



Health and Environmental
Sciences Department

Tertiary-Amyl Methyl Ether (TAME)

Toxicity to the Freshwater Alga, *Selenastrum capricornutum*

FEBRUARY 1995

TOXICOLOGY REPORT NUMBER 402
CAIS ABSTRACT NO. 41-5415



Documents

Document Sharing Hub



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.

API ENVIRONMENTAL MISSION AND GUIDING ENVIRONMENTAL PRINCIPLES

The members of the American Petroleum Institute are dedicated to continuous efforts to improve the compatibility of our operations with the environment while economically developing energy resources and supplying high quality products and services to consumers. The members recognize the importance of efficiently meeting society's needs and our responsibility to work with the public, the government, and others to develop and to use natural resources in an environmentally sound manner while protecting the health and safety of our employees and the public. To meet these responsibilities, API members pledge to manage our businesses according to these principles:

- ❖ To recognize and to respond to community concerns about our raw materials, products and operations.
- ❖ To operate our plants and facilities, and to handle our raw materials and products in a manner that protects the environment, and the safety and health of our employees and the public.
- ❖ To make safety, health and environmental considerations a priority in our planning, and our development of new products and processes.
- ❖ To advise promptly, appropriate officials, employees, customers and the public of information on significant industry-related safety, health and environmental hazards, and to recommend protective measures.
- ❖ To counsel customers, transporters and others in the safe use, transportation and disposal of our raw materials, products and waste materials.
- ❖ To economically develop and produce natural resources and to conserve those resources by using energy efficiently.
- ❖ To extend knowledge by conducting or supporting research on the safety, health and environmental effects of our raw materials, products, processes and waste materials.
- ❖ To commit to reduce overall emission and waste generation.
- ❖ To work with others to resolve problems created by handling and disposal of hazardous substances from our operations.
- ❖ To participate with government and others in creating responsible laws, regulations and standards to safeguard the community, workplace and environment.
- ❖ To promote these principles and practices by sharing experiences and offering assistance to others who produce, handle, use, transport or dispose of similar raw materials, petroleum products and wastes.

FOREWORD

API PUBLICATIONS NECESSARILY ADDRESS PROBLEMS OF A GENERAL NATURE. WITH RESPECT TO PARTICULAR CIRCUMSTANCES, LOCAL, STATE, AND FEDERAL LAWS AND REGULATIONS SHOULD BE REVIEWED.

API IS NOT UNDERTAKING TO MEET THE DUTIES OF EMPLOYERS, MANUFACTURERS, OR SUPPLIERS TO WARN AND PROPERLY TRAIN AND EQUIP THEIR EMPLOYEES, AND OTHERS EXPOSED, CONCERNING HEALTH AND SAFETY RISKS AND PRECAUTIONS, NOR UNDERTAKING THEIR OBLIGATIONS UNDER LOCAL, STATE, OR FEDERAL LAWS.

NOTHING CONTAINED IN ANY API PUBLICATION IS TO BE CONSTRUED AS GRANTING ANY RIGHT, BY IMPLICATION OR OTHERWISE, FOR THE MANUFACTURE, SALE, OR USE OF ANY METHOD, APPARATUS, OR PRODUCT COVERED BY LETTERS PATENT. NEITHER SHOULD ANYTHING CONTAINED IN THE PUBLICATION BE CONSTRUED AS INSURING ANYONE AGAINST LIABILITY FOR INFRINGEMENT OF LETTERS PATENT.

Copyright © 1995 American Petroleum Institute

ACKNOWLEDGMENTS

THE FOLLOWING PEOPLE ARE RECOGNIZED FOR THEIR CONTRIBUTIONS OF TIME AND EXPERTISE DURING THIS STUDY AND IN THE PREPARATION OF THIS REPORT:

API STAFF CONTACT

Richard Rhoden, Ph.D., Health and Environmental Sciences Department

MEMBERS OF THE AIR/FUELS TOXICOLOGY HEALTH EFFECTS WORKGROUP

Russell D. White, Ph.D., *Chairman*, Chevron Research & Technology, Inc.

Rudolph J. Breglia, Ph.D., BP America, Inc.

Charles R. Clark, Ph.D., Unocal

Wayne Daughtrey, Ph.D., Exxon Biomedical Sciences, Inc.

David Duncan, Texaco, Inc.

Francis J. Koschier, Ph.D., ARCO

Charles A. Lapin, Ph.D., ARCO

David C. Logan, M.D., Mobil Corporation

John H. Mitchell, M.D., Amoco Corporation

Susan A. Rodney, Ph.D., Texaco, Inc.

Ceinwen A. Schreiner, Ph.D., Stonybrook Laboratories

David R. Steup, Ph.D., Shell Oil Company

Michael S. Wells, Ph.D., Amoco Corporation

American Petroleum Institute
Health and Environmental Sciences Department

QUALITY ASSURANCE/GLP COMPLIANCE STATEMENT

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

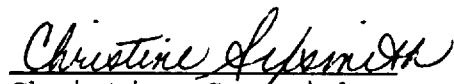
Testing Facility: Springborn Laboratories, Inc.

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.

<u>Date(s) of Inspection/Review</u>	<u>Type of Inspection</u>	<u>Date of Report to Management</u>
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
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

790 Main Street • Wareham, Massachusetts 02571-1075 • (508) 295-2550 • Telex 4436041 • Facsimile (508) 295-8107

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.



LETTERS AND REPORTS: Springborn Laboratories, Inc. letters and reports are issued for the exclusive use of the clients to whom they are addressed. No quotations from reports or use of the Springborn Laboratories, Inc. name is permitted except as expressly authorized in writing. Letters and reports apply only to the specific materials, products or processes examined or surveyed and are not necessarily indicative of the qualities of apparently identical or similar materials, products or processes. The liability of Springborn Laboratories, Inc. with respect to services rendered shall be limited to the amount of the consideration paid for such services and not include any consequential damages.

Documents
Document Sharing Hub

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

 **Documents**
Document Sharing Hub

**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**

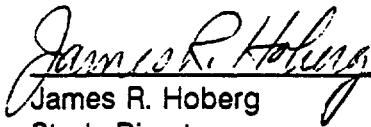
12 August 1994

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.

 3/12/94
James R. Hoberg Date
Study Director

Springborn Laboratories, Inc.

TABLE OF CONTENTS

	PAGE
GOOD LABORATORY PRACTICES COMPLIANCE STATEMENT	2
LIST OF TABLES	5
LIST OF FIGURES	6
SUMMARY	7
1.0 INTRODUCTION	9
2.0 MATERIALS AND METHODS	9
2.1 Protocol	9
2.2 Test Material	9
2.3 Test Organism	10
2.4 Test Dilution Water	11
2.5 Test Concentrations	11
2.6 Preparation of Test Solutions	11
3.0 TEST PROCEDURES	12
3.1 Test Initiation	12
3.2 Test Monitoring	12
3.2.1 Algal Growth	12
3.2.2 Recovery	13
3.2.3 Test Conditions	13
3.2.4 Chemical Analysis	14
4.0 STATISTICAL ANALYSIS	14
5.0 RESULTS AND DISCUSSION	15
5.1 Preliminary Testing	15
5.2 Definitive Testing	17
PROTOCOL DEVIATIONS	20
QUALITY ASSURANCE UNIT STATEMENT	21
REFERENCES	22
TABLES	23
FIGURE	29

Springborn Laboratories, Inc.**Documents**

Document Sharing Hub

SIGNATURES AND APPROVAL	31
6.0 APPENDIX I - STUDY PROTOCOL	32
7.0 APPENDIX II - CERTIFICATE OF ANALYSIS	43
8.0 APPENDIX III - DILUTION WATER ANALYSIS	45
9.0 APPENDIX IV - ANALYTICAL METHODOLOGY	48
10.0 APPENDIX V - EXCERPTED COPIES OF RAW DATA	62

Springborn Laboratories, Inc.

LIST OF TABLES

	PAGE
Table 1. Composition of algal growth medium (AAP medium) used during this study.	24
Table 2. Conductivity, pH, temperature and light intensity measured during the 96-hour exposure of <i>Selenastrum capricornutum</i> to TAME.	25
Table 3. Concentrations of TAME measured in the exposure solutions during the 96-hour toxicity test with <i>Selenastrum capricornutum</i>	26
Table 4. Cell densities ($\times 10^4$ cells/mL) of <i>Selenastrum capricornutum</i> after 24, 48, 72 and 96 hours of exposure to TAME.	27
Table 5. EC10, EC50 and EC90 values for TAME calculated from results (cell density) of the 96-hour toxicity test with <i>Selenastrum capricornutum</i>	28
Table 1A. Analytical results for the recovery of TAME from AAP media	55
Table 2A. Analytical results for the recovery of TAME from filtered seawater	56
Table 3A. Analytical results for the recovery of TAME from freshwater (reconstituted to increase hardness)	57
Table 4A. Repeatability of TAME analysis from ASTM Type II water at 0.026 mg/L	58

 Springborn Laboratories, Inc.

LIST OF FIGURES

	PAGE
Figure 1. Graphical illustration of the relationship between cell densities and time during the 96-hour toxicity test exposing <i>Selenastrum capricornutum</i> to TAME.	30
Figure 1A. A representative chromatogram of TAME purge and trap GC/FID analysis	59
Figure 2A. A representative linear regression analysis from standard TAME analysis	60
Figure 3A. A representative polynomial regression analysis from standard TAME analysis	61

Springborn Laboratories, Inc.

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

Springborn Laboratories, Inc.

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).

Springborn Laboratories, Inc.

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

Springborn Laboratories, Inc.

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 $\text{pH } 7.5 \pm 0.1$ 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 $24 \pm 1^\circ\text{C}$ 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.

Springborn Laboratories, Inc.

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

Springborn Laboratories, Inc.

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 111×10^4 cells/mL, was aseptically introduced into each flask. This inoculum provided the required initial cell density of approximately 1.0×10^4 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.

Springborn Laboratories, Inc.

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.

Springborn Laboratories, Inc.

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 $89.7 \pm 2.3\%$ 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.

Springborn Laboratories, Inc.

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 Bartlett's Test (Hornig 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

Springborn Laboratories, Inc.

densities in the treatment levels (0.10, 1.0, 10, 100 and 1000 mg A.I./L) averaged 83, 66, 53, 55 and 50×10^4 cells/mL, respectively. The control solutions averaged 93×10^4 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 1.0×10^4 cells/mL and two were inoculated with an initial cell density of 0.10×10^4 cells/mL. Following 96 hours of exposure, cell densities in the replicate flasks inoculated with 1.0×10^4 cells/mL averaged 23, 22, 17, 3.8 and 1.3×10^4 cells/mL representing 45, 48, 60, 91 and 97% reduction respective to the control (42×10^4 cells/mL). Following 96 hours of exposure, cell densities in the replicate flasks inoculated with 0.10×10^4 cells/mL averaged 10, 11, 5.6, 5.1 and 0×10^4 cells/mL, representing 29, 21, 60, 64 and 100% reduction respective to the control (14×10^4 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 1.0×10^4 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,

Springborn Laboratories, Inc.