Data Evaluation Report on the Acute Toxicity of Dimethyl Disulfide to Algae, *Pseudokirchneriella subcapitata*

PMRA Submission Number {.....} EPA MRID Number 470528-17

Data Requirement:	PMRA DATA CODE	{.....}
	EPA DP Barcode	D338050
	OECD Data Point	OECD Guideline No. 201
	EPA MRID	470528-17
	EPA Guideline	OPPTS 850.5400 (123-2)

Test material: Dimethyl Disulfide TC **Purity:** 998.89 g/kg (99.9%)
Common name: DMDS
Chemical name: IUPAC: Dimethyl Disulfide
CAS name: Dimethyl Disulfide
CAS No.: 624-92-0
Synonyms: None Reported

Primary Reviewer: John Marton **Signature:**
Staff Scientist, Cambridge Environmental, Inc. **Date:** 05/15/07

Secondary Reviewer: Teri S. Myers **Signature:**
Senior Scientist, Cambridge Environmental, Inc. **Date:** 06/04/07

Secondary Reviewer: James Felkel 10/30/08 **Date:** {.....}
{EPA/OECD/PMRA}

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

Reference/Submission No.: {.....}

Company Code	{.....}	[For PMRA]
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EPA PC Code	029088	

Date Evaluation Completed: {dd-mm-yyyy}

CITATION: Scheerbaum, Dirk. 2007. Dimethyl Disulfide TC: Alga, Growth Inhibition Test with *Pseudokirchneriella subcapitata*, 72 h. Unpublished study performed by Dr. U. Noack-Laboratorien, Käthe-Paulus-Str. 1, D-31157 Sarstedt. Laboratory report number SPO106301. Study sponsored by Arkema/Thiochemistry Business Unit, Departement Securite Environnement Produit, France. Study completed January 8, 2007.

DISCLAIMER: This document provides guidance for EPA and PMRA reviewers on how to complete a data evaluation record after reviewing a scientific study concerning the acute toxicity of a pesticide to aquatic nonvascular plants. It is not intended to prescribe conditions to any external party for conducting this study nor to establish absolute criteria regarding the assessment of whether the study is scientifically sound and whether the study satisfies any applicable data requirements. Reviewers are expected to review and to determine for each study, on a case-by-case basis, whether it is scientifically sound and provides sufficient information to satisfy applicable data requirements. Studies that fail to meet any of the conditions may be accepted, if appropriate; similarly, studies that meet all of the conditions may be rejected, if appropriate. In sum, the reviewer is to take into account the totality of factors related to the test methodology and results in determining the acceptability of the study.

This Data Evaluation Record may have been revised by the Environmental Fate and Effects Division subsequent to signing by Cambridge Environmental Inc. personnel.

EXECUTIVE SUMMARY:

In a 72-hour acute toxicity study, cultures of *Pseudokirchneriella subcapitata* (HINDAK SAG 61.81) were exposed to Dimethyl Disulfide TC at serial dilution factors of 1:16, 1:8, 1:4, 1:2 and 1:1 of an initial 100 mg/L stock solution under static conditions; a negative control was also included. The measured values at 0-hours were 5.73, 11.6, 21.9, 44.0 and 86.7 mg ai/L. The measured values at 72-hours were <LOQ, 7.2, 19.2, 39.8 and 78.9 mg ai/L. A mean measured concentration could not be determined for the 1:16 dilution factor and the mean measured concentrations for the serial dilutions of 1:8, 1:4, 1:2 and 1:1 were 9.40, 20.6, 41.9 and 82.8 mg ai/L, respectively. The NOAEC and EC_{50}/IC_{50} values based on cell density were 5.73 and 17 mg ai/L, respectively. The % growth inhibition, based on cell densities at 72 hours, in the treated algal culture as compared to the control ranged from -5 to 99%. Biomass was the most sensitive endpoint, with an EC_{50} of 15 mg ai/L; the NOAEC (and EC_{05}) for biomass was lower than the lowest treatment level (i.e., 5.73 mg ai/L), which inhibited growth 10%, relative to the control.

Cells in the 44.0 and 86.7 mg ai/L treatment groups were observed to be enlarged. No major pH shifts were observed.

This study was only conducted for 72 hours, instead of the recommended 96-120 hours for an algal study. According to a US EPA memo (October 21, 1994) entitled, "Closure on Nontarget Plant Phytotoxicity Policy Issues", three-day OECD studies will be accepted for review as Tier I screening studies only. Furthermore, a NOAEC/EC_{05} could not be determined for biomass, the most sensitive endpoint, due to significant inhibition at all treatment levels. As a result, this toxicity study is classified as scientifically sound, but does not satisfy the guideline requirement for a Tier II nonvascular plant toxicity study with freshwater unicellular green algae.

Results Synopsis

Test Organism: *Pseudokirchneriella subcapitata* (SAG 61.81)
Test Type (Flow-through, Static, Static Renewal): Static

Cell Density:
EC_{05}: 6.6 mg ai/L 95% C.I.: 4.6-9.4 mg ai/L
EC_{50}: 17 mg ai/L 95% C.I.: 14-20 mg ai/L
NOAEC: 5.73 mg ai/L
Probit Slope: 4.08±0.474

Biomass:
EC_{05}: <5.73 mg ai/L 95% C.I.: N/A
EC_{50}: 15 mg ai/L 95% C.I.: 11-20 mg ai/L
NOAEC: <5.73 mg ai/L
Probit Slope: 2.11±0.237

Growth Rate:
EC_{05}: Not Determined 95% C.I.: N/A
EC_{50}: Not Determined 95% C.I.: N/A
NOAEC: 11.6 mg ai/L
Probit Slope: Not Determined
Endpoint(s) Affected: Cell Density, Biomass and Growth Rate

I. MATERIALS AND METHODS:

GUIDELINE FOLLOWED: This study was reported to be conducted following guidelines outlined in OECD-Guideline No. 201 for Testing of Chemicals; Directive 92/69/EC Method C.3, Official Journal of the European Communities No. L383A/179; and OPPTS Draft Guideline No. 850.5400. These guidelines are reported to be in accordance with the Japanese (METI) guideline.

The following deviations from OPPTS 850.5400 are noted in this review:

1. The duration of the definitive test (72-hours) was shorted than recommended (96-hours).
2. The pretest health of the algal culture was not reported.
3. The reported pH at test initiation (7.77-8.05) was higher than recommended for this species (7.5±0.1).
4. The source and type of dilution water were not reported.
5. The results of a periodic screening analysis of the dilution water were not reported.
6. The reviewer's analysis of biomass (the most sensitive endpoint) indicated that the NOAEC and EC_{05} values for this endpoint were both <5.73 mg ai/L, the lowest treatment concentration.

According to a US EPA memo (October 21, 1994) entitled, "Closure on Nontarget Plant Phytotoxicity Policy Issues", three-day OECD studies will be accepted for review as Tier I screening studies only. Furthermore, a NOAEC/EC_{05} could not be determined for biomass, the most sensitive endpoint. These deviations impacted the acceptability of this study.

COMPLIANCE: Signed and dated No Data Confidentiality, GLP and Quality Assurance statements were provided; the study was declared to be conducted in compliance with the present OECD, EC and German principles of Good Laboratory Practice.

A. REPORTED MATERIALS:

1. Test material Dimethyl Disulfide TC

Description: Light Yellow Liquid

Lot No./Batch No. : 17-08-04 (Batch Number)

Purity: 998.89 g/kg (99.9%)

Stability of compound under test conditions: Analytical verification of the test material in the dilution water was conducted at 0 and 72 hours. The 72-hour mean measured concentrations yielded recoveries of 85-96% of nominal for the 1:8, 1:4, 1:2 and 1:1 serial dilutions. No test material was detected in the 1:16 serial dilution treatment level at 72-hours. The recoveries of the 72-hour measured values were 62, 88, 90 and 91% of the initial measured values for the 1:8, 1:4, 1:2 and 1:1 serial dilution treatment levels, respectively.

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

Storage conditions of

test chemicals: Stored at room temperature and protected from moisture and light.

Physicochemical properties of Dimethyl Disulfide TC.

Parameter	Values	Comments
Water solubility at 20EC	Insoluble	
Vapor pressure	20°C: 28 hPa (mbar) 25°C: 38 hPa (mbar)	
UV absorption	Not Reported	
PKa	Not Reported	
Kow	Not Reported	

2. Test organism:

Name: Unicellular Green Algae, *Pseudokirchneriella subcapitata* (formerly *S. 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: HINDAK SAG 61.81
Source: In-house cultures, originally obtained from Sammlung von Algenkulturen (SAG), Pflanzenphsiologisches Institut der Universitat Gottingen, Nikolausberger Weg 18, D-37073 Gottingen
Age of inoculum: 3 Days Old
Method of cultivation: Nutrient Medium Z according to Luttge et al. (1994)

B. REPORTED STUDY DESIGN:

1. Experimental Conditions

a. Range-finding study: The study author reported the definitive test concentrations were selected based on the results of a range-finding (Non-GLP) study. The 72-hour range-finding test was conducted using a saturated solution and dilution factors of 1:10 and 1:100. Biomass was inhibited -5, 10 and 100% in the 1:100, 1:10 and saturated solution levels, respectively. Rate related inhibitions were 1, 1 and 100 in the 1:100, 1:10 and saturated solution levels, respectively.

b. Definitive Study

Table 1: Experimental Parameters

Parameter	Details	Remarks *Criteria*
Acclimation period: Culturing media and conditions: (same as test or not) Health: (any mortality observed)	Continuous 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.*
Test system Static/static renewal Renewal rate for static renewal	Static N/A	*EPA expects the test concentrations to be renewed every 3 to 4 days (one renewal for the 7 day test, 3-4 renewals for the 14 day test).*
Incubation facility	Environmental chamber	
Duration of the test	72 hours After 72 hours of exposure, algae from the control and mean measured 20.6-82.8 mg ai/L treatment levels (0.2-1 mL algae suspension from each replicate) were reported to be transferred into fresh untreated medium and allowed to grow for an additional 6-12 days to determine if the effects of the test material were reversible.	*EPA requires: 96-120 hours* *OECD: 72 hours*

Parameter	Details	Remarks *Criteria*
Test vessel Material: *(glass/stainless steel)* Size: Fill volume:	Glass Not Reported 119 mL With regard to the volatility of the test material, glass flasks without headspace were used to reduce losses of the test material. Aluminum tops with PTFE seals were used to close the test vessels.	*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**):	Nutrient medium Z (Luttge, *et al*, 1994) 7.77-8.05 8.07-9.11 Yes $NaHCO_3$ N/A To enable sufficient growth under conditions without headspace, 0.25 g NaHCO3/L and 2.6656 g MES/L (corresponding to 12.5 mmol) were added. MES= 2-Morpholinoethanesulfonic acid Monohydrat	*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 and chelating agents (e.g. EDTA) in the nutrient medium for optimum cell growth. Lower concentrations of chelating agents (down to one-third of the normal concentration recommended for AAP medium) may be used in the nutrient medium used for test solution preparation if it is suspected that the chelator will interact with the test material. ASTM reference, E1415-91and D 3978-80 (reapproved 1987).*
If non-standard nutrient medium was used, detailed composition provided (Yes/No)	Yes	

Parameter	Details	Remarks *Criteria*
Dilution water source/type: pH: salinity (**for marine algae**): water pretreatment (if any): Total Organic Carbon: particulate matter: metals: pesticides: chlorine:	 Not Reported 7.77-8.05 (at test initiation) N/A None Reported Not Reported Not Reported Not Reported Not Reported Not Reported	*EPA pH: Skeletonema costatum= ~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)	A 100 mg/L stock solution was prepared initially and then diluted to factors of 1:16, 1:8, 1:4, 1:2 and 1:1 for the nominal treatment levels.	
Aeration or agitation	Agitation (~70 rpm)	
Initial cells density	0.5-1.0x10^4 cells/mL	*EPA requires an initial number of 3,000 - 10,000 cells/mL. For Anabaena flos-aquae, 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.*

Parameter	Details	Remarks *Criteria*
Number of replicates Control: Solvent control: Treatments:	6 N/A 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.*
Test concentrations Nominal: Measured:	Negative control and a 100 mg/L stock solution serially diluted to 1:16, 1:8, 1:4, 1:2 and 1:1 ratios 0-Hour Measured Values: <0.010 (<LOQ; control), 5.73, 11.6, 21.9, 44.0 and 86.7 mg ai/L 72-Hour Measured Values: <0.010 (<LOQ; control), <LOQ, 7.2, 19.2, 39.8 and 78.9 mg ai/L	As no test material was detected in the lowest treatment level at 72-hours and the measured value of the 1:8 dilution factor at 72 hours was only 62% of the initial measured value, the reviewer used the initial measured values for all results and analyses. *EPA requires at least 5 test concentrations, with each at least 60% of the next higher one.* *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)	N/A	
Method and interval of analytical verification	All dilution levels and control replicates were analytically verified using SPME/GC-MS/MS at 0 and 72 hours.	

Parameter	Details	Remarks *Criteria*
Test conditions Temperature: Photoperiod: Light intensity and quality:	22.9-23.5°C Continuous 83.0-97.2 $\mu E \cdot m^{-2} \cdot s^{-1}$	*EPA temperature: Skeletonema: 20EC, Others: 24-25EC; 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:	 Potassium Dichromate 0.1, 0.2, 0.4, 0.8 and 1.6 mg/L	
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 density, biomass, growth rate and phytotoxic effects.	*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.*

Parameters	Details	Remarks Criteria
Measurement technique for cell density and other end points	Cell density was reported to be measured using Chlorophyll-a-fluorescence, excitation at 435 nm, emission at 685 nm, with dilution water as the background signal. Biomass and growth rates were calculated based on the cell density determinations for each replicate.	No self fluorescence was reported to be found up to 82.8 mg ai/L (saturated solution). *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	Cell density was determined at test initiation and every 24 hours thereafter.	*EPA and OECD: every 24 hours.*
Other observations, if any	None	
Indicate whether there was an exponential growth in the control	Yes. Mean control cell density was 7878 cells/mL at test initiation and 619269 cells/mL at 72-hours.	*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?	Yes	

II. RESULTS and DISCUSSION:

A. REPORTED INHIBITORY EFFECTS:

At test termination, mean cell density was 619269 cells/mL in the negative control and 651653, 469185, 210179, 11541 and 6723 cells/mL in the initial measured 5.73, 11.6, 21.9, 44.0 and 86.7 mg ai/L treatment levels, respectively, yielding inhibitions of -5, 24, 66, 98 and 99%, respectively, relative to the negative control.

At test termination, the mean biomass integral was 551491 in the negative control and 495732, 381794, 174829, 49748 and 58493 in the initial measured 5.73, 11.6, 21.9, 44.0 and 86.7 mg ai/L treatment levels, respectively, yielding inhibitions of 10, 31, 68, 91 and 89%, respectively, relative to the negative control.

At test termination, mean growth rate was 1.460 $days^{-1}$ in the negative control and 1.469, 1.358, 1.091, 0.100 and -0.081 $days^{-1}$ in the initial measured 5.73, 11.6, 21.9, 44.0 and 86.7 mg ai/L treatment levels, respectively, yielding

inhibitions of -0.66, 7, 25, 92 and 98%, respectively, relative to the negative control

Cells in the 44.0 and 86.7 mg ai/L treatment groups were observed to be enlarged. No major pH shifts were observed.

Table 3: Effect of Dimethyl Disulfide on algal cell density *Pseudokirchneriella subcapitata*

Initial Measured Concentrations mg ai/L	Initial Dell Density ($x10^4$ cells/mL)	Cell Density (cells/mL) at			
		24 Hours	48 Hours	72 hours	
				Cell Count	% Inhibition [a]
Negative control	0.5-1.0	44299	217252	619269	N/A
5.73	0.5-1.0	37655	151945	651653	-5
11.6	0.5-1.0	39195	127701	469185	24
21.9	0.5-1.0	37501	51933	210179	66
44.0	0.5-1.0	44541	19131	11541	98
86.7	0.5-1.0	38117	36709	6723	99
Reference chemical (if used)	N.R.	N.R.	N.R.	N.R.	N.R.

[a] Negative percent inhibition indicates promoted growth
N/A- Not Applicable
N.R.- Not Reported

Table 4: Effect of Dimethyl Disulfide on algal growth and biomass *Pseudokirchneriella subcapitata*

Initial Measured Concentrations mg ai/L	Initial Cell Density ($x\ 10^4$ cells/mL)	Mean Growth Rate		Mean Biomass Integral	
		0-72 Hours	Percent Inhibition[a]	0-72 Hours	Percent Inhibition[a]
Negative control	0.5-1.0	1.460	N/A	551491	N/A
5.73	0.5-1.0	1.469	-0.66	495732	10
11.6	0.5-1.0	1.358	7	381794	31
21.9	0.5-1.0	1.091	25	174829	68
44.0	0.5-1.0	0.100	92	49748	91
86.7	0.5-1.0	-0.081	98	58493	89

[a] Negative percent inhibition indicates promoted growth

Table 5: Statistical endpoint values.

Statistical Endpoint	Cell Density	Growth Rate	Biomass
NOAEC or EC_{05} (mg ai/L)	N.R.	9.40	5.73
EC_{50} (mg ai/L)	N.R.	25.6	14.3
IC_{50} or EC_{50} (mg ai/L) (95% C.I.)	N.R.	23.6-27.8	12.8-15.9
Other (IC_{25}/EC_{25})	N.R.	N.R.	N.R.
Reference chemical, if used NOAEC IC_{50}/EC_{50}	N.R.	EC_{50}: 0.93 (0.87-0.98) mg/L	EC_{50}: 0.50 (0.48-0.52) mg/L

N.R.- Not Reported

After test termination, samples containing algae from the control, and initial measured 21.9, 44.0 and 86.7 mg ai/L treatment groups were placed into clean dilution water and were observed for a period of 6-12 days to determine if the effects of the test material were reversible. Following 6 days of recovery, growth rates were reported to be 0.79 and 0.80 $days^{-1}$ in the negative control, 0.80-0.82 $days^{-1}$ in the 21.9 mg ai/L treatment group, 1.19 $days^{-1}$ in the 44.0 mg ai/L treatment group and 0.30-0.38 $days^{-1}$ in the 86.7 mg ai/L treatment group. Relative to the observed growth rates for these three treatment levels during the definitive test (1.091, 0.100 and -0.081 $days^{-1}$, respectively), the study author concluded that the effects of the test material are reversible, indicating the possibility of recovery.

The in-life portion of the definitive toxicity test was conducted from September 5 to September 8, 2006.

B. REPORTED STATISTICS:

Analyzed endpoints included biomass and growth rate. EC_{50} values were calculated by sigmoidal dose-response regression. Calculation of the confidence intervals for the LC_{50} was reported to be done using standard procedures according to Clopper and Pearson (1937). NOAEC values were determined using One Way ANOVA and Dunnett's tests. When running the ANOVA, a Normality and Equal Variance test were done first. P-values for both tests were 0.05.

C. VERIFICATION OF STATISTICAL RESULTS:

Statistical Method(s): Cell density, biomass integral and growth rates were analyzed to determine if a significant difference existed at any treatment level relative to the negative control. Prior to analysis, data sets for all three endpoints were analyzed for normality using Chi-square and Shapiro-Wilks tests and for homogeneity of variance using Hartley and Bartlett's tests. Cell density and biomass integral did not meet these assumptions of ANOVA; therefore, NOAEC values were determined using the non-parametric Kruskal-Wallis test. As the growth rate data met the assumptions of ANOVA, the NOAEC value was determined using the parametric Bonferroni's and Williams tests. All NOAEC determinations were made via Toxstat statistical software. The reviewer then attempted to determine ECx values and probit slopes using the probit analysis via Nuthatch statistical software. All analyses were made using the initial measured concentrations.

Cell Density:
EC_{05}: 6.6 mg ai/L 95% C.I.: 4.6-9.4 mg ai/L
EC_{50}: 17 mg ai/L 95% C.I.: 14-20 mg ai/L
NOAEC: 5.73 mg ai/L
Probit Slope: 4.08±0.474

Biomass:
EC_{05}: <5.73 mg ai/L 95% C.I.: N/A
EC_{50}: 15 mg ai/L 95% C.I.: 11-20 mg ai/L
NOAEC: <5.73 mg ai/L
Probit Slope: 2.11±0.237

Growth Rate:
EC_{05}: Not Determined 95% C.I.: N/A
EC_{50}: Not Determined 95% C.I.: N/A
NOAEC: 11.6 mg ai/L
Probit Slope: Not Determined

D. STUDY DEFICIENCIES:

Deficiencies in this study included the short study duration (72 hours vs. the recommended 96-120 hours) and the inability to define a NOAEC or EC_{05} for biomass, the most sensitive endpoint. These deficiencies impacted the acceptability of this study and the results should be used for Tier I screening purposes only.

E. REVIEWER'S COMMENTS:

As the recovery of the 1:8 dilution treatment level at 72 hours was <70% of the initial measured concentration and no test material was detected at 72 hours in the 1:16 dilution treatment level, the reviewer conducted all analyses using the initial measured concentrations. The study author used the initial measured concentration for the 1:16 dilution treatment level and the mean measured concentrations for the remaining treatment levels. Therefore, the reviewer's results are reported in the Executive Summary and Conclusions sections of this DER.

The study author's analysis of biomass detected a significant difference at the initial measured 11.6-86.7 mg ai/L treatment levels. However, as a dose-responsive pattern of inhibition was observed, the reviewer felt that the 10% inhibition in biomass at the 5.73 mg ai/L treatment level was biologically significant. Therefore, the reviewer visually determined the NOAEC value to be less than the lowest treatment concentration.

F. CONCLUSIONS:

The study is considered scientifically sound, but is considered acceptable only as a Tier I screening study. The NOAEC and EC50 values, based on biomass, the most sensitive endpoint, were <5.73 and 15 mg ai/L, respectively.

Cell Density:
EC_{05}: 6.6 mg ai/L 95% C.I.: 4.6-9.4 mg ai/L
EC_{50}: 17 mg ai/L 95% C.I.: 14-20 mg ai/L
NOAEC: 5.73 mg ai/L
Probit Slope: 4.08±0.474

Biomass:
EC_{05}: <5.73 mg ai/L 95% C.I.: N/A
EC_{50}: 15 mg ai/L 95% C.I.: 11-20 mg ai/L
NOAEC: <5.73 mg ai/L
Probit Slope: 2.11±0.237

Growth Rate:
EC_{05}: Not Determined 95% C.I.: N/A
EC_{50}: Not Determined 95% C.I.: N/A
NOAEC: 11.6 mg ai/L
Probit Slope: Not Determined
Endpoint(s) Affected: Cell Density, Biomass and Growth Rate

III. REFERENCES:

EPA OPPTS draft No. 850.5400 (1996)

Clopper and Pearson (1934). Biometrika 26:404-413 cited in GraphPad Prism Statistics Guide 4.0

Luttge, U., E. Schnepf, A. Lauchli, and T. Nagata (editors, 1994): Botanica Acta, Journal of the German Botanical Society, No. 3 Volume 107 page 111-186 (June 19964), Thieme-Verlag.

SANCO/3029/99 rev. 4, Residues: Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/474 (11/07/00).

APPENDIX I. OUTPUT OF REVIEWER'S STATISTICAL VERIFICATION:

Cell density (cells/mL), 72-hours; mg ai/L
File: 2817cd Transform: NO TRANSFORMATION

Chi-square test for normality: actual and expected frequencies

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	1.407	5.082	8.022	5.082	1.407
OBSERVED	0	7	7	6	1

Calculated Chi-Square goodness of fit test statistic = 2.5446
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.

Cell density (cells/mL), 72-hours; mg ai/L
File: 2817cd Transform: NO TRANSFORMATION

Shapiro Wilks test for normality

D = 44566712256.000

W = 0.915

Critical W (P = 0.05) (n = 21) = 0.908
Critical W (P = 0.01) (n = 21) = 0.873

Data PASS normality test at P=0.01 level. Continue analysis.

Cell density (cells/mL), 72-hours; mg ai/L
File: 2817cd Transform: NO TRANSFORMATION

Hartley test for homogeneity of variance

Calculated H statistic (max Var/min Var) = 960.04
Closest, conservative, Table H statistic = 184.0 (alpha = 0.01)

Used for Table H ==> R (# groups) = 6, df (# reps-1) = 3
Actual values ==> R (# groups) = 6, df (# avg reps-1) = 2.50
(average df used)

Data FAIL homogeneity test. Try another transformation.

NOTE: This test requires equal replicate sizes. If they are unequal but do not differ greatly, the Hartley test may still be used as an approximate test (average df are used).

Cell density (cells/mL), 72-hours; mg ai/L
File: 2817cd Transform: NO TRANSFORMATION

Bartletts test for homogeneity of variance

Calculated B statistic = 22.14
Table Chi-square value = 15.09 (alpha = 0.01)
Table Chi-square value = 11.07 (alpha = 0.05)

Average df used in calculation ==> df (avg n - 1) = 2.50
Used for Chi-square table value ==> df (#groups-1) = 5

Data FAIL homogeneity test at 0.01 level. Try another transformation.

NOTE: If groups have unequal replicate sizes the average replicate size is used to calculate the B statistic (see above).

Cell density (cells/mL), 72-hours; mg ai/L
File: 2817cd Transform: NO TRANSFORMATION

KRUSKAL-WALLIS ANOVA BY RANKS - TABLE 1 OF 2

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	RANK SUM
1	neg control	619269.000	619269.000	99.000
2	5.73	651653.000	651653.000	54.000
3	11.6	469185.000	469185.000	33.000
4	21.9	210179.000	210179.000	24.000
5	44.0	11541.000	11541.000	13.000
6	86.7	6723.000	6723.000	8.000

Calculated H Value = 18.108 Critical H Value Table = 11.070
Since Calc H > Crit H REJECT Ho:All groups are equal.

Cell density (cells/mL), 72-hours; mg ai/L
File: 2817cd Transform: NO TRANSFORMATION

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

GROUP	IDENTIFICATION	TRANSFORMED MEAN	ORIGINAL MEAN	GROUP 6	5	4	3	1	2
6	86.7	6723.000	6723.000	\					
5	44.0	11541.000	11541.000	.	\				
4	21.9	210179.000	210179.000	.	.	\			
3	11.6	469185.000	469185.000	.	.	.	\		
1	neg control	619269.000	619269.000	*	.	.	.	\	
2	5.73	651653.000	651653.000	*	.	.	.	.	\

* = significant difference (p=0.05) . = no significant difference
Table q value (0.05,6) = 2.936 Unequal reps - multiple SE values

Estimates of EC%

Parameter	Estimate	95% Bounds Lower	95% Bounds Upper	Std.Err.	Lower Bound /Estimate
EC5	6.6	4.6	9.4	0.075	0.70
EC10	8.1	5.9	11.	0.065	0.73
EC25	11.	8.9	14.	0.050	0.79
EC50	17.	14.	20.	0.035	0.84

Slope = 4.08 Std.Err. = 0.474

!!!Poor fit: p = 0.0082 based on DF= 3.0 15.

2817CD : Cell density (cells/mL), 72-hours; mg ai/L

Observed vs. Predicted Treatment Group Means

Dose	#Reps.	Obs. Mean	Pred. Mean	Obs. -Pred.	Pred. %Control	%Change
0.00	6.00	6.19e+05	6.36e+05	-1.72e+04	100.	0.00
5.73	3.00	6.52e+05	6.18e+05	3.39e+04	97.1	2.94
11.6	3.00	4.69e+05	4.70e+05	-957.	73.9	26.1
21.9	3.00	2.10e+05	1.99e+05	1.11e+04	31.3	68.7
44.0	3.00	1.15e+04	2.69e+04	-1.53e+04	4.22	95.8
86.7	3.00	6.72e+03	1.08e+03	5.64e+03	0.170	99.8

Biomass integral, 0-72 hours; mg ai/L
File: 2817bi Transform: NO TRANSFORMATION

Chi-square test for normality: actual and expected frequencies

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	1.407	5.082	8.022	5.082	1.407
OBSERVED	0	7	7	6	1

Calculated Chi-Square goodness of fit test statistic = 2.5446
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.

Biomass integral, 0-72 hours; mg ai/L
File: 2817bi Transform: NO TRANSFORMATION

Shapiro Wilks test for normality

D = 16320692173.500

W = 0.971

Critical W (P = 0.05) (n = 21) = 0.908
Critical W (P = 0.01) (n = 21) = 0.873

Data PASS normality test at P=0.01 level. Continue analysis.

Biomass integral, 0-72 hours; mg ai/L
File: 2817bi Transform: NO TRANSFORMATION

Hartley test for homogeneity of variance

Calculated H statistic (max Var/min Var) = 12718.22
Closest, conservative, Table H statistic = 184.0 (alpha = 0.01)

Used for Table H ==> R (# groups) = 6, df (# reps-1) = 3
Actual values ==> R (# groups) = 6, df (# avg reps-1) = 2.50
(average df used)

Data FAIL homogeneity test. Try another transformation.

NOTE: This test requires equal replicate sizes. If they are unequal but do not differ greatly, the Hartley test may still be used as an approximate test (average df are used).

Biomass integral, 0-72 hours; mg ai/L
File: 2817bi Transform: NO TRANSFORMATION

Bartletts test for homogeneity of variance

Calculated B statistic = 22.05
Table Chi-square value = 15.09 (alpha = 0.01)
Table Chi-square value = 11.07 (alpha = 0.05)

Average df used in calculation ==> df (avg n - 1) = 2.50
Used for Chi-square table value ==> df (#groups-1) = 5

Data FAIL homogeneity test at 0.01 level. Try another transformation.

NOTE: If groups have unequal replicate sizes the average replicate size is used to calculate the B statistic (see above).

Biomass integral, 0-72 hours; mg ai/L
File: 2817bi Transform: NO TRANSFORMATION

KRUSKAL-WALLIS ANOVA BY RANKS - TABLE 1 OF 2

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	RANK SUM
1	neg control	551490.500	551490.500	108.000
2	5.73	495732.000	495732.000	45.000
3	11.6	381794.000	381794.000	33.000
4	21.9	174829.000	174829.000	24.000
5	44.0	49748.000	49748.000	9.000
6	86.7	58493.000	58493.000	12.000

Calculated H Value = 18.390 Critical H Value Table = 11.070
Since Calc H > Crit H REJECT Ho:All groups are equal.

Biomass integral, 0-72 hours; mg ai/L
File: 2817bi Transform: NO TRANSFORMATION

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

GROUP	IDENTIFICATION	TRANSFORMED MEAN	ORIGINAL MEAN	GROUP 0 0 0 0 0 0 5 6 4 3 2 1
5		44.0	49748.000	49748.000 \
6		86.7	58493.000	58493.000 . \
4		21.9	174829.000	174829.000 . . \
3		11.6	381794.000	381794.000 . . . \
2		5.73	495732.000	495732.000 . . . . \
1	neg control		551490.500	551490.500 * * . . . \

* = significant difference (p=0.05) . = no significant difference
Table q value (0.05,6) = 2.936 Unequal reps - multiple SE values

Estimates of EC%

Parameter	Estimate	95% Bounds Lower	Upper	Std.Err.	Lower Bound /Estimate
EC5	2.5	1.3	4.8	0.14	0.52
EC10	3.7	2.1	6.5	0.12	0.57
EC25	7.2	4.7	11.	0.088	0.65
EC50	15.	11.	20.	0.060	0.75

Slope = 2.11 Std.Err. = 0.237

!!!Poor fit: p < 0.001 based on DF= 3.00 15.0

2817BI : Biomass integral, 0-72 hours; mg ai/L

Observed vs. Predicted Treatment Group Means

Dose	#Reps.	Obs. Mean	Pred. Mean	Obs. -Pred.	Pred. %Control	%Change
0.00	6.00	5.51e+05	5.68e+05	-1.64e+04	100.	0.00
5.73	3.00	4.96e+05	4.61e+05	3.48e+04	81.2	18.8
11.6	3.00	3.82e+05	3.37e+05	4.47e+04	59.4	40.6
21.9	3.00	1.75e+05	2.07e+05	-3.23e+04	36.5	63.5
44.0	3.00	4.97e+04	9.20e+04	-4.23e+04	16.2	83.8
86.7	3.00	5.85e+04	3.06e+04	2.79e+04	5.39	94.6

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

!!!Warning: EC10 not bracketed by doses evaluated.

Growth rate (day-1), 0-72 hours; mg ai/L
File: 2817gr Transform: NO TRANSFORMATION

Chi-square test for normality: actual and expected frequencies

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	1.407	5.082	8.022	5.082	1.407
OBSERVED	1	6	5	9	0

Calculated Chi-Square goodness of fit test statistic = 5.8496
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.

Growth rate (day-1), 0-72 hours; mg ai/L
File: 2817gr Transform: NO TRANSFORMATION

Shapiro Wilks test for normality

D = 0.162

W = 0.954

Critical W (P = 0.05) (n = 21) = 0.908
Critical W (P = 0.01) (n = 21) = 0.873

Data PASS normality test at P=0.01 level. Continue analysis.

Growth rate (day-1), 0-72 hours; mg ai/L
File: 2817gr Transform: NO TRANSFORMATION

Hartley test for homogeneity of variance

Calculated H statistic (max Var/min Var) = 10.65
Closest, conservative, Table H statistic = 184.0 (alpha = 0.01)

Used for Table H ==> R (# groups) = 6, df (# reps-1) = 3
Actual values ==> R (# groups) = 6, df (# avg reps-1) = 2.50
(average df used)

Data PASS homogeneity test. Continue analysis.

NOTE: This test requires equal replicate sizes. If they are unequal but do not differ greatly, the Hartley test may still be used as an approximate test (average df are used).

Growth rate (day-1), 0-72 hours; mg ai/L
File: 2817gr Transform: NO TRANSFORMATION

Bartletts test for homogeneity of variance

Calculated B statistic = 6.24
Table Chi-square value = 15.09 (alpha = 0.01)
Table Chi-square value = 11.07 (alpha = 0.05)

Average df used in calculation ==> df (avg n - 1) = 2.50
Used for Chi-square table value ==> df (#groups-1) = 5

Data PASS homogeneity test at 0.01 level. Continue analysis.

NOTE: If groups have unequal replicate sizes the average replicate size is used to calculate the B statistic (see above).

Growth rate (day-1), 0-72 hours; mg ai/L
File: 2817gr Transform: NO TRANSFORMATION

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	5	8.268	1.654	150.364
Within (Error)	15	0.162	0.011	
Total	20	8.431		

Critical F value = 2.90 (0.05,5,15)
Since F > Critical F REJECT Ho:All groups equal

Growth rate (day-1), 0-72 hours; mg ai/L
File: 2817gr Transform: NO TRANSFORMATION

BONFERRONI T-TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	neg control	1.460	1.460		
2	5.73	1.469	1.469	-0.133	
3	11.6	1.358	1.358	1.364	
4	21.9	1.091	1.091	4.969	*
5	44.0	0.100	0.100	18.336	*
6	86.7	-0.081	-0.081	20.777	*

Bonferroni T table value = 2.60 (1 Tailed Value, P=0.05, df=15,5)

Growth rate (day-1), 0-72 hours; mg ai/L
File: 2817gr Transform: NO TRANSFORMATION

BONFERRONI T-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	neg control	6			
2	5.73	3	0.193	13.2	-0.010
3	11.6	3	0.193	13.2	0.101
4	21.9	3	0.193	13.2	0.369
5	44.0	3	0.193	13.2	1.360
6	86.7	3	0.193	13.2	1.541

Growth rate (day-1), 0-72 hours; mg ai/L
File: 2817gr Transform: NO TRANSFORMATION

WILLIAMS TEST (Isotonic regression model) TABLE 1 OF 2

GROUP	IDENTIFICATION	N	ORIGINAL MEAN	TRANSFORMED MEAN	ISOTONIZED MEAN
1	neg control	6	1.460	1.460	1.463
2	5.73	3	1.469	1.469	1.463
3	11.6	3	1.358	1.358	1.358
4	21.9	3	1.091	1.091	1.091
5	44.0	3	0.100	0.100	0.100
6	86.7	3	-0.081	-0.081	-0.081

Growth rate (day-1), 0-72 hours; mg ai/L
File: 2817gr Transform: NO TRANSFORMATION

WILLIAMS TEST (Isotonic regression model) TABLE 2 OF 2

IDENTIFICATION	ISOTONIZED MEAN	CALC. WILLIAMS	SIG P=.05	TABLE WILLIAMS	DEGREES OF FREEDOM
neg control	1.463				
5.73	1.463	0.045		1.75	k= 1, v=15
11.6	1.358	1.375		1.84	k= 2, v=15
21.9	1.091	5.010	*	1.87	k= 3, v=15
44.0	0.100	18.488	*	1.88	k= 4, v=15
86.7	-0.081	20.949	*	1.89	k= 5, v=15

s = 0.104
Note: df used for table values are approximate when v > 20.