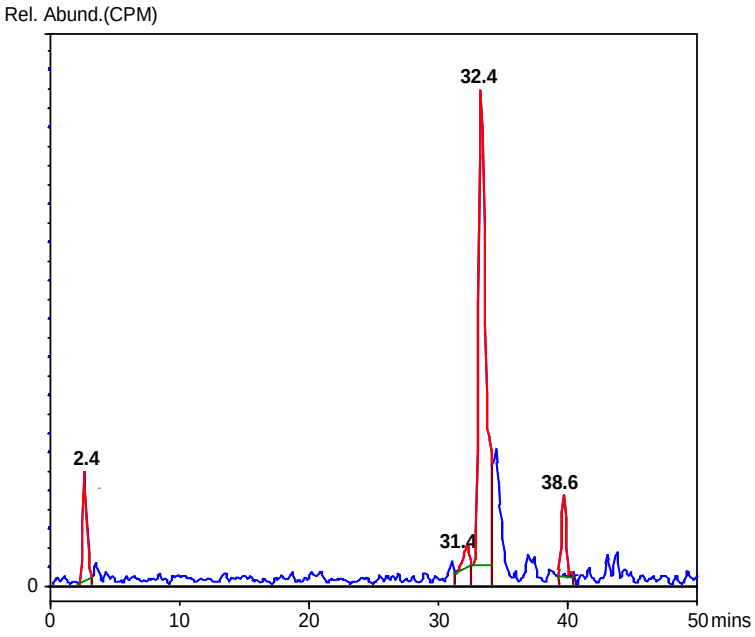

Figures 4A-4B

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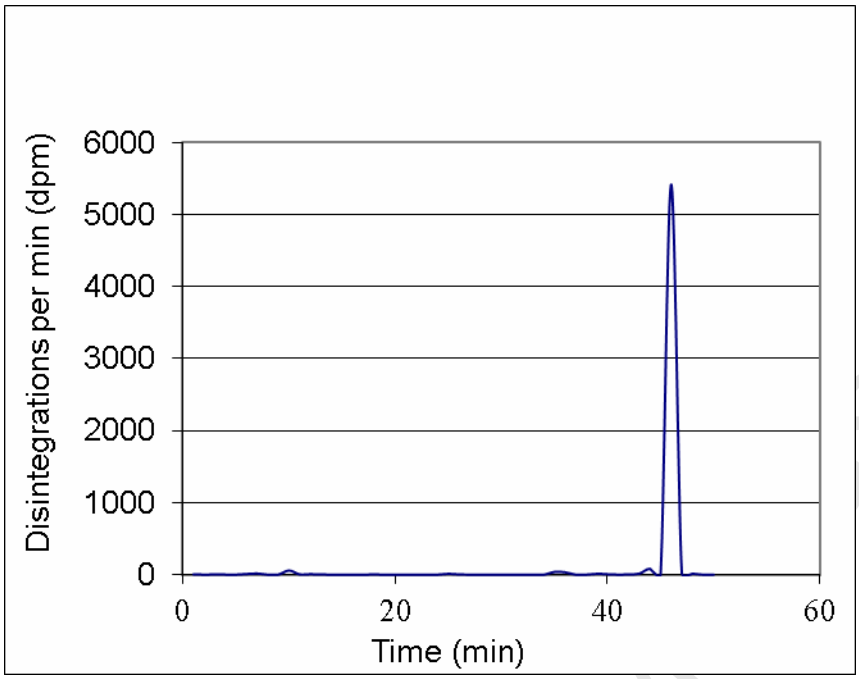

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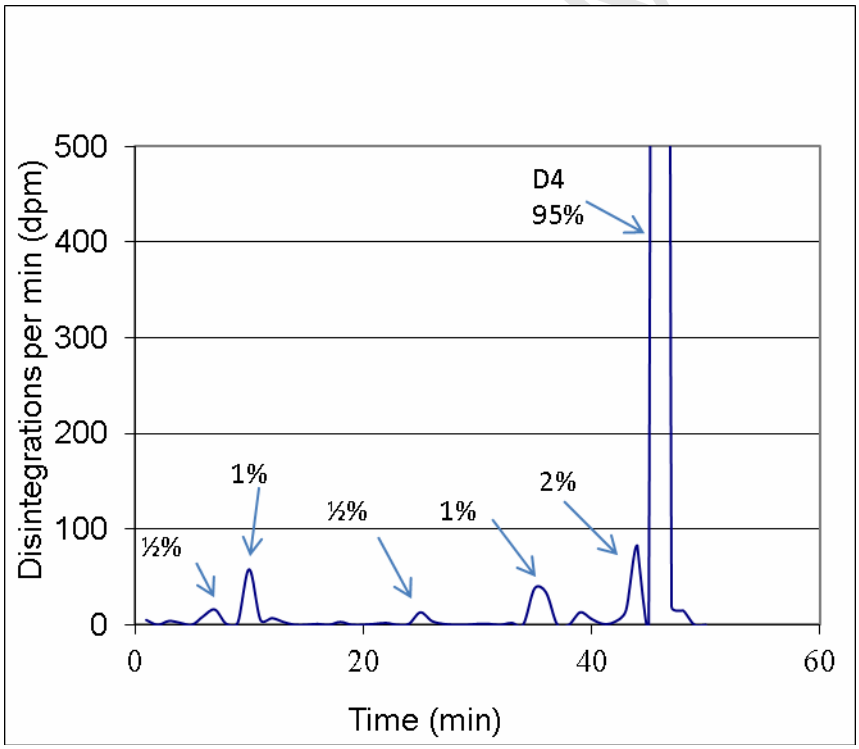
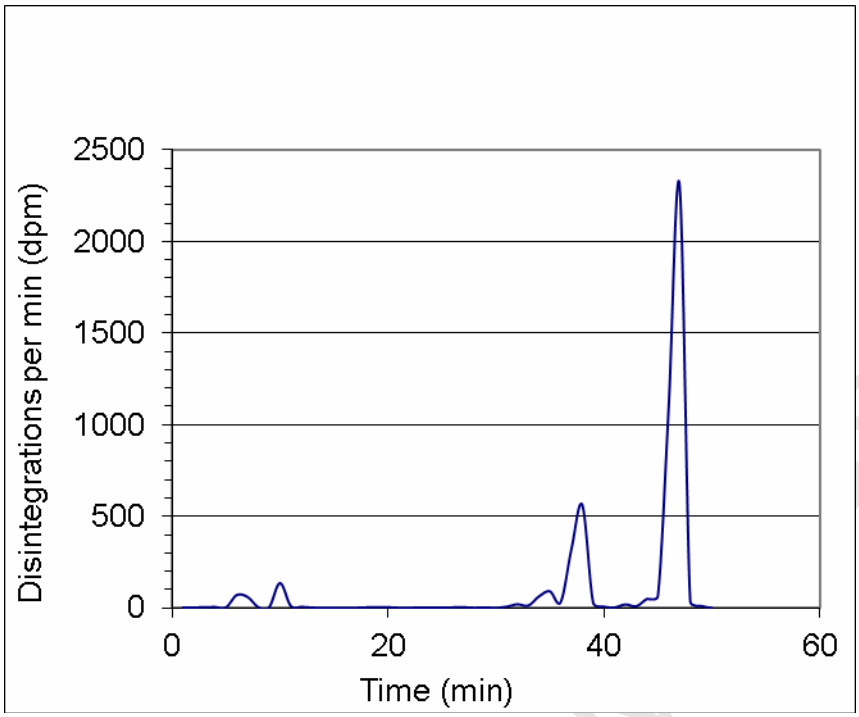


Figures 4C-4D

C



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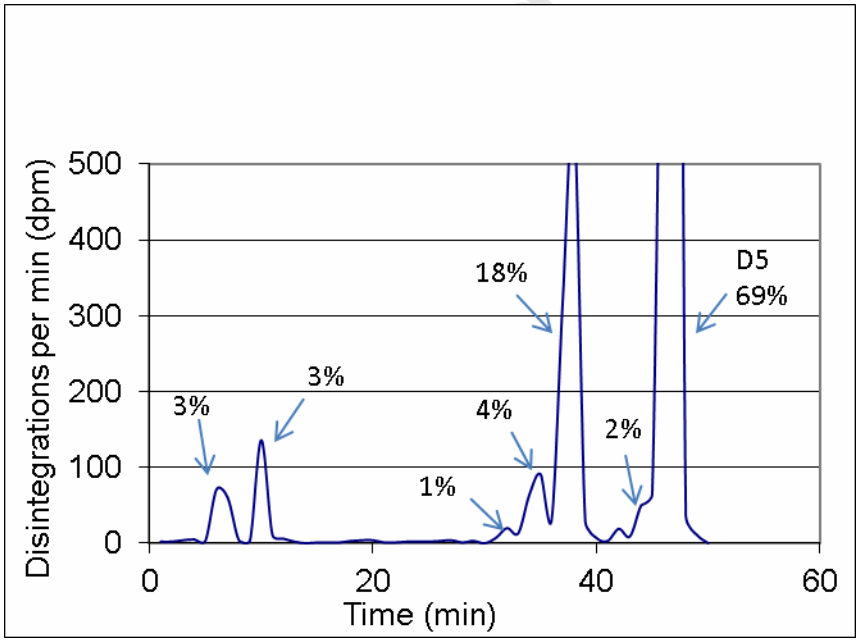

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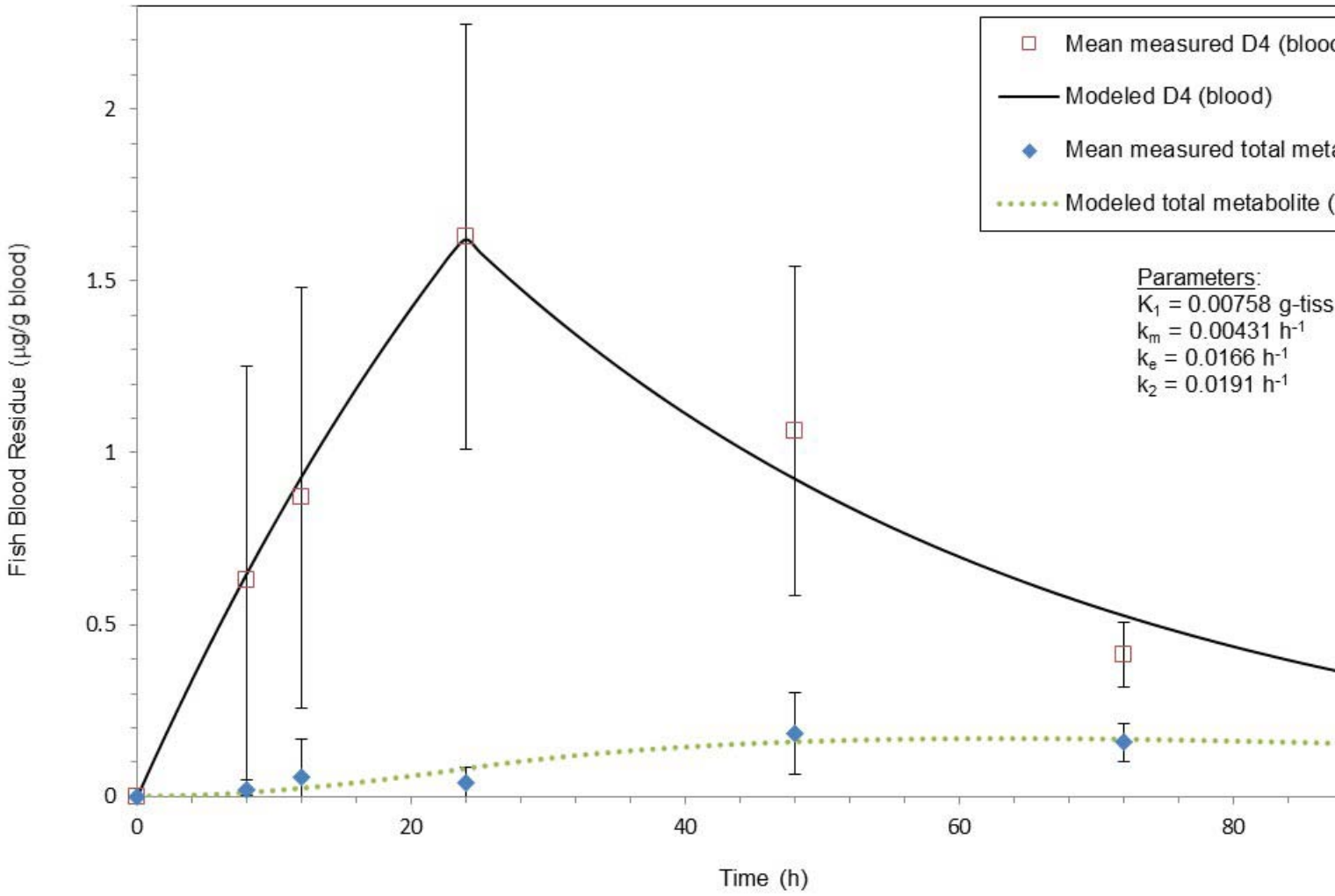

Figure 5B.

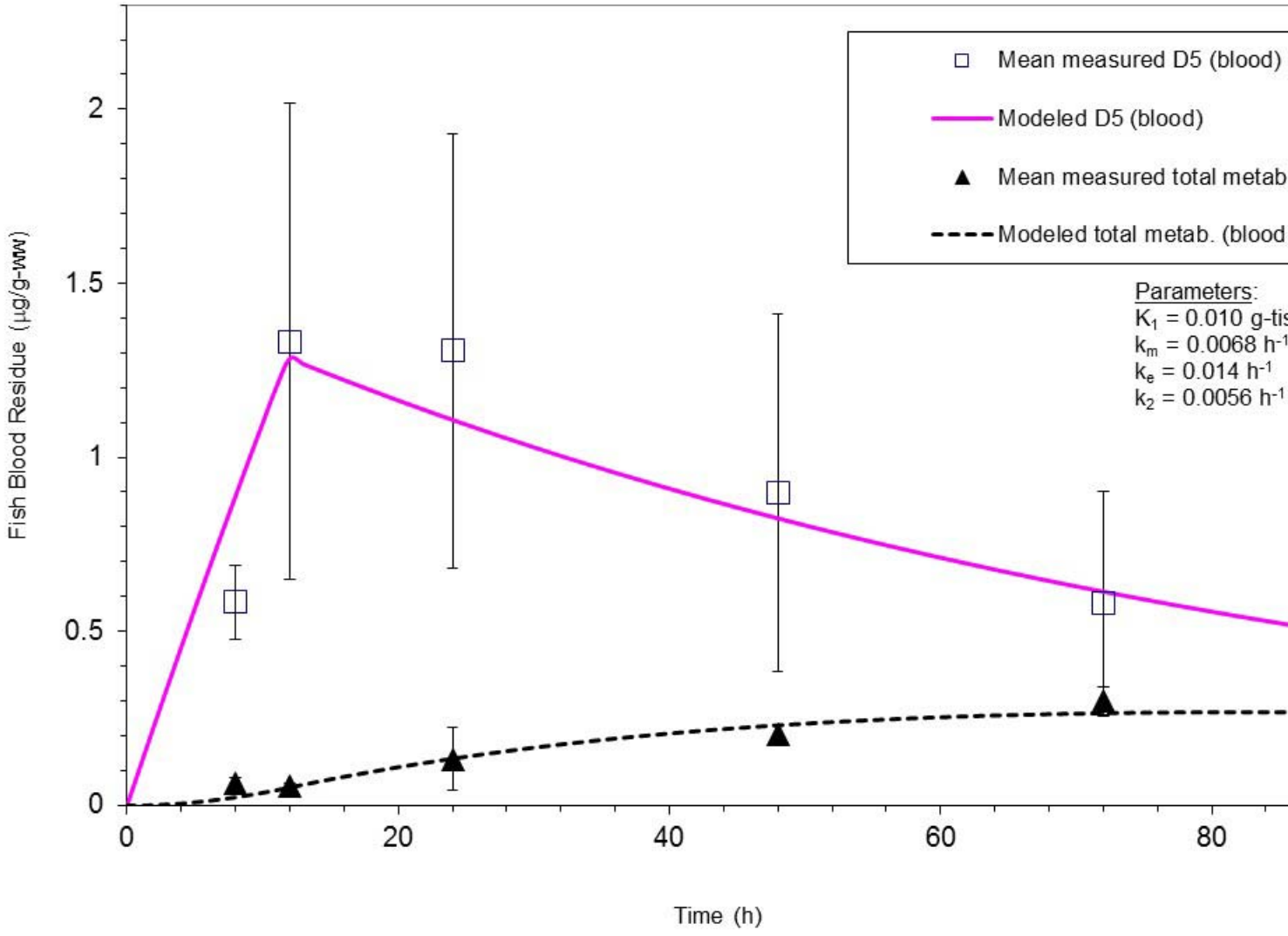

Table 1. Percentage of the administered dose recovered at 96 h post-dosing of rainbow trout with a targeted dose level of 15 mg D_4 or D_5/kg bw via a single oral gavage in a corn oil vehicle.

	[^{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	16237	12996	80				

2	17413	14408	83	6	13197	8851	67
3	12445	11164	90	7	10205	8589	84
4	9170	5955	65	8	13319	11032	83
Mean	13816	11131	79	Mean	12240	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 of 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 of 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

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.

	14C-D4			14C-D5	
Fish ID	Radioactivity AUC µg eq-h/g	Parent AUC µg-h/g	Fish ID	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 of 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 µg/g	Metabolite µg eq/g	Parent % of Total	Parent µg/g	Metabolite µg eq/g	Parent % of Total	Parent µg/g	Metabolite µg eq/g	Pa % T
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	0.02	0.23	0.21	0.04	0.05	0.04	0.

	Digestive Tract			Fat		
Fish ID	Parent µg/g	Metabolite µg eq/g	Parent % of Total	Parent µg/g	Metabolite µg 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	0.02	10.36	0.00	0.01

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 of 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 μg/g	Metabolite μg eq/g	Parent % of Total	Parent μg/g	Metabolite μg eq/g	Parent % of Total	Parent μg/g	Metabolite μg eq/g	Pa % T
6	0.201	66.58	0.301	0.198	0.39	33.7	0.770	0.85	47
7	BLQ		0.00	1.45	0.44	76.6	2.95	0.99	74
8	2.55	170.53	1.47	1.01	0.70	58.8	1.97	1.45	57
Mean	1.38		0.592	0.885	0.50	56.4	1.90	1.1	60
SE				0.366	0.10	0.1	0.63	0.2	0.

	Digestive Tract			Fat		
Fish ID	Parent μg/g	Metabolite μg eq/g	Parent % of Total	Parent μg/g	Metabolite μg eq/g	Parent % of Total
6	1.50	0.28	84.5	14.6	0.0	111
7	27.6	16.70	62.3	24.3	0.0	114

8	2.54	0.62	80.4	18.4	0.0	116
Mean	10.5	5.9	75.7	19.1	0.0	114
SE	8.5	5.4	0.1	2.82	0.0	0.0

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.