TRADE SECRET

Study Title

H-25914: Influence on Growth and Growth Rate of the Green Alga *Selenastrum capricornutum*

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STUDY COMPLETED ON: August 18, 2003

PERFORMING LABORATORY: E.I. du Pont de Nemours and Company
Haskell Laboratory for Health and Environmental Sciences
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LABORATORY PROJECT ID: DuPont-13348

[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 18 August 2003
Barbra D. Ferrell, B.S. Date
Associate Scientist

Issued by Study Director: Robert A. Hoke 18 August 2003
Robert A. Hoke, Ph.D. Date
Senior Research Ecotoxicologist and Manager

STUDY INFORMATION

Substance Tested: [redacted]

Synonyms/Codes: • H-25914
[redacted]

Submitter's Notebook Number(s): [redacted]

Haskell Number: 25914

CAS Registry Number: [redacted]

Composition: [redacted]

Purity: [redacted]

Known Impurities: [redacted]

Physical Characteristics: Amber liquid

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: July 22, 2003 / (see report cover page)

SUMMARY

A study was conducted to determine the effect of H-25914 on the growth and growth rate of the green alga *Selenastrum capricornutum.*[a] The algae were exposed to nominal concentrations of 0.1, 1, 10, and 100 mg H-25914/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}, and the 95% confidence interval (C.I.),[b] based on nominal concentrations is as follows:(1)

Healthy Cell Count

EC_{50}	1.17 mg/L (95% C.I. = 0.75 to 1.59 mg H-25914/L)

H-25914 exhibited medium concern(2) under TSCA and was "toxic" to aquatic organisms (R51),(3) classified as dangerous for the environment, and assigned the symbol "N" under the EU Directive in a 72-hour, acute test using the green alga *Selenastrum capricornutum.*

a Also known as *Pseudokirchneriella subcapitata.*

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

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.[4,5] 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[4] 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

Four nominal test concentrations (0.1, 1, 10, and 100 mg H-25914/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. To achieve the desired nominal concentration of approximately 10,000 *Selenastrum capricornutum* cells/mL at test initiation, an approximate 0.340 mL (= 340 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 2 wells of the hemacytometer and 8 squares from each grid area (16 total squares) were selected for counting. All cells located in the 16 squares 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, scenescent, 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 0.1, 1, 10, and 100 mg/L test solutions were clear and colorless 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 6885 lumens/m^2 (= lux). The shaking speed was 98 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 7.51 to 7.56, and at test termination ranged from 7.77 to 8.10.

CONCLUSIONS

The 72-hour EC_{50}, including the 95% confidence interval (C.I.),[a] based on nominal concentrations is listed below:[(1)]

Healthy Cell Count

EC_{50}	1.17 mg/L (95% C.I. = 0.75 to 1.59 mg H-25914/L)

H-25914 exhibited medium concern[(2)] under TSCA and was "toxic" to aquatic organisms (R51),[(3)] classified as dangerous for the environment, and assigned the symbol "N" 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.

REFERENCES

1. Slob, W., (2002). Dose-response modeling of continued endpoints. Toxicol. Sci., 66, 298-312.

2. 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.

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

4. 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.

5. 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.

TABLES

TABLE 1

pH MEASUREMENTS OF TEST SOLUTIONS

Nominal Concentration (mg/L)	Test Initiation (0 hours) pH	Test Termination (72 hours) pH
Blank Control	7.56	7.77
0.1	7.53	8.10
1	7.53	7.98
10	7.51	7.78
100	7.51	7.80

TABLE 2

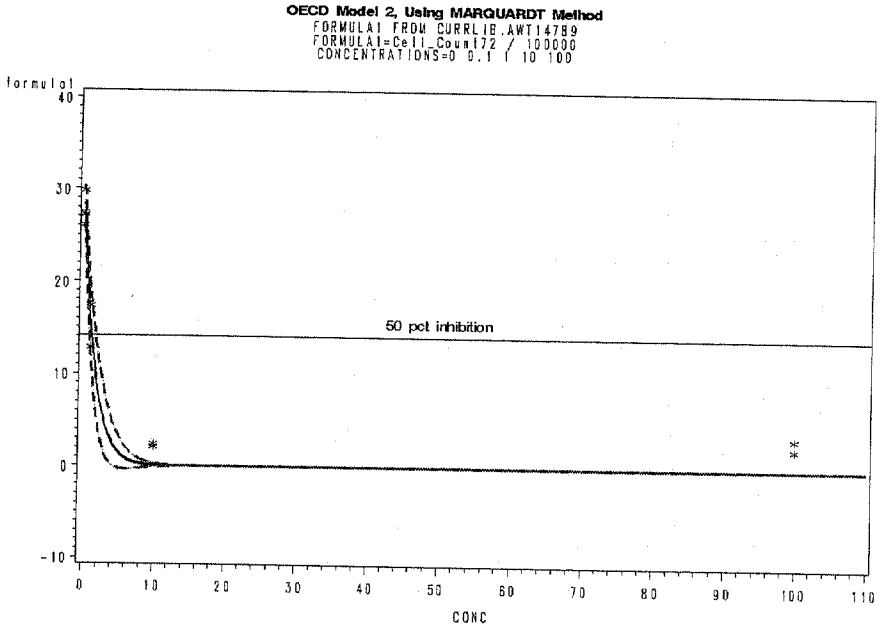
CELL COUNT DATA SUMMARY

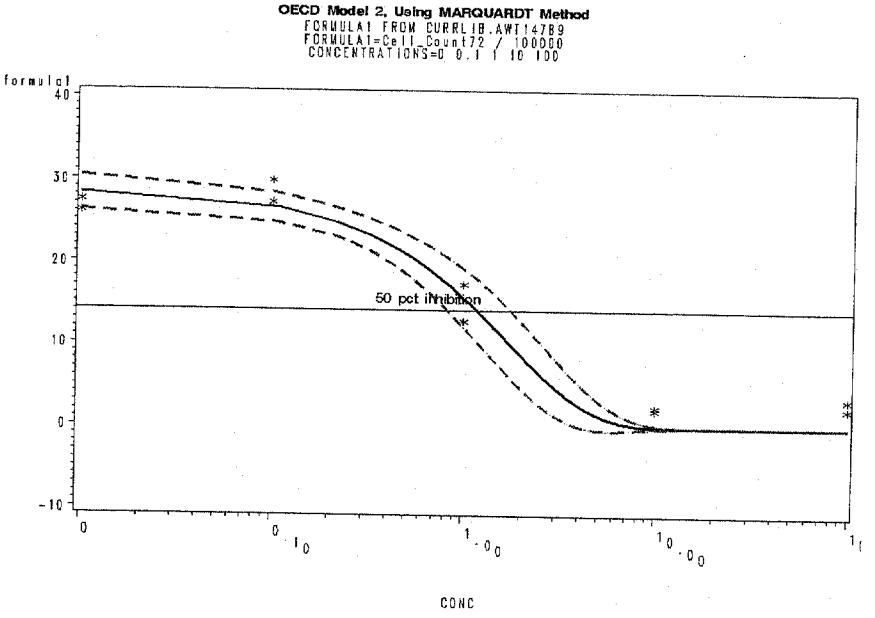
Nominal Concentration	Day 0 Healthy Cell Count (cells/mL)	Day 3 Healthy Cell Count (cells/mL)
Blank Control		
Replicate 1	10,000	2,600,000
Replicate 2	10,000	2,740,000
0.1 mg/L		
Replicate 1	10,000	2,980,000
Replicate 2	10,000	2,720,000
1 mg/L		
Replicate 1	10,000	1,730,000
Replicate 2	10,000	1,280,000
10 mg/L		
Replicate 1	10,000	240,000
Replicate 2	10,000	210,000
100 mg/L		
Replicate 1	10,000	230,000
Replicate 2	10,000	340,000

FIGURE

FIGURE 1

72-HOUR HEALTHY CELL COUNT DOSE-RESPONSE CURVE





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 4.
b Includes magnesium from both magnesium sulfate and magnesium chloride.

APPENDIX B

72-Hour Healthy Cell Count Statistics

72-HOUR HEALTHY CELL COUNT STATISTICS

Results of Jonckheere test to determine NOEC

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Check for Number of Reps in FORMULA1
Maximum Number of Reps in Any Treatment Group or Control
Maximum Number of Reps per Group is 2
Exact Permutation Methods Recommended

Analysis of [redacted] Data
Test for TREND IN EITHER DIRECTION in Response=FORMULA1
ANALYSIS OF Cell Count at 72 Hours USING ALPHA=0.05
FULL DATA

KEY
_JT_ IS JONCKHEERE STATISTIC
Z_JT IS STANDARDIZED JONCKHEERE STATISTIC
XPR_JT IS P-VALUE FOR UPWARD TREND
XPL_JT IS P-VALUE FOR DOWNWARD TREND
P-VALUES ARE FOR TIE-CORRECTED TEST WITH CONTINUITY CORRECTION FACTOR
SIGNIF RESULTS ARE FOR D ALTERNATIVE HYPOTHESIS

Jonckheere Trend Test on Dose 0 + Lowest 4 Doses thru 100 PPM

____JT____       Z_JT      XPL_JT      XPR_JT  SIGNIF  DOSE
       8.5  -2.556039  0.0051146           .  **          5


Jonckheere Trend Test on Dose 0 + Lowest 3 Doses thru 10 PPM

____JT____       Z_JT      XPL_JT      XPR_JT  SIGNIF  DOSE
         5  -2.298393  0.0123016           .  **          4


Jonckheere Trend Test on Dose 0 + Lowest 2 Doses thru 1 PPM

____JT____       Z_JT      XPL_JT      XPR_JT  SIGNIF  DOSE
       4.5  -1.192079  0.1666667           .              3
```

Thus, the NOEC=1 ppm.

EC50 Estimatation

OBSERVED PERCENT CHANGE FROM CONTROL MEAN
FORMULA1 FROM [redacted]

Obs	CONC	MEAN	PCTCHNG	N
1	0.0	26.70	0	2
2	0.1	28.50	7	2
3	1.0	15.05	-44	2
4	10.0	2.25	-92	2
5	100.0	2.85	-89	2

OBSERVED & PREDICTED PERCENT CHANGE FROM CONTROL MEAN
FORMULA1 FROM [redacted]
FORMULA1=Cell_Count72 / 100000

Obs	CONC	N	MEAN	PRED	PCTCHNG	PREDPCTCHNG
1	0.0	2	26.70	28.2210	0	0
2	0.1	2	28.50	26.5972	7	-6
3	1.0	2	15.05	15.6034	-44	-45
4	10.0	2	2.25	0.0753	-92	-100
5	100.0	2	2.85	0.0000	-89	-100

EC50 using Method=MARQUARDT, OECD Model 2
Covariance Matrix and Standard Error of Parameter Estimates

NAME	y0	EC50	SE
EC50	-0.08165	0.045333	0.21291
y0	0.81932	-0.081648	0.90516

Model 2 Parameter Estimates, EC50 and 95% Confidence Bounds

y0	EC50	LB	UB
28.2210	1.16972	0.75241	1.58703

Model 2 Evaluation Measures

SSREG	SSERR	SSTOTC	SSTOTU	RSQUARE
3195.49	21.5787	150.621	3217.07	0.85673

note
Graph descriptions include the following:
formula1: EC50 OECD MODEL 2 FIT BY MARQUARDT,