THE CHRONIC TOXICITY OF TETRABROMOBISPHENOL A (TBBPA) TO *Daphnia magna* UNDER FLOW-THROUGH CONDITIONS

Submitted To:

Brominated Flame Retardant Industry Panel
c/o Great Lakes Chemical Corporation
West Lafayette, IN 47906

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FINAL

GOOD LABORATORY PRACTICE STATEMENT

The data and report presented for this study were produced and compiled in accordance with all pertinent EPA Good Laboratory Practice regulations except in the case of stability, characterization and verification of test substance identity. Maintenance of records on the test substance is the responsibility of the test Sponsor.

DC Surprenant 8/15/89

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1.0 SUMMARY

The purpose of this study was to estimate the chronic toxicity of Tetrabromobisphenol A (TBBPA) to daphnids (*Daphnia magna*) under flow-through conditions. *Daphnia magna* were continuously exposed for 21 days to nominal concentrations of TBBPA ranging from 2.0 to 0.13 mg/L, a solvent (acetone) control and a dilution water control. Observations on organism survival, reproduction, immobilized young and growth were recorded during the exposure to determine the effects of TBBPA on these standard performance criteria. Procedures used during this study were consistent with the requirements published in the EPA's Tetrabromobisphenol A Final Test Rule (Federal Register Volume 52, No. 128, 6 July 1987).

Weekly radiometric analyses of the test solutions for ^{14}C-TBBPA established that the diluter system generally functioned properly throughout the test period and maintained mean measured concentrations of test material which averaged 40% of the nominal levels. Measured concentrations of TBBPA (as active ingredient) in the exposure solutions followed the expected concentration gradient and were generally consistent between replicate treatments and sampling intervals. The exposure levels based on mean measured concentrations were: 0.98, 0.30, 0.19, 0.10 and 0.056 mg/L of TBBPA. Weekly analyses of the highest treatment level (2.0 mg/L nominal) using high pressure liquid chromatography (HPLC) resulted in measured concentrations which were generally consistent between replicate vessels and sampling intervals. Based on HPLC analyses, the average measured concentration of filtered (0.45 μm teflon filter) samples was 0.65 $\pm$ 0.19 mg/L TBBPA and of unfiltered samples was 0.93 $\pm$ 0.24 mg/L TBBPA. Throughout the exposure period, a small amount of precipitated test material was present in the diluter system's mixing chamber. No undissolved TBBPA (e.g., precipitate, film on solution surface) was observed in any of the treatment levels or control solutions.

After 21 days exposure, both the control and solvent control daphnids survived and reproduced at rates which met the minimum standard criteria established by the US EPA under TSCA Guidelines (i.e., ≥ 80% survival, ≥ 60 offspring per female). Statistical comparison of the performance of the dilution water control and solvent control organisms established that no significant differences existed between the two control groups. All comparisons to determine treatment level effects were made using pooled (control and solvent control) data.

At the termination of the 21-day study, daphnid survival in all concentrations (0.98 - 0.056 mg/L) ranged from 95 - 100%. This was statistically comparable to the survival of the pooled control organisms (98%).

Reproduction, as determined by cumulative numbers of offspring per female at test termination was the most sensitive indicator of the toxicity of TBBPA to *Daphnia magna* in the concentration range tested. Reproduction in the highest test concentration (0.98 mg/L TBBPA) was 21 offspring per female and was significantly less ($P \leq 0.05$) than the reproduction of the pooled control organisms (60 offspring/female). Reproduction among daphnids exposed to the remaining test concentrations of TBBPA (0.30 - 0.056 mg/L) ranged from 44 to 58 offspring per female which was statistically similar to the reproduction of the pooled control organisms.

Organism growth, as determined by the measurement of individual body lengths at test termination, was not adversely affected by exposure to TBBPA concentrations ≤ 0.98 mg/L. Average organism body lengths ranged from 4.0 - 4.4 mm in the treatment level solutions (0.98 - 0.056 mg/L TBBPA) which was statistically comparable to the lengths of the control (pooled data) organisms (4.1 mm).

Based on the effect of TBBPA on daphnid reproduction, the Maximum Acceptable Toxicant Concentration (MATC) of this test material to *Daphnia magna* was > 0.30 mg/L and < 0.98 mg/L (geometric mean MATC = 0.54 mg/L).

2.0 INTRODUCTION

2.1 Objective

The objective of this study was to determine the chronic effects of Tetrabromobisphenol A (TBBPA) on the survival, reproduction and growth of the daphnid (*Daphnia magna*). The study was performed under continuous exposure, flow-through conditions for a period of 21 days (one generation). Exposure concentrations were analytically confirmed on day 0, 7, 14 and 21. Test results were used to estimate the Maximum Acceptable Toxicant Concentration (MATC) defined as the concentration range encompassing the highest mean measured concentration that had no significant ($P \leq 0.05$) effect on the test organism performance and the lowest mean measured concentration that significantly affected the exposed organisms. The MATC is usually expressed as the geometric mean of the tested no effect and effect concentrations, and is estimated from the most sensitive of the performance criteria used, e.g., adult survival, number of offspring produced, growth (measured as body length).

2.2 Rationale

Chronic toxicity tests with freshwater invertebrates, particularly representative fish food organisms like daphnids, are often used to evaluate the toxicological properties of pesticides and other organic chemicals. Species such as *Daphnia magna* are valued as indicator organisms to evaluate effects on survival as well as possible effects on the reproduction of aquatic organisms (Biesinger et al., 1974; Macek et al., 1976a; Macek et al., 1976b; Maki and Johnson, 1975; Nebeker and Puglisi, 1974; Schober and Lampert, 1977; Winner and Farrell, 1976). Daphnids have also been shown to be sensitive organisms for indicating the toxicity of a wide variety of test substances. Kenaga (1978) derived comparisons between test organism sensitivity using 75 insecticides and herbicides and concluded that daphnids and shrimp were the most sensitive form of test organism among the aquatic invertebrates, birds, rats, fish and honeybees tested. This sensitivity, in combination with the organisms' small size, ease of culture and relatively short life-cycle, has established the

21-day flow-through test as a standard for evaluating the potential chronic effects of chemicals on aquatic invertebrates.

3.0 MATERIALS AND METHODS

3.1 Protocol

This study was conducted according to the procedures outlined in the protocol entitled "Protocol for Conducting a Flow-Through Life Cycle Toxicity Test with *Daphnia magna* following TSCA Guidelines", SLI Protocol #020188/DM-LC.BFRIP. The test was conducted from 5 - 26 January 1989, at the Environmental Sciences Division of Springborn Laboratories, Inc., (SLI), Wareham, Massachusetts. All raw data generated and the final report are stored at the above location.

3.2 Test Material

The Tetrabromobisphenol A (tested as 100% active ingredient), a white powder, was received in five aliquots from the Brominated Flame Retardant Industry Panel. Appendix I presents the description of the material received and the procedure used to formulate a composite super stock solution [labeled (^{14}C) and unlabeled] of the test article. Prior to testing, the TBBPA was stored at room temperature (~ 20°C) in a ventilated cabinet. Test concentrations are expressed as milligrams of TBBPA per liter of solution (mg/L).

3.3 Stock Solution

A diluter stock solution of 119.9 mg/mL was prepared by diluting 10 milliliters (mL) of a ^{14}C-TBBPA superstock (4.29 mg/mL which was blown to dryness with nitrogen) with the nonlabled TBBPA superstock (119 mg/mL) to a volume of 50 mL (see Appendix I for labeled and nonlabled superstock preparation).

3.4 Test Organisms

The daphnids (*Daphnia magna*) used in this toxicity test were obtained from populations cultured at Springborn Laboratories, Inc. (SLI). Daphnids were cultured under static

renewal conditions (20 ± 2°C) in well water fortified to a total hardness of approximately 160 - 180 mg/L $CaCO_3$. Water used to culture these organisms was from the same source as the dilution water used during the chronic exposure. Daphnids were fed daily a combination of green algae (*Ankistrodesmus falcatus*) and a yeast suspension. Selco®, a commercial mixture of fatty acids and protein, was added weekly to each vessel. Offspring produced during the 24 hour period prior to the start of the 21 day exposure were used to initiate the study. Records of daphnid culture are retained in SLS's culture log entitled "Invertebrate Culture Log; *Daphnia magna*, Volume V" (1 January - 31 December 1989).

3.5 Test Dilution Water

Culture and test dilution water were prepared in 1900-liter batches by fortifying well water according to the formula for hard water (ASTM, 1980) and filtering it through an Amberlite XAD-7 resin column to remove any potential organic contaminants. Generally, several batches of water were prepared each week. The frequency at which the dilution water was prepared depended on the requirements of the laboratory. Fortified water was discarded if not used within 14 days of preparation. This water generally had a total hardness and alkalinity as calcium carbonate ($CaCO_3$) of 160 - 180 mg/L and 110 - 130 mg/L, respectively, a pH range of 7.9 - 8.3, a temperature of 20 ± 1°C and a specific conductivity of 400 - 600 micromhos per centimeter (μmhos/cm). Water quality parameters were measured on each batch of fortified water prior to use. The ability of several daphnid species to survive and reproduce over several generations of culture in this water source and periodic scans for PCB and pesticide contamination confirmed that the dilution water was of acceptable quality.

3.6 Test Conditions

A 200-mL Mount and Brungs (1967) proportional diluter, calibrated to provide 50 percent dilutions between adjacent nominal concentrations, delivered the dilution water and the TBBPA to the test vessels during the chronic toxicity test. The diluter was constructed

entirely of glass and silicone tubing, stoppers and sealant. The diluter system was equipped with a 20-mL gas-tight Glenco® syringe which delivered 0.0066 mL of the ^{14}C-TBBPA stock solution (119.9 mg/mL) into 387 mL of dilution water in the system's mixing chamber during each diluter cycle. In order to completely solubilize the test material in the dilution water the solution within the mixing chamber was continuously ultrasonicated and stirred using a water driven magnetic stirrer. This 387-mL solution (nominal concentration of 2.0 mg/L) served as the highest treatment level from which calibrated volumes were diluted to provide the 50% nominal concentration gradient (2.0 to 0.13 mg/L). Five-centimeter (cm) lengths of 1-millimeter (inside diameter) glass capillary tubing were inserted through silicone stoppers in the mixing/splitting chambers of the diluter and into the test solution delivery tubes. This tubing served to restrict the flow of the test solutions, minimizing potentially stressful turbulence in the test vessels and providing equal distribution of the test solutions to the replicate vessels.

Test vessels were glass battery jars having a volume capacity of 1.8 liters. Test solutions drained from each vessel through a 3.0 x 8.0 cm notch on the upper edge of the jars. The drains were covered with a Nitex® 40-mesh screen to prevent loss of the daphnids. Test vessels were loosely covered with plastic wrap throughout the duration of the exposure period. In addition to the five concentrations of TBBPA, dilution water control and solvent (acetone) control solutions were maintained during the study. The solvent control solutions contained the greatest amount of acetone present in any treatment level (0.17 µL/L). The solvent (acetone, CAS# 67-64-1) used to prepare the test material stock solution and the solvent control solutions was from the same source. All treatments and the controls were maintained in duplicate. Test solutions were delivered to the vessels at an approximate rate of 6 aquarium volumes per 24-hour period in order to provide a 90% test solution replacement rate of approximately 9 hours (Sprague, 1969). The test area was illuminated with Durotest Optima and Gro-Lux® fluorescent lights at an intensity of 30 to 100 footcandles and a photoperiod of 16 hours light and 8 hours darkness. The study was

conducted in an air-temperature controlled room designed to maintain the test solution temperatures at 20 ± 1°C.

Selection of TBBPA concentrations for the 21-day chronic exposure was based on the results of preliminary tests previously conducted at SLI. Forty *Daphnia magna* (≤ 24 hours old) were impartially selected and distributed to each test concentration (20 per replicate) to initiate the chronic test. Adult survival and measurements of offspring production were made on days 1, 2, 3, 4 and three times per week from day 7 through 21. The offspring were removed, counted and discarded. Test vessels were brushed to remove algal growth and the solution filtered through a fine mesh net a minimum of twice each week. At test termination, the body length of all surviving daphnids in each treatment level and the controls was determined. Organism lengths were determined by measuring the daphnids from the top of the helmet to the base of the spine. During the measuring process the organisms were viewed using a Bausch & Lomb dissecting microscope equipped with an ocular micrometer.

The test organisms were fed a diet consisting of a suspension of Fleischmann's yeast (5 mg/mL), a suspension of green algae (*Ankistrodesmus falcatus*; 4 x 10^7 cells/mL) and Selco® (a commercial mixture of proteins and fatty acids, 0.6 mg/mL). During the exposure, the food was introduced at a rate of 0.5 mL of yeast suspension, 3.0 mL of algal suspension and 1.0 mL of Selco® food supplement three times daily on weekdays and twice daily on weekends.

3.7 Analytical Measurements

The test solution temperature was measured daily in one replicate vessel of each treatment level and each control solution throughout the 21-day exposure. In addition, the test solution temperature was continuously monitored in one replicate vessel of the solvent control solution throughout the study using a Taylor® Max/Min thermometer. The dissolved oxygen concentration of the test solutions was measured every weekday in one replicate

vessel of each treatment level and the controls. Total hardness, alkalinity, specific conductivity and pH of the test solutions were monitored weekly in one replicate vessel from each treatment level and the control(s) solutions. The pH, dissolved oxygen concentration and temperature were also measured once a week in all replicate vessels of each treatment level and the controls. The dissolved oxygen concentration was measured using a YSI Model #57 dissolved oxygen meter. A LaMotte Model HA pH meter and a Fisher Model #956 were used for pH measurements. Daily temperature measurement in each treatment level and the controls was measured with a Brooklyn alcohol thermometer. Specific conductivity was monitored with a YSI Model #33 conductivity-salinity meter. Total hardness and alkalinity of the test solutions were determined according to APHA et. al. (1985). Light intensity was measured with a General Electric type 214 light meter.

During the in-life phase of the test, water samples were removed and analyzed for ^{14}C-TBBPA on days 0, 7, 14 and 21 from two replicate vessels of each test concentration and the controls. All samples were analyzed for ^{14}C-TBBPA using a radiometric procedure as described in Appendix II (part A). A method validation recovery study conducted at SLI prior to the initiation of the chronic test established a mean recovery (radiometric analyses) of 102 ± 5.03% of the ^{14}C-TBBPA from freshwater. In addition, the concentration of TBBPA in the highest treatment level was confirmed by high pressure liquid chromatography (HPLC) at each sampling interval. Analyses of these samples were performed using the HPLC method described in Appendix II (part B). A method validation recovery study conducted at SLI prior to the initiation of the chronic test established a mean recovery (HPLC) of 96.1 ± 6.64% of TBBPA from freshwater.

Three Quality Assurance (QA) samples, formulated in test dilution water at a concentration unknown to the analyst, were also prepared at each sampling interval. The results of the analyses of these QA samples were used to judge the precision and quality control maintained during each analytical process (radiometric, HPLC).

3.8 Statistical Analyses

3.8.1 MATC Calculation

At the termination of the study, data obtained on organism survival, reproduction and growth were statistically analyzed to establish significant treatment level effects. Analyses were performed using the mean organism response in each replicate vessel rather than individual response values. All statistical conclusions were made at the 95% level of certainty except in the case of the Chi-Square Goodness of Fit Test and the Bartlett's Test, in which the 99% level of certainty was applied. The following procedures were used:

1) Significant differences in the percentage survival were determined after transformation (e.g. arcsine square-root percentage) of the data.

2) A one-way, single classification analysis of variance (ANOVA) was conducted for each endpoint to compare the performance of the control organisms with that of the solvent control organisms. These comparisons indicated that the presence of acetone in the exposure solutions did not affect survival, reproduction or growth of the test organisms. Consequently, for all parameters, data for the dilution water control and the solvent control were pooled. Treatment effects were established by comparison with the pooled control data.

3) The Chi-Square Goodness of Fit Test (Horning and Weber, 1985) was conducted and compared the observed sample distribution with a normal distribution. The assumption that observations are normally distributed must be validated before subsequent analyses, following parametric procedures, can be performed. If the data is not normally distributed, then a non-parametric procedure is used for subsequent analyses.

4) As a check on the assumption of homogeneity of variance implicit in parametric statistics, data for each endpoint were analyzed using Bartlett's Test (Horning and Weber, 1985).

5) For each endpoint, the performance at each dose level of TBBPA was compared with the performance of the pooled control using the Williams' Test (Williams, 1971, 1972), the Dunnett's Test (Dunnett, 1955,1964), or the Kruskal-Wallis Test (Zar, 1985; Sokal and Rohlf, 1981). The Williams' Test and the Dunnetts' Test are parametric procedures. The Williams' Test is preferred for chronic toxicity tests and is more powerful than the Dunnett's Test (Rand and Petrocelli, 1985). However the Williams' Test, by design, assumes a dose response due to increasing concentration of toxicant. If this assumption is violated, then the Dunnett's Test may be more appropriate. The Kruskal-Wallis Test is a non-parametric procedure and is used if the data are not normally distributed or the group variances are not homogeneous (i.e., if the chi-square test for normality or the Bartlett's Test fail).

6) Survival data were analyzed before data for growth and reproduction; dose levels that caused significant survival effects were excluded from the analysis of growth and reproduction.

A computer program was used to perform the computations. The theoretical threshold concentration expected to produce no deleterious effects at the 95% level of certainty was estimated as the Maximum Acceptable Toxicant Concentration (MATC). The MATC is equal to the geometric mean of the limits set by the lowest test concentration that showed a statistically significant effect (Lowest Observed Effect Concentration, LOEC) and the highest test concentration that showed no statistically significant difference from the control (No Observed Effect Concentration, NOEC). Based on these data, the MATC of TBBPA to *Daphnia magna* was estimated.

3.8.2 EC50 Calculation

The concentrations tested and the corresponding biological response data (immobilization) derived from the toxicity test were also used to estimate 4-, 7-, 14- and 21-day median effect concentrations (EC50) and 95% confidence intervals. The EC50 is defined as the concentration of test material in dilution water which caused immobilization of 50% of the test organism population at the stated time interval. Since, during this study, no concentration tested caused immobilization of ≥ 50%, the EC50 value at each time interval was empirically estimated as being greater than the highest concentration of TBBPA tested.

4.0 RESULTS

4.1 Preliminary Testing

Prior to the performance of the definitive chronic study, a preliminary-range finding test was conducted at Springborn Laboratories, Inc. During this preliminary test, daphnids (≤ 24 hours old at initiation) were exposed, under flow-through conditions, to nominal concentrations of 5.0, 2.5, 1.3, 0.63 and 0.31 mg/L of TBBPA. Following 16 days of exposure, 100% immobilization of daphnids was recorded in the highest treatment level (5.0 mg/L). Immobilization of ≤ 20% was observed among daphnids exposed to the remaining nominal test concentrations (2.5 - 0.31 mg/L). During the same period, reproduction, determined by cumulative number of offspring per female, averaged 4 offspring per female in the 2.5 mg/L level which was significantly reduced as compared to the reproduction in the solvent control solutions (11 offspring/female). Reproduction in the remaining treatment levels (1.3 - 0.31 mg/L) was generally consistent with the reproduction by the solvent control organisms. Based on these data, the following nominal concentrations were selected for the definitive chronic study: 2.0, 1.0, 0.5, 0.25 and 0.13 mg/L TBBPA.

4.2 Water Quality

The results of water quality determinations made during the daphnid chronic exposure demonstrate that the dissolved oxygen concentration, pH, specific conductivity, total hardness and alkalinity of the test solutions were generally unaffected by the concen-

trations of TBBPA tested (Table 1). Continuous temperature monitoring in one replicate (A) of the solvent control demonstrated that the test solution temperature ranged from 18 to 22°C during the exposure period. Daily measurement of the temperature in each treatment level solution established that the average temperature was 20 ± 1°C. Water quality conditions established for the test remained within acceptable ranges for the survival, reproduction and growth of *Daphnia magna*.

4.3 Exposure Monitoring

Analyses of solutions during the pretest period established that the concentrations of ^{14}C-TBBPA in the exposure solutions were generally consistent and that the delivery apparatus maintained the expected concentration gradient. Throughout the exposure period, a small amount of precipitate was present in the diluter system's mixing chamber, however, no undissolved TBBPA (e.g., film of material on the surface of the test solution, precipitate) was observed in any of the test solutions. This precipitate was removed from the mixing chamber at a minimum of twice daily to prevent malfunction of the diluter system.

During the in-life phase of the study, weekly radiometric analyses of the test solutions demonstrated that based on mean measured concentrations of ^{14}C-TBBPA, an exposure gradient of approximately 50% dilutions was maintained during the 21-day study. Analyses of the five treatment levels resulted in mean measured concentrations which averaged 40% of the nominal treatment levels. Aquaria concentrations achieved were stable and generally consistent throughout the study (Table 2). Coefficients of variation averaged 31% for all mean measured concentrations. The treatment levels based on mean measured concentrations were 0.98, 0.30, 0.19, 0.10 and 0.056 mg/L TBBPA (Table 3). Figure 1 illustrates the relationship between the nominal treatment levels and the mean measured concentrations of TBBPA during the chronic toxicity test. Analyses of the QA samples on days 0, 7, 14 and 21 (radiometric analyses) resulted in measured concentrations (TBBPA) which averaged 106 ± 4.27% of the nominal fortified concentrations and were consistent

with the mean recovery established during the method validation study (Appendix II, Part I). On exposure day 0, analyses of the solvent control solutions resulted in measured concentrations which were unusually high and exceeded the minimum detection limit established for this study. It is believed that improper handling of the sample resulted in the high recovery and that this was not indicative of the exposure conditions of the solvent control for this sampling interval. At the highest nominal concentration tested , 2.0 mg/L, agreement between the radiometric and HPLC analyses was generally consistent and reproducible. Analyses of the highest treatment level (HPLC analyses) resulted in a mean measured concentration of 0.65 ± 0.19 mg/L for filtered samples and 0.94 ± 0.24 mg/L for unfiltered samples (Table 4). Based on these data, it was established that the satisfactory control of the exposure solutions was maintained throughout the 21-day test period. Analyses of the Quality Assurance (QA) samples during the HPLC analyses resulted in measured concentrations (TBBPA) which averaged 91.8 ± 11.3% (filtered samples) and 103 ± 3.6% (unfiltered samples) of the nominal concentrations added (Table 4). Based on the analyses of the QA samples (radiometric and HPLC analyses), it was established that satisfactory precision and quality control were maintained during the analysis of TBBPA in the exposure solutions.

Figure 1 illustrates the relationship between the nominal treatment levels and the mean measured concentrations of TBBPA during the chronic toxicity study.

4.4 Biological Observations

At test termination, biological performance of the two control groups (solvent control and dilution water control) was statistically comparable and indicated that the presence of solvent (acetone; 0.17 µL/L) had no adverse effect on the survival, reproduction and growth of the test organisms during the study. Based on these analyses, all statistical comparisons of the biological end points at the various treatment levels were performed using pooled data from the solvent control and dilution water control groups.

A summary of the survival data from the chronic exposure of *Daphnia magna* to TBBPA is presented in Table 5 and illustrated in Figure 2. At the termination of the 21-day study, survival of daphnids at all treatment levels of TBBPA tested (2.0 - 0.13 mg/L) ranged from 95 to 100% and was statistically similar to the survival of the pooled control organisms (98%). Since no concentrations tested caused immobilization of ≥ 50%, the EC50 value at each time interval was empirically estimated as being greater than the highest concentration of TBBPA tested (0.98 mg/L).

A summary of the cumulative number of offspring produced and organism growth (as body length) by daphnids exposed to TBBPA is presented in Tables 6 and 7, respectively. Control daphnids had begun to release offspring by test day 7. By test termination, pooled control daphnids had produced an average of 60 ± 7 offspring and had an average body length of 4.1 ± 0.2 mm. Time to first offspring released, total number of offspring released and growth of the control organisms were within normal performance expectations established by the US EPA (1985). The time for release of first brood offspring by daphnids in any of the exposure solutions was not adversely affected by the concentration of TBBPA tested. Reproduction, as determined by cumulative number of offspring per female, was the most sensitive indicator of the toxicity of TBBPA to *Daphnia magna* in the concentration range tested. Reproduction in the highest treatment level (0.98 mg/L) was 21 offspring per female which was significantly less ($P \leq 0.05$) than reproduction by the pooled control organisms (60 offspring/female). Reproduction ranged between 44 and 58 offspring per female in the remaining tested concentrations (0.30, 0.19, 0.1 and 0.056 mg/L) which was statistically comparable to that of the pooled control. During this 21-day study, no young were observed to be immobilized in any of the tested treatment levels (0.98 to 0.056 mg/L).

After 21 days of exposure, organism growth, determined as body length, was not adversely affected in the concentration range of TBBPA tested. Organism growth ranged from 4.0 to 4.4 mm at all treatment levels (0.98 - 0.056 mg/L) and was statistically similar to the growth of the pooled control organisms (4.1 mm). The concentration-effect relationship

for daphnid reproductive performance and growth following 21 days of exposure to TBBPA is presented in Figures 3 and 4, respectively.

Based on the observed effect of TBBPA on daphnid reproduction, the Maximum Acceptable Toxicant Concentration (MATC) of this test material to *Daphnia magna* was > 0.30 mg/L and < 0.98 mg/L (geometric mean MATC = 0.54 mg/L).

PROTOCOL DEVIATION

The protocol states that periodic analyses of representative samples of dilution water are conducted to ensure the absence of potential toxicants, including pesticides, PCBs and selected toxic metals, at concentrations which may be harmful to the mayflies. During this study, periodic analyses of representative samples of dilution water were conducted to ensure the absence of potential toxicants at concentrations which may be harmful to the daphnids.

It is our opinion that this deviation did not affect the results of this study.

SPRINGBORN LABORATORIES, INC.

D C Surprenant 8/15/89

Donald C. Surprenant Date
Study Director

QUALITY ASSURANCE STATEMENT

The raw data and final report for "The Chronic Toxicity of Tetrabromobisphenol A (TBBPA) to *Daphnia magna* Under Under Flow-Through Conditions" were inspected by the Springborn Laboratories, Inc., Environmental Sciences Division, Quality Assurance Unit (QAU) to assure compliance with the study protocol, laboratory standard operating procedures and the pertinent EPA Good Laboratory Practice Regulations. Dates of study inspections and dates reported to Study Director and to Management are given below.

It is the opinion of the QAU that this report accurately reflects the raw data collected during this study.

Phase Inspection	Inspection Date	Reported to Study Director	Reported to Management
10/3/88	3/20/89	7/18/89	8/15/89
	3/21/89	7/20/89	
	3/22/89	8/14/89	
	3/23/89	8/15/89	
	7/17/89		
	7/18/89		
	7/20/89		
	8/10/89		
	8/11/89		
	8/14/89		
	8/15/89		

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Date

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TABLES

Table 1. Water quality analysis of test solutions during the 21-day chronic exposure of daphnids (*Daphnia magna*) to TBBPA.

	Mean (Standard Deviation)					
Nominal Concentration (mg/L)	Dissolved[a] Oxygen (mg/L)	Temperature[b] (°C)	Total[c] Hardness (mg/L $CaCO_3$)	Total[c] Alkalinity (mg/L $CaCO_3$)	Specific[c] Conductance (μmhos/cm)	pH[d] Range
2.0	8.0(0.4)	20(0.7)	170(5)	120(2)	498(5)	8.1 - 8.2
1.0	8.3(0.4)	20(0.7)	170(8)	120(1)	498(5)	8.1 - 8.2
0.5	8.3(0.4)	20(0.7)	170(3)	120(1)	498(5)	8.1 - 8.2
0.25	8.3(0.5)	20(0.7)	170(5)	120(3)	498(5)	8.1 - 8.2
0.13	8.6(0.4)	20(0.7)	170(5)	120(1)	498(5)	8.1 - 8.2
Solvent Control	8.5(0.4)	20(0.7)	170(3)	120(2)	498(5)	8.1 - 8.2
Control	8.7(0.5)	20(0.7)	170(2)	120(1)	498(5)	8.1 - 8.2

[a]N = 28
[b]N = 34 (based on daily measurement of each treatment level and control, Brooklyn alcohol thermometer)
[c]N = 4
[d]N = 16

Table 2. Results of the analysis of test solutions for ^{14}C-TBBPA during the 21-day exposure of *Daphnia magna*.

Nominal Concentration (mg/L)	Measured Concentration (mg/L)			
	Day 0	Day 7	Day 14	Day 21
2.0	0.87	1.0	0.84	1.2
	0.65	1.0	0.83	1.3
1.0	0.29	0.41	0.19	0.028[a]
	0.29	0.40	0.21	<0.025[a]
0.5	0.14	0.20	0.18	0.26
	0.17	0.19	0.18	0.24
0.25	0.068	0.098	0.11	0.13
	0.072	0.10	0.12	0.13
0.13	0.037	0.058	0.058	0.078
	0.042	0.054	0.055	0.069
Solvent Control	0.047[b]	<0.025	<0.025	<0.025
	0.037[b]	<0.025	<0.025	<0.025
Control	<0.025	<0.025	<0.025	<0.025
	<0.025	<0.025	<0.025	<0.025
QA #1[c]	0.858(0.810)[d]	0.144(0.146)	0.269(0.243)	3.09(2.92)
QA #2	0.440(0.405)	0.576(0.567)	0.435(0.405)	0.402(0.405)
QA #3	1.72(1.62)	1.70(1.62)	1.83(1.62)	0.683(0.648)

[a] Measured value was not used in the calculation of the mean measured concentration for this treatment level due to diluter malfunction.

[b] It is believed that improper handling of the sample resulted in a high recovery and that this concentration was not indicative of the exposure conditions for this sampling interval.

[c] QA = Quality Assurance Sample.

[d] Value in parentheses represents nominal fortified concentration.

Table 3. Mean measured concentrations of ^{14}C-TBBPA established during the 21-day chronic exposure of *Daphnia magna*.

Nominal Concentration (mg/L)	Mean Measured Concentration (SD)[a] (mg/L)	Percent of Nominal	N
2.0	0.98(0.23)	49	8
1.0	0.30(0.090)	30	6
0.5	0.19(0.040)	38	8
0.25	0.10(0.024)	40	8
0.13	0.056(0.013)	43	8

[a] SD = Standard Deviation

Table 4. Measured concentrations of TBBPA during the 21-day chronic exposure of *Daphnia magna*. Results are based on High Pressure Liquid Chromatography (HPLC).

Nominal Concentration (mg/L)	Measured Concentrations (mg/L)				
	Day 0	Day 7	Day 14	Day 21	Mean
2.0[a]	0.70 0.58	0.68 0.56	0.37 0.49	0.93 0.90	0.65[f]
QA #1[ab]	0.520(0.500)	0.468(0.500)	0.312(0.500)	0.935(1.00)	NA
QA #2	1.04(1.00)	0.857(1.00)	0.669(0.750)	1.37(1.50)	NA
QA #3	2.07(2.00)	1.47(1.60)	1.74(2.00)	1.90(2.00)	NA
2.0[d]	0.92 0.63	1.0 0.98	0.74 0.70	1.3 1.2	0.94[f]
QA#1[bd]	0.502(0.500)[c]	0.531(0.500)	0.494(0.500)	1.09(1.00)	NA
QA #2	0.982(1.00)	1.03(1.00)	0.761(0.750)	1.58(1.50)	NA
QA #3	2.07(2.00)	1.74(1.60)	2.01(2.00)	2.07(2.00)	NA
Stock[de]	113	119	110	109	113

[a] Samples passed through a 0.45 μm teflon filter prior to analysis.
[b] QA = Quality Assurance Sample.
[c] Value in parentheses represents nominal fortified concentration.
[d] Samples were not filtered prior to analysis.
[e] Stock concentration: 120 mg/mL.
[f] N = 8

Table 5. Weekly mean percentage survival of daphnids (*Daphnia magna*) during the 21-day chronic exposure to TBBPA.

Mean Measured Concentration (mg/L)	Mean Percentage Survival		
	Day 7	Day 14	Day 21
0.98	95	95	95
0.30	100	100	100
0.19	100	100	100
0.10	100	100	100
0.056	100	98	98
Solvent Control	100	100	98
Control	100	100	98
Pooled Control	100	100	98

Table 6. Cumulative number of offspring produced per female *Daphnia magna* during the 21-day chronic exposure to TBBPA.

Mean Measured Concentration (mg/L)	Mean Cumulative Number of Offspring/Female							
	Day: 7	8	11	14	15	18	20	21
0.98	0	2	11	13	15	21	21	21[a]
0.30	0	8	22	23	24	36	38	56
0.19	0	5	19	20	21	30	35	44
0.10	0	7	24	26	29	41	47	58
0.056	0	6	23	30	31	37	42	55
Solvent Control	0	5	18	25	26	41	45	58
Control	0	4	19	26	33	45	53	62
Pooled Control	0	4	18	25	29	43	49	60

[a] Significantly ($P \leq 0.05$) different as compared to the pooled control data.

Table 7. Mean total body length of *Daphnia magna* after 21 days of exposure to TBBPA.

Mean Measured Concentration (mg/L)	Mean Organism Body Length (SD)[a] (mm)
0.98	4.0(0.3)
0.30	4.4(0.2)
0.19	4.2(0.2)
0.10	4.3(0.2)
0.056	4.4(0.1)
Solvent Control	4.2(0.2)
Control	4.0(0.2)
Pooled Control	4.1(0.2)

[a] SD = Standard Deviation

FIGURES

Figure 1. Graphical illustration of the relationship between mean measured concentrations and the nominal test concentrations and the nominal test concentrations during the 21-day chronic exposure of *Daphnia magna* to TBBPA.

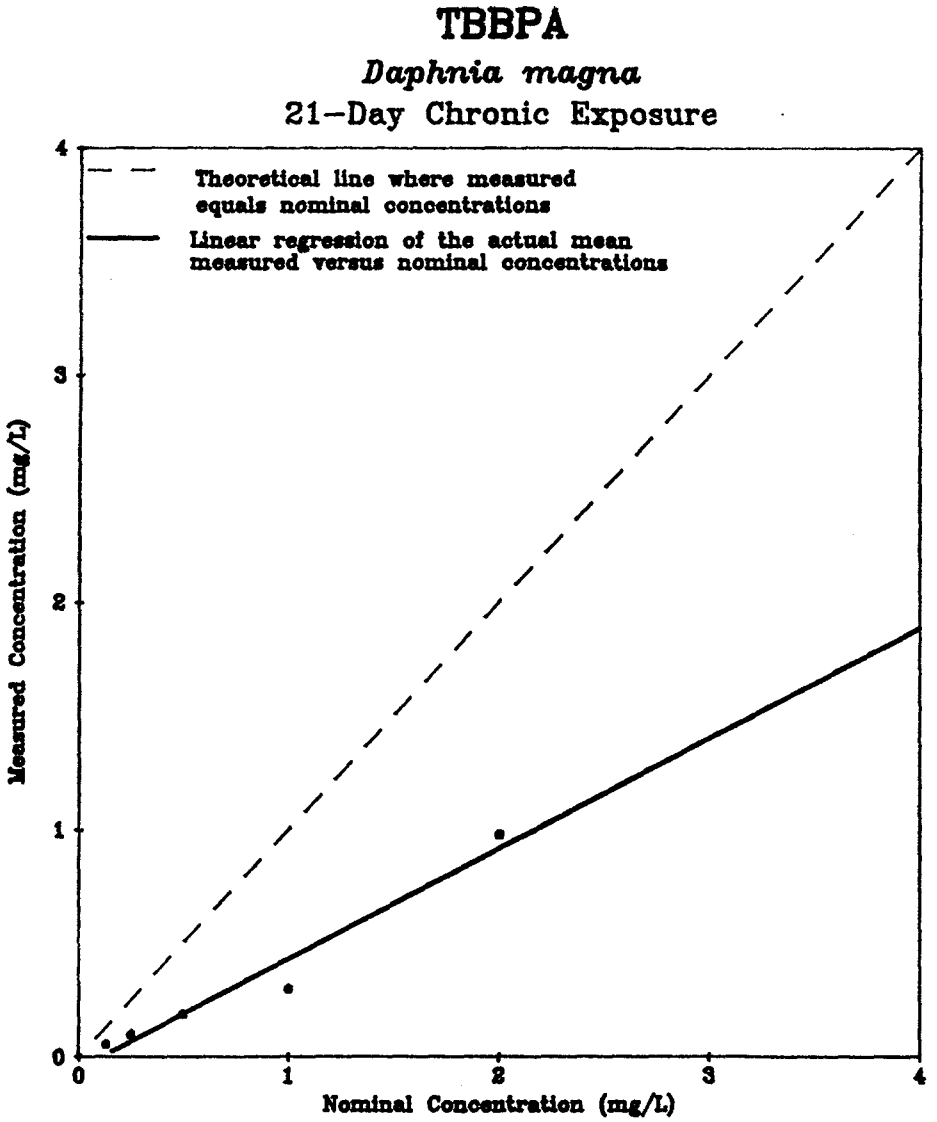


Figure 2. Mean percent survival of *Daphnia magna* exposed to mean measured concentrations of TBBPA during a 21-day chronic toxicity study.

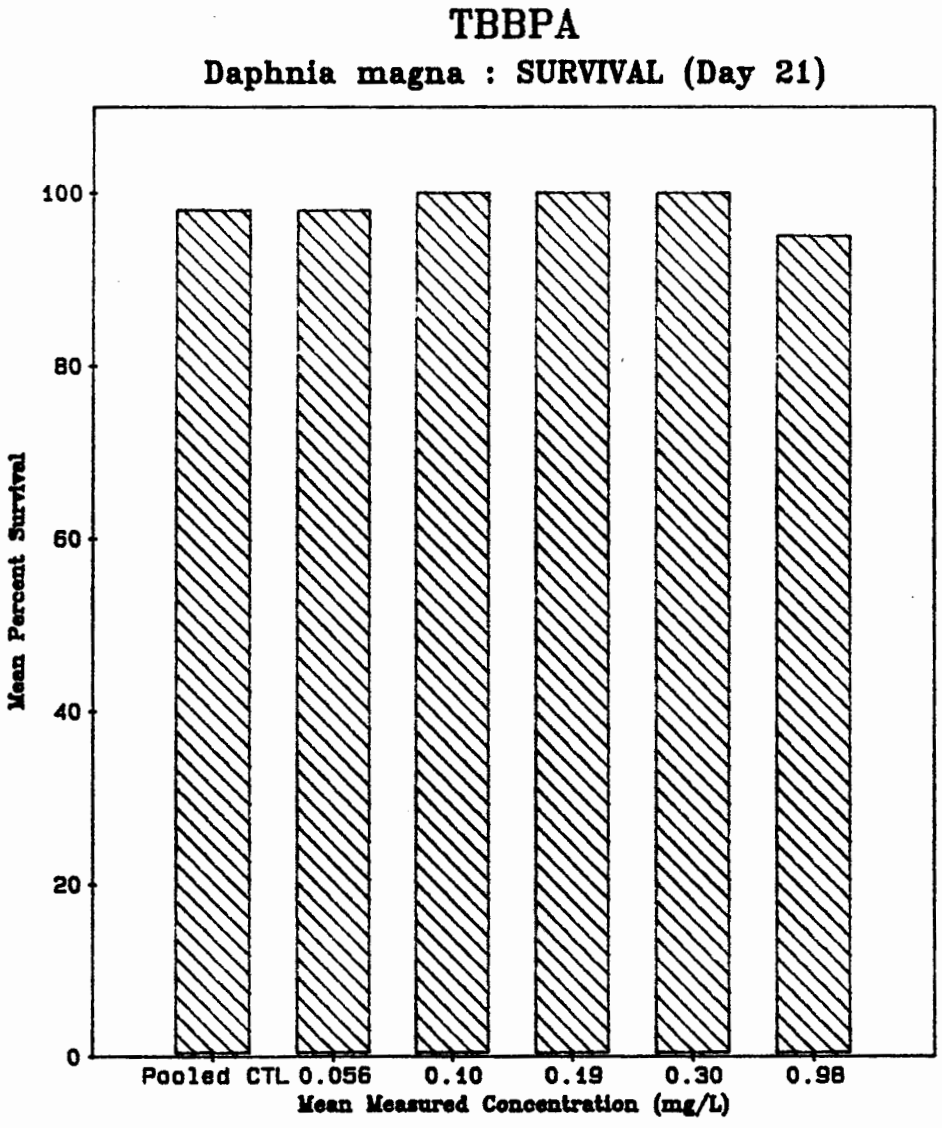


Figure 3. **Cumulative number of offspring per female of *Daphnia magna* exposed to mean measured concentrations of TBBPA during a 21-day chronic toxicity study.**

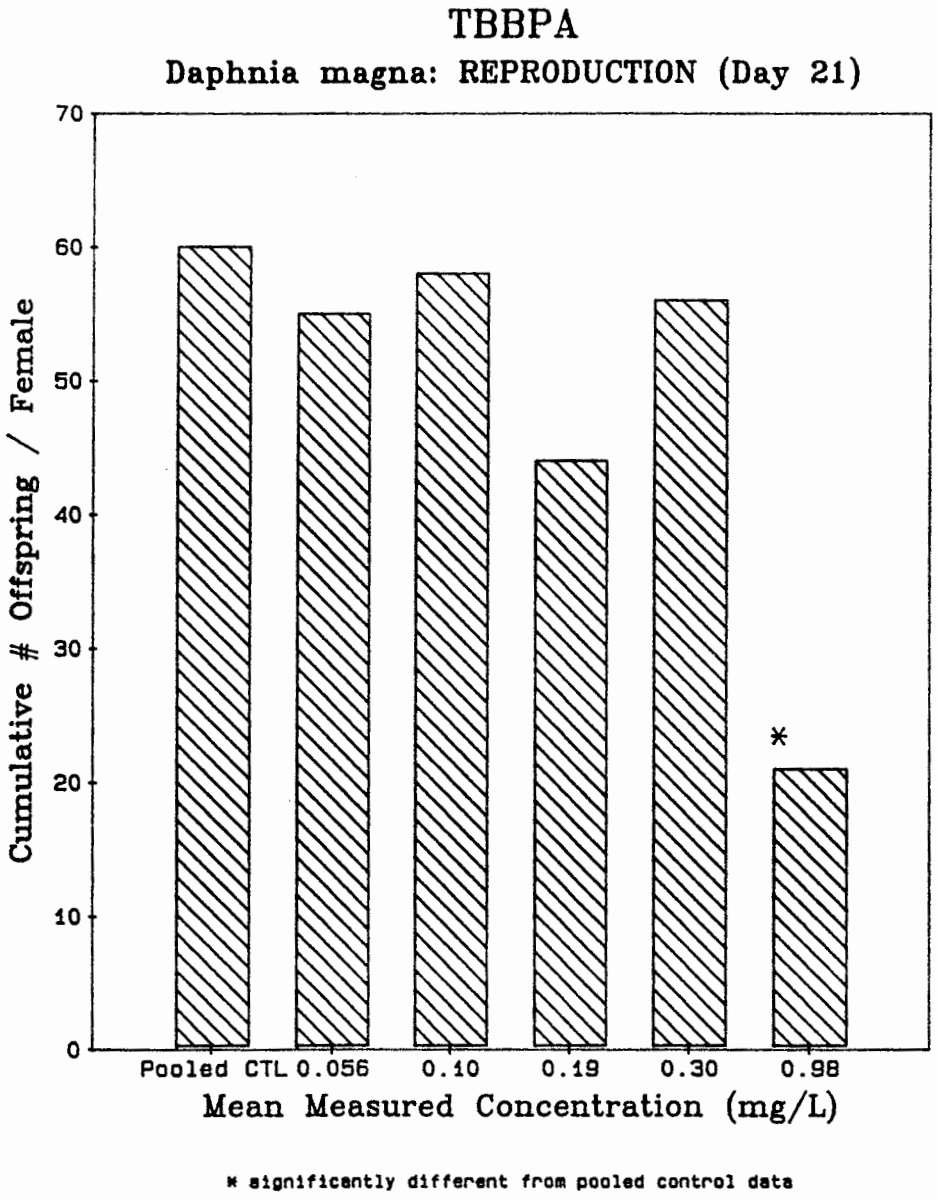
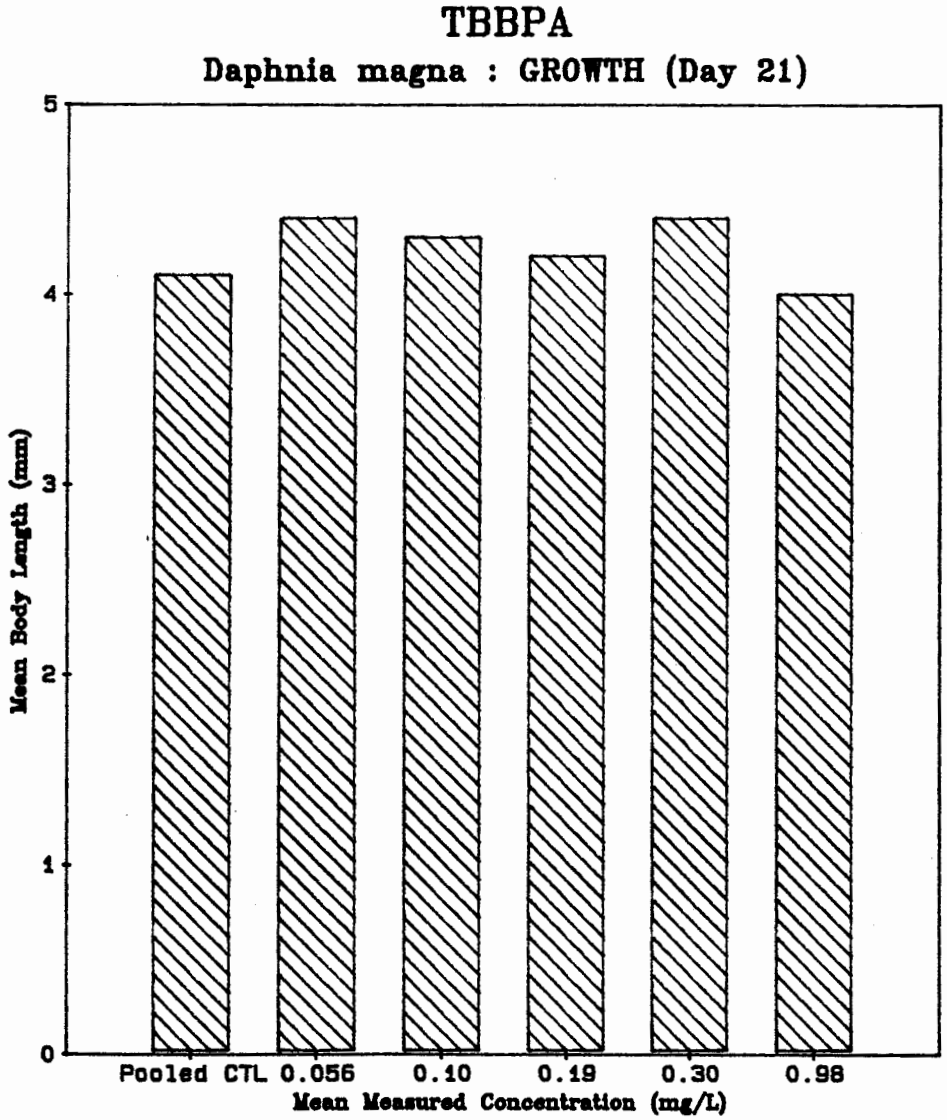


Figure 4. **Mean growth of *Daphnia magna* exposed to mean measured concentrations of TBBPA during a 21-day chronic toxicity study.**



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APPENDIX I - TEST MATERIAL

Description of the test material (Tetrabromobisphenol A) and procedures used to prepare the super stock solutions of the test article.

Description of the five sample lots of TBBPA (nonlabeled) received at Springborn Laboratories, Inc., from the Bromine Flame Retardant Industry Panel.

Manufacturer	Lot Number	Date Received
Bromine Compounds Ltd.	7137	12/22/87
Great Lakes Chemical Corp.	114-21H-16B	1/19/88
Ethyl Corporation	25739-36	2/5/88
Bromine Compounds Ltd.	7137	3/17/88
Ethyl Corporation	25813-96	4/29/88

The following table lists the quantity of each sample used to formulate the TBBPA (nonlabeled) super stock solution used during the 21-day exposure of daphnids (*Daphnia magna*).

Manufacturer	Lot Number	Quantity Used
Bromine Compounds Ltd.	7137	20.0257 grams
Great Lakes Chemical Corp.	114-21H-16B	40.0146 grams
Ethyl Corporation	25739-36	20.0052 grams
Bromine Compounds Ltd.	7137	20.0070 grams
Ethyl Corporation	25813-96	20.0135 grams

The above quantity of each sample was mixed in a 1000-mL glass volumetric flask, then diluted to volume with acetone to produce a stock solution of 119 mg/mL TBBPA. Stock solution concentration is based on 100% active ingredient.

The following is a description of the procedure used to prepare the super stock solution of ^{14}C-labeled TBBPA for the 21-day exposure of daphnids (*Daphnia magna*) to TBBPA.

Manufacturer	Specific Activity	Date Received
Chemsyn Science Laboratories Lot #CSL-88-164-72-31 (tested as 100% A.I.)	15.5 mCi/mmole	8/18/88

The entire volume (429 mg) of ^{14}C-labeled TBBPA was diluted with acetone to volume in a 100-mL glass volumetric flask to produce a stock solution of 4.29 mg ^{14}C-TBBPA/mL

The following is a description of the procedure used to prepare the diluter stock solutions for the 21-day exposure of daphnids (*Daphnia magna*).

Nominal[a] Concentration	Date Prepared	mL of Super Stock[b] (Labeled)	mL of Super Stock[c] (nonlabeled)
119.9 mg/mL	1/3/89	10[d]	50

[a] Nominal concentration represents the total amount of labeled and nonlabeled material.
[b] Super stock concentration (labeled) = 4.29 mg/mL.
[c] Super stock concentration (nonlabeled) = 119 mg/mL.
[d] Labeled super stock was blown to dryness with nitrogen before it was diluted to volume in a 50 mL volumetric flask with the nonlabled super stock.

APPENDIX II - ANALYTICAL METHODOLOGY

Part I - RADIOASSAY METHODOLOGY

1.0 OBJECTIVE:

This study was designed to validate a procedure for quantitative analysis of ^{14}C-Tetrabromo[ring-u-^{14}C]bisphenol A (TBBPA) in freshwater.

2.0 SUMMARY:

Water samples were filtered prior to analysis by liquid scintillation counting subsequent to the addition of scintillation cocktail.

3.0 EQUIPMENT AND REAGENTS:

3.1 Equipment

1. Scintillation vials: 22 mL, Wheaton.
2. Syringes: Hamilton, assorted volumes and micro mate

   10 mL.
3. Liquid scintillation counter: Beckman LS 1801.
4. Pipets: volumetric, class A, 5 mL.
5. Wheaton vials: 100 mL with crimp caps and Teflon^{R} septa.
6. Volumetric flasks: assorted sizes
7. Acrodisc filters: Gelman Acrodisc-CR, 0.45 micron, Teflon

3.2 Reagents

1. Monophase S^R - Packard Instrument Company.
2. Radiolabeled (^{14}C) Tetrabromo[ring[-u-^{1}4C]bisphenol A, 85 mg, S.A. = 12.9 mCi/mmole Lot # CSL-88-164-21-10 supplied by Chemsyn Science Laboratories, Lenexa, KS; as 100% active ingredient.

4.0 PROCEDURE

4.1 Superstock Preparation

A 1.624 mg/mL superstock was prepared by quantitative transfer of the radiolabeled material (81.2 mg) into a total volume of 50.0 mL acetone.

4.2 Determination of Specific activity

Specific activity was determined by spiking 10 µL of the 1.624 mg/mL stock into a scintillation vial containing 15 mL of Monophase S^R (prepared in triplicate). The ^{14}C-activity of each sample was measured by placing the vial in a Beckman LS 1801 scintillation counter and recording disintegrations per minute (dpm). The dpm for each sample were divided by the total µg spiked to yield dpm/µg. This measured specific activity, used for calculation of all test and quality assurance samples, was a mean of the above triplicate analyses. The mean measured specific activity was determined to be 52742 dpms/µg (100% of theoretical).

4.3 Sample Preparation and Analysis

Samples (approximately 7.0 mL) were taken with a 10-mL syringe. An Acrodisc filter was then placed on the end of the syringe and filtered into a beaker. Five mL was then volumetrically transferred into a scintillation vial.

Counting efficiencies of all experimental samples were determined using an external standard and factory prepared calibration curve. All test samples were counted for a maximum of 100 minutes or until a 2 sigma error of 5% was attained. Using this criterion and the calculations described in Standard Methods for the Examination of Water and Wastewater (APHA et al.), it was determined at the 95% confidence level that a minimum net counts per minute (cpm) for all samples of 53 cpm (background 43.48 cpm) had an associated error of less than 10%. This percentage was the maximum acceptable error and was associated with the minimum net cpm of that sample; the counting error decreased as the sample activity increased.

The minimum detectable ^{14}C-residue concentration was dependent on counting efficiency, volume of each sample and the minimum net cpm acceptable. Three quality assurance (QA) samples were prepared and analyzed at each sampling interval. Analysis of QA samples was used to determine the accuracy of the analytical procedure used.

The calculation used in determining the concentration of ^{14}C-residues in the test sample was:

$$\frac{\text{Net dpm}}{(\text{Specific Activity of } ^{14}\text{C-TBBPA}) \; (\text{Sample size}) \; (\%\ ^{14}\text{C-activity of the stock}) \; \text{mL}}$$

where:

Net dpm = disintegrations calculated by instrument after background calculation

Specific Activity of ^{14}C-TBBPA = 52742 dpm/μg

Sample size = Initial volume of sample (mL)

% ^{14}C-activity of the stock = 100%

5.0 RESULTS

Analytical results for the recovery of ^{14}C-TBBPA from freshwater are presented in Table 1A.

Table 1A. Analytical results for the recovery of ^{14}C-TBBPA from freshwater.

Nominal Fortified Concentration (mg/L)		Sample Volume (mL)	Concentration Recovered (mg/L)	% Recovered
10.6	A	10.0	11.6	109
	B	10.0	11.5	108
	C	10.0	10.6	100
5.2	A	10.0	5.47	105
	B	10.0	5.41	104
	C	10.0	4.88	93.8
0.309	A	10.0	0.331	107
	B	10.0	0.325	105
	C	10.0	0.295	95.5
0.162	A	10.0	0.156	96.3
	B	10.0	0.165	102
	C	10.0	0.164	101
Control	A	10.0	<0.000219	NA
	B	10.0	<0.000219	NA
	C	10.0	<0.000219	NA

Average recovery = 102% ± 5.03.

Part II - HIGH PRESSURE LIQUID CHROMATOGRAPHY METHODOLOGY

1.0 OBJECTIVE:

This study was designed to validate a procedure for quantitative analysis of Tetrabromobisphenol A (TBBPA) in freshwater.

2.0 SUMMARY:

All samples were analyzed by HPLC by direct aqueous injection, following 0.45 micron (Teflon) filtration.

3.0 EQUIPMENT AND REAGENTS

3.1 Equipment

1. Balance:S/P 182, four-place analytical balance
2. Beakers: Pyrex, assorted sizes
3. Flasks: Volumetric, assorted sizes
4. Instrument: Waters Model 510 liquid chromatograph solvent pump equipped with Waters Intelligent Sample Processor Model 710B, Kratos Model 757 variable wavelength detector and Hewlett-Packard Model 3388 A Integrator
5. Pipets: Volumetric (Class A), assorted sizes

6. Serum bottles: Wheaton, assorted sizes, with teflon-lined lids and metal crimp tops
7. Syringes: Hamilton, assorted sizes
8. Filters: Gelman Acrodisc-CR 0.45 micron

3.2 Reagents

1. Acetonitrile, Burdick and Jackson, HPLC grade, UV cutoff @188 nm
2. TBBPA, Lot No. 7173, Bromine Compounds, Ltd.; as 100% active ingredient
3. Water, Burdick and Jackson, HPLC grade

4.0 PROCEDURE

4.1 Preparation of Stock

Approximately (ca.) 0.1 gram (g) of TBBPA was weighed on a balance in a 100-milliliter (mL) volumetric flask and solubilized in acetonitrile. The TBBPA stock solution (ca. 1 µg A.I./µL) was stored refrigerated (4 - 10°C) in a 100-mL amber serum vial with a teflon-lined lid. This stock was then used, with appropriate dilution, for quantitation and fortification.

4.2 Sample Fortification

Method validation/recovery samples were prepared using freshwater. The aqueous samples were fortified with dilutions of the TBBPA stock solution. The fortification levels

produced were 0.500, 1.00, and 3.00 μg A.I./mL (three replicates at each level). An additional three freshwater samples (100 mL) were left unfortified to be utilized as blank control samples.

4.3 High Pressure Liquid Chromatography

High Pressure Liquid Chromatographic (HPLC) analysis was conducted utilizing the following instrumental conditions:

Instrument: Waters Model 510 liquid chromatograph solvent pump equipped with Waters Intelligent Sample Processor Model 710B, Kratos Model 757 Variable Wave length Detector and Hewlett-Packard Model 3388A Integrator

Column: Phenomenex Ultremex C_{18} (5μm) 250 mm (length) x 4.6 mm ID

Mobile Phase: 80% Acetonitrile: 20% HPLC grade water

Flow: 1.50 mL/minute

Pressure: 1000 p.s.i.

Chart Speed: 0.3 cm/minute

Wavelength: 230 nm

Injection Volume:25 μL

Instrument Sensitivity: 0.10 AUFS

Peak Width: 0.1 seconds

Attenuation: 2^1

Threshold: 1

4.4 Analysis

The TBBPA stock was diluted with 50/50 acetonitrile-HPLC grade water to prepare appropriate HPLC calibration standards of 200, 350, 500, 1000, and 1500 μg A.I./L for the method validation. Analyses of the samples and standards were performed by programmed injection. A standard curve was constructed by plotting the peak height observed versus the concentration (μg A.I./L) of the standard injected.

Linear regression analysis was used to determine the concentration of TBBPA found in the sample.

4.5 Calculations

The following equation was utilized in calculating analytical results:

Analytical Result (μg A.I./L) = A x D.F.

where:

Analytical Result = concentration of TBBPA

A = concentration (μg A.I./L) of sample from the regression analysis

D.F. = dilution factor, ratio of final volume (mL) of the sample to volume (mL) of sample used

5.0 RESULTS AND DISCUSSION:

Analytical results for the recovery of TBBPA from freshwater are presented in Table 2A.

A representative chromatogram showing the recovery of TBBPA from freshwater is presented in Figure 1A.

The linear regression analysis for the standards used in the recovery study is shown in Figure 2A.

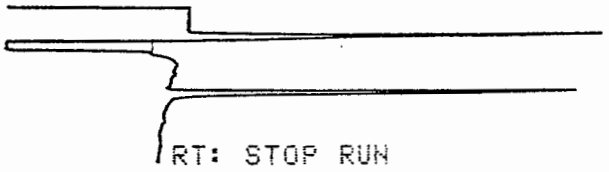
Table 2A. Analytical results for the recovery of TBBPA from freshwater.

Sample ID (mg/mL)		Volume (mL)	Concentration Recovered (mg/mL)	% Recovered
3.00	A	100	2.67	89.0
	B	100	2.70	90.0
	C	100	2.54	84.7
1.00	A	100	0.989	98.9
	B	100	0.994	99.4
	C	100	0.971	97.1
0.500	A	100	0.518	104
	B	100	0.515	103
	C	100	0.492	98.4
Control	A	100	<2.22	N/A
	B	100	<2.22	N/A
	C	100	<2.22	N/A

Average recovery: 96.1% (±6.64).

Theoretical minimum detectable concentration is <2.22 mg/mL.

Figure 1A. Chromatogram showing TBBPA recovery from freshwater.



[hp] 3388A MANUAL INJECTION @ 14:11 JAN 12, 1989
AREA %

RT	AREA	TYPE	WIDTH	HEIGHT	BASELINE	AREA %
0.00			BASELINE @ START RUN = 0.12			
0.00			THRESHOLD @ START RUN = 4			
0.00			PEAK WIDTH @ START RUN = 0.15			
0.01			RT: INTG → OFF			
3.50			RT: INTG → ON			
3.81	358.39	BB	0.129	43.64	-1.46	100.000

TOTAL AREA = 358.39
MULTIPLIER = 1

2.0 Bug/ml
1-89-317

Figure 2A. **The Linear Regression Analysis for TBBPA standards used in the recovery study.**