

AR226-3271

453

**TRADE SECRET*****Study Title***

H-25435: Influence on Growth and Growth Rate of the Green  
*Alga Selenastrum capricornutum*

Laboratory Project ID: DuPont-11604

**TEST GUIDELINES:** OECD Guideline for Testing Chemicals  
Section 2: Effects on Biotic Systems, Number 201 (1984)

Commission Directive 92/69/EEC  
EEC Method C3 (1992)

**AUTHOR:** Terry Lee Sloman, B.S.

**STUDY COMPLETED ON:** March 31, 2003

**PERFORMING LABORATORY:** E.I. du Pont de Nemours and Company  
Haskell Laboratory for Health and Environmental Sciences  
Elkton Road, P.O. Box 50  
Newark, Delaware 19714-0050

**WORK REQUEST NUMBER:** [REDACTED]

**SERVICE CODE NUMBER:** [REDACTED]

### GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with U.S. EPA TSCA (40 CFR part 792) Good Laboratory Practice Standards, which are consistent with the OECD Principles of Good Laboratory Practice (as revised in 1997) published in ENV/MC/CHEM(98)17 and MAFF Japan Good Laboratory Practice Standards (59 NohSan Number 3850) except for the item documented below. The item listed does not impact the validity of the study.

The test substance is commercially available and was characterized by the supplier prior to the initiation of this study. An analysis was provided, although the characterization was not performed under Good Laboratory Practice Standards, based on our knowledge of the performing laboratory and its experience with analyzing this chemical, we have no reason to doubt the validity of the analysis.

Applicant / Sponsor: E.I. du Pont de Nemours and Company  
Wilmington, Delaware 19898  
U.S.A.

Study Director: Terry Lee Sloman 31-MAR-2003  
Terry Lee Sloman, B.S. Date  
Associate Scientist

Applicant / Sponsor: \_\_\_\_\_  
DuPont Representative Date

## QUALITY ASSURANCE STATEMENT

Haskell Sample Number(s):

25435

Dates of Inspections:

Protocol: January 7, 2003

Conduct: January 7, 2003

Records, Reports: March 17, 19, 20, 2003

Dates Findings Reported to:

Study Director: March 20, 2003

Management: March 31, 2003

Reported by:

Denise M. Janney

Denise M. Janney  
Quality Assurance Auditor

31-Mar-2003

Date

### CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Analytical Reported by: Bogdan Szostek 31-MAR-2003  
Bogdan Szostek, Ph.D.  
Senior Research Chemist  
Date

Statistical Reported by: John W. Green 31-Mar-2003  
John W. Green, Ph.D., Ph.D.  
Principal Research Statistician  
Date

Issued by Study Director: Terry Lee Sloman 31-MAR-2003  
Terry Lee Sloman, B.S.  
Associate Scientist  
Date

## TABLE OF CONTENTS

|                                                                   | Page      |
|-------------------------------------------------------------------|-----------|
| <b>GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT .....</b>        | <b>2</b>  |
| <b>QUALITY ASSURANCE STATEMENT .....</b>                          | <b>3</b>  |
| <b>CERTIFICATION .....</b>                                        | <b>4</b>  |
| <b>LIST OF TABLES .....</b>                                       | <b>6</b>  |
| <b>LIST OF FIGURES .....</b>                                      | <b>6</b>  |
| <b>LIST OF APPENDICES .....</b>                                   | <b>6</b>  |
| <b>STUDY INFORMATION .....</b>                                    | <b>7</b>  |
| <b>STUDY PERSONNEL.....</b>                                       | <b>8</b>  |
| <b>SUMMARY .....</b>                                              | <b>9</b>  |
| <b>INTRODUCTION.....</b>                                          | <b>10</b> |
| <b>MATERIALS AND METHODS .....</b>                                | <b>10</b> |
| A. Test Guidelines .....                                          | 10        |
| B. Test Substance .....                                           | 10        |
| C. Test System .....                                              | 10        |
| D. Test Design .....                                              | 11        |
| E. Test Preparation .....                                         | 11        |
| F. Experimental Design.....                                       | 12        |
| G. Test Solutions .....                                           | 12        |
| H. Test Conditions - Definitive and Recovery Tests .....          | 12        |
| I. <i>Selenastrum capricornutum</i> Growth Measurement .....      | 13        |
| J. Statistical Analyses .....                                     | 13        |
| K. Analysis of Test Solutions - Definitive Test.....              | 14        |
| <b>RESULTS AND DISCUSSION .....</b>                               | <b>19</b> |
| A. Environmental Conditions - Definitive and Recovery Tests ..... | 19        |
| B. Test Substance Stability and Analytical Verification .....     | 19        |
| C. <i>Selenastrum capricornutum</i> Growth.....                   | 20        |
| <b>CONCLUSIONS .....</b>                                          | <b>21</b> |
| <b>RECORDS AND SAMPLE STORAGE .....</b>                           | <b>21</b> |
| <b>REFERENCES.....</b>                                            | <b>22</b> |
| <b>TABLES.....</b>                                                | <b>23</b> |
| <b>FIGURES.....</b>                                               | <b>32</b> |
| <b>APPENDICES .....</b>                                           | <b>41</b> |

## LIST OF TABLES

|                                                                         | Page |
|-------------------------------------------------------------------------|------|
| 1. pH MEASUREMENTS OF TEST SOLUTIONS.....                               | 24   |
| 2. CHAMBER LIGHT INTENSITY, SHAKING SPEED, AND TEMPERATURE RANGE .....  | 24   |
| 3. MEASURED CONCENTRATIONS OF H-25435 IN TEST SOLUTIONS.....            | 25   |
| 4. HEALTHY CELL COUNT DATA SUMMARY - DEFINITIVE TEST .....              | 28   |
| 5. AREA UNDER THE GROWTH CURVE DATA SUMMARY - DEFINITIVE TEST .....     | 29   |
| 6. GROWTH RATE DATA SUMMARY - DEFINITIVE TEST.....                      | 30   |
| 7. 72-HOUR (DAY 3) STATISTICAL ANALYSES SUMMARY - DEFINITIVE TEST ..... | 31   |

## LIST OF FIGURES

|                                                                                                    | Page |
|----------------------------------------------------------------------------------------------------|------|
| 1. REPRESENTATIVE ANALYTICAL CALIBRATION STANDARD CURVE FOR H-25435: [REDACTED]<br>[REDACTED]..... | 33   |
| 2. REPRESENTATIVE CHROMATOGRAM OF A CALIBRATION STANDARD SOLUTION .....                            | 35   |
| 3. REPRESENTATIVE CHROMATOGRAM OF THE BLANK CONTROL SOLUTION .....                                 | 37   |
| 4. REPRESENTATIVE CHROMATOGRAM OF A TEST CONCENTRATION SOLUTION .....                              | 39   |

## LIST OF APPENDICES

|                                                                                 | Page |
|---------------------------------------------------------------------------------|------|
| A. AAP NUTRIENT MEDIUM CONSTITUENTS.....                                        | 42   |
| B. 72-HOUR HEALTHY CELL COUNT STATISTICS FOR THE DEFINITIVE TEST .....          | 44   |
| C. 72-HOUR AREA UNDER THE GROWTH CURVE STATISTICS FOR THE DEFINITIVE TEST ..... | 48   |
| D. 72-HOUR GROWTH RATE STATISTICS FOR THE DEFINITIVE TEST .....                 | 53   |

### STUDY INFORMATION

Substance Tested: [REDACTED]

Synonyms/Codes: [REDACTED]

- H-25435

Haskell Number: 25435

Composition: [REDACTED]

Known Impurities: [REDACTED]

Physical Characteristics: Amber brown liquid

Stability: The test substance appeared to be stable under the conditions of the study.

Sponsor: E. I. du Pont de Nemours and Company  
Wilmington, Delaware 19898  
U.S.A.

Study Initiated/Completed: January 6, 2003 / (see report cover page)

### STUDY PERSONNEL

Study Director: Terry Lee Sloman, B.S.  
Management: Robert A. Hoke, Ph.D.  
Primary Technician: Barbra D. Ferrell, B.S.

Analytical Chemist: Bogdan Szostek, Ph.D.  
Management: S. Mark Kennedy, Ph.D.

Statistical Analysis: John W. Green, Ph.D., Ph.D.  
Management: Janice L. Connell, M.S., B.A., C.I.H.

Ecotoxicology Report Preparation: Wanda F. Dinbokowitz  
Management: Nancy S. Selzer, M.S.

## SUMMARY

A study was conducted to determine the effect of H-25435 on the growth and growth rate of the green alga *Selenastrum capricornutum*.<sup>a</sup> The algae were exposed to nominal concentrations of 6.25, 12.5, 25, 50, and 100 mg H-25435/liter of nutrient medium (ppm).

For the definitive (dose-response) test, the organisms were exposed for 72 hours (3 days) without test medium renewal. The effect was expressed as percent inhibition in growth based on healthy cell count (cell density), area under the growth curve, and growth rate relative to the blank (normal culture medium) control for the 72-hour (day 3) interval of the test.

The 72-hour results<sup>b</sup> based on nominal concentrations are as follows:

### Healthy Cell Count

|                  |                    |
|------------------|--------------------|
| EC <sub>50</sub> | > 100 mg H-25435/L |
| NOEC             | > 100 mg H-25435/L |

### Area Under the Growth Curve

|                  |                    |
|------------------|--------------------|
| EC <sub>50</sub> | > 100 mg H-25435/L |
| NOEC             | > 100 mg H-25435/L |

### Growth Rate

|                  |                    |
|------------------|--------------------|
| EC <sub>50</sub> | > 100 mg H-25435/L |
| NOEC             | > 100 mg H-25435/L |

None of the definitive test concentrations exhibited a 50% or greater growth inhibition based on healthy cell count relative to the blank control. Therefore, a recovery test was not performed.

a Also known as *Pseudokirchneriella subcapitata*.

b The EC<sub>50</sub> is defined as the "effective concentration" producing a 50% inhibition of growth relative to the control. The NOEC is defined as the highest concentration of test substance that has no statistically significant effect on the measured parameter relative to the control.

## INTRODUCTION

This study was conducted to determine the effect of H-25435 on the growth and growth rate of the green alga *Selenastrum capricornutum*.<sup>a</sup>

## MATERIALS AND METHODS

### A. Test Guidelines

The study design complies with the following test guidelines:

- Organisation for Economic Co-Operation and Development (OECD) (1984). 201 Effects on Biotic Systems. *Guideline for Testing of Chemicals*.
- European Economic Communities (EEC) (1992). Directive 92/69/EEC Annex V, Part C.3, Algal Inhibition Test. *Methods for the Determination of Toxicity*.

### B. Test Substance

The test substance, H-25435, was supplied by the sponsor as an amber brown liquid. It was received October 17, 2002; the expiration date is April 17, 2003.

### C. Test System

*Selenastrum capricornutum*, a freshwater, unicellular, non-motile, green alga, was used in this study.

#### 1. Original Culture Source

The original culture source was the Department of Botany - Culture Collection of Algae - The University of Texas at Austin - Austin, Texas 78713-7640.

#### 2. Culture Maintenance

The culture method for *Selenastrum capricornutum* was based on published literature.<sup>(1-2)</sup> Prior to the study, *Selenastrum capricornutum* cultures were maintained under the same environmental conditions used in the study. The organisms were cultured in sterilized 250 mL Erlenmeyer flasks containing approximately 50 mL of filtered (= filter-sterilized) AAP nutrient medium and were aseptically transferred to fresh medium every 3 to 7 days. The flasks were fitted with foam stoppers to permit gas exchange.

<sup>a</sup> Also known as *Pseudokirchneriella subcapitata*.

## D. Test Design

The test design used for the *Selenastrum capricornutum* definitive and recovery tests is described below:

| Organism                         | Nutrient Medium | Flask Volume (mL) | Solution Volume (mL) | Volume Ratio |
|----------------------------------|-----------------|-------------------|----------------------|--------------|
| <i>Selenastrum capricornutum</i> | AAP             | 250               | 50                   | 5:1          |

## E. Test Preparation (Appendix A)

### 1. AAP Nutrient Medium Preparation for *Selenastrum capricornutum*

AAP nutrient medium<sup>(2)</sup> was prepared by adding 1 mL of each of the 6 macronutrient stock solutions and 1 mL of the micronutrient stock solution to approximately 800 mL of Milli-Q<sup>®</sup> (deionized) water, with mixing after each addition. The volume of the medium was brought to 1 liter with additional Milli-Q<sup>®</sup> water. Appropriate proportions (1 mL of each stock solution for each 1 liter of medium) were used to prepare the larger volumes of the medium required for the definitive and recovery tests.

The medium pH was adjusted to 7.46 with 0.1N hydrochloric acid. For both the definitive and recovery tests, the medium was filter-sterilized using Corning<sup>®</sup> pre-sterilized filtration systems each with a 0.22  $\mu$ m cellulose acetate filter. The containers with the resulting filter-sterilized AAP nutrient medium for use in the definitive and recovery tests were stored in the refrigerator in the dark at approximately 4°C and acclimated to room temperature prior to use. Any remaining unused medium was properly stored.

### 2. Test Solution Preparation

Prior to the initiation of the definitive test, method development was conducted to determine the appropriate concentrations to be used in the definitive test.

A primary stock solution was prepared by dissolving the test substance in 1000 mL filter-sterilized AAP nutrient medium for a nominal concentration of 100 mg H-25435/L (= 100 ppm). A working stock solution was prepared by diluting the primary stock solution with filter-sterilized AAP nutrient medium to make a nominal concentration of 100 mg H-25435/L (= 100 ppm). Aliquots of the filter-sterilized AAP nutrient medium were used for the blank (normal culture medium) control solution. Test solutions were prepared using aliquots of the nominal 100 mg/L working stock solution and diluting with filter-sterilized AAP nutrient medium to make nominal concentrations of 6.25, 12.5, 25, and 50 mg H-25435/L. Aliquots of the nominal 100 mg/L working stock solution were used for the 100 mg H-25435/L test concentration solution and the abiotic (stability) control solution.

### 3. Test Culture Preparation

For each definitive control and test solution, 3 aliquots (50 mL each) were placed in separate sterilized 250 mL Erlenmeyer flasks fitted with sterilized foam stoppers. Each flask, excluding the abiotic control, was randomly assigned a number to eliminate bias while counting. To achieve the desired nominal concentration of approximately 10,000 *Selenastrum capricornutum* cells/mL at test initiation, an approximate 0.370 mL (= 370  $\mu$ L) aliquot of algal inoculum from a logarithmically growing stock culture was aseptically transferred to each flask, except the abiotic control.

## F. Experimental Design

### 1. Definitive Test

The organisms were exposed for 72 hours (3 days), without test medium renewal. Test flasks were arranged on a lighted shelf in a chamber in a non-systematic design and were re-positioned each working day. Each blank control, test concentration, and abiotic control was tested as 3 replicates. Cell counts were made approximately 24, 48, and 72 hours after the definitive test initiation (0-hour or day 0).

### 2. Recovery Test

None of the definitive test concentrations exhibited a 50% or greater growth inhibition based on healthy cell count relative to the blank control. Therefore, a recovery test was not performed.

## G. Test Solutions

Solutions used in the definitive test were as follows:

Blank Control      AAP nutrient medium containing no H-25435

Treatment          nominal 6.25, 12.5, 25, 50, and 100 mg H-25435/liter nutrient medium

Abiotic Control    nominal 100 mg H-25435/liter nutrient medium; (no  
*Selenastrum capricornutum*)

## H. Test Conditions - Definitive and Recovery Tests

The control and test flasks were placed in a chamber (Hotpack model 06084) and air temperature in the chamber was recorded continuously with a continuous temperature recorder (Dickson Model SL445C7). The algae were incubated for 72 hours without test medium renewal for the definitive test. Illumination was supplied by cool-white fluorescent tubes. The **target** environmental parameters are described in the following table.

| Test       | Initial<br>Population | Illumination<br>(lumens/m <sup>2</sup> = lux) | Photo-<br>Period<br>(hours) | Shaking<br>Speed<br>(rpms) | Temperature<br>(°C) |
|------------|-----------------------|-----------------------------------------------|-----------------------------|----------------------------|---------------------|
| Definitive | 10,000 cells/mL       | 6000 to 10,000                                | 24                          | 100                        | 24 ± 2              |

## I. *Selenastrum capricornutum* Growth Measurement

### 1. Definitive Test

*Selenastrum capricornutum* growth measurement was determined by visually counting the number of cells taken from an approximate 0.2 mL sample from each flask at approximately 24, 48, and 72 hours from the definitive test initiation. The counts were conducted using a hemacytometer and a compound microscope. An aliquot of each sample was loaded into the hemacytometer and 16 grids were selected for counting. All cells located in the 16 grids were counted and recorded as healthy or unhealthy. The total number of cells counted per grid was multiplied by 10,000 to determine the number of cells per milliliter. Cells outside these 16 grids were not counted nor included in the total number. Observations and counts of healthy and unhealthy cells (e.g., deformed, senesced, stunted) were recorded in the study records. Most counts were made at approximately the same time each day. Statistical calculations of the EC<sub>50</sub> and NOEC were based on mean healthy cell counts and nominal concentrations.

### 2. Recovery Test

None of the definitive test concentrations exhibited a 50% or greater growth inhibition based on healthy cell count relative to the blank control. Therefore, a recovery test was not performed.

## J. Statistical Analyses

The mean healthy cell count, area under the growth curve, and growth rate at the 72-hour interval for each test concentration were expressed relative to the blank control. All calculations were based on the nominal concentrations.

The area under the growth curves for each interval were calculated according to the following formula:

$$A = [0.5 \times t_1 \times (N_1 - N_0)] + [0.5 \times (t_2 - t_1) \times (N_2 + N_1 - 2N_0)] \\ + \dots + [0.5 \times (t_n - t_{n-1}) \times (N_n + N_{n-1} - 2N_0)]$$

where  $t_1$ ,  $t_2$ ,  $t_{n-1}$ , and  $t_n$  are times of observation measured from the initiation of the test and  $N_0$ ,  $N_1$ ,  $N_{n-1}$ , and  $N_n$  are the initial and subsequent healthy cell counts (cells/mL) corresponding to the observation times.

The growth rate ( $\mu$ ) for each test concentration and for the controls were calculated according to the following formula:

$$\mu = \frac{\ln(N_n / N_0)}{t_n}$$

where  $t_n$  is the time of observation measured from the initiation of the test and  $N_0$  and  $N_n$  are the initial and subsequent healthy cell counts (cells/mL) corresponding to the observation time.

The percent growth inhibition, % I, was calculated according to the following formula:

$$\% I = \frac{C - T}{C} \times 100$$

where C is equal to the mean healthy cell count, area under the growth curve, or growth rate for the blank control at a selected sampling interval and T is the mean healthy cell count, area under the growth curve, or growth rate for each test concentration at a selected sampling interval. Negative values of inhibition indicate stimulation of growth.

The "effective concentration" ( $EC_{50}$ ) was defined as the concentration producing a 50% inhibition of growth relative to the control. The  $EC_{50}$  value and associated 95% confidence limits were determined by weighted least-squares non-linear regression of the log of the test concentration against the measured parameter. The NOEC, defined as the highest concentration of test substance that had no significant effect on the measured parameter relative to the blank control, was determined from a trend test (Jonckheere-Terpstra or Williams) applied in a step-down manner. All tests of significance were at  $\alpha = 0.05$ .<sup>(3-4)</sup>

## K. Analysis of Test Solutions - Definitive Test

### 1. Sample Collection

The concentrations of H-25435 in the working stock (nominal 100 mg H-25435/L), blank control, and test concentration (nominal 6.25, 12.5, 25, and 50 mg H-25435/L) solutions were verified at test initiation (day 0). An aliquot from each of the blank control and test concentration solutions was taken prior to the addition of *Selenastrum capricornutum*. The pH was determined for each of the working stock, blank control, and test concentration solutions.

The concentrations of H-25435 in the blank control, test concentration (nominal 6.25, 12.5, 25, 50, and 100 mg H-25435/L), and abiotic control (nominal 100 mg/L H-25435/L) solutions were verified at test termination (day 3). These samples were prepared by pooling all 3 replicates for each of the control and test concentration solutions and taking an aliquot from each of the pooled samples. The pH was determined for each of these day 3 samples.

The concentrations of in each of the test solutions were determined by high performance liquid chromatography/mass spectrometry (HPLC/MS).

## 2: Sample Treatment

An aliquot from the blank control, working stock, and test concentration were sampled at the study start (day 0) and submitted for analysis. An aliquot from the blank control, test concentration, and abiotic control were sampled at the end of the exposure (day 3) and submitted for analysis. Back-up solutions for each sample also were provided. All samples were analyzed on the day of receipt. Day 0 samples were also analyzed on day 1. Centrifugation and settling of algae were investigated as methods for analyzing day 3 samples. Settling of algae was the preferred method based on analytical recoveries of the test material. The day 3 samples were placed in a refrigerator and stored for 3 days to facilitate the settling of algae. After 3 days of storage the supernatants of the refrigerated day 3 samples were taken and analyzed. Aliquots of all test solutions were transferred to autosampler vials for analysis by LC/MS. The samples were diluted before analysis with AAP nutrient medium as follows: 6.25 mg/L (1:2), 12.5 mg/L (1:5), 25 mg/L (1:10), 50 mg/L (1:25), and 100 mg/L (1:50).

### 3. Instrumentation and Conditions

HPLC Instrument: Model 2795, Waters  
MS Instrument: Quattro Micro, Micromass

#### LC Parameters:

Column: Luna 3u C18(2), 20 mm x 2mm  
Mobile Phase: A- 50 mM ammonium acetate in water  
C- methanol

#### Gradient:

| Time (min) | %C  | Flow (mL/min) |
|------------|-----|---------------|
| 0          | 30  | 0.25          |
| 5          | 100 | 0.25          |
| 10         | 100 | 0.25          |
| 10.1       | 30  | 0.50          |
| 12         | 30  | 0.50          |
| 12.1       | 30  | 0.25          |

Column Temperature: 30°C  
Injection Volume: 50 µL  
Column Switch: 2 and 10 min

#### MS Parameters:

Ionization mode: Electrospray, negative ions  
Capillary voltage: 3.2 kV  
Cone voltage: 30 V  
Source Temperature: 120°C  
Desolvation Temperature: 450°C  
Data Acquisition Function: C6-P-C6 bis ester ion: SIR of 789 m/z; 0-10 min  
C6-P-IPA mixed ester ion: SIR of 485 m/z; 0-10 min  
C6-P mono ester ion: SIR of 443 m/z; 0-10 min

### 4. Quantitation

H-25435 is a [REDACTED] The [REDACTED] present in H-25435 can be subdivided into three distinct groups: [REDACTED] One chemical species was selected from each group and monitored by LC/MS though the study. The selected species chemical structures, names, and abbreviations used were as follows:

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

The test substance H-25435 [REDACTED] was used as the reference compound. The test substance H-25435 has not been characterized to determine actual contents of the three species that were monitored during the study. The concentrations of individual chemical species that were monitored during the study were represented as the total concentrations of the H-25435.

A primary stock solution of the reference compound, H-25435 [REDACTED], was made by dissolving approximately 60 mg of H-25435 in 500 mL of AAP nutrient medium. Approximately 30 minutes of sonication was required to completely dissolve the test substance. Calibration standards were prepared on each test day by dilution with AAP nutrient medium at concentrations ranging from 0.288 to 5.75 mg/L. Duplicate injections of calibration solutions were made and peak areas were determined by electronic integration.

The calibration curves were generated by regression analysis of the peak area data obtained from chromatographic analysis of the calibration solutions. Data for test solutions was fitted to the appropriate calibration curves to determine the concentrations of each of monitored species. The

limit of detection (LOD) and limit of quantitation (LOQ) were determined by calculating the average noise level in chromatograms of blank control samples and comparing them to the signal of a calibration standard of known concentration. Two chromatograms were examined for noise-related peaks near the retention time of the analyte. The LOD was calculated as 3 times the concentration equivalent of the mean noise level. The LOQ was calculated as 10 times the concentration equivalent of the mean noise level.

## RESULTS AND DISCUSSION

### A. Environmental Conditions - Definitive and Recovery Tests (Tables 1-2)

- For the definitive test, the pH measurements of the test solutions ranged from 7.23 to 7.48 at the 0-hour and from 7.66 to 8.49 at the 72-hour interval.
- For the definitive test, light intensity for the areas used in the chamber ranged from 8580 to 9440 lumens/m<sup>2</sup> (= lux). The mean light intensity was 9022 lumens/m<sup>2</sup>.
- For the definitive test, the shaking speed was 107 revolutions per min (rpm).
- For the definitive test, the temperature from the digital readout on the temperature recorder ranged from 25.1 to 25.3°C for the duration of the study.

### B. Test Substance Stability and Analytical Verification (Table 3, Figures 1-4)

#### 1. Chromatographic Results

[REDACTED] eluted as a well-resolved peak with a retention time of approximately 6.8 minutes. [REDACTED] eluted as a well-resolved peak with a retention time of approximately 5.9 minutes. [REDACTED] eluted as a well-resolved peak with a retention time of approximately 5.3 minutes. Figure 1 shows a representative calibration curve obtained for [REDACTED]. A chromatogram of a typical standard is shown in Figure 2. Figures 3 and 4 show chromatograms of a blank control solution and test concentration solution, respectively.

The LOD and LOQ for [REDACTED] were determined to be 0.001 mg/L and 0.003 mg/L of H-25435, respectively. The LOD and LOQ for [REDACTED] were determined to be 0.012 mg/L and 0.04 mg/L of H-25435, respectively. The LOD and LOQ for [REDACTED] were determined to be 0.0036 mg/L and 0.012 mg/L of H-25435, respectively.

#### 2. Test Solution Results

The mean, measured concentration of [REDACTED] in the day 0 working stock (= 100 mg H-25435/L test concentration, = abiotic control) was 85 mg/L. This represents 85% recovery of the active ingredient. The mean, measured concentration of [REDACTED] in the day 0 working stock (=100 mg H-25435/L test concentration, = abiotic control) was 94 mg/L. This represents 94% recovery of the active ingredient. The mean, measured concentration of [REDACTED] in the day 0 working stock (=100 mg H-25435/L test concentration, = abiotic control) was 102 mg/L. This represents 102% recovery of the active ingredient. These data indicated the

stock solution was prepared at the desired concentration and confirmed the expected test concentration was achieved. The mean, measured concentrations of [REDACTED] in the day 0 nominal 6.25, 12.5, 25, and 50 mg/L test concentration solutions were 5.0, 11, 21, and 44 mg/L, respectively. This represents 80, 88, 84, 88 % recovery of the active ingredient, respectively. The mean, measured concentrations of [REDACTED] in the day 0 nominal 6.25, 12.5, 25, and 50 mg/L test concentration solutions were 6.2, 15, 27, and 50 mg/L, respectively. This represents 99, 120, 108, 100 % recovery of the active ingredient, respectively. The mean, measured concentrations of [REDACTED] in the day 0 nominal 6.25, 12.5, 25, and 50 mg/L test concentration solutions were 6.9, 13, 26, and 52 mg/L, respectively. This represents 110, 104, 104, 104 % recovery of the active ingredient, respectively. These data indicated the stock and test concentration solutions were prepared at the desired nominal concentrations. After 3 days, the mean, measured concentrations of [REDACTED] in the nominal 6.25, 12.5, 25, 50, and 100 mg/L test concentrations, and abiotic control solutions were 2.5, 5.9, 12, 20, and 45 mg/L and 44 mg/L, respectively. After 3 days, the mean, measured concentrations of [REDACTED] in the nominal 6.25, 12.5, 25, 50, and 100 mg/L test concentrations, and abiotic control solutions were 5.3, 12, 24, 54, and 100 mg/L and 95 mg/L, respectively. After 3 days, the mean, measured concentrations of [REDACTED] in the nominal 6.25, 12.5, 25, 50, and 100 mg/L test concentrations, and abiotic control solutions were 1.6, 7.6, 19, 43, and 86 mg/L and 110 mg/L, respectively. The blank control solutions contained no detectable concentrations of [REDACTED] on both day 0 and day 3.

Using the mean of the day 0 and day 3 results, the concentrations of [REDACTED], active ingredient of H-25435, of the nominal 6.25, 12.5, 25, 50, 100 mg/L test concentration and the abiotic control solutions were 3.75, 8.45, 16.5, 32, 65, and 64.5 mg/L, respectively. This represents 60, 68, 66, 64, 65, and 65% recovery of the active ingredient, respectively.

Using the mean of the day 0 and day 3 results, the concentrations of [REDACTED], active ingredient of H-25435, of the nominal 6.25, 12.5, 25, 50, 100 mg/L test concentration and the abiotic control solutions were 5.75, 13.5, 25.5, 52, 97, and 94.5 mg/L, respectively. This represents 92, 108, 102, 104, 97, and 95% recovery of the active ingredient, respectively.

Using the mean of the day 0 and day 3 results, the concentrations of [REDACTED], active ingredient of H-25435, of the nominal 6.25, 12.5, 25, 50, 100 mg/L test concentration and the abiotic control solutions were 4.25, 10.3, 22.5, 47.5, 94, and 106 mg/L, respectively. This represents 68, 82, 90, 95, 94, and 106% recovery of the active ingredient, respectively.

**C. *Selenastrum capricornutum* Growth**  
(Tables 4-8, Figures 5-7, Appendices C-E)

**1. Definitive Test**

The organisms were exposed for 72 hours without test medium renewal. The effects were expressed in terms of percent inhibition in growth based on healthy cell count, area under the

growth curve, and growth rate relative to the blank control for the 72-hour interval of the test. The 72-hour results are as follows:

**Healthy Cell Count**

|                  |                    |
|------------------|--------------------|
| EC <sub>50</sub> | > 100 mg H-25435/L |
| NOEC             | > 100 mg H-25435/L |

**Area Under the Growth Curve**

|                  |                    |
|------------------|--------------------|
| EC <sub>50</sub> | > 100 mg H-25435/L |
| NOEC             | > 100 mg H-25435/L |

**Growth Rate**

|                  |                    |
|------------------|--------------------|
| EC <sub>50</sub> | > 100 mg H-25435/L |
| NOEC             | > 100 mg H-25435/L |

**2. Recovery Test**

None of the definitive test concentrations exhibited a 50% or greater growth inhibition based on healthy cell count relative to the blank control. Therefore, a recovery test was not performed.

**CONCLUSIONS**

There was no significant inhibitory effect on the growth and growth rate of *Selenastrum capricornutum* when exposed to nominal concentrations of 6.25, 12.5, 25, 50, and 100 mgH-25435/L for 72 hours. Therefore, the EC<sub>50</sub> and NOEC for all 3 endpoints are greater than 100 mg H-25435/L.

**RECORDS AND SAMPLE STORAGE**

All data and records for analytical characterizations conducted by the sponsor will be retained by the sponsor.

Specimens (if applicable), raw data, and the final report will be retained at Haskell Laboratory, Newark, Delaware, the DuPont Crop Protection Archives, Newark, Delaware, or at Iron Mountain Records Management, Wilmington, Delaware.

## REFERENCES

1. Miller, W.E.; Greene, J.C.; Shiroyama, T. The *Selenastrum capricornutum* Printz Algal Assay Bottle Test; U.S. Environmental Protection Agency; U.S. Government Printing Office: Washington, DC, 1978; EPA-600/9-78-018.
2. American Society for Testing and Materials (ASTM). (1990). "Standard Guide for Conducting Static 96-h Toxicity Tests with Microalgae" in *ASTM Annual Book of Standards*, E1218-90. Vol. 11.04, Philadelphia, PA.
3. Snedecor, G.W. and Cochran, W.G. (1967). Statistical Methods, 6th edition. The Iowa State University Press.
4. Tamhane, A.C., Dunnett, C.W., Green, J.W., and Wetherington, J.D. (2001). Multiple test procedures for identifying the maximum safe dose. *J. Amer. Statist. Assoc.* 96, 835-843.

**TABLES**

**TABLE 1**  
**pH MEASUREMENTS OF TEST SOLUTIONS**

| Nominal<br>H-25435<br>Concentration | pH                                                   |                                                   |
|-------------------------------------|------------------------------------------------------|---------------------------------------------------|
|                                     | Exposure Initiated:<br>07-Jan-2003<br>0-Hour (Day 0) | Exposure Ended:<br>10-Jan-2003<br>72-Hour (Day 3) |
| Blank Control                       | 7.48                                                 | 7.66                                              |
| 6.25 mg/L                           | 7.39                                                 | 7.78                                              |
| 12.5 mg/L                           | 7.38                                                 | 8.43                                              |
| 25 mg/L                             | 7.32                                                 | 8.49                                              |
| 50 mg/L                             | 7.28                                                 | 8.48                                              |
| 100 mg/L                            | 7.23                                                 | 8.47                                              |
| Abiotic Control<br>(100 mg/L)       | 7.23                                                 | 7.66                                              |

**TABLE 2**  
**CHAMBER LIGHT INTENSITY, SHAKING SPEED, AND TEMPERATURE RANGE**

| Test       | Mean Light Intensity<br>at Test Initiation<br>(lumens/m <sup>2</sup> = lux) | Oscillations<br>(rpms) | Temperature<br>(°C) |
|------------|-----------------------------------------------------------------------------|------------------------|---------------------|
| Definitive | 9022                                                                        | 107                    | 25.1 to 25.2        |

**TABLE 3**  
**MEASURED CONCENTRATIONS OF H-25435 IN TEST SOLUTIONS**

| Sample<br>Description <sup>a</sup> | Total, Nominal<br>Formulation<br>Concentration<br>(mg/L) | Nominal<br>Concentration <sup>b</sup><br>(mg/L) | Mean, Measured<br>Concentration<br>(mg/L) |                 | Mean <sup>c</sup> | Mean %<br>Recovery |
|------------------------------------|----------------------------------------------------------|-------------------------------------------------|-------------------------------------------|-----------------|-------------------|--------------------|
|                                    |                                                          |                                                 | Day 0                                     | Day 3           |                   |                    |
| Blank Control                      | 0                                                        | 0                                               | ND <sup>d</sup>                           | ND              | ND                | NA <sup>e</sup>    |
| Working Stock<br>(100 mg/L)        | 100                                                      | 100                                             | 85                                        | NS <sup>f</sup> | NA                | NA                 |
| 6.25 mg/L                          | 6.25                                                     | 6.25                                            | 5.0                                       | 2.5             | 3.75              | 60                 |
| 12.5 mg/L                          | 12.5                                                     | 12.5                                            | 11                                        | 5.9             | 8.45              | 68                 |
| 25 mg/L                            | 25                                                       | 25                                              | 21                                        | 12              | 16.5              | 66                 |
| 50 mg/L                            | 50                                                       | 50                                              | 44                                        | 20              | 32                | 64                 |
| 100 mg/L                           | 100                                                      | 100                                             | 85 <sup>g</sup>                           | 45              | 65                | 65                 |
| Abiotic Control<br>(100 mg/L)      | 100                                                      | 100                                             | 85 <sup>g</sup>                           | 44              | 64.5              | 65                 |

a Defined in terms of mg H-25435/liter nutrient medium.

b H-25435 was assumed to be 100% for analytical purposes.

c Mean of day 0 and day 3 concentrations.

d ND stands for not detected. The limit of detection was calculated as 0.001 mg/L.

e NA stands for not applicable.

f NS stands for not submitted.

g No separate analysis was conducted since the working stock, the day 0 100 mg/L test concentration, and the day 0 abiotic control were aliquots of the same solution. Therefore, the measured concentration obtained for the working stock can be used for the 100 mg/L test concentration and the abiotic control.

**TABLE 3 (Continued)**  
**MEASURED CONCENTRATIONS OF H-25435 IN TEST SOLUTIONS**

| Sample<br>Description <sup>a</sup> | Total, Nominal<br>Formulation<br>Concentration<br>(mg/L) | Nominal<br>Concentration <sup>b</sup><br>(mg/L) | Mean, Measured<br>Concentration<br>(mg/L) |                 |                   | Mean %<br>Recovery |
|------------------------------------|----------------------------------------------------------|-------------------------------------------------|-------------------------------------------|-----------------|-------------------|--------------------|
|                                    |                                                          |                                                 | Day 0                                     | Day 3           | Mean <sup>c</sup> |                    |
| Blank Control                      | 0                                                        | 0                                               | ND <sup>d</sup>                           | ND              | ND                | NA <sup>e</sup>    |
| Working Stock<br>(100 mg/L)        | 100                                                      | 100                                             | 94                                        | NS <sup>f</sup> | NA                | NA                 |
| 6.25 mg/L                          | 6.25                                                     | 6.25                                            | 6.2                                       | 5.3             | 5.75              | 92                 |
| 12.5 mg/L                          | 12.5                                                     | 12.5                                            | 15                                        | 12              | 13.5              | 108                |
| 25 mg/L                            | 25                                                       | 25                                              | 27                                        | 24              | 25.5              | 102                |
| 50 mg/L                            | 50                                                       | 50                                              | 50                                        | 54              | 52                | 104                |
| 100 mg/L                           | 100                                                      | 100                                             | 94 <sup>g</sup>                           | 100             | 97                | 97                 |
| Abiotic Control<br>(100 mg/L)      | 100                                                      | 100                                             | 94 <sup>g</sup>                           | 95              | 94.5              | 95                 |

a Defined in terms of mg H-25435/liter nutrient medium.

b H-25435 was assumed to be 100% for analytical purposes.

c Mean of day 0 and day 3 concentrations.

d ND stands for not detected. The limit of detection was calculated as 0.001 mg/L.

e NA stands for not applicable.

f NS stands for not submitted.

g No separate analysis was conducted since the working stock, the day 0 100 mg/L test concentration, and the day 0 abiotic control were aliquots of the same solution. Therefore, the measured concentration obtained for the working stock can be used for the 100 mg/L test concentration and the abiotic control.

**TABLE 3 (Continued)**  
**MEASURED CONCENTRATIONS OF H-25435 IN TEST SOLUTIONS**

| Sample<br>Description <sup>a</sup> | Total, Nominal<br>Formulation<br>Concentration<br>(mg/L) | Nominal<br>Concentration <sup>b</sup><br>(mg/L) | Mean, Measured<br>Concentration<br>(mg/L) |                 | Mean <sup>c</sup> | Mean %<br>Recovery |
|------------------------------------|----------------------------------------------------------|-------------------------------------------------|-------------------------------------------|-----------------|-------------------|--------------------|
|                                    |                                                          |                                                 | Day 0                                     | Day 3           |                   |                    |
| Blank Control                      | 0                                                        | 0                                               | ND <sup>d</sup>                           | ND              | ND                | NA <sup>e</sup>    |
| Working Stock<br>(100 mg/L)        | 100                                                      | 100                                             | 102                                       | NS <sup>f</sup> | NA                | NA                 |
| 6.25 mg/L                          | 6.25                                                     | 6.25                                            | 6.9                                       | 1.6             | 4.25              | 68                 |
| 12.5 mg/L                          | 12.5                                                     | 12.5                                            | 13                                        | 7.6             | 10.3              | 82                 |
| 25 mg/L                            | 25                                                       | 25                                              | 26                                        | 19              | 22.5              | 90                 |
| 50 mg/L                            | 50                                                       | 50                                              | 52                                        | 43              | 47.5              | 95                 |
| 100 mg/L                           | 100                                                      | 100                                             | 102 <sup>g</sup>                          | 86              | 94                | 94                 |
| Abiotic Control<br>(100 mg/L)      | 100                                                      | 100                                             | 102 <sup>g</sup>                          | 110             | 106               | 106                |

a Defined in terms of mg H-25435/liter nutrient medium.

b H-25435 was assumed to be 100% for analytical purposes.

c Mean of day 0 and day 3 concentrations.

d ND stands for not detected. The limit of detection was calculated as 0.001 mg/L.

e NA stands for not applicable.

f NS stands for not submitted.

g No separate analysis was conducted since the working stock, the day 0 100 mg/L test concentration, and the day 0 abiotic control were aliquots of the same solution. Therefore, the measured concentration obtained for the working stock can be used for the 100 mg/L test concentration and the abiotic control.

**TABLE 4**  
**HEALTHY CELL COUNT DATA SUMMARY - DEFINITIVE TEST**

| Nominal<br>Total<br>Formulation<br>Concentration | Rep. | Exposure Initiated:                              |           | Exposure Ended:        |              |
|--------------------------------------------------|------|--------------------------------------------------|-----------|------------------------|--------------|
|                                                  |      | Day 0: 7 January 2003                            |           | Day 3: 10 January 2003 |              |
|                                                  |      | Healthy Cell Count (cells/mL) by Exposure Period |           |                        |              |
|                                                  |      | 0-Hour                                           | 24-Hour   | 48-Hour                | 72-Hour      |
| Blank Control                                    | 1    | 10,000                                           | 80,000    | 470,000                | 3,940,000    |
|                                                  | 2    | 10,000                                           | 100,000   | 640,000                | 2,740,000    |
|                                                  | 3    | 10,000                                           | 60,000    | 720,000                | 3,640,000    |
| Mean                                             |      | 10,000                                           | 80,000    | 610,000                | 3,440,000    |
| Standard Error                                   |      | 0.00                                             | 11,547.01 | 73,711.15              | 360,555.13   |
| Std. Dev.                                        |      | 0.00                                             | 20,000.00 | 127,671.45             | 624,499.80   |
| Variance                                         |      | 0.00E+00                                         | 4.00E+08  | 1.63E+10               | 3.90E+11     |
| 6.25 mg/L                                        | 1    | 10,000                                           | 70,000    | 700,000                | 3,990,000    |
|                                                  | 2    | 10,000                                           | 70,000    | 960,000                | 3,710,000    |
|                                                  | 3    | 10,000                                           | 80,000    | 340,000                | 1,760,000    |
| Mean                                             |      | 10,000                                           | 73,333    | 666,667                | 3,153,333    |
| Standard Error                                   |      | 0.00                                             | 3,333.33  | 179,752.92             | 701,339.99   |
| Std. Dev.                                        |      | 0.00                                             | 5,773.50  | 311,341.18             | 1,214,756.49 |
| Variance                                         |      | 0.00E+00                                         | 3.33E+07  | 9.69E+10               | 1.48E+12     |
| % Inhibition                                     |      | 0.00                                             | 8.33      | -9.29                  | 8.33         |
| 12.5 mg/L                                        | 1    | 10,000                                           | 110,000   | 520,000                | 3,770,000    |
|                                                  | 2    | 10,000                                           | 80,000    | 500,000                | 3,570,000    |
|                                                  | 3    | 10,000                                           | 90,000    | 920,000                | 5,200,000    |
| Mean                                             |      | 10,000                                           | 93,333    | 646,667                | 4,180,000    |
| Standard Error                                   |      | 0.00                                             | 8,819.17  | 136,788.56             | 513,257.57   |
| Std. Dev.                                        |      | 0.00                                             | 15,275.25 | 236,924.74             | 888,988.19   |
| Variance                                         |      | 0.00E+00                                         | 2.33E+08  | 5.61E+10               | 7.90E+11     |
| % Inhibition                                     |      | 0.00                                             | -16.67    | -6.01                  | -21.51       |
| 25 mg/L                                          | 1    | 10,000                                           | 60,000    | 530,000                | 3,710,000    |
|                                                  | 2    | 10,000                                           | 70,000    | 910,000                | 4,050,000    |
|                                                  | 3    | 10,000                                           | 100,000   | 540,000                | 3,450,000    |
| Mean                                             |      | 10,000                                           | 76,667    | 660,000                | 3,736,667    |
| Standard Error                                   |      | 0.00                                             | 12,018.50 | 125,033.33             | 173,717.52   |
| Std. Dev.                                        |      | 0.00                                             | 20,816.66 | 216,564.08             | 300,887.58   |
| Variance                                         |      | 0.00E+00                                         | 4.33E+08  | 4.69E+10               | 9.05E+10     |
| % Inhibition                                     |      | 0.00                                             | 4.17      | -8.20                  | -8.62        |
| 50 mg/L                                          | 1    | 10,000                                           | 100,000   | 770,000                | 3,900,000    |
|                                                  | 2    | 10,000                                           | 170,000   | 920,000                | 2,040,000    |
|                                                  | 3    | 10,000                                           | 90,000    | 670,000                | 4,010,000    |
| Mean                                             |      | 10,000                                           | 120,000   | 786,667                | 3,316,667    |
| Standard Error                                   |      | 0.00                                             | 25,166.11 | 72,648.32              | 639,122.66   |
| Std. Dev.                                        |      | 0.00                                             | 43,588.99 | 125,830.57             | 1,106,992.92 |
| Variance                                         |      | 0.00E+00                                         | 1.90E+09  | 1.58E+10               | 1.23E+12     |
| % Inhibition                                     |      | 0.00                                             | -50.00    | -28.96                 | 3.59         |
| 100 mg/L                                         | 1    | 10,000                                           | 90,000    | 760,000                | 4,360,000    |
|                                                  | 2    | 10,000                                           | 80,000    | 400,000                | 3,600,000    |
|                                                  | 3    | 10,000                                           | 120,000   | 580,000                | 3,600,000    |
| Mean                                             |      | 10,000                                           | 96,667    | 580,000                | 3,853,333    |
| Standard Error                                   |      | 0.00                                             | 12,018.50 | 103,923.05             | 253,333.33   |
| Std. Dev.                                        |      | 0.00                                             | 20,816.66 | 180,000.00             | 438,786.20   |
| Variance                                         |      | 0.00E+00                                         | 4.33E+08  | 3.24E+10               | 1.93E+11     |
| % Inhibition                                     |      | 0.00                                             | -20.83    | 4.92                   | -12.02       |

**TABLE 5**  
**AREA UNDER THE GROWTH CURVE DATA SUMMARY - DEFINITIVE TEST**

|                                                  |      | Exposure Initiated:                              |              | Exposure Ended:        |  |
|--------------------------------------------------|------|--------------------------------------------------|--------------|------------------------|--|
|                                                  |      | Day 0: 7 January 2003                            |              | Day 3: 10 January 2003 |  |
| Nominal<br>Total<br>Formulation<br>Concentration | Rep. | Area Under the Growth Curve Based on             |              |                        |  |
|                                                  |      | Healthy Cell Count (cells/mL) by Exposure Period |              |                        |  |
|                                                  |      | 0-24 Hour                                        | 0-48 Hour    | 0-72 Hour              |  |
| Blank Control                                    | 1    | 840,000                                          | 7,200,000    | 59,880,000             |  |
|                                                  | 2    | 1,080,000                                        | 9,720,000    | 50,040,000             |  |
|                                                  | 3    | 600,000                                          | 9,720,000    | 61,800,000             |  |
| Mean                                             |      | 840,000                                          | 8,880,000    | 57,240,000             |  |
| Standard Error                                   |      | 138,564.06                                       | 840,000.00   | 3,642,416.78           |  |
| Std. Dev.                                        |      | 240,000.00                                       | 1,454,922.68 | 6,308,850.93           |  |
| Variance                                         |      | 5.76E+10                                         | 2.12E+12     | 3.98E+13               |  |
| 6.25 mg/L                                        | 1    | 720,000                                          | 9,720,000    | 65,760,000             |  |
|                                                  | 2    | 720,000                                          | 12,840,000   | 68,640,000             |  |
|                                                  | 3    | 840,000                                          | 5,640,000    | 30,600,000             |  |
| Mean                                             |      | 760,000                                          | 9,400,000    | 55,000,000             |  |
| Standard Error                                   |      | 40,000.00                                        | 2,084,610.28 | 12,228,295.06          |  |
| Std. Dev.                                        |      | 69,282.03                                        | 3,610,650.91 | 21,180,028.33          |  |
| Variance                                         |      | 4.80E+09                                         | 1.30E+13     | 4.49E+14               |  |
| % Inhibition                                     |      | 9.52                                             | -5.86        | 3.91                   |  |
| 12.5 mg/L                                        | 1    | 1,200,000                                        | 8,520,000    | 59,760,000             |  |
|                                                  | 2    | 840,000                                          | 7,560,000    | 56,160,000             |  |
|                                                  | 3    | 960,000                                          | 12,840,000   | 86,040,000             |  |
| Mean                                             |      | 1,000,000                                        | 9,640,000    | 67,320,000             |  |
| Standard Error                                   |      | 105,830.05                                       | 1,623,822.65 | 9,417,515.60           |  |
| Std. Dev.                                        |      | 183,303.03                                       | 2,812,543.33 | 16,311,615.49          |  |
| Variance                                         |      | 3.36E+10                                         | 7.91E+12     | 2.66E+14               |  |
| % Inhibition                                     |      | -19.05                                           | -8.56        | -17.61                 |  |
| 25 mg/L                                          | 1    | 600,000                                          | 7,440,000    | 58,080,000             |  |
|                                                  | 2    | 720,000                                          | 12,240,000   | 71,520,000             |  |
|                                                  | 3    | 1,080,000                                        | 8,520,000    | 56,160,000             |  |
| Mean                                             |      | 800,000                                          | 9,400,000    | 61,920,000             |  |
| Standard Error                                   |      | 144,222.05                                       | 1,453,822.55 | 4,831,894.04           |  |
| Std. Dev.                                        |      | 249,799.92                                       | 2,518,094.52 | 8,369,085.97           |  |
| Variance                                         |      | 6.24E+10                                         | 6.34E+12     | 7.00E+13               |  |
| % Inhibition                                     |      | 4.76                                             | -5.86        | -8.18                  |  |
| 50 mg/L                                          | 1    | 1,080,000                                        | 11,280,000   | 67,080,000             |  |
|                                                  | 2    | 1,920,000                                        | 14,760,000   | 50,040,000             |  |
|                                                  | 3    | 960,000                                          | 9,840,000    | 65,760,000             |  |
| Mean                                             |      | 1,320,000                                        | 11,960,000   | 60,960,000             |  |
| Standard Error                                   |      | 301,993.38                                       | 1,460,410.90 | 5,473,280.55           |  |
| Std. Dev.                                        |      | 523,067.87                                       | 2,529,505.88 | 9,480,000.00           |  |
| Variance                                         |      | 2.74E+11                                         | 6.40E+12     | 8.99E+13               |  |
| % Inhibition                                     |      | -57.14                                           | -34.68       | -6.50                  |  |
| 100 mg/L                                         | 1    | 960,000                                          | 10,920,000   | 72,120,000             |  |
|                                                  | 2    | 840,000                                          | 6,360,000    | 54,120,000             |  |
|                                                  | 3    | 1,320,000                                        | 9,480,000    | 59,400,000             |  |
| Mean                                             |      | 1,040,000                                        | 8,920,000    | 61,880,000             |  |
| Standard Error                                   |      | 144,222.05                                       | 1,345,808.31 | 5,342,059.53           |  |
| Std. Dev.                                        |      | 249,799.92                                       | 2,331,008.37 | 9,252,718.52           |  |
| Variance                                         |      | 6.24E+10                                         | 5.43E+12     | 8.56E+13               |  |
| % Inhibition                                     |      | -23.81                                           | -0.45        | -8.11                  |  |

**TABLE 6**  
**GROWTH RATE DATA SUMMARY - DEFINITIVE TEST**

| Nominal<br>Total<br>Formulation<br>Concentration |                | Exposure Initiated:                              |           | Exposure Ended:        |  |
|--------------------------------------------------|----------------|--------------------------------------------------|-----------|------------------------|--|
|                                                  |                | Day 0: 7 January 2003                            |           | Day 3: 10 January 2003 |  |
|                                                  |                | Growth Rate Based on                             |           |                        |  |
|                                                  |                | Healthy Cell Count (cells/mL) by Exposure Period |           |                        |  |
| Rep.                                             |                | 0-24 Hour                                        | 0-48 Hour | 0-72 Hour              |  |
| Blank Control                                    | 1              | 0.0866                                           | 0.0802    | 0.0830                 |  |
|                                                  | 2              | 0.0959                                           | 0.0866    | 0.0780                 |  |
|                                                  | 3              | 0.0747                                           | 0.0891    | 0.0819                 |  |
|                                                  | Mean           | 0.0857                                           | 0.0853    | 0.0810                 |  |
|                                                  | Standard Error | 0.0061                                           | 0.0027    | 0.0015                 |  |
| Std. Dev.                                        | 0.0106         | 0.0046                                           | 0.0026    |                        |  |
| Variance                                         | 1.13E-04       | 2.11E-05                                         | 6.90E-06  |                        |  |
| 6.25 mg/L                                        | 1              | 0.0811                                           | 0.0885    | 0.0832                 |  |
|                                                  | 2              | 0.0811                                           | 0.0951    | 0.0822                 |  |
|                                                  | 3              | 0.0866                                           | 0.0735    | 0.0718                 |  |
|                                                  | Mean           | 0.0829                                           | 0.0857    | 0.0791                 |  |
|                                                  | Standard Error | 0.0018                                           | 0.0064    | 0.0036                 |  |
| Std. Dev.                                        | 0.0032         | 0.0111                                           | 0.0063    |                        |  |
| Variance                                         | 1.01E-05       | 1.23E-04                                         | 3.99E-05  |                        |  |
| % Inhibition                                     | 3.27           | -0.47                                            | 2.35      |                        |  |
| 12.5 mg/L                                        | 1              | 0.0999                                           | 0.0823    | 0.0824                 |  |
|                                                  | 2              | 0.0866                                           | 0.0815    | 0.0816                 |  |
|                                                  | 3              | 0.0916                                           | 0.0942    | 0.0869                 |  |
|                                                  | Mean           | 0.0927                                           | 0.0860    | 0.0836                 |  |
|                                                  | Standard Error | 0.0039                                           | 0.0041    | 0.0016                 |  |
| Std. Dev.                                        | 0.0067         | 0.0071                                           | 0.0029    |                        |  |
| Variance                                         | 4.51E-05       | 5.06E-05                                         | 8.16E-06  |                        |  |
| % Inhibition                                     | -8.13          | -0.82                                            | -3.29     |                        |  |
| 25 mg/L                                          | 1              | 0.0747                                           | 0.0827    | 0.0822                 |  |
|                                                  | 2              | 0.0811                                           | 0.0940    | 0.0834                 |  |
|                                                  | 3              | 0.0959                                           | 0.0831    | 0.0812                 |  |
|                                                  | Mean           | 0.0839                                           | 0.0866    | 0.0823                 |  |
|                                                  | Standard Error | 0.0063                                           | 0.0037    | 0.0006                 |  |
| Std. Dev.                                        | 0.0109         | 0.0064                                           | 0.0011    |                        |  |
| Variance                                         | 1.18E-04       | 4.11E-05                                         | 1.21E-06  |                        |  |
| % Inhibition                                     | 2.14           | -1.52                                            | -1.61     |                        |  |
| 50 mg/L                                          | 1              | 0.0959                                           | 0.0905    | 0.0829                 |  |
|                                                  | 2              | 0.1181                                           | 0.0942    | 0.0739                 |  |
|                                                  | 3              | 0.0916                                           | 0.0876    | 0.0832                 |  |
|                                                  | Mean           | 0.1019                                           | 0.0908    | 0.0800                 |  |
|                                                  | Standard Error | 0.0082                                           | 0.0019    | 0.0031                 |  |
| Std. Dev.                                        | 0.0142         | 0.0033                                           | 0.0053    |                        |  |
| Variance                                         | 2.02E-04       | 1.09E-05                                         | 2.79E-05  |                        |  |
| % Inhibition                                     | -18.82         | -6.41                                            | 1.19      |                        |  |
| 100 mg/L                                         | 1              | 0.0916                                           | 0.0902    | 0.0844                 |  |
|                                                  | 2              | 0.0866                                           | 0.0769    | 0.0818                 |  |
|                                                  | 3              | 0.1035                                           | 0.0846    | 0.0818                 |  |
|                                                  | Mean           | 0.0939                                           | 0.0839    | 0.0827                 |  |
|                                                  | Standard Error | 0.0050                                           | 0.0039    | 0.0009                 |  |
| Std. Dev.                                        | 0.0087         | 0.0067                                           | 0.0015    |                        |  |
| Variance                                         | 7.54E-05       | 4.46E-05                                         | 2.25E-06  |                        |  |
| % Inhibition                                     | -9.53          | 1.64                                             | -2.10     |                        |  |

**TABLE 7**  
**72-HOUR (DAY 3) STATISTICAL ANALYSES SUMMARY - DEFINITIVE TEST**

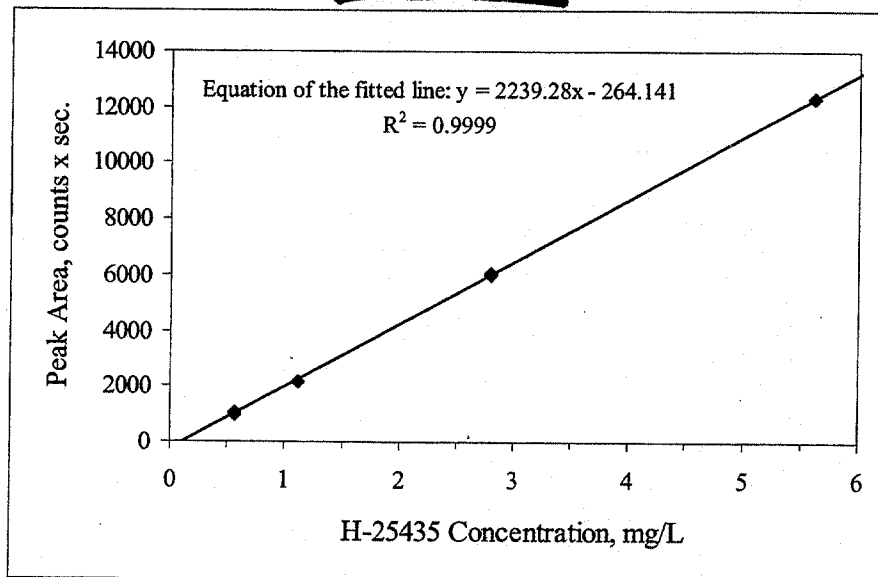
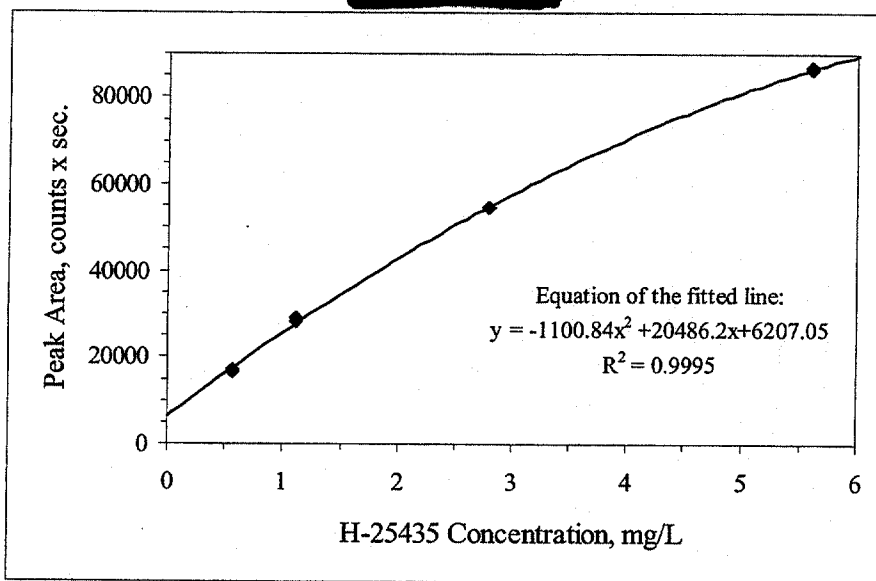
|                                        | Nominal<br>H-25435<br>Concentration | Mean<br>Response | Standard<br>Error | N | Test Concentration<br>Response (mg/L) |       |
|----------------------------------------|-------------------------------------|------------------|-------------------|---|---------------------------------------|-------|
|                                        |                                     |                  |                   |   | Level                                 | Value |
| <b>Healthy Cell<br/>Count</b>          | Blank Control                       | 3,440,000        | 360,555           | 3 |                                       |       |
|                                        | 6.25 mg/L                           | 3,153,333        | 701,340           | 3 | EC <sub>50</sub> <sup>b</sup>         | >100  |
|                                        | 12.5 mg/L                           | 4,180,000        | 513,258           | 3 |                                       |       |
|                                        | 25 mg/L                             | 3,736,667        | 173,718           | 3 | NOEC                                  | >100  |
|                                        | 50 mg/L                             | 3,316,667        | 639,123           | 3 |                                       |       |
|                                        | 100 mg/L                            | 3,853,333        | 253,333           | 3 |                                       |       |
| <b>Area Under the<br/>Growth Curve</b> | Blank Control                       | 57,240,000       | 3,642,417         | 3 |                                       |       |
|                                        | 6.25 mg/L                           | 55,000,000       | 12,228,295        | 3 | EC <sub>50</sub>                      | >100  |
|                                        | 12.5 mg/L                           | 67,320,000       | 9,417,516         | 3 |                                       |       |
|                                        | 25 mg/L                             | 61,920,000       | 4,831,894         | 3 | NOEC                                  | >100  |
|                                        | 50 mg/L                             | 60,960,000       | 5,473,281         | 3 |                                       |       |
|                                        | 100 mg/L                            | 61,880,000       | 5,342,060         | 3 |                                       |       |
| <b>Growth Rate</b>                     | Blank Control                       | 0.0810           | 0.0015            | 3 |                                       |       |
|                                        | 6.25 mg/L                           | 0.0791           | 0.0036            | 3 | EC <sub>50</sub>                      | >100  |
|                                        | 12.5 mg/L                           | 0.0836           | 0.0016            | 3 |                                       |       |
|                                        | 25 mg/L                             | 0.0823           | 0.0006            | 3 | NOEC                                  | >100  |
|                                        | 50 mg/L                             | 0.0800           | 0.0031            | 3 |                                       |       |
|                                        | 100 mg/L                            | 0.0827           | 0.0009            | 3 |                                       |       |

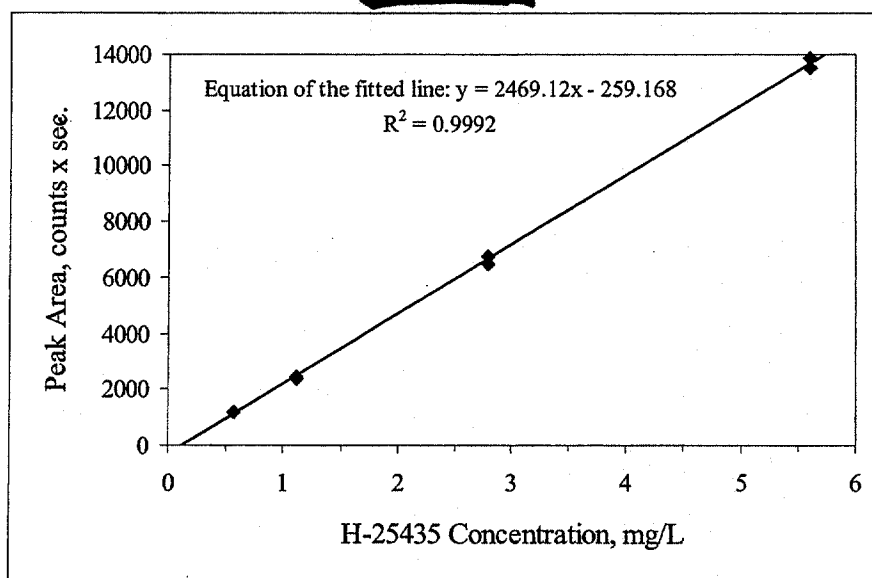
a Confidence Interval

b EC<sub>50</sub> was calculated using Probit analysis.

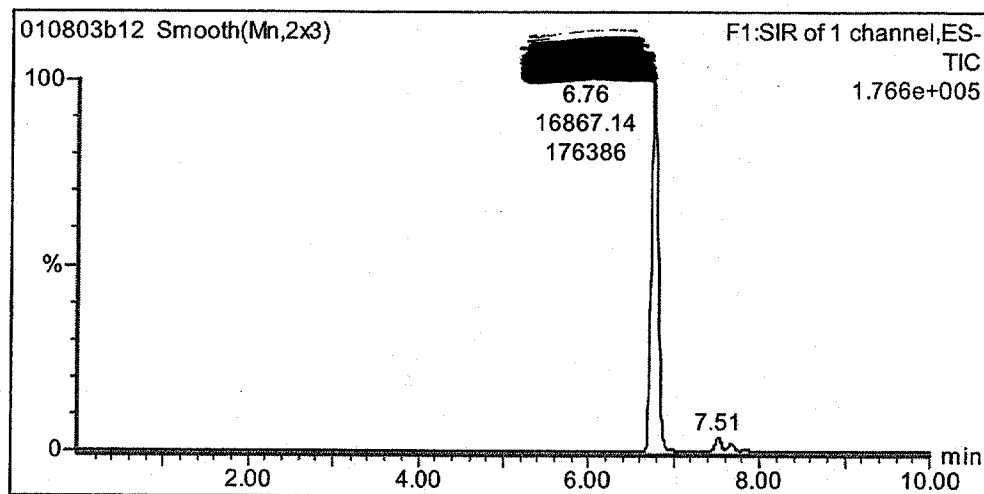
## FIGURES

**FIGURE 1**  
**REPRESENTATIVE ANALYTICAL CALIBRATION STANDARD CURVE FOR**  
**H-25435:** [REDACTED]

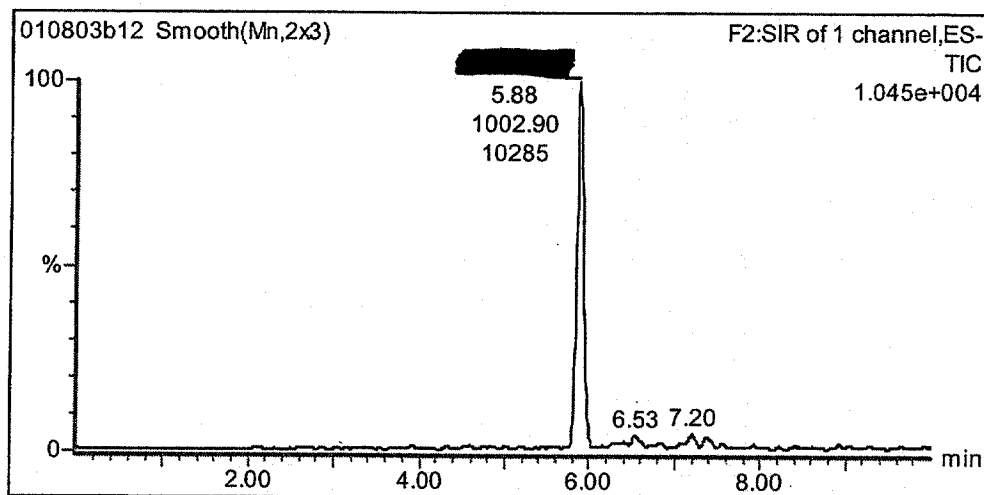




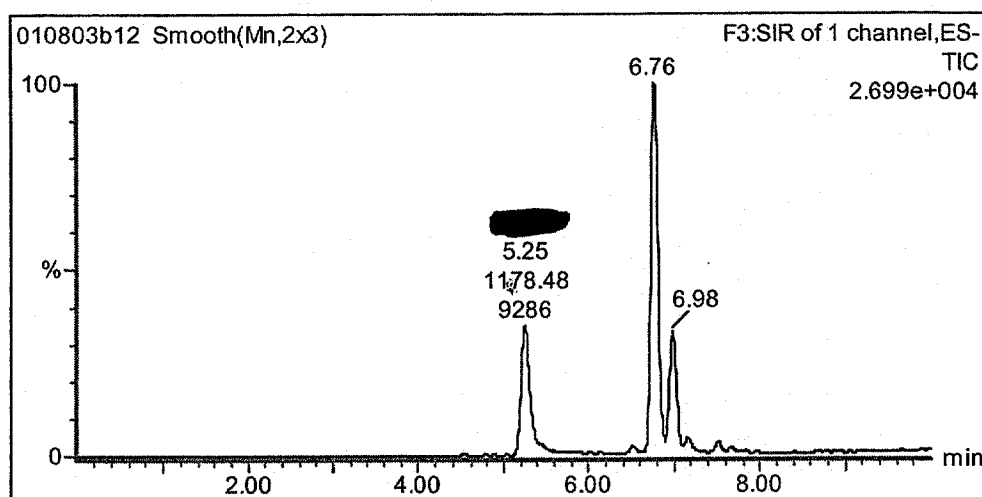
**FIGURE 2**  
**REPRESENTATIVE CHROMATOGRAM OF A CALIBRATION STANDARD SOLUTION**



[REDACTED] elutes at a retention time of approximately 6.8 minutes. Calibration standard solution contains H-25435 at a concentration of 0.56 mg/L.

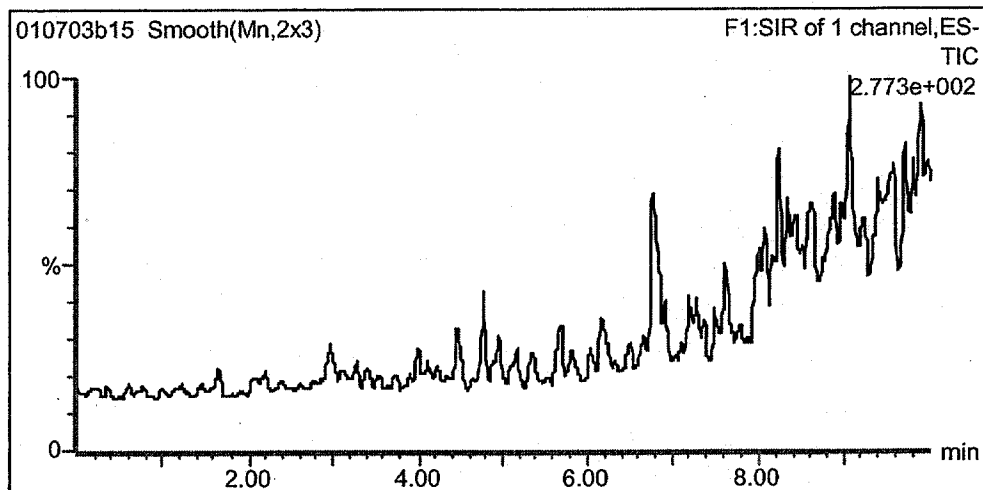


[REDACTED] elutes at a retention time of approximately 5.9 minutes. Calibration standard solution contains H-25435 at a concentration of 0.56 mg/L.

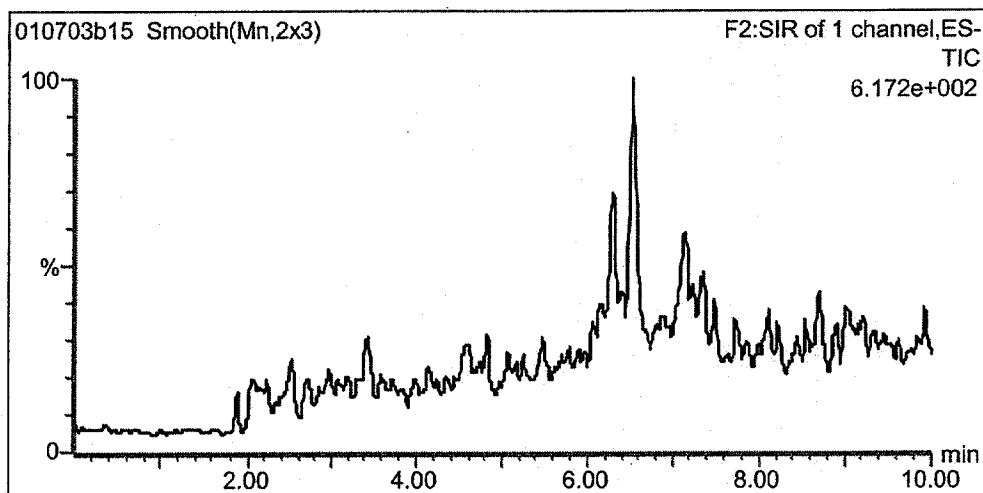


[Redacted] elutes at a retention time of approximately 5.3 minutes. Calibration standard solution contains H-25435 at a concentration of 0.56 mg/L.

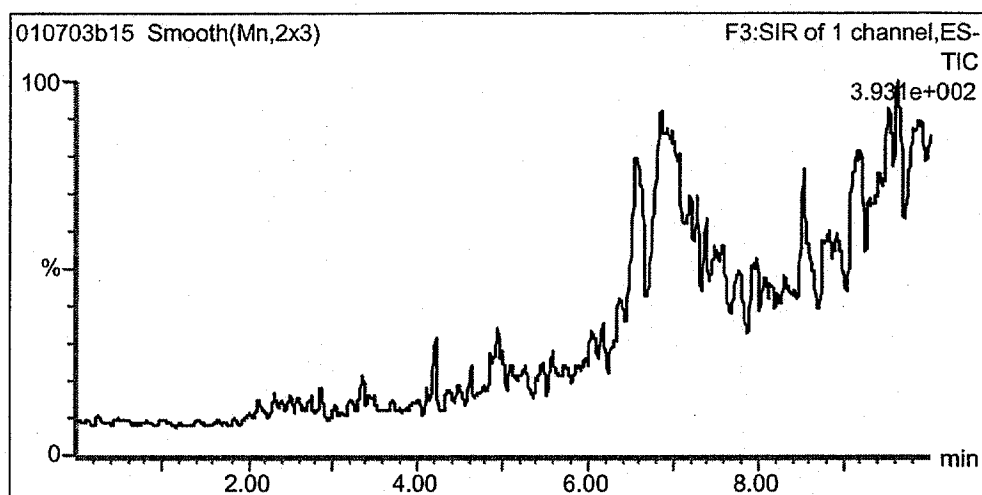
**FIGURE 3**  
**REPRESENTATIVE CHROMATOGRAM OF THE BLANK CONTROL SOLUTION**



██████████ would elute at a retention time of approximately 6.8 minutes.

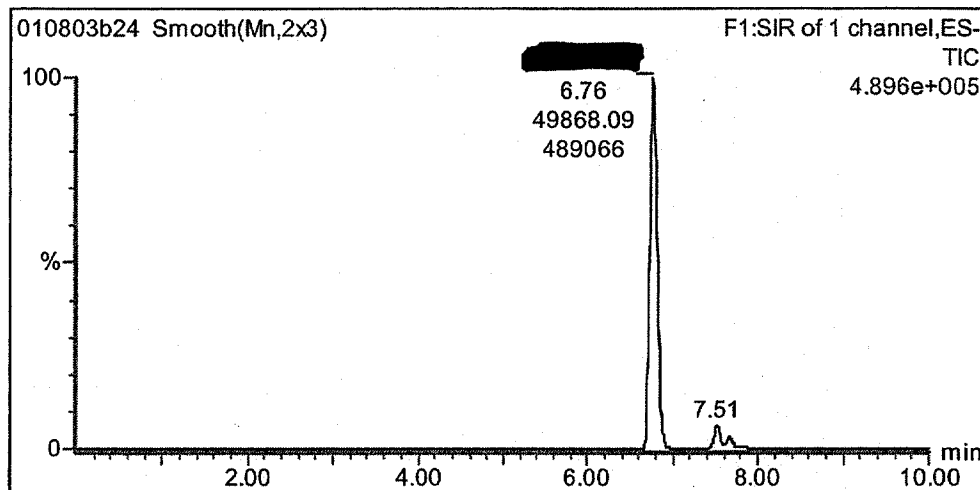


██████████ would elute at a retention time of approximately 5.9 minutes.

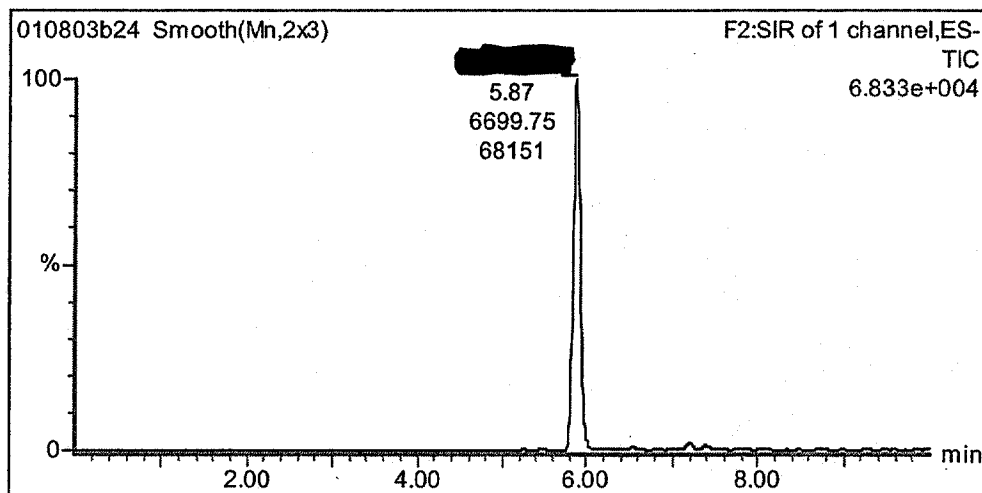


██████████ would elute at a retention time of approximately 5.3 minutes.

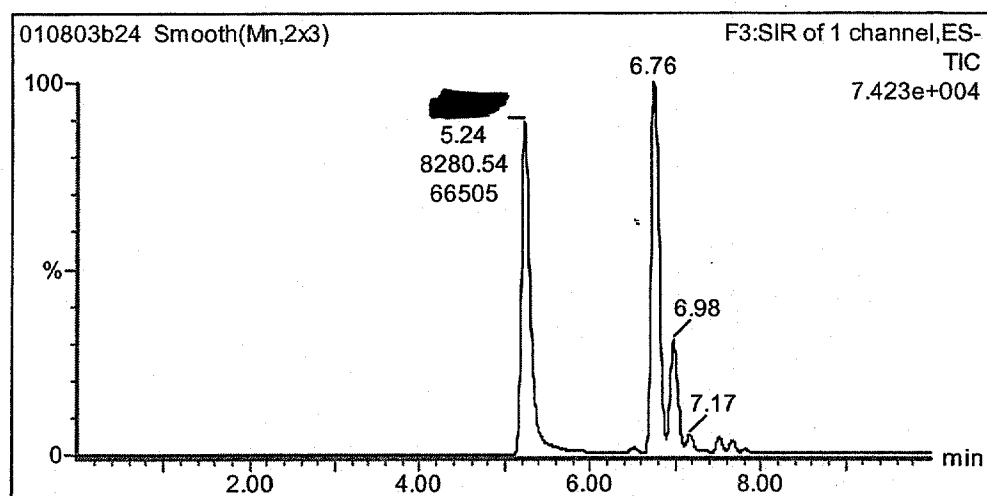
**FIGURE 4**  
**REPRESENTATIVE CHROMATOGRAM OF A TEST CONCENTRATION SOLUTION**



[REDACTED] elutes at a retention time of approximately 6.8 minutes. Test solution sample contains H-25435 at a nominal concentration of 6.25 mg/L. The sample was diluted 1:2 before analysis.



[REDACTED] elutes at a retention time of approximately 5.9 minutes. Test solution sample contains H-25435 at a nominal concentration of 6.25 mg/L. The sample was diluted 1:2 before analysis.



██████████ elutes at a retention time of approximately 5.3 minutes. Test solution sample contains H-25435 at a nominal concentration of 6.25 mg/L. The sample was diluted 1:2 before analysis.

## APPENDICES

## **APPENDIX A**

### **AAP Nutrient Medium Constituents**

### AAP NUTRIENT MEDIUM CONSTITUENTS

| Stock Solutions <sup>a</sup>                         |                      | Final Prepared Medium |                      |
|------------------------------------------------------|----------------------|-----------------------|----------------------|
| Component                                            | Concentration (g/L)  | Component             | Concentration (mg/L) |
| Macronutrient Stock #1                               |                      |                       |                      |
| NaNO <sub>3</sub>                                    | 25.500               | Na                    | 11.001               |
|                                                      |                      | N                     | 4.200                |
| Macronutrient Stock #2                               |                      |                       |                      |
| NaHCO <sub>3</sub>                                   | 15.000               | C                     | 2.143                |
|                                                      |                      | K                     | 0.469                |
| Macronutrient Stock #3                               |                      |                       |                      |
| K <sub>2</sub> HPO <sub>4</sub>                      | 1.044                | P                     | 0.186                |
|                                                      |                      | Mg                    | 2.904 <sup>b</sup>   |
| Macronutrient Stock #4                               |                      |                       |                      |
| MgSO <sub>4</sub> •7H <sub>2</sub> O                 | 14.700               | Ca                    | 1.202                |
|                                                      |                      | S                     | 1.911                |
| Macronutrient Stock #5                               |                      |                       |                      |
| MgCl <sub>2</sub> •6H <sub>2</sub> O                 | 12.164               |                       |                      |
| Macronutrient Stock #6                               |                      |                       |                      |
| CaCl <sub>2</sub> •2H <sub>2</sub> O                 | 4.410                |                       |                      |
| Stock Solutions <sup>a</sup>                         |                      | Final Prepared Medium |                      |
| Component                                            | Concentration (mg/L) | Component             | Concentration (µg/L) |
| Micronutrient Stock                                  |                      |                       |                      |
| H <sub>3</sub> BO <sub>3</sub>                       | 185.520              | B                     | 32.460               |
| MnCl <sub>2</sub> •4 H <sub>2</sub> O                | 415.610              | Mn                    | 115.374              |
| ZnCl <sub>2</sub>                                    | 3.271                | Zn                    | 1.570                |
| CoCl <sub>2</sub> •6 H <sub>2</sub> O                | 1.428                | Co                    | 0.354                |
| CuCl <sub>2</sub> •2H <sub>2</sub> O                 | 0.012                | Cu                    | 0.004                |
| Na <sub>2</sub> MoO <sub>4</sub> •2 H <sub>2</sub> O | 7.260                | Mo                    | 2.878                |
| FeCl <sub>3</sub> •6 H <sub>2</sub> O                | 160.000              | Fe                    | 33.051               |
| Na <sub>2</sub> EDTA•2 H <sub>2</sub> O              | 300.000              |                       |                      |

a As reported in Reference 2.

b Includes magnesium from both magnesium sulfate and magnesium chloride.

## **APPENDIX B**

### **72-Hour Healthy Cell Count Statistics for the Definitive Test**

---

**72-HOUR HEALTHY CELL COUNT STATISTICS FOR THE DEFINITIVE TEST**

---

OBSERVED PERCENT CHANGE FROM CONTROL MEAN  
RESPONSE: COUNT from DATASET: ██████████ AWR11604

| Obs | Rate<br>(mg/L) | MEAN       | PCTCHNG | N |
|-----|----------------|------------|---------|---|
| 1   | 0              | 3440000    | 0       | 3 |
| 2   | 6.25           | 3153333.33 | -8      | 3 |
| 3   | 12.5           | 4180000    | 22      | 3 |
| 4   | 25             | 3736666.67 | 9       | 3 |
| 5   | 50             | 3316666.67 | -4      | 3 |
| 6   | 100            | 3853333.33 | 12      | 3 |

STATISTICAL ANALYSIS LIST FILE  
REPORT CREATED ON 19:00 WEDNESDAY 22JAN03  
ECOTOX MEASUREMENTS FROM DATASET AWR11604  
GROUP STATISTICS FOR Count BY DOSE

| dose | doseval<br>(mg/L) | COUNT | MEAN       | MEDIAN  | STD_DEV    | STD_ERR   |
|------|-------------------|-------|------------|---------|------------|-----------|
| 1    | 0                 | 3     | 3440000.00 | 3640000 | 624499.80  | 360555.13 |
| 2    | 6.25              | 3     | 3153333.33 | 3710000 | 1214756.49 | 701339.99 |
| 3    | 12.5              | 3     | 4180000.00 | 3770000 | 888988.19  | 513257.57 |
| 4    | 25                | 3     | 3736666.67 | 3710000 | 300887.58  | 173717.52 |
| 5    | 50                | 3     | 3316666.67 | 3900000 | 1106992.92 | 639122.66 |
| 6    | 100               | 3     | 3853333.33 | 3600000 | 438786.20  | 253333.33 |

STATISTICAL ANALYSIS LIST FILE  
REPORT CREATED ON 19:00 WEDNESDAY 22JAN03  
SHAPIRO-WILK TEST OF NORMALITY OF Count  
AQUATIC SELENASTRUM: FULL DATA

| STD       | SKEW     | KURT     | SW_STAT | P_VALUE | SIGNIF |
|-----------|----------|----------|---------|---------|--------|
| 699952.38 | -0.54723 | -0.46879 | 0.94643 | 0.37177 |        |

STATISTICAL ANALYSIS LIST FILE  
REPORT CREATED ON 19:00 WEDNESDAY 22JAN03  
LEVENE TEST FOR Count - FULL Model  
ANALYSIS OF VARIANCE ON FULL DATA SET

| Effect | DF | LEVENE  | P_VALUE | SIGNIF |
|--------|----|---------|---------|--------|
| DOSE   | 5  | 0.26667 | 0.92276 |        |

The data was found to be normally distributed with equal variances.  
An analysis of variance will be performed.

STATISTICAL ANALYSIS LIST FILE  
REPORT CREATED ON 19:00 WEDNESDAY 22JAN03  
ANALYSIS OF SELENASTRUM - FULL DATA  
DOSES 0, 6.25, 12.5, 25, 50, 100 mg/L  
ECOTOX OF AQUATIC SELENASTRUM

| Obs | Class | Levels | Values      |
|-----|-------|--------|-------------|
| 1   | dose  | 6      | 1 2 3 4 5 6 |

OVERALL F-TESTS FOR ANOVA  
Count FROM FULL DATA, Weight=NO WEIGHT, Variance Ratio=0.33

| Obs | Effect | Num<br>DF | Den<br>DF | FValue | ProbF  |
|-----|--------|-----------|-----------|--------|--------|
| 1   | dose   | 5         | 12        | 0.63   | 0.6838 |

LSMEANS - DOSE MEANS ADJUSTED FOR THE EFFECTS OF SELENASTRUM  
Count FROM FULL DATA, Weight=NO WEIGHT, Variance Ratio=0.33

| Obs | LEVEL | LSMEAN     | SE        | DDF |
|-----|-------|------------|-----------|-----|
| 1   | 0     | 3440000.00 | 480996.27 | 12  |
| 2   | 6.25  | 3153333.33 | 480996.27 | 12  |
| 3   | 12.5  | 4180000.00 | 480996.27 | 12  |
| 4   | 25    | 3736666.67 | 480996.27 | 12  |
| 5   | 50    | 3316666.67 | 480996.27 | 12  |
| 6   | 100   | 3853333.33 | 480996.27 | 12  |

ESTIMATED DOSE EFFECTS & DUNNETT FOR Two-sided ALTERNATIVE  
USING ALPHA=.05 FOR COMPARISONS TO CONTROL  
Count FROM FULL DATA, Weight=NO WEIGHT, Variance Ratio=0.33

| Estimate   | SIGNIF | Dunnett<br>2-sided<br>p-value | Test Group<br>Mean | N |
|------------|--------|-------------------------------|--------------------|---|
| DOSE TREND |        | 0.60905                       | .                  | . |
| DOSE QUAD  |        | 0.71144                       | .                  | . |
| DOSE 2-1   |        | 0.99052                       | 3153333.33         | 3 |
| DOSE 3-1   |        | 0.71946                       | 4180000.00         | 3 |
| DOSE 4-1   |        | 0.98899                       | 3736666.67         | 3 |
| DOSE 5-1   |        | 0.99982                       | 3316666.67         | 3 |
| DOSE 6-1   |        | 0.95690                       | 3853333.33         | 3 |

STATISTICAL ANALYSIS LIST FILE  
REPORT CREATED ON 19:00 WEDNESDAY 22JAN03  
ANALYSIS OF ECOTOX USING ALPHA=.05

FULL DATA  
TEST

Jonckheere Trend Test on Dose 0  
and Lowest 5 Doses through Dose 100 mg/L  
Analysis of Count

KEY

ZC IS JONCKHEERE STATISTIC COMPUTED WITH TIE CORRECTION  
ZCCF IS ZC WITH CONTINUITY CORRECTION FACTOR  
P1UPCF IS P-VALUE FOR UPWARD TREND  
P1DNCF IS P-VALUE FOR DOWNWARD TREND  
P-VALUES ARE FOR TIE-CORRECTED TEST WITH CONTINUITY CORRECTION FACTOR  
SIGNIF RESULTS ARE FOR A TWO-SIDED ALTERNATIVE HYPOTHESIS

|      |          |           |           |           |        |
|------|----------|-----------|-----------|-----------|--------|
| JONC | ZC       | ZCCF      | P1UPCF    | P1DNCF    | SIGNIF |
| 74.5 | 0.539566 | 0.5781065 | 0.2815961 | 0.6918234 |        |

NOTE

Jonckheere test results are included in the summary table.  
Group means should be examined to check for lack-of-fit  
to a linear trend before trend test results are accepted.

## **APPENDIX C**

### **72-Hour Area Under the Growth Curve Statistics for the Definitive Test**

---

**72-HOUR AREA UNDER THE GROWTH CURVE STATISTICS FOR THE  
DEFINITIVE TEST**

---

| Obs | Rate<br>(mg/L) | MEAN     | PCTCHNG | N |
|-----|----------------|----------|---------|---|
| 1   | 0              | 57240000 | 0       | 3 |
| 2   | 6.25           | 55000000 | -4      | 3 |
| 3   | 12.5           | 67320000 | 18      | 3 |
| 4   | 25             | 61920000 | 8       | 3 |
| 5   | 50             | 60960000 | 6       | 3 |
| 6   | 100            | 61880000 | 8       | 3 |

STATISTICAL ANALYSIS LIST FILE  
REPORT CREATED ON 19:00 WEDNESDAY 22JAN03  
ECOTOX MEASUREMENTS FROM DATASET AWR11604  
GROUP STATISTICS FOR Area BY DOSE

| dose | doseval<br>(mg/L) | COUNT | MEAN     | MEDIAN   | STD_DEV     | STD_ERR     |
|------|-------------------|-------|----------|----------|-------------|-------------|
| 1    | 0                 | 3     | 57240000 | 59880000 | 6308850.93  | 3642416.78  |
| 2    | 6.25              | 3     | 55000000 | 65760000 | 21180028.33 | 12228295.06 |
| 3    | 12.5              | 3     | 67320000 | 59760000 | 16311615.49 | 9417515.60  |
| 4    | 25                | 3     | 61920000 | 58080000 | 8369085.97  | 4831894.04  |
| 5    | 50                | 3     | 60960000 | 65760000 | 9480000.00  | 5473280.55  |
| 6    | 100               | 3     | 61880000 | 59400000 | 9252718.52  | 5342059.53  |

STATISTICAL ANALYSIS LIST FILE  
REPORT CREATED ON 19:00 WEDNESDAY 22JAN03  
SHAPIRO-WILK TEST OF NORMALITY OF Area  
AQUATIC SELENASTRUM: FULL DATA

| OBS | STD         | SKEW     | KURT      | SW_STAT | P_VALUE | SIGNIF |
|-----|-------------|----------|-----------|---------|---------|--------|
| 18  | 10846462.15 | -0.31113 | -0.052253 | 0.96885 | 0.77585 |        |

STATISTICAL ANALYSIS LIST FILE  
REPORT CREATED ON 19:00 WEDNESDAY 22JAN03  
LEVENE TEST FOR Area - FULL Model  
ANALYSIS OF VARIANCE ON FULL DATA SET

| Effect | DF | LEVENE  | P_VALUE | SIGNIF |
|--------|----|---------|---------|--------|
| DOSE   | 5  | 0.26034 | 0.92624 |        |

NOTE

The data was found to be normally distributed with equal variances.  
An analysis of variance will be performed

STATISTICAL ANALYSIS LIST FILE  
REPORT CREATED ON 19:00 WEDNESDAY 22JAN03  
ANALYSIS OF SELENASTRUM - FULL DATA  
DOSES 0, 6.25, 12.5, 25, 50, 100 mg/L  
ECOTOX OF AQUATIC SELENASTRUM

| Obs | Class | Levels | Values      |
|-----|-------|--------|-------------|
| 1   | dose  | 6      | 1 2 3 4 5 6 |

OVERALL F-TESTS FOR ANOVA  
Area FROM FULL DATA, Weight=NO WEIGHT, Variance Ratio=0.33

| Obs | Effect | Num<br>DF | Den<br>DF | FValue | ProbF  |
|-----|--------|-----------|-----------|--------|--------|
| 1   | dose   | 5         | 12        | 0.33   | 0.8862 |

LSMEANS - DOSE MEANS ADJUSTED FOR THE EFFECTS OF SELENASTRUM  
Area FROM FULL DATA, Weight=NO WEIGHT, Variance Ratio=0.33

| Obs | LEVEL | LSMEAN   | SE         | DDF |
|-----|-------|----------|------------|-----|
| 1   | 0     | 57240000 | 7453518.18 | 12  |
| 2   | 6.25  | 55000000 | 7453518.18 | 12  |
| 3   | 12.5  | 67320000 | 7453518.18 | 12  |
| 4   | 25    | 61920000 | 7453518.18 | 12  |
| 5   | 50    | 60960000 | 7453518.18 | 12  |
| 6   | 100   | 61880000 | 7453518.18 | 12  |

ESTIMATED DOSE EFFECTS & DUNNETT FOR Two-sided ALTERNATIVE  
USING ALPHA=.05 FOR COMPARISONS TO CONTROL  
Area FROM FULL DATA, Weight=NO WEIGHT, Variance Ratio=0.33

| Estimate   | SIGNIF | Dunnett<br>2-sided<br>p-value | Test<br>Group<br>Mean | N |
|------------|--------|-------------------------------|-----------------------|---|
| DOSE TREND |        | 0.57778                       | .                     | . |
| DOSE QUAD  |        | 0.59486                       | .                     | . |
| DOSE 2-1   |        | 0.99961                       | 55000000              | 3 |
| DOSE 3-1   |        | 0.80095                       | 67320000              | 3 |
| DOSE 4-1   |        | 0.98811                       | 61920000              | 3 |
| DOSE 5-1   |        | 0.99571                       | 60960000              | 3 |
| DOSE 6-1   |        | 0.98854                       | 61880000              | 3 |

STATISTICAL ANALYSIS LIST FILE  
REPORT CREATED ON 19:00 WEDNESDAY 22JAN03  
ANALYSIS OF ECOTOX USING ALPHA=.05  
FULL DATA  
TEST

Jonckheere Trend Test on Dose 0  
and Lowest 5 Doses through Dose 100 mg/L  
Analysis of Area

KEY

ZC IS JONCKHEERE STATISTIC COMPUTED WITH TIE CORRECTION  
ZCCF IS ZC WITH CONTINUITY CORRECTION FACTOR  
P1UPCF IS P-VALUE FOR UPWARD TREND  
P1DNCF IS P-VALUE FOR DOWNWARD TREND  
P-VALUES ARE FOR TIE-CORRECTED TEST WITH CONTINUITY CORRECTION FACTOR  
SIGNIF RESULTS ARE FOR A TWO-SIDED ALTERNATIVE HYPOTHESIS

| JONC | ZC | ZCCF | P1UPCF | P1DNCF | SIGNIF |
|------|----|------|--------|--------|--------|
|------|----|------|--------|--------|--------|

|      |           |           |           |           |  |
|------|-----------|-----------|-----------|-----------|--|
| 71.5 | 0.3085257 | 0.3470914 | 0.3642613 | 0.6064045 |  |
|------|-----------|-----------|-----------|-----------|--|

=====

NOTE

Jonckheere test results are included in the summary table.  
Group means should be examined to check for lack-of-fit  
to a linear trend before trend test results are accepted.

## **APPENDIX D**

### **72-Hour Growth Rate Statistics for the Definitive Test**

---

**72-HOUR GROWTH RATE STATISTICS FOR THE DEFINITIVE TEST**

---

| Obs | Rate<br>(mg/L) | MEAN       | PCTCHNG | N |
|-----|----------------|------------|---------|---|
| 1   | 0              | 0.08096667 | 0       | 3 |
| 2   | 6.25           | 0.07906667 | -2      | 3 |
| 3   | 12.5           | 0.08363333 | 3       | 3 |
| 4   | 25             | 0.08226667 | 2       | 3 |
| 5   | 50             | 0.08       | -1      | 3 |
| 6   | 100            | 0.08266667 | 2       | 3 |

STATISTICAL ANALYSIS LIST FILE  
REPORT CREATED ON 19:00 WEDNESDAY 22JAN03  
ECOTOX MEASUREMENTS FROM DATASET AWR11604  
GROUP STATISTICS FOR G\_Rate BY DOSE

| dose | doseval<br>(mg/L) | COUNT | MEAN     | MEDIAN | STD_DEV    | STD_ERR    |
|------|-------------------|-------|----------|--------|------------|------------|
| 1    | 0                 | 3     | 0.080967 | 0.0819 | .002627420 | .001516941 |
| 2    | 6.25              | 3     | 0.079067 | 0.0822 | .006312950 | .003644783 |
| 3    | 12.5              | 3     | 0.083633 | 0.0824 | .002857155 | .001649579 |
| 4    | 25                | 3     | 0.082267 | 0.0822 | .001101514 | .000635959 |
| 5    | 50                | 3     | 0.080000 | 0.0829 | .005284884 | .003051229 |
| 6    | 100               | 3     | 0.082667 | 0.0818 | .001501111 | .000866667 |

STATISTICAL ANALYSIS LIST FILE  
REPORT CREATED ON 19:00 WEDNESDAY 22JAN03  
SHAPIRO-WILK TEST OF NORMALITY OF G\_Rate  
AQUATIC SELENASTRUM: FULL DATA

| OBS | STD        | SKEW     | KURT    | SW_STAT | P_VALUE | SIGNIF |
|-----|------------|----------|---------|---------|---------|--------|
| 18  | .003186676 | -0.90046 | 0.43426 | 0.91952 | 0.12677 |        |

STATISTICAL ANALYSIS LIST FILE  
REPORT CREATED ON 19:00 WEDNESDAY 22JAN03  
LEVENE TEST FOR G\_Rate - FULL Model  
ANALYSIS OF VARIANCE ON FULL DATA SET

| Effect | DF | LEVENE  | P_VALUE | SIGNIF |
|--------|----|---------|---------|--------|
| DOSE   | 5  | 0.37769 | 0.85454 |        |

NOTE  
The data was found to be normally distributed with equal variances.  
An analysis of variance will be performed.

STATISTICAL ANALYSIS LIST FILE  
REPORT CREATED ON 19:00 WEDNESDAY 22JAN03  
ANALYSIS OF SELENASTRUM - FULL DATA  
DOSES 0, 6.25, 12.5, 25, 50, 100 mg/L  
ECOTOX OF AQUATIC SELENASTRUM

| Obs | Class | Levels | Values      |
|-----|-------|--------|-------------|
| 1   | dose  | 6      | 1 2 3 4 5 6 |

OVERALL F-TESTS FOR ANOVA

G\_Rate FROM FULL DATA, Weight=NO WEIGHT, Variance Ratio=0.33

| Obs | Effect | Num<br>DF | Den<br>DF | FValue | ProbF  |
|-----|--------|-----------|-----------|--------|--------|
| 1   | dose   | 5         | 12        | 0.62   | 0.6858 |

LSMEANS - DOSE MEANS ADJUSTED FOR THE EFFECTS OF SELENASTRUM  
G\_Rate FROM FULL DATA, Weight=NO WEIGHT, Variance Ratio=0.33

| Obs | LEVEL | LSMEAN   | SE         | DDF |
|-----|-------|----------|------------|-----|
| 1   | 0     | 0.080967 | .002189833 | 12  |
| 2   | 6.25  | 0.079067 | .002189833 | 12  |
| 3   | 12.5  | 0.083633 | .002189833 | 12  |
| 4   | 25    | 0.082267 | .002189833 | 12  |
| 5   | 50    | 0.080000 | .002189833 | 12  |
| 6   | 100   | 0.082667 | .002189833 | 12  |

ESTIMATED DOSE EFFECTS & DUNNETT FOR Two-sided ALTERNATIVE  
USING ALPHA=.05 FOR COMPARISONS TO CONTROL  
G\_Rate FROM FULL DATA, Weight=NO WEIGHT, Variance Ratio=0.33

| Estimate   | SIGNIF | Dunnett<br>2-sided<br>p-value | Test<br>Group<br>Mean | N |
|------------|--------|-------------------------------|-----------------------|---|
| DOSE TREND |        | 0.59763                       | .                     | . |
| DOSE QUAD  |        | 0.82636                       | .                     | . |
| DOSE 2-1   |        | 0.95528                       | 0.079067              | 3 |
| DOSE 3-1   |        | 0.85419                       | 0.083633              | 3 |
| DOSE 4-1   |        | 0.99068                       | 0.082267              | 3 |
| DOSE 5-1   |        | 0.99756                       | 0.080000              | 3 |
| DOSE 6-1   |        | 0.97113                       | 0.082667              | 3 |

STATISTICAL ANALYSIS LIST FILE  
REPORT CREATED ON 19:00 WEDNESDAY 22JAN03  
ANALYSIS OF ECOTOX USING ALPHA=.05  
FULL DATA  
TEST

Jonckheere Trend Test on Dose 0  
and Lowest 5 Doses through Dose 100 mg/L  
Analysis of G\_Rate

KEY

ZC IS JONCKHEERE STATISTIC COMPUTED WITH TIE CORRECTION  
ZCCF IS ZC WITH CONTINUITY CORRECTION FACTOR  
P1UPCF IS P-VALUE FOR UPWARD TREND  
P1DNCF IS P-VALUE FOR DOWNWARD TREND  
P-VALUES ARE FOR TIE-CORRECTED TEST WITH CONTINUITY CORRECTION FACTOR  
SIGNIF RESULTS ARE FOR A TWO-SIDED ALTERNATIVE HYPOTHESIS

| JONC | ZC        | ZCCF    | P1UPCF    | P1DNCF   | SIGNIF |
|------|-----------|---------|-----------|----------|--------|
| 74   | 0.5013542 | 0.53992 | 0.2946261 | 0.678242 |        |

NOTE

Jonckheere test results are included in the summary table.  
Group means should be examined to check for lack-of-fit  
to a linear trend before trend test results are accepted.