

Data Evaluation Report on the acute toxicity of Chlorsulfuron on the Freshwater Alga *Anabaena flos-aquae*
PMRA Submission #: {.....} EPA MRID #: 45832903

Data Requirement: PMRA DATA CODE {.....}
EPA DP Barcode ~~D287772~~ ~~XXXXXXXXXX~~
OECD Data Point {.....} OK
EPA MRID 45832903
EPA Guideline 123-2

Test material: Chlorsulfuron Purity: 97.79%
Common name: DPX-W4189
Chemical name: IUPAC: Bensenesulfonamide, 2-chloro-N-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl) amino carbonyl]-
CAS name: Not reported
CAS No.: 64902-72-3
Synonyms: DPX-W4189-257

Primary Reviewer: Rebecca Bryan
Staff Scientist, Dynamac Corporation

Signature: *Rebecca Bryan*
Date: 2/5/03

QC Reviewer: Dana Worcester
Staff Scientist, Dynamac Corporation

Signature: *Dana Worcester*
Date: 2/5/03

Primary Reviewer: Norman Birchfield, ERB I
{EPA/OECD/PMRA}

Date: {.....}

Secondary Reviewer(s): *Dan Balluff*
{EPA/OECD/PMRA}

Signature: *Daniel Balluff*
Date: *3/3/03*

Company Code {.....} [For PMRA]
Active Code {.....} [For PMRA]
EPA PC Code 118601

Date Evaluation Completed: {dd-mmm-yyyy}

CITATION: R.L. Boeri, D.C. Wyskiel, and T.J. Ward. 2001. Chlorsulfuron (DPX-W4189) Technical: Influence on Growth and Growth Rate of the Alga, *Anabaena flos-aquae*. Unpublished study performed by T.R Wilbury Laboratories, Inc., Marblehead, MA, Laboratory Study No. 2044-DU, and sponsored by E.I. du Pont de Nemours and Company, Study Number 4466. Experimental start date November 10, 2000 and experimental termination date November 15, 2000. The final report issued January 3, 2003.

EXECUTIVE SUMMARY:

In a 120-hour acute toxicity study, 7-day old cultures of *Anabaena flos-aquae* were exposed to Chlorsulfuron under static conditions. Nominal concentrations were 0 (negative control), 0.25, 0.50, 1.0, 2.0, and 4.1. Mean measured concentrations were <LOQ, negative control, 0.236, 0.485, 0.961, 1.92, and 3.95 mg/L. After 120 hours, mean cell density percent inhibition was 22, 57, 97, and >99% in the 0.485, 0.961, 1.92, and 3.95 mg/L treatment groups, respectively, compared to the dilution water control. Mean growth rate percent inhibition was 6, 16, 60, and 80% in the 0.485, 0.961, 1.92, and 3.95 mg/L treatment groups, respectively, compared to the dilution water control. Mean area under the growth curve (biomass) percent inhibition was 19, 33, 70, 80, 95% in the 0.236, 0.485, 0.961, 1.92, and 3.95 mg/L treatment groups, respectively, compared to the dilution water control. No toxic effects were observed. Biomass was the most sensitive endpoint. The NOEC based on cell density and biomass was 0.236 mg/L. The EC₅₀ based on biomass was 0.609 mg/L.

The study is scientifically sound and satisfies the guidelines for a freshwater algae study with *Anabaena flos-aquae* (U.S. EPA Guideline 122-2). This study is classified as Core.

Results Synopsis

Test Organism: *Anabaena flos-aquae*

Test Type: Static

Cell Density:

NOEC: 0.236 mg/L

LOEC: 0.485 mg/L

EC₅₀: 0.78 mg/L 95% C.I.: 0.65 to 0.95

Growth rate:

NOEC: 0.485 mg/L

EC₅₀: 1.77 95% C.I.: 1.55 to 2.02

Area Under the Growth Curve (Biomass):

NOEC: 0.236 mg/L

EC₅₀: 0.609 95% C.I.: 0.472 to 0.785

Endpoint(s) Affected: Cell density, growth rate, and biomass

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: The test was based on the following guidelines: OECD Guideline no. 201 and U.S. EPA Pesticide Assessment Guidelines, Growth and Reproduction of Aquatic Plants-Tier 2, Subdivision J, §123-2. No deviations from U.S. EPA Guideline 123-2 were noted.

COMPLIANCE: Signed and dated GLP, Quality Assurance and No Data Confidentiality statements were provided.

A. MATERIALS:

1. Test Material Chlorsulfuron

Description: White powder

Lot No./Batch No. : DPX-W4189-257

Purity: 97.79%

Stability of Compound

Under Test Conditions: The day 0 measured concentrations were 96-99% of nominal and the day 5 measured concentrations were 92-100% of nominal. In the stability study, percent recovery range for the nominal 0.10 and 10 mg/L samples was 91-96% (Table 4, p. 23). All OECD requirements were not reported.

(OECD requires water solubility, stability in water and light, pKa, Pow, vapor pressure of test compound)

Water (algal medium) solubility: 9.93 mg/L at approximately 24°C

Storage conditions of test chemicals: Not reported

2. Test organism:

Name: *Anabaena flos-aquae*

EPA requires a nonvascular species: For tier I testing, only one species, S. capricornutum, to be tested; for tier II testing, S. costatum, A. flos-aquae, S. capricornutum, and a freshwater diatom is tested

OECD suggests the following species are considered suitable: S. capricornutum, S. subspicatus, and C. vulgaris. If other species are used, the strain should be reported

Strain: UTEX 1444

Source: University of Texas at Austin, Culture Collection of Algae

Age of inoculum: 7 days old

Method of cultivation: Sterile enriched freshwater medium

B. STUDY DESIGN:

a) Range-finding Study: Three 120-hour static range finding studies were conducted. The first was conducted at nominal concentrations of 0.0010, 0.10, 0.50, 0.10, and 0.50 mg/L, the second was at 0.98, 4.9, 9.8, 49, and 122 mg/L, and the third was at 0.050, 0.10, 0.50, 1.0, and 10 mg/L. In the third test, after 120 hours, the percent growth reductions compared to the control were 18, 35, and >99% in the 0.10, 1.0, and 10 mg/L treatment groups, respectively. There was increased growth for the 0.050 or 0.50 mg/L treatment groups.

b) Definitive Study

Table 1 . Experimental Parameters

Parameter	Details	Remarks
		Criteria
Acclimation period: culturing media and conditions: (same as test or not) health: (any toxicity observed)	At least 14 days Sterile enriched freshwater medium; same as test Not reported.	<hr/> <i>EPA recommends two week acclimation period.</i> <i>OECD recommends an amount of algae suitable for the inoculation of test cultures and incubated under the conditions of the test and used when still exponentially growing, normally after an incubation period of about 3 days. When the algal cultures contain deformed or abnormal cells, they must be discarded.</i>
Test system static/static renewal: renewal rate for static renewal:	Static	
Incubation facility	Incubator	
Duration of the test	120 hours	<hr/> <i>EPA requires: 96 - 120 hours</i> <i>OECD: 72 hours</i>
Test vessel material: (glass/polystyrene) size: fill volume:	Glass Erlenmeyer flasks loosely capped with inverted glass beakers 250 mL 50 mL	<hr/> <i>OECD recommends 250 ml conical flasks are suitable when the volume of the test solution is 100 ml or use a culturing apparatus.</i>

Parameter	Details	Remarks
		Criteria
Details of growth medium name: pH at test initiation: pH at test termination: Chelator used: Carbon source: Salinity (for marine algae):	Sterile enriched freshwater medium 7.3 7.4-7.5 Yes NaHCO ₃ N/A	The EDTA containing-compound was Disodium (Ethylenedinitrilo) tetraacetic acid. <hr/> OECD recommends the medium pH after equilibration with air is ~8 with less than .001 mmol/l of chelator if used. EPA recommends 20X-AAP medium.
If non-standard nutrient medium was used, detailed composition provided (Yes/No)	N/A	
Dilution water source: type: pH: salinity (for marine algae): water pretreatment (if any): Total Organic Carbon: particulate matter: metals: pesticides: chlorine:	Laboratory water Deionized 7.5 ± 0.1 N/A None 0.45 mg/L (water analysis) <10 mg/L See Table 1, p. 19 Not detected Not reported	<hr/> EPA pH: <i>Skeletonema costatum</i> = ~8.0 Others = ~7.5 from beginning to end of the test. EPA salinity: 30-35 ppt. EPA is against the use of dechlorinated water. OECD: pH is measured at beginning of the test and at 72 hours, it should not normally deviate by more than one unit during the test.
Indicate how the test material is added to the medium (added directly or used stock solution)	Stock solution	
Aeration or agitation	Agitation, 100 rpm	Agitation is not recommended for this test species. <hr/> EPA recommends agitation at 100 cycles per min and sonication for <i>Anabaena</i> . Aeration is not recommended.

Parameter	Details	Remarks
		Criteria
Initial cells density	Approximately 3,000 cells/mL	<p><i>EPA requires an initial number of 3,000 - 10,000 cells/mL. For <i>Anabaena flos-aquae</i>, cell counts on day 2 are not required.</i></p> <p><i>OECD recommends that the initial cell concentration be approximately 10,000 cells/ml for <i>S. capricornutum</i> and <i>S. subspicatus</i>. When other species are used the biomass should be comparable.</i></p>
Number of replicates control: solvent control: treated ones:	3 N/A 3	<p><i>EPA requires a negative and/or solvent control with 3 or more replicates per doses. <i>Navicula sp.</i> tests should be conducted with four replicate.</i></p> <p><i>OECD preferably three replicates at each test concentration and ideally twice that number of controls. When a vehicle is used to solubilize the test substance, additional controls containing the vehicle at the highest concentration used in the test cultures should be included in the test.</i></p>

Parameter	Details	Remarks
		Criteria
Test concentrations nominal: measured:	0 (negative control), 0.25, 0.50, 1.0, 2.0, and 4.0 mg/L (corrected for purity) <0.00525 (<LOD, negative control), 0.236, 0.485, 0.961, 1.92, and 3.95 mg/L	Mean measured concentrations were determined from 0 hour and 120 hour samples. <i>EPA requires at least 5 test concentrations, with each at least 60% of the next higher one.</i> <i>OECD recommends at least five concentrations arranged in a geometric series, with the lowest concentration tested should have no observed effect on the growth of the algae. The highest concentration tested should inhibit growth by at least 50% relatively to the control and, preferably, stop growth completely.</i>
Solvent (type, percentage, if used)	N/A	
Method and interval of analytical verification	HPLC; 0 and 120 hours	
Test conditions temperature: photoperiod: light intensity and quality:	24.5-24.6°C Continuous 1,900 to 2,000 lux	<i>EPA temperature: <u>Skeletonema</u>: 20°C, Others: 24-25°C; EPA photoperiod: <u>S. costatum</u> 14 hr light/ 10 hr dark, Others: Continuous; EPA light: <u>Anabaena</u>: 2.0 Klux (±15%), Others: 4 - 5 Klux (±15%)</i> <i>OECD recommended the temperature in the range of 21 to 25°C maintained at ± 2°C and continuous uniform illumination provided at approximately 8000 Lux measured with a spherical collector.</i>
Reference chemical {if used} name: concentrations:	N/A	
Other parameters, if any	None	

2. Observations:

Table 2: Observation parameters

Parameters	Details	Remarks/Criteria
Parameters measured including the growth inhibition/other toxicity symptoms	Cell count, growth rate, mean area under the growth curve (biomass), and toxic effects	<i>EPA recommends the growth of the algae expressed as the cell count per mL, biomass per volume, or degree of growth as determined by spectrophotometric means.</i>
Measurement technique for cell density and other end points	Haemocytometer	<i>EPA recommends the measurement technique of cell counts or chlorophyll a</i> <i>OECD recommends the electronic particle counter, microscope with counting chamber, fluorimeter, spectrophotometer, and colorimeter. (note: in order to provide useful measurements at low cell concentrations when using a spectrophotometer, it may be necessary to use cuvettes with a light path of at least 4 cm).</i>
Observation intervals	Every 24 hours	<i>EPA and OECD: every 24 hours.</i>
Other observations, if any	None	
Indicate whether there was exponential growth in the control	Yes, dilution water control group cell density at test termination was 380X greater than the dilution water control group cell density at test initiation.	<i>EPA requires control cell count at termination to be $\geq 2X$ initial count or by a factor of at least 16 during the test.</i> <i>OECD: cell concentration in control cultures should have increased by a factor of at least 16 within three days.</i>
Were raw data included?	Yes	

II. RESULTS and DISCUSSION:**A. INHIBITORY EFFECTS:**

After 120 hours, mean cell density percent inhibition was 22, 57, 97, and >99% in the 0.485, 0.961, 1.92, and 3.95 mg/L treatment groups, respectively, compared to the dilution water control. Mean growth rate percent inhibition was 6, 16, 60, and 80% in the 0.485, 0.961, 1.92, and 3.95 mg/L treatment groups, respectively, compared to the dilution water control. Mean area under the growth curve (biomass) percent inhibition was 19, 33, 70, 80, 95% in the 0.236, 0.485, 0.961, 1.92, and 3.95 mg/L treatment groups, respectively, compared to the dilution water control. No toxic effects were observed.

Table 3: Effect of Chlorsulfuron on Freshwater alga, *Anabaena flos-aquae*

Treatment measured and nominal concentration ^a (mg/L)	Initial cell density (cells/mL)	Mean Cell density (cells/mL) at		
		24 hours	120 hours	
			cell count	% inhibition
Dilution water control	~3,000	111,000	1,141,000	--
0.236 (0.25)	~3,000	55,000	1,161,000	-2
0.485 (0.50)	~3,000	53,000	893,000	22
0.961 (1.0) ^b	~3,000	60,000	485,000	57
1.92 (2.0)	~3,000	51,000	33,000	97
3.95 (4.0)	~3,000	40,000	<10,000	>99
Reference chemical (if used)	N/A	N/A	N/A	N/A

^a Nominal concentrations are in parentheses.

Table 4: Effect of Chlorsulfuron on the Freshwater alga, *Anabaena flos-aquae*

Mean Measured and Nominal ^a Treatment Concentrations (mg/L)	Initial cell density (cells/mL)	Mean Growth Rate at 120 hours	% inhibition (Mean Growth Rate at 120 hours) ^b	Mean Area Under Growth Curve at 120 hours	% inhibition (Mean Area Under Growth Curve at 120 hours) ^b
Dilution water control	~3,000	0.050	--	58,032,000	--
0.236 (0.25)	~3,000	0.050	0	47,232,000	19
0.485 (0.50)	~3,000	0.047	6	39,072,000	33
0.961 (1.0)	~3,000	0.042	16	17,232,000	70
1.92 (2.0)	~3,000	0.020	60	5,712,000	80

Mean Measured and Nominal ^a Treatment Concentrations (mg/L)	Initial cell density (cells/mL)	Mean Growth Rate at 120 hours	% inhibition (Mean Growth Rate at 120 hours) ^b	Mean Area Under Growth Curve at 120 hours	% inhibition (Mean Area Under Growth Curve at 120 hours) ^b
3.95 (4.0)	~3,000	0.010	80	3,036,000	95
Reference chemical (if used) -	Not reported	Not reported	Not reported	Not reported	Not reported

^a Nominal concentrations are in parentheses.

^b Percent inhibition was reviewer-calculated from percent of control data.

Table 5: Statistical endpoint values.

Statistical Endpoint	Biomass	Growth rate	Cell density
NOEC or EC ₀₅ (mg/L)	0.236	0.485	0.236
EC ₅₀ (mg/L) (95% C.I.)	0.609 (0.472 to 0.785)	1.77 (1.55 to 2.02)	0.807 (0.663 to 0.982)
other: EC ₂₅ (mg/L) (95% C.I.)	0.307 (<0.236 to 0.441)	0.987 (<0.798 to 1.22)	0.557 (0.422 to 0.737)
Reference chemical, if used NOAEC IC ₅₀ /EC ₅₀	N/A	N/A	N/A

N/A = Not applicable.

B. REPORTED STATISTICS:

Statistical Method: The growth rate and area under the growth curve calculation is described on page 15. The EC₂₅ and EC₅₀ values were calculated using the cell number, growth rate, and area under the growth curve data by a weighted least squares non-linear regression technique by Bruse and Versteeg. The NOEC was determined using a one-way analysis of variance and Dunnett's test (TOXSTAT 3.3).

Cell Density:

NOEC: 0.236 mg/L

EC₅₀: 0.807 mg/L 95% C.I.: 0.663 to 0.982

Growth rate:

NOEC: 0.485 mg/L

EC₅₀: 1.77 95% C.I.: 1.55 to 2.02

Area Under the Growth Curve (Biomass):

NOEC: 0.236 mg/L

EC₅₀: 0.609 95% C.I.: 0.472 to 0.785

Endpoint(s) Affected: Cell density, growth rate, and biomass

C. VERIFICATION OF STATISTICAL RESULTS:

Statistical Method: Statistical Method: Data for cell density satisfied assumptions of normality, but not variance homogeneity using both Hartley or Bartlett's tests for homogeneity of variances. The Kruskal Wallis test with Dunn's multiple comparison confirmed that the highest treatment group was significantly different from the control. Data from the highest level was excluded from the analysis of growth rate because all values were reported as <10,000. The untransformed data were analyzed using ANOVA followed by William's test via TOXSTAT statistical software. The EC_{50} value was determined using the probit method via Nuthatch statistical software.

Cell Density:

NOEC: 0.236 mg/L
LOEC: 0.485 mg/L Probit Slope: 4.10 ± 0.506
 EC_{50} : 0.78 mg/L 95% C.I.: 0.65 to 0.95

Growth rate:

NOEC: 0.485 mg/L
 EC_{50} : 0.987 95% C.I.: <0.236 to 0.441

Area Under the Growth Curve (Biomass):

NOEC: 0.236 mg/L
 EC_{50} : 0.307 95% C.I.: <0.236 to 0.441

Endpoint(s) Affected: Cell density, growth rate, and biomass

D. STUDY DEFICIENCIES:

The deviations did not affect the acceptability or validity of the study.

E. REVIEWER'S COMMENTS:

The reviewer's conclusions regarding the NOEC and LOEC were identical to those of the study author.

The analytical samples at 120 hours were pooled from each replicate and centrifuged for approximately 20 minutes (nominal 3,100 rpm). Samples were analyzed within three days of collection.

The test solutions were carbon-filtered before disposal at test end.

An algastatic test was conducted with the maximally inhibited test concentration (p. 13).

F. CONCLUSIONS: The study is scientifically sound and satisfies the guidelines for a freshwater algae study with *Anabaena flos-aquae* (U.S. EPA Guideline 122-2). This study is classified as Core.

Cell Density:

NOEC: 0.236 mg/L

LOEC: 0.485 mg/L

EC₅₀: 0.78 mg/L 95% C.I.: 0.65 to 0.95

Growth rate:

NOEC: 0.485 mg/L

EC₅₀: 0.987 95% C.I.: <0.236 to 0.441

Area Under the Growth Curve (Biomass):

NOEC: 0.236 mg/L

EC₅₀: 0.307 95% C.I.: <0.236 to 0.441

Endpoint(s) Affected: Cell density, growth rate, and biomass

III. REFERENCES:

Organisation for Economic Co-Operation and Development (OECD). Guideline for Testing Chemicals. Section 2: Effects on Biotic Systems. Method 201, Algal Growth Inhibition Test, Adopted 4 April, 1984.

American Society for Testing and Materials (ASTM). 1986. Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians. E 729-80a. Annual Book of ASTM Standards, Vol. 11.04.

U.S. Environmental Protection Agency (EPA). 1978. The *Selenastrum capricornutum* Printz Algal Assay Bottle Test. EPA-600/9-78-018. Environmental Research Laboratory, Corvallis, Oregon.

U.S. Environmental Protection Agency (EPA). 1988. Pesticide Assessment Guidelines. Subdivision E. Hazard Evaluation: Wildlife and Aquatic Organisms. Ecological Effects Branch, Hazard Evaluation Division, Office of Pesticide Programs, Washington, D.C. Draft, March 1988.

U.S. Environmental Protection Agency (EPA). 1989. Pesticide Assessment Guidelines. Subdivision J. 123-2: Growth and Reproduction of Aquatic Plants-Tier 2. Ecological Effects Branch, Hazard Evaluation Division, Office of Pesticide Programs, Washington, D.C.

Bruce, R.D., and J.D. Versteeg. 1992. A Statistical Procedure for Modeling Continuous Toxicity Data. Environmental Toxicology and Chemistry Vol. 11. No. 10, pp. 1485-1494.

Gulley, D.D., A.M. Boelter, and H.L. Bergman. 1990. TOXSTAT Version 3.3. Fish Physiology and Toxicology Laboratory, University of Wyoming, Laramie, Wyoming.

Organisation for Economic Co-Operation and Development (OECD). 1997. OECD Principles of Good Laboratory Practice. [C(97)186/Final].

MAFF. 1984. 59 NohSan, No. 3850. Good Laboratory Practice Standards.

U.S. Environmental Protection Agency (EPA). 1993. 40 CFR Part 160. Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA); Good Laboratory Practice Standards. Code of Federal Regulations, Title 40 Part 160. Final Rule.

APPENDIX I. OUTPUT OF REVIEWER'S STATISTICAL VERIFICATION:

Cell density
 File: 2903stat Transform: NO TRANSFORM

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	4	2783133599988.000	695783399997.000	35.925
Within (Error)	10	1936753333344.000	193675333334.406	
Total	14	2976808933332.000		

Critical F value = 3.48 (0.05,4,10)
 Since F > Critical F REJECT Ho: All groups equal

Cell density
 File: 2903stat Transform: NO TRANSFORM

DUNNETTS TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	Control	1141333.333	1141333.333		
2	0.236	1161333.333	1161333.333	-0.176	
3	0.485	893333.333	893333.333	2.183	
4	0.961	485333.333	485333.333	5.773	*
5	1.92	32333.333	32333.333	9.760	*

Dunnett table value = 2.47 (1 Tailed Value, P=0.05, df=10,4)

Cell density
 File: 2903stat Transform: NO TRANSFORM

DUNNETTS TEST - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	Control	3			

Data Evaluation Report on the acute toxicity of Chlorsulfuron on the Freshwater Alga *Anabaena flos-aquae*

PMRA Submission #:{.....}

EPA MRID #: 45832903

2	0.236	3	280665.143	24.6	-20000.000
3	0.485	3	280665.143	24.6	248000.000
4	0.961	3	280665.143	24.6	656000.000
5	1.92	3	280665.143	24.6	1109000.000

Cell density

File: 2903stat

Transform: NO TRANSFORM

WILLIAMS TEST (Isotonic regression model) TABLE 1 OF 2

GROUP	IDENTIFICATION	N	ORIGINAL MEAN	TRANSFORMED MEAN	ISOTONIZED MEAN
1	Control	3	1141333.333	1141333.333	1151333.333
2	0.236	3	1161333.333	1161333.333	1151333.333
3	0.485	3	893333.333	893333.333	893333.333
4	0.961	3	485333.333	485333.333	485333.333
5	1.92	3	32333.333	32333.333	32333.333

Cell density

File: 2903stat

Transform: NO TRANSFORM

WILLIAMS TEST (Isotonic regression model) TABLE 2 OF 2

IDENTIFICATION	ISOTONIZED MEAN	CALC. WILLIAMS	SIG P=.05	TABLE WILLIAMS	DEGREES OF FREEDOM
Control	1151333.333				
0.236	1151333.333	0.088		1.81	k= 1, v=10
0.485	893333.333	2.183	*	1.91	k= 2, v=10
0.961	485333.333	5.773	*	1.94	k= 3, v=10
1.92	32333.333	9.760	*	1.96	k= 4, v=10

s = 139167.285

Note: df used for table values are approximate when v > 20.