CONFIDENTIAL

SPONSOR
Rhodia Organique - Life Science Systems
190 avenue Thiers
69457 Lyon CEDEX 06
France

TEST ITEM
BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM

STUDY TITLE
ALGAL INHIBITION TEST

STUDY DIRECTOR
Jacques L'Haridon

STUDY COMPLETION DATE
23 August 2002

PERFORMING LABORATORY
CIT
BP 563 - 27005 Evreux - France

LABORATORY STUDY NUMBER
22920 EAA

CONTENTS

STATEMENT OF THE STUDY DIRECTOR AND CIT SCIENTIFIC MANAGEMENT

The study was performed in compliance with the principles of Good Laboratory Practice as described in:

- OECD Principles on Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM (98) 17.
- Décret N° 98-1312 du 31 décembre 1998 concernant les Bonnes Pratiques de Laboratoire, (Journal Officiel du 1er janvier 1999) Ministère de l'Economie, des Finances et de l'Industrie.
- Commission Directive 1999/11/EC of 8 March 1999 adapting to technical progress the Principles of Good Laboratory Practice as specified in Council Directive 87/18/EEC on the harmonisation of laws, regulations and administrative provisions relating to the application of the Principles of Good Laboratory Practice and the verification of their applications for tests on chemical substances (OJ No. L 77 of 23.3.1999).

I declare that this report constitutes a true and faithful record of the procedures undertaken and the results obtained in the performance of the study.

This study was performed at CIT, BP 563, 27005 Evreux, France.

Ecotoxicology

J. L'Haridon Date: 23 August 2002
Doctor of Ecotoxicology
Study Director

R. Groult Date: 23 August 2002
Doctor of Chemistry
Scientific Management

OTHER SCIENTIST INVOLVED IN THIS STUDY

For Pharmacy: X. Manciaux
Doctor of Pharmacy

STATEMENT OF QUALITY ASSURANCE UNIT

Type of inspections	Dates		
	Inspections	Reported to Study Director (*)	Reported to Management (*)
Study plan	22 February 2002	22 February 2002	22 February 2002
Study	4 march 2002	7 march 2002	8 march 2002
Report	24 July 2002	30 July 2002	30 July 2002

In addition to the above-mentioned inspections, at about the same time as the study described in this report, "process-based" and routine facility inspections of critical procedures relevant to this study type were also made by the Quality Assurance Unit.
The findings of these inspections were reported to the Study Director and to CIT Management.

The inspections were performed in compliance with CIT Quality Assurance Unit procedures and Principles of Good Laboratory Practice.

The reported methods and procedures were found to describe those used and the results to constitute an accurate and complete reflection of the study raw data.

C. Galli-Kar Date: 23 August 2002
Ing. Biol.
Head of Quality Assurance Unit

(*) The dates indicated correspond to the dates of signature of audit reports by Study Director and Management.

SUMMARY

At the request of Rhodia Organique - Life Science Systems, Lyon, France, the acute toxicity of the test item BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM (CAS number 90076-65-6) was evaluated in the algal strain *Scenedesmus subspicatus* using a 72-hour static test according to OECD guideline (No. 201, 7th June 1984) and Commission Directive 92/69/EEC (C.3, 31st July 1992).
The criterion measured is the EC50 (Median Effective Concentration), a statistically derived concentration which can be expected to cause a reduction in algal growth of 50% in comparison with the control. This criterion is evaluated as:

- The ErC50 - the concentration of test item resulting in 50% reduction of the specific growth rate with respect to the control. Specific growth rate can be defined as the increase of the natural log of the cell number (ln no. of cells/mL) over specific intervals (e.g. 0 to 24, 0 to 48 and 0 to 72 hours).
- The EbC50 - the concentration of test item resulting in 50% reduction of the biomass (or growth) with respect to the control. Biomass, in this case may be defined as the increase of the cell number per mL of solution x time in hours and therefore represents an area ($cell.mL^{-1}.h$).

Methods

The test item was dissolved in reconstituted water (LC) prepared from deionized water with a conductivity < 10 µS/cm. The total hardness of the reconstituted water is 34 ± 17 mg/L as $CaCO_3$ and the pH is 7.5 ± 0.3.

A preliminary test which included a limit and a range-finding tests preceded the definitive test.

Preliminary test
. Limit test
Three replicate solutions of algae with an initial dilution of 1 x 10^4 cells/mL were exposed to a nominal concentration of 100 mg/L and a further group of six replicate algal solutions without test item was used as the control. The growth of the cell culture in each flask was monitored by counting the number of cells at 24, 48 and 72 hours.

. Range-finding test
Four groups of two replicate flasks were exposed to nominal concentrations of 0.01, 0.1, 1 and 10 mg/L of the test item under the same conditions of light and temperature.

Definitive test
Seven concentrations of BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM were used together with a control at: 0, 5, 10, 20, 40, 80, 160 and 320 mg/L.
Three replicates were exposed to each concentration and six replicates to dilution water only (to act as the control) for 72 hours. Observations of cell growth were recorded at 24, 48 and 72 hours.

Environmental parameters were:
- pH: 7.53 to 8.00,
- temperature: 22.6°C to 23.7°C,
- lighting: 6690 lux to 7390 lux.

Test solutions were agitated throughout the test.

Chemical analysis
Chemical analysis was undertaken to measure the concentration of the test item in each test solution of the definitive test, except for the control and the 5 mg/L solution, since this nominal concentration was lower than the limit of quantification (10 mg/L).

Results

Preliminary test
. Limit test
Inhibitions of the growth rate and growth at 100 mg/L nominal were 34% and 77%, respectively, relative to the control, at T72 hours.

. Range-finding test
Growth rate inhibitions at 0.01, 0.1, 1 and 10 mg/L nominal were 0%, 0%, 1% and 1%, respectively, relative to the control, at T72 hours.
Growth inhibitions at 0.01, 0.1, 1 and 10 mg/L nominal were 0%, 3%, 7% and 7%, respectively, relative to the control, at T72 hours.

Therefore, the 72-hour ErC50 was estimated to be > 100 mg/L and the 72-hour EbC50 between 10 and 100 mg/L.

Definitive test
An analytical problem did not allow to determine the evolution of the actual concentration in the 10 mg/L solution.
However, all measured concentrations in the other test solutions (20, 40, 80, 160 and 320 mg/L), with or without algae, were within ± 20% of the corresponding nominal values at the beginning and the end of the test.
The study results are therefore based on nominal concentrations.

EC50s were calculated according to Probit analysis and the confidence interval limits according to Fieller's method.

The ErC50 at each of the measured growth intervals was as follows:

Time (h)	ErC50 (mg/L)	95% confidence limits (mg/L)
24	318	225 - 513
48	275	172 - 558
72	178	143 - 232

The 72-hour EbC50 was as follows:

Time (h)	EbC50 (mg/L)	95% confidence limits (mg/L)
72	36.0	29.5 - 43.7

The No Observed Effect Concentration (NOEC) at 72 hours was calculated as 5 mg/L ($p = 0.05$) based on the specific growth rate and biomass data.

Conclusion

The two validity criteria of the test were respected:
- the cell concentration in the control cultures increased by a factor of at least 16 within 3 days,
- the variation coefficient of the control cell density was ≤ 15%.

Under our experimental conditions, the 72-hour ErC50 and the 72-hour EbC50 of BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM in a static test system are 178 and 36.0 mg/L, respectively, for *Scenedesmus subspicatus*.

The NOEC at 72 hours is 5 mg/L based on the specific growth rate and biomass data.

1. INTRODUCTION

The objective of this study was to assess the acute toxicity of BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM, to *Scenedesmus subspicatus*, in a 72-hour static test complying with the OECD guideline (No. 201, 7th June 1984) and Commission Directive 92/69/EEC (C.3, 31st July 1992).

The criterion measured is the EC50 (Median Effective Concentration), a statistically derived concentration of the test item in water which can be expected to cause a reduction of algal growth of 50% in comparison with the control. This criterion is evaluated as:

- The ErC50 - the concentration of test item resulting in 50% reduction of the specific growth rate with respect to the control.
- The EbC50 - the concentration of test item resulting in 50% reduction of the biomass (or growth) with respect to the control.

2. MATERIALS AND METHODS

2.1 TEST ITEM

2.1.1 Identification

- supplier: Rhodia
- name:
  - Study plan: BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM
  - other name: TFSI Li
  - labeling: TFSi Li
- batch number:
  - labeling: 01 324 01
  - Study plan: 01-324-01
- description: white powder
- containers: four glass flasks
- date of receipt: 22 November 2001
- storage conditions: at room temperature and protected from humidity (from 8 January 2002)
- purity: ≥ 99.5%.

Data relating to the characterisation of the test item are documented in an analytical certificate (presented in appendix 1) provided by the Sponsor.

2.1.2 Preparation of the test solutions

The stock solutions, for the preliminary and definitive tests, were prepared by dissolving the test item directly in LC reconstituted water (see § 2.2.2).

The conditions of preparation of the stock solutions are reported in the following table:

	Quantity of test item (mg)	Volume of LC reconstituted water (mL)	Concentration of the test item (mg/L)	Duration of the agitation (minutes)
Preliminary test	100	1000	100	5
Definitive test	320	1000	320	5

The agitation was continued until the stock solutions were used to prepare the test solutions.

Test solutions were prepared by further dilution of the stock solution with LC reconstituted water to provide a geometric series of concentrations:
- 0, 0.01, 0.1, 1, 10 and 100 mg/L for the preliminary test,
- 0, 5, 10, 20, 40, 80, 160 and 320 mg/L for the EC50 test.

Glass test vessels (250 mL Erlenmeyer flasks) containing algae and test solutions (or dilution water in the case of the controls) were filled directly from the test solution containers immediately after preparation and test solutions remained unchanged throughout the study.
The pH of the test solutions remained within acceptable limits (between 6 and 9 and in the range ± 0.5 unit of the test water) after preparation and there was no adjustment of pH before addition of the algae.

For the limit and definitive tests, a further group of vessels was prepared for chemical analysis by adding test solutions (except for the control) but no algal pre-culture.

2.2 TEST SYSTEM

2.2.1 Algae

Species:	*Scenedesmus subspicatus.*
Strain No.:	CCAP 276/20.
Reason for this choice:	species commonly used in Europe for aquatic toxicity testing and recommended in OECD and EEC guidelines.
Cultured at:	CIT.
Origin:	Culture Collection of Algae and Protozoa, Institute of Freshwater Ecology, Far Sawrey, Ambleside, Cumbria, LA22 OLP, UK.
Culture method:	the algae are cultured under sterile conditions and maintained at exponential growth rate.

2.2.2 Environmental conditions during culture

During the culture period the conditions were as follows:

Water:	reconstituted water (LC), see appendix 2. A primary stock solution is made up every month from which a final culture medium is prepared. This solution is autoclaved at which point it has a stock life of one week. Before use, the pH is verified at 7.5 ± 0.3 or adjusted until this pH is obtained. The hardness of this solution is approximately 34 ± 17 mg/L as $CaCO_3$.
Culture period:	algae are cultivated for 7 days before harvesting for use in a new culture.
Temperature:	between 21°C and 25°C in water. Algae are maintained at ± 2°C during the acclimation period. Temperature is checked daily.
Illumination:	24 hours per day of constant illumination maintained at approximately 2000 lux throughout the culture period. For lighting details see § 2.2.3.

Aeration: the cultures are constantly aerated with air filtered using a 0.22 µm porosity filter to maximize the CO_2 availability (and thereby the cell growth) while maintaining the sterility.

Preculture loading: new cultures are loaded at a concentration of 1 x 10^4 cells/mL. Each weekly culture is prepared under sterile conditions using autoclaved medium while week old cell cultures are checked for contamination before being transferred to fresh medium.

2.2.3 Environmental conditions during the test

Test water: reconstituted-LC (see § 2.2.2).

Temperature: controlled daily in a flask containing test water but no algae, run alongside the test. Between 21°C and 25°C and did not vary by more than ± 2°C during the test.

Illumination: continuous. A set of 15 and 30 W Osram Fluora® fluorescent tubes between "white" and "daylight" type (spectral range 400 to 700 nm) were set approximately 40 cm above the cultures. These emit light measured using a lux meter equipped with a spherical collector at a level corresponding to half the height of the cultures in their conical flasks (corresponding to an irradiance of 252 mW/m^2 for 15 W and 476 mW/m^2 for 30 W tubes x no. of bulbs = 3724 mW/m^2). The light intensity at each position to be occupied by a culture flask is measured regularly. The color temperature of the fluorescent lamps is between 4400 K and 5000 K.

Duration of test: 72 hours.

Transfer: the number of cells in the week-old culture was counted and the quantity of algal pre-culture to be added to the fresh test medium calculated to give a test solution concentration of 1 x 10^4 cells/mL. The algal pre-cultures were then added where appropriate to the 100 mL test solutions and all the 250 mL conical flasks stoppered with sterile cotton wool wrapped with lint and transferred to the agitator.

Culture homogeneity: the solutions were agitated throughout the test.

pH: the pH values of the control and of all the test concentrations (except for the range-finding concentrations) were measured at the beginning and the end of the tests. These values did not deviate by more than 1.5 units throughout the tests.

Hardness: water hardness was measured once in LC reconstituted water before the start of the test.

Forced aeration: was not used during the test but the cultures were constantly agitated.

2.3 TREATMENT

2.3.1 Study design

A preliminary test which included a limit and a range-finding tests preceded the definitive test. The duration of each test was 72 hours.

Preliminary test

Limit test	Concentration (mg/L)	Number of replicates	
		with algae	without algae
Control	0	6	0
Treated	100	3	1

Range-finding test	Concentration (mg/L)	Number of replicates	
		with algae	without algae
Treated	0.01	2	0
Treated	0.1	2	0
Treated	1	2	0
Treated	10	2	0

Definitive test

EC50	Concentration (mg/L)	Number of replicates	
		with algae	without algae
Group 1 (control)	0	6	0
Group 2	5	3	1
Group 3	10	3	1
Group 4	20	3	1
Group 5	40	3	1
Group 6	80	3	1
Group 7	160	3	1
Group 8	320	3	1

2.3.2 Time schedule

Experimental starting date (first day of treatment): 4 March 2002,
Experimental completion date: 11 April 2002.

2.4 OBSERVATIONS

The number of cells in solutions was calculated at T0 as 1 x 10^4 cells/mL. For the remaining observation times (T24, T48 and T72 hours), cell numbers were determined using a Malassez cell counter.

An observation of less than 1 x 10^4 cells/mL is below the sensitivity of the Malassez cell counter. Possible observations of this type were considered as 1 x 10^4 cells/mL - i.e. the test item was taken to be algaestatic at this test concentration rather than algaecidal, as no further experiment was undertaken to determine whether algae exposed to algaestatic concentrations were capable of growth post-exposure.

2.5 CHEMICAL ANALYSIS

Each sample contained 5 mL.

2.5.1 Test solutions analysis

2.5.1.1 Preliminary test

At T0 hour, a sample was taken from the limit test solution container (100 mg/L) and refrigerated at +4°C and –20°C.
At T24 and T48 hours, samples were taken from each limit test solution replicate, pooled (4 mL in total) and refrigerated at +4°C and –20°C.
Further samples were also taken at T24 and T48 hours from the limit test solution which contained no algae.

Although samples were taken during the test, no chemical analysis was performed on these samples because the test item was found to be toxic after 72 hours.

2.5.1.2 Definitive test

At T0 hour, samples were taken from each test solution container, except for the control, and refrigerated at +4°C and –20°C until analysis.
At T24, T48 and T72 hours, samples were taken from all test replicate groups at each concentration (except for the control), pooled (5mL in total) and refrigerated at +4°C and –20°C until analysis.
Further samples were also taken at T24, T48 and T72 hours from the solutions which contained no algae and had been run alongside the test to determine the influence of adsorption (at the surface of algae cells) and/or bioaccumulation on the possible decrease of test item concentration throughout the test.

Chemical analysis was only performed on samples (stored at +4°C) taken at the beginning and the end (T72 hours) of the test.

The analytical procedure is presented in appendix 3.

2.6 DATA EVALUATION

2.6.1 Determination of the ErC50 and the EbC50

Specific growth rate can be defined as the natural log of the cell number (ln no. of cells/mL) over specific intervals (e.g. 0 to 24, 0 to 48 and 0 to 72 hours) (see appendix 4 for details). The concentration causing a reduction of growth rate to 50% that of the control (ErC50) is obtained for each observation time (T24, T48, and T72 hours) by calculation from the average specific growth rate at each concentration.
Biomass (or growth), in this case may be defined as the number of cells per mL of solution x time in hours and therefore represents an area (cells.mL^{-1}.h). The biomass at 72 hours is determined using the trapezoïdal method from the number of cells observed at T0, T24, T48 and T72 hours. The concentration causing a reduction of biomass at 72 hours to 50% that of the control (EbC50) is then calculated.
When for at least two concentrations, inhibition is > 0% and < 100%, the EC50 is calculated according to Probit analysis (i.e. Finney's method, published by E. Weber, combined with Bliss's method). The confidence interval limits are calculated statistically according to Fieller's method.
When at only one concentration, inhibition is > 0% and < 100%, the EC50 is also calculated by Probit analysis. In this case, the highest concentration causing no inhibition and the lowest concentration producing 100% inhibition are used as confidence limits.

If at all concentrations, inhibition is 0% or 100%, the EC50 corresponds to the geometric mean of the highest concentration causing no inhibition and the lowest concentration producing 100% inhibition.

2.6.2 Determination of the No Observed Effect Concentration (NOEC)

After checking of the normality of the data with Chi-square and Shapiro-Wilks tests (normal at $p = 0.01$) as well as the variance homogeneity (Bartlett test), the NOEC is determined by ANOVA (the Bonferroni T-test) using the individual replicates of the area under the curve and the specific growth rate.

2.7 ARCHIVING

The study documentation generated during the course of the study is archived at CIT, 27005 Evreux, France, for 10 years after the end of the *in vivo* phase of the study.

The archived study materials include:
. Study plan and possible amendments,
. raw data,
. correspondence,
. final report and possible amendments.

On completion of this period, the archived study materials will be returned to the Sponsor, or may be archived at CIT for a further period (at additional cost).
In addition, raw data not specific to the study including, but not limited to, records of environmental data and equipment calibration, will also be archived at CIT and retained for at least 30 years.

2.8 STUDY PLAN ADHERENCE

The study was performed in accordance with the Study plan No. 22920 EAA and subsequent amendments. There were no deviations from the agreed Study plan.

3. RESULTS

3.1 PRELIMINARY TEST

3.1.1 Water quality

The test solution was a translucent colorless solution at all test item concentrations.

The minimum and maximum parameters measured during the preliminary test were:
- pH: 7.39 and 7.87,
- temperature: 24.0°C and 24.1°C,
- lighting: 6470 lux and 7550 lux.

The temperature and pH data are presented in table 1.

3.1.2 Chemical analysis

No chemical analysis was undertaken since the test item was found to be toxic in this preliminary test.

3.1.3 Growth inhibition

The cell growth data are presented in table 2.

. Limit test

Inhibitions of the growth rate and growth at 100 mg/L nominal were 34% and 77%, respectively, relative to the control, at T72 hours.

. Range-finding test

Growth rate inhibitions at 0.01, 0.1, 1 and 10 mg/L nominal were 0%, 0%, 1% and 1%, respectively, relative to the control, at T72 hours.
Growth inhibitions at 0.01, 0.1, 1 and 10 mg/L nominal were 0%, 3%, 7% and 7%, respectively, relative to the control, at T72 hours.

Therefore, the 72-hour ErC50 was estimated to be > 100 mg/L and the 72-hour EbC50 between 10 and 100 mg/L.

3.2 DEFINITIVE TEST

3.2.1 Water quality

The test solution was a translucent colorless solution at all test item concentrations.

The minimum and maximum parameters measured during the EC50 test were:
- pH: 7.53 and 8.00,
- temperature: 22.6°C and 23.7°C,
- lighting: 6690 lux and 7390 lux.

The temperature and pH data are presented in table 3.

3.2.2 Chemical analysis

Results are presented in tables A, appendix 3.

An analytical problem did not allow to determine the evolution of the actual concentration in the 10 mg/L solution.
However, all measured concentrations in the other test solutions (20, 40, 80, 160 and 320 mg/L), with or without algae, were within ± 20% of the corresponding nominal values at the beginning and the end of the test.
The study results are therefore based on nominal concentrations.

3.2.3 Growth inhibition

The results are presented in tables 4a, b and c and in table 5.
The 24, 48 and 72-hour ErC50s and the 72-hour EbC50 were estimated from the % inhibition of specific growth rate (ErC50s) and biomass (EbC50).
All these EC50s were calculated by computer as the spread of inhibition across the concentrations was sufficient for the software to undertake the calculation.

The ErC50 at each of the measured growth intervals was as follows:

Time (h)	ErC50 (mg/L)	95% confidence limits (mg/L)
24	318	225 - 513
48	275	172 - 558
72	178	143 - 232

The 72-hour EbC50 was as follows:

Time (h)	EbC50 (mg/L)	95% confidence limits (mg/L)
72	36.0	29.5 - 43.7

The EC50 calculations are reported in appendix 5.

The No Observed Effect Concentration (NOEC) at 72 hours was calculated as 5 mg/L ($p = 0.05$) based on the specific growth rate and biomass data.

4. CONCLUSION

The two validity criteria of the test were respected:
- the cell concentration in the control cultures increased by a factor of at least 16 within 3 days,
- the variation coefficient of the control cell density was ≤ 15%.

Under our experimental conditions, the 72-hour ErC50 and the 72-hour EbC50 of BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM in a static test system are 178 and 36.0 mg/L, respectively, for *Scenedesmus subspicatus*.

The NOEC at 72 hours is 5 mg/L based on the specific growth rate and biomass data.

5. REFERENCES

Bliss, C.I.: The determination of dosage-mortality curves from small number. Quart. J. Pharm. 11, 192-216 (1938).

Fieller: A fundamental formula in the statistics of biological assay and some applications. Quarterly Journal of Pharmacy and Pharmacology, 117-123 (1944).

Weber, E: Grunden der biologischen Statistik, Gustav Fisher Verlag, Stuttgart, 1972.

Table 1: Preliminary test - temperature and pH

Nominal concentration (mg/L)	pH at T 0 hour	pH at T 72 hours
0	7.39	7.87
100	7.52	7.57

Temperature measured between 0 and 2 hours: 24.0°C
Temperature at 24 hours (measured in the same test vessel): 24.1°C
Temperature at 48 hours (measured in the same test vessel): 24.1°C
Temperature at 72 hours (measured in the same test vessel): 24.0°C

Table 2: Preliminary test cell growth data

Group	Nominal concentration (mg/L)	Replicate	Cells/mL at 24 hours (x 10^4)	Cells/mL at 48 hours (x 10^4)	Cells/mL at 72 hours (x 10^4)
1	0	1	6.8	28.5	121.0
1	0	2	7.3	27.0	122.0
1	0	3	4.7	25.4	114.0
1	0	4	4.2	22.2	104.0
1	0	5	5.9	26.5	120.0
1	0	6	4.9	27.7	103.0
2	0.01	1	4.7	25.5	123.0
2	0.01	2	4.1	21.4	122.0
3	0.1	1	4.6	20.0	110.0
3	0.1	2	4.1	25.0	126.0
4	1	1	4.4	25.5	109.0
4	1	2	3.5	25.5	103.0
5	10	1	4.5	25.0	109.0
5	10	2	3.8	21.6	112.0
6	100	1	3.6	6.2	21.2
6	100	2	4.5	8.3	26.5
6	100	3	4.5	6.4	20.5

Variation coefficient of the control cell density at 72 hours: 7.55%

Table 3: Definitive test - temperature and pH

Nominal concentration (mg/L)	pH at T 0 hour	pH at T 72 hours
0	7.76	8.00
5	7.74	7.61
10	7.79	7.53
20	7.76	7.58
40	7.77	7.81
80	7.70	7.57
160	7.71	7.65
320	7.69	7.70

Temperature measured between 0 and 2 hours: 23.7°C
Temperature at 24 hours (measured in the same test vessel): 22.6°C
Temperature at 48 hours (measured in the same test vessel): 23.0°C
Temperature at 72 hours (measured in the same test vessel): 22.8°C

Table 4a: T24 hours - number of cells and calculation of the specific growth rate at each concentration

Nominal concentration (mg/L)	Rep.	Total No. of cells at 24 hours (10^4/mL)	Ln No. of cells	Mean Ln No. of cells	Specific growth rate	Inhibition of growth rate (%)
0	1	3.1	10.34	10.42	0.05	
	2	3.6	10.49			
	3	3.1	10.34			
	4	2.9	10.27			
	5	3.9	10.57			
	6	3.7	10.52			
5	1	3.7	10.52	10.39	0.05	2.82
	2	3.2	10.37			
	3	2.9	10.27			
10	1	2.8	10.24	10.26	0.04	13.22
	2	3.0	10.31			
	3	2.8	10.24			
20	1	3.1	10.34	10.30	0.05	10.52
	2	2.7	10.20			
	3	3.1	10.34			
40	1	3.0	10.31	10.39	0.05	2.64
	2	3.2	10.37			
	3	3.6	10.49			
80	1	2.7	10.20	10.14	0.04	23.10
	2	3.2	10.37			
	3	1.9	9.85			
160	1	2.4	10.09	9.83	0.03	48.65
	2	1.8	9.80			
	3	1.5	9.62			
320	1	1.7	9.74	9.81	0.03	50.45
	2	1.7	9.74			
	3	2.1	9.95			

Ln number of cells at T0 (10 000 cells) = 9.21

Rep. : replicate

Table 4b: T48 hours - number of cells and calculation of the specific growth rate at each concentration

Nominal concentration (mg/L)	Rep.	Total No. of cells at 48 hours (10^4/mL)	Ln No. of cells	Mean Ln No. of cells	Specific growth rate	Inhibition of growth rate (%)
0	1	13.7	11.83	11.87	0.06	
	2	13.1	11.78			
	3	18.1	12.11			
	4	12.7	11.75			
	5	17.3	12.06			
	6	11.9	11.69			
5	1	8.6	11.36	11.65	0.05	8.34
	2	13.0	11.77			
	3	13.4	11.81			
10	1	8.1	11.30	11.36	0.04	19.23
	2	8.0	11.29			
	3	9.7	11.48			
20	1	6.7	11.11	11.10	0.04	28.75
	2	7.7	11.25			
	3	5.7	10.95			
40	1	6.8	11.13	10.99	0.04	33.01
	2	5.4	10.90			
	3	5.7	10.95			
80	1	6.9	11.14	11.15	0.04	27.19
	2	7.1	11.17			
	3	6.8	11.13			
160	1	4.6	10.74	10.95	0.04	34.74
	2	6.1	11.02			
	3	6.5	11.08			
320	1	3.9	10.57	10.28	0.02	59.67
	2	2.0	9.90			
	3	3.2	10.37			

Ln number of cells at T0 (10 000 cells) = 9.21

Rep. : replicate

Table 4c: T72 hours - number of cells and calculation of the specific growth rate at each concentration

Nominal concentration (mg/L)	Rep.	Total No. of cells at 72 hours (10^4/mL)	Ln No. of cells	Mean Ln No. of cells	Specific growth rate	Inhibition of growth rate (%)
0	1	55.0	13.22	13.31	0.06	
	2	67.0	13.41			
	3	55.5	13.23			
	4	64.5	13.38			
	5	68.5	13.44			
	6	54.0	13.20			
5	1	45.3	13.02	13.24	0.06	1.82
	2	69.0	13.44			
	3	56.5	13.24			
10	1	39.3	12.88	13.02	0.05	7.11
	2	48.5	13.09			
	3	48.3	13.09			
20	1	39.4	12.88	12.81	0.05	12.13
	2	36.0	12.79			
	3	35.0	12.77			
40	1	35.0	12.77	12.77	0.05	13.23
	2	35.6	12.78			
	3	34.8	12.76			
80	1	26.3	12.48	12.34	0.04	23.80
	2	21.8	12.29			
	3	20.6	12.24			
160	1	11.5	11.65	11.52	0.03	43.58
	2	10.6	11.57			
	3	8.5	11.35			
320	1	3.0	10.31	10.32	0.02	72.90
	2	2.6	10.17			
	3	3.6	10.49			

Ln number of cells at T0 (10 000 cells) = 9.21

Rep. : replicate

Variation coefficient of the control cell density at 72 hours: 10.9%

Table 5: Number of cells (10^4 cells x mL^{-1}) at each observation time and calculation of the area under the growth curve (biomass) at each concentration. Area = cell x mL^{-1} x hour

		Total number of cells			Growth					Inhibition (%)
Nominal concentration (mg/L)	Rep.	at 24 hours	at 48 hours	at 72 hours	0 to 24 hours	24 to 48 hours	48 to 72 hours	0 to 72 hours	Average (0-72 hours)	
0	1	3.10	13.70	55.00	25.20	177.60	800.40	1003.20	1097.40	
	2	3.60	13.10	67.00	31.20	176.40	937.20	1144.80		
	3	3.10	18.10	55.50	25.20	230.40	859.20	1114.80		
	4	2.90	12.70	64.50	22.80	163.20	902.40	1088.40		
	5	3.90	17.30	68.50	34.80	230.40	1005.60	1270.80		
	6	3.70	11.90	54.00	32.40	163.20	766.80	962.40		
5	1	3.70	8.60	45.30	32.40	123.60	622.80	778.80	981.60	10.55
	2	3.20	13.00	69.00	26.40	170.40	960.00	1156.80		
	3	2.90	13.40	56.50	22.80	171.60	814.80	1009.20		
10	1	2.80	8.10	39.30	21.60	106.80	544.80	673.20	759.60	30.78
	2	3.00	8.00	48.50	24.00	108.00	654.00	786.00		
	3	2.80	9.70	48.30	21.60	126.00	672.00	819.60		
20	1	3.10	6.70	39.40	25.20	93.60	529.20	648.00	613.60	44.09
	2	2.70	7.70	36.00	20.40	100.80	500.40	621.60		
	3	3.10	5.70	35.00	25.20	81.60	464.40	571.20		
40	1	3.00	6.80	35.00	24.00	93.60	477.60	595.20	583.20	46.86
	2	3.20	5.40	35.60	26.40	79.20	468.00	573.60		
	3	3.60	5.70	34.80	31.20	87.60	462.00	580.80		
80	1	2.70	6.90	26.30	20.40	91.20	374.40	486.00	443.60	59.58
	2	3.20	7.10	21.80	26.40	99.60	322.80	448.80		
	3	1.90	6.80	20.60	10.80	80.40	304.80	396.00		
160	1	2.40	4.60	11.50	16.80	60.00	169.20	246.00	245.60	77.62
	2	1.80	6.10	10.60	9.60	70.80	176.40	256.80		
	3	1.50	6.50	8.50	6.00	72.00	156.00	234.00		
320	1	1.70	3.90	3.00	8.40	43.20	58.80	110.40	93.60	91.47
	2	1.70	2.00	2.60	8.40	20.40	31.20	60.00		
	3	2.10	3.20	3.60	13.20	39.60	57.60	110.40		

Rep. : replicate

Variation coefficient of the control cell density at 72 hours: 10.9%

APPENDICES

1. Analytical certificate

Rhodia

RHODIA H.P.C.I.I.

USINE DE CLAMECY

LABORATOIRE DE CONTROLE ANALYTIQUE

PRODUIT : BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM (TFSILi)

REFERENCE : 01-324-01 **ORIGINE : Atelier F1**	**ANALYSE** **Demandée le : 20 Novembre 2001** **Rendue le : 20 Novembre 2001**

	Unité	RESULTATS	SPECIFICATIONS
Teneur en eau	ppm	200	Pour information
Titre en TFSILi	%	≥ 99.5	Pour information

Conclusions du contrôle analytique :

PRODUIT CONFORME

Approbation :

D.MATYKOWSKI
Responsable L.C.A.

2. Substances required for the preparation of LC reconstituted water

	Final concentrations
Solution No. 1:	
- calcium nitrate ($Ca(NO_3)_2.4H_2O$)	40 mg/L
Solution No. 2:	
- potassium nitrate (KNO_3)	100 mg/L
Solution No. 3:	
- magnesium sulphate ($MgSO_4.7H_2O$)	30 mg/L
Solution No. 4:	
- monohydrogen potassium phosphate (K_2HPO_4).....…	40 mg/L
Trace element solutions:	
Solution No. 5:	
- copper sulphate ($CuSO_4.5H_2O$)	15 µg/L
- ammonium heptamolybdate [$(NH_4)_6Mo_7O_{24}.4H_2O$]	30 µg/L
- zinc sulphate ($ZnSO_4.7H_2O$)	30 µg/L
- cobalt chloride ($CoCl_2.6H_2O$)	30 µg/L
- manganese nitrate ($Mn(NO_3)_2.4H_2O$)	30 µg/L
- citric acid ($C_6H_8O_2.H_2O$)	30 µg/L
- boric acid (H_3BO_3)	30 µg/L
Solution No. 6:	
- iron citrate (III) ($C_6H_5FeO_7$)	0.616 mg/L
- iron sulphate (II) ($FeSO_4.7H_2O$)	0.3125 mg/L
- iron chloride (III) ($FeCl_3.6H_2O$)	0.3125 mg/L

All solutions are made up with injectable grade deionized water (conductivity < 10 μScm^{-1}).
Prepared solutions are kept for no more than 1 week after autoclaving.
Final pH = 7.5 ± 0.3; hardness = 34 mg/L ± 17 mg/L as $CaCO_3$.

3. Chemical analysis of test solutions

CHEMICAL ANALYSIS

Principle

An aliquot of each sample was diluted and analyzed by Ion Chromatography with Electrochemical Detection (Conductimetry). The concentrations of BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM were determined from a calibration curve of peak area against concentration of BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM in standard solutions.

Sample preparation

All samples were mixed and centrifuged (only samples with algae), (4000 rpm, 15 min, +4°C). An aliquot of each sample was injected without dilution (except for 160 and 320 mg/L which were diluted in Milli-Q water to achieve concentrations in the range from 10 to 100 mg/L of test item.

Chromatographic conditions

Pump : GP 50 Gradient Pump (Dionex)

Mobile phase : phase A: aqueous sodium hydroxide solution 50 mM*
phase B: Milli-Q water
phase C: acetonitrile

* Aqueous sodium hydroxide solution 50 mM: 2.6 mL of sodium hydroxide solution (Fisher Chemicals, Ref. S/4930/05) was added to 1 L of Milli-Q Water.

Time (min)	phase A (%)	phase B (%)	phase C (%)	curve
0	10	85	5	6
5	40	0	60	6
12	40	0	60	-
13	10	85	5	-
25	10	85	5	-

Flow rate : 1 mL/min

Precolumn : Ionpac ATC-3, 4 mm (Dionex)

Column : Ionpac AS16 (Dionex)
length = 250 mm, inner diameter = 4 mm

Temperature : 30°C

Detector : ED 50 Electrochemical Detector (Dionex)
Conductimetry after eluent neutralization

Neutralization : ASRS Ultra (Dionex), electric current 70 mA

Injector : A50 (Dionex), at 10°C
Injected volume : 25 µL

Data acquisition software : Multichrom 2 (Fisons Instruments)

Retention time : BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM, approx. 10 min

Analysis time : 40 min

Calibration curve

Peak areas were determined for standard solutions ranging from 10 to 100 mg/L of BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM (six levels). A calibration curve was obtained by plotting test item peak areas against concentrations.

The regression analysis of the calibration data gave an equation of the following form:

$$Y = aX + b$$

where Y = Test item peak area (µVs)
X = concentration of test item (mg/L)
a = slope value
b = intercept

Assay

Samples of BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM were analyzed by Ion Chromatography with Electrochemical Detector (Conductimetry).

One dilution was prepared for each sample and one injection (of 25 µL-aliquots) was performed.

The peak areas were determined for each sample. The concentration of BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM in each sample was calculated using the equation obtained from the calibration data.

All the results are expressed as mg/L of BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM.

Results

Measured concentrations of BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM are presented in table A.

Table A: Concentration of BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM in the definitive test solutions (mg/L)

Nominal	Measured (1)					
	0 Hour		72 Hours			
	(3)		(2)		(3)	
5	na	nc	na	nc	na	nc
10	vd	nc	vd	nc	vd	nc
20	18.6	(93)	16.9	(85)	15.9	(80)
40	34.8	(87)	34.6	(87)	32.1	(80)
80	66.6	(83)	73.0	(91)	69.5	(87)
160	138	(86)	128	(80)	156	(98)
320	311	(97)	297	(93)	289	(90)

(1): numbers in brackets represent percentages of the nominal concentrations

(2): samples with algae

(3): samples without algae

na: not analyzed

nc: not calculated

vd: value discarded (due to an analytical trouble)

VALIDATION OF THE ANALYTICAL METHOD

The validation of the analytical method was performed according to CIT Standard Operating Procedures.
The specificity, limit of quantification, linearity, repeatability of injections, accuracy and precision (Coefficient of variation: CV %) of the analytical method were determined.

Specificity

The specificity of the analytical method was demonstrated as follows:
- analysis of a standard solution of BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM in Milli-Q water,
- analysis of Milli-Q water without dilution,
- analysis of test water (Milli-Q water, purified water, LC, M4 or dechlorited: deionized water) without dilution.

No relevant interference between the test item peak and Milli-Q water was observed on chromatograms.

Limit of quantification

The limit of quantification of the analytical method was established as 10 mg/L for a standard solution of BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM.
This limit corresponds to a limit of quantification of 10 mg/L for the test item in aqueous phase and organic phase.

Linearity

Linearity was checked by analysis of three different sets of six standard solutions containing 10, 20, 30, 50, 75 and 100 mg/L of BIS TRIFLUOROMETHANESULFONIMIDE LITHIUM in Milli-Q water.

Satisfactory linearity was demonstrated in the range 10 to 100 mg/L since the coefficients of determination obtained were higher than 0.999.
A summary of back-calculated concentrations is presented in table B.

Repeatability of injections

Replicate analysis (n = 10) of a solution containing 100 mg/L of the test item gave satisfactory results since the coefficient of variation obtained were as follows:
. 2% based on peak height,
. 1% based on peak area.

Based on these results, the repeatability of injections of the analytical method was validated taking into account peak area.

Accuracy and precision

Six analyses of solution containing 1000 and 18000 mg/L of the test item in purified water were carried out. Samples were diluted appropriately with Milli-Q water before analysis.
The accuracy and the precision (CV%) obtained were as follows:

Concentration (mg/L)		Dilution	CV	Accuracy
Nominal	Mean measured*	factor	(%)	(%)
1000	10600	50	1	106
18000	19700	400	1	109

*: mean values of six determinations

Conclusion

The analytical method was validated and considered to be suitable for the analysis of the samples of the study.

Table B: Back-calculated concentrations for standard solutions of BIS TRIFLUOROMETHANE-SULFONIMIDE LITHIUM

	First linearity		Second linearity		Third linearity		All values together		
Nominal concentration (mg/L)	Measured concentration (mg/L)	Deviation %	Measured concentration (mg/L)	Deviation %	Measured concentration (mg/L)	Deviation %	Mean	CV %	Deviation %
10.0	11.3	*13*	9.99	*0*	10.5	*5*	10.6	6	*6*
20.0	19.3	*-4*	19.1	*-4*	20.1	*1*	19.5	3	*-2*
30.0	28.2	*-6*	30.5	*2*	29.0	*-3*	29.2	4	*-3*
50.0	51.3	*3*	51.5	*3*	50.0	*0*	50.9	2	*2*
75.0	75.4	*1*	73.4	*-2*	75.8	*1*	74.9	2	*0*
100	99.7	*0*	100	*0*	99.6	*0*	99.8	0	*0*
R^2	0.9988		0.9989		0.9992				

R^2: coefficient of determination

Deviation: Deviation (%) from nominal concentration

4. Comparison of areas under the growth curves

COMPARISON OF AREAS UNDER THE GROWTH CURVES (Biomass)

The areas between the growth curves and the horizontal line $N = N_0$ were calculated according to the formula:

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

where,

A = area
N_0 = number of cells/mL at time t_0 (beginning of the test),
N_1 = measured number of cells/mL at time t_1
N_n = measured number of cells/mL at time t_n
t_1 = time of first measurement after beginning of test
t_n = time of n^{th} measurement after beginning of test
n = number of measurements taken after the beginning of the test.

The percentage inhibition of the cell growth at each test item concentration (I_A) was calculated according to the formula:

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

where,
A_c = area between the control growth curve and the horizontal line $N = N_0$
A_t = area between the growth curve at the concentration t and the horizontal line $N = N_0$

I_A values are plotted on semilogarithmic paper or on semilogarithmic probit paper against the corresponding concentrations. If plotted on probit paper, the points are fitted by a straight line, either by eye or by a computed regression.

The EC50 is estimated from the regression line by reading off the concentration that is equivalent to a 50% inhibition ($I_A = 50\%$). To denote this value unambiguously in relation to this method of calculation, it is proposed to use the symbol E_bC_{50} is quoted with the appropriate exposure period, e.g. E_bC_{50}(0-72h).

COMPARISON OF GROWTH RATES

The average specific growth rate (μ) for exponentially growing cultures was calculated as:

$$\mu = \frac{Ln\ N_n - Ln\ N_0}{t_n - t_0}$$

where t_0 is the time at the beginning of the test.

The percentage inhibition of specific growth rate at each test item concentration ($I_\mu t$) was calculated according to the formula:

$$I_{\mu t} = \frac{\mu_c - \mu_t}{\mu_c} \times 100$$

where,
μ_c = mean control specific growth rate
μ_t = mean specific growth rate for the test concentration t

The percentage reduction of average specific growth rate at each test item concentration compared with the control value is plotted against the logarithm of the concentration. The EC50 may be red from the resulting graph. To denote unambiguously the EC50 derived by this method it is proposed to use the symbol E_rC_{50}. The times of measurement must be indicated, e.g. if the value relates to times 0 and 72 hours, the symbol becomes E_rC_{50}(0-72h).

Note: specific growth rate is a logarithmic term and small changes in growth rate may lead to great changes in biomass. E_bC and E_rC values are therefore not numerically comparable.

CALCULATION OF THE NOEC

The No Observed Effect Concentration was determined by first checking for normality (i.e. Chi-square test or Shapiro-Wilks test) as well as variance homogeneity (i.e. Bartlett test) and then by analysis of variance (i.e. Bonferroni T-test), using the individual replicate values of the areas under the growth curves or the specific growth rates.

5. EC50 estimations

24h ErC50 ESTIMATION

Concentration mg/L	Percentage inhibition
0	
5	3
10	13
20	11
40	3
80	23
160	49
320	50

Slope: 0.448 Equation: Y = 0.448 x X + 2.418

24h ErC50	LOWER LIMIT		UPPER LIMIT		
318	(225	-	513)		mg/L

Significance: 95%

48h ErC50 ESTIMATION

Concentration mg/L	Percentage inhibition
0	
5	8
10	19
20	29
40	33
80	27
160	35
320	60

Slope: 0.292 Equation: Y = 0.292 x X + 3.360

48h ErC50	LOWER LIMIT		UPPER LIMIT		
275	(172	-	558)		mg/L

Significance: 95%

72h ErC50 ESTIMATION

Concentration mg/L	Percentage inhibition
0	
5	2
10	7
20	12
40	13
80	24
160	44
320	73

Slope: 0.603 Equation: Y = 0.603 x X + 1.878

72h ErC50	LOWER LIMIT		UPPER LIMIT		
178	(143	-	232)		mg/L

Significance: 95%

72h EbC50 ESTIMATION

Concentration mg/L	Percentage inhibition
0	
5	11
10	31
20	44
40	47
80	60
160	78
320	91

Slope: 0.526 Equation: Y = 0.526 x X – 0.517

72h ErC50	LOWER LIMIT		UPPER LIMIT		
36.0	(29.5	-	43.7)	mg/L	

Significance: 95%