Data Evaluation Report on the Acute Toxicity of Pyroxsulam Technical (XDE-742) to Algae, *Anabaena flos-aquae*

PMRA Submission Number 2006-4727; 1283247 EPA MRID Number 469084-~~xx~~ 21 APVMA ATS 40362

[DM1] [DM2]

Data Requirement:

PMRA DATA CODE	Fresh water algae: 9.8.2
EPA DP Barcode	D332116
OECD Data Point	201
EPA Guideline	850.5400 (123-2)

Test material: Pyroxsulam (provisionally approved, ISO 175, Compendium of Pesticide Common Names, http://www.alanwood.net/pesticides/pyroxsulam.html) or XR-742

Purity (%): 98%

Common name: XDE-742

Chemical name: 3-pyridinesulfonamide,N,-(5,7-dimethoxy[1,2,4]triazolo[1,5-a]pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl).

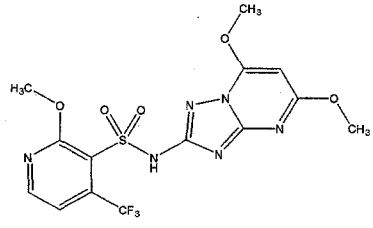
IUPAC: N-(5,7-dimethoxy[1,2,4]triazolo[1,5-a]pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl)pyridine-3-sulfonamide

CAS name: N-(5,7-dimethoxy[1,2,4]triazolo[1,5-a]pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl)-3-pyridinesulfonamide

CAS No.: 422556-08-9

Synonyms: XR-742, X666742

Chemical Structure:



Primary Reviewer: Daryl Murphy D. Murphy 22/05/07 **Date:** 10 May 2007
Australian Government Department of the Environment, Water, Heritage and the Arts (DEWHA)

Secondary Reviewers: Phil Sinclair/Jack Holland 22/2/08 **Date:** 20 May 2007
Australian Government Department of the Environment and Water Resources

Émilie Larivière (#1269) **Date:** 15 June 2007
Environmental Assessment Directorate, PMRA Émilie Larivière 05/03/08

Christopher Salice 4/05/08 **Date:** 6 July, 2007
Environmental Protection Agency, Environmental Fate and Effects Division

Company Code DWE
Active Code JUA
Use Site Category: 13, 14
EPA PC Code 108702

CITATION: Hoberg J. R. 2005: XDE-742 Growth Inhibition Test with Freshwater Bluegreen Alga (*Anabaena flos-aquae*). Springborn Smithers Laboratories, 790 Main Street, Wareham, Massachusetts. Springborn Smithers Study No. 12550.6366, Sponsor Protocol/Project No. 050284. The Dow Chemical Company Midland, Michigan 48674 for Dow AgroSciences, Indianapolis, Indiana 46268. 30 June 2005. Unpublished report.

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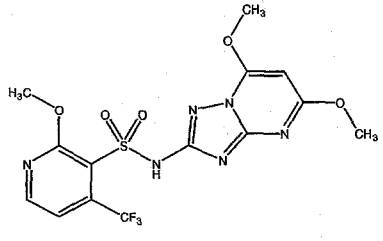
PMRA Submission Number 2006-4727; 1283247 EPA MRID Number 469084-31 APVMA ATS 40362

Data Requirement:	PMRA DATA CODE Fresh water algae: 9.8.2 EPA DP Barcode D332116 OECD Data Point 201 EPA Guideline 850.5400 (123-2)
Test material:	Pyroxsulam (provisionally approved, ISO 175, Compendium of Pesticide Common Names, http://www.alanwood.net/pesticides/pyroxsulam.html) or XR-742
Purity (%):	98%
Common name:	XDE-742
Chemical name:	3-pyridinesulfonamide,N,-(5,7-dimethoxy[1,2,4]triazolo[1,5-a]pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl).
IUPAC:	N-(5,7-dimethoxy[1,2,4]triazolo[1,5-a]pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl)pyridine-3-sulfonamide
CAS name:	N-(5,7-dimethoxy[1,2,4]triazolo[1,5-a]pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl)-3-pyridinesulfonamide
CAS No.:	422556-08-9
Synonyms:	XR-742, X666742

Chemical Structure:



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EXECUTIVE SUMMARY:

The purpose of this study was to determine the effect of pyroxsulam on the growth of the freshwater blue-green alga, *Anabaena flos-aquae*. In this 120 hour acute toxicity study, the cultures of *Anabaena flos-aquae* were exposed to pyroxsulam at concentrations of 0.41, 1.0, 2.6, 6.4, 16, 40 and 100 mg/L (nominal) corresponding to 0.36, 0.89, 2.2, 5.4, 13, 28 and 85 mg pyroxsulam/L (measured) under static conditions.

Concentrations of pyroxsulam were determined at 0 and 120 hours. All mean exposure levels were >80% nominal, except at 40 mg pyroxsulam/L, where a low recovery at 0-hour resulted in a mean measured value of 69% of nominal. A recovery of 83% for the same solution at 120 hours was taken as an indication that this exposure level had been prepared correctly.

Statistical evaluation of the data was performed using mean measured concentrations. Treatment groups were set in triplicate (the medium control group contained six replicates) with each replicate having an initial cell density of approximately 10,000 cells/mL. Temperature during the exposure period was 23°C. The light intensity ranged from 1600 to 3200 lux. The pH values ranged from 5.0 to 7.4 at test initiation (showing the test solution pH was affected by the test substance, with pH inversely proportional to test concentration) and 5.1 to 7.6 at test termination. The pH of the control media increased by 0.1 pH unit from pH 7.4 to pH 7.5.

Anabaena flos-aquae develops aggregates of nested chains of cells which should be broken up when microscope counting or an electronic particle counter is used for determination of biomass. In the study, the solutions were vigorously pipetted multiple times to break up the filaments and achieve a more homogeneous suspension prior to removing a sample for cell counts. However, the effectiveness of this procedure, compared to the recommended sonication, syringing etc, in disrupting the filaments was not demonstrated and there was a high variability noted in cell counts. Additionally, examination of the cell counts reported gives some doubt that logarithmic growth was truly achieved, and results were highly variable between replicates. Consequently, the study is classified as **INVALID.**

The study does not satisfy the guideline requirements for an acute toxicity study with the unicellular green alga, *Anabaena flos-aquae.*

I. MATERIALS AND METHODS

GUIDELINES FOLLOWED:

The toxicity test was performed according to the protocol entitled "Growth Inhibition Test with Freshwater Blue-Green Alga, Anabaena flos-aquae", Springborn Smithers Laboratories Protocol No.: 032405/120-hour Anabaena/Dow. The methods described in this protocol were stated to meet the requirements specified in the:

- US EPA FIFRA Subdivision J Guidelines 122-2 and 123-2. 1982 (Pesticide Assessment Guidelines, Subdivision J. Hazard Evaluation: Nontarget Plants. Report No. EPA 540/9-82-020, PB83-153940. U.S. Environmental Protection Agency, Washington, D.C.),
- OECD Guideline for Testing of Chemicals. Alga, Growth Inhibition Test #201. Adopted 7 June 1984. Organization for Economic Cooperation and Development. Paris, France and
- Official Journal of the European Communities. 1992. Methods for the determination of Ecotoxicity. C.3 Algal Inhibition Test. L383A Volume 35, 29 December 1992.

OECD 201 was originally adopted in 1984 with a revised version adopted in 2006. The study report has been assessed primarily against the 2006 version with its requirements largely met. However, as the study was conducted in 2004, before the changes to OECD 201 test guideline were announced in 2006, deviations from the current OECD Guideline are generally considered acceptable unless otherwise noted.

COMPLIANCE:

The data and report for "XDE-742 - Growth Inhibition Test with Freshwater Blue-Green Alga (*Anabaena flos-aquae*)" were produced and compiled in accordance with all pertinent OECD and U.S. EPA Good Laboratory Practice regulations (40 CFR, Part 160), namely:

- OECD Good Laboratory Practice in the Testing of Chemicals. Paris. France, as revised 1997, and
- U.S. EPA. Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards; Final Rule (40 CFR, Part 160). U.S. Environmental Protection Agency, Washington, DC.

with the following exceptions: routine dilution water contaminant screening analyses for pesticides, PCBs and toxic metals were conducted using standard U.S. EPA procedures by GeoLabs, Inc., Braintree, Massachusetts using standard U.S. EPA procedures and were considered facility records under Springborn Smithers Laboratories' SOP 7.92. Since the analyses were conducted following standard validated methods, these exceptions were stated to have had no impact on the study results.

Signed and dated No Data Confidentiality Claims, Good Laboratory Practice Compliance and Quality Assurance statements were provided in the test report.

A. MATERIALS:

1. Test Material:	XDE-742, i.e. pyroxsulam
Description:	Solid
Lot No./Batch No.:	E0952-52-01/ TSN103826
SSL No.:	02-22 (number assigned for sample received at Springborn Smithers, Snow Camp, North Carolina) and 103-15 (number assigned to aliquot transferred to Springborn Smithers, Wareham, Massachusetts). (This sample of test substance was used to prepare test solutions and quality control samples. Concentrations were adjusted for the purity of the test substance and are presented as active constituent/ingredient)
Purity:	98%
Stability of Compound Under Test Conditions:	**Stable.** Analytical verification of the test material was conducted at 0- and 120 hours. Initial mean recoveries were 82-89% of nominal (excluding one at 55%) and after 102 h were 82-89% for the test solutions.
Storage conditions of test chemicals:	Room temperature in the dark. (The reference substance used in this study was also pyroxsulam (Lot number E0728-78A, TSN 102482, SSL No. 112-18 and of 100% purity). Upon receipt at Springborn Smithers, the reference substance was stored at room temperature in the original container in a dark ventilated cabinet. This

sample of reference substance was used to prepare calibration standards during testing).

Physicochemical properties of pyroxsulam.

The physicochemical properties shown in Table 1 are taken from the Study Profile Template (Mercer, 2006) which noted that the UV data were unavailable at the time of publication of the Study Profile Template.

Table 1. Summary of physicochemical properties of pyroxsulam.

Parameter	Values	Comments
Water solubility at 20°C		
pH 4	0.0164 g/L	Turner (2004a)
pH 6	0.0626 g/L	Turner (2004a)
pH 7	3.2 g/L	Turner (2004a)
Vapour pressure	<1E-7	Madsen (2003)
UV absorption	Not available	
pKa	4.670	Cathie (2004)
Kow		
pH 4	12.1 (log Pow = 1.08)	Turner (2004b)
pH 7	0.097 (log Pow = -1.01)	Turner (2004b)
pH 9	0.024 (log Pow = -1.60)	Turner (2004b)

Note: The Kow values shown in the study profile template were misordered. The correct values (confirmed by examination of Turner (2004b) in Madsen (2006)) are shown above in the physicochemical properties of pyroxsulam table.

2. Test organism:

Name: Freshwater blue-Green Algae,
Species: *Anabaena flos-aquae*
Strain: LB2557
Class: Cyanophyceae
Source: University of Texas, Austin, Texas
(Algae maintained in stock culture at Springborn Smithers)
Age of inoculum: 2-5 Days
Method of cultivation: Algal assay procedure (AAP) medium prepared with sterile deionised water. The inoculum used to initiate the toxicity test with XDE-742 was taken from a stock culture that had been transferred to fresh medium two to five days before testing.

B. STUDY DESIGN:

1. Experimental Conditions

a) Range-finding Study:

A preliminary range-finding exposure was conducted at nominal concentrations of 0 (control), 0.0010, 0.010, 0.10, 1.0, 10 and 100 mg/L of pyroxsulam and solvent (solvent not identified) control. Following 120 hours of exposure, cell densities in the 0.0010, 0.010, 0.10, 1.0, 10 and 100 mg/L treatment levels averaged 46, 28, 50, 13, 0 and 0 x 10^4 cells/mL, respectively. The control and solvent control averaged 19 and 112 x 10^4 cells/mL, respectively. The cell density observed in one replicate of the solvent control at test termination was unusually high relative to the remaining control replicates. Excluding this replicate, the mean pooled control cell density was 29 x 10^4 cells/mL.

b. Definitive Study

Based on the range finding data, concentrations of 0.12, 0.31, 0.77, 1.9, 4.8 and 12 mg/L of pyroxsulam were selected for the definitive exposure. However, due to higher than expected analytical recoveries in the two lowest concentrations (i.e. 148 and 265% of nominal) and the lack of a well-defined dose response, the first definitive study was terminated.

A second definitive study (described below) was then started using nominal concentrations of 0.41, 1.0, 2.6, 6.4, 16, 40 and 100 mg/L of pyroxsulam (concentrations were adjusted for the purity of the test substance).

The definitive study was conducted under static exposure conditions with test vessels (250 mL Erlenmeyer flasks, six replicate vessels for controls (A to F) and three replicate vessels per exposure concentration (A, B and C)) containing Algal Assay Procedure Medium (AAP) dosed at nominal (target) pyroxsulam concentrations of 0 (control, AAP medium), 0.41, 1.0, 2.6, 6.4, 16, 40 and 100 mg pyroxsulam/L.

In order to estimate the impact that the presence of algal biomass had on the test substance concentration, an additional replicate flask (D) of the 6.4 mg pyroxsulam/L (nominal) treatment level was prepared. This flask, which was not inoculated with algae, was analysed at 120 hours of exposure for pyroxsulam concentration. The results of this analysis were compared with the results for the 6.4 mg pyroxsulam/L solution containing algae.

The definitive test was conducted from 27 April to 2 May 2005 when replicate test vessels were inoculated with approximately 10,000 cells/mL. Inoculations were made approximately two hours after preparation of the test solutions and addition to the exposure flasks (100 mL/flask). The exposure phase was carried out aseptically under static conditions for five days (120 hours) in an environmental chamber at 24 ± 2°C with continuous light and constant shaking. Algal cell densities were determined at 24, 48, 72, 96 and 120 hours (test termination) using a haemocytometer and a compound microscope. Observations of the health of the algal cells were made at each 24-hour interval. Following each observation interval, the test flasks were assigned new random positions based on computer-generated random numbers. Since *Anabaena flos-aquae* grows in filaments, the solutions were vigorously pipetted multiple times to break up the filaments and achieve a more homogeneous suspension prior to removing a sample for cell counts at the 24-, 48-, 72-, 96- and 120-hour intervals. Analyses of test solutions to determine pyroxsulam concentrations were conducted at test initiation and test termination (120 hours).

The algal endpoints analysed were growth rate (day^{-1}), cell density (algal cell counts/mL), and cell biomass (area under the growth curve, expressed in the study report as = cell count X 10^4 cells X days/mL or as cell count X 10^4 cells//mL).

In the following two tables' Criteria columns (and elsewhere as relevant) entries in italics are those given in the PMRA's Draft Evaluation Report template for acute toxicity to algae. In its examination of the initial drafts of the aquatic invertebrate DERs, the PMRA advised (email of 3/07/2007) that the criteria in the templates were

understood to have come from old US guidelines and that failure to comply with these template requirements would not be a deficiency. Provided the equivalent and more recent OPPTS and/or OECD guideline requirements are met, this is agreed with.

Table 2. Experimental parameters

Parameter	Details	Remarks *Criteria*
Acclimation period:	Stock culture transferred to fresh medium 2-5 days prior to test initiation to provide the test inoculum.	Acclimitisation requirement considered met. OECD 201 states that an inoculum culture in the test medium is prepared 2-4 days before start of the test wi the inoculum culture incubated under the same conditions as the test cultures. US EPA OPPTS 850.5400 states that the test begins when algae (inocul from 3– to 7–day–old stock cultures are placed in the test chambers containing test solutions having the appropriate concentrations of the test substance. This guideline also states that toxicity testing should not be performed until algal cultures are shown to be actively growing (i.e. capable of logarithmic growth within the test period) in at least two subcultures lasting 7 days each prior to the start of the definitive test. For this DER, priority has been assigned to the first US EPA guideline statement (i.e. the stock culture can be 3 to 7 days old). *EPA recommends two week acclimation period.* *OECD recommends an amount of algae suitable for th 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 days. When the algal cultures contain deformed or abnormal cells, they must be discarded.*
Culturing media and conditions: (same as test or not)	1xAAP, same as test The stock cultures were maintained within the following conditions: a shaking rate of 100 ± 10 rpm, a temperature of 24 ± 2°C and continuous illumination at the surface of the medium with an intensity range of 1600 to 3200 lux. Lighting was supplied by fluorescent bulbs. Culture flasks were agitated continuously on an orbital shaker. Temperature was controlled using an environmental chamber. Comparison of the AAP medium's formulation	Requirement considered met. The test was conducted in an environmental chamber under conditions equivalent to those used in the stock culture.

	given in the study report shows it to be equivalent to the AAP formulation reported in OECD 201 except for the study having used sodium selenate at 1.88 µg/L. This was noted as a required additional nutrient. OECD 201 does not identify this chemical as a constituent of either the AAP or OECD TG 201 medium except in the case of diatom species where it is allowed at 0.01 µg/L.	
Health: (any mortality observed)	Healthy (assumed on basis of satisfactory growth in the controls in the test and the observation that at test termination (120 hours), cells exposed to the treatment levels tested and the control were observed to be normal).	Requirement considered met.
Test system Static/static renewal Renewal rate for static renewal	Static N/A (not applicable)	Requirement considered met. OECD 201 does not refer to static or static renewal but can be interpreted as referring to them as no mention is made of renewal of test solutions. US EPA OPPTS 850.5400 defines a static system as o in which old nutrient medium is not renewed or replaced. It does not refer to renewal intervals. *EPA expects the test concentrations to be renewed eve 3 to 4 days (one renewal for the 7 day test, 3-4 renewa for the 14 day test).*
Incubation facility	Environmental chamber	Requirement considered met. OECD 201 refers to use of a cabinet or chamber as recommended, in which the chosen incubation temperature can be maintained at ± 2°C. US EPA OPPTS 850.5400 refers to use of a growth chamber or controlled environment room that can hold the test containers and maintain the necessary growth parameters (e.g. temperature, lighting).
Duration of the test	120-hours	See deviations/deficiencies table, page 33 of this DER with respect to the duration of the test. OECD 201 notes that test duration is normally 72 hou but that shorter or longer test durations may be used provided that all validity criteria in the guideline can b met. US EPA OPPTS 850.5400 refers to counting of algal

		cells at 24, 48, 72 and 96 hours. No reference to a 12(hour exposure was identified. *EPA requires: 96-120 hours* *OECD: 72 hours*
st vessel terial: ss/stainless) : volume:	Glass (sterilized Erlenmeyer flasks) 250 mL 100 mL	Requirement considered met. OECD 201 states the test vessels will normally be glass flasks of dimensions that allow a sufficient volume of culture for measurements during the test and a sufficient mass transfer of carbon dioxide from the atmosphere and that the liquid volume must be sufficient for analytical determinations. USEPA OPPTS 850.5400 states that Erlenmeyer flasks should be used for test containers. The flasks may be of any volume between 125 and 500 mL as long as the same size is used throughout a test and the test solution volume does not exceed 50 percent of the flask volume. The guideline also requires all glassware to be cleaned and sterilised before use. *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	AAP medium, modified by addition of sodium selenate at 1.88 µg/L.	See deviations/deficiencies table, page 33 of this DER with respect to use of sodium selenate. OECD 201 refers to AAP medium and provides a comparison (Annex 3) of the US EPA AAP medium and the OECD 201 medium. The guideline identifies both as suitable growth media. OECD 201 states that sodium selenate is to be used only in medium for stock cultures of diatom species. US EPA OPPTS 850.5400 does not specifically refer to media composition, instead referring to other sources for this information. *EPA recommends 20X-AAP medium*

<table>
<tr>
<td>pH at test initiation:</td>
<td>Control medium pH was 7.4 (the AAP medium was adjusted to a pH of 7.6 ± 0.1 after its preparation).

Pyroxsulam containing solutions had pH values of 5.0 to 7.0 (see below) with the pH becoming more acidic as the pyroxsulam concentration increased.</td>
<td>pH requirements considered met.

The pH of the higher test concentrations (40 and 100 mg/L) was effected by the test substance.

OECD 201 states the pH of the control medium should not increase by more than 1.5 units during the test.

US EPA OPPTS 850.5400 states that, for Anabaena, the pH of the nutrient medium should be 7.5 (±0.1) at the start of the test. This guideline goes on to note that if the test chemical is highly acidic and reduces the pH of the test solution below 5.0 at the first measurement, appropriate adjustments to pH should be considered, and the test solution measured for pH on each day of the test. Such action was not taken in the study, presumably because the pH did not fall below 5 in the control test concentrations although the pH did reach 5.0 in the 100 mg pyroxsulam/L test concentration. The relatively low initial pH is considered consistent with the guidelines.</td>
</tr>
<tr>
<td>pH at test termination:</td>
<td>The pH values at times 0 and 120 hours were:

<table>
<tr><th rowspan="2">Concentration*</th><th colspan="2">pH</th></tr>
<tr><th>0 h</th><th>120 h</th></tr>
<tr><td>Control</td><td>7.4</td><td>7.5</td></tr>
<tr><td>0.41</td><td>7.0</td><td>7.6</td></tr>
<tr><td>1.0</td><td>7.0</td><td>7.5</td></tr>
<tr><td>2.6</td><td>7.0</td><td>7.4</td></tr>
<tr><td>6.4</td><td>7.0</td><td>7.4</td></tr>
<tr><td>16</td><td>6.8</td><td>7.3</td></tr>
<tr><td>40</td><td>6.3</td><td>7.0</td></tr>
<tr><td>100</td><td>5.0</td><td>5.1</td></tr>
</table>
* Nominal concentrations, mg pyroxsulam/L.

The effect of the pyroxsulam at concentrations of ≥ 16 mg/L on the initial pH is clearly seen in the above table. There was a pH increase in all concentrations over time except at 100 mg pyroxsulam/L where the absence of an increase is attributed to the inhibition of algal growth over the 120 hours (100% inhibition at 96 and 120 h).</td>
<td>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).</td>
</tr>
<tr>
<td>Chelator used:</td>
<td>Yes, Na_2EDTA used in AAP medium</td>
<td>Requirement considered met.

OECD 201 identifies the presence of the chelator, disodium ethylene-diaminetetraacetic acid in both the AAP and OECD growth media.</td>
</tr>
</table>

		US EPA OPPTS 850.5400 states that chelating agents are included in the nutrient medium for optimum cell growth. No chelating agents are to be included in the nutrient medium used for test solution preparation if it is suspected that the chelating agent will interact with the test chemical. No data or comments have been identified in the test report to suggest that such an interaction might occur.
Carbon source:	Not specifically reported. Acceptable growth in the controls indicates a suitable carbon source was available, presumably via ambient carbon dioxide.	Requirement considered met. OECD 201 and US EPA OPPTS 850.5400 do not make specific reference to a carbon source.
Salinity (for marine algae):	N/A	Parameter is not relevant for freshwater algae.
If non-standard nutrient medium was used, detailed composition provided (Yes/No)	Yes, a non-standard medium was used with its full composition provided. The medium is considered non-standard because OECD 201 indicates that sodium selenate is only used for the diatom species.	Requirement considered met. (see previous comment under "Details of growth medium name").
lution water urce/type:	The dilution water source used to prepare the test medium was not identified. The water was, however, sterilised and deionised.	Dilution water source/type requirement considered met. OECD 201 includes reference to ISO 8692, ISO/DIS 14442 and ISO 5667/16 water quality standards and states that deionised or distilled water are to be used in preparation of the AAP algal growth media. While the OECD medium recipe refers to "water", there is also a reference to use of deionised water to prepare both the AAP and OECD media. US EPA OPPTS 850.5400 states that water of sufficient quality (e.g. ASTM Type I water) is to be used in the preparation of the nutrient medium. Neither guideline requires identification of the source of the dilution water.

pH:	Not provided but the culture medium prepared with this dilution water had its pH adjusted to pH 7.5 ± 0.1	pH requirements considered met. OECD 201 indicates the pH of the US EPA AAP medium is 7.5 and that of the OECD medium, 8.1. US EPA OPPTS 850.5400 states that the pH of nutrient medium in which algae are subcultured is to be 7.5 (± 0.1) for *Anabaena*. *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.*
Salinity (for marine algae):	N/A	Parameter is not relevant for freshwater algae.
Water pretreatment (if any):	The dilution water used to prepare the AAP medium was deionised and sterilized.	Requirement considered met. The use of sterilization and deionization processes is considered in accord with the OECD and US EPA guidelines' intentions.
Total Organic Carbon:	A representative sample of AAP medium was analysed monthly for total organic carbon (TOC) concentration. In April 2005, the TOC was 0.53 mg/L.	Requirements for TOC, particulate matter, metals, pesticides and chlorine are considered met, primarily on the basis of the successful growth of the control algae, the maintenance of the stock culture and the information that some analyses are conducted periodically.

Particulate matter:	Not reported	
Metals: Pesticides:	The study report noted that representative samples of the dilution water source used in the preparation of the culture medium were analysed periodically for the presence of pesticides, PCBs and toxic metals by GeoLabs, Inc., Braintree, Massachusetts. None of these compounds have been detected at concentrations that are considered toxic in any of the water samples analysed in agreement with ASTM guidelines (2002).	
Chlorine:	Not reported	
Indicate how the test material is added to the medium (added directly or used stock solution)	Stock solutions were prepared in AAP medium. The highest concentration was prepared first and then serially diluted to obtain stock solutions for all other treatment levels. The primary stock solution was made by placing 0.2042 g of pyroxsulam (0.2001 g as active ingredient) in a 2000-mL volumetric flask and bringing it to volume with AAP medium. Concentrations were adjusted for the purity of the test substance. It is noted that while the preliminary range-finding study used an unidentified solvent, this definitive study did not do so. Given that there was difficulty in getting all the pyroxsulam dissolved (see adjacent column), this is a little surprising.	Requirement considered met. The 100 mg pyroxsulam/L primary stock solution was observed to be cloudy and white in colour with a large amount of visible undissolved test substance. The primary stock solution was stirred and sonicated for approximately three hours, and then stirred overnight. On the following morning, the solution was observed to be clear and colourless with a small amount of visible undissolved material. The soluble portion of this solution was siphoned from the volumetric flask, and was used to prepare the nominal test concentrations. All test solutions were clear and colourless with no visible undissolved test substance. Untreated algal medium was used to prepare the control.
Aeration or agitation	Agitation orbital shaker at 100 rpm	Requirement considered met. Both OECD 201 and US EPA OPPTS 850.5400 refer to the use of shaking. OECD 201 referring to an orbital or reciprocate shaker being used at about 150 revolutions/minute. This guideline also notes that, alternatively, intermittent agitation may be used to reduce the tendency of *Anabaena* to form clumps. The US EPA guideline refers to shaking test containers on a rotary shaking apparatus at 100 rpm.

Initial cells density	10,000 cells/mL (for each replicate)	Requirement considered met. OECD 201 recommends an initial cell count of 10^4 cells of *A. flos-aquae*/mL while US EPA OPPTS 850.5400 refers to test chambers containing approximately 1 X 10^4 *S. A. flos-aquae* cells. *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.*
Number of replicates Control: Solvent control: Treatments:	6, inoculated with algae. N/A 3, inoculated with algae. In order to estimate the impact that the presence of algal biomass had on the test substance concentration, an additional replicate flask of the 6.4 mg pyroxsulam/L (nominal) treatment level was prepared. This flask, which was not inoculated with algae, was analysed at 120 hours of exposure for pyroxsulam concentration.	Requirement considered met. OECD 201 states that the test design should include three replicates at each test concentration and that the number of control replicates must be at least three, and ideally should be twice the number of replicates used for each test concentration. US EPA OPPTS 850.5400 states that a minimum of three replicates is required for each test concentration. A solvent control was not used. *EPA requires a negative and/or solvent control with 3 or more replicates per doses.* *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:	0 (negative control), 0.41, 1.0, 2.6, 6.4, 16, 40 and 100 mg pyroxsulam/L (As noted above, an	See deviations/deficiencies table, page 33 of this DER with respect to the ratio of the test concentrations.

Measured:

additional 6.4 mg pyroxsulam/L treatment level was prepared but not inoculated with algae).

The nominal test concentrations were in ratios of 2.4 to 2.6.

The nominal, 0 and 120 hour and mean (0 and 120 hour) concentrations of pyroxsulam that were determined are shown in the following table.

Nominal and mean measured concentrations as mg pyroxsulam/L.

Nominal	0 h	120 h	Mean
Control	<0.014	0.095[a]	NA[c]
0.41	0.36	0.36	0.36
1.0	0.89	0.89	0.89
2.6	2.2	2.2	2.2
6.4	5.2	5.5/ 5.6[b]	5.4
16	13	13	13
40	22	33	28
100	85	84	85

a. At 120 hours (and at resample event the following day), a peak in the control was observed at the same retention time for pyroxsulam. However, the study author considered it unlikely that the exposure solution contained pyroxsulam since the concentration of test substance was less than detectable limits at 0 hour. The analytical sample may have been contaminated during sample processing, or this is an artefact with a similar retention time. The reviewer does not disagree with such interpretation.
b. Result of the additional sample without algae present to determine biological uptake/degradation.
c. NA = not applicable.

As a percent of the nominal concentrations, the mean measured concentrations were:

Nominal	Mean	% of Nominal
Control	Not applicable	Not applicable
0.41	0.36	**88**
1.0	0.89	**89**
2.6	2.2	**86***
6.4	5.4	**84**
16	13	**83***
40	28	**69***
100	85	**85**

Note: Mean measured concentrations and percent of nominal were calculated using actual analytical data and not the rounded (2 significant figures) data presented in this table. Consequently, values marked with an asterisk differ slightly from those calculated from the values in the table.

OECD 201 states that for the final definitive test at least five concentrations, arranged in a geometric series with a factor not exceeding 3.2 [the factor used ranged from 2.4 to 2.6], should be selected. For test substances showing a flat concentration response curve a higher factor may be justified.

US EPA OPPTS 850.5400 states that algae should be exposed to five or more concentrations of the test chemical in a geometric series in which the ratio is between 1.5 and
2.0 (e.g. 2, 4, 8, 16, 32, and 64 mg/L).

For the treatment blank (nominally 6.4 mg pyroxsulam/L), the initial measured concentration was 5.2 mg pyroxsulam/L and after 120 h the concentration was 5.6 mg pyroxsulam/L, which was similar to inoculated treatment.

		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; a solvent was not used in the definitive test.	The parameter is not relevant as a solvent was not used. OECD 201 and US EPA OPPTS 850.5400 allow, but do not require, the use of solvents.
Method and interval of analytical verification	Test solutions were analyzed for the presence of pyroxsulam technical at 0 and 120 hours using HPLC. All exposure solution and QC samples were analysed for pyroxsulam by high performance liquid chromatography using ultraviolet detection (HPLC/UV at 254 nm) based on methodology validated at Springborn Smithers. The method validation study established an average recovery of 100 ± 1.85% (for fortified concentrations of 0.05, 2.0 and 35.0 mg pyroxsulam/L with a range of 97.9 to 103% of the fortified concentration) from Algal Assay Procedure (AAP) medium. The acceptable range for evaluating QC sample recovery was set at 70.0% to 120%. The analytical method's limit of quantitation was set at 0.0155 mg pyroxsulam/L.	Requirement considered met. The conditions and procedures were the same as those described for the method validation. Representative chromatograms from the analysis of a calibration standard, recovery sample and a control sample were presented with the first two clearly identifying a peak attributed to pyroxsulam and with the control chromatogram not having the equivalent peak present. Linear regression analysis of detector response versus concentration was shown to be linear over the concentration range of 0.00 to 3.00 mg pyroxsulam/L (r^2 = 0.99998).

Test conditions:		
Temperature:	Minimum/maximum 23/23°C	Temperature requirements considered met. OECD 201 states that these cultures should be maintained at a temperature in the range of 21 to 24°C, controlled at ± 2°C. US EPA OPPTS 850.5400 states that the test temperature is to be 24°C for *Anabaena*, with excursions from the test temperature should be no greater than ± 2°C. *OECD recommended the temperature in the range of 21 to25°C maintained at ± 2°C* *EPA temperature: Skeletonema: 20°C, Others: 24-25°C;*
Photoperiod:	Continuous illumination.	Requirement considered met. OECD 201 refers to the cultures being allowed unrestricted exponential growth under nutrient sufficient conditions and continuous light. US EPA OPPTS 850.5400 refers to test chambers containing *Anabaena* having to be illuminated continuously. *OECD recommended continuous uniform illumination* *EPA photoperiod: S. costatum 14 hr light/ 10 hr dark, Others: Continuous.*
Light intensity and quality	1600-3200 lux with the lighting supplied by fluorescent bulbs. Measured lux values over the 120 hours were:	Parameter considered met. OECD 201 states that some species, in particular *Anabaena flos-aquae*, grow well at lower light intensities (i.e. compared to the value of 60-120 $\mu E \cdot m^{-2} s^{-1}$ referred to in the guideline) and may be damaged at high intensities. For such species an average light intensity in

	Time (hours) / Light intensity (lux) **0** 1700-2500 **24** 1600-3200 **48** 1700-2600 **72** 1700-2500 **96** 1600-3200 **120** 1600-3200 Test flasks were randomly placed on the shaking table at test initiation based upon computer generated random numbers. Following each observation interval, the test flasks were assigned new random positions based on computer-generated random numbers. The photosynthetically active radiation (PAR) of the test area measured at test initiation ranged from 25-33 $\mu Em^2/s$. The expression Photosynthetically Active Radiation, often abbreviated PAR, designates the spectral range of solar light from 400 to 700 nanometers that is useful to terrestrial plants in the process of photosynthesis.	the range 40-60 $\mu E \cdot m^{-2} \cdot s^{-1}$ should be selected. The guideline notes that this range corresponds approximately to 2960-4440 lux (60-120 $\mu E \cdot m^{-2}$ s^{-1} was identified as corresponding to approximately 4440-8880 lux). US EPA OPPTS 850.5400 states fluorescent lights providing 2200 lux for *Anabaena* are to be used. *OECD recommended continuous uniform illumination provided at approximately 8000 Lux measured with a spherical collector.* *EPA light: Anabaena: 2.0 Klux (±15%), Others: 4 - 5 Klux (±15%)* PAR is defined in terms of photon (quantum) flux, specifically, the number of moles of photons in the radiant energy between 400 nm and 700 nm. One mole of photons is 6.0222 x 10^{23} photons (6.0222 x 1023 is Avogadro's Number). The *Photosynthetic Photon Flux Density* (PPFD), i.e., the photon irradiance, is expressed in moles per square meter and per second (formerly, Einsteins per square meter and per second). (www.sylvania.com/content/display.scfx?id=003680197)
Reference chemical (if used) name: concentrations:	 N/A N/A	Requirement considered met. A reference chemical was not used. OECD 201 notes that a reference substance may be tested as a means of checking test procedures and that this should be done at least twice a year. US EPA OPPTS 850.5400 also states that positive controls using zinc chloride as a reference chemical should also be run periodically. The study report could profitably have presented the most recent results from reference chemicals test against algae in their laboratory.

Other parameters, if any	Conductivity measured at test initiation and termination in the treatment and control solutions ranged from 80 to 90 µmhos/cm.	Requirement considered met. The observation of cell normality would not apply to the 85 mg pyroxsulam/L nominal replicates as a 0 cell density was reported at 120 hours.

2. Observations:

Table 3. Observation parameters

Parameters	Details	Remarks Criteria
Parameters measured including the growth inhibition/other toxicity symptoms	Cell density (cells/mL), biomass and growth rate. Morphological observations were conducted at study termination. pH, temperature, light intensity, conductivity and concentrations of pyroxsulam in the test solutions were also determined over the course of the study.	The parameters determined are acceptable. OECD 201 refers to growth and growth inhibition being quantified from measurements of the algal biomass as a function of time. US EPA OPPTS 850.5400 refers to enumeration of the algal cells to determine inhibition or stimulation of growth and the pattern of growth in test containers compared to controls. *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	Cell density calculated using a haemocytometer and microscope (with counter chamber (Mercer, 2006)). Growth rate was determined by comparing the change in cell density from day 0 to day 3. The cumulative biomass was determined by plotting the daily cell density and determining the area under the curve.	Meets Guidelines criteria. OECD 201 refers to cell counts being made using an electronic particle counter, a microscope with counting chamber, or a flow cytometer. Other biomass surrogates can be measured using a flow cytometer, fluorimeter, spectrophotometer or colorimeter. US EPA OPPTS 850.5400 refers to the algal growth response being determined by an indirect (spectrophotometry, electronic cell counters, dry weight, etc.) or a direct (actual microscopic cell count of at least 400 cells per flask) method. *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	24, 48, 72, 96 and 120 hours.	Requirement considered met. OECD 201 refers to algal biomass being determined at least daily. US EPA OPPTS 850.5400 states that at the end of 96 h, and, if possible, at the end of 24, 48, and 72 h, the algal growth response (number or weight of algal cells per millilitre) in all test containers and controls is to be determined. *EPA and OECD: every 24 hours.*
Other observations, if any	*Anabaena flos-aquae* was identified as growing in filaments which required vigorous pipetting multiple times to break-up the filaments to achieve a more homogeneous	See deviations/deficiencies table, page 33 of this DER with respect to methodology used to break up algal chains. OECD 201 refers to *Anabaena flos-*

	suspension before removing a sample for cell counts at 24, 48, 72, 96 and 120 hours. Observation of cells exposed to the treatment levels and controls at test termination were reported as showing the cells were normal.	*aquae* developing aggregates of nested chains of cells whose size may vary with culturing conditions. The guideline states it may be necessary to break up these aggregates when microscope counting or an electronic particle counter is used for determination of biomass. The guideline notes that sonication of sub-samples may be used to break up chains to reduce count variability. US EPA OPPTS 850.5400 similarly states that a particle counter or microscopic counting cannot be used for *Anabaena* unless the filaments are broken up and dispersed using a syringe, ultrasonic bath, or blender.
Indicate whether there was exponential growth in the control	Yes according to OECD 201 requirements, but see also page 28 of this DER where other reasons for believing consistent exponential growth did not occur in the controls are discussed. Cell density in the control increased by a factor of 48.5 by test termination (120 h) and 25.0 at 72 h, with a mean 0-72 h growth rate of 1.08 day^{-1}. Algal growth in the controls at 96 hours ranged from 4.75 to 36.13 X 10^4 cells/mL with an average value of 20.98 X 10^4 cells/mL; i.e. an average factor of ~21. A plotting of mean control cell counts against time using the Microsoft Excel Chart Wizard function and fitting the data points to an exponential curve (data and curve shown on page 45 of this DER) returned an r^2 value of 0.7809, a value taken as indicative of some deviation	OECD 201 states that the biomass of the controls must have increased exponentially by a factor of at least 16 within the 72-hour test period. This corresponds to a specific growth rate of 0.92 day^{-1}. For *Anabaena flos-aquae*, OECD 201 states that the growth rate most frequently observed in OECD medium at a light intensity of 70 $\mu E/m^2/sec$ at 21°C is 1.1-1.4 day^{-1}. US EPA OPPTS 850.5400 states that algal growth in controls should reach the logarithmic growth phase by 96 h (at which time the number of algal cells should be approximately 1.5 X 10^6/mL for *Skeletonema* or 3.5 X 10^6/mL for *Selenastrum* [no value given for *A. flos-aquae*]. If logarithmic growth cannot be demonstrated, the test is to be repeated. *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.*

	from an exponential growth curve.	
Water quality was acceptable? (Yes/No)	Yes	Parameter considered met on basis of successful growth of the controls and details provided on the medium's preparation.
Were raw data included?	As laboratory notes, no. However, the tabulated data presented was made up of individual replicate values which could be used to verify the study report's results. The study report notes that all original raw data, the protocol and the original final report produced during this study are stored at Toxicology and Environmental Research and	Parameter considered met. While raw data were not submitted, the tabulated results presented were sufficient to allow statistical analysis by the reviewer.. While US EPA OPPTS 850.5400 states that the sponsor must submit to the EPA all data developed by the test including those that are suggestive or predictive of acute phytotoxicity, advice from the US EPA was that,

	Consulting of The Dow Chemical Company, Midland, Michigan.	because the tabulated results presented in the study report were sufficient to allow statistical analysis, the guideline would be considered met.

II. RESULTS and DISCUSSION:

A. INHIBITORY EFFECTS:

Cell density

At test termination (120 h), cell density percent reductions were 3, 39, 40, 42, 53, 81 and 100%, when compared to the negative control, at the mean-measured 0.36, 0.89, 2.2, 5.4, 13, 28 and 85 mg pyroxsulam/L treatment levels, respectively. Biomass was determined as -47, -37, -49, 10, -33, 70 and 114 (negative figures indication stimulation of growth) percent inhibition compared to the controls after 72 hours and mean growth rate was reduced 8, 8, -2, 17, 5, 37 and 94%, at the mean-measured 0.36, 0.89, 2.2, 5.4, 13, 28 and 85 mg pyroxsulam/L treatments, respectively compared to the control mean. The 72 h EC_{50} values for cell density, biomass and growth rate were 23 (4.9-30), 22 (8.9-28) and 41 (25-51) mg pyroxsulam/L, respectively with the 95% confidence limits shown in brackets. The NOEC was 13 mg pyroxsulam/L for cell density, biomass and growth rate.

The analytical result of the 120-hour sample from the 6.4 mg/L nominal treatment level (mean measured concentration was 5.2 mg pyroxsulam/L) without algae present was 5.6 mg pyroxsulam/L. The equivalent test solution with algae present was 5.5 mg pyroxsulam/L, indicating that the presence of algae in the test solution had no effect on the concentrations of pyroxsulam present in solution.

The effects of pyroxsulam on the growth of the blue green alga, *Anabaena flos-aquae*, under the test conditions are shown in Table 4 by the cell density counts at 24, 48, 72 and 96 hours and the % inhibition after 96 hours.

At 24 hours, five of the control replicates had mean counts of 0.00 X 10^4 cells/mL while the sixth had a count of 1.63 X 10^4 cells/mL which gave the average result of 0.27 X 10^4 cells/mL. A similar result was reported for the nominal 13 mg pyroxsulam/L replicates (two replicates with zero counts, one with 2.50 X 10^4 cells/mL for a mean of 0.83 X 10^4 cells/mL. At nominal concentrations of 0.36, 0.89, 5.4, 28 and 85 mg pyroxsulam/L, all replicates had counts of 0.00 X 10^4 cells/mL. In the 2.2 mg pyroxsulam/L replicates, the individual cell counts were 0.13, 10.25 and 3.63 X 10^4 cells/mL with a mean of 4.67 X 10^4 cells/mL. In cases where zero cell counts were reported, the study report stated cells had been observed in the replicate flasks before pipetting.

The low cell counts after 24 hours indicate a lag phase had occurred in some of the pyroxsulam exposed algae.

Table 4. Effect of Pyroxsulam Technical on algal growth, Blue-Green Algae (*Anabaena flos-aquae*). Mean cell densities are shown with standard deviations in brackets.

Treatment Measured concentration mg pyroxsulam/L and (nominal).	Approximate initial cell Density (cells/mL)	Mean Cell density (x10^4 cells/mL) at					
		24 h	48 h	72 h	96 h	120 h	
						Cell density	% inhibition
Control	1 x10^4	0.27 (0.66)	7.02 (5.66)	25.00 (11.18)	20.98 (11.70)	48.52 (24.14)	N/A
0.36 (0.41)	1 x10^4	0.00 (0.00)	17.17 (2.31)	18.13 (3.68)	23.67 (19.82)	47.21 (34.41)	3
0.89 (1.0)	1 x10^4	0.00 (0.00)	15.5 (11.32)	18.29 (6.66)	15.67 (6.26)	29.58 (6.01)	39
2.2 (2.6)	1 x10^4	4.67 (5.14)	9.08 (2.27)	24.63 (4.98)	19.42 (9.00)	29.00 (13.15)	40
5.4 (6.4)	1 x10^4	0.00 (0.00)	8.08 (7.01)	17.71 (12.24)	5.75 (7.33)	28.25 (26.29)	42
13 (16)	1 x10^4	0.83 (1.44)	9.75 (8.77)	26.17 (17.80)	10.33 (3.14)	22.75 (16.87)	53
28 (40)	1 x10^4	0.00 (0.00)	3.17 (3.88)	8.13 (4.20)	6.79 (3.98)	9.21* (9.77)	81
85 (100)	1 x10^4	0.00 (0.00)	0.04 (0.07)	0.58 (1.01)	0.00 (0.00)	0.00* (0.00)	100
Reference chemical (if used)	N/A						

Results marked with an asterisk, *, are statistically significantly different from the pooled control (Williams Test).

The cell counts at 24 hours are noticeable for their indication of a lack of strong growth in the control and, with the exception of the 2.2 mg/L results, all the pyroxsulam containing replicates had cell counts below the initial cell density of 1 X 10^4 cells/mL. The drop in cell density in most cases at 96 hours compared to 72 hours and the recovery by 120 hours is also noticeable.

The algal growth curves (cell density against time) for *Anabaena flos-aquae* exposed to pyroxsulam are shown in Figure 1.

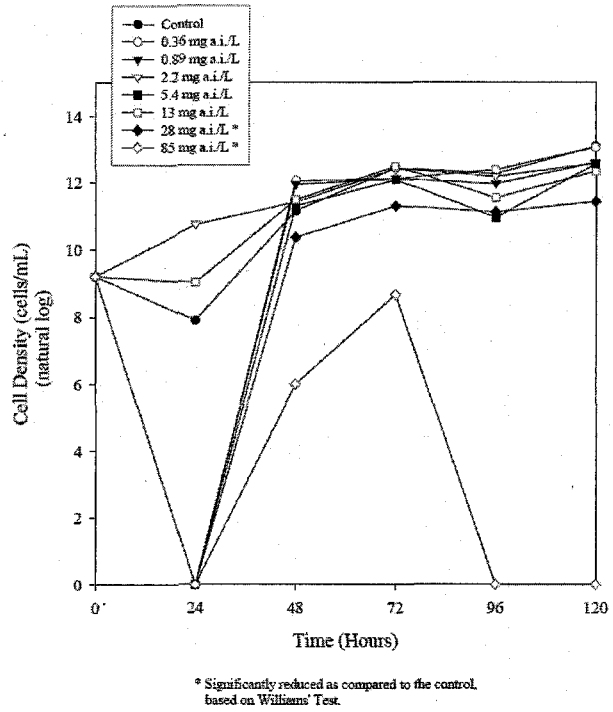

Figure 1. Algal growth curves (cell density against time) for *Anabaena flos-aquae* exposed to pyroxsulam (from Hoberg, 2005).

A sustained exponential growth is not considered to be demonstrated by the data in this figure.

The EC25 and EC50 values for pyroxsulam calculated from cell density results of the 120 hour toxicity test with *Anabaena flos-aquae* were:

Time	Parameter	EC25	EC50
24 hour results	**EC (mg pyroxsulam/L)**	**3.5**	4.9
	95% confidence limits	3.0 – 17	3.8 – 21
48 hour results	**EC (mg pyroxsulam/L)**	13	21
	95% confidence limits	0.89 – 21	3.8 – 32
72 hour results	**EC (mg pyroxsulam/L)**	16	23
	95% confidence limits	2.7 – 21	4.9 – 30
96 hour results	**EC (mg pyroxsulam/L)**	2.6	4.4
	95% confidence limits	0.57 – 4.3	0.89 – 29
120 hour results	**EC (mg pyroxsulam/L)**	0.69	11
	95% confidence limits	0.41 – 14	0.76 – 21

The 120 hour NOEC was 13 mg pyroxsulam/L.

Mean specific growth rates and biomass (area under growth curve)

The mean specific growth rates per day and the mean areas under the growth curves reported following exposure of *Anabaena flos-aquae* to pyroxsulam are shown in Table 5 with respective percent inhibition results also shown.

Table 5. Effect of pyroxsulam on growth of the blue-green algae *Anabaena flos-aquae*. **Standard deviations are shown in brackets.**

Treatment measured and (nominal) concentrations (mg pyroxsulam/L)	Approximate initial cell density	Mean Growth Rate (per day)		Mean Biomass (area under the growth curve, reported as cell count X 10^4 cells/mL)	
		0-72 hours	Percent Inhibition	0-72 hours	Percent Inhibition
Control	1 x10^4	1.08 (0.17)	N/A	16.23 (9.72)	N/A
0.36 (0.41)	1 x10^4	0.99 (0.07)	8	21.87 (3.80)	-47
0.89 (1.0)	1 x10^4	0.99 (0.12)	8	20.44 (12.56)	-37
2.2 (2.6)	1 x10^4	1.10 (0.07)	-2	22.19 (7.56)	-49
5.4 (6.4)	1 x10^4	0.90 (0.33)	17	13.44 (4.29)	10
13 (16)	1 x10^4	1.03 (0.36)	5	19.81 (14.68)	-33
28 (40)	1 x10^4	0.68* (0.21)	37	4.40* (5.45)	70
85 (100)	1 x10^4	0.06* (0.11)	94	-2.04* (0.55)	114

Results marked with an asterisk, *, are statistically significantly different from the pooled control (Dunnett's Test, p≤0.05).

Consistent with the lack of cell growth in the controls and certain of the pyroxsulam concentrations tests (*vide supra* under "Cell density"), five of the control replicates had zero growth rates over the 0-24 hour period, the sixth had a value of 0.45 day^{-1}, to give a control mean of 0.07 day^{-1}. Growth rates of zero over the 0-24 hours period were seen in all the replicates for the 0.36, 0.89, 5.4, 28 and 85 mg pyroxsulam/L (mean measured) concentrations. Replicate values for the 13 mg pyroxsulam/L exposure were 0.0, 0.0 and 0.84 day^{-1} to give a mean of 0.28 day^{-1}. Replicate values for the 2.2 mg pyroxsulam/L exposure were -1.91, 2.24 and 1.18 day^{-1} over the 0-24 hour period, with a mean of 0.47 day^{-1}. Corresponding to the zero cell counts, calculated biomass (area under curve) results gave a biomass result of -0.54 X 10^4 cells/mL.

The 120 and 72 hour EC50 and NOEC results are summarised in Table 6.

Table 6. Statistical endpoint values (mg pyroxsulam/L).

Statistical Endpoint	Cell Density (120 h)	Growth Rate 0-72 h	Biomass 0-72 h
NOEC	13	13	13
EC_{50} (95% C.I.)	11 (0.76-21)	41 (25-51)	22 (8.9-28)

Toxicity values were determined based on mean-measured concentrations.

The average growth rates (day^{-1}) over 0 to 72 hours for *Anabaena flos-aquae* exposed to pyroxsulam are shown in Figure 2.

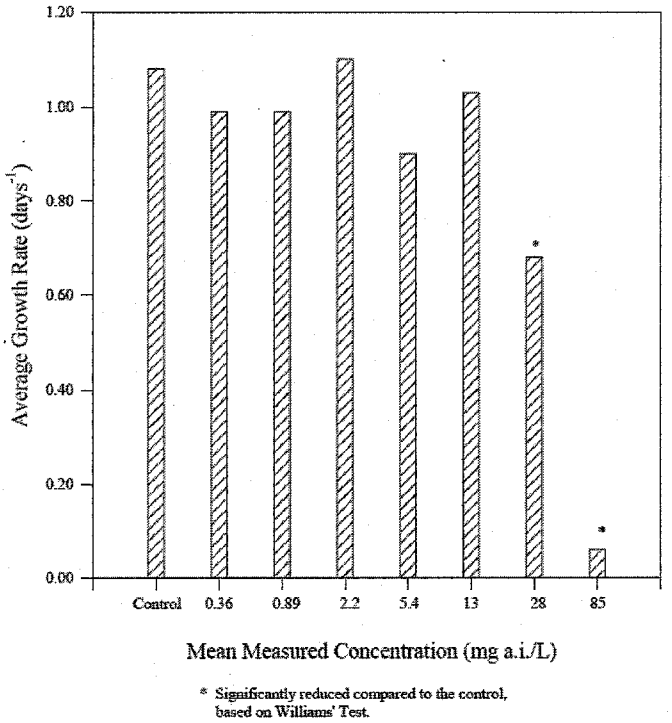

Figure 2. Algal average growth rate (0-72 hours) histograms (average growth rate as days^{-1} against mean measured pyroxsulam concentrations) for *Anabaena flos-aquae* exposed to pyroxsulam (from Hoberg, 2005).

The total biomass (total area under the growth curve 0 to 72 hours) results for *Anabaena flos-aquae* exposed to pyroxsulam are shown in Figure 3.

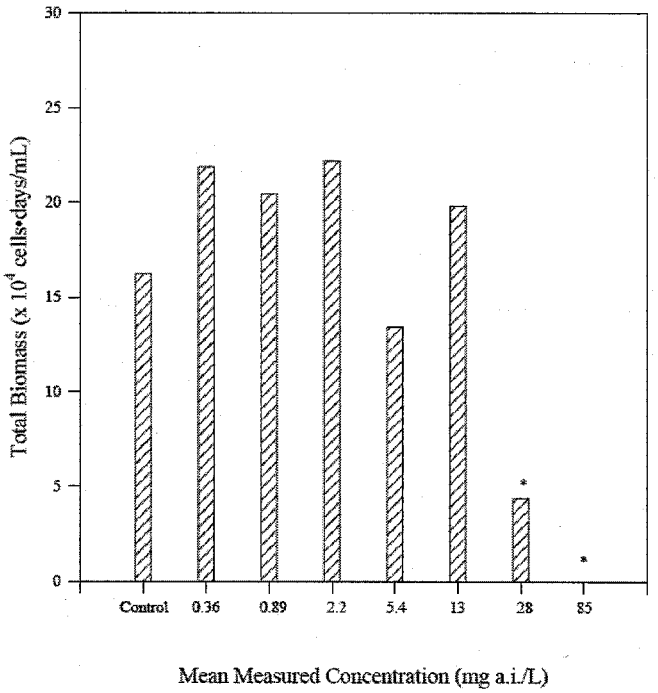

Figure 3. Algal biomass histograms (biomass, as cell count X 10,000 cells/mL against mean measured pyroxsulam concentration) for *Anabaena flos-aquae* exposed to pyroxsulam (from Hoberg, 2005). Results in the figure that are marked with an asterisk were identified by Hoberg as statistically significantly different from the control mean based on William's test.

Validity of test

OECD 201 requires that, for the test to be valid, the following performance criteria should be met:

- the biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72-hour test period;
- the mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2 and 2-3, for 72-hour tests) in the control cultures (See Annex 1 under "coefficient of variation") must not exceed 35%; and
- the coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7% in tests with *Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus*. For other less frequently tested species, the value should not exceed 10%.

In contrast, OECD 201 (1984), the guideline version the study followed, does requires only that the cell concentration in the control cultures should have increased by a factor of at least 16 within three days.

US EPA OPPTS 850.5400 states that algal growth in controls should reach the logarithmic growth phase by 96 h (at which time the number of algal cells should be approximately 1.5 X 10^6/mL for *Skeletonema* or 3.5 X 10^6/mL for *Selenastrum* but does not give a cell count for *Anabaena flos-aque*. No reference to coefficient of variation requirements was identified in this US EPA guideline.

With respect to exponential growth, this requirement is not considered by the reviewer to have been met although Table 3, on page 20 of this DER under the parameter "Indicate whether there was an exponential growth in the control" does indicate OECD guideline requirements were met. The reason for this decision are the apparent absence of sustained logarithmic growth over the exposure period as shown by the %CV values for growth rates (as shown in Table 7) with the variability of cell counts readily seen in Table 4, page 23 of this DER and the fitting of the mean cell count data against an exponential growth curve (pages 20 and 45 of this DER refer).

The 0-24, 24-48, 48-72, 72-96 and 96-120 hour control replicate growth rates were calculated from the initial (10,000 cells/mL), 24, 48, 72 and 96 hour cell density counts using the growth rate formula shown under "Verification of Statistical Results" on page 30 of this DER. The values and calculated statistics, including the overall mean % coefficient of variation (%CV) are as shown in Table 7:

Table 7. Reviewer-calculated control growth rates for the 0-24 to 96-120 hour periods and associated means, standard deviations and percentage coefficients of variation for *A. flos-aquae* exposed to pyroxsulam.

	Reviewer-calculated growth rates (/day) for the control replicates				
Replicate	**0-24 h**	**24-48 h**	**48-72 h**	**72-96 h**	**96-120 h**
1	Not calculated because of low (0) cell counts in 5 of the 6 replicates at 24 hours	Not calculated because of low (0) cell counts in 5 of the 6 replicates at 24 hours	1.14	-0.81	1.74
2			1.73	-0.17	0.82
3			1.95	-1.57	2.74
4			0.00	1.24	-3.59
5			0.86	-0.65	0.73
6	0.49	1.58	0.73	0.33	0.62
Mean	Not determined	Not determined	1.07	-0.27	0.51
Standard deviation			0.71	0.98	2.16
%CV			66.31	-360.36	425.16

The results indicate some growth issues took place in the study and the OECD 201 (2006) requirements with regard to the percentage coefficient of variance are not met. In particular, growth between 72 and 96 hours basically stopped (see in Table 4, page 23 of this DER). Similarly the %CV values reported indicate high variability in the results.

The 0-72 hour %CV was 15.9 (mean 1.04, standard deviation 0.16, page 42 shows the data and ToxCalc determinations). The %CV using the study's 0-72 hour growth rate mean of 1.08 and standard deviation of 0.17 is 15.7%, which exceeds the 2006 OECD 201 guideline recommendation of the %CV over the study period not exceeding 10%.

During the whole test period, i.e. 0-120 hour and using reviewer calculated values (calculations not shown in this DER), the %CV is 49.5%.

Although the study was conducted following the 1984 version of the OECD 201 guideline, these %CV are considered a deficiency as the values determined indicate there were some serious problems with the study. While the %CV limits may not apply because of the use of the 1984 OECD guideline, the study was conducted in 2005, when such requirements were known and could have been taken into account. Consequently, the study is considered deficient because of the high variability recorded in the controls (see table of deviations on page 33 of this DER under "Validity of test").

B. REPORTED STATISTICS:

Based on the results of statistical analysis performed for 120-hour cell density, 72-hour total biomass and average growth rate, the No-Observed-Effect Concentration (NOEC), the highest test concentration which demonstrated no statistically adverse effect ($p < 0.05$) when compared to the control data, was determined. The data were first checked for normality using Shapiro-Wilks' Test (Weber et al., 1989) and for homogeneity of variance using Bartlett's Test (Horning and Weber, 1985). If the data sets passed the tests for homogeneity and normality, then Williams' Test (Williams, 1971, 1972) was used to determine the NOEC. If the data did not pass the tests for homogeneity and normality, then Kruskal-Wallis' Test was used to determine the NOEC. All statistical determinations were made at the 95% level of certainty, except in the case of Shapiro-Wilks' and Bartlett's Tests, where the 99% level of certainty was applied. A computer program, TOXSTAT® (Gulley *et al.,* 1996) was used to perform these calculations.

C. VERIFICATION OF STATISTICAL RESULTS:

Statistical Method(s):
Cell density

Replicate data for cell density were tested (ToxCalc™ v5.0.23j. Copyright 1994-2005 Tidepool Scientific Software, McKinleyville, CA 95519 USA) for normality and homogeneity, by respectively, the Shapiro-Wilk's and Bartlett's tests and for difference between the mean cell counts and mean specific growth rates of the pyroxsulam exposed algae and the mean of the controls by Bonferroni's t test or William's test. Differences between the mean specific growth rate and biomass results of the pyroxsulam exposed algae and that of the controls were tested by, respectively, Bonferroni's t test and William's test. All NOEC values were determined using the ToxCalc package.

The cell density in each test flask was calculated for each daily interval by dividing the number of cells counted by the number of fields examined for each cell count. Duplicate counts were averaged for replicate at each time interval. Means and standard deviations for cell density for each treatment and the control were calculated from replicate values.

Using the Microsoft Excel Data Analysis function "Descriptive Statistics", the means and standard deviations of the cell count data were determined using the cell count data in the study report. The re-calculated values were considered equivalent to those reported, with only small differences observed – accepted as most probably the result of the re-calculations using the results reported in the study report whereas the study report values were identified as calculated from original raw data and not the rounded results given in the study report. The only noticeable difference was the recalculated standard deviation for the mean cell count at 120 hours which was 26.62 compared to the reported 24.14. The mean values were the same, namely 48.52.

The reported and reviewer-calculated cell density 120-hour endpoints were:

120 hour endpoints:	**EC 25**	**EC50**	**NOEC**
Reported (Hoberg, 2005)	0.69 (0.41-14)	11 (0.76-21)	13
Recalculated	0.68 (0.00-25.3)	10.9 (0.00-26.8)	13

The 96 hour cell density endpoints were also recalculated with the following results:

96 hour endpoints:	**EC 25**	**EC50**	**NOEC**
Reported (Hoberg, 2005)	2.6 (0.57-4.3)	4.4 (0.89-29)	Not reported.
Recalculated	2.47 (0.00-4.67)	4.35 (0.00-24.1)	28

The recalculated values for these two time intervals are considered equivalent to those reported by Hoberg (2005) and the most sensitive endpoint is identified as cell density at 120 hours.

The 24, 48 and72 hour EC values for cell densities reported by Hoberg (2005) were not verified by re-calculation.

The ToxCalc calculations for cell counts are shown on page 38 of this DER.

Growth rate

The growth rate (μ) for each replicate flask was calculated for the period from test initiation to each observation time using the following equation:

$$\mu = \frac{\ln X_t - \ln X_0}{t_t - t_0}$$

where:

μ	=	specific growth rate ($days^{-1}$)
ln	=	natural logarithm
X_0	=	initial cell density in cells/mL
X_t	=	cell density at the specified time interval in cells/mL
t_0	=	time of test initiation
t_t	=	time of observation interval in days (i.e., 1, 2, 3...)

The re-calculated growth rate values were considered equivalent to those reported, with only small differences observed – accepted as most probably the result of the re-calculations using the results reported in the study report whereas the study report values were identified as calculated from original raw data and not the rounded results given in the study report.

The re-calculated growth rates (day^{-1}) with the associated means and standard deviations and those reported in the study report are shown in Table 10, page 41 of this report.

The ToxCalc program for determination of specific growth rate in alga was used with the above replicate results to re-calculate the ErC50 0-72 hour results. The program used the reported 0-72 hour cell density counts for the determination.

The reported and reviewer-calculated 0-72 hour growth rate endpoints were:

0-72 hour endpoints:	**ErC50 (95% confidence limits)**	**NOEC**
Reported (Hoberg, 2005)	41 (25-51)	13
Recalculated	41.4 (16.03-57.88)	13

The two sets of values for the ErC50 and NOEC results are considered equivalent.

The ToxCalc calculations and results are shown on page 42 of this DER.

Calculated biomass (area under the growth curve)

The biomass (area under the growth curve) for each replicate vessel was calculated for the exposure period between 0 and 72 hours using the following equation:

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1) + \ldots + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1})$$

where:

A	=	area under the growth curve (units: x 10^4 cells•days/mL)
N_0	=	calculated number of cells/mL at time t_0
N_1	=	measured number of cells/mL at t_1
N_n	=	measured number of cells/mL at time t_n
t_1	=	time of first measurement after beginning of test
t_n	=	time of n^{th} measurement after beginning of test
n	=	number of measurements taken after test initiation

The 72 hour biomass-area under the growth curve values were recalculated using the Microsoft Excel package with the 0-72 hour results shown on page 43 of this DER.

The re-calculated growth rate values were of similar magnitude to those reported, with only small differences observed. This is again probably the result of the re-calculations using the results reported in the study report whereas the study report values were identified as calculated from original raw data and not the rounded results given in the study report. The calculated growth rates (day^{-1}) with the associated means and standard deviations and those reported in the study report are shown in Table 11, page 43 of this report.

The ToxCalc program was used with the recalculated replicate results to re-calculate the EbC50 0-72 hour results. The reported and reviewer-calculated 0-72 hour growth rate endpoints were:

0-72 hour endpoints:	**EbC50 (95% confidence limits)**	**NOEC**
Reported (Hoberg, 2005)	22 (8.9-28)	13
Recalculated	21.3 (0.0-35.4)	13

These results were determined by use of the log (x + 1) transformation with the data analysed excluding the 85 mg pyroxsulam/L results for the testing for normality and equality of variance and testing for significance of differences between the pyroxsulam and control mean results using William's test. The EC value was determined from all test concentrations.

The ToxCalc analysis identified the NOEC as 13 mg pyroxsulam/L and the 28 and 85 mg pyroxsulam/L results as statistically significantly different from the control mean.

The re-calculated EbC50 results are considered to have confirmed the reported EbC50 value with the differences seen again attributed to the re-calculated results using the reported cell counts while the study report's values used the original raw data.

The ToxCalc calculations and results are shown on page 43 of this DER.

Summary of algal endpoints calculated by the reviewer:

The reviewer re-calculated the 120-hour cell density, 0-72 hour growth rate and 0-72 hour biomass end points reported in the study report with the values obtained equivalent to those in the study report. The most sensitive endpoint was cell density at 120 hours.

There was considerable variation of cell counts between replicates in control, e.g. after 48 hours the measured cell counts ranged from 0.00 to 16.75 x 10^4 cell /mL. This also applies to the treatments. However, at some sample intervals there was limited variation, e.g. control at 72 hours cell counts varied from 10.5 to 39.6 x 10^4 cell /mL. This sample variation appears to be possibly due to the fact that the filaments in the test solutions were not adequately separated. This high variation has increased the standard deviation in the data and overall this has reduced the sensitivity of the statistical analysis used.

The endpoints reported in the study report and those re-calculated in the assessment of the study are shown in Table 8. The table includes the 96 hour cell count results reported by the study report and as re-calculated by the reviewer. The 24, 48 and 72 hour cell count endpoints from the study report are also shown in the table, although they were not verified by recalculation.

Table 8. Reported and recalculated toxicity endpoints.

Toxicity endpoint	Mean measured pyroxsulam concentration, mg/L (95% confidence limits)	
	As presented in the study report	As calculated using the ToxCalc program
24 hour cell density		
EC50	4.9 (3.8-21)	Not recalculated
NOEC	Not reported	
48 hour cell density		
EC50	21 (3.8-32)	Not recalculated
NOEC	Not reported	
72 hour cell density		
EC50	23 (4.9-30)	Not recalculated
NOEC	Not reported	
96 hour cell density		
EC50	4.4 (0.89 – 29)	4.3 (0.0-24.1)
NOEC	Not reported	28
120 hour cell density		
EC50	11 (0.76-21)	10.9 (0.0-26.8)
NOEC	13	28
0-72 hour biomass		
EbC50	22 (8.9-28)	21.3 (0.0-35.4)
NOEC	13	13
0-72 hour mean specific growth rate		
ErC50	41 (25-51)	41.4 (16-58)
NOEC	13	13

D. STUDY DEFICIENCIES:

Table 9 summarises deficiencies and deviations from the OECD 201 and US EPA OPPTS 850.5400 Guidelines

Table 9. Deviations from Guidelines and other deficiencies

Parameter	Study reported results	OECD 201	US EPA OPPTS 850.5400 Algal Toxicity, Tiers I and II
Culturing media and conditions: (same as test or not)	Sodium selenate used in the test medium. This chemical is not included in the AAP formulation provided in OECD 201 for species other than diatoms. The references to AAP medium should have been to "modified AAP medium".	OECD 201 indicates that the AAP medium should only have been treated with sodium selenate if stock cultures of diatom species were being cultured. This was not the case with the present study.	Sodium selenate is not referred to in the US EPA guideline.
Duration of the test	120-hours	OECD 201 notes that test duration is normally 72 hours but that shorter or longer test durations may be used provided that all validity criteria in the guideline can be met.	US EPA OPPTS 850.5400 refers to counting of algal cells at 24, 48, 72 an 96 hours. No reference to a 120 hour exposure was identified.
Details of growth medium name	AAP medium, modified by addition of sodium selenate at 1.88 µg/L.	OECD 201 refers to AAP medium and provides a comparison (Annex 3) of the US EPA AAP medium and the OECD 201 medium. The guideline identifies both as suitable growth media. OECD 201 states that sodium selenate is to be used only in medium for stock cultures of diatom species.	US EPA OPPTS 850.5400 does not specifically refer to media compositio instead referring to other sources for this information.
Test concentrations Nominal:	The ratio between the nominal test concentrations ranged from 2.4 to 2.6. Nominal concentrations were: 0 (control), 0.41, 1.0, 2.6, 6.4, 16, 40 and 100 mg pyroxsulam/L.	For the final definitive test at least five concentrations, arranged in a geometric series with a factor not exceeding 3.2, should be selected.	Algae should be exposed to five or more concentrations of the test chemical in a geometric series in which the ratio is between 1.5 and 2.0 (e.g. 2, 4, 8, 16, 32, and 64 mg/L).
Other observations, if any	*Anabaena flos-aquae* was identified as growing in filaments which required vigorous pipetting multiple times to break-up the filaments to achieve a more homogeneous	OECD 201 refers to *Anabaena flos-aquae* developing aggregates of nested chains of cells which may be necessary to break up when microscope counting or an electronic particle counter is used for	US EPA OPPTS 850.5400 states that a particle counter or microscopic counting cannot be used for *Anabaena* unless the filaments are broken up and dispersed using a syringe, ultrasonic bath, or blender.

	suspension before removing a sample for cell counts.	determination of biomass. The guideline notes that sonication of sub-samples may be used to break up chains to reduce count variability. Pipetting is not identified in this list.	
Validity of test	The %CV values for the 0-24 to the 96-120 hour period specific growth rates of the controls ranged from -360% [at 72-96 hours] through "not determinable" [at 0-24 hours] to 425% [at 96-120 hours] with such values indicative of non-compliance with the 35% limit set by the 2006 OECD 201 guideline (see Table 7 for calculations). The 0-72 hour %CV was 15.9% while the 0-120 hour %CV was 49.5%.	OECD 201 (2006) requires that, for the test to be valid, the mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2 and 2-3, for 72-hour tests) in the control cultures must not exceed 35%. The OECD guideline states that the coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7% in tests with *Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus*. For other less frequently tested species, the value should not exceed 10%.	No %CV requirement.

Of these deviations or deficiencies, the variability of the cell count results is a major concern (as indicated under the "Validity of test" entry). This and the lack of sustained logarithmic growth are indicative of some experimental problem and taken as sufficient reasons to class the study as invalid.

The possibility that the *Anabaena flos-aquae* filaments were not necessarily sufficiently broken up by the pipetting procedure used would be of major concern if this alga was found to be the most sensitive to pyroxsulam and such an event could be linked to the variability of cell counts. Following the 2005 OECD 201 reference to the use of sonication to break up chains to reduce count variability could have reduced this source of variability.

While the use of sodium selenate is probably acceptable (although OECD 201 indicates it is only to be used in the AAP medium for stock cultures of diatom species), an early reference to its use in the study report would have been preferable, e.g. under item 2.3 Test Organism in the study report where the AAP medium is discussed.

The other issues identified are not considered to have adversely affected the either the study's conduct or outcomes.

E. REVIEWER'S COMMENTS:

The study is classified as **INVALID** due to the problem of cell counting that was noted above and because sustained logarithmic growth was not achieved and results were highly variable between replicates.

Results are therefore not reported in the Executive Summary or the Conclusions Sections of this DER, and should not be used in a risk assessment.

The inclusion of sodium selenate in the growth medium and its identification as an additional nutrient required could profitably been identified much earlier and more conspicuously in the study report. Additionally, the information in OECD 201 would indicate that the AAP medium should only have been treated with sodium selenate if stock cultures of diatom species were being cultured, although brief comparison with published data suggests concentrations were below toxic levels.

F. CONCLUSIONS:

This study is classified as **INVALID** because sustained logarithmic growth was not achieved and results were highly variable between replicates. Results of this study should not be used in a risk assessment.

This study is of limited utility due to the uncertainty related to the problems of cell counting and exponential growth in the controls. Some useful information can be obtained from this study and used in a risk assessment (ErC50 expected to be of the order of 41 mg pyroxsulam/L) and the low toxicity of the test substance has been adequately demonstrated.

The 0-72 hour ErC50 of ~41 mg pyroxsulam/L is approximately two orders of magnitude greater than the 0-72 hour ErC50 value of 0.695 mg pyroxsulam/L determined in the DER for the effect of pyroxsulam on the freshwater green alga, *Pseudokirchneriella subcapitata.* This is indicative of pyroxsulam's toxicity to *Anabaena flos-aquae* being much less than to *Pseudokirchnerialla.*

A new study would not be expected to provide significant additional information.

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APPENDIX I. OUTPUT OF REVIEWER'S STATISTICAL VERIFICATION:

Algal Cell Count at 120 hours (page 29 of this DER refers)

(1) ToxCalc analysis of the 120 hour algal cell count data (based on mean measured pyroxsulam concentrations but excluding the 85 mg pyroxsulam/L results for normality and equality with the control mean value) presented in the study report gave the following results. Cell counts are as cells/mL.

Conc mg/L	1	2	3	4	5	6
B-Control	623800	687500	733800	10000	432500	423800
0.36	833800	433800	148800			
0.89	357500	237500	292500			
2.2	431300	267500	171300			
5.4	0	327500	520000			
13	183800	85000	413800			
28	2500	196300	77500			
85	0	0	0			

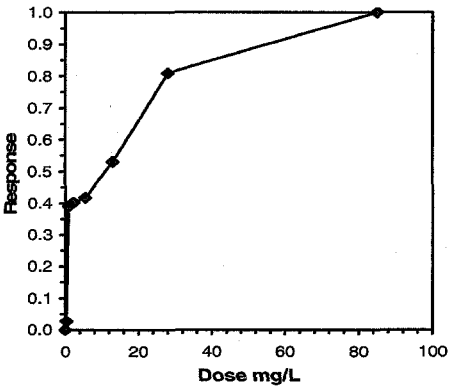
			Transform: Untransformed						1-Tailed		Isotonic	
Conc mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Mean	N-Mean
B-Control	485233	1.0000	485233	10000	733800	54.859	6				485233	1.0000
0.36	472133	0.9730	472133	148800	833800	72.883	3	0.083	2.655	419088	472133	0.9730
0.89	295833	0.6097	295833	237500	357500	20.305	3	1.200	2.655	419088	295833	0.6097
2.2	290033	0.5977	290033	171300	431300	45.325	3	1.237	2.655	419088	290033	0.5977
5.4	282500	0.5822	282500	0	520000	93.064	3	1.284	2.655	419088	282500	0.5822
13	227533	0.4689	227533	85000	413800	74.146	3	1.633	2.655	419088	227533	0.4689
28	92100	0.1898	92100	2500	196300	106.104	3	2.491	2.655	419088	92100	0.1898
85	0	0.0000	0	0	0	0.000	3				0	0.0000

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)	0.96854	0.884	-0.4829	0.59843
Bartlett's Test indicates equal variances (p = 0.37)	6.51178	16.8119		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Bonferroni t Test Treatments vs B-Control	28	85	48.7852	3.57143	419088	0.86368	7E+10	5E+10	0.2675	6, 17

Linear Interpolation (200 Resamples)

Point	%	SD	95% CL(Exp)		Skew
IC05	0.394	1.478	0.000	1.460	7.9253
IC10	0.466	1.622	0.000	4.165	7.5437
IC15	0.539	2.092	0.000	9.195	5.5515
IC20	0.612	2.501	0.000	12.691	4.6419
IC25	0.685	2.978	0.000	14.484	3.4551
IC40	1.950	5.306	0.000	27.657	1.4648
IC50	10.914	6.263	0.000	26.798	0.8644



Note: These 120 hour cell density statistics are based on non-inclusion of the 85 mg pyroxsulam/L results in the analysis for normality and equality with the control mean. The ECx (reported as IC50 in the statistical output) values are based on the use of all test concentrations (see following ToxCalc output).

Algal Cell Count at 120 hours (continued)

(2) ToxCalc analysis of the 120 hour algal cell count data (cells/mL) using all the test concentrations (as mean measured concentrations of 0.36 to 85 mg pyroxsulam/L) presented in the study report gave the following results.

Conc-mg/L	1	2	3	4	5	6
D-Control	623800	687500	733800	10000	432500	423800
0.36	833800	433800	148800			
0.89	357500	237500	292500			
2.2	431300	267500	171300			
5.4	0	327500	520000			
13	183800	85000	413800			
28	2500	196300	77500			
85	0	0	0			

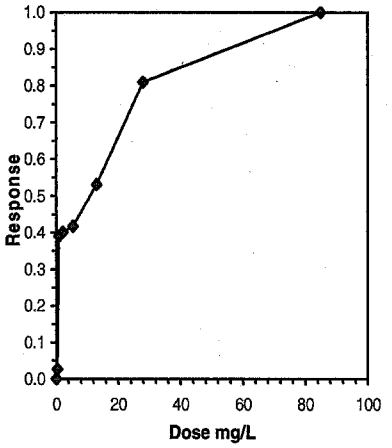
			Transform: Untransformed						1-Tailed			Isotonic	
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Mean	N-Mean
D-Control	485233	1.0000	485233	10000	733800	54.859	6				485233	1.0000
0.36	472133	0.9730	472133	148800	833800	72.883	3	0.088	1.730	258307	472133	0.9730
0.89	295833	0.6097	295833	237500	357500	20.305	3	1.268	1.795	268012	295833	0.6097
2.2	290033	0.5977	290033	171300	431300	45.325	3	1.307	1.820	271745	290033	0.5977
5.4	282500	0.5822	282500	0	520000	93.064	3	1.358	1.825	272491	282500	0.5822
13	227533	0.4689	227533	85000	413800	74.146	3	1.726	1.835	273984	227533	0.4689
*28	92100	0.1898	92100	2500	196300	106.104	3	2.633	1.840	274731	92100	0.1898
*85	0	0.0000	0	0	0	0.000	3	3.250	1.840	274731	0	0.0000

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)	0.95971	0.894	-0.5082	1.04373
Equality of variance cannot be confirmed				

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Williams' Test	13	28	19.0788		274731	0.56618	1E+11	4.5E+10	0.0732	7, 19
Treatments vs D-Control										

Linear Interpolation (200 Resamples)

Point	mg/L	SD	95% CL(Exp)		Skew
IC05	0.394	0.713	0.000	0.909	7.4134
IC10	0.466	1.277	0.000	4.043	7.8067
IC15	0.539	1.621	0.000	5.251	6.6118
IC20	0.612	2.165	0.000	10.244	4.8325
IC25	0.685	2.779	0.000	12.430	3.7591
IC40	1.950	5.229	0.000	25.318	1.5663
IC50	10.914	6.412	0.000	25.725	0.8720



In this situation, the 28 and 85 mg pyroxsulam/L 120 hour results are identified as statistically significantly different to the mean value of the control at that time (as was recorded in the study report). However, equality of variance was not able to be confirmed. The reported and re-calculated 120 hour toxicity endpoints for cell density are:

120 hour endpoints:	**EC 25**	**EC50**	**NOEC**
Reported (Hoberg, 2005)	0.69 (0.41-14)	11 (0.76-21)	13
Recalculated	0.68 (0.00-25.32)	10.9 (0.00-25.72)	13

Note: EC25, EC50 and NOEC results are expressed as mg pyroxsulam/L.

Algal Cell Count at 96 hours

(3) ToxCalc analysis of the 96 hour algal cell count data (as cells/mL and based on mean measured pyroxsulam concentrations including the 85 mg pyroxsulam/L results for normality and equality with the control mean value) presented in the study report gave the following results.

Conc-mg/L	1	2	3	4	5	6
D-Control	110000	303800	47500	361300	207500	228800
0.36	48800	443800	217500			
0.89	96300	221300	152500			
2.2	285000	192500	105000			
5.4	140000	0	32500			
13	138800	92500	78800			
28	75000	103800	25000			
85	0	0	0			

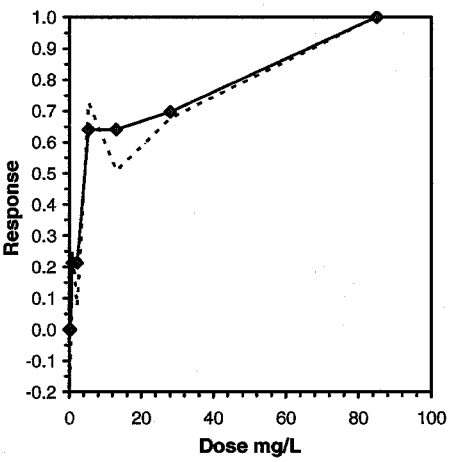
			Transform: Untransformed						1-Tailed		Isotonic	
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Mean	N-Mean
D-Control	209817	1.0000	209817	47500	361300	55.767	6				223258	1.0000
0.36	236700	1.1281	236700	48800	443800	83.734	3	-0.383	2.697	189164	223258	1.0000
0.89	156700	0.7468	156700	96300	221300	39.953	3	0.757	2.697	189164	175433	0.7858
2.2	194167	0.9254	194167	105000	285000	46.358	3	0.223	2.697	189164	175433	0.7858
5.4	57500	0.2740	57500	0	140000	127.429	3	2.172	2.697	189164	80433.3	0.3603
13	103367	0.4927	103367	78800	138800	30.417	3	1.518	2.697	189164	80433.3	0.3603
28	67933.3	0.3238	67933.3	25000	103800	58.694	3	2.023	2.697	189164	67933.3	0.3043
*85	0	0.0000	0	0	0	0.000	3	2.991	2.697	189164	0	0.0000

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)	0.96456	0.894	0.11749	1.03543
Equality of variance cannot be confirmed				

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Bonferroni t Test	28	85	48.7852		189164	0.90157	2.4E+10	9.8E+09	0.06088	7, 19
Treatments vs D-Control										

Linear Interpolation (200 Resamples)

Point	mg/L	SD	95% CL(Exp)	Skew
IC05	0.4837	0.5910	0.0000	3.6917	2.6677
IC10	0.6074	0.7546	0.0000	4.0795	1.9877
IC15	0.7311	0.8852	0.0000	4.4857	1.6345
IC20	0.8548	1.0332	0.0000	5.0292	1.2054
IC25	2.4691	1.1670	0.0000	4.6723	0.8811
IC40	3.5972	2.2197	0.0000	7.6502	3.7973
IC50	4.3492	4.2136	0.0000	24.0987	3.2957



The reported and re-calculated 96 hour toxicity endpoints are:

96 hour endpoints:	EC 25	EC50	NOEC
Reported (Hoberg, 2005)	2.6 (0.57-4.3)	4.4 (0.89-29)	Not reported.
Recalculated	2.47 (0.00-4.67)	4.35 (0.00-24.1)	28

Note: EC25, EC50 and NOEC results are expressed as mg pyroxsulam/L.

Algal Growth Rate
(page 30 of this DER refers)

Re-calculated algal growth rates (days^{-1}) and their comparison with the values reported in the study report (Hoberg, 2005) are shown in Table 10 (page 30 and following of this DER refers).

Table 10. Comparison of re-calculated algal growth rates (day^{-1}) with those reported by Hoberg (2005).

Time interval		0-24 h		0-48 h		0-72 h	
		Hoberg (2005)	Re-calculated value	Hoberg (2005)	Re-calculated value	Hoberg (2005)	Re-calculated value
Control	A	0.00	*0.00*	1.06	*1.03*	1.11	*1.07*
	B	0.00	*0.00*	0.95	*0.93*	1.24	*1.19*
	C	0.00	*0.00*	0.61	*0.59*	1.08	*1.04*
	D	0.00	*0.00*	0.00	*0.00*	0.81	*0.78*
	E	0.00	*0.00*	1.45	*1.41*	1.27	*1.23*
	F	0.45	*0.49*	1.06	*1.03*	0.96	*0.93*
	Mean (SD)	**0.07 (0.18)**	**0.08 (0.20)**	**0.86 (0.50)**	***0.83 (0.48)***	**1.08 (0.17)**	***1.04 (0.17)***
0.36	A	0.00	*0.00*	1.50	*1.46*	1.01	*0.98*
	B	0.00	*0.00*	1.38	*1.34*	0.91	*0.88*
	C	0.00	*0.00*	1.50	*1.46*	1.06	*1.02*
	Mean (SD)	**0.00 (0.00)**	**0.00 (0.00)**	**1.46(0.07)**	***1.42 (0.07)***	**0.99 (0.07)**	***0.96 (0.07)1.42 (0.07)***
0.89	A	0.00	*0.00*	0.50	*0.48*	0.87	*0.84*
	B	0.00	*0.00*	1.63	*1.59*	0.98	*0.94*
	C	0.00	*0.00*	1.54	*1.50*	1.12	*1.08*
	Mean (SD)	0.00 (0.00)	0.00 (0.00)	1.22 (0.63)	*1.19 (0.61)*	0.99 (0.12)	*0.95 (0.12)*
2.2	A	-1.91	*-2.04*	1.19	*1.15*	1.01	*0.98*
	B	2.14	*2.33*	1.22	*1.19*	1.14	*1.11*
	C	1.18	*1.29*	0.96	*0.94*	1.14	*1.10*
	Mean (SD)	0.47 (2.12)	*0.53 (2.28)*	1.12 (0.14)	*1.09 (0.14)*	1.10 (0.07)	*1.06 (0.07)*
5.4	A	0.00	*0.00*	0.00	*0.00*	1.16	*1.12*
	B	0.00	*0.00*	1.27	*1.23*	0.53	*0.51*
	C	0.00	*0.00*	1.30	*1.26*	1.03	*0.99*
	Mean (SD)	**0.00 (0.00)**	***0.00 (0.00)***	**0.86 (0.74)**	***0.83 (0.72)***	**0.90 (0.33)**	***0.87 (0.32)***
13	A	0.00	*0.00*	1.46	*1.42*	1.21	*1.17*
	B	0.00	*0.00*	1.29	*1.25*	1.26	*1.22*
	C	0.84	*0.92*	0.00	*0.00*	0.61	*0.59*
	Mean (SD)	**0.28 (0.49)**	***0.31 (0.53)***	**0.92 (0.80)**	***0.89 (0.77)***	**1.03 (0.36)**	***0.99 (0.35)***
28	A	0.00	*0.00*	1.04	*1.01*	0.86	*0.83*
	B	0.00	*0.00*	0.36	*0.35*	0.74	*0.71*
	C	0.00	*0.00*	0.00	*0.00*	0.46	*0.44*
	Mean (SD)	**0.00 (0.00)**	***0.00 (0.00)***	**0.46 (0.53)**	***0.45 (0.51)***	**0.68 (0.21)**	**0.66 (0.20)**
85	A	0.00	*0.00*	0.00	*0.00*	0.00	*0.00*
	B	0.00	*0.00*	-1.07	*-1.02*	0.19	*0.19*
	C	0.00	*0.00*	0.00	*0.00*	0.00	*0.00*
	Mean (SD)	**0.00 (0.00)**	**0.00 (0.00)**	**-0.36 (0.62)**	**-0.34 (0.59)**	**0.06 (0.11)**	**0.06 (0.11)**

Algal Growth Rate (continued)
(page 30 of this DER refers)

The ToxCalc analysis of the 0-72 hour mean specific growth rate data (days^{-1}) in the table gave the following results.

Conc-mg/L	1	2	3	4	5	6
D-Control	1.0696	1.1945	1.0415	0.7838	1.2265	0.9320
0.36	0.9794	0.8828	1.0208			
0.89	0.8387	0.9444	1.0796			
2.2	0.9794	1.1063	1.1033			
5.4	1.1210	0.5109	0.9924			
13	1.1705	1.2223	0.5905			
28	0.8319	0.7134	0.4406			
85	0.0000	0.1865	0.0000			

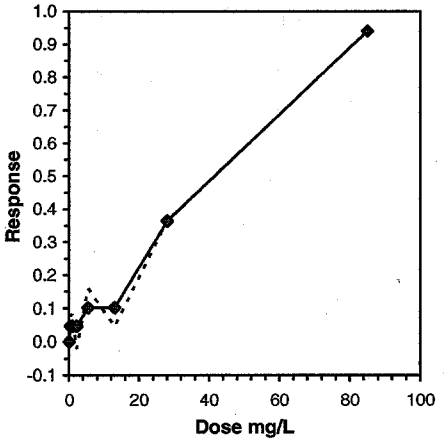
			Transform: Untransformed					1-Tailed			Isotonic	
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Mean	N-Mean
D-Control	1.0413	1.0000	1.0413	0.7838	1.2265	15.886	6				1.0413	1.0000
0.36	0.9610	0.9228	0.9610	0.8828	1.0208	7.371	3	0.575	2.697	0.3772	0.9927	0.9533
0.89	0.9542	0.9163	0.9542	0.8387	1.0796	12.652	3	0.623	2.697	0.3772	0.9927	0.9533
2.2	1.0630	1.0208	1.0630	0.9794	1.1063	6.813	3	-0.155	2.697	0.3772	0.9927	0.9533
5.4	0.8748	0.8400	0.8748	0.5109	1.1210	36.770	3	1.191	2.697	0.3772	0.9346	0.8975
13	0.9944	0.9550	0.9944	0.5905	1.2223	35.273	3	0.335	2.697	0.3772	0.9346	0.8975
*28	0.6619	0.6357	0.6619	0.4406	0.8319	30.313	3	2.713	2.697	0.3772	0.6619	0.6357
*85	0.0622	0.0597	0.0622	0.0000	0.1865	173.205	3	7.001	2.697	0.3772	0.0622	0.0597

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)	0.93965	0.894	-0.8017	0.29663
Bartlett's Test indicates equal variances (p = 0.29)	8.53926	18.4753		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Bonferroni t Test Treatments vs D-Control	13	28	19.0788		0.37716	0.36219	0.35111	0.03912	6.7E-05	7, 19

Linear Interpolation (200 Resamples)

Point	mg/L	SD	95% CL(Exp)		Skew
IC05	2.391	6.056	0.000	24.033	0.7079
IC10	5.257	6.798	0.000	27.618	0.0638
IC15	15.721	6.774	0.000	27.458	-0.2867
IC20	18.586	6.817	0.000	32.617	-0.4464
IC25	21.450	7.268	0.000	37.080	-0.5831
IC40	31.530	7.035	11.120	49.809	-0.3229
IC50	41.427	7.150	16.026	57.884	-0.5051



The reported and re-calculated 0-72 hour toxicity endpoints for growth rate are:

0-72 hour endpoints:	ErC50	NOEC
Reported (Hoberg, 2005)	41 (25-51)	13
Recalculated	41.4 (16.03-57.88)	13

Note: EC25, EC50 and NOEC results are expressed as mg pyroxsulam/L.

Algal Biomass (Area Under the Growth Curve)
(page 31 of this DER refers)

Re-calculated algal biomass (area under the growth curve) for the period 0 to 72 hours (i.e. total biomass) and their comparison with the values reported in the study report (Hoberg, 2005) are shown in Table 11 (page 31 of this DER refers).

Table 11. Reported and re-calculated algal total biomass after 72 hours.

Mean measured concentration, mg pyroxsulam/L	Replicate	Total biomass (0-72 h) as reported by Hoberg, 2005 using raw data (X 10^4 cells/mL)	Total biomass (0-72 h) as re-calculated by the reviewer using the summary data reported by Hoberg, 2005 (X 10^4 cells/mL)
Control	**A**	16.62	17.76
	B	20.64	21.88
	C	11.47	12.13
	D	2.67	2.75
	E	31.78	34.07
	F	14.19	15.20
Mean and standard deviation		**16.23(9.72)**	**17.30 (10.44)**
0.36	**A**	23.43	25.44
	B	17.54	19.07
	C	24.63	26.69
Mean and standard deviation		**21.87(3.80)**	**23.73 (4.09)**
0.89	**A**	5.94	6.32
	B	27.41	29.88
	C	27.96	30.25
Mean and standard deviation		**20.44(12.56)**	**22.15 (13.71)**
2.2	**A**	15.86	17.07
	B	30.56	32.32
	C	20.15	21.32
Mean and standard deviation		**22.19(7.56)**	**23.57 (7.87)**
5.4	**A**	11.46	11.94
	B	10.50	11.57
	C	18.36	19.82
Mean and standard deviation		**13.44(4.29)**	**14.44 (4.66)**
13	**A**	29.07	31.25
	B	27.46	29.32
	C	2.88	2.94
Mean and standard deviation		**19.81(14.68)**	**21.17 (15.82)**
28	**A**	10.24	12.07
	B	3.52	3.75
	C	-0.56	-0.63
Mean and standard deviation		**4.40(5.45)**	**5.06 (6.45)**
85	**A**	-2.36	-2.50
	B	-1.40	-1.50
	C	-2.36	-2.50
Mean and standard deviation		**-2.04(0.55)**	**-2.17 (0.58)**

Algal Biomass (Area Under the Growth Curve)

(page 31 of this DER refers)

The ToxCalc analysis of the 0-72 hour area under the growth curve (biomass) data in the table gave the following results. Biomass area = cell count X 10^4 cells/mL.

Conc-mg/L	1	2	3	4	5	6
D-Control	17.755	21.880	12.125	2.750	34.065	15.200
0.36	25.440	19.065	26.690			
0.89	6.320	29.880	30.250			
2.2	17.070	32.315	21.320			
5.4	11.940	11.565	19.815			
13	31.250	29.315	2.940			
28	12.065	3.750	0.000			
85	0.000	0.000	0.000			

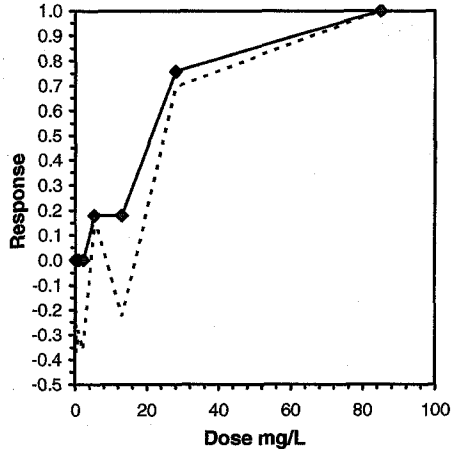
			Transform: Log (X + 1)					1-Tailed			Isotonic	
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Mean	N-Mean
D-Control	17.296	1.0000	1.1798	0.5740	1.5449	28.006	6				21.686	1.0000
0.36	23.732	1.3721	1.3890	1.3024	1.4423	5.446	3	-0.899	1.730	0.4027	21.686	1.0000
0.89	22.150	1.2807	1.2830	0.8645	1.4949	28.249	3	-0.643	1.795	0.4178	21.686	1.0000
2.2	23.568	1.3627	1.3761	1.2570	1.5226	9.806	3	-0.643	1.820	0.4236	21.686	1.0000
5.4	14.440	0.8349	1.1765	1.0992	1.3184	10.458	3	-0.026	1.825	0.4248	17.804	0.8210
13	21.168	1.2239	1.1952	0.5955	1.5085	43.469	3	-0.026	1.835	0.4271	17.804	0.8210
*28	5.272	0.3048	0.5976	0.0000	1.1161	94.083	3	2.501	1.840	0.4283	5.272	0.2431
*85	0.000	0.0000	0.0000	0.0000	0.0000	0.000	3	5.069	1.840	0.4283	0.000	0.0000

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)	0.90342	0.894	-0.8466	0.75365
Equality of variance cannot be confirmed				

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Williams' Test Treatments vs D-Control	13	28	19.0788		9.48677	0.627	0.70841	0.10836	5.1E-04	7, 19

Linear Interpolation (200 Resamples)

Point	mg/L	SD	95% CL(Exp)		Skew
IC05	3.094	4.066	0.000	20.859	1.9611
IC10	3.988	4.945	0.000	22.381	1.2830
IC15	4.881	5.659	0.000	23.834	0.6682
IC20	13.545	6.023	0.000	20.658	0.2456
IC25	14.842	6.221	0.000	21.954	-0.0738
IC40	18.736	5.969	0.000	27.715	-0.6889
IC50	21.331	5.316	0.000	35.417	-0.2673



The reported and re-calculated 0-72 hour toxicity endpoints for growth rate are:

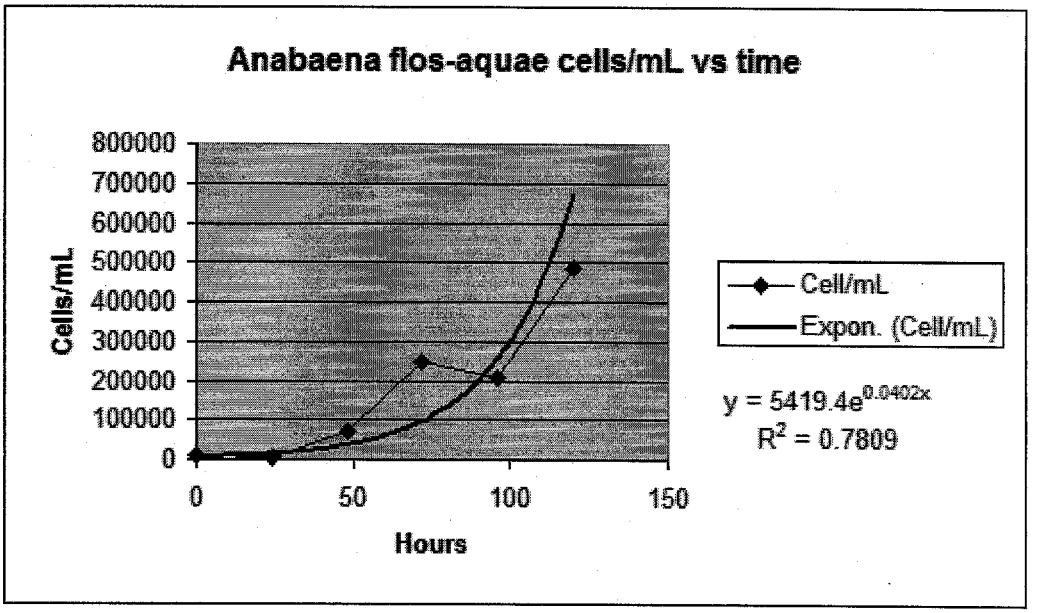
0-72 hour endpoints:	EbC50	NOEC
Reported (Hoberg, 2005)	22 (8.9-28)	13
Recalculated	21 (0-35.4)	13

Note: EC25, EC50 and NOEC results are expressed as mg pyroxsulam/L.

Exponential growth in the controls (page 20 of this DER refers)

To investigate the goodness of fit of the mean control cell counts over time with exponential growth, the mean control cell counts were plotted against time using the Microsoft Excel Chart Wizard function and the resultant curve fitted to an exponential curve. The data used and the Excel output is shown below:

Hours	Cell/mL*
0	10000
24	2700
48	70200
72	250000
96	209800
120	485200



*** Mean control cell counts at the respective times.**

An r^2 value of 0.7809 is indicative of some divergence from exponential growth in the controls over the 0 to 120 hour exposure period.