Data Evaluation Report on the acute toxicity of Hallcomid M-8-10 to Green Alga *Selenastrum capricornutum*
PMRA Submission #:{.....} EPA MRID #: 45369710

Data Requirement:

PMRA DATA CODE	{.....}
EPA DP Barcode	D284964
OECD Data Point	{.....}
EPA MRID	45369710
EPA Guideline	123-2

DRAFT COPY

Test material: Hallcomid M-8-10 **Purity:** 94.4%
Common name: Hallcomid M-8-10
Chemical name: IUPAC: Not reported
CAS name: Not reported
CAS No.: Not reported
Synonyms: Not reported

Primary Reviewer: Dana Worcester
Staff Scientist, Dynamac Corporation
Signature:
Date: 6/9/03

QC Reviewer: Teri Myers
Staff Scientist, Dynamac Corporation
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Date: 6/9/03

Primary Reviewer:
{EPA/OECD/PMRA}
Date:

Secondary Reviewer(s):
{EPA/OECD/PMRA}
Date:

Reference/Submission No.

Company Code:
Active Code:
EPA PC Code: 999999

Date Evaluation Completed:

CITATION: Anderson, J.P.E. 1993. Influence of Hallcomid M-8-10 on the Growth of the Green Alga, *Selenastrum capricornutum*. Unpublished study performed and sponsored by Bayer AG, Leverkusen, Germany and submitted by The C.P. Hall Company, Chicago, IL. GLP Study Number: E 323 0716-2. Final report issued October 18, 1993.

EXECUTIVE SUMMARY:

In a 72 hour acute toxicity study, cultures of *Selenastrum capricornutum* were exposed to Hallcomid M-8-10 under static conditions. Nominal concentrations were 1.8, 3.2, 5.6, 10.0, 18.0, 32.0 and 56.0 mg/L. The test material, Hallcomid M-8-10 contains four ingredients; mean measured concentrations, determined for three of the isomers, averaged 89-98% of the nominal concentrations . Because measured concentrations do not represent all components of Hallcomid M-8-10, toxicity values are based on the nominal concentrations. The 72-hour percent inhibition for biomass in the 1.8, 3.2, 5.6, 10.0, 18.0, 32.0 and 56.0 mg/L treatment groups was 17.7, 38.3, 35.3, 68.7, 92.2, 96.1 and 98.4%, respectively, compared to the control. The 72-hour percent inhibition for the growth rate in the 1.8, 3.2, 5.6, 10.0, 18.0, 32.0 and 56.0 mg/L treatment groups was 4.2, 10.3, 8.0, 23.7, 58.4, 80.2, and 87.2%, respectively, compared to the control. Based on the EC_{50} (5.47 mg/L), biomass was the most sensitive endpoint; the NOEC for this endpoint was <1.80 mg/L.

This toxicity study is scientifically sound, but it does not satisfy the guideline requirements for an aquatic plant study with *Selenastrum capricornutum*, because there were numerous deviations from US EPA guideline recommendations (Subdivision J, §123-2). Most importantly, this study was conducted for 72-hours and US EPA will review three day OECD studies as Tier I screening studies only. As a result, this study is classified as Supplemental.

Results Synopsis

Test Organism: *Selenastrum capricornutum*
Test Type: Static

Cell density; reviewer reported:

EC_{05}: 1.5 mg/L	95% C.I.: 1.0-2.4 mg/L
NOEC: 18 mg/L	Probit Slope: 2.75 ± 0.245
EC_{50}: 6.1 mg/L	95% C.I.: 5.0-7.5 mg/L

Growth Rate; study author reported:

EC_{05}: Not determined	95% C.I.: N/A
NOEC: 1.80 mg/L	Probit Slope: Not reported
EC_{50}: 16.06 mg/L	95% C.I.: 7.95-32.45 mg/L

Area Under the Growth Curve (Biomass); study author reported:

EC_{05}: Not determined	95% C.I.: N/A
NOEC: <1.80 mg./L	Probit Slope: Not reported
EC_{50}: 5.47 mg/L	95% C.I.: 2.64-11.34 mg/L

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

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: EEC Directive 79/831/EWG, Annex V, C.3, Algal Inhibition Test (1992), ISO Guideline No. 8692 (1989) and OECD Guideline No. 201 (1984). The following deviations from 123-2 were noted:

The following deviations are noted:

1. The study was conducted for 72 hours. US EPA requires a test duration of 96-120 hours for Tier II studies. Three day OECD studies will be reviewed as Tier I screening studies only.

2. The light intensity (8.0 Klux) was substantially higher than recommended by the US EPA for this algal species (4-5 Klux ± 15%).

3. The stability of the test substance was not reported. Measured concentrations (day 0) were only reported for 3 of the four active ingredient isomers.

4. The acclimation period used in the study was 3 days. US EPA recommends a two-week acclimation period.

5. The agitation rate was 3 revolutions per minute. EPA recommends 100 cycles per minute for this species.

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

A. MATERIALS:

1. Test Material Hallcomid M-8-10

Description: Clear, yellow liquid

Lot No./Batch No.: 233290307

Purity: 94.4%

Stability of Compound Under Test Conditions: Not reported, day 0 mean measured concentrations were 95% of nominal.

OECD requires water solubility, stability in water and light, pK_a, P_{ow}, and vapor pressure of the test compound. OECD requirements were not reported.

Water Solubility: 0.17%

Storage conditions of test chemicals: Not reported

2. Test organism:

Name: *Selenastrum capricornutum*

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. capricorntum, 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: ATCC 22662, CCAP 278/4
Source: American Type Culture Collection, Rockville, MD
Age of inoculum: Not reported
Method of cultivation: Synthetic test water

B. STUDY DESIGN:

a) Range-finding Study: A range finding study was conducted. No further details were reported.

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)	3 days Synthetic test water; same as test Not reported	*EPA recommends two week acclimation period.* *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.*

Parameter	Details	Remarks *Criteria*
Test system static/static renewal: renewal rate for static renewal:	Static	
Incubation facility	Temperature controlled chamber	
Duration of the test	72 Hours	*EPA requires: 96 - 120 hours* *OECD: 72 hours*
Test vessel material: (glass/polystyrene) size: fill volume:	Glass 300 mL 150 mL	*OECD recommends 250 ml conical flasks are suitable when the volume of the test solution is 100 ml or use a culturing apparatus.*
Details of growth medium name: pH at test initiation: pH at test termination: Chelator used: Carbon source: Salinity **(for marine algae):**	Sterile Freshwater Media 8.31 8.91 100 µg/L $Na_2EDTA{\cdot}2H_2O$ 50 mg/L $NaHCO_3$ N/A	*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 and chelators.*
If non-standard nutrient medium was used, detailed composition provided (Yes/No)	Yes (p. 8)	
Dilution water source/type: pH: salinity **(for marine algae):** water pretreatment (if any): Total Organic Carbon: particulate matter: metals: pesticides: chlorine:	Synthetic test water Purified water with analytical grade salts 7.9-9.0 N/A Not reported Not reported Not reported Not reported Not reported Not reported	*EPA pH: <u>Skeletonema costatum</u>= ~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.*

Parameter	Details	Remarks *Criteria*
Indicate how the test material is added to the medium (added directly or used stock solution)	Stock solution	
Aeration or agitation	Agitation, 3 revolutions per minute	*EPA recommends agitation only for Selenastrum at 100 cycles per min and Skeletonema at ~60 cycles per min. Aeration is not recommended.*
Initial cells density	10,000 cells/mL	*EPA requires an initial number of 3,000 - 10,000 cells/mL. For Selenastrum capricornutum, cell counts on day 2 are not required.* *OECD recommends that the initial cell concentration be approximately 10,000 cells/ml for S. capricornutum and S. subspicatus. When other species are used the biomass should be comparable.*
Number of replicates control: solvent control: treated ones:	 6 0 3	*EPA requires a negative and/or solvent control with 3 or more replicates per doses. Navicula sp.tests should be conducted with four replicate.* *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.*
Test concentrations nominal: measured:	 1.8, 3.2, 5.6, 10.0, 18.0, 32.0 and 56.0 mg/L Not reported	Measured concentrations for only 3 of the 4 active ingredient isomers was provided. *EPA requires at least 5 test concentrations, with each at least 60% of the next higher one.*

Parameter	Details	Remarks
		Criteria
		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.
Solvent (type, percentage, if used)	None	
Method and interval of analytical verification	HPLC; 0 and 72 hours	
Test conditions temperature: photoperiod: light intensity and quality:	 23 ± 2°C Continuous illumination 8.0 ± 20% Klux, fluorescent lighting	*EPA temperature: Skeletonema: 20°C, Others: 24-25°C; EPA photoperiod: S. costatum 14 hr light/ 10 hr dark, Others: Continuous; EPA light: Anabaena: 2.0 Klux (±15%), Others: 4 - 5 Klux (±15%)* *OECD recommended the temperature in the range of 21 to25°C maintained at ± 2°C and continuous uniform illumination provided at approximately 8000 Lux measured with a spherical collector.*
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	Cell density, growth rate, and biomass	

symptoms		*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.*
Measurement technique for cell density and other end points	Extinction values were determined at a wave length of 578 nm using a single-beam photometer. Cell numbers were computed from the extinction values using the formula provided on pages 9 and 10.	*EPA recommends the measurement technique of cell counts or chlorophyll a* *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).*
Observation intervals	Every 24 hours	*EPA and OECD: every 24 hours.*
Other observations, if any	None	
Indicate whether there was exponential growth in the control	Yes, cell density in the control group at 72 hours was 168X of the initial cell density.	*EPA requires control cell count at termination to be ≥2X initial count or by a factor of at least 16 during the test.* *OECD: cell concentration in control cultures should have increased by a factor of at least 16 within three days.*
Were raw data included?	Replicate data were provided for extinction values and cell numbers.	

II. RESULTS and DISCUSSION:

A. INHIBITORY EFFECTS:

Percent inhibition in cell density in the nominal 1.8, 3.2, 5.6, 10.0, 18.0, 32.0 and 56.0 mg/L treatment groups was 20, 42, 34, 71, 95, 98 and 99%, respectively, compared to the control at test termination. The 72-hour percent inhibition for biomass in the 1.8, 3.2, 5.6, 10.0, 18.0, 32.0 and 56.0 mg/L treatment groups was 17.7, 38.3, 35.3, 68.7, 92.2, 96.1 and 98.4%, respectively, compared to the control. The 72-hour percent inhibition for the growth rate in the 1.8, 3.2, 5.6, 10.0, 18.0, 32.0 and 56.0 mg/L treatment groups was 4.2, 10.3, 8.0, 23.7, 58.4, 80.2 and 87.2%, respectively, compared to the control. Deformed cells were noted in the treatment groups ≥3.2 mg/L.

Table 3: Effect of Hallcomid M-8-10 on algal growth *Selenastrum capricornutum*

Treatment (record) measured and nominal concentration (mg/L)[1]	**Initial cell density**	**Mean Cell density (cells/mL) at**			
		24 hours	**48 hours**	**72 hours**	
				cell count	**% inhibition[2]**
Control	10,000	72,200	372,000	1,689,600	--
1.8	10,000	60,900	326,300	1,356,000	20
3.2	10,000	55,900	254,600	987,800	42
5.6	10,000	45,900	239,500	1,114,600	34
10.0	10,000	39,100	134,900	491,800	71
18.0	10,000	28,500	50,300	87,300	95
32.0	10,000	32,800	26,600	26,000	98
56.0	10,000	18,600	16,100	18,600	99
Reference chemical (if used)	N/A				

[1] Measured concentrations were not provided for all active ingredient constituents.
[2] Percent inhibition was calculated relative to the control.

Table 4: Effect of Hallcomid M-8-10 on the Green alga *Selenastrum capricornutum*

Treatment (record measured and nominal concentration (mg/L)[1]	Initial cell density (cells/mL)	Mean Growth Rate per day (72 hours)	% Inhibition (Mean Growth Rate per day)	Mean Area Under Growth Curve (72 hours)	% Inhibition (Mean Area Under Growth Curve)
Negative Control	10,000	1.74	--	3040	--
1.8	10,000	1.66	4.2	2502	17.7
3.2	10,000	1.56	10.3	1876	38.3
5.6	10,000	1.60	8.0	1968	35.3
10.0	10,000	1.32	23.7	952	68.7
18.0	10,000	0.72	58.4	238	92.2
32.0	10,000	0.34	80.2	118	96.1
56.0	10,000	0.22	87.2	50	98.4
Reference chemical (if used)	N/A				

[1] Measured concentrations were not provided for all active ingredient constituents.

Table 5: Statistical endpoint values.

Statistical Endpoint	Growth rate	Biomass	Cell density
NOEC or EC_{05} (mg/L)	1.80	<1.80	Not reported
EC_{50} (mg/L) (95% C.I.)	16.06 (7.95-32.45)	5.47 (2.64-11.34)	Not reported
EC_{25} (mg/L) (95% C.I.)	N/A	N/A	N/A
other (Effect Threshold)	2.40	<1.80	N/A
Reference chemical, if used EC_{50} (mg/L)	1.34	0.70	N/A

B. REPORTED STATISTICS:

Statistical Method: The EC_{50} values were calculated by probit analysis. The NOEC and LOEC were calculated using Dunnett's Test.

C. VERIFICATION OF STATISTICAL RESULTS:

Statistical Method: Data for cell density were normally distributed, but the variances were not homogeneous. As a result, the NOEC was determined using the non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparison test via TOXSTAT statistical software. The EC_{05} and EC_{50} were determined using the Probit method via Nuthatch statistical software. Toxicity values were estimated using the nominal concentrations because mean measured concentrations could not be determined for the test material (only 3 of the 4 components were measured at time 0). The reviewer did not verify results for biomass and growth rate because replicate data were not provided for these endpoints.

Cell density:

EC_{05}: 1.5 mg/L	95% C.I.: 1.0-2.4 mg/L
NOEC: 18 mg/L	Probit Slope: 2.75 ± 0.245
EC_{50}: 6.1 mg/L	95% C.I.: 5.0-7.5 mg/L

Endpoint(s) Affected: Cell density

D. STUDY DEFICIENCIES:

This 72-hour study was conducted according to OECD guidelines. According to a memo issued by US EPA on October 21, 1994 titled "Closure on Nontarget Plant Phytotoxicity Policy Issues," three day OECD studies will be reviewed as Tier I screening studies only. As a result, this study is classified as Supplemental.

E. REVIEWER'S COMMENTS:

The reviewer's conclusions could not be compared to those of the study author because the reviewer determined the toxicity values for cell density, while the study author determined toxicity values for biomass and growth rate. Based on the EC_{50} values that were calculated, biomass was the most sensitive endpoint.

The test material, Hallcomid M-8-10 contains four ingredients; mean measured concentrations determined for three of the isomers averaged 89-98% of the nominal concentrations . Because measured concentrations do not represent all components of Hallcomid M-8-10, toxicity values are based on the nominal concentrations.

A reference test using potassium dichromate at nominal concentrations of 0.18, 0.32, 0.56, 1.0, and 1.8 mg/L was conducted in March 1993. The results of this study were reported on page 10.

F. CONCLUSIONS: This toxicity study is scientifically sound, but it does not satisfy the guideline requirements for an aquatic plant study with *Selenastrum capricornutum*, because there were numerous deviations from US EPA guideline recommendations (Subdivision J, §123-2). Most importantly, this study was conducted for 72-hours and US EPA will review three day OECD studies as Tier I screening studies only. As a result, this study is classified as Supplemental.

Cell density; reviewer reported:

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Growth Rate; study author reported:

EC_{05}: Not determined	95% C.I.: N/A
NOEC: 1.80 mg/L	Probit Slope: Not reported
EC_{50}: 16.06 mg/L	95% C.I.: 7.95-32.45 mg/L

Area Under the Growth Curve (Biomass); study author reported:

EC_{05}: Not determined	95% C.I.: N/A
NOEC: <1.80 mg./L	Probit Slope: Not reported
EC_{50}: 5.47 mg/L	95% C.I.: 2.64-11.34 mg/L

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

III. REFERENCES:

Bringman, G. and R. Kuhn. 1980. Comparison of the toxicity thresholds of water pollutants to bacteria, algae, and protozoa in the cell multiplication inhibition test. Water Research 14: 231-241.

Dorgerloh, M. 1993. Kaliumdichromat-Growth Inhibition of Green Algae (*Selenastrum capricornutum*). Unpublished Research Report of the Bayer Ag, No. DOM93020, 15 June 1993.

EEC Directive 79/831/EWG, Annex V, C.3, Algal Inhibition Test (Feb. 1992).

Finney, D.J. 1952. "Statistical Methods in Biological Assay", London.

GLP Standards (OECD, C (81) 30 (Final), May 12, 1981, Bundesanzeiger, February 4, 1983; and "Grundsätze der Guten Laborpraxis (GLP)", ChemG, dated March 14, 1990, (Germany).

ISO-Guideline No. 8692: 1989 (E) "Water Quality - Fresh Water Algal Growth Inhibition Test with *Scenedesmus subspicatus* and *Selenastrum capricornutum*" (15 Nov. 1989).

Litchfield, J.F. and F.A. Wilcox. 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. 31: 99-113.

OECD-Guideline No. 201 (1984), "OECD-Guideline for Testing of Chemical", "Algal Growth Inhibition Test" (7 June 1984).

Ratte, H.T. 1993. "Easy Assay, Algae Growth Inhibition", Version 4.0, 1 April 1993, RWTH Aachen, 52066 Aachen, Germany.

APPENDIX I. OUTPUT OF REVIEWER'S STATISTICAL VERIFICATION:

cell number
File: 9710cd Transform: NO TRANSFORM

KRUSKAL-WALLIS ANOVA BY RANKS - TABLE 1 OF 2

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	RANK SUM
1	control	169.000	169.000	147.000
2	1.80	135.333	135.333	60.000
3	3.20	98.667	98.667	43.000
4	5.60	111.667	111.667	50.000
5	10.00	49.333	49.333	33.000
6	18.00	8.667	8.667	24.000
7	32.00	3.000	3.000	15.000
8	56.00	1.667	1.667	6.000

Calculated H Value = 25.454 Critical H Value Table = 14.070
Since Calc H > Crit H REJECT Ho:All groups are equal.

cell number
File: 9710cd Transform: NO TRANSFORM

DUNNS MULTIPLE COMPARISON - KRUSKAL-WALLIS - TABLE 2 OF 2

GROUP	IDENTIFICATION	TRANSFORMED MEAN	ORIGINAL MEAN	GROUP 8 7 6 5 3 4 2 1
8	56.00	1.667	1.667	\
7	32.00	3.000	3.000	. \
6	18.00	8.667	8.667	. . \
5	10.00	49.333	49.333	. . . \
3	3.20	98.667	98.667	. . . . \
4	5.60	111.667	111.667	. . . . . \
2	1.80	135.333	135.333	. . . . . \
1	control	169.000	169.000	* * . . . . . \

* = significant difference (p=0.05) . = no significant difference
Table q value (0.05,8) = 3.124 Unequal reps - multiple SE values

EC_{05} and EC_{50} values
9710CD : cell number

Williams Test

[One-Sided Test for Decrease, alpha = 0.050000]

Dose	Isotone Means	T-bar	P-value	Significance

0	169	.		
1.8	135	6.798	<0.005	*
3.2	105	12.89	<0.005	*
5.6	105	12.89	<0.005	*
10	49.3	24.16	<0.005	*
18	8.67	32.37	<0.005	*
32	3	33.52	<0.005	*
56	1.67	33.79	<0.005	*

"*"=Significant; "N.S."=Not Significant.

Estimates of EC%

Parameter	Estimate	95% Bounds		Std.Err. /Estimate	Lower Bound
		Lower	Upper		
EC5	**1.5**	**1.0**	**2.4**	**0.089**	**0.66**
EC10	2.1	1.4	3.0	0.078	0.69
EC25	3.5	2.6	4.6	0.060	0.75
EC50	**6.1**	**5.0**	**7.5**	**0.043**	**0.82**

Slope = 2.75 Std.Err. = 0.245

!!!Poor fit: p < 0.001 based on DF= 5.00 19.0

9710CD : cell number

Observed vs. Predicted Treatment Group Means

Dose	#Reps.	Obs. Mean	Pred. Mean	Obs. -Pred.	Pred. %Control	%Change
0.00	6.00	169.	160.	8.69	100.	0.00
1.80	3.00	135.	149.	-13.4	92.8	7.21
3.20	3.00	98.7	125.	-26.4	78.0	22.0
5.60	3.00	112.	86.9	24.8	54.2	45.8
10.0	3.00	49.3	44.7	4.67	27.9	72.1
18.0	3.00	8.67	15.8	-7.17	9.88	90.1
32.0	3.00	3.00	3.87	-0.865	2.41	97.6
56.0	3.00	1.67	0.658	1.01	0.410	99.6

!!!Warning: EC5 not bracketed by doses evaluated.