TETRABROMOBISPHENOL A:
A 96-HOUR FLOW-THROUGH ACUTE TOXICITY TEST WITH THE RAINBOW TROUT (*Oncorhynchus mykiss*)

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER : 439A-123

OECD GUIDELINE 203

and

U.S. EPA OPPTS 850.1075

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STUDY INITIATION DATE: February 28, 2003

STUDY COMPLETION DATE: July 8, 2003

SUBMITTED TO :

American Chemistry Council's
Brominated Flame Retardant Industry Panel
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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel

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This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency (40 CFR Parts 160 and/or 792, 17 August 1989); OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98)17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999).

STUDY DIRECTOR :

Amy S. Blankinship, M.S.
Aquatic Biologist

7-8-03
Date

QUALITY ASSURANCE STATEMENT

This study was examined for compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency (40 CFR Parts 160 and/or 792, 17 August 1989); OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98)17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999). The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Laboratory Management were as follows:

		DATE REPORTED TO:	
ACTIVITY:	DATE CONDUCTED:	STUDY DIRECTOR:	MANAGEMENT:
Test Substance Preparation	February 28, 2003	March 3, 2003	March 5, 2003
Matrix Fortification	March 3, 2003	March 3, 2003	March 5, 2003
Matrix Fortification	March 5, 2003	March 5, 2003	March 7, 2003
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Marshall T. Hynson
Quality Assurance Program Supervisor

7/8/2003
Date

REPORT APPROVAL

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SUMMARY

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TITLE : Tetrabromobisphenol A: A 96-Hour Flow-Through Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*)

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TEST DATES :	Study Initiation :	February 28, 2003
	Experimental Start (OECD) :	February 28, 2003
	Experimental Start (EPA) :	March 3, 2003
	Biological Termination :	March 7, 2003
	Experimental Termination :	March 7, 2003
LENGTH OF EXPOSURE :	96 Hours	
TEST ORGANISMS :	Rainbow Trout (*Oncorhynchus mykiss*)	
SOURCE OF TEST ORGANISMS :	Thomas Fish Company Anderson, California 96007	
AGE OF TEST ORGANISMS :	Juveniles	
MEASUREMENTS OF 10 NEGATIVE CONTROL FISH :	Mean Wet Weight : 0.54 g	Range : 0.31 – 0.70 g
	Mean Total Length : 4.1 cm	Range : 3.5 – 4.7 cm

TEST CONCENTRATIONS :

Nominal	Mean Measured
Negative Control	< LOQ
Solvent Control	< LOQ
1.2 mg a.i./L	1.1 mg a.i./L
1.8 mg a.i./L	1.7 mg a.i./L

RESULTS : (Based on mean measured concentrations)

96-Hour LC50 :	1.1 mg a.i./L
95% Confidence Interval :	Not Applicable
No Mortality Concentration :	< 1.1 mg a.i./L
NOEC :	< 1.1 mg a.i./L

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for the American Chemistry Council's Brominated Flame Retardant Industry Panel at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the definitive toxicity test was conducted from March 3, to 7, 2003. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 439A-123 in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to determine if the acute toxicity of tetrabromobisphenol A (TBBPA) to the rainbow trout, *Oncorhynchus mykiss*, during a 96-hour exposure period under flow-through test conditions would be >1.0 mg active ingredient (a.i.)/L.

EXPERIMENTAL DESIGN

Rainbow Trout were exposed to two test concentrations, a negative (dilution water) control, and a solvent (0.1 mL dimethyl formamide/L) control for 96 hours under flow-through conditions. Two replicate test chambers were maintained in each treatment and control group, with 10 fish in each test chamber for a total of 20 fish per concentration. Two nominal test concentrations were selected by the Sponsor to be assured that at least one mean measured concentration would be > 1.0 mg a.i./L. Nominal test concentrations were 1.2 and 1.8 mg (a.i.)/L. Mean measured test concentrations were determined from samples of test water collected from each treatment and control group at the beginning of the test, at approximately 48 hours, and at test termination.

Delivery of the test substance was initiated approximately 66 hours prior to test initiation in order to achieve equilibrium of the test substance in the test chambers. The test organisms were impartially assigned to test chambers at test initiation. Observations of mortality and other signs of toxicity were made approximately 5.5, 24, 48, 72 and 96 hours after test initiation. The cumulative percent mortality observed in the treatment groups was used to estimate LC50 values at 24, 48, 72 and 96 hours. The no mortality concentration and the no-observed-effect-concentration (NOEC) were determined by visual interpretation of the mortality and observation data.

MATERIALS AND METHODS

The study was conducted based on the procedures outlined in the protocol, "Tetrabromobisphenol A: A 96-Hour Flow-Through Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*)" (Appendix 4). The protocol was based on procedures outlined in the OECD Guidelines for Testing of Chemicals, 203: *Fish, Acute Toxicity Test* (1); U.S. Environmental Protection Agency Series 850 – Ecological Effects Test Guidelines, OPPTS Number 850.1075, *Fish Acute Toxicity Test, Freshwater and Marine* (2); and ASTM Standard E729-88a: *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (3).

Test Substance

The test substance used in the study consisted of a composite of TBBPA samples received from three manufacturers (Great Lakes Chemical Corporation (lot# 1008JE04B), Albermarle Corporation (lot# 25115T-1), and Bromine Compounds, Ltd. (lot# 010040)) between August 16 – 31, 2001. The composite sample was prepared by Wildlife International, Ltd. and assigned Wildlife International identification number 5754. Subsamples of the composite were shipped to Albermarle Corporation for characterization and purity analysis. The test substance, a white powder, was identified as: TBBPA composite; CAS Number 79-94-7. The reported purity was 99.17%, but the sponsor did not indicate an expiration date (Appendix 5). The test substance was stored under ambient conditions.

Test Organism

The rainbow trout, *Oncorhynchus mykiss*, was selected as the test species for this study. Rainbow trout are representative of an important group of aquatic vertebrates, and were selected for use in the test based upon past history of use in the laboratory. All fish used in the test were from the same source and year class. The fish were hatched on January 12, 2003 and were obtained in the swim-up stage from Thomas Fish Company, Anderson, California. Identification of the species was verified by the supplier.

The fish were held for at least 14 days prior to the test in water from the same source and at approximately the same temperature as used during the test. During the 14-day period preceding the test, water temperatures ranged from 10.5 to 12.0°C, measured with a hand-held liquid-in-glass thermometer. The pH of the water ranged from 8.2 to 8.5, measured with a Fisher Scientific Accumet

Model 915 pH meter. Dissolved oxygen ranged from 9.4 to 10.3 mg/L (≥87% of saturation), measured with a Yellow Springs Instruments Model 51B dissolved oxygen meter. The fish were acclimated to test conditions for approximately 50 hours prior to test initiation. During the acclimation period, no mortalities occurred, and the fish showed no signs of disease or stress. At test initiation, the fish were collected from the acclimation tank and impartially distributed two at a time to the test chambers until each contained 10 fish.

During the holding period, the rainbow trout were fed daily a commercially-prepared diet supplied by Zeigler Brothers, Inc., Gardners, Pennsylvania. The fish were not fed for at least two days prior to the test or during the test.

The length of the longest fish measured at the end of the test was no more than twice the length of the shortest. The average total length of 10 negative control fish measured at the end of the test was 4.1 cm, with a range of 3.5 to 4.7 cm. The average wet weight (blotted dry) of 10 negative control fish measured at the end of the test was 0.54 grams, with a range of 0.31 to 0.70 grams. Loading was defined as the total wet weight of fish per liter of test water that passed through the test chamber in 24 hours, and was 0.060 g fish/L/day. Instantaneous loading was 0.36 g fish/L of test water present in the test chambers at any given time.

Dilution Water

The water used for culturing and testing was freshwater obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The well water is characterized as moderately-hard water. The specific conductance, hardness, alkalinity and pH of the well water during the four-week period immediately preceding the test are presented in Appendix 1.

The well water was passed through a sand filter to remove particles greater than approximately 25 µm, and pumped into a 37,800-L storage tank where the water was aerated with spray nozzles. Prior to use, the water was filtered to 0.45 µm to remove microorganisms and particles. The results of periodic analyses performed to measure the concentrations of selected organic and inorganic constituents in the well water used by Wildlife International, Ltd. are presented in Appendix 2.

Test Apparatus

A continuous-flow diluter was used to deliver each concentration of the test substance, a solvent control and a negative control. A syringe pump (Harvard Apparatus, South Natick, Massachusetts) was used to deliver the two test substance stock solutions and dimethyl formamide (DMF) for the solvent control into mixing chambers assigned to each treatment and the solvent control. The syringe pump was calibrated prior to the test. The stock solutions were mixed with dilution water (Wildlife International, Ltd. well water) in the mixing chambers in order to obtain the desired test concentrations. The flow of dilution water to the mixing chambers was controlled by rotameters which were calibrated before the test. The flow of test water from each mixing chamber was split and allowed to flow into replicate test chambers. The proportion of test water that was split into each replicate was checked prior to the test to ensure that flow rates varied by no more than ±10% of the mean of the two replicates.

The diluter was adjusted so that each test chamber received approximately six volume additions of test water every 24 hours. The general operation of the diluter was checked visually at least two times per day during the test and at least once at the beginning and end of the test.

Test chambers were 25-L stainless steel aquaria filled with approximately 15 L of test water. The depth of the test water in a representative chamber was approximately 17.6 cm. The test chambers were covered with nylon mesh to prevent the rainbow trout from escaping. Test chambers were indiscriminately positioned in a temperature-controlled water bath set to maintain the desired test temperature. The water bath was enclosed in a plexiglass ventilation hood in order to minimize any potential for cross-contamination. The test chambers were labeled with the project number, test concentration and replicate.

Preparation of Test Concentrations

One stock solution was prepared for each of the two concentrations tested. A primary stock solution was prepared in DMF at a nominal concentration of 18 mg a.i./mL. An aliquot of the primary stock solution was proportionally diluted with DMF to prepare an additional stock solution at a concentration of 12 mg a.i./mL. The primary stock solution was mixed by inversion and sonication, and was clear and colorless in appearance. The additional stock was mixed by inversion and was clear and colorless in appearance. The two stock solutions were injected into the diluter mixing

chambers (at a rate of 12.5 µL/minute) where they were mixed with well water (at a rate of 125 mL/minute) to achieve the desired test concentrations. The solvent control was prepared by injecting DMF into the mixing chamber for the solvent control. The concentration of DMF in the solvent control and all TBBPA treatment groups was 0.1 mL/L. The test solutions appeared clear and colorless in the test chambers at test initiation and termination. However, a slight white precipitate on the mixing chambers was observed for the test solutions in the diluter mixing chambers at test initiation and also a surface slick in addition to the precipitation was observed at test termination. The test solutions were adjusted for active ingredient (99.17%).

Analytical Sampling

Samples were collected from both replicate test chambers in each treatment and control group at test initiation, at the approximate mid-point of the test, and at test termination to measure concentrations of the test substance. All samples were collected at mid-depth, placed in glass vials, and processed immediately for analysis.

Analytical Method

The analytical method used for the analysis of TBBPA in freshwater was developed at Wildlife International, Ltd. (Appendix 3). The analytical method consisted of dilution of the aqueous samples in 50% (v/v) methanol in NANOpure® water solution, and analysis by direct injection high performance liquid chromatography mass spectrometry (HPLC/MS).

Concentrations of TBBPA were determined by HPLC/MS using a Hewlett-Packard Model 1100 High Performance Liquid Chromatograph interfaced with a Perkin-Elmer SCIEX API 100 Mass Spectrometer. Chromatographic separations were achieved with a Keystone Betasil C_{18} column (50 mm × 2 mm, 3-µm particle size) fitted with a Keystone Javelin C_{18} guard column (20 mm x 2 mm). A flow chart for the analysis of TBBPA is provided in Appendix 3.1, and typical instrumental parameters are summarized in Appendix 3.2.

Calibration standards of TBBPA, ranging in concentration from 0.0100 to 0.100 mg a.i./L, were prepared in 50% (v/v) methanol in NANOpure® water solution using a stock solution of TBBPA in methanol (Appendix 3.3). Linear regression equations were generated using the peak area for each standard versus the respective concentrations of the calibration standards. The concentration of TBBPA

in the samples was determined by substituting the peak area responses into the applicable linear regression equation. An example of the calculations for a representative sample is included in Appendix 3.4.

The limit of quantitation (LOQ) for the freshwater analyses was set at 0.250 mg a.i./L, calculated as the product of the lowest calibration standard (0.0100 mg a.i./L) and the dilution factor of the matrix blank samples (25).

Quality control samples were prepared in freshwater and analyzed concurrently with test samples at each sampling interval to assess the performance of the analytical methodology. Three matrix blank samples were analyzed to determine possible interferences. No interferences were observed at or above the LOQ in the matrix blanks during the sample analyses (Appendix 3.5). Samples of freshwater were fortified at 1.00, 1.50, and 2.00 mg a.i./L using the appropriate fortification stock solution of TBBPA in methanol (Appendix 3.3), and were analyzed concurrently with the samples. The measured concentrations for the matrix fortification samples ranged from 85.1 to 104% of nominal concentrations (Appendix 3.5).

A representative calibration curve for TBBPA is presented in Appendix 3.6. Representative chromatograms of low and high-level calibration standards are presented in Appendices 3.7 and 3.8, respectively. A representative chromatogram of a matrix blank sample is presented in Appendix 3.9, and a representative chromatogram of a matrix fortification sample is presented in Appendix 3.10. A representative chromatogram of a test sample is presented in Appendix 3.11.

Environmental Conditions

Fluorescent light bulbs that emit wavelengths similar to natural sunlight (Colortone® 50) were used for illumination of the culture and test chambers during holding, acclimation and testing. A photoperiod of 16 hours of light and 8 hours of darkness was controlled with an automatic timer. A 30-minute transition period of low light intensity was provided when lights went on and off to avoid sudden changes in lighting. Light intensity at test initiation was 239 lux at the surface of the water of one representative test chamber.

The target test temperature during the study was 12 ± 1°C. Temperature was measured in each test chamber at the beginning and end of the test using a liquid-in-glass thermometer. Temperature also was measured continuously during the test in one negative control test chamber using a Fulscope ER/C Recorder, which was verified prior to test initiation with a liquid-in-glass thermometer. Dissolved oxygen and pH were measured in alternating test chambers at the beginning and end of the test and at approximately 24-hour intervals during the test. When a treatment replicate reached 100% mortality, measurements of DO, pH and temperature were made at that time and then discontinued. Hardness, alkalinity and specific conductance were measured in the dilution water at the beginning of the test.

Light intensity was measured using a SPER Scientific Model 840006C light meter. Dissolved oxygen was measured using a Thermo Orion Model 850Aplus dissolved oxygen meter, and measurements of pH were made using a Thermo Orion Model 525Aplus meter. Specific conductance was measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Hardness and alkalinity measurements were made by titration based on procedures in *Standard Methods for the Examination of Water and Wastewater* (4).

Observations

Observations were made periodically to determine the number of mortalities. The number of individuals exhibiting signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 5.5, 24, 48, 72 and 96 hours after test initiation.

Statistical Analyses

The mortality data at 48, 72 and 96 hours were analyzed using the computer program of C. E. Stephan (5). The program was designed to calculate the LC50 value and the 95% confidence interval by probit analysis, the moving average method, and binomial probability with nonlinear interpolation (6, 7, 8). Due to insufficient morality at 24-hours in this study, a LC50 value at 24-hours could not be calculated. The binomial probability method was used to calculate the 48, 72, and 96-hour LC50 values. Also, due to the mortality in the treatment groups, 95% confidence intervals could not be calculated for the 72 and 96-hour LC50 values. The no mortality concentration and the no-observed-effect-concentration (NOEC) were determined by visual interpretation of the mortality and observation data.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Nominal concentrations selected for use in this study were 1.2 and 1.8 mg a.i./L. Results of analyses to measure concentrations of TBBPA in the samples collected during the test ranged from 75 to 106% of nominal (Table 1). When the measured concentrations of the test samples collected at test initiation, at approximately 48 hours and at test termination were averaged, the mean measured concentrations for the study were 1.1 and 1.7 mg a.i./L, representing 92 and 94% of nominal concentrations, respectively. The results of the study were based on the mean measured test concentrations.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH of the water in each test chamber are presented in Table 2. Water temperatures were within the 12 ± 1°C range established for the test. Dissolved oxygen (DO) concentrations in the treatment groups remained at or above 8.8 mg/L (81% of saturation) throughout the test. Measurements of pH ranged from 8.0 to 8.2. The measurements of hardness, alkalinity and specific conductance in the dilution water at test initiation were typical of Wildlife International, Ltd. well water (Table 3).

Daily observations for mortality and signs of toxicity during the test are presented in Table 4. Rainbow trout in the negative and solvent control groups appeared normal throughout the test. Percent mortality at test termination in the 1.1 and 1.7 mg a.i./L treatment groups was 45 and 100%, respectively. Rainbow trout remaining in the 1.1 mg a.i./L treatment group at test termination were observed to be either lying on the bottom of the test chamber or showing signs of lethargy compared to the controls. Consequently, the no-mortality concentration and the NOEC were < 1.1 mg a.i./L. LC50 values at 24, 48, 72 and 96 hours were calculated from the mortality data and are presented in Table 5.

CONCLUSIONS

Rainbow trout, *Oncorhynchus mykiss*, were exposed to two concentrations of Tetrabromobisphenol A (TBBPA) under flow-through conditions for 96 hours. The 96-hour LC50 value was 1.1 mg a.i./L, but 95% confidence intervals could not be calculated. The 96-hour no-mortality concentration and the NOEC were both < 1.1 mg a.i./L, the lowest test concentration tested.

REFERENCES

1 **Organization for Economic Cooperation and Development**. 1993. *Guideline 203: Fish, Acute Toxicity Test.* OECD Guidelines for Testing of Chemicals. Updated Guideline adopted on 17 July 1992.

2 **U.S. Environmental Protection Agency.** 1996. *Fish Acute Toxicity Test, Freshwater and Marine.* Series 850 – Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1075:

3 **ASTM Standard E729-88a**. 1994. *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians*. American Society for Testing and Materials.

4 **APHA, AWWA, WPCF.** 1998. *Standard Methods for the Examination of Water and Wastewater.* 20th Edition, American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.

5 **Stephan, C.E.** 1978. U.S. EPA, Environmental Research Laboratory, Duluth, Minnesota. Personal communication.

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8 **Finney, D.J.** 1971. *Statistical Methods in Biological Assay.* Second edition. Griffin Press, London.

Table 1

Measured Concentrations of Tetrabromobisphenol A (TBBPA) in Test Samples

Nominal Test Concentration (mg a.i./L)	Sample ID (439A-123-)	Sampling Time (Hours)	Measured Concentration (mg a.i./L)[1,2]	Percent of Nominal[2]	Mean Measured Concentration (mg a.i./L)	Mean Percent of Nominal
Negative Control	1	0	< LOQ	--	--	--
0.0	2	0	< LOQ	--		
	9	48	< LOQ	--		
	10	48	< LOQ	--		
	17	96	< LOQ	--		
	18	96	< LOQ	--		
Solvent Control	3	0	< LOQ	--	--	--
0.0	4	0	< LOQ	--		
	11	48	< LOQ	--		
	12	48	< LOQ	--		
	19	96	< LOQ	--		
	20	96	< LOQ	--		
1.2	5	0	0.978	81.5	1.1	92
	6	0	0.902	75.2		
	13	48	1.15	95.6		
	14	48	1.14	94.8		
	21	96	1.15	95.5		
	22	96	1.13	94.2		
1.8	7	0	1.60	89.1	1.7	94
	8	0	1.35	75.2		
	15	48	1.91	106		
	16	48	1.90	106		
	--	96[3]	--	--		
	--	96[3]	--	--		

[1] The limit of quantitation (LOQ) was 0.250 mg a.i./L calculated as the product of the lowest calibration standard (0.0100 mg a.i./L) and the dilution factor of the matrix blanks (25).
[2] Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly.
[3] Samples not collected due to 100% mortality at 48 hours.

Table 2

Temperature, Dissolved Oxygen and pH of Water in the Test Chambers

Mean Measured Test Concentration (mg a.i./L)	Replicate	0 Hour			24 Hours		48 Hours		72 Hours		96 Hours		
		Temp[1] (°C)	DO[2] (mg/L)	pH	DO (mg/L)	pH	DO (mg/L)	pH	DO (mg/L)	pH	Temp (°C)	DO (mg/L)	pH
Negative Control	A	12.3	9.6	8.2	--	--	9.1	8.0	--	--	11.9	9.2	8.0
	B	12.3	--	--	9.3	8.0	--	--	8.8	8.1	11.8	--	--
Solvent Control	A	12.3	9.4	8.2	--	--	9.2	8.1	--	--	11.9	9.1	8.2
	B	12.3	--	--	9.3	8.1	--	--	8.9	8.2	11.9	--	--
1.1	A	12.0	9.4	8.2	--	--	9.1	8.1	--	--	11.6	9.3	8.2
	B	12.0	--	--	9.2	8.1	--	--	9.0	8.2	11.5	--	--
1.7	A	12.2	9.3	8.2	--	--	9.1[3]	8.1[3]	--	--	--[4]	--[4]	--[4]
	B	12.2	--	--	9.1	8.1	9.1[3]	8.1[3]	--[4]	--[4]	--[4]	--	--

[1] Manual temperature measurements. Temperature measured continuously during the test ranged from approximately 11.5 to 12.5°C, measured to the nearest 0.5°C.

[2] A dissolved oxygen concentration of 8.1 mg/L represents 75% saturation at 12°C in freshwater.

[3] Temperature measured in Replicate A and B was 11.9°C and 11.9°C, respectively. Temperature, DO, and pH measured in each replicate due to 100% mortality.

[4] Measurement discontinued due to 100% mortality.

Table 3

Hardness, Alkalinity and Specific Conductance
Measured in Dilution Water at Test Initiation

Parameter	Day 0
Hardness (mg/L as $CaCO_3$)	128
Alkalinity (mg/L as $CaCO_3$)	181
Specific Conductance (µmhos/cm)	270

Table 4

Cumulative Percent Mortality and Observed Effects

Mean Measured Test Concentration (mg a.i./L)	Rep.	No. Exposed	5.5 Hours		24 Hours		48 Hours		72 Hours		96 Hours		Cumulative Percent Mortality
			No. Dead[1]	Effects[2]	No. Dead	Effects	No. Dead	Effects	No. Dead	Effects	No. Dead	Effects	
Negative Control	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
Solvent Control	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	
1.1	A	10	0	10 AN	0	9 C, 1 R	0	1 AN, 3 C, 3 R, 3 N	3	2 C, 4 R, 1 N	5	2 C, 3 R	45
	B	10	0	10 AN	0	2 AN, 8 C	0	8 C, 1 R, 1 N	3	2 C, 3 R, 2 N	4	3 C, 3 R	
1.7	A	10	0	5 AN, 5 C	1	9 R	10	--	10	--	10	--	100
	B	10	0	3 AN, 7 C	1	9 R	10	--	10	--	10	--	

[1] Cumulative number of dead fish.
[2] Observed Effects: AN = appear normal; C = lethargic; R = lying on bottom of test chamber, N = loss of equilibrium.

Table 5

LC50 Values

Time	LC50 (mg a.i./L)	95% Confidence Interval (mg a.i./L)	Statistical Method
24 Hours	> 1.7	--[1]	NA[2]
48 Hours	1.4	1.1 – 1.7	Binomial Probability
72 Hours	1.2	--[1]	Binomial Probability
96 Hours	1.1	--[1]	Binomial Probability

[1] 95% confidence limits could not be calculated from the data.
[2] NA = not applicable; <50% mortality/immobility precluded statistical calculation of an EC50 value.

Appendix 1

Specific Conductance, Hardness, Alkalinity and pH of Well Water Measured During the 4-Week Period Immediately Preceding the Test

Parameter	Mean	Range
Specific Conductance (μmhos/cm)	310 (N = 4)	310 – 310
Hardness (mg/L as $CaCO_3$)	131 (N = 4)	124 – 140
Alkalinity (mg/L as $CaCO_3$)	180 (N = 4)	170 – 184
pH	8.2 (N = 4)	8.1 – 8.4

Appendix 2

Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Well Water[1]

Pesticides and Organics			
Component	Measured Concentration (ppb or ng/g)	Component	Measured Concentration (ppb or ng/g)
Aldicarb sulfone	< 50	Isofenphos	< 50
Aldicarb sulfoxide	< 50	Leptophos	< 50
Azinphos-ethyl	< 50	Linuron	< 50
Azinphos-methyl	< 50	Methidathion	< 50
Bifenox	< 50	Methiocarb	< 50
Bitertanol	< 50	Methomyl	< 50
Bromacil	< 50	Methoxychlor	< 250
Bromoxynil octanoic acid ester	< 50	Mirex	< 50
Captafol	< 50	Monocrotophos	< 50
Carbaryl	< 50	Myclobutanil	< 50
3-Hydroxy Carbofuran	< 50	Napropamide	< 50
Carbofuran	< 50	Norflurazon	< 50
Carbophenothion	< 50	Oxadiazon	< 50
cis-Chlordane	< 50	Oxamyl	< 50
trans-Chlordane	< 50	Oxyfluorfen	< 50
Chlorfenson	< 50	Paraoxon	< 50
trans-Chlorfenvinphos	< 50	cis-Permethrin	< 50
Chlorobenzilate	< 50	Perthane	< 50
Chloropropylate	< 50	Phosalone	< 50
Chloroxuron	< 50	Phosphamidon	< 50
Coumaphos	< 50	Piperalin	< 50
Crotoxyphos	< 50	Profenfos	< 50
Cyanazine	< 50	Promecarb	< 50
Cyfluthrin I	< 50	Propanil	< 50
Cypermethrin I	< 50	Propargite	< 50
o,p'-DDD	< 50	Propoxur	< 50
p,p'-DDE	< 50	Pyrethrin I	< 50
p,p'-DDD	< 50	Quinalphos	< 50
o,p'-DDT	< 50	Quinomethionate	< 50
p,p'-DDT	< 250	Quizalofop-ethyl	< 50
DEF	< 50	Sulprofos	< 50
Diclofop methyl	< 50	Tetrachlorovinphos	< 50
Dicrotophos	< 50	Tetradifon	< 50
Dieldrin	< 50	Thiobendazole	< 50
Diphenamid	< 50	Tilt I	< 50
Diuron	< 50	Tilt II	< 50
Endosulfan II	< 50	Trimethyl carbamate	< 50
Endrin	< 50		
Endrin ketone	< 50		
EPN	< 50		
Ethion	< 50		
Fenamiphos	< 50		
Fenarimol	< 50		
Fenobucarb	< 50		
Fenpropathrin	< 50		
Fensulfothion	< 50		
Fluzifop-P-butyl	< 50		

[1]Analyses performed by Exygen Research on samples collected on July 31, 2002.

Appendix 2 (Continued)

Analyses of Pesticides, Organics and Metals in Wildlife International, Ltd. Well Water[1]

Metals			
	(ppm or mg/L)		(ppm or mg/L)
Aluminum	< 0.204	Manganese	< 0.0153
Arsenic	< 0.0102	Mercury	< 0.0002
Beryllium	< 0.0051	Molybdenum	< 0.0005
Cadmium	< 0.0051	Nickel	< 5.1
Calcium	28.2	Potassium	5.45
Chromium	< 0.0102	Selenium	0.009
Cobalt	< 5.1	Silver	< 0.0102
Copper	< 0.0255	Sodium	18.6
Iron	< 5.1	Zinc	< 0.0204
Magnesium	11.6		

[1]Analyses performed by Exygen Research on samples collected on July 31, 2002.

Appendix 3

The Analysis of Tetrabromobisphenol A (TBBPA) in Freshwater

Appendix 3.1

Analytical Method Flowchart for the Analysis of TBBPA in Freshwater

METHOD OUTLINE FOR THE ANALYSIS OF TBBPA IN FRESHWATER

Prepare quality control samples in freshwater using gas-tight syringes and volumetric flasks. The matrix blank will be freshwater.

↓

Dilute submitted samples and quality control samples with 50% (v/v) methanol NANOpure® water solution using gas-tight syringes and volumetric flasks.

↓

Transfer samples to autosampler vials and submit samples for HPLC/MS analysis.

Appendix 3.2

Typical HPLC/MS Operational Parameters

INSTRUMENT:	Hewlett-Packard Model 1100 High Performance Liquid Chromatograph with a Perkin-Elmer SCIEX API 100 Mass Spectrometer configured with a Heated Nebulizer ion source. Operated in negative selective ion monitoring mode.
ANALYTICAL COLUMN:	Keystone Betasil C_{18} column (50 mm × 2 mm, 3-µm particle size)
GUARD COLUMN:	Keystone Javelin C_{18} column (20 mm × 2 mm)
OVEN TEMPERATURE:	40°C
STOP TIME:	5.00 minutes
FLOW RATE:	0.250 mL/minute
MOBILE PHASE:	Solvent A: 0.1% formic acid in NANOpure®water (20%) Solvent B: Methanol (80%)
INJECTION VOLUME:	100 µL
TBBPA RETENTION TIME:	Approximately 3.4 minutes
TBBPA MONITORED MASS:	542.7 amu

Appendix 3.3

Analytical Stocks and Standards Preparation

A primary stock solution of TBBPA was prepared by weighing 0.1008 g of the test substance on an analytical balance. The test substance (99.17% purity) was transferred to a 100-mL volumetric flask and brought to volume using methanol. The primary stock solution contained 1.00 mg a.i./mL of TBBPA. Secondary stocks (0.100, 0.0100, and 0.00100 mg a.i./mL TBBPA in methanol) were prepared from the primary stock by serial dilution. The primary and secondary stocks were used to fortify the quality control samples and to prepare calibration standards. Calibration standards for TBBPA were prepared in 50% (v/v) methanol in NANOpure® water solution. The following shows the dilution scheme for the calibration standards.

Stock Concentration (mg a.i./mL)	Aliquot (mL)	Final Volume (L)	Standard Concentration (mg a.i./L)
0.0100	0.100	0.100	0.0100
0.0100	0.250	0.100	0.0250
0.0100	0.500	0.100	0.0500
0.0100	0.750	0.100	0.0750
0.0100	1.00	0.100	0.100

Appendix 3.4

Example Calculations for a Representative Sample

The analytical result and percent recovery for sample number 439A-123-5, nominal concentration of 1.2 mg a.i./L, were calculated using the following equations:

$$\text{TBBPA (mg a.i./L) in sample} = \frac{\text{Peak area - (Y-intercept)}}{\text{Slope}} \times \text{Dilution factor}$$

Peak Area = 589965
Y-intercept = 2567.6514
Slope = 15013852
Initial Volume (V_i): = 0.200 mL
Final Volume (V_f): = 5.00 mL
Dilution Factor (V_f/V_i): = 25

$$\text{TBBPA (mg a.i./L) in sample} = \frac{589965 - (2567.6514)}{15013852} \times 25$$

$$= 0.978 \text{ mg a.i./L}$$

$$\text{Percent of nominal concentration} = \frac{\text{TBBPA in sample (mg a.i./L)}}{\text{TBBPA nominal concentration (mg a.i./L)}} \times 100$$

$$= \frac{0.978}{1.2} \times 100$$

$$= 81.5\%$$

Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly.

Appendix 3.5

Quality Control Samples of TBBPA in Freshwater

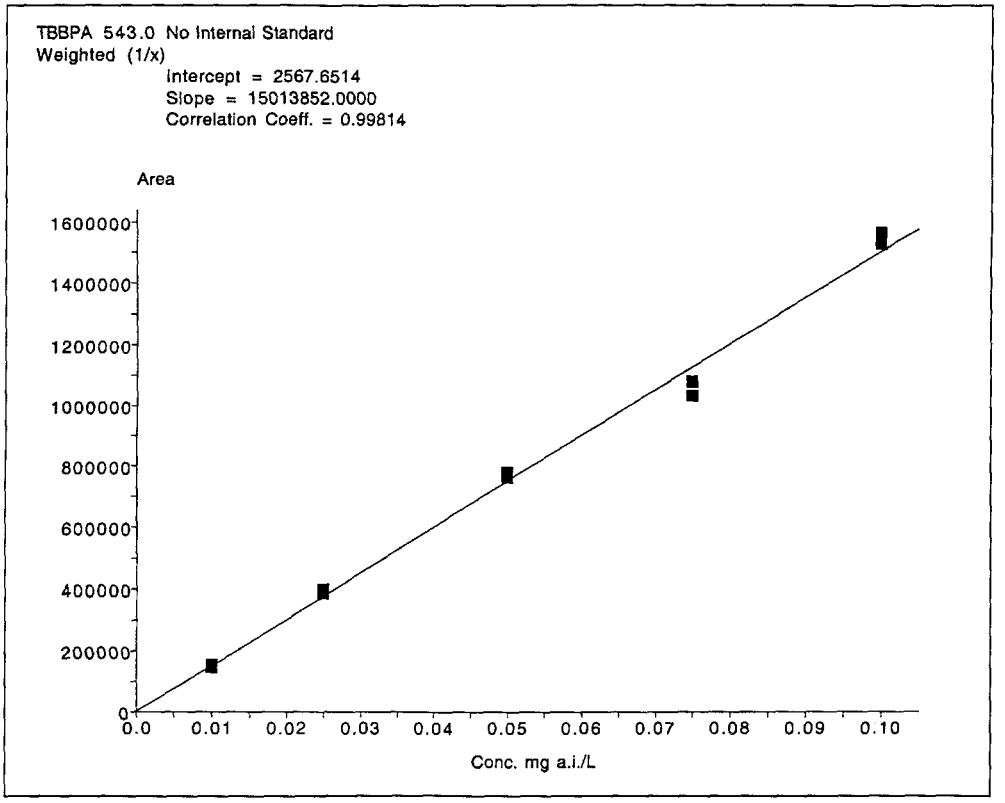
Sample Number	Concentration (mg a.i./L)		Percent Recovery [2]
	Fortified	Measured [1,2]	
439A-123-MAB-1	0.0	< LOQ	--
439A-123,124-MAB-2	0.0	< LOQ	--
439A-123,124-MAB-3	0.0	< LOQ	--
439A-123-MAS-1	1.00	0.884	88.4
439A-123-MAS-2	1.50	1.28	85.1
439A-123-MAS-3	2.00	2.03	101
439A-123,124-MAS-4	1.00	0.994	99.4
439A-123,124-MAS-5	1.50	1.50	100
439A-123,124-MAS-6	2.00	2.08	104
439A-123,124-MAS-7	1.00	1.00	100
439A-123,124-MAS-8	1.50	1.51	100
439A-123,124-MAS-9	2.00	2.02	101

[1] The limit of quantitation (LOQ) was 0.250 mg a.i./L calculated as the product of the lowest calibration standard (0.0100 mg a.i./L) and the dilution factor of the matrix blanks (25).

[2] Results were generated using MacQuan version 1.6 software. Manual calculations may differ slightly.

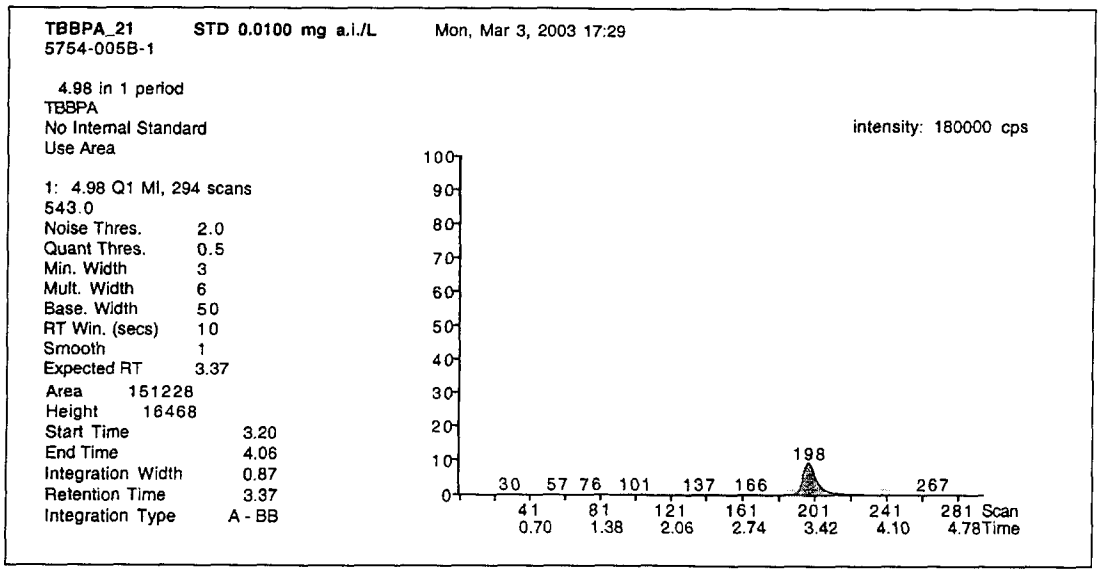
Appendix 3.6

Representative Calibration Curve for TBBPA



Appendix 3.7

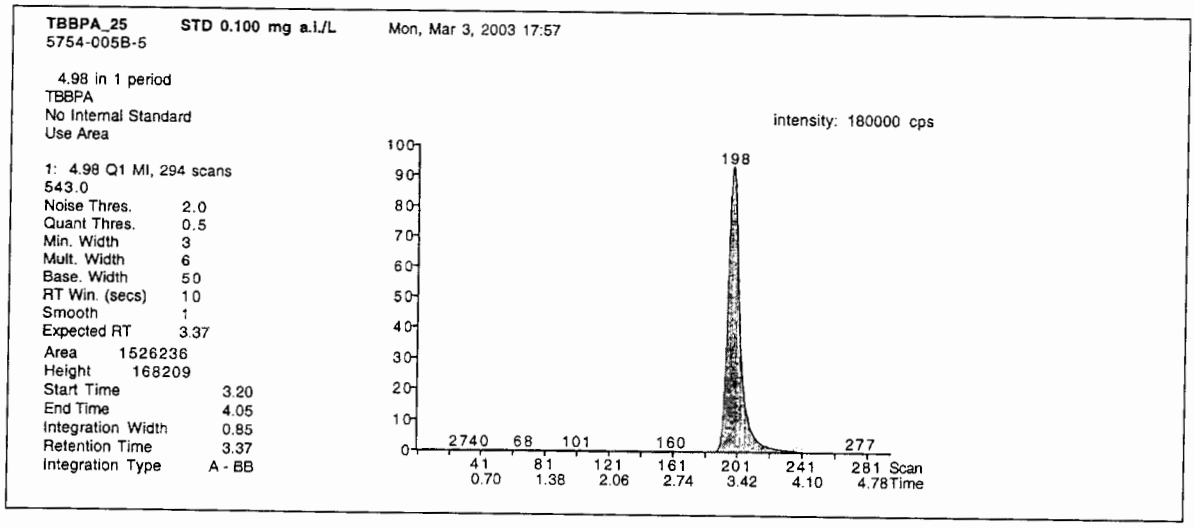
Representative Chromatogram of a Low-level TBBPA Calibration Standard



Nominal concentration: 0.0100 mg a.i./L

Appendix 3.8

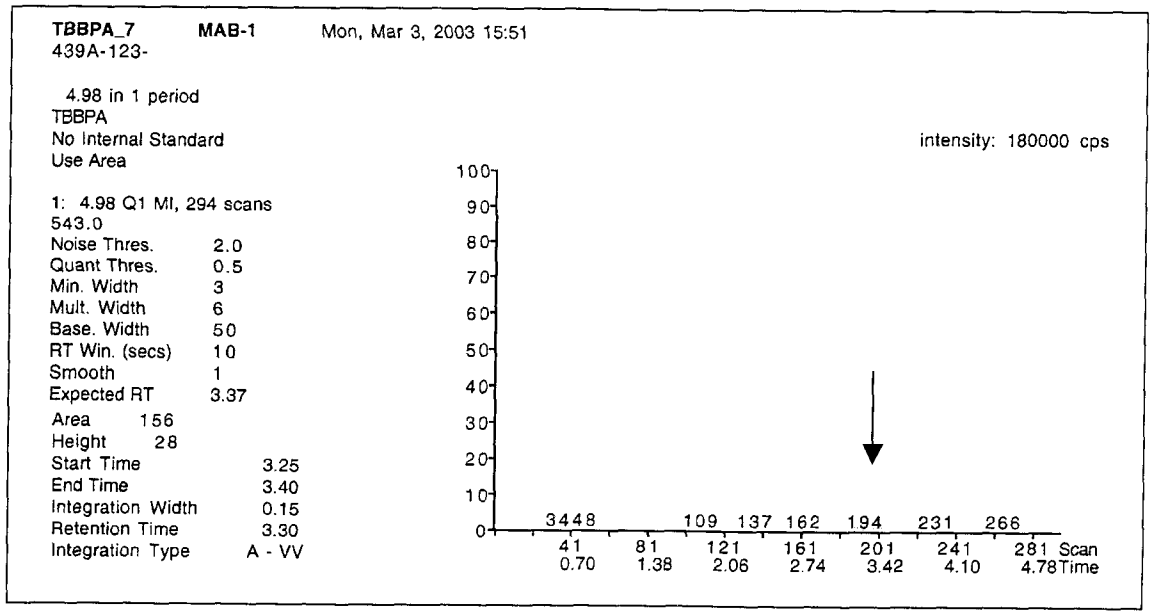
Representative Chromatogram of a High-level TBBPA Calibration Standard



Nominal concentration: 0.100 mg a.i./L

Appendix 3.9

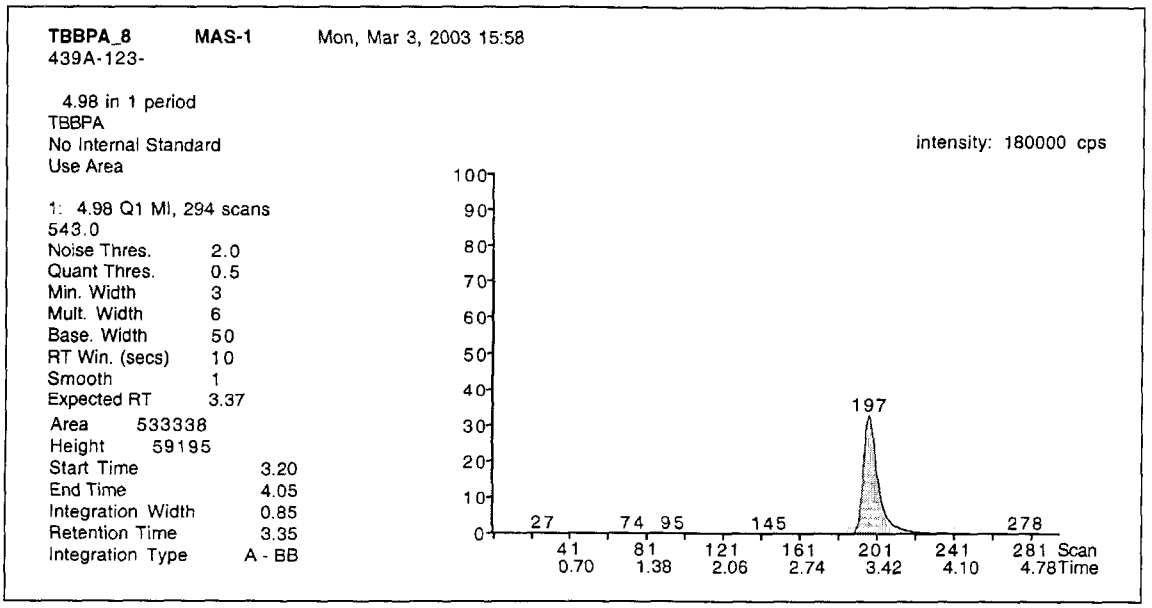
Representative Chromatogram of a Matrix Blank Sample



Sample number 439A-123-MAB-1. Dilution factor = 25X.
The arrow indicates the approximate retention time of TBBPA.

Appendix 3.10

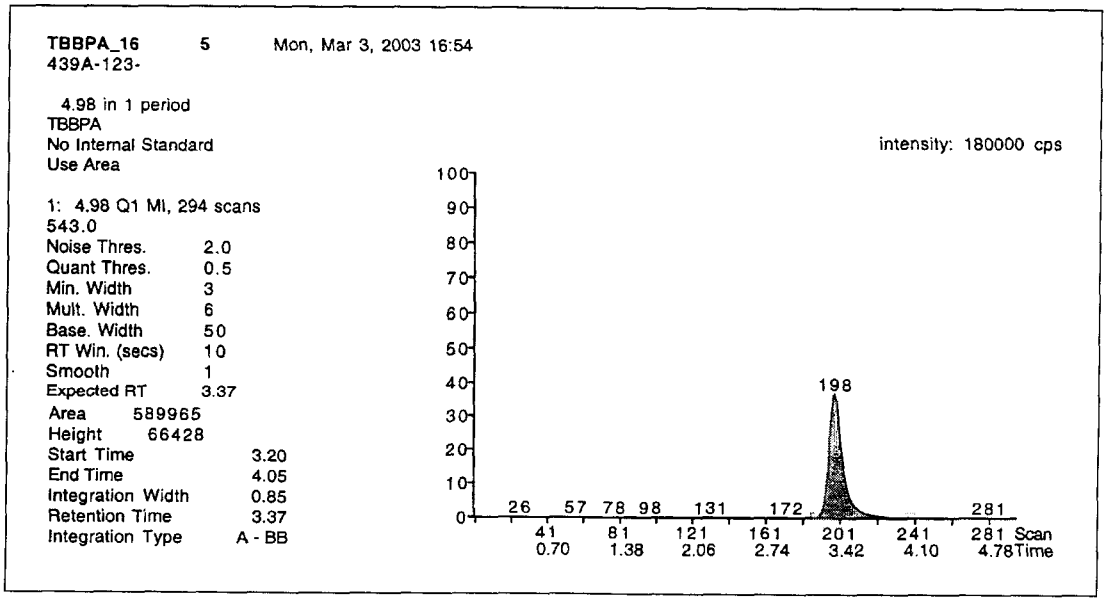
Representative Chromatogram of a Matrix Fortification Sample



Sample number: 439A-123-MAS-1, nominal concentration 1.00 mg a.i./L.
Dilution factor = 25X.

Appendix 3.11

Representative Chromatogram of a Test Sample



Sample number: 439A-123-5, Day 0, nominal concentration 1.2 mg a.i./L.
Dilution factor = 25X.

Appendix 4

Protocol and Amendment

PROTOCOL

TETRABROMOBISPHENOL A: A 96-HOUR FLOW-THROUGH ACUTE TOXICITY TEST WITH THE RAINBOW TROUT (*Oncorhynchus mykiss*)

OECD Guideline 203

U.S. EPA OPPTS Number 850.1075

Submitted to

American Chemistry Council's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601
(410) 822-8600

February 13, 2003

Wildlife International, Ltd.

- 2 -

TETRABROMOBISPHENOL A: A 96-HOUR FLOW-THROUGH ACUTE TOXICITY TEST WITH THE RAINBOW TROUT (*Oncorhynchus mykiss*)

SPONSOR:	American Chemistry Council's Brominated Flame Retardant Industry Panel 1300 Wilson Boulevard Arlington, Virginia 22209
SPONSOR'S REPRESENTATIVE:	Ms. Wendy Sherman
TESTING FACILITY:	Wildlife International, Ltd. 8598 Commerce Drive Easton, Maryland 21601
STUDY DIRECTOR:	Amy Blankinship, Biologist Wildlife International, Ltd.
LABORATORY MANAGEMENT:	Henry O. Krueger, Ph.D. Director of Aquatic Toxicology/Terrestrial Plants & Insects

FOR LABORATORY USE ONLY

Proposed Dates:

Experimental Start Date: 3/3/03 Experimental Termination Date: 3/7/03

Project No.: 439A-123

Test Concentrations: Negative Control, Solvent Control (0.1mL DMF/L), 1.2 mg a.i./L, 1.8 mg a.i./L

Test Substance No.: 5754 Reference Substance No. (if applicable): —

PROTOCOL APPROVAL

Amy Blankinship 2/28/03
STUDY DIRECTOR DATE

H. Krueger 2/28/03
LABORATORY MANAGEMENT DATE

Susan Lewis for Wendy Sherman 2/26/03
SPONSOR'S REPRESENTATIVE DATE

PROTOCOL NO.: 439/021303/RBT-96H2/OECD-OPPTS/SUB439

Wildlife International, Ltd.

INTRODUCTION

Wildlife International, Ltd. will conduct a flow-through acute toxicity test with the rainbow trout (*Oncorhynchus mykiss*) for the Sponsor at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The study will be performed based on procedures in *OECD Guideline for Testing of Chemicals 203: Fish Acute Toxicity Test* (1); U.S. EPA Series 850 - Ecological Effects Test Guidelines OPPTS Number 850.1075: *Fish Acute Toxicity Test, Freshwater and Marine* (2); and ASTM Standard E729-88a: *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (3). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site or at an alternative location to be specified in the final report.

OBJECTIVE

The objective of this study is to determine the acute effects of Tetrabromobisphenol A (TBBPA) on the rainbow trout (*Oncorhynchus mykiss*) during a 96-hour exposure period under flow-through test conditions.

EXPERIMENTAL DESIGN

Rainbow trout will be exposed to two test concentrations, (1.2 and 1.8 ppm), a negative (dilution water) control and a solvent control for 96 hours. Two replicate test chambers will be maintained in each treatment and control group, with 10 rainbow trout in each chamber for a total of 20 rainbow trout per test concentration.

Nominal test concentrations were selected in consultation with the Sponsor based upon information such as known toxicity data, physical/chemical properties of the test substance or other relevant information. Water samples will be collected from appropriate test chambers at specified intervals for analysis of the test substance. Results of analyses will be used to calculate mean measured test concentrations.

To control bias, rainbow trout will be impartially assigned to exposure chambers at test initiation. No other potential sources of bias are expected to affect the results of the study. Observations of mortality and other clinical signs will be made throughout the 96-hour test period. Cumulative percent

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mortality observed in the treatment groups will be used to calculate, when possible, LC50 values at 24, 48, 72 and 96 hour intervals. The no mortality concentration and the no-observed-effect concentration (NOEC) will be determined by visually interpreting the clinical observation data.

MATERIALS AND METHODS

Test Substance

The test substance consisted of a composite of TBBPA samples received from three manufacturers. The material's identity and date received from each of the manufacturers is given below:

Manufacturer	Lot/Batch	Date Received	Wildlife International Ltd. Identification Number
Great Lakes Chemical Corporation	1008JE04B	August 16, 2001	5722
Albemarle Corporation	25115T-1	August 16, 2001	5721
Bromine Compounds, Ltd.	010040	August 31, 2001	5733

The composite test substance was assigned Wildlife International Ltd. identification number 5754 and was stored under ambient conditions. Subsamples of the composite test substance were shipped to Albemarle Corporation for characterization and purity analyses. The results of the analyses indicated the composite test substance was homogeneous and contained TBBPA with a purity of 99.17%. The test substance was stored at room temperature.

The Sponsor is responsible for all information related to the test substance and agrees to accept any unused test substance and/or test substance containers remaining at the end of the study.

Preparation of Test Concentrations

The test substance will be administered to the test organism in water. This route of administration was selected because it represents the most likely route of exposure to aquatic organisms.

The test substance will be mixed directly with dilution water or may be first mixed with a solvent. If a solvent is used, the test substance will be dissolved in the solvent to form a stock solution that will subsequently be added to dilution water. Reverse osmosis water will be the solvent of choice, although dimethyl formamide, triethylene glycol, methanol, ethanol or acetone may be used. If an organic solvent is required, a solvent control will be included in the test in addition to a negative (dilution water) control. The concentration of the organic solvent will not exceed 0.1 mL/L, when possible. The organic solvent

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concentration in the solvent control will be equal to the highest solvent concentration in test chambers containing the test substance.

Test Organism

The rainbow trout (*Oncorhynchus mykiss*) has been selected as the test species for this study. Rainbow trout are representative of an important group of aquatic vertebrates, and have been selected for use in the test based upon past use history in the laboratory. Fish will be from the same source and year class, and the length of the longest fish measured will be no more than twice that of the shortest. Fish will weigh between 0.1 and 3.0 grams (wet weight blotted dry), and the total weight in each test chamber will not exceed 0.5 grams per liter of solution passing through the chamber in 24 hours nor 5 g/L at any time. The recommended total length of fish is 5.0 ± 1.0 cm. Total lengths and wet weights of the individual fish in one negative control replicate will be measured at the end of the test and will be considered representative of the length and weight of all fish used in the study. Fish and/or fish eggs will be obtained from a supplier or hatchery, and the identity of the species will be verified by the supplier, or by Wildlife International, Ltd. personnel using appropriate taxonomic keys, such as Eddy (4).

Rainbow trout will be held for at least 14 days prior to the test in water from the same source and at approximately the same temperature as used during the test. Variations in water temperature will not exceed 3°C in any 72-hour period during holding, and dissolved oxygen must be > 80% of saturation during holding. If mortality of the test fish exceeds 3%, or the fish show signs of disease or stress during a two-day period immediately preceding the test, they will be euthanized or held for an additional 14-day period to ensure that the fish are healthy. At test initiation, the rainbow trout will be collected from holding or acclimation tanks and transferred to the test chambers.

During the holding period, the test fish will be fed at least once daily. The diet will consist of live or frozen brine shrimp nauplii (*Artemia sp.*), frozen brine shrimp, and/or commercial food. Fish will not be fed for at least two days prior to the test or during the test. Specifications for acceptable levels of contaminants in fish diets have not been established. However, there are no known levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the test.

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Dilution Water

Water used for the holding and testing of rainbow trout will be obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The water will be passed through a sand filter and pumped into a 37,800-L storage tank where the water will be aerated with spray nozzles. Prior to use the water will be filtered to 0.45 µm in order to remove fine particles. Water used for holding and testing is characterized as moderately hard. Typical values for hardness, alkalinity, pH and specific conductance are approximately:

Hardness, mg/L as $CaCO_3$	145
Alkalinity, mg/L as $CaCO_3$	190
pH	8.1
Specific Conductance, µmhos/cm	330

Hardness, alkalinity, pH and specific conductance will be measured weekly to monitor the consistency of the well water. Means and ranges of the measured parameters for the four-week period preceding the test will be provided in the final report. Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of the well water and results of the most recent GLP-compliant analyses will be summarized in the final report.

Test Apparatus

A continuous-flow diluter will be used to provide each concentration of the test substance, a negative (dilution water) control, and a solvent control, when necessary. A syringe pump, peristaltic pump, or a similar device will be used to deliver the test substance to mixing chambers where the test substance will be mixed with dilution water. The flow of dilution water into each mixing chamber will be controlled using rotameters. After mixing, test solutions will be split to each replicate chamber. The proportion of water split to each replicate will be checked prior to the test to ensure that these flow rates vary by no more than ±10% of the mean of the two replicates. Test chambers will be 25-L, Teflon®-lined polyethylene or stainless steel chambers filled with approximately 15 L of water. Test chambers will be indiscriminately positioned in a temperature-controlled water bath designed to maintain a temperature of 12 ± 1°C. The water bath will be enclosed in a plexiglass ventilation hood in order to minimize potential cross-contamination between test systems. Test chambers will be labelled with the project number, test concentration and replicate.

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In tests where solvent controls are required, the solvent will be injected into a mixing chamber where it will be diluted to the appropriate concentration with dilution water. The concentration of solvent in the solvent control will be equal to that in the highest treatment level.

The diluter will be adjusted so that each test chamber receives at least 5 volume additions of test solution every 24 hours. Test substance stock delivery pumps and rotameters will be calibrated before each study, and the delivery of test substance to test chambers will begin at least 4 hours prior to the test in order to establish equilibrium concentrations of the test substance. The general operation of the diluter will be checked visually at least two times per day during the test and at least once at the beginning and end of the test.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during holding, acclimation, and testing will be provided by fluorescent tubes that emit wavelengths similar to natural sunlight (e.g., Colortone® 50). A photoperiod of 16 hours of light and 8 hours of dark will be controlled with an automatic timer. A 30-minute transition period of low light intensity will be provided when lights go on and off to avoid sudden changes in light intensity. Light intensity will be measured at test initiation with a SPER Scientific Ltd. light meter or equivalent.

The target test temperature will be 12 ± 1°C. Temperature will be measured in all replicates at the beginning and end of the test using a liquid-in-glass thermometer. Temperature also will be measured with a continuous recorder in one negative control chamber. Recorder measurements will be verified with a liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen will be measured in alternate replicates of each treatment and control group at test initiation and at approximately 24-hour intervals thereafter using a Thermo Orion Model 850Aplus dissolved oxygen meter, or equivalent. In the event that dissolved oxygen levels fall below 75% saturation, dissolved oxygen measurements will be made in every test chamber and appropriate actions will be taken after consultation with the Sponsor. Measurements of pH will be made in alternate replicates of each treatment and control group at test initiation and at approximately 24-hour intervals thereafter using a Thermo Orion Model 720Aplus pH meter, or equivalent. If a treatment replicate reaches 100% mortality, dissolved oxygen, pH, and temperature measurements will be taken in that replicate at that time, and then discontinued.

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Hardness, alkalinity, and specific conductance will be measured in the dilution water at test initiation. Hardness and alkalinity measurements will be made by titration using procedures based on methods in *Standard Methods for the Examination of Water and Wastewater* (5). Specific conductance will be measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter, or equivalent. Additional water quality measurements may be taken as deemed necessary by study personnel. The reason for the additional measurements will be documented in the raw data and summarized in the final report.

Biological Measurements

Observations of mortality and clinical signs of toxicity will be made between 0-24 hours and at 24, 48, 72 and 96 hours ± 1 hour. Lethality is defined as the lack of visible movement (e.g. lack of fin or opercular movement) in the fish after gentle prodding. All clinical observations including abnormal behavior will be noted.

Sampling for Analytical Measurements

Water samples will be collected from each test chambers at the beginning of the test and at 48 and 96 hours (±1 hour) to determine concentrations of the test substance. In the event that 100% mortality occurs in any treatment, then sampling of that treatment will terminate following the next sampling interval. Samples will be collected at mid-depth from each test chamber and analyzed immediately, or placed in an appropriate storage container (e.g., glass or polypropylene bottle) and stored under refrigeration until analyzed. The sample scheme is summarized below:

PROPOSED NUMBERS OF VERIFICATION SAMPLES

Experimental Group	0 Hour	48 Hours	96 Hours
Control	2	2	2
Solvent Control (if needed)	2	2	2
Level 1- 1.2 ppm	2	2	2
Level 2- 1.8 ppm	2	2	2
Totals	8	8	8

Total Number of Verification Samples = 24

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The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study. The actual number of samples collected and/or analyzed will be documented in the raw data. At the discretion of the Study Director, water samples from one or more appropriate test chambers and/or stock solutions will be collected and analyzed if an analytical error in sampling or analysis is suspected, or if a malfunction in the test substance delivery system occurs. The reason for the additional samples will be documented in the raw data and summarized in the final report.

Analytical Chemistry

Chemical analysis of the samples will be performed by Wildlife International, Ltd. The analytical method used will be based upon chromatographic methodology provided by the Sponsor and/or developed at Wildlife International, Ltd. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

Data Analysis

When the dose-response pattern allows calculation of an LC50 value, the data will be analyzed using the computer software of C.E. Stephan (6). The program was designed to calculate the LC50 value and the 95% confidence interval by probit analysis, the moving average method, or binomial probability with nonlinear interpolation (7,8,9). The LC50 value will be calculated, when possible, using mortality data collected at 24, 48, 72 and 96 hours. Additional analysis of data may be conducted if deemed appropriate by the Study Director. The results of the analysis will be documented in the raw data and summarized in the final report. The no-mortality concentration and the no-observed-effect concentration (NOEC) will be determined by visually interpreting the clinical observation data.

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International, Ltd. will include, but will not be limited to:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Dates of initiation and termination of the test.
4. Test organism holding and acclimation records.

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5. Stock solution calculation and preparation, if applicable.
6. Observations.
7. Water chemistry results (e.g., alkalinity and hardness).
8. The methods used to analyze test substance concentrations and the results of analytical measurements, if applicable.
9. Statistical calculations, if applicable.
10. Test conditions (light intensity, photoperiod, etc.).
11. Calculation and preparation of test concentrations.
12. Copy of final report.

FINAL REPORT

A final report of the results of the study will be prepared by Wildlife International, Ltd. The report will include, but not be limited to the following, when applicable:

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures, as stated in the approved protocol, including all changes to the protocol.
5. The test substance identification including name, chemical abstract number or code number, strength, purity, composition, and other information provided by the Sponsor.
6. Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor.
7. A description of the methods used to conduct the test.
8. A description of the test organisms, including the source, scientific name, age or life stage, lengths and weights of a representative group of test organisms and feed types.
9. A description of the preparation of the test solutions.
10. The methods used to allocate organisms to test chambers and begin the test, the number of organisms and chambers per treatment, the duration of the test and environmental conditions during the test.
11. A description of circumstances that may have affected the quality or integrity of the data.

Wildlife International, Ltd.

12. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
13. A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses. A graph plotting the concentration-mortality curve at 96 hours. If the data are conducive to evaluation by probit analysis, the slope of the concentration-response curve will be reported.
14. Statistical methods used to evaluate the data, if applicable.
15. The signed and dated reports of each of the individual scientists or other professionals involved in the study.
16. The location where raw data and final report are to be stored.
17. A statement prepared by the Quality Assurance Unit listing the dates that study inspections and audits were made and the dates of any findings reported to the Study Director and Management.
18. If it is necessary to make corrections or additions to a final report after it has been accepted, such changes will be made in the form of an amendment issued by the Study Director. The amendment will clearly identify the part of the final report that is being amended and the reasons for the amendment, and will be signed by the Study Director.

CHANGES TO PROTOCOL

Planned changes to the protocol will be in the form of written amendments signed by the Study Director and approved by the Sponsor's Representative. Amendments will be considered as part of the protocol and will be attached to the final protocol. Any other changes will be in the form of written deviations signed by the Study Director and filed with the raw data. All changes to the protocol will be indicated in the final report.

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160 and/or Part 792); OECD Principles of Good Laboratory Practices (ENV/MC/CHEM (98) 17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999). Each study conducted by Wildlife International, Ltd. is routinely examined by the Wildlife International, Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory

Wildlife International, Ltd.

Practices will be prepared for all portions of the study conducted by Wildlife International, Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site or at an alternative location to be specified in the final report.

Wildlife International, Ltd.

REFERENCES

1 **Organization for Economic Cooperation and Development.** 1993. *Guideline 203: Fish, Acute Toxicity Test.* OECD Guideline for Testing of Chemicals. Updated Guideline adopted on 17 July 1992.

2 **U.S. Environmental Protection Agency.** 1996. *Fish Acute Toxicity Test, Freshwater and Marine.* Series 850-Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1075.

3 **ASTM Standard E729-88a.** 1994. *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians.* American Society for Testing and Materials.

4 **Eddy, S.** 1974. *The Freshwater Fishes.* Wm. C. Brown Company Publishers, Dubuque, Iowa.

5 **APHA, AWWA, WPCF.** 1998. *Standard Methods for the Examination of Water and Wastewater.* 20th Edition, American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.

6 **Stephan, C.E.** 1978. U.S. EPA, Environmental Research Laboratory, Duluth, Minnesota. Personal communication.

7 **Thompson, W.R.** 1947. *Bacteriological Reviews.* Vol. II, No. 2. Pp. 115-145.

8 **Stephan, C.E.** 1977. "Methods for Calculating an LC50," *Aquatic Toxicology and Hazard Evaluations.* American Society for Testing and Materials. Publication Number STP 634, pp 65-84.

9 **Finney, D.J.** 1971. *Statistical Methods in Biological Assay.* Second edition. Griffin Press, London.

Wildlife International, Ltd.

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Tetrabromobisphenol A: A 96-Hour Flow-Through Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*)

PROTOCOL NO.: 439/021303/RBT-96H2/OECD-OPPTS/SUB439 **AMENDMENT NO.:** 1

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel **PROJECT NO.:** 439A-123

EFFECTIVE DATE: July 3, 2003

AMENDMENT: **Objective**, Page 3:

CHANGE: The objective of this study is to determine the acute effects of Tetrabromobisphenol A (TBBPA) on the rainbow trout (*Oncorhynchus mykiss)* during a 96-hour exposure period under flow-through test conditions.

TO: The objective of this study is to determine if the acute toxicity of Tetrabromobisphenol A (TBBPA) to the rainbow trout, *Oncorhynchus mykiss*, during a 96-hour exposure period under flow-through test conditions would be > 1.0 mg active ingredient (a.i.)/L.

REASON: To add additional wording requested by the sponsor.

Amy Hankins 7-8-03

STUDY DIRECTOR DATE

7-8-03

LABORATORY MANAGEMENT DATE

Appendix 5

Analytical Report for Tetrabromobisphenol A (TBBPA)

ALBEMARLE CORPORATION
RESEARCH AND DEVELOPMENT DEPARTMENT

FINAL REPORT ON THE CHEMICAL CHARACTERIZATION (IDENTITY AND PURITY) OF TETRABROMOBISPHENOL-A (TBBPA) IN SUPPORT OF "A 48-HOUR FLOW-THROUGH ACUTE TOXICITY TEST WITH THE CLADOCERAN (Daphnia magna) AND A 96-HOUR FLOW-THROUGH ACUTE TOXICITY TEST WITH RAINBOW TROUT (Oncorhynchus mykiss)", CONDUCTED BY WILDLIFE INTERNATIONAL, LTD.

I.	Protocol Number:	TBBPA-05-29-2003
II.	Sponsor:	American Chemistry Council Brominated Flame Retardant Industry Panel 1300 Wilson Boulevard Arlington, Virginia 22209 Study Monitor: Wendy K. Sherman
III.	Analytical Testing Facilities:	Albemarle Corporation Process Development Center Gulf States Road Baton Rouge, LA 70805 Study Chemist: Paul F. Ranken, Ph. D.
IV.	Date of Study Initiation: Date of Study Completion:	June 2, 2003 June 16, 2003
V.	Test Article:	A sample of the test article, an end of study sample of Tetrabromobisphenol-A (WIL Test Substance 5754), was analyzed at the Albemarle Process Development Center. WIL Test Substance 5754 is a composite of commercial product from Albemarle Corporation, Great Lakes Chemical Corporation and the Dead Sea Bromine Group. Wildlife International Ltd., Easton, MD 21601, prepared the composite.
VI.	Objective/Methodology:	This study was initiated to confirm the identity of the test article and to demonstrate the purity of the test article. Fourier Transform Infrared Spectroscopy using SOP No. ARS-284-R4 confirmed the identity of the test article. In this procedure, the test article infrared spectrum was compared to a standard reference spectrum of TBBPA (Aldrich TBBPA, lot 03120DI). High Performance Liquid Chromatography (HPLC)

		using SOP No. ARS-443-R2 determined the purity (area % TBBPA) of the test article. The test article was further characterized by measuring the concentration (area%) of three potential impurities: tribromophenol, tribromobisphenol-A and o,p'-tetrabromobisphenol-A. Chain of Custody and sample handling were conducted according to established standard operating procedures.
VII.	Results:	The Table contains the test article analytical data from the study. Fourier Transform Infrared Spectroscopy confirmed the identity of the test article. High Performance Liquid Chromatography (HPLC) determined the purity of the test article to be 99.27%. Further characterization of the test article was accomplished by measuring the concentration of the three expected impurities. There were no circumstances that may have affected the quality or integrity of the data.
VIII.	Regulatory Requirements:	The study conformed to the requirements of EPA TSCA (40 CFR Part 792) Good Laboratory Practice Regulations and the OECD [C(97)186/Final] Good Laboratory Practice Regulations.
IX.	Data/Record Retention:	All log books, spectra and reports will be forwarded to the Quality Assurance Unit (QAU) for a final review prior to filing in the designated Health and Environment archives at Albemarle Corporation, Health and Environment Department, 451 Florida Street, Baton Rouge, LA 70801.
X.	Protocol Amendment:	The protocol was amended on June 12, 2003 to reflect a change in the approval date. The approval date originally listed by the study chemist was incorrectly listed as June 30, 2003. The amendment shows the correct approval date of June 2, 2003.

Paul F. Ranken, Ph.D.
STUDY CHEMIST

June 16, 2003
DATE

Table 1. CONCLUSIONS AND TEST ARTICLE ANALYTICAL DATA FOR TBBPA, WIL Test Substance #5754

CHEMICAL NAME: Tetrabromobisphenol-A
C.A.S. No.: 79-94-7
MOLECULAR FORMULA: $C_{15}H_{12}Br_4O_2$
PHYSICAL FORM: White Powder
CHEMICAL STRUCTURE:

ANALYSIS	RESULTS				ANALYSIS DATES	ANALYST
	Area %					
	TBBPA	2,4,6-Tribromophenol	o,p-tetrabromobisphenol-A	Tribromobisphenol-A		
HPLC	99.27	0.01	0.04	0.68	06/07/03	J.S. Amoyave
FT-IR	The sample FT-IR spectrum matched that of the Aldrich reference spectrum. All spectra are on file with the original data.				06/06/03	W.T. Cobb
CONCLUSION: Based on these analytical data, the test article identity was confirmed as tetrabromobisphenol-A. The test article was shown to have a purity of 99.27%.						

CHEMICAL CHARACTERIZATION ANALYTICAL PROTOCOL
FOR DETERMINING THE IDENTITY AND PURITY
OF TETRABROMOBISPHENOL-A (TBBPA) IN SUPPORT OF "A 48-HOUR FLOW-THROUGH ACUTE TOXICITY TEST WITH THE CLADOCERAN (Daphnia magna) AND A 96-HOUR FLOW-THROUGH ACUTE TOXICITY TEST WITH RAINBOW TROUT (Oncorhynchus mykiss)", CONDUCTED BY WILDLIFE INTERNATIONAL, LTD.

PROTOCOL AMENDMENT

Date:	June 16, 2003
Section to be changed:	Section X, page 2
Change:	Date of protocol approval is June 2, 2003.
Reason for change:	The protocol approval date originally listed by the Study Chemist was incorrectly given as June 30, 2003. The amendment shows the correct approval date of June 2, 2003.

June 16, 2003
Date:

Paul F. Ranken
Paul F. Ranken
Study Chemist

Appendix 6

Personnel Involved in the Study

The following key Wildlife International, Ltd. personnel were involved in the conduct or management of this study:

1. Henry O. Krueger, Ph.D., Director of Aquatic Toxicology/Terrestrial Plants and Insects
2. Willard B. Nixon, Ph.D., Director of Chemistry
3. Cary A. Sutherland, Laboratory Supervisor
4. Raymond L. Van Hoven, Scientist
5. Frank J. Lezotte, Chemist
6. Amy S. Blankinship, Aquatic Biologist