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CHEMICAL MANUFACTURERS ASSOCIATION

FYI-0698-1333

COURTNEY M. PRICE  
VICE PRESIDENT  
CHEMSTAR

May 18, 1998

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 environmental, health and safety research that it manages. In keeping with this policy, the following recently completed report is enclosed:

Hexabromocyclododecane (HBCD) A Flow-Through Life-Cycle Toxicity Test with the Cladoceran (*Daphnia Magna*).

This report does not include confidential information.

If you have any questions, please call Wendy K. Sherman of my staff at 703-741-5639.

Sincerely yours,

*Courtney M. Price*



FYI-98-001333

Enclosure

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PERMETHYL-BROMOCYCLODIPICOLATE  
A FLOW-THROUGH LIFE-CYCLE TEST  
WITH THE CLADOCERAN (*Daphnia magna*)

FINAL REPORT

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 439A-108

OECD Guideline 202  
and  
TSCA Title 40 of the Code of Federal Regulations  
Part 797, Section 1330

AUTHORS:

Kurt R. Drottar  
Henry O. Krueger, Ph.D.

STUDY INITIATION DATE: October 28, 1997

STUDY COMPLETION DATE: April 30, 1998

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

WIL

WILDLIFE INTERNATIONAL LTD.

PROJECT NO.: 439A-108

**GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT**

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

**TITLE:** Hexabromocyclododecane (HBCD): A Flow-Through Life-Cycle Toxicity Test with the Cladoceran (*Daphnia magna*)

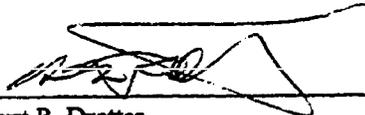
**WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER:** 439A-108

**STUDY COMPLETION:** April 30, 1998

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 and OECD Principles of Good Laboratory Practice, OCDE/GD (92) 32, Environment Monograph No. 45, Paris 1992.

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

**STUDY DIRECTOR:**

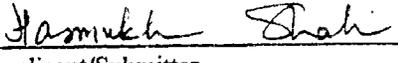
  
\_\_\_\_\_  
Kurt R. Drott  
Senior Aquatic Biologist

4/30/98  
DATE

**SPONSOR APPROVAL:**

  
\_\_\_\_\_  
Sponsor

5-3-1998  
DATE

  
\_\_\_\_\_  
Applicant/Submitter

5-3-1998  
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 and OECD Principles of Good Laboratory Practice, OCDE/GD (92) 32, Environment Monograph No. 45, Paris 1992. 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	January 5, 1998	January 6, 1998	January 8, 1998
Matrix Fortifications	January 7, 1998	January 7, 1998	January 8, 1998
Water Chemistry Measurements	January 21, 1998	January 21, 1998	January 22, 1998
Analytical Sampling	January 21, 1998	January 21, 1998	January 22, 1998
Biological Data and Draft Report	March 18 to 20, 1998	March 20, 1998	March 23, 1998
Analytical Data and Draft Report	March 20, 1998	March 20, 1998	March 30, 1998
Final Report	April 30, 1998	April 30, 1998	April 30, 1998

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

4-30-98  
 DATE

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WILDLIFE INTERNATIONAL LTD.

PROJECT NO.: 439A-108

- 4 -

REPORT APPROVAL

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

TITLE: Hexabromocyclododecane (HBCD): A Flow-Through Life-Cycle Toxicity Test with the Cladoceran (*Daphnia magna*)

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 439A-108

STUDY DIRECTOR:



Kurt R. Drottar  
Senior Aquatic Biologist

4/30/98

DATE

MANAGEMENT:



Henry O. Krueger, Ph.D.  
Director, Aquatic Toxicology and  
Non-Target Plants

4/30/98

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-108
TEST SUBSTANCE:	Hexabromocyclododecane (HBCD)
STUDY:	Hexabromocyclododecane (HBCD). A Flow-Through Life- Cycle Toxicity Test with the Cladoceran ( <i>Daphnia magna</i> )
MEAN MEASURED TEST CONCENTRATIONS:	Negative Control, Solvent Control, 0.87, 1.6, 3.1, 5.6 and 11 µg HBCD/L
TEST DATES:	Experimental Start - January 7, 1998 Biological Termination - January 28, 1998 Experimental Termination - January 30, 1998
LENGTH OF TEST:	21 Days

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

HBCD WATER SOLUBILITY:	3.4 µg HBCD/L
NO-OBSERVED-EFFECT- CONCENTRATION:	3.1 µg HBCD/L (~ equivalent to water solubility)
LOWEST-OBSERVED- EFFECT-CONCENTRATION:	5.6 µg HBCD/L (> water solubility)
MAXIMUM ACCEPTABLE TOXICANT CONCENTRATION:	4.2 µg HBCD/L (> water solubility)

## INTRODUCTION

This study was conducted by Wildlife International Ltd. for Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel at the Wildlife International Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the test was conducted from January 7, 1998 to January 28, 1998. Raw data generated by Wildlife International Ltd. and a copy of the final report are filed under Project Number 439A-108 in archives located on the Wildlife International Ltd. site.

## OBJECTIVE

The objective of this study was to evaluate the chronic toxicity of Hexabromocyclododecane (HBCD) on the survival, growth and reproduction of the cladoceran (*Daphnia magna*) during a 21-day exposure period under flow-through test conditions.

## EXPERIMENTAL DESIGN

Daphnids were exposed to a geometric series of five test concentrations, a solvent control and a negative (well water) control. Two replicate test chambers were maintained in each treatment and control group. Each treatment and control group consisted of four test compartments containing ten daphnids. Nominal test concentrations were selected by the Sponsor. Nominal test concentrations selected were 0.85, 1.7, 3.4, 6.8 and 13.6  $\mu\text{g}$  HBCD/L. Mean measured test concentrations were determined from samples of test water collected from each test chamber at test initiation, Days 7 and 14 and at test termination.

Delivery of the test substance was initiated approximately 43 hours prior to the introduction of the daphnids to the test water in order to achieve equilibrium of the test substance in the test chambers. Daphnids were impartially assigned to exposure chambers at test initiation. Observations of mortality, reproduction and other sublethal signs of toxicity were made every Monday, Wednesday and Friday during the test and at the end of the test. The no-observed-effect-concentration (NOEC) and lowest-observed-effect-concentration (LOEC) were determined by examination of the mortality, growth and reproduction data. The maximum acceptable toxicant concentration (MATC) was calculated as the geometric mean of the NOEC and LOEC.

### MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, "Hexabromocyclododecane (HBCD): A Flow-Through Life-Cycle Toxicity Test with the Cladoceran (*Daphnia magna*)". The protocol was based on procedures outlined in OECD Guideline 202, *Daphnia sp.*, *Acute Immobilisation and Reproduction Test* (1); Title 40 of the Code of Federal Regulations, Part 797, Section 1350, *Daphnia Chronic Toxicity Test* (2); and ASTM Standard E 1193-87, *Standard Guide for Conducting Renewal Life-Cycle Toxicity Tests with Daphnia magna* (3).

#### Test Substance

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

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

An equal part (300 g) of each of the manufacturer's HBCD material was placed in a 2-L, high density polyethylene (HDPE) bottle. The bottle was placed on a reciprocating shaker for two hours. The composite test substance was assigned Wildlife International Ltd. identification number 3577. Subsamples of the composite test substance were shipped to Albemarle Corp. for 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 expiration date of the composite sample was 12/31/98. The test substance was stored under ambient conditions.

#### Preparation of Test Concentrations

Nominal test concentrations were 0.85, 1.70, 3.40, 6.80 and 13.6  $\mu\text{g}$  HBCD/L. One stock solution was prepared for each of the concentrations tested. A primary stock was prepared by dissolving the test substance in dimethylformamide (DMF) at a concentration of 136 mg HBCD/L. Four additional stock solutions were prepared at concentrations of 68, 34, 17 and 8.5 mg HBCD/L by proportional dilution of the primary stock solution with DMF. The five stock solutions were injected into the diluter mixing chambers (at a rate of 0.0125 mL/minute) where they were mixed with dilution water (at a rate of 125 mL/minute) to achieve the desired test concentrations. Dimethylformamide was injected into the mixing chamber for the solvent control group. The concentration of DMF in the solvent control and all HBCD treatment groups was 0.1 mL/L. Test concentrations were not adjusted for the purity of the active ingredient in the test substance.

#### Test Organism

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

Adult daphnids were cultured in water from the same source and at approximately the same temperature as used during the test in Wildlife International Ltd. well water supplemented with selenium. Daphnids in the cultures were held for at least 22 days prior to collection of the juveniles for testing. The adults showed no signs of disease or stress during the holding period. During the 14-day holding period preceding the test, water temperatures ranged from 19.7 to 20.8°C. The pH of the water ranged from 8.3 to 8.5, and dissolved oxygen ranged from 8.2 to 8.8 mg/l. Instrumentation used for water measurements are described in the *Environmental Conditions* section of this report.

Neonate daphnids were obtained for testing from individual adult daphnids. The progeny from 15 adults were used in the test. At test initiation, the juvenile daphnids were collected from the cultures and placed in glass beakers. The daphnids were then transferred to the test chambers using a wide-bore pipet. All transfers were conducted underneath the air/water interface. Daphnids in the cultures and those used in the test were fed a mixture of yeast, Cerophyll®, and trout chow, as well as a suspension of the freshwater green alga, *Scenedesmus capricornutum*. During the test, daphnids were fed three times daily and once on the last day of the test.

### Test Apparatus

A continuous-flow diluter was used to deliver each concentration of the test substance, a solvent control, and a negative (well water) control. Syringe pumps (Harvard Apparatus) were used to deliver the five test substance stocks and the solvent for the solvent control into mixing chambers assigned to each treatment and control group. The stock solutions were diluted with well water in the mixing chambers in order to obtain the desired test concentrations. The flow of dilution water to the mixing chambers was controlled by rotameters. Rotameters were calibrated prior to test initiation and at weekly intervals thereafter during the test. The flow of test water from each mixing chamber was split and allowed to flow into replicate test chambers. The proportion of test water that was split into each replicate was checked prior to the test and at weekly intervals thereafter during the test to ensure that flow rates varied by no more than  $\pm 10\%$  of the mean for the two replicates.

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

Test compartments were constructed from 500-mL glass beakers approximately 7.0 cm in diameter and 13 cm in height. Nylon mesh screen covered two holes on opposite sides of each test compartment to allow test solution to flow in and out of the test compartments. The test compartments were placed in 8-L stainless steel test chambers filled with approximately 6.5 L of test water. The depth of the test solution in a representative test compartment and test chamber was 9.0 cm and 17 cm, respectively. Test chambers were impartially positioned in a temperature-controlled water bath designed to maintain a temperature of  $20 \pm 1^\circ\text{C}$ . The water bath was enclosed in a plexiglass ventilation hood in order to minimize potential for cross-contamination. The test chambers were labeled with the project number, test concentration and replicate. Test compartments were also uniquely identified.

### Dilution Water

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

The well water was passed through a sand filter to remove particles greater than approximately 25  $\mu\text{m}$ , and pumped into a 37,800-L storage tank where the water was aerated with spray nozzles. Prior to delivery to the diluter system, the water again was filtered to 0.2  $\mu\text{m}$  and passed through a UV sterilizer to remove particles and microorganisms, respectively. 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 II.

#### Environmental Conditions

Lighting used to illuminate the cultures and test chambers during culturing and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (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 325 lux at the surface of the water.

Temperature was measured in each test chamber at the beginning and end of the test and at weekly intervals during the test using a liquid-in-glass thermometer. Temperature also was measured continuously in one negative control replicate using a Fulscope ER/C Recorder. The target test temperature during the study was  $20 \pm 1^\circ\text{C}$ . The pH was measured in alternate replicates of each treatment and control group at test initiation and at least three times per week thereafter. Dissolved oxygen was measured in alternate replicates of each treatment and control group daily during the first week of the test and at least three times per week thereafter. Hardness, alkalinity, specific conductance and total organic carbon (TOC) were measured in alternating replicates of the negative control at the beginning of the test and at weekly intervals thereafter.

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.

#### Biological Observations and Measurements

Observations of each first-generation daphnid were made daily during the test for survival, the onset of reproduction and clinical signs of toxicity. The criteria for death included absence of heartbeat, white opaque coloration, lack of movement of appendages, and lack of response to gentle prodding. Immobilization was defined as a lack of movement except for minor spontaneous random movement of the appendages. An example of a sublethal sign of toxicity included lethargy. The presence of eggs in the brood pouch, aborted eggs, males or ephippia were also recorded daily.

With the onset of reproduction, the number of live and dead second-generation daphnids were counted then discarded every Monday, Wednesday and Friday during the test and at test termination. The number of immobile second-generation daphnids was also recorded. At the end of the test, the length, measured from the apex of the head to the base of the spine, and dry weight of each live first-generation daphnid also were determined.

#### Statistical Analyses

EC<sub>50</sub> values for mortality/immobility and reproduction could not be calculated because of the concentration-response observed during the test (i.e. for one of the test durations, a 50% decrease in survival or reproduction).

Statistical analyses were performed on the survival of the first-generation daphnids, the number of young produced per reproductive day and the length and dry weight of the surviving first-generation daphnids. Reproductive days were defined as the number of days a daphnid was alive from the first brood release of any daphnid in the test to the end of the test. In this study, the first reproductive day was Day 7.

Survival was analyzed at 24 hours, 48 hours and 96 hours, 7 days, 14 days and 21 days using 2 X 2 contingency tables. If the negative and solvent controls were not significantly different, then they were pooled for comparisons among the HBCD treatment groups. Analyses of reproduction, length and dry weight were evaluated for normality and homogeneity of variance using the Shapiro-Wilk's test and Bartlett's test, respectively. The negative and solvent controls were compared using Student's t-test. If no statistical differences were found, then the data was pooled for comparisons among the HBCD treatment groups. Analysis of variance and the Bonferroni t-test were used to determine if statistical differences existed among the controls and the HBCD treatment groups.

All statistical procedures were performed on a personal computer using SPSS/PC + Version 2.0 (5) or TOXSTAT Version 3.5 statistical software (6).

#### Analytical Chemistry

During the definitive test, water samples were collected from each replicate test chamber of each treatment and control group at test initiation and weekly thereafter to measure concentrations of the test substance. The samples were collected in glass scintillation vials and were extracted as soon as possible without storage. Analytical procedures used in the extraction and analysis of the samples are provided in Appendix III.

### RESULTS AND DISCUSSION

#### Measurement of Test Concentrations

Results of analyses to measure concentrations of HBCD in water samples collected during the in-life phase of the test are presented in Table I and in the analytical chemistry report (Appendix III). Nominal concentrations selected for use in this study were 0.85, 1.70, 3.40, 6.80 and 13.6  $\mu\text{g}$  HBCD/L. The mean measured test concentrations achieved in the test were 0.87, 1.6, 3.1, 5.6 and 11  $\mu\text{g}$  HBCD/L, which represented 102, 94, 91, 82 and 81% of the nominal concentrations, respectively. Mean measured concentrations were used in the calculations of the NOEC, LOEC and MATC.

#### Physical and Chemical Measurements of Water

Measurements of temperature, dissolved oxygen, and pH are presented in Tables 2, 3 and 4, respectively. Water temperatures were within the  $20 \pm 1^\circ\text{C}$  range established for the test. Dissolved oxygen concentrations were  $\geq 79\%$  of saturation throughout the test. Measurements of pH ranged from 8.1 to 8.4.

Measurements of specific conductance, hardness, alkalinity and TOC of the negative control treatment are given in Table 5. No apparent differences were observed for any of these parameters throughout the exposure period.

#### Survival and Biological Observations

A summary of the observations of mortality, immobility and sublethal signs of toxicity are shown in Table 6. All surviving daphnids appeared normal and healthy during the test. After 21 days of exposure, mortality in the negative control and solvent control was 0.0 and 2.5%, respectively. A 2 X 2 contingency table showed that mortality in the negative control and solvent control were not significantly different ( $p > 0.05$ ) and the controls were pooled for comparisons among the HBCD treatment groups. Mortality in the HBCD treatment groups ranged from 0 percent in the 3.1  $\mu\text{g}$  HBCD/L treatment group to 12.5% in the 11  $\mu\text{g}$  HBCD/L treatment group. Statistical analysis of mortality using 2 X 2 contingency tables showed that mortality in all HBCD treatment groups was not significantly different ( $p > 0.05$ ) from the pooled controls.

Based on the lack of sufficient mortality/immobility during this study, EC50 values could not be calculated. Consequently, the 24-hour, 48-hour, 96-hour, 7-day, 14-day and 21-day EC50 values based on mortality/immobility were  $>11 \mu\text{g}$  HBCD/L, the highest concentration tested.

#### Reproduction

A summary of the mean number of live young produced per reproductive day is presented in Table 7. A complete listing of young produced and the number of reproductive days for each replicate test chamber is provided in Appendix IV. Daphnids in the negative and solvent controls produced an average of 3.62 and 3.85 young per reproductive day, respectively. Student's t-test showed that reproduction in the negative and solvent control were not significantly different ( $p > 0.05$ ) and the controls were pooled for comparisons among the HBCD/L treatment groups. Reproduction in the HBCD treatment groups ranged from 1.86 young per reproductive day in the 1.6  $\mu\text{g}$  HBCD/L treatment group to 2.84 young per reproductive day in the 11  $\mu\text{g}$  HBCD/L treatment group. The Bonferroni t-test showed that reproduction was significantly reduced in the 11  $\mu\text{g}$  HBCD/L treatment group as compared to the pooled controls ( $p \leq 0.05$ ).

Although reproduction was significantly reduced in the 11  $\mu\text{g}$  HBCD/L treatment group, EC50 values based on reproduction could not be calculated. Reproduction was not decreased by a factor of 50% in any HBCD treatment group. Consequently, the 24-hour, 48-hour, 96-hour, 7-day, 14-day and 21-day EC50 values based on reproduction were  $>11 \mu\text{g}$  HBCD/L, the highest concentration tested.

### Growth

A summary of the mean lengths and mean dry weights of the surviving first-generation daphnids are presented in Table 8. Individual measurements are provided in Appendix V. The mean length and mean dry weight of first-generation daphnids in the negative control and solvent control were 4.09 mm and 0.69 mg, and 4.07 mm and 0.68 mg, respectively. Student's t-test showed that mean length and mean dry weight were not significantly different ( $p > 0.05$ ) in the negative and solvent control groups and the controls were pooled for comparisons among the HBCD treatment groups. The Bonferroni t-test showed that daphnids exposed to 11  $\mu\text{g}$  HBCD/L had significantly reduced mean lengths and dry weights as compared to the pooled controls ( $p \leq 0.05$ ). In addition, mean length was also significantly reduced in daphnids exposed to 5.6  $\mu\text{g}$  HBCD/L ( $p \leq 0.05$ ).

### CONCLUSIONS

No statistically significant effects on survival, reproduction and growth were observed in *Daphnia magna* exposed for 21 days to  $\leq 3.1$   $\mu\text{g}$  HBCD/L. HBCD's water solubility was determined in an earlier study in this laboratory to be 3.4  $\mu\text{g}/\text{L}$ . Therefore, HBCD's 21 day NOEC in *Daphnia magna* was approximately equivalent to HBCD's water solubility.

Daphnids exposed to 11  $\mu\text{g}$  HBCD/L for 21 days, the highest dose tested, had statistically significantly reduced lengths, dry weights and fewer young. Daphnids exposed to 5.6  $\mu\text{g}$  HBCD/L for 21 days had significantly reduced mean lengths.

Under the conditions of this study and based on mean daphnid length, the NOEC was 3.1  $\mu\text{g}$  HBCD/L. The LOEC was 5.6  $\mu\text{g}$  HBCD/L for 21 days. The MATC, the geometric mean of the NOEC and LOEC, was calculated to be 4.2  $\mu\text{g}$  HBCD/L. HBCD's 21 day NOEC, LOEC and MATC are either above or approximately equivalent to HBCD's water solubility.

## REFERENCES

- 1 **OECD.** 1984. Guideline 202: *Daphnia sp. Acute Immobilisation Test and Reproduction Test.*
- 2 **Title 40 Code of Federal Regulations.** 1994. Part 797, Section 1330: *Daphnia Chronic Toxicity Test.*
- 3 **American Society for Testing and Materials.** 1991. *Standard Guide for Conducting Renewal Life-Cycle Toxicity Tests with Daphnia magna*, ASTM E1193-87. Philadelphia, PA.
- 4 **APHA, AWWA, WPCF.** 1995. *Standard Methods for the Examination of Water and Wastewater.* 17th Edition, American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.
- 5 **SPSS Inc.** 1988. SPSS/PC+ Version 2.0. Chicago, Illinois.
- 6 **West, Inc. and D.D. Gulley.** 1996. TOXSTAT, Version 3.5. Western EcoSystems Technology, Inc. Cheyenne, Wyoming.

Table 1

## Summary of Analytical Chemistry Data

Sponsor: Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel Test Substance: Hexabromocyclododecane (HBCD) Test Organism: Cladoceran, <i>Daphnia magna</i> Dilution Water: Well Water					
Nominal Test Concentration ( $\mu\text{g HBCD/L}$ )	Time	Replicate	Measured Concentration ( $\mu\text{g HBCD/L}$ )	Mean Measured Concentration ( $\mu\text{g HBCD/L}$ )	Mean Percent of Nominal
Negative Control	Day 0	A	<LOQ	--	--
		B	<LOQ		
	Day 7	A	<LOQ		
		B	<LOQ		
	Day 14	A	<LOQ		
		B	<LOQ		
	Day 21	A	<LOQ		
		B	<LOQ		
Solvent Control	Day 0	A	<LOQ	--	--
		B	<LOQ		
	Day 7	A	<LOQ		
		B	<LOQ		
	Day 14	A	<LOQ		
		B	<LOQ		
	Day 21	A	<LOQ		
		B	<LOQ		
0.85	Day 0	A	0.90	0.87	102
		B	0.93		
	Day 7	A	0.84		
		B	0.81		
	Day 14	A	0.99		
		B	1.02		
	Day 21	A	0.74		
		B	0.72		
1.70	Day 0	A	1.64	1.6	94
		B	1.85		
	Day 7	A	1.63		
		B	1.63		
	Day 14	A	1.83		
		B	1.75		
	Day 21	A	1.34		
		B	1.45		

<sup>1</sup> The limit of quantitation (LOQ) was set at 0.50  $\mu\text{g HBCD/L}$ , the lowest fortification level in this study.

Table 1 (Continued)

Summary of Analytical Chemistry Data<sup>1</sup>

Sponsor: Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel					
Test Substance: Hexabromocyclododecane (HBCD)					
Test Organism: Cladoceran, <i>Daphnia magna</i>					
Dilution Water: Well Water					
Nominal Test Concentration ( $\mu\text{g HBCD/L}$ )	Time	Replicate	Measured Concentration ( $\mu\text{g HBCD/L}$ )	Mean Measured Concentration ( $\mu\text{g HBCD/L}$ )	Mean Percent of Nominal
3.40	Day 0	A	3.27	3.1	91
		B	3.63		
	Day 7	A	3.07		
		B	2.98		
	Day 14	A	3.34		
		B	3.34		
	Day 21	A	2.76		
		B	2.69		
6.80	Day 0	A	6.11	5.6	82
		B	5.81		
	Day 7	A	5.48		
		B	5.75		
	Day 14	A	6.38		
		B	5.68		
	Day 21	A	5.05		
		B	4.75		
13.6	Day 0	A	10.6	11	81
		B	11.0		
	Day 7	A	10.4		
		B	9.82		
	Day 14	A	11.9		
		B	12.3		
	Day 21	A	10.5		
		B	10.1		

<sup>1</sup> The limit of quantitation (LOQ) was set at 0.50  $\mu\text{g HBCD/L}$ , the lowest quantification level in this study

Table 2

Temperature of Water in the Test Chambers<sup>1</sup>

Sponsor:		Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel			
Test Substance:		Hexabromocyclododecane (HBCD)			
Test Organism:		Cladoceran, <i>Daphnia magna</i>			
Dilution Water:		Well Water			
Mean Measured Test Concentration ( $\mu\text{g}$ HBCD/L)	Replicate	Temperature ( $^{\circ}\text{C}$ )			
		Day 0	Day 7	Day 14	Day 21
Negative Control	A	20.2	19.9	19.9	19.9
	B	20.2	19.9	19.9	19.9
Solvent Control	A	20.2	19.9	19.9	19.9
	B	20.1	19.9	20.0	20.0
0.87	A	20.2	19.9	20.0	20.0
	B	20.2	19.9	20.0	19.9
1.6	A	20.1	19.8	20.0	19.9
	B	20.2	19.9	20.0	19.9
3.1	A	20.2	19.9	20.0	19.9
	B	20.2	19.9	20.0	19.9
5.6	A	20.2	20.0	20.1	20.1
	B	20.3	20.0	20.1	20.0
11	A	20.3	20.0	20.1	20.1
	B	20.3	20.0	20.1	20.0

<sup>1</sup> Temperature measured continuously during the test ranged from 19.0 to 20.5 $^{\circ}\text{C}$ .

Table 3

Dissolved Oxygen Content of Water in the Test Chambers

		Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel Hexabromocyclododecane (HBCD) <sup>1</sup>															
		Cladoceran, <i>Daphnia magna</i> Well Water															
Mean Measured Test Concentration (µg HBCD/L)	Test Chamber Replicate	Dissolved Oxygen (mg/L) <sup>1</sup>															
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 9	Day 12	Day 14	Day 16	Day 19	Day 21		
Negative Control	A	8.7	--	8.6	--	8.6	--	8.5	--	8.6	--	8.6	--	8.6	--	8.7	--
	B	--	8.7	--	8.5	--	8.6	--	8.7	--	8.6	--	8.6	--	8.4	--	8.4
Subvey Control	A	8.7	--	8.6	--	8.5	--	8.4	--	8.6	--	8.4	--	8.4	--	7.5	--
	B	--	8.7	--	8.6	--	8.5	--	8.7	--	8.4	--	7.9	--	7.6	--	7.6
0.87	A	8.8	--	8.6	--	8.5	--	8.4	--	8.6	--	8.2	--	8.2	--	7.4	--
	B	--	8.7	--	8.6	--	8.5	--	8.7	--	8.4	--	8.0	--	7.7	--	7.7
1.6	A	8.7	--	8.6	--	8.5	--	8.4	--	8.6	--	8.2	--	8.2	--	7.6	--
	B	--	8.7	--	8.5	--	8.5	--	8.7	--	8.4	--	7.7	--	7.6	--	7.8
3.1	A	8.7	--	8.6	--	8.5	--	8.4	--	8.7	--	8.2	--	8.2	--	7.6	--
	B	--	8.7	--	8.5	--	8.5	--	8.7	--	8.4	--	7.9	--	7.9	--	7.8
5.6	A	8.7	--	8.6	--	8.5	--	8.4	--	8.6	--	8.1	--	8.1	--	7.3	--
	B	--	8.7	--	8.6	--	8.5	--	8.7	--	8.4	--	7.9	--	7.9	--	7.4
11	A	8.7	--	8.6	--	8.5	--	8.4	--	8.6	--	8.2	--	8.2	--	7.2	--
	B	--	8.7	--	8.5	--	8.5	--	8.7	--	8.4	--	8.1	--	8.1	--	7.5

<sup>1</sup> A dissolved oxygen concentration of approximately 5.4 mg/L represents 60% of saturation at 20°C in freshwater.

Table 4  
pH of Water in the Test Chambers

		Dissolved Oxygen (mg/L) <sup>1</sup>										
Mean Measured Test Concentration (µg HBCD/L)	Test Chamber Replicate	Day 0	Day 2	Day 5	Day 7	Day 9	Day 12	Day 14	Day 16	Day 19	Day 21	
		Sponsor: Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel Test Substance: Hexabromocyclodecane (HBCD) Test Organism: Cladoceran, <i>Daphnia magna</i> Dilution Water: Well Water										
Negative Control	A	8.2	--	8.2	--	8.2	--	8.3	--	8.3	--	8.3
	B	--	8.1	--	8.3	--	8.2	--	8.3	--	8.3	--
Solvent Control	A	8.2	--	8.2	--	8.2	--	8.3	--	8.3	--	8.3
	B	--	8.1	--	8.3	--	8.2	--	8.4	--	8.3	--
0.87	A	8.2	--	8.2	--	8.2	--	8.3	--	8.3	--	8.3
	B	--	8.1	--	8.3	--	8.2	--	8.4	--	8.3	--
1.6	A	8.2	--	8.2	--	8.2	--	8.3	--	8.3	--	8.3
	B	--	8.1	--	8.3	--	8.2	--	8.4	--	8.3	--
3.1	A	8.2	--	8.2	--	8.2	--	8.3	--	8.3	--	8.3
	B	--	8.1	--	8.3	--	8.2	--	8.4	--	8.3	--
5.6	A	8.2	--	8.2	--	8.2	--	8.3	--	8.3	--	8.3
	B	--	8.1	--	8.3	--	8.2	--	8.4	--	8.3	--
11	A	8.2	--	8.2	--	8.2	--	8.3	--	8.4	--	8.3
	B	--	8.1	--	8.3	--	8.2	--	8.4	--	8.3	--

Table 5

Specific Conductance, Hardness, Alkalinity and Total Organic Carbon in the Negative Control

Sponsor:	Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel			
Test Substance:	Hexabromocyclododecane (HBCD)			
Test Organism:	Cladoceran, <i>Daphnia magna</i>			
Dilution Water:	Well Water			
Negative Control				
	Time			
Parameter	Day 0, Rep A	Day 7, Rep B	Day 14, Rep A	Day 21, Rep A
Specific Conductance ( $\mu$ mhos/cm)	310	315	320	310
Hardness (mg/L as CaCO <sub>3</sub> )	128	132	128	132
Alkalinity (mg/L as CaCO <sub>3</sub> )	176	178	178	176
TOC (mg C/L)	< 1	< 1	< 1	< 1

Table 6  
 Summary of Cumulative Percent Mortality and Treatment-Related Effects<sup>1</sup>

Sponsor: Test Substance: Test Organism: Dilution Water:	Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel Hexabromocyclododecane (HBCD)											
	Cumulative Observations to 24 Hours			Cumulative Observations to 48 Hours			Cumulative Observations to 96 Hours					
Mean Measured Test Concentration (µg HBCD/L)	% Mortality	% Immobile	Effects	% Mortality	% Immobile	Effects	% Mortality	% Immobile	Effects	% Mortality	% Immobile	Effects
Negative Control	0	0	40 AN	0	0	40 AN	0	0	40 AN	0	0	40 AN
Solvent Control	0	0	40 AN	0	0	40 AN	0	0	40 AN	0	0	40 AN
0.87	0	0	40 AN	0	0	40 AN	0	0	40 AN	0	0	40 AN
1.6	0	0	40 AN	0	0	40 AN	0	0	40 AN	0	0	40 AN
3.1	0	0	40 AN	0	0	38 AN; 2C	0	0	40 AN	0	0	40 AN
5.6	0	0	40 AN	0	0	40 AN	0	0	40 AN	0	0	40 AN
11	0	0	40 AN	0	0	40 AN	0	0	40 AN	0	0	40 AN

<sup>1</sup> Observed Effects: AN = All organisms appear normal and no unusual behavior observed; C = Lethargy.

Table 6 (Continued)  
 Summary of Cumulative Percent Mortality and Treatment-Related Effects<sup>1</sup>

Sponsor Test Substance Test Organism Dilution Water	Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel Hexabromocyclododecane (HBCDD) Cladoceran, <i>Daphnia magna</i> Well Water	Cumulative Observations to Day 7			Cumulative Observations to Day 14			Cumulative Observations to Day 21		
		% Mortality	% Immobile	Effects	% Mortality	% Immobile	Effects	% Mortality	% Immobile	Effects
Mean Measured Test Concentration (µg HBCDD/L)										
Negative Control		0	0	40 AN	2.5	0	39 AN	5.0	0	38 AN
Solvent Control		0	0	40 AN	0	0	40 AN	2.5	0	39 AN
0.87		0	0	40 AN	0	0	40 AN	2.5	0	39 AN
1.6		0	0	40 AN	2.5	0	39 AN	2.5	0	39 AN
3.1		0	0	40 AN	0	0	40 AN	0	0	40 AN
5.6		0	0	40 AN	0	0	40 AN	7.5	0	37 AN
11		2.5	0	39 AN	5.0	0	38 AN	12.5	0	35 AN

<sup>1</sup> Observed Effects: AN = All organisms appear normal and no unusual behavior observed.

Table 7

Summary of *Daphnia magna* Reproduction During the Life-Cycle Toxicity Test

Mean Measured Test Concentration ( $\mu\text{g HBCD/L}$ )	Replicate	Replicate Mean Number of Young per Reproductive Day	Mean Number of Young per Reproductive Day ( $\pm\text{SD}$ )
Negative Control	A	3.6611	3.62 ( $\pm 0.056$ )
	B	3.5819	
Solvent Control	A	3.9100	3.85 ( $\pm 0.084$ )
	B	3.7912	
0.87	A	3.7667	3.72 ( $\pm 0.068$ )
	B	3.6700	
1.6	A	3.8100	3.86 ( $\pm 0.069$ )
	B	3.9078	
3.1	A	3.9333	3.83 ( $\pm 0.14$ )
	B	3.7333	
5.6	A	3.7517	3.51 ( $\pm 0.34$ )
	B	3.2685	
11	A	2.3922	2.84 ( $\pm 0.63$ ) <sup>1</sup>
	B	3.2784	

<sup>1</sup> Indicates a significant difference from the pooled control using Bonferroni t-test ( $p \leq 0.05$ ).

NOTE: The mean live young/adult daphnid ( $\pm\text{SD}$ ) in the pooled control group was 3.74 ( $\pm 0.14$ )

Table 8

Summary of Length and Dry Weight of Surviving  
First-Generation Daphnids

Sponsor: Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel					
Test Substance: Hexabromocyclododecane (HBCD)					
Test Organism: Cladoceran, <i>Daphnia magna</i>					
Dilution Water: Well Water					
Mean Measured Test Concentration ( $\mu\text{g}$ HBCD/L)	Replicate	Replicate Total Length (mm)	Total Length (mm) Mean ( $\pm$ SD)	Replicate Dry Weight (mg)	Dry Weight (mg) Mean ( $\pm$ SD)
Negative Control	A	4.1132	4.09 ( $\pm$ 0.035)	0.6984	0.69 ( $\pm$ 0.016)
	B	4.0632		0.6763	
Solvent Control	A	4.0750	4.07 ( $\pm$ 0.0083)	0.6800	0.68 ( $\pm$ 0.00078)
	B	4.0632		0.6811	
0.87	A	4.1000	4.08 ( $\pm$ 0.024)	0.7020	0.70 ( $\pm$ 0.0044)
	B	4.0658		0.6958	
1.6	A	4.0400	4.05 ( $\pm$ 0.014)	0.6610	0.67 ( $\pm$ 0.019)
	B	4.0605		0.6874	
3.1	A	4.0325	4.04 ( $\pm$ 0.0053)	0.6565	0.68 ( $\pm$ 0.034)
	B	4.0400		0.7040	
5.6	A	4.0194	4.01 ( $\pm$ 0.014) <sup>1</sup>	0.6772	0.66 ( $\pm$ 0.025)
	B	4.0000		0.6416	
11	A	3.9056	3.87 ( $\pm$ 0.054) <sup>1</sup>	0.5889	0.56 ( $\pm$ 0.037) <sup>1</sup>
	B	3.8294		0.5359	

<sup>1</sup> Indicates a significant difference from the pooled controls using the Bonferroni t-test ( $p \leq 0.05$ ).

NOTE: The mean length and mean dry weight of the pooled control group was 4.08 ( $\pm$  0.024) mm and 0.68 ( $\pm$  0.0098) mg.

## APPENDIX I

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

Sponsor:	Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel	
Test Substance:	Hexabromocyclododecane (HBCD)	
Test Organism:	Cladoceran, <i>Daphnia magna</i>	
Dilution Water:	Well Water	
	Mean	Range
Specific Conductance ( $\mu$ mhos/cm)	306 (N = 4)	305 - 310
Hardness (mg/L as CaCO <sub>3</sub> )	130 (N = 4)	128 - 132
Alkalinity (mg/L as CaCO <sub>3</sub> )	177 (N = 4)	176 - 178
pH	8.3 (N = 4)	8.3

APPENDIX II  
Analyses of Pesticides, Organics, Metals and Other Inorganics  
in Wildlife International Ltd. Well Water<sup>1</sup>

ANALYSIS	MEASURED CONCENTRATION	
<b>Miscellaneous Measurements</b>		
Total Dissolved Solids	286	mg/L
Ammonia Nitrogen	< 0.050	mg/L
Total Organic Carbon <sup>2</sup>	< 1.0	mg/L
Total Cyanide	< 10.0	µg/L
<b>Organochlorines and PCBs</b>		
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)	< 0.006	µg/L
Chlordane	< 0.025	µg/L
DDD, pp'	< 0.006	µg/L
DDE, pp'	< 0.005	µg/L
DDT, pp'	< 0.008	µg/L
Dieldrin	< 0.005	µg/L
Endosulfan, A	< 0.005	µg/L
Endosulfan, B	< 0.005	µg/L
Endosulfan Sulfate	< 0.018	µg/L
Endrin	< 0.010	µg/L
Endrin Aldehyde	< 0.005	µg/L
Heptachlor	< 0.005	µg/L
Methoxychlor	< 0.007	µg/L
Heptachlor Epoxide	< 0.005	µg/L
Toxaphene	< 0.500	µg/L
PCB-1016	< 0.260	µg/L
PCB-1221	< 0.260	µg/L
PCB-1232	< 0.260	µg/L
PCB-1242	< 0.720	µg/L
PCB-1248	< 0.720	µg/L
PCB-1254	< 0.720	µg/L
PCB-1260	< 0.720	µg/L
<b>Metals and Other Inorganics</b>		
Aluminum <sup>3</sup>	< 100	µg/L
Arsenic <sup>3</sup>	< 25.0	µg/L
Beryllium <sup>3</sup>	< 0.50	µg/L
Cadmium <sup>3</sup>	< 1.0	µg/L
Calcium <sup>3</sup>	35.0	mg/L
Chromium <sup>3</sup>	< 2.0	µg/L
Cobalt <sup>3</sup>	< 1.0	µg/L
Copper <sup>3</sup>	< 20.0	µg/L
Iron <sup>3</sup>	< 100	µg/L
Lead <sup>3</sup>	< 10.0	µg/L
Magnesium <sup>3</sup>	13.5	mg/L
Manganese <sup>3</sup>	< 1.0	µg/L
Mercury	< 0.20	µg/L
Molybdenum <sup>3</sup>	< 2.0	µg/L
Nickel <sup>3</sup>	< 2.0	µg/L
Potassium <sup>3</sup>	6.62	mg/L
Selenium <sup>3</sup>	< 25.0	µg/L
Silver <sup>3</sup>	< 1.0	µg/L
Sodium <sup>3</sup>	21.3	mg/L
Zinc <sup>3</sup>	< 20.0	µg/L

<sup>1</sup> Analyses performed by QST Environmental, Gainesville, Florida for samples collected on November 3 through November 7, 1997.

<sup>2</sup> Analyses performed by Wildlife International Ltd. for the sample collected on November 5, 1997.

<sup>3</sup> Analyses performed by Wildlife International Ltd. for samples collected on November 5 through 7, 1997.

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PROJECT NO.: 439A-108

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APPENDIX III

THE ANALYSIS OF HEXABROMOCYCLODODECANE (HBCD) IN FRESHWATER

IN SUPPORT OF

WILDLIFE INTERNATIONAL LTD. PROJECT NO.: 439A-108

APPENDIX III

REPORT APPROVAL

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

TITLE: Hexabromocyclododecane (HBCD): A Flow-Through Life-Cycle Toxicity Test with the Cladoceran (*Daphnia magna*)

WILDLIFE INTERNATIONAL LTD. PROJECT NUMBER: 439A-108

PRINCIPAL INVESTIGATOR:

Willard B. Nixon for Nels H. Mahle  
Nels H. Mahle, Ph.D.  
Senior Scientist

4/30/98  
DATE

MANAGEMENT:

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

4/30/98  
DATE

## APPENDIX III

Introduction

Freshwater samples were collected from a flow-through life-cycle aquatic toxicity study designed to determine the effects of hexabromocyclododecane (HBCD) to the cladoceran (*Daphnia magna*). This study was conducted by Wildlife International Ltd. and identified as Project Number 439A-108. The analyses of these water samples were performed at Wildlife International Ltd. by high performance liquid chromatography (HPLC) with negative ion atmospheric pressure ionization mass spectrometry. Samples were received and analyzed between January 6, 1998 and February 10, 1998.

Test Substance

The test substance, hexabromocyclododecane (HBCD), was a composite of three materials (Wildlife International Ltd. identification numbers 3462, 3519 and 3551) prepared on February 19, 1996. The composite was assigned Wildlife International Ltd. identification number 3577. The test substance was used to prepare analytical standards and matrix fortification samples.

Analytical Method

The analytical method consisted of extracting the matrix blanks, fortified matrix samples and freshwater samples two times with 100 mL of dichloromethane (DCM). The extracts were rotary evaporated to dryness. The residues were then reconstituted with 90% acetonitrile/10% NANOpure® water. When necessary, the extracts were diluted with 90% acetonitrile/10% NANOpure® water to bring the concentration within the range of the calibration curve. Concentrations of HBCD were determined using a liquid chromatograph-mass spectrometer. The ionization source was an atmospheric pressure ionization (API) manufactured by Sciex Corporation. The instrument parameters are summarized in Table 1 and the method flow chart is provided in Figure 1.

Calibration Curve, Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Calibration standards of the hexabromocyclododecane (HBCD), ranging in concentration from 0.10 to 1.0 µg HBCD/mL, were analyzed with each set of samples. The Sciex MacQuan software program (version 1.5) was used for quantitation. Linear regression equations were generated using concentrations of the calibration standards versus the respective peak area responses. A representative calibration curve is

## APPENDIX III

shown in Figure 2. The correlation coefficient for the curve is 0.999. The concentration of HBCD in the samples was determined by substituting the peak area responses into the following linear regression equation:

$$\text{HBCD in sample } (\mu\text{g HBCD/mL}) = ((\text{Peak Area} - \text{y-intercept})/\text{slope}) \times \text{Dilution Factor}$$

The HBCD ( $\mu\text{g/mL}$ ) found in each sample was divided by the nominal concentration of each sample (fortified level,  $\mu\text{g/mL}$ ). This ratio times 100 is the percent recovery of the method at that level of fortification.

$$\% \text{ Recovery} = \frac{\text{Measured HBCD Concentration } (\mu\text{g/mL}) \times 100}{\text{Nominal HBCD Concentration } (\mu\text{g/mL})}$$

A representative ion chromatogram for the low calibration standard ( $0.1 \mu\text{g HBCD/mL}$ ) is shown in Figure 3. A representative ion chromatogram for the high calibration standard ( $1.0 \mu\text{g HBCD/mL}$ ) is shown in Figure 4. The shaded area indicates the integrated peak area used in the calculations.

The instrument limit of detection (LOD) for this study was set based upon the injection volume ( $30 \mu\text{L}$ ) and the lowest standard concentration ( $0.10 \mu\text{g HBCD/mL}$ ). The LOD was set at 3 ng injected on-column. The method limit of quantitation (LOQ) for these analyses was set at  $0.50 \mu\text{g HBCD/L}$ , (500 parts-per-trillion), the lowest concurrent fortification level.

#### Matrix Blank and Fortification Samples

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

Freshwater samples were fortified at 0.5, 5.0 and  $15 \mu\text{g/L}$  and analyzed concurrently with the samples to determine the mean procedural recovery (Table 3). A representative ion chromatogram of a matrix fortification is presented in Figure 6. The overall mean procedural recovery for this study was 109%. Sample concentrations were adjusted for the daily mean procedural recovery.

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## APPENDIX III

RESULTSSample Analysis

Freshwater samples were collected from the flow-through life-cycle toxicity study with the cladoceran (*Daphnia magna*) at pre-test, January 6, 1998, at test initiation (Day 0), January 7, 1998; at Day 7, January 14, 1998; at Day 14, January 21, 1998 and at test termination (Day 21), January 28, 1998. The pre-test samples were not run at the request of the Study Director. The measured concentration of HBCD in the samples collected at initiation of exposure of the test organisms (Day 0) ranged from 78 to 110% of the nominal concentrations (Table 4). Samples collected at Day 7 and Day 14 had measured concentration ranges of 72 to 98 and 84 to 120% of nominal concentrations, respectively. Samples collected at test termination (Day 21) ranged from 70 to 87% of nominal concentrations. A representative ion chromatogram of a sample at Day 0 is shown in Figure 7.

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Table 1

## Typical LC/MS Operational Parameters

MASS SPECTROMETER	Perkin-Elmer Sciex API 100 LC Mass Spectrometer
ION SOURCE:	Atmospheric Pressure Ionization (API) Heated Nebulizer
ION POLARITY:	Negative Ion Mode
ION SOURCE CURRENT:	2 microamps
ION SOURCE TEMPERATURE:	350°C
IONS MONITORED:	638.3 and 640.4 amu
LIQUID CHROMATOGRAPH:	Waters Model 2690 Separation Module Liquid Chromatograph
ANALYTICAL COLUMN:	Metachem Inertsil C-8 (2.0 mm ID, 25 cm, 5 µm particle size)
MOBILE PHASE:	90% acetonitrile/10% water
FLOW RATE:	250 µL/minute
INJECTION VOLUME:	30 microliters
RUN TIME:	12 minutes

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Table 2

## Matrix Blanks Analyzed Concurrently During Sample Analysis

Sample		Measured Concentration of Hexabromocyclododecane (HBCD) ( $\mu\text{g/L}$ ) <sup>1</sup>
Number (439A-108-)	Type	
MAB-1	Matrix Blank	< LOQ
MAB-2	Matrix Blank	< LOQ
MAB-3	Matrix Blank	< LOQ
MAB-4	Matrix Blank	< LOQ

<sup>1</sup> The limit of quantitation (LOQ) was set at 0.50  $\mu\text{g}$  HBCD/L, the lowest fortification level in this study.

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## APPENDIX III

Table 3

## Matrix Fortifications Analyzed Concurrently During Sample Analysis

Sample Number (439A-108-)	Concentration of HBCD ( $\mu\text{g/L}$ )		Percent Recovery <sup>1</sup>	Mean Percent Recovery <sup>1</sup>
	Fortified	Measured		
MAS-1	0.50	0.60	120	105
MAS-2	5.00	4.96	99.2	
MAS-3	15.0	14.3	95.6	
MAS-4	0.50	0.57	114	110
MAS-5	5.00	5.48	110	
MAS-6	15.0	16.0	107	
MAS-7	0.50	0.59	118	109
MAS-8	5.00	5.55	111	
MAS-9	15.0	14.9	99.6	
MAS-10	0.50	0.63	127	113
MAS-11	5.00	5.17	104	
MAS-12	15.0	16.3	109	
			Overall Mean =	109
			Standard Deviation =	9.2
			n =	12

<sup>1</sup> Results were generated using Excel 4.0 in the full precision mode. Manual calculations may differ slightly.

APPENDIX III

Table 4

Concentrations of Hexabromocyclododecane (HBCD) in Freshwater Samples from a Daphnia Flow-Through Life-Cycle Toxicity Test

Nominal Test Concentration ( $\mu\text{g/L}$ )	Sample Number (439A-108-)	Sampling Time (Days)	HBCD Concentration ( $\mu\text{g/L}$ )		Mean Percent of Nominal <sup>3</sup>
			Measured <sup>1</sup>	Corrected <sup>2</sup>	
0.0 (Negative Control)	1	0	<LOQ	<LOQ	--
	2	0	<LOQ	<LOQ	--
	15	7	<LOQ	<LOQ	--
	16	7	<LOQ	<LOQ	--
	29	14	<LOQ	<LOQ	--
	30	14	<LOQ	<LOQ	--
	43	21	<LOQ	<LOQ	--
	44	21	<LOQ	<LOQ	--
0.0 (Solvent Control)	3	0	<LOQ	<LOQ	--
	4	0	<LOQ	<LOQ	--
	17	7	<LOQ	<LOQ	--
	18	7	<LOQ	<LOQ	--
	31	14	<LOQ	<LOQ	--
	32	14	<LOQ	<LOQ	--
	45	21	<LOQ	<LOQ	--
	46	21	<LOQ	<LOQ	--

<sup>1</sup> The limit of quantitation (LOQ) was set at 0.50  $\mu\text{g}$  HBCD/L, the lowest fortification level in this study.

<sup>2</sup> Corrected for daily procedural recoveries (see Table 3).

<sup>3</sup> Results were generated using Excel 4.0 in the full precision mode. Manual calculations may differ slightly.

APPENDIX III

Table 4 (Continued)

Concentrations of Hexabromocyclododecane (HBCD) in Freshwater Samples from a Daphnia Flow-Through Life-Cycle Toxicity Test

Nominal Test Concentration ( $\mu\text{g/L}$ )	Sample Number (439A-108-)	Sampling Time (Days)	HBCD Concentration ( $\mu\text{g/L}$ )		Mean Percent of Nominal <sup>3</sup>
			Measured <sup>1</sup>	Corrected <sup>2</sup>	
0.85	5	0	0.95	0.90	106
	6	0	0.98	0.93	110
	19	7	0.92	0.84	98
	20	7	0.90	0.81	96
	33	14	1.08	0.99	116
	34	14	1.12	1.02	120
	47	21	0.84	0.74	87
	48	21	0.84	0.74	87
1.7	7	0	1.72	1.64	97
	8	0	1.93	1.85	109
	21	7	1.79	1.63	96
	22	7	1.79	1.63	96
	35	14	2.00	1.83	108
	36	14	1.92	1.75	103
	49	21	1.53	1.35	80
	50	21	1.63	1.45	85

<sup>1</sup> The limit of quantitation (LOQ) was set at 0.50  $\mu\text{g}$  HBCD/L, the lowest fortification level in this study.

<sup>2</sup> Corrected for daily procedural recoveries (See Table 3).

<sup>3</sup> Results were generated using Excel 4.0 in the full precision mode. Manual calculations may differ slightly.

APPENDIX III

Table 4 (Continued)  
 Concentrations of Hexabromocyclododecane (HBCD) in Freshwater Samples from a Daphnia Flow-Through Life-Cycle Toxicity Test

Nominal Test Concentration ( $\mu\text{g/L}$ )	Sample Number (439A-108-)	Sampling Time (Days)	HBCD Concentration ( $\mu\text{g/L}$ )		Mean Percent of Nominal <sup>3</sup>	
			Measured <sup>1</sup>	Corrected <sup>2</sup>		
3.4	9	0	3.42	3.27	96	
	10	0	3.80	3.63	107	
	23	7	3.37	3.07	90	
	24	7	3.27	2.98	88	
	37	14	3.66	3.34	98	
	38	14	3.66	3.34	98	
	51	21	3.11	2.76	81	
	52	21	3.03	2.69	79	
	6.8	11	0	6.41	6.11	90
		12	0	6.09	5.81	85
25		7	6.03	5.48	81	
26		7	6.32	5.75	85	
39		14	6.98	6.38	94	
40		14	6.22	5.68	84	
53		21	5.71	5.05	74	
54		21	5.36	4.75	70	
					Mean	3.13
					Mean	5.63

<sup>1</sup> The limit of quantitation (LOQ) was set at 0.50  $\mu\text{g HBCD/L}$ , the lowest fortification level in this study.

<sup>2</sup> Corrected for daily procedural recoveries (See Table 3).

<sup>3</sup> Results were generated using Excel 4.0 in the full precision mode. Manual calculations may differ slightly.

APPENDIX III

Table 4 (Continued)  
 Concentrations of Hexabromocyclododecane (HBCD) in Freshwater Samples from a Daphnia Flow-Through Life-Cycle Toxicity Test

Nominal Test Concentration ( $\mu\text{g/L}$ )	Sample Number (439A-108-)	Sampling Time (Days)	HBCD Concentration ( $\mu\text{g/L}$ )		Mean Percent of Nominal <sup>3</sup>
			Measured <sup>1</sup>	Corrected <sup>2</sup>	
13.6	13	0	11.1	10.6	78
	14	0	11.5	11.0	81
	27	7	11.5	10.4	77
	28	7	10.8	9.82	72
	41	14	13.0	11.9	87
	42	14	13.4	12.3	90
	55	21	11.9	10.5	77
	56	21	11.4	10.1	74

<sup>1</sup> The limit of quantitation (LOQ) was set at 0.50  $\mu\text{g HBCD/L}$ , the lowest fortification level in this study.

<sup>2</sup> Corrected for daily procedural recoveries (See Table 3).

<sup>3</sup> Results were generated using Excel 4.0 in the full precision mode. Manual calculations may differ slightly.

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## APPENDIX III

**METHOD OUTLINE FOR THE ANALYSIS OF HEXABROMOCYCLODODECANE  
(HBCD) IN FRESHWATER (DIRECT INJECTION)**

Add 100 mL of dichloromethane (DCM) to each separatory funnel that contains an aqueous sample.

↓

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

↓

Shake each separatory funnel for approximately one minute, let stand to separate, then transfer the DCM layer into a 250-mL round-bottom flask.

↓

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

↓

Evaporate the combined DCM extracts to dryness under vacuum using rotary evaporation with the water bath at ambient temperature (20-25°C). If necessary, use nitrogen to reduce solvent closer to complete dryness.

↓

Add appropriate volume of mobile phase with Class A pipet.

↓

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

↓

Transfer the sample into a chromatography vial for analysis.

↓

Run the samples by reverse phase liquid chromatography - negative ion atmospheric pressure ionization mass spectrometry.

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

APPENDIX III

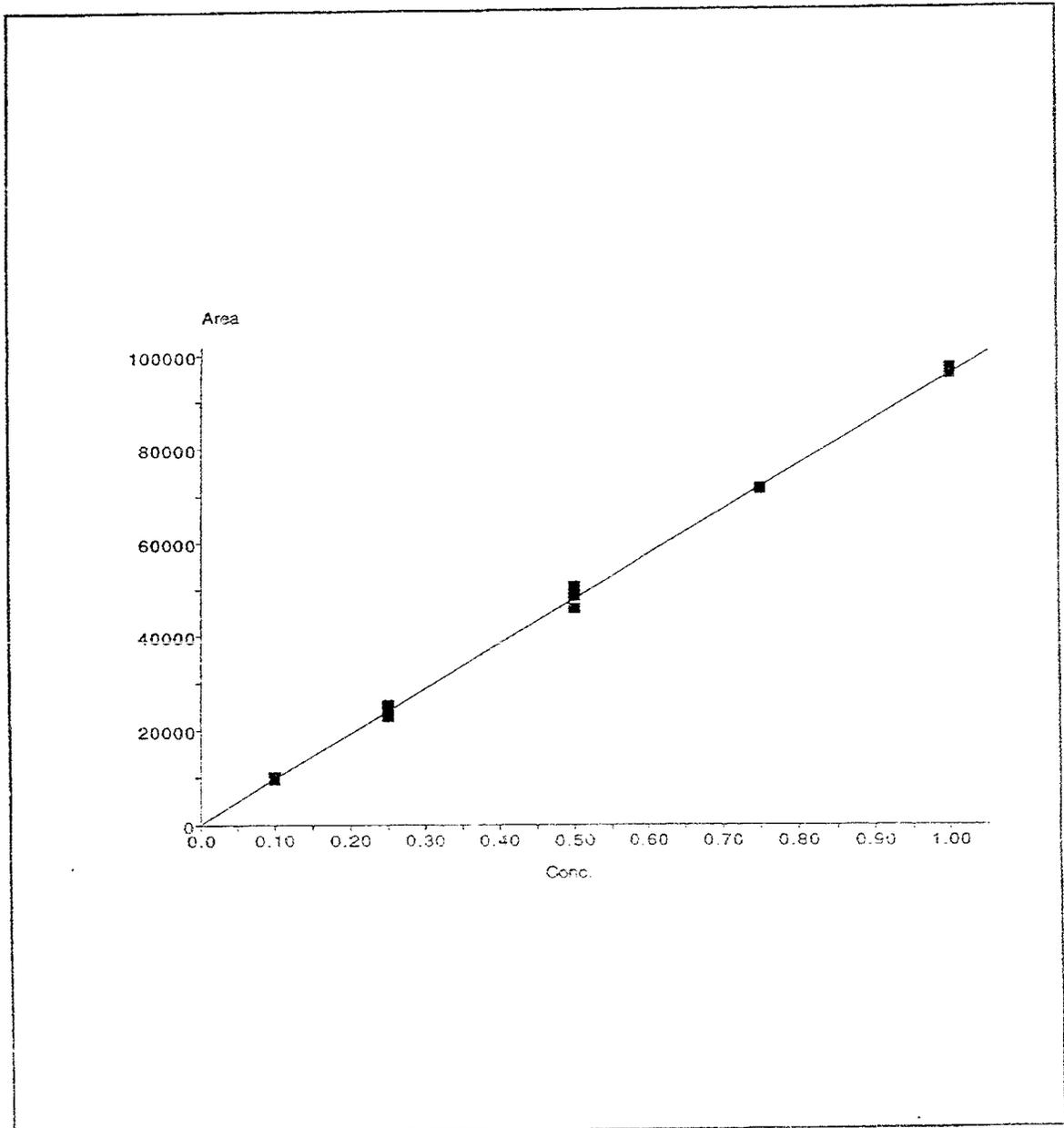


Figure 2. A representative calibration curve for HBCD Slope = 95582.703; Intercept = 239.611;  $r = 0.999$ .

APPENDIX III

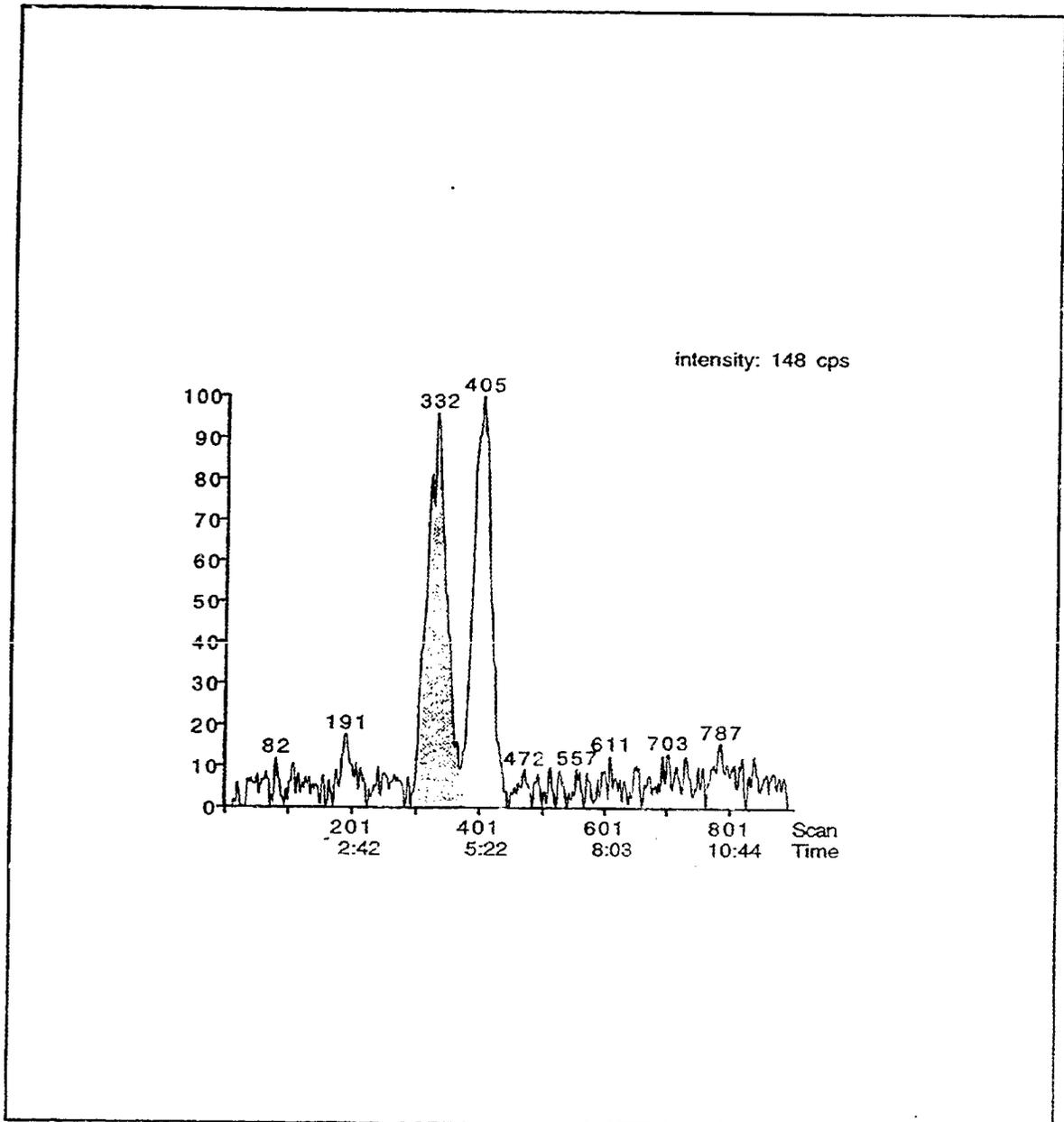


Figure 3. A representative ion chromatogram of a 0.10  $\mu\text{g}/\text{mL}$  HBCD standard (3 ng on-column).

APPENDIX III

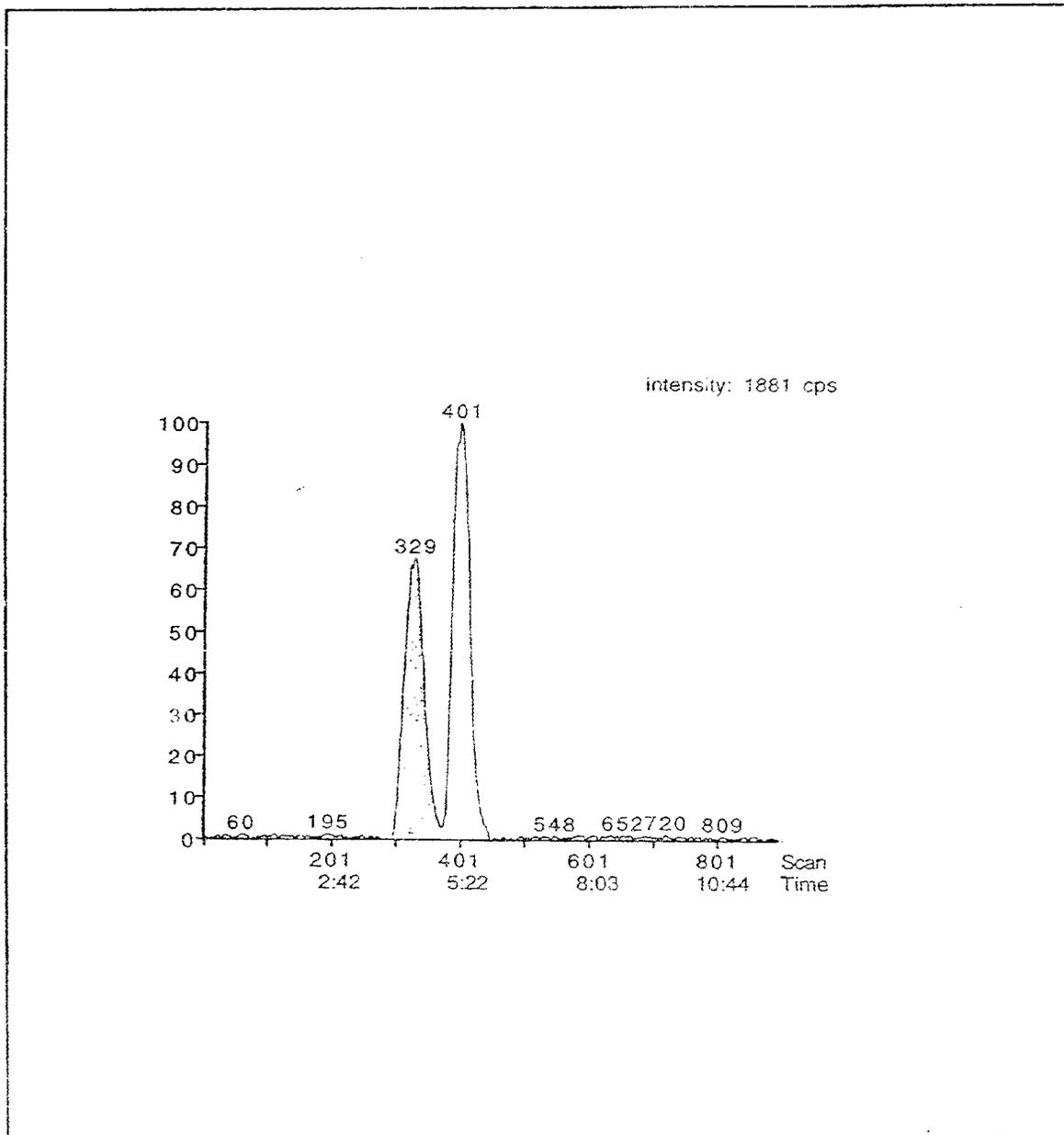


Figure 4. A representative ion chromatogram of a 1.00 µg/mL HBCD standard (30 ng on-column).

APPENDIX III

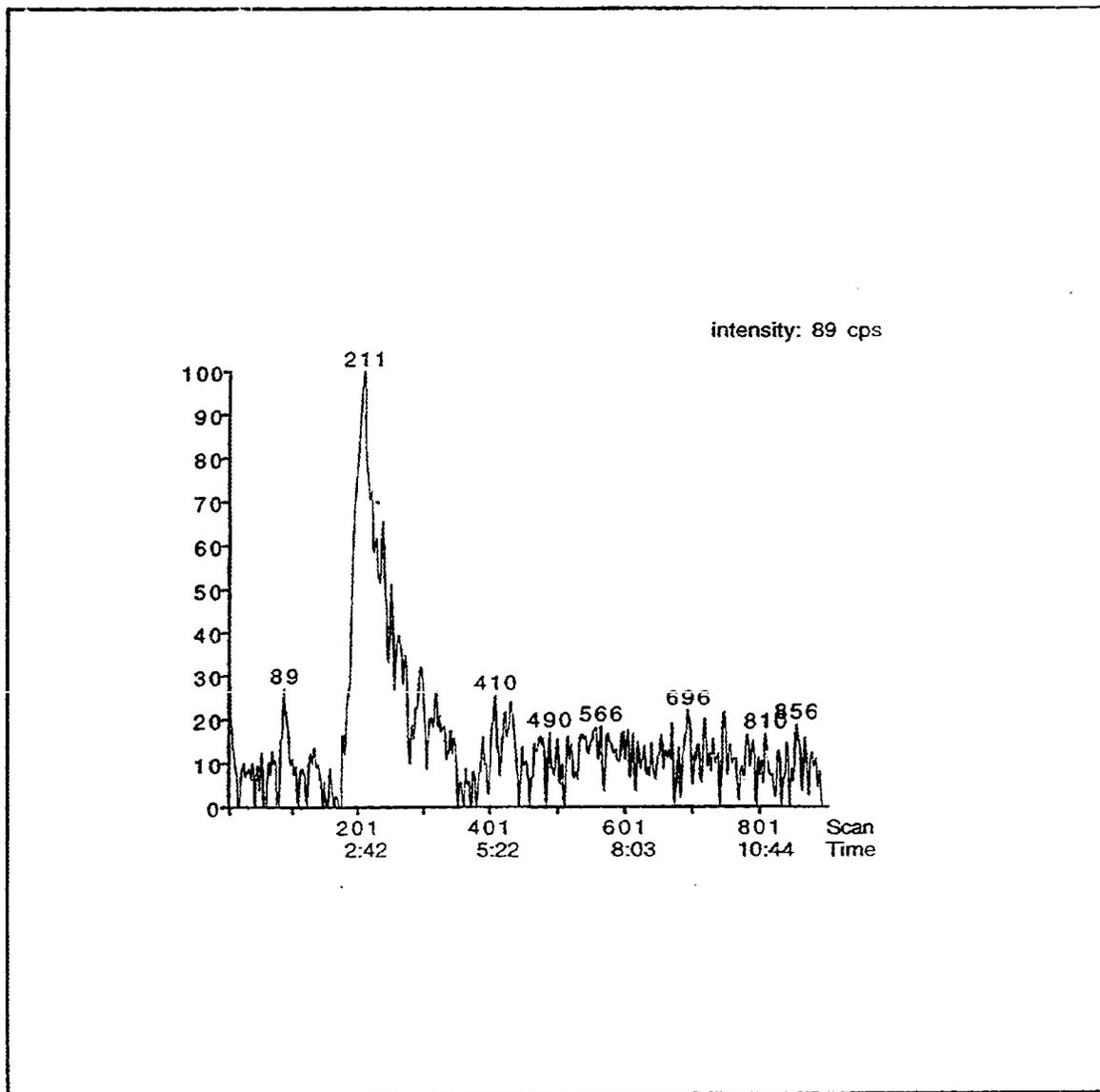


Figure 5. A representative ion chromatogram of a matrix blank, 439A-108-MAB-3.

APPENDIX III

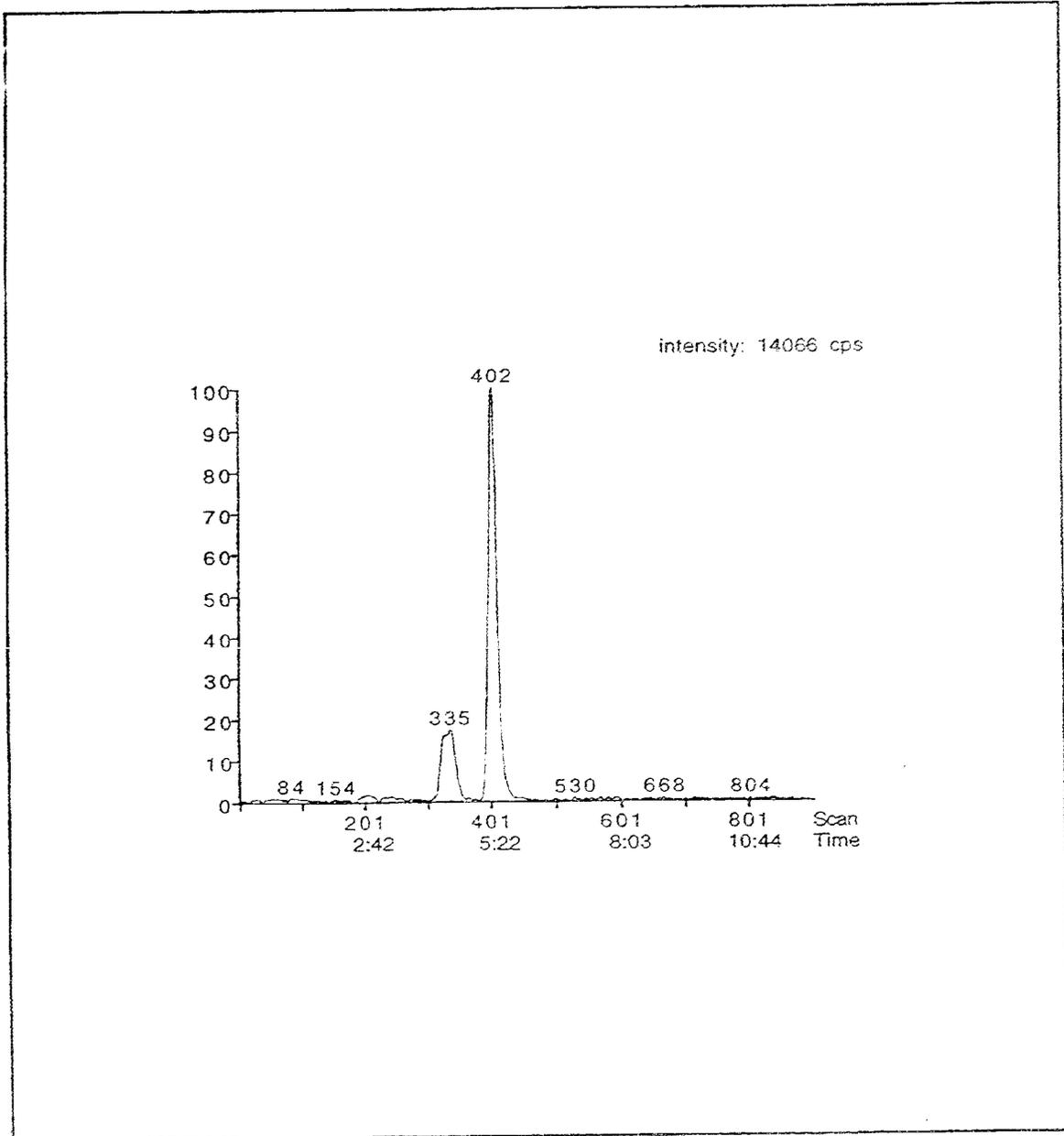


Figure 6. A representative ion chromatogram of a matrix fortification, 439A-108-MAS-2 (5.00  $\mu\text{g/L}$ ).

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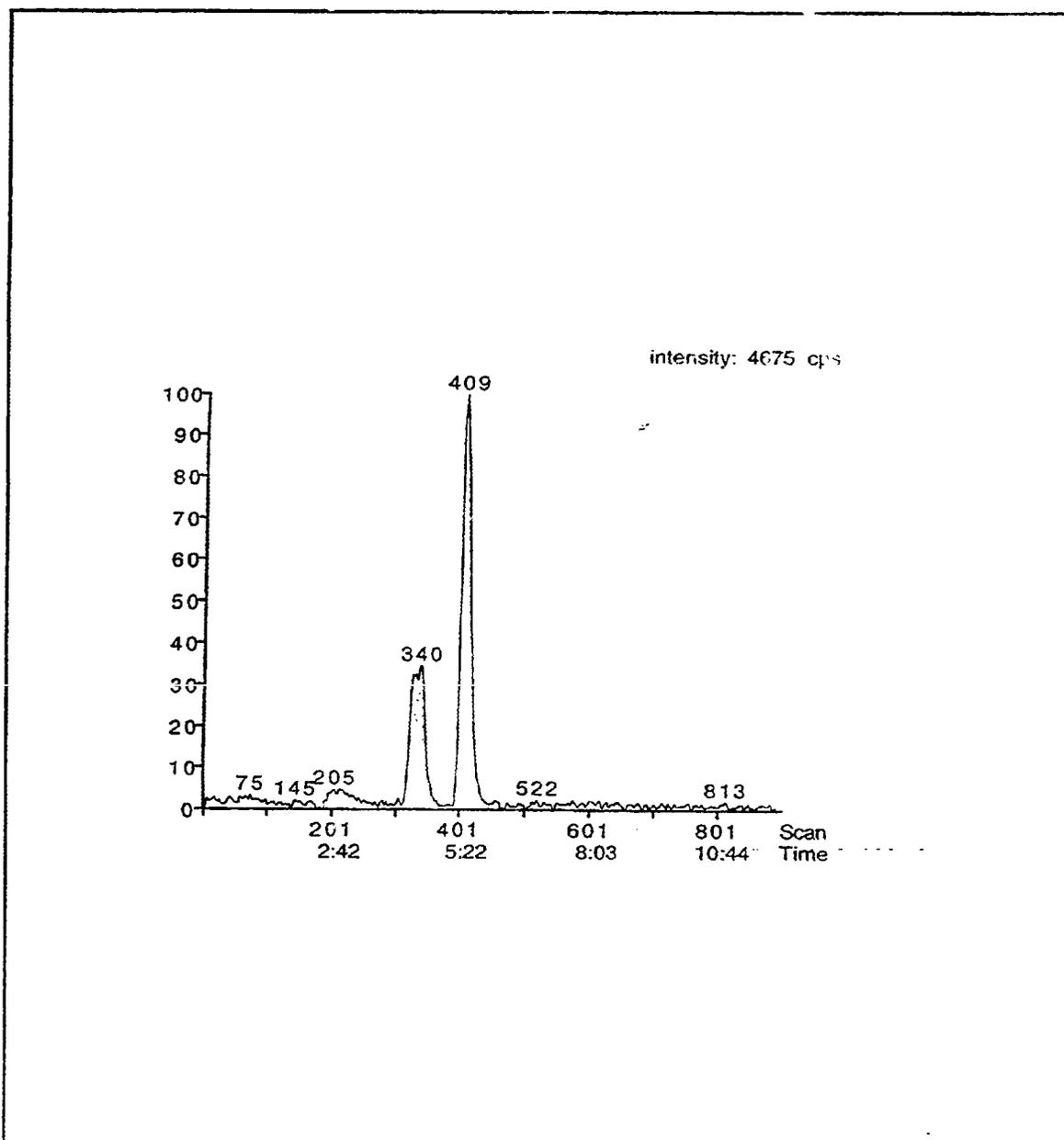


Figure 7. A representative ion chromatogram of a sample on Day 0, 439A-108-9 (3.4  $\mu\text{g/L}$  nominal concentration).

## APPENDIX IV

## Neonate Production

Sponsor:	Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel			
Test Substance:	Hexabromocyclododecane (HBCD)			
Test Organism:	Cladoceran, <i>Daphnia magna</i>			
Dilution Water:	Well Water			
Mean Measured Test Concentra- tion ( $\mu\text{g}$ HBCD/L)	Replicate	Number of Young Produced	Number of Reproductive Days <sup>1</sup>	Number of Young per Reproductive Day
Negative Control	A	1,091	298	3.6611
	B	1,028	287	3.5819
Solvent Control	A	1,173	300	3.9100
	B	1,126	297	3.7912
0.87	A	1,130	300	3.7667
	B	1,090	297	3.6700
1.6	A	1,143	300	3.8100
	B	1,145	293	3.9078
3.1	A	1,180	300	3.9333
	B	1,120	300	3.7333
5.6	A	1,118	298	3.7517
	B	974	298	3.2685
11	A	677	283	2.3922
	B	954	291	3.2784

<sup>1</sup>The number of reproductive days is the number of days the adult was alive from the first brood release of any daphnid in the test to the end of the test.

APPENDIX V

Length and Dry Weight of Surviving First-Generation Daphnids

Sponsor:		Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel				
Test Substance:		Hexabromocyclododecane (HBCD)				
Test Organism:		Cladoceran, <i>Daphnia magna</i>				
Dilution Water:		Well Water				
Mean Measured Test Concentration ( $\mu\text{g HBCD/L}$ )	Replicate	Compartment	Individual Length (mm)	Mean Length (mm)	Individual Dry Weights (mg)	Mean Dry Weights (mg)
Negative Control	A	A	4.00	4.1132	0.65	0.6984
			4.15		0.73	
			4.10		0.76	
			4.15		0.64	
			4.05		0.58	
			4.25		0.78	
			4.10		0.69	
		4.05	0.59			
		4.15	0.82			
		B	4.15		0.71	
			4.20		0.84	
			4.05		0.66	
			4.10		0.71	
			3.95		0.51	
	4.10		0.68			
	4.15		0.76			
	B	C	4.05	4.0632	0.54	0.6763
			4.25		0.83	
			4.15		0.79	
			4.00		0.58	
			3.95		0.53	
			4.10		0.70	
			4.15		0.78	
		D	3.95		0.63	
			4.00		0.59	
			4.10		0.79	
			4.05		0.61	
			3.95		0.56	
4.15			0.81			
4.10			0.72			
4.00	0.55					
3.95	0.61					
4.10	0.64					
4.15	0.32					
4.20	0.86					
4.10	0.71					
4.05	0.69					
4.15	0.67					

## APPENDIX V (Continued)

## Length and Dry Weight of Surviving First-Generation Daphnids

Sponsor: Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel								
Test Substance: Hexabromocyclododecane (HBCD)								
Test Organism: Cladoceran, <i>Daphnia magna</i>								
Dilution Water: Well Water								
Mean Measured Test Concentration ( $\mu\text{g HBCD/L}$ )	Replicate	Compartment	Individual Length (mm)	Mean Length (mm)	Individual Dry Weights (mg)	Mean Dry Weights (mg)		
Solvent Control	A	A	4.10	4.0750	0.69	0.6800		
			4.05		0.63			
			4.15		0.73			
			4.15		0.79			
			4.25		0.85			
			4.05		0.60			
			4.10		0.67			
			4.15		0.75			
			4.05		0.58			
			4.00		0.57			
			B		B		3.95	0.53
							3.90	0.56
							4.05	0.62
							4.10	0.71
	4.00	0.65						
	3.90	0.64						
	4.10	0.72						
	4.15	0.71						
	4.20	0.84						
	4.10	0.76						
	B	C		4.10		4.0632	0.72	0.6811
				4.00			0.63	
				3.95			0.61	
				4.10			0.69	
			4.00	0.57				
			4.10	0.69				
			4.15	0.78				
			4.10	0.71				
4.20			0.84					
D			D	4.10	0.65			
				4.00	0.54			
				4.15	0.79			
				3.95	0.53			
				3.90	0.58			
	4.05	0.65						
	4.10	0.76						
	4.05	0.69						
	4.15	0.69						
	4.05	0.72						

## APPENDIX V (Continued)

## Length and Dry Weight of Surviving First-Generation Daphnids

Sponsor: Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel							
Test Substance: Hexabromocyclododecane (HBCD)							
Test Organism: Cladoceran, <i>Daphnia magna</i>							
Dilution Water: Well Water							
Mean Measured Test Concentration ( $\mu\text{g HBCD/L}$ )	Replicate	Compartment	Individual Length (mm)	Mean Length (mm)	Individual Dry Weights (mg)	Mean Dry Weights (mg)	
0.87	A	A	4.25	4.1000	0.81	0.7020	
			4.00		0.67		
			4.20		0.79		
			4.05		0.60		
			4.15		0.79		
			4.10		0.74		
			3.95		0.54		
			4.15		0.76		
			4.20		0.85		
			4.15		0.77		
			4.10		0.59		
			4.05		0.63		
			4.15		0.74		
			4.25		0.90		
		B	C	3.90	4.0658	0.54	0.6958
	3.95			0.51			
	4.00			0.62			
	4.10			0.67			
	4.20			0.82			
	4.10			0.70			
	4.10			0.74			
	4.15			0.82			
	3.95			0.63			
	4.00			0.57			
	4.20			0.76			
	3.95			0.53			
	4.15			0.84			
	4.10			0.72			
	D	D	4.05	4.0658	0.68	0.6958	
4.15			0.73				
4.00			0.59				
3.90			0.52				
4.15			0.79				
4.05			0.64				
4.00			0.62				
3.95			0.61				
4.10			0.73				
4.15			0.89				
4.15	0.81						

APPENDIX V (Continued)

Length and Dry Weight of Surviving First-Generation Daphnids

Sponsor: Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel							
Test Substance: Hexabromocyclododecane (HBCD)							
Test Organism: Cladoceran, <i>Daphnia magna</i>							
Dilution Water: Well Water							
Mean Measured Test Concentration ( $\mu\text{g HBCD/L}$ )	Replicate	Compartment	Individual Length (mm)	Mean Length (mm)	Individual Dry Weights (mg)	Mean Dry Weights (mg)	
1.6	A	A	3.90	4.040	0.57	0.6610	
			4.00		0.62		
			4.05		0.60		
			4.15		0.75		
			4.00		0.63		
			4.10		0.73		
			3.95		0.66		
			4.10		0.69		
			4.15		0.75		
			4.20		0.78		
			3.85		0.54		
			4.10		0.70		
			4.00		0.62		
			4.05		0.69		
	4.10	0.75					
	3.95	0.56					
	4.00	0.63					
	4.10	0.60					
	3.95	0.63					
	4.10	0.72					
		B	C	4.15	4.0605	0.82	0.6674
				3.95		0.62	
				4.00		0.57	
				4.05		0.66	
				3.95		0.54	
				4.05		0.65	
				4.10		0.71	
				4.00		0.62	
4.15				0.82			
4.20				0.87			
4.15				0.74			
4.05				0.67			
4.10				0.59			
3.85				0.56			
3.95	0.62						
4.00	0.60						
4.10	0.71						
4.20	0.80						
4.15	0.79						

APPENDIX V (Continued)

Length and Dry Weight of Surviving First-Generation Daphnids

Mean Measured Test Concentration (µg HBCD/L)	Replicate	Compartment	Individual Length (mm)	Mean Length (mm)	Individual Dry Weights (mg)	Mean Dry Weights (mg)
3.1	A	A	4.10	4.0325	0.68	0.6565
			4.10		0.72	
			4.05		0.67	
			4.00		0.60	
			3.85		0.52	
			3.95		0.59	
			4.05		0.57	
			4.00		0.63	
		4.15	0.80			
		4.15	0.84			
		4.10	0.76			
		4.00	0.69			
		4.05	0.61			
		3.95	0.62			
		4.10	0.73			
		3.80	0.47			
	3.95	0.59				
	4.10	0.72				
	4.15	0.70				
	4.05	0.62				
	4.15	0.84				
	4.20	0.86				
	4.00	0.70				
	3.95	0.56				
	4.10	0.75				
	4.15	0.77				
	3.10	0.40				
	4.05	0.57				
	4.00	0.62				
	4.20	0.80				
	4.10	0.66				
	4.00	0.65				
4.15	0.79					
4.05	0.71					
4.20	0.82					
3.95	0.61					
4.00	0.59					
4.10	0.67					
4.15	0.82					
4.20	0.89					

## APPENDIX V (Continued)

## Length and Dry Weight of Surviving First-Generation Daphnids

Sponsor:		Chemical Manufacturers Association's Brominated Flame Retardant Industry Panel				
Test Substance:		Hexabromocyclododecane (HBCD)				
Test Organism:		Cladoceran, <i>Daphnia magna</i>				
Dilution Water:		Well Water				
Mean Measured Test Concentration ( $\mu\text{g HBCD/L}$ )	Replicate	Compartment	Individual Length (mm)	Mean Length (mm)	Individual Dry Weights (mg)	Mean Dry Weights (mg)
5.6	A	A	3.95	4.0194	0.62	0.6772
			4.10		0.69	
			3.80		0.54	
			4.05		0.72	
			4.15		0.83	
			3.85		0.53	
			4.05		0.60	
		4.10	0.73			
		B	3.80		0.51	
			3.85		0.62	
			4.10		0.80	
			4.00		0.59	
			4.15		0.79	
			3.95		0.62	
	4.05		0.61			
	B	C	4.15	4.0000	0.83	0.6416
			4.10		0.72	
			4.15		0.84	
			4.00		0.65	
			3.80		0.52	
			4.10		0.73	
			4.05		0.59	
		3.85	0.50			
		3.95	0.65			
		4.00	0.60			
		3.95	0.63			
		4.15	0.71			
		D	4.20		0.82	
4.10			0.80			
3.90	0.54					
3.75	0.47					
4.10	0.78					
4.00	0.60					
4.15	0.84					
4.05	0.58					
3.90	0.52					
4.00	0.62					

## APPENDIX V (Continued)

## Length and Dry Weight of Surviving First-Generation Daphnids

Mean Measured Test Concentration ( $\mu\text{g HBCD/L}$ )	Replicate	Compartment	Individual Length (mm)	Mean Length (mm)	Individual Dry Weights (mg)	Mean Dry Weights (mg)				
11	A	A	4.10	3.9056	0.79	0.5889				
			3.85		0.49					
			3.95		0.52					
			4.10		0.75					
			4.00		0.64					
			4.15		0.79					
			3.65		0.42					
			3.75		0.40					
			4.10		0.81					
			B		B		A	3.95	0.59	
								4.05	0.63	
								4.10	0.73	
								3.65	0.40	
								3.70	0.42	
	3.95	0.56								
	3.70	0.50								
	3.55	0.49								
	4.00	0.67								
	3.65	0.50								
	B	C	C	3.55	3.8294	0.40	0.5359			
				4.00		0.60				
				3.75		0.48				
				3.60		0.42				
				4.10		0.79				
				4.05		0.68				
				3.85		0.53				
				D		D		D	4.00	0.65
									3.65	0.41
3.75									0.47	
4.10									0.75	
4.00									0.54	
3.90									0.53	
3.70									0.42	
3.80	0.51									
3.65	0.43									

APPENDIX VI

Protocol, Amendment and Deviations

PROTOCOL

HEXABROMOCYCLODODECANE (HBCD): A FLOW-THROUGH LIFE-CYCLE  
TOXICITY TEST WITH THE CLADOCERAN (*Daphnia magna*)

OECD Guideline 202

and

TSCA Title 40 of the Code of Federal Regulations  
Part 797, Section 1330

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



October 10, 1997

PROTOCOL NO.: 439/101097/DAP-LC2/SUB439

WILDLIFE INTERNATIONAL LTD.

HEXABROMOCYCLODODECANE (HBCD): A FLOW-THROUGH LIFE-CYCLE TOXICITY TEST WITH THE CLADOCERAN (*Daphnia magna*)

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

SPONSOR'S REPRESENTATIVE: Dr. Hasmukh Shah

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

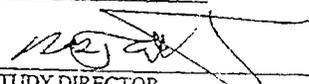
STUDY DIRECTOR: Kurt Drottar  
Senior Aquatic Biologist

LABORATORY MANAGEMENT: Henry O. Krueger, Ph.D.  
Director of Aquatic Toxicology & Non-Target Plants

FOR LABORATORY USE ONLY

Proposed Dates:	
Experimental Start Date: _____	Experimental Termination Date: _____
Project No.: <u>439A-108</u>	Study Room: _____
Test Concentrations: <u>Negative Control, Solvent Control, 0.85, 1.70, 3.40, 6.80 &amp; 13.6 µg/L</u>	
Test Substance No.: <u>3577</u>	Int/Date: _____

PROTOCOL APPROVAL

  
STUDY DIRECTOR

10/22/97  
DATE

  
LABORATORY MANAGEMENT

10/28/97  
DATE

  
SPONSOR'S REPRESENTATIVE

October 22, 1997  
DATE

**WILDLIFE INTERNATIONAL LTD.****INTRODUCTION**

Wildlife International Ltd. will conduct a flow-through life cycle toxicity test with the cladoceran, *Daphnia magna*, for the Sponsor at the Wildlife International Ltd. aquatic toxicology facility in Easton, Maryland. The study will be performed based on procedures in OECD Guideline 202, *Daphnia sp., Acute Immobilisation and Reproduction Test* (1); Title 40 of the Code of Federal Regulations, Part 797, Section 1330, *Daphnia Chronic Toxicity Test* (2); and ASTM Standard E1193-87 *Standard Guide for Conducting Renewal Life-Cycle Toxicity Tests with Daphnia magna* (3). Raw data for all work performed at Wildlife International Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International Ltd. site or at an alternative location to be specified in the final report.

**PURPOSE**

The purpose of this study is to determine what effects the test substance may have on the survival, growth and reproduction of the cladoceran, *Daphnia magna*, under flow-through test conditions for a period of 21 days.

**EXPERIMENTAL DESIGN**

Daphnids will be exposed to a geometric series of five nominal test concentrations (0.85, 1.70, 3.40, 6.80 and 13.6 µg/L HBCD, a negative (dilution water) control and a solvent control for 21 days. Ten neonate daphnids (< 24 hours old) will be placed in each of two test compartments per test chamber. Test chambers will be replicated so that a total of 40 daphnids are exposed to each treatment and control group.

The nominal test concentrations were selected in consultation with the Sponsor and are based upon information such as known toxicity data and physical/chemical properties of the test substance. Water samples from appropriate test chambers will be collected at specified intervals for analysis and determination of the actual test substance concentration. Results of the analyses will be used to calculate actual mean test concentrations. Both nominal and actual mean measured test concentrations will be determined and reported.

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To control bias, neonate daphnids will be impartially assigned to exposure chambers at test initiation. Observations of the survival and reproduction of the first-generation daphnids will be made approximately three times per week (e.g., Monday, Wednesday and Friday). The results of the test will be used to calculate the EC50 (death and immobilization) and EC50 (reproduction) values when possible at 24 hours, 48 hours, 96 hours, 7 days, 14 days and at the end of the test. The EC50 is defined as the concentration estimated to cause death and/or immobilization in 50% of the daphnids or reduce reproduction by 50% of that in the control daphnids. In addition, the results of the test will be used to calculate the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC). The NOEC is defined as the highest concentration that produces no statistically significant effect on survival, reproduction or growth. The LOEC is defined as the lowest concentration that produces a statistically significant effect on survival, reproduction or growth. The maximum acceptable toxicant concentration (MATC) will be calculated as the geometric mean of the NOEC and LOEC.

MATERIALS AND METHODSTest Substance

The test substance consists 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:

Manufacturer	Lot/Batch	Date Received	Wildlife International Ltd. ID No.
Great Lakes Chemical Corp.	635297G-1	October 26, 1995	3452
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 and is being stored under ambient conditions. Another subsample of the composite test substance will be shipped to Albemarle Corp. for characterization and homogeneity analyses to demonstrate stability and confirm its activity for the purpose of the study. Initial analyses of subsamples of the

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composite 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%

Preparation of Test Concentrations

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

The test substance will be mixed with dimethyl formamide. The test substance will be dissolved in the solvent to form a stock solution that will subsequently be added to the dilution water. A solvent control group will be included in the experimental design along with a negative (dilution water) control group. The concentration of the organic solvent will not exceed 0.1 mL/L, when possible. The solvent concentration in the solvent control group will be equal to the highest solvent concentration in test chambers containing the test substance.

Test Organism

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

Daphnids will be cultured in water from the same source and at approximately the same temperature as will be used during the test, except that the culture water is supplemented with

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

Daphnids in the cultures will be fed once daily. The diet will be a mixture of yeast, Cerophyll®, and trout chow (YCT), supplemented with a suspension of the freshwater green alga *Selenastrum capricornutum*. Feed (YCT) provided to daphnids will be analyzed at least once annually to ensure that there are no contaminants at levels known to be capable of interfering with the study. Although specifications for acceptable levels of contaminants in daphnid diets have not been established, however there are no known levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the test.

Neonates will be obtained for testing from at least three individual adults. Prior to test initiation, the neonates will be collected from cultures and transferred to glass beakers or directly to the test compartments. The daphnids will be released into the test compartments below the water surface using a wide-bore pipette. Daphnids will be fed daily during the test using the same diet as used in the cultures. The frequency of feeding will be documented in the raw data and the final report.

Dilution Water

Water used for the culturing and testing of daphnids will be obtained from a well approximately 40 meters deep located on the Wildlife International Ltd. site. The water will be passed through a sand filter and pumped into a 37,800-L storage tank where the water will be aerated with spray nozzles. Prior to use the water will be filtered to 0.2  $\mu\text{m}$  and passed through an ultraviolet sterilizer in order to remove fine particles and microorganisms, respectively. Water used for culturing and testing is characterized as moderately hard. Typical values for hardness, alkalinity, pH and specific conductance are approximately:

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Hardness, mg/L as CaCO <sub>3</sub>	145
Alkalinity, mg/L as CaCO <sub>3</sub>	190
pH	8.1
Specific Conductance, $\mu$ mhos/cm	330

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

Test Apparatus

A continuous-flow diluter will be used to provide each concentration of the test substance, a negative (dilution water) control, and a solvent control, when necessary. A syringe pump, peristaltic pump, or a similar device will be used to deliver the test substance to mixing chambers where the test substance will be mixed with dilution water. The flow of dilution water into each mixing chamber will be controlled using rotameters. The rotameters will be calibrated prior to the test and verified and/or calibrated at least once a week during the test. After mixing, test solutions will be split to each replicate chamber. The proportion of water split to each replicate will be checked prior to the study and approximately weekly thereafter to ensure that these flow rates vary by no more than  $\pm 10\%$  of the mean flow rate of the two replicates. Daphnids will be held in test compartments suspended in 25-L stainless steel chambers. Test chambers will hold approximately 22-L of water. Test compartments will be constructed from 500-mL glass beakers, approximately 7.0 cm in diameter and 13 cm in height. Nylon mesh screen will cover two, approximately 4-cm holes on opposite sides of each compartment to permit test solution to flow into and out of the compartment. Two compartments will be suspended in each replicate test chamber. Test chambers will be indiscriminately positioned in a temperature-controlled water bath to maintain a temperature of  $20 \pm 1^\circ\text{C}$ . The water bath will be enclosed in a plexiglass ventilation hood in order to minimize potential cross contamination between test systems. Test chambers will be labelled with the project number, test concentration and replicate.

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

Environmental Conditions

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

The target test temperature will be  $20 \pm 1^\circ\text{C}$ . Temperature will be measured in each test chamber at the beginning of the test and at weekly intervals during the test using a hand-held thermometer. Temperature also will be measured with a continuous recorder in one negative control replicate. Recorder measurements will be verified with a liquid-in-glass thermometer prior to test initiation and at least weekly thereafter.

Dissolved oxygen will be measured in alternate replicates of the treatment and control group(s) at test initiation, daily during the first week of the test, and at least three times per week (e.g. Monday, Wednesday and Friday) thereafter, using a Yellow Springs Instrument Model 51B dissolved oxygen meter, or equivalent. In the event that dissolved oxygen levels fall below 60% saturation, dissolved oxygen measurements will be made in every test chamber and appropriate actions will be taken after consultation with the Sponsor. Measurements of pH will be made in alternate replicates of each treatment and control group at test initiation and at least three times per week (e.g., Monday, Wednesday and Friday) thereafter using a Fisher Accumet Model 915 pH meter, or equivalent. If a treatment group reaches 100% mortality, dissolved oxygen, pH and temperature measurements will be taken at that time, then discontinued.

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Hardness, alkalinity, specific conductance and total organic carbon (TOC) will be measured in alternate replicates of the negative (dilution water) control at test initiation and at weekly intervals until test termination. Hardness and alkalinity measurements will be made by titration using procedures based on methods in *Standard Methods for the Examination of Water and Wastewater* (5). Specific conductance will be measured using a Yellow Springs Instrument Model 73 Salinity-Conductivity-Temperature meter, or equivalent. Total organic carbon will be analyzed with a Shimadzu Model 5000 TOC analyzer.

Biological Observations

The first generation daphnids will be observed approximately three times per week (e.g., Monday, Wednesday and Friday) during the test for survival, the onset of reproduction, and clinical signs of toxicity. The criteria for death include absence of heartbeat, white opaque coloration, lack of movement of appendages and lack of response to gentle prodding. Immobilization is defined as a lack of movement except for the spontaneous random movement of the appendages. Examples of clinical signs of toxicity include inability to maintain position in the water column, uncoordinated swimming and cessation of feeding. The presence of eggs in the brood pouch, aborted eggs, males, or ephippia also will be recorded daily.

The number of second-generation daphnids will be counted and recorded approximately three times per week. The number of second-generation daphnids which are immobile also will be recorded. At each observation, live first-generation daphnids will be retained, and the second-generation daphnids will be discarded. At the end of the test, surviving first-generation daphnids will be measured for length (distance from the apex of the head to the base of the spine) and dry weight.

Sampling for Analytical Measurements

Water samples will be collected from each test chamber of the low and high level concentrations prior to the start of exposure, and from all levels at the beginning of the test, at approximately weekly intervals during the test, and at the end of the test to determine concentrations of the test substance. In the event that 100% mortality occurs in any treatment, then sampling of that treatment will terminate following the next sampling interval. Samples will be collected at mid-depth from each test chamber and analyzed immediately, or placed in a glass storage container and stored

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under refrigeration until analyzed. If refrigeration storage of collected samples is used until analyses can be performed, standards of 0.5, 5.0 and 15  $\mu\text{g/L}$  will also accompany them during storage and will be processed, as well as fresh standards at the time the samples are analyzed. The sample scheme is summarized below:

PROPOSED NUMBERS OF SAMPLES					
Experimental Group	Pre-test <sup>a</sup>	Day 0	Day 7	Day 14	Day 21
Control	-	2	2	2	2
Solvent Control (if needed)	-	2	2	2	2
Level 1-Low Concentration	2 <sup>b</sup>	2	2	2	2
Level 2	-	2	2	2	2
Level 3	-	2	2	2	2
Level 4	-	2	2	2	2
Level 5-High Concentration	2	2	2	2	2
<b>TOTALS</b>	<b>4</b>	<b>14</b>	<b>14</b>	<b>14</b>	<b>14</b>

<sup>a</sup>Pre-test samples will be collected after conditioning of the diluter. More than one pre-test sampling interval may be required, depending upon the Sponsor's needs, and additional sampling and results will be documented in the raw data and included in the final report.

<sup>b</sup>One sample from the A and B replicate test chambers.

Total Number of Verification Samples = 60

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study. At the discretion of the Study Director, water samples also will be collected from at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system.

#### Analytical Chemistry

Chemical analysis of the samples will be performed by Wildlife International Ltd. using LC/MS methodology. Sample preparation is summarized in Appendix B. The methodology used to prepare and analyze the test samples will be documented in the raw data and summarized in the final report.

Data Analyses

EC50 values with 95% confidence intervals will be calculated when possible by probit analysis, the moving average-angle method, or binomial probability (6, 7, 8, 9) using mortality/immobilization and reproduction data collected at 24 hours, 48 hours, 96 hours, 7 days, 14 days, and at the end of the test. Survival data also will be evaluated at those same times to identify those treatments statistically different from the control group using 2 X 2 contingency tables or a similar statistical comparison test. If a solvent control group is used in addition to a negative control group, these two groups will be compared by a 2 X 2 contingency table. If no statistical differences are found, then the data of the two control groups may be pooled. If statistical differences are found, then either the negative or solvent control groups will be used to evaluate the treatment-related effects.

Reproduction and growth (length and dry weights) data will be evaluated for normality and homogeneity of variances. Transformations will be used when necessary to correct for non-normality or heterogeneity of variances. If a solvent control group is used in addition to a negative control group, these two groups will be compared by a Student's t-test. If no statistical differences are found, then the data of the two control groups may be pooled. If statistical differences are found, then either the negative (dilution water) or solvent control group will be used to evaluate the treatment-related effects.

When the reproduction and growth data are considered to be normal with homogeneous variances, an analysis of variance (ANOVA) will be used to determine whether or not statistical differences exist among the experimental groups. If statistical differences are found, then a means comparison test (e.g., Dunnett's test, Bonferroni's t-test, or an alternate test) will be used to identify those treatments differing from the control group(s). The NOEC, the LOEC, and the MATC will be determined using the results of the statistical analyses of the survival, reproduction and growth data. When transformations fail to correct for non-normality or heterogeneous variances, then nonparametric analyses will be used to evaluate treatment-related effects.

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**RECORDS TO BE MAINTAINED**

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

1. A copy of the signed protocol.
2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Dates of initiation and termination of the test.
4. Length and dry weight measurements.
5. Reproduction parameters.
6. Test organism culture records.
7. Results of rangefinding tests, when applicable.
8. Stock solution calculation and preparation.
9. Daily observations.
10. Water chemistry calculations (e.g., hardness and alkalinity).
11. If applicable, the methods used to analyze test substance concentrations and the results of analytical measurements.
12. Statistical calculations.
13. Test conditions and physical/chemical measurements.
14. Calculation and preparation of test concentrations.
15. Copy of final report.

**FINAL REPORT**

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

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed. It is the responsibility of the Sponsor to provide the final date that data are recorded for chemistry pathology and/or supporting evaluations that may be generated at other laboratories.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures, as stated in the approved protocol, including changes in the original protocol.

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5. The test substance identification, including name, chemical abstract number or code number, strength, purity, composition, and other information provided by the Sponsor.
6. Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor or contracted to Wildlife International Ltd.
7. A description of the methods used to conduct the test.
8. A description of the test organisms, including the source, scientific name, age, life stage, feed types, light intensity, and photoperiod.
9. A description of the preparation of the test solutions, the methods used to allocate organisms to test chambers and begin the test, the number of organisms and chambers per treatment, and the duration of the test.
10. A description of circumstances that may have affected the quality or integrity of the data.
11. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
12. A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses.
13. Statistical methods used to evaluate the data.
14. The signed and dated reports of each of the individual scientists or other professionals involved in the study.
15. The location where raw data and final report are to be stored.
16. A statement prepared by the Quality Assurance Unit listing the dates that study inspections and audits were made and the dates of any findings reported to the Study Director and Management.
17. If it is necessary to make corrections or additions to a final report after it has been accepted, such changes will be made in the form of an amendment issued by the Study Director. The amendment will clearly identify the part of the final report that is being amended and the reasons for the amendment, and will be signed and dated by the Study Director.

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#### CHANGING OF PROTOCOL

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

#### GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 792) and OECD Principles of Good Laboratory Practices (OCDE/GD (92) 32, Environment Monograph No. 45). Each study conducted by Wildlife International Ltd. is routinely examined by the Wildlife International Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International Ltd. site, or at an alternative location to be specified in the final report.

REFERENCES

- 1 **OECD.** 1984. Guideline 202: *Daphnia sp.*, Acute Immobilisation Test and Reproduction Test.
- 2 **Title 40 Code of Federal Regulations.** 1994. Part 797, Section 1330: *Daphnia* Chronic Toxicity Test.
- 3 **ASTM Standard E1193-87.** 1988. *Standard Guide for Conducting Renewal Life-Cycle Toxicity Tests with Daphnia magna.* American Society for Testing and Materials.
- 4 **Pennak, R.W.** 1978. *Freshwater Invertebrates of the United States.* 2nd Ed. 365 p.
- 5 **APHA, AWWA, WPCF.** 1995. *Standard Methods for the Examination of Water and Wastewater.* 17th Edition, American Public Health Association. American Water Works Association. Water Pollution Control Federation, New York.
- 6 **Finney, I. J.** 1971. *Statistical Methods in Biological Assay.* Second Edition. Griffin Press, London.
- 7 **Thompson, W.R.** *Bacteriological Reviews.* Vol. II, Nos. 2, pp. 115-145.
- 8 **Stephan, C.E.** 1977. "Methods for Calculating and LC50". *Aquatic Toxicology and Hazard Evaluations.* American Society for Testing and Materials. Publication Number STP 634, pp. 65-84.
- 9 **Stephan, C.E.** 1978. U.S. Environmental Protection Agency, Environmental Research Laboratory, Duluth, Minnesota. Personal communication.

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APPENDIX I  
IDENTIFICATION OF TEST SUBSTANCE BY SPONSOR

To be Completed by Sponsor

I. Test Substance Identity (name to be used in the report): HBCD

Reference Standard (if applicable): Analytical Standard: N/A

Internal Standard: N/A

Test Substance Sample Code or Batch Number: Wildlife International Ltd, Identification No. 3577

Test Substance Purity (% Active Ingredient): 93.67 - HBCD Expiration Date: \_\_\_\_\_

II. Test Substance Characterization

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

III. Test Substance Storage Conditions

Please indicate the recommended storage conditions at Wildlife International Ltd.

Ambient

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

Other pertinent stability information: \_\_\_\_\_

IV. Test Concentrations:

\_\_\_\_\_ Adjust test concentration to 100% a.i. based upon the purity (%) given above.

X Do not adjust test concentration to 100% a.i. Test the material AS IS.

V. Toxicity Information:

Mammalian: Rat LD50 \_\_\_\_\_ Mouse LD50 \_\_\_\_\_

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

\_\_\_\_\_

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

VI. Classification of the Compound:

\_\_\_\_\_ Insecticide \_\_\_\_\_ Herbicide \_\_\_\_\_ Fungicide

\_\_\_\_\_ Microbial Agent \_\_\_\_\_ Economic Poison

Other: \_\_\_\_\_

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APPENDIX II

Samples will be analyzed based upon the following procedures:

Add 100 mL of dichloromethane (DCM) to each separatory funnel that contains an aqueous sample.



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



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



Use a rotary evaporator to reduce the extract to a small volume.



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



Evaporate the extract to complete dryness.



Add appropriate volume of mobile phase with a Class A pipet.



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



Transfer the sample into a chromatography vial for analysis using LC/API-MS.

A copy of the above method will be maintained in the raw data. The actual methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.



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PROJECT NO.: 439A-108  
Page 1 of 1

DEVIATION TO STUDY PROTOCOL

STUDY TITLE: HEXABROMOCYCLODODECANE (HBCD): A FLOW-THROUGH LIFE-CYCLE TOXICITY TEST WITH THE CLADOCERAN (*Daphnia magna*)

PROTOCOL NO.: 439/101097/DAP-LC2/SUB439

DEVIATION NO.: 1

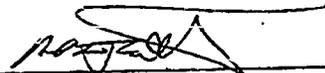
SPONSOR: Chemical Manufacturers Association

PROJECT NO.: 439A-108

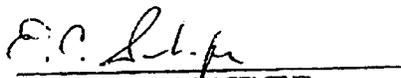
DATE OF DEFACTO DEVIATION: January 6, 1998

DEVIATION: The protocol specifies that pre-test samples will be collected and analyzed. The pre-test samples were collected, however, they were not analyzed.

REASON: The analytical method required two days to perform. Pre-test samples were collected on the day prior to test initiation. Due to the length of time it required to obtain the results of the analyses, pre-test samples would not have provided any valuable information. Consequently, the Study Director decided not to analyze the pre-test samples. It is the best judgment of the Study Director that this deviation did not adversely affect the results of the study.

  
STUDY DIRECTOR

3/10/98  
DATE

  
LABORATORY MANAGEMENT

3/10/98  
DATE

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DEVIATION TO STUDY PROTOCOL

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PROTOCOL NO.: 439/101097/DAP-LC2/SUB439

DEVIATION NO.: 2

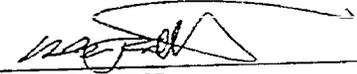
SPONSOR: Chemical Manufacturers Association

PROJECT NO.: 439A-108

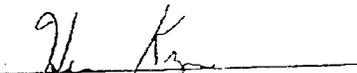
DATE OF DEFACTO DEVIATION: January 7, 1998

DEVIATION: The protocol states that if refrigeration storage of collected samples is used until analyses can be performed, standards of 0.5, 5.0 and 15 µg/L will also accompany them during storage and will be processed, as well as fresh standards at the time the samples are analyzed. Matrix fortifications were prepared and stored with the samples until analysis, however, fresh fortifications were not prepared on the day of analysis.

REASON: Fresh matrix fortifications were inadvertently not prepared. Based on the recovery of the stored matrix fortifications (95 to 126 percent of nominal), it is the best judgment of the Study Director that this deviation did not adversely affect the results of the study.

  
STUDY DIRECTOR

3/26/98  
DATE

  
LABORATORY MANAGEMENT

3/26/98  
DATE

CCD-ID  
RECEIVED  
05/28/98

## APPENDIX VII

## Personnel Involved in the Study

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

1. Henry O. Krueger, Ph.D., Director, Aquatic Toxicology and Non-Target Plants
2. Willard B. Nixon, Ph.D., Manager, Analytical Chemistry
3. Nels H. Mahle, Ph.D., Senior Scientist
4. Kurt R. Drottar, Senior Aquatic Biologist
5. Mark A. Mank, Aquatic Laboratory Supervisor

**CERTIFICATE OF AUTHENTICITY**

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

Data produced 09 27 1999 Mary Furber  
(Month) (Day) (Year) Camera Operator

Place Syracuse New York  
(City) (State)

