FYI-94-001050

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October 21, 1994

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Dear FYI Coordinator:

In accordance with API's policy of providing the federal government with copies of research designed to determine whether any chemical substance or mixture manufactured, processed or distributed by API member companies may cause a risk of injury to health or the environment, we are enclosing a copy of the following amended final report:

(Identification no: FYI not assigned) Tert-Amyl Methyl Ether (TAME): Toxicity to the Freshwater Alga (Selenastrum capricornutum). Amended Final Report.

Please note that this information is provided in accordance with the full disclosure policy of API and does not constitute a formal submission as required by a test rule. This document does not contain confidential information. If you have any questions, please communicate with me.

Sincerely,

Robert T. Drew

Robert T. Drew, Ph.D.

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Springborn Laboratories, Inc.
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12 August 1994

Richard A. Rhoden, Ph.D.
American Petroleum Institute
1220 L Street, Northwest
Washington, DC 20005

Dear Dr. Rhoden:

Enclosed please find ten copies and the original of SLI amended final report #93-11-5065 entitled "Tert-Amyl Methyl Ether (TAME) - Toxicity to the Freshwater Alga, *Selenastrum capricornutum*."

This report has been amended based upon comments received from Christine Sexsmith dated 29 April 1994. Please do not hesitate to contact James R. Hoberg or myself with any questions or comments.

Very truly yours,

SPRINGBORN LABORATORIES, INC.

Susan P. Shepherd DCS

Susan P. Shepherd
Coordinator, Data Management
and Reporting Unit

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Enclosures (11)

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AMENDMENT TO THE STUDY FINAL REPORT

Final Report Title: Tert-Amyl Methyl Ether (TAME) - Toxicity to the Freshwater Alga, *Selenastrum capricornutum*

Amendment No. 1 **Amendment Date:** 12 August 1994

SLI Study No. 12627.0002.6106.430

SLI Report No. 93-11-5065

Study Sponsor: American Petroleum Institute

Study Director: James R. Hoberg

Final Report Modifications and/or Additions:

page 2 - In the third sentence, "maintenance of records" was changed to "maintenance of these records"

page 12 - In the second complete sentence, "vessel control" was changed to "control vessel"

page 15 - The statistical section was clarified to explain that EC values could not be calculated at the 48-hour interval

page 18 - Solubility and volatility information was added to the end of the first paragraph.

- Reference to section 2.6 of the report was added to the eighth sentence.

page 27 - A column for nominal concentrations was added to Table 4.
- Footnote "f" was added to the table.
- The 96 hour cell density value for the B replicate of the 0.52 mg A.I./L treatment level was corrected to read "9"

page 28 - The 48-hour EC10, EC50 and EC90 results were removed from the table.

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Appendix II - The Certificate of Analysis for Lot No. 07905KZ was removed as this lot was not used during testing.

Approval Signatures:

James R. Hoberg 8/12/94
James R. Hoberg
Study Director

Doreen S. Newhouse 12 Aug 94
Doreen S. Newhouse
Supervisor, Quality Assurance Unit

Springborn Laboratories

TERT-AMYL METHYL ETHER (TAME) TOXICITY TO THE FRESHWATER ALGA, *Selenastrum capricornutum*

GUIDELINE REFERENCE NUMBER: 797-1050

Submitted to:

American Petroleum Institute
1220 L Street, Northwest
Washington, D.C. 20005

SLI Report #93-11-5065

SLI Study #12827.0692.6106.430

Study Director: James R. Hoberg

Springborn Laboratories, Inc.
Environmental Sciences Division
790 Main Street
Wareham, Massachusetts 02571

12 August 1994

AMENDED FINAL REPORT

GOOD LABORATORY PRACTICES COMPLIANCE STATEMENT

The data and report prepared for "Tert Amyl Methyl Ether (TAME) - Toxicity To The Freshwater Alga, *Selenastrum capricornutum*" were produced and compiled in accordance with all pertinent U.S. EPA Good Laboratory Practice Regulations (40 CFR, Part 792) with the following exceptions: routine water screening analyses for pesticides, PCBs and metals are conducted using standard U.S. EPA procedures by Lancaster Laboratories, Lancaster, PA. These data were not collected in accordance with Good Laboratory Practice procedures (i.e. no distinct protocol, Study Director, etc.). Stability, characterization and verification of the test material identity and maintenance of these records on the test material are the responsibility of the Study Sponsor. At the termination of the testing program, all remaining test material will be sent to the Study Sponsor. Archival of a sample of the test material is the responsibility of the Study Sponsor.

SPRINGBORN LABORATORIES, INC.

James R. Hoberg 5/12/94

James R. Hoberg Date
Study Director

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SUMMARY

Tert-Amyl Methyl Ether (TAME) - Toxicity to the Freshwater Alga, *Selenastrum capricornutum*

SPONSOR:	American Petroleum Institute 1220 L Street, Northwest Washington, DC 20005
PROTOCOL TITLE:	"Protocol for Conducting a 96-Hour Acute Toxicity Test with the Alga, *Selenastrum capricornutum*, Following TSCA 797-1050," Springborn Laboratories Protocol #:091192/TSCA 797.1050 and Protocol Amendment #1 dated 18 November 1993. This protocol conforms to U.S. EPA TSCA Guideline §797-1050 (U.S. EPA, 1985, 1987)
REPORT NUMBER:	93-11-5065
STUDY NUMBER:	12827.06S2.6106.430
TEST MATERIAL:	Tert-Amyl Methyl Ether (TAME) Lot #02814BZ
DATE RECEIVED:	17 August 1993
DESCRIPTION:	A clear liquid, 98.8% active ingredient (Certificate of Analysis, Appendix II).
EXPERIMENTAL START DATE:	18 November 1993
EXPERIMENTAL TERMINATION DATE:	22 November 1993
TEST ORGANISM:	*Selenastrum capricornutum*, inoculum - three days since previous transfer, source - Springborn culture
DILUTION WATER:	Algal Assay Procedure (AAP) medium
TEST CONDITIONS:	96-hour duration, 25 °C, continuous illumination at 3200 to 4800 lux (300 to 450 footcandles), shaking rate of 100 rpm
NOMINAL TEST CONCENTRATIONS:	0.040, 0.080, 0.16, 0.31, 0.63, 1.3, 2.5 and 5.0 mg A.I./L

MEAN MEASURED CONCENTRATIONS: 0.017, 0.037, 0.067, 0.23, 0.48, 0.52, 1.4 and 3.7 mg A.I./L

EFFECT CRITERION: Inhibition of cell growth (culture density relative to the control)

RESULTS: The 96-hour EC50 value was calculated to be 0.11 mg A.I./L (95% confidence limits of 0.018 and 0.64 mg A.I./L). The 96-hour No-Observed-Effect Concentration (NOEC) was determined to be 0.017 mg A.I./L.

Results from the supplemental exposure conducted at test termination demonstrated that TAME, at a concentration of 3.7 mg A.I./L has an algistatic (reversible) rather than algicidal (non-reversible) effect on the growth of *Selenastrum capricornutum* once it is diluted to a non-inhibitory concentration (0.040 mg A.I./L).

1.0 INTRODUCTION

The objective of this study was to determine the effect of Tert-Amyl Methyl Ether (TAME) on the growth of the freshwater alga *Selenastrum capricornutum*. The exposure system was specifically modified to minimize the loss of test material due to volatilization. The results are based on mean measured concentrations and are reported as the 96-hour EC10, EC50 and EC90 values (i.e., the concentrations of test material that reduce culture density by 10, 50 and 90%, respectively, as compared with the control). The study was initiated on 16 October 1992, the day the Study Director signed the protocol, and was completed on the day the Study Director signed the final report. The experimental phase of the 96-hour acute toxicity test was conducted from 18 to 22 November 1993 at the Environmental Sciences Division of Springborn Laboratories, Inc. (SLI), Wareham, Massachusetts. A final report for this study was issued to American Petroleum Institute dated 24 February 1994. This amended final report, 12 August 1994, incorporates changes as made in Final Report Amendment #1.

2.0 MATERIALS AND METHODS

2.1 Protocol

The toxicity test was performed according to the Springborn protocol entitled "Protocol for Conducting a 96-Hour Acute Toxicity Test with the Alga, *Selenastrum capricornutum*, Following TSCA 797-1050," Springborn Laboratories Protocol #:091192/TSCA 797.1050, and Protocol Amendment #1 dated 18 November 1993 (Appendix I). This protocol conforms to U.S. EPA TSCA Test Guideline §797-1050 (U.S. EPA, 1985, 1987) with modifications approved by the U.S. EPA to minimize volatilization of the test material from the exposure vessels.

2.2 Test Material

A sample of Tert-Amyl Methyl Ether (TAME), CAS #994-05-8, Lot 02814BZ, a clear liquid was received from Experimental Pathology Labs, Inc., Herndon, Virginia, on 17 August 1992. This sample was identified by Aldrich Chemical Company to contain 98.8% active ingredient (Certificate of Analysis, Appendix II). Upon receipt at Springborn, the sample was stored in a dark, ventilated cabinet at room temperature (approximately 20 °C). This sample was used to

prepare test solutions for the preliminary and definitive exposures. Additionally, it was used for analytical standards and quality control samples. Test concentrations are expressed as milligrams of TAME as active ingredient per liter of solution (mg A.I./L).

2.3 Test Organism

The alga used in this toxicity test was the freshwater green alga *Selenastrum capricornutum*, strain 1648, class Chlorophyceae. The alga was originally obtained from the Carolina Biological Supply Company, Burlington, North Carolina, and was maintained in stock culture at Springborn.

The culture medium used was Algal Assay Procedure (AAP) medium prepared with sterile, deionized water. The components used to formulate the AAP medium are presented in Table 1. Representative samples of the water source used to prepare the deionized water for AAP medium preparation were analyzed for the presence of pesticides, PCBs and toxic metals (Appendix III). None of these compounds have been detected at concentrations that are considered toxic in any of the water samples analyzed in agreement with U.S. EPA guidelines. In addition, a representative sample of AAP medium is analyzed monthly for total organic carbon (TOC) concentration. The TOC concentration of the AAP medium was 0.90 mg/L for the month of November 1993.

The pH of the AAP medium was adjusted to pH 7.5 ± 0.1 with either 0.10 *N* hydrochloric acid or 0.10 *N* sodium hydroxide. Stock cultures were grown in 125 mL glass flasks containing 50 mL of medium. The flasks were covered with stainless steel caps which permitted gas exchange.

The stock cultures were maintained under the following test conditions: shaking rate of approximately 100 rpm, temperature of 24 ± 1 °C with continuous illumination at the solutions' surface with a light intensity of approximately 6000 to 6500 lux (560 to 600 footcandles), for a minimum of three days prior to test initiation (SLI Algae Daily Log, 1993). Temperature was controlled using an environmental chamber. Lighting was supplied by Duro-Test, Inc. Vita-Lite® fluorescent lights. Culture flasks were agitated continuously on orbital shakers.

Stock cultures were transferred to fresh medium approximately twice weekly. The inoculum used to initiate the toxicity test with TAME was obtained from a stock culture that had been transferred to fresh medium three days prior to testing.

2.4 Test Dilution Water

The AAP medium used to prepare the exposure solutions during the preliminary and definitive tests was formulated in the same manner as the culture medium except 500 mg/L sodium bicarbonate was added to the medium to provide sufficient sodium bicarbonate for cell growth in a closed test system. Several liters of test medium were prepared and equilibrated to test temperature. The initial pH of this medium was 8.1 and was adjusted to 7.6 with 0.1 *N* HCl prior to use.

2.5 Test Concentrations

Based on the results of three range-finding tests conducted at SLI from 15 to 19 October 1992 and 14 to 18 December 1992, nominal test concentrations of 0.040, 0.080, 0.16, 0.31, 0.63, 1.3, 2.5 and 5.0 mg A.I./L of TAME were selected for the definitive exposure.

2.6 Preparation of Test Solutions

A 500 mg A.I./L stock solution was prepared by diluting 0.2530 g (0.2500 g as A.I.) of test material with 500 mL of AAP medium containing 500 mg/L of sodium bicarbonate (test medium). Test solutions were then prepared by diluting the appropriate amount of the 500 mg A.I./L stock solution to a volume of 4000 mL with test medium resulting in the desired nominal test solution concentrations. Additional untreated test medium was prepared and designated as the control. In addition, a reference control was established, using standard AAP medium without the addition of sodium bicarbonate and the closed system vessels, in order to demonstrate that the algal culture used to initiate the definitive test met standard performance criterion (i.e., log growth phase after 96 hours of exposure).

Sterile Erlenmeyer flasks, twelve flasks per treatment level and the controls, were conditioned prior to use by rinsing with the appropriate exposure solution. Approximately 275 mL of the

appropriate test solution was then placed in each of the conditioned replicate flasks. A set of control vessels was established which contained test medium.

An additional reference control vessel was established containing standard AAP medium without the addition of sodium bicarbonate or TAME. This vessel was maintained under the standard conditions used for algal culture (e.g., 100 mL solution in a 250-mL flask with gas exchange cap, see section 2.3). The control vessels were maintained under the same conditions as treatment level vessels but contained no TAME. All test vessels were completely filled with test solution (i.e., zero headspace) and tightly capped with a silicone stopper to prevent volatilization of the test material.

3.0 TEST PROCEDURES

3.1 Test Initiation

Within 40 minutes after the test solutions were prepared and added to the replicate flasks, 2.5 mL of an inoculum of *Selenastrum capricornutum* cells, at a density of 111 x 10^4 cells/mL, was aseptically introduced into each flask. This inoculum provided the required initial cell density of approximately 1.0 x 10^4 cells/mL.

3.2 Test Monitoring

3.2.1 Algal Growth. At each 24-hour interval during the definitive test, cell counts were conducted on three replicate vessels of each treatment level and control (single vessel for reference control) using a hemacytometer (Neubauer Improved) and an Olympus compound microscope. One sample (approximately 0.5 mL) was removed from each flask for counting. Following counting, the sample and the replicate solution from which the sample was removed were discarded. One or more hemacytometer fields, each 0.10 x 0.10 cm in surface area and 0.010 cm deep and containing 0.00010 mL of culture, were examined for each sample until four fields were counted. Observations of the health of the cells were made and recorded at each 24-hour interval. The test and control solutions observed at each 24-hour interval were discarded after use, due to the potential loss of test material from the test vessels.

3.2.2 Recovery. At test termination, approximately 2.2 mL was removed from the composite of the three replicate solutions of the highest test concentration (nominal concentration, 5.0 mg A.I./L) which most severely inhibited algal growth. The subsample was diluted with sterile AAP medium (pH adjusted to 7.5) to prepare a subculture with a nominal test material concentration equal to a test concentration which did not inhibit cell growth during the definitive exposure (nominal concentration, 0.040 mg A.I./L). This subculture was used to determine if the effects of the test material on algae were algistatic, in which case cells would resume growth in the subculture, or algicidal, no growth would occur in the subculture. The subculture was incubated under closed system test conditions for up to 9 days or until growth was observed.

3.2.3 Test Conditions. The test was conducted in an environmental chamber designed to maintain the following test conditions: a temperature range of 24 ± 1 °C, continuous lighting with a light intensity within the range of 3200 to 5400 lux (300 to 500 footcandles) and a shaking table rate of 100 ± 10 rpms.

Temperature was measured continuously with a Taylor Thermometer Company, Inc. minimum/maximum thermometer located in a flask of water adjacent to the test flasks in the environmental chamber. The shaking rate of the orbital shakers was recorded daily. Light intensity of the test area was measured with a General Electric Type 214 light meter at 0-hour and each 24-hour interval of the exposure period. Light intensity was measured in footcandles and converted to lux based on 1 footcandle equals 10.76 lux. Test flasks were randomly positioned based upon computer-generated random numbers on a Lab-Line orbital shaker Model #3520 at test initiation and after each observation interval.

Water quality parameters (pH and specific conductivity) were measured at test initiation and at the termination of the 96-hour exposure period. Measurements at 0-hour were conducted on the test solution remaining in the 4000-mL flask after the individual test flasks had been filled. At test termination, after cell counts were completed, the three remaining replicate vessels for each test concentration and the control were individually composited and a portion of each composite solution was transferred to a 100-mL beaker for pH and conductivity measurements.

Each of these water quality parameters was also measured in the reference control solution at test initiation and termination. Test solution pH was measured with a LaMotte Model HA pH meter, and specific conductivity was measured with a Yellow Springs Instrument (YSI) Model #33 salinity-conductivity-temperature meter.

3.2.4 Chemical Analysis. At test initiation (0 hour) and test termination (96 hours), a single sample from each test solution and the controls was analyzed for TAME concentration. Samples analyzed at 0 hour were removed from each test solution as it was prepared prior to division into the replicate test vessels. Samples analyzed at 96 hours were removed from the individual composite solutions of the three replicates of each test concentration and the control(s). In addition, three Quality Control (QC) samples were prepared at each interval using fresh algal growth medium. QC samples were prepared at nominal concentrations similar to the concentration range tested. Each set of QC samples remained with the corresponding set of exposure solution samples throughout the analysis. The results of these analyses were used to judge the precision and quality control maintained during the analytical process. All samples were analyzed for TAME concentration using a gas chromatography (GC) procedure according to the methodology presented in Appendix IV. A method validation study conducted at Springborn prior to initiation of the definitive test, established a mean recovery of TAME of 89.7 ± 2.3% from AAP medium. Conditions and procedures used throughout the analysis of exposure solution samples and QC samples during this study were similar to those described in Appendix IV.

4.0 STATISTICAL ANALYSIS

The cell density of each flask sampled at each 24-hour interval was calculated by dividing the number of cells counted by the total volume of culture examined. Means and standard deviations for cell density were calculated for each treatment level and the control(s) from individual replicate values.

For the observed values for cell density, the highest test concentration that caused no statistical adverse effect, the No-Observed-Effect Concentration (NOEC), was determined using Williams' Test (Williams, 1971, 1972). The data were first checked for normality using Shapiro-Wilks Test (Weber, *et al*, 1989) and for homogeneity of variance using Bartletts's Test (Horning and Weber, 1985). All statistical determinations were made at the 95% level of certainty, with the exception of Shapiro-Wilks' and Bartlett's Tests where the 99% level of certainty was applied.

EC10, EC50 and EC90 values (the concentration of test material which reduced cell densities by 10, 50 and 90%, respectively) were calculated based on cell density after 72- and 96-hours of exposure. EC values could not be calculated at the 24- and 48-hour observation intervals due to the lack of a well-defined concentration-response. The EC10, EC50 and EC90 values and their 95% confidence limits were determined by linear regression of response (percent reduction of cell density as compared with the control data) vs. mean measured exposure concentration over the range of test concentrations where a clear exposure-response relationship was observed. Four linear regressions were estimated based on (a) untransformed data, (b) untransformed response vs. logarithm-transformed concentration, (c) probit-transformed response vs. logarithm-transformed concentration, and (d) probit-transformed response vs. untransformed concentration. The regression that best fit the data was selected based on the highest coefficient of determination (r^2). This regression equation was then applied to estimate the EC values and their 95% confidence limits, using the method of inverse prediction (Sokal and Rohlf, 1981).

5.0 RESULTS AND DISCUSSION

Copies of pertinent raw data produced during this study are presented in Appendix V.

5.1 Preliminary Testing

A preliminary range-finding exposure was conducted in an open test system (flasks were covered with stainless steel caps which permitted gas exchange) from 15 to 19 October 1992 at nominal TAME concentrations of 0.10, 1.0, 10, 100 and 1000 mg A.I./L. Two exposure vessels were established for each concentration and a control. Following 96-hours of exposure, cell

densities in the treatment levels (0.10, 1.0, 10, 100 and 1000 mg A.I./L) averaged 63, 66, 53, 55 and 50 x 10^4 cells/mL, respectively. The control solutions averaged 93 x 10^4 cells/mL. The cell densities observed in the treatment flask represented 11, 29, 43, 41 and 46% reductions respective to the control.

A second preliminary exposure was conducted in a closed system (flasks with no headspace were tightly capped with silicone stoppers) from 14 to 18 December 1992 at nominal TAME concentrations of 0.10, 1.0, 10, 100 and 1000 mg A.I./L. Four exposure vessels were established for each concentration and the control. Two exposure vessels at each test concentration were inoculated with an initial cell density of 1.0 x 10^4 cells/mL and two were inoculated with an initial cell density of 0.10 x 10^4 cells/mL. Following 96 hours of exposure, cell densities in the replicate flasks inoculated with 1.0 x 10^4 cells/mL averaged 23, 22, 17, 3.8 and 1.3 x 10^4 cells/mL representing 45, 48, 60, 91 and 97% reduction respective to the control (42 x 10^4 cells/mL). Following 96 hours of exposure, cell densities in the replicate flasks inoculated with 0.10 x 10^4 cells/mL averaged 10, 11, 5.6, 5.1 and 0 x 10^4 cells/mL, representing 29, 21, 60, 64 and 100% reduction respective to the control (14 x 10^4 cells/mL). Results of these preliminary investigations established that exposure, under closed system conditions, to 1000 mg A.I./L TAME reduced algal growth by 97 to 100%. Exposure to the same nominal concentration of TAME (1000 mg A.I./L), under standard (open) system conditions, resulted in a 46% reduction of algal growth. This difference in concentration-effect relationships is believed due to the difference in exposure conditions. That is, due to the volatility of the test material, the closed system maintained a higher concentration of test material for a longer period of time. Although cell growth is limited within a capped system due to the lack of carbon dioxide exchange with the atmosphere, inoculation at a lower cell density, which provided a more substantial growth curve before carbon dioxide becomes limited, provided toxicity data similar to that of a closed system inoculated at 1.0 x 10^4 cells/mL. Based on these data, and consultation with the Study Sponsor and the U.S. EPA, nominal TAME concentrations of 0.040, 0.080, 0.16, 0.31, 0.63, 1.3, 2.5 and 5.0 mg A.I./L were selected for the definitive test. Exposure vessels during the definitive test were tightly-capped to provide "closed system" conditions. Closed system conditions were maintained during the definitive study to provide conditions which would maintain the highest exposure level,

relative to nominal, for the longest period of time (i.e., 96 hours). Based on a request from the U.S. EPA, the initial cell density of 1.0×10^4 cells/mL was required. Additionally, to extend the level of bicarbonate in the closed test system, an additional 500 mg/L of sodium bicarbonate was required to be added to the test medium.

5.2 Definitive Testing

Conductivity, pH, temperature and light intensity measurements recorded during the definitive test are presented in Table 2. Conductivity of the exposure and control solutions ranged from 500 to 600 µmhos/cm at test initiation which is within the expected range for AAP medium with additional sodium bicarbonate. Conductivity of exposure and control solutions measured 500 µmhos/cm at test termination. The conductivity of the reference control solution was 80 µmhos/cm throughout the exposure period, which is within the expected range for standard AAP medium. At test initiation, the pH of the control and reference control solutions was 7.5 and 7.6, respectively. The pH of the test solutions was 8.0 for each concentration. Based on the results of the control analysis, the presence of TAME at the concentrations tested increased the pH of the test medium. At test termination, pH increased in each test solution and control and ranged from 8.2 to 9.1. An increase in pH is common in static algal cultures and is due to photosynthesis by the algae. Continuous temperature monitoring established that the solution temperature was maintained at 25 °C throughout the study period. The shaking rate was maintained throughout the exposure at a constant rate of 100 rpm. Light intensity of the test area ranged from 3200 to 4800 lux (300 to 450 footcandles).

The results of the analysis of the exposure solutions for TAME during the definitive exposure are presented in Table 3. Analysis for test material concentration resulted in measured concentrations which decreased (approximately 25 to 72%) between 0 and 96 hours and averaged 56% of the nominal treatment levels. However, these analyses demonstrated that the closed test system employed for this study maintained a substantial amount of the test material throughout the exposure period. Based on mean measured concentrations, the treatment levels were defined as 0.017, 0.037, 0.067, 0.23, 0.48, 0.52, 1.4 and 3.7 mg A.I./L. Analyses of the Quality Control samples resulted in measured concentrations which were generally consistent

with the predetermined recovery range (Appendix V) and averaged 94.6% (n = 6) of the nominal fortified levels (0.0400 to 10.0 mg A.I./L). Based on the results of the recoveries for the QC samples, it was determined that the appropriate quality control was maintained during the analysis of the exposure solution samples. The measured concentrations of TAME established during this study are believed to accurately represent the exposure conditions maintained during the 96-hour test. Although the water solubility limit of TAME is 1.2% (12,000 mg/L, which is well above the highest nominal concentration tested, i.e., 5.0 mg A.I./L), the volatility of TAME (Reid vapor pressure, 1.5 psi) is suspected to be responsible for the loss of test material during the exposure period..

Cell densities determined at each observation interval are presented in Table 4. Figure 1 displays the growth curve for each treatment and control during the 96-hour exposure phase. Cell fragments, thin cell walls and bloated cells were observed in the five highest treatment levels (0.23, 0.48, 0.52, 1.4 and 3.7 mg A.I./L, respectively) at test termination. Cell fragments and bloated cells were observed in the 0.067 mg A.I./L treatment level. Bloated cells were observed in the remaining treatment levels tested 0.017 and 0.037 mg A.I./L and in the control at test termination. Cells exposed to the reference control solution were observed to be normal. Control cultures averaged 65 x 10^4 cells/mL at test termination. Cell densities in the reference control (see section 2.6) averaged 63 x 10^4 cells/mL at test termination. Cell densities in the treatment levels (0.017, 0.037, 0.067, 0.23, 0.48, 0.52, 1.4 and 3.7 mg A.I./L) averaged 68, 47, 30, 16, 12, 7, 4 and 3 x 10^4 cells/mL, respectively, at test termination. Statistical analysis (William's Test) demonstrated a significant difference for cell density in the 0.037, 0.067, 0.23, 0.48, 0.52, 1.4 and 3.7 mg A.I./L treatment levels when compared to the cell density (65 x 10^4 cells/mL) of the control solutions. Based on these results, the 96-hour No-Observed-Effect Concentration (NOEC) for cell density was determined to be 0.017 mg A.I./L. Table 5 presents the EC10, EC50 and EC90 values calculated throughout the exposure period. The 96-hour EC50 value based on cell density was calculated by linear regression to be 0.11 mg A.I./L (95% confidence limits of 0.018 to 0.64 mg A.I./L). The 96-hour EC10 and EC90 values were calculated to be 0.012 and 1.0 mg A.I./L, respectively (95% confidence limits of 0.0016 to 0.072 mg A.I./L and 0.18 to 6.5 mg A.I./L, respectively).

At test termination, a 2.2 mL subsample was removed from the composite of the three replicate solutions of the 3.7 mg A.I./L test concentration. The subsample was diluted to 275 mL (calculated TAME concentration of 0.040 mg A.I./L, nominal) with fresh AAP medium, yielding 0.021 x 10^4 cells/mL for determination of growth recovery. After two days in fresh medium, 15 x 10^4 cells/mL were observed. Consequently, the growth of *Selenastrum capricornutum* after transfer to fresh medium (dilution to 0.040 mg A.I./L) indicates that TAME has an algistatic, rather than algicidal, effect at 3.7 mg A.I./L.

PROTOCOL DEVIATIONS

1. The protocol states that a preliminary range-finding test will be conducted without replication and suggests that it will be conducted with flasks fitted with gas exchange caps and utilizing the standard exposure conditions listed for the definitive test. During this test, several preliminary range-finding exposures were conducted which contained replicated test solutions in order to improve the reliability of the test results. Additionally, due to the volatility of the test material, the exposure conditions (e.g., larger flasks and test solutions, capped vessels, addition of sodium bicarbonate) were modified at the request of the U.S. EPA to minimize the loss of the material from the test system (see Protocol Amendment #1).

2. The protocol states that the test organism will be cultured at a light intensity of 3200 to 5400 lux. The definitive test was inadvertently initiated with a culture maintained at 6000 to 6500 lux. This range is within acceptable light intensity ranges for the culture of *Selenastrum capricornutum* (e.g., OECD, 1984).

It is our opinion that these deviations do not affect the results or interpretation of this test.

SPRINGBORN LABORATORIES, INC.

James R. Hoberg 8/12/94

James R. Hoberg Date
Study Director

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QUALITY ASSURANCE UNIT STATEMENT

The raw data and report for "Tert-Amyl Methyl Ether (TAME) – Toxicity To The Freshwater Alga *Selenastrum capricornutum*" were inspected by the Quality Assurance Unit (QAU) at Springborn Laboratories, Inc. Environmental Sciences Division to determine adherence with the study protocol and laboratory standard operating procedures. In addition, inspection of certain phases of the in-life portion of the study was performed. Dates of study inspections, dates reported to Study Director and to Management are listed below.

Based on these inspections, it was determined that this report accurately reflects the raw data collected during this study.

Inspection Date	Reported to Study Director	Reported to Management
11/19/93	11/19/93	11/19/93
12/4/93	12/6/93	12/17/93
12/9/93	12/9/93	12/17/93
2/23/94	2/23/94	2/24/94
8/11/94	8/11/94	8/12/94
8/12/94	8/12/94	8/12/94

SPRINGBORN LABORATORIES, INC.

Doreen S. Newhouse 12 Aug 94

Doreen S. Newhouse Date
Supervisor, Quality Assurance Unit

REFERENCES

Horning, W.B. and C.I. Weber. 1985. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio. EPA/600/4-85/014.

Miller W.E., J.C. Green and T. Shiroyama. 1978. The *Selenastrum capricornutum* Printz algal assay bottle test. EPA 600/9-78-018. U.S. Environmental Protection Agency, Washington, D.C.

OECD. 1984. Guideline for Testing of Chemicals. Alga, Growth Inhibition Test #201. Adopted 7 June 1984. Organization for Economic Cooperation and Development. Paris, France.

Sokal, R.R. and F.J. Rohlf. 1981. *Biometry* 2nd Edition. W.H. Freeman and Co., New York, NY. 859 pp.

U.S. EPA. 1982. *Pesticide Assessment Guidelines, Subdivision J, Hazard Evaluation: Nontarget Plants*. EPA 540/9-82-020, 27 October 1982. U.S. EPA, Washington, DC.

U.S. EPA. 1985, 1987. Toxic Substances Control Test Act Guidelines. Federal Register 50(188): 39252-39516, September 27, 1985. Amended May 20, 1987.

Weber, C.I., W.H. Peltier, T.J. Norberg-King, W.B. Horning II, F.A. Kessler, J.R. Menkedick, T.W. Neiheisel, P.A. Lewis, D.J. Klemm, Q.H. Pickering, E.L. Robinson, J.M. Lazorchak, L.J. Wymer and R.W. Freyberg (eds.). 1989. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 2nd ed. EPA/-600/4/89/001. Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH.

Williams, D.A. 1971. A test for differences between treatment means when survival dose levels are compared with a zero dose control. *Biometrics* 27: 103-117.

Williams, D.A. 1972. A comparison of several dose levels with a zero control. *Biometrics* 28: 519-531.

TABLES

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Table 1. Composition of algal growth medium (AAP medium) used during this study.

Compound	Concentration
$NaNO_3$	25.5 mg/L
$MgCl_2 \cdot 6H_2O$	12.16 mg/L
$CaCl_2 \cdot 2H_2O$	4.41 mg/L
$MgSO_4 \cdot 7H_2O$	14.7 mg/L
$K_2HPO_4 \cdot 3H_2O$	1.368 mg/L
$NaHCO_3$[a]	15.0/515 mg/L
H_3BO_3	185.5 µg/L
Na_2SeO_4[b]	1.88 µg/L
$MnCl_2 \cdot 4H_2O$	415.4 µg/L
$ZnCl_2$	3.270 µg/L
$CoCl_2 \cdot 6H_2O$	1.43 µg/L
$CuCl_2 \cdot 2H_2O$	0.012 µg/L
$Na_2MoO_4 \cdot 2H_2O$	7.26 µg/L
$FeCl_3 \cdot 6H_2O$	159.8 µg/L
$Na_2EDTA \cdot 2H_2O$	300.0 µg/L

Final pH adjusted to 7.5 ± 0.1

[a] Based on a request from the U.S. EPA, an additional 500 mg/L of sodium bicarbonate ($NaHCO_3$) was added to the test medium to extend the amount of bicarbonate in the closed test system. The reference control contained standard AAP medium.

[b] Additional nutrient required, personal communication. Dr. R.R.L. Guillard, June 1991.

Source: Miller, W.E., J.C. Greene and T. Shiroyama. 1978. The *Selenastrum capricornutum* Printz algal assay bottle test. EPA 600/9-78-018. U.S. Environmental Protection Agency, Washington, D.C.

Table 2. Conductivity, pH, temperature and light intensity measured during the 96-hour exposure of *Selenastrum capricornutum* to TAME.

Nominal Concentration (mg A.I./L)	pH		Conductivity (µmhos/cm)	
	0-Hour	96-Hour	0-Hour	96-Hour
Reference Control[a]	7.5	8.2	80	80
Control	7.6	9.0	500	500
0.040	8.0	9.1	500	500
0.080	8.0	9.0	600	500
0.16	8.0	8.9	600	500
0.31	8.0	8.9	600	500
0.63	8.0	8.8	600	500
1.3	8.0	8.9	600	500
2.5	8.0	8.9	600	500
5.0	8.0	8.9	600	500

Minimum/Maximum Temperature (°C)

0 - 24-Hour	24 - 48-Hour	48 - 72-Hour	72 - 96-Hour
25/25	25/25	25/25	25/25

Light Intensity

	24-Hour	48-Hour	72-Hour	96-Hour
footcandles:	300 - 450	300 - 450	300 - 450	300 - 450
lux:	3200 - 4800	3200 - 4800	3200 - 4800	3200 - 4800

[a] Medium without additional of sodium bicarbonate (500 mg/L, see Table 1).

Table 3. Concentrations of TAME measured in the exposure solutions during the 96-hour toxicity test with *Selenastrum capricornutum*.

Nominal Concentration (mg A.I./L)	Measured Concentration (mg A.I./L)		
	0-Hour	96-Hour[a]	Mean[a]
Control	<0.010	<0.010	NA[b]
0.040	0.023	0.010	0.017
0.080	0.043	0.032	0.037
0.16	0.083	0.050	0.067
0.31	0.27	0.19	0.23
0.63	0.75	0.21	0.48
1.3	0.77	0.27	0.52
2.5	1.6	1.2	1.4
5.0	5.7	1.6	3.7
QC #1[c]	0.0470 (0.0400)[d]	0.085 (0.0800)	
QC #2	0.979 (1.00)	0.718 (1.00)	
QC #3	7.96 (10.0)	5.35 (5.00)	

[a] Calculated values are based on actual analytical results and not on rounded values (two significant figures) presented in this table.
[b] NA = Not Applicable
[c] QC = Quality Control sample
[d] Nominal concentration of each Quality Control sample is presented in parentheses.

Table 4. Cell densities (x 10^4 cells/mL) of *Selenastrum capricornutum* after 24, 48, 72 and 96 hours of exposure to TAME.

Concentration Nominal (mg AI/L)	Concentration Mean Measured		Cell Density (x 10^4 cells/mL) 24-Hour	48-Hour	72-Hour	96-Hour
Reference Control	Reference Control		2	8	19	63
Control	Control	A	3	38	16	69
		B	2	35	28	64
		C	2	35	28	63
		Mean(SD)[a]	2(1)	38(2)[f]	24(5)[d]	65(3)[d]
0.040	0.017	A	2	7	9	53
		B	3	8	12	83
		C	2	10	12	69
		Mean(SD)[a]	2(<1)	8(2)	11(2)[bd]	69(15)[d]
0.080	0.037	A	3	5	8	48
		B	4	2	10	46
		C	2	5	17	47
		Mean(SD)[a]	3(1)	4(2)	11(5)[bd]	47(1)[de]
0.16	0.067	A	1	6	12	37
		B	2	6	6	26
		C	1	3	21	28
		Mean(SD)[a]	1(<1)[b]	5(2)	13(7)[bd]	30(6)[bde]
0.31	0.23	A	2	10	9	17
		B	1	7	13	19
		C	2	4	9	13
		Mean(SD)[a]	1<1)[b]	7(3)	10(2)[bc]	16(3)[bcde]
0.63	0.46	A	1	7	7	11
		B	1	5	7	12
		C	1	10	9	14
		Mean(SD)[a]	1(<1)[b]	7(2)	8(1)[bc]	12(2)[bcde]
1.3	0.52	A	3	4	5	7
		B	1	5	4	9
		C	1	6	6	6
		Mean(SD)[a]	2(1)[b]	5(1)[b]	5(1)[bc]	7(1)[bcde]
2.5	1.4	A	1	3	2	3
		B	1	4	3	5
		C	<1	5	4	4
		Mean(SD)[a]	1(1)[b]	4(1)[b]	3(1)[bc]	4(1)[bcde]
5.0	3.7	A	1	4	3	2
		B	1	2	2	3
		C	2	3	2	4
		Mean(SD)[a]	1(1)[b]	3(1)[b]	2(1)[bc]	3(1)[bcde]

a Mean and standard deviation (SD) were calculated from original raw data, not from the rounded values presented in this table.
b Cell fragments were observed.
c Thin cell walls were observed.
d Blasted cells were observed.
e Significantly reduced as compared to the control based on Williams' Test.
f The cell density recorded for the control at 48 hours was higher than expected (i.e., 38 x 10^4 cells/mL). The expectation of 8 x 10^4 cells/mL is based on the historical performance of this species and the performance of the reference control organisms during this study. Due to the uncertainty of the accuracy of the cell density determined at 48-hours, these data were not used to calculate EC50 values at this time interval. Cell densities measured for the control at subsequent intervals (i.e., 72 and 96 hours) were consistent with standard expectations for this species.

Table 4. EC10, EC50 and EC90 values for TAME calculated from results (cell density) of the 96-hour toxicity test with *Selenastrum capricornutum*.

$\bar{X}$ = Mean Measured Concentration (mg A.I./L).
Y = Percent Inhibition (in cell density, compared with control)

24- and 48-Hour Results[a]

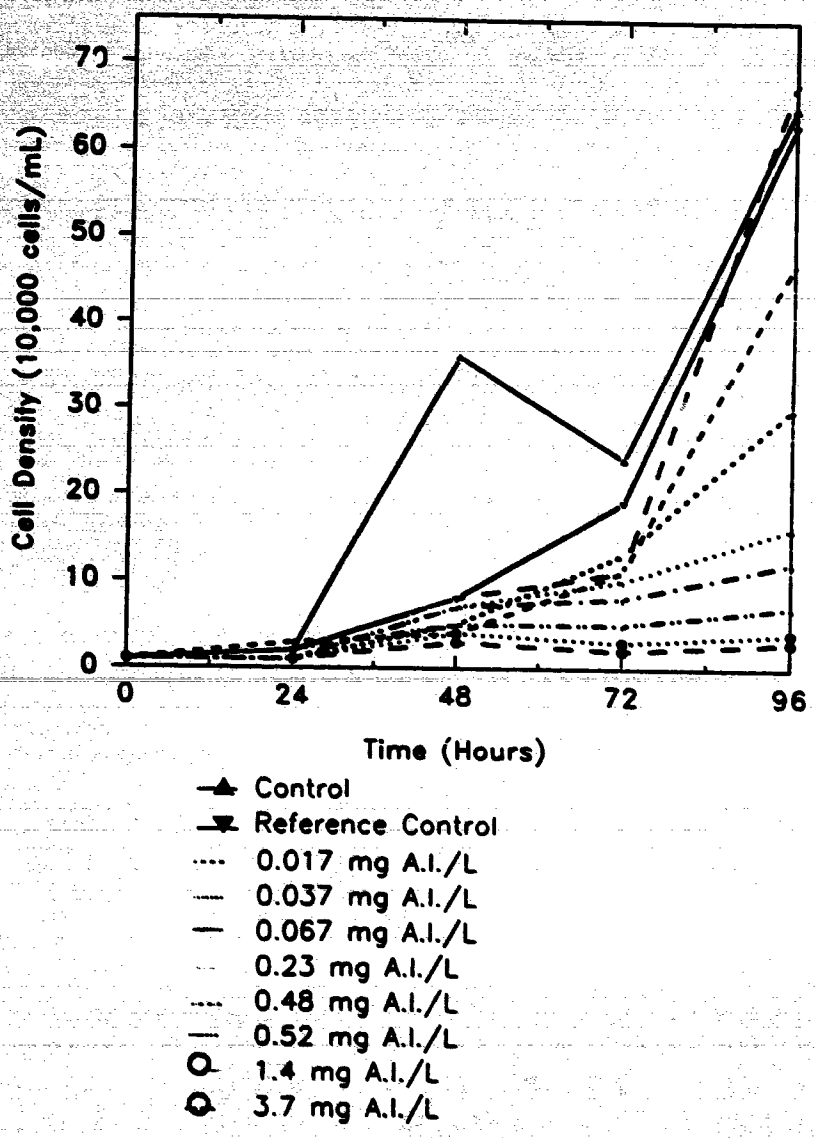
72-Hour Results	EC10	EC50	EC90
EC value (mg A.I./L):	0.0055	0.12	2.5
95% Confidence Limits:	0.00030-0.039	0.014-0.69	0.41-21
Regression Equation:	Probit (Y) = 5.9 + 0.97 Log(X)		
r^2:	0.73		
N:	18		
Concentration Range[b]:	0.067 - 3.7 mg A.I./L		

96-Hour Results	EC10	EC50	EC90
EC value (mg A.I./L):	0.012	0.11	1.0
95% Confidence Limits:	0.0016-0.072	0.018-0.64	0.18-6.5
Regression Equation:	Y = 90 + 42 Log(X)		
r^2:	0.83		
N:	24		
Concentration Range[b]:	0.017 - 3.7 mg A.I./L		

[a] EC values could not be calculated due to the lack of a well-defined concentration-response at this interval.
[b] Exposure-response relationship was judged to be linear over this concentration range; values for this concentration range were included in the linear regression.
[c] Confidence limit could not be calculated.

FIGURE

Figure 1. Graphical illustration of the relationship between cell densities and time during the 96-hour toxicity test exposing *Selenastrum capricornutum* to TA892.



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This final report has been signed in accordance with SLI SOP No. 4.3.07(01).