TETRABROMOBISPHENOL A:
A 48-HOUR FLOW-THROUGH ACUTE TOXICITY TEST WITH THE CLADOCERAN (*Daphnia magna*)

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER : 439A-124

OECD GUIDELINE 202

and

U.S. EPA OPPTS NUMBER 850.1010

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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
1300 Wilson Boulevard
Arlington, Virginia 22209

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8598 Commerce Drive
Easton, Maryland 21601
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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

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

TITLE: Tetrabromobisphenol A: A 48-Hour Flow-Through Acute Toxicity Test with the Cladoceran (*Daphnia magna)*

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This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 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 in 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:
Protocol	March 4, 2003	March 4, 2003	March 7, 2003
Test Substance Preparation	March 4, 2003	March 5, 2003	March 5, 2003
Matrix Fortification	March 5, 2003	March 5, 2003	March 7, 2003
Environmental Conditions, Sample Collection, and Water Chemistry	March 7, 2003	March 7, 2003	March 7, 2003
Biological Data and Draft Report	March 19, 2003	March 19, 2003	March 20, 2003
Analytical Data and Draft Report	March 19, 2003	March 19, 2003	March 20, 2003
Final Report	July 2, 2003	July 2, 2003	July 3, 2003

All inspections were study-based unless otherwise noted.

Susan L. Coleman

Susan L. Coleman, B.A.
Senior Quality Assurance Representative

7-8-03

Date

REPORT APPROVAL

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SUMMARY

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

TITLE: Tetrabromobisphenol A : A 48-Hour Flow-Through Acute Toxicity Test with the Cladoceran (*Daphnia magna)*

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 439A-124

TEST DATES:	Experimental Start (OECD):	March 4, 2003
	Experimental Start (EPA):	March 5, 2003
	Biological Termination:	March 7, 2003
	Experimental Termination:	March 7, 2003

LENGTH OF EXPOSURE: 48 Hours; Flow-Through Conditions

TEST ORGANISMS: Cladoceran (*Daphnia magna*)

SOURCE OF TEST ORGANISMS: Wildlife International, Ltd. Cultures
Easton, Maryland 21601

AGE OF TEST ORGANISMS: Neonates <24 hours at test start

TEST CONCENTRATIONS:	Nominal	Mean Measured
	Negative Control	<LOQ
	Solvent Control	<LOQ
	1.2 mg a.i./L	1.2 mg a.i./L
	1.8 mg a.i./L	1.8 mg a.i./L

RESULTS (Based on Mean Measured Concentrations):

48-Hour EC50:	> 1.8 mg a.i./L
95% Confidence Interval :	Not Applicable
No Mortality/Immobility Concentration:	1.8 mg a.i./L

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for American Chemistry Council's Brominated Flame Retardant Industry Panel at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The definitive toxicity test was conducted from March 5 to 7, 2003. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 439A-124 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 cladoceran, *Daphnia magna*, during a 48-hour exposure period under flow-through test conditions would be > 1.0 mg active ingredient (a.i.)/L.

EXPERIMENTAL DESIGN

Daphnids were exposed to two test concentrations, a negative control (dilution water), and a solvent control (0.1 mL dimethyl formamide/L). Two replicate test chambers were maintained in each treatment and control group, with 10 daphnids in each test chamber for a total of 20 daphnids per concentration. Two nominal test concentrations were selected by the Sponsor to be reasonably assured that at least one mean measured concentration would be > 1.0 mg a.i./L. Nominal test concentrations selected 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 test initiation and termination.

Delivery of the test substance was initiated approximately 17 hours prior to test initiation in order to achieve equilibrium of the test substance in the test chambers. Daphnids were impartially assigned to exposure chambers at test initiation. Observations of mortality, immobility and other signs of toxicity were made approximately 4.5, 24 and 48 hours after test initiation. Cumulative percent mortality and immobility observed in the treatment groups were used to estimate EC50 values at 24 and 48 hours. The no-mortality/immobility concentration was determined by visual interpretation of the mortality and immobility data.

MATERIALS AND METHODS

The study was conducted based on the procedures outlined in the protocol, "Tetrabromobisphenol A: A 48-Hour Flow-Through Acute Toxicity Test with the Cladoceran (*Daphnia magna*)". The protocol was based on procedures outlined in OECD Guidelines for Testing of Chemicals, 202: *Daphnia sp. Acute Immobilization Test and Reproduction Test* (1); U.S. Environmental Protection Agency Series 850 – Ecological Effects Test Guidelines (draft) OPPTS Number 850.1010, *Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids* (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. The test substance was stored under ambient conditions.

Test Organism

The cladoceran, *Daphnia magna*, was selected as the test species for this study. Daphnids are representative of an important group of aquatic invertebrates and were selected for use in the test based upon past history of use and ease of culturing in the laboratory. Daphnid neonates used in the test were less than 24-hours old and were obtained from cultures maintained by Wildlife International, Ltd., Easton, Maryland.

Adult daphnids were cultured in water from the same source and at approximately the same temperature as used during the test. The adult daphnids used to supply neonates for the test were held for 22 days prior to collection of the juveniles for testing. The adults showed no signs of disease or stress during the holding period. During the 2-week holding period immediately preceding the test, water temperatures ranged from 19.8 to 21.0°C, measured with a hand-held liquid-in-glass

thermometer. The pH of the water ranged from 8.3 to 9.0, measured with a Fisher Scientific Accumet Model 915 pH meter. Dissolved oxygen ranged from 8.2 to 9.0 mg/L (≥91% of saturation), measured with a Yellow Springs Instruments Model 51B dissolved oxygen meter. Daphnids in the cultures were fed daily a mixture of yeast, Cerophyll®, and trout chow, as well as a suspension of the freshwater green alga, *Selenastrum capricornutum*. The adults were fed prior to test initiation, but neonates were not fed during the test.

Neonate daphnids were obtained for testing from five individual adult daphnids. At test initiation, the juvenile daphnids were collected from the cultures and indiscriminately transferred one or two at a time to transfer chambers (e.g., 10 mL glass beakers) until each chamber contained ten daphnids. All transfers were made below the water surface using wide-bore pipettes. The transfer chambers were indiscriminately assigned to the test chambers and were placed inside the test compartments. The daphnids were released into the test compartments by gently submerging the compartments in the test solution.

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 well water was filtered to 0.45 µm and then passed through an ultraviolet (UV) sterilizer to remove microorganisms and fine 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 (dimethyl formamide (DMF)) control, and a negative (well water) control. A syringe pump (Harvard Apparatus, South Natick, Massachusetts) was used to deliver the two test substance stock

solutions and 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 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 prior to 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 for the two replicates.

The diluter was adjusted so that each test chamber received approximately 13 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 9-L glass aquaria filled with approximately 7 L of test water. The depth of the test water in a representative chamber was approximately 14.8 cm. Each test chamber contained one test compartment constructed from a glass cylinder approximately 50 mm in diameter, with 425 µm nylon screen attached to the bottom using silicone sealant. The cylinder was inverted inside a 150-mL glass beaker, which was submerged in the test chamber. Test chambers were indiscriminately positioned in a temperature-controlled environmental chamber set to maintain the desired test temperature. 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 concentration of 18 mg a.i./mL. The primary stock solution was mixed by inversion and sonication, and was clear and colorless. 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 stock solution was mixed by inversion, and was clear and colorless. The two stock solutions were injected into the diluter mixing chambers (at a rate of 12.50 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 only 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. All test solutions appeared clear and colorless in the diluter mixing chambers and in the test chambers at test initiation and termination.

Analytical Sampling

Samples were collected from both replicate test chambers in each treatment and control group at test initiation and termination to measure concentrations of the test substance. All test solution samples were collected at mid-depth, placed in glass vials, and analyzed as soon as possible without storage.

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. Two 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 98.8 to 103% 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

Lighting used to illuminate the cultures and test chambers during holding, acclimation and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50). 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 479 lux at the surface of the water of one representative test chamber.

The target test temperature during the study was 20 ± 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 of each

treatment and control group at the beginning and end of the test, and at approximately 24-hours. Hardness, alkalinity, specific conductance and total organic carbon (TOC) 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 to determine the numbers of dead and immobile organisms. Immobility was defined as a lack of movement by the organism except for minor activity of the appendages. The number of individuals exhibiting signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 4.5, 24 and 48 hours after test initiation. Prior to test termination, observations were conducted without removing the test compartments from the test solution in order to minimize disturbance of the daphnids. Therefore, the 4.5 and 24-hour observations were estimates, with exact counts of mortality/immobility conducted at test termination.

Statistical Analyses

The absence of mortality or immobility in this study precluded the statistical calculation of EC50 values. Therefore, the 24 and 48-hour EC50 values were estimated to be greater than the highest concentration tested. 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 Tetrabromobisphenol A (TBBPA) in the test solution samples collected during the test ranged from 95 to 104% of nominal (Table 1). When the measured concentrations of the test samples collected at test initiation and termination were averaged, the mean

measured concentrations for the study were 1.2 and 1.8 mg a.i./L, representing 100 and 100% 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 20 ± 1°C range established for the test. Dissolved oxygen concentrations remained ≥8.6 mg/L (95% of saturation) throughout the test. Measurements of pH ranged from 8.1 to 8.2 during the test. The measurements of hardness, alkalinity, specific conductance and TOC in the dilution water at test initiation were typical of Wildlife International, Ltd. well water (Table 3).

Daily observations of mortality and signs of toxicity observed during the test are presented in Table 4. There were two immobile daphnids in the negative control group at test termination. Current OECD and OPPTS guidelines state that up to 10% immobility/death is acceptable for control performance, therefore the two immobile daphnids (10%) in the negative control is acceptable. All other daphnids that were observed in the negative and solvent control groups appeared normal throughout the test. All daphnids that were observed in the 1.2 and 1.8 mg a.i./L treatment groups appeared normal throughout the test. Consequently, the no-mortality concentration and the NOEC were 1.8 mg a.i./L. EC50 values at 24 and 48 hours were estimated to be >1.8 mg a.i./L (Table 5).

CONCLUSIONS

The 48-hour EC50 value for the cladoceran, *Daphnia magna*, exposed to Tetrabromobisphenol A (TBBPA) under flow-through conditions was > 1.8 mg a.i./L, the highest test concentration tested. The NOEC and no mortality/immobility concentration was 1.8 mg a.i./L.

REFERENCES

1 **Organisation for Economic Cooperation and Development**. 1984. OECD Guidelines for Testing of Chemicals. Guideline 202: *Daphnia sp. Acute Immobilization Test and Reproduction Test.* Updated Guideline, adopted April, 1984.

2 **U.S. Environmental Protection Agency.** 1996. OPPTS Number 850.1010: *Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids*. Series 850 – Ecological Effects Test Guidelines (*draft*).

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.

Table 1

Measured Concentrations of Tetrabromobisphenol A (TBBPA) in Test Samples

Nominal Test Concentration (mg a.i./L)	Sample ID (439A-124-)	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	--		
Solvent Control	3	0	< LOQ	--	--	--
0.0	4	0	< LOQ	--		
	11	48	< LOQ	--		
	12	48	< LOQ	--		
1.2	5	0	1.24	103	1.2	100
	6	0	1.25	104		
	13	48	1.18	98.7		
	14	48	1.19	99.3		
1.8	7	0	1.85	103	1.8	100
	8	0	1.82	101		
	15	48	1.74	96.7		
	16	48	1.71	95.3		

[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.

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		
		Temp.[1] (°C)	DO[2] (mg/L)	pH	DO (mg/L)	pH	Temp. (°C)	DO (mg/L)	pH
Negative Control	A	20.2	8.6	8.1	--	--	20.1	8.7	8.2
	B	20.2	--	--	8.7	8.1	20.1	--	--
Solvent Control	A	20.0	8.6	8.1	--	--	20.0	8.7	8.2
	B	20.0	--	--	8.7	8.1	20.0	--	--
1.2	A	20.1	8.7	8.1	--	--	20.0	8.8	8.2
	B	20.1	--	--	8.7	8.1	20.0	--	--
1.8	A	19.8	8.7	8.1	--	--	19.8	8.8	8.2
	B	19.8	--	--	8.7	8.2	19.8	--	--

[1] Manual temperature measurements. Temperature measured continuously during the test was approximately 20.0 to 20.5°C, measured to the nearest 0.5°C.

[2] A dissolved oxygen concentration of 6.8 mg/L represents 75% saturation at 20.0°C in freshwater.

Table 3

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

Parameter	Day 0
Hardness (mg/L as $CaCO_3$)	132
Alkalinity (mg/L as $CaCO_3$)	185
Specific Conductance (µmhos/cm)	330
Total Organic Carbon (mg C/L)	<1

Table 4

Cumulative Percent Mortality, Immobility and Treatment-Related Effects

Mean Measured Concentration (mg a.i./L)	Replicate	Daphnia/ Replicate	4.5 Hours[1]			24 Hours[1]			48 Hours			Percent Immobile and Dead
			Cumulative Dead	Number Immobile	Effects[2]	Cumulative Dead	Number Immobile	Effects[2]	Cumulative Dead	Number Immobile	Effects[2]	
Negative Control	A	10	0	0	AN	0	0	AN	0	1	9 AN	10
	B	10	0	0	AN	0	0	AN	0	1	9 AN	
Solvent Control	A	10	0	0	AN	0	0	AN	0	0	10 AN	0
	B	10	0	0	AN	0	0	AN	0	0	10 AN	
1.2	A	10	0	0	AN	0	0	AN	0	0	10 AN	0
	B	10	0	0	AN	0	0	AN	0	0	10 AN	
1.8	A	10	0	0	AN	0	0	AN	0	0	10 AN	0
	B	10	0	0	AN	0	0	AN	0	0	10 AN	

[1] Daphnids were observed without removing the test compartments from the test solutions. Therefore, observations at 4.5 and 24-hours are estimates of mortality and immobility.
[2] Observed Effects: AN = appear normal.

Table 5

EC50 Values

Time	EC50 (mg a.i./L)	95% Confidence Interval (mg a.i./L)	Statistical Method
24 Hours	>1.8	--[1]	NA[2]
48 Hours	>1.8	--[1]	NA[2]

[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)	313 (N = 4)	310 – 320
Hardness (mg/L as $CaCO_3$)	131 (N = 4)	124 – 140
Alkalinity (mg/L as $CaCO_3$)	184 (N = 4)	182 – 186
pH	8.1 (N = 4)	8.1 – 8.2

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 3.6 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-124-5, 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 = 606118
Y-intercept = 3517.6104
Slope = 12188727
Initial Volume (V_i): = 0.200 mL
Final Volume (V_f): = 5.00 mL
Dilution Factor (V_f/V_i): = 25

$$\text{TBBPA (mg a.i./L) in sample} = \frac{606118 - (3517.6104)}{12188727} \times 25$$

$$= 1.236 \text{ mg a.i./L}$$

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

$$= \frac{1.236}{1.2} \times 100$$

$$= 103\%$$

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

Appendix 3.5

Quality Control Samples of TBBPA in Freshwater

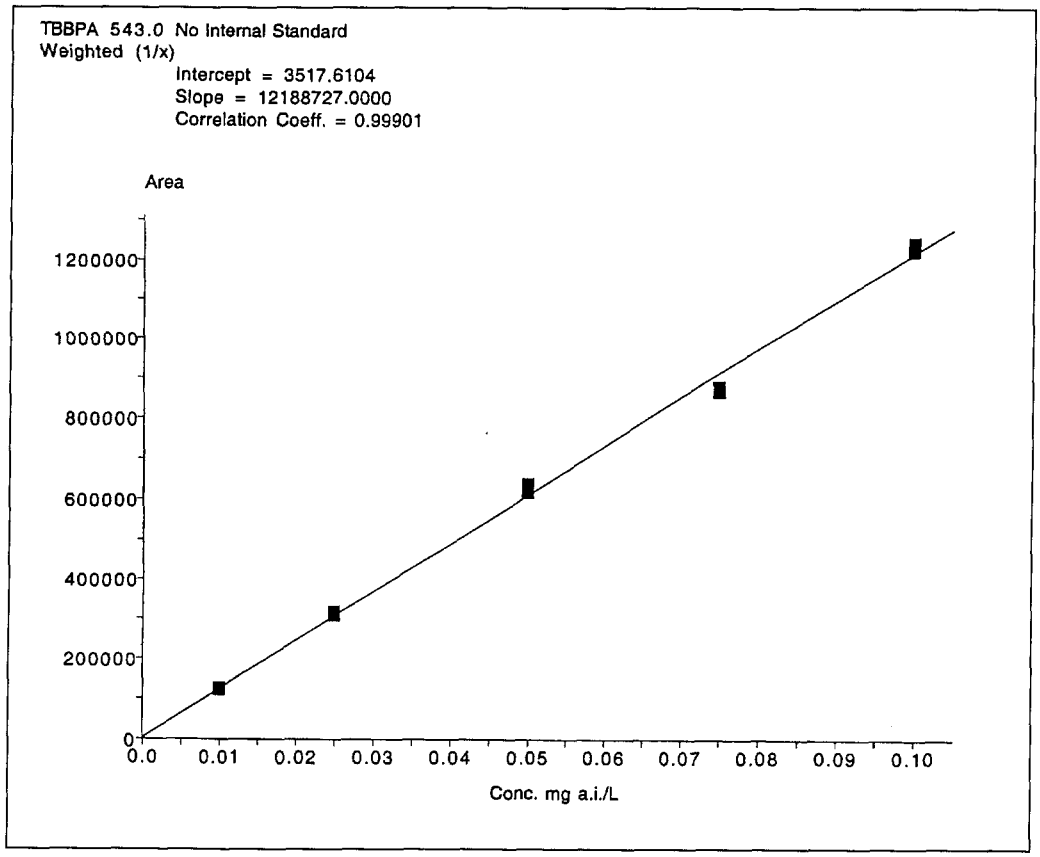
Sample Number (439A-123,124-)	Concentration (mg a.i./L)		Percent Recovery [2]
	Fortified	Measured [1,2]	
MAB-2	0.0	< LOQ	--
MAB-3	0.0	< LOQ	--
MAS-4	1.00	0.988	98.8
MAS-5	1.50	1.49	99.2
MAS-6	2.00	2.02	101
MAS-7	1.00	1.01	101
MAS-8	1.50	1.50	100
MAS-9	2.00	2.05	103

[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.

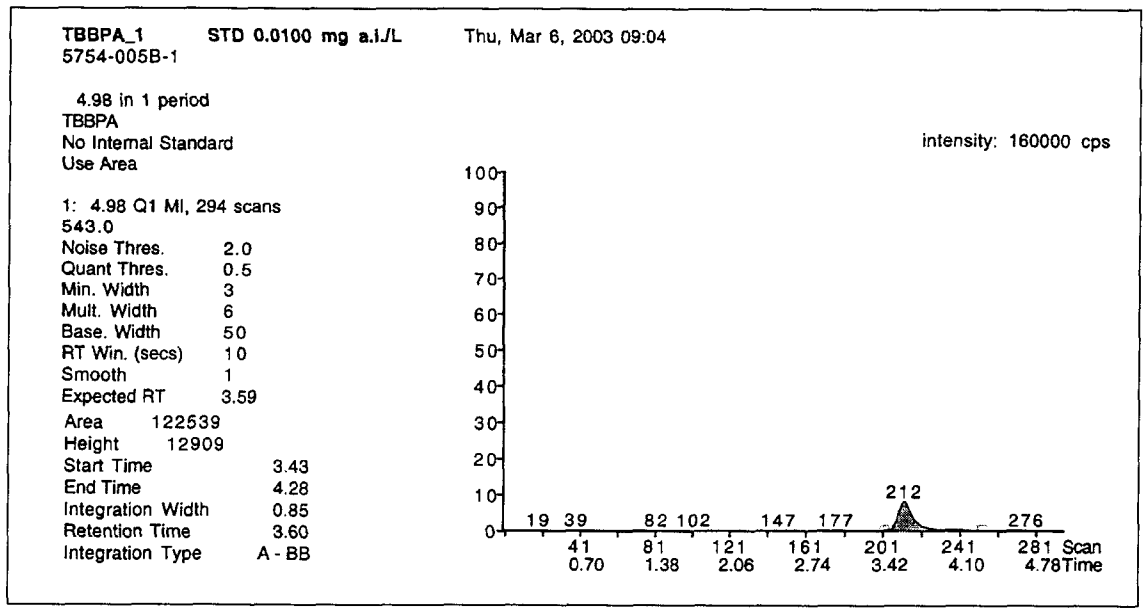
Appendix 3.6

Representative Calibration Curve for TBBPA



Appendix 3.7

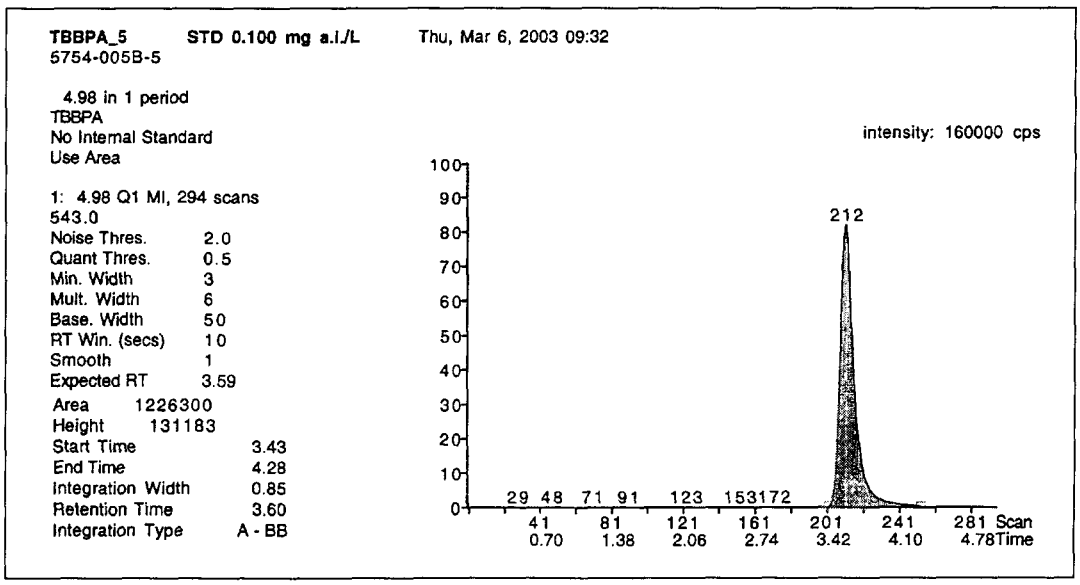
Representative Chromatogram of a Low-level TBBPA Calibration Standard



Nominal concentration: 0.0100 mg a.i./L

Appendix 3.8

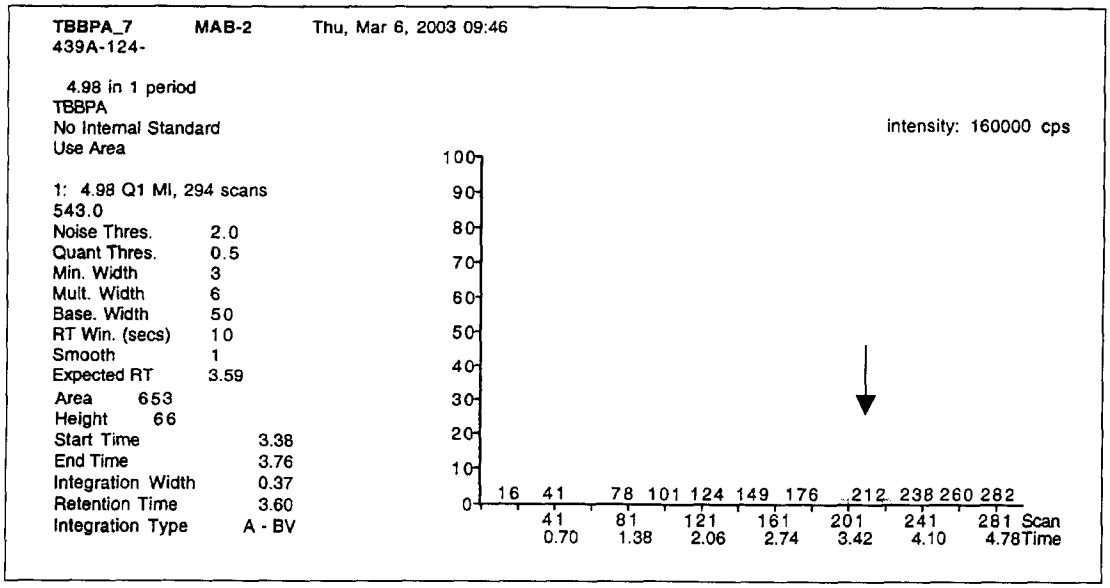
Representative Chromatogram of a High-level TBBPA Calibration Standard



Nominal concentration: 0.100 mg a.i./L

Appendix 3.9

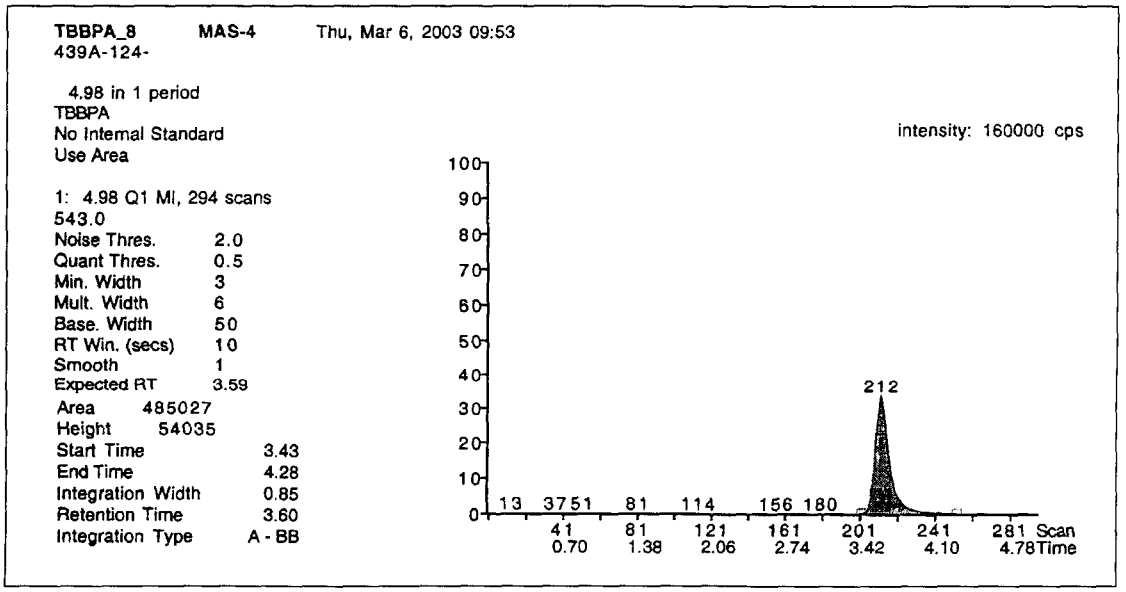
Representative Chromatogram of a Matrix Blank Sample



Sample number 439A-123,124-MAB-2. Dilution factor = 25X.
The arrow indicates the approximate retention time of TBBPA.

Appendix 3.10

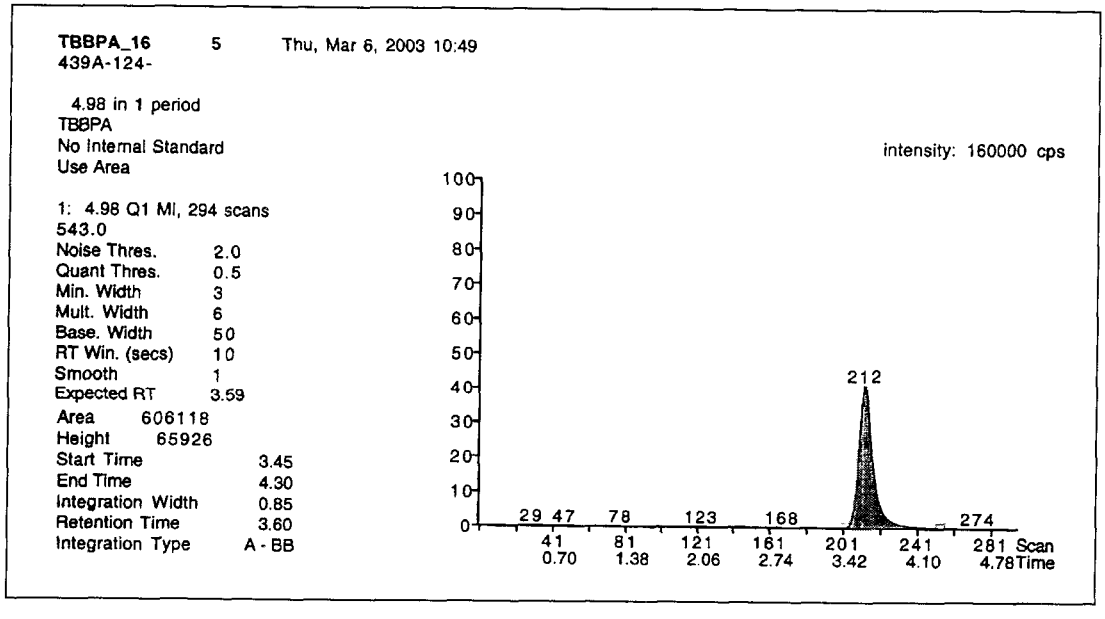
Representative Chromatogram of a Matrix Fortification Sample



Sample number: 439A-123,124-MAS-4, nominal concentration 1.00 mg a.i./L.
Dilution factor = 25X.

Appendix 3.11

Representative Chromatogram of a Test Sample



Sample number: 439A-124-5, Day 0, nominal concentration 1.2 mg a.i./L.
Dilution factor = 25X.

Appendix 4

Protocol and Amendments

PROTOCOL

TETRABROMOBISPHENOL A: A 48-HOUR FLOW-THROUGH ACUTE TOXICITY TEST WITH THE CLADOCERAN (*Daphnia magna*)

OECD Guideline 202

U.S. EPA OPPTS Number 850.1010

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.

TETRABROMOBISPHENOL A: A 48-HOUR FLOW-THROUGH ACUTE TOXICITY TEST WITH THE CLADOCERAN (*Daphnia magna*)

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: 3/5/03 Experimental Termination Date: 3/7/03

Project No.: 439A-124

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

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

PROTOCOL APPROVAL

Amy B Blankinship 2/28/03
STUDY DIRECTOR DATE

[signature] 2/28/03
LABORATORY MANAGEMENT DATE

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

Wildlife International, Ltd.

INTRODUCTION

Wildlife International, Ltd. will conduct a flow-through acute toxicity test with the cladoceran, *Daphnia magna*, for the Sponsor at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The study will be performed based on procedures in the OECD Guideline for Testing of Chemicals, 202: *Daphnia sp. Acute Immobilization Test and Reproduction Test* (1); U.S. EPA Series 850 - Ecological Effects Test Guidelines OPPTS Number 850.1010: *Aquatic Invertebrate Acute Toxicity Test* (2); and ASTM Standard E-729-88a: *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 cladoceran, *Daphnia magna*, under flow-through test conditions for a period of 48 hours.

EXPERIMENTAL DESIGN

Daphnids will be exposed to a two test concentrations (1.2 and 1.8 ppm), a negative (dilution water) control and a solvent control for 48 hours. Two replicate test chambers will be maintained in each treatment and control group, with 10 neonate daphnids in each chamber so that a total of 20 neonate daphnids are exposed in each treatment and control group.

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, neonate daphnids will be impartially assigned to transfer chambers at test initiation. No other potential sources of bias are expected to affect the results of the study. EC50 values will be calculated, when possible, based on the number of dead or immobilized daphnids observed in each test concentration after each 24-hour interval of exposure.

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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 the 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 experimental design along with a negative (dilution water) control group. The concentration of the organic solvent will not exceed 0.1-mL/L, when possible. The solvent concentration in the solvent control will be equal to the highest solvent concentration in test chambers containing the test substance.

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Test Organism

The cladoceran, *Daphnia magna*, has been selected as the test species for this study. Daphnids are representative of an important group of aquatic invertebrates, and have been selected for use in the test based upon past use history and ease of culturing in the laboratory. Daphnid neonates to be used in the test will be less than 24 hours old and will be obtained from cultures maintained at Wildlife International, Ltd., Easton, Maryland. The identity of the species will be verified by the supplier of the original culture or by Wildlife International, Ltd. using appropriate taxonomic keys such as Pennak (4).

Daphnids will be cultured in water from the same source and at approximately the same temperature as will be used during the test. Daphnids in the cultures producing neonates for the test will be held for at least 10 days prior to collection of the neonates for testing. Adult daphnids in the culture will produce an average of at least 3 young per adult per day over the 7 day period prior to the test. Neonates from daphnids that show signs of disease or stress will not be used as test organisms. Daphnids in holding that produce ephippia also will not be used to supply neonates for testing.

Daphnids in the cultures will be fed at least once daily. The diet will be a mixture of yeast, Cerophyll®, and trout chow (YCT), supplemented with a suspension of the freshwater green alga *Selenastrum capricornutum*. Adults are fed during the 24-hour period prior to test initiation, but neonates will not be fed during the test. Specifications for acceptable levels of contaminants in daphnid 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.

Neonates will be obtained for testing from at least three individual adults that have produced at least one previous brood. Prior to test initiation, the neonates will be collected from cultures and transferred to small containers. The daphnids will be released into the test compartments below the water surface using a wide-bore pipette.

Dilution Water

The water used for culturing and testing 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, and may be UV-sterilized. Water used

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for culturing 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 in the 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.

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 test, 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.

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Test compartments will be constructed from glass beakers 6.5 cm in diameter and 12 cm in height. Nylon screens will be attached to two holes in the side of each glass beaker. The beakers will be suspended in 8-L, Teflon®-lined polyethylene or stainless steel chambers filled with approximately 6.5 L of test solution. Test chambers will be indiscriminately positioned in a temperature-controlled water bath to maintain a temperature of 20 ± 1°C. Test chambers will be labelled with project number, test concentration and replicate.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during culturing 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 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 20 ± 1°C. Temperature will be measured in each test chamber 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 and pH will be measured in alternate replicates of each treatment and control group at test initiation and at approximately 24-hour intervals during the test. In the event that dissolved oxygen concentrations fall below 75% of saturation, dissolved oxygen measurements will be taken in every test chamber and appropriate actions will be taken after consultation with the Sponsor. Dissolved oxygen concentrations will be measured with a Thermo Orion Model 850Aplus dissolved oxygen meter, or equivalent and pH will be measured with 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.

Hardness, alkalinity, specific conductance and total organic carbon (TOC) will be measured in the dilution water at the beginning of the test. Hardness and alkalinity measurements will be made by titration using procedures based on methods in *Standard Methods for the Examination of Water and*

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Wastewater (5). Specific conductance will be measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter, or equivalent. Total organic carbon will be measured on a Shimadzu Model 5000 TOC analyzer, 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, immobilization and clinical signs of toxicity will be made between 0-24 hours, and at 24 and 48 hours ± 1 hour. Immobilization is defined as a lack of movement by the test organism except for minor activity of the appendages.

Sampling for Analytical Measurements

Water samples will be collected from each test chambers at the beginning and at the end of the test 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 Hours	48 Hours
Control	2	2
Solvent Control (if needed)	2	2
Level 1- 1.2 ppm	2	2
Level 2- 1.8 ppm	2	2
	8	8

Total Number of Verification Samples = 16

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. 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

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sampling or analysis is suspected, or if a malfunction in the test substance delivery system occurs. The reason for the additional samples will be described by the Study Director and documented in the raw data and final report.

Analytical Measurements

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 EC50 value, the data will be analyzed using the computer software of C.E. Stephan (6). The program was designed to calculate the EC50 value and the 95% confidence interval by probit analysis, the moving average method, or binomial probability with nonlinear interpolation (7,8,9). The EC50 value will be calculated, when possible, using mortality/immobility data collected at 24 and 48 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.

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International, Ltd. will include, but 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. Daphnid history and culture records.
5. Results of rangefinding tests, when applicable.
6. Stock solution calculation and preparation.
7. Daily observations.
8. Water chemistry results (e.g., hardness and alkalinity).
9. If applicable, the methods used to analyze test substance concentrations and the results of analytical measurements.

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10. Statistical calculations.
11. Test conditions (light intensity, photoperiod, etc.).
12. Calculation and preparation of test concentrations.
13. 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 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 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, and the duration of the test.
11. A description of circumstances that may have affected the quality or integrity of the data.
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 response curve at 48

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- 11 -

hours, when sufficient data exists. If the data is conducive to evaluation by probit analysis, the slope of the concentration-response curve will be reported.

14. Statistical methods used to evaluate the data.
15. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
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 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.

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REFERENCES

1 **Organization for Economic Cooperation and Development.** 1984. Guideline 202: *Daphnia sp. Acute Immobilization Test and Reproduction Test.* OECD Guideline for Testing of Chemicals. Updated Guideline, adopted April, 1984.

2 **U.S. Environmental Protection Agency.** 1996. *Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids.* Series 850-Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1010.

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 **Pennak, R.W.** 1978. Freshwater Invertebrates of the United States. 2nd Ed. 365 p.

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

8 **Thompson, W.R.** 1974. *Bacteriological Reviews.* Vol. II, No. 2. Pp. 115-145.

9 **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.

Wildlife International, Ltd.

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Tetrabromobisphenol A: A 48-Hour Flow-Through Acute Toxicity Test with the Cladoceran (*Daphnia magna*)

PROTOCOL NO.: 439/021303/DAP-48H2/OECD-OPPTS/SUB439 **AMENDMENT NO.:** 1

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel **PROJECT NO.:** 439A-124

EFFECTIVE DATE: March 3, 2003

AMENDMENT: Test Apparatus, Pages 6 and 7:

CHANGE: Test compartments will be constructed from glass beakers 6.5 cm in diameter and 12 cm in height. Nylon screens will be attached to two holes in the side of each glass beaker. The beakers will be suspended in 8-L, Teflon®-lined polyethylene or stainless steel chambers filled with approximately 6.5L of test solution. Test chambers will be indiscriminately positioned in a temperature-controlled water bath to maintain a temperature of 20 ± 1°C.

TO: Test compartments will be constructed from glass cylinders approximately 50 mm in diameter, with 425-µm nylon or Teflon® screen attached to the bottom using silicone sealant. The cylinders will be inverted inside 150-mL glass beakers. The beakers will be submerged in 9-L glass aquaria filled with approximately 7 L of test solution. Test chambers will be indiscriminately positioned in a temperature-controlled environmental chamber set to maintain a temperature of 20 ± 1°C.

REASON: This change is in an effort to help prevent the Daphnids from becoming trapped on air bubbles that form on the test chambers and compartments in the test apparatus.

AMENDMENT: Test Organism, Page 5:

CHANGE: Prior to test initiation, the neonates will be collected from cultures and transferred to small containers. The daphnids will be released into the test compartments below the water surface using a wide-bore pipette.

TO: Prior to test initiation, the neonates will be collected from cultures and impartially transferred 1 or 2 at a time to small glass containers until each contains 10 neonates. Each transfer container will be impartially assigned to a test chamber, and placed inside the test compartment. The neonates will be released into the test compartment by gradually submerging the test compartments in the test chamber. The test compartments and transfer containers will remain submerged in the test chamber throughout the exposure period.

REASON: To clarify how the organisms will be distributed to the test chambers so that the neonates will not become trapped in the air/water interface during the test.

AMENDMENT: Biological Measurements, Page 8:

ADD: Observations for the 0-24 and 24-hour intervals will be conducted without removing the test compartments from the test solution. Therefore, these observations will be estimates, rather than exact counts. An exact count of mortalities, immobile organisms and effects will be made at test termination.

REASON: Numbers will be estimated in order to minimize disturbance of the test organisms in the compartments during the test.

Amy Blankinship 3-5-03

STUDY DIRECTOR DATE

LABORATORY MANAGEMENT 3/10/03

DATE

Wildlife International, Ltd.

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Tetrabromobisphenol A: A 48-Hour Flow-Through Acute Toxicity Test with the Cladoceran (*Daphnia magna*)

PROTOCOL NO.: 439/021303/DAP-48H2/OECD-OPPTS/SUB439 **AMENDMENT NO.:** 2

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel **PROJECT NO.:** 439A-124

EFFECTIVE DATE: March 6, 2003

AMENDMENT: References, Page 12:

CHANGE: **Thompson, W.R.** 1974. *Bacteriological Reviews*. Vol. II, No. 2. Pp. 115-145.

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

REASON: Year is incorrect.

Amy Blankinship — 3.10.03
STUDY DIRECTOR — DATE

[signature] — 3-12-03
LABORATORY MANAGEMENT — DATE

QA Reviewed by [signature] 3.7.03

Wildlife International, Ltd.

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Tetrabromobisphenol A: A 48-Hour Flow-Through Acute Toxicity Test with the Cladoceran (*Daphnia magna*)

PROTOCOL NO.: 439/021303/DAP-48H2/OECD-OPPTS/SUB439 **AMENDMENT NO.:** 3

SPONSOR: American Chemistry Council's Brominated Flame Retardant Industry Panel **PROJECT NO.:** 439A-124

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 cladoceran, *Daphnia magna*, under flow-through test conditions for a period of 48 hours.

TO: The objective of this study is to determine if the acute toxicity of Tetrabromobisphenol A (TBBPA) to the cladoceran, *Daphnia magna*, during a 48-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

K Kan 7-8-03
LABORATORY MANAGEMENT DATE

Reviewed by QA (SLC) 7-3-03

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 June 16, 2003

Paul F. Ranken, Ph.D. DATE
STUDY CHEMIST

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: $C_{15}H_{10}Br_4O_2$
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. Arroyave
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