THE TOXICITY OF TETRABROMOBISPHENOL A (TBBPA) TO FATHEAD MINNOW (*Pimephales promelas*) EMBRYOS AND LARVAE

Submitted to:

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

SLI Report #89-2-2937

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FINAL

GOOD LABORATORY PRACTICES COMPLIANCE STATEMENT

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

DC Surprenant 8/17/89

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1.0 SUMMARY

Fathead minnow (*Pimephales promelas*) embryos and larvae were continuously exposed for 35 days (30 days post-hatch) to mean measured Tetrabromobisphenol A (TBBPA) concentrations ranging from 0.31 to 0.024 mg A.I./L, a dilution water control and a solvent (18 µL/L acetone) control. Observations were made on survival of organisms at hatch and survival and growth (wet weight and total length) of larvae after 30 days post-hatch exposure.

Fathead minnow survival at the completion of the hatching period (day 5) in the highest mean measured test concentration of TBBPA (0.31 mg A.I./L) was 28% and was significantly less ($P \leq 0.05$) than the survival of the control organisms (pooled control and solvent control data, 84%). The survival of embryos exposed to mean measured concentrations ≤ 0.16 mg A.I./L ranged from 74 to 90% and was unaffected as compared to the survival of the control organisms.

All larvae, exposed to the highest test concentration (0.31 mg A.I./L) died within the initial 7 days of the post-hatch exposure period. The survival of larvae exposed to the remaining concentrations of TBBPA (0.16 - 0.024 mg A.I./L) ranged from 87 to 93% and was comparable to the survival of the control (pooled data; 93%) larvae.

Statistical comparison of the growth data (total length and wet weight) determined at test termination (30 days post hatch) established that surviving fish at all treatment levels grew at rates comparable to the control larvae. The mean total length and wet weight of larvae exposed to mean measured TBBPA concentrations ≤ 0.16 mg A.I./L ranged from 24 to 25 mm, and 112 to 126 mg, respectively, and was statistically comparable to the growth of control larvae (pooled data, 25 mm and 111 mg, respectively).

Based on significant adverse ($P \leq 0.05$) effects on embryo survival and larvae survival, the Maximum Acceptable Toxicant Concentration (MATC) of TBBPA for fathead minnows was estimated to be > 0.16 A.I./L and < 0.31 A.I./L (geometric mean MATC = 0.22 mg A.I./L).

2.0 INTRODUCTION

2.1 Objective

The objective of this study was to determine the effects of Tetrabromobisphenol A (TBBPA) on fathead minnow (*Pimephales promelas*) embryos and larvae during continuous aqueous exposure. Test concentrations were selected based on the results of preliminary range-finding exposures of juvenile fathead minnows to TBBPA which were performed at Springborn Laboratories Inc. (SLI). The definitive embryo/larval exposure was initiated within 18 hours after egg fertilization and continued through 35 days (30 days post-hatch). The effects on embryo survival at hatch and on survival and growth (wet weight and total length) of larvae at test termination were measured and used to estimate the Maximum Acceptable Toxicant Concentration (MATC). The MATC is defined as the range encompassing the highest test concentration that had no significant effect ($P \leq 0.05$) and the lowest concentration that had a significant effect on the test organisms. The MATC is usually expressed as the geometric mean of these tested no effect and effect concentrations and is estimated from the most sensitive of the performance criteria used, e.g., organism survival at hatch, larval survival, larval growth at test termination.

2.2 Rationale

Macek and Sleight (1977) and McKim (1977) described fish embryo/larvae investigations as providing reasonably accurate short-term predictions of potential long-term chemical toxicity to fish. In the majority of the chronic toxicity studies reported by the authors, and of those performed at this laboratory, the embryos and larvae were generally the most sensitive life stages to chemical exposure. Rarely was reproduction or survival and growth of second generation larvae reduced at exposure levels lower than those that reduced

survival or growth of the first generation larvae. The authors demonstrated that for the majority of chemicals, the shorter and more economical embryo/larvae tests yielded estimates of safe concentrations very similar to those derived from full life-cycle chronic toxicity studies.

3.0 MATERIALS AND METHODS

3.1 Protocol

The embryo-larval test was conducted according to the protocol entitled "Protocol For Conducting Early Life Stage Toxicity Tests with Fathead Minnow (*Pimephales promelas*)" (Protocol #020188/FM.ELS-BFRIP), a protocol amendment #1, dated 2 December 1988 prepared by Springborn Laboratories, Inc., and EPA's Tetrabromobisphenol - A Final Test Rule (Federal Register, Volume 52, No. 128, 6 July 1987). This test was conducted from 21 November to 26 December 1988, at Springborn Laboratories, Inc., Wareham, Massachusetts. The raw data and final report produced during this study 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 22.3 mg/mL was prepared by diluting 5.2 milliliters (mL) of a ^{14}C-TBBPA superstock (4.29 mg/mL which was blown to dryness with nitrogen) with 9.2 mL of the nonlabeled TBBPA superstock (119 mg/mL) to a volume of 50 mL (see Appendix I for labeled and nonlabeled superstock preparation).

3.4 Test System

A modified intermittent flow proportional diluter, similar to that described by Mount and Brungs (1967) with a 50% dilution factor, was used to prepare and deliver the selected test concentration range of TBBPA to the exposure aquaria during the 35-day study. The dilution and control water was well water which was pumped into an epoxy-coated concrete reservoir where it was supplemented with Town of Wareham untreated well water and aerated. Weekly characterization of the well water established the total hardness and alkalinity ranges as $CaCO_3$ were 26 - 32 mg/L and 21 - 29 mg/L, respectively; the pH range was 7.0 - 7.4 and the specific conductivity range was 90 - 130 μmhos/cm during the study period.

A 50-mL gas tight syringe with a stainless steel needle, extended with polyethylene tubing, was mechanically activated during each diluter cycle to inject 0.0348 of the 22.3 mg/mL (TBBPA) stock solution into the diluter's chemical mixing chamber. The mixing chamber was positioned over a magnetic stirrer which continuously mixed the contents. The solution contained in the mixing chamber constituted the highest test concentration (0.40 mg A.I./L TBBPA, nominal) and was subsequently diluted (50%) to provide the range of nominal exposure concentrations. The diluter was calibrated to deliver five concentrations of TBBPA ranging from 0.40 to 0.025 mg A.I./L, a dilution water control, and a solvent (18 μL/L of acetone) control to duplicate test aquaria. The solvent control solutions contained a concentration of acetone which equaled the solvent level in the highest TBBPA treatment level. A mechanical injector similar to that used to deliver the TBBPA stock solution was used to deliver acetone to the solvent control aquaria. Each glass test aquarium measured 39 x 20 x 25 cm with a 19.5-cm high side drain that maintained a constant exposure solution volume of 15 L. The diluter continually delivered the control and test solutions to the exposure aquaria at a rate sufficient to provide approximately 6.3 aquarium volumes per 24-hour period, with a 90% replacement time of approximately 9 hours (Sprague, 1969). Illumination was provided by Durotest Vitalite^R fluorescent lights centrally located above the test aquaria. Sixteen hours of light at 20 - 100 footcandles at the water

surface were provided each day. The aquaria were impartially positioned in a water bath containing circulating water designed to maintain the test solution temperatures at 25 ± 1°C.

3.5 Embryo-Larval Exposure

The exposure of fathead minnow embryos and larvae to TBBPA was initiated with fertilized embryos obtained from the fathead minnow culture unit maintained at SLI. Sixty embryos were impartially selected and distributed to each of 14 embryo incubation cups, one of which was then suspended in each duplicate test aquarium per exposure concentration and the control(s). Embryo incubation cups were glass jars (5 cm O.D., 8 cm high) with 40-mesh Nitex® screen bottoms. A rocker arm apparatus, as described by Mount (1968), was used to gently oscillate the incubation cups in the test solutions. Dead embryos were counted daily until hatching was complete. Hatching was deemed complete (exposure day 5) when no more than 10% unhatched viable embryos remained in any egg incubation cup. Calculations of percentage survival of organisms at hatch were based on the number of live larvae and embryos per incubation cup after hatching was completed compared to the number of embryos per cup on test day 0. Any embryos which were heavily infected with fungus were discarded and the number discarded was subtracted from the initial number of embryos used as a basis for the calculation of percent survival.

To initiate the 30-day post-hatch larval exposure, 30 live larvae were impartially selected from the surviving larvae in each incubation cup on test day 5 and placed into their respective exposure aquaria.

Larvae were fed live brine shrimp (*Artemia salina*) nauplii three times daily on weekdays and twice daily on weekends and holidays. Aquaria were brushed and siphoned when necessary (generally several times per week) to remove excess food and fecal matter. Behavior and appearance of larvae were observed and recorded daily, and larval survival was estimated twice weekly. At 30 days post-hatch exposure (test termination), the percent-

age larval survival was determined. The surviving larvae were anesthetized with MS-222 (tricaine methanesulfonate) and measured for mean total length, and mean wet weight. The larvae were measured and weighed individually to calculate the mean and standard deviation of total length and wet weight.

3.6 Water Quality Measurements

Dissolved oxygen concentration, pH and temperature were measured in every aquarium daily. The temperature was continuously monitored in one replicate of the dilution water control. Dissolved oxygen concentration was measured using a YSI Model #57 dissolved oxygen meter with a combination (temperature/dissolved oxygen) electrode polarographic probe. A LaMotte Model HA pH meter was used for pH measurements. Temperature (daily measurement) was measured with a Brooklyn alcohol thermometer. Continuous monitoring of the control solution temperature was performed using a Taylor^{R} Min-Max thermometer. Total hardness and total alkalinity as $CaCO_3$ (APHA et al., 1985) and specific conductance were measured on day 0 and weekly thereafter in alternating replicates of all the highest and lowest treatment levels, the dilution water control, and the solvent control. Specific conductance was measured using a YSI Model #33 conductivity meter.

3.7 Analytical Measurements

The control and the high, middle, and low test concentrations were sampled twice and analyzed for TBBPA concentrations prior to the start of the definitive exposure. Results of these pretest analyses were used to judge whether sufficient quantities of test material were being delivered and maintained in the exposure aquaria to initiate the study. During the in-life phase of the definitive study, water samples were removed from the test solutions on test days 0, 4, 11, 16, 18, 25, 29, 32 and 35 for analysis of TBBPA. Each exposure solution sample was collected from the approximate midpoint of the aquarium with a volumetric pipet. The analytical procedures used to analyze the exposure solution samples, are presented in the methodology described in Appendix II (Part I). Prior to the initiation of the definitive test, a method validation recovery study was conducted at SLI and established an

average recovery of TBBPA from freshwater equal to 102 ± 5.03%. In addition, the concentration of TBBPA in the highest treatment level was confirmed by high pressure liquid chromatography (HPLC) on days 11, 14, 15, 18, 25, 29, 32 and 35. Analyses of these samples were performed using the HPLC method described in Appendix II (Part II). 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

At the termination of the study, data obtained on embryo survival at hatch, larval survival and larval growth (wet weight and total length) at test termination were statistically analyzed. Analyses were performed using the mean organism response in each replicate aquarium 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 hatchability, survival 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 than 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 for homogenity fail). For this study, all data sets met the assumptions for normality and therefore the Williams' Test was used to establish treatment level effects.

6) Larval survival data were analyzed before larval length and weight; dose levels that caused significant survival effects were excluded from the analysis of larval growth.

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 fathead minnow juvenile stages was estimated.

4.0 RESULTS

4.1 Selection of Test Concentrations

A flow-through acute (144 hours) toxicity test, conducted at SLI (SLI Report #88-10-2834), exposed juvenile fathead minnow to nominal concentrations of TBBPA ranging from 1.0 mg A.I./L to 0.18 mg A.I./L (65% dilution factor). The diluter system which prepared and delivered the test material to the exposure aquaria functioned properly throughout the preliminary test period. Throughout the exposure period, a small amount of precipitate was present in the diluter system's mixing chamber; however no undissolved material (e.g., precipitate, film on the solution surface) was observed in any of the exposure vessels. Results of the analyses of the exposure solutions, at 0, 96 and 144 hours, defined the test concentrations as: 0.63, 0.45, 0.32, 0.26 and 0.19 mg A.I./L TBBPA. Following 144 hours of exposure, 100% mortality was observed at the highest test concentration (0.63 mg A.I./L), 30% mortality was observed at a concentration of 0.45 mg A.I./L, while no mortalities were observed among organisms exposed to nominal TBBPA concentrations $\leq$ 0.32 mg/L. During the same period, fathead minnows at the 0.32 mg A.I./L treatment level exhibited sublethal effects (e.g. partial loss of equilibrium, darkened pigmentation). Organisms exposed to TBBPA concentrations $\leq$ 0.26 mg A.I./L were generally unaffected during the acute exposure. Based on the data generated during this acute test, the 144-hour LC50 was calculated to be 0.49 mg A.I./L TBBPA.

Subsequently, a preliminary range-finding test was conducted exposing juvenile fathead minnows to nominal concentrations of 0.40, 0.20, 0.10, 0.050 and 0.025 mg A.I./L TBBPA. After 10 days of exposure, fathead minnows in the 0.40 mg A.I./L TBBPA treatment level exhibited lethargic behavior. Organisms exposed to ≤ 0.20 mg A.I./L TBBPA were unaffected during this 10-day exposure. Based on these data, the following nominal concentrations were selected for the definitive early life stage exposure: 0.40, 0.20, 0.10, 0.050 and 0.025 mg A.I./L of TBBPA.

4.2 Water Quality Determinations

A summary of the water quality parameters measured during the 35-day exposure of fathead minnow embryos and larvae is presented in Table 1. At the concentrations of TBBPA tested, mean dissolved oxygen, pH, alkalinity and total hardness varied minimally and were not affected by the established concentration gradient of test material. A mean (standard deviation) temperature of 24°C (±0°C) was maintained in the exposure solutions throughout the exposure period. The results of the water quality measurements made during this study established that conditions maintained throughout the 35-day exposure were satisfactory for the promotion of fathead minnow embryo hatchability, larval survival and growth.

4.3 Exposure Monitoring

During the in-life phase of the study, weekly analyses (radiometric) of the test solutions demonstrated that based on mean measured concentrations of TBBPA an exposure concentration gradient of approximately 50% was generally maintained during the 35-day study. Analyses of the five treatment levels resulted in mean measured concentrations which averaged 84% of the nominal concentrations. Aquaria concentrations achieved were stable and generally consistent throughout the study (Table 2). Coefficients of variation averaged 30% for all mean measured concentrations. The test concentrations, based on mean measured concentrations, were 0.31, 0.16, 0.084, 0.040 and 0.024 mg A.I./L (Table 3).

Figure 1 illustrates the relationship between the nominal treatment levels and the mean measured concentrations of TBBPA during the 35-day test. Throughout the exposure period, a small amount of precipitate was present in the diluter system's mixing chamber; however, no visible sign of insoluble material (e.g., film of material on the surface of the test solution, precipitate) was observed in any of the test solutions. In the concentration range of TBBPA tested, 0.40 to 0.025 mg A.I./L nominal, agreement between the measured and nominal concentration was generally consistent and reproducible, indicating satisfactory control of the exposure conditions throughout the test period. Analyses of the Quality Assurance (QA) samples (Table 2) resulted in measured concentrations (TBBPA) which ranged from 89.5 to 123% of nominal which was consistent with the predetermined recovery range (Appendix II). Based on these results, it was determined that satisfactory precision and quality control were maintained during the analysis of TBBPA in the exposure solutions.

Analyses of the highest treatment level (0.40 mg A.I./L) for TBBPA using high pressure liquid chromatography (HPLC) established that parent TBBPA was present in the exposure solutions. Analysis (HPLC) performed on days 14, 15, 18, 29 and 35 resulted in measured concentrations ranging from 0.17 to 0.27 mg A.I./L TBBPA. On days 11, 25 and 32, measurements for TBBPA resulted in concentrations which were below the established detection limit (0.071 to 0.10 mg A.I./L). The lower than expected measured concentrations determined for days 11, 25 and 32 are believed to be related to the level of suspended solids in the exposure vessels. Conditions which may have influenced the concentration of suspended solids in the exposure solutions are feeding or cleaning (brushing the vessel walls) prior to sampling. Since the exposure solution samples (day 11, 25 and 32) were filtered prior to analyses, TBBPA adhering to suspended solids were removed prior to analyses. Qulaity Assurance samples, analyzed concurrently with the exposure solution samples, generally met the standard acceptance criteria established by this laboratory.

4.4 Biological Observations

A summary of the biological results of the continuous 35-day exposure of fathead minnow embryos and larvae to measured concentrations of TBBPA ranging from 0.31 to 0.024 mg A.I./L are presented in Table 4.

Fathead minnow survival at the completion of the hatching period (day 5) in the highest concentration of TBBPA (0.31 mg A.I./L) was 28% and was significantly less ($P \leq 0.05$) than the survival of the control organisms (pooled data, 84%). The survival of embryos exposed to mean measured concentrations $\leq$ 0.16 mg A.I./L ranged from 74 to 90% and were unaffected as compared to the control organisms (Figure 2).

Since embryo survival was significantly affected in the highest concentration (0.31 mg A.I./L) of TBBPA, fewer larvae were available for release at this test concentration at the initiation of the post-hatch stage of the study. Following two days of post-hatch exposure, 100% mortality was observed in the highest test concentration. Following 30 days post-hatch exposure, the survival of larvae in the remaining test concentrations of TBBPA (0.16 - 0.24 mg A.I./L) ranged from 87 to 93% and was statistically comparable ($P \leq 0.05$) to the survival of the control larvae (pooled data, 93%) (Figure 3).

Statistical comparison of the growth data (total length and wet weight) determined at test termination (30 days post hatch) established that surviving fish at all treatment levels grew at rates comparable to the control larvae. The mean total length and wet weight of larvae exposed to mean measured concentrations of TBBPA $\leq$ 0.16 mg A.I./L ranged from 24 to 25 mm and 112 to 126 mg, respectively and was statistically comparable to the growth of control larvae (pooled data, 25 mm and 111 mg, respectively).

The Lowest Observed Effect Concentration (LOEC) was determined to be 0.31 mg A.I./L, based on significantly reduced embryo and larval survival of fathead minnow exposed to TBBPA. The No Observed Effect Concentration (NOEC) established for this

study was 0.16 mg A.I./L TBBPA. Based on these results, the Maximum Acceptable Toxicant Concentration (MATC) for this material and fathead minnows is estimated to be >0.16 mg A.I./L and <0.31 mg A.I./L (geometric mean MATC = 0.22 mg A.I./L).

PROTOCOL DEVIATIONS

1. The protocol states that the dilution water will have a specific conductivity range of 120 - 170 mg/L as $CaCO_3$. During this study, the specific conductivity ranged from 90 - 130 μmhos/cm.

2. The protocol states that the normal pH range of the gravity feed tank water should be 6.9 - 7.2. The pH range of the weekly measured gravity feed tank water was 7.0 - 7.4.

3. The protocol states that 40 fish will be exposed per treatment level during the exposure period. During this study, 30 fish were exposed per treatment level.

4. The protocol states that when hatch is designated as complete that 40 fry are transfered to their respective aquaria. During this study, 30 fry were selected and transferred to their respective aquaria.

5. The protocol states that the water quality conditions (dissolved oxygen, pH and temperature) during the acclimation period will be monitored and reported. For this study, the test organisms were transferred directly, as eggs, from the culture unit to the test system.

It is the opinion of SLI Study Director that these deviations did not affect the results of this study.

DC Surprenant 8/17/89

Donald C. Surprenant Date
Study Director

QUALITY ASSURANCE STATEMENT

The raw data and final report for "The Toxicity of Tetrabromobisphenol A (TBBPA) to Fathead Minnow (*Pimephales promelas*) Embryos and Larvae" 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
12/9/88	3/28/89 3/30/89 3/31/89 8/14/89 8/15/89 8/17/89	8/17/89	8/17/89

Janice E. Dean 8/17/89

Janice E. Dean
Quality Assurance Unit

Date

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TABLES

Table 1. Water quality determinations made during the 35-day exposure (30 days post hatch) of fathead minnow (Pimephales promelas) embryos and larvae to TBBPA.

Nominal Concentration (mg A.I./L)	Mean Dissolved Oxygen (mg/L)	Mean Temperature (^{o}C)	Mean Total Hardness (mg/L as $CaCO_3$)	Mean Total Alkalinity (mg/L as $CaCO_3$)	Specific Conductivity Range ($\mu mhos/cm^3$)	pH Range
0.40	8.3 (0.39)	24 (0.0)	28 (2.9)	23 (3.3)	120 - 140	7.0 - 7.6
0.20	8.1 (0.44)	24 (0.0)	———	———	———	7.0 - 7.6
0.10	8.2 (0.37)	24 (0.0)	———	———	———	7.0 - 7.6
0.050	8.3 (0.41)	24 (0.0)	———	———	———	7.0 - 7.6
0.025	8.3 (0.54)	24 (0.0)	28 (5.2)	24 (1.5)	140 - 140	7.0 - 7.9
Solvent Control	8.1 (0.46)	24 (0.0)	28 (3.3)	24 (2.9)	140 - 140	7.0 - 7.9
Control	8.6 (0.47)	24 (0.0)	29 (4.5)	24 (2.0)	120 - 140	7.0 - 8.2

[a] Values in parentheses represent standard deviation.

Table 2. Measured concentrations and the results of the analyses of the exposure solutions for ^{14}C-TBBPA during the early life stage test with the fathead minnow (Pimephales promelas).

Nominal Concentration (mg A.I./L)		Measured Concentration (mg A.I./L)								
		Day 0	Day 4	Day 11	Day 16	Day 18	Day 25	Day 29	Day 32	Day 35
0.40	A	0.46	0.37	0.28	0.32	0.22	0.18	0.33	0.33	0.35
	B	0.41	0.30	0.30	0.33	0.23	0.18	0.34	0.35	0.35
0.20	A	0.21	0.16	0.13	—	0.13	0.12	—	0.19	0.19
	B	0.20	0.16	0.18	—	0.12	0.098	—	0.18	0.20
0.10	A	0.11	0.087	0.075	—	0.072	0.050	—	0.090	0.096
	B	0.11	0.087	0.071	—	0.058	0.066	—	0.093	0.099
0.05	A	0.054	0.037	0.032	—	0.027	0.023	—	0.051	0.050
	B	0.059	0.042	0.041	—	0.034	0.024	—	0.047	0.054
0.025	A	0.025	0.018	0.049	0.020	0.021	0.013	—	0.021	0.024
	B	0.028	0.018	0.059	0.020	0.015	0.012	—	0.022	0.026
Solvent Control	A	<0.0090	<0.0090	<0.0090	—	<0.0090	<0.0090	—	<0.0089	<0.0089
	B	0.24	<0.0090	<0.0090	—	<0.0090	<0.0090	—	<0.0089	<0.0089
Control	A	<0.0090	<0.0090	<0.0090	—	<00090	<0.0090	—	<0.0089	<0.0089
	B	<0.0090	<0.0090	<0.0090	—	<0.0090	<0.0090	—	<0.0089	<0.0089

Table 2. Continued.

Nominal Concentration (mg A.I./L)	Measured Concentration (mg A.I./L)								
	Day 0	Day 4	Day 11	Day 16	Day 18	Day 25	Day 29	Day 32	Day 35
QA #1	0.0271 (0.0243)[a]	0.0399 (0.0324)	0.262 (0.259)	0.537 (0.486)	0.0323 (0.0275)	0.239 (0.227)	0.233 (0.227)	0.270 (0.260)	0.509 (0.487)
QA #2	0.0384 (0.0324)	0.0281 (0.0243)	0.525 (0.486)	0.0292 (0.0259)	0.0295 (0.0243)	0.530 (0.487)	0.319 (0.292)	0.0308 (0.0259)	0.0263 (0.0243)
QA #3	0.145 (0.162)	0.354 (0.325)	0.0343 (0.0292)	0.0282 (0.0243)	0.269 (0.244)	0.0300 (0.0259)	0.465 (0.487)	0.036 (0.0324)	0.0365 (0.0324)

[a] Value in parentheses represents nominal fortified concentration.

Table 3. **Summary (mean measured concentrations) of the analyses of the exposure solutions for TBBPA during the early life-stage exposure of fathead minnow (*Pimephales promelas*) embryos and larvae.**

Nominal Concentration (mg A.I./L)	Mean Measured Concentration (S.D.) (mg A.I./L)	Percent of Nominal
0.40	0.31 (0.074)	78
0.20	0.16 (0.038)	80
0.10	0.084 (0.019)	84
0.050	0.040 (0.012)	80
0.025	0.024 (0.012)	96

Table 4. **Survival of organisms at hatch (test day 5) and survival, total length and wet weight of fathead minnow (*Pimephales promelas*) larvae after 30 days post-hatch exposure to TBBPA.**

Mean Measured Concentration (mg A.I./L)		Larvae (30 Days Post-Hatch) Survival of Organisms at Hatch (%)	Larvae Survival (%)	Mean Total Length (S.D.) (mm)	Mean Wet Weight (S.D.) (mg)
0.31	A	35	0	——	——
	B	20	0	——	——
	Mean	28[a]	0[a]	——	——
0.16	A	71	93	24 (2.4)	108 (34)
	B	83	83	24 (2.1)	118 (34)
	Mean	77	88	24 (2.3)	113 (34)
0.084	A	90	90	25 (1.5)	125 (23)
	B	89	90	25 (1.8)	124 (29)
	Mean	90	90	25 (1.7)	125 (26)
0.040	A	88	80	24 (1.8)	133 (29)
	B	92	93	25 (2.3)	121 (32)
	Mean	90	87	25 (2.1)	127 (31)
0.024	A	76	100	24 (2.1)	116 (25)
	B	71	87	24 (1.2)	125 (18)
	Mean	74	93	24 (1.7)	120 (22)
Solvent Control	A	67	100	24 (1.9)	94 (29)
	B	97	93	25 (2.1)	119 (34)
	Mean	82	97	24 (2.2)	106 (34)
Control	A	88	87	25 (1.9)	110 (35)
	B	86	93	25 (1.9)	122 (31)
	Mean	87	90	25 (1.9)	116 (33)
Pooled[b] Controls		84	93	25	111

[a] Indicates significantly different ($P \leq 0.05$) from the control (pooled control and solvent control) data.

[b] Pooled mean dilution water control and solvent control data.

FIGURES

Figure 1. Relationship between mean measured concentrations and the nominal test concentrations during the early life stage exposures of fathead minnow (*Pimephales promelas*) to TBBPA.

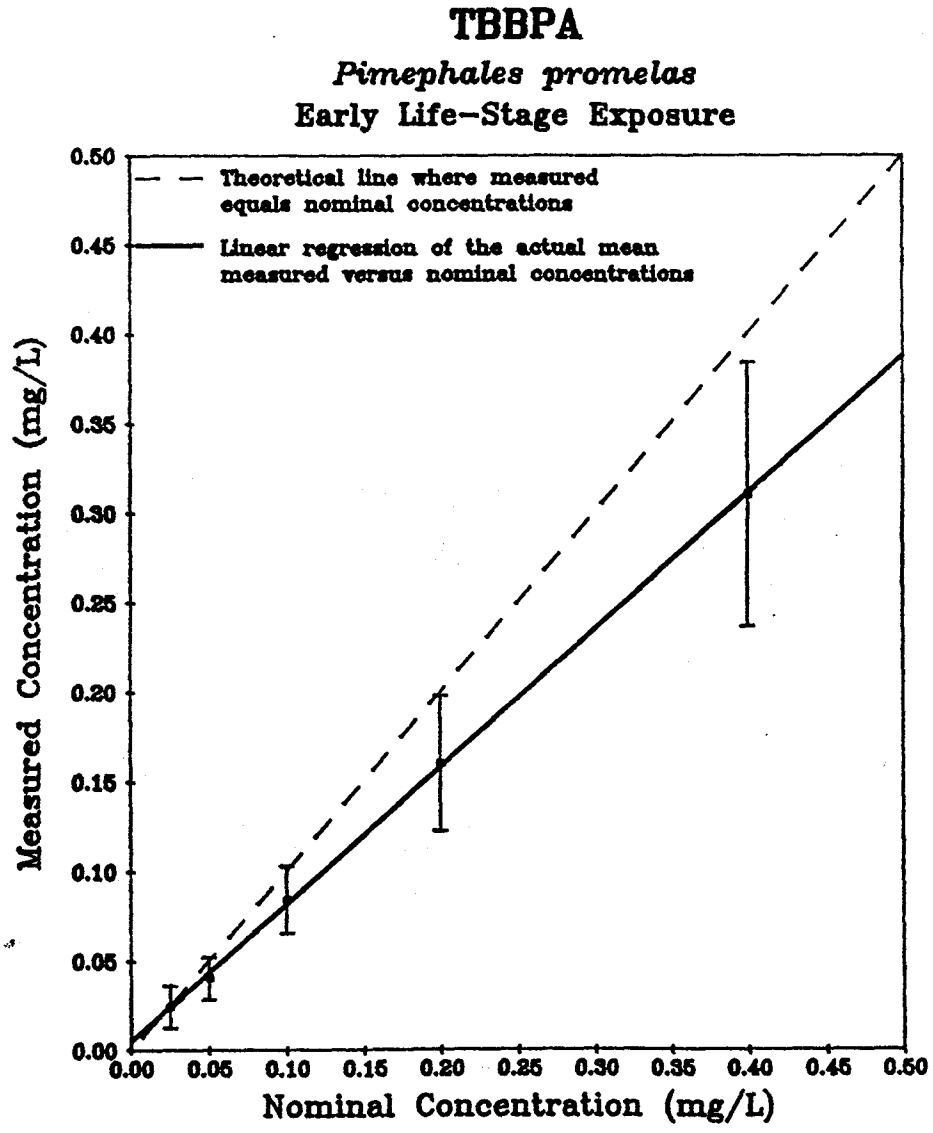
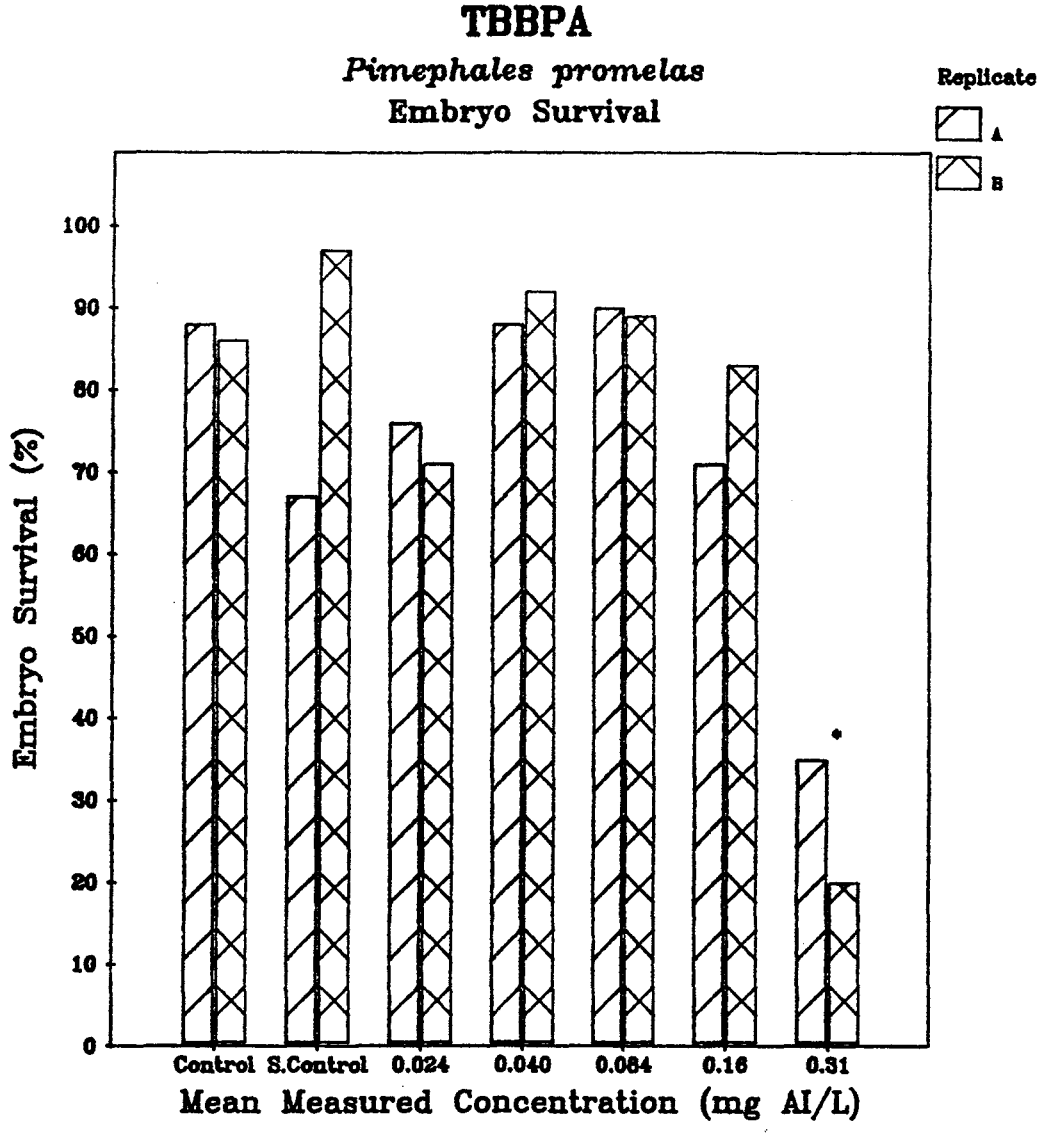
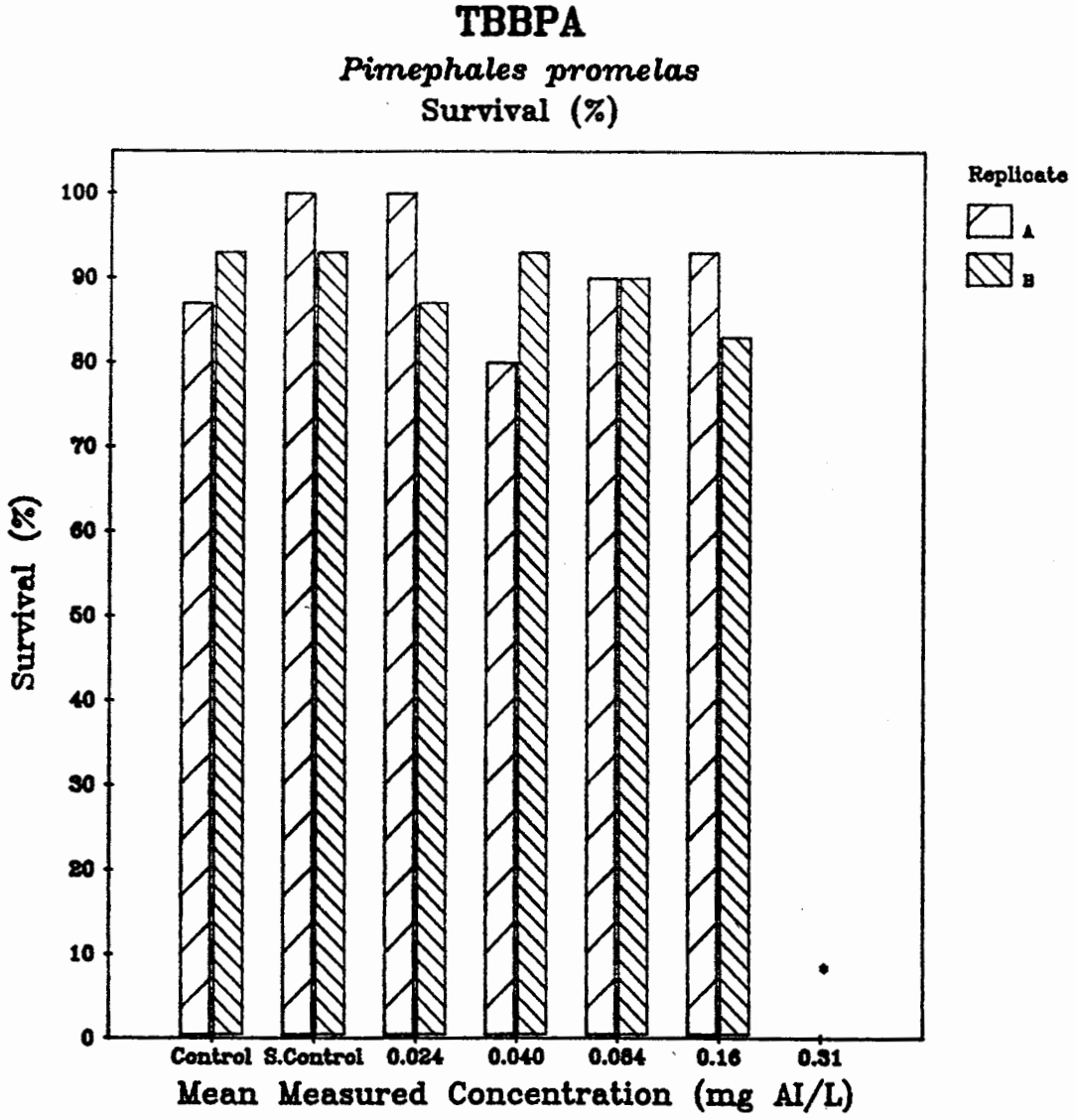


Figure 2. Embryo survival during the early life stage exposure of fathead minnow (*Pimephales promelas*) to TBBPA.



* Indicates significantly different as compared to the pooled control data.

Figure 3. **Larval survival during the early life stage exposure of fathead minnow (*Pimephales promelas*) to TBBPA.**



* Indicates significantly different as compared to the pooled control data.

Figure 4. Mean total length of organisms during the early life stage exposure of fathead minnow (*Pimephales promelas*) to TBBPA.

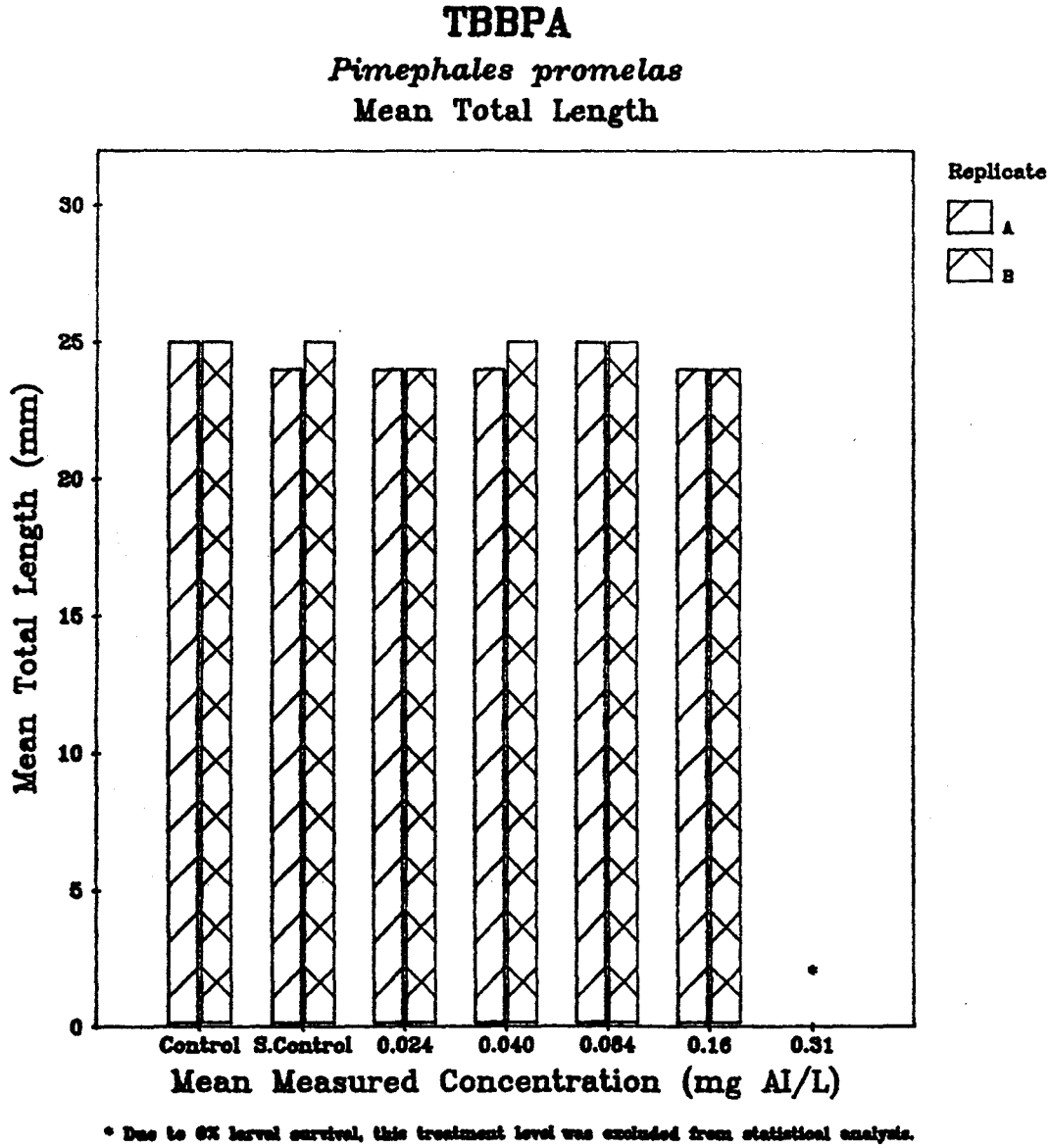
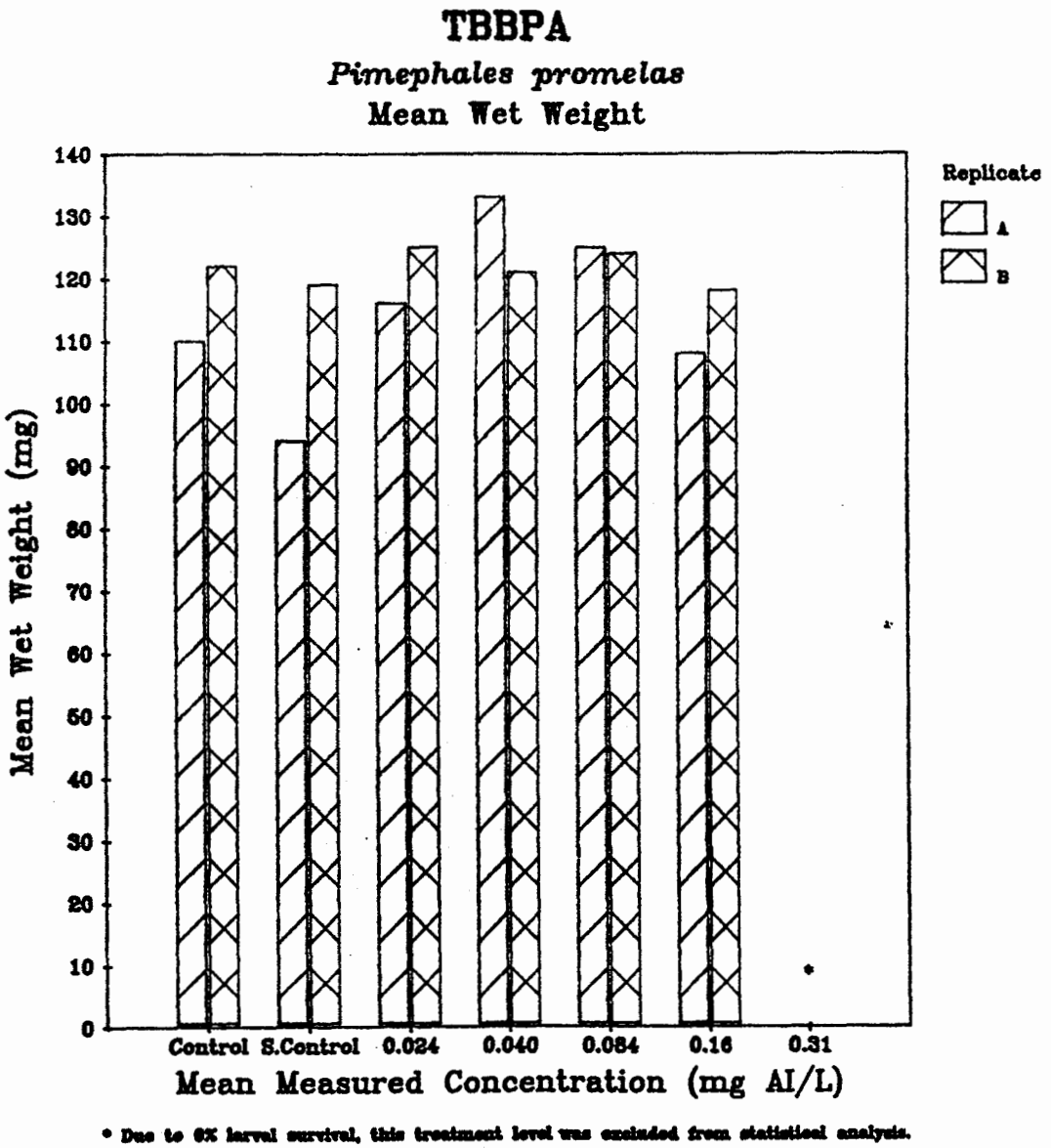


Figure 5. **Mean total weight of organisms during the early life stage exposure of fathead minnow (*Pimephales promelas*) to TBBPA.**



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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 Brominated 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 35-day exposure of fathead minnows (*Pimephales promelas*).

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 35-day exposure of fathead minnows (*Pimephales promelas*) to TBBPA.

Manufacturer	Specific Activity	Date Received
Chemsyn Science Laboratories Lot #CSL-88-164-21-10 (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 35-day exposure of fathead minnows (*Pimephales promelas*).

Nominal[a] Concentration	mL of Super Stock[b] (Labeled)	Super Stock[c] (Nonlabeled)
22.35 mg/mL	5.2[d]	9.2

[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 nonlabeled 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, Teflo

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:

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

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.

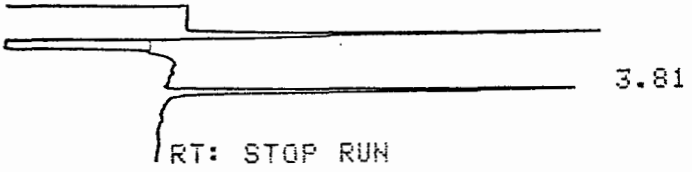
Table 2A. Analytical results for the recovery of TBBPA from freshwater.

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.