TRADE SECRET

Study Title

H-25434: Influence on Growth and Growth Rate of the Green Alga *Selenastrum capricornutum*

Laboratory Project ID: DuPont-11410

AUTHOR: Terry Lee Sloman, B.S.

STUDY COMPLETED ON: October 4, 2002

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]

STUDY CODE NUMBER: [redacted]

CERTIFICATION

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

Work Completed by: Barbra D. Ferrell — 4 October 2002
Barbra D. Ferrell, B.S. — Date
Associate Scientist

Issued by Study Director: Terry Lee Sloman — 4 October 2002
Terry Lee Sloman, B.S. — Date
Associate Scientist

STUDY INFORMATION

Substance Tested: [redacted]

Synonyms/Codes: • H-25434

Haskell Number: 25434

Composition: [redacted]

Known Impurities: [redacted]

Physical Characteristics: Off-white, milky semi-opaque aqueous dispersion

Stability: The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.

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

Study Initiated/Completed: September 24, 2002 / (see report cover page)

In-Life Initiated/Completed: September 24, 2002 / September 27, 2002

SUMMARY

A study was conducted to determine the effect of H-25434 on the growth and growth rate of the green alga *Selenastrum capricornutum*. The algae were exposed to nominal concentrations of 0.01, 0.1, 1, 10, 100, and 1000 mg H-25434/Liter of nutrient medium (ppm).

The algae 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 (also referred to as cell density) relative to the blank (culture medium) control for the 72-hour (day 3) interval of the test.

The 72-hour EC_{50}, including the 95% confidence intervals (C.I.),[a] based on nominal concentrations are as follows:[b,c,d]

Healthy Cell Count

EC_{50}	36.2 mg/L (95% C.I. not calculated)

H-25434 exhibited medium concern[e] under TSCA and classified "harmful" to aquatic organisms (R52)[f] under the EU Directive in a 72-hour, acute test using the green alga *Selenastrum capricornutum*.

a The EC_{50} is defined as the "effective concentration" producing a 50% inhibition of growth relative to the control.

b BBN Software Products Corp., (1998). An RS/1® RPL procedure conducting Williams' test and probit analysis.

c McCullogh, L.P. and Nelder, J.A. (1989). *Generalized Linear Models*, 2nd edition, Chapter 9, pp 328. Chapman and Hall, London.

d Snedecor, G.W. and Cochran, W.G. (1967). *Statistical Methods*, 6th edition, Table A4, pp 469. The Iowa State University Press, Ames.

e Smrchek, J., Clements, R., Morcock, R., and Rabert, W. (1993). Assessing ecological hazard under TSCA: methods and evaluation of data. *Environmental Toxicology and Risk Assessment, ASTM STP 1179*. (W.G. Landis, J.S. Hughes, and M.A. Lewis, Eds.), pp 22-39. American Society for Testing and Materials, Philadelphia.

f Official Journal of the European Communities, Volume 36, 4 May 1993, pp. 68, section 5.2.1.

MATERIALS AND METHODS

A. Test System

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

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

The culture method for *Selenastrum capricornutum* was based on published literature.[1,2] 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) synthetic algal-assay procedure (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.

B. Test Design

The test design used for the *Selenastrum capricornutum* study is described below:

Organism	Nutrient Medium	Flask Volume (mL)	Solution Volume (mL)	Volume Ratio
Selenastrum capricornutum	AAP	250	50	5:1

C. AAP Nutrient Medium Preparation for *Selenastrum capricornutum* (Appendix A)

AAP nutrient medium[1] 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® (deionized) water, with mixing after each addition. The volume of the medium was brought to 1 liter with additional Milli-Q® 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 study.

The medium pH was adjusted to approximately 7.5. The medium was filter-sterilized using Corning® pre-sterilized filtration systems each with a 0.22 µm cellulose acetate filter.

1. Test Solution Preparation

Six nominal test concentrations (0.01, 0.1, 1, 10, 100, and 1000 mg H-25434/L AAP nutrient medium) were used in this study. Aliquots of the filter-sterilized AAP nutrient medium were used for the blank (normal culture medium) control solution.

2. Test Culture Preparation

For each blank control and test solution, 2 aliquots (50 mL each) were placed in separate sterilized 250 mL Erlenmeyer flasks fitted with sterilized foam stoppers. Each flask was labeled with a study number, test substance, concentration, replicate number, date, and experimenter's initials. To achieve the desired nominal concentration of approximately 10,000 *Selenastrum capricornutum* cells/mL at test initiation, an approximate 0.208 mL (= 208 µL) aliquot of algal inoculum from a logarithmically growing stock culture was aseptically transferred to each flask.

D. Experimental Design and Test Conditions

The test flasks were placed on a shaker table in a chamber in a non-systematic design and were re-positioned each working day. Air temperature in the chamber was recorded continuously with a continuous temperature recorder. Each blank control and test concentration was tested as 2 replicates. The algae were incubated for approximately 72 hours without test medium renewal and cell counts were made. Illumination was supplied by cool-white fluorescent tubes. The **target** environmental parameters are described in the following table.

Initial Population	Illumination (lumens/m² = lux)	Photo-Period (hours)	Shaking Speed (rpms)	Temperature (°C)
10,000 cells/mL	6000 to 10,000	24	100	24 ± 2

E. *Selenastrum capricornutum* Growth Measurement

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 72 hours from study 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. All cells located in the 16 grids were counted and recorded as healthy or unhealthy. The total number of cells counted 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. The statistical calculation of the EC_{50} was based on mean healthy cell counts and nominal concentrations.

RESULTS

Based on visual observations, the blank control solution and the 0.01, 0.1, 1, 10, and 100 mg/L test solutions were clear and had no color before addition of algal cells. However, the 1000 mg/L test solution was cloudy before addition of algal cells. After the addition of algal cells, the test and blank control flasks were placed on a shaker table in a chamber with a temperature of approximately 25°C. The algae were incubated for 72 hours (3 days) without medium renewal. Illumination was supplied by cool-white fluorescent tubes. The mean light intensity in the chamber was 7534 lumens/m^2 (= lux). The shaking speed was 100 revolutions per minute (rpm). The pH measurement of the culture medium was approximately 7.5 at study start. The pH measurements of the test solutions at test initiation ranged from 7.29 to 7.63, and at test termination ranged from 6.72 to 7.05.

CONCLUSIONS

The 72-hour EC_{50}, including the 95% confidence intervals (C.I.),[a] based on nominal concentrations are as follows:[3,4,5]

Healthy Cell Count

EC_{50}	36.2 mg/L (95% C.I. not calculated)

H-25434 exhibited medium concern[b] under TSCA and classified "harmful" to aquatic organisms (R52)[c] under the EU Directive in a 72-hour, acute test using the green alga *Selenastrum capricornutum.*

a The EC_{50} is defined as the "effective concentration" producing a 50% inhibition of growth relative to the control.

b Smrchek, J., Clements, R., Morcock, R., and Rabert, W. (1993). Assessing ecological hazard under TSCA: methods and evaluation of data. *Environmental Toxicology and Risk Assessment, ASTM STP 1179.* (W.G. Landis, J.S. Hughes, and M.A. Lewis, Eds.), pp 22-39. American Society for Testing and Materials, Philadelphia.

c Official Journal of the European Communities, Volume 36, 4 May 1993, pp. 68, section 5.2.1.

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. BBN Software Products Corp., (1998). An RS/1® RPL procedure conducting Williams' test and probit analysis.

4. McCullogh, L.P. and Nelder, J.A. (1989). *Generalized Linear Models*, 2nd edition, Chapter 9, pp 328. Chapman and Hall, London.

5. Snedecor, G.W. and Cochran, W.G. (1967). *Statistical Methods*, 6th edition, Table A4, pp 469. The Iowa State University Press, Ames.

6. Smrchek, J., Clements, R., Morcock, R., and Rabert, W. (1993). Assessing ecological hazard under TSCA: methods and evaluation of data. *Environmental Toxicology and Risk Assessment, ASTM STP 1179*. (W.G. Landis, J.S. Hughes, and M.A. Lewis, Eds.), pp 22-39. American Society for Testing and Materials, Philadelphia.

7. Official Journal of the European Communities, Volume 36, 4 May 1993, pp. 68, section 5.2.1.

TABLES

TABLE 1
pH MEASUREMENTS OF TEST SOLUTIONS

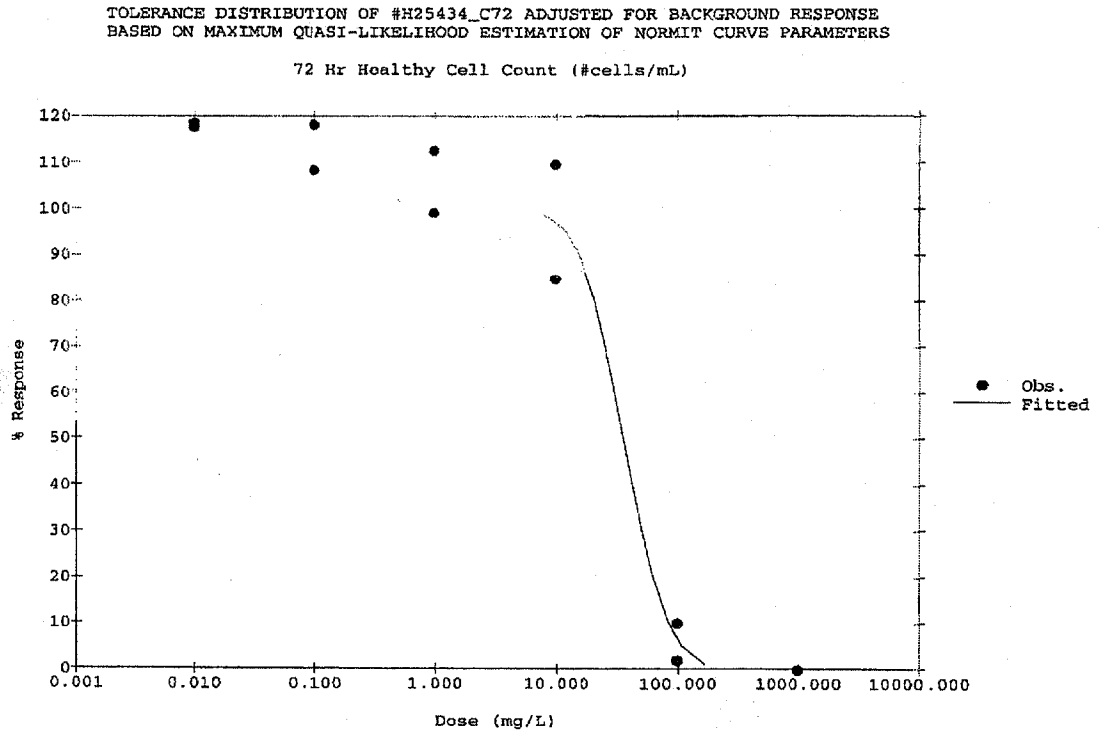
Nominal Concentration (mg/L)	Test Initiation (0 hours) pH	Test Termination (72 hours) pH
Blank Control	7.63	6.72
0.01	7.39	6.93
0.1	7.33	6.98
1	7.35	7.03
10	7.32	7.05
100	7.32	6.97
1000	7.29	6.92

TABLE 2
DATA SUMMARY

Nominal Concentration	Day 0 Healthy Cell Count (cells/mL)	Day 3 Healthy Cell Count (cells/mL)
Blank Control		
Replicate 1	10,000	2,640,000
Replicate 2	10,000	2,110,000
0.01 mg/L		
Replicate 1	10,000	2,820,000
Replicate 2	10,000	2,800,000
0.1 mg/L		
Replicate 1	10,000	2,810,000
Replicate 2	10,000	2,580,000
1 mg/L		
Replicate 1	10,000	2,680,000
Replicate 2	10,000	2,360,000
10 mg/L		
Replicate 1	10,000	2,610,000
Replicate 2	10,000	2,020,000
100 mg/L		
Replicate 1	10,000	50,000
Replicate 2	10,000	240,000
1000 mg/L		
Replicate 1	10,000	0
Replicate 2	10,000	0

FIGURE

FIGURE 1
72-HOUR HEALTHY CELL COUNT DOSE-RESPONSE CURVE



Quasi-likelihood assumed: Variance = 8.630834e+10*Mean^0.
Normit(%) = 5.456969 + -3.501484*Log10(Dose) with 95% confidence limits.

APPENDICES

APPENDIX A

AAP Nutrient Medium Constituents

AAP NUTRIENT MEDIUM CONSTITUENTS

Stock Solutions[a]		Final Prepared Medium	
Component	**Concentration (g/L)**	**Component**	**Concentration (mg/L)**
Macronutrient Stock #1			
$NaNO_3$	25.500	Na	11.001
		N	4.200
Macronutrient Stock #2			
$NaHCO_3$	15.000	C	2.143
		K	0.469
Macronutrient Stock #3			
K_2HPO_4	1.044	P	0.186
		Mg	2.904[b]
Macronutrient Stock #4			
$MgSO_4 \cdot 7H_2O$	14.700	Ca	1.202
		S	1.911
Macronutrient Stock #5			
$MgCl_2 \cdot 6H_2O$	12.164		
Macronutrient Stock #6			
$CaCl_2 \cdot 2H_2O$	4.410		

Stock Solutions[a]		Final Prepared Medium	
Component	**Concentration (mg/L)**	**Component**	**Concentration (µg/L)**
Micronutrient Stock			
H_3BO_3	185.520	B	32.460
$MnCl_2 \cdot 4\ H_2O$	415.610	Mn	115.374
$ZnCl_2$	3.271	Zn	1.570
$CoCl_2 \cdot 6\ H_2O$	1.428	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2\ H_2O$	7.260	Mo	2.878
$FeCl_3 \cdot 6\ H_2O$	160.000	Fe	33.051
$Na_2EDTA \cdot 2\ H_2O$	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

72-HOUR HEALTHY CELL COUNT STATISTICS

WILLIAMS TEST OF #@ALGAE@SCREEN@H25434
72 Hr Healthy Cell Count (#cells/mL)

W.T. Rank	Dose	Mean Response	Std Error	Transform Mean	Transform Std Error	n	$\hat{m}$	$\bar{t}$
0	0.00	2375000	265000	6.372943	0.048661	2		
0	0.01	2810000	10000	6.448704	0.001546	2	6.448704	-0.404801
0	0.10	2695000	115000	6.430163	0.018543	2	6.430163	-0.305735
0	1.00	2520000	160000	6.400523	0.027611	2	6.400523	-0.147366
0	10.00	2315000	295000	6.360996	0.055645	2	6.360996	0.063836
1	100.00	145000	95000	5.039593	0.340619	2	5.039593	7.124321
2	1000.00	0	0	-0.301030	0.000000	2	-0.301030	35.660201

W.T. Rank	$\bar{t}$ crit.
0	
0	1.895
0	2.002
0	2.039
0	2.058
1	2.069
2	2.076

One-sided test at the 95% CL for a decreasing trend.
Common logarithm transformation, i.e. Y = log10(X + 0.5), applied to the data.
MSE = 0.035027 with 7 df.
Ref: Williams, D. A. Biometrics 28: 519-531 (1972).

QUASI-LIKELIHOOD ESTIMATES OF NORMIT PARAMETERS FOR #H25434_C72

72 Hr Healthy Cell Count (#cells/mL)

Parameter	Estimate	Standard Error	Corr. with Background	Corr. with Intercept	Corr. with Slope
Background	2375000.000000	265000.000000	1.000000	0.000000	0.000000
Intercept	5.456969	3.053138		1.000000	-0.977014
Slope	-3.501484	1.650424			1.000000

Statistic:	Chisquare	df	Significance	Dispersion	t-value	(t*CVslope)^2
Value	1.0357e+12	12.000000	0.001	8.630834e+10	2.178813	1.054695

Quantile (%)	Estimate	Lower 95%CI	Upper 95%CI
1	167.056252	-	-
5	106.716749	-	-
10	84.038052	-	-
20	62.926684	-	-
25	56.377164	-	-
30	51.078573	-	-
40	42.739333	-	-
50	36.180413	-	-
60	30.628047	-	-
70	25.627620	-	-
75	23.219016	-	-
80	20.802340	-	-
90	15.576542	-	-
95	12.266325	-	-
99	7.835817	-	-

Quasi-likelihood assumed: Variance = 8.630834e+10*Mean^0.
Convergence achieved. Relative change in quasi-likelihood = 2.438883e-08
Variances and covariances have been multiplied by the dispersion
parameter of 8.630834e+10.

DuPont Haskell Laboratory for Health
and Environmental Sciences

November 4, 2002

cc: MR [redacted]
Quality Assurance

TO: MR FILE [redacted] POCKET H-25425

FROM: J. C. Maslanka JCM

TEST SUBSTANCE STABILITY OF H-25425

Medical Research Project Number:	[redacted]
Haskell Sample Number:	25425
Haskell Reference Standard Number:	25425
Analytical Test Code:	[redacted]
Analytical Report Number:	HA-2002-094
Notebook References:	[redacted]

Attached is the analytical report for the study identified above. This analysis was performed to satisfy protocol requirements for test substance stability but may also be used for other purposes.

TEST SUBSTANCE STABILITY OF H-25425

Medical Research Project Number: [redacted]
Haskell Sample Number: 25425
Analytical Report Number: HA-2002-094

A sample of H-25425 was submitted on October 14, 2002 and analyzed on October 15, 2002. The percentage of active ingredient (a.i.) was measured to be [redacted] with a range of [redacted] of nominal for replicate analyses (n = 3). The sponsor reported a purity of [redacted] when the sample was submitted.

ACKOWLEGEMENTS

Sample preparation by Sheila A. Riley (Chemistry Associate). Sample analysis by Bogdan Szostek (Senior Research Chemist).

SIGNATURE

Report by: Janet C. Maslanka 4-Nov-2002
Janet C. Maslanka — Date
Analytical Staff Chemist

Date Issued: 4- Nov- 2002

METHODS

Analysis for the percent of a.i. in a sample of H-25425 was by high-performance liquid chromatography (HPLC) with a Gel Permeation Column (GPC) and refractive index detector according to the following method. Sonication was used to completely dissolve the test substance and the analytical standard stock solution.

SAMPLE PREPARATION & ANALYSIS

Aliquots (0.0413, 0.0407 and 0.0412 grams) of H-25425 were dissolved in tetrahydrofuran (10 mL) to give nominal concentrations of 4130, 4070, and 4120 ppm, respectively.

CHROMATOGRAPHIC CONDITIONS

Instrument:	Hewlett-Packard Model 1100 HPLC
Column:	Styragel® HR 4; 7.8 mm x 300 mm (GPC column)
Mobile Phase	100% tetrahydrofuran (THF)
Flow Rate:	1.0 mL/min
Injection Volume:	50 µl
Column Temperature:	35°C
Detection:	Refractive Index

CALIBRATION & QUANTITATION

A separate sample of H-25425 test substance was designated as the analytical reference standard (93.17% pure). A stock solution of H-25425 in tetrahydrofuran was used to make calibration solutions that bracketed the test solutions. Peak heights from replicate HPLC/GPC/RI analysis of each calibration solution were used to construct a calibration curve by least-squares regression. Measured concentrations for each test solution were determined by applying respective peak heights to the calibration curve.

RESULTS

H-25425 eluted from the HPLC column as a resolved peak with a retention time of about 8.582 ± 0.01 minutes. The sponsor reported a purity of [redacted] when the sample was submitted.

Table I. The percent of a.i. in the H-25425 sample analyzed October 15, 2002.

Aliquot	ppm H-25425 Nominal	ppm H-25425 Measured	Percent Nominal
1	4130	3730	90.3
2	4070	3720	91.4
3	4120	3790	92.0
Average % Nominal			**91.2**
Standard Deviation			**± 0.85**
Coefficient of Variation			**1%**