

TRADE SECRET

Study Title

H-24750: Influence on Growth and Growth Rate of the Green
Alga Selenastrum capricornutum

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STUDY COMPLETED ON: May 20, 2003

PERFORMING LABORATORY: E.I. du Pont de Nemours and Company
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WORK REQUEST NUMBER: [REDACTED]

STUDY CODE NUMBER: [REDACTED]

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CERTIFICATION

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

Work Completed by:

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20 May 2003
Date

Issued by Study Director:

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STUDY INFORMATION

9th Collective Nomenclature: [REDACTED]

Synonyms/Codes: [REDACTED]

• H-24750

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

Haskell Number: 24750

CAS Registry Number: [REDACTED]

Purity: [REDACTED]

Known Impurities: [REDACTED]

Physical Characteristics: White Solid

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: April 15, 2003 / (see report cover page)

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SUMMARY

A study was conducted to determine the effect of H-24750 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, 100, and 1000 mg H-24750/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 EC₅₀, including the 95% confidence intervals (C.I.),^b based on nominal concentrations are as follows:^c

Healthy Cell Count

EC₅₀ 10.6 mg/L (95% C.I. = 5.8 to 15.5)

NOEC 1 mg/L

H-24750 exhibited medium concern^d under TSCA and was classified as "harmful" to aquatic organisms (R52)^e under the EU Directive in a 72-hour, acute test using the green alga *Selenastrum capricornutum*.

a Also known as *Pseudokirchneriella subcapitata*.

b The EC₅₀ is defined as the "effective concentration" producing a 50% inhibition of growth relative to the control.

c Slob, W., (2002). Dose-response modeling of continuous endpoints. *Toxicological Science*, V66; pp 298-312.

d Smrcek, 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.

e 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
<i>Selenastrum capricornutum</i>	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

Five nominal test concentrations (0.1, 1, 10, 100, and 1000 mg H-24750/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 0.475 mL (= 475 μ L) 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 an environmental 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 without test medium renewal and cell counts were made after 72 hours. Illumination was supplied by cool-white fluorescent tubes. The target environmental parameters are described in the following table.

Initial Population	Illumination (lumens/m ² = lux)	Photo- Period (hours)	Shaking Speed (rpms)	Temperature (°C)
10,000 cells/mL	6000 to 10,000	24	100	24 $\pm$ 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 each side of the hemacytometer and 8 grids from each side 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 was multiplied by 10,000 to determine the number of cells per milliliter. Observations and counts of healthy and unhealthy cells (e.g., deformed, senescent, stunted) were recorded in the study records. The statistical calculation of the EC₅₀ was based on mean healthy cell counts and nominal test substance concentrations.

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RESULTS

Based on visual observations, the blank control solution and the 0.1, 1, 10, and 100 mg/L test solutions were clear and had no color before addition of algal cells. The 1000 mg/L test solution was clear and had undissolved particles of test material and a surface film. 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 without medium renewal. Illumination was supplied by cool-white fluorescent tubes. The mean light intensity in the chamber was 7564 lumens/m² (= lux). The shaking speed was 96 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 3.20 to 7.47, and at test termination ranged from 3.33 to 7.86.

CONCLUSIONS

The 72-hour EC₅₀, including the 95% confidence intervals (C.I.)^a based on nominal test substance concentrations and the NOEC are as follows:⁽³⁾

Healthy Cell Count

EC ₅₀	10.6 mg/L (95% C.I. = 5.8 to 15.5)
NOEC	1 mg/L

H-24750 exhibited medium concern ⁽⁴⁾ under TSCA and was classified as "harmful" to aquatic organisms (R52)⁽⁵⁾ under the EU Directive in a 72-hour, acute test using the green alga *Selenastrum capricornutum*.

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a The EC₅₀ is defined as the "effective concentration" producing a 50% inhibition of growth relative to the control.

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. Slob, W., (2002). Dose-response modeling of continuous endpoints. *Toxicological Science*, V66; pp 298-312.
4. Smrcek, 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.
5. Official Journal of the European Communities, Volume 36, 4 May 1993, pp. 68, section 5.2.1.

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TABLES

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TABLE 1
pH MEASUREMENTS OF TEST SOLUTIONS

Nominal Concentration (mg/L)	Test Initiation (0 hours) pH	Test Termination (72 hours) pH
Blank Control	7.41	7.74
0.1	7.47	7.78
1	7.42	7.84
10	7.33	7.86
100	6.33	6.99
1000	3.20	3.33

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TABLE 2
DATA SUMMARY

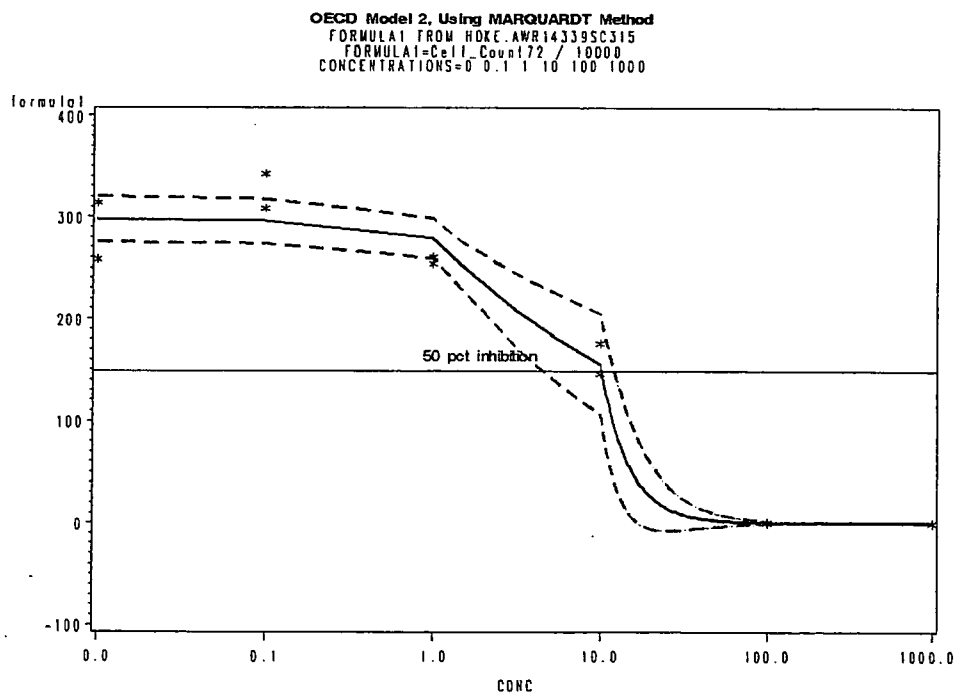
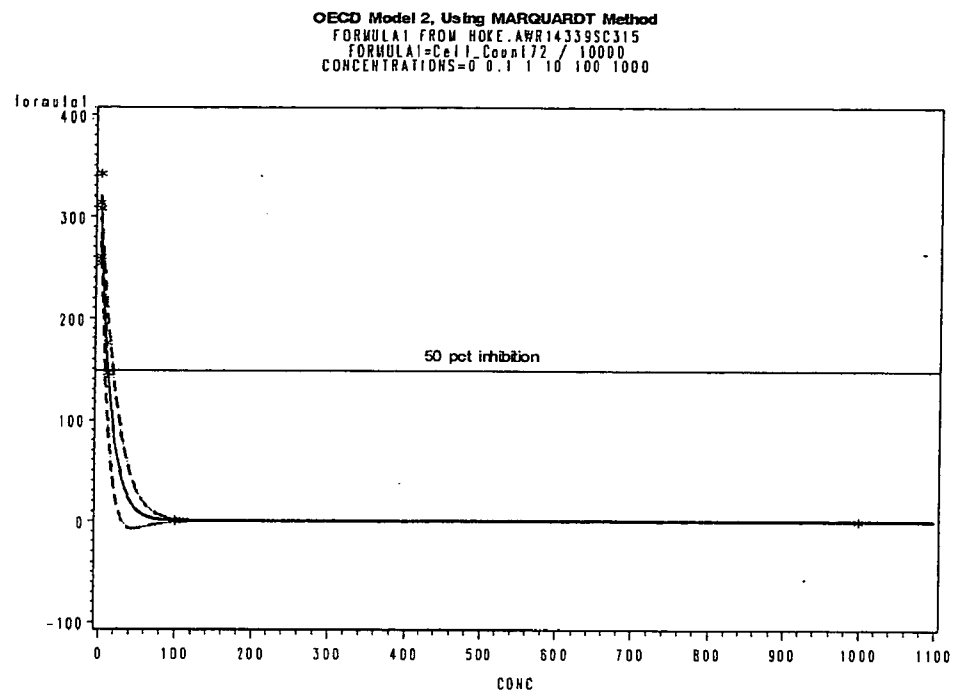
Nominal Concentration	Day 0 Healthy Cell Count (cells/mL)	Day 3 Healthy Cell Count (cells/mL)
Blank Control		
Replicate 1	10,000	3,140,000
Replicate 2	10,000	2,580,000
0.1 mg/L		
Replicate 1	10,000	3,080,000
Replicate 2	10,000	3,420,000
1 mg/L		
Replicate 1	10,000	2,540,000
Replicate 2	10,000	2,600,000
10 mg/L		
Replicate 1	10,000	1,460,000
Replicate 2	10,000	1,760,000
100 mg/L		
Replicate 1	10,000	10,000
Replicate 2	10,000	0
1000 mg/L		
Replicate 1	10,000	0
Replicate 2	10,000	0

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FIGURE

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



APPENDICES

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APPENDIX A

AAP Nutrient Medium Constituents

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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 ₂ HPO ₄	1.044	P	0.186
		Mg	2.904 ^b
Macronutrient Stock #4			
MgSO ₄ •7H ₂ O	14.700	Ca	1.202
		S	1.911
Macronutrient Stock #5			
MgCl ₂ •6H ₂ O	12.164		
Macronutrient Stock #6			
CaCl ₂ •2H ₂ O	4.410		

Stock Solutions ^a		Final Prepared Medium	
Component	Concentration (mg/L)	Component	Concentration (µg/L)
Micronutrient Stock			
H ₃ BO ₃	185.520	B	32.460
MnCl ₂ •4 H ₂ O	415.610	Mn	115.374
ZnCl ₂	3.271	Zn	1.570
CoCl ₂ •6 H ₂ O	1.428	Co	0.354
CuCl ₂ •2H ₂ O	0.012	Cu	0.004
Na ₂ MoO ₄ •2 H ₂ O	7.260	Mo	2.878
FeCl ₃ •6 H ₂ O	160.000	Fe	33.051
Na ₂ EDTA•2 H ₂ 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

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72-HOUR HEALTHY CELL COUNT STATISTICS

NOEC Determination Using StatXact

!K Independent Samples:Jonckhere-Terpstra Test

>>> KI JT/EX /RO=CONC /CO=COUNT72 All concentrations present

Datafile: C:\AQUATICS\hoke [REDACTED] stxdata.cy3

JONCKHEERE-TERPSTRA TEST [That the 6 populations are identically distributed]

Statistic based on the 12 observations:

Mean	Std-dev	Observed(JT(x))	Standardized(JT*(x))
30.00	7.129	5.000	-3.507

Asymptotic p-value:

One-sided: Pr { JT*(X) .LE.	-3.507 }	=	0.0002
Two-sided: 2 * One-sided		=	0.0005

Exact p-values:

One-sided: Pr { JT*(X) .LE.	-3.507 }	=	0.0001
Pr { JT*(X) .EQ.	-3.507 }	=	0.0000
Two-sided: Pr { JT*(X) .GE.	3.507 }	=	0.0001

!K Independent Samples:Jonckhere-Terpstra Test

>>> KI JT/EX /RO=CONC /CO=COUNT72 1000 mg/L omitted

Datafile: C:\AQUATICS\hoke [REDACTED] stxdata.cy3

JONCKHEERE-TERPSTRA TEST [That the 5 populations are identically distributed]

Statistic based on the 10 observations:

Mean	Std-dev	Observed(JT(x))	Standardized(JT*(x))
20.00	5.477	4.000	-2.921

Asymptotic p-value:

One-sided: Pr { JT*(X) .LE.	-2.921 }	=	0.0017
Two-sided: 2 * One-sided		=	0.0035

Exact p-values:

One-sided: Pr { JT*(X) .LE.	-2.921 }	=	0.0012
Pr { JT*(X) .EQ.	-2.921 }	=	0.0007
Two-sided: Pr { JT*(X) .GE.	2.921 }	=	0.0024

!K Independent Samples:Jonckhere-Terpstra Test

>>> KI JT/EX /RO=CONC /CO=COUNT72 1000 and 100 mg/L omitted

Datafile: C:\AQUATICS\hoke [REDACTED] stxdata.cy3

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JONCKHEERE-TERPSTRA TEST [That the 4 populations are identically distributed]

Statistic based on the 8 observations:

Mean	Std-dev	Observed(JT(x))	Standardized(JT*(x))
12.00	3.916	4.000	-2.043

Asymptotic p-value:

One-sided: Pr { JT*(X) .LE.	-2.043 }	=	0.0205
Two-sided: 2 * One-sided		=	0.0411

Exact p-values:

One-sided: Pr { JT*(X) .LE.	-2.043 }	=	0.0262
Pr { JT*(X) .EQ.	-2.043 }	=	0.0139
Two-sided: Pr { JT*(X) .GE.	2.043 }	=	0.0524

!K Independent Samples:Jonckhere-Terpstra Test

>>> KI JT/EX /RO=CONC /CO=COUNT72 1000, 100, and 10 mg/L omitted

Datafile: C:\AQUATICS\hoke [REDACTED] stxdata.cy3

JONCKHEERE-TERPSTRA TEST [That the 3 populations are identically distributed]

Statistic based on the 6 observations:

Mean	Std-dev	Observed(JT(x))	Standardized(JT*(x))
6.000	2.517	4.000	-0.7947

Asymptotic p-value:

One-sided: Pr { JT*(X) .LE.	-0.7947 }	=	0.2134
Two-sided: 2 * One-sided		=	0.4268

Exact p-values:

One-sided: Pr { JT*(X) .LE.	-0.7947 }	=	0.2889
Pr { JT*(X) .EQ.	-0.7947 }	=	0.1222
Two-sided: Pr { JT*(X) .GE.	0.7947 }	=	0.5778

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EC50 Estimation Using OECD Model 2

OBSERVED & PREDICTED PERCENT CHANGE FROM CONTROL MEAN
FORMULA1 FROM HOKE
FORMULA1=Cell_Count72 / 10000

Obs	CONC	N	MEAN	PRED	PCTCHNG	PREDPCTCHNG
1	0.0	2	286.0	297.537	0	0
2	0.1	2	325.0	295.603	14	-1
3	1.0	2	257.0	278.759	-10	-6
4	10.0	2	161.0	155.034	-44	-48
5	100.0	2	0.5	0.439	-100	-100
6	1000.0	2	0.0	0.000	-100	-100

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

NAME	y0	EC50	SE
EC50	-8.778	6.06778	2.4633
y0	102.717	-8.77808	10.1350

Model 2 Parameter Estimates, EC50 and 95% Confidence Bounds

y0	EC50	LB	UB
297.537	10.6328	5.80478	15.4609

Model 2 Evaluation Measures

SSREG	SSERR	SSTOTC	SSTOTU	RSQUARE
521335.79	5301.12	21827.68	526636.92	0.75714

note

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

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