



Health and Environmental
Sciences Department

Tert-Amyl Methyl Ether (TAME)— Acute Toxicity to Daphnids (*Daphnia magna*) under Flow- through Conditions

TSCA Guideline §797.1300

FEBRUARY 1995

TOXICOLOGY REPORT NUMBER 406
CAIS ABSTRACT NO. 42-1519



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



Documents
Document Sharing Hub

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) - Acute Toxicity to Daphnids under Flow-Through Conditions

Testing Facility: Springborn Laboratories, Inc.

SLI Study Number: 12827.0692.6102.115

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, as well as those listed on the Springborn GLP Compliance Statement.

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
2/16-17/93	Draft Report Audit Re-issued	2/19/93 4/29/94
10/15/94	Revised Final Draft Report Acceptance	10/15/94
12/20/94	Final Report Acceptance	12/20/94

Christine Sexsmith

Christine Sexsmith
Quality Assurance Coordinator

12/20/94

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.

**TERT-AMYL METHYL ETHER (TAME) -
ACUTE TOXICITY TO DAPHNIDS (*Daphnia
magna*) UNDER FLOW-THROUGH
CONDITIONS**

TSCA GUIDELINE § 797.1300

Submitted to:

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

SLI Report # 92-12-4545

SLI Study # 12827.0692.6102.115

Study Director: Arthur E. Putt

**Springborn Laboratories, Inc.
Environmental Sciences Division
790 Main Street
Wareham, Massachusetts 02571-1075**

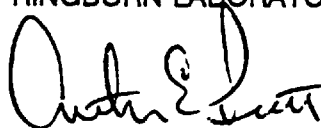
19 December 1994

FINAL REPORT

GOOD LABORATORY PRACTICES COMPLIANCE STATEMENT

The data and report for **"Tert-Amyl Methyl Ether (TAME) - Acute Toxicity To Daphnids (*Daphnia magna*) Under Flow-Through Conditions"** were produced and compiled in accordance with all pertinent EPA Good Laboratory Practice Regulations (40 CFR, Part 792) with the following exceptions: routine water and food contaminant 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.). Storage 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. Maintenance of a sample of the test material is the responsibility of the Study Sponsor.

SPRINGBORN LABORATORIES, INC.



12/19/94

Arthur E. Putt
Study Director

Date

Springborn Laboratories, Inc.**Documents**

Document Sharing Hub

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 Organisms	10
2.4 Test Dilution Water	11
2.5 Test Conditions	11
2.6 Test Concentrations	12
2.7 Exposure Solution Preparation	12
3.0 TEST PROCEDURES	13
3.1 Test Initiation	13
3.2 Test Monitoring	13
3.3 Water Quality Measurements	13
3.4 Analytical Measurements	14
4.0 STATISTICS	15
5.0 RESULTS	15
5.1 Preliminary Test	15
5.2 Definitive Test	16
PROTOCOL DEVIATION	18
QUALITY ASSURANCE UNIT STATEMENT	19
REFERENCES	20
SIGNATURES AND APPROVAL	30
6.0 APPENDIX I - STUDY PROTOCOL	31

Springborn Laboratories, Inc.

**Documents**

Document Sharing Hub

7.0 APPENDIX II - CERTIFICATE OF ANALYSIS	43
8.0 APPENDIX III - CULTURE FOOD ANALYSIS	46
9.0 APPENDIX IV - DILUTION WATER ANALYSIS	51
10.0 APPENDIX V - ANALYTICAL METHODOLOGY	54

Springborn Laboratories, Inc.

LIST OF TABLES

	Page
Table 1. The water quality parameters (i.e., pH, dissolved oxygen, temperature) measured in replicate exposure solutions during the 48-hour flow-through toxicity test exposing daphnids (<i>Daphnia magna</i>) to TAME	22
Table 2. Total hardness, total alkalinity and specific conductance determined at the initiation (0-hour) of the 48-hour flow-through exposure of daphnids (<i>Daphnia magna</i>) to TAME	23
Table 3. Concentrations of TAME measured in replicate (A,B) test solutions during the 48-hour flow-through exposure of daphnids (<i>Daphnia magna</i>)	24
Table 4. Mean measured concentrations tested, corresponding cumulative percent of immobilized daphnids (<i>Daphnia magna</i>) and observations made during the 48-hour flow-through exposure to TAME	25
Table 5. The EC50 values (95% confidence interval) and No-Observed-Effect Concentration (NOEC) for the 48-hour flow-through exposure of daphnids (<i>Daphnia magna</i>) to TAME	26
Table 1A. Analytical results for the recovery of TAME from AAP media ...	61
Table 2A. Analytical results for the recovery of TAME from filtered seawater	62
Table 3A. Analytical results for the recovery of TAME from freshwater (reconstituted to increase hardness)	63
Table 4A. Repeatability of TAME analysis from ASTM Type II water at 0.026 mg/L	64

 Springborn Laboratories, Inc.

LIST OF FIGURES

	Page
Figure 1. Graphical illustration of the relationship between the mean measured concentrations (analyses at 0- and 48-hours) and the nominal treatment levels during the 48-hour flow-through exposure of daphnids (<i>Daphnia magna</i>) to TAME	28
Figure 2. The 48-hour concentration-response (immobilization) curve for daphnids (<i>Daphnia magna</i>) exposed to TAME	29
Figure 1A. A representative chromatogram of TAME purge and trap GC/FID analysis	65
Figure 2A. A representative linear regression analysis from standard TAME analysis	66
Figure 3A. A representative polynomial regression analysis from standard TAME analysis	67

Springborn Laboratories, Inc.

SUMMARY

The purpose of this study was to estimate the acute toxicity (EC50) of Tert-Amyl Methyl Ether (TAME) to *Daphnia magna* under flow-through conditions. The EC50 is defined as the concentration of test material estimated to cause immobilization among 50% of a test population at the stated exposure interval. Twenty organisms (ten per replicate) were exposed in duplicate test vessels to five concentrations of TAME and a dilution water control for 48 hours. During the test, nominal concentrations of 690, 410, 250, 150 and 89 mg A.I./L were maintained in the exposure vessels by introducing approximately 6.0 test chamber volumes per day of newly prepared test solution via intermittent-flow proportional diluter apparatus. Each replicate solution was sampled and analyzed for TAME concentration at 0-hour (initiation) and 48-hours (termination) of the exposure period. Based on the results of these analyses, the mean measured exposure concentrations were defined as 120, 83, 55, 28 and 15 mg A.I./L. Biological observations and observations of the physical characteristics of the exposure solutions were made and recorded at test initiation, 3, 6, 24 and 48 hours. Throughout the exposure period, no visible signs of undissolved test material (e.g., precipitate) were observed in either the diluter system or in the exposure solutions.

At test termination (48-hours), immobilization of 90% was observed among daphnids exposed to the highest mean measured concentration tested (120 mg A.I./L). In addition, sublethal effects (e.g., lethargy) were observed among all of the mobile daphnids exposed to this treatment level. No immobilization or sublethal effects were observed among daphnids exposed to the remaining concentrations tested (83, 55, 28 and 15 mg A.I./L). The EC50 values and the corresponding 95% confidence intervals determined throughout the exposure period are summarized in the following table. The No-Observed-Effect Concentration (NOEC) through 48-hours of exposure was established to be 83 mg A.I./L.

Springborn Laboratories, Inc.

TEST RESULTS

EC50 (mg A.I./L) ^{ab}				No-Observed- Effect Concentration Through 48 Hours (mg A.I./L) ^a
3-Hour ^c	6-Hour ^c	24-Hour ^c	48-Hour ^d	
> 120	> 120	> 120	100 (83 - 120)	83

- ^a Based on mean measured concentrations of TAME (as active ingredient).
- ^b Corresponding 95% confidence interval is presented in parentheses.
- ^c EC50 value empirically estimated to be greater than the highest concentration tested.
- ^d EC50 value estimated by nonlinear interpolation; 95% confidence interval calculated by binomial probability.

Springborn Laboratories, Inc.

1.0 INTRODUCTION

The purpose of this study was to estimate the acute toxicity (EC50) of Tert-Amyl Methyl Ether (TAME) to daphnids (*Daphnia magna*) under flow-through conditions. The EC50 is defined as the concentration of test material in dilution water which causes immobilization of 50% in the exposed test population after a fixed period of time. This value is often used as a relative indicator of potential acute hazards resulting from the release of the test substance into aquatic environments. The study was initiated on 26 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 48-hour definitive toxicity test was conducted from 10 - 12 December 1992 at Springborn Laboratories, Inc. (SLI), Environmental Sciences Division, Wareham, Massachusetts. All original raw data and the final report produced for this study are stored at SLI.

2.0 MATERIALS AND METHODS

2.1 Protocol

Procedures used in this acute toxicity study followed those described in the SLI protocol entitled "Protocol for Conducting a Flow-Through Acute Toxicity Test with *Daphnia magna* Following TSCA §797.1300", SLI Protocol #: 091192/TSCA 797.1300 DM-FA and Protocol Amendment # 1 dated 15 December 1992 (Appendix I). The methods described in this protocol generally follow the standard procedures described in the EPA/OTS guidelines for testing the effects of chemicals on daphnids and meet the TSCA guidelines as specified in the appropriate Registration Standard. This protocol is intended to meet premanufacture notice ("PMN") registration requirements.

2.2 Test Material

Two samples of Tert-Amyl Methyl Ether (TAME) (CAS # 994-05-8), a clear liquid, were received from Experimental Pathology Labs, Inc., Herndon, Virginia. The first sample, Lot # 02814BZ, was received at SLI on 17 August 1992 and was used to prepare exposure solutions during the preliminary exposure, analytical standards during the method validation/recovery study and to prepare Quality Control samples during the definitive exposure. The sample was identified

Springborn Laboratories, Inc.



by Aldrich Chemical Company to contain 98.8% active ingredient A.I. (Certificate of Analysis, Appendix II). The second sample, Lot # 07905KZ, was received at SLI on 2 November 1992 and was used to prepare exposure solutions during the definitive exposures. The sample was identified by Aldrich Chemical Company to contain 98.7% active ingredient A.I. (Certificate of Analysis, Appendix II). Upon receipt at SLI, the samples of test material were stored in a dark, ventilated cabinet at room temperature (approximately 20 °C). Test concentrations are expressed as milligrams of test material (as active ingredient) per liter of test solution and are reported as mg A.I./L.

At the request of the Study Sponsor, mass spectral analysis was conducted on the initial batch of TAME received at program initiation, and the additional batches received throughout the course of the program. The purpose of the mass spectral analysis evaluation was to determine test material integrity throughout the duration of the program. Initial evaluation of test material (i.e., lot # 02814BZ) was conducted on 3 December 1992. Following completion of the flow-through acute toxicity test with mysids (SLI Report # 94-5-5269), spectral analysis was conducted on 20 July 1994 on each of the remaining two lots (lot # 02814BZ and lot # 07905KZ). The spectral analysis conducted on 20 July 1994 on the two remaining lots in comparison to the initial spectral analyses of lot # 02814BZ established that negligible change in test material composition had occurred during storage at Springborn Laboratories, Inc. (i.e., approximately 24 months).

2.3 Test Organisms

The *Daphnia magna* used in this toxicity test were obtained from laboratory cultures maintained at Springborn Laboratories, Inc., Wareham, Massachusetts. The culture water was prepared by fortifying well water based on the formula for hard water (U.S. EPA, 1975) and filtering it through an Amberlite XAD-7 resin column and a carbon filter. Two weeks prior to test initiation, this water had total hardness and total alkalinity as calcium carbonate (CaCO_3) of 160 mg/L and 110 mg/L, respectively, a pH of 8.1, a specific conductivity range of 400 to 500 $\mu\text{mhos/cm}$ and a dissolved oxygen concentration of greater than 60% of saturation. The daphnid culture area received a regulated photoperiod of 16 hours of light and 8 hours of

Springborn Laboratories, Inc.

darkness. Light intensity in the culture area ranged from 32 - 48 footcandles (Invertebrate Culture Log, Vol. VIII). A waterbath in the culture area was used to maintain the culture solution temperature at 20 ± 2 °C. Daphnids were fed a combination of a trout food suspension and a unicellular green algae (*Ankistrodesmus falcatus*) once daily. The food solution contained 5.0 mg/mL trout food and approximately 4×10^7 cells/mL of algae. Representative samples of the food source were analyzed for the presence of pesticides, PCBs and toxic metals (Appendix III). Food sources were considered to be of acceptable quality since the total concentration of pesticides measured was less than 0.3 mg/kg (ASTM, 1985).

2.4 Test Dilution Water

The dilution water used during this study was from the same source as the culture water described above and had a total hardness and total alkalinity (CaCO_3) of 160 and 110 mg/L, respectively, a pH range of 8.1 - 8.2 and a specific conductivity of 500 $\mu\text{mhos/cm}$ (IWQ Log Book, Vol. 13). During holding and prior to use, the dilution water was continuously aerated. Representative samples of the dilution water source were analyzed for the presence of pesticides, PCBs and toxic metals (Appendix IV). None of these compounds have been detected at concentrations that are considered toxic in any of the water samples analyzed, in agreement with ASTM standard practice (ASTM, 1985). In addition, representative samples of the dilution water source were analyzed monthly for total organic carbon (TOC) concentrations. The results of these analyses demonstrated that the TOC concentration of the dilution water source ranged from 0.97 - 2.2 mg/L for the months of June - November 1992 (TOC and TSS master log, Volume I). Daphnid cultures are maintained in water from the same source as the dilution water utilized in this study and have successfully survived and reproduced over several generations. This, in combination with the previously mentioned analyses, confirms the acceptability of this dilution water for bioassays.

2.5 Test Conditions

The toxicity test was conducted using an exposure system consisting of an intermittent-flow proportional diluter (Mount and Brungs, 1967) and a set of 12 exposure vessels. The test system was designed to provide five concentrations of the test material and a dilution water

Springborn Laboratories, Inc.



Documents
Document Sharing Hub

control. Exposure vessels were maintained in an area illuminated with Duro-Test® Cool-White and Duro-Test® Vitalite fluorescent lights at an intensity of 45 - 70 footcandles. The photoperiod was the same as that of the culture area. Sudden transitions from light to dark and vice versa were avoided. The test was conducted in a temperature controlled room and waterbath which were designed to maintain test solution temperatures at 20 ± 2 °C. Two replicate vessels were established for each treatment level and the control. Exposure vessels were labeled to identify the nominal test material concentration and designated replicate.

2.6 Test Concentrations

Selection of nominal TAME concentrations for the 48-hour definitive flow-through toxicity test with daphnids was based on toxicity information developed at SLI through preliminary testing.

2.7 Exposure Solution Preparation

Prior to test initiation, a 50 mL Glenco® gas-tight syringe in conjunction with a Sage syringe pump (Model # 355) was calibrated to deliver 0.416 mL/cycle of the test material (760 mg/mL) directly to the diluter system's chemical mixing chamber, which also received 0.458 L of dilution water per cycle. The mixing chamber was positioned over a magnetic stirrer which continuously mixed the contents of the mixing chamber and aided in the solubilization of the test material. The solution contained in the mixing chamber constituted the highest nominal treatment level (690 mg A.I./L) and was subsequently diluted (60% dilution factor) to provide the remaining nominal test concentrations (410, 250, 150 and 89 mg A.I./L).

During each cycle of the diluter system, approximately 50 mL of exposure solution was delivered to each replicate test vessel. The system cycled approximately 216 times each day. The diluter system was calibrated prior to test initiation by measuring delivery volumes of toxicant and dilution water. During the study, visual checks of the diluter system and analysis of the exposure solutions for TAME concentration were used to verify proper operation of the diluter system. The exposure system was in proper operation 9 days prior to test initiation to allow equilibration of the test material in the diluter apparatus and exposure vessels. Four glass

Springborn Laboratories, Inc.

capillary tubes with an approximate length of five centimeter (cm) and a diameter of 1-millimeter (mm) (inside diameter) were inserted through silicone stoppers in the mixing/splitting chambers of the diluter and into the test solution delivery tubes. This tubing served to restrict the flow of the test solutions, minimizing potentially stressful turbulence in the exposure vessels and provided equal distribution of the solutions to the replicate vessels. Each glass test vessel maintained a constant solution volume of 1.8 L and a solution depth of approximately 13 cm. Each replicate vessel received approximately 6.0 solution volume replacements per day. The function of the diluter system (e.g., flow rates, stock consumption) was monitored daily and a visual check was made twice daily to verify proper performance during the study period.

3.0 TEST PROCEDURES

3.1 Test Initiation

The test was initiated when 10 daphnids ($\leq$ 24 hours old) were impartially selected and introduced to each replicate exposure vessel (20 per treatment level and the control).

3.2 Test Monitoring

The number of immobilized daphnids observed in each replicate test vessel was recorded at 3-, 6-, 24- and 48-hours during the exposure period. Daphnids were determined immobile if, after gentle prodding, no movement except for minor appendages was observed (i.e., absence of movement within the solution's water column). Biological observations (e.g., abnormal behavior or appearance of the test organisms) and observations of the physical characteristics of the test solutions (e.g. precipitate, film on the surface of the test solution) were also made and recorded at test initiation and at 3-, 6-, 24- and 48-hours of exposure. Daphnids were not fed during the 48 hour definitive exposure.

3.3 Water Quality Measurements

Dissolved oxygen concentration, temperature and pH were measured once daily in both replicate vessels of each treatment level and the control throughout the exposure period. Total hardness, total alkalinity and specific conductance were measured at test initiation in one replicate vessel of each treatment level and control solution. Total hardness concentrations

Springborn Laboratories, Inc.

presented in this report were measured by the EDTA titrimetric method and total alkalinity concentrations were determined by potentiometric titration to an endpoint of pH 4.5 (APHA *et al.*, 1985) using a Jenco Model 601A pH meter and combination electrode. Specific conductivity was measured with a Yellow Springs Instrument Company (YSI) Model #33 salinity-conductivity-temperature meter and probe; the pH was measured with a Jenco Model 601A pH meter and combination electrode; the dissolved oxygen concentration was measured with a YSI Model #57 dissolved oxygen meter and probe; and the daily solution temperature was measured with a Fisher alcohol thermometer. Continuous temperature monitoring was performed in one replicate (B) of the 690 mg A.I./L test solution (nominal) using the Omega Data Acquisition System (ODAS). Light intensity was measured with a General Electric type 214 light meter.

3.4 Analytical Measurements

Both replicate solutions of the high, middle, and low treatment levels and the control were sampled and analyzed for TAME concentration prior to the start of the definitive exposure. Results of these pretest analyses were used to judge whether sufficient quantities of TAME were being delivered to the test vessels and the appropriate test concentrations were being maintained in order to initiate the definitive exposure. During the in-life phase of the definitive study, water samples were removed from both replicate test solutions of each treatment level and the control at 0- and 48-hours of exposure for analysis of TAME concentration. Each exposure solution sample was collected from the approximate midpoint of the test vessel with a volumetric pipet. In addition, three Quality Control (QC) samples were prepared at each sampling interval and remained with the samples of the exposure solutions throughout the analytical process. Results of the analyses of the QC samples were used to judge the precision and the quality control maintained during the analysis of exposure solution samples. All samples were analyzed for TAME using a gas chromatography (GC) procedure according to the methodology described in Appendix V. A method validation recovery study, conducted at SLI prior to the initiation of the definitive test, established an average recovery of TAME of $102 \pm 10\%$ from hard reconstituted water.

Springborn Laboratories, Inc.

4.0 STATISTICS

The mean measured concentrations tested (based on 0- and 48-hour analyses) and the corresponding effect (immobilization) data derived from the definitive toxicity test were used to estimate the median lethal concentration (EC50) and 95% confidence interval for each 24-hour interval of the exposure period. The EC50 is defined as the concentration of the test material in dilution water which caused immobilization of 50% of the test animals population at the stated time interval. When no concentration caused $\geq 50\%$ immobilization of the test population, then the EC50 value was empirically estimated as being greater than the highest mean measured concentration tested. If at least one test concentration caused immobilization of greater than or equal to 50% of the test population, then a computer program (Stephan, 1977, 1982) was used to calculate the EC50 values and 95% confidence interval.

Three statistical methods were available in the computer program: moving average angle analysis, probit analysis, and nonlinear interpolation with 95% confidence intervals calculated by binomial probability. Moving average angle and probit analyses yield statistically sound results only if at least two concentrations produce a immobilization of between 0 and 100% of the test organism population. The selection of reported EC50 values and 95% confidence intervals was based upon an examination of the data base and the results of the computer analysis. Selection criteria included the establishment of a concentration-effect (immobilization) relationship, the number of concentrations causing partial responses, and the span of responses bracketing the EC50 value. If two or more statistical methods produced acceptable results, then the method which yielded the smallest 95% confidence interval was selected. The No-Observed-Effect Concentration (NOEC) during the 48 hour exposure period was also determined. The NOEC is defined as the highest concentration tested at and below which there were no toxicant related immobilization or physical and behavioral abnormalities, (e.g., lethargy, flared carapace), with respect to the control organisms.

5.0 RESULTS

5.1 Preliminary Test

Prior to initiating the definitive study, a 48-hour preliminary range-finding test was conducted at SLI. During this 48-hour preliminary test, daphnids were exposed under flow-

Springborn Laboratories, Inc.

through conditions to nominal concentrations of TAME ranging from 690 - 89 mg A.I./L. All test vessels during this preliminary exposure were uncovered. At test termination (48-hours), 100% immobilization was observed among daphnids exposed to the highest nominal concentration tested (690 mg A.I./L). Immobilization of 10% was observed among mobile daphnids exposed to the 410 mg A.I./L, nominal treatment level. Sublethal effects (i.e., lethargy) were observed among all of the mobile daphnids exposed to the 410 mg A.I./L nominal treatment level and one mobile daphnid in the 250 mg A.I./L treatment level. No immobilization or sublethal effects were observed among daphnids exposed to the remaining nominal concentrations tested (150 and 89 mg A.I./L). Based on these results, nominal concentrations of 690, 410, 250, 150 and 89 mg A.I./L were selected for the definitive exposure.

5.2 Definitive Test

Results for the water quality parameters (pH, dissolved oxygen and temperature) measured at 0-, 24- and 48-hours of the definitive exposure are summarized in Table 1. Total hardness, total alkalinity and specific conductance measured at test initiation are presented in Table 2. Throughout the exposure period, the water quality parameters measured were unaffected by the concentrations of TAME tested and remained within acceptable ranges for the survival of *Daphnia magna*. Daily and continuous (replicate B of the 690 mg A.I./L treatment level, nominal) temperature monitoring of the test solutions established that the test solution temperature ranged from 18 - 20 °C throughout the exposure period.

The diluter system which prepared and delivered the test solutions to the exposure aquaria functioned properly during the pretest period and throughout the 48-hour study. Analyses of the solutions during the pretest period established that the concentration of TAME in the exposure solutions was consistent between replicate solutions and that the delivery apparatus maintained the expected concentration gradient (approximately 60% dilution factor). Analyses of the pretest samples resulted in measured concentrations which averaged 19% of nominal. Throughout the exposure period, no visible signs of undissolved test material (e.g., precipitate) was observed in either the diluter system or in the exposure solutions.

Springborn Laboratories, Inc.

The results of the analysis of the exposure solutions for TAME concentration during the in-life portion of the definitive exposure are presented in Table 3. Throughout the exposure period, analytical measurements between replicate solutions and sampling intervals were consistent and established the expected concentration gradient of test material (i.e., 60% dilutions). Mean measured concentrations averaged 19% of the nominal concentrations and defined the treatment levels as 120, 83, 55, 28 and 15 mg A.I./L. The relatively low recovery obtained for the tested treatment levels (mean = 19%) is believed due to the volatile nature of the test material. However, the mean measured concentrations established were sufficient to produce a biological response (immobilization). Coefficients of variation averaged 11% for all mean measured concentrations. Analysis of the Quality Control samples resulted in measured concentrations which were consistent with the predetermined recovery range (Appendix V) and averaged 100% of the nominal fortified levels (695 - 49.7 mg A.I./L). Based on the results of these analyses, it was established that the appropriate quality control was maintained during the analyses of the exposure solutions.

The relationship between the nominal treatment levels and the mean measured concentrations established by the diluter apparatus during this study is illustrated in Figure 1.

The mean measured concentrations tested, the corresponding percent of immobilized daphnids and observations recorded during the 48-hour test are presented in Table 4. At test termination (48-hours), immobilization of 90% was observed among daphnids exposed to the highest mean measured concentration tested (120 mg A.I./L). In addition, sublethal effects (e.g., lethargy) were observed among all of the mobile daphnids exposed to this treatment level. No immobilization or sublethal effects were observed among daphnids exposed to the remaining concentrations tested (83, 55, 28 and 15 mg A.I./L). The 48-hour concentration-response (immobilization) curve established for this study is presented in Figure 2. The slope of this curve was calculated to be 3.3683. Table 5 summarizes the 3-, 6-, 24- and 48-hour EC50 values, corresponding 95% confidence intervals and presents the No-Observed-Effect Concentration (NOEC) through 48 hours. The 48-hour EC50 value was estimated by nonlinear interpolation to be 100 mg A.I./L with a 95% confidence interval calculated by binomial probability to be 83 - 120 mg A.I./L. The NOEC established for this study was determined to be 83 mg A.I./L.

Springborn Laboratories, Inc.

PROTOCOL DEVIATION

The study protocol states that the calibration of the diluter system is checked prior to test initiation and at test termination. For this study, the diluter calibration check at test termination was inadvertently missed. The diluter calibration was confirmed to be functioning properly by the consistency between measured concentrations at 0 and 48 hours.

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

SPRINGBORN LABORATORIES, INC.

 12/19/14

Arthur E. Putt
Study Director

Date

Springborn Laboratories, Inc.