American Petroleum Institute

Health and Environmental Sciences Department

STEP
Strategies for Today's Environmental Partnership

Tertiary-Amyl Methyl Ether (TAME)

Toxicity to the Freshwater Alga, *Selenastrum capricornutum*

FEBRUARY 1995

TOXICOLOGY REPORT NUMBER 402
CAIS ABSTRACT NO. 41-5415

One of the most significant long-term trends affecting the future vitality of the petroleum industry is the public's concerns about the environment. Recognizing this trend, API member companies have developed a positive, forward-looking strategy called STEP: Strategies for Today's Environmental Partnership. This program aims to address public concerns by improving our industry's environmental, health and safety performance; documenting performance improvements; and communicating them to the public. The foundation of STEP is the API Environmental Mission and Guiding Environmental Principles.

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QUALITY ASSURANCE/GLP COMPLIANCE STATEMENT

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

Testing Facility: Springborn Laboratories, Inc.

SLI Study Number: 12827.0692.6106.430

This study was reviewed by API Quality Assurance personnel under the direction of API Management on the dates indicated below for compliance with EPA (TSCA) Good Laboratory Practice (GLP) regulations. These studies were conducted in accordance with EPA GLP regulations, with the exceptions* listed below.

Copies of reports by API Quality Assurance personnel are available upon written request to the Director of the Health and Environmental Sciences Department of the American Petroleum Institute or his designee.

Date(s) of Inspection/Review	Type of Inspection	Date of Report to Management
8/28/92	Protocol Evaluation	8/28/92
12/2/93	Protocol Amendment Review	12/2/93
4/27-28/94	Draft Report Audit	4/29/94
8/15/94	Final Report Acceptance	8/15/94

Christine Sexsmith
Christine Sexsmith
Quality Assurance Coordinator

8/15/94
Date

*Test article characterization, other than that received by the manufacturer (98.8% pure TAME) was not performed, storage stability for this test article is not known, and the method of fabrication is maintained with the test article supplier.

Springborn Laboratories, Inc.
Environmental Sciences Division
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AMENDMENT TO THE STUDY FINAL REPORT

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

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

SLI Study No. 12827.0692.6106.430

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.

Springborn Laboratories

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

TABLE OF CONTENTS

LIST OF TABLES

PAGE

LIST OF FIGURES

PAGE

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: 12827.0692.6106.430

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 $\pm$ 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 $\pm$ 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 83, 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

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

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TABLES

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 A.I./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	39	18	69
		B	2	35	28	64
		C	2	35	26	63
		Mean(SD)[a]	2(1)	36(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]	68(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	8	8	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	0.48	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] Bloated 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., 36 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 5. EC10, EC50 and EC90 values for TAME calculated from results (cell density) of the 96-hour toxicity test with *Selenastrum capricornutum*.

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

24- and 48-Hour Results[a]

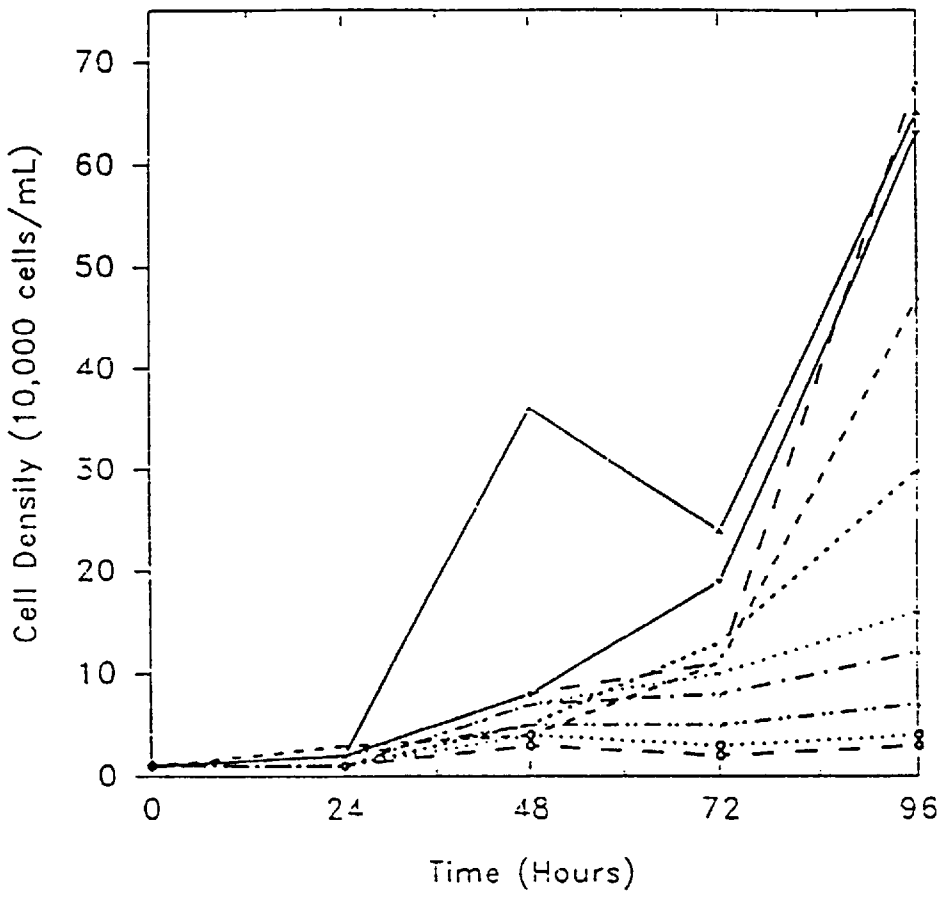
72-Hour Results	EC10	EC50	EC90
EC value (mg A.I./L):	0.0055	0.12	2.5
95% Confidence Limits:	0.00030-0.039	0.014-0.69	0.41-21
Regression Equation:	Probit (Y) = 5.9 + 0.97 Log(X)		
r^2:	0.73		
N:	18		
Concentration Range[b]:	0.067 - 3.7 mg A.I./L		

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 TAME.**



—▲— Control
—▼— Reference Control
— — 0.017 mg A.I./L
— — — 0.037 mg A.I./L
- - - - - 0.067 mg A.I./L
. . . . . 0.23 mg A.I./L
—·—·— 0.48 mg A.I./L
—·—·— 0.52 mg A.I./L
. . . o . . . 1.4 mg A.I./L
- —o— - 3.7 mg A.I./L

SIGNATURES AND APPROVAL

SUBMITTED BY:

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

PREPARED BY:

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

Carlene Thomas 8-12-94
Carlene Thomas date
Principal Investigator formerly Carlene Hardy

Robert B. Foster for EC 8/12/94
Edward Chalmers date
Analytical Chemist

Susan P. Shepherd 12 Aug 94
Susan P. Shepherd date
Coordinator, Data Management
and Reporting Unit

APPROVED BY:

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

Donald C. Surprenant 8/12/94
Donald C. Surprenant date
Program Manager,
Environmental Toxicology

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

6.0 APPENDIX I - STUDY PROTOCOL

Springborn Laboratories, Inc.
Environmental Sciences Division
790 Main Street • Wareham, Massachusetts 02571 • (508) 295-2550 • Telex 4436041 • Facsimile (508) 295-8107

TEST PROTOCOL

PROTOCOL TITLE: Protocol for Conducting a 96-Hr Acute Toxicity Test with the Alga, *Selenastrum capricornutum* Following TSCA 797-1050.

TO BE COMPLETED BY THE STUDY SPONSOR:

Study Sponsor: American Petroleum Institute

Address: 1220 L Street, Northwest

Washington, D.C. 20005 **Phone:** (202) 682-8300

Sponsor Protocol/Project No.:

Test Substance: Tert-Amyl Methyl Ether, ("TAME")

Purity: 94% **CAS# or LOT#:** 02814BZ

Additional Comments and/or Modifications:

Sponsor Approval 10/6/92 Date

TO BE COMPLETED BY SLI PRIOR TO TEST INITIATION:

Testing Facility: Springborn Laboratories, Inc. Project #: 12827.~~6542~~.0692.0 6106.430

Study Director: J. Hoberg

Test Concentrations: To be supplied by Amendment

Solvent Used: NA CAS# or LOT#: NA

Proposed Schedule: (Start) 1 Nov 92 (Completion) 1 Dec 92

Additional Comments and/or Modifications: ① IE, JCH 11/9/92

Study Director 10/16/92 Date

PROTOCOL FOR CONDUCTING A 96-HR ACUTE TOXICITY TEST WITH THE ALGA, *SELENASTRUM CAPRICORNUTUM* FOLLOWING TSCA 797-1050

OBJECTIVE

The objective of this test is to determine the effects of a test material on growth (culture density) of *Selenastrum capricornutum*, a freshwater green alga, under static conditions. Test results are reported as 24-, 48-, 72- and 96-hour EC10, EC50 and EC90 values, i.e., the concentration of test material that reduces the growth of the test population by 10, 50 and 90%, respectively. This protocol conforms to EPA Toxic Substances Control Act Test Guideline §797.1050 (Fed. Reg. 50(188):39321-39323, 27 September 1985, as amended in Fed. Reg. 52(97):19056-19082, 20 May 1987).

MATERIALS AND METHODS

TEST ORGANISMS:

1. Species. The test organism is the freshwater green alga *Selenastrum capricornutum* (Chlorophyta).

2. Culture Conditions. The algae are cultured in a synthetic growth medium (see below) in mechanically shaken (approximately 100 rpm) Erlenmeyer flasks under continuous illumination of approximately 5,000 lux. Algal cultures are maintained in an environmental chamber at a temperature of 24±1°C. New cultures are obtained from Carolina Biological Supply Company, Burlington, North Carolina. Transfers are made regularly into fresh medium to provide five- to eight-day-old cultures for test inoculations.

PHYSICAL SYSTEM:

1. Glassware Preparation. All glassware used in testing is thoroughly scrubbed with detergent and rinsed with tap water. This is followed by rinsing with a 10% solution (volume) of reagent nitric acid (HNO_3) followed by a rinse with reagent grade acetone, and a final rinse with distilled water. The cleaned glassware is stored in closed cabinets.

2. Medium Preparation. Stock solutions used in the preparation of algal growth medium are prepared by adding appropriate amounts of nutrients (Table I) to sterile, deionized water. The stocks are stored in the dark at approximately 4°C to minimize photochemical changes, and are renewed every three months. The same stock solutions are used for all media prepared for a given test. The test medium is a synthetic algal assay growth medium prepared by adding appropriate volumes of stock solutions to deionized water. The medium is then autoclaved and allowed to come to room temperature before use. The pH of each batch of medium is adjusted to 7.5 ± 0.1 with dilute hydrochloric acid or sodium hydroxide before use.

3. Test Containers. Test vessels are 125- or 250-mL Erlenmeyer flasks fitted with stainless steel caps which permit gas exchange. Flasks and caps are autoclaved before use. To provide the proper surface-to-volume ratio, the amount of liquid in each 125- or 250-mL flask is approximately 50 or 100 mL, respectively. Selection of the test medium volume depends on the sample requirements for chemical analysis of the test material. Test containers are conditioned prior to use.

CHEMICAL SYSTEM:

1. Test Material. Upon arrival at Springborn Laboratories, the external packaging of the test material is inspected for damage. The packaging is removed and the primary storage container is also inspected for leakage or damage. The sample identity and percent active ingredient are recorded and, unless different arrangements are made with the study Sponsor, the material is stored in the dark at approximately 20°C until used.

2. Analytical Measurements. Test concentrations are analytically confirmed in a sample of each test solution collected at test initiation (before distribution of test solutions into test vessels), and a composite sample from the three replicate vessels of each treatment at test termination. At each sampling interval, three Quality Control (QC) spikes of concentration similar to the test solution concentrations are analyzed.

TESTING PROCEDURE:

1. Range-Finding Test. To assist in selection of test concentrations, a preliminary test is conducted without replication using widely spaced test concentrations. If the highest concentration tested (water saturation concentration or 1000 mg/L) results in growth reduction of less than 50%, a definitive test is conducted consisting of one test concentration at either the water saturation concentration or 1000 mg/L.

2. Definitive Test. At least five concentrations, a control, and a solvent control (if appropriate) are tested, with three replicates of each treatment. Except for the control, the nominal concentration of test material in each treatment is approximately 50% of the next higher one. Test concentrations are selected in consultation with the sponsor. Test vessels are conditioned by rinsing with the appropriate test solution. Appropriate volumes of the test solutions are then placed in each flask. Algae are transferred aseptically from the stock culture to each test vessel, with the inoculum volume calculated to yield approximately 10^4 cells/mL. Test flasks are impartially positioned on a shaker in an environmentally controlled room. Agitation is approximately 100 rpm and temperature is maintained at 24±1°C. Cool white fluorescent bulbs provide continuous illumination of approximately 5,000 lux at the surface of the test solutions.

3. Monitoring. Test monitoring includes measurement of culture density in each test vessel at 24, 48, 72 and 96 hours after the start of the test; measurement of conductivity and pH at the

start and finish of the test; measurement of light intensity and shaking rate daily; and continuous measurement of temperature.

Algal culture density is determined by cell counts using a hemacytometer and a compound microscope. One sample is taken from each test vessel, and one count is made on each sample. Whenever feasible, approximately 400 cells per replicate are counted in order to obtain ±10% accuracy at the 95% confidence level. The control cultures should be in logarithmic growth phase at 96 hours. At each observation, records are made of any unusual appearances of the algal cells including cell size, shape, color, occurrence of flocculation, adherence to glass walls, and/or aggregation.

At test termination the following procedure is used to differentiate between algistatic and algicidal effects. A 0.5-mL sample of culture is taken from each replicate of the lowest test concentration which completely inhibited algal growth. (If algal growth is not completely inhibited at any test concentration, the highest test concentration that inhibited growth is used.) These samples are combined in a new test vessel, and sufficient fresh algal growth medium is added to dilute the test chemical to a concentration that does not affect growth. This subculture is incubated under test conditions for up to 9 days, during which time it is examined microscopically each day to determine whether growth has resumed. As soon as new growth is observed the subculture test is discontinued.

STATISTICAL ANALYSIS.

For each 24-hour observation interval, EC10, EC50 and EC90 values (the concentrations of test material which reduced culture density by 10, 50 and 90%, respectively) and 95% confidence limits are determined by linear regression of response (percent reduction of culture density, as compared with controls) vs. exposure concentration. Four linear regression curves are computed based on (a) untransformed data, (b) untransformed response vs. logarithm-transformed concentration, (c) probit-transformed response vs. untransformed concentration, and (d) probit-transformed response vs. logarithm-transformed concentration. The regression line which provides the best fit of the untransformed or transformed data is selected based on the highest coefficient of determination, i.e., r^2. This regression equation is then applied to calculate the EC10, EC50 and EC90 values and their 95% confidence limits, using the method of inverse prediction (Sokal and Rohlf, 1981). A computer program developed and validated at Springborn Laboratories is used to assist in these computations.

REPORTING

The raw data and final draft of the report are reviewed by the Quality Assurance Unit and Study Director. All values of chemical and water quality measurements are reported to various levels of significance depending on the accuracy of the measuring devices employed during any one process. A single copy of the draft report will initially be submitted to the study Sponsor for review. Upon acceptance by the Sponsor, three copies of the final report will be submitted. All reports include, but are not limited to, the following information.

- Springborn Laboratories report number and study number, and sponsor protocol and project numbers.
- Laboratory and site, dates of testing, and personnel involved in the study—Quality Assurance Unit, Program Coordinator (if applicable), Study Director, Principal Investigator(s).
- Identification of test material by sponsor I.D. and sample No., percent active ingredient, color, form, and date received at Springborn Laboratories.
- Reference to laboratory notebook or other file containing the raw data.
- Date the definitive test was conducted.
- Scientific name of the test organism, source, and culturing information.
- Test container volume, test solution volume, and inoculum culture density.
- Description of exposure solution preparation scheme.
- Description of test conditions.
- Criteria for determination of toxic effects and general observations on nonquantifiable effects.
- A table of culture density measurements for each 24-hour interval.
- Data on test temperatures, specific conductivity, pH and illumination.
- The EC10, EC50 and EC90 values and 95% confidence limits at 24, 48, 72 and 96 hours, when possible, and calculation methods used.
- Results of post-exposure subculture of growth-inhibited cells in fresh medium.
- Reference (or inclusion as an appendix) of any analytical procedures used, if applicable.
- Analytical results of test solution measurements and QA samples, if applicable.
- Table of analytical precision and accuracy data from a recovery study of the test material in algal growth medium.
- Deviations from the protocol not addressed in protocol amendments will be listed, together with a discussion of their impact on the study, signed by the Principal Investigator and Study Director.
- Good Laboratory Practice (GLP) compliance statement, signed by the Study Director.
- Dates of Quality Assurance reviews and reports to management of GLP compliance, signed by the QA unit.

SPECIAL PROVISIONS

GOOD LABORATORY PRACTICES (GLP): All test procedures, documentation, records, and reports will comply with the U. S. Environmental Protection Agency's Good Laboratory Practices as promulgated under the Toxic Substances Control Act (FEDERAL REGISTER, Part III, 17 August, 1989).

TEST MATERIAL DISPOSAL: After 60 days of the issuance of the final test report, the test material will be returned to the Sponsor's project officer, at Sponsor expense, unless different arrangements are made.

REFERENCES

U.S. EPA. 1985. Algal Acute Toxicity Test. Fed. Reg. Vol. 50, No. 188, Section 797.1050. 27 September 1985, updated June 19, 1987.

Sokal, R.R., and F.J. Rohlf. 1981. Biometry. 2nd Ed. Chapter 14, pp. 496-498. W.H. Freeman and Co., New York. 859 pp.

TABLE I

MBL Algal Growth Medium

Compound	Final Concentration (mg/L)
$CaCl_2$ $2H_2O$	36.76
$MgSO_4$ $7H_2O$	36.97
$NaHCO_3$	12.60
K_2HPO_4	8.71
$NaNO_3$	85.01
$FeCl_3$ $6H_2O$	1.575
$CuSO_4$ $5H_2O$	0.01
$CoCl_2$ $6H_2O$	0.01
$ZnSO_4$ $7H_2O$	0.022
$MnCl_2$ $4H_2O$	0.18
Na_2MoO_4 $2H_2O$	0.006
H_3BO_3	1.0
Na_2 EDTA[a]	4.36

[a] EDTA is included in medium for algal cultures but not included in medium for testing purposes.

SOURCE: H.W. Nichols. 1973. Growth media—freshwater. pp. 7-24 IN: J. Stein. ed., *Handbook of Phycological Methods. Culture Methods and Growth Measurements.* Cambridge University Press.

Springborn Laboratories, Inc.
Environmental Sciences Division
790 Main Street • Wareham, Massachusetts 02571 • (508) 295-2550 • Telex 4436041 • Facsimile (508) 295-8107

PROTOCOL AMENDMENT

AMENDMENT #: 1

DATE: 18 November 1993

PROTOCOL TITLE: Protocol for Conducting a 96-Hr Acute Toxicity Test with the Alga, *Selenastrum capricornutum* Following TSCA 797-1050.

SPECIES: *S. capricornutum*

STUDY SPONSOR: American Petroleum Institute

TEST MATERIAL: Tert-Amyl Methyl Ether, ("TAME")

SLI STUDY NO: 12827.0592.5106.430

AMENDMENT(S):

Based on the potential for the test material to volatilize from the test system, the following modifications have been made in the study protocol. These modifications are in agreement with current U.S. EPA requirements for testing a volatile substance in a static algal test system.

1. The protocol states that 250 mL test vessels will contain 100 mL of test solution and be capped with a stainless steel gas-exchange caps. During this study, each test vessel (Erlenmeyer flask) will be completely filled with test solution (zero headspace) and then tightly capped with a silicone stopper to prevent volatilization of the test substance. The solution volume will be approximately 275 mL.

2) The protocol states that three replicate flasks will be established for each concentration or control. During this study, the number of replicate test vessels will be increased to 12 per concentration. The additional test vessels will allow three vessels at each interval (ie., 24, 48, 72 and 96 hours) to be sampled, monitored for cell density and discarded, since a substantial amount of TAME will be lost from the system once opened.

3) The protocol states that Marine Biological (MBL) freshwater medium will be used as the culture and dilution water. The test organism is currently maintained in Algal Assay Procedure (AAP) medium, a similar medium to MBL medium. Consequently, the test dilution water will be AAP medium. The composition of AAP medium is presented in the attached table.

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4) Due to testing in a capped system, dissolved bicarbonate will become the limiting factor for cell growth. Consequently, to provide sufficient enough algal growth to evaluate the toxicity of the test substance, an additional 500 mg/L of sodium bicarbonate ($NaHCO_3$) will be added to the AAP medium.

5) In additional to the control (AAP medium plus 500 mg/L sodium bicarbonate but without TAME), a reference control will be initiated in standard AAP medium. The reference control will consist of three 125 mL Erlenmeyer flasks fitted with gas-exchange caps and containing 50 mL of AAP medium without additional sodium bicarbonate. This culture will demonstrate that the algal culture used to inoculate the toxicity test will meet the standard performance criterion (i.e., the control be in log phase growth after 96 hours) when cultured under standard conditions.

6) The protocol states an additional exposure will be conducted at termination of the toxicity test to determine if the test substance has algistatic or algicidal properties. This exposure will also be conducted in a capped flask system as described above for the definitive test.

7) The protocol states the light intensity of the culture and test area will be approximately 5000 lux. To clarify the acceptable range of light intensity, the study will be conducted within a light intensity range of 3200 - 5400 lux (300 - 500 footcandles).

8) The definitive test will be conducted at the following nominal concentrations: 5.0, 2.5, 1.3, 0.63, 0.31, 0.16, 0.080 and 0.040 mg A.I./L.

Approval Signatures: James R. Hoberg 18 Nov 93

James R. Hoberg
SLI Study Director

Date

Richard A. Rhoden 12/9/93

Richard A. Rhoden, Ph.D.
Study Sponsor Representative

Date

Springborn

Table 1. **ALGAL ASSAY PROCEDURE (AAP) MEDIUM**

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 ($K_2HPO_4 \cdot 3H_2O$)	1.044 (1.368) mg/L
$NaHCO_3$	15.0 mg/L
H_3BO_3	185.5 µg/L
Na_2SeO_4[a]	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
$Na_2MoO_4 \cdot 2H_2O$	7.25 µg/L
$FeCl_3 \cdot 6H_2O$	159.3 µg/L
$Na_2SiO_3 \cdot 9H_2O$[b]	20.0 mg/L
$Na_2EDTA \cdot 2H_2O$	300.0 µg/L

Final pH is adjusted to 7.5 ± 0.1

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

[b] $Na_2SiO_3 \cdot 9H_2O$ is added to medium used for diatoms (*N. peliculosa*) only.

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

7.0 APPENDIX II - CERTIFICATE OF ANALYSIS

aldrich chemical co.
chemists helping chemists in research & industry

SPRINGBORN LABORTORIES
508 295 8107
PAULA LECONTE

PO NBR:

PRODUCT INFORMATION

PRODUCT NUMBER: 28309-6 LOT NUMBER: 02814BZ

PRODUCT NAME: TERT-AMYL METHYL ETHER, 94%

FORMULA: C6H14O FORMULA WEIGHT: 102.18

APPEARANCE	COLORLESS LIQUID
REFRACTIVE INDEX AT 20 DEG C	1.3885
INFRARED SPECTRUM	CONFORMS TO STRUCTURE AND STANDARD AS ILLUSTRATED ON PAGE 268A OF EDITION I, VOLUME 3 OF "THE ALDRICH LIBRARY OF FT-IR SPECTRA".
GAS LIQUID CHROMATOGRAPHY	98.8 %

Aldrich Chemical Company
DAVID SWESSEL
NOVEMBER 11, 1992

aldrich chemical co.
P.O. Box 355, Milwaukee, Wisconsin 53201 USA • (414) 273-3850

8.0 APPENDIX III - DILUTION WATER ANALYSIS

Well[1] Water Sample*		
Date Collected: 7/29/93 Date Reported: 9/17/93		
Pesticide Screen I;II;III	Result As Received	Limit of Quantitation
Alpha BHC	< 0.01 µg/l	0.01
Beta BHC	< 0.01 µg/l	0.01
Gamma BHC - Lindane	< 0.01 µg/l	0.01
Delta BHC	< 0.01 µg/l	0.01
Heptachlor	< 0.01 µg/l	0.01
Aldrin	< 0.01 µg/l	0.01
Heptachlor Epoxide	< 0.01 µg/l	0.01
DDE	< 0.01 µg/l	0.01
DDD	< 0.01 µg/l	0.01
DDT	< 0.01 µg/l	0.01
HCB	< 0.01 µg/l	0.01
Mirex	< 0.01 µg/l	0.01
Methoxychlor	< 0.05 µg/l	0.05
Dieldrin	< 0.01 µg/l	0.01
Endrin	< 0.01 µg/l	0.01
Telodrin	< 0.01 µg/l	0.01
Chlordane	< 0.3 µg/l	0.3
Toxaphene	< 4. µg/l	4.
PCBs	< 1. µg/l	1.
Ronnel	< 0.01 µg/l	0.01
Ethion	< 0.02 µg/l	0.02
Trithion	< 0.05 µg/l	0.05
Diazinon	< 0.1 µg/l	0.1
Methyl Parathion	< 0.02 µg/l	0.02
Ethyl Parathion	< 0.02 µg/l	0.02
Malathion	< 0.05 µg/l	0.05
Endosulfan I	< 0.01 µg/l	0.01
Endosulfan II	< 0.01 µg/l	0.01
Endosulfan Sulfate	< 0.03 µg/l	0.03
[1] Well water supplemented by Town of Wareham water		
* Analyzed by Lancaster Laboratories, Inc.		

Well[1] Water Sample*		
Date Collected: 8/9/93 Date Reported: 8/26/93		
Analysis	Result As Received	Limit of Quantitation
Mercury	< 0.00020 mg/l	0.00020
Arsenic	< 0.20 mg/l	0.20
Selenium	< 0.20 mg/l	0.2
Boron	< 0.040 mg/l	0.04
Thallium	< 0.30 mg/l	0.3
Aluminum	< 0.20 mg/l	0.2
Antimony	< 0.20 mg/l	0.2
Barium	< 0.10 mg/l	0.1
Beryllium	< 0.010 mg/l	0.01
Cadmium	< 0.010 mg/l	0.01
Calcium	7.71 mg/l	0.2
Chromium	< 0.050 mg/l	0.05
Cobalt	< 0.050 mg/l	0.05
Copper	< 0.020 mg/l	0.02
Iron	< 0.10 mg/l	0.1
Lead	< 0.10 mg/l	0.1
Magnesium	2.31 mg/l	0.1
Manganese	< 0.010 mg/l	0.01
Molybdenum	< 0.10 mg/l	0.1
Nickel	< 0.050 mg/l	0.05
Potassium	1.07 mg/l	0.5
Silver	< 0.020 mg/l	0.02
Sodium	14.0 mg/l	0.4
Titanium	< 0.010 mg/l	0.01
Vanadium	< 0.010 mg/l	0.01
Zinc	<0.040 mg/l	0.04
Total Organic Carbon ***	< 1. mg/L	1.
[1] Well water supplemented by Town of Wareham water		
* Analyzed by Lancaster Laboratories, Inc.		
*** Represents "non-purgeable TOC"		

9.0 APPENDIX IV - ANALYTICAL METHODOLOGY

SUMMARY

An analytical methodology is presented for the measurement of TAME (Tert-Amyl Methyl Ether) in AAP media, filtered seawater and freshwater (reconstituted to increase hardness). All water samples were analyzed either by direct sampling into a purge and trap liquid sample concentrator or vial sampling system. The water phase was stripped of TAME with a high flow of helium gas and trapped on an active support material. The TAME was then thermally desorbed from the support and transferred through a heated line onto a gas chromatograph for separation and quantitation. TAME was detected utilizing a flame ionization detector. Quantitation was performed using various fitting techniques both on and off the instrument.

Mean recovery from AAP media was 89.7 ± 2.3%, 104 ± 11% for filtered seawater and 102 ± 10 for freshwater. Repeatability of TAME analysis showed a 5.4% relative standard deviation (%RSD) at 0.026 mg/L from water.

EQUIPMENT AND REAGENTS

Equipment

1. Balance: Mettler AE 200 182, four-place analytical
2. Volumetric flask: grade A, assorted sizes
3. Wheaton vials with teflon-lined crimp top lids, assorted sizes
4. Syringes: Hamilton, assorted sizes, gas tight and valved
5. Absorbent Trap: 25 cm x 0.125 O.D. stainless steel column packed with 1 cm 3% OV -1, 15 cm tenax and 8 cm silica gel.
6. Purge and Trap Liquid Sample Concentrator: Tekmar model LSC-2000
7. Vial Sampling System: Tekmar Model ALS2050
8. Gas chromatograph: Hewlett-Packard 5890A equipped with a capillary injection port and 105 m x 0.53 mm I.D. 3 μm film RT_x 502.2 column and Flame Ionization detector.

Reagents

1 Methanol: reagent grade solvent

2. TAME: Lot # 02814BZ, was received from Experimental Pathology Labs, Inc., on 17 August 1992 and was identified by the Sponsor to be 98.8% pure.

3. Water: All solutions were prepared using water from a Sybron/Barnstead NANOpure II® (meets ASTM Type IIA specifications) filtered and sterilized water purification system. The filtered sterilized water typically shows greater than 16.7 Mohm-cm resistivity and less than 1 mg/L total organic carbon.

4. AAP Media

5. Filtered seawater

6. Hard Reconstituted water

PROCEDURE

Preparation of Stock Solution

Primary standards were prepared by placing approximately nine and a half milliliters (mL) of methanol into a 10 mL volumetric flask. The flask was allowed to stand unstoppered to allow any methanol along the neck to evaporate and was weighed to the nearest 0.1 milligram (mg). TAME was immediately added to the flask using a microliter syringe, making sure the primary material fell directly into the alcohol. The vessel was reweighed, diluted to the mark, stoppered, and finally mixed by inverting the flask several times.

The solution was transferred to a 10 mL crimp top bottle with a Teflon lined lid and stored in a refrigerator until used. This stock was used with further dilution for sample fortification and standard(s) preparation. All stock solutions and dilutions were stored in Wheaton vials with Teflon lined crimp tops in a refrigerator.

Preparation of Standards for Purge & Trap

Secondary standards (104, 26.0 and 5.20 mg/L in methanol) were drawn into a microliter syringe and spiked directly into water in a 5 mL gas tight Luer lock syringe. These aqueous standards were added directly to the purge vessel and analyzed immediately. Calibration and

check standards were prepared just prior to analysis. Standards were prepared in a 5 mL gas-tight syringe using TAME working standards. Examples of formulation working standard formulation are outlined below:

Stock Concentration (mg/L)	Volume Taken (μL)	Nominal Concentration (mg/L)
5.20	25.0	0.026
26.0	25.0	0.130
26.0	50.0	0.260
26.0	100	0.520
26.0	250	1.30
104	250	5.20
104	500	10.4

Sample Fortification

Method validation/recovery samples were prepared using AAP media, filtered seawater and freshwater (reconstituted to increase hardness). Samples were fortified with dilutions of the TAME stock in volumetric flasks and loaded onto a automatic liquid sample autosampler (LSC 2050). The fortified levels produced were 0.052, 4.16 and 10.4 mg/L TAME in AAP media, 0.026, 4.16 and 10.4 mg/L in filtered seawater and 49.7, 248 and 695 mg/L in freshwater (reconstituted to increase hardness). Three replicates at each level were prepared for each experiment along with three unfortified matrix blanks.

Liquid Sampler

Samples were loaded into 40 mL vials. Vials were placed in vial sampler. Five milliliters sample was transferred from the vial samples into the purge vessel attached in-line with the activated sorbent support matrix (EPA method 624 trap) and the stripping program initiated with a high flow of helium (60 mL/min) bubbled through the vessel. The sorbent trapped gaseous TAME from the helium carrier gas. This approach was effective because the compound is highly volatile. After the water phase had been stripped for four or six minutes, the sorbent trap was heated and TAME stripped into the carrier and brought through a heated capillary transfer line

(0.53 mm I.D fused silica) onto the top of the gas chromatographic column located in a capillary injection port of the gas chromatograph.

TAME was separated chromatographically using a temperature program after splitless injection from the purge and trap liquid sample concentrator.

Liquid Sample Concentrator: Tekmar LSC-2000.

Programmed Purge & Trap Conditions

Standby Temperature: 40 ° C

	Time (minutes)	Temperature (° C)
Purge:	4 or 6	< 40
Desorption Preheat:	NA	175
Desorption:	4.0	180
Bake:	8.0	225

Heating Zones

	Temperature °C
Valve:	200
Mount:	40
Transfer Line:	200

Gas Chromatography

Gas chromatographic analysis was conducted utilizing a directly coupled liquid sample concentrator (purge and trap) into the capillary injection port. The samples were introduced by programmed injection from the purge and trap. The refocusing of sample entered the column occurred at the head of the column as a function of the film thickness of the RT_x 502.2 column.

Gas Chromatograph: Hewlett Packard 5890A gas chromatograph equipped with a split/splitless capillary injection port operated in the splitless mode.

Column:	105 m x 0.53 mm ID x 3 μm film
	Temperature (°C): Injector: 200
	column temperature programmed: 40 - 250
	Rate: 10 °C per minute from 40 to 70 °C
	25 °C per minute from 70 - 250 °C
Gas (mL/minute):	Helium
	Carrier Gas: ca. 9
Makeup gas(mL/minute):	Helium (28)
	Run Time: 16 minutes
	Retention Time: ca. 12.4 minutes

Integrator: Hewlett Packard 3396A II programmable integrator

Analysis

TAME was analyzed utilizing purge and trap thick film capillary (0.53 mm I.D.) gas chromatography flame ionization detection (GC/FID). Water samples were loaded onto the purge vessel (5 mL) of the LSC-2000 using a 5 mL gas tight syringe or vial transfer line from the vial sampler. The purge program was initiated and the systems allowed to sequence through the preprogrammed methods (purge and trap, gas chromatograph and integrator).

RESULTS AND DISCUSSION

Analytical results for the recovery of TAME from AAP media, filtered seawater and freshwater (reconstituted to increase hardness) are presented in Table 1A, 2A and 3A, respectively. System performance was tested for system repeatability in water. Results of repeatability studies are presented in Table 4A. Run time for samples was approximately 27 minutes. Samples were introduced through the capillary injection port operated in the splitless mode onto the gas chromatographic column. The split vent was closed for the 4 minutes of

desorb on the purge and trap. Figure 1A is a representative chromatogram of TAME analysis by purge and trap GC-FID.

TAME analysis was generally linear (correlation coefficient, r^2, greater than 0.98) from 0.25 mg/L TAME in water through 5.0 mg/L (Figure 2A). Detector response was not linear, rather there is a notable curve apparent in detector response from 0.026 though 10.4 mg/L TAME (Figure 3A). The integrator had software to fit calibration data to polynomial fit. Recovery samples for AAP media and filtered seawater were calculated using a least squares polynomial analysis performed on the height response. Recovery from freshwater (reconstituted to increase hardness) samples were calculated using a least squares linear regression analysis performed on the height response.

The reports generated by the integrator were categorized in a report with concentration (mg/L) calibrated from a 5-mL sample. Check standards were evaluated periodically and providing up-to-date evaluation of system calibration. Calibration was monitored utilized a series of stock standards in methanol. Evaluation was based on the trend of results and the reported value for that standard. Working standards were prepared around the concentration range of interest and stored along with other operating information on the integrator. Calibration could be conducted using linear, polynomial or point to point fitting techniques.

Table 1A. Analytical results for the recovery of TAME from AAP media.

Nominal Concentration (mg/L)	Volume Purged (mL)	Concentration Recovered (mg/L)	Percent Recovered (%)
10.4	5.00	8.92	85.8
10.4	5.00	9.17	88.1
10.4	5.00	9.39	90.3
4.16	5.00	3.79	91.1
4.16	5.00	3.88	93.2
4.16	5.00	3.84	92.3
0.052	5.00	0.0462	88.9
0.052	5.00	0.0462	88.9
0.052	5.00	0.0462	88.9
Control	5.00	< 0.026	NA
Control	5.00	< 0.026	NA
Control	5.00	< 0.026	NA

Mean Recovery: 89.7 ± 2.3%

The minimum detectable concentration was 0.026 mg/L for a 5.00 mL sample which is the lowest standard used in the polynomial fit.

Table 2A. Analytical results for the recovery of TAME from filtered seawater.

Nominal Concentration (mg/L)	Volume Purged (mL)	Concentration Recovered (mg/L)	Percent Recovered (%)
10.4	5.00	10.0	96.3
10.4	5.00	12.1	116
10.4	5.00	12.1	117
10.4	5.00	11.9	114
4.16	5.00	3.79	91.1
4.16	5.00	3.78	90.9
4.16	5.00	3.79	91.2
0.026	5.00	0.027	105
0.026	5.00	0.027	105
0.026	5.00	0.028	109
Control	5.00	< 0.026	NA
Control	5.00	< 0.026	NA
Control	5.00	< 0.026	NA

Mean Recovery: 104 ± 11%

The minimum detectable concentration was 0.026 mg/L for a 5.00 mL sample which is the lowest calibration standard used in the polynomial fit.

Table 3A. Analytical results for the recovery of TAME from freshwater (reconstituted to increase hardness).

Nominal Concentration (mg/L)	Dilution Factor	Volume Purged (mL)	Concentration Recovered (mg/L)	Percent Recovered (%)
695	200	5.00	694	99.8
695	200	5.00	693	99.6
695	200	5.00	705	101
248	100	5.00	268	108
248	100	5.00	258	104
248	100	5.00	265	107
49.7	20.0	5.00	50.9	102
49.7	20.0	5.00	44.9	90.3
49.7	20.0	5.00	51.7	104
Control	1.00	5.00	< 0.248	NA
Control	1.00	5.00	< 0.248	NA
Control	1.00	5.00	< 0.248	NA

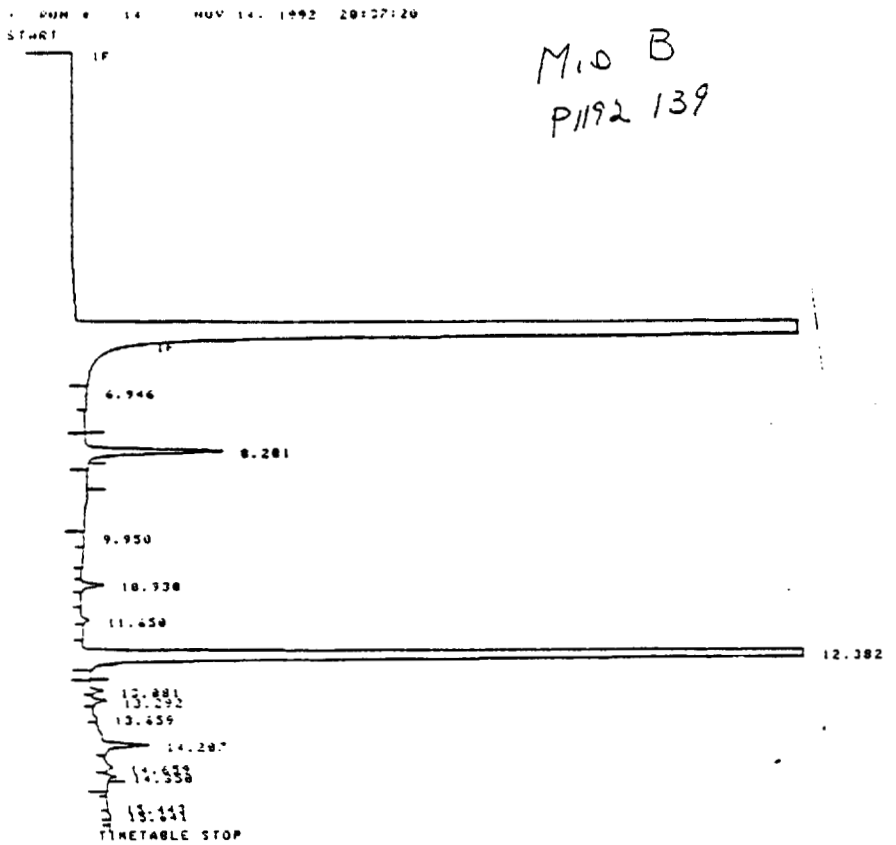
Mean Recovery: 102 ± 10%

The minimum detectable concentration was 0.248 mg/L for a 5.00 mL sample which is the lowest standard used in the linear regression analysis.

Table 4A. Repeatability of TAME analysis from ASTM Type II water at 0.026 mg/L.

Replicate	Area	Height
1	47510	5725
2	54711	6099
3	46909	5631
4	36628	5646
5	36305	5699
6	55640	6292
7	54256	6365
Mean:	47423	5922
Std Dev.:	8243	320
% RSD:	17.4	5.4

Figure 1A. A representative chromatogram of TAME purge and trap GC/FID analysis.



RUN# 14 NOV 14, 1992 20:37:20

METHOD NAME: M:\TAMEP\GTP.MET

IDENTIFIER 1: 31400039449

~~CORRECTION~~ TAME
ER QA 1846.92

ESTD-HEIGHT

RT	TYPE	AREA	WIDTH	HEIGHT	CAL#	AMT	NAME
8.201	PB	156375	.107	24263		.000	
10.930	PP	18711	.082	3817		.000	
12.382	PB	3787178	.103	612842	1R	3.596	TAME
13.242	VF	11181	.071	2618		.000	
14.207	VV	69806	.129	8923		.000	
14.459	VV	17942	.164	1825		.000	

TOTAL HEIGHT= 654298
MUL FACTOR=1.0000E+00

Figure 2A. **A representative linear regression analysis from standard TAME analysis.**

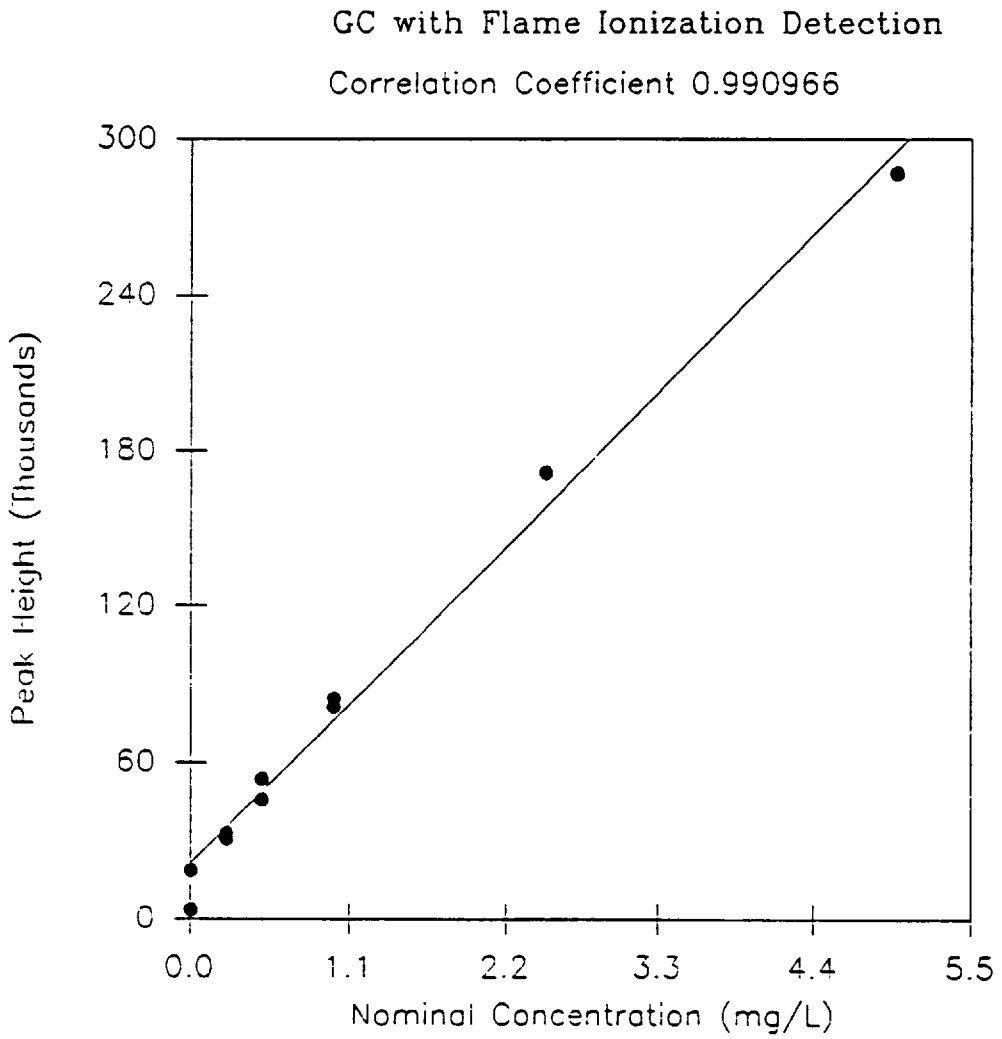


Figure 3A. **A representative polynomial regression analysis from standard TAME analysis.**

#1 TAME

12827-0692-6100-250 PAGE 6
METHOD VALIDATION

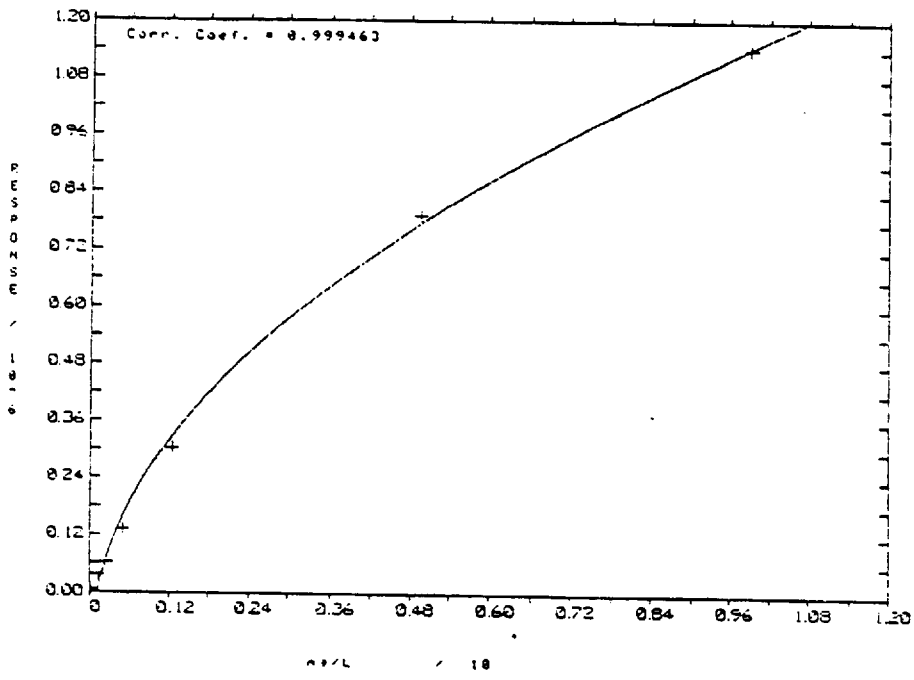
```
WELCOME TO THE HP3396 CALIBRATION CURVE PLOTTING PROGRAM Rev. B.02.00

At any prompt:     'Q' [ENTER] Quits
                   'S' [ENTER] Starts Over

Load which method or calib. file [Current active]:

Plot the calibration curve for which CAL # [All]:

                    mg/L       vs. Response for Cal #  1
mg/L        = +1.16E-01 +1.37E-06 (RESPONSE) +6.26E-12 (RESPONSE^2)
```




```
Plot additional peaks (Y=/N): N


* EDIT: CALIB  2

1 = CALIB PROCEDURE
2 = RETENTION TIME WINDOWS
3 = TABLE ENTRIES
4 = PEAK GROUPS
5 = CALIB OPTIONS
```

10.0 APPENDIX V - EXCERPTED COPIES OF RAW DATA

4

Springborn Laboratories, Inc.

QC Table

****

Sponsor: American Petroleum Institute
Test Material: Tame
Project No.: 12827-692-6106-430
Test Type: Selenastrum acute
Sample Date(s): 0hr (18-Nov-93) & 96hr (22-Nov-93)
Data Entered By: JPS

****

Sample ID	Nominal Concentration (mg/L)	Interval	Analytical Result (mg/L)	Mean Analytical Result (mg/L)	Mean % of Nominal
1193-1329	0.00	0 HR	<0.010	NA	NA
1193-1342	0.00	96 HR	<0.010	NA	NA
1193-1329	0.040	0 HR	0.023	0.017	42.0
1193-1341	0.040	96 HR	0.010		
1193-1328	0.08	0 HR	0.043	0.037	46.7
1193-1340	0.08	96 HR	0.032		
1193-1327	0.16	0 HR	0.083	0.067	41.6
1193-1339	0.16	96 HR	0.050		
1193-1326	0.31	0 HR	0.27	0.23	75.2
1193-1338	0.31	96 HR	0.19		
1193-1325	0.63	0 HR	0.75	0.48	75.8
1193-1337	0.63	96 HR	0.21		
1193-1324	1.3	0 HR	0.77	0.52	40.2
1193-1336	1.3	96 HR	0.27		
1193-1323	2.5	0 HR	1.6	1.4	56.5
1193-1335	2.5	96 HR	1.2		
1193-1322	5.0	0 HR	5.7	3.7	73.3
1193-1334	5.0	96 HR	1.6		
			Total Mean % of Nominal:		56.4

Springborn Laboratories, Inc.

QC Table

Sponsor: American Petroleum Institute
Test Material: Tame
Project No.: 12827-692-6106-430
Test Type: Selenastrum acute
Sample Date(s): Ohr (18-Nov-93) & 96hr (22-Nov-93)
Data Entered By: JPS

Sample ID	Nominal Concentration (mg/L)	Interval	Analytical Result (mg/L)	Mean Analytical Result (mg/L)	Mean % of Nominal
1193-1332 qc	1.00	0 HR	0.979	0.849	78.4
1193-1344 qc	1.00	96 HR	0.718		
1193-1333 qc	10.0	0 HR	7.96	6.65	93.3
1193-1345 qc	5.00	96 HR	5.35		
1193-1331 qc	0.04	0 HR	0.047	0.0659	112.0
1193-1343 qc	0.08	96 HR	0.085		
			Total Mean % of Nominal:		94.6

ALGAL GROWTH CALCULATIONS Page 37 Study No. 12827-0592-6106-430

DENSITY CALCULATIONS

Cell density (10^4 cells/mL) is calculated from raw data by the formula:
Cell Density = #Cells/(#Fields * Field Volume).
Means and standard deviations for each treatment group are calculated.
Field Volume is the volume of a hemocytometer grid 0.1 x 0.1 x 0.01 cm.

FIELD VOLUME = 1 x 10^(-4) cm3 = 0.0001 mL

	24	48	72	96
Control REP A	3.25	38.75	18.00	69.00
Control REP B	2.25	34.75	27.50	63.75
Control REP C	1.75	35.25	26.00	63.25
Mean	2.42	36.25	23.83	65.33
S.D.	0.76	2.18	5.11	3.19
0.040 mg/L REP A	2.00	7.00	8.50	53.00
0.040 mg/L REP B	2.50	7.75	11.75	83.25
0.040 mg/L REP C	2.00	10.25	12.25	68.50
Mean	2.17	8.33	10.83	⑥68.25
S.D.	0.29	1.70	2.04	15.13
0.080 REP A	3.00	5.25	7.50	47.75
0.080 REP B	4.00	2.25	10.00	45.50
0.080 REP C	2.00	4.50	16.50	46.75
Mean	3.00	4.00	11.33	①46.67
S.D.	1.00	1.56	4.65	1.13
0.16 REP A	1.25	5.50	11.50	37.25
0.16 REP B	1.75	7.50	7.75	25.50
0.16 REP C	1.00	2.75	20.75	27.75
Mean	1.33	5.25	①13.33	①30.17
S.D.	0.38	2.38	6.69	6.24
0.31 REP A	1.50	10.00	8.50	17.25
0.31 REP B	1.25	6.75	12.75	18.50
0.31 REP C	1.50	3.50	9.25	13.00
Mean	1.42	6.75	①10.17	①16.25
S.D.	0.14	3.25	2.27	2.88
0.63 REP A	0.75	7.25	7.25	10.75
0.63 REP B	0.50	5.25	7.00	11.75
0.63 REP C	1.00	9.50	8.75	14.25
Mean	0.75	⑤7.33	⑤7.67	①12.25
S.D.	0.25	⑤2.13	0.95	1.80
1.3 REP A	2.75	3.50	5.00	6.50
1.3 REP B	0.75	4.50	4.25	8.75
1.3 REP C	1.25	5.75	5.50	6.25
Mean	1.58	⑤4.58	⑥4.92	①7.17
S.D.	1.04	⑤1.13	0.63	1.38

① Include in EC calculations—JRH 11-28-93.
② Do not calculate EC's due to lack a a clear - conc. response—JRH 11-28-93
③ Exclude 48 hr. data from EC's based on ②—JRH 5-9-94.

LV
11/23/93

ALGAL GROWTH CALCULATIONS Page_____ Study No. 12827-0592-6106-430

2.5	REP A	1.25	2.75	2.25	3.25
2.5	REP B	0.75	4.00	3.00	4.75
2.5	REP C	0.25	4.75	3.50	3.50
	Mean	0.75	3.83	2.92	3.83
5.0	S.D.	0.50	1.01	0.63	0.80
10.0	REP A	0.50	3.50	2.75	2.00
10.0	REP B	0.75	2.00	2.25	2.50
10.0	REP C	1.50	2.50	1.50	3.50
	Mean	0.92	2.67	2.17	2.67
	S.D.	0.52	0.76	0.63	0.76

11/23/93

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