SafePharm Laboratories

C6-2AL:

ALGAL INHIBITION TEST

SPL PROJECT NUMBER: 1742/023

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QUALITY ASSURANCE REPORT

This study type is classed as short-term. The standard test method for this study type ("General Study Plan" in OECD terminology) was reviewed for compliance once only on initial production. Inspection of the routine and repetitive procedures that constitute the study is carried out as a continuous process designed to encompass the major phases at or about the time this study was in progress.

This report has been audited by Safepharm Quality Assurance Unit, and is considered to be an accurate account of the data generated and of the procedures followed.

In each case, the outcome of QA evaluation is reported to the Study Director and Management on the day of evaluation. Audits of study documentation, and process inspections appropriate to the type and schedule of this study were as follows:

	09 June 2003	Standard Test Method Compliance Audit
	12 October 2004	Test Material Preparation
	12 October 2004	Test System Preparation
	12 October 2004	Exposure
	22 October 2004	Assessment of Response
	04, 28 October 2004	Chemical Analysis
§	17 January 2005	Draft Report Audit
§	Date of QA Signature	Final Report Audit

§ Evaluation specific to this study

..... DATE: -7 OCT 2005

For Safepharm Quality Assurance Unit*

***Authorised QA Signatures:**

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GLP COMPLIANCE STATEMENT

The work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

These international standards are acceptable to the Regulatory agencies of the following countries: Australia, Austria, Belgium, Canada, the Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Israel, Italy, Japan, Republic of Korea, Luxembourg, Mexico, The Netherlands, New Zealand, Norway, Poland, Portugal, Slovenia, South Africa, Spain, Sweden, Switzerland, Turkey, the United Kingdom, and the United States of America.

This report fully and accurately reflects the procedures used and data generated.

..... Date: 05 OCT 2005

H Vryenhoef BSc
Study Director

The analytical data presented in this report were compiled by me or under my supervision and accurately reflect the data obtained.

..... Date: - 5 OCT 2005

Dr J McKenzie CChem MRSC
Head of Analytical Services

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SUMMARY

Introduction. A study was performed to assess the effect of the test material on the growth of the green alga *Scenedesmus subspicatus*. The method followed that described in the OECD Guidelines for Testing of Chemicals (1984) No 201, "Alga, Growth Inhibition Test" referenced as Method C.3 of Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC).

Methods. Following a preliminary range-finding test, *Scenedesmus subspicatus* was exposed to solutions of the test material at mean measured concentrations of 1.3, 2.3, 3.1, 6.7 and 13 mg/l (three replicate flasks per concentration) for 72 hours, under constant illumination and shaking at a temperature of 24 ± 1°C. The test material solutions were prepared by stirring an excess (50 mg/l) of test material in culture medium for a period of time and then removing any undissolved test material by filtration. This "saturated" solution was then further diluted, as necessary, to provide the remaining test groups.

Samples of the algal populations were removed daily and cell concentrations determined for each control and treatment group, using a Coulter® Multisizer Particle Counter.

The test material was suspected to be volatile and hence testing was conducted in completely filled, stoppered test vessels in order to minimise possible losses due to volatilisation. Following the recommendations of published data (Herman *et al* 1990 and Mayer *et al* 2000) in order to prevent inhibition of growth due to the restriction of gaseous exchange, additional sodium bicarbonate was added to the culture medium to provide a source of carbon dioxide for algal growth.

Results. Analysis of the test preparations at 0 and 72 hours showed measured test concentrations to range from 1.15 to 15.2 mg/l. Analysis of a fourth test replicate for each test concentration at 72 hours that had remained unopened throughout the test duration showed measured test concentrations to range from 2.24 to 14.7 mg/l indicating that no losses due to volatility occurred.

Exposure of *Scenedesmus subspicatus* to the test material, based on the mean measured test concentrations, gave an E_bC_{50} (72 h) value of 3.8 mg/l; 95% confidence limits 3.4 - 4.3 mg/l and

an E_rC_{50} (0 - 72 h) value of 7.8 mg/l; 95% confidence limits 6.8 - 8.9 mg/l. The No Observed Effect Concentration was 1.3 mg/l.

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ALGAL INHIBITION TEST

1. INTRODUCTION

This report contains a description of the methods used and results obtained during a study to investigate the effect of the test material on the growth of the green alga *Scenedesmus subspicatus*. The method followed the recommendations of the OECD Guidelines for Testing of Chemicals (1984) No 201, "Alga, Growth Inhibition Test" referenced as Method C.3 of Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC).

The test material was suspected to be volatile and hence testing was conducted in completely filled, stoppered test vessels in order to minimise possible losses due to volatilisation. Following the recommendations of published data (Herman *et al* 1990 and Mayer *et al* 2000) in order to prevent inhibition of growth due to the restriction of gaseous exchange, additional sodium bicarbonate was added to the culture medium to provide a source of carbon dioxide for algal growth.

Scenedesmus subspicatus is a freshwater unicellular alga, representative of primary producers found in natural waters and can therefore be considered as an important non-target organism in freshwater ecosystems.

The range-finding test was conducted between 27 September 2004 and 1 October 2004 and the definitive test between 11 October 2004 and 15 October 2004.

In view of the difficulties associated with the evaluation of aquatic toxicity of poorly water soluble test materials, a modification of the standard method for the preparation of aqueous media was performed. An approach endorsed by several important regulatory authorities in the EU and elsewhere (ECETOC 1996 and OECD 2000), is to expose organisms to a saturated solution of the test material in cases where the test material is of high purity and is poorly soluble in water and in the permitted auxiliary solvents and surfactants. Using this approach, a saturated solution was prepared by stirring an excess (50 mg/l) of test material with culture medium for 24 hours and then removing any undissolved test material by filtration through a pre-conditioned filter (0.2 µm) to give a saturated solution with a nominal concentration of 13 mg/l.

2. TEST MATERIAL AND EXPERIMENTAL PREPARATION

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	C6-2AL
Chemical name	:	1H,1H,2H,2H-tridecafluoro-1-octanol
Description	:	clear colourless liquid
Batch number	:	001002
Date received	:	12 January 2004
Storage conditions	:	room temperature, in the dark

The integrity of supplied data relating to the identity, purity and stability of the test material is the responsibility of the Sponsor.

2.2 Experimental Preparation

Due to the low aqueous solubility and high purity of the test material the test concentration used in the definitive test was a saturated solution prepared from an initial test material dispersion at a concentration of 50 mg/l.

An amount of test material (550 mg) was dispersed in 11 litres of culture medium with the aid of propeller stirring at approximately 1500 rpm for a period of 24 hours. After 24 hours the stirring was stopped and the undissolved test material removed by filtration (0.2 µm Gelman Acrocap filter, first approximate 1 litre discarded in order to pre-condition the filter) to give a saturated solution with a nominal concentration of 13 mg/l. A series of dilutions was made from this saturated solution to give further stock solutions of 6.5, 3.3, 1.6 and 0.81 mg/l. An aliquot (2 litres) of each of the stock solutions was separately inoculated with algal suspension (10 ml) to give the mean measured test concentrations of 1.3, 2.3, 3.1, 6.7 and 13 mg/l.

Media preparation trials (see Section 3.3.1) conducted indicated that no significant loss of dissolved test material due to its volatile nature occurred when filtering a 50 mg/l test material dispersion through a pre-conditioned filter. It was therefore considered justifiable to use filtration as a method of removing dispersed test material to produce a saturated solution of the test material.

The concentration and stability of the test material in the test solutions were verified by chemical analysis at 0 and 72 hours (see Appendix 1).

3. METHODS

3.1 Test Species

The test was carried out using *Scenedesmus subspicatus* strain CCAP 276/20. Liquid cultures of *Scenedesmus subspicatus* were obtained from the Culture Collection of Algae and Protozoa (CCAP), Institute of Freshwater Ecology, The Ferry House, Far Sawrey, Ambleside, Cumbria. Cultures were maintained in the laboratory by the periodic replenishment of culture medium (Section 3.2). The culture was maintained in the laboratory at a temperature of 21 ± 1°C under continuous illumination (intensity approximately 7000 lux) and constant aeration.

3.2 Culture Medium

The culture medium used to maintain the stock cultures is defined in Appendix 2.

The culture medium used for the range-finding and definitive tests was the same as that used to maintain the stock culture however additional sodium bicarbonate (500 mg/l) was added after preparation in order to provide a source of CO_2 required for algal growth in sealed test vessels.

3.3 Procedure

3.3.1 Media Preparation Trials

Media preparation trials were conducted for the Acute Toxicity to *Daphnia magna* test (Safepharm Laboratories Project Number: 1742/022) using a solvent spike method of preparation for the test material. However the results obtained indicated that using a solvent spike method of preparation was not suitable for this test material as low and inconsistent measured concentrations were obtained.

Therefore, additional media preparation trials were conducted for the Acute Toxicity to Rainbow Trout (Safepharm Laboratories Project Number: 1742/021) to determine if the production of a saturated solution would be the most appropriate method of preparation for the test material. An amount of test material (550 mg) was dispersed in 11 litres of dechlorinated tap water with the aid of propeller stirring at approximately 1500 rpm to give an initial test material dispersion of 50 mg/l. This was stirred for a period of 24 hours. Samples were taken from the saturated solution for chemical analysis after centrifugation (at either 10000 *g* or 40000 *g*) and after filtration through a 0.2 µm Gelman Suporcap filter (after either the initial 1 or 2 litres discarded to pre-condition the filters).

3.3.2 Range-finding test

The test concentrations to be used in the definitive test were determined by a preliminary range-finding test. The range-finding test was conducted by exposing *Scenedesmus subspicatus* cells to a series of nominal test concentrations of 0.13, 1.3 and 13 mg/l* for a period of 72 hours.

An amount of test material (550 mg) was dispersed in 11 litres of culture medium with the aid of propeller stirring at approximately 1500 rpm for a period of 24 hours. After 24 hours the stirring was stopped and the undissolved test material removed by filtration (0.2 µm Gelman Acrocap filter, first approximate 1 litre discarded in order to pre-condition the filter) to give a saturated solution with a nominal concentration of 13 mg/l*. A series of dilutions was made from this saturated solution to give further stock solutions of 1.3 and 0.13 mg/l. An aliquot (900 ml) of each of the stock solutions was separately inoculated with algal suspension (4.5 ml) to give the required test concentrations of 0.13, 1.3 and 13 mg/l.

The test was conducted in 250 ml glass conical flasks completely filled and sealed with ground glass stoppers to reduce losses due to volatility. Two replicate flasks were prepared for each control and test concentration.

The control group was maintained under identical conditions but not exposed to the test material.

At the start of the range-finding test a sample of each test and control culture was removed and the cell density determined using a Coulter® Multisizer Particle Counter. The flasks were then sealed with ground glass stoppers and incubated (INFORS Multitron® Version 2 incubator) at 24 ± 1°C under continuous illumination (intensity approximately 7000 lux) and constantly shaken at approximately 150 rpm for 72 hours.

After 72 hours the cell density of each flask was determined using a Coulter® Multisizer Particle Counter.

3.3.3 Definitive test

Based on the results of the range-finding test conducted, the test material solutions for the definitive test were prepared by stirring an excess (50 mg/l) of test material in culture medium at approximately 1500 rpm for 24 hours. After 24 hours undissolved test material was removed by filtration (0.2 µm Gelman Acrocap Filter, first approximate 1 litre discarded in order to pre-condition the filter) to give a saturated solution of the test material from which a series of

* Concentrations based on the results of preliminary analysis of a saturated solution prepared in a similar manner

dilutions was made to give the mean measured test concentrations of 1.3, 2.3, 3.1, 6.7 and 13 mg/l.

3.3.3.1 Preparation of the test material

For the purpose of the definitive test the required amount of test material was added to each test vessel using the method described in Section 2.2.

3.3.3.2 Exposure conditions

As in the range-finding test 250 ml glass conical flasks were used. Three flasks each completely filled with test preparation were used for the control and each treatment group.

The control group was maintained under identical conditions but not exposed to the test material.

Pre-culture conditions gave an algal suspension in log phase growth characterised by a cell density of 2.57×10^6 cells per ml. This suspension was diluted to a cell density of 1.92×10^6 cells per ml prior to use. Inoculation of 2 litres of test medium with 10 ml of this algal suspension gave an initial cell density of 10^4 cells per ml and had no significant dilution effect.

The flasks were sealed with ground glass stoppers and incubated (INFORS Multitron® Version 2 incubator) at 24 ± 1°C under continuous illumination (intensity approximately 7000 lux) and constantly shaken at approximately 150 rpm for 72 hours.

Samples were taken at 0, 24, 48 and 72 hours and the cell densities determined using a Coulter® Multisizer Particle Counter.

Despite the use of completely filled test vessels, there was a possibility that losses of test material may occur over the test period due to the requirement to open the test vessels on a daily basis in order to remove samples for the determination of algal cell density. Therefore additional test material vessels were prepared and incubated alongside the definitive test to provide samples for chemical analysis at 72 hours from vessels that had not been opened throughout the test period.

3.3.3.3 Physico-chemical measurements

The pH of each control and test flask was determined at initiation of the test and after 72 hours exposure. The pH was measured using a WTW pH 320 pH meter. The temperature within the incubator was recorded daily.

3.3.3.4 Verification of test concentrations

Water samples were taken from the control and each test group (replicates R_1 - R_3 pooled) at 0 and 72 hours for quantitative analysis. Duplicate samples were taken at each occasion and stored frozen (approximately -20°C) for further analysis.

The method of analysis, stability, recovery and test solution analyses are described in Appendix 1.

3.3.4 Evaluation of data

3.3.4.1 Comparison of areas under the growth curves

The area under the curve is taken to be an index of growth and was calculated using the following equation:

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

where

A	=	area
N_0	=	cell concentration at the start of the test
N_1	=	cell concentration at t_1
N_n	=	cell concentration at t_n
t_1	=	time of first measurement (hours from start)
t_n	=	time of nth measurement (hours from start)

Percentage inhibition of growth at each test concentration (I_A) was calculated by comparing the area under the test curve (A_t) with that under the control curve (A_c) using the following equation:

$$I_A = \frac{A_c - A_t}{A_c} \times 100$$

3.3.4.2 Comparison of growth rates

The average maximum growth rate (μ) for each culture was also calculated, from the straight section of the growth curve (Figure 1), using the following equation:

$$\mu = \frac{\ln N_n - \ln N_1}{t_n - t_1}$$

3.3.4.3 Statistical analysis

One way analysis of variance incorporating Bartlett's test for homogeneity of variance (Sokal and Rohlf 1981) and Dunnett's multiple comparison procedure for comparing several treatments with a control (Dunnett 1955) was carried out on the area under the growth curve data at 72 hours for the control and all test concentrations to determine any statistically significant differences between the test and control groups. All statistical analyses were performed using the SAS computer software package (SAS 1999 - 2001).

4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Safepharm archives for five years, after which instructions will be sought as to further retention or disposal.

5. RESULTS

5.1 Media Preparation Trials

Media preparation trials were conducted for the Acute Toxicity to *Daphnia magna* test (Safepharm laboratories Project Number: 1742/022) using a solvent spike method of preparation for the test material. However the results obtained indicated that using a solvent spike method of preparation was not suitable for this test material as low and inconsistent measured concentrations were obtained.

A further media preparation trial was conducted for the Acute Toxicity to Rainbow Trout test (Safepharm Laboratories Project Number: 1742/021) using a saturated solution method of preparation. Filtration of the test material dispersions through a 0.2 µm Gelman SuporCap filter showed measured concentrations of 13 and 12 mg/l, discarding the first approximate 1 litre or 2 litres respectively in order to pre-condition the filter. Centrifugation of the test material dispersions at either 10000 *g* or 40000 *g* showed measured test concentrations of 13 mg/l.

Based on these results it was considered justifiable to prepare the test media using a 24-Hour preparation period followed by filtration (0.2 µm), with the first approximately 1 litre discarded, to remove excess dispersed test material and produce a saturated solution

5.2 Range-finding Test

The cell densities and percentage inhibition of growth values from the exposure of *Scenedesmus subspicatus* to the test material during the range-finding test are given in Table 1.

The results showed no effect on growth at the test concentrations of 0.13 and 1.3 mg/l. However, growth was observed to be reduced at 13 mg/l*.

Based on this information the test material solutions for the definitive test were prepared by stirring an excess (50 mg/l) of the test material in culture medium for a period of time and then removing any undissolved test material by filtration to give a saturated solution with a nominal concentration of 13 mg/l. This saturated solution was then further diluted, as necessary, to produce the remaining test concentrations.

* Concentration based on the results of preliminary analysis of a saturated solution prepared in a similar manner

5.3 Definitive Test

5.3.1 Verification of test concentrations

Due to the low aqueous solubility and high purity of the test material the test material solutions were prepared by stirring an excess (50 mg/l) of test material in culture medium for a period of time and then removing any undissolved test material by filtration. This saturated solution was then further diluted, as necessary, to produce the remaining test groups.

Analysis of the test preparations at 0 and 72 hours (see Appendix 1) showed measured test concentrations to range from 1.15 to 15.2 mg/l. At both 0 and 72 hours the measured test concentrations of the bottom two test concentrations were in excess of nominal, the reason for which was not clear from the data obtained. However, the measured test concentrations obtained followed a concentration dependent increase indicating that the serial dilutions performed from the initial saturated solution were correct.

Analysis of a fourth test replicate at 72 hours (see Appendix 1) that had remained unopened throughout the test duration showed measured test concentrations to range from 2.24 to 14.7 mg/l indicating that no losses due to volatility occurred. Once again the measured test concentrations were in excess of nominal though they followed a concentration dependent decrease with each serial dilution performed.

Given this it was considered appropriate to base the results on the mean measured test concentrations in order to give a "worst case" analysis of the data. The following results were calculated based on the mean measured test concentrations:

E_bC_{50} (72 h) = 3.8 mg/l; 95% confidence limits 3.4 - 4.3 mg/l
E_rC_{50} (0 - 72 h) = 7.8 mg/l; 95% confidence limits 6.8 - 8.9 mg/l
No Observed Effect Concentration (NOEC) = 1.3 mg/l

5.3.2 Growth data

From the data given in Tables 2 and 3, it is clear that both the growth (r) and the biomass (b) of *Scenedesmus subspicatus* (CCAP 276/20) were affected by the presence of the test material over the 72-Hour exposure period.

The mean cell densities versus time for the definitive test are presented in Figure 1.

The percentage inhibition values (I_A) (Table 3) were plotted against the mean measured test concentration (Figure 2), a line fitted using the Xlfit 3 software package (IDBS 2002) and the EC_{50} value with respect to the area under the growth curve, E_bC_{50} (72 h) determined from the equation for the fitted line.

The percentage inhibition values (I_μ) (Table 3) were plotted against the mean measured test concentration (Figure 3), a line fitted using the Xlfit 3 software package (IDBS 2002) and the EC_{50} value with respect to the growth rate, E_rC_{50} determined from the equation for the fitted line.

The 95% confidence limits were calculated using the method of Litchfield and Wilcoxon (Litchfield and Wilcoxon 1949).

The following data show that the cell concentration of the control cultures increased by a factor of 66 during the test in line with the OECD Guideline that states the enhancement must be at least by a factor of 16 after 72 hours:

Mean cell density of control at 0 hours : 9.34×10^3 cells per ml
Mean cell density of control at 72 hours : 6.13×10^5 cells per ml

5.3.3 Observations on cultures

All test and control cultures were inspected microscopically at 72 hours. After 72 hours there no abnormalities detected in the control or test cultures at 1.3, 2.3, 3.1 and 6.7 mg/l, however few intact cells were observed to be present in the test cultures at 13 mg/l.

5.3.4 Physico-chemical measurements

The pH values of each test and control flask are given in Table 2. Temperature was maintained at 24 ± 1°C throughout the test.

The pH values of the control cultures (see Table 2) were observed to increase from pH 7.9 at 0 hours to pH 9.7 at 72 hours. This increase was considered to be due to the amount of carbon dioxide required by the large number of algal cells in the log phase of growth (see Figure 1) exceeding the transfer rate of CO_2 from the gaseous phase to the aqueous phase. In this situation CO_2 required for photosynthesis and growth would be derived from bicarbonate in solution which results in an increase in the pH of the culture. The increase in pH after 72 hours was in excess of that recommended in the EEC Guidelines (1.5 pH units after 72 hours). This is considered to have had no adverse effect on the results of the study given that the increase in cell concentration in the control cultures exceeded the validation criterion given in the Test Guidelines.

5.3.5 Observations on test material solubility

At the start of the test all control and test cultures were observed to be clear colourless solutions. After the 72-Hour test period all control, 1.3, 2.3 and 3.1 mg/l test cultures were observed to be bright green dispersions. The 6.7 mg/l test cultures were observed to be green dispersions whilst the 13 mg/l test cultures were observed to be very pale green dispersions.

6. CONCLUSION

The effect of the test material on the growth of *Scenedesmus subspicatus* has been investigated over a 72-Hour period and based on the mean measured test concentrations gave an E_bC_{50} (72 h) value of 3.8 mg/l; 95% confidence limits 3.4 - 4.3 mg/l and an E_rC_{50} (0 - 72 h) value of 7.8 mg/l; 95% confidence limits 6.8 - 8.9 mg/l. The No Observed Effect Concentration at 72 hours was 1.3 mg/l.

7. REFERENCES

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Table 1 Cell Densities and Percentage Inhibition of Growth from the Range-finding Test

Nominal Test Concentration (mg/l)		Cell Densities* (Cells per ml)		
		0 Hours	72 Hours	% Inhibition (Area Under Curve at 72 h)
Control	R_1	1.05E+04	2.27E+05	-
	R_2	1.01E+04	2.45E+05	
	Mean	1.03E+04	2.36E+05	
0.13	R_1	1.06E+04	5.45E+05	[123]
	R_2	9.35E+03	4.82E+05	
	Mean	9.97E+03	5.13E+05	
1.3	R_1	1.07E+04	3.64E+05	[64]
	R_2	8.76E+03	3.97E+05	
	Mean	9.72E+03	3.81E+05	
13	R_1	8.44E+03	8.65E+03	99
	R_2	9.67E+03	1.24E+04	
	Mean	9.05E+03	1.05E+04	

* Cell densities represent the mean number of cells per ml calculated from the mean of the cell counts from 3 counts for each of the replicate flasks.

$R_1 - R_2$ = Replicates 1 and 2

[Increase in growth compared to controls]

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Table 2 Cell Densities and pH Values in the Definitive Test

Mean Measured Test Concentration (mg/l)		pH	Cell Densities* (cells per ml)				pH
		0 h	0 h	24 h	48 h	72 h	72 h
Control	R_1	7.9	9.30E+03	2.95E+04	1.22E+05	6.45E+05	9.7
	R_2	7.9	9.89E+03	3.31E+04	8.17E+04	6.32E+05	9.7
	R_3	7.9	8.84E+03	3.37E+04	1.06E+05	5.62E+05	9.7
	Mean		9.34E+03	3.21E+04	1.03E+05	6.13E+05	
1.3	R_1	7.9	9.94E+03	3.60E+04	1.08E+05	5.78E+05	9.8
	R_2	7.9	9.48E+03	3.50E+04	9.91E+04	5.97E+05	9.9
	R_3	7.9	1.09E+04	3.46E+04	1.12E+05	7.07E+05	9.9
	Mean		1.01E+04	3.52E+04	1.06E+05	6.27E+05	
2.3	R_1	7.9	1.09E+04	2.65E+04	1.00E+05	5.83E+05	9.8
	R_2	7.9	1.02E+04	2.31E+04	7.89E+04	5.19E+05	9.8
	R_3	7.9	9.96E+03	2.89E+04	8.74E+04	5.79E+05	9.9
	Mean		1.03E+04	2.62E+04	8.89E+04	5.60E+05	
3.1	R_1	7.9	9.17E+03	1.16E+04	6.03E+04	3.77E+05	9.6
	R_2	7.9	1.03E+04	9.08E+03	5.35E+04	3.64E+05	9.6
	R_3	7.9	9.96E+03	1.19E+04	6.49E+04	3.94E+05	9.7
	Mean		9.80E+03	1.09E+04	5.96E+04	3.78E+05	
6.7	R_1	7.9	1.06E+04	4.53E+03	3.92E+04	1.53E+05	9.5
	R_2	7.9	1.01E+04	3.21E+03	3.60E+04	1.45E+05	9.5
	R_3	7.9	1.05E+04	4.45E+03	3.77E+04	1.50E+05	9.5
	Mean		1.04E+04	4.06E+03	3.77E+04	1.49E+05	
13	R_1	7.9	1.17E+04	1.34E+03	5.16E+03	2.78E+03	8.5
	R_2	7.9	9.76E+03	4.29E+03	4.31E+03	2.42E+03	8.4
	R_3	7.9	9.46E+03	8.20E+02	5.54E+03	3.36E+03	8.4
	Mean		1.03E+04	2.15E+03	5.00E+03	2.85E+03	

* Cell densities represent the mean number of cells per ml calculated from the mean of the cell counts from 3 counts for each of the replicate flasks.

$R_1 - R_3$ = Replicates 1 to 3

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Table 3 Inhibition of Growth Rate and Biomass

Mean Measured Test Concentration (mg/l)	Area Under Curve at 72 h	% Inhibition	Growth Rate (0-72 h)	% Inhibition
Control	1.00E+07	-	0.058	-
1.3	1.03E+07	[3]	0.057	2
2.3	8.86E+06	12	0.055	5
3.1	5.64E+06	44	0.051	12
6.7	2.17E+06	78	0.037	36
13	-4.12E+05	104	-0.018	131

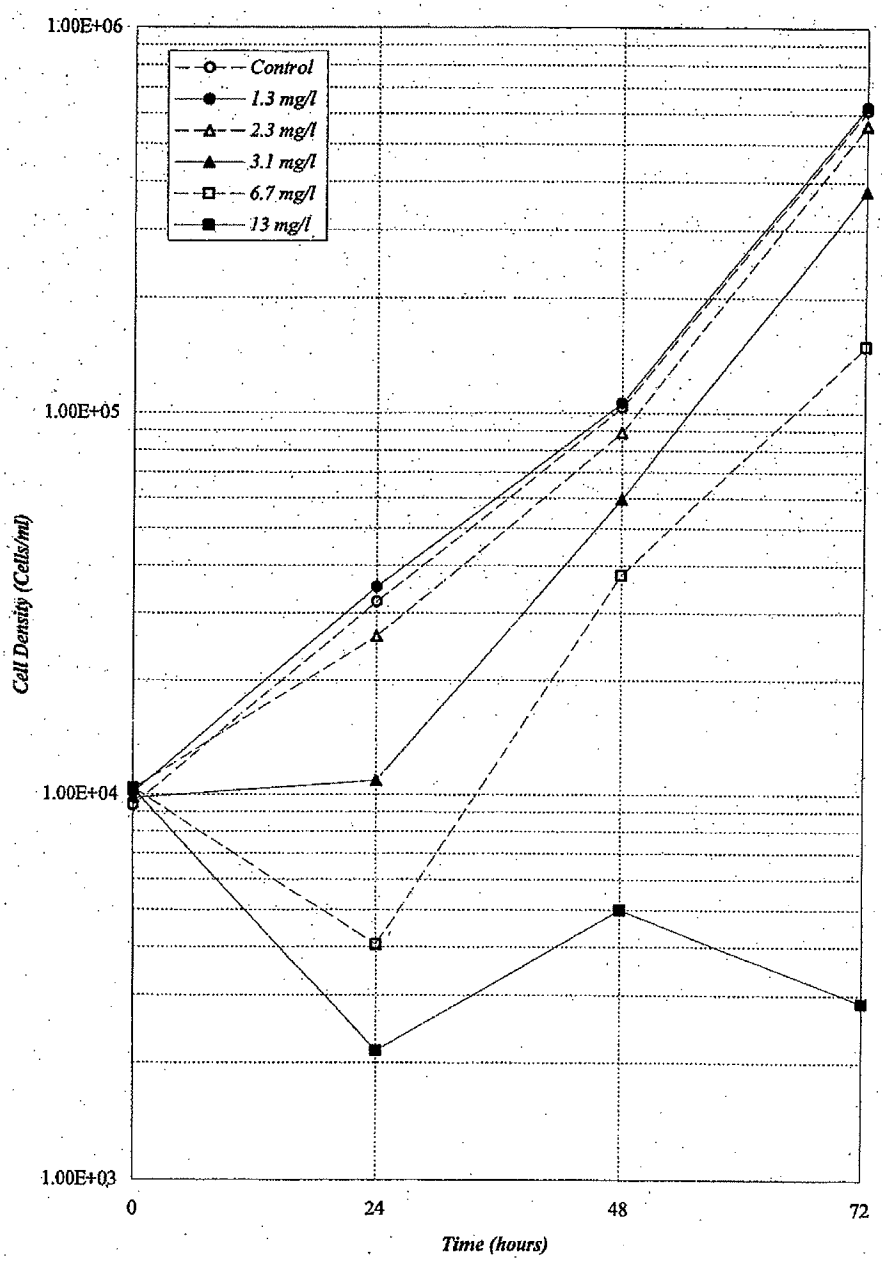
[Increase in growth as compared to the controls]

E_bC_{50} (72 h) from Figure 2 = 3.8 mg/l

E_rC_{50} (0 – 72 h) from Figure 3 = 7.8mg/l

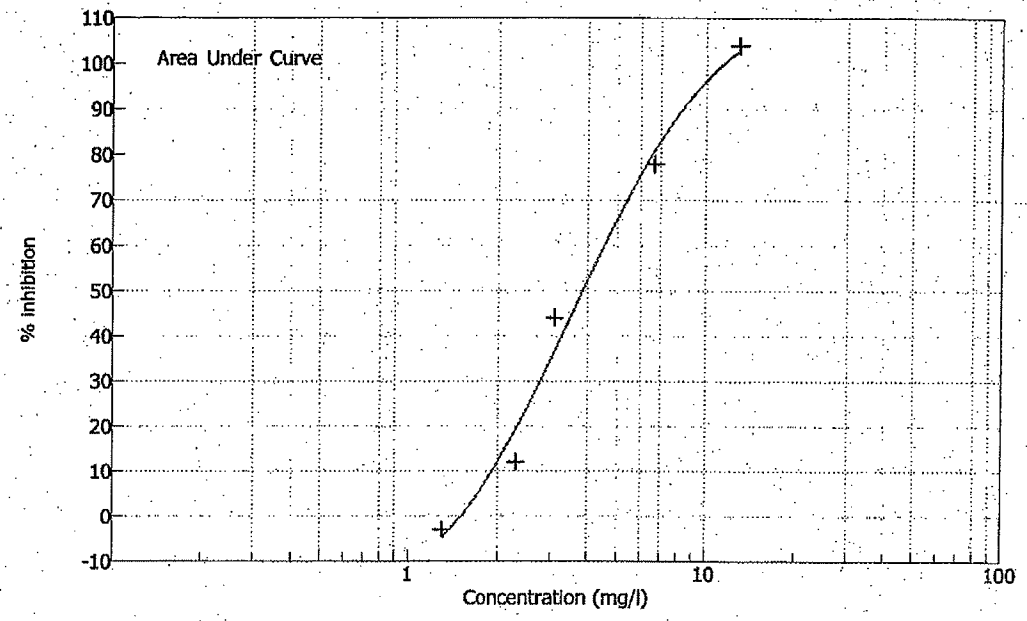
C6-2AL: ALGAL INHIBITION TEST

Figure 1 Mean Cell Densities v Time for the Definitive Test



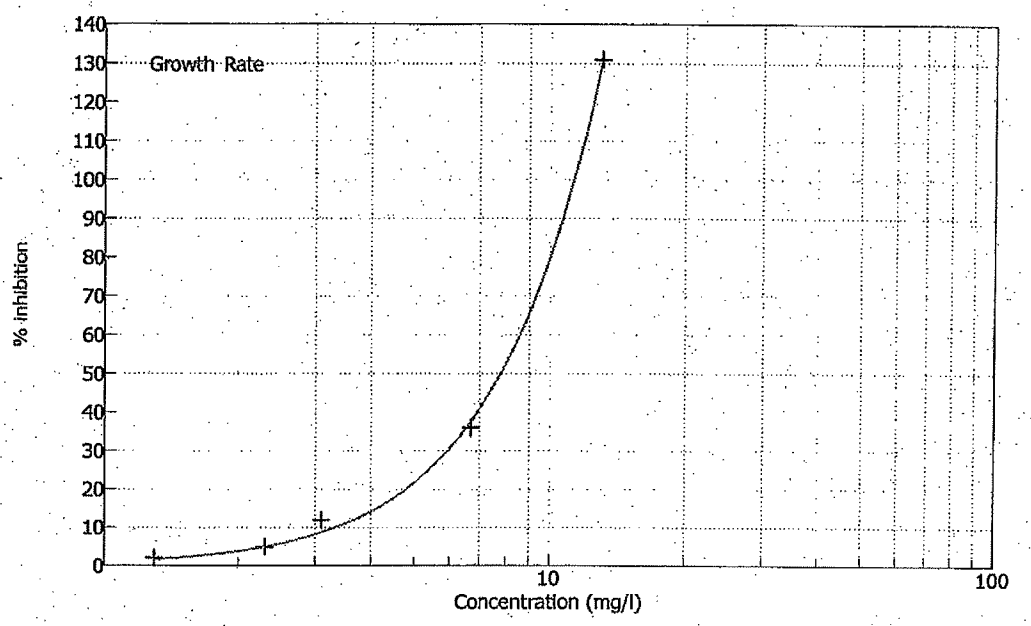
C6-2AL: ALGAL INHIBITION TEST

Figure 2 Inhibition of Biomass Based on Mean Measured Test Concentrations



C6-2AL: ALGAL INHIBITION TEST

Figure 3 Inhibition of Growth Rate Based on Mean Measured Test Concentrations



C6-2AL: ALGAL INHIBITION TEST

Appendix 1 Verification of Test Concentrations

1. METHOD OF ANALYSIS

1.1 Introduction

The test material concentration in the test samples was determined by gas chromatography (GC) using an external standard. The test material gave a chromatographic profile consisting of a single peak.

The method was developed by the Department of Analytical Services, Safepharm Laboratories Limited.

1.2 Sample Preparation

A Strata X solid phase extraction (SPE) cartridge packed with glass wool, to remove algae, was sequentially pre-conditioned with methanol and water. A volume of test sample was eluted through the cartridge and the cartridge kept wet. The test material was eluted from the cartridge with methanol and made to volume to give final theoretical concentrations of approximately 13 to 16.5 mg/l.

1.3 Standards

Standard solutions of test material were prepared in methanol at a nominal concentration of 20 mg/l.

1.4 Procedure

The standards and samples were analysed by GC using the following conditions:

GC System	:	Agilent Technologies 5890, incorporating autosampler and workstation
Column	:	DB-5 and ZB-5(30 m x 0.53 mm id, 5 µm film)
Oven temperature program	:	initial 60 °C for 2 minutes rate 1 10 °C/minute final 150°C

C6-2AL: ALGAL INHIBITION TEST

Appendix 1 (continued) Verification of Test Concentrations

Injector temperature	:	200°C
Detector temperature	:	250°C
Carrier gas and pressure	:	nitrogen at 5 psi
Injection volume	:	1 µl
Injection mode	:	Splitless purge on at 1 minutes
Detector	:	flame ionisation detector (FID)
Retention time	:	approximately 7 minutes

2. VALIDATION

2.1 Linearity

A range of standard solutions covering 1.0 to 98 mg/l (5 % to 490 % of the standard concentration) was analysed.

Linearity was confirmed (percentage fit, R^2 =0.9998) ranging from 1.0 to 98 mg/l.

The results are presented graphically on page 26.

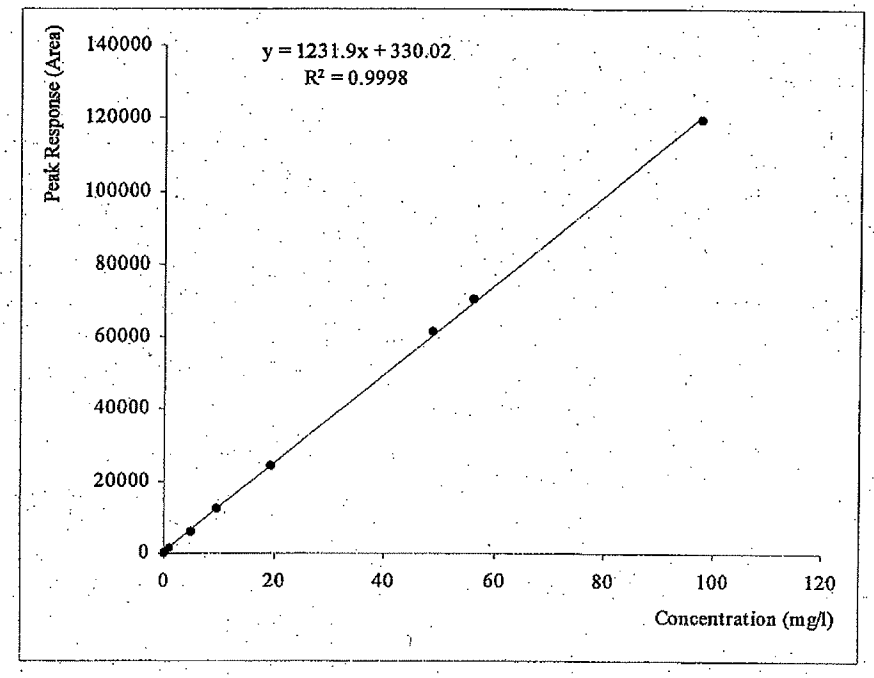
2.2 Spiked Recoveries

The recovery samples were prepared by addition of a standard solution of test material to a sample of test medium. A standard solution was accurately prepared by dissolving the test material in methanol. An accurate volume of the standard solution was added to a known volume of test medium to achieve the required concentration of test material in test medium.

C6-2AL: ALGAL INHIBITION TEST

Appendix 1 (continued) Verification of Test Concentrations

Linearity of Detector Response



C6-2AL: ALGAL INHIBITION TEST

Appendix 1 (continued) Verification of Test Concentrations

Fortification (mg/l)	Recoveries		
	(mg/l)	(%)	Mean %
0.970	0.448	46	43
0.970	0.381	39	
0.970 plus algae	0.453	47	47
3.88	2.11	54	57
3.88	2.32	60	
15.5	10.9	71	72
15.5	11.3	73	
15.5 plus algae	9.19	59	59

The recovery results are outside the accepted limits of 80-120%. Procedural recoveries were run alongside the test samples at each level. The method has been considered to be sufficiently accurate for the purposes of this test. All test sample results have been corrected for recovery.

The presence of algal cells was considered to have no significant effect on the recovery of the test material from the medium.

2.3 Saturated Solution Recoveries

The recovery samples were prepared by direct addition of the test material to a sample of test medium. The dispersion was stirred using a propeller set at 1500 rpm for 24 hours at 21°C. After the stirring period, undissolved test material was removed by filtration through a pre-conditioned 0.2 µm Gelman Acrocap filter.

Nominal concentration (mg/l)	Recoveries		
	(mg/l)	(%)	Mean %
0.810	0.557	69	76
0.810	0.676	83	
0.810 plus algae	0.489	60	60
3.30	1.83	55	60
3.30	2.10	64	
13.0	7.15	55	57
13.0	7.78	60	
13.0 plus algae	6.43	49	49

Appendix 1 (continued) Verification of Test Concentrations

The method has been considered to be sufficiently accurate for the purposes of this test. All test sample results have been corrected for recovery.

The presence of algal cells was considered to have no significant effect on the recovery of the test material from the medium.

2.4 Limit of Quantitation

The limit of quantitation has been assessed down to 0.017 mg/l.

3 STABILITY

3.1 Spiked Stability

A range of preliminary test samples, was prepared, analysed initially and then after storage in sealed glass vessels at ambient temperature in light and dark conditions for approximately 72 hours (equivalent to the test exposure period). In addition a test sample was tested for stability without prior mixing (sonication) the sample bottle to assess for losses due to adsorption and/or insolubility.

Nominal concentration (mg/l)	0.810	3.30	13.0
Concentration found initially (mg/l)	0.415	2.22	11.1
Concentration found after storage in light conditions (mg/l)	0.127	1.52	9.10
Expressed as a percent of the initial concentration	31	69	82
Concentration found after storage in dark conditions (mg/l)	0.305	1.92	9.90
Expressed as a percent of the initial concentration	73	87	89
Concentration found after storage in dark conditions (mg/l) – unsonicated sample	0.310	NA	9.24
Expressed as a percent of the initial concentration	75	-	83

The test samples have shown some evidence of instability in the test medium at the lower levels. The unsonicated stability vessel showed no evidence of insolubility or adherence to glass.

NA = Not applicable

Appendix 1 (continued) Verification of Test Concentrations

3.2 Saturated Solution Stability

A range of preliminary test samples was prepared, as in Section 2.3, analysed initially and then after storage in at ambient temperature in light and dark conditions for approximately 72 hours (equivalent to the test exposure period). In addition a test sample was tested for stability without prior mixing (sonication) the test sample bottle to assess for losses due to adsorption and/or insolubility.

Nominal concentration (mg/l)	0.810	3.30	13.0
Concentration found initially (mg/l)	0.617	1.97	7.47
Concentration found after storage in light conditions (mg/l)	0.477	1.56	6.77
Expressed as a percent of the initial concentration	77	80	91
Concentration found after storage in dark conditions (mg/l)	0.492	1.59	6.44
Expressed as a percent of the initial concentration	80	81	86
Concentration found after storage in dark conditions (mg/l) – unsonicated sample.	0.403	NA	6.68
Expressed as a percent of the initial concentration	65	-	90

The test samples have shown some evidence of instability in the test medium at the lower levels.

The unsonicated stability vessel showed no evidence of insolubility or adherence to glass.

3.3 Volatility Stability

A range of preliminary test samples was prepared, as in Section 2.3, analysed initially and then after storage in open glass vessels at ambient temperature in dark conditions for approximately 72 hours (equivalent to the test exposure period). All samples were found to be less than the limit of quantitation.

NA = Not applicable

C6-2AL: ALGAL INHIBITION TEST

Appendix 1 (continued) Verification of Test Concentrations

4. RESULTS

Sample	Nominal Concentration (mg/l)	Concentration Found (mg/l)*	Expressed as a Percent of the Nominal Concentration (%)
0 Hours	Control	<LOQ	-
	0.810	1.51	187
	1.60	2.07	129
	3.30	3.29	100
	6.50	5.84	90
	13.0	10.8	83
72 Hours Open vessel	Control	<LOQ	-
	0.810	1.15	142
	1.60	2.46	154
	3.30	2.83	86
	6.50	7.52	116
	13.0	15.2	117
72 Hours unopened vessel	Control	<LOQ	-
	0.810	2.24	277
	1.60	2.69	168
	3.30	4.42	134
	6.50	9.24	142
	13.0	14.7	113

5. DISCUSSION

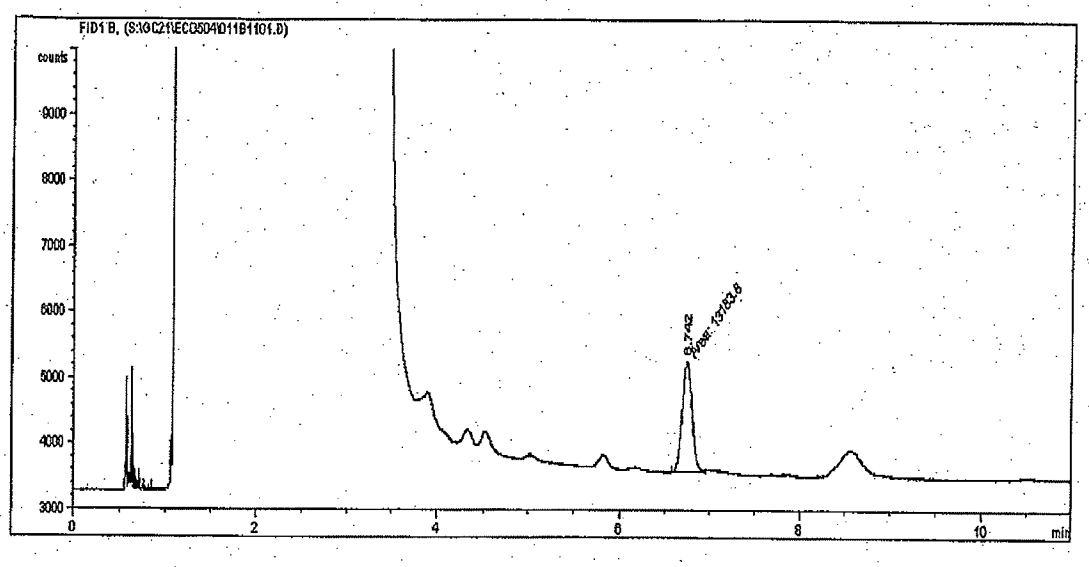
The detection system was found to have acceptable linearity. The analytical procedure had acceptable recoveries of test material in test medium. A method of analysis was validated and proven to be suitable for use.

* Results corrected for corresponding procedural recovery
LOQ = Limit of quantitation

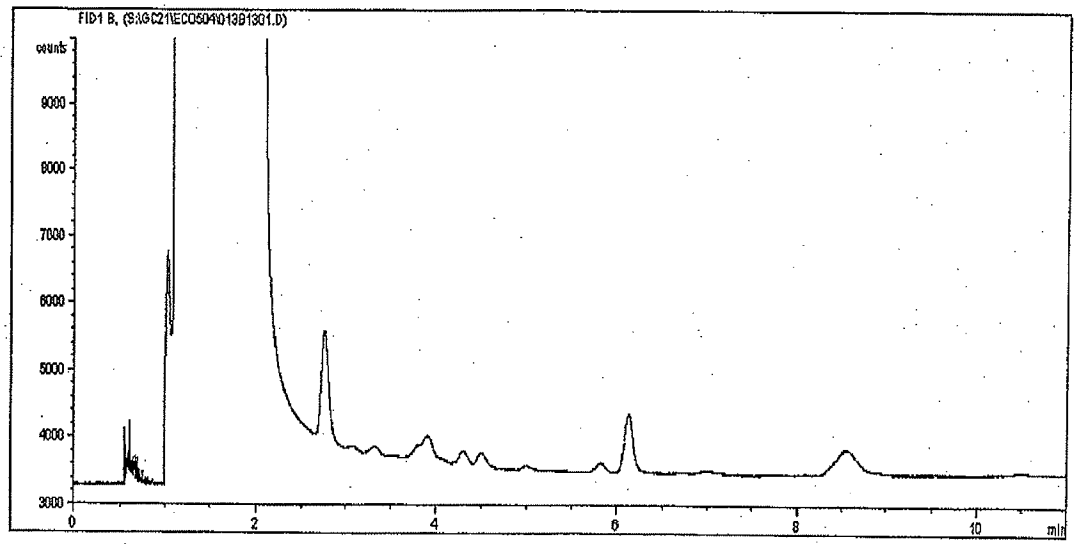
C6-2AL: ALGAL INHIBITION TEST

Appendix 1 (continued) Verification of Test Concentrations

6. TYPICAL CHROMATOGRAPHY



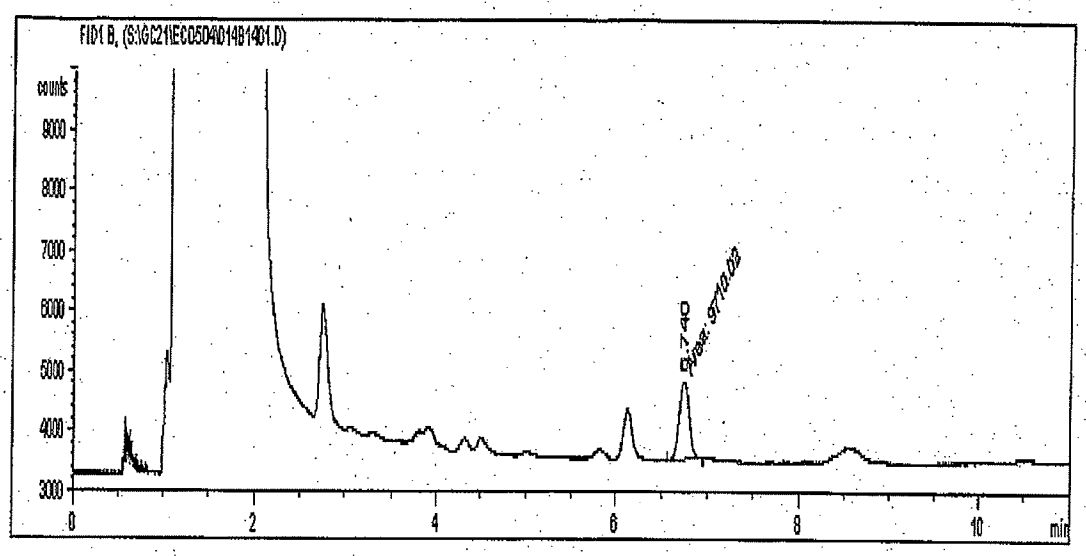
Standard 20 mg/l



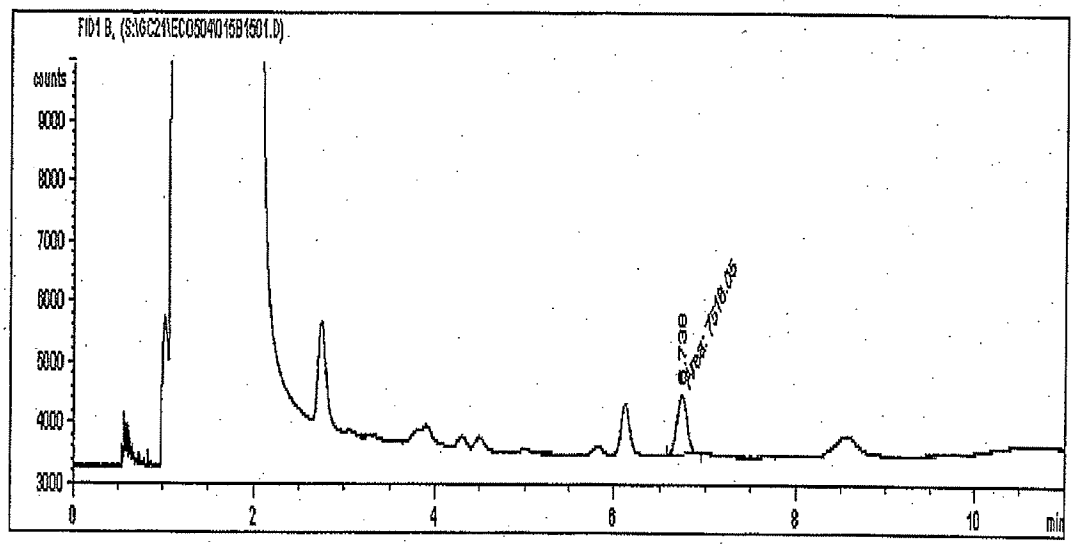
Control Sample

C6-2AL: ALGAL INHIBITION TEST

Appendix 1 (continued) Verification of Test Concentrations



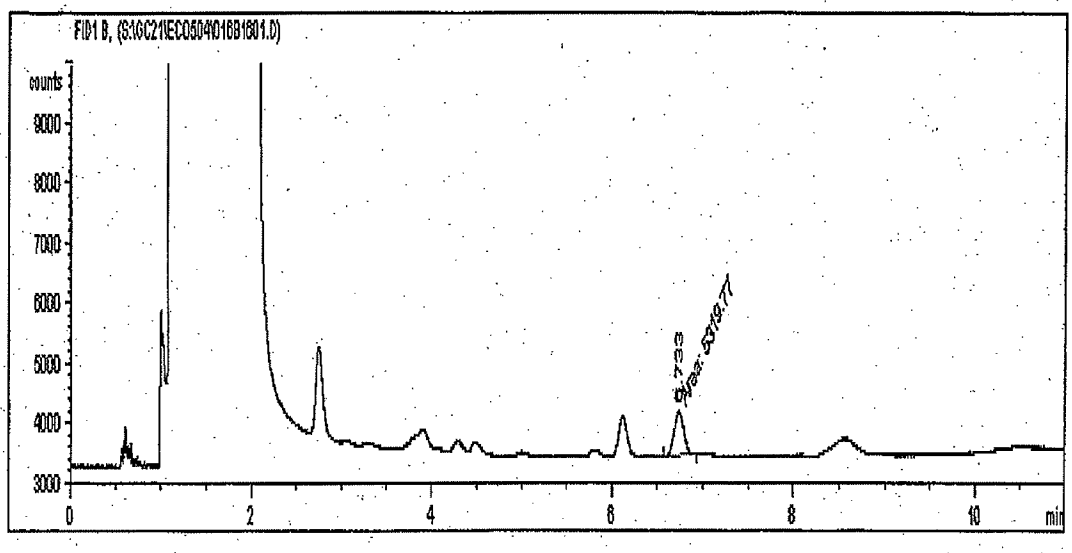
Test Sample 0.81 mg/l



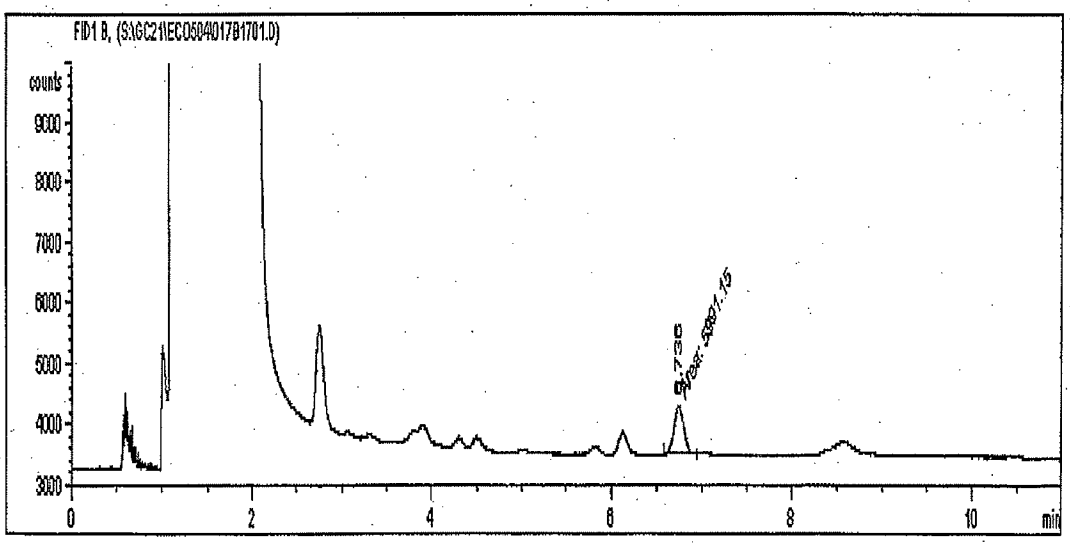
Test Sample 1.6 mg/l

C6-2AL: ALGAL INHIBITION TEST

Appendix 1 (continued) Verification of Test Concentrations



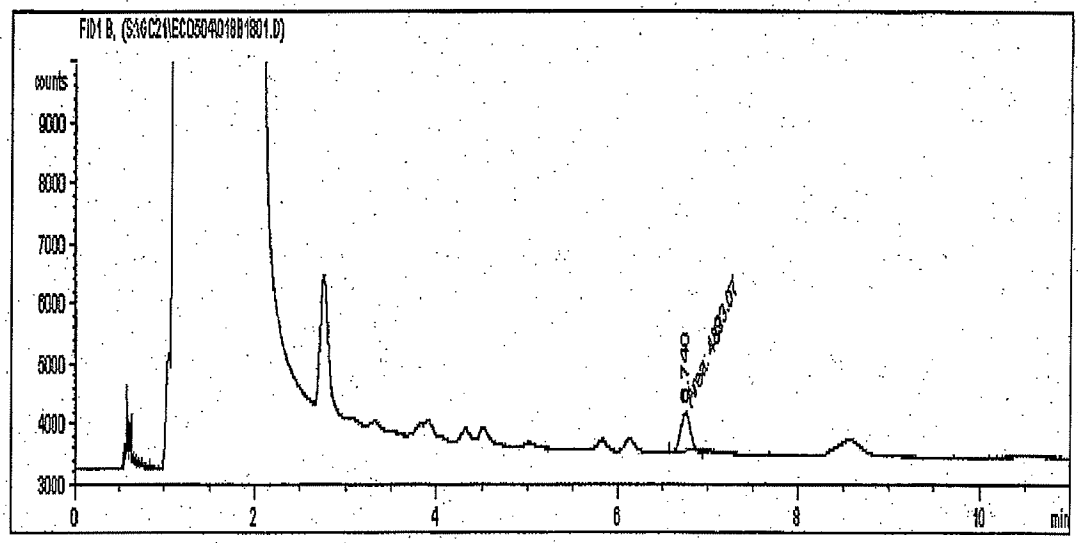
Test Sample 3.3 mg/l



Test Sample 6.5 mg/l

C6-2AL: ALGAL INHIBITION TEST

Appendix 1 (continued) Verification of Test Concentrations



Test Sample 13 mg/l

C6-2AL: ALGAL INHIBITION TEST

Appendix 2 Culture Medium

$NaNO_3$	25.5	mg/l
$MgCl_2.6H_2O$	12.164	mg/l
$CaCl_2.2H_2O$	4.41	mg/l
$MgSO_4.7H_2O$	14.7	mg/l
K_2HPO_4	1.044	mg/l
$NaHCO_3$	15.0	mg/l
H_3BO_3	0.1855	mg/l
$MnCl_2.4H_2O$	0.415	mg/l
$ZnCl_2$	0.00327	mg/l
$FeCl_3.6H_2O$	0.159	mg/l
$CoCl_2.6H_2O$	0.00143	mg/l
$Na_2MoO_4.2H_2O$	0.00726	mg/l
$CuCl_2.2H_2O$	0.000012	mg/l
$Na_2EDTA.2H_2O$	0.30	mg/l
$Na_2SeO_3.5H_2O$	0.000010	mg/l

The culture medium was prepared using reverse osmosis purified deionised water* and the pH adjusted to 7.5 ± 0.1 with 0.1N NaOH or HCl. The prepared media was sterilised by 0.2 µm membrane filtration.

* Elga Optima 15+ or Elga Purelab Option R-15 BP

Appendix 3 Statement of GLP Compliance in Accordance with Directive 88/320/EEC

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY
SafePharm Limited
Shardlow Business Park,
London Road,
Shardlow,
Derbyshire,
DE72 2GD

TEST TYPE
Analytical/Clinical
Chemistry
Environmental tox.
Environmental fate
Mutagenicity
Phys./Chem. tests
Toxicology

DATE OF INSPECTION

2nd December 2002

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Roger G. Alexander
13/2/03

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority