SafePharm Laboratories

Dibromomethane:

ALGAL GROWTH INHIBITION TEST

SPL PROJECT NUMBER: 0466/0265

OECD GUIDELINE NO. 201; US CODE OF FEDERAL REGULATIONS TITLE 40, PART 797, SECTION 1050; US EPA PESTICIDE ASSESSMENT GUIDELINES, SUB-DIVISION J, SECTION 122-2, US EPA DRAFT ECOLOGICAL EFFECTS TEST GUIDELINE OPPTS 850.5400 AND ASTM E 1218-04

AUTHORS: H Vryenhoef
D Mullee

CO-SPONSORS:

Bromine Compounds Ltd.
Makleff House
P.O.B. 180
BEER-SHEVA 84101
ISRAEL

Albemarle Corporation
Health, Safety and Environment
451 Florida Street
Baton Rouge
LA 70801
UNITED STATES OF AMERICA

TEST FACILITY:

Safepharm Laboratories Limited
Shardlow Business Park
Shardlow
Derbyshire
DE72 2GD
UK

Telephone: +44 (0) 1332 792896

Facsimile: +44 (0) 1332 799018

CONTAINS NO CBI

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:

	12 September 2006	Standard Test Method Compliance Audit
	19 February 2007	Test Material Preparation
	20 February 2007	Test System Preparation
	20 February 2007	Exposure
	14 February 2007	Assessment of Response
	06, 23 February 2007	Chemical Analysis
§	22 March 2007	Draft Report Audit
§	Date of QA Signature	Final Report Audit

§ Evaluation specific to this study

..... DATE: 15 MAY 2007

For Safepharm Quality Assurance Unit*

***Authorised QA Signatures:**

Head of Department:	JR Pateman CBiol MIBiol DipRQA AIQA FRQA
Deputy Head of Department:	JM Crowther MIScT MRQA
Senior Audit Staff:	JV Johnson BSc MRQA; G Wren ONC MRQA

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.

Specifically these GLP standards are acceptable to the United States Environmental Protection Agency, fulfilling the requirements of 40CFR Part 792, as amended.

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

..... H. Vryenhoef ..... Date: ..... 15 MAY 2007 .....

H Vryenhoef BSc
Study Director

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Dibromomethane:

ALGAL GROWTH INHIBITION TEST

SUMMARY

Introduction. A study was performed to assess the effect of the test material on the growth of the green alga *Pseudokirchneriella subcapitata*. 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), US CFR Title 40 Part 797 Section 1050, US EPA Pesticide Assessment Guidelines, Sub-Division J, Section 122-2, the US EPA Draft Ecological Effects Test Guideline OPPTS 850.5400 and ASTM E 1218-04.

Methods. Following a preliminary range-finding test, *Pseudokirchneriella subcapitata* was exposed to an aqueous solution of the test material at concentrations of 10, 32, 100, 320 and 1000 mg/l (three replicate flasks per concentration) for 96 hours, under constant illumination and shaking at a temperature of 24 ± 1°C.

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

Results. In terms of growth rate, exposure of *Pseudokirchneriella subcapitata* to the test material gave an E_rC_{50} (0 - 72 h) value of 190 mg/l* and an E_rC_{50} (0 - 96 h) value of 210 mg/l*. The No Observed Effect Concentration was 32 mg/l.

In terms of yield, exposure of *Pseudokirchneriella subcapitata* to the test material gave an E_yC_{50} (0 - 72 h) value of 100 mg/l; 95% confidence limits 84 - 130 mg/l and an E_yC_{50} (0 - 96 h) value of 130 mg/l; 95% confidence limits 120 - 140 mg/l. The No Observed Effect Concentration was 32 mg/l.

In terms of biomass integral, exposure of *Pseudokirchneriella subcapitata* to the test material gave an E_bC_{50} (0 - 72 h) value of 96 mg/l; 95% confidence limits 79 - 120 mg/l and an E_bC_{50}

* It was not possible to calculate 95% confidence limits for these E_rC_{50} values as the data generated did not fit the models available for the calculation of confidence limits.

(0 - 96 h) value of 110 mg/l; 95% confidence limits 96 - 140 mg/l. The No Observed Effect Concentration was 32 mg/l.

Analysis of the test preparations at 0 hours showed measured test concentrations to be near nominal with the exception of the 32 mg/l test sample which showed a measured concentration of 79% of nominal. Analysis of the test preparations at 96 hours showed a slight decline in measured test concentrations in the range 64% to 101% of nominal.

Due to the volatile nature of the test material, additional test replicates were prepared at 0 hours and incubated alongside the test to provide samples for analysis at 96 hours from unopened vessels. Analysis of these preparations showed measured test concentrations to range from 81% to 106% of nominal. As these measured concentrations were near nominal it was considered that the decline seen in the 96 hour test samples was through losses due to volatility.

Given this decline in measured test concentrations it was considered justifiable to base the results on the geometric mean measured test concentrations in order to give a "worst case" analysis of the data.

In terms of growth rate, exposure of *Pseudokirchneriella subcapitata* to the test material based on the geometric mean measured test concentrations gave an E_rC_{50} (0 - 72 h) value of 140 mg/l* and an E_rC_{50} (0 - 96 h) value of 150 mg/l*. The No Observed Effect Concentration was 23 mg/l.

In terms of yield, exposure of *Pseudokirchneriella subcapitata* to the test material based on the geometric mean measured test concentrations gave an E_yC_{50} (0 - 72 h) value of 76 mg/l; 95% confidence limits 62 - 93 mg/l and an E_yC_{50} (0 - 96 h) value of 95 mg/l; 95% confidence limits 87 - 100 mg/l. The No Observed Effect Concentration was 23 mg/l.

In terms of biomass integral, exposure of *Pseudokirchneriella subcapitata* to the test material based on the geometric mean measured test concentrations gave an E_bC_{50} (0 - 72 h) value of 72 mg/l; 95% confidence limits 59 – 87 mg/l and an E_bC_{50} (0 - 96 h) value of 87 mg/l; 95% confidence limits 74 - 100 mg/l. The No Observed Effect Concentration was 23 mg/l.

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* It was not possible to calculate 95% confidence limits for these E_rC_{50} values as the data generated did not fit the models available for the calculation of confidence limits.

A re-growth experiment was conducted which indicated that the test material was algistatic in effect.

Exposure of *Pseudokirchneriella subcapitata* to the reference material, zinc chloride, gave an E_bC_{50} (0 - 72 h) value of 0.14 mg/l; 95% confidence limits 0.12 - 0.16 mg/l, an E_bC_{50} (0 - 96 h) value of 0.17 mg/l* and an E_rC_{50} (0 - 96 h) value of 0.39 mg/l*. The No Observed Effect Concentration was 0.10 mg/l.

The results from the positive control with zinc chloride were within the normal range for this reference material.

Dibromomethane:

ALGAL GROWTH 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 *Pseudokirchneriella subcapitata*. 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), US CFR Title 40 Part 797 Section 1050, US EPA Pesticide Assessment Guidelines, Sub-Division J, Section 122-2, the US EPA Draft Ecological Effects Test Guideline OPPTS 850.5400 and ASTM E 1218-04.

Pseudokirchneriella subcapitata 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 12 October 2006 and 16 October 2006 and the definitive test between 29 January 20007 and 2 February 2007. The re-growth test was conducted between 2 February 2007 and 10 February 2007.

The positive control (Safepharm Laboratories Project Number: 0039/0883) was conducted between 30 October 2006 and 3 November 2006.

2. TEST MATERIAL AND EXPERIMENTAL PREPARATION

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	Dibromomethane
CAS No.		74-95-3
Description	:	clear colourless liquid
Purity	:	99.4% (see Appendix 1)
Date received	:	14 September 2006
Storage conditions	:	room temperature in the dark

Individual samples of dibromomethane were supplied by both Co-Sponsors. The two test materials were then mixed in equal proportions and the resulting mixture analysed to determine its purity under Safepharm Laboratories Ltd Project Number: 0466/0267. The Certificate of Analysis generated under Safepharm Laboratories Ltd Project Number: 0466/0267, including the original details provided by each Co- Sponsor, the analytical parameters and the resulting test

material purity is given in Appendix 1. This mixed material was used for all experimental testing detailed in this report. The integrity of supplied data relating to the identity and stability of the test material is the responsibility of the Co-Sponsors. The purity of the test material has been addressed under Safepharm Laboratories Project Number: 0466/0267. The Certificate of Analysis generated is included as Appendix 1.

2.2 Experimental Preparation

For the purpose of the definitive test, the test material was dissolved directly in culture medium.

Amounts of test material (2000 and 640 mg) were each separately dissolved in culture medium with the aid of ultrasonication for approximately 20 minutes and the volumes adjusted to 2 litres to give 1000 and 320 mg/l stock solutions respectively. A series of dilutions was made from these stock solutions to give further stock solutions of 100, 32 and 10 mg/l. An aliquot (1800 ml) of each of the stock solutions was separately inoculated with algal suspension (9.0 ml) to give the required test concentrations of 10, 32, 100, 320 and 1000 mg/l.

The stock solutions and each of the prepared concentrations were inverted several times to ensure adequate mixing and homogeneity.

The concentration and stability of the test material in the test preparations were verified by chemical analysis at 0 and 96 hours (see Appendix 2).

2.3 Reference Material

A positive control (Safepharm Laboratories Project Number 0039/0883) used zinc chloride (Fisher Chemicals Lot No 0694439) as the reference material. An amount of reference material (50 mg) was dissolved in culture medium and the volume adjusted to 500 ml to give a 100 mg/l stock solution from which a series of dilutions were made to give a further stock solutions of 10, 2.0, 0.64, 0.20, 0.064 and 0.020 mg/l. An aliquot (250 ml) of each of the 0.020, 0.064, 0.20, 0.64 and 2.0 mg/l stock solutions was separately mixed with algal suspension (250 ml) to give the required test concentrations of 0.010, 0.032, 0.10, 0.32 and 1.0 mg/l.

3. METHODS

3.1 Test Species

The test was carried out using *Pseudokirchneriella subcapitata* strain CCAP 278/4 (formerly known as *Selenastrum capricornutum*). Liquid cultures of *Pseudokirchneriella subcapitata* were

obtained from the Culture Collection of Algae and Protozoa (CCAP), Dunstaffnage Marine Laboratory, Oban, Argyll, Scotland. 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 for both the range-finding and definitive tests was the same as that used to maintain the stock culture with the addition of 500 mg/l of sodium bicarbonate to counteract the increase in pH due to algal growth in an enclosed system.

The culture medium is defined in Appendix 3.

3.3 Procedure

3.3.1 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 *Pseudokirchneriella subcapitata* cells to a series of nominal test concentrations of 0.10, 1.0, 10, 100 and 1000 mg/l for a period of 96 hours.

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 test material was dissolved directly in culture medium.

An amount of test material (2000 mg) was dissolved in culture medium with the aid of ultrasonication for approximately 10 minutes and the volume adjusted to 1 litre to give a 2000 mg/l stock solution from which a series of dilutions was made to give further stock solutions of 200, 20, 2.0 and 0.20 mg/l. An aliquot (500 ml) of each of the stock solutions was separately mixed with algal suspension (500 ml) to give the required test concentrations of 0.10, 1.0, 10, 100 and 1000 mg/l.

The stock solutions and each of the prepared concentrations were inverted several times to ensure adequate mixing and homogeneity.

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 96 hours.

After 96 hours the cell density of each flask was determined using a Coulter® Multisizer Particle Counter.

3.3.2 Definitive test

Based on the results of the range-finding test and in order to ensure that both EC_{50} and NOEC values were obtained the following test concentrations were assigned to the definitive test: 10, 32, 100, 320 and 1000 mg/l.

3.3.2.1 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.

3.3.2.2 Exposure conditions

As in the range-finding test completely filled 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.

Due to the volatile nature of the test material an additional replicate was prepared for the control and each treatment group at 0 hours and incubated alongside the test to provide samples at 96 hours from unopened vessels.

Pre-culture conditions gave an algal suspension in log phase growth characterised by a cell density of 6.77×10^6 cells per ml. This suspension was diluted to a cell density of 1.98×10^6 cells per ml prior to use. Inoculation of 900 ml of test medium with 4.5 ml of this algal suspension gave an initial cell density of 10^4 cells per ml and had no significant dilution effect on the final test concentration.

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 96 hours.

Samples were taken at 0, 24, 48, 72 and 96 hours and the cell densities determined using a Coulter® Multisizer Particle Counter.

3.3.2.3 Physico-chemical measurements

The pH of each control and test flask was determined at initiation of the test and after 96 hours exposure. The pH was measured using a WTW pH 320 pH meter. The temperature within the incubator was recorded hourly using the incubator's internal pt-100 temperature probe and Multicom Software package (see Figure 14).

3.3.2.4 Verification of test concentrations

Samples were taken from the control and each test group (replicates R_1 - R_3 pooled) at 0 and 96 hours for quantitative analysis. Duplicate samples were taken at each occasion and stored at approximately -20°C for further analysis if necessary.

Given the volatile nature of the test material a fourth test replicate was prepared at each test concentration and incubated alongside the test remaining unopened until the end of the test. A sample of each of these additional test replicates was taken for chemical analysis at 96 hours.

The method of analysis, stability, recovery and test preparation analyses are described in Appendix 2.

3.3.3 Evaluation of data

3.3.3.1 Comparison of growth rates

The average specific growth rate for a specified period is calculated as the logarithmic increase in biomass from the equation:

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

where:

μ = average specific growth rate from time t_1 to t_n
N_1 = cell concentration at t_1
N_n = cell concentration at t_n
t_1 = time of first measurement
t_n = time of nth measurement

The average specific growth rate over the test duration was calculated for each replicate control and test material vessel using the nominally inoculated cell concentration as the starting value rather than the measured starting value in order to increase the precision of the calculation.

In addition the average daily growth rate (days 0-1, 1-2, 2-3 and 3-4) was calculated for the control cultures and the results examined in order to determine whether the growth rate remained constant.

Percentage inhibition of growth rate for each replicate test material vessel was calculated using the following equation:

$$I_r = \frac{\mu_c - \mu_t}{\mu_c} \times 100$$

where:

I_r = percentage inhibition of average specific growth rate
μ_c = mean average specific growth rate for the control cultures
μ_t = average specific growth rate for the test culture

3.3.3.2 Comparison of Yield

Yield is calculated as the increase in biomass over the exposure period using the following equation:

$$Y = N_n - N_0$$

where:

Y = Yield
N_0 = cell concentration at the start of the test
N_n = cell concentration at the end of the test

For each test concentration and control the mean value for yield along with the standard deviation is calculated. Percentage inhibition of yield is calculated using the following equation:

$$I_y = \frac{(Y_c - Y_t)}{Y_c} \times 100$$

where

I_y	=	percentage inhibition of yield
Y_c	=	mean value for yield in the control group
Y_t	=	mean value for yield for the treatment group

3.3.3.3 Comparison of biomass integral

The biomass integral (area under the growth curve) 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	=	nominal cell concentration at start of test
N_1	=	measured cell concentration at t_1
N_n	=	measured cell concentration at t_n
t_1	=	time of first measurement after beginning of test
t_n	=	time of nth measurement after beginning of test

Percentage inhibition of the biomass integral for each replicate test material vessel was calculated using the following equation:

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

where:

I_A	=	percentage inhibition of the biomass integral
A_c	=	mean log-biomass integral for the control cultures
A_t	=	biomass integral for the test culture

3.3.3.4 Determination of EC_x values

For each individual test vessel (mean values for yield), percentage inhibition (arithmetic axis) was plotted against test concentration (logarithmic axis) and a line fitted by computerised interpolation using the Xlfit software package (IDBS). EC_x values were then determined from the equation for the fitted line.

Where appropriate 95% confidence limits for the EC_{50} values were calculated, using the simplified method of evaluating dose-effect experiments of Litchfield and Wilcoxon (1949).

3.3.3.5 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 96 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).

3.3.3.6 Geometric mean measured test concentrations

The geometric mean measured test concentrations of the samples were calculated as follows using the measured test concentrations of replicates R_1 - R_3 pooled:

$$GM = \sqrt{C_0 \times C_1}$$

where

GM	=	geometric mean measured test concentration (mg/l)
C_0	=	measured concentration at the start of the test (mg/l)
C_1	=	measured concentration at the end of the test (mg/l)

3.4 Positive Control

A positive control (Safepharm Laboratories Project Number: 0039/0883) using zinc chloride as the reference material was conducted using test concentrations of 0.010, 0.032, 0.10, 0.32 and 1.0 mg/l.

The required amount of reference material was added to the test vessels using the method described in Section 2.3.

Exposure conditions for the positive control were the same as used in the definitive test.

3.4.1 Evaluation of data for the positive control

3.4.1.1 Comparison of growth rates

Average specific growth rates and inhibition of growth rate were calculated as in Section 3.3.3.1.

3.4.1.2 Comparison of Yield

Yield and percentage inhibition of yield were calculated as in Section 3.3.3.2.

3.4.1.3 Comparison of biomass integral

The biomass integral (area under the growth curve) and inhibition of the biomass integral were calculated as in Section 3.3.3.3.

3.4.1.4 Determination of EC_x values

For each individual test vessel (mean values for yield), percentage inhibition (arithmetic axis) was plotted against test concentration (logarithmic axis) and a line fitted by computerised interpolation using the Xlfit software package (IDBS).

Where appropriate 95% confidence limits for the EC_{50} values were calculated, using the simplified method of evaluating dose-effect experiments of Litchfield and Wilcoxon (1949).

3.4.1.5 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 96 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 Range-finding Test

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

The results showed no significant effect on growth at the test concentrations of 0.10, 1.0 and 10 mg/l. However, growth was observed to be reduced at 100 and 1000 mg/l.

Based on this information test concentrations of 10, 32, 100, 320, and 1000 mg/l were selected for the definitive test.

5.2 Definitive Test

5.2.1 Growth data

Cell density values determined at each sampling time and pH values at 0 and 96 hours are given in Table 2. Daily specific growth rates for the control cultures are given in Table 3. Growth rates, yield and biomass integral values for the control and test cultures after 96 hours and percentage inhibition values are given in Table 4.

The mean cell densities versus time for the definitive test are presented in Figure 1. Percentage inhibition values are plotted against test concentration in Figures 2, 3, 4, 5, 6 and 7.

5.2.2 Validation criteria

The following data show that the cell concentration of the control cultures increased by a factor of 110 after 72 hours and 205 after 96 hours. This increase was in line with the OECD Guideline that states the enhancement must be at least by a factor of 16 after 72 hours and the EPA Guideline that states the enhancement must be at least by a factor of 100 after 96 hours.

Mean cell density of control at 0 hours	:	1.07×10^4 cells per ml
Mean cell density of control at 72 hours	:	1.17×10^6 cells per ml
Mean cell density of control at 96 hours	:	2.18×10^6 cells per ml

The coefficient of variation for average specific growth rate for the control cultures over the test period (0 - 96 h) was 4% and hence satisfied the validation criterion given in the OECD/EPA Guidelines which that this must not exceed 7%.

The coefficient of variation for yield for the control cultures over the test period (0 - 96 h) was 14% and hence satisfied the validation criterion given in the EPA Guideline which that this must not exceed 20%.

5.2.3 Growth data

From the data given in Tables 2 and 4, it is clear that the growth rate (r), yield (y) and the biomass integral (b) of *Pseudokirchneriella subcapitata* (CCAP 278/4) were affected by the presence of the test material over the 96-Hour exposure period.

Accordingly the following results were determined from the data:

5.2.3.1 Inhibition of growth rate

E_rC_{10} (0 - 72 h) : 93 mg/l
E_rC_{50} (0 - 72 h) : 190 mg/l*
E_rC_{90} (0 - 72 h) : 370 mg/l
E_rC_{10} (0 - 96 h) : 120 mg/l
E_rC_{50} (0 - 96 h) : 210 mg/l*
E_rC_{90} (0 - 96 h) : 350 mg/l

where E_rC_x is the test concentration that reduced growth rate by x%.

Statistical analysis of the growth rate data was carried out for the control and all test concentrations using 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). There were no statistically significant differences between the control and 32 mg/l test concentration ($P \geq 0.05$), however all other test concentrations were significantly different ($P < 0.05$) and, therefore the "No Observed Effect Concentration" (NOEC) based on growth rate was 32 mg/l.

5.2.3.2 Inhibition of yield

E_yC_{10} (0 - 72 h) : 50 mg/l
E_yC_{50} (0 - 72 h) : 100 mg/l; 95% confidence limits 84 - 130 mg/l
E_yC_{90} (0 - 72 h) : 290 mg/l

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* It was not possible to calculate 95% confidence limits for the E_rC_{50} values as the data generated did not fit the models available for the calculation of confidence limits.

E_yC_{10} (0 - 96 h) : 88 mg/l
E_yC_{50} (0 - 96 h) : 130 mg/l; 95% confidence limits 120 - 140 mg/l
E_yC_{90} (0 - 96 h) : 210 mg/l

where E_yC_x is the test concentration that reduced yield by x%.

Statistical analysis of the yield data was carried out as in Section 5.2.2.1. There were no statistically significant differences between the control and 32 mg/l test concentration ($P \geq 0.05$), however all other test concentrations were significantly different ($P<0.05$) and, therefore the "No Observed Effect Concentration" (NOEC) based on yield was 32 mg/l.

5.2.3.3 Inhibition of biomass integral

E_bC_{10} (0 - 72 h) : 40 mg/l
E_bC_{50} (0 - 72 h) : 96 mg/l; 95% confidence limits 79 - 120 mg/l
E_bC_{90} (0 - 72 h) : 230 mg/l
E_bC_{10} (0 - 96 h) : 58 mg/l
E_bC_{50} (0 - 96 h) : 110 mg/l; 95% confidence limits 96 - 140 mg/l
E_bC_{90} (0 - 96 h) : 280 mg/l

where E_bC_x is the test concentration that reduced biomass integral by x%.

Statistical analysis of the biomass integral data was carried out as in Section 5.2.2.1. There were no statistically significant differences between the control and 32 mg/l test concentration ($P \geq 0.05$), however all other test concentrations were significantly different ($P<0.05$) and, therefore the "No Observed Effect Concentration" (NOEC) based on biomass integral was 32 mg/l.

5.2.4 Observations on Cultures

All test and control cultures were inspected microscopically at 96 hours. After 96 hours there were no abnormalities detected in any of the control, 10, 32 and 100 mg/l test cultures. However, only a few intact cells were observed to be present in the 320 and 1000 mg/l test cultures.

5.2.5 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 96-Hour test period all control, 10, 32 and 100 mg/l test cultures were observed to be

bright green dispersions whilst the 320 and 1000 mg/l test cultures were observed to be clear colourless solutions.

5.2.6 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 7.8 at 0 hours to pH 9.8 – 9.9 at 96 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 a sealed test system. 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 96 hours was in excess of that recommended in the Test Guidelines (1.5 pH units after 96 hours). This was 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.

5.2.7 Verification of test concentrations

Analysis of the test preparations at 0 hours showed measured test concentrations to be near nominal with the exception of the 32 mg/l test sample which showed a measured concentration of 79% of nominal. Analysis of the test preparations at 96 hours showed a slight decline in measured test concentrations in the range 64% to 101% of nominal.

Due to the volatile nature of the test material, additional test replicates were prepared at 0 hours and incubated alongside the test to provide samples for unopened vessel analysis at 96 hours. Analysis of these preparations showed measured test concentrations to range from 81% to 106% of nominal. As these measured concentrations were near nominal it was considered that the decline seen in the 96 hour test samples was through losses due to volatility. Whilst all efforts were made to prevent losses due to volatility it was not possible to completely close the test system due to the need to open the test vessels after each 24-hour period in order to take samples for cell density determination.

Current regulatory advice is that in cases where a decline in measured concentrations is observed, geometric mean measured concentrations should be used for calculating EC_{50} values. It was

therefore considered justifiable to base the results on the geometric mean measured test concentrations in order to give a "worst case" analysis of the data. The geometric mean measured test concentrations were determined to be:

Nominal Test Concentration (mg/l)	Geometric Mean Measured Test Concentration (mg/l)	Expressed as a % of the Nominal Test Concentration
10	11	110
32	23	72
100	77	77
320	230	72
1000	830	83

The following results were determined from the data based on the geometric mean measured test concentrations:

Growth rate

E_rC_{10} (0 - 72 h) : 72 mg/l
E_rC_{50} (0 - 72 h) : 140 mg/l*
E_rC_{90} (0 - 72 h) : 260 mg/l
E_rC_{10} (0 - 96 h) : 92 mg/l
E_rC_{50} (0 - 96 h) : 150 mg/l*
E_rC_{90} (0 - 96 h) : 250 mg/l
No Observed Effect Concentration (NOEC) = 23 mg/l

Yield

E_yC_{10} (0 - 72 h) : 37 mg/l
E_yC_{50} (0 - 72 h) : 76 mg/l; 95% confidence limits 62 - 93 mg/l
E_yC_{90} (0 - 72 h) : 230 mg/l
E_yC_{10} (0 - 96 h) : 69 mg/l
E_yC_{50} (0 - 96 h) : 95 mg/l; 95% confidence limits 87 - 100 mg/l
E_yC_{90} (0 - 96 h) : 140 mg/l
No Observed Effect Concentration (NOEC) = 23 mg/l

* It was not possible to calculate 95% confidence limits for the E_rC_{50} values as the data generated did not fit the models available for the calculation of confidence limits.

Biomass integral

E_bC_{10} (0 - 72 h) : 32 mg/l
E_bC_{50} (0 - 72 h) : 72 mg/l; 95% confidence limits 59 - 87 mg/l
E_bC_{90} (0 - 72 h) : 170 mg/l
E_bC_{10} (0 - 96 h) : 47 mg/l
E_bC_{50} (0 - 96 h) : 87 mg/l; 95% confidence limits 74 - 100 mg/l
E_bC_{90} (0 - 96 h) : 200 mg/l
No Observed Effect Concentration (NOEC) = 23 mg/l

The use of the geometric mean measured test concentrations in the calculation of the EC_{50} and NOEC values had no significant effect on the outcome of the study given that the EC_x values were of a similar magnitude to those obtained based on nominal test concentrations.

5.2.8 Re-growth experiment

A re-growth experiment was performed after 96 hours exposure to determine the algicidal or algistatic effect of the test material. Aliquots (0.5 ml) were removed for each replicate culture and the replicates pooled for each concentration. Fresh sterile culture medium (100 ml) was added to ensure that the test concentrations were reduced to below the inhibiting level. The sub-cultures were incubated at 24 ± 1°C for a period of 192 hours. Re-growth occurred in the control, 10 and 32 and 100 mg/l test cultures after 48 hours, in the 320 mg/l test cultures after 168 hours and in the 1000 mg/l test cultures after 192 hours. These results indicated that the test material was algistatic in effect.

5.3 Positive Control

The cell densities from exposure of *Pseudokirchneriella subcapitata* (CCAP 278/4) to the reference material; zinc chloride are given in Table 5 and Figure 15 and the inhibition of growth values are given in Table 6 and Figures 16, 17, 18, 19, 20 and 21.

Accordingly the following results were determined from the data:

E_bC_{50} (0 - 72 h) : 0.14 mg/l; 95% confidence limits 0.12 - 0.16 mg/l
E_bC_{50} (0 - 96 h) : 0.17 mg/l*

* It was not possible to calculate 95% confidence limits for these EC_{50} values as the data generated did not fit the models available for the calculation of confidence limits.

E_yC_{50} (0 - 72 h) : 0.16 mg/l; 95% confidence limits 0.14 - 0.18 mg/l
E_yC_{50} (0 - 96 h) : 0.62 mg/l; 95% confidence limits 0.54 - 0.71 mg/l

E_rC_{50} (0 - 72 h) : 0.30 mg/l; 95% confidence limits 0.24 - 0.37 mg/l
E_rC_{50} (0 - 96 h) : 0.39 mg/l*
No Observed Effect Concentration (NOEC) based on biomass integral: 0.10 mg/l.

The results from the positive control with zinc chloride were within the normal range for this reference material.

6. CONCLUSION

The effect of the test material on the growth of *Pseudokirchneriella subcapitata* has been investigated and based on nominal concentrations over a 72-Hour period gave an E_rC_{50} value of 190 mg/l* an E_yC_{50} value of 100 mg/l; 95% confidence limits 84 - 130 mg/l and an E_bC_{50} value of 96 mg/l; 95% confidence limits 79 - 120 mg/l. Over a 96-Hour period the effect of the test material gave an E_rC_{50} value of 210 mg/l* an E_yC_{50} value of 130 mg/l; 95% confidence limits 120 - 140 mg/l and an E_bC_{50} value of 110 mg/l; 95% confidence limits 96 - 140 mg/l. The No Observed Effect Concentration (NOEC) was 32 mg/l.

The effect of the test material based on the geometric mean measured test concentrations over a 72-Hour period gave an E_rC_{50} value of 140 mg/l* an E_yC_{50} value of 76 mg/l; 95% confidence limits 62 - 93 mg/l and an E_bC_{50} value of 72 mg/l; 95% confidence limits 59 - 87 mg/l. Over a 96-Hour period the effect of the test material gave an E_rC_{50} value of 150 mg/l* an E_yC_{50} value of 95 mg/l; 95% confidence limits 87 - 100 mg/l and an E_bC_{50} value of 87 mg/l; 95% confidence limits 74 - 100 mg/l. The No Observed Effect Concentration (NOEC) was 23 mg/l.

A re-growth experiment showed the test material was algistatic in effect.

7. REFERENCES

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* It was not possible to calculate 95% confidence limits for the E_rC_{50} values as the data generated did not fit the models available for the calculation of confidence limits.

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Dibromomethane: ALGAL GROWTH INHIBITION TEST

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

Nominal Concentration (mg/l)		Cell Densities* (cells per ml)		Inhibition Values (%)	
		0 Hours	96 Hours	Growth Rate	Yield/Biomass Integral
Control	R_1	1.34E+04	1.51E+06	-	-
	R_2	1.31E+04	2.05E+06		
	Mean	1.33E+04	1.78E+06		
0.10	R_1	1.41E+04	1.62E+06	4	10
	R_2	1.47E+04	1.58E+06		
	Mean	1.44E+04	1.60E+06		
1.0	R_1	1.34E+04	1.58E+06	2	8
	R_2	1.36E+04	1.70E+06		
	Mean	1.35E+04	1.64E+06		
10	R_1	1.40E+04	1.57E+06	4	11
	R_2	1.36E+04	1.59E+06		
	Mean	1.38E+04	1.58E+06		
100	R_1	1.32E+04	1.32E+06	6	24
	R_2	1.41E+04	1.39E+06		
	Mean	1.37E+04	1.35E+06		
1000	R_1	1.41E+04	3.57E+04	84	99
	R_2	1.38E+04	2.29E+04		
	Mean	1.40E+04	2.93E+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 and R_2 = Replicates 1 and 2

Dibromomethane: ALGAL GROWTH INHIBITION TEST

Table 2 Cell Densities and pH Values in the Definitive Test

Nominal Concentration (mg/l)		pH	Cell Densities* (cells per ml)					pH
		0 h	0 h	24 h	48 h	72 h	96 h	96 h
Control	R_1	7.8	1.16E+04	6.67E+04	3.31E+05	1.20E+06	1.99E+06	9.9
	R_2	7.8	1.05E+04	5.38E+04	3.48E+05	1.01E+06	2.02E+06	9.9
	R_3	7.8	9.90E+03	5.84E+04	3.45E+05	1.30E+06	2.54E+06	9.8
	Mean		1.07E+04	5.96E+04	3.41E+05	1.17E+06	2.18E+06	
10	R_1	7.7	9.84E+03	5.03E+04	3.32E+05	1.44E+06	2.62E+06	10.6
	R_2	7.7	8.15E+03	5.23E+04	3.31E+05	1.57E+06	2.61E+06	10.6
	R_3	7.7	9.88E+03	5.47E+04	3.26E+05	1.93E+06	2.60E+06	10.6
	Mean		9.29E+03	5.24E+04	3.30E+05	1.64E+06	2.61E+06	
32	R_1	7.7	1.03E+04	5.66E+04	2.92E+05	1.32E+06	2.45E+06	10.6
	R_2	7.7	1.03E+04	5.47E+04	2.86E+05	1.31E+06	2.46E+06	10.6
	R_3	7.7	9.98E+03	5.22E+04	2.67E+05	1.16E+06	2.37E+06	10.6
	Mean		1.02E+04	5.45E+04	2.82E+05	1.26E+06	2.43E+06	
100	R_1	7.7	1.07E+04	2.61E+04	1.21E+05	6.64E+05	1.76E+06	10.3
	R_2	7.7	9.76E+03	3.23E+04	1.26E+05	6.49E+05	1.67E+06	10.3
	R_3	7.7	1.01E+04	3.37E+04	1.21E+05	6.52E+05	1.67E+06	10.3
	Mean		1.02E+04	3.07E+04	1.23E+05	6.55E+05	1.70E+06	
320	R_1	7.7	1.15E+04	1.22E+04	1.19E+04	2.53E+04	2.28E+04	7.8
	R_2	7.7	1.22E+04	1.47E+04	1.25E+04	1.64E+04	1.83E+04	7.8
	R_3	7.7	1.29E+04	1.47E+04	1.40E+04	1.73E+04	2.24E+04	7.8
	Mean		1.22E+04	1.39E+04	1.28E+04	1.97E+04	2.12E+04	
1000	R_1	7.6	1.17E+04	1.30E+04	1.38E+04	1.64E+04	1.50E+04	7.8
	R_2	7.6	1.22E+04	1.30E+04	1.35E+04	1.61E+04	1.52E+04	7.8
	R_3	7.6	1.14E+04	1.33E+04	1.47E+04	1.30E+04	1.44E+04	7.8
	Mean		1.18E+04	1.31E+04	1.40E+04	1.52E+04	1.49E+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_3 = Replicates 1 to 3

Dibromomethane: ALGAL GROWTH INHIBITION TEST

Table 3 Daily Specific Growth Rates for the Control Cultures in the Definitive Test

		Daily Specific Growth Rate			
		Day 0 - 1	Day 1 - 2	Day 2 - 3	Day 3 - 4
Control	R_1	0.079	0.070	0.052	0.021
	R_2	0.070	0.081	0.043	0.029
	R_3	0.073	0.077	0.053	0.028
	Mean	0.074	0.076	0.049	0.026

$R_1 - R_3$ = Replicates 1 to 3

Dibromomethane: ALGAL GROWTH INHIBITION TEST

Table 4 Inhibition of Growth Rate, Yield and Biomass Integral in the Definitive Test

Nominal Concentration (mg/l)		Growth Rate (cells/ml/hour)		Yield (cells/ml)		Biomass Integral	
		0 – 96 h	% Inhibition	0 – 96 h	% Inhibition*	0 – 96 h	% Inhibition
Control	R_1	0.055		1.97E+06		6.35E+07	
	R_2	0.055	-	2.01E+06	-	5.93E+07	-
	R_3	0.058		2.53E+06		7.30E+07	
	Mean	0.056		2.17E+06		6.53E+07	
	SD	0.002		2.80E+05		6.27E+06	
10	R_1	0.058	[4]	2.61E+06		7.70E+07	[18]
	R_2	0.058	[4]	2.60E+06		8.01E+07	[23]
	R_3	0.058	[4]	2.59E+06		8.89E+07	[36]
	Mean	0.058	[4]	2.60E+06	[20]	8.20E+07	[26]
	SD	0.000		9.47E+03		6.17E+06	
32	R_1	0.057	[2]	2.44E+06		7.10E+07	[9]
	R_2	0.057	[2]	2.45E+06		7.08E+07	[8]
	R_3	0.057	[2]	2.36E+06		6.55E+07	0
	Mean	0.057	[2]	2.42E+06	[11]	6.91E+07	[6]
	SD	0.000		4.84E+04		3.13E+06	
100	R_1	0.054	4	1.75E+06		4.13E+07	37
	R_2	0.053	5	1.66E+06		4.00E+07	39
	R_3	0.053	5	1.66E+06		4.00E+07	39
	Mean	0.053	5	1.69E+06	22	4.04E+07	38
	SD	0.001		5.18E+04		7.22E+05	
320	R_1	0.009	84	1.12E+04		6.39E+05	99
	R_2	0.006	89	6.10E+03		4.34E+05	99
	R_3	0.008	86	9.54E+03		5.45E+05	99
	Mean	0.008	86	8.96E+03	100	5.39E+05	99
	SD	0.002		2.62E+03		1.03E+05	
1000	R_1	0.004	93	3.28E+03		3.85E+05	99
	R_2	0.004	93	3.01E+03		3.72E+05	99
	R_3	0.004	93	2.97E+03		3.19E+05	100
	Mean	0.004	93	3.09E+03	100	3.59E+05	99
	SD	0.000		1.70E+02		3.52E+04	

* In accordance with test guidelines only the mean value for yield for each test concentration is calculated
R_1 – R_3 = Replicates 1 to 3
SD = Standard Deviation
[Increase in growth compared to controls]

Dibromomethane: ALGAL GROWTH INHIBITION TEST

Table 5 Cell Densities and pH Values in the Positive Control with Zinc Chloride

Nominal Concentration (mg/l)		pH	Cell Densities* (cells per ml)					pH
		0 h	0 h	24 h	48 h	72 h	96 h	96 h
Control	R_1	7.4	1.18E+04	2.39E+04	1.13E+05	8.94E+05	1.23E+06	7.7
	R_2	7.4	1.05E+04	2.48E+04	1.14E+05	9.06E+05	1.21E+06	7.7
	R_3	7.4	1.02E+04	2.30E+04	1.39E+05	8.81E+05	1.19E+06	7.7
	Mean		1.08E+04	2.39E+04	1.22E+05	8.94E+05	1.21E+06	
0.010	R_1	7.4	9.62E+03	2.53E+04	1.34E+05	9.84E+05	1.31E+06	7.7
	R_2	7.4	1.03E+04	2.56E+04	1.47E+05	9.86E+05	1.28E+06	7.8
	R_3	7.4	9.84E+03	2.56E+04	1.42E+05	1.02E+06	1.29E+06	7.8
	Mean		9.92E+03	2.55E+04	1.41E+05	9.96E+05	1.29E+06	
0.032	R_1	7.4	1.08E+04	2.76E+04	1.39E+05	1.02E+06	1.48E+06	7.7
	R_2	7.4	9.77E+03	2.51E+04	1.43E+05	9.51E+05	1.41E+06	7.7
	R_3	7.4	9.47E+03	2.41E+04	1.35E+05	1.13E+06	1.59E+06	7.7
	Mean		1.00E+04	2.56E+04	1.39E+05	1.03E+06	1.49E+06	
0.10	R_1	7.4	9.72E+03	2.34E+04	1.06E+05	6.84E+05	1.56E+06	7.6
	R_2	7.4	9.68E+03	2.42E+04	9.86E+04	6.55E+05	1.56E+06	7.5
	R_3	7.4	9.36E+03	2.36E+04	1.10E+05	6.86E+05	1.77E+06	7.6
	Mean		9.59E+03	2.37E+04	1.05E+05	6.75E+05	1.63E+06	
0.32	R_1	7.3	9.80E+03	1.25E+04	1.86E+04	8.16E+04	1.38E+05	7.1
	R_2	7.3	9.45E+03	1.26E+04	1.78E+04	5.24E+04	8.37E+04	7.1
	R_3	7.3	9.07E+03	1.34E+04	1.63E+04	5.63E+04	1.04E+05	7.1
	Mean		9.44E+03	1.28E+04	1.75E+04	6.34E+04	1.08E+05	
1.0	R_1	7.1	9.38E+03	1.05E+04	1.25E+04	2.29E+04	4.66E+04	7.0
	R_2	7.1	9.74E+03	1.04E+04	9.46E+03	1.72E+04	4.02E+04	7.0
	R_3	7.1	9.25E+03	1.04E+04	1.03E+04	1.46E+04	1.78E+04	7.0
	Mean		9.46E+03	1.04E+04	1.07E+04	1.82E+04	3.49E+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_3 = Replicates 1 to 3

Table 6 **Inhibition of Growth Rate, Yield and Biomass Integral in the Positive Control with Zinc Chloride**

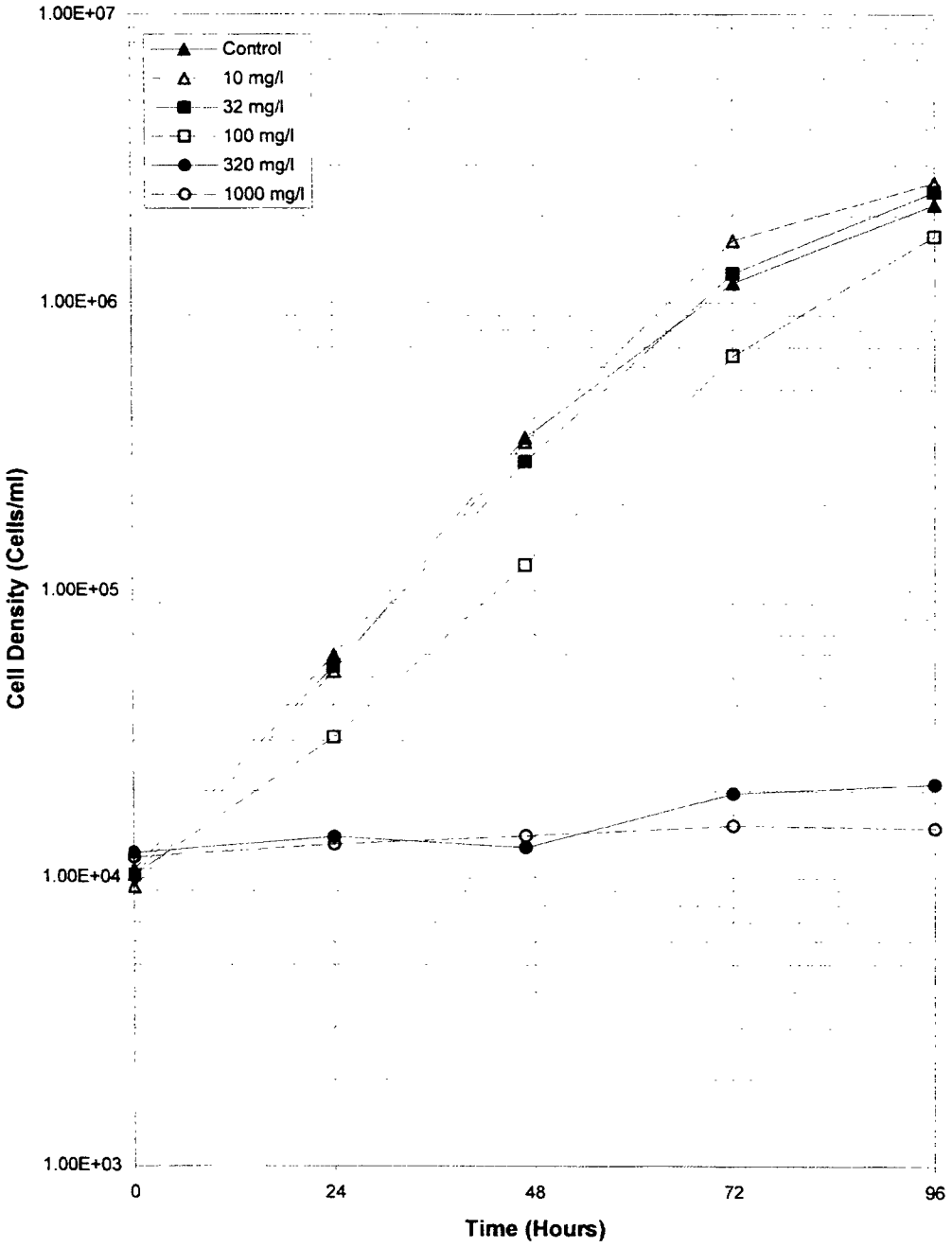
Nominal Concentration (mg/l)	Area Under Curve at 72 h	% Inhibition	Growth Rate (0 – 72 h)	% Inhibition	Yield at 72 h	% Inhibition
Control	1.36E+07	-	0.061	-	8.81E+05	-
0.010	1.54E+07	[13]	0.064	[5]	9.87E+05	[12]
0.032	1.58E+07	[16]	0.064	[5]	1.02E+06	[16]
0.10	1.06E+07	22	0.059	3	6.65E+05	25
0.32	9.24E+05	93	0.026	57	5.40E+04	94
1.0	1.60E+05	99	0.0087	86	8.77E+03	99

Nominal Concentration (mg/l)	Area Under Curve at 96 h	% Inhibition	Growth Rate (0 - 96h)	% Inhibition	Yield at 96 h	% Inhibition
Control	3.85E+07	-	0.049	-	1.20E+06	-
0.010	4.26E+07	[11]	0.051	[4]	1.28E+06	[8]
0.032	4.59E+07	[19]	0.052	[6]	1.48E+06	[23]
0.10	3.81E+07	1	0.054	[10]	1.62E+06	[35]
0.32	2.76E+06	93	0.025	49	9.91E+05	17
1.0	5.70E+05	99	0.013	73	2.54E+04	98

[Increase in growth compared to controls]

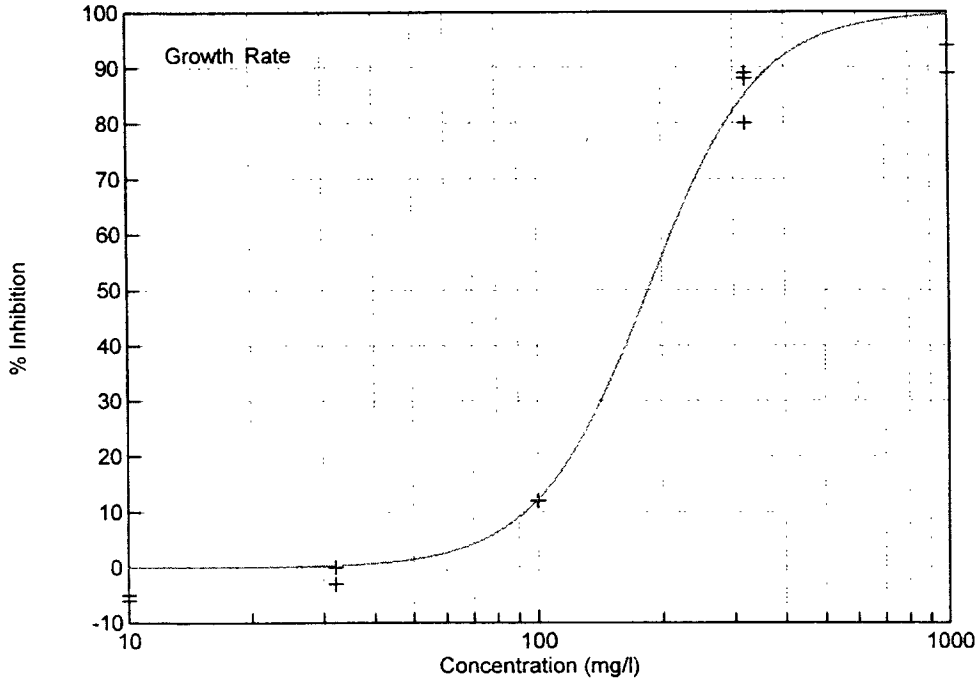
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 1 Mean Cell Densities v Time for the Definitive Test



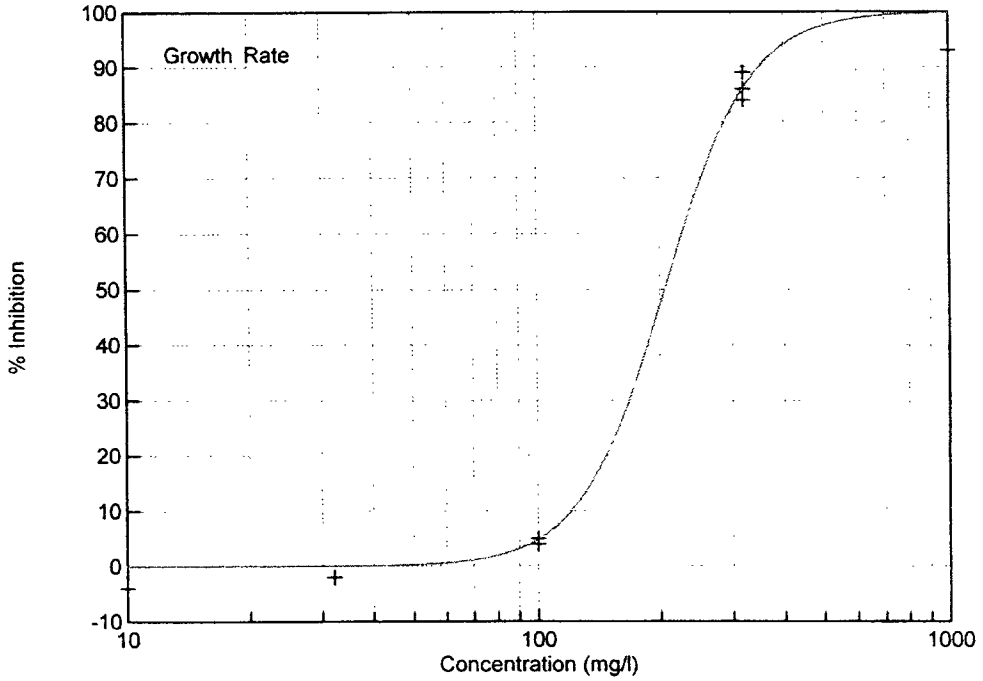
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 2 Inhibition of Growth Rate at 72 Hours Based on Nominal Test Concentrations



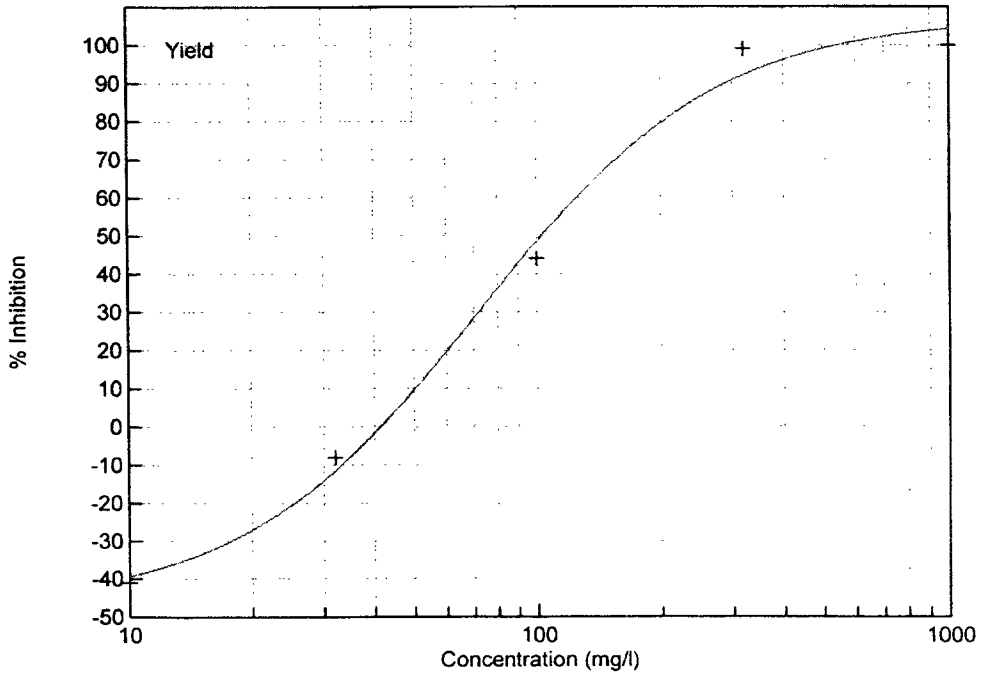
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 3 Inhibition of Growth Rate at 96 Hours Based on Nominal Test Concentrations



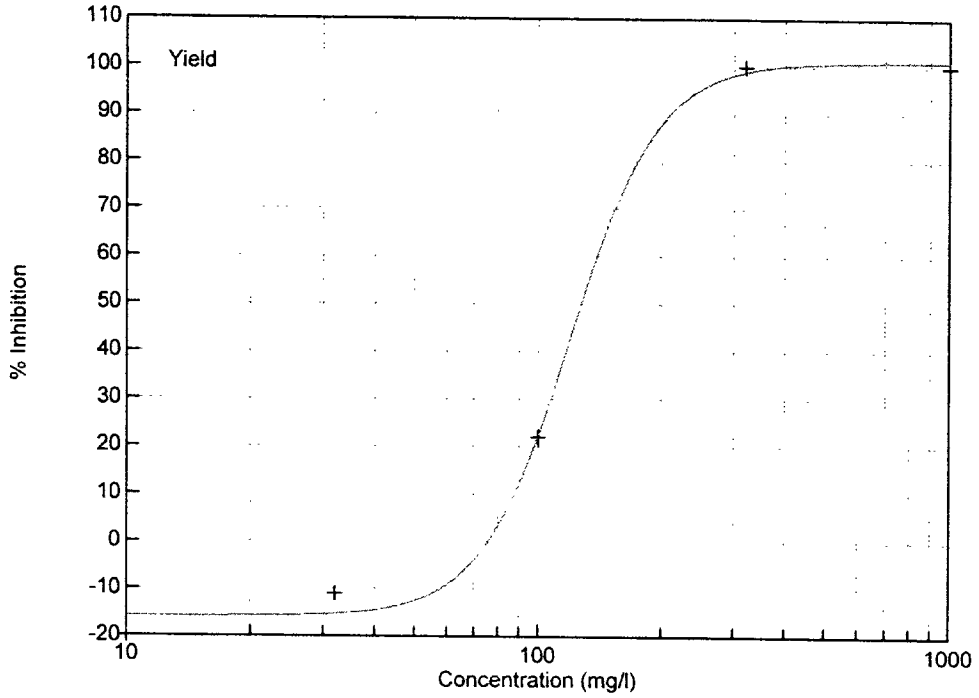
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 4 Inhibition of Yield at 72 Hours Based on Nominal Test Concentrations



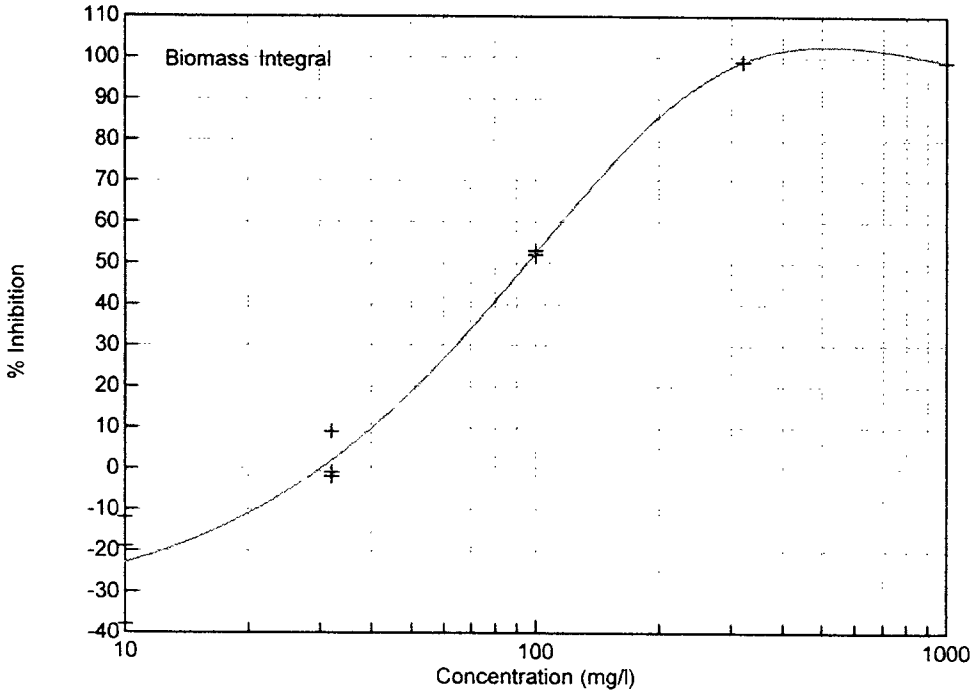
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 5 Inhibition of Yield at 96 Hours Based on Nominal Test Concentrations



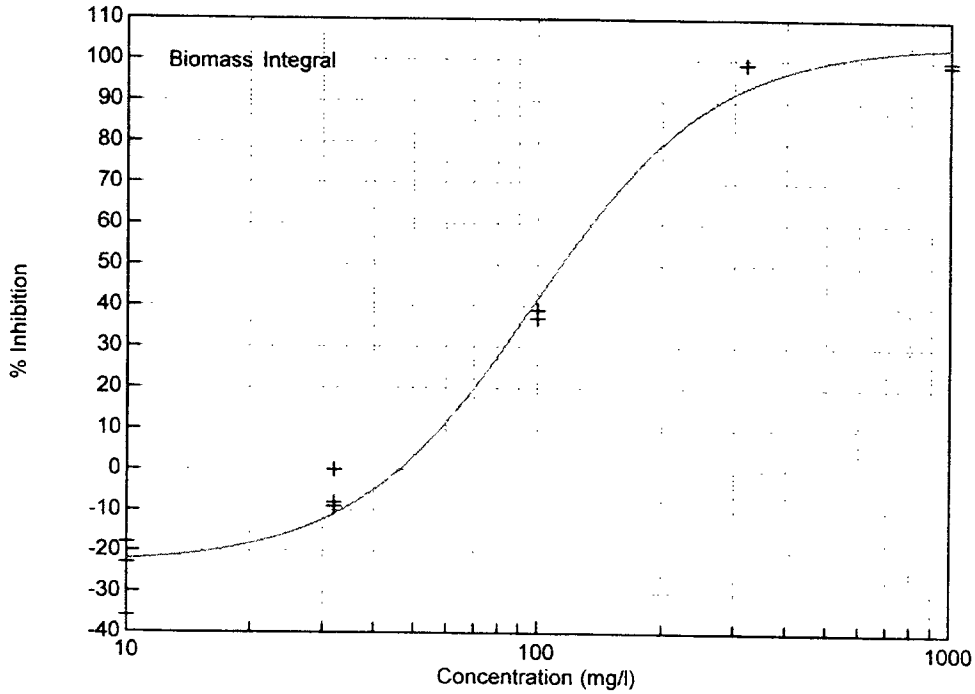
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 6 **Inhibition of Biomass Integral at 72 Hours Based on Nominal Test Concentrations**



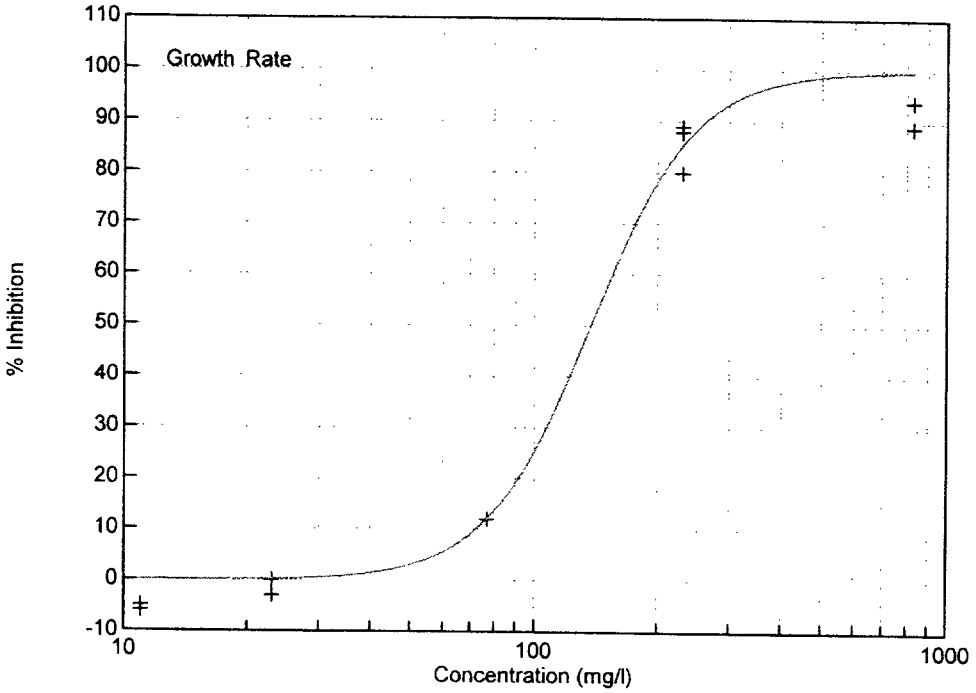
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 7 **Inhibition of Biomass Integral at 96 Hours Based on Nominal Test Concentrations**



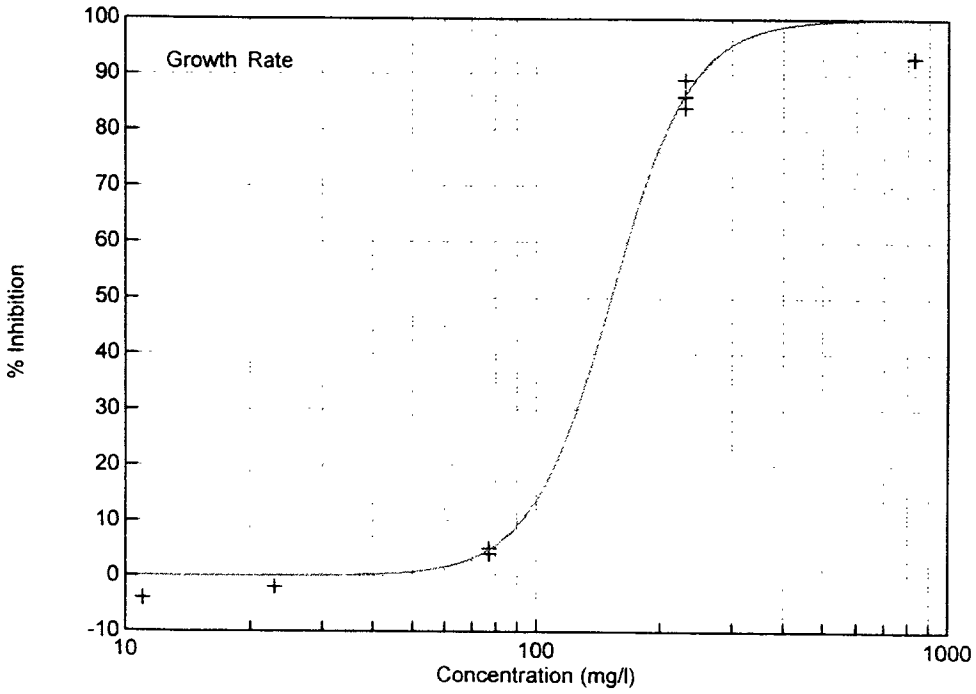
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 8 **Inhibition of Growth Rate at 72 Hours Based on Geometric Mean Measured Test Concentrations**



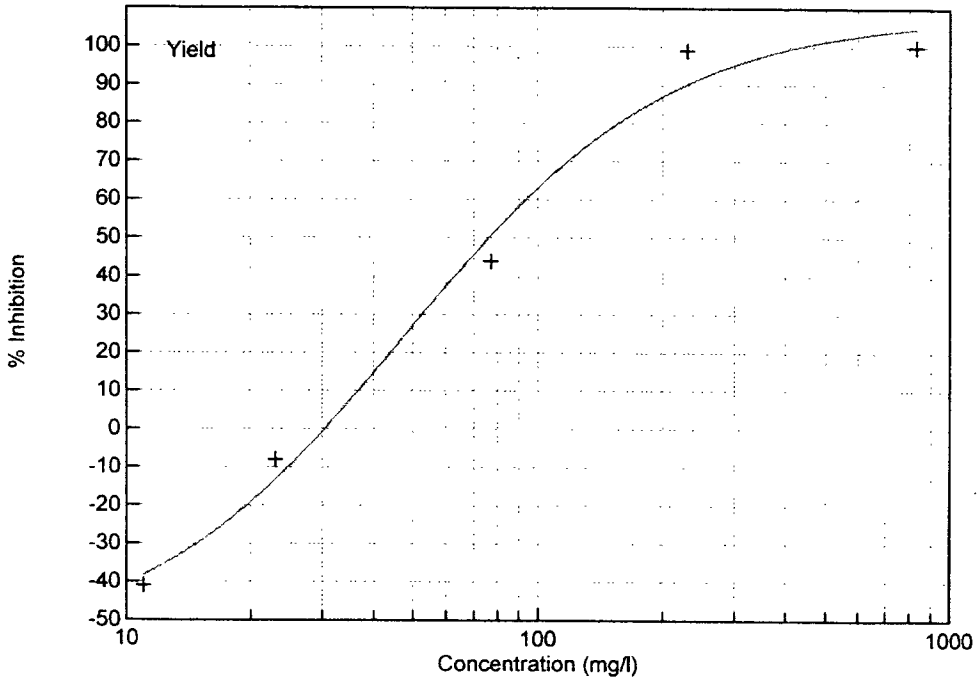
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 9 **Inhibition of Growth Rate at 96 Hours Based on Geometric Mean Measured Test Concentrations**



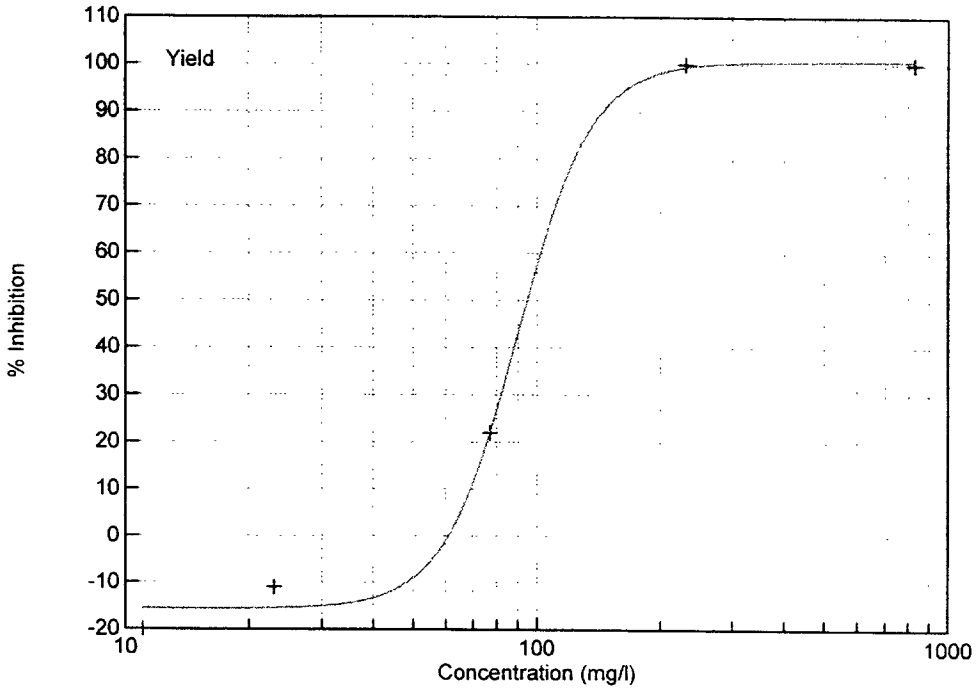
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 10 Inhibition of Yield at 72 Hours Based on Geometric Mean Measured Test Concentrations



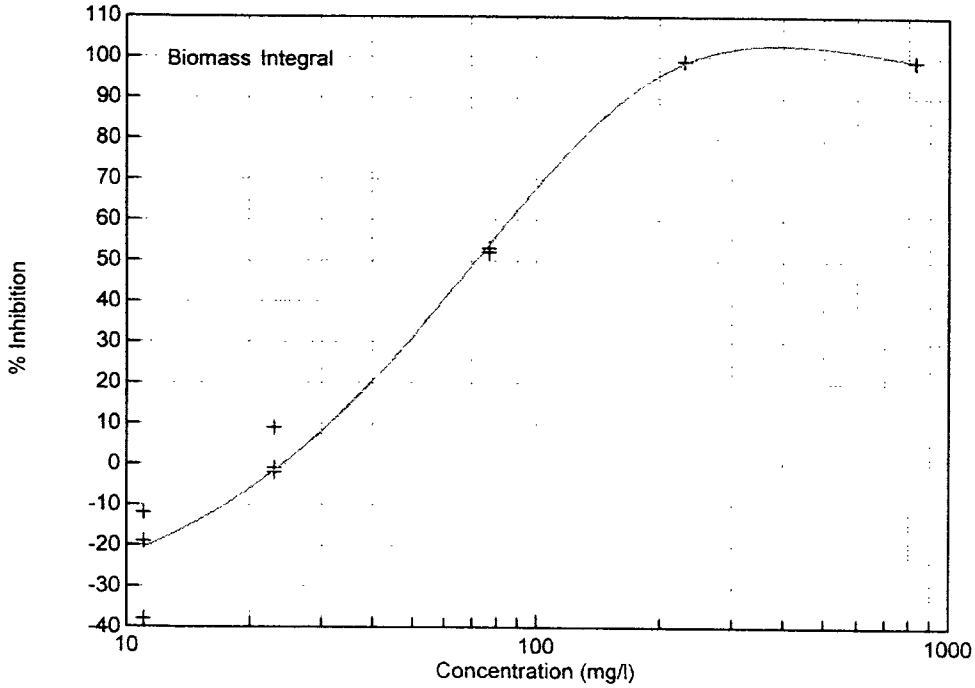
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 11 Inhibition of Yield at 96 Hours Based on Geometric Mean Measured Test Concentrations



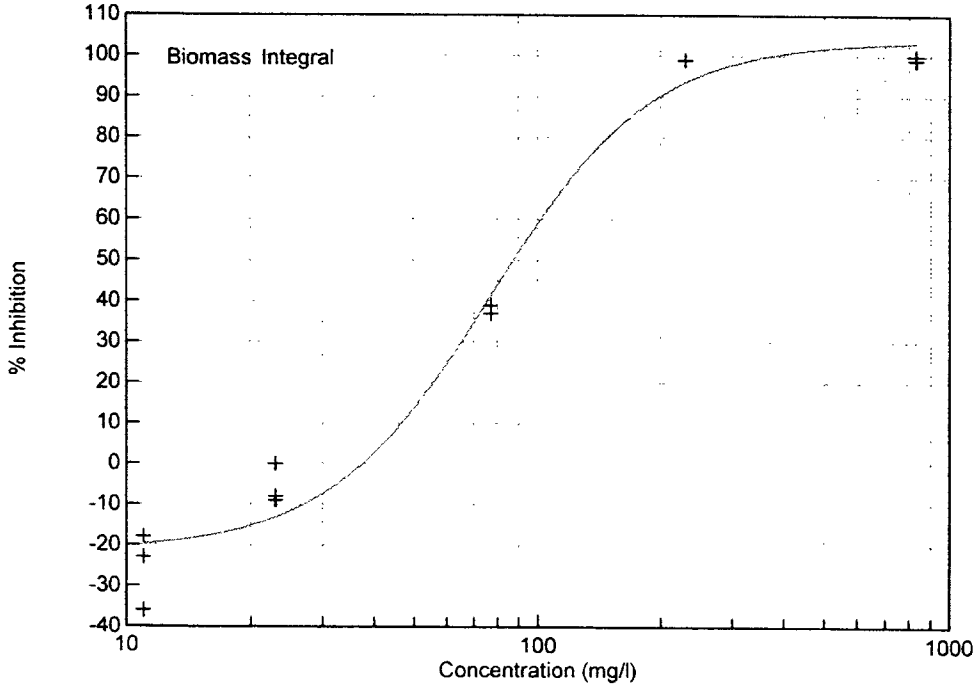
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 12 Inhibition of Biomass Integral at 72 Hours Based on Geometric Mean Measured Test Concentrations



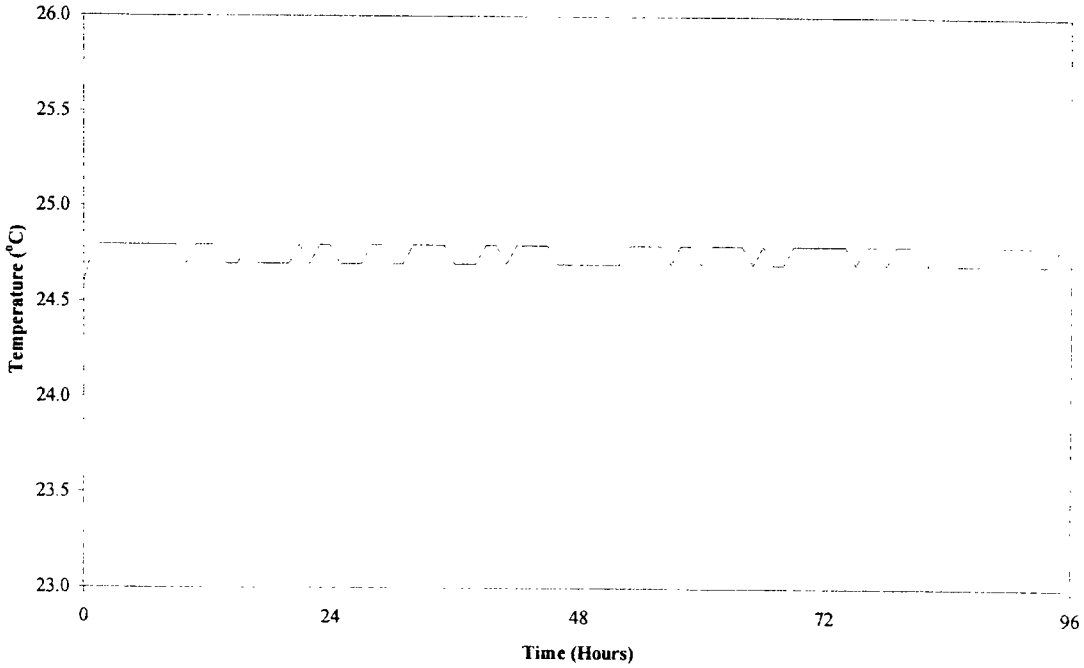
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 13 Inhibition of Biomass Integral at 96 Hours Based on Geometric Mean Measured Test Concentrations



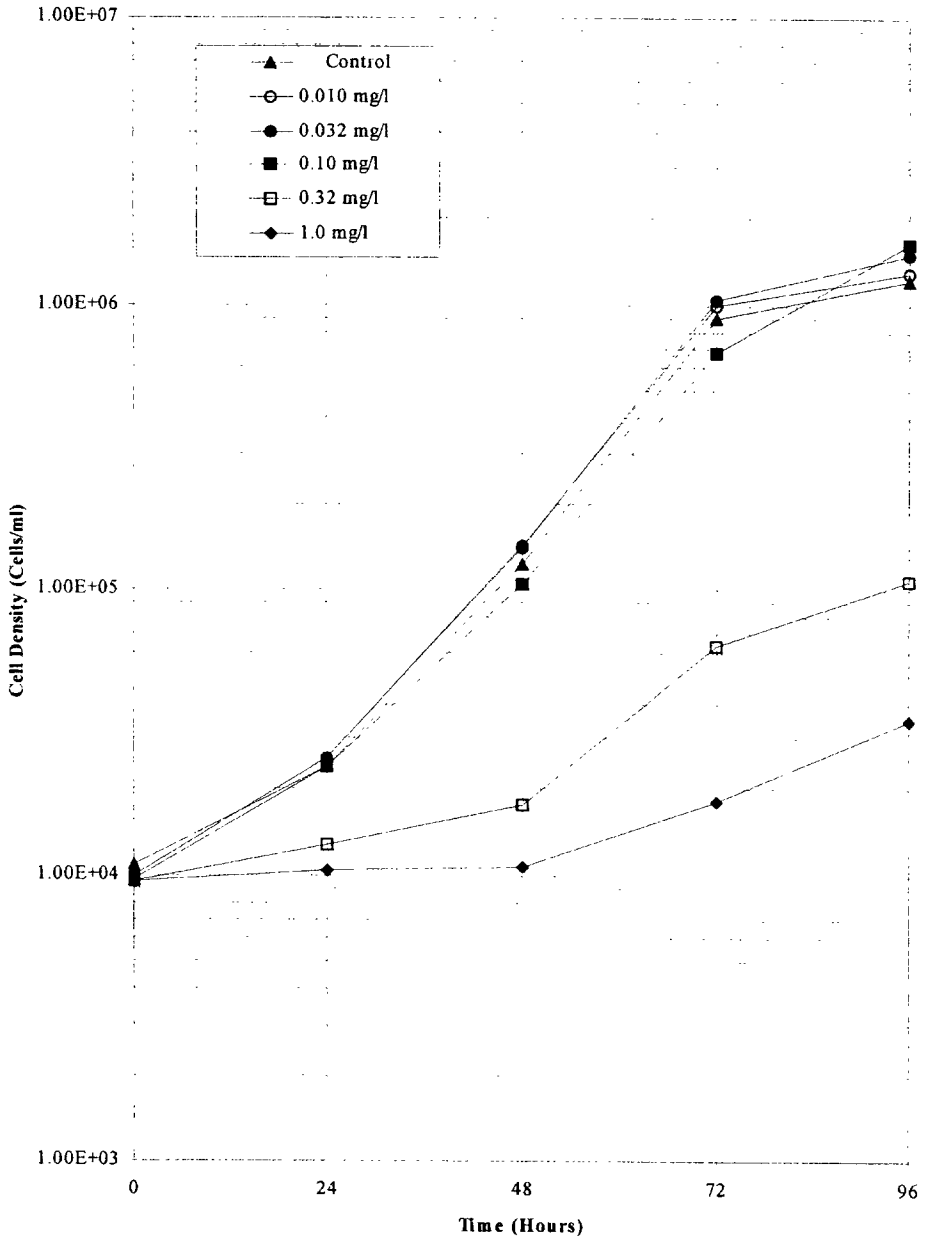
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 14 **Temperature Measurements Taken Throughout the Test Period**



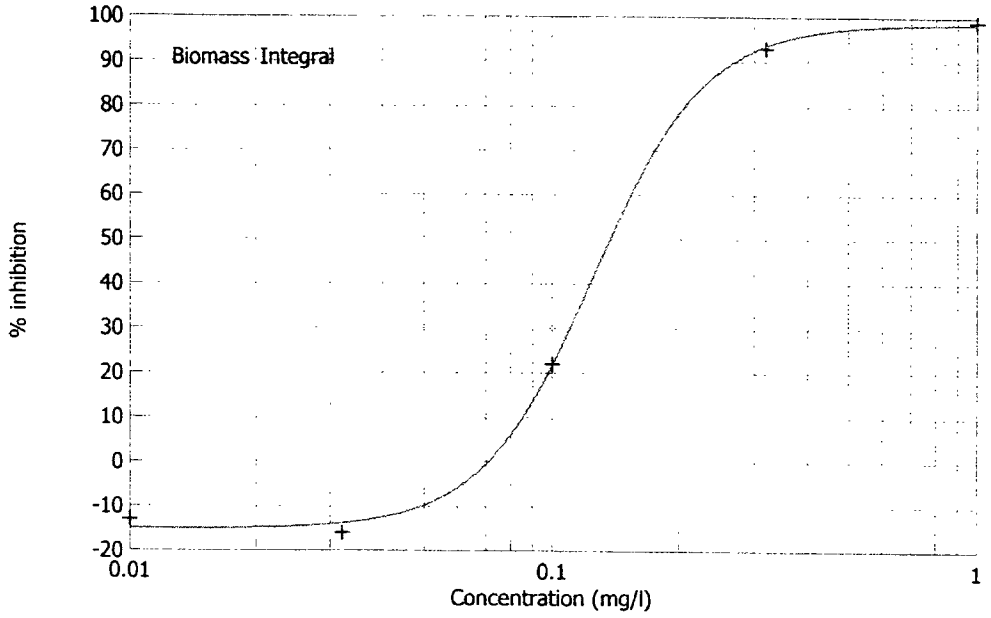
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 15 **Mean Cell Densities v Time for the Positive Control with Zinc Chloride**



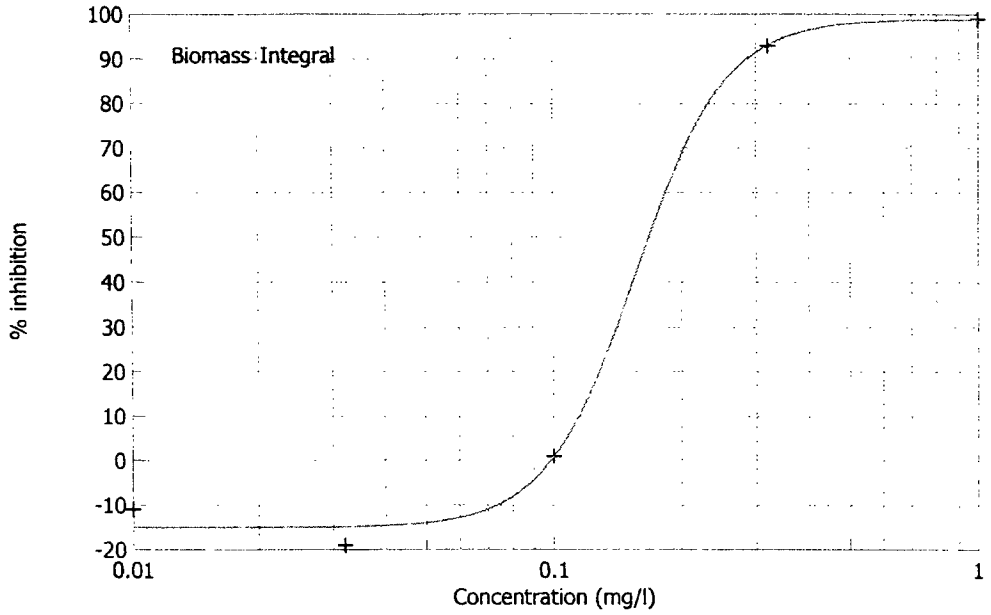
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 16 Inhibition of Biomass Integral at 72 Hours for the Positive Control with Zinc Chloride



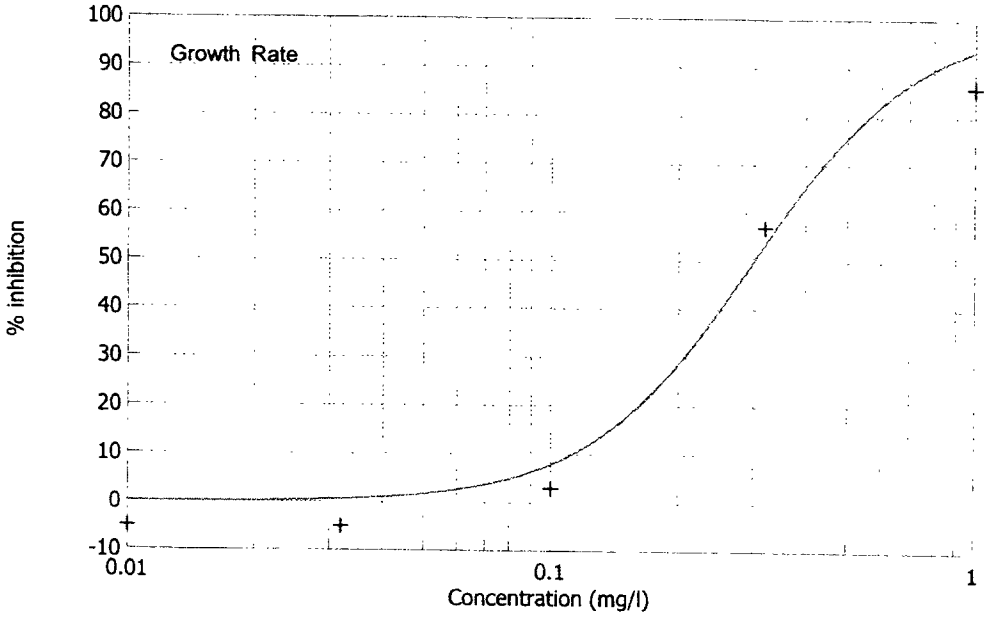
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 17 Inhibition of Biomass Integral at 96 Hours for the Positive Control with Zinc Chloride



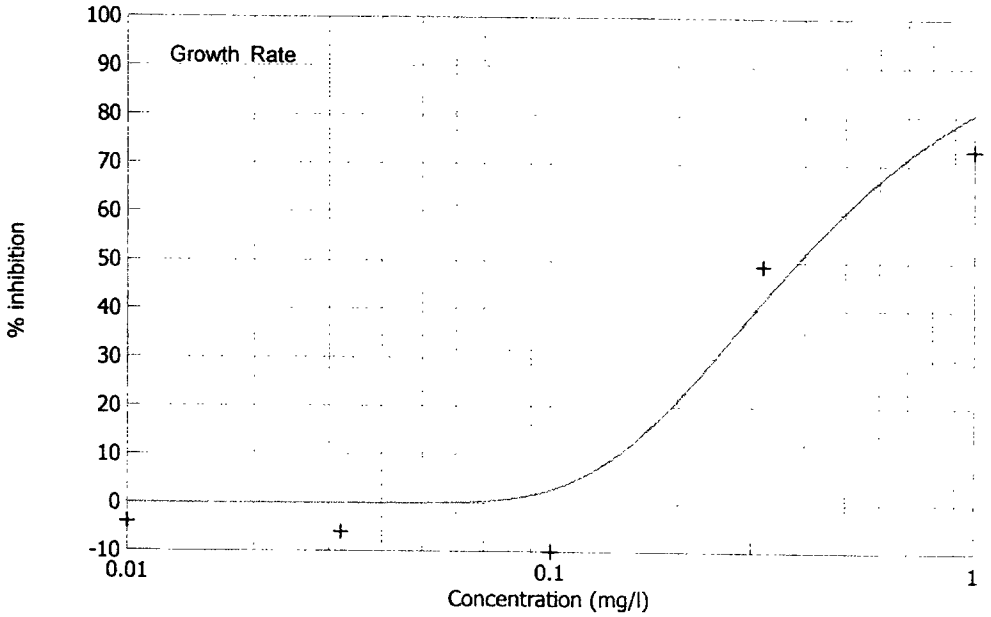
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 18 **Inhibition of Growth Rate (0 – 72 hours) for the Positive Control with Zinc Chloride**



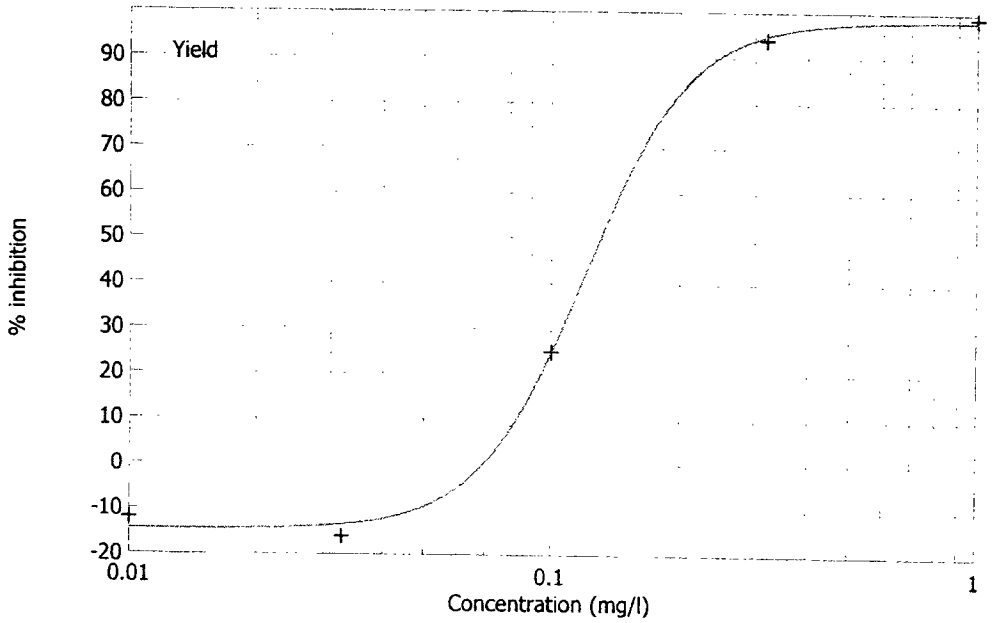
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 19 **Inhibition of Growth Rate (0 – 96 hours) for the Positive Control with Zinc Chloride**



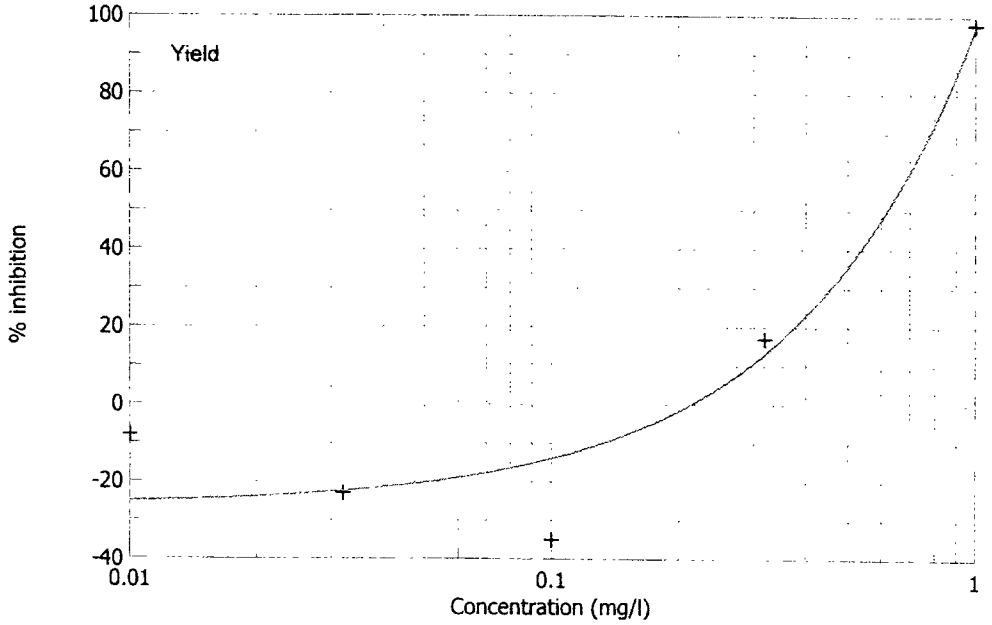
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 20 **Inhibition of Yield (0 – 72 hours) for the Positive Control with Zinc Chloride**



Dibromomethane: ALGAL GROWTH INHIBITION TEST

Figure 21 **Inhibition of Yield (0 – 96 hours) for the Positive Control with Zinc Chloride**



Dibromomethane: ALGAL GROWTH INHIBITION TEST

Appendix 1 Certificate of Analysis

SafePharm Laboratories

SafePharm Laboratories Ltd
Shardlow Business Park
London Road, Shardlow
Derbyshire DE72 2GD
Tel: 01332 792896 Fax: 01332 799018
Website: www.safepharm.co.uk

MIXING AND ANALYSIS OF TEST MATERIAL SAMPLES

CERTIFICATE OF ANALYSIS

TEST MATERIAL	:	Dibromomethane	
PROJECT NUMBER	:	0466/0267	
CO-SPONSORS	:	Bromine Compounds Ltd. Makleff House P.O.B. 180 BEER-SHEVA 84101 ISRAEL	Albemarle Corporation Health, Safety and Environment 451 Florida Street Baton Rouge LA 70801 UNITED STATES OF AMERICA
TEST DATES	:	14 September 2006 to 28 September 2006	

Test Material Details:

Supplier	:	Bromine Compounds Ltd	Albemarle Corporation
Name	:	Dibromomethane	Dibromomethane
Description	:	clear, colourless liquid	clear, colourless liquid
Lot number	:	20050298	DBM082506
Date Received	:	12 September 2006	01 September 2006
Storage Conditions	:	room temperature, in the dark	room temperature, in the dark

Testing Details:

The above two test materials were mixed in equal proportions and the purity of the resulting mixture determined by gas chromatography (GC) area normalisation. The purity was determined to be 99.4%.

Analytical Conditions:

Column	:	ZB-624 (30 m x 0.25 mm id, 1.40 µm film)
Oven temperature program	:	initial temperature : 40°C rate: 5°C/minute final temperature: 260°C for 5 minutes
Injector temperature	:	250°C
Flame ionisation detector temperature	:	300°C
Injection volume	:	1 µl
Retention time	:	approximately 9.5 minutes for Dibromomethane

Registered in England No. 3009027

Dibromomethane: ALGAL GROWTH INHIBITION TEST

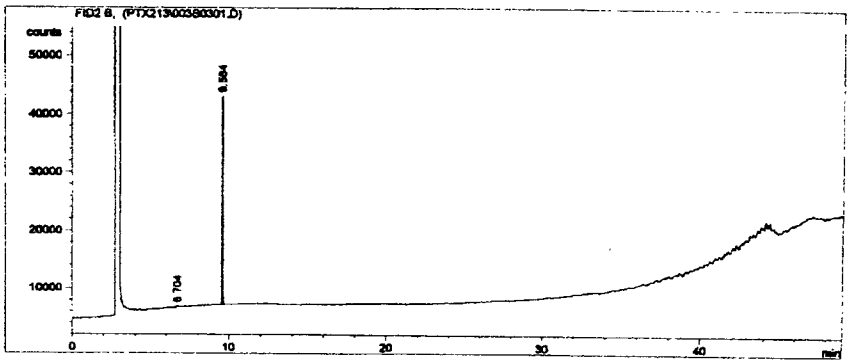
Appendix 1 (continued) Certificate of Analysis

SafePharm Laboratories

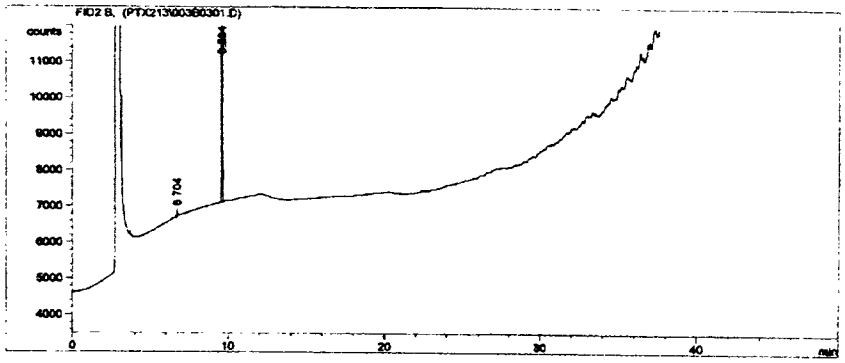
SafePharm Laboratories Ltd
Shardlow Business Park
London Road, Shardlow
Derbyshire DE72 2GD
Tel: 01332 792896 Fax: 01332 799018
Website: www.safepharm.co.uk

Typical Chromatography

Sample Solution, 6.14 x 10^3 mg/l in Methanol



Sample Solution (Expanded Scale)



Registered in England No. 3009027

Dibromomethane: ALGAL GROWTH INHIBITION TEST

Appendix 1 (continued) Certificate of Analysis

SafePharm Laboratories

SafePharm Laboratories Ltd
Shardlow Business Park
London Road, Shardlow
Derbyshire DE72 2GD
Tel: 01332 792896 Fax: 01332 799018
Website: www.safepharm.co.uk

I the undersigned, hereby declare that this certificate of analysis accurately reflects the original data generated in SPL Project Number 0466/0267.

This study was performed in compliance with the UK GLP Regulations 1999 (as amended in 2004) and this certificate accurately reflects the results obtained. The original data and the Certificate of Analysis will be kept for five years in the archives of Safepharm Laboratories Ltd.

Signed: ..... Date: 02 NOV 2006
B J O'Connor BSc (Hons) MRSC
Study Director

Studies of this type are monitored and reported by Safepharm Laboratories Quality Assurance Unit as part of a routine sampling programme: 14, 26 September 2006.

The certificate was audited on 02 November 2006 by Safepharm Laboratories Quality Assurance Unit. It is considered to be an accurate account of the data generated and of the results produced.

Signed: ..... Date: 02 NOV 2006
For Safepharm Quality Assurance Unit

Registered in England No. 3009027

Appendix 2 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 volume of test sample was diluted with acetone to give a final theoretical concentration of 5 to 500 mg/l.

1.3 Standards

Standard solutions of test material were prepared in acetone: water* (50:50 v/v) at a nominal concentration of 50 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	:	ZB-5 (30 m x 0.53 mm id, 5 μm film)

* Prepared by ELGA Purelab Option R-15 BP water purification

Appendix 2 (continued) Verification of Test Concentrations

Oven temperature program	:	initial 40°C for 2 minutes rate 1 3°C/minute to 70°C rate 2 20°C/minute final 130°C for 2 minutes
Injector temperature	:	300°C
Detector temperature	:	300°C
Carrier gas and pressure	:	nitrogen at approximately 5 psi
Injection volume	:	2 µl
Injection mode	:	splitless purge on at 1 minute
Detector	:	flame ionisation detector (FID)
Retention time	:	approximately 6 to 9 minutes

2. VALIDATION

2.1 Linearity

A range of standard solutions covering 2.0 to 610 mg/l (40% of the lowest working sample concentration to 122% of the highest working sample concentration) was analysed.

Linearity was confirmed (R^2 = 0.9994) in the range 0 to 610 mg/l.

The results are presented graphically on page 59.

2.2 Recoveries

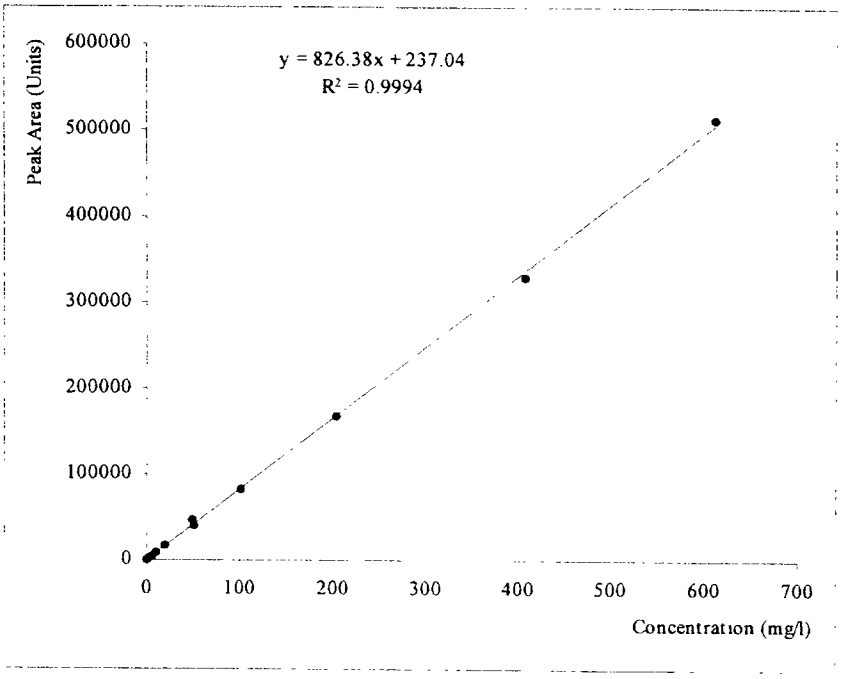
A range of preliminary test samples, accurately fortified at known concentrations of test material, was prepared and analysed.

The recovery samples were prepared by direct addition of the test material to a sample of test medium.

Dibromomethane: ALGAL GROWTH INHIBITION TEST

Appendix 2 (continued) Verification of Test Concentrations

Linearity of Detector Response



Dibromomethane: ALGAL GROWTH INHIBITION TEST

Appendix 2 (continued) Verification of Test Concentrations

Further portions of the test samples were analysed following the addition of algal cells to assess the effects of algae on the recovery of test material from test media.

Fortification (mg/l)	Recoveries		
	(mg/l)	(%)	Mean %
11.1	14.8	134	129
11.1	13.9	125	
11.1 plus algae	13.0	117	-
111	99.2	90	89
111	98.6	89	
1110	981	89	88
1110	961	87	
1110 plus algae	833	75	-

The lowest test level recovery was just above the normal upper acceptance limit of 120%. This may have been due to the closeness to the limit of quantification giving poorer accuracy. However, the other two levels were acceptable and the method has been considered to be sufficiently accurate for the purposes of this test overall. The test sample results have not 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 Limit of Quantitation

The limit of quantitation has been assessed down to 4.1 mg/l.

Appendix 2 (continued) Verification of Test Concentrations

3. 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 96 hours (equivalent to the test exposure period). In addition test samples were tested for stability without prior mixing (sonication) the test sample bottles to assess for losses due to adsorption and/or insolubility and also in open vessels to assess for loses due to volatility.

Nominal concentration (mg/l)	10	100	1000
Concentration found initially (mg/l)	14.3	98.9	971
Concentration found after storage in light conditions (mg/l)	8.08	68.8	765
Expressed as a percent of the initial concentration	56	70	79
Concentration found after storage in dark conditions (mg/l)	8.20	65.8	747
Expressed as a percent of the initial concentration	57	67	77
Concentration found after storage in dark conditions (mg/l) – unsonicated sample	7.98	68.8	732
Expressed as a percent of the initial concentration	56	70	75
Concentration found after storage in dark conditions (mg/l) – open vessel samples	1.84	3.27	532
Expressed as a percent of the initial concentration	13	3	55

Whilst apparent instability in both light and dark conditions was observed, closer inspection of the results obtained from analysis of the unopened test samples (see Section 4) would suggest that the losses seen were more likely attributable to the volatile nature of the test material rather than apparent instability. Furthermore, inspection of the chromatography generated indicated that no degradation products were present.

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

The results of the open stability vessel showed evidence of volatility.

Dibromomethane: ALGAL GROWTH INHIBITION TEST

Appendix 2 (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	-
	10	11.5	115
	32	25.4	79
	100	85.6	86
	320	259	81
	1000	927	93
96 Hours	Control	<LOQ	-
	10	10.1	101
	32	21.1	66
	100	68.4	68
	320	205	64
	1000	743	74
96 Hours unopened vessels	Control	<LOQ	-
	10	10.6	106
	32	26.0	81
	100	87.5	87
	320	260	81
	1000	955	96

5. DISCUSSION

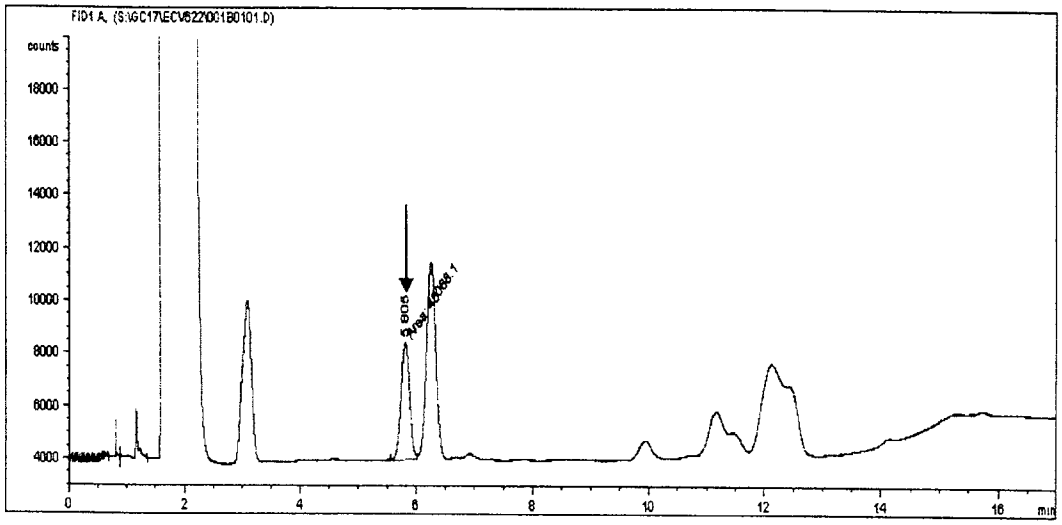
The detection system was found to have acceptable linearity. The analytical procedure had acceptable recoveries of test material in test medium. A method of analysis was validated and proven to be suitable for use.

LOQ = Limit of quantitation

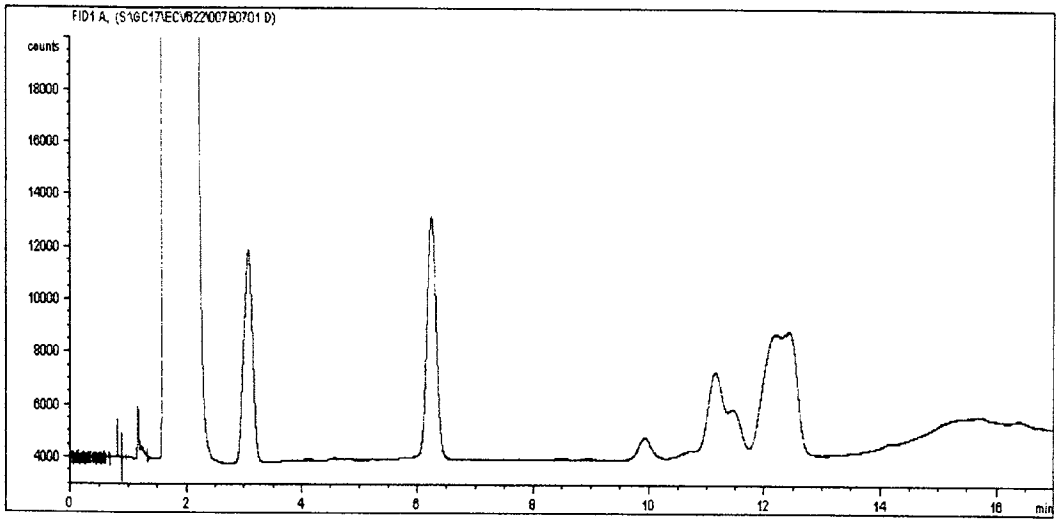
Dibromomethane: ALGAL GROWTH INHIBITION TEST

Appendix 2 (continued) Verification of Test Concentrations

6. TYPICAL CHROMATOGRAPHY



Standard 50 mg/l



Control Sample*

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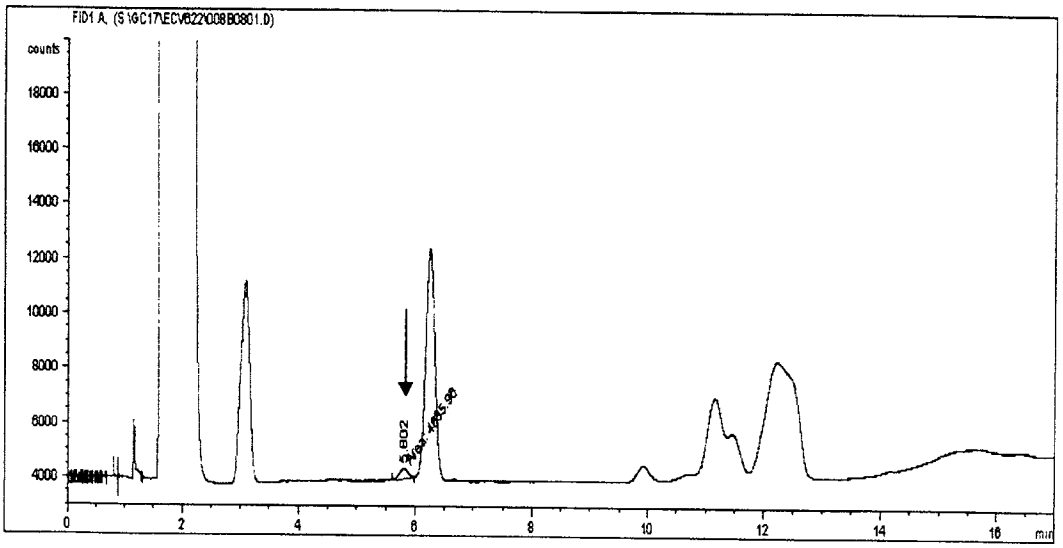
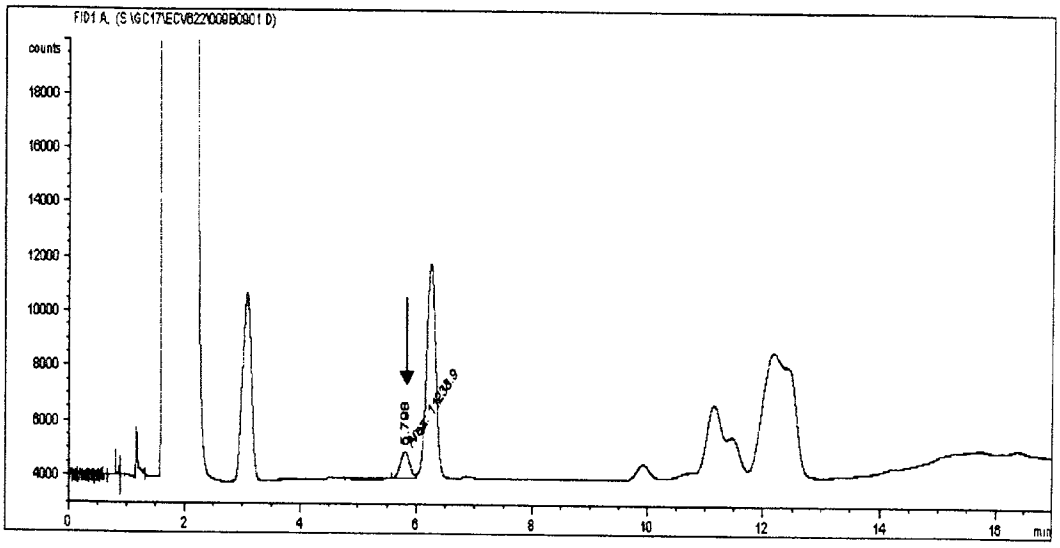
* The peak observed at approximately 6 minutes was considered to be an excipient of the control water and not test material as results from analysis of the control samples showed there to be no test material present.

Dibromomethane: ALGAL GROWTH INHIBITION TEST

Appendix 2 (continued) Verification of Test Concentrations



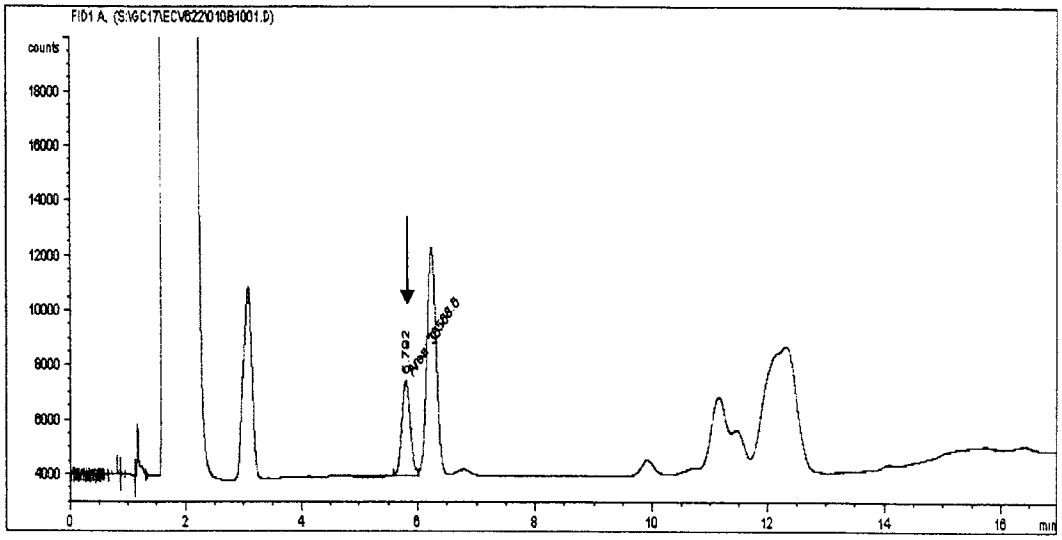
Test Sample 10 mg/l



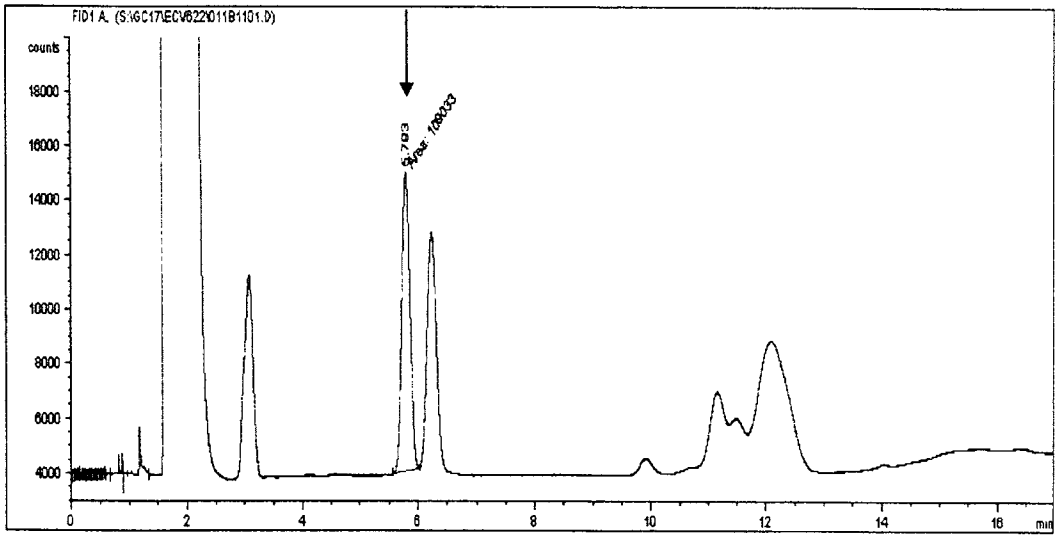
Test Sample 32 mg/l

Dibromomethane: ALGAL GROWTH INHIBITION TEST

Appendix 2 (continued) Verification of Test Concentrations



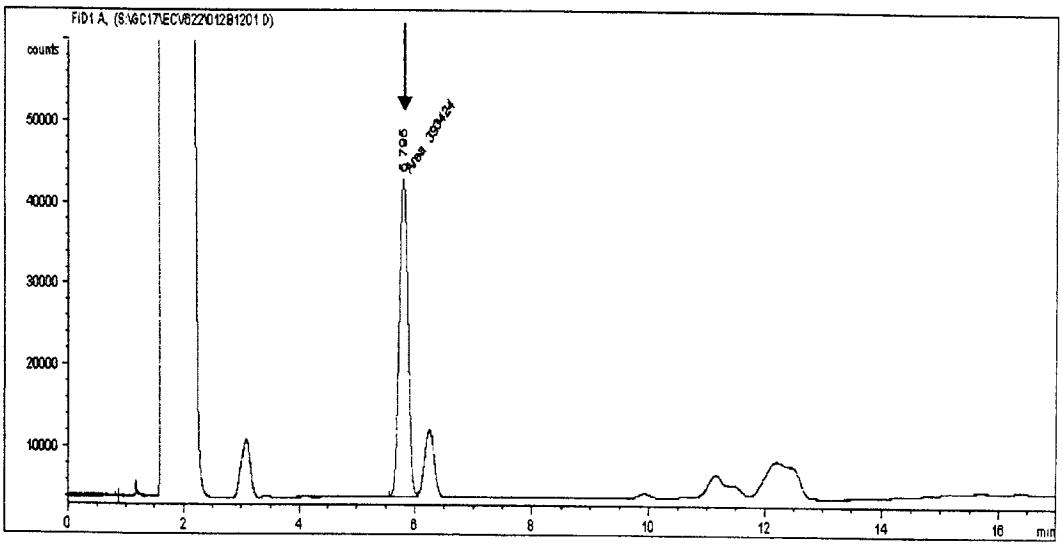
Test Sample 100 mg/l



Test Sample 320 mg/l

Dibromomethane: ALGAL GROWTH INHIBITION TEST

Appendix 2 (continued) Verification of Test Concentrations



Test Sample 1000 mg/l

Appendix 3 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.

For the purposes of the range-finding and definitive test, additional sodium bicarbonate (500 mg/l) was added to the prepared culture medium prior to use.

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

Appendix 4 Statement of GLP Compliance in Accordance with Directive 2004/9/EC

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

LABORATORY	TEST TYPE
SafePharm Laboratories Ltd. **Shardlow Business Park** **London Road** **Shardlow** **Derby** **DE72 2GD**	**Analytical Chemistry** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Phys/Chem Testing** **Toxicology**

DATE OF INSPECTION

30th August 2005

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

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

Bryan J Wright 21/11/05.

Mr. Bryan J. Wright
Head, UK GLP Monitoring Authority