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CHEMICAL MANUFACTURERS ASSOCIATION



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June 27, 1997

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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

Dear Dr. Goldman:

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

1. HEXABROMOCYCLODODECANE (HBCD): A 96-Hour Toxicity Test with the Freshwater Alga (*Selenastrum capricornutum*);
2. HEXABROMOCYCLODODECANE (HBCD): A 96-Hour Flow-Through Acute Toxicity Test with the Rainbow Trout;
3. HEXABROMOCYCLODODECANE (HBCD): Determination of *n*-Octanol/Water Partition Coefficient;
4. HEXABROMOCYCLODODECANE (HBCD): Determination of the Water Solubility; and
5. Analytical Method Verification for the Determination of hexabromocyclododecane (HBCD) in Well Water.

These reports do not include confidential information.

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

Sincerely Yours,

Courtney M. Price
Vice President, CHEMSTAR

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

FINAL REPORT

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 439A-101

TSCA Title 40 of the Federal Code of Regulations
Part 797, Section 1400
and
Organisation for Economic Cooperation and Development
OECD Guideline 203

AUTHORS:

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

STUDY INITIATION DATE: May 15, 1996

STUDY COMPLETION DATE: June 3, 1997

Submitted to

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

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WILDLIFE INTERNATIONAL LTD.

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



GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

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

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

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 439A-101

STUDY COMPLETION: June 3, 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

6-3-97
DATE

SPONSOR:

Hammukh Shah
Sponsor

6-4-97
DATE

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QUALITY ASSURANCE

This study was examined for 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. 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	April 1, 1997	April 1, 1997	April 2, 1997
Analytical Sampling	April 9, 1997	April 9, 1997	April 9, 1997
Biological Data and Draft Report	May 2 and 5, 1997	May 5, 1997	May 6, 1997
Analytical Data and Draft Report	May 14 and 15, 1997	May 15, 1997	May 27, 1997
Final Report	June 3, 1997	June 3, 1997	June 3, 1997



 Lisa T. Drottar
 Quality Assurance Representative

6-3-97

 DATE

REPORT APPROVAL

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

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

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 439A-101

STUDY DIRECTOR:

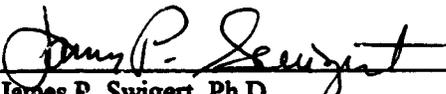


William C. Graves
Senior Aquatic Biologist

6.3.97

DATE

MANAGEMENT:



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

6/3/97

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-101
TEST SUBSTANCE:	Hexabromocyclododecane (HBCD)
STUDY:	Hexabromocyclododecane (HBCD): A 96-Hour Flow-Through Acute Toxicity Test with the Rainbow Trout (<i>Oncorhynchus mykiss</i>)
NOMINAL TEST CONCENTRATIONS:	Negative Control, Solvent Control, 1.5, 2.2, 3.2, 4.6 and 6.8 µg/L
MEAN MEASURED TEST CONCENTRATIONS:	Negative Control, Solvent Control, 0.75, 1.5, 2.3, 2.3 and 2.5 µg/L
TEST DATES:	Experimental Start - April 7, 1997 Biological Termination - April 11, 1997 Experimental Termination - April 23, 1997
LENGTH OF TEST:	96 Hours

TEST ORGANISM:	Rainbow Trout (<i>Oncorhynchus mykiss</i>)
SOURCE OF TEST ORGANISMS:	Troutlodge, Inc. P. O. Box 1290 Sumner, WA 98390
AGE OF TEST ORGANISMS:	Juveniles
MEASUREMENTS OF 10 NEGATIVE CONTROL FISH:	
WEIGHT (g):	Mean = 2.4; Range = 1.6 to 3.6
TOTAL LENGTH (mm):	Mean = 55; Range = 50 to 61

96-HOUR LC50:	>6.8 µg/L (nominal, 2X HBCD's water solubility of 3.4 µg/L) (>2.5 µg/L mean measured concentration)
NO MORTALITY CONCENTRATION:	6.8 µg/L (nominal, 2X HBCD's water solubility of 3.4 µg/L) (>2.5 µg/L mean measured concentration)
NO-OBSERVED-EFFECT-CONCENTRATION:	6.8 µg/L (nominal, 2X HBCD's water solubility of 3.4 µg/L) (>2.5 µg/L mean measured concentration)

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-lake phase of the test was conducted from April 7, 1997 to April 11, 1997. Raw data generated by Wildlife International Ltd. and a copy of the final report are filed under Project Number 439A-101 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 rainbow trout (*Oncorhynchus mykiss*) during a 96-hour exposure period under flow-through test conditions.

EXPERIMENTAL DESIGN

Rainbow trout were exposed to one of five test concentrations, a solvent control, or the negative (well water) control. Two replicate test chambers were maintained in each treatment and control group. Ten rainbow trout were used in each test chamber for a total of 20 rainbow trout 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 $6.8 \mu\text{g/L}$. Due to co-eluting artifacts at 96 hours, mean measured test concentrations were determined analytically from samples of test water collected from each treatment and control group at the beginning of the test, and at approximately 48 hours.

Delivery of the test substance was initiated approximately 6 days prior to the introduction of the fish to the test water in order to achieve equilibrium of the test substance in the test chambers. The fish were indiscriminately assigned to exposure chambers at test initiation. Observations of

mortality and other clinical signs were made approximately 1, 24, 48, 72, and 96 hours after test initiation. Cumulative percent mortality observed in the treatment groups was used to estimate LC50 values at 24, 48, 72 and 96 hours. The no mortality concentration and no-observed-effect-concentration (NOEC) were determined by visual interpretation of the mortality and clinical observation data.

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, Hexabromocyclododecane (HBCD): A 96-Hour Flow-Through Acute Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*) (Appendix V). The protocol was based on procedures outlined in TSCA Title 40 of the Code of Federal Regulations, Part 797, Section 140C: *Fish Acute Toxicity Test* (1); OECD Guidelines for Testing of Chemicals, 203: *Fish Acute Toxicity 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 identity and date 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
Libemarle Corp.	33449-15X	December 2, 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

- 10 -

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 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 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 stirred with a glass 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 once 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 DMF 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 rainbow trout, *Oncorhynchus mykiss*, was selected as the test species for this study. The rainbow trout is representative of an important group of aquatic vertebrates and was selected for use in the test based upon past history of use and ease of culturing in the laboratory. Rainbow trout used

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in the test originated as eyed eggs from Troutlodge, Inc., Sumner, Washington and were held in the culture facility of Wildlife International Ltd. until they achieved the proper size for testing. Identification of the species was certified by the supplier.

The rainbow trout were cultured in water from the same source and at approximately the same temperature as that used during the test. The fish were held for approximately 4.5 months prior to testing. The fish were acclimated to test conditions for approximately 52 hours prior to test initiation. During the holding and acclimation periods the fish showed no signs of disease or stress. During the 14-day holding period preceding the test, water temperatures ranged from 11.5 to 12.5°C. The pH of the water ranged from 8.1 to 8.4 and dissolved oxygen ranged from 8.8 to 10.0 mg/L. Instrumentation used for water measurements are described in the *Environmental Conditions* section of this report. At test initiation, the rainbow trout were collected from the acclimation tank and transferred to the test chambers.

During the holding period, the rainbow trout were fed a commercially-prepared diet supplied by Zeigler Brothers, Inc., Gardners, Pennsylvania. The fish were not fed during the acclimation period (at least 48 hours prior to the test) or during the test.

All fish used in the test were from the same source and year class, and the total length of the longest fish was no more than twice the length of the shortest. The average length of 10 negative control fish measured at the end of the test was 55 mm with a range of 50 to 61 mm. The average wet weight (blotted dry) of 10 negative control fish at the end of the test was 2.4 grams with a range of 1.6 to 3.6 grams. Loading was defined as the total wet weight of fish per liter of test water that passed through the test chamber in 24 hours, and was determined to be 0.27 g fish/L/day. Instantaneous loading was 1.6 g fish/L of test water present in the test chambers at any given time.

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

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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 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. 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 6 volume additions of test water every 24 hours. The stock solution delivery pumps and the dilution water rotameters were calibrated before the test, and the general operation of the diluter was checked visually at least two times per day during the test phase and at termination.

Test chambers were 25-L Teflon®-lined stainless steel aquaria filled with approximately 15 L of test water. The depth of the test water in a representative chamber (29 cm x 29 cm x 30 cm) was 17.8 cm. Test chambers were indiscriminately positioned in a temperature-controlled water bath designed to maintain a temperature of $12 \pm 2^\circ\text{C}$. The water bath was enclosed in a plexiglass ventilation hood in order to minimize any potential for cross-contamination. The test chambers were labeled with the project number, test concentration and replicate.

Dilution Water

The water used for holding 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 measurements 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 μm , and pumped into a 37,800-L storage tank and 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 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 336 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 $12 \pm 2^\circ\text{C}$. Dissolved oxygen and pH measurements were made on water samples collected from each replicate test chamber at test initiation and at approximately 48 and 96 hours after test initiation. Hardness, alkalinity, specific conductance, acidity and total organic carbon (TOC) were measured in the dilution water at test initiation and termination.

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 using a Shimadzu Model 5000 TOC Analyzer. Light intensity measurements were made using an SPER Scientific Ltd. light meter.

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Observations

Observations were made to determine the number of mortalities. The number of individuals exhibiting clinical signs of toxicity and/or abnormal behavior also were evaluated. Observations were made approximately 1, 24, 48, 72, and 96 hours after test initiation.

Statistical Analyses

In this study, an LC50 could not be statistically defined due to the lack of an adequate dose response pattern. Therefore, the LC50 values were estimated from the biological response data. The no mortality concentration and NOEC were also determined by visual interpretation of the mortality and clinical observation data.

Analytical Chemistry

Samples of test media solutions (test samples) were collected from each replicate test chamber at 0, 48 and 96 hours to measure concentrations of the test substance. Test samples also were collected from the lowest and highest treatment groups prior to test initiation. The test samples were collected in glass graduated cylinders. The test 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 test 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 eluent system. For this reason, the highest test concentration selected for the test was twice the water solubility limit (i.e., 6.8 $\mu\text{g/L}$). The series of five nominal

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test concentrations used in the test were 1.5, 2.2, 3.2, 4.6 and $7 \mu\text{g/L}$. In this way, the solubility limit of HBCD was bracketed by the five concentrations.

Results of the analysis of HBCD concentrations in water samples collected during the toxicity test are presented in Table 1 and in the analytical chemistry report (Appendix IV). One set of pretest samples was collected from the highest and lowest test concentrations and analyzed. The values are shown in Table 1 as Day -4. All pretest samples yielded concentrations that were considerably 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, middle and end of the test. In general, concentrations of HBCD made on samples collected at Day 0 and Day 2 were variable and failed to correspond to the dilution series expected from the nominal concentrations. All diluter operational records were checked and no evidence of any malfunctions or errors were found. Concentrations measured in the Day 4 samples were artificially high due to co-eluting artifacts at the retention time of HBCD. Attempts were made to separate the co-eluting artifacts during a reanalysis of the original Day 4 sample extracts, but the resulting chromatography showed those same interferences.

While the pattern of measured HBCD was unexpected, the results suggest that the exposure solutions were at the solubility limit of HBCD in the diluter system. The variability in the measured concentrations could have been influenced by the temperature of the exposure water (12°C), the flow-through design, or the hydrophobic nature of HBCD (as evidenced by its nonpolar alkane structure and extremely low water solubility). These factors could explain both the failure of the measured values to correspond to the nominal concentrations and the variability observed in the measured concentrations. Overall, it appears that the solubility limit of HBCD, under the conditions that it was applied in this test, is within the range of 2.0 to $3.0 \mu\text{g/L}$. The values obtained in the Day 4 samples were not reflective of the true conditions due to the co-eluting artifacts, and therefore, were not used in the study.

Observations and Measurements

Measurements of temperature, dissolved oxygen, and pH are presented in Table 2. Temperatures were within the limits of the $12 \pm 2^\circ\text{C}$ range established for the test. Dissolved oxygen concentrations $\geq 78\%$ of saturation were observed throughout the test. Water pH was consistent with values for moderately-hard water. Measurements of pH ranged from 8.2 to 8.3. Total organic carbon values were $< 1.0 \text{ mg C/L}$ at test initiation and termination.

Daily observations of mortality and other signs of toxicity observed during the test are shown in Table 3. Rainbow trout in the negative control and solvent control groups appeared healthy and normal throughout the test. All rainbow trout in the 1.5, 2.2, 3.2, 4.6 and 6.8 $\mu\text{g/L}$ (nominal) (0.75, 1.5, 2.3, 2.3 and 2.5 $\mu\text{g/L}$ mean measured concentrations) treatment groups also appeared normal throughout the test with no mortalities or overt signs of toxicity. Based on these results, the LC50 values at 24, 48, 72 and 96 hours were estimated to be $> 6.8 \mu\text{g/L}$ (nominal) ($> 2.5 \mu\text{g/L}$ mean measured concentration), the highest concentration tested.

CONCLUSIONS

The 96-hour LC50 value for rainbow trout exposed to hexabromocyclododecane (HBCD) was $> 6.8 \mu\text{g/L}$ (nominal) ($> 2.5 \mu\text{g/L}$ mean measured concentration), the highest concentration tested and twice HBCD's water solubility (3.4 $\mu\text{g/L}$). Based on the mortality and observation data, the 96-hour no mortality concentration and the no-observed-effect-concentration were 6.8 $\mu\text{g/L}$ (nominal) (2.5 $\mu\text{g/L}$ mean measured concentration).

REFERENCES

- 1 **TSCA Title 40 of the Code of Federal Regulations.** 1994. Part 797, Section 1400: *Fish Acute Toxicity Test.*
- 2 **Organisation for Economic Cooperation and Development.** 1984. OECD Guideline for Testing of Chemicals, 203: *Fish Acute Toxicity 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 ($\mu\text{g/L}$) Hexabromocyclododecane (HBCD)					Mean Measured Concentration ³ ($\mu\text{g/L}$)
		Pretest Day -4	Day 0 ¹	Day 2 ¹	Day 4	Day 4 Repeat Analysis ²	
Negative Control	A	--	<0.571	<0.571	--	--	--
	B	--	<0.571	<0.571	--	--	--
Solvent Control	A	--	<0.571	<0.571	--	--	--
	B	--	<0.571	<0.571	--	--	--
1.5	A	<0.571	<0.571	0.735	6.31	6.03	0.75
	B	<0.571	0.947	0.573	6.76	6.29	
2.2	A	--	1.46	2.2 ¹	6.97	6.51	1.5
	B	--	1.71	0.732	6.95	6.42	
3.2	A	--	2.37	1.87	3.63	20.6	2.3
	B	--	3.96	1.04	3.30	4.20	
4.6	A	--	9.65 ³	1.61	5.72	4.67	2.3
	B	--	2.13	3.04	3.56	3.68	
6.8	A	0.85	3.53	1.86	3.61	4.59	2.5
	B	0.95	2.71	1.70	3.70	4.09	

¹ Values were corrected for a mean procedural recovery of 114%.

² Values for confirmatory purposes only, not included in the mean measured calculations.

³ Mean measured concentration did not include pretest values and Day 4 values.

⁴ The limit of quantitation was 0.400 mg/L, based upon the product of the lowest standard (100 $\mu\text{g/L}$) and the dilution factor of the matrix blanks (0.004).

⁵ Outlier, not used in the overall mean measured concentration.

Table 2

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

Nominal Test Concentration ($\mu\text{g/L}$)		0 Hour ¹			48 Hours		96 Hours ²		
Replicate	Temp ³ ($^{\circ}\text{C}$)	DO ⁴ (mg/L)	pH	DO (mg/L)	pH	Temp ($^{\circ}\text{C}$)	DO (mg/L)	pH	
									Sponsor: CMA's Brominated Flame Retardant Industry Panel
Negative Control	A	11.9	9.8	8.3	9.2	8.2	12.0	9.2	8.2
	B	11.9	9.9	8.3	9.1	8.2	12.0	9.2	8.2
Solvent Control	A	11.8	9.8	8.3	9.4	8.2	12.0	9.0	8.2
	B	11.8	9.8	8.3	9.3	8.2	12.0	9.0	8.2
1.5 (0.75)	A	11.7	9.8	8.3	9.3	8.2	12.0	8.6	8.2
	B	11.7	9.8	8.3	9.3	8.2	12.0	8.8	8.2
2.2 (1.5)	A	11.5	9.7	8.3	9.3	8.2	11.5	8.8	8.2
	B	11.5	9.7	8.3	9.2	8.2	11.5	8.8	8.2
3.2 (2.3)	A	11.6	9.8	8.3	9.3	8.2	11.5	8.8	8.2
	B	11.6	9.7	8.3	9.4	8.2	11.5	8.8	8.2
4.6 (2.3)	A	11.6	9.7	8.3	9.1	8.2	11.6	8.6	8.2
	B	11.6	9.8	8.3	9.2	8.2	11.6	8.6	8.2
6.8 (2.5)	A	11.5	9.6	8.3	9.2	8.2	11.6	8.4	8.2
	B	11.5	9.6	8.3	9.3	8.2	11.6	8.4	8.2

¹ The 0-hour dilution water measurements for hardness, alkalinity, specific conductance and acidity were 136 mg/L as CaCO₃, 178 mg/L as CaCO₃, 270 $\mu\text{mhos/cm}$ and 21 mg/L as CaCO₃, respectively.

² The 96-hour dilution water measurements for hardness, alkalinity, specific conductance and acidity were 136 mg/L as CaCO₃, 172 mg/L as CaCO₃, 270 $\mu\text{mhos/cm}$ and 19 mg/L as CaCO₃, respectively.

³ Temperature measured continuously during the test ranged from approximately 12.0 to 12.5 $^{\circ}\text{C}$.

⁴ A dissolved oxygen concentration of 8.4 mg/L represents 73% saturation at 12 $^{\circ}\text{C}$ 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	No. Exposed	1 Hour		24 Hours		48 Hours		72 Hours		96 Hours		Cumulative Percent Mortality
			No. Dead ²	Effects ²	No. Dead	Effects							
Negative Control	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
Solvent Control	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
1.5 (0.75)	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
2.2 (1.5)	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
3.2 (2.3)	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
4.6 (2.3)	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
6.8 (2.5)	A	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0
	B	10	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0	10 AN	0

¹ Cumulative number of dead fish.

² Observed Effects: AN = Appears Normal

Note: Values in parentheses are mean measured test concentrations.

APPENDIX I

Rangefinding Results

Sponsor: CMA's Brominated Flame Retardant Industry Panel
 Test Substance: Hexabromocyclododecane (HBCD)
 Test Organism: Rainbow Trout, *Oncorhynchus mykiss*
 Dilution Water: Well Water

Nominal Test Concentration ($\mu\text{g/L}$)	No. Dead in 24 Hours/Cumulative No. Dead/No. Exposed (Observed) ¹			
	24 Hours	48 Hours	72 Hours	96 Hours
Negative Control	0/0/5 (5 AN)	0/0/5 (5 AN)	0/0/5 (5 AN)	0/0/5 (5 AN)
Solvent Control	0/0/5 (5 AN)	0/0/5 (5 AN)	0/0/5 (5 AN)	0/0/5 (5 AN)
0.16	0/0/5 (5 AN)	0/0/5 (5 AN)	0/0/5 (5 AN)	0/0/5 (5 AN)
0.54	0/0/5 (5 AN)	0/0/5 (5 AN)	0/0/5 (5 AN)	0/0/5 (5 AN)
1.8	0/0/5 (5 AN)	0/0/5 (5 AN)	0/0/5 (5 AN)	0/0/5 (5 AN)
6.0	0/0/5 (5 AN)	0/0/5 (5 AN)	0/0/5 (5 AN)	0/0/5 (5 AN)
20	0/0/5 (5 AN)	0/0/5 (5 AN)	0/0/5 (5 AN)	0/0/5 (5 AN)

¹ Observations: AN = Appears Normal.

**HEXABROMOCYCLODODECANE (HBCD):
DETERMINATION OF n-OCTANOL/WATER PARTITION COEFFICIENT**

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 439C-104

**OPPTS 830.7560 Partition Coefficient (n-Octanol/Water),
Generator Column Method**

AUTHORS:

**Jon A. MacGregor
Willard B. Nixon, Ph.D.**

STUDY INITIATION DATE: March 11, 1997

STUDY COMPLETION DATE: May 23, 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

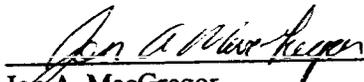
TITLE: Hexabromocyclododecane (HBCD): Determination of n-Octanol/Water Partition Coefficient

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 439C-104

STUDY COMPLETION: May 23, 1997

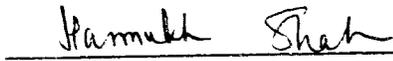
This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Part 792, 17 August 1989; OECD Principles of Good Laboratory Practices, (OECD)C(81)30 (Final)Annex 2; and Japan MHW/MITI 59 Kikyoku No. 85; EA, Kankiken No.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

STUDY DIRECTOR:


Jon A. MacGregor
Senior Chemist

5-23-97
DATE

SPONSOR APPROVAL:


Sponsor

6-5-97
DATE

- 3 -

QUALITY ASSURANCE

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

TITLE: Hexabromocyclododecane (HBCD): Determination of n-Octanol/Water Partition Coefficient

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 439C-104

This study was examined for 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. 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:
Stock Preparation and Sample Preparation	March 11, 1997	March 18, 1997	March 20, 1997
Draft Report and Data	May 12, 1997	May 12, 1997	May 14, 1997
Final Report	May 23, 1997	May 23, 1997	May 23, 1997

Susan L. Hopper
Susan L. Hopper
Senior Quality Assurance Representative

5-23-97
DATE

REPORT APPROVAL

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

TITLE: Hexabromocyclododecane (HBCD): Determination of n-Octanol/Water Partition Coefficient

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 439C-104

This report was reviewed by the individuals involved in the conduct and management of the study, and was found to be an accurate reflection of the methods used, data collected and results of the study.

STUDY DIRECTOR:



Jon A. MacGregor
Senior Chemist

5-23-97

DATE

MANAGEMENT:



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

5-23-97

DATE

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SUMMARY

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

SPONSOR'S REPRESENTATIVE: Hasmukh Shah, Ph.D.

LOCATION OF STUDY, RAW DATA AND A COPY OF THE FINAL REPORT: Wildlife International Ltd.
Easton, Maryland 21601

WILDLIFE INTERNATIONAL LTD.

PROJECT NUMBER: 439C-104

TEST SUBSTANCE: Hexabromocyclododecane (HBCD)

STUDY: Hexabromocyclododecane (HBCD): Determination of the n-Octanol/Water Partition Coefficient

TEST DATES: Experimental Start - March 11, 1997
Experimental Termination - March 18, 1997

SUMMARY: The \log_{10} octanol/water partition coefficient (K_{ow}) of HBCD was determined to be 5.625 at $25 \pm 0.05^\circ\text{C}$ using the generator column method.

- 8 -

INTRODUCTION

This study was conducted by Wildlife International Ltd. for Chemical Manufacturers Association's (CMA) Brominated Flame Retardant Industry Panel at the Wildlife International Ltd. analytical chemistry facility in Easton, Maryland. Tests were performed using the generator column method. Samples were eluted from a generator column and analyzed from March 11 to March 18, 1997. Raw data generated by Wildlife International Ltd. and a copy of the final report are filed under Project Number 439C-104 in archives located on the Wildlife International Ltd. site.

OBJECTIVE

The objective of this study was to determine the n-octanol/water partition coefficient of hexabromocyclododecane (HBCD) at 25.0°C using a generator column method.

EXPERIMENTAL DESIGN

A single generator column was prepared for the definitive test. The column was packed with Chromosorb W HP support and loaded with an approximate 0.2% solution of the test substance in octanol. Dilutions of the test substance solution in octanol were analyzed. The column temperature was maintained at $25 \pm 0.05^\circ\text{C}$ and reagent water saturated with octanol was pumped through it at approximately 1 mL per minute to elute the test substance. Samples of the eluate were collected and analyzed to determine the concentration of the test substance in the aqueous fractions.

MATERIALS AND METHODS

This study was conducted according to procedures outlined in the protocol, "Hexabromocyclododecane (HBCD): Determination of n-Octanol/Water Partition Coefficient" (Appendix I). The protocol was based on procedures outlined in OPPTS 830.7560 (1). The generator column method was used to determine the partition coefficient of the test substance.

Test Substance

The test substance consisted of a composite of hexabromocyclododecane (HBCD) samples received from three manufacturers. The materials identity and date 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.	G35297G-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 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.

Reagent Water

The reagent water used in this study met the specifications for ASTM Type II water. The water was obtained from a well located on the Wildlife International Ltd. site. The well water was

pumped through a series of filters to remove microorganisms and particles greater than $0.2 \mu\text{m}$. The water was further purified using a Culligan® Hi-Flo 1 Water Softener, a Culligan® S-Series Reverse Osmosis System, and a Barnstead NANOpure® ultrapure water system. The resistivity of the purified reagent water used for this study was at least 17.3 megohm-cm. The reagent water was saturated with octanol prior to use in this study.

Solvents

1-Octanol (certified), Fisher Chemical (Fairlawn, NJ), catalog number A 402-4, was used to prepare a stock solution of the test substance. Mixtures of acetonitrile (ACN) and reagent water were used to prepare calibration standards, samples, and mobile phases for high pressure liquid chromatography (HPLC). The acetonitrile was supplied by either Burdick and Jackson (Muskegon, MI, catalog number 015-4) or Fisher Chemical (catalog number A996-4). Dichloromethane (DCM), Burdick and Jackson, catalog number 300-4 was used as an extraction solvent for this study. All solvents used for this study were either suitable for HPLC and residue analysis or certified reagents.

Preparation of Generator Column

The generator column was supplied by At-Mar Glass Co. (Kennett Square, PA). The glass column was 20 cm long with an internal diameter of ~6 mm, and was joined to a section with an internal diameter of ~9 mm. The entire column was enclosed in a water jacket for temperature control. A diagram of the generator column is presented in Figure 1.

The inert support material used in the generator column was Chromosorb WHP (100 - 120 mesh), and was supplied by Supelco Inc. (Bellefonte, PA, catalog number 2-0159). A small plug of silanized glass wool was placed in the column just above the enlarged section, the column was filled with the support material, and another plug of glass wool was added to the top of the column. Gentle tapping and vibration were used to facilitate packing of the column.

A stock solution of the composite test substance was prepared by combining 50 mg of test substance with 25 g of octanol and sonicating for approximately 20 minutes to dissolve the test substance. The nominal concentration of the stock solution was 0.2% test substance by weight or 1.65 mg/mL, based on the density of octanol (0.827 g/mL). Three subsamples of the stock solution were diluted in acetonitrile/water (50:50, v/v) and analyzed to determine the concentration of test substance in the octanol. An aliquot of the stock solution (15 mL) was also used to charge the generator column. The stock solution was loaded onto the support material by applying gentle suction at the bottom of the column with the aid of a vacuum pump apparatus.

The column was then back-flushed with reagent water saturated with octanol by applying gentle suction at the top of the column. End fittings were attached to the column after it had been flushed.

Apparatus Configuration

A Nesslab Model IC-515 Constant Temperature Water Bath (Nesslab Instruments, Inc., Portsmouth, NH) was used to maintain the test temperature ($25.0 \pm 0.05^\circ\text{C}$) throughout the experiment. The constant temperature bath was filled with water. The temperature of the water bath was monitored using a SAMA CP-45 high-resolution thermometer (Ever Ready Thermometer Company, West Paterson, NJ).

A Teel Model 1P680A submersible pump (Dayton Electric Mfg. Co., Chicago, IL) was placed in the constant temperature bath, and was used to pump a continuous stream of water through the jacket surrounding the generator column in order to maintain a constant temperature.

A 2-L Erlenmeyer flask was used as a reservoir for the reagent water saturated with octanol that was being pumped through the generator column. The flask was submerged in the constant temperature bath so that the top of the flask remained above the surface of the water in the constant temperature bath. The inlet line of a Waters Model 510 solvent delivery system (Waters Associates, Milford, MA) was placed in the 2-L flask, and the top of the flask covered with aluminum foil. The

pump was used to control the flow rate of the reagent water through the generator column. The supply of reagent water in the 2-L flask was replenished intermittently during the experiment as needed.

The eluate from the generator column was directed to 2-L separatory funnels. The individual samples were collected manually. A diagram of the apparatus configuration is presented in Figure 2.

Aqueous Sample Collection

The pump was set to deliver approximately 1 mL of reagent water per minute through the generator column. The system was allowed to equilibrate for approximately one hour prior to aqueous sample collection. The eluate was collected dropwise into 2-L separatory funnels containing 100 mL of the extraction solvent (DCM). Three consecutive individual aqueous samples were collected at approximately 17 hour intervals into three separate 2-L separatory funnels. The volumes of sample collected ranged from 870 to 890 mL. The separatory funnels were stored on a laboratory bench under ambient conditions for one to three days prior to extraction and analysis.

Analytical Method

The analytical method consisted of extracting the aqueous samples with DCM, evaporating the DCM, and reconstituting the sample residues in acetonitrile/water (50:50, v/v). A flow chart of the method is presented in Figure 3. Dichloromethane (100 mL) was added to each separatory funnel prior to sample collection. After collection, each separatory funnel was stoppered and shaken for approximately one minute. The DCM layer was transferred to a round-bottom flask and evaporated to near dryness. The sample was extracted with another 100 mL portion of DCM. The volume of the aqueous sample was then measured using a graduated cylinder. The DCM extracts were combined in the round-bottom flask and evaporated to near dryness. Any remaining octanol was then evaporated from the round-bottom flask under a stream of nitrogen at 80°C. The samples were brought to a final volume of 1.0 mL using ACN/H₂O (50:50, v/v) and placed in autosampler vials for analysis.

The analytical method used for samples of the octanol stock solution consisted of diluting the samples using ACN/H₂O (50:50). Subsamples of 0.100 mL were diluted to a final volume of 50 mL and mixed. Aliquots of the diluted solutions were placed in autosampler vials for analysis.

Concentrations of HBCD in the samples were determined using a Waters high pressure liquid chromatography system (HPLC). The HPLC system consisted of a Waters 616 Pump and 600S Controller, a Waters 717plus Autosampler, and a Waters 486 Tuneable Absorbance Detector. Chromatographic separations were achieved using an Inertsil C₈ column (250 mm x 2.0 mm, 5 μm particle size) supplied by MetaChem Technologies, Inc. (Torrance, CA). Acetonitrile and water was used as the mobile phase utilizing gradient elution conditions. The instrument parameters are summarized in Table 1.

Preparation of Quality Control Samples

Fortification solutions of HBCD were prepared in acetonitrile at concentrations of 10 mg/L and 100 mg/L. These standards were used to fortify reagent water matrix samples at 1.0 and 10.0 μg HBCD/L. One matrix blank and two matrix fortifications (1 ppb and 10 ppb) were prepared and analyzed along with the aqueous samples collected from the generator column.

Calibration Curve and Quantitation

A stock solution of the test substance was prepared in tetrahydrofuran (THF) at a concentration of 1.00 mg/mL. This stock was used to prepare calibration standards in ACN/H₂O (50:50, v/v). Calibration standards ranged in concentration from 0.5 to 10.0 mg HBCD/L. A set of calibration standards was analyzed before and after the set of samples, and a standard was injected after every three samples during the analytical run. A calibration curve was constructed from the linear regression equation using the respective concentration versus peak area responses of the calibration standards (Figure 4). Representative chromatograms of low and high calibration standards are shown in Figures 5 and 6, respectively. The concentration of HBCD in the samples was determined by substituting the peak area responses into the applicable linear regression equation generated from the calibration curve as follows:

- 14 -

HBCD in sample ($\mu\text{g/L}$) = [(Peak area - Y-intercept/slope)* Dilution factor

Molar Concentration (M) = $\frac{\text{Measured HBCD Concentration (g/L)}}{\text{Molecular Weight (HBCD)}} \times 1$

% Recovery = $\frac{\text{Measured HBCD concentration (}\mu\text{g/L)}}{\text{Nominal HBCD concentration (}\mu\text{g/L)}}$

The instrument limit of detection (LOD) for this study was set based upon the injection volume (200 μL) and the lowest calibration standard concentration (0.5 mg/L). The LOD was set at 100 ng of HBCD injected.

The limit of quantitation (LOQ) was 0.500 $\mu\text{g HBCD/L}$ calculated as the product of the lowest calibration standard (500 $\mu\text{g HBCD/L}$) and the dilution factor of the matrix blank sample (0.0010).

RESULTS AND DISCUSSION

Quality Control Samples

No interferences were observed at or above the LOQ in the matrix blank sample. A chromatogram of the matrix blank is shown in Figure 7.

The percent recovery of the 1.00 and 10.0 $\mu\text{g HBCD/L}$ matrix fortifications were 104 and 85%. The mean recovery was calculated as 95% of nominal concentration. Representative chromatograms of the 1.0 and 10.0 $\mu\text{g HBCD/L}$ matrix fortification samples are shown in Figures 8 and 9, respectively.

Column Elution

The temperature of the water bath remained constant at 25.0°C throughout the experiment (Table 2).

- 15 -

The nominal flow rate of reagent water through the generator column was measured prior to the start of sample collection. Flow rates were also calculated based on the volume and collection time of each sample that was analyzed. The pump setting was 1.0 mL/min. and the flow rate was measured at 1.0 mL/min. The calculated flow rates for samples averaged 0.87 mL/min. and ranged from 0.86 to 0.87 mL/min. (Table 3).

The results from the analyses of aqueous samples eluted from the generator column are presented in Table 3. A representative chromatogram is shown in Figure 10. The mean concentration of HBCD measured in these samples was 3.97 μg HBCD/L, or 6.19×10^{-9} M (molecular weight of HBCD is 641.7 g/mole).

The results from the analyses of the octanol stock solution are presented in Table 4. A representative chromatogram is shown in Figure 11. The mean concentration of HBCD measured in these samples was 1.67 g HBCD/L, or 2.61×10^{-3} M (molecular weight of HBCD is 641.7 g/mole).

CONCLUSIONS

The octanol/water partition (K_{ow}) coefficient was calculated from the following equation:

$$K_{ow} = \frac{\text{Measured Concentration in Octanol (M)}}{\text{Measured Concentration in Aqueous Samples (M)}}$$

Based on the results from octanol samples collected from the stock solution and aqueous samples collected from the generator column, the mean octanol/water partition coefficient (K_{ow}) for HBCD was determined to be 4.22×10^5 ($\log_{10} K_{ow}$ is 5.625).

REFERENCES

1. **U.S. Environmental Protection Agency.** 1996. Product Properties Test Guidelines, OPPTS 830.7560, Partition Coefficient (n-Octanol/Water), Generator Column Method. Washington, D.C.

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Table 1

Typical HPLC Operational Parameters

INSTRUMENT:	Waters 616 Pump, 600S Controller and 717plus Autosampler Equipped with Millennium Version 2.15 software		
DETECTOR:	Waters 486 Tuneable Absorbance Detector		
ANALYTICAL COLUMN:	Inertsil C ₈ Column (250 mm x 2.0 mm, 5- μ m particle size)		
COLUMN TEMPERATURE:	40°C		
MOBILE PHASE:	Channel A: ACN/H ₂ O (30:70) Channel C: ACN/H ₂ O (95:5)		
FLOW RATE:	0.5 mL/min.		
GRADIENT TABLE:	<u>Time</u>	<u>% A</u>	<u>% C</u>
	0.0	50	50
	3.0	50	50
	8.0	45	55
	13.0	40	60
	18.0	30	70
	24.0	0	100
	25.0	50	50
	30.0	50	50
INJECTION VOLUME:	200 μ L		
WAVELENGTH:	295 nm		
HBCD PEAK RETENTION TIME:	approximately 19 minutes		

Table 2

Water Bath Test Temperatures

Date & Time of Observation	Water Bath Temperature (°C)
3/11/97 5:00 pm	25.0
3/12/97 6:15 am	25.0
3/13/97 2:45 am	25.0
3/13/97 7:45 pm	25.0

Table 3

Results for Aqueous Samples Collected from Generator Column

Sample ID (439C-104-)	Peak Area (uV*sec)	Sample Volume (mL)	Final Volume (mL)	Collection Time (min.)	Calculated Flow Rate (mL/min.)	Measured Concentration ($\mu\text{g HBCD/L}$)	Measured Concentration (M)
1	102976	870	1.0	1005	0.87	2.36	3.63×10^{-9}
2	182810	880	1.0	1020	0.86	4.14	6.45×10^{-9}
3	241270	890	1.0	1020	0.87	5.41	8.43×10^{-9}
						$\bar{x} = 3.97$	$\bar{x} = 6.19 \times 10^{-9}$
						SD = 1.53	SD = 2.39×10^{-9}

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Table 4

Results for Octanol Stock Solution Samples

Sample ID (439C-104-)	Peak Area (uV*sec)	Sample Volume (mL)	Final Volume (mL)	Measured Concentration (μ g HBCD/L)	Measured Concentration (M)
0-1	168002	0.10	50.0	1675000	2.61×10^{-3}
0-2	168585	0.10	50.0	1681000	2.62×10^{-3}
0-3	167246	0.10	50.0	1667000	2.60×10^{-3}
				$\bar{x} = 1674000$	$\bar{x} = 2.61 \times 10^{-3}$
				SD = 7024	SD = 1.00×10^{-3}

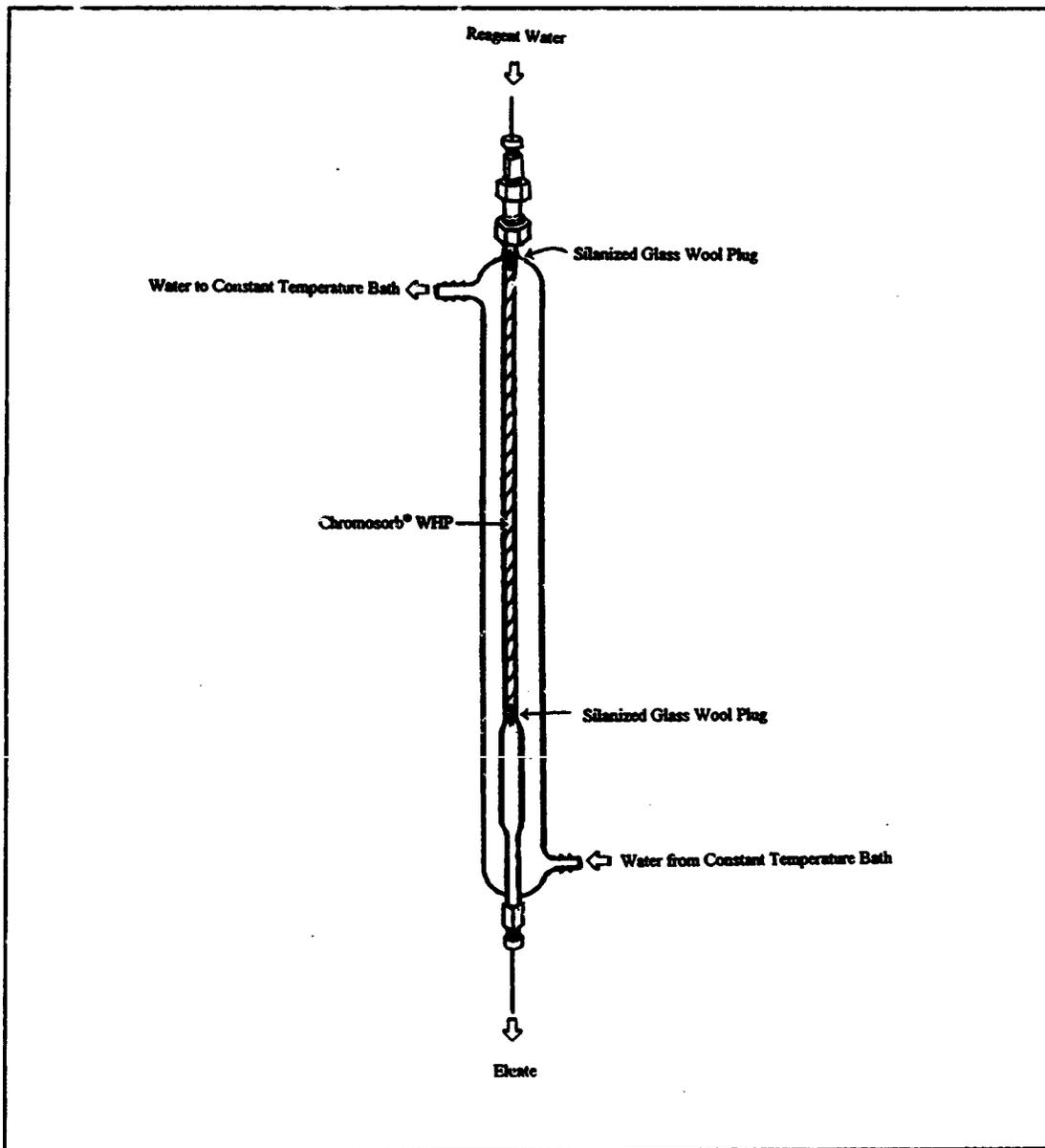


Figure 1. Diagram of generator column.

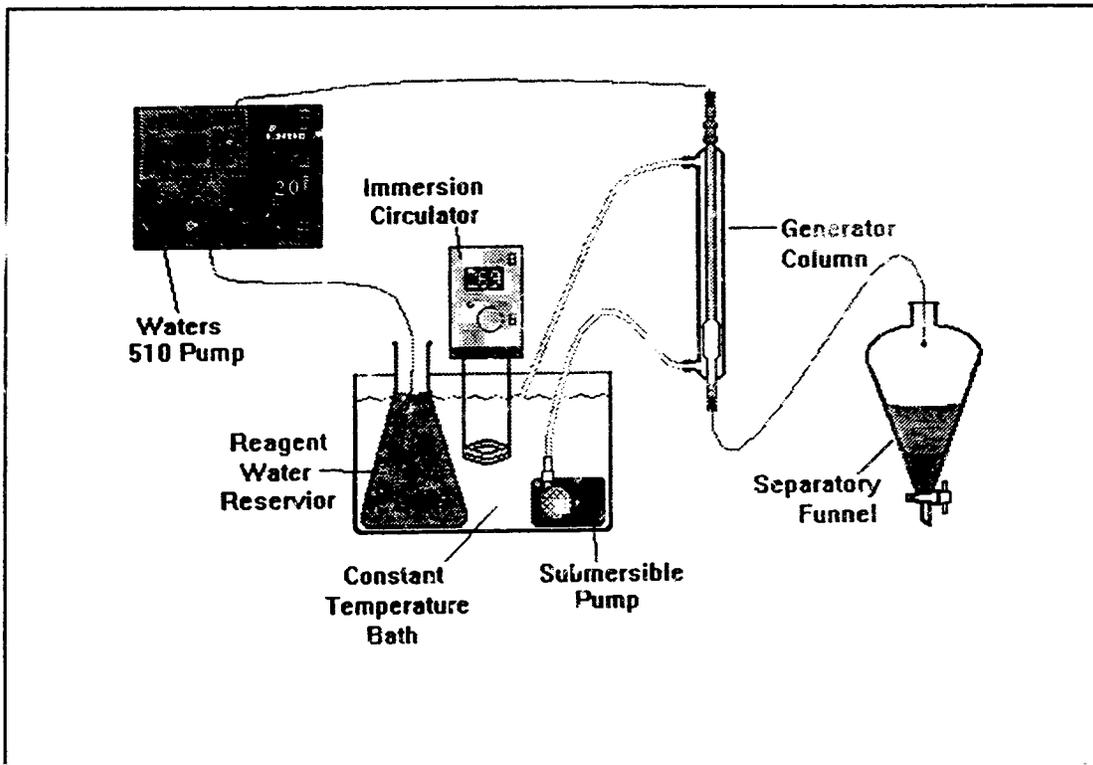


Figure 2. Diagram of apparatus configuration.

ANALYTICAL METHOD FLOW CHART

Add 100 mL of dichloromethane (DCM) to each separatory funnel containing a matrix blank or fortification sample

↓

Shake each separatory funnel for about one minute, let stand to separate, then transfer a portion of the DCM layer into a 125-mL round-bottom flask.

↓

Evaporate the extract to a small volume on a rotovap at 30 to 40°C.

↓

Continue adding portions of the DCM extract and evaporating to a small volume.

↓

Repeat the partition steps with an additional 100 mL of DCM.

↓

Measure the volume of the sample after extraction using a 1-L graduated cylinder.

↓

Evaporate the extract to complete dryness.

↓

Add 1.0 mL of acetonitrile/water (50:50) to the round-bottom flask using a class A pipet.

↓

Swirl and vortex or sonicate the round-bottom flask to reconstitute the sample.

↓

Transfer the sample into a vial for analysis using HPLC/UV.

Figure 3. An analytical method flow chart.

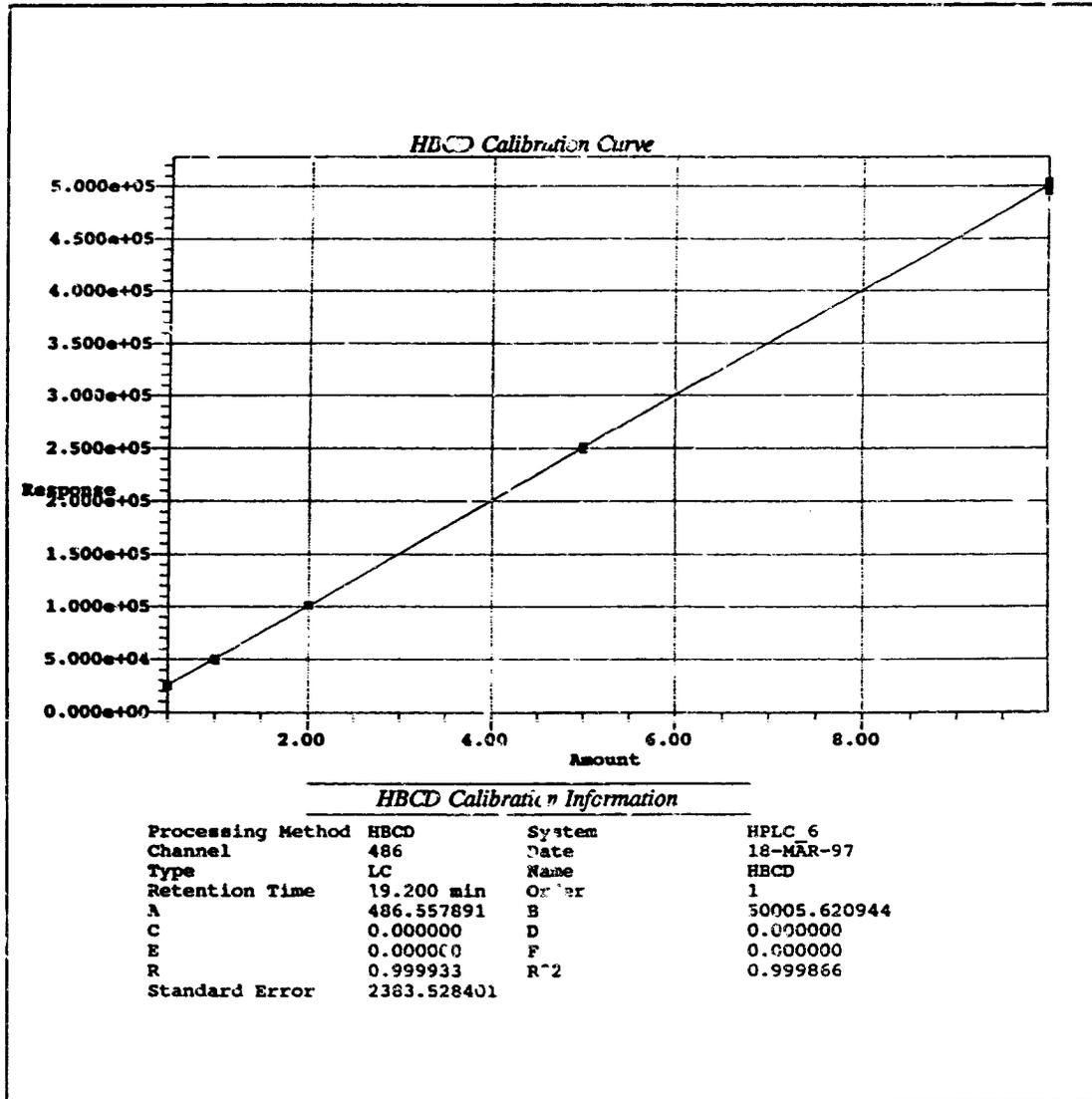


Figure 4. Representative calibration curve.

**ANALYTICAL METHOD VERIFICATION FOR THE DETERMINATION OF
HEXABROMOCYCLODODECANE (HBCD) IN WELL WATER**

FINAL REPORT

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 439C-107

AUTHORS:

**Timothy Z. Kendall, M.S.
Willard B. Nixon, Ph.D.**

STUDY INITIATION DATE: March 5, 1997

STUDY COMPLETION DATE: June 4, 1997

Submitted to

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



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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

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

TITLE: Analytical Method Verification for the Determination of Hexabromocyclododecane (HBCD) in Well Water

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 439C-107

STUDY COMPLETION: June 4, 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:

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

June 4, 1997
DATE

SPONSOR APPROVAL

Hasmukh Shah
Sponsor

June 6, 1997
DATE

QUALITY ASSURANCE

This study was examined for 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. 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:
Sample Preparation	March 6, 1997	March 6, 1997	March 12, 1997
Draft Report and Data	March 17 and 18, 1997	March 18, 1997	March 21, 1997
Final Report	June 4, 1997	June 4, 1997	June 4, 1997



 Lisa T. Drottar
 Quality Assurance Representative

6-4-97

 DATE

REPORT APPROVAL

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

TITLE: Analytical Method Verification for the Determination of Hexabromocyclododecane (HBCD) in Well Water

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 439C-107

This report was reviewed by the individuals involved in the conduct and management of the study, and was found to be an accurate reflection of the methods used, data collected and results of the study.

STUDY DIRECTOR:

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

June 4, 1997
DATE

MANAGEMENT:

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

June 4, 1997
DATE

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SUMMARY

SPONSOR:	Chemical Manufacturers Association's 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:	439C-107
TEST SUBSTANCE:	Hexabromocyclododecane (HBCD)
STUDY:	Analytical Method Verification for the Determination of Hexabromocyclododecane (HBCD) in Well Water
FORTIFIED TEST CONCENTRATIONS:	1.00, 2.00, 5.00 and 20.0 µg/L
TEST DATES:	Experimental Start - March 6, 1997 Experimental Termination - March 7, 1997

TEST SYSTEM:	Well Water
--------------	------------

SUMMARY:	Well water samples were fortified to reflect concentrations of 1.00, 2.00, 5.00 and 20.0 µg/L of hexabromocyclododecane (HBCD). The fortified samples were extracted and the extracts analyzed using gradient elution high performance liquid chromatography and UV detection. Sample recoveries ranged from 95.4 to 99.1% of nominal concentrations with a mean percent recovery of 97.1%. No interferences at or above the limit of quantitation of 0.400 µg/L were observed during the sample analyses. Based on these recoveries, the methodology was considered suitable to analyze HBCD in well water.
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INTRODUCTION

Verification samples were fortified and analyzed to evaluate the performance of a method for the analysis of hexabromocyclododecane (HBCD) in well water. The study was conducted by Wildlife International Ltd. and identified as Project Number 439C-107. The analyses of water samples were performed at Wildlife International Ltd. using high performance liquid chromatography (HPLC) with UV detection. All raw data generated by Wildlife International Ltd. and a copy of the final report are filed under Project Number 439C-107 in archives located on the Wildlife International Ltd. site.

MATERIALS AND METHODS

Test Substance

The test substance used for the analytical portion of the study was the industry composite of HBCD designated as Wildlife International Ltd. identification number 3577. This was the same composite used for the biological portion of the study. The composite test substance was used to prepare both matrix fortification samples and calibration standards.

Test System

The water used for testing was well water obtained from a well approximately 45 meters deep located on the Wildlife International Ltd. site. The well water is characterized as moderately-hard water. Specific conductance, hardness, alkalinity and pH of well water during the four-week period immediately preceding the test are presented in Appendix I. The results of analyses performed to measure the concentrations of selected contaminants in well water used by Wildlife International Ltd. are presented in Appendix II.

Analytical Method

The analytical method used for the analysis of hexabromocyclododecane (HBCD) in well water utilized a solvent partition (extraction) procedure. Aqueous samples ranging from 100 to

1000 mL were partitioned with 100 mL of dichloromethane by shaking (in a separatory funnel) for approximately one minute. The phases were allowed to separate and the lower organic phase was drained into a 500-mL roundbottom flask. An additional 100 mL of dichloromethane was added to the aqueous sample and the partition was repeated. The dichloromethane extracts were combined in the roundbottom flask and the solvent reduced to approximately 1 - 2 milliliters using rotary evaporation at approximately 40°C. The concentrated extracts were evaporated to dryness under a gentle stream of nitrogen. To each flask was added four milliliters of 50% CH₃CN : 50% H₂O and the flasks were swirled in order to ensure complete dissolution of the HBCD residues. An aliquot of each sample extract was transferred to an autosampler vial and submitted for HPLC analysis.

Concentrations of HBCD in the test sample extracts were determined by high performance liquid chromatography (HPLC) using a Hewlett-Packard Model 1090 high performance liquid chromatograph equipped with a Waters Model 486 UV/VIS detector. Chromatographic separations of the peak representing HBCD was achieved using two Zorbax Rx C₁₈ Columns (150 mm length x 4.6 mm ID) in series and gradient elution chromatography. Instrumental parameters have been 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 the series of test samples. Linear regression equations were generated using the respective concentrations of the calibration standards versus the peak area responses. A representative calibration curve for hexabromocyclododecane (HBCD) is presented in Figure 2. The concentration of hexabromocyclododecane (HBCD) in the test samples was determined by substituting the respective peak area responses into the applicable linear regression equation. Representative chromatograms of low and high calibration standards for hexabromocyclododecane (HBCD) are shown in Figures 3 and 4, respectively.

The instrument limit of detection (LOD) for this study was set based upon the injection volume (250 µL) and the lowest detectable calibration standard concentration (100 µg/L). The LOD

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was set at 25 ng injected on-column. The limit of quantitation (LOQ) for the method validation analyses was set at 0.400 $\mu\text{g/L}$ as determined by the product of the lowest standard (100 $\mu\text{g/L}$) and the dilution factor of the matrix blanks (0.00400).

RESULTS

Reagent and Matrix Blank Samples

In addition to the fortified test samples, three reagent blanks and three matrix blanks were analyzed to determine the possibility of interferences. No interferences in these test samples were observed at or above the LOQ during the sample analyses (Table 2). A representative chromatogram of a matrix blank is presented in Figure 5.

Fortification Samples

During the method verification, well water test samples were fortified in triplicate at 1.00, 2.00, 5.00 and 20.0 $\mu\text{g/L}$ of hexabromocyclododecane (HBCD) using stock solutions prepared in acetonitrile. Well water test samples fortified at 1.00, 2.00, 5.00 and 20.0 $\mu\text{g/L}$ of hexabromocyclododecane (HBCD) yielded mean recoveries of 97.3, 96.0, 96.7 and 98.3% of nominal concentrations, respectively (Table 2). Representative chromatograms of low and high-level matrix fortifications in well water are presented in Figures 6 and 7, respectively.

CONCLUSIONS

The method for the analysis of hexabromocyclododecane (HBCD) in well water was verified for concentrations ranging from 1.00 to 20.0 $\mu\text{g/L}$ of HBCD. The recoveries of hexabromocyclododecane ranged from 95.4 to 99.1% of nominal concentrations. The method is suitable for the analysis of hexabromocyclododecane (HBCD) in well water taken from aquatic toxicity studies.

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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 (150 mm x 4.6 mm, 5 μ m particle size) x 2 in series			
OVEN TEMPERATURE:	40°C			
FLOW RATE:	1.00 mL/minute			
PUMP MODE:	Gradient			
MOBILE PHASE:	Solvent A:(30% acetonitrile:70% water) Solvent B:(95% acetonitrile:5% water)			
	Time (min.)	%A	%B	Curve
	0.01	45.0	55.0	--
	2.00	45.0	55.0	Hold
	12.0	0.0	100	Linear
	22.0	0.0	100	Hold
	22.1	45.0	55.0	Step
	27.0	45.0	55.0	Hold
INJECTION VOLUME:	250 μ L			
HEXABROMOCYCLO- DODECANE (HBCD) PEAK RETENTION TIME:	14.6 minutes			
PRIMARY ANALYTICAL WAVELENGTH:	210 nm			

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Table 2
Method Verification Recoveries of Hexabromocyclododecane (HBCD)
in Well Water

Sample		Hexabromocyclododecane (HBCD) Concentration ($\mu\text{g/L}$)		Percent Recovery
Number (439C-107-)	Type	Fortified	Measured	
VREB-1	Reagent Blank	0.0	<LOQ ¹	--
VREB-2	Reagent Blank	0.0	<LOQ	--
VREB-3	Reagent Blank	0.0	<LOQ	--
VMAB-1	Matrix Blank	0.0	<LOQ	--
VMAB-2	Matrix Blank	0.0	<LOQ	--
VMAB-3	Matrix Blank	0.0	<LOQ	--
VMAS-1	Matrix Fortification	1.00	0.966	96.6
VMAS-2	Matrix Fortification	1.00	0.961	96.1
VMAS-3	Matrix Fortification	1.00	0.991	99.1
VMAS-4	Matrix Fortification	2.00	1.93	96.5
VMAS-5	Matrix Fortification	2.00	1.91	95.4
VMAS-6	Matrix Fortification	2.00	1.92	96.2
VMAS-7	Matrix Fortification	5.00	4.86	97.2
VMAS-8	Matrix Fortification	5.00	4.84	96.8
VMAS-9	Matrix Fortification	5.00	4.80	96.1
VMAS-10	Matrix Fortification	20.0	19.6	97.8
VMAS-11	Matrix Fortification	20.0	19.7	98.4
VMAS-12	Matrix Fortification	20.0	19.8	98.8
Mean =				97.1
Standard Deviation =				1.19
n =				12

¹ The limit of quantitation was set at 0.400 $\mu\text{g/L}$ as determined by the product of the lowest standard (100 $\mu\text{g/L}$) and the dilution factor of the matrix blanks (0.00400).

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

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**METHOD OUTLINE FOR THE PROCESSING OF
HECD IN WELL WATER**

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

↓

Prepare recovery samples by directly fortifying well water (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 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 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 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.

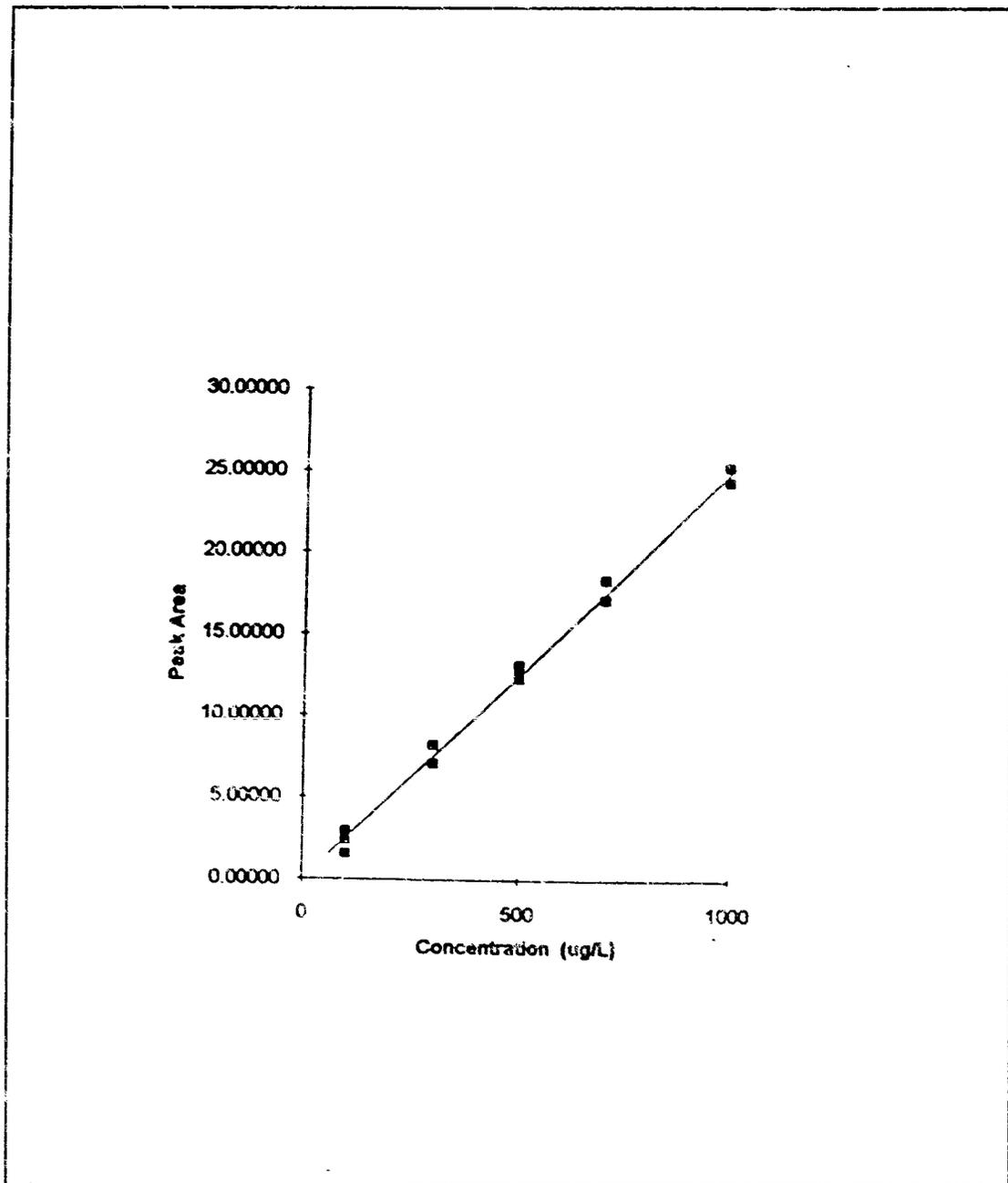


Figure 2. A representative calibration curve for hexabromocyclododecane (HBCD).
Slope = 0.02; Intercept = 0.03537; $r^2 = 0.9956$.

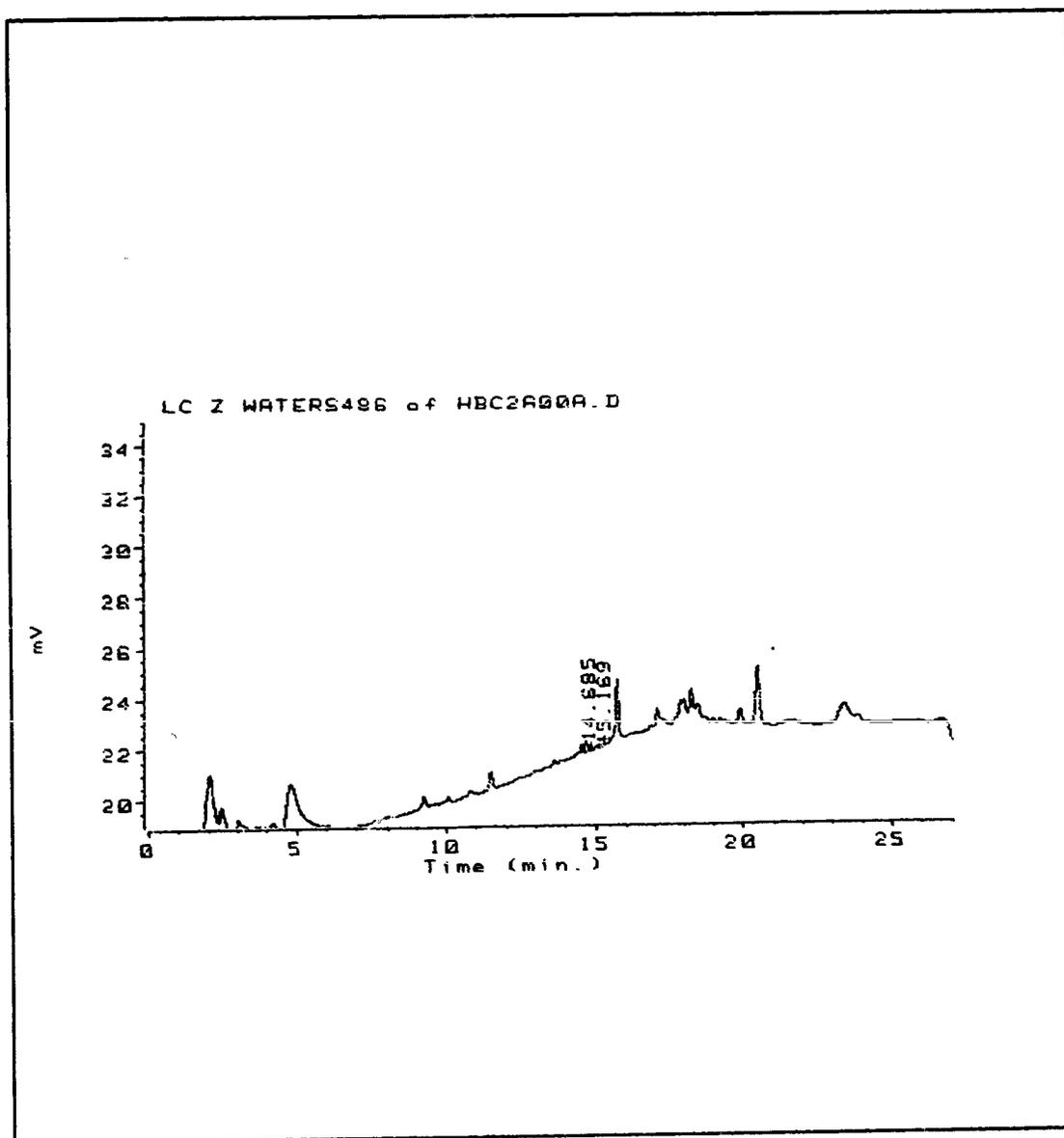


Figure 3. A representative chromatogram of a low-level (100 µg/L) hexabromocyclododecane (HBCD) standard (25 ng on-column).

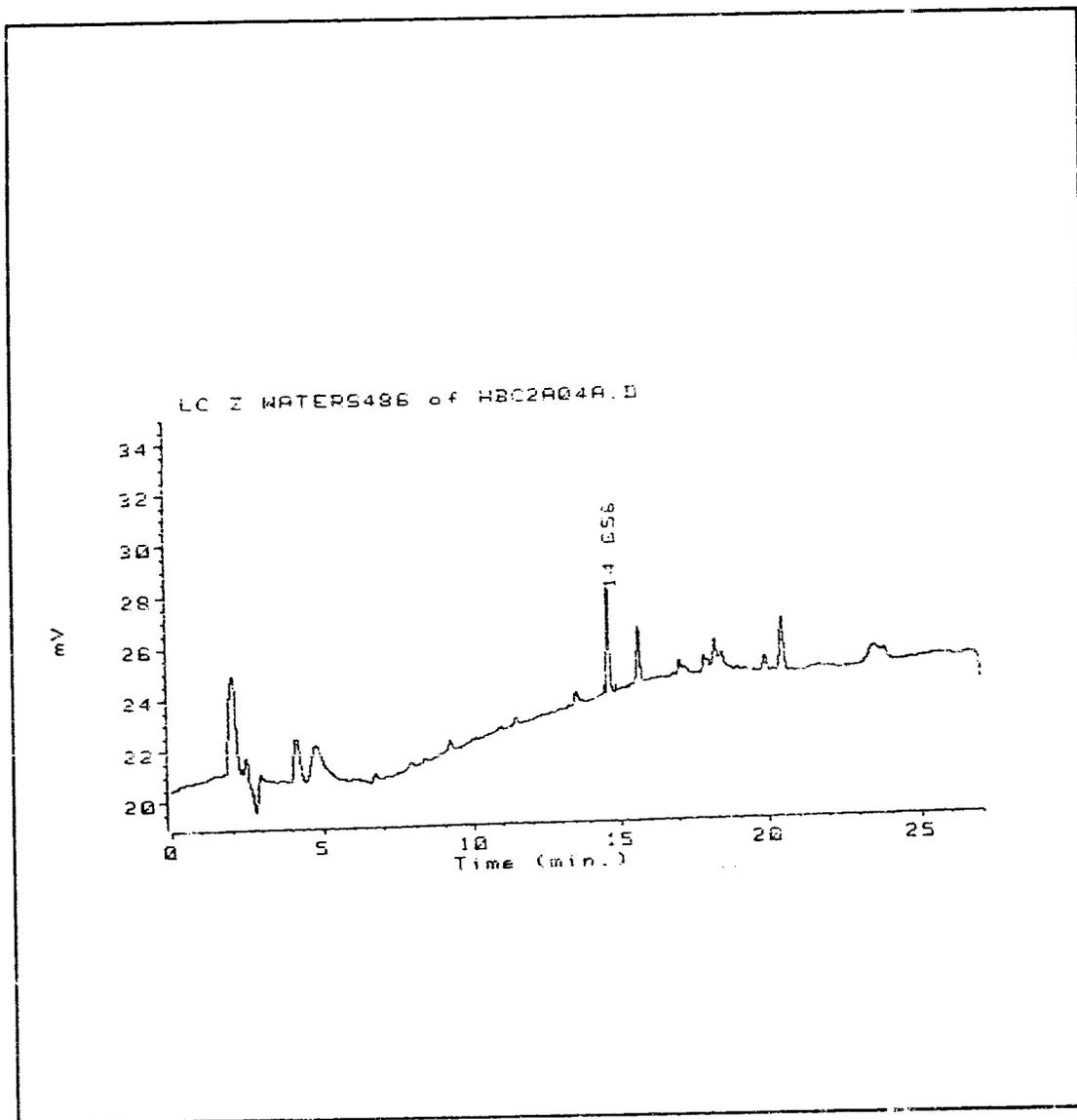


Figure 4. A representative chromatogram of a high-level (1000 $\mu\text{g/L}$) hexabromocyclododecane (HBCD) standard (250 ng on-column).

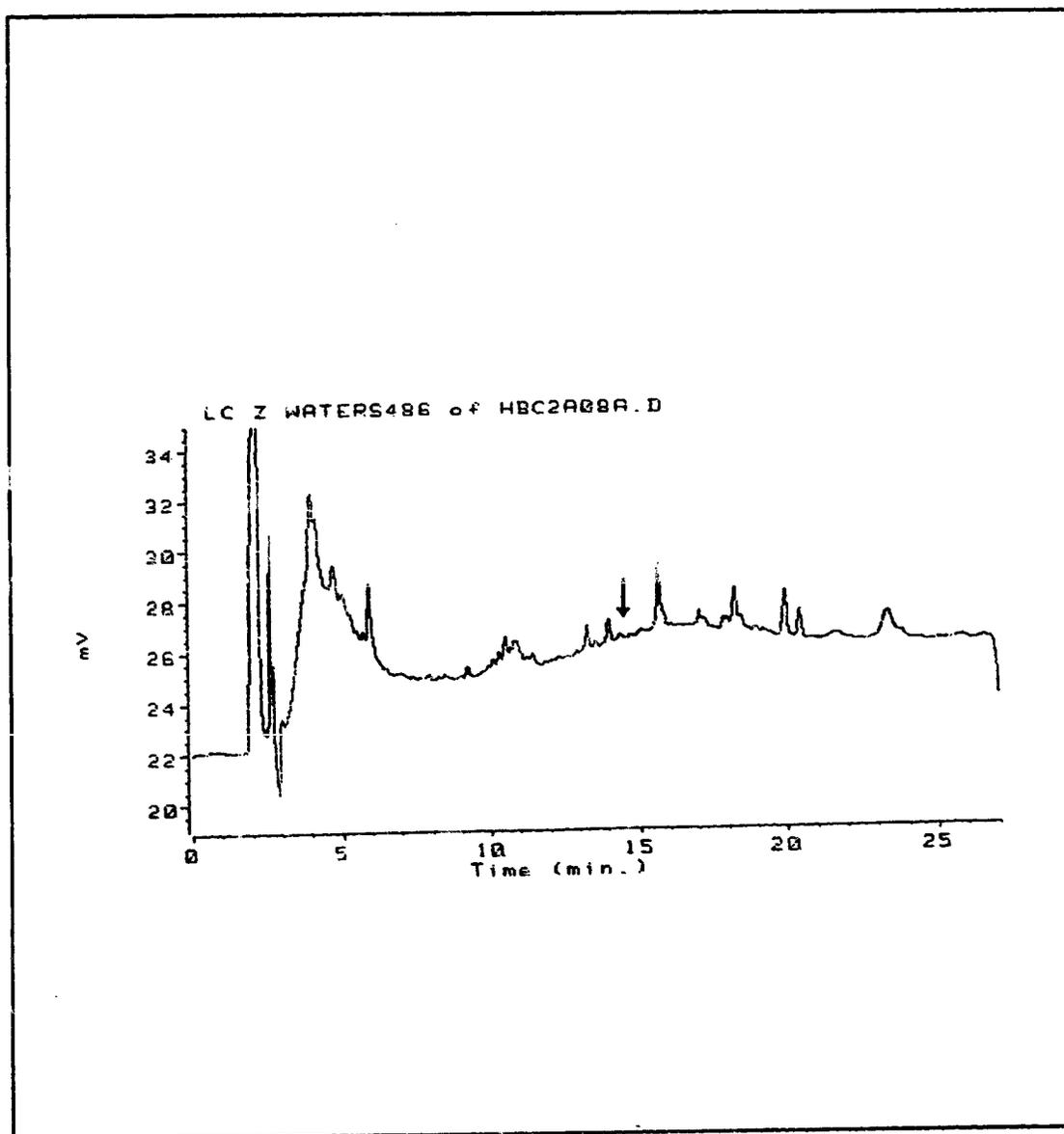


Figure 7. A representative chromatogram of a well water matrix blank (439C-107-VMAB-1). The arrow indicates the retention time of hexabromocyclododecane (HBCD).

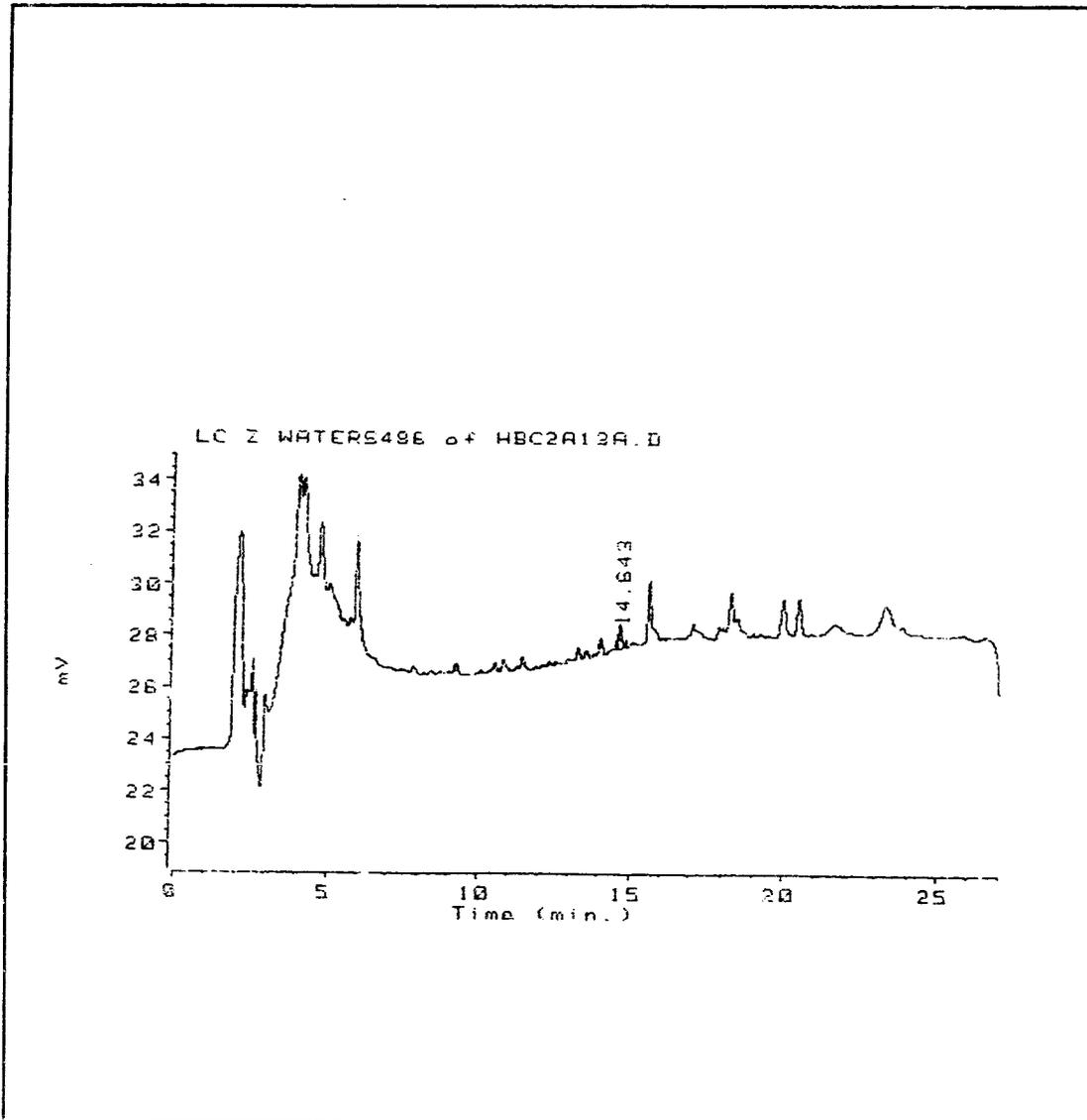


Figure 6. A representative chromatogram of a low-level (1.00 $\mu\text{g/L}$) well water hexabromocyclododecane (HBCD) matrix fortification (439C-107-VMAS-2).

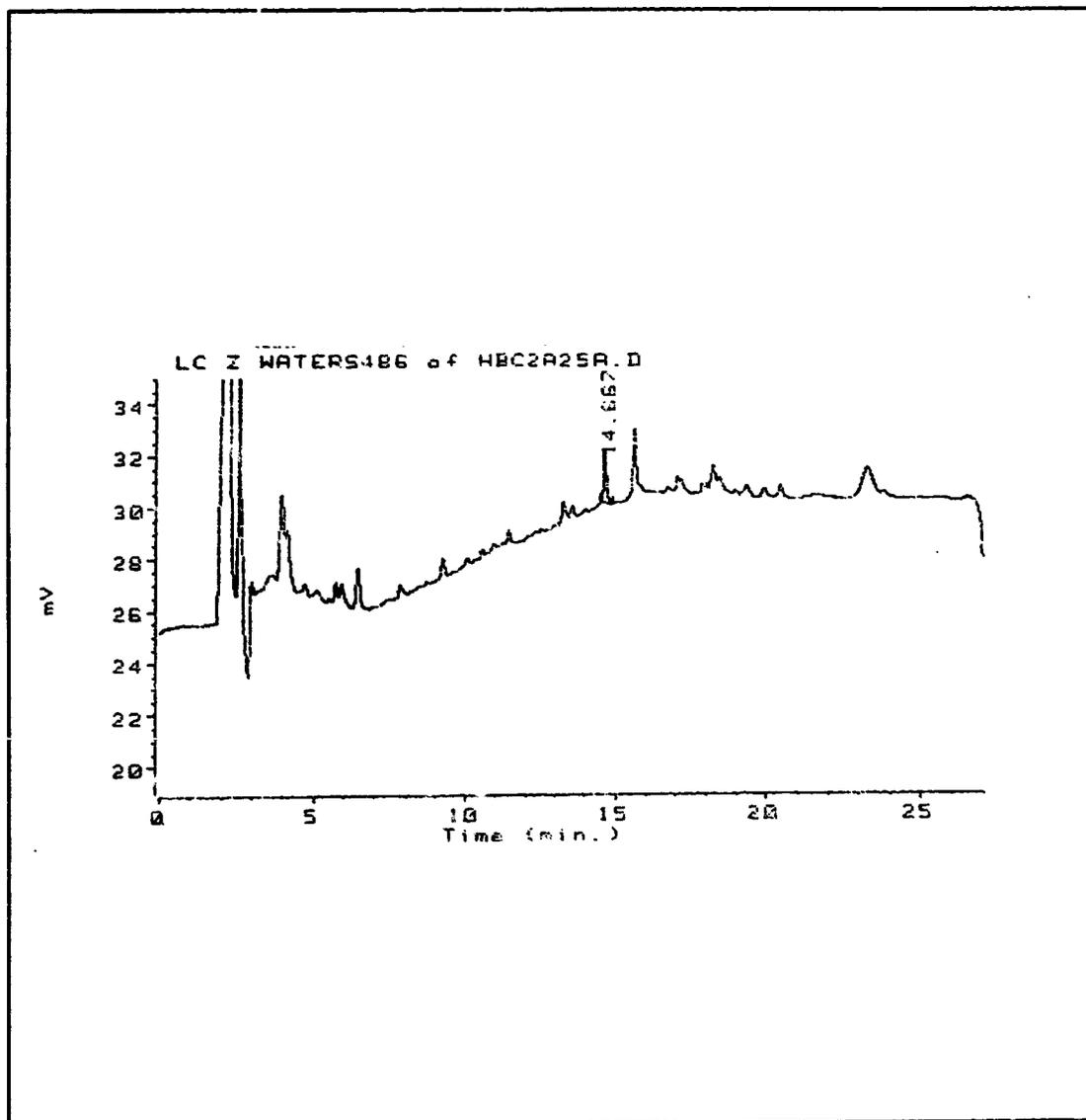


Figure 7. A representative chromatogram of a high-level ($20.0 \mu\text{g/L}$) well water hexabromocyclododecane (HBCD) matrix fortification (439C-107-VMAS-12).

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APPENDIX I

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

	Mean	Range
Specific Conductance (μ mhos/cm)	299 (N = 4)	295 - 300
Hardness (mg/L as CaCO ₃)	132 (N = 4)	124 - 136
Alkalinity (mg/L as CaCO ₃)	176 (N = 4)	172 - 180
pH	8.3 (N = 4)	8.2 - 8.4

APPENDIX II
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)
 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 PCB's

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, 1996.

APPENDIX III

Protocol and Amendments

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**HEXABROMOCYCLODODECANE (HBCD):
DETERMINATION OF THE WATER SOLUBILITY**

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 439C-105

**U.S. EPA 40 CFR Ch. 1 § 796.1860 Water Solubility (Generator Column Method)
OECD Guideline 105 Water Solubility (Column Elution Method)**

AUTHORS:

**Joel I. Stenzel
Barbara J. Markley, Ph.D.**

STUDY INITIATION DATE: February 19, 1996

STUDY COMPLETION DATE: June 13, 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): Determination of the Water Solubility

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 439C-105

STUDY COMPLETION: June 13, 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:

Joel I. Stenzel
Joel I. Stenzel, B.S.
Senior Chemist

6/13/97
DATE

SPONSOR APPROVAL:

Hamukh Shah
Sponsor

6-18-97
DATE

QUALITY ASSURANCE

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

TITLE: Hexabromocyclododecane (HBCD): Determination of the Water Solubility

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 439C-105

This study was examined for 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. 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 Composite Preparation	February 19, 1996	February 19, 1996	February 21, 1996
Generator Column Preparation	February 18, 1997	February 18, 1997	February 27, 1997
Draft Report and Data	April 1 to April 3, 1997	April 3, 1997	April 8, 1997
Final Report	June 12, 1997	June 13, 1997	June 13, 1997

Susan L. Hopper
Susan L. Hopper
Senior Quality Assurance Representative

6-13-97
DATE

REPORT APPROVAL

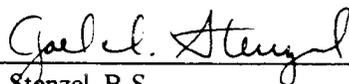
SPONSOR: Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel

TITLE: Hexabromocyclododecane (HBCD): Determination of the Water Solubility

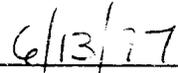
WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 439C-105

This report was reviewed by the individuals involved in the conduct and management of the study, and was found to be an accurate reflection of the methods used, data collected and results of the study.

STUDY DIRECTOR:



Joel I. Stenzel, B.S.
Senior Chemist



DATE

MANAGEMENT:



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



DATE

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0-1-9-6

SUMMARY

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

WILDLIFE INTERNATIONAL LTD.	
PROJECT NUMBER:	439C-105
TEST SUBSTANCE:	Hexabromocyclododecane (HBCD)
STUDY:	Hexabromocyclododecane (HBCD): Determination of the Water Solubility
TEST DATES:	Experimental Start - May 3, 1996 Experimental Termination - March 12, 1997

SUMMARY:	The solubility of HBCD in water at 25.0°C was determined to be 3.4 $\mu\text{g/l}$ (ppb) using a column elution method.
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INTRODUCTION

This study was conducted by Wildlife International Ltd. for Chemical Manufacturers Association's (CMA) Brominated Flame Retardant Industry Panel at the Wildlife International Ltd. analytical chemistry facility in Easton, Maryland. Tests were performed using a column elution method. Samples were eluted from a generator column and analyzed from February 26 to March 12, 1997. Raw data generated by Wildlife International Ltd. and a copy of the final report are filed under Project Number 439C-105 in archives located on the Wildlife International Ltd. site.

OBJECTIVE

The objective of this study was to determine the solubility limit of the test substance, hexabromocyclododecane (HBCD), in water at 25.0°C using a column elution method.

EXPERIMENTAL DESIGN

A preliminary test was conducted to estimate the solubility of the test substance in reagent water at room temperature. A generator column was prepared for the definitive test. The column temperature was maintained at 25.0 °C and reagent water was pumped through it at approximately 2 mL per minute to elute the test substance. Samples of the eluate were collected and analyzed to determine the saturation concentration of the test substance. The flow rate of reagent water through the column was reduced to approximately half the original flow rate and the saturation concentration was determined again.

MATERIALS AND METHODS

This study was conducted according to procedures outlined in the protocol, "Hexabromocyclododecane (HBCD): Determination of the Water Solubility" (Appendix I). The protocol was based on procedures outlined in OECD Guidelines, Method 105 (1); and EPA 40 CFR

§ 796.1860 (2). The column elution method was used to determine the water solubility limit of the test substance.

Test Substance

The test substance consisted of a composite of hexabromocyclododecane (HBCD) samples received from three manufacturers. The materials identity and date received from each of the manufacturers are 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 Corporation for 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.

Reagent Water

The reagent water used in this study met the specifications for ASTM Type II water. The water was obtained from a well located on the Wildlife International Ltd. site. The well water was pumped through a series of filters to remove microorganisms and particles greater than $0.2 \mu\text{m}$. The water was further purified using a Culligan® Hi-Flo 1 Water Softener, a Culligan® S-Series Reverse Osmosis System, and a Barnstead NANOpure® ultrapure water system. The resistivity of the purified reagent water used for this study was at least 17.3 megohm-cm.

Preliminary Test

A preliminary test was performed to estimate the solubility of the test substance in reagent water. Approximately 100 mg of the composite test substance was placed in a vessel. A pre-determined volume of reagent water was added, and the vessel was shaken. The solubility of the test substance was assessed by visual observations. The process was repeated twice by adding successively larger volumes of reagent water.

Solvents

Tetrahydrofuran (THF) (Fisher Chemical, Fairlawn, NJ catalog number T425-1) was used for preparation of stock solutions of the test substance. Mixtures of acetonitrile (ACN) and reagent water were used for preparation of calibration standards and samples, and as mobile phases for high pressure liquid chromatography (HPLC). The acetonitrile was supplied by either Burdick and Jackson (Muskegon, MI, catalog number 015-4) or Fisher Chemical (catalog number A996-4). Dichloromethane (DCM) (Burdick and Jackson, catalog number 300-4) was used as an extraction solvent for this study. All solvents used for this study were suitable for HPLC and residue analysis.

Preparation of Generator Column

The inert carrier material used to charge the generator column was glass beads. The glass beads were supplied from J. T. Baker (Phillipsburg, NJ, catalog number 7467-01), and identified on the label as Empore, Filter Aid 400, high density glass beads. Approximately 25 mL (~74 g) of glass beads were rinsed with acetone, acetonitrile and dichloromethane to remove any potential contaminants, then transferred to a round-bottom flask.

A subsample of the composite test substance (300 mg) was weighed into a 500-mL Erlenmeyer flask. Dichloromethane was added to the 250-mL mark. The flask was swirled until

the test substance appeared to be completely dissolved. The test substance solution was transferred to the round-bottom flask containing the glass beads.

A rotary evaporator was used to remove the solvent and coat the test substance onto the glass beads. The water bath of the rotary evaporator was set at 35 °C. The trap was emptied after the solvent had evaporated, and the flask was replaced on the rotary evaporator for at least another 30 minutes, to ensure all the solvent had been removed. The contents of the round-bottom flask then were transferred to a 150-mL beaker. Approximately 20 mL of reagent water were added, and the beaker was swirled and sonicated to remove air bubbles and homogenize the slurry.

The generator column was an Adjusta-Chrom[®], jacketed, recycling column supplied by Ace Glass Inc. (Vineland, NJ, catalog number 5819). The glass column, 300 mm long with an internal diameter of 10 mm, was equipped with Teflon[®] plungers and glass filter discs at both ends. A small plug of silanized glass wool was placed in the bottom of the column, the plunger inserted, and end fittings fastened to the bottom. The slurry of glass beads coated with test substance was poured into the top of the column. Another small plug of silanized glass wool was placed on the top of the column, the plunger inserted, and end fittings attached. The height of the material packed in the column was approximately 26 cm. A diagram of the generator column is presented in Figure 1.

Apparatus Configuration

A Nesslab Model IC-515 Immersion Circulator (Nesslab Instruments, Inc., Portsmouth, NH) was used to maintain the test temperature (25.0 ± 0.1 °C) of the constant temperature bath throughout the experiment. The constant temperature bath was filled with water. The temperature of the water bath was monitored using either an ASTM 45C kinematic viscosity thermometer or a SAMA CP-45 high-resolution thermometer (Ever Ready Thermometer Company, West Paterson, NJ).

A Teel Model IP680A submersible pump (Dayton Electric Mfg. Co., Chicago, IL) was placed in the constant temperature bath. The submersible pump was used to pump a continuous stream of water through the jacket surrounding the generator column to maintain a constant temperature.

A 2-L Erlenmeyer flask was used as a reservoir for the reagent water being pumped through the generator column. The flask was filled with reagent water and submerged in the constant

temperature bath so that the top of the flask remained above the surface of the water in the constant temperature bath. The inlet line of a Waters Model 510 solvent delivery system (Waters Associates, Milford, MA) was placed in the 2-L flask, and the top of the flask covered with aluminum foil. The pump was used to control the flow rate of the reagent water through the generator column. The supply of reagent water in the 2-L flask was replenished intermittently during the experiment.

The eluate from the generator column was directed to 2-L separatory funnels. The individual samples were collected manually. A diagram of the apparatus configuration is presented in Figure 2.

Sample Collection

The pump was initially set to deliver approximately 2 mL of reagent water per minute through the generator column. The eluate was collected dropwise into 2-L separatory funnels containing 100 mL of the extraction solvent (DCM). The separatory funnels were labelled with a sequential sample number. Individual samples were collected at approximately eight hour intervals, the time required to collect the volume of sample required for analysis. After 30 consecutive samples were collected, the flow rate was changed to approximately half the original flow rate, and sample collection was resumed at approximately 16-hour intervals. The separatory funnels were stored on a laboratory bench under ambient conditions for one to three days prior to extraction and analysis.

Analytical Method

The analytical method consisted of extracting the samples with DCM, evaporating the DCM, and reconstituting the samples in ACN/H₂O (50:50). A flow chart of the method is presented in Figure 3. Dichloromethane (100 mL) was added to each separatory funnel prior to sample collection. After collection, each separatory funnel was stoppered and shaken for approximately one minute. The DCM layer was transferred to a round-bottom flask and evaporated to near dryness. The aqueous sample was extracted with another 100 mL portion of DCM. The second DCM extract was added to the round-bottom flask, and evaporated to dryness. The volume of the aqueous sample remaining was then measured using a graduated cylinder. The extracts were brought to a final volume of 1.0 mL using ACN/H₂O (50:50) and placed in vials for analysis.

Concentrations of HBCD in the samples were determined using a Waters high pressure liquid chromatography system (HPLC). The HPLC was equipped with a Waters 616 Pump and 600S

Controller, a Waters 717plus Autosampler, and a Waters 486 Tuneable Absorbance Detector. Chromatographic separations were achieved using an Inertsil C₈ column (250 mm x 2.0 mm, 5 μm particle size) supplied by MetaChem Technologies, Inc (Torrance, CA). A gradient mixture of acetonitrile and water was used as the mobile phase. The instrument parameters are summarized in Table 1.

Preparation of Quality Control Samples

Fortification standards of HBCD in ACN/H₂O (50:50) were prepared at concentrations of 1.0 mg/L and 10.0 mg/L. These standards were used to fortify reagent water samples (matrix spikes) at 1.0 and 10.0 μg HBCD/L (ppb). A matrix blank and two matrix fortifications (1 ppb and 10 ppb) were prepared each day that samples from the generator column were analyzed. A reagent blank (ACN/H₂O) was also analyzed with each sample set.

Calibration and Quantitation

A stock solution of the test substance was prepared in THF at a concentration of 1.0 mg/mL. Calibration standards of HBCD were prepared from the stock in ACN/H₂O (50:50), and ranged in concentration from 0.5 to 10.0 mg HBCD/L. A complete set of calibration standards was analyzed before and after each set of samples, and a standard injected after a maximum of every five samples. Representative chromatograms of low and high calibration standards are shown in Figures 4 and 5, respectively. The peak area for the major component was used to determine the detector response. No attempt was made to quantitate the minor components of the test substance, since peaks for these components could not be detected at the lowest calibration standard concentration (0.5 mg/L). The instrument limit of detection (LOD) for this study was not experimentally determined, but was set based upon the injection volume (200 μL) and the lowest calibration standard concentration (0.5 mg/L). The LOD thus was set at 100 ng of HBCD injected.

Calibration curves were calculated based on linear regression equations using the respective concentration versus peak area responses of the calibration standards. A representative linear calibration curve is presented in Figure 6.

RESULTS AND DISCUSSION

Preliminary Test

The test substance at approximately 100 mg did not appear to be soluble in 10 mL, 100 mL or 1 L of reagent water. Most of the test substance was observed to settle to the bottom of the bottle. The solubility was estimated to be much less than 100 mg/L, based on visual observations.

Quality Control Samples

No interferences were observed at or above the limit of detection ($0.5 \mu\text{g HBCD/L}$) in any of the matrix blank samples. A representative chromatogram of a matrix blank is shown in Figure 7. The peak area response for the matrix blanks was always below the response of the lowest calibration standard. There were also no interferences observed in the reagent blank (ACN/H₂O) samples. A representative chromatogram of a reagent blank is shown in Figure 8.

The mean recovery from ten matrix samples fortified at 10 ppb was 105% (standard deviation 2.0), and ranged from 103% to 108%. A representative chromatogram of a 10-ppb matrix fortification is shown in Figure 9. The mean recovery from ten matrix samples fortified at 1 ppb was 104% (standard deviation 5.2), and ranged from 100% to 110%. A representative chromatogram of a 1-ppb matrix fortification is shown in Figure 10. The 1 ppb concentration was considered the limit of quantitation (LOQ).

Column Elution

The temperature of the water bath ranged from 24.9 to 25.1°C during the experiments (Table 2), with the exception of one observation. The temperature of the water bath recorded on March 2 at 4:00 pm was 25.4°C.

The nominal flow rate of reagent water through the generator column was initially set at 2.0 mL/min. The initial flow rate was measured at 2.0 mL/min. prior to the start of sample collection. Samples were collected at this flow rate until the solubility plateau was achieved. Flow rates were calculated for each sample based on the sample volumes and collection times. The calculated flow rates for samples collected at the initial flow rate averaged 1.96 mL/min. and ranged from 1.88 to 1.98 mL/min. (Table 3). After the solubility plateau was achieved, the flow rate was reduced to approximately half the initial flow rate. The reduced flow rate was measured at

1.0 mL/min. prior to resuming sample collection. The calculated flow rates for samples collected at the reduced flow rate averaged 0.92 mL/min. and ranged from 0.91 to 0.93 mL/min. (Table 4).

The results from the analyses of samples eluted at a nominal flow rate of 2.0 mL/min. are presented in Table 3 and Figure 11. All of the samples were analyzed to determine the solubility concentration of HBCD. The solubility limit was considered to have been achieved when at least five consecutive samples gave similar results. The results from samples 26 to 30 met these criteria and were considered to have reached the solubility plateau. A representative chromatogram is shown in Figure 12. The mean concentration of HBCD measured in these samples was 3.4 μg HBCD/L (ppb), with a standard deviation of 0.23.

The results from the analyses of samples eluted at a nominal flow rate of 1.0 mL/min. are presented in Table 4 and Figure 13. The results from samples 32 to 37 were considered to have reached the solubility plateau. Sample number 35 was excluded from the solubility calculations because a portion of the DCM extract from this sample was spilled. A representative chromatogram is shown in Figure 14. The mean concentration of HBCD measured in these samples was 3.3 μg HBCD/L (ppb) with a standard deviation of 0.20.

CONCLUSIONS

Based on the results from samples collected at both flow rates from the generator column, the solubility of HBCD in water was determined to be 3.4 μg HBCD/L (ppb) at 25.0°C.

REFERENCES

1. **Organisation for Economic Cooperation and Development.** 1981. Guideline for Testing of Chemicals, Method 105, "Water Solubility" (Column Elution Method - Flask Method).
2. **U.S. Environmental Protection Agency.** 1991. 40 CFR § 796.1860, Water Solubility (generator column method). Washington, D.C.

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Table 1

Typical HPLC Operational Parameters

INSTRUMENT:	Waters 616 Pump, 600S Controller and 717plus Autosampler Equipped with Millennium Version 2.15 software		
DETECTOR:	Waters 486 Tuneable Absorbance Detector		
ANALYTICAL COLUMN:	Inertsil C ₈ Column (250 mm x 2.0 mm, 5 μm particle size)		
COLUMN TEMPERATURE:	40°C		
MOBILE PHASE:	Channel A: ACN/H ₂ O (30:70) Channel C: ACN/H ₂ O (95:5)		
FLOW RATE:	0.5 mL/min.		
GRADIENT TABLE:	<u>Time</u>	<u>% A</u>	<u>% C</u>
	0.0	50	50
	3.0	50	50
	8.0	45	55
	13.0	40	60
	18.0	30	70
	24.0	0	100
	25.0	50	50
	30.0	50	50
INJECTION VOLUME:	200 μL		
WAVELENGTH:	205 nm		
HBCD PEAK RETENTION TIME:	approximately 19 minutes		

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Table 2

Water Bath Test Temperatures

Date & Time of Observation	Temperature (°C)
2/25/97 4:20 pm	25.1
2/26/97 12:03 am	25.0
8:03 am	25.0
5:12 pm	25.1
2/27/97 12:00 am	25.1
7:59 am	25.0
4:05 pm	25.1
11:56 pm	25.1
2/28/97 8:03 am	25.0
4:02 pm	25.0
3/01/97 12:00 am	25.0
8:00 am	25.0
4:32 pm	25.1
3/02/97 12:03 am	25.1
8:04 am	25.0
4:00 pm	25.4
11:55 pm	25.0
3/03/97 8:05 am	25.0
4:20 pm	25.1
11:59 pm	25.0
3/04/97 8:08 am	25.0
4:12 pm	25.0
11:59 pm	25.0
3/05/97 8:03 am	25.0
4:08 pm	25.0
11:57 pm	25.0
3/06/97 8:08 am	25.0
4:02 pm	25.0
3/07/97 12:22 am	25.0
8:05 am	25.0
4:00 pm	25.0
3/08/97 8:19 am	25.0
3/09/97 12:00 am	25.0
4:08 pm	25.0
3/10/97 8:03 am	25.0
3/11/97 12:01 am	25.0
4:14 pm	25.0
3/12/97 7:59 am	24.9
3/13/97 12:03 am	25.0

Table 3

Results for Samples Collected at a Nominal Flow Rate of 2.0 mL/min.

Sample ID (439C-105-)	Sample Volume (mL)	Collection Time (min.)	Flow Rate (mL/min.)	Measured Concentration (μg HBCD/L)
1	905	467	1.94	6.2
2	940	476	1.97	5.1
3	1075	553	1.94	4.8
4	800	404	1.98	4.6
5	940	484	1.94	4.0
6	945	481	1.96	3.5
7	920	472	1.95	4.0
8	960	486	1.98	3.6
9	900	479	1.88	3.8
10	940	478	1.97	3.8
11	950	480	1.98	3.5
12	1000	513	1.95	3.3
13	890	450	1.98	3.9
14	940	482	1.95	3.7
15	940	475	1.98	3.7
16	940	479	1.96	3.0
17	960	486	1.98	2.2
18	975	495	1.97	2.5
19	935	475	1.97	2.2
20	905	456	1.98	3.3
21	950	484	1.96	3.1
22	920	467	1.97	2.2
23	950	484	1.96	2.9
24	955	485	1.97	3.1
25	925	472	1.96	2.6
26	945	488	1.94	3.4
27	930	474	1.96	3.6
28	970	500	1.94	3.5
29	890	463	1.92	3.4
30	930	481	1.93	3.0

Table 4

Results for Samples Collected at a Nominal Flow Rate of 1.0 mL/min.

Sample ID (439C-105-)	Sample Volume (mL)	Collection Time (min.)	Flow Rate (mL/min.)	Measured Concentration (μg HBCD/L)
31	880	948	0.93	2.6
32	860	940	0.91	3.5
33	890	968	0.92	3.4
34	880	955	0.92	3.5
35	885	958	0.92	2.0 ¹
36	900	973	0.92	3.1
37	875	947	0.92	3.1

¹Sample results excluded from solubility plateau due to spillage.

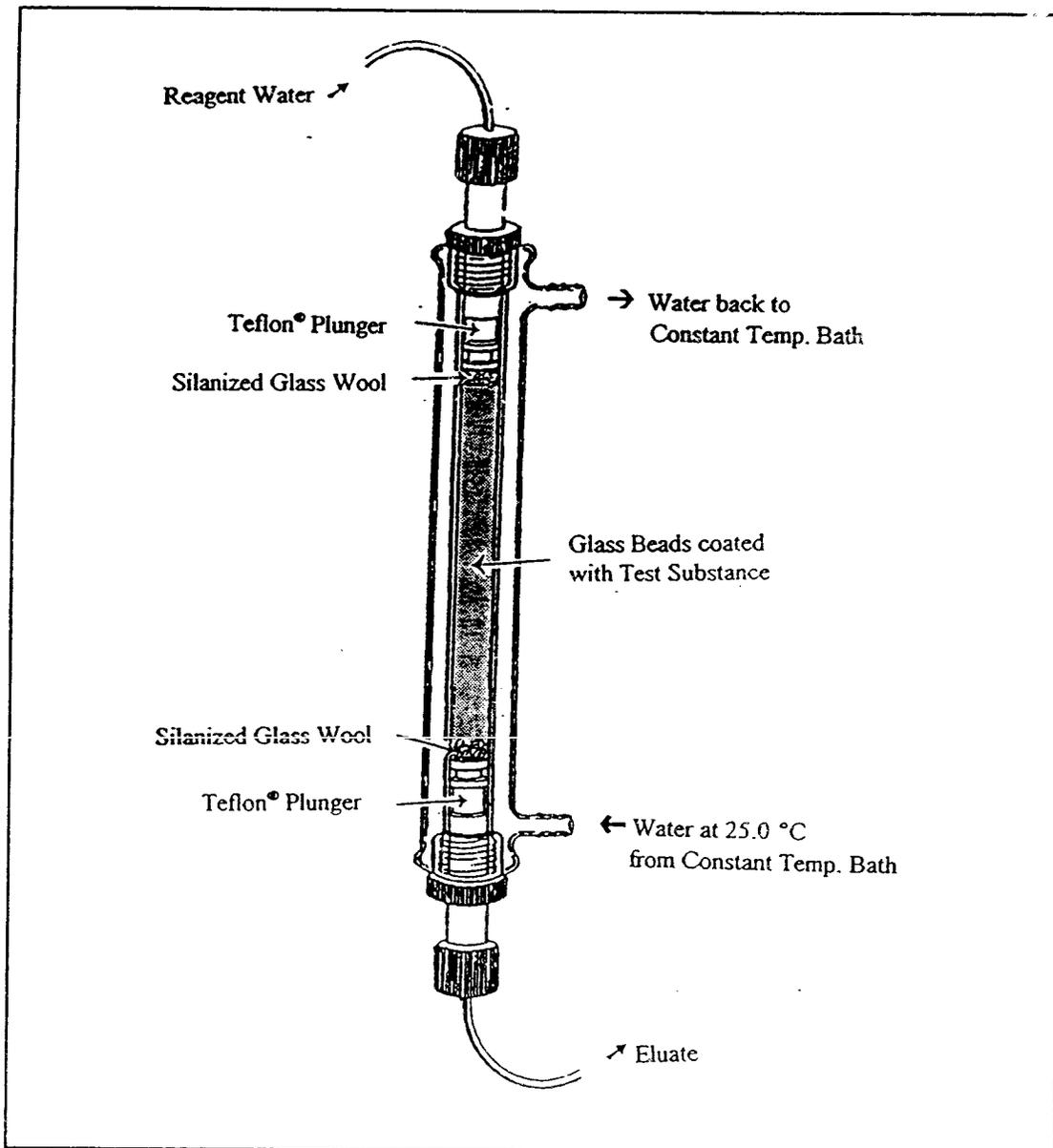


Figure 1. Diagram of generator column.

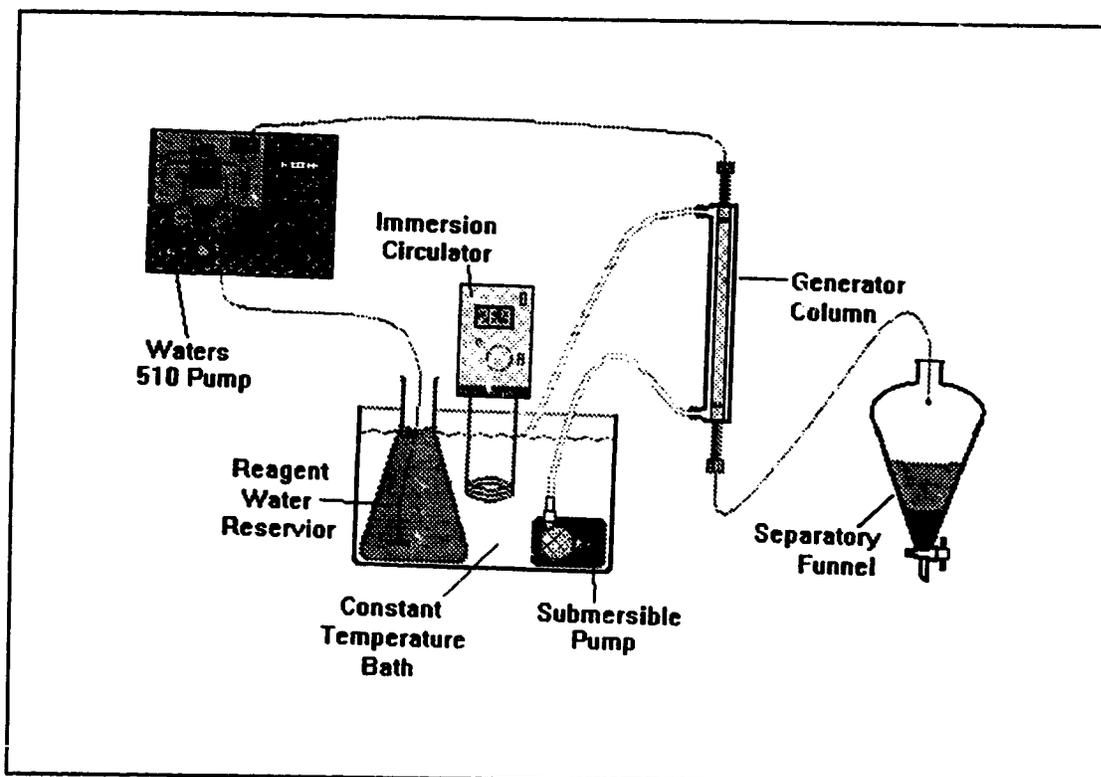


Figure 2. Diagram of apparatus configuration.

ANALYTICAL METHOD FLOW CHART

Add 100 mL of dichloromethane (DCM) to each separatory funnel in preparation for sample collection, and collect samples from generator column.



Prepare matrix blank and fortification samples, and add 100 mL of DCM.



Shake each separatory funnel for about one minute, let stand to separate, then transfer a portion of the DCM layer into a 125-mL round-bottom flask.



Evaporate the extract to a small volume on a rotovap at 30 to 40°C.



Continue adding portions of the DCM extract and evaporating to a small volume.



Repeat the partition steps with an additional 100 mL of DCM.



Measure the volume of the aqueous sample after extraction using a 1-L graduated cylinder.



Evaporate the extract to complete dryness.



Add 1.0 mL of acetonitrile/water (50:50) to the round-bottom flask using a class A pipet.



Swirl and vortex or sonicate the round-bottom flask to reconstitute the sample.



Transfer the sample into a vial for analysis using HPLC/UV.

Figure 3. Analytical method flow chart.

HEXABROMOCYCLODODECANE (HBCD)
A 96-HOUR TOXICITY TEST WITH THE
FRESHWATER ALGA (*Selenastrum capricornutum*)

FINAL REPORT

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 439A-103

TSCA, Title 40 of the Code of Federal Regulations
Part 797, Section 1050
and
Organisation for Economic Cooperation and Development
OECD Guideline 201

AUTHORS:

Cindy A. Roberts
James P. Swigert, Ph.D.

STUDY INITIATION DATE: February 16, 1996

STUDY COMPLETION DATE: June 3, 1997

Submitted to

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



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Easton, Maryland 21601
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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

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

TITLE: Hexabromocyclododecane (HBCD): A 96-Hour Toxicity Test with the Freshwater Alga (*Selenastrum capricornutum*)

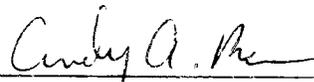
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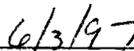
STUDY COMPLETION: June 3, 1997

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, with the following exceptions:

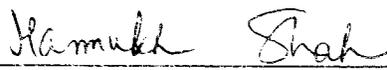
The stability of the test substance under storage conditions at the test site was not conducted in accordance with Good Laboratory Practices.

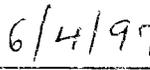
STUDY DIRECTOR:


Cindy A. Roberts
Senior Aquatic Biologist


DATE

SPONSOR:


Sponsor

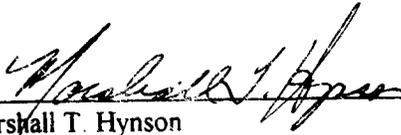

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QUALITY ASSURANCE

This study was examined for 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. 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 and Test Initiation	April 10, 1997	April 10, 1997	April 11, 1997
Matrix Fortification	April 10, 1997	April 10, 1997	April 10, 1997
Data and Draft Report	May 7 and 8, 1997	May 8, 1997	May , 1997
Final Report	June 3, 1997	June 3, 1997	June 3, 1997



Marshall T. Hynson
Quality Assurance Program Supervisor

4/3/97

DATE

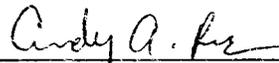
REPORT APPROVAL

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

TITLE: Hexabromocyclododecane (HBCD): A 96-Hour Toxicity Test with the Freshwater Alga (*Selenastrum capricornutum*)

WILDLIFE INTERNATIONAL LTD PROJECT NUMBER: 439A-103

STUDY DIRECTOR:


Cindy A. Roberts
Senior Aquatic Biologist

6/3/97
DATE

MANAGEMENT:


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

6/3/97
DATE

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SUMMARY

SPONSOR:	Chemical Manufacturers Association's 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-103
TEST SUBSTANCE:	Hexabromocyclododecane (HBCD)
STUDY:	Hexabromocyclododecane (HBCD): A 96-Hour Toxicity Test with the Freshwater Alga (<i>Selenastrum capricornutum</i>)
NOMINAL TEST CONCENTRATIONS:	Negative Control, Solvent Control, (0.1 mL DMF/L) 1.5, 2.2, 3.2, 4.6 and 6.8 µg HBCD/L
MEAN MEASURED TEST CONCENTRATIONS:	Negative Control; Solvent Control and 3.7 µg HBCD/L
SOLUBILITY µg HBCD/L IN WELL WATER AT 25°C)	3.4 µg HBCD/L
TEST DATES:	Experimental Start - April 10, 1997 Exposure Termination - April 14, 1997 Experimental Termination - April 26, 1997
LENGTH OF TEST:	96 Hours

TEST ORGANISM:	Freshwater Alga (<i>Selenastrum capricornutum</i>)
SOURCE OF TEST ORGANISMS:	Wildlife International Ltd. Easton, Maryland 21601

SUMMARY (continued)

CELL DENSITY	
96-HOUR EC10:	>6.8 μg HBCD/L (nominal, 2 X HBCD's water solubility of 3.4 $\mu\text{g}/\text{L}$) (>3.7 μg HBCD/L mean measured concentration)
96-HOUR EC50:	>6.8 μg HBCD/L (nominal 2 X HBCD's water solubility of 3.4 $\mu\text{g}/\text{L}$) (>3.7 μg HBCD/L mean measured concentration)
96-HOUR EC90:	>6.8 μg HBCD/L (nominal, 2 X HBCD's water solubility of 3.4 $\mu\text{g}/\text{L}$) (>3.7 μg HBCD/L mean measured concentration)
NO-OBSERVED-EFFECT-CONCENTRATION:	6.8 μg HBCD/L (nominal, 2 X HBCD's water solubility of 3.4 $\mu\text{g}/\text{L}$) (3.7 μg HBCD/L mean measured concentration)

AREA UNDER THE GROWTH CURVE	
96-HOUR EC10:	>6.8 μg HBCD/L (nominal, 2 X HBCD's water solubility of 3.4 $\mu\text{g}/\text{L}$) (>3.7 μg HBCD/L mean measured concentration)
96-HOUR EC50:	>6.8 μg HBCD/L (nominal, 2 X HBCD's water solubility of 3.4 $\mu\text{g}/\text{L}$) (>3.7 μg HBCD/L mean measured concentration)
96-HOUR EC90:	>6.8 μg HBCD/L (nominal, 2 X HBCD's water solubility of 3.4 $\mu\text{g}/\text{L}$) (>3.7 μg HBCD/L mean measured concentration)
NO-OBSERVED-EFFECT-CONCENTRATION:	6.8 μg HBCD/L (nominal, 2 X HBCD's water solubility of 3.4 $\mu\text{g}/\text{L}$) (3.7 μg HBCD/L mean measured concentration)

INTRODUCTION

This study was conducted by Wildlife International Ltd. at their aquatic toxicology facility in Easton, Maryland for Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel. The definitive test was conducted from April 10, 1997 to April 14, 1997. Raw data generated by Wildlife International Ltd. and a copy of the final report are filed under Project Number 439A-103 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 freshwater green alga, *Selenastrum capricornutum*.

EXPERIMENTAL DESIGN

The freshwater alga, *Selenastrum capricornutum*, was exposed to one of five test concentrations, a solvent control or the negative (culture medium) control under static conditions for 96 hours. Three replicate test chambers were maintained for each treatment and control group. 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 range finding toxicity test (Appendix I). Nominal test concentrations selected were 1.5, 2.2, 3.2, 4.6 and $6.8 \mu\text{g HBCD/L}$.

Test solutions were inoculated with 1.0 mL of an inoculum with an approximate density of 1.0×10^6 cells/mL to achieve a final cell density of 1.0×10^4 cells/mL. Samples of the test solutions were collected from each replicate test chamber at approximately 24-hour intervals during the test to determine cell densities. Cell densities and area under the growth curve values were determined for each replicate and were used to calculate percent inhibition values relative to the

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controls over the 96-hour exposure period. EC10, EC50 and EC90 values were calculated, if possible, based upon cell densities and area under the growth curve values for each 24-hour interval. The no-observed-effect-concentration (NOEC) was determined based upon statistical evaluation of the cell densities and area under the growth curve values. A recovery phase was not needed in this experiment.

MATERIALS AND METHODS

This study was conducted according to procedures outlined in the protocol (Appendix VII), Hexabromocyclododecane (HBCD): A 96-Hour Toxicity Test with the Freshwater Alga (*Seienastrum capricornutum*). The protocol was based on procedures outlined in Title 40 of the Code of Federal Regulations, Part 797, Section 1050, *Algal Acute Toxicity Test* (1); and OECD Guideline for Testing of Chemicals, 201: *Alga, Growth Inhibition Test* (2).

Test Substance

The test substance consisted of a composite of hexabromocyclododecane (HBCD) samples received from three manufacturers. The materials identity and date 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.

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Subsamples of the composite test substance were shipped to Albemarle Corp. for 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.

Test Solution Preparation

A primary stock solution was prepared by dissolving HBCD in dimethylformamide (DMF). The concentration of the stock was 0.068 mg HBCD/mL. The stock solution was inverted at least 20 times to aid in dissolution of the hexabromocyclododecane (HBCD). Aliquots of both the primary and secondary stocks were diluted with DMF to prepare a total of four additional stock solutions at concentrations of 0.046, 0.032, 0.022 and 0.015 mg HBCD/mL. Two-hundred and fifty microliter aliquots of the primary stock and the four secondary stocks were diluted to 2.5 liters with culture medium and mixed vigorously for approximately one minute with a glass stir rod in one gallon glass jars to prepare the 1.5, 2.2, 3.2, 4.6 and 6.8 μg HBCD/L nominal test concentrations, respectively. Stock concentrations and the resultant test concentrations were prepared on a total product basis. A solvent control was prepared by diluting 250 μL of DMF to 2.5 L with culture medium to yield a solvent concentration equivalent to that in the treatment groups.

Test Organism

The freshwater alga, *Selenastrum capricornutum*, was selected as the test species for this study. The species is representative of an important group of freshwater algae and was selected for use in the test based upon a past history of use and ease of culturing in the laboratory. Original algal cultures were obtained from UTEX - The Culture Collection of Algae at the University of Texas at Austin and have been maintained in culture medium at Wildlife International Ltd., Easton, Maryland. Algal cells used in this test were obtained from Wildlife International Ltd. cultures that

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had been actively growing in culture medium for at least two weeks prior to test initiation. The control organisms were expected to exhibit exponential growth over the 96-hour exposure period. Exponential growth phase, defined as the period of growth where the algal cells are dividing at a constant rate, is indicated by the linear section of the growth curve (Figure 1).

Culture Medium

The algal cells were cultured and tested in freshwater algal medium (1). Stock nutrient solutions were prepared by adding reagent-grade chemicals to Wildlife International Ltd. well water purified by reverse osmosis. The test medium was prepared by adding the appropriate volumes of stock nutrient solutions to purified well water (Appendix II). The pH of the medium was adjusted to 7.5 ± 0.1 using 10% HCl and the medium was sterilized by filtration ($0.22 \mu\text{m}$) prior to use. Analyses are performed at least once annually to determine the concentrations of selected organic and inorganic constituents in the well water. The results of analyses performed to measure the concentrations of selected contaminants in well water used by Wildlife International Ltd. are presented in Appendix III.

Test Apparatus

Test chambers were sterile 250-mL Erlenmeyer flasks plugged with foam stoppers, and containing 100 mL of test or control algal medium. The test chambers were labeled with the project number, test concentration and replicate, and were indiscriminately positioned on a mechanical shaker in an environmental chamber designed to maintain the desired test temperature throughout the test. The test chambers were shaken continuously at 100 rpm.

Abiotic (no algae) analytical test solutions were maintained in one gallon glass jars covered with parafilm.

Environmental Conditions

Test flasks and the one gallon glass jars containing the remaining aliquots of abiotic (no algae) test solutions prepared at test initiation were held in an environmental chamber at a temperature of $24 \pm 2^\circ\text{C}$. The temperature of a container of water adjacent to the test chambers in the environmental chamber was recorded twice daily during the test using a hand-held mercury thermometer.

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The algae and abiotic test solutions were held under continuous cool-white fluorescent lighting throughout the test. The target light intensity was 4310 ± 431 lux. Light intensity was measured at the four corners and the middle of the shaker table at test initiation using a SPER Scientific Model 840006 light meter.

The pH of the medium prepared for each treatment and control group was measured at test initiation and termination using a Fisher Accumet Model 915 pH meter. Samples for pH measurement at test initiation were collected from individual batches of test solution prepared for each treatment and control group. At test termination, samples of test solution were collected from the pooled replicates of the treatment and control groups for pH measurement.

Algal Growth Measurements

Samples of approximately 2 mL were collected from each treatment and control vessel at approximate 24-hour intervals during the 96-hour exposure and were either counted that day or were held under refrigerated conditions until cell counts could be performed.

Cell counts were performed using an electronic particle counter (Coulter Electronics, Inc.). Prior to conducting cell counts, the linearity of the instrument response was determined at settings previously established for *Selenastrum capricornutum*. A primary counting standard containing *Selenastrum capricornutum* cells was prepared, the concentration was verified using a hemacytometer and a microscope, and the standard was subsequently diluted to provide a series of counting standards for the determination of instrument linearity. Theoretical concentrations were assigned to each secondary counting standard based upon the verified concentration of the primary counting standard and the dilution ratio. The cell densities of the counting standards were measured using the electronic particle counter and were compared to the theoretical concentrations by performing a least squares regression analysis. Cell counts for samples collected during the test were conducted once instrument linearity was demonstrated (i.e., the R-squared value obtained through the regression analysis was ≥ 0.970). The R-squared values for cell density determinations performed during this test ranged from 0.999 to 1.0. A single aliquot of each sample collected during the test was diluted with an electrolyte solution (Isoton®), and three 0.5-mL volumes of the dilution were counted. The cell density of the sample was determined by adjusting the mean cell count (cells/mL) obtained using the particle counter based upon the Y-intercept and slope calculated through the regression analysis. The following equation was used:

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$$\text{Cell Density of Sample (Cells/mL)} = \frac{\text{Mean Cell Count (Cells/mL)} - \text{Y Intercept}}{\text{Slope}}$$

Statistical Analyses

Cell densities, mean cell densities, area under the growth curve values and percent inhibition values were calculated using "Microsoft Excel Version 4.0" (3), while evaluations of EC values and no observed effect levels were evaluated using "ICPIN Version 2.0" (4) and "TOXSTAT Release 3.2" (5), respectively.

Mean cell density values and mean area under the growth curve values in the negative and solvent control groups were compared using "t" tests. Based upon these results, all statistical evaluations for cell density were made relative to the solvent control, and all statistical evaluations for area under the growth curve were made relative to the pooled controls.

Percent inhibition based on cell density was calculated for each treatment group as the percent reduction in cell density relative to the mean cell density in the solvent control replicates. Percent inhibition was calculated for each treatment group as the percent reduction in area under the growth curve relative to the mean area under the growth curve value for the pooled control replicates. The following formula was used:

$$\text{Percent Inhibition} = \frac{\text{Mean Control Response} - \text{Mean Treatment Response}}{\text{Mean Control Response}} \times 100$$

Cell densities and area under the growth curve values were analyzed statistically using the computer program ICPIN (4) to calculate the EC10, EC50 and EC90 values (i.e., the theoretical test concentrations that would produce a 10, 50 or 90% reduction in cell density or area under the growth curve, respectively) and 95% confidence limits, if possible, for the 96-hour test period. This

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program was designed to calculate the EC values and 95% confidence limits by linear interpolation. Cell densities and area under the growth curve values were evaluated for normality and homogeneity of variances using the Shapiro Wilk's and the Bartlett's test, respectively. Cell densities in the treatment groups were compared to cell density values in the solvent control using Dunnett's test (5). Area under the growth curve values for the treatment groups were compared to the pooled controls using Bonferroni's "t" test (5). Results of the statistical analyses were used to determine the no-observed-effect-concentration (NOEC).

Analytical Chemistry

Samples of the test medium (test samples) were collected from each treatment and control group at the beginning and end of the test to measure concentrations of the test substance. Samples of test medium collected on Day 0 were taken from individual batches of test medium prepared at test initiation. Samples of the abiotic test solutions from each treatment group were collected on Day 4 as well as the composited test medium from each of the three replicates in the 6.8 μg HBCD/L treatment group (nominal concentration). The composite sample of the 6.8 μg HBCD/L containing algal cells was filtered (0.45 μm) to remove algal cells prior to analysis. The test samples were collected in a 1000-mL glass graduated cylinder or into glass separatory funnels and were extracted as soon as possible without storage. The samples were analyzed according to an analytical method that was previously verified in freshwater by Wildlife International Ltd. The analytical methodology was verified in culture medium concurrently with the analysis of definitive test samples. Analytical procedures used in the analysis of the samples are provided in Appendix IV.

RESULTS AND DISCUSSION

Measurements of Test Concentrations

The selection of exposure concentrations of hexabromocyclododecane (HBCD) took into consideration the water solubility limit and a finding of no acute toxicity from an exploratory

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rangefinding test. The water solubility limit was determined in a generator column study to be $3.4 \mu\text{g/L}$ (6). However, there was a potential to have a slight enhancement of HBCD's water solubility due to the use of dimethylformamide (DMF) as a solvent. For this reason, the highest test concentration selected for the test was approximately twice the defined solubility limit (i.e., $6.8 \text{ HBCD } \mu\text{g/L}$). The series of five nominal test concentrations used in the test were 1.5, 2.2, 3.2, 4.6 and $6.8 \text{ HBCD } \mu\text{g/L}$. In this way, the solubility limit of HBCD was bracketed by the five concentrations.

Analytical measurements were performed to verify exposure concentrations of HBCD in the test medium. Results of those analyses are presented in Table 1 and in the analytical chemistry report (Appendix IV). Nominal concentrations selected for use in this study were 1.5, 2.2, 3.2, 4.6 and $6.8 \mu\text{g HBCD/L}$. Samples collected on Day 0 showed measured values of 1.30, 2.25, 3.38, 4.28 and $6.44 \mu\text{g HBCD/L}$, representing 87, 102, 106, 93 and 95% of nominal, respectively. The measured values in the abiotic test solutions on Day 4 were <0.571 , 1.20, 1.90, 1.64 and $2.47 \mu\text{g HBCD/L}$, representing -- (non-quantifiable), 55, 59, 36 and 36% of nominal, respectively. The measured concentration of HBCD in the $6.8 \mu\text{g HBCD/L}$ (nominal concentration) treatment group was <1.94 on Day 4. When measured concentrations of samples collected in the $6.8 \mu\text{g HBCD/L}$ treatment group at Days 0 and 4 were averaged, the mean measured concentration for this treatment group was $3.7 \mu\text{g HBCD/L}$ representing 54% of nominal. Nominal concentrations were used for statistical evaluations. The mean measured concentration is also presented.

Observations and Measurements

Measurements of temperature are presented in Table 2. The temperatures ranged from 23.6 to 25.9°C and were within the range established for the test ($24 \pm 2^\circ\text{C}$). The light intensity ranged from 3870 to 4230 lux at test initiation. Measurements of pH ranged from 7.4 to 7.5 on Day 0 and ranged from 8.0 to 8.4 at 96 hours (Table 3).

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The effect of HBCD upon *Selenastrum capricornutum* was determined by evaluating difference in cell densities over a 96-hour exposure period. Cell densities were then used to calculate area under the growth curve values, and percent inhibition values were calculated for both cell density and area under the growth curve data. Mean cell densities and mean area under the growth curve values and their corresponding percent inhibition values are presented in Tables 4 and 5, respectively. Cell densities and area under the growth curve values for each individual replicate over the exposure period are presented in Appendices V and VI, respectively.

Changes in cell density over time in the negative and solvent control groups indicated that exponential growth was achieved during the test period (Figure 1). Cell densities and areas under the growth curve of *Selenastrum* in all control and treatment groups increased at approximately the same rate and achieved similar values by the end of the test. Any differences between the treatments and the control groups were slight and neither dose-responsive nor statistically significant ($p > 0.05$). No EC10, EC50 and EC90 values could be defined in this test due to the lack of growth inhibition at the solubility limit of HBCD. EC estimates for cell density and area under the growth curve are presented in Tables 6 and 7, respectively.

Visual and Microscopic Observations

There were no noticeable changes in cell color, size or morphology in any of the treatment groups when compared to the control. No evidence of clumping, flocculation or adherence of the algae to the test flask was found during the visual and microscopic examination of algal cells from each treatment or control group.

CONCLUSIONS

Selenastrum capricornutum exposed to HBCD over a range of concentrations covering twice the HBCD aqueous solubility limit failed to demonstrate any inhibition in reproduction and growth.

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Visual and microscopic examination of algal cells in all groups also failed to reveal any evidence of treatment level effects. The 96-hour EC10, EC50 and EC90 endpoints could not be calculated due to insufficient growth inhibition, but all values were estimated to be greater than 6.8 μg HBCD/L, the maximum nominal test concentration used in the study (>3.7 μg HBCD/L mean measured). The no-observed-effect-concentration (NOEC) under the conditions of the study is 6.8 $\mu\text{g}/\text{L}$ (nominal) (3.7 μg HBCD/L mean measured).

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REFERENCES

1. **Title 40 of the Code of Federal Regulations**, Part 797, Section 1050. 1988. *Algal Acute Toxicity Tests*.
2. **OECD**. 1984. Guideline for Testing of Chemicals, 201: *Alga, Growth Inhibition Test*.
3. **Microsoft Corporation**. "Microsoft Excel Version 4.0." Copyright 1985 - 1992.
4. **Norberg-King, T.J.** 1993. *A Linear Interpolation Method for Sublethal Toxicity: The Inhibition Concentration (IC_p) Approach*. Version 2.0. U.S. Environmental Protection Agency. National Effluent Toxicity Assessment Center. Duluth, Minnesota. Technical Report 03-93.
5. **Gulley, D.D.** "TOXSTAT Release 3.2," The University of Wyoming, July 1990.
6. **Stenzel, J.L. and B.J. Markley.** 1997. Hexabromocyclododecane (HBCD) Determination of the Water Solubility. Wildlife International Ltd. Project Number 439C-105. Pp. 56.

Table 1
Summary of Analytical Chemistry Data

Nominal Test Concentration ($\mu\text{g/L}$)	Sampling Time (Hours)	Measured Concentration	Mean Measured ¹ Concentration	Corrected ²	Rounded Mean Value	Percent of Nominal
0.0 (Negative Control)	0 ³	<0.571	--	--	--	--
	96 ⁴	<0.571	--	--	--	--
0.0 (Solvent Control)	0 ³	<0.571	--	--	--	--
	96 ⁴	<0.571	--	--	--	--
1.5	0 ³	1.47	--	1.30	--	87
	96 ⁴	<0.571	--	--	--	--
2.2	0 ³	2.54	--	2.25	--	102
	96 ⁴	1.36	--	1.20	--	55
3.2	0 ³	3.82	--	3.38	--	106
	96 ⁴	2.15	--	1.90	--	59
4.6	0 ³	4.84	--	4.28	--	93
	96 ⁴	1.85	--	1.64	--	36
6.8	0 ³	7.28	--	6.44	--	95
	96 ⁴	2.79	4.13	2.47	--	36
	96 ⁵	<1.94 ⁶	4.13	3.65	3.7	54

¹ The mean measured concentration₀ was calculated as the mean of the Day 0 measured value and one half of the product of the low standard and the dilution factor of the sample (< 1.94).

² Values were corrected for a mean procedural recovery of 113%.

³ Day 0 samples were collected from individual batches of test solution prepared at test initiation.

⁴ Abiotic test solution

⁵ Reflects pooled sample which contained algal cells throughout duration of the test.

⁶ This sample reflects a nonquantifiable level due to limited sample availability at test termination.

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Table 2
Temperature Measurements

Sponsor:	Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel	
Test Substance:	Hexabromocyclododecane (HBCD)	
Test Organism:	Freshwater Alga, <i>Selenastrum capricornutum</i>	
Dilution Water:	Freshwater Algal Medium	
	Temperature (°C)	
Time (Days)	Measurement 1	Measurement 2 ¹
0	23.6	25.1
24	25.9	24.3
48	24.5	24.4
72	24.5	24.3
96	24.2	24.1

¹ Temperature Measurement 2 was taken at least 4 hours after Measurement 1, with the exception of 96 hours.

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Table 3
pH Measurements

Sponsor:		Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel	
Test Substance:		Hexabromocyclododecane (HBCD)	
Test Organism:		Freshwater Alga, <i>Selenastrum capricornutum</i>	
Dilution Water:		Freshwater Algal Medium	
Nominal Test Concentration (μg HBCD/L)	pH Measurements		
	0 Hours ¹	96 Hours ²	
Negative Control	7.4	8.0	
Solvent Control	7.5	8.2	
1.5	7.5	8.2	
2.2	7.5	8.2	
3.2	7.5	8.4	
4.6	7.5	8.3	
6.8 (3.7)	7.5	8.3	

¹ 0-hour samples were collected from individual batches of test solution prepared for the treatment and control groups at test initiation.
² 96-hour samples were collected from pooled replicates of the treatment and control group.
 Note: Value in parentheses is mean measured test concentration.

Table 4

Mean Cell Densities and Percent Inhibition Values for Each 24-Hour Interval During the Test

Sponsor: Test Substance: Test Organism: Dilution Water:	Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel Hexabromocyclododecane (HBCD) Freshwater Alga, <i>Selenastrum capricornutum</i> Freshwater Algal Medium											
	Nominal Test Concentration (μ g HBCD/L)	24 Hours			48 Hours			72 Hours			96 Hours	
	Cell Density	Percent Inhibition		Cell Density	Percent Inhibition		Cell Density	Percent Inhibition		Cell Density	Percent Inhibition	
Negative Control	36,587	--	--	125,021	--	--	580,092	--	--	1,414,468	--	--
Solvent Control	34,225	--	--	136,027	--	--	570,558	--	--	1,594,460	--	--
1.5	39,177	-14	2.7	132,395	-15	2.7	571,999	-0.25	-0.25	1,610,515	-1.0	-1.0
2.2	39,725	-16	-15	157,104	-19	11	510,265	11	11	1,730,498	-8.5	-8.5
3.2	37,158	-8.6	-19	162,299	-14	-7.5	613,406	-7.5	-7.5	1,629,684	-2.2	-2.2
4.6	40,905	-20	-14	154,397	-4.2	2.0	559,010	2.0	2.0	1,636,447	-2.6	-2.6
6.8 (3.7)	33,105	3.3	-4.2	141,790	16	7.8	479,394	16	16	1,470,507	7.8	7.8

Note: Value in parentheses is mean measured test concentration.
Percent inhibition values calculated using mean cell densities of the solvent control group.

Table 5

Mean Area Under the Growth Curve and Percent Inhibition Values
for Each 24-Hour Interval During the Test

Sponsor: Test Substance: Test Organism: Dilution Water:	Nominal Test Concentration (µg HBCD/L)	24 Hours		48 Hours		72 Hours		96 Hours	
		Mean Area	Percent ¹ Inhibition						
Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel Hexabromocyclododecane (HBCD)									
Freshwater Alga, <i>Selenastrum capricornutum</i>									
Freshwater Algal Medium									
Negative Control		319,049	--	2,018,346	--	10,239,704	--	33,034,429	--
Solvent Control		290,697	--	2,093,716	--	10,332,731	--	36,072,938	--
Pooled Control		304,873	--	2,056,031	--	10,286,218	--	35,003,683	--
1.5		350,121	-15	2,168,987	-5.5	10,381,716	-0.93	36,331,878	-3.8
2.2		356,696	-17	2,478,634	-21	10,247,056	0.38	36,896,216	-5.4
3.2		325,891	-6.9	2,479,367	-21	11,547,831	-12	38,224,916	-9.2
4.6		370,864	-22	2,474,489	-20	10,795,375	-5.0	36,900,866	-5.4
6.8 (3.7)		277,263	9.1	2,136,001	-3.9	9,350,204	9.1	32,509,011	9.9

Note: Value in parentheses is mean measured concentration.

¹ Percent inhibition was calculated relative to the pooled control replicates.

Table 6

EC10, EC50 and EC90 Values Based on Cell Density Over the 96-Hour Exposure Period

Sponsor: Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel
 Test Substance: Hexabromocyclododecane (HBCD)
 Test Organism: Freshwater Alga, *Selenastrum capricornutum*
 Dilution Water: Freshwater Algal Medium

Time	EC10 ($\mu\text{g HBCD/L}$)	95% Confidence Limits ($\mu\text{g HBCD/L}$)	EC50 ($\mu\text{g HBCD/L}$)	95% Confidence Limits ($\mu\text{g HBCD/L}$)	EC90 ($\mu\text{g HBCD/L}$)	95% Confidence Limits ($\mu\text{g HBCD/L}$)
24 Hours	> 6.8 (nominal) (> 3.7 mean measured concentration)	NA	> 6.8 (nominal) (> 3.7 mean measured concentration)	NA	> 6.8 (nominal) (> 3.7 mean measured concentration)	NA
48 Hours	> 6.8 (nominal) (> 3.7 mean measured concentration)	NA	> 6.8 (nominal) (> 3.7 mean measured concentration)	NA	> 6.8 (nominal) (> 3.7 mean measured concentration)	NA
72 Hours	> 6.8 (nominal) (> 3.7 mean measured concentration)	NA	> 6.8 (nominal) (> 3.7 mean measured concentration)	NA	> 6.8 (nominal) (> 3.7 mean measured concentration)	NA
96 Hours	> 6.8 (nominal) (> 3.7 mean measured concentration)	NA	> 6.8 (nominal) (> 3.7 mean measured concentration)	NA	> 6.8 (nominal) (> 3.7 mean measured concentration)	NA

NA - Statistical method not used. Estimation made by visual interpretation of the cell density data.

Table 7

EC10, EC50 and EC90 Values Based on Area Under the Growth Curve Over the 96-Hour Exposure Period

Sponsor: Chemical Manufacturers' Association's Brominated Flame Retardant Industry Panel						
Test Substance: Hexabromocyclododecane (HBCD)						
Test Organism: Freshwater Alga, <i>Selenastrum capricornutum</i>						
Dilution Water: Freshwater Algal Medium						
Time	EC10 (µg HBCD/L)	95% Confidence Limits (µg HBCD/L)	EC50 (µg HBCD/L)	95% Confidence Limits (µg HBCD/L)	EC90 (µg HBCD/L)	95% Confidence Limits (µg HBCD/L)
24 Hours	> 3.7 mean measured concentration)	NA	> 6.8 (nominal) (> 3.7 mean measured concentration)	NA	> 5.8 (nominal) (> 3.7 mean measured concentration)	NA
48 Hours	> 3.7 mean measured concentration)	NA	> 6.8 (nominal) (> 3.7 mean measured concentration)	NA	> 6.8 (nominal) (> 3.7 mean measured concentration)	NA
72 Hours	> 3.7 mean measured concentration)	NA	> 6.8 (nominal) (> 3.7 mean measured concentration)	NA	> 6.8 (nominal) (> 3.7 mean measured concentration)	NA
96 Hours	> 3.7 mean measured concentration)	NA	> 6.8 (nominal) (> 3.7 mean measured concentration)	NA	> 6.8 (nominal) (> 3.7 mean measured concentration)	NA

NA - Statistical method not used. Estimation made by visual interpretation of the cell density data.

APPENDIX I

Rangefinding Toxicity Data

Sponsor: Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel														
Test Substance: Hexabromocyclododecane (HBCD)														
Test Organism: Freshwater Alga, <i>Selenastrum capricornutum</i>														
Dilution Water: Freshwater Algal Medium														
Nominal Concentration (μ g HBCD/L)	Hemocytometer Cell Counts													
	1	2	3	4	5	6	7	8	9	10	Mean	DF		
Negative Control	6	9	5	7	10	8	6	8	6	6	7.1	20	1,420,000	--
Solvent Control	10	8	10	5	8	8	6	6	5	5	7.1	20	1,420,000	--
20	5	8	6	5	8	7	7	4	10	12	7.2	20	1,440,000	-1.4

*Percent Inhibition = (control cell density - treatment cell density) / control cell density X 100.

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APPENDIX II

Freshwater Algal Medium¹

Sponsor:	Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel
Test Substance:	Hexabromocyclododecane (HBCD)
Test Organism:	Freshwater Alga, <i>Selenastrum capricornutum</i>
Dilution Water:	Freshwater Algal Medium

Compound	Nominal Concentration
MgCl ₂ •6H ₂ O	12.16 mg/L
CaCl ₂ •2H ₂ O	4.40 mg/L
H ₃ BO ₃	0.1856 mg/L
MnCl ₂ •4H ₂ O	0.416 mg/L
ZnCl ₂	3.28 µg/L
FeCl ₃ •6H ₂ O	0.1598 mg/L
CoCl ₂ •6H ₂ O	1.428 µg/L
Na ₂ MoO ₄ •2H ₂ O	7.26 µg/L
CuCl ₂ •2H ₂ O	0.012 µg/L
NaNO ₃	25.50 mg/L
MgSO ₄ •7H ₂ O	14.70 mg/L
K ₂ HPO ₄	1.044 mg/L
NaHCO ₃	15.0 mg/L

¹ The pH was adjusted, as necessary, to between 7.4 and 7.6 using 0.1 N NaOH or dilute HCl prior to use in the study.

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

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, S	<	0.005	µg/L
Endosulfan Sulfate	<	0.005	µg/L
Endrin	<	0.005	µg/L
Endrin Aldrin, is	<	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	µg/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 sample collection on August 14, 1996.