



FYI-1097-1300

CHEMICAL MANUFACTURERS ASSOCIATION



FYI-97-001300

June 20, 1997

Dr. Lynn Goldman
Assistant Administrator
Office of Prevention, Pesticides and Toxic Substances TS-7101
Environmental Protection Agency
401 M Street, SW, Room 637, East Tower
Washington, DC 20460



3498000002

Dear Dr. Goldman:

The Chemical Manufacturers Association makes available to the public and appropriate government agencies final reports of environmental, health and safety research that it manages. In keeping with this policy, the following recently completed report is enclosed:

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

This report does not include confidential information.

If you have any questions, please call Has Shah of my staff at 703-741-5637.

Sincerely,

Carol R. Stack, Ph.D.
Acting Vice-President
CHEMSTAR

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Enclosure

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

FINAL REPORT

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 439A-102

TSCA Title 40 of the Federal Code of Regulations
Part 797, Section 1300
and
Organisation for Economic Cooperation and Development
OECD Guideline 202, Part I

AUTHORS:

William C. Graves
James P. Swigert, Ph.D.

STUDY INITIATION DATE: May 15, 1996

STUDY COMPLETION DATE: May 21, 1997

Submitted to

Chemical Manufacturers Association's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209



WILDLIFE INTERNATIONAL LTD.

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

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel

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

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 439A-102

STUDY COMPLETION: May 21, 1997

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Part 792, 17 August 1989; OECD Principles of Good Laboratory Practices, (OECD)C(81)30 (Final)Annex 2; and Japan MHW/MITI 59 Kikyoku No. 85; EA, Kankiken No. 233: MHW, Eisei No. 38 and MITI, 63 Kikyoku No. 823.

STUDY DIRECTOR:

William C Graves
William C. Graves
Senior Aquatic Biologist

5-21-97
DATE

SPONSOR:

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Sponsor

6-8-97
DATE

- 3 -

QUALITY ASSURANCE

This study was examined for compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFP Part 792, 17 August 1989; OECD Principles of Good Laboratory Practices, (OECD)C(81)30 (Final)Annex 2; and Japan MHW/MITI 59 Kikyoku No. 85; EA, Kankiken No. 233; MHW, Eisei No. 38 and MITI, 63 Kikyoku No. 823. 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:

ACTIVITY:	DATE CONDUCTED:	DATE REPORTED TO:	
		STUDY DIRECTOR:	MANAGEMENT:
Test substance preparation	March 31, 1997	March 31, 1997	April 1, 1997
Analytical sample collection and analytical sample preparation	April 4, 1997	April 4, 1997	April 4, 1997
Biological Data and Draft Report	April 24 and 25, 1997	April 25, 1997	April 25, 1997
Analytical Data and Draft Report	April 29 and 30, 1997	April 30, 1997	May 1, 1997
Final Report	May 21, 1997	May 21, 1997	May 21, 1997


 Lisa T. Drottar
 Quality Assurance Representative

5-2-97
 DATE

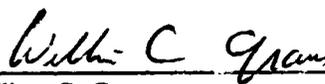
REPORT APPROVAL

SPONSOR: Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel

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

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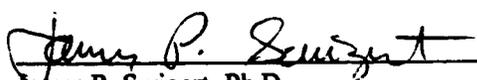
STUDY DIRECTOR:



William C. Graves
Senior Aquatic Biologist

5-21-97
DATE

MANAGEMENT:



James P. Swigert, Ph.D.
Manager, Aquatic Toxicology

5/21/97
DATE

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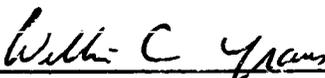
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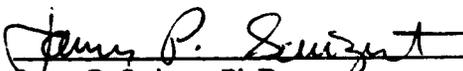


William C. Graves
Senior Aquatic Biologist

5-21-97

DATE

MANAGEMENT:



James P. Swigert, Ph.D.
Manager, Aquatic Toxicology

5/21/97

DATE

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SUMMARY

SPONSOR:	Chemical Manufacturers Association's (CMA) Brominated Flame Retardant Industry Panel
SPONSOR'S REPRESENTATIVE:	Dr. Hasmukh Shah
LOCATION OF STUDY, RAW DATA AND A COPY OF THE FINAL REPORT:	Wildlife International Ltd. Easton, Maryland 21601

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER:	439A-102
TEST SUBSTANCE:	Hexabromocyclododecane (HBCD)
STUDY:	Hexabromocyclododecane (HBCD): A 48-Hour Flow-Through Acute Toxicity Test with the Cladoceran (<i>Daphnia magna</i>)
NOMINAL TEST CONCENTRATIONS:	Negative Control, Solvent Control, 1.5, 2.2, 3.2, 4.6 and 6.8 $\mu\text{g/L}$
MEAN MEASURED TEST CONCENTRATIONS:	Negative Control, Solvent Control, 2.4, 1.8, 2.1, 2.3 and 3.2 $\mu\text{g/L}$
TEST DATES:	Experimental Start - April 4, 1997 Biological Termination - April 6, 1997 Experimental Termination - April 10, 1997
LENGTH OF TEST:	48 Hours

TEST ORGANISM:	Neonate Cladocerans (<i>Daphnia magna</i>)
SOURCE OF TEST ORGANISMS:	Wildlife International Ltd. cultures Easton, Maryland 21601
AGE OF TEST ORGANISMS:	< 24 hours at test initiation

48-HOUR EC50:	>6.8 $\mu\text{g/L}$ (nominal) (3.2 $\mu\text{g/L}$ mean measured concentration) (2 x HBCD's water solubility of 3.4 $\mu\text{g/L}$)
NO MORTALITY/IMMOBILITY CONCENTRATION:	6.8 $\mu\text{g/L}$ (nominal) (3.2 $\mu\text{g/L}$ mean measured concentration) (2 x HBCD's water solubility of 3.4 $\mu\text{g/L}$)
NO-OBSERVED-EFFECT-CONCENTRATION:	6.8 $\mu\text{g/L}$ (nominal) (3.2 $\mu\text{g/L}$ mean measured concentration) (2 x HBCD's water solubility of 3.4 $\mu\text{g/L}$)

INTRODUCTION

This study was conducted by Wildlife International Ltd. at their aquatic toxicology facility in Easton, Maryland for Chemical Manufacturers Association's (CMA) Brominated Flame Retardant Industry Panel. The in-life phase of the test was conducted from April 4, 1997 to April 6, 1997. Raw data generated by Wildlife International Ltd. and a copy of the final report are filed under Project Number 439A-102 in archives located on the Wildlife International Ltd. site.

OBJECTIVE

The objective of this study was to evaluate the acute toxicity of hexabromocyclododecane (HBCD) to the cladoceran (*Daphnia magna*) during a 48-hour exposure period under flow-through test conditions.

EXPERIMENTAL DESIGN

Daphnids were exposed to either one of five test concentrations, a solvent control or the negative (well water) control. Two replicate test chambers were maintained for each treatment and control group. Ten daphnids were used in each test chamber for a total of 20 daphnids per test concentration. Nominal test concentrations were selected in consultation with the Sponsor, and were based upon the solubility of the test substance in water (3.4 $\mu\text{g/L}$) and the results of an exploratory rangefinding toxicity test (Appendix I). Nominal test concentrations selected were 1.5, 2.2, 3.2, 4.6 and 5.8 $\mu\text{g/L}$. Mean measured test concentrations were analytically determined from samples of test water collected from each treatment and control group at the beginning and end of the test.

Delivery of the test substance was initiated approximately 4 days prior to the introduction of the daphnids to the test water in order to achieve equilibrium of the test substance in the test chambers. Daphnids were indiscriminately assigned to exposure chambers at test initiation. Observations of mortality/immobility and other clinical signs were made approximately 2, 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 and the no-observed-effect-concentration (NOEC) were determined by visual interpretation of the mortality, immobility and clinical observation data.

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, Hexabromocyclododecane (HBCD): A 48-Hour Flow-Through Acute Toxicity Test with the Cladoceran (*Daphnia magna*) (Appendix V). The protocol was based on procedures outlined in TSCA Title 40 of the Code of Federal Regulations, Part 797, Section 1300: *Daphnid Acute Toxicity Test* (1); Part I of OECD Guideline for Testing of Chemicals, 202: *Daphnia* sp. *Acute Immobilisation Test and Reproduction Test* (2); and ASTM Standard E729-88a, *Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians* (3).

Test Substance

The test substance consisted of a composite of hexabromocyclododecane (HBCD) samples received from three manufacturers. The materials identities and dates received from each of the manufacturers is given below:

<u>Manufacturer</u>	<u>Lot/Batch</u>	<u>Date Received</u>	Wildlife International Ltd. <u>ID No.</u>
Great Lakes Chemical Corp.	635297G-1	October 26, 1995	3462
Albemarle Corp.	33449-15X	December 20, 1995	3519
Bromine Compounds Ltd.	950303	February 5, 1996	3551

An equal part (300 g) of each of the manufacturer's HBCD material was placed in a 2-L, high density polyethylene (HDPE) bottle. The bottle was placed on a reciprocating shaker for two hours. The composite test substance was assigned Wildlife International Ltd. identification number 3577. Subsamples of the composite test substance were shipped to Albemarle Corp. for

- 10 -

characterization and homogeneity analyses. The analyses were performed on March 20, 1996. The results of the analyses indicated the composite test substance was homogeneous and contained the following components:

HBCD - beta isomer	8.5%
HBCD - alpha isomer	6.0%
<u>HBCD - gamma isomer</u>	<u>79.1%</u>
Total HBCD	93.6%

The composite test substance was stored under ambient conditions.

Preparation of Test Concentrations

One stock solution was prepared for each of the five concentrations tested. The first stock solution was prepared by dissolving hexabromocyclododecane (HBCD) in dimethylformamide (DMF) at a concentration of 0.068 mg hexabromocyclododecane (HBCD)/mL. The stock solution was inverted and mixed with a glass stir rod to aid in dissolution of the hexabromocyclododecane (HBCD). Aliquots of the stock solution were diluted with DMF to prepare four additional secondary stock solutions at concentrations of 0.046, 0.032, 0.022 and 0.015 mg/mL. Stock solutions were prepared one time during the test period. The five stocks were injected into the diluter mixing chambers where they were mixed with well water to achieve the desired test concentrations. The resultant test concentrations were not adjusted for purity of the active ingredient in the test substance. The solvent concentration in the treatment and solvent control groups was 0.10 mL/L. All test solutions in the test chambers appeared clear and colorless.

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 that used during the test in Wildlife International Ltd. well water except supplemented with selenium. Daphnids in the cultures were held for 15 to 29 days prior to collection of the juveniles for testing. The adults showed no signs of disease or stress during the holding period. During the 14-day holding period preceding the test, water temperatures ranged from 20.2 to 21.4°C. The pH of the water ranged from 8.0 to 8.5, and dissolved oxygen ranged from 8.2 to 9.0 mg/L. Instrumentation used for water measurements are described in the *Environmental Conditions* section of this report.

Neonate daphnids were obtained for testing from individual adult daphnids. The progeny from seven adults were used in the test. During the 14-day period immediately preceding the test, the adults produced an average of at least 3 young per adult per day. At test initiation, the neonate daphnids were collected from the cultures and placed in glass beakers. The daphnids were then transferred to the test chambers. All transfers were made below the water surface using wide-bore pipettes. Daphnids in the cultures were fed 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.

Test Apparatus

A continuous-flow diluter was used to deliver each concentration of the test substance, a solvent control, and a negative (dilution water) control. Syringe pumps (Harvard Apparatus) were used to deliver the five test substance stock solutions and the solvent for the solvent control into mixing chambers assigned to each treatment level and the solvent control. The stock solutions were diluted with 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. Rotameters were calibrated prior to test initiation. 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 $\pm 10\%$ of the mean for the two replicates.

The diluter was adjusted so that each test chamber received approximately 14 volume additions of test water every 24 hours. The stock solution delivery pumps were calibrated before the test, and the general operation of the diluter was checked visually at least two times per day during the test and once at the end of the test.

Test compartments were constructed from 300-mL glass beakers approximately 8 cm in diameter and 13 cm in height. Nytex® screen was attached to an opening on each side of the test compartments to allow water to flow in and out of the test compartments. The beakers were suspended in 8-L stainless steel test chambers filled with approximately 6.5 L of test water. The depth of the test water in a representative test chamber was 18 cm, whereas the depth of the test water in a representative test compartment was 7.2 cm. Test chambers were indiscriminately positioned in a temperature-controlled water bath designed to maintain a temperature of $20 \pm 1^\circ\text{C}$. The water bath was enclosed in a plexiglass ventilation hood in order to minimize potential for cross-contamination. Test chambers were labeled with the project number, test concentration and replicate.

Dilution Water

The water used for culturing and testing was freshwater obtained from a well approximately 45 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 II.

The well water was passed through a sand filter to remove particles greater than approximately $25 \mu\text{m}$, and pumped into a 37,800-L storage tank where the water was aerated with spray nozzles. Prior to use, the water again was filtered to remove microorganisms and particles. The results of periodic analyses performed to measure the concentrations of selected contaminants in well water used by Wildlife International Ltd. are presented in Appendix III.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during culturing and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (ColorTemp® 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 were on and off to avoid sudden changes in lighting. Light intensity at test initiation was approximately 242 lux at the surface of the water.

Temperature was measured in each test chamber at the beginning and end of the test using a hand-held thermometer. Temperature also was measured continuously in one negative control replicate using a Fulscope ER/C Recorder. The target test temperature during the study was $20 \pm 2^\circ\text{C}$. The pH and dissolved oxygen content of the water in alternate replicate test chambers of each treatment and control group were measured at 24-hour intervals. Hardness, alkalinity, specific conductance and total organic carbon (TOC) were measured in the dilution water at test initiation.

Measurements of pH were made using a Fisher Accumet Model 915 pH meter, and dissolved oxygen was measured using a Yellow Springs Instrument Model 51B dissolved oxygen 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). Total organic carbon was measured on a Shimadzu Model 5000 TOC Analyzer. Light intensity measurements were made using an SPER Scientific Ltd. light meter.

Observations

Observations were made to determine the number of mortalities and immobile organisms. The number of individuals exhibiting clinical signs of toxicity and/or abnormal behavior were evaluated. Observations were made approximately 2, 24, and 48 hours after test initiation.

Statistical Analyses

In this study, an EC50 could not be statistically defined due to the lack of an adequate dose response pattern. Therefore, the EC50 values were estimated from the biological response data. The no mortality/immobility concentration and NOEC were also determined by visual interpretation of the mortality/immobility and clinical observation data.

Analytical Chemistry

Samples were collected from each replicate test chamber at the beginning and end of the test to measure concentrations of the test substance. Water samples also were collected from the lowest and highest treatment groups prior to test initiation. The samples were collected in glass graduated cylinders. The samples were then transferred to glass separatory funnels and extracted as soon as possible without storage. Analytical procedures used in the extraction and analysis of the samples are provided in Appendix IV.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

The selection of exposure concentrations took into consideration the water solubility limit and a finding of no acute toxicity from an exploratory rangefinding test. The water solubility limit was determined in a generator column elution study (5) to be 3.4 $\mu\text{g/L}$. However, there was a potential to have a slight enhancement of HBCD's water solubility due to the use of dimethylformamide (DMF) as a vehicle in the diluter system. For this reason, the highest test concentration selected for the test was twice the defined solubility limit (i.e., 6.8 $\mu\text{g/L}$). The series of five nominal test concentrations used in the test were 1.5, 2.2, 3.2, 4.6 and 6.8 $\mu\text{g/L}$. In this way, the solubility limit of HBCD was bracketed by the five concentrations.

Results of the analyses of HBCD concentrations in water samples collected during the toxicity test are presented in Table I and in the analytical chemistry report (Appendix IV). Two sets of pretest samples were collected from the highest and lowest test concentrations and analyzed.

These are shown in Table 1 as Day -3 and -2 respectively. The results of those analyses indicated that exposure concentrations were steady, although somewhat lower than the expected concentrations. The toxicity test was initiated and measurements of the HBCD concentrations in all test chambers were made at the beginning and end of the test. However, those measurements indicated that HBCD concentrations were generally similar across all treatment levels. One replicate of the 4.6 and 6.8 $\mu\text{g/L}$ (nominal) treatments failed to show any measurable HBCD in the 48-hour samples. No reason for these anomalies was clear because all diluter check records and analytical process steps failed to suggest a cause for those results. While the pattern of measured HBCD concentrations was unexpected, the results may reflect a phenomenon in the delivery system whereby HBCD adsorbed to the physical surfaces of the diluter system. This is possibly due to the hydrophobic nature of HBCD (e.g., as evidenced by its nonpolar alkane structure and extremely low water solubility). This characteristic could have enabled the inert surfaces (e.g., stainless steel and Teflon[®]) of the diluter system to eventually become saturated with HBCD. As this process progressed, an equilibrium was established whereby a contribution from the surface back to the water was established. The result of this new equilibrium was that concentrations of HBCD in the dilution water were approximately the solubility of HBCD in Wildlife International Ltd. well water under flow-through conditions.

Observations and Measurements

Measurements of temperature, dissolved oxygen, and pH are presented in Table 2. Water temperatures were within the $20 \pm 2^\circ\text{C}$ range established for the test. Dissolved oxygen concentrations $\geq 97\%$ of saturation were observed throughout the test. Measurements of water pH ranged from 8.1 to 8.4. Total organic carbon in the dilution water at test initiation was $< 1.0 \text{ mg C/L}$.

Daily observations of mortality and other signs of toxicity observed during the test are shown in Table 3. Daphnids in the negative control and solvent control groups appeared healthy and normal throughout the test. With the exception of one aberrant mortality in the 4.6 $\mu\text{g/L}$ (nominal) (2.3 $\mu\text{g/L}$ mean measured concentration) treatment group, all daphnids in the 1.5, 2.2, 3.2, 4.6 and 6.8 $\mu\text{g/L}$ (nominal) (2.4, 1.8, 2.1, 2.3 and 3.2 $\mu\text{g/L}$ mean measured concentrations) treatment groups

appeared normal throughout the test with no mortalities or overt signs of toxicity. Based on these results, EC50 values for 24 and 48 hours were estimated to be greater than 6.8 $\mu\text{g/L}$ (nominal) (3.2 $\mu\text{g/L}$ mean measured concentration), the highest concentration tested.

CONCLUSIONS

The 48-hour EC50 value for daphnids exposed to hexabromocyclododecane (HBCD) was >6.8 $\mu\text{g/L}$ (nominal) (3.2 $\mu\text{g/L}$ mean measured concentration), the highest concentration tested and twice HBCD's water solubility (3.4 $\mu\text{g/L}$). Based on the mortality, immobility and observation data, the 48-hour no mortality/immobility concentration and the no-observed-effect-concentration was 6.8 $\mu\text{g/L}$ (nominal) (3.2 $\mu\text{g/L}$ mean measured concentration).

REFERENCES

- 1 **TSCA Title 40 of the Code of Federal Regulations.** 1994. Part 797, Section 1300: *Daphnid Acute Toxicity Test.*
- 2 **Organisation for Economic Cooperation and Development.** 1984. Guideline for Testing of Chemicals, 202: *Daphnia sp. Acute Immobilisation Test and Reproduction Test.*
- 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.** 1995. *Standard Methods for the Examination of Water and Wastewater.* 19th Edition, American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.
- 5 **Stenzel, J. I. and B. J. Markley.** 1997. Hexabromocyclododecane (HBCD). Determination of the Water Solubility. Wildlife International Ltd. Project Number 439C-105.

Table 1

Summary of Analytical Chemistry Data

Nominal Test Concentration ($\mu\text{g/L}$)	Replicate	Measured Concentration ($\mu\text{g/L}$)				Mean Measured Concentration ($\mu\text{g/L}$)
		Day -3	Day -2	Day 0	Day 2	
Negative Control	A	--	--	<LOQ ¹	<LOQ	--
	B	--	--	<LOQ	<LOQ	--
Solvent Control	A	--	--	<LOQ	<LOQ	--
	B	--	--	<LOQ	<LOQ	--
1.5	A	0.615	-- ²	2.17	2.48	2.4
	B	0.499	0.514	2.26	2.50	
2.2	A	--	--	1.74	1.75	1.8
	B	--	--	1.85	1.70	
3.2	A	--	--	2.16	2.48	2.1
	B	--	--	1.55	2.27	
4.6	A	--	--	2.73	1.55	2.3
	B	--	--	2.47	-- ³	
6.8	A	4.02	4.00	2.99	3.41	3.2
	B	3.93	3.82	3.33	-- ³	

¹ The limit of quantitation (LOQ) was based upon the product of the lowest standard (100 $\mu\text{g/L}$ and the dilution factor of the control samples.

² Chromatography contained co-eluting peaks. This value was 6.63 $\mu\text{g/L}$ (442% of nominal), and was considered anomalous.

³ Samples resulted in no quantifiable residues. Results excluded from the calculation of the mean.

Table 2

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

		0 Hour ¹			24 Hours		48 Hours		
Nominal Test Concentration ($\mu\text{g/L}$)	Replicate	Temp ² ($^{\circ}\text{C}$)	DO ³ (mg/L)	pH	DO (mg/L)	pH	Temp ($^{\circ}\text{C}$)	DO (mg/L)	pH
Negative Control	A	19.8	8.8	8.1	-	-	20.0	8.9	8.2
	B	19.8	-	-	9.0	8.2	20.0	-	-
Solvent Control	A	19.8	8.9	8.1	-	-	20.0	8.9	8.3
	B	19.8	-	-	9.0	8.4	20.0	-	-
1.5 (2.4)	A	19.9	8.9	8.1	-	-	19.9	8.9	8.3
	B	19.9	-	-	9.1	8.4	19.9	-	-
2.2 (1.8)	A	19.8	8.8	8.1	-	-	20.0	8.9	8.3
	B	19.8	-	-	9.0	8.4	20.0	-	-
3.2 (2.1)	A	19.9	8.8	8.1	-	-	20.0	8.8	8.3
	B	19.9	-	-	9.1	8.4	20.0	-	-
4.6 (2.3)	A	19.8	8.8	8.1	-	-	20.0	8.9	8.3
	B	19.8	-	-	9.0	8.4	20.0	-	-
6.8 (3.2)	A	19.9	8.8	8.1	-	-	20.0	8.9	8.3
	B	19.9	-	-	9.0	8.4	20.0	-	-

¹ The 0-hour dilution water measurements for hardness, alkalinity and specific conductance were 132 mg/L as CaCO_3 , 176 mg/L as CaCO_3 , and 320 $\mu\text{mhos/cm}$, respectively.

² Temperature measured continuously during the test ranged from approximately 19.5 to 20.0 $^{\circ}\text{C}$.

³ A dissolved oxygen concentration of 8.8 mg/L represents 97% saturation at 20 $^{\circ}\text{C}$ in freshwater.

Note: Values in parentheses are mean measured test concentrations

Table 3
Cumulative Percent Mortality and Treatment-Related Effects¹

Nominal Test Concentration (µg/L)	Replicate	Daphnia/ Replicate	2 Hours			24 Hours			48 Hours			Percent Immobile and Dead
			Cumulative Dead	Number Immobile	Effects	Cumulative Dead	Number Immobile	Effects	Cumulative Dead	Number Immobile	Effects	
Negative Control	A	10	0	0	10 AN	0	0	10 AN	0	0	10 AN	0
	B	10	0	0	10 AN	0	0	10 AN	0	0	10 AN	0
Solvent Control	A	10	0	0	10 AN	0	0	10 AN	0	0	10 AN	0
	B	10	0	0	10 AN	0	0	10 AN	0	0	10 AN	0
1.5 (2.4)	A	10	0	0	10 AN	0	0	10 AN	0	0	10 AN	0
	B	10	0	0	10 AN	0	0	10 AN	0	0	10 AN	0
2.2 (1.8)	A	10	0	0	10 AN	0	0	10 AN	0	0	10 AN	0
	B	10	0	0	10 AN	0	0	10 AN	0	0	10 AN	0
3.2 (2.1)	A	10	0	0	10 AN	0	0	10 AN	0	0	10 AN	0
	B	10	0	0	10 AN	0	0	10 AN	0	0	10 AN	0
4.6 (2.3)	A	10	0	0	10 AN	0	0	10 AN	0	0	10 AN	5
	B	10	0	0	10 AN	0	0	10 AN	0	0	10 AN	0
6.8 (3.2)	A	10	0	0	10 AN	0	0	10 AN	0	0	10 AN	0
	B	10	0	0	10 AN	0	0	10 AN	0	0	10 AN	0

¹ Observed Effects: AN = Appears Normal.

Note: Values in parentheses are mean measured test concentrations.

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APPENDIX I

Rangefinding Results

Sponsor:	CMA's Brominated Flame Retardant Industry Panel	
Test Substance:	Hexabromocyclododecane (HBCD)	
Test Organism:	Cladoceran, <i>Daphnia magna</i>	
Dilution Water:	Well Water	
Nominal Test Concentration ($\mu\text{g/L}$)	No. Dead in 24 Hours/Cumulative No. Dead ¹ /No. Exposed (Observed) ²	
	24 Hours	48 Hours
Negative Control	0/0/5 (5 AN)	0/0/5 (5 AN)
Solvent Control	0/0/5 (5 AN)	0/0/5 (5 AN)
0.16	0/0/5 (5 AN)	0/0/5 (5 AN)
0.54	0/0/5 (5 AN)	0/0/5 (5 AN)
1.8	0/0/5 (5 AN)	0/0/5 (5 AN)
6.0	0/0/5 (5 AN)	0/0/5 (5 AN)
20	0/0/5 (5 AN)	0/0/5 (5 AN)

¹ Cumulative number dead and immobile.
² Observations: AN = Appears Normal.

APPENDIX II

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

Sponsor:	CMA's Brominated Flame Retardant Industry Panel	
Test Substance:	Hexabromocyclododecane (HBCD)	
Test Organism:	Cladocera, <i>Daphnia magna</i>	
Dilution Water:	Well Water	
	Mean	Range
Specific Conductance (μ mhos/cm)	303 (N = 4)	300 - 310
Hardness (mg/L as CaCO ₃)	134 (N = 4)	132 - 136
Alkalinity (mg/L as CaCO ₃)	179 (N = 4)	178 - 180
pH	8.3 (N = 4)	8.3 - 8.4

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APPENDIX III
Analyses of Pesticides, Organics, Metals and Other Inorganics
in Wildlife International Ltd. Well Water¹

Sponsor:	CMA's Brominated Flame Retardant Industry Panel		
Test Substance:	Hexabromocyclododecane (HBCD)		
Test Organism:	Cladoceran, <i>Daphnia magna</i>		
Dilution Water:	Well Water		
ANALYSIS		MEASURED CONCENTRATION	
Miscellaneous Measurements			
Total Dissolved Solids		261	mg/L
Ammonia Nitrogen	<	0.050	mg/L
Total Organic Carbon ²	<	1.0	mg/L
Total Cyanide	<	5.0	µg/L
Organochlorines and PCBs			
Aldrin	<	0.005	µg/L
Alpha BHC	<	0.005	µg/L
Beta BHC	<	0.005	µg/L
Delta BHC	<	0.005	µg/L
Gamma BHC (Lindane)	<	0.005	µg/L
Chlordane	<	0.025	µg/L
DDD, pp'	<	0.005	µg/L
DDE, pp'	<	0.005	µg/L
DDT, pp'	<	0.005	µg/L
Dieldrin	<	0.005	µg/L
Endosulfan, A	<	0.005	µg/L
Endosulfan, B	<	0.005	µg/L
Endosulfan Sulfate	<	0.005	µg/L
Endrin	<	0.005	µg/L
Endrin Aldehyde	<	0.005	µg/L
Heptachlor	<	0.005	µg/L
Methoxychlor	<	0.005	µg/L
Heptachlor Epoxide	<	0.005	µg/L
Toxaphene	<	0.500	µg/L
PCB-1016	<	0.250	µg/L
PCB-1221	<	0.250	µg/L
PCB-1232	<	0.250	µg/L
PCB-1242	<	0.250	µg/L
PCB-1248	<	0.250	µg/L
PCB-1254	<	0.250	µg/L
PCB-1260	<	0.250	µg/L
Metals and Other Inorganics			
Aluminum		69.9	µg/L
Arsenic	<	2.5	µg/L
Beryllium	<	4.0	µg/L
Boron		142	µg/L
Cadmium	<	5.0	µg/L
Calcium		34.3	mg/L
Chromium	<	10.0	µg/L
Cobalt	<	20.0	µg/L
Copper		40.4	µg/L
Iron	<	45.0	µg/L
Lead	<	2.0	µg/L
Magnesium		13.5	mg/L
Manganese	<	5.0	µg/L
Mercury	<	0.20	µg/L
Molybdenum	<	10.0	µg/L
Nickel	<	15.0	µg/L
Potassium		6.16	mg/L
Selenium	<	2.5	µg/L
Silver	<	5.0	µg/L
Sodium		21.6	mg/L
Zinc	<	30.0	µg/L

¹ Analyses performed by Environmental Science & Engineering, Inc., Gainesville, Florida for samples collected on August 21, 1996.

² Analyses performed by Wildlife International Ltd. for the sample collected on August 14, 1995.

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APPENDIX IV

**THE ANALYSIS OF HEXABROMOCYCLODODECANE (HBCD)
IN FRESHWATER (WELL WATER)**

IN SUPPORT OF

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 439A-102

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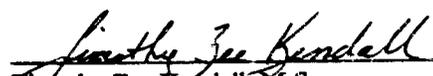
REPORT APPROVAL

SPONSOR: Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel

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

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 439A-102

PRINCIPAL INVESTIGATOR:



Timothy Zee Kendall, M.S.
Laboratory Supervisor, Analytical Chemistry

5/21/97

DATE

MANAGEMENT:



Willard B. Nixon, Ph.D.
Manager, Analytical Chemistry

5/21/97

DATE

APPENDIX IV

Introduction

Fresh samples of the test media solutions (test samples) were collected from a flow-through acute aquatic toxicity study designed to determine the effects of hexabromocyclododecane (HBCD) to the cladoceran (*Daphnia magna*). This study was conducted by Wildlife International Ltd. and identified as Project Number 439A-102. The analyses of these test samples were performed at Wildlife International Ltd. by high performance liquid chromatography (HPLC) with UV detection. The analytical method was verified on March 6, 1997 at Wildlife International Ltd. Test samples were received for analysis between April 1 and April 6, 1997 and extracted on each sample receipt date.

Test Substance

The hexabromocyclododecane (HBCD) test substances were received at Wildlife International Ltd. on October 26, 1995, December 20, 1995 and February 5, 1996 and assigned Wildlife International Ltd. identification numbers 3462, 3519 and 3551, respectively. On February 19, 1996, equal parts (300 g) of each were placed in a 2-L high density polyethylene (HDPE) bottle and placed on a reciprocating shaker for two hours. The ensuing composite test substance was assigned Wildlife International Ltd. identification number 3577. Results from the analyses of this composite showed the mixture to be homogeneous and the reported purity to be 93.6%. The composite test substance was used to prepare calibration standards and matrix fortifications and was stored under ambient conditions.

Analytical Method

The analytical method consisted of rinsing all glassware with a 10% phosphoric acid solution followed by NANOpure® water. The separatory funnels and roundbottom flasks were rinsed with dichloromethane. The requisite volume of test solution was siphoned into a graduated cylinder and transferred directly to a separatory funnel. A 100 mL aliquot of dichloromethane was added to the sample. The solution was shaken (with venting) for approximately one minute after which the two

APPENDIX IV

liquid phases were allowed to separate. The lower organic phase was drained into a 500-mL roundbottom flask. An additional 100 mL of dichloromethane was added to the aqueous sample contained in the separatory funnel. The extraction/separation process was repeated and the organic extract was combined in the roundbottom flask with the first extract. Using a waterbath maintained at a temperature of between 40 - 50°C, the organic extract was rotary evaporated to a volume of approximately 1-2 mL. The residual dichloromethane was evaporated to dryness under a gentle stream of nitrogen. The hexabromocyclododecane (HBCD) residues were dissolved using 50% acetonitrile:50% water. The diluted extract was transferred to autosampler vials and submitted for analysis by high performance liquid chromatography.

Concentrations of hexabromocyclododecane (HBCD) in the test samples were determined by high performance liquid chromatography using a Hewlett-Packard Model 1090 High Performance Liquid Chromatograph (HPLC) equipped with a Waters Model 486 UV/VIS detector. Separations were achieved using in series, two Zorbax Rx C₈ columns (15 cm x 4.6 mm ID, 5 μm). The instrument parameters are summarized in Table 1 and a method flow chart is provided in Figure 1.

Calibration Curve, Limit of Detection, and Limit of Quantitation

Calibration standards of hexabromocyclododecane (HBCD), ranging in concentration from 100 to 1000 μg/L, were analyzed with each series of test samples. Linear regression equations were generated using the peak area responses versus the respective concentrations of the calibration standards. A representative calibration curve is presented in Figure 2. The concentration of hexabromocyclododecane (HBCD) in the test samples was determined by substituting the area responses into the applicable linear regression equation. Representative chromatograms of low and high calibration standards are shown in Figures 3 and 4, respectively.

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The instrument limit of detection (LOD) for this study was set based upon the injection volume (250 μ L) and the lowest standard concentration (100 μ g/L). The LOD was set at 25 ng injected on-column. The method limit of quantitation (LOQ) for these analyses was set at 0.400 μ g/L based upon the product of the lowest standard (100 μ g/L) and the dilution factor of the control samples (0.004).

Matrix Blank and Fortification Samples

Along with the actual test sample analyses, two matrix blanks were analyzed to determine possible interference. No interferences were observed at or above the LOQ during the test sample analyses (Table 2). A representative chromatogram of a matrix blank is presented in Figure 5.

Well water samples were fortified at 1.00, 3.00 and 10.0 μ g/L and analyzed concurrently with the test samples to determine the mean procedural recovery (Table 3). Measured concentrations for the test samples were corrected for the mean procedural recovery of 95.1%. A representative chromatogram of a matrix fortification is presented in Figure 6.

RESULTS

Sample Analysis

Difficulties were encountered during the test sample analyses of HBCD in well water. The processing portion of the method required that a relatively large volume of water (500 - 1000 mL) be extracted using dichloromethane to achieve the desired LOQ. Additionally, a large injection volume (250 μ L) and a non-selective wavelength (210 nm) were required in the analytical method since HBCD is a comparatively weak chromophore. Several measures were used in order to minimize contamination in the extracts including acid washing of glassware, isolation of glassware by test concentration and manual integration of the HBCD peak. Generally, these procedures appeared adequate to resolve the peak representing HBCD from adjacent components. However, some results yielded excessively high recoveries which could not be remedied (see Tables 4-6).

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Prior to initiation of the test, pre-test samples were collected and analyzed for diluter verification (Tables 4 and 5). Test samples were collected from the flow-through acute toxicity test with the cladoceran (*Daphnia magna*) at test initiation (0 Hour) and at test termination (48 Hours). The measured concentrations of hexabromocyclododecane (HBCD) in the pre-test samples ranged from 33 to 59% of the nominal concentrations (Tables 4 and 5). The measured concentrations of hexabromocyclododecane (HBCD) in the test samples collected at initiation of exposure (Day 0) of the test organisms ranged from 44 to 151% of the nominal concentrations (Table 6). Test samples collected at test termination (48 Hours), had a measured concentration range of below detection to 167% of the nominal values. A representative chromatogram of a sample is shown in Figure 7.

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Table 1

Typical HPLC Operational Parameters

INSTRUMENT:	Hewlett-Packard Model 1090 High Performance Liquid Chromatograph equipped with a Waters Model 486 UV/VIS Detector		
ANALYTICAL COLUMN:	Zorbax Rx C ₈ Column (15 cm x 4.6 mm ID, 5 μm); 2 in series		
STOP TIME:	27 minutes		
FLOW RATE:	1.00 mL/minute		
OVEN TEMPERATURE:	40°C		
MOBILE PHASE:	Solvent A: 30% acetonitrile : 70% H ₂ O Solvent B: 95% acetonitrile : 5% H ₂ O		
GRADIENT TABLE:	<u>Time</u>	<u>% A</u>	<u>% B</u>
	0.01	45	55
	2.00	45	55
	12.0	--	100
	22.0	--	100
	22.1	45	55
	27.0	45	55
INJECTION VOLUME:	250 μL		
HEXABROMOCYCLO- DODECANE (HBCD) PEAK RETENTION TIME:	Approximately 14.7 minutes		
PRIMARY ANALYTICAL WAVELENGTH:	210 nm		

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Table 2

Matrix Blanks Analyzed Concurrently During Test Sample Analysis

Sample		Measured Concentration of Hexabromocyclododecane (HBCD) ($\mu\text{g/L}$) ¹
Number (439A-102-)	Type	
MAB-1	Matrix Blank	< LOQ
MAB-2	Matrix Blank	< LOQ

¹ The limit of quantitation (LOQ) was set at 0.400 $\mu\text{g/L}$ and was based upon the product of the lowest standard (100 $\mu\text{g/L}$) and the dilution factor of the control samples (0.004).

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Table 3

Matrix Fortifications Analyzed Concurrently During Test Sample Analysis

Sample Number (439A-102-)	Concentrations of Hexabromocyclododecane (HBCD) ($\mu\text{g/L}$)		Percent Recovery
	Fortified	Measured	
MAS-1	1.00	0.972	97.2
MAS-2	3.00	2.92	97.4
MAS-3	10.0	8.98	89.8
MAS-4	1.00	0.933	93.3
MAS-5	3.00	2.83	94.2
MAS-6	10.0	9.85	98.5
		Mean =	95.1%
		Standard Deviation =	3.27
		n =	6

Analytical results were generated using Excel 4.0 in the full precision mode. Manual calculations may differ slightly.

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Table 4

Measured Concentrations of Hexabromocyclododecane (HBCD) in First Pre-test Diluter Verification Samples

Nominal Test Concentration ($\mu\text{g/L}$)	Sample Number (439A-102-PT-)	Sampling Time Prior to Test (Days)	Measured Concentration of Hexabromocyclododecane (HBCD) ($\mu\text{g/L}$)	Mean	Mean Percent of Nominal
1.5	1	3	0.615	0.56	37
	2	3	0.499		
6.8	3	3	4.02	4.0	59
	4	3	3.93		

Fortification recoveries ranged from 99 to 109%.

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Table 5

Measured Concentrations of Hexabromocyclododecane (HBCD) in Second Pre-test Diluter Verification Samples

Nominal Test Concentration ($\mu\text{g/L}$)	Sample Number (439A-102-PT-)	Sampling Time Prior to Test (Days)	Measured Concentration of Hexabromocyclododecane (HBCD) ($\mu\text{g/L}$)	Mean	Mean Percent of Nominal
1.5	5	2	6.63 ¹	0.51	34
	6	2	0.514		
6.8	7	2	4.00	3.9	57
	8	2	3.82		

¹ Sample was rejected due to co-elution with an adjacent peak.

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Table 6
 Measured Concentrations of Hexabromocyclododecane (HBCD) in Well Water Samples
 from a Daphnia Flow-Through Acute Toxicity Test

Nominal Test Concentration ($\mu\text{g/L}$)	Sample Number (439A-102-)	Sampling Time (Hours)	Hexabromocyclododecane (HBCD) Concentration ($\mu\text{g/L}$)		Mean Percent of Nominal
			Measured ¹	Corrected ²	
0.0 (Negative Control)	1	0	< LOQ	--	--
	2	0	< LOQ	--	--
	15	48	< LOQ	--	--
	16	48	< LOQ	--	--
0.0 (Solvent Control)	3	0	< LOQ	--	--
	4	0	< LOQ	--	--
	17	48	< LOQ	--	--
	18	48	< LOQ	--	--
1.5	5	0	2.06	2.17	160
	6	0	2.15	2.26	
	19	48	2.36	2.48	
	20	48	2.38	2.50	
2.2	7	0	1.65	1.74	82
	8	0	1.76	1.85	
	21	48	1.66	1.75	
	22	48	1.62	1.70	

¹ The limit of quantitation (LOQ) was based upon the product of the lowest standard (100 $\mu\text{g/L}$) and the dilution factor of the control samples (0.004).

² Values were corrected for a mean procedural recovery of 95.1%.

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Table 6 (Continued)
 Measured Concentrations of Hexabromocyclododecane (HBCD) in Well Water Samples
 from a Daphnia Flow-Through Acute Toxicity Test

Nominal Test Concentration ($\mu\text{g/L}$)	Sample Number (439A-102-)	Sampling Time (Hours)	Hexabromocyclododecane (HBCD) Concentration ($\mu\text{g/L}$)		Mean Percent of Nominal
			Measured ¹	Corrected ²	
3.2	9	0	2.05	2.16	66
	10	0	1.47	1.55	
	23	48	2.36	2.48	
	24	48	2.16	2.27	
4.6	11	0	2.60	2.73	50
	12	0	2.35	2.47	
	25	48	1.47	1.55	
	26	48	<0.800 ³	--	
6.8	13	0	2.84	2.99	47
	14	0	3.17	3.33	
	27	48	3.24	3.41	
	28	48	<0.800 ³	--	

¹ The limit of quantitation (LOQ) was based upon the product of the lowest standard (100 $\mu\text{g/L}$) and the dilution factor of the control samples (0.004).

² Values were corrected for a mean procedural recovery of 95.1%.

³ Samples resulted in no quantifiable residues. Results were excluded from the calculation of the mean.

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**METHOD OUTLINE FOR THE ANALYSIS
OF HEXABROMOCYCLODODECANE (HBCD) IN WELL WATER**

Rinse all glassware with 10% H_3PO_4 followed by NANOpure® water. Rinse separatory funnels and roundbottom flasks with dichloromethane.

Prepare quality control samples by directly fortifying freshwater (contained in separatory funnels) with an appropriate HBCD stock solution.

Collect each sample (from the appropriate test chamber) into a graduated cylinder and transfer directly into a separatory funnel.

Using a graduated cylinder, add 100 mL of dichloromethane to each sample. Stopper and shake each sample (with venting) for approximately one minute. Allow the organic and aqueous layer to separate. Drain the dichloromethane (lower) layer into a 500-mL roundbottom flask.

Add an additional 100 mL of dichloromethane to the separatory funnel containing the aqueous sample. Shake and partition as described above and combine each extract in its respective roundbottom flask; the total volume should be approximately 200 mL.

Rotary evaporate each sample to approximately 1-2 mL using a waterbath maintained at 40-50°C. Do not rotary evaporate the extract to dryness.

Evaporate the samples to dryness under a gentle stream of nitrogen.

Add the requisite volume of 50% CH_3CN : 50% H_2O to each flask and swirl to ensure solvation of the residues.

Transfer the diluted samples to autosampler vials and submit for HPLC/UV analysis.

Figure 1. Analytical method flow chart for the analysis of hexabromocyclododecane (HBCD) in well water.

APPENDIX IV

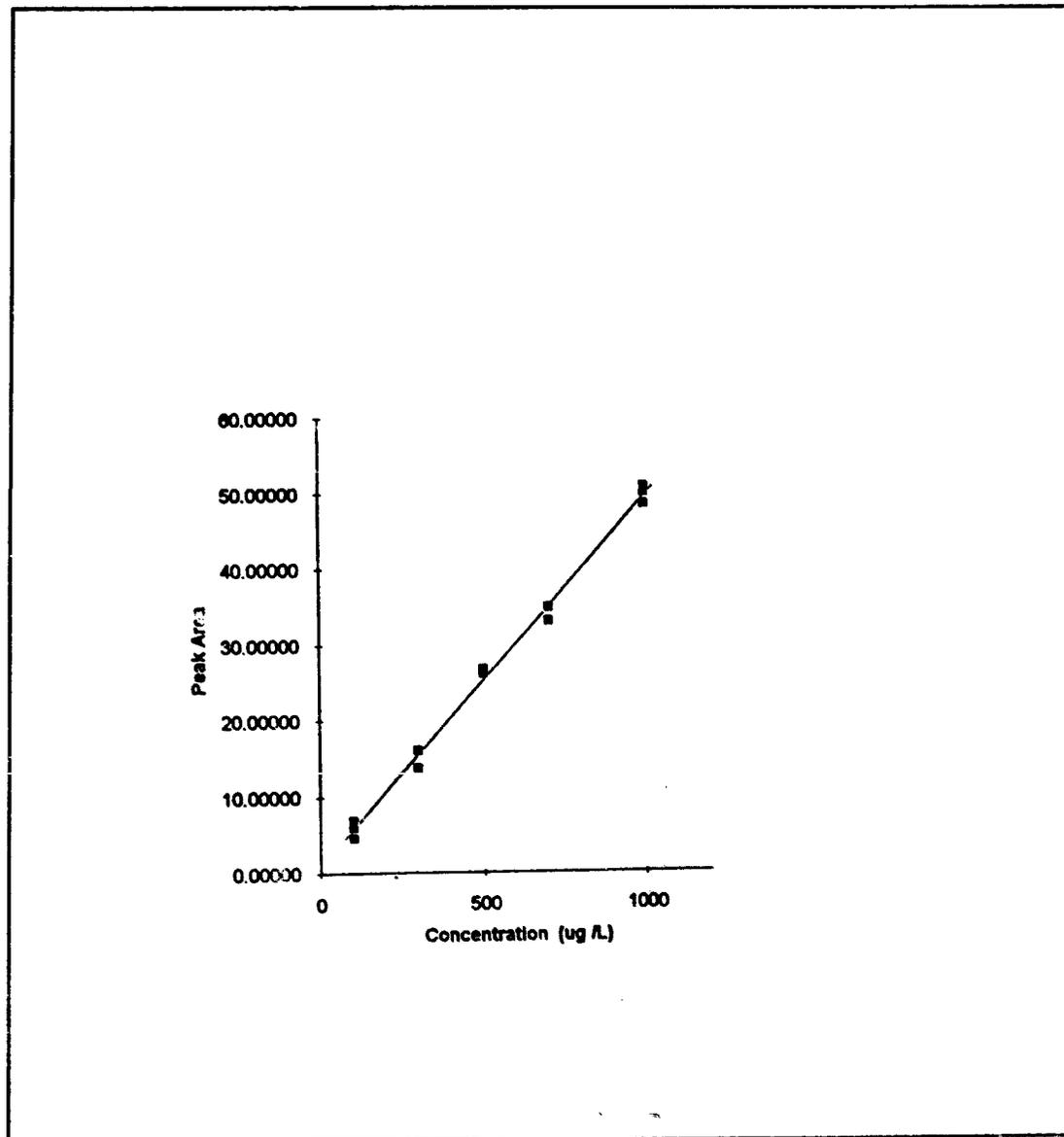


Figure 2. A representative calibration curve for hexabromocyclododecane (HBCD).
Slope = 0.05; Intercept = 0.95901; $r^2 = 0.9949$.

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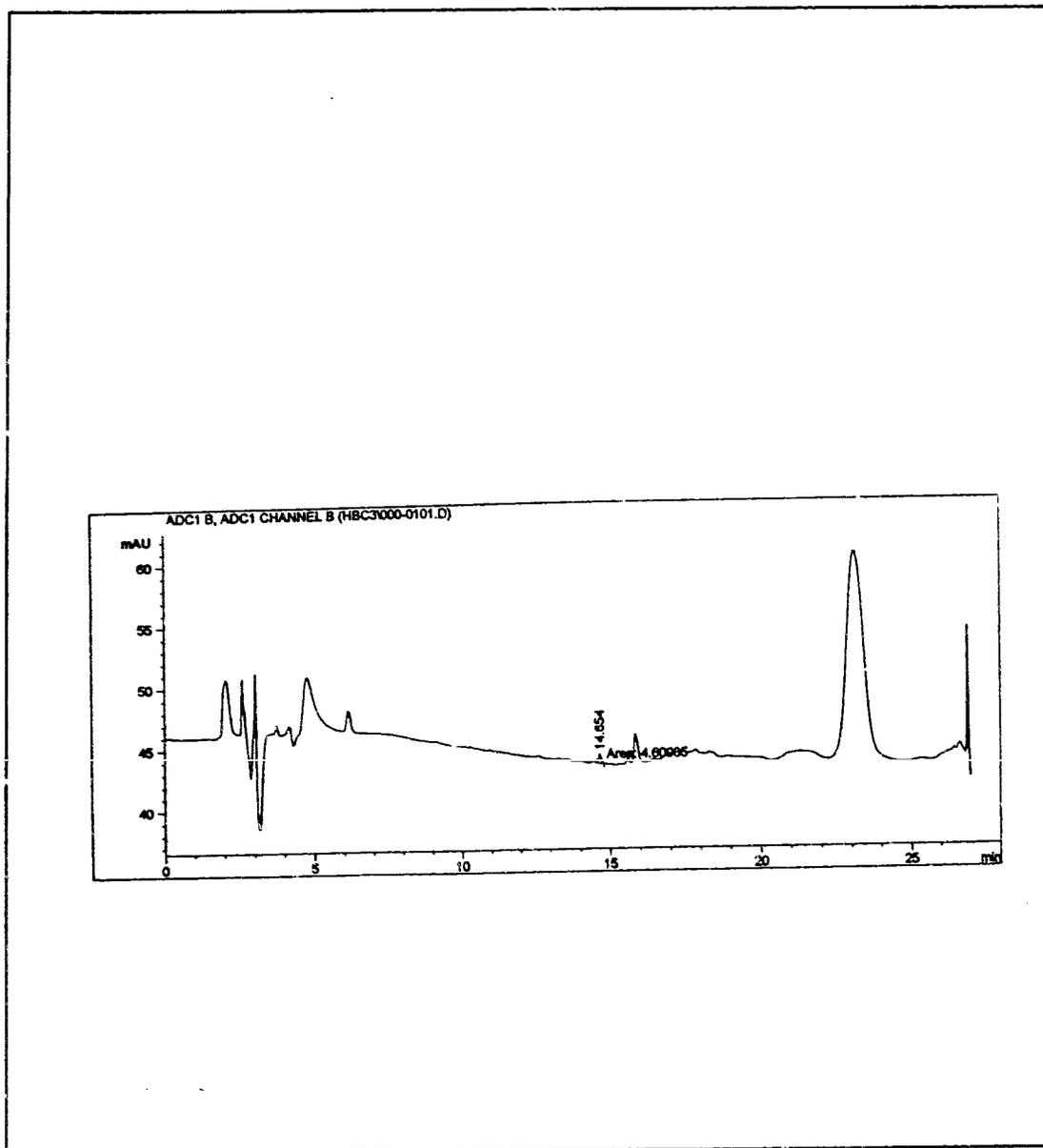


Figure 3. A representative chromatogram of a .00 $\mu\text{g/L}$ hexabromocyclododecane (HBCD) standard (25 ng on-column).

APPENDIX IV

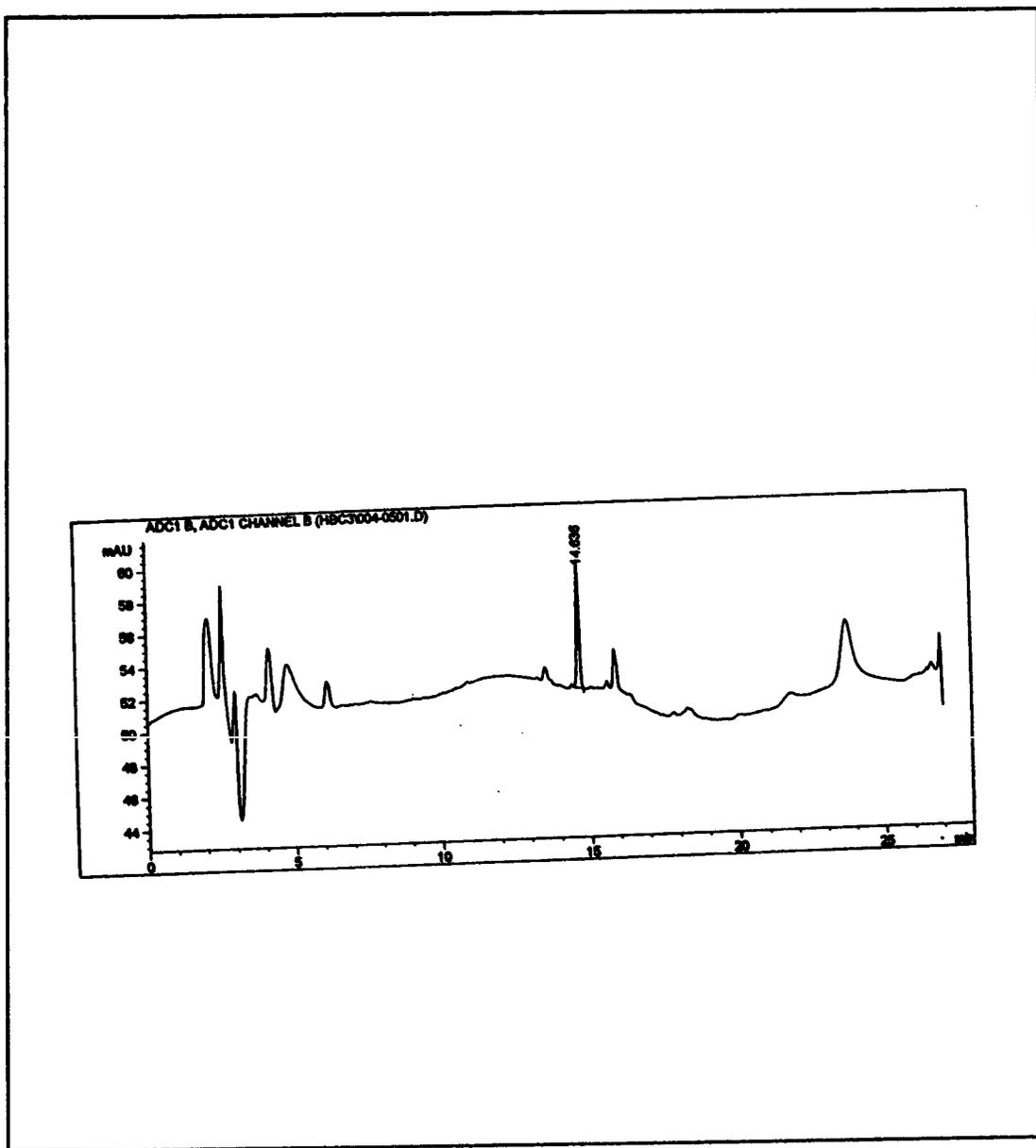


Figure 4. A representative chromatogram of a 1000 $\mu\text{g/L}$ hexabromocyclododecane (HBCD) standard (250 ng on-column).

APPENDIX IV

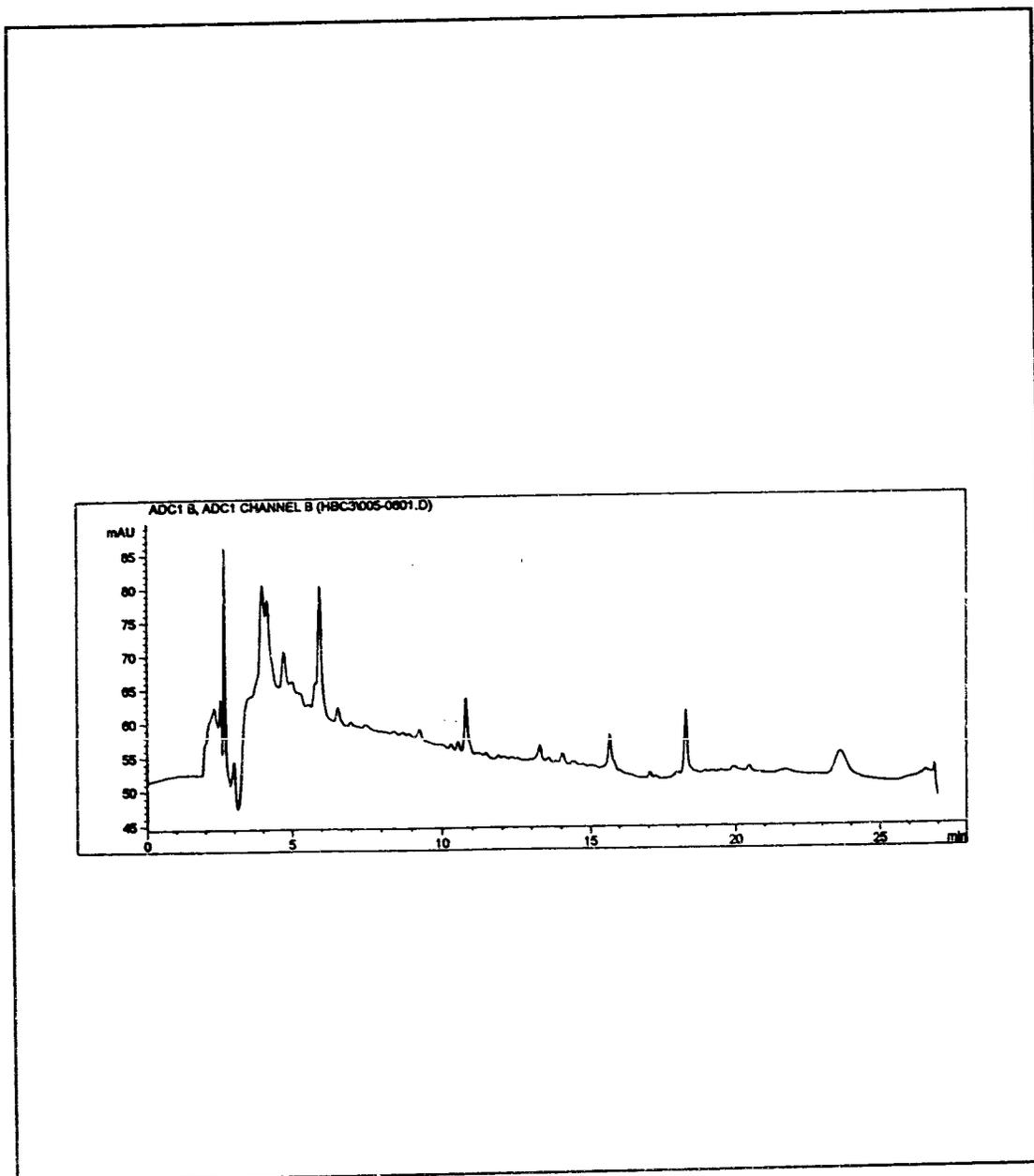


Figure 5. A representative chromatogram of a matrix blank, 439A-102-MAB-1.

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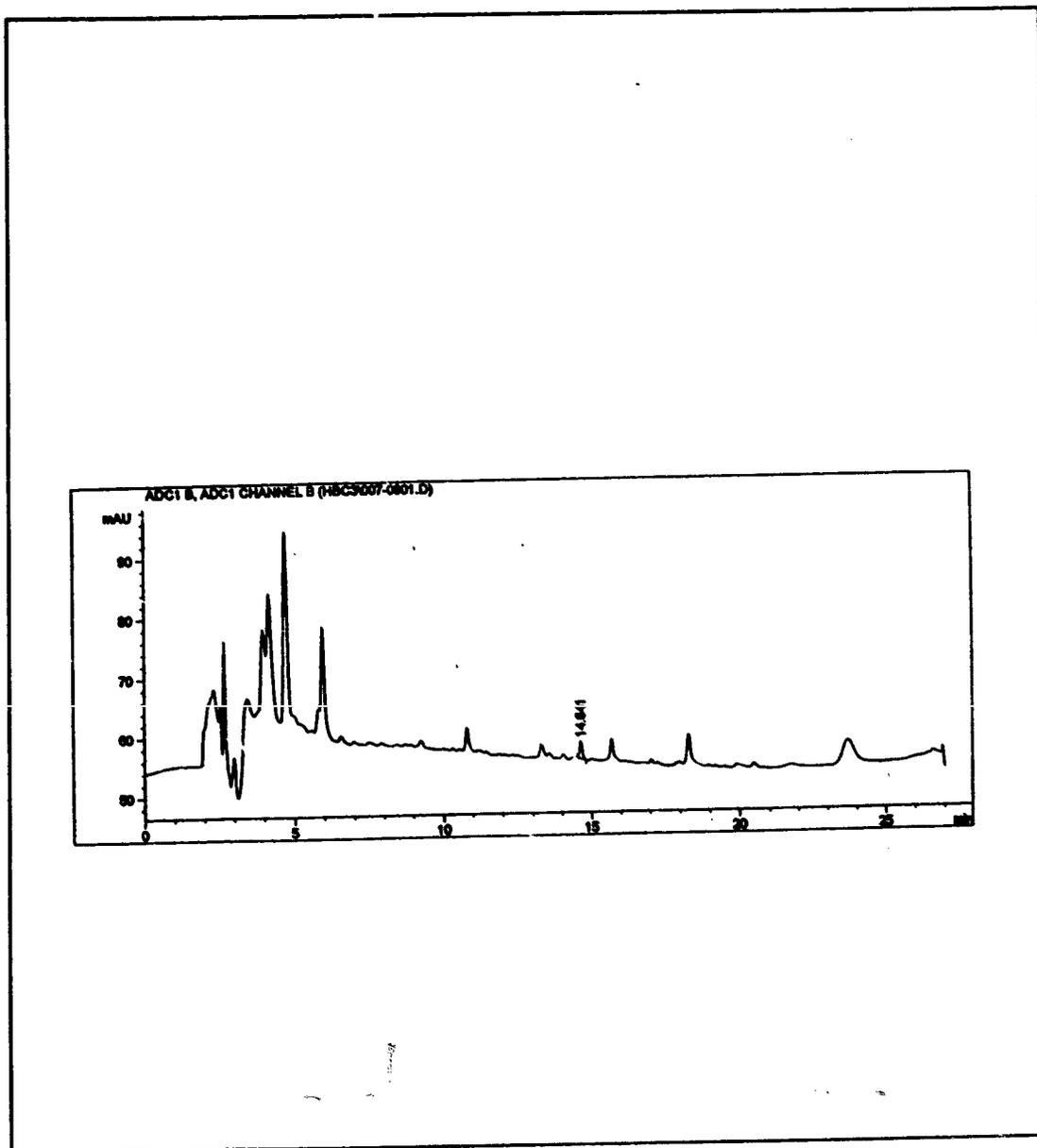


Figure 6. A representative chromatogram of a matrix fortification, 439A-102-MAS-2 (3.00 $\mu\text{g/L}$).

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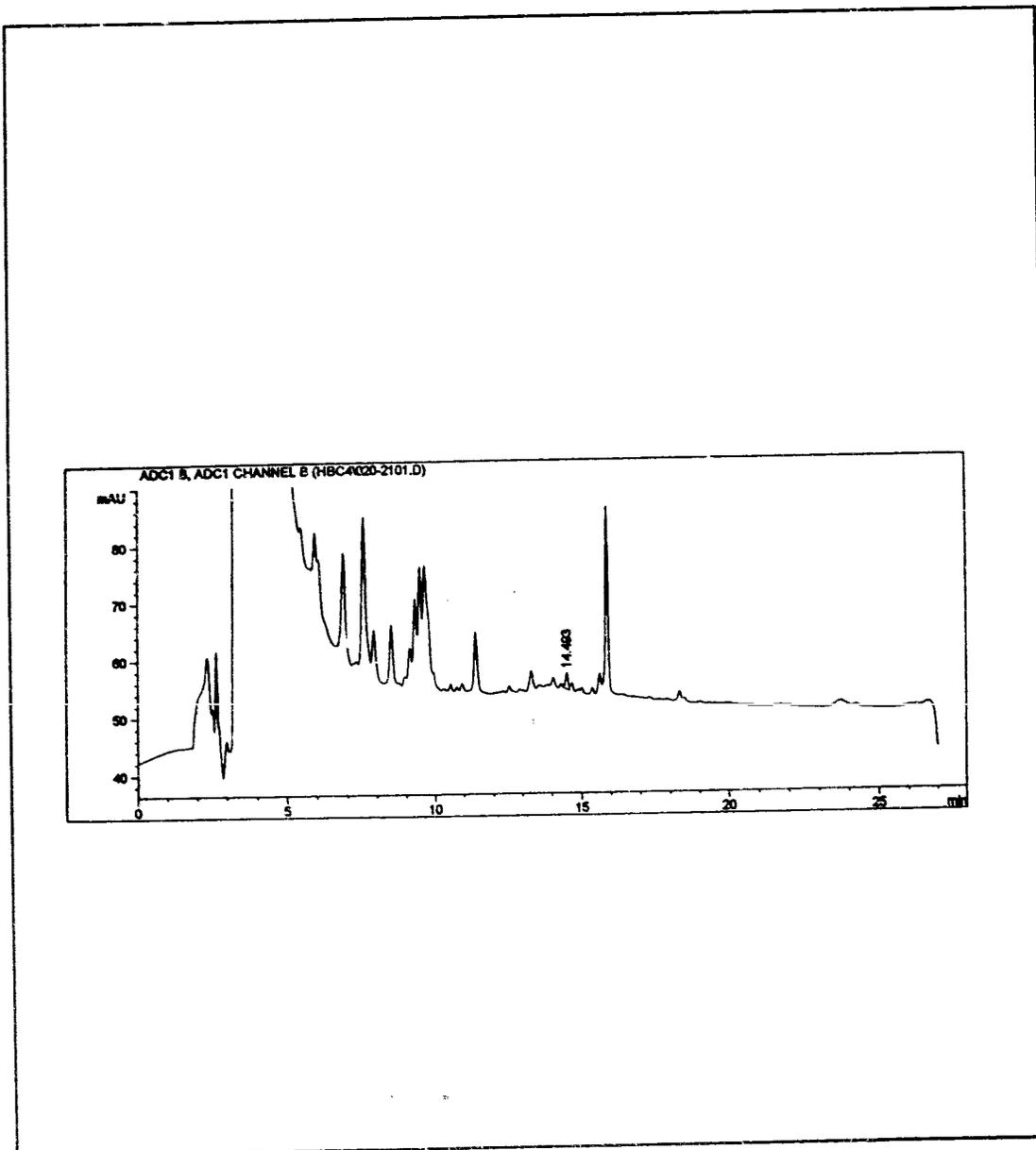


Figure 7. A representative chromatogram of a test sample, 439A-102-24 (3.2 $\mu\text{g/L}$ nominal concentration).

0045

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APPENDIX V

Protocol, Protocol Amendments and Deviation

07.45

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WILDLIFE INTERNATIONAL LTD.

PROJECT NO.: 439A-102
Page 1 of 2

AMENDMENT TO STUDY PROTOCCL

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

PROTOCOL NO.: 439/021296/DAP-48H2/SUB439

AMENDMENT NO.: 1

SPONSOR: Chemical Manufacturers Association's
Brominated Flame Retardant Industry Panel

PROJECT NO.: 439A-102

EFFECTIVE DATE: March 25, 1997

AMENDMENT: Page 2:

Add: Proposed Dates:
Experimental Start Date: April 1, 1997
Experimental Termination Date: April 4, 1997
Study Room: Diluter #2
Test Concentrations: 1.5, 2.2, 3.2, 4.6, 6.8 $\mu\text{g/L}$ and Negative and Solvent Controls

REASON:

The above information was not available at the time the protocol was signed and is needed to complete the protocol.

AMENDMENT: Page 8, Biological Measurements, 1st sentence:

Change: Observations of mortality, immobilization and clinical signs of toxicity will be made between at 24 hour intervals (± 1 hour) throughout the test.

To: Observations of mortality, immobilization and clinical signs of toxicity will be made between 0 and 24 hours and at 24 and 48 hours (± 1 hour).

REASON:

Clarification of the frequency of biological measurements performed during the test.

APPENDIX V

WILDLIFE INTERNATIONAL LTD.

PROJECT NO.: 439A-102
Page 2 of 2

AMENDMENT: Page 9, Sampling for Analytical Measurements, 1st paragraph, 5th sentence:

Delete: An assessment of the stability of the test substance in well water and in carrier solvent will be performed under a separate protocol (Analytical Method Verification for the Determination of Hexabromocyclododecane (HBCD) in Well Water; Wildlife International Ltd. Project #439/021296/METVER.FW/SUB439).

REASON:

Stability of HBCD will be demonstrated during the aquatic...

AMENDMENT: Page 10, Analytical Chemistry, 1st sentence:

Change: Chemical analysis of the samples will be performed by Wildlife International Ltd. The analytical method used will be based upon methodology provided by the Sponsor (Appendix II).

To: Chemical analysis of the samples will be performed by Wildlife International Ltd. The analytical method used will be based upon method outline provided in Appendix II.

REASON:

Clarification of the method used in the test.

Willie G... 3-26-97
STUDY DIRECTOR DATE

Janey Stewart 3/26/97
LABORATORY MANAGEMENT DATE

Harmukh Shah 3/28/97
SPONSOR'S REPRESENTATIVE DATE

SADJ

QA Reviewed: 3-24-97 LD

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APPENDIX V

WILDLIFE INTERNATIONAL LTD.

PROJECT NO.: 439A-102
Page 1 of 2

AMENDMENT TO STUDY PROTOCOL

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

PROTOCOL NO.: 439/021296/DAP-48H2/SUB439

AMENDMENT NO.: 2

SPONSOR: Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel

PROJECT NO.: 439A-102

EFFECTIVE DATE: March 25, 1997

AMENDMENT: Add the following page as Appendix II.

REASON:

To complete Appendix II (Analytical Method).

Will C Guss 3-26-97
STUDY DIRECTOR DATE

Cheryl Switzer 3/26/97
LABORATORY MANAGEMENT DATE

Harmukh Shah 3/28/97
SPONSOR'S REPRESENTATIVE DATE

SADJ

QA Reviewed: 3-24-97

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PROJECT NO.: 439A-102

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METHOD OUTLINE FOR THE PROCESSING
OF HBCD IN WELL WATER

Rinse all glassware with 10% H₃PO₄, followed by NANOpure® water.
Rinse separatory funnels and round-bottom flasks with dichloromethane.

Prepare recovery samples by directly fortifying freshwater (contained in
separatory funnels) with an appropriate HBCD stock solution.

Using a graduated cylinder, add 100 mL of dichloromethane to each sample. Stopper and shake each
sample (with venting) for approximately one minute. Allow the organic and aqueous layers
to separate. Drain the dichloromethane (lower) layer into a 500-mL round-bottom flask.

Add an additional 100 mL of dichloromethane to the separatory funnel containing the aqueous
sample. Shake and partition as described above and combine each extract in its respective
round-bottom flask; the total volume should be approximately 200 mL.

Rotary evaporate each sample to approximately 1-2 mL using a waterbath
maintained at 40-50°C. Do not evaporate the extract to dryness.

Evaporate the samples to dryness under a gentle stream of nitrogen.

Add the requisite volume of 50% CH₃CN:50% H₂O to each flask and swirl
to ensure solvation of residues.

Transfer the diluted samples to autosampler vials and submit for HPLC/UV analysis.

QA Revised: 4/3-2-97

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APPENDIX V

PROTOCOL

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

TSCA Title 40 of the Code of Federal Regulations
Part 797, Section 1300

and

Organisation for Economic Cooperation and Development
OECD Guideline 202, Part I

Submitted to

Chemical Manufacturers Association'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 12, 1996

PROTOCOL NO.: 439/021296/DAP-48H2/SUB439

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WILDLIFE INTERNATIONAL LTD.

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

SPONSOR: Chemical Manufacturers Association's
Brominated Flame Retardant Industry Panel
1300 Wilson Boulevard
Arlington, Virginia 22209

SPONSOR'S REPRESENTATIVE: Dr. Hasnukh Shah

TESTING FACILITY: Wildlife International Ltd.
8598 Commerce Drive
Easton, Maryland 21601

STUDY DIRECTOR: William C. Graves, Senior Aquatic Biologist

LABORATORY MANAGEMENT: James P. Swigert, Ph.D.
Manager of Aquatic Toxicology

FOR LABORATORY USE ONLY

Proposed Dates:	
Experimental Start Date: _____	Experimental Termination Date: _____
Project No.: <u>439A-102</u>	Study Room: _____
Test Concentrations: _____	Int./Date: _____
Test Substance No.: _____	Receipt Date: _____

PROTOCOL APPROVAL

William C. Graves 5/15/96
STUDY DIRECTOR DATE

James P. Swigert 5/15/96
LABORATORY MANAGEMENT DATE

Hasnukh Shah 2-14-96
SPONSOR'S REPRESENTATIVE DATE

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APPENDIX V

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 upon procedures in TSCA Title 40 of the Code of Federal Regulations, Part 797, Section 1300: *Daphnid Acute Toxicity Test* (1); Part I of OECD Guideline for Testing of Chemicals, 202: *Daphnia sp., Acute Immobilisation Test and Reproduction 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.

OBJECTIVES

The objective of this study is to determine the acute effects of a test substance on the cladoceran, *Daphnia magna*, during a 48-hour exposure period under flow-through test conditions.

EXPERIMENTAL DESIGN

Daphnids will be exposed to a geometric series of at least five test concentrations, a negative (dilution water) control and if necessary, 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 for a total of 20 neonate daphnids per test concentration.

Nominal test concentrations will be selected in consultation with the Sponsor, and will be based upon information such as the results of range-finding toxicity data, known toxicity data, physical/chemical properties of the test substance or other relevant information. Target concentrations will not exceed 1000 mg/L or the solubility limit of the test substance in water (whichever is lower). Generally, each concentration of test substance used in the definitive test will be at least 50% of the next higher treatment, unless information concerning the concentration-effect curve indicates that a different dilution factor would be more appropriate. Water samples from appropriate test chambers will be collected at specified intervals for analysis of the test substance. Results of chemical analyses will be used to calculate mean measured test concentrations.

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WILDLIFE INTERNATIONAL LTD.

To control bias, neonate daphnids 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. EC50 values will be calculated based on the number of dead or immobilized daphnids observed in each test concentration after each 24-hour interval of exposure.

MATERIALS AND METHODS**Test Substance**

The test substance will be a composite sample of hexabromocyclododecane (HBCD) produced by three manufacturers (Albemarle Corporation, Ameribrom LTD, and Great Lakes Chemical Corporation). The composite HBCD sample will be composed of equal parts of each of the three manufacturers' product. Prior to use in the study, equal weights of test substance from each manufacturer will be placed in an appropriate plastic container and mixed on a laboratory shaker for a minimum of two hours to form a composite HBCD sample for use as the test substance. Subsamples of the composite sample will be collected from the left and right sides of the top, middle and bottom of the container. The subsamples will be analyzed by Albemarle Corporation to determine the homogeneity of the mixture. An additional sample of the composite will be collected indiscriminately from the mixture and analyzed by Albemarle Corporation to characterize the test substance. If additional mixing or other procedures are necessary based on the results of the analysis, details of the procedure will be amended to the protocol. The mean HBCD percent of the homogeneity samples will be used for the study.

Information on the characterization of test, control or reference substances is required by Good Laboratory Practice Standards (GLP), 40 CFR, Part 792.31. The Sponsor is responsible for providing Wildlife International Ltd. written verification that the HBCD composite sample has been characterized according to GLP's prior to its use in the study. If written verification of GLP test substance characterization is not provided to Wildlife International Ltd., it will be noted in the compliance statement of the final report. The attached form IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR (Appendix I) will be used to provide information necessary for GLP compliance.

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.

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WILDLIFE INTERNATIONAL LTD.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 N,N-dimethylformamide, triethylene glycol, methanol, ethanol, or acetone may be used. The concentration of organic solvent will not exceed 0.1 mL/L, when possible. If an organic solvent is required, a solvent control will be included in the experimental design along with a negative (dilution water) control. The solvent concentration in the solvent control group will be equal to the highest solvent concentration in test chambers containing the test substance.

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 Wildlife International Ltd. personnel 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, except culture water is supplemented with selenium. Adult 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. During the 10-day holding period the adults will produce an average of at least 3 young per adult per day. 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 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

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neonates will not be fed during the test. Feed (YCT) provided to daphnids in the cultures will be analyzed at least once annually by a GLP compliant laboratory to ensure that there are no contaminants at levels known to be capable of interfering with the study. 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. At test initiation, the neonates will be collected from cultures and transferred to glass beakers. Wide-bore pipettes will be used to transfer the daphnids from the beakers to the test compartments.

Dilution Water

The water used for culturing and testing will be obtained from a well approximately 45 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 0.2 μ m in order to remove fine particles. Water used 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, μ mhos/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. A list of the parameters routinely measured along with detection limits is presented in Table 1.

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WILDLIFE INTERNATIONAL LTD.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 study to ensure that these flow rates vary by no more than $\pm 10\%$ of the mean of the two replicates. Test chambers will be Teflon[®]-lined, 8-L polyethylene aquaria or non-lined stainless steel chambers holding approximately 6.5 L of water. Test chambers will be indiscriminately positioned in a temperature-controlled water bath designed to maintain a temperature of $20 \pm 2^\circ\text{C}$. 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. Daphnids will be held in test compartments suspended in each test chamber. Compartments will be constructed from glass beakers (8 cm diameter and 13 cm height) having screen-covered openings (Nytex) on opposite sides of each beaker.

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 collecting the pre-test samples 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 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 darkness will be controlled with an automatic

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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 $20 \pm 2^\circ\text{C}$. Temperature will be measured in each test chamber at the beginning and end of the test using a hand-held thermometer. Temperature also will be measured with a continuous recorder in one negative control chamber. Recorder measurements will be verified with a hand-held thermometer prior to test initiation.

Dissolved oxygen will be measured in alternate replicates of the treatment and control groups at test initiation and at approximately 24-hour intervals thereafter using a Yellow Springs Instrument Model 51B dissolved oxygen meter, or equivalent. In the event that dissolved oxygen levels fall below 60% 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 Fisher Accumet Model 915 pH meter, or equivalent. If a treatment group reaches 100% mortality, dissolved oxygen, pH, and temperature measurements will be taken at the next scheduled interval, then discontinued.

Hardness, pH, 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 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.

Biological Measurements

Observations of mortality, immobilization and clinical signs of toxicity will be made between at 24 hour intervals (± 1 hour) throughout the test. The criteria for death include lack of movement, absence of respiratory movements, and lack of reaction to gentle prodding. Immobilization is defined as a lack of movement by the test organism except for minor activity of the appendages.

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Sampling for Analytical Measurements

Prior to test initiation, water samples will be collected from each test chamber of both the low and high level concentrations. Water samples also will be collected from each test chamber 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. An assessment of the stability of the test substance in well water and in carrier solvent will be performed under a separate protocol (Analytical Method Verification for the Determination of Hexabromocyclododecane (HBCD) in Well Water; Wildlife International Ltd. Project #439/021296/METVER.FW/SUB439). The sample scheme is summarized below:

PROPOSED NUMBERS OF VERIFICATION SAMPLES			
Experimental Group	Pretest	0 Hours	48 Hours
Control	-	2	2
Solvent Control (if needed)	-	2	2
Level 1-Low Concentration	2	2	2
Level 2	-	2	2
Level 3	-	2	2
Level 4	-	2	2
Level 5-High Concentration	2	2	2
Totals	4	14	14

Total Number of Verification Samples = 32

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 may be collected from at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system.

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WILDLIFE INTERNATIONAL LTD.**Analytical Chemistry**

Chemical analysis of the samples will be performed by Wildlife International Ltd. The analytical method used will be based upon methodology provided by the Sponsor (Appendix II). 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 and 9). The EC50 value will be calculated using mortality/immobility data collected at 24 and 48 hours. The no-mortality/immobility 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 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. History of the test organism, culture and holding records.
5. Stock solution calculation and preparation.
6. Daily observations.
7. Water chemistry results (e.g., hardness and alkalinity).
8. If applicable, the methods used to analyze test substance concentrations and the results of analytical measurements.
9. Statistical calculations.
10. Test conditions (light intensity, photoperiod, etc.).
11. Calculation and preparation of test concentrations.
12. Copy of final report.

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WILDLIFE INTERNATIONAL LTD.**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. Name and address of the Sponsor.
3. Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates.
4. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
5. Objectives and procedures, as stated in the approved protocol, including a copy of the protocol, and amendments and deviations to the protocol.
6. The test substance identification including name, chemical abstract number or code number, strength, purity, composition, and other information provided by the Sponsor.
7. Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor.
8. A description of the methods used to conduct the test.
9. A description of the test organisms, including the source, scientific name, age or life stage, feed types, results of latest feed analyses, light intensity and photoperiod.
10. A description of the preparation of the test solutions.
11. 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.
12. A description of circumstances that may have affected the quality or integrity of the data.
13. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
14. 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 for each period, an EC50 is calculated. If the data is conducive to evaluation by probit analysis, the slope of the concentration-response curve will be reported.
15. Statistical methods used to evaluate the data.
16. The signed and dated reports of each of the individual scientists or other professionals involved in the study.
17. The location where raw data and final report are to be stored.

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18. A statement prepared by the Quality Assurance Unit listing the dates that study inspections and audits were made and findings reported to the Study Director and Management.
19. 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 IN PROTOCOL

Planned changes to the protocol will be in the form of written amendments signed by the Study Director and 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 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 792); OECD (ISBN 92-84-12367-9); and Japan MAFF (59 NohSan, Notification No. 3850, Agricultural Production Bureau). 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. 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 TSCA Title 40 of the Code of Federal Regulations. 1994. Part 797, Section 1300: *Daphnid Acute Toxicity Test*.
- 2 Organisation for Economic Cooperation and Development. 1984. Guideline for Testing of Chemicals, 202: *Daphnia sp. Acute Immobilization Test and Reproduction Test*.
- 3 ASTM Standard E729-88a. 1991. *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. 1985. *Standard Methods for the Examination of Water and Wastewater*. 16th 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.

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WILDLIFE INTERNATIONAL LTD.

TABLE I

PESTICIDES, ORGANICS, METALS AND OTHER INORGANICS ANALYZED
IN WILDLIFE INTERNATIONAL LTD. WELL WATER

ANALYSIS	TARGET LIMIT OF DETECTION
Miscellaneous Measurements	
Total Dissolved Solids	4 mg/L
Ammonia Nitrogen	0.02 mg/L
Total Organic Carbon	1 mg/L
Total Cyanide	0.001 mg/L
Organochlorines and PCBs	
Aldrin	≤ 0.01 µg/L
Alpha BHC	≤ 0.01 µg/L
Beta BHC	≤ 0.01 µg/L
Delta BHC	≤ 0.01 µg/L
Gamma BHC (Lindane)	≤ 0.01 µg/L
Chlordane	≤ 0.03 µg/L
DDD, pp'	≤ 0.01 µg/L
DDE, pp'	≤ 0.01 µg/L
DDT, pp'	≤ 0.01 µg/L
Dieldrin	≤ 0.01 µg/L
Endosulfan, A	≤ 0.01 µg/L
Endosulfan, B	≤ 0.01 µg/L
Endosulfan Sulfate	≤ 0.01 µg/L
Endrin	≤ 0.01 µg/L
Endrin Aldehyde	≤ 0.01 µg/L
Heptachlor	≤ 0.01 µg/L
Methoxychlor	≤ 0.01 µg/L
Heptachlor Epoxide	≤ 0.01 µg/L
Toxaphene	≤ 0.06 µg/L
PCB-1016	≤ 0.3 µg/L
PCB-1221	≤ 0.3 µg/L
PCB-1232	≤ 0.3 µg/L
PCB-1242	≤ 0.3 µg/L
PCB-1248	≤ 0.3 µg/L
PCB-1254	≤ 0.3 µg/L
PCB-1260	≤ 0.3 µg/L
Actual analysis is based on available methodologies at the testing facility. Results from each annual analysis with the limit of detection for each chemical is retained in the Wildlife International Ltd. archives.	

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TABLE I (Continued)

PESTICIDES, ORGANICS, METALS AND OTHER INORGANICS ANALYZED
IN WILDLIFE INTERNATIONAL LTD. WELL WATER

ANALYSIS	TARGET LIMIT OF DETECTION
Organophosphorus & Organonitrogen Pesticides	
Azinphos Methyl	< 3 µg/L
Chlorpyrifos	< 0.3 µg/L
Coumaphos	< 3 µg/L
Demeton	< 0.3 µg/L
Diazinon	< 0.3 µg/L
Dichlorvos	< 0.3 µg/L
Dimethoate	< 0.3 µg/L
Disulfoton	< 2 µg/L
EPN	< 0.5 µg/L
Ethoprop	< 0.5 µg/L
Fenthion	< 0.5 µg/L
Fensulfothion	< 3 µg/L
Malathion	< 0.5 µg/L
Merphos	< 0.5 µg/L
Mevinphos	< 0.5 µg/L
Monochrotophos	< 3 µg/L
Naled	< 2 µg/L
Methylparathion	< 0.3 µg/L
Parathion	< 0.3 µg/L
Phorate	< 0.3 µg/L
Ronnel	< 2 µg/L
Stirofos	< 0.6 µg/L
Sulfotepp	< 0.3 µg/L
Sulprofos	< 0.3 µg/L
Tepp	< 0.3 µg/L
Tokuthion	< 0.3 µg/L
Trichloronate	< 0.3 µg/L
Actual analysis is based on available methodologies at the testing facility. Results from each annual analysis with the limit of detection for each chemical is retained in the Wildlife International Ltd. archives.	

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TABLE I (Continued)

PESTICIDES, ORGANICS, METALS AND OTHER INORGANICS ANALYZED
IN WILDLIFE INTERNATIONAL LTD. WELL WATER

ANALYSIS	TARGET LIMIT OF DETECTION
Chlorophenoxy Acid Herbicides	
2,4-D, Total	< 0.02 µg/L
2,4-DB	< 0.02 µg/L
2,4,5-T Water	< 0.02 µg/L
2,4,5-TP/Silvex	< 0.02 µg/L
Delapron	< 0.02 µg/L
Dicamba (Banvel)	< 0.02 µg/L
Dichloronoprop	< 0.02 µg/L
Dinoseb	< 0.02 µg/L
MCPA	< 0.4 µg/L
MCPP	< 0.4 µg/L
Metals and Other Inorganics	
Aluminum	< 40 µg/L
Arsenic	< 3 µg/L
Beryllium	< 5 µg/L
Cadmium	< 5 µg/L
Calcium	< 500 µg/L
Chromium	< 5 µg/L
Copper	< 5 µg/L
Iron	< 45 µg/L
Lead	< 3 µg/L
Magnesium	< 3 µg/L
Manganese	< 3 µg/L
Mercury	< 3 µg/L
Nickel	< 10 µg/L
Potassium	< 500 µg/L
Selenium	< 3 µg/L
Silver	< 5 µg/L
Sodium	< 500 µg/L
Zinc	< 15 µg/L
Molybdenum	< 10 µg/L

Actual analysis is based on available methodologies at the testing facility. Results from each annual analysis with the limit of detection for each chemical is retained in the Wildlife International Ltd. archives.

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APPENDIX I
IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR

To be Completed by Sponsor

- I. Test Substance Identity (name to be used in the report): _____
 Reference Standard (if applicable): Analytical Standard: _____
 Internal Standard: _____
 Test Substance Sample Code or Batch Number: _____
 Test Substance Purity (% Active Ingredient): _____ Expiration Date: _____

II. Test Substance Characterization

Have the identity, strength, purity and composition or other characteristics which appropriately define the test substance and reference standard been determined prior to its use in this study in accordance with GLP Standards? Yes ___ No ___

III. Test Substance Storage Conditions

Please indicate the recommended storage conditions at Wildlife International Ltd.

Has the stability of the test substance under these storage conditions been determined in accordance with GLP Standards? Yes ___ No ___

Other pertinent stability information: _____

- IV. Test Concentrations: Adjust test concentration to 100% a.i. based upon the purity (%) given above.
 Do not adjust test concentration to 100% a.i. Test the material AS IS.

V. Toxicity Information:

Mammalian: Rat LD50 _____ Mouse LD50 _____

Aquatic: Invertebrate Toxicity (EC/LC50) Fish Toxicity (LC50)

Other Toxicity Information (including findings of chronic and subchronic tests):

VI. Classification of the Compound:

_____ Insecticide _____ Herbicide _____ Fungicide
 _____ Microbial Agent _____ Economic Poison

Other: _____

PROTOCOL NO.: 439/021296/DAP-48H2/SUB439

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APPENDIX V

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APPENDIX II

Analytical Method to be Provided by Sponsor

PROTOCOL NO.: 439/021296/DAP-48H2/SUB#39

APPENDIX V

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DEVIATION TO STUDY PROTOCOL

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

PROTOCOL NO.: 439/021296/DAP-48H2/SUB439

DEVIATION NO.: 1

SPONSOR: Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel

PROJECT NO.: 439A-102

DATE OF DEFACTO DEVIATION: April 4, 1997

DEVIATION:

Daphnids were not exposed to a geometric series of test concentrations.

REASON:

The dose levels requested by the Sponsor were not a geometric series of test concentrations.

IMPACT:

This deviation should not adversely impact the outcome or interpretation of the results of the study.

Will C. Gunn
STUDY DIRECTOR

4-9-97
DATE

John Seuzgen
LABORATORY MANAGEMENT

4/9/97
DATE

11159

APPENDIX VI

Personnel Involved in the Study

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

1. James P. Swigert, Ph.D., Manager, Aquatic Toxicology
2. Barbara J. Markley, Ph.D., Manager, Analytical Chemistry
3. Willard B. Nixon, Ph.D., Manager, Analytical Chemistry
4. Edward C. Schaefer, Manager, Biodegradation
5. Timothy Z. Kendall, Laboratory Supervisor, Analytical Chemistry
6. William C. Graves, Senior Aquatic Biologist
7. Mark A. Mank, Aquatic Laboratory Supervisor

CERTIFICATE OF AUTHENTICITY

THIS IS TO CERTIFY that the microimages appearing on this microfiche are accurate and complete reproductions of the records of U.S. Environmental Protection Agency documents as delivered in the regular course of business for microfilming.

Date produced 8 25 98 Barbara Smith
(Month) (Day) (Year) Camera Operator

Place Syracuse New York
(City) (State)

