6-3-04

DP Barcode: 299970 **MRID No: 460626-35**

DATA EVALUATION RECORD
ALGAL TOXICITY TEST
GUIDELINE OPPTS 850.5400 (TIERS I AND II)

1. **CHEMICAL:** PXTS **PC Code No.:** 006929

2. **TEST MATERIAL:** PXTS TECHNICAL **Purity:** 100%
   Batch No.: 1685-23, Bottle #2
   Exp. Date March 28, 2005
   EPA File Symbol: 75799-R

3. **CITATION:**

   Author: Debbie Desjardins (Study Director), Raymond L. Van Hoven and Henry O. Krueger
   Title: PXTS: A 96-Hour Toxicity Test With the Freshwater Alga (*Anabaena flos-aquae*)
   Study Completion Date: December 20, 2003
   Laboratory: Wildlife, International, Ltd.
   8598 Commerce Drive
   Easton, Maryland 21601
   Sponsor: Akzo Nobel Functional Chemicals LLC
   5 Livingstone Avenue
   Dobbs Ferry, New York 10522
   Laboratory Report ID: Wildlife International, Ltd. Project No. 497A-117
   MRID No.: 460626-35

4. **REVIEWED BY:** Srinivas Gowda, Biologist
   US EPA/OPP/AD/RASSB/Team 1

   Signature: Srinivas Gowda **Date:** 05/13/04

5. **APPROVED BY:** Norm Cook, Chief
   US EPA/OPP/AD/RASSB

   Signature: Norman J. Cook **Date:** 6/3/04

6. **STUDY PARAMETERS:**

Definitive Test Duration: 96-hr
Type of Concentrations: Nominal and Mean Measured (highest concentration)

7. **CONCLUSIONS:**

Results Synopsis (based on nominal concentrations):

Cell Density	Reported	Verified
96-hr		
EC_{50}:	**>125 µg/L**	**>125 µg/L**
(95 %CI)	**Not calculable**	**Not calculable**
NOEC:	**125 µg/L**	**125 µg/L**

Study results were based on the nominal concentrations and the initial mean measured concentration of the highest test solution. After 72 and 96 hours of exposure, there were no apparent treatment-related effects upon growth. After 96 hours, there were signs of aggregation/flocculation and long chains of algae, which is considered normal for *Anabaena flos-aquae*. There were no signs of adherence to the test chambers.

8. **ADEQUACY OF THE STUDY**

A. **Classification:** Supplemental.

B. **Rationale:** This study did not determine an EC_{50} value. A range finding test was not conducted to establish test solution concentrations for the definitive test.

C. **Repairability:** This study may be upgraded to core if the registrant submits a valid range finding study for *Anabaena flos-aquae* and provides additional description of good faith efforts made to solubilize PXTS.

9. **GUIDELINE DEVIATIONS**

- The study was conducted using the Wildlife International, Ltd protocol which is based on OECD Guideline 201, harmonized OPPTS Test Guideline 850.5400, and EC Guideline L383A - C.3. The OECD and EC Guideline criteria may differ from the OPPTS Guideline (850.5400) that was used in preparing this Data Evaluation Record.

- The pH of the stock nutrient solution was adjusted to 8.0 using 10% HCL and sterilized by filtration. The OPPTS guideline recommends a pH of 7.5 ± 0.1 for *Anabaena*.
- Photosynthetically-active radiation was not reported.
- The physical-chemical properties of the test chemical were not reported.
- Only the highest concentration (125 µg/L) samples could be analyzed due to limits of the analytical method. Therefore, the results of the study were based on the nominal test concentrations, the measured high dose chamber concentrations, and the analyses of the stock solutions.
- The test concentrations did not bracket the EC_{50}. The study was conducted at concentrations above the known limit of solubility (below 12.5 µg/L) using a solvent to raise the solubility of the test substance above the saturation level, at the request of the EPA.
- Growth was inhibited by <90% at the highest concentration (-12 at 96-M).
- A positive control was not included as a part of the study.

10. SUBMISSION PURPOSE: Registration

11. MATERIALS AND METHODS

A. Test Organisms

Guideline Criteria	Reported Information
Species • *Selenastrum capricornatum (Raphidocelis subcapitata)* • *Skeletonema costatum* • *Anabaena flos-aquae* • *Navicula pelliculosa*	(p. 12) • *Anabaena flos-aquae*
Initial Number of Cells • 10,000 cells/mL (*Selenastrum, Anabaena, Navicula)* • 77,000 cells/mL (*Skeletonema*)	(p. 11) • Approximately 10,000 cells/mL at test initiation.

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Guideline Criteria	Reported Information
Stock Culture • 3 to 7 days old	(p.12) • Inocula for the test was prepared from a 3 day old culture.
Nutrients • Standard formula (ASTM E1218-20) • pH 7.5 ± 0.1 (*Selenastrum, Navicula, Anabaena*), 8.1 ± 0.1 (*Skeletonema*) • Freshly prepared	(p. 13) • Algal cells cultured and tested in freshwater algal medium (ASTM 1218-90E) • Stock nutrient solutions prepared by mixing reagent-grade chemicals with purified well water. The nutrient solutions then added to purified well water to prepare the test medium. • The pH was adjusted to 8.0 ± 0.1 using 10% HCL and sterilized by filtration.

B. Test System

Guideline Criteria	Reported Information
Solvent • Upper limit - 0.5 mL/L	(p. 14) • 0.1 mL/L of acetone was used to raise the solubility of the test substance above the saturation level.
Temperature • 24° ± 2°C (*Selenastrum, Navicula, Anabaena*) • 20° ± 2°C (*Skeletonema*) • Recorded hourly	(p. 13 and 23) • Test chambers were held in an environmental chamber at 24 ± 2°C (range: 22.5 to 24.1°C). • The temperature was monitored continuously in the chamber and twice daily in a container of water adjacent to test chambers.

Guideline Criteria	Reported Information
Light Intensity • 4.3 K lx (± 10%) (*Selenastrum, Skeletonema, Navicula*) • 2.2 K lx (± 10%) (*Anabaena*) • Photosynthetically active radiation approx. 66.5 ± 10% µEin/m^2/sec	(p. 13 and 19) • 1990 to 2250 lux (measurements taken at five locations surrounding the test flasks). • Photosynthetically active radiation not reported.
Photoperiod • 14-hr light/10-hr dark (*Skeletonema*) • Continuous (*Selenastrum, Navicula, Anabaena*)	(p. 13) • Continuous - 24-hr light/0-hr dark.
pH • 7.5 ± 0.1 (*Selenastrum, Navicula, Anabaema*) • 8.1 ± 0.1 (*Skeletonema*) • Measured at beginning and end of test	(p. 13 and 24) • pH = 8.1 (0-hr) • pH = 7.8 - 8.0 (96-hr) • At test initiation, pH was measured in the individual batches of test solution prepared for each treatment. At test termination, the pH was measured in pooled samples of test solution collected from each of the replicates of each treatment and control.
Oscillation Rates • 100 cycles/min (*Selenastrum*) • 60 cycles/min (*Skeletonema*)	(p. 13) • Test flasks were shaken continuously at approximately 100 rpm.
Test Containers • 125-500 mL Erlenmeyer flasks • Cleaned/sterilized (solvent and acid) and conditioned • Test solution volume ≤ 50% of flask volume	(p.13) • Sterile 250-mL Erlenmeyer flasks, plugged with foam stoppers, and containing the test solution of each respective treatment. • 100 mL test solution (<50% of flask volume).

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Guideline Criteria	Reported Information
Dilution Water • Sufficient quality (e.g., ASTM Type I) • Saltwater - commercial or modified synthetic formulation added to distilled/deionized water (30 ppt or 24-35 g/kg)	(p. 13) • Purified well water (NANOpure® water)

C. **Test Design**

Guideline Criteria	Reported Information
Range-Finding Test • Water solubility and physical-chemical properties of test chemical determined? • Validated analytical method developed? • Lowest dose at detection limit, upper dose at saturation concentration or 1000 mg/L • If < 50% reduction in growth at highest dose, no definitive test required	(p. 11) • Physical-chemical properties of the test chemical were not reported. • A validated analytical method was developed. • Range-finding test was not mentioned. • The final test was conducted at concentrations above the known limit of solubility (below 12.5 µg/L) using a solvent to raise the solubility of the test substance above the saturation level, at the request of the EPA.
Dose Range • 1.5X -2X progression	(p. 14) • Approximately 2X progression

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Guideline Criteria	Reported Information
Doses • 5 or more concentrations of test substance in a geometric series • >90% growth inhibited or stimulated at highest concentration or concentrations bracket expected EC_{50}	(p. 9 and 26) • Five concentrations: Nominal = 7.8, 16, 31, 63, 125 µg/L. Mean measured =160 µg/L Only the highest concentration (125 µg ai/L) could be analyzed due to limits of the analytical method, the maximum amount of water that can be removed from the test chambers, and the complexity of the algal medium. • <90% growth inhibited at the highest concentration (-12% at 96-hr)
Controls • Negative and/or solvent each test • Positive - zinc chloride (periodically)	(p.9) • Negative and solvent control • No positive control
Replicates Per Dose • 3 or more (4 or more for *Navicula*)	(p. 11) • 3 replicates per dose, plus a negative and solvent control.
Duration of Test • 96-hr	(p. 11) • 96-hr
Growth • Logarithmic growth (controls) by 96-hr or repeat test • 1.5 x 10^6 cells/mL (*Skeletonema*) • 3.5 x 10^6 cells/mL (*Selenastrum*)	(p. 19 , 26 and 30) • Logarithmic growth in control by 96-hr • Mean of 2.7 x 10^6 cells/mL at 96-hr. in the pooled control. • Increase by factor of 270.
Daily Observations?	• Yes (p. 16 and 26)
Method of Observations • Direct - microscopic cell count of at least 400 cells/flask • Indirect - spectrophotometry, electronic cell counter, dry weight, etc; calibrated by microscopic count • Qualitative and descriptive	(p. 16 and 19) • Cell counts were performed using a hemacytometer and microscope. • Growth of cells were assessed for aggregations or flocculation of cells adherence of cells to the test chamber, and atypical cell morphology.

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Guideline Criteria	Reported Information
Cell Separation • Syringe, ultrasonic bath, or blender; limited sonification (*Anabaena*) • Manual or rotary shaking only (*Selenastrum, Skeletonema, Navicula*)	(p. 13 and 16) • Mechanical shaking in an environmental chamber. • Prior to counting, sample solutions drawn in and out of a syringe three times to shorten length of cell chains. Samples diluted using electron solution (Isoton®), as need.
Algistatic and algicidal effects differentiated?	(p. 19) • Algistatic and algicidal effects not differentiated. After 72 and 96 hours of exposure, there were no apparent treatment-related effects upon growth. After 96 hours, there were signs of aggregation/flocculation and long chains of algae, which is considered normal for *Anabaena flos-aquae*. There were no signs of adherence to the test chambers.
Maximum Labeled Rate	• Not reported.

12. REPORTED RESULTS

Guideline Criteria	Reported Information
Quality assurance and GLP compliance statements included in report?	• Yes (p. 3 and 4)
Detailed information on test organisms included (scientific name, method of verification, strain, and source)?	(p. 12) • Yes • Original algal cultures obtained from the University of Toronto Culture Collection and maintained at Wildlife International, Ltd., Easton, Maryland.
Growth in controls reported?	• Yes (p. 26)

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Guideline Criteria	Reported Information
Description of test system and test design included?	• Yes (p. 13)
Initial and final chemical concentrations and pH measured?	• Yes (p. 11, 22, 24)
Initial, 24-, 48-, 72- and 96-hr cell densities measured? % of inhibition or growth and other adverse effects reported?	• Yes • Yes (p. 26)
96-hr EC_{50} and when sufficient data generated 24-, 48-, and 72-hr EC_{50}, and 95% C.I. reported?	• Yes, 72- and 96- hour EC_{50} values were determined. 95% C.I. were not calculable. (p. 10)
Raw data included?	• Yes (p. 47-49)
Methods and data records reported?	• Yes (p. 12)
<u>Statistical Analysis</u> • Mean and standard deviation calculated and plotted? • Goodness-of-fit determined?	(p. 26-31) • Only mean calculated and plotted. • Yes

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Dose Response

Mean Cell Density and Percent Inhibition

Nominal Conc. at Test Initiation (µg/L)	24-Hour		48-Hour		72-Hour		96-Hour	
	Mean Cell Density (cell/mL)	Percent Inhibition	Mean Cell Density (cell/mL)	Percent Inhibition	Mean Cell Density (cell/mL)	Percent Inhibition	Mean Cell Density (cell/mL)	Percent Inhibition
Negative Control	20,667	--	230,333	--	748,333	--	3,100,000	--
Solvent Control	23,667	--	183,000	--	561,667	--	2,213,333	--
Pooled Control	22,167	--	206,667	--	655,000	--	2,656,667	--
7.8	50,333	-127	173,667	16	525,000	20	2,483,333	-12
16	14,333	35	221,333	-7.1	430,000	34	2,850,000	-29
31	45,667	-106	169,000	18	625,000	4.6	2,293,333	-3.6
63	48,000	-117	180,000	13	915,000	-40	2,670,000	-21
125	39,000	-76	164,000	21	558,333	15	2,483,333	-12

[1] Percent Inhibition was calculated relative to the pooled control replicates using SAS Version 8.02.
[2] Percent Inhibition was calculated relative to the solvent control replicates using SAS Version 8.02.
*[1] No statistically significant differences ($p>0.05$) at 72 hours from the pooled control replicates using Dunnett's test.
*[2] No statistically significant differences ($p>0.05$) at 96 hours from the solvent control replicates using Dunnett's test.
p. 26

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Mean Area Under the Growth Curve (Biomass) and Percent Inhibition

Nominal Test Conc. at Test Initiation (µg/L)	0-24 Hours		0-48 Hours		0-72 Hours		0-96 Hours	
	Mean Area	Percent Inhibition	Mean Area	Percent Inhibition	Mean Area	Percent Inhibition	Mean Area	Percent Inhibition[2]
Negative Control	156,000	--	2,928,000	--	14,432,000	--	60,372,000	--
Solvent Control	192,000	--	2,432,000	--	11,128,000	--	44,188,000	--
Pooled Control	174,000	--	2,680,000	--	12,780,000	--	52,280,000	--
7.8	524,000	-201	2,972,000	-11	11,116,000	13	46,976,000	-6.3
16	52,000	70	2,640,000	1.5	10,216,000	20	49,336,000	-12
31	428,000	-146	2,764,000	-3.1	12,052,000	5.7	46,832,000	-6.0
63	456,000	-162	2,952,000	-10	15,852,000	-24	58,632,000	-33
125	348,000	-100	2,544,000	5.1	10,972,000	14	47,232,000	-6.9

[1] Percent Inhibition was calculated relative to the pooled control replicates using SAS Version 8.02.
[2] Percent Inhibition was calculated relative to the solvent control replicates using SAS Version 8.02.
*1 No statistically significant difference ($p>0.05$) at 72 hours from the pooled control replicates using Dunnett's test.
*2 No statistically significant difference ($p>0.05$) at 92 hours from the solvent control replicates using Dunnett's test.
p. 27.

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Mean Growth Rate and Percent Inhibition

Nominal Concentration at Test Initiation (µg/L)	0-24-hour		0-48-hour		0-72-hour		0-96-hour	
	Mean Growth Rate	Percent Inhibition	Mean Growth Rate	Percent Inhibition	Mean Growth Rate	Percent Inhibition	Mean Growth Rate	Percent Inhibition
Negative Control	0.0248	--	0.0626	--	0.0595	--	0.0597	--
Solvent Control	0.0336	--	0.0600	--	0.0559	--	0.0561	--
Pooled Control	0.0292	--	0.0613	--	0.0577	--	0.0579	--
7.8	0.0561	-92	0.0583	4.9	0.0550	4.7	0.0566	2.4
16	0.0136	53	0.0642	-4.8	0.0516	11	0.0588	-1.6
31	0.0557	-91	0.0579	5.5	0.0559	3.2	0.0566	2.3
63	0.0650	-123	0.0564	8.0	0.0619	-7.2	0.0577	0.37
125	0.0481	-65	0.0570	7.1	0.0557	3.6	0.0574	1.0

[1] Percent inhibition was calculated relative to the pooled control replicates using SAS Version 8.02.
* No statistically significant difference ($p<0.05$) at 72 and 96 hours from the pooled control replicates using Dunnett's Test.
p. 28

Statistical Results

Statistical Method: Cell density, growth rate, and area under the growth curve were analyzed statistically by linear interpolation (SAS, Version 8.02) to determine EC_{50} values and corresponding 95% confidence limits for each 24-hour exposure interval. To determine the NOEC at 72 and 96 hours, cell density and the area under the growth curve data were first evaluated for normality and homogeneity of variance using Shapiro-Wilk's and Levene's tests, respectively, and were compared to the control using Dunnett's test ($p=0.05$).

EC_{50}, E_bC_{50} and E_rC_{50} Values (µg/L) Values Over the 96-hr Exposure Period

Time	Cell Density			Area Under the Growth Curve (Biomass)			Growth Rate		
	EC_{50} (µg/L)	95% C.I.	NOEC (µg/L)	EC_{50} (µg/L)	95% C.I.	NOEC (µg/L)	EC_{50} (µg/L)	95% C.I.	NOEC (µg/L)
24-hr	>125	–[1]	--	>125	–[1]	--	>125	–[1]	--
48-hr	>125	–[1]	--	>125	–[1]	--	>125	–[1]	--
72-hr	>125	–[1]	125	>125	–[1]	125	>125	–[1]	125
96-hr	>125	–[1]	125	>125	–[1]	125	>125	–[1]	125

[1] 95% Confidence limits could not be calculated with the data obtained.
p. 20 and 29

13. VERIFICATION OF STATISTICAL RESULTS

Statistical Method:

NOEC Determination

The 72 hour and 96 hour data were first checked for normality and homogeneity using the Shapiro-Wilks' Test and Bartletts Test, respectively. Data were normally distributed; therefore, the NOECs were determined using the Bonferroni T-Test.

EC_{50} Determination

The EC_{50}, E_bC_{50} and E_rC_{50} values and 95% confidence limits were calculated for cell densities, biomass and growth rate. The EC values were determined using EPA's Linear Interpolation Method for Sublethal Toxicity: The Inhibition Concentration (ICp) Approach.

EC_{50}, E_bC_{50} and E_rC_{50} Values (µg/L) Values Over the 96-hr Exposure Period

Time	Cell Density			Area Under the Growth Curve			Growth Rate		
	EC_{50} (µg/L)	95% C.I.	NOEC (µg/L)	EC_{50} (µg/L)	95% C.I.	NOEC (µg/L)	EC_{50} (µg/L)	95% C.I.	NOEC (µg/L)
24-hr	>125	–[1]	--	>125	–[1]	--	>125	–[1]	--
48-hr	>125	–[1]	--	>125	–[1]	--	>125	–[1]	--
72-hr	>125	–[1]	125	>125	–[1]	125	>125	–[1]	–[2]
96-hr	>125	–[1]	125	>125	–[1]	125	>125	–[1]	–[2]

[1] 95% Confidence limits could not be calculated with the data obtained.
[2] The NOEC could not be verified because the mean square values are zero, and an F value could not be calculated.

14. REVIEWER'S COMMENTS:

- Verified NOEC values are the same as reported in the Study, with the exception of the growth rate NOEC that could not be verified because the mean square values are zero and an F value could not be calculated.
- Verified EC_{50} values are the same as those reported in the Study.

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