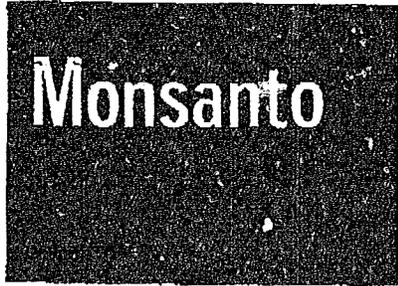


CODING FORMS FOR SRC INDEXING

Microfiche No.	OTS0559903		
New Doc ID	88000000142	Old Doc ID	8EHQ-0400-14703
Date Produced	11/11/80	Date Received	04/18/00
		TSCA Section	8E
Submitting Organization	TCC CONSORTIUM		
Contractor	MONSANTO CO		
Document Title	INITIAL SUBMISSION: BIOCONCENTRATION, EXCRETION AND METABOLISM 3,4,4'-TRICHLOROCARBANILIDE (TCC) IN CHANNEL CATFISH, W/TSCA HLTH & SFTY STUDY CVR SHT DATED 4/12/00		
Chemical Category	UREA, N-(4-CHLOROPHENYL)-N'-(3,4-DICHLOROPHENYL)-		



A 04



REVISION 11/11/80

MIC - D&P - R&D  
(CO./DIV./DEPT./LOCATION)

Final REPORT  
(TYPE OF REPORT)

REPORT NO.: MSL-1277

JOB/PROJECT NO.: 03-000-760.11-7603495

DATE: October 15, 1980

TITLE: BIOCONCENTRATION, EXCRETION AND METABOLISM OF  
3,4,4'-TRICHLOROCARBANILIDE (TCC) IN CHANNEL  
CATFISH

AUTHORS: Charles Lakinger and Richard A. Kimerle  
T. A. Taulli

ABSTRACT: In bioconcentration studies with channel catfish, TCC was shown to have a whole fish wet weight BCF of 137 and 13 for muscle. These BCF's are much lower than one would expect for a chemical such as TCC. An explanation for the low BCF's is possible because a concurrent metabolism study was conducted. TCC was metabolized to hydroxylated TCC and the sulfate and glucuronide conjugates which are apparently much more rapidly eliminated than TCC. Excretion was primarily biliary via the alimentary canal with significant amounts also excreted in the urine. Very little excretion took place across the gills. This fish metabolism pattern was quite similar to that published for mammalian systems.

These data suggest that TCC would not bioconcentrate from water to fish to any significant degree and that significant food chain biomagnification is not likely to occur, especially at the anticipated low exposures.

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REPORT NUMBER MSL-1277

AUTHOR(S) Charles Lakinger and Richard A. Kimerle

ABSTRACT (To be used on Report Notice)

In bioconcentration studies with channel catfish, TCC was shown to have a whole fish wet weight BCF of 137 and 13 for muscle. These BCF's are much lower than one would expect for a chemical such as TCC. An explanation for the low BCF's is possible because a concurrent metabolism study was conducted. TCC was metabolized to hydroxylated TCC and the sulfate and glucuronide conjugates which are apparently much more rapidly eliminated than TCC. Excretion was primarily biliary via the alimentary canal with significant amounts also excreted in the urine. Very little excretion took place across the gills. This fish metabolism pattern was quite similar to that published for mammalian systems.

These data suggest that TCC would not bioconcentrate from water to fish to any significant degree and that significant food chain biomagnification is not likely to occur, especially at the anticipated low exposures. Please list on the reverse side SUGGESTED SUBJECTS OR KEYWORDS to be used in indexing and retrieving this report. For instance, a tentative process for Pydraul<sup>®</sup> 230A hydraulic fluid might have the following keywords suggested by the author:

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Bioconcentration  
Biomagnification  
Food Chain  
Fish  
Residue  
Metabolism  
Excretion

INTRODUCTION

TCC®, 3,4,4'-trichlorocarbamide, is widely used as a bacteriostatic agent in bar soaps. The potential exists for TCC to be found in natural waters as a result of disposal in domestic sewage. A number of similarities exist in physical and chemical properties of TCC and other chlorinated hydrocarbons which have been a source of environmental concern. Of serious concern with this type of compound is food chain biomagnification. Chemical residues can occur in fish and birds in excess of what can be tolerated by the organism or a consumer of the organism. However, when a chemical is metabolized and rapidly eliminated it generally indicates a low potential for causing food chain biomagnification problems.

Realistic aquatic hazard assessment of TCC necessitates an extensive data base because TCC possesses the types of chemical and physical properties which have been associated with problem chemicals. In addition to traditional acute toxicity and chronic toxicity data, contemporary hazard assessment recognizes the need for metabolic studies which determine how a chemical is metabolized, identify the products of metabolism, and define the routes and rates of chemical excretion. Such studies are becoming increasingly important as toxicologists now attempt to understand residue dynamics and metabolism. The purpose of this study is to expand the fish toxicological data base on TCC. One part verifies a suggested bioaccumulation pattern and depuration pattern and compares the relation between these patterns in large and small catfish (Ictalurus punctatus). Another aspect focuses on the routes of elimination of a TCC body burden via urine, anal water, gill water, and solid anal excreta. A third facet of the study focuses on HPLC identification of the <sup>14</sup>C labeled components in the various excretion products. A fourth part of the study focuses on the identification of the <sup>14</sup>C residues of TCC in various fish tissues at equilibrium and after 240 hours of clearance. Finally a comparison of fish metabolism data with mammalian data is made to determine whether or not a common metabolic pattern exists.

EXPERIMENTAL SUMMARY

CHANNEL CATFISH BIOCONCENTRATION STUDY #1

Forty small channel catfish (Ictalurus punctatus) were continuously exposed to 23µg/l <sup>14</sup>C TCC for four days in a 100 gallon aquarium. Fish were sacrificed at eight times during uptake and dissected into tissues which were oven dried. Samples of each tissue were completely oxidized to <sup>14</sup>CO<sub>2</sub> and counted in a scintillation counter to determine <sup>14</sup>C-TCC uptake. The hand plotted curves gave uptake at equilibrium and bioconcentration factors for each tissue.

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CHANNEL CATFISH BIOCONCENTRATION STUDY #2A

One-hundred small channel catfish were continuously exposed to 14.8µg/l <sup>14</sup>C-TCC for six days in a 100 gallon aquarium and allowed to depurate for five days in dilution water. Fish were sacrificed at ten times during uptake and seven times during depuration. Tissues were dissected, dried and weighed. Samples of all dried tissues were completely oxidized to <sup>14</sup>CO<sub>2</sub> and counted in a scintillation counter to determine <sup>14</sup>C-TCC uptake at equilibrium, bioconcentration factors for each tissue, and clearance rate.

CHANNEL CATFISH BIOCONCENTRATION STUDY #2B

Thirteen large channel catfish were also exposed to <sup>14</sup>C-TCC in study #2A above. Eight of these large fish were sacrificed at intervals during uptake, dissected, and tissues oven dried. The remaining five fish were sacrificed during depuration, dissected, and tissues oven dried. Tissue samples were completely oxidized to <sup>14</sup>CO<sub>2</sub> and counted in a scintillation counter to determine <sup>14</sup>C-TCC uptake at equilibrium, bioconcentration factor for each tissue, and clearance rate.

CHANNEL CATFISH METABOLISM STUDY #3

Large channel catfish were continuously exposed to 35.4µg/l <sup>14</sup>C-TCC in a 160 liter aquarium. After 24 hours exposure, two fish were sacrificed and dissected, and tissues were freeze dried. The other four fish were catheterized and transferred to metabolism chambers which separated the excretion products into the anterior gill chamber, posterior anal chamber, and urine collection vessel. Urine, anal water, gill water, and solid anal excreta were collected at various time intervals during depuration when the chambers were also cleaned and refilled with fresh water. After 240 hours of clearance, the three surviving fish were sacrificed and dissected, and tissues were freeze dried. All samples of tissue, urine, anal water, gill water, and anal excreta were analyzed both by counting <sup>14</sup>C activity and by HPLC for the distribution of intact TCC and TCC metabolites.

MATERIALS AND METHODSEXPERIMENTAL FACILITIES

All TCC fish bioconcentration/metabolism experiments were conducted in the aquatic toxicology laboratory of Environmental Sciences in "N" Building, Room 104. Bioconcentration aquaria and metabolism chambers were held on shelves at room temperature of 22 ± 2°C. Light was provided by 3,48 inch fluorescent tubes, approximately three feet above the aquaria. A 16 hour on, 8 hours off light regime was provided.

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WATER

The water used for fish holding, bioconcentration, and metabolism studies was from "N" Building's dechlorinated city water system. The acceptability of the water supply has been established in studies by Adams (1978a-b). The alkalinity, hardness, and pH ranged from 38-42 mg/l, 123-142 mg/l, and 7.1 to 7.7, respectively.

FISH

Small channel catfish, *Ictalurus punctatus*, weighing 4.5 - 13 g, and large channel catfish, weighing 165 - 400 g, were obtained from Osage Catfisheries, Inc., Osage Beach, Missouri. All fish were maintained in flowing dechlorinated tap water in a 50 gallon or 100 gallon tank for at least one week before use. Before placing fish in the holding tanks, the tanks were scrubbed with cleanser containing sodium hypochlorite, sterilized for 1 hour with 5.25% sodium hypochlorite (bleach), and thoroughly rinsed. One day after arrival, fish were treated with 200 mg/l of 25% formalin and 0.1 mg/l of malachite green for 1 hour on 3 alternate days. Small fish were fed Trout Chow #3 (Ralston Purina); however, they were not fed two days prior to experiments and were not fed during a study. Large catfish did not feed in the laboratory.

TCC STOCKS AND EXPOSURE

The unlabelled TCC stock solution for all studies was prepared by adding 1 g of TCC (Lot #QM769) to 250 ml of triethylene glycol (TEG), thus yielding a concentration of 4 mg TCC/ml TEG. The <sup>14</sup>C labelled TCC was from Lot 103 of the Monsanto Company. Verification of purity of this sample using HPLC and liquid scintillation counting showed it to be 98.2% radio tagged purity. The specific activity was calculated to be 31,935 dpm/μg TCC. Working stocks for each experiment were prepared by diluting 1/10 <sup>14</sup>C-TCC, from individual vials, and 9/10 unlabelled TCC from the 4 mg/ml TEG stock, to 1000 ml TEG in a 1 l volumetric flask.

Working stock TCC solutions were metered into the aquaria for all bioconcentration studies using a Manostat pump. Prior to placing fish in the aquaria, TCC was manually spiked to the desired concentration. After fish were in the aquaria there was a rapid uptake of TCC during the first few hours of exposure necessitating spiking of additional TCC in order to maintain the desired exposure concentrations. The dilution water flow was controlled by a calibrated flow meter. The rate of flow in all bioconcentration studies was 2 to 4 aquarium volume exchanges per day.

CHANNEL CATFISH BIOCONCENTRATION STUDY #1

Forty small catfish (2-4 inches long, 6-13 grams wet weight) were exposed to a nominal TCC concentration of 23 μg/l in a 338l aquarium with the use of the flow through water/toxicant delivery system. TCC from the 72 μg/l TEG working stock was delivered at a rate of 0.1 ml/minute

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and mixed with the 470 ml/minute of dilution water. The measured exposure concentration was based on 19 duplicated water samples taken at intervals during the 154 hours of exposure. The average measured exposure concentration was 23  $\mu\text{g}/\ell$ . Three catfish were removed at times 0, 1, 4, 7.5, 23.5, 53, 68, and 92 hours during the uptake and equilibrium phases. Data on  $^{14}\text{C}$  content were obtained for each fish separately. The DPM's were converted to  $\mu\text{g}$  of TCC per kg of fish tissue and the data were hand plotted to obtain the TCC concentration at equilibrium and the bioconcentration factor by the Plateau Method (ASTM, 1978). Notebook pages 1641311-1641335.

#### CHANNEL CATFISH BIOCONCENTRATION STUDY #2A

One hundred small channel catfish (2-4 inches long, 5-11 grams wet weight) were exposed to a nominal TCC concentration of 15  $\mu\text{g}/\ell$  in a 338  $\ell$  aquarium with the use of a continuous flow through water/toxicant delivery system. TCC from the 65  $\mu\text{g}/\text{ml}$  working stock was delivered at a rate of 0.13 ml/minute and mixed with 470 ml/minute of dilution water. The measured exposure concentration was based on 16 duplicated water samples taken over the 145 hours of exposure. The calculated average was 14.8  $\mu\text{g}/\ell$ . Three catfish were removed at times 0, 1, 4, 8, 24, 56, 72, 96, 119, and 145 hours during the uptake and equilibrium phases and at times 146, 149, 153, 169, 193, 220, and 265 hours during the depuration phase. Data on  $^{14}\text{C}$  content were obtained for each fish separately. The DPM's were converted to  $\mu\text{g}$  of TCC per kg of fish tissue and the data were hand plotted to obtain the TCC concentration at equilibrium and the bioconcentration factor by the Plateau Method. The data were also analyzed by the computer program BIOFAC (Blau and Agin, 1978) to obtain the uptake rate ( $K_1$ ), the depuration rate ( $K_2$ ), the bioconcentration factor ( $\text{BCF} = K_1/K_2$ ), and the computer plotted bioconcentration curves. Notebook pages 1641336-1641360.

#### CHANNEL CATFISH BIOCONCENTRATION STUDY #2B

Thirteen large channel catfish, weighing 165-410g, were exposed to TCC in the same 338  $\ell$  aquarium with the small catfish of Study #2A. One fish was sacrificed at times 0, 1, 4, 9, 24, 45, 56, and 96 hours during the uptake and equilibrium phases of bioconcentration and at times 97, 100, 104, 120 and 144 hours during the depuration phase. The data were analyzed by the Plateau Method and by BIOFAC. Notebook pages 1641361-1641387.

#### CHANNEL CATFISH METABOLISM STUDY #3

The methods of Schmidt and Kimerle, 1979, were used to study the metabolism and elimination of TCC from channel catfish. Six large fish, weighing 300 to 350 grams, were exposed for 24 hours to 35.4  $\mu\text{g}/\ell$  of TCC in a 160 liter aquarium in a continuous flow through water/toxicant delivery system. TCC from the 51  $\mu\text{g}/\text{ml}$  TEG working stock was delivered at a rate of 0.4 ml/minute and mixed with 450 ml/minute of dilution water. The exposure concentration was measured to be 35.4  $\mu\text{g}/\ell$ , using an average of 18 time-weighted counts of the  $^{14}\text{C}$  activity in the aquarium water taken at intervals during the exposure period.

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After exposure for 24 hours, two fish were removed and tissues analyzed for equilibrium concentrations of TCC. The remaining four fish were anesthetized in MS-222 (tricaine methane sulfonate), catheterized, and confined for 240 hours (10 days) in individual metabolism chambers partitioned into an anterior gill compartment and a separate posterior anal compartment. Each chamber was marked with a different color of tape and the fish in the chamber was identified by this color; thus the "Blue Fish" referred to the specimen in the chamber marked with blue tape.

Urine was collected in graduated cylinders at about 12 hour intervals for the first 4 days of depuration (at 9, 24, 35, 47, 57, 72, 83, and 95 hours) and then at 24 hour intervals for the next 6 days (at 120, 144, 168, 192, 216, and 240 hours). Duplicate 0.5 ml samples were counted for  $^{14}\text{C}$  activity to determine the TCC excreted in urine. At these times anal chambers were completely drained and gill chambers were drained about one-third and duplicate 2 ml samples were counted for  $^{14}\text{C}$  activity to determine the TCC excreted in these waters. About 3.8 liters of anal water and 2.6 liters of gill water were collected and preserved for future analysis in gallon glass jugs at all of the indicated times except 35 and 57 hours. Each time chambers were drained and sampled for  $^{14}\text{C}$  activity the chambers were also cleaned. Anal chambers which had been completely drained were refilled with fresh water which was then allowed to flow in and out for 5 to 10 minutes. Gill chambers which could be drained only about one-third were filled with fresh water and drained three times and then fresh water was allowed to circulate in and out for 5 to 10 minutes. Several times after flushing, water samples counted for  $^{14}\text{C}$  activity showed no activity indicating that the chamber cleaning process had been effective.

No urine was collected from one fish after 35 hours in a chamber. This fish was terminated and eliminated from the data base. The remaining three fish which contributed excretion samples during the 240 hours of depuration were then sacrificed and prepared for analysis of TCC concentration.

Eight selected two ml samples of urine were extracted with ethyl acetate; six selected 250 ml samples each of anal water and gill water were extracted with tetrahydrofuran. One gram samples of 5 different tissues from the two fish sacrificed at equilibrium and from the three fish held in the metabolism chambers were extracted with tetrahydrofuran. Solid anal excretia found at intervals in anal chambers were air dried and five selected ones were extracted with tetrahydrofuran. Duplicate portions of each extract were counted to obtain the percent efficiency of the extraction. Duplicate portions of each residue after extraction were counted to obtain a material balance. The extracts were analyzed by high pressure liquid chromatography using a reversed phase column for separation of intact TCC and TCC metabolites. Notebook pages 1626102-1626200 and 1861601-1861700.

#### SAMPLE PREPARATION

Fish Tissue Samples All fish were sacrificed and dissected into gall bladder, liver, viscera, muscle, and remains. In the bioconcentration studies, small tissues were placed in paper combusto-cones, large tissues in appropriate size glass jars for oven drying. The dried tissues were ground with a glass rod in the jars or were ground with a mortar and pestle.

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In the metabolism study, tissues were put in appropriate size glass beakers for freezing in crushed dry ice. The beakers were covered with several layers of cheesecloth and frozen tissues were freeze dried in a Thermovact Industries Corp. Model FD-3-V-15 Freeze Drier. After freeze drying, tissues were ground in a Waring Commercial Blender and stored at room temperature in covered glass jars. Some tissue samples were later extracted and their residues were air dried and ground with a mortar and pestle. Milligram size samples of tissue for burning were weighed on a Mettler ME22 balance, used with a Mettler BE22 balance control and a Mettler BA25 readout unit. Larger samples were weighed on a Mettler Type H5 160 gram capacity analytical pan balance.

Fish Excretion Product Samples Gill and anal water samples were collected in gallon glass jugs and stored at room temperature. Urine samples were collected and measured in 10 ml graduated cylinders and transferred to appropriated size screw cap vials and jars for storage in a refrigerator. All water and urine samples were preserved with a 5% water solution of mercuric chloride, using 1 ml solution for every liter of sample.

#### RADIO-TRACER METHODOLOGY

Ground samples of dried fish tissue were completely oxidized to  $^{14}\text{CO}_2$  in a Packard Tri-carb Sample Oxidizer and the  $\text{CO}_2$  collected in a mixture of 12 ml Carbo-sorb (Packard) and 13 ml Permafluor V (Packard). Sample sizes for burning were mostly in the following ranges: muscle 120 - 300 mg, remains 175 - 325 mg, liver 20 - 125 mg, viscera 30 - 150 mg, gall bladder 0.2 - 4 mg for small fish and not over 25 mg for large fish. If this much tissue was not present, the whole organ was used. The tissue sample size of gall bladder was most critical; samples larger than the suggested maximum sometimes resulted in sooty burns. Dried tissue residues after extraction contained ammonium sulfate used in the extraction and the sample size was reduced about one-third and an estimated equal amount of cellulose powder (Packard) was mixed with the sample for burning. For scintillation counting, 2 ml samples of aquarium water and 0.5 ml samples of THF and ethyl acetate extracts were added to Insta-gel (Packard) so that sample and Insta-gel totaled about 18 ml. Two ml samples of extraction residues of urine, gill water, anal water, and anal excreta were added to 10 ml of Insta-gel and several ml of tap water were added and hand shaken vigorously until a homogeneous gel formed. Counting of radioactive samples was for 10 minutes by a Nuclear Chicago Isocap/300 Liquid Scintillation System for Study #1, Study #2A, Study #2B, and urine, gill water, and anal water counts of Study #3. All other counts for Study #3 were made by a Searle Analytic 81 Liquid Scintillation Counter. One set of samples counted in both systems showed no significant difference in results.

#### SAMPLE EXTRACTION

Extraction From Urine A two ml urine sample, measured in a 2 ml pipette, was put in an 8 dram vial with 1 gram of sodium chloride and shaken until the sodium chloride was dissolved. Five ml of ethyl acetate (distilled in glass, Burdick and Jackson Laboratories), measured in a 5 ml

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pipette, were added to the urine and shaken for a total of 5 minutes, four minutes by hand and 1 minute on a Vortex-Genie Mixer. After centrifuging for 10 minutes, the ethyl acetate extract on top was removed with a pasteur pipette and put into a separate 8 dram vial. The extraction was repeated on the urine residue with another 5 ml of ethyl acetate using the same procedure. Both extracts were combined and stored in a refrigerator. Duplicate 1 ml samples of urine, ethyl acetate extract, and urine residue after extraction were counted to calculate percent extraction and a material balance. Notebook page 1826135.

Extraction from Freeze Dried Fish Tissue One gram of dried ground tissue was added to 5 ml of saturated ammonium sulfate solution (175 g ammonium sulfate dissolved in 250 ml of Milli Q water) in an 8 dram vial and shaken for 2 minutes with a Mini-Shaker (Fisher). The resulting tissue slurry was hand shaken for 5 minutes with 10 ml THF. After centrifuging for 15 minutes, the THF extract on top was removed with a pasteur pipette. Several muscle and remains extracts were slightly cloudy and filtered through a 2 micron tip (Supelco Inc., Bellefonte, PA. 16823, Catalog No. 5-8473) on a pasteur pipette. Several scoops (about 3-5 grams) of sodium sulfate were added to the extract, which was hand shaken for one minute and passed through Whatman No. 1 filter paper. A second extraction with 10 ml THF was performed on the tissue slurry in the same manner. Both THF extracts were combined and stored in a refrigerator. The remaining tissue residue was air dried at room temperature with a stream of air blowing over the tissue residue to speed drying. Dried residues were ground to a powder in a mortar and pestle. Samples of original ground freeze dried tissue and of ground dried tissue residue after extraction were burned and counted along with samples of each extract. These counts were used to calculate percent extraction and a material balance. Notebook pages 1826189 and 1861677.

Extraction from Anal Water and Gill Water Two-hundred fifty milliliters (250 ml) of water taken from either the anterior gill compartment or the posterior anal compartment of the fish metabolism chamber were placed in a 500 ml separatory funnel. One-hundred seventy-five grams (175g) of ammonium sulfate (granular, certified A.C.S.) were dissolved in the water to get a saturated solution. For the first extraction fifty milliliters (50 ml) of tetrahydrofuran (THF, uv, distilled in glass, Burdick and Jackson Laboratories) were added to the water solution in the funnel and hand shaken for five minutes. After one minute the lower water layer was drained off and saved for repeated extraction. The THF was drained into a screw cap bottle and several scoops (about 6-8 grams) of sodium sulfate (anhydrous, certified A.C.S.) were added to the THF and hand shaken for one minute to absorb any water. The THF was then passed through Whatman No. 1 filter paper. A second, third, and fourth extraction were made, each with 50 ml THF, and following the same procedure. The extracts were combined and stored in a refrigerator. Sample counts of original anal water and gill water, of extracts, and of residues after extraction were used to calculate percent extraction and to obtain a material balance. Notebook pages 1826155 and 1826156.

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Extraction from Anal Excreta Solid anal excreta collected from the anal chambers were placed in paper combusto-cones (Packard) and air dried. For extraction, a cone was cut into small pieces and placed in an eight dram vial. Five milliliters (5 ml) of saturated ammonium sulfate solution (175 grams of ammonium sulfate in 250 ml Milli Q water) were added and the vial contents were shaken by hand for one minute and then on a Mini-Shaker (Fisher) for one minute. The contents were then homogenized with 10 ml of THF, using a Tekmar Tissuemizer (SDT 182) for 2 minutes, centrifuged for 15 minutes, and the extract removed with a pasteur pipette. Duplicate 0.5 ml extract samples were counted for  $^{14}\text{C}$ . The residue was air dried and ground with a mortar and pestle. The entire pulverized residue was divided into several smaller samples which were mixed with an equal amount of cellulose powder (Packard), were oxidized to  $^{14}\text{CO}_2$ , and counted. These counts were used to calculate percent extraction and a material balance. Notebook pages 1826193-1826195.

#### SAMPLE ANALYSIS

Instrumentation Extracts of urine, anal water, gill water, solid anal excreta, tissues from fish at equilibrium, and tissues from fish after 240 hours of depuration were analyzed for TCC by reversed phase chromatography (Tauli, 1977) on a Waters Associates High Pressure Liquid Chromatograph to determine the presence and distribution of intact TCC and TCC metabolites. The analytical column was a 3.9 mm ID x 30 cm reversed phase column packed with  $\mu$  Bondapak  $\text{C}_{18}$ . The guard column was 3.9 mm ID x 2.1 cm, packed with Bondapak  $\text{C}_{18}$ /Corasil. A Model 660 Waters Associates Solvent Programmer produced a linear solvent gradient, initially 20% acetonitrile/80% water and changing to 90% acetonitrile/10% water in 30 minutes. A constant flow of 2.0 ml/min was employed. One-hundred  $\mu\text{l}$  samples were introduced onto the column via a Waters WISP 710B. Column effluent was monitored with a Waters 450 Wavelength Detector at 265 nm at 0.1 AUFS for all samples except liver, viscera, muscle, and remains extracts from fish after 240 hours depuration. These oily, highly UV absorbing extracts were monitored at 0.4 AUFS. The monitored output was charted on a Houston Instruments Model B5117-1 Omni Scribe Recorder at a chart speed of 0.5 cm/inch. However, the emergence of TCC and metabolites was determined by counting the total HPLC effluent fractions. Thirty 2 ml fractions were taken at one minute intervals in an Instrument Specialities Model 328 Fraction Collector. Fifteen ml of Packard Instagel were added to the fractions, all of which were assayed for radioactivity in a Searle Analytic 81 Liquid Scintillation Counter.

Urine Samples The volume of ethyl acetate extract aliquot recommended below was evaporated to dryness under a nitrogen purge in a Reacti-Vial (Pierce Chemical) in a Thermolyne Dri-Bath maintained at 60°C. The dry sample was reconstituted by adding the appropriate THF volume, then shaken on a Mini-Shaker for one minute, and centrifuged for 10 minutes. The supernatant liquid was used for HPLC injection and  $^{14}\text{C}$  counting.

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<u>Anticipated DPM/ml Urine</u>	<u>Aliquot of Ethyl Acetate Extract</u>	<u>Reconstitution Volume</u>	<u>Estimated DPM in 100 µl Injected</u>
1000 to 10,000	8 ml	0.2 ml	4,000 - 40,000
10,000 to 50,000	4 ml	1.0 ml	4,000 - 20,000
>50,000	2 ml	1.0 ml	10,000 minimum

HPLC injection volume was 100 µl. With a 0.2 ml reconstitution volume, 50 µl was available for counting in the scintillation counter; with a 1.0 ml reconstitution, two 100 µl samples were counted. These counts were used to determine the recovery of the HPLC. Notebook page 1861607.

Anal Water and Gill Water Samples After four 50 ml THF extractions from a 250 ml anal or gill water sample, the combined extracts were transferred to a Kuderna-Danish Evaporative Concentrator and reduced to 4 to 5 ml using a hot water bath at 90 to 94°C. After cooling, the concentrated extract was transferred to a Reacti-Vial. The Kuderna-Danish flask and lower tube were washed with THF and the washings added to the Reacti-Vial. The extract was evaporated to dryness under a nitrogen purge in a Thermolyne-Dri-Bath at 60°C. The dry sample was reconstituted by adding the THF volume recommended below, then was mixed on a Mini-Shaker for one minute, and centrifuged for 10 minutes. The supernatant liquid was used for HPLC injection and <sup>14</sup>C counting.

<u>Anticipated DPM/ml Water</u>	<u>Reconstitution Volume</u>	<u>Estimated DPM in 100 µl Injection</u>
10 to 50	0.2 ml	1,250 to 6,250
>50	1.0 ml	1,250 minimum

HPLC injection volume was always 100 µl. With a 0.2 ml reconstitution volume, 50 µl was available for <sup>14</sup>C counting; with a 1.0 ml reconstitution volume two 100 µl samples were counted. Notebook page 1861624.

Tissue Samples From Fish Sacrificed at Equilibrium Sample aliquots from THF tissue extracts were dried, reconstituted, and prepared for HPLC injection in the same manner as urine extracts. All extracts had high DPM's ranging from about 9,000 to 30,000, so 4 ml aliquots, dried and reconstituted to 1 ml with THF, were used. Notebook page 1861659.

Tissue Samples From Fish After 240 Hours Depuration Initially, one gram of tissue (or less if one gram was not available) was used for extraction. Sample extract aliquots were dried, reconstituted, and prepared for injection in the same manner as urine extracts. Extract DPM's from gallbladder, liver and viscera were high, ranging from about 40,000 DPM/ml to about 2,000,000 DPM/ml. Aliquot and reconstitution volumes were calculated from these extract DPM's to deliver an estimated 3000 to 30,000 DPM's in a 100 µl HPLC injection volume as shown in the table below. Liver and viscera aliquots did not dry completely but remained as a very viscous, amber liquid of less than 0.1 ml. The THF reconstituted sample, however, was suitable for HPLC injection, since the addition of THF gave the sample a watery consistency.

<u>Extract DPM/ml</u>	<u>Sample Aliquot Volume</u>	<u>Reconstitution Volume</u>	<u>Estimated DPM in 100 µl Injection</u>
>50,000	1 ml	0.5 ml	10,000 minimum
10,000 - 50,000	3 ml	0.5 ml	6,000 - 30,000
4,000 - 10,000	6 ml	0.5 ml	4,800 - 12,000
1,000 - 4,000	12 ml	0.4 ml	3,000 - 12,000
100 - 1,000	Entire Extract (Estimated 16 ml)	0.2 ml	800 - 8,000

Extract DPM's from muscle and remains were low (about 100 to 200 DPM/ml) and THF extractions were tried on larger tissue samples of 3 to 6 grams. When concentrated, the extracts did not dry completely but remained as a very viscous amber liquid in amounts varying from 0.5 ml to about 1 ml. The THF needed to reconstitute these "dried" samples was so great that it diluted the sample and decreased again the DPM/ml. It is recommended, therefore, that 1 gram of tissue be used for extraction and the above table of aliquot volumes and reconstitution volumes be followed, using a 200 µl injection volume. Notebook pages 1861677-1861685.

Anal Excretia Samples The entire THF extract or an aliquot of it was dried completely in a Reacti-Vial and reconstituted with THF according to the schedule below. The same procedure used with urine samples was followed with these samples. Notebook page 1861636.

<u>DPM/ml Extract</u>	<u>Aliquot Volume</u>	<u>Reconstitution Volume</u>	<u>Estimated DPM in 100 µl Injection</u>
>10,000	2 ml	1 ml	2,000 minimum
2000 to 10,000	5 ml	0.5 ml	2000 to 10,000
300 to 2,000	Entire Extract (usually 7 ml)	0.2 ml	1500 to 7,000

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DEFINITIONS

In parts of this report, the results of "TCC analyses" in water, tissues, and excretion products are reported as  $^{14}\text{C}$ -TCC. This means the  $^{14}\text{C}$  activity of the sample was counted and multiplied by the specific activity for TCC stocks of 31,935 DPM/ $\mu\text{g}$  TCC. Therefore,  $^{14}\text{C}$ -TCC results represent maximums. When metabolites were present they too were counted by  $^{14}\text{C}$  and included as "TCC" in the total.

In the metabolism section of this report HPLC analyses enabled the intact TCC to be quantitatively differentiated, and so designated, from the metabolites. Specificity of TCC $\text{\textcircled{C}}$  and TCC-metabolites was based on retention time agreement with available authentic standards.

RESULTSBIOCONCENTRATION

In Table 1 the TCC bioconcentration at equilibrium and bioconcentration factors (BCF) are shown for gall bladder, liver, viscera, muscle, remains, and whole fish. Three experiments are summarized: small catfish exposed to 23  $\mu\text{g}$  TC/ $\ell$ , small catfish exposed to 14.8  $\mu\text{g}/\ell$ , and large catfish exposed to 14.8  $\mu\text{g}/\ell$ . For small catfish at 23  $\mu\text{g}/\ell$  exposure, gall bladder shows the highest bioconcentration, 842,000  $\mu\text{g}/\text{kg}$ , while muscle shows the lowest with 428  $\mu\text{g}/\text{kg}$ . The other tissues vary from 1,240  $\mu\text{g}/\text{kg}$  for remains to 26,360  $\mu\text{g}/\text{kg}$  for viscera. Similarly the BCF, calculated by the Plateau Method, is highest for gall bladder at 36,240 and lowest for muscle at 18 with other tissues ranging from 54 for remains to 1,135 for viscera. For small catfish exposed to 14.8  $\mu\text{g}/\ell$ , gall bladder at 1,916,000  $\mu\text{g}/\text{kg}$  and muscle at 1048  $\mu\text{g}/\ell$  are again the highest and lowest bioconcentrations, with other tissues ranging from 1,947  $\mu\text{g}/\text{kg}$  for muscle to 34,811  $\mu\text{g}/\text{kg}$  for viscera. Likewise, the BCF, calculated by the Plateau Method, is highest for gall bladder at 127,700 and lowest for muscle at 70 with a range of 130 for remains to 2321 for viscera. For large catfish exposed to 14.8  $\mu\text{g}/\ell$  the same pattern exists for bioconcentration with gall bladder highest at 1,540,000  $\mu\text{g}/\text{kg}$ , muscle lowest at 620  $\mu\text{g}/\text{kg}$ , and other tissues ranging from 1,498  $\mu\text{g}/\text{kg}$  for remains to 48,727 for liver. The Plateau Method BCF is highest for gall bladder at 102,700, lowest for muscle at 41 and ranging from 100 for remains to 3248 for liver. BCF values calculated by the computer program BIOFAC are very similar to those calculated by the Plateau Method. Gall bladder consistently had bioconcentration and BCF values that were two or three orders of magnitude higher than other tissues. Muscle had the lowest bioconcentration and BCF values, of the same order of magnitude as remains and one to two orders of magnitude lower than liver and viscera. Tissues are ranked by BCF and bioconcentration in this order: gall bladder > viscera > liver > remains > muscle. Only in large catfish was there a deviation from this pattern with the ranking of viscera and liver reversed. However, these data were not replicated as the small catfish studies which are considered more reliable with three replicates.

Table 2 shows the BIOFAC calculated uptake rate, depuration rate, bioconcentration factor (BCF), and half life of clearance for two studies, one with small catfish and one with large catfish. Tissues for which the

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values were calculated are gall bladder, liver, viscera, muscle, and remains. The uptake rate was the highest for gall bladder (3490) and lowest for the remains and muscle (6,18) in both large catfish and small catfish. Viscera and liver ranged from 18 to 422 in large and small catfish. Depuration rates for muscle were highest in both large and small catfish, 0.230 and 0.220 respectively. These rates were lowest in viscera of large catfish (0.004) and in gall bladder of small catfish (0.017). Bioconcentration factors followed the same pattern in both large and small catfish with gall bladder showing the highest values, 102,700 and 201,104 respectively. These values were 2 orders of magnitude higher than the values for the next highest tissues, the viscera and liver, with BCF's from 1284 to 4668 in both large and small catfish. Remains and muscle BCF value ranged from 50 to 162 for large and small catfish, respectively. Tissues are ranked as follows for BCF values: gall bladder > viscera > liver > remains > muscle. Half-life of clearance was shortest in large and small catfish muscle. In large catfish, values were greatest for viscera (182 hours), followed by remains (14) and then liver (7), with no value calculated for gall bladder. In small catfish, gall bladders had the longest half life of clearance (40 hours) followed by viscera (22), then liver (14) and then remains (10).

The uptake, equilibrium, and clearance data for  $^{14}\text{C}$  activity in the organs and tissues are shown for experiment 2A in Figures 1 through 5. The plot was accomplished by the BIOFAC program.

Tables 3A and 3B show the milligrams of TCC and the percents of the total TCC body burden in large catfish organs at equilibrium and after 240 hours of depuration. Gall bladder, liver, viscera, muscle and remains were the body parts sampled. Fish 1 and Fish 2 were exposed to 14.8  $\mu\text{g}$  TCC/l of water for 24 hours and were sacrificed at equilibrium before any depuration occurred. Remains and muscle contained most of the TCC body burden with 48 and 26 percent, respectively. The liver contained 14%, viscera 8%, and the gall bladder contained the least percent of the total body burden, 4%. The order of ranking for percent of the body burden in the various tissues was from highest to lowest was remains > muscle > liver > viscera > gall bladder.

After 240 hours of depuration the TCC distribution changed dramatically (Table 3B). In the three fish analyzed gall bladder contained most of the total  $^{14}\text{C}$  body burden, 60%, viscera contained 15%, remains 9%, liver 5%, and muscle 2%. The rank order of these tissues from highest to lowest was: gall bladder > viscera > remains > liver > muscle, almost in reverse of the order during equilibrium.

Figures 6A and 6B show the mg quantities in the tissues at equilibrium and after depuration.

#### EXCRETION

Table 4 shows the distribution of  $^{14}\text{C}$ -TCC remaining in the tissue of, and excreted from, three large catfish after 240 hours of depuration. Approximately 20 percent (0.94 mg) remained in the tissue after 240 hours of depuration and 80 percent was excreted by the fish. Urine in the 3 fish contained a mean of 1.82 mg or 40% of the total body burden.

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An additional 33% (1.52 mg) was eliminated into the anal water. Seven percent of the  $^{14}\text{C}$ -TCC (0.34 mg) was excreted via the gills. Solid anal excretia accounted for only 0.03 mg or about 1% of the total body burden.

Figure .6C graphically shows the distribution of the excreted  $^{14}\text{C}$ -TCC in urine, anal water, gill water, and anal excretia.

Table 5 presents the amount of  $^{14}\text{C}$ -TCC excreted by 3 fish in urine, anal water, and gill water for 14 time intervals sampled during 240 hours of depuration. Largest amounts were excreted in urine during the early time intervals. From time 0 to 9 hours, 0.36 mg was excreted; from 9 to 24 hours, 0.58 mg was excreted; from time 24 hours to 35 hours, 0.41 mg was excreted; and from time 35 to 47 hours, 0.25 mg was excreted. After 47 hours  $^{14}\text{C}$ -TCC excreted in urine was less than 0.1 mg in each of the remaining 10 time intervals. The total amount in the urine at the end of 240 hours was 1.82 mg. The pattern of TCC excretion into anal water was the reverse of the urine excretion pattern. In the early time intervals, very little  $^{14}\text{C}$ -TCC appeared in anal water, with amounts measuring less than 0.01 mg. In the later time intervals when yellow stringy anal excretia appeared in the anal chambers, the  $^{14}\text{C}$ -TCC amounts in the water were much greater and showed much variability. From 120 to 144 hours 0.41 mg was excreted; from 144 to 168 hours, 0.20 mg was excreted; from time 168 to 192 hours, 0.11 mg was excreted; from 192 to 216 hours 0.18 mg was excreted; and from time 216 to 240 hours 0.16 mg was excreted. The total amount excreted into anal water was 1.52 mg. Excretion of  $^{14}\text{C}$ -TCC into gill water was quite low and relatively uniform in each time interval during the 240 hours of depuration. Values in the intervals were most commonly in the hundredths of mg. The total amount of TCC excreted into gill water after 240 hours was a 0.34 mg.

Figure 7 presents the average excretion of  $^{14}\text{C}$ -TCC via urine, anal water, and gill water.

Table 6 shows the accumulated excretion of  $^{14}\text{C}$ -TCC in urine, anal water, and gill water during 240 hours of depuration by three large catfish, the "blue" fish, the "yellow" fish and the "green" fish. The mean total TCC accumulation in each of 14 time intervals was used to arrive at each individual interval's percent of the total accumulation. TCC excreted in urine was very high in the first four time intervals. By the end of 47 hours (2 days) 88% of the TCC excreted in urine had been given off with only 11% excreted in the remaining 193 hours. The TCC excretion in anal water was the reverse of the urine excretion pattern. Only 2% of anal water excretion occurred in the first 95 hours (3 days); the rest was excreted later, closely linked to the excretion of yellow, stringy material in the anal chamber. Excretion into gill water was always less than excretion via urine and anal water, mostly only hundredths of mg in each time interval. Gill water excretion tended to be somewhat higher although fairly uniform in the early part of depuration with 56% excreted in the first 72 hours (3 days). For the remaining 7 days the amounts excreted were much less but still were fairly evenly excreted.

Figure 8 presents the pattern of accumulated excretion of  $^{14}\text{C}$ -TCC via the routes of urine, anal water, and gill water over the 240 hours of sample collection.

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METABOLISM

Table 7 summarizes the extraction efficiency of samples of fish excretion products and of fish tissues prepared for HPLC analysis. Urine, gill water, anal water, and anal excretia were the excretion products extracted. The tissues were taken from two fish sacrificed at equilibrium after 24 hours of exposure to 35.4 µg/l TCC and from 3 fish with the same exposure sacrificed after 240 hours of depuration. Excretion products were extracted most uniformly, with 73-98% extraction from 8 urine samples, 75-102% from 6 gill water samples and 78-90% from 6 anal water samples. Mean values were 82% for urine, 86% for gill water, and 84% for anal water. Anal excretia extractions showed more variation ranging from 55-84%, with a mean of 71% extraction for 5 samples. Tissue extractions showed the greatest variation, reflecting the increase in variability between the different tissues; namely, gall bladder, liver, viscera, muscle, and remains. Extractions from the tissues of two fish sacrificed at equilibrium ranged from 47-102% with a mean of 77% for 10 samples. All 5 tissues of fish that underwent depuration for 240 hours were extracted with a range from 39-111% and with a mean of 71% for 10 samples. When only muscle and remains from these fish were extracted a second time, the extractions ranged from 65-93% with a mean of 75% for the 4 samples.

Table 8 shows the results of HPLC analysis for intact TCC and three metabolites in the gall bladder, liver, viscera, muscle, and remains from 2 fish which were sacrificed at equilibrium after exposure to 35.4 µg/l TCC. Glucuronide conjugates of TCC, sulfate conjugates of TCC, hydroxyl derivatives of TCC, and intact TCC were separated and evaluated by HPLC. Gall bladder showed considerable glucuronides (mean value 49%) and sulfates (mean value 32%), with some intact TCC (mean value 14%) and very little hydroxy-TCC (mean value 4%). Liver contained almost half intact TCC (mean value 48%), considerable glucuronides (mean value 28%) and sulfates (mean value 22%), and very little hydroxy-TCC (mean value 3%). Viscera contained even more intact TCC (mean value 66%), some glucuronides (mean value 15%) and sulfates (mean value 14%) and also very little hydroxy-TCC (mean value 2%). Muscle was almost all intact TCC (mean value 97%) and only about 1% each of glucuronides, sulfates, and hydroxy-TCC. Remains were very similar to muscle with almost all intact TCC (mean value 93%) and very little glucuronides (mean value 2%), sulfates (mean value 4%), and hydroxy-TCC (about 1%). These data are summarized in Figure 9A.

Table 9 shows the results of HPLC analysis for intact TCC and the metabolites in the gall bladder, liver, viscera, muscle, and remains from 2 of the fish which were sacrificed after 240 hours of depuration. Glucuronide conjugates of TCC, sulfate conjugates of TCC, hydroxyl derivatives of TCC, and intact TCC were separated and evaluated by HPLC. Gall bladder contained considerable glucuronides (mean value 40%) and sulfates (mean value 44%) with some hydroxy-TCC (mean value 16%), and almost no intact TCC (mean value 0.2%). Liver was almost all sulfates (mean value 80%) with little glucuronides and hydroxy-TCC (mean values 11% and 9% respectively, and no intact TCC. Viscera paralleled liver with mostly sulfates (mean value 68%), some glucuronides and hydroxy-TCC (mean values 17% and 16%), and only a trace of intact TCC (mean value 0.2%). Muscle was almost entirely sulfates (mean value 96%) a slight amount of intact

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TCC (mean value 2%), and no glucuronides or hydroxy-TCC. Remains has a distribution similar to muscle with mostly sulfates (mean value 79%), little hydroxy-TCC mean value 15%, and almost no glucuronides (mean value 1%) or intact TCC (mean value 5.5%). Figure 9B summarizes these data.

Table 10 presents the HPLC analysis for intact TCC and metabolites in selected samples of urine, anal water, and gill water. Urine samples selected for analysis were high in  $^{14}\text{C}$  counts in the first half of depuration. The first part of the table shows the distribution in 6 urine samples from one fish (the "Blue" fish) taken at 5 hours, 11.5 hours, and then at about 12 hour intervals to 57 hours of depuration. Glucuronides were consistently abundant, ranging from 60% to 83%. There were lesser amounts of sulfates, ranging from 7% to 21%, and a similar distribution of hydroxy-TCC, ranging from 7% to 22%. Very little intact TCC was present, the amount usually 1% or less except for 7% at 57 hours. The urine data for the blue, green, and yellow fish, hours 9-24, are presented to demonstrate the variability which can exist in these types of data. The 3 fish were very similar in TCC metabolites present. Glucuronides were consistently high, 65%, 83% and 84%. Moderate amounts of sulfates, 18%, 10%, 8% and of hydroxy-TCC, 16%, 4%, and 8% were present. Very little intact TCC was found, 1%, 2%, and an amount too small to be detected.

Anal water samples were selected for analysis on the basis of high  $^{14}\text{C}$  counts, found only in samples from 95-240 hours of depuration. Glucuronides were prominent, but tended to decrease with time, falling from 49% at 120 hours to 28% at 240 hours. Sulfates were present in appreciable but almost consistently smaller amounts and also seemed to decrease, going from 34% at 120 hours to 11% at 240 hours. Hydroxyls seemed to be just the reverse, rising from 16% at 120 hours to 55% at 240 hours. Intact TCC was consistently very low, ranging from 0.3% at 120 hours to 4% at 240 hours.

Gill water samples selected for analysis were some of the highest in  $^{14}\text{C}$  counts; however, all were low relative to other excretion samples. Glucuronides and hydroxyls were both fairly prominent but quite variable, ranging from 13% to 52% for glucuronides and 20% to 52% for hydroxyls and when one was higher the other was lower. Sulfates were common and did not vary as widely, ranging from 15% to 38%. Intact TCC was usually low, 1%-4%, although the green fish at 24 hours had 13%.

The distribution of TCC and TCC metabolites in 5 samples of anal excretia is shown in Table 11. The excretia were yellow, stringy, fragile solids found in anal chambers in the second half of the 10 day depuration period. The 5 samples selected for analysis were high in  $^{14}\text{C}$  counts. Glucuronides were present in sizeable amounts, ranging from 20% in the "blue" fish at 216 hours to 57% in the "green" fish at 240 hours. Sulfates were also prominent, ranging from 21% in the "green" fish at 240 hours to 40% in the "green" fish at 216 hours. Hydroxy-TCC showed more variability, ranging from 9% in the "green" fish at 216 hours to 50% in the "blue" fish at 216 hours. Intact TCC was low, 0% in 2 samples and 7%, 9%, and 10% in the other 3 samples.

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The summary of the distribution of TCC and its metabolites in the various excretion products is presented in Table 12 and Figure 10.

## DISCUSSION

### BIOCONCENTRATION

Bioconcentration studies of  $^{14}\text{C}$ -TCC by small channel catfish (*Ictalurus punctatus*) confirmed the results of a previous study (Schmidt, et al, 1980). The  $^{14}\text{C}$  activity from exposure to  $^{14}\text{C}$ -TCC concentrated in all tissues, particularly the gall bladder, and was rapidly eliminated from the fish during depuration. An abbreviated bioconcentration study was also performed on large catfish of the size that were later used in metabolism chamber studies. These data were needed to design the metabolism study. The results of this study indicated that uptake, bioconcentrating and depuration in large catfish were all very comparable with the values for small catfish as shown in Table 2.

Bioconcentration curves for individual fish tissues sometimes showed BCF values higher than equilibrium early in uptake just before equilibrium was achieved. These elevated BCF peaks were quickly followed by lower values which remain relatively constant resulting in equilibrium. The phenomenon was most readily observed in muscle and remains curves in Figures 1 and 2; the data for liver, viscera and gall bladder were too scattered to detect the initial BCF peak. The elevated BCF peak values followed by lower equilibrium values may result from an enzyme induction mechanism triggered by TCC uptake in catfish. Xenobiotics that enter the body can be inducers of the enzymes that metabolize them, as Hodgson and Guthrie (1980) point out. Perhaps the early elevated BCF values resulted from initial low amounts of certain enzymes essential for TCC metabolism. When enough time had elapsed the TCC taken in induced greater production of the TCC metabolism enzymes.

The  $^{14}\text{C}$  bioconcentration data suggest a metabolic route. The TCC enters the fish via the gills, is transported to various tissues by the blood and is metabolized by the liver. The metabolites are transported by the blood to the gall bladder for storage and eventual excretion with bile into the anal water and to the kidney for excretion in the urine. A small amount of the metabolites are removed from the blood as it passes through the gills and these metabolites are excreted into the gill water.

Work done with rhesus monkeys (Hiles, et al, 1978) showed that in infant monkeys highest levels of accumulation of TCC were in liver, gall bladder and in the intestinal tract. In adult monkeys Hiles reports a similar distribution pattern. The 12 hour steady state plasma level in monkeys was very close to the 9-12 hour time to reach equilibrium in catfish tissues.

A comparison of whole fish Bioconcentration Factors at several exposure concentrations and in large and small catfish showed no significant differences.

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<u>Catfish Size</u>	<u>TCC Exposure Concentration (ug/l)</u>	<u>Whole Fish BCF (Plateau Method)</u>
Large	35.4	422 (56) <sup>1</sup>
Large	35.4	401 (23)
Large	14.8	698 (414)
Small	23	214 (43)
Small	14.8	751 (236)
Small	0.33	245 <sup>2</sup>

<sup>1</sup> Standard deviation<sup>2</sup> Schmidt, et al (1980)EXCRETION

Because TCC is quite lipophilic, the BCF values for muscle might have been predicted to be higher than they actually were, with a longer time to reach equilibrium. Since gall bladder had the highest BCF, however, metabolism into polar conjugates and their rapid excretion in bile and urine seems likely. The BCF curves for muscle, liver, and remains (Figures 1, 3 and 5) show most clearly rapid excretion with a biphasic regression during depuration. The gall bladder and viscera curves (Figures 2 and 4) suggest biphasic excretion but show it less clearly. Hiles and Birch (1978a) showed that the removal of total <sup>14</sup>C-TCC from the plasma of humans and its excretion in urine occurred in a biphasic manner. The removal of <sup>14</sup>C-TCC from rhesus monkey plasma (Hiles, et al 1978c) and from rat plasma (Hiles and Birch 1978b) was also biphasic.

The excretion data in Table 4 and Figures 7 and 8 indicate that there are two major excretion routes, the urine and the bile. Table 4 shows that these routes were almost equally effective with about 40% of the total body burden excreted in bile (anal excretion is probably due to biliary excretion) and 32% of the total body burden excreted in urine. Excretion in urine was very rapid initially as shown in Tables 5 and 6 and Figure 7, with 88% of the urine-excreted TCC lost in the first 47 hours of depuration and with only small amounts in the urine thereafter. TCC excretion into anal water was the opposite of urinary excretion. Almost 60% of anal excretion occurred in the last 5 days of depuration, with TCC excretion closely correlated with the appearance of yellow, stringy anal excreta in the anal chambers. Excretion into the gill chambers was relatively constant and always low, accounting for only 7% of the total body burden. Anal excretion must be interpreted cautiously, however, since the large catfish used in the study would not eat for the three weeks they were held in the laboratory prior to the start of the study and they were not fed during the 11 days of the study. If the fish had eaten, biliary excretion might have been greater and more uniform.

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Biliary excretion was the major route of excretion in the adult and infant rhesus monkey, accounting for more than 80% of the  $^{14}\text{C}$  from IV dosed TCC eliminated (Hiles, et. al. 1978c). Fecal elimination accounted for 70% of the administered dose in humans and was complete 120 hours after dosing (Hiles and Birch 1978a). In rats, one study showed 86-89% of the radioactivity was contained in bile; another study found that 77-82% of the absorbed radioactivity was in bile 72 hours after exposure (Hiles 1977). All of these studies showed urine to be of lesser importance, accounting for less than 20% of the excretion in monkeys, 27% of the dose in humans, and 4-6% and 13-16% of the excretion in rats.

Figure 6 summarizes clearly the distribution of  $^{14}\text{C}$ -TCC in fish tissue. At equilibrium most (74%) of the  $^{14}\text{C}$  was held in the muscle tissue and remains which served as a vast reservoir. Tissues associated with metabolism like liver and viscera accounted for 14% and 8% respectively of the  $^{14}\text{C}$  materials. Gall bladder, the storage site for metabolites, was low in  $^{14}\text{C}$ , holding only 4%. After 10 days of depuration, only 20% of the original  $^{14}\text{C}$  labeled compounds were still in the tissues. Of this 20%, 11% of the  $^{14}\text{C}$  body burden was in muscle and remains, liver and viscera held 5% and 15% respectively, and gall bladder contained the overwhelming majority, 69%, of the  $^{14}\text{C}$ . The data support the idea of a depletion of the muscle and remains reservoir of intact TCC taken in by the fish during uptake and equilibrium. During depuration most of the material is metabolized by liver and stored in the gall bladder. Excretion accounted for 80% of the  $^{14}\text{C}$ . The majority of the excretion products are eliminated almost equally by urine and anal excretion, with only a small fraction excreted by gills. The following material balance based on Table 3 supports the validity of the data. If the measured mean total  $^{14}\text{C}$ -TCC in the tissues at equilibrium is 4.56 mg and the measured mean amount remaining after depuration is 0.94 mg, the predicted excretion would be 3.62 mg. The measured excretion, 3.71 mg, was almost identical to the predicted value.

#### METABOLISM

HPLC analysis of excretion products and of fish tissues supplied data on the fate of the  $^{14}\text{C}$ -TCC to which the large catfish were exposed in Study #3. Table 7 shows that extraction efficiency was high in urine, gill water, and anal water (mean values 82%, 86% and 84% respectively) and would probably be raised in anal excretia (mean value 71%) if more than one extraction were performed on each sample. Tissue extraction was slightly lower in fish tissues (mean value of 77% from the fish sacrificed at equilibrium, mean value of 71% from the fish that underwent depuration), and more extractions are recommended for all tissues to raise the percent extraction. One gram of tissue seemed to be an optimum amount for extraction, larger amounts proved to be difficult to work with due to the oils in the tissue. The high extractions suggest that the HPLC analysis is representative of the selected tissues and excretion products. HPLC analysis of tissues from fish at equilibrium, given in Table 8 and Figure 9, showed that muscle tissue and remains had essentially intact TCC, serving as a extensive storage reservoir. The lipophilic nature of TCC probably accounts for its entry into these tissues where it binds to the membranes. Very few metabolites were present in muscle and remains, suggesting that few enzymes are present for metabolism. Liver and viscera have mostly intact TCC (48% and 66% respectively) but the increased metabolic activity of these tissues is reflected in the presence of metabolites, mostly TCC

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conjugated to glucuronides and TCC conjugated to sulfates with very little hydroxylated TCC. This distribution suggests that the first step in TCC metabolism is the hydroxylation of the molecule followed by glucuronide and sulfate conjugation via the hydroxide bridge. The gall bladder has very little intact TCC or hydroxylated TCC but has 49% glucuronides and 32% sulfates, which would be expected if it is a storage site for metabolites. A sample of extract from the gall bladder of Fish 1 was analyzed twice by HPLC; the results of the two analyses varied by only 1-2%, indicating excellent reproducibility.

The HPLC analyses of tissues from fish after 240 hours of depuration, shown in Table 9 and Figure 9 gives a strikingly different distribution of TCC and metabolites. Muscle and remains, initially the vast reservoir of intact TCC, now contain very little, supporting the idea of rapid metabolism. If depuration continued even longer than 10 days, this reservoir would probably disappear completely. The overwhelmingly large percent is now TCC conjugated to sulfates with almost no glucuronides. This distribution supports the biphasic pattern of depuration by suggesting that glucuronides, found in the earliest rapid phase of depuration, have now been removed, with only the sulfates produced in the later slow phase of depuration remaining. The small mg amounts of sulfates remaining, however, indicate that these metabolites have been mostly removed from these storage reservoirs. Liver and viscera, high metabolic sites, also contain an overwhelmingly large percent of sulfates, but now small but significant amounts of glucuronides and hydroxyl TCC. This distribution also supports the idea that sulfates are the primary metabolite in the later slow phase of biphasic depuration although the small milligram amounts indicate they are being removed. Other metabolites are still present in these two sites, due to their earlier formation and removal. The absence of intact TCC is additional evidence of rapid metabolism. Gall bladder, a final storage reservoir, still contains some glucuronides, slightly more sulfates, a small amount of hydroxylated TCC, and no intact TCC, further indications of rapid TCC metabolism. The higher milligram amounts of the three metabolites suggest that their excretion is still occurring.

Table 10 summarizes HPLC analysis of the excretion products urine, anal water, and gill water, providing additional support to the suggested pattern of catfish metabolism. Urine excretion was high in  $^{14}\text{C}$ -TCC only in the first 2½ days of depuration as shown in Figure 7. During this interval HPLC analyses showed that intact TCC was very low, indicating again its rapid metabolism. Glucuronide conjugates were overwhelmingly the main metabolite, both in percent and in milligram amounts, suggesting again that it was the product of the initial rapid phase of biphasic depuration. The downward trend in glucuronide percent and milligrams would be expected as it is being excreted over a 57 hour interval. The analysis of urine samples from three fish taken in the same time interval reveals a fairly uniform pattern of percent distribution of urine metabolites with slightly more variation in mg amounts of the metabolites.

HPLC analysis of anal excretia, shown in Table 11, was done to compare their distribution of intact TCC and metabolites with the distribution in anal water shown in Table 10. The two sets of data vary considerably and suggest that excretion into anal water results from more than the solid excretia found in the chambers at times of high  $^{14}\text{C}$ -TCC activity.

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Table 12 and Figure 10 summarize the distribution of TCC and metabolites in the various excretion compartments. A few conclusions are obvious; (1) very little intact TCC is excreted in any compartment, (2) anal water and urine account for most  $^{14}\text{C}$  excretion, (3) glucuronides are the major metabolite and (4) excretion across the gills is a minor route of excretion.

#### MAMMALIAN METABOLISM OF TCC

Metabolite distribution during depuration has been studied in several mammals. In human plasma (Hiles and Birch 1978a) and in adult and infant rhesus monkey plasma (Hiles et al, 1978c) glucuronides were the principal metabolites in the early rapid phase of biphasic depuration and sulfates in the later slow phase. Analysis of rat plasma (Hiles and Birch 1978b) showed sulfates as the principal metabolite of the rapid phase and metabolites strongly bound to proteins in the slow phase. Studies of human, rat, and rabbit plasma by Tauli (1976) identified a sulfate conjugate as the principal metabolite.

Bile was the major route of excretion in monkeys and rats. An analysis of monkey bile (Hiles et al, 1978c) and two studies of rat bile (Hiles and Birch 1978b) (Jeffcoat et al 1977) identified the metabolites as mostly glucuronides with small amounts of sulfates and little hydroxyls. The distribution of metabolites in catfish anal water is somewhat similar. Anal excretion is assumed to be the result of biliary action.

Human urine (Hiles and Birch 1978a) contained glucuronides as the main metabolite in the rapid phase of biphasic depuration and 7 different compounds in the slow phase. Glucuronides were identified as the main metabolite in another study of human urine and in the analysis of monkey urine, with a complex metabolic mixture in rat urine (Birch and Hiles 1978). The data in these studies agrees with the predominance of glucuronides in catfish urine.

#### DATE RELIABILITY

For the two small catfish bioconcentration studies the protocol recommended by the American Society for Testing and Materials (ASTM 1978) was followed when data were collected and when data were analyzed by the Plateau Method and by BIOFAC. Replicate samples of 3 fish were taken at each time during uptake, equilibrium, and depuration and calculated means were used in analysis. For the bioconcentration study with large catfish, only one fish was sampled at each time due to the difficulty of maintaining a sizeable population of large fish in the laboratory. Thus the degree of confidence in the data in this large catfish study would be less than for the small catfish study.

For the large catfish metabolism study replicate samples were used. Two fish were sacrificed at equilibrium and the tissues from both fish were analyzed for  $^{14}\text{C}$  content and by HPLC.  $^{14}\text{C}$  analysis was done using duplicates of each fish tissue, of urine, anal water and gill water and means were used in data analysis. In a previous preliminary study 50% of the fish were alive and catheterized for a 96 hour experiment (Schmidt et al. 1979). To be reasonably sure of getting replicate samples of

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excretion products from 3 fish, a minimum of 6 fish in metabolism chambers was planned for. Since the work involved in getting samples and cleaning chambers is too extensive for 6 fish set up simultaneously, it was planned to collect data in two data runs. The first time 4 fish in chambers were set up and 3 of the 4 fish survived and gave off excretion products so a second set up was not needed.

Schmidt (1978) reported that in some tests he maintained fish in an apparently healthy physiological condition for 240 hours. The fish in this metabolism study were in an apparently stable physiological state based on several observations. They excreted almost uniform amounts of urine during all 240 hours of depuration. Their skin coloring remained normal, they developed no serious external sores, there were no signs of external parasites, they appeared to be disease free before the study began, and the rate of operculum opening and closing stayed constant. After an initial adjustment period of about 12 hours, there was no hyperactivity of the fish in the chambers when people passed by and when chambers were drained to collect water samples and to clean the chambers.

Table 5 gives data from 3 fish, with mean values and standard deviation values for the mg of  $^{14}\text{C}$ -TCC in the excretion products at 14 time intervals. Standard deviations are high for the anal water samples. However, the amount of TCC in the water sample is very closely correlated with the presence or absence of anal excreta in the chamber. Collecting data on more than 3 fish would lead to even wider variations in the data, so 3 fish seem to be a desirable number of replicates. Gill water contained so little  $^{14}\text{C}$ -TCC in the samples that there was considerable variation in these low values, resulting in standard deviations equal to or greater than the mean values. However, these samples are not biologically significant for this catfish study due to their low content; gill water is really unnecessary in this study to understand TCC excretion in catfish. Standard deviations were low for the 14 urine samples with coefficients of variance (standard deviation expressed as a percent of the mean) ranging from 9% to 61% for the first 5 samples when most of the TCC was excreted. Therefore these urine samples are the most pertinent data on excretion products, suggesting that replicate samples on 3 fish are adequate.

Extraction from excretion products was repeated a second time on several samples with the results shown here, indicating excellent reproducibility of results.

<u>Fish</u>	<u>Excretion Product</u>	<u>Depuration Time (Hours)</u>	<u>First Extraction</u>	<u>Second Extraction</u>
Yellow	Gill Water	120	78%	81%
Yellow	Gill Water	72	93%	85%
Blue	Anal Water	216	91%	86%

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HPLC results were replicated in several ways. Tissue analysis was done on samples from 2 fish and the percents of metabolites were reported as the means shown in Tables 8 and 9. Samples of urine from 3 fish at time interval 9-24 hours were analyzed and the results in Table 10 are quite similar. When a gall bladder sample from Fish 1 was analyzed twice separately, the results were almost identical.

A number of weaknesses can be found in this study. The fish that were held in the metabolism chamber were unfed whereas those in the uptake/deuration study were fed. Fish that are actively ingesting and defecating probably would metabolize and eliminate a chemical faster than unfed fish. This would especially be true when biliary excretion was known to be an important route of elimination. Perhaps the delayed excretion in anal water (Figure 7) observed in the unfed fish in the metabolism chamber would not have been so late (hours 100-150 of deuration) had the fish been fed.

A need also exist to better understand the cause of the obvious biphasic deuration pattern observed in the uptake/deuration study. Samples of fish tissues and excretion products need to be taken earlier during deuration.

#### ENVIRONMENTAL SIGNIFICANCE OF METABOLISM

TCC has a number of properties that would suggest it could be a problem chemical in the environment as a result of bioconcentration and food chain biomagnification. However, analysis by HPLC of  $^{14}\text{C}$  residues in organs and tissues indicates the actual content of TCC to be lower than  $^{14}\text{C}$  analyses would indicate (Table 13). Table 14 and Figure 11 present a comparison of dry and wet weight  $^{14}\text{C}$  BCFs, with intact TCC dry and wet weight BCFs. A whole fish BCF of 137 is not a concern compared to the 1000 to 5000 which is usually the magnitude for raising a concern. These low bioconcentration numbers, which result from metabolism and rapid elimination, reduce the probability of TCC causing any food chain biomagnification problems. However, it should always be remembered that residues in aquatic organisms will be influenced by the exposure concentration. TCC's anticipated low exposure, coupled with the laboratory water to fish BCF data and the ability of fish to metabolize TCC, suggest TCC would not be a problem. Actual field data would be needed to confirm this position.

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