AR226-3789

SANITIZED

Commercial-in-Confidence

Chemex reference: ENV8010/020737

The growth inhibition of the marine alga *Skeletonema costatum* by

[ ]

Report for
Notox B.V

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Report issued by:

Chemex Environmental International Limited
Unit J
Broad Lane Industrial Estate
Cottenham
Cambridge
CB24 8SW
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Sponsor:

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April 2007

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Graphs

Appendices

Compliance with Good Laboratory Practice standards

I, the undersigned, hereby declare that the study described in this report was performed under my supervision, and that the final report fully and accurately reflects the raw data generated during the conduct of the study, in compliance with international codes of Good Laboratory Practice including:

- Section II of Annex 1 to the European Parliament and council Directive 2004/10/EC and Annex 1 to the European Parliament and council Directive 2004/9/EC (Official Journal No. L 50) and embodied within:
- *The UK Good Laboratory Practice Regulations 1999 (The United Kingdom GLP Regulations 1999, Statutory Instrument 3106)* as amended by:
- *The UK Good Laboratory Practice (Codification Amendments Etc.) Regulations 2004 (Statutory Instrument No 994)*

These principles are in accordance with the OECD Principles of Good Laboratory Practice, revised 1997 (ENV/MC/CHEM(98)17).

..... 23 April 2007

Tom Fisher
Study Director

Date

Quality Assurance audit statement

Studies of this type are subject to random process-based inspections by the Chemex Quality Assurance Unit.

Date of relevant inspection: 13 & 16 February 2007

Date relevant inspection reported: 16 February 2007

Date of study plan inspection: 28 February 2007

This report has been audited by the Chemex Quality Assurance Unit. It is considered to be an accurate description of the procedures and practices employed during the course of the study and an accurate presentation of the findings.

Date audit started: 20 April 2007

Date audit reported: 23 April 2007

Signed: .....

David Stittle
Quality Manager

Date: 23 April 2007

All reports are individually audited.

Reports are issued to Management on a monthly basis.

Raw data used to derive results included in this report, supplied by either the sponsor or sub-contractor (where applicable), are not audited by Chemex Environmental International Limited.

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Summary

This section summarises aquatic toxicity test results obtained by Chemex Environmental International Limited on a sample as detailed below:

Test commissioned by:	Notox B.V
Substance under test:	[ ]
Chemex reference:	Sample: ECO020737 Study: ENV8010
Test species:	*Skeletonema costatum*, strain 1077/5
Test type:	Acute toxicity: 72 hour EC_{50}
Protocol:	Static test conditions according to SOP E209 based on ISO/DIS BS EN ISO 10253: 1998 "Water quality – Marine Algal Growth Inhibition Test with *Skeletonema costatum and Phaeodactylum tricornutum.*
Test period:	From 2 to 5 April 2007
Test concentrations:	0, 2.2, 4.5, 10, 22, 45 and 100mg/l
Test performed at:	Chemex Environmental International Limited Unit J Broad Lane Industrial Estate Cottenham Cambridge CB24 8SW England

Results:

Period of exposure (hours)	E_bC_{50} value mg/l biomass (95% confidence limits)	Period of exposure (hours)	E_rC_{50} value mg/l growth rate (95% confidence limits)
0 to 48	14mg/l (10 – 19mg/l)	0 to 48	23mg/l (21 – 25mg/l)
0 to 72	13mg/l (10 – 17mg/l)	0 to 72	24mg/l (21 – 27mg/l)
$NOEC_b$ (biomass)	4.5mg/l (0-72 hours)		
$NOEC_r$ (growth rate)			

1. Introduction

This report contains a description of the methods used and the results obtained during a study to investigate the marine algal growth inhibition of [ ] to *Skeletonema costatum*. The objective of this study was to determine the 72 hour median effective concentration (EC_{50}) which is defined as the concentration which inhibits the growth of the marine algae by 50% after a 72 hour exposure period at 20°C.

2. Materials and methods

Unless otherwise specified all methods mentioned in this report are according to Chemex Environmental International Limited standard procedures. All records of measurements and observations made during this test will be collated and held in the Chemex Environmental International Limited archives at Unit J, Broad Lane Industrial Estate, Cottenham, Cambridge CB24 8SW, England.

2.1 Test material

Identification:	
Supplied by:	Notox B.V
Date of receipt:	27 February 2007
Chemex reference:	ECO020737
Required storage conditions:	Store in original containers at room temperature
Actual storage conditions:	Room temperature in dark
Quoted stability:	Stable
Quoted appearance:	Amber viscous liquid/paste
Observed appearance:	Amber viscous liquid/paste
Quoted odour:	Not Stated
Quoted solubility in water:	Complete

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2.2 Test organism

Species:	*Skeletonema costatum* strain: 1077/5
Source:	Culture Collection of Algae and Protozoa Dunstaffnage Marine Laboratory, Scotland
Culture conditions:	Temperature: 20 ± 1°C Illumination: 6000 - 10000 lux continuous white light Shaking: 200rpm
Culture media:	Natural seawater with added nutrients according to the ISO standard, with concentrations of iron and EDTA at the levels recommended by VKI (Danish Water Quality Institute) (see Appendix 2).

2.3 Preparation of test solutions

The behaviour of [ ] was examined in seawater according to the method described in the Harmonised Offshore Chemical Notification Format (OSPAR 1995).

A nominal 1,000mg/l solution was prepared in dilution water (see section 2.4), shaken vigorously and allowed to stand for one hour.

A faintly grey solution was obtained. Test concentrations were prepared by mixing specified quantities of the sample with culture medium to obtain the required concentrations.

A preliminary study had identified the 72 hour EC_{50} as being between 10 and 32mg/l and therefore definitive test concentrations were prepared as 0, 2.2, 4.5, 10, 22, 45 and 100mg/l.

2.4 Test methods and conditions

Static test conditions according to ISO draft method (1991) "WATER QUALITY Marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*" (ISO/DIS 10253).

Chemex SOP reference:	E209 "Marine Algal Growth Inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*"
Test period:	2 to 5 April 2007
Test volume:	200ml
Test vessel:	250ml conical flask
Number of replicates:	6 control flasks, 3 replicates at each test concentration
Seawater:	Collected on 6 December 2006 from the CEFAS laboratories at Burnham-on-Crouch directly from the River Crouch estuary.
Algal test inoculum:	From a pre-culture growing in exponential phase under conditions described in 2.2 above. Inoculum level adjusted to give an initial cell density of 1×10^4 cells/ml.
Test conditions:	Temperature: 20 ± 1°C Illumination: 6000 - 10000 lux continuous white light Shaking: 200 rpm
Water quality measurements:	The pH (to 0.1) and temperature (to 0.5°C) were measured on the pooled replicates for each test and control solution immediately prior to initiating the test and at the end of the full 72 hour test period.
Observations/frequency:	The cell density was determined at 24, 48 and 72 hours on a small volume removed from each replicate flask. The cell counts were made using a haemocytometer and microscope.
Calculation and expression of results:	Growth curves for each test concentration were plotted as logarithm of the mean cell density against time. The inhibition of growth was calculated using both the biomass (area under the growth curve) and the growth rate method. Where possible, the EC_{50} values were estimated graphically and 95% confidence limits calculated according to the method of ToxCalc™ Version 5.0 "Comprehensive Toxicity Data Analysis and Database Software", copyright 1994-1996.

3. Results

3.1 Water quality data

Nominal concentration (mg/l)	Mean pH values		Temperature °C	
	Start of test	End of test	Start of test	End of test
0	8.0	8.6	20.0	20.0
2.2	8.0	8.7	20.0	20.0
4.5	8.0	8.7	20.0	20.0
10	8.0	8.3	20.0	20.0
22	8.0	8.0	20.0	20.0
45	8.0	8.0	20.0	20.0
100	8.0	8.0	20.0	20.0

3.2 Percent inhibition by biomass integral and growth rate

Nominal concentration (mg/l)	Percent inhibition by biomass integral		Percent inhibition by growth rate	
	48 hours	72 hours	48 hours	72 hours
2.2	-6	-8	2	-3
4.5	0	2	-1	1
10	39	45	13	17
22	68	73	32	35
45	100	100	100	100
100	100	100	97	100

Note: Negative inhibition values indicate an increase in growth compared with controls.

3.3 EC_{50} values by biomass integral (E_bC_{50} value) and growth rate (E_rC_{50} value)

Period of exposure (hours)	E_bC_{50} value mg/l biomass (95% confidence limits)	Period of exposure (hours)	E_rC_{50} value mg/l growth rate (95% confidence limits)
0 to 48	14mg/l (10 – 19mg/l)	0 to 48	23mg/l (21 – 25mg/l)
0 to 72	13mg/l (10 – 17mg/l)	0 to 72	24mg/l (21 – 27mg/l)
$NOEC_b$ (biomass)	4.5mg/l (0-72 hours)		
$NOEC_r$ (growth rate)			

4. Discussion

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The 72 hour EC_{50} o[redacted] to *Skeletonema costatum* was estimated as 13mg/l using the biomass integral and 24mg/l by growth rate calculation. The 48 hour EC_{50} was 14mg/l by biomass integral and 23mg/l by growth rate. The 72 hour NOEC was estimated as 4.5mg/l as determined by the Bonferroni T test.

The growth curves illustrated in Graph 1 show that growth inhibition increases with increasing concentration, demonstrating a clear dose response to the test material. The inhibition values given in 3.2 support these observations.

The test organism sensitivity was also monitored during this study using the recommended reference substance 3,5 DCP. The algae were exposed to a 1.5mg/l concentration of 3,5 DCP for 72 hours under test incubation conditions and therefore the 42% inhibition by growth rate represents an acceptable level of sensitivity (ie. 72 hour inhibition was within the range of 20 to 80%).

The water quality measurements of the test solutions are given in 3.1 and were within acceptable limits. The cell density of the control during the 72 hour test period increased by a factor of 67, which is satisfactory.

There was no chemical analytical confirmation of the actual dissolved concentrations. The dissolved concentrations were likely to be as given in this report as the sample was soluble in water.

5. References

ISO/DIS BS EN ISO 10253: 1998 "Water quality – Marine Algal Growth Inhibition Test with *Skeletonema costatum and Phaeodactylum tricornutum.*

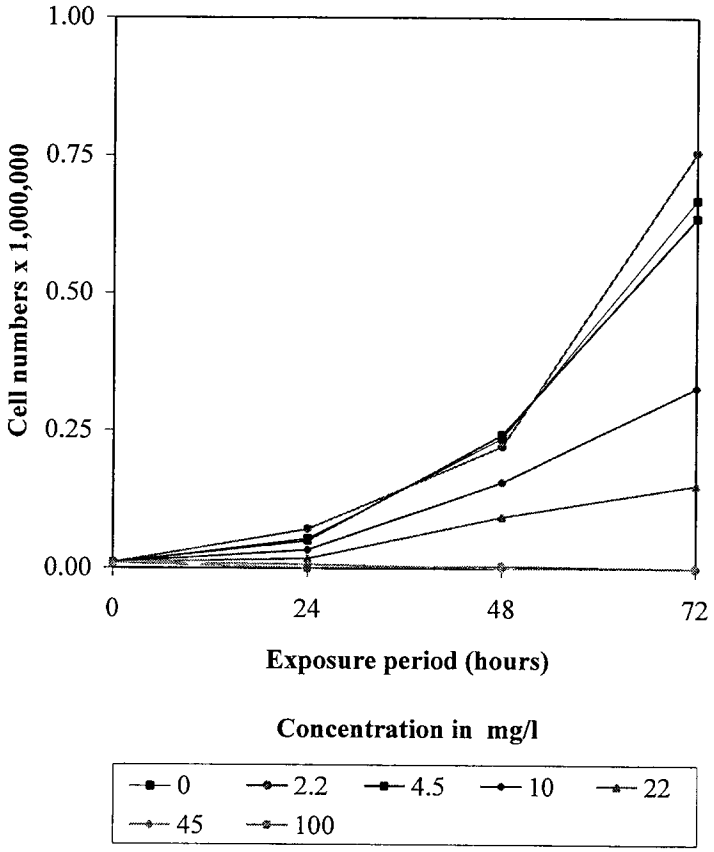
VKI – "Addendum to PARCOM Ring Toxicity Test with Marine Unicellular Algae" (1991) (Danish Water Quality Institute).

OSPAR – Oslo and Paris Conventions for the Prevention of Marine Pollution Programmes and Measures Committee (PRAM) Harmonised Offshore Chemical Notification Format 1995.

ToxCalc™ Version 5.0 "Comprehensive Toxicity Data Analysis and Database Software",copyright 1994-1996.

Graph 1

Growth curves for control and test concentrations



Statistical data

$NOEC_b$ (biomass) = 4.5mg/l

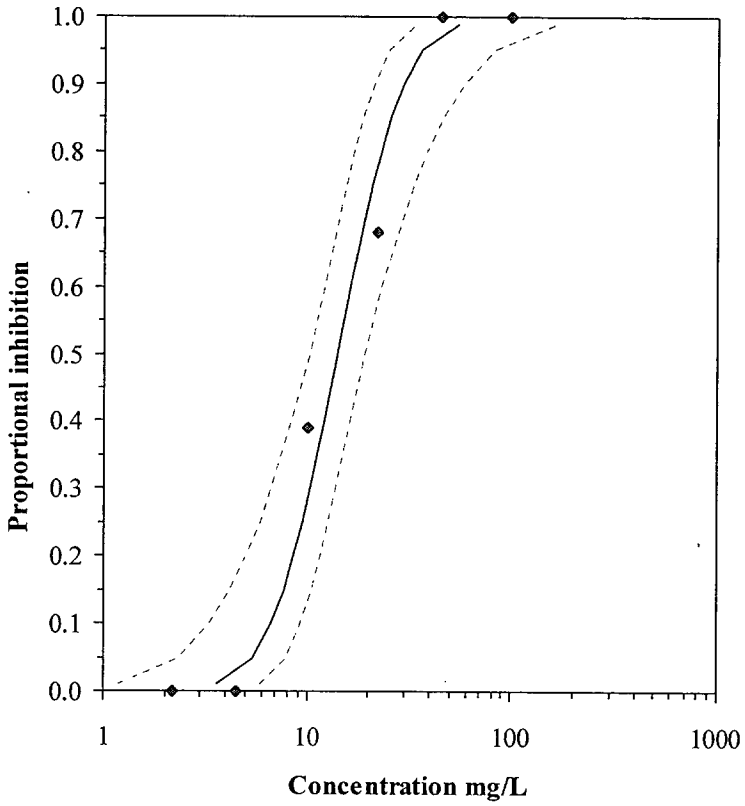
$NOEC_r$ (growth rate) = 4.5mg/l

LOEC = 10mg/l

As determined by the Bonferroni T test, based on the 0-72 hour values.

Graph 2

Estimation of 0 to 48 hours E_bC_{50} value



---------- Denotes 95% confidence limits

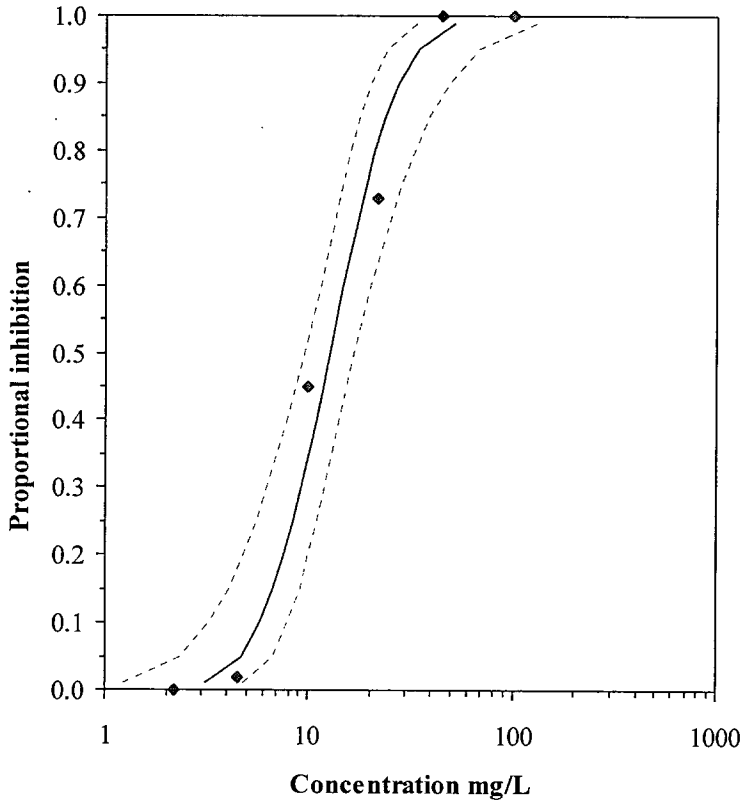
Statistical data

Estimated EC_{50} value = 14mg/l
(as determined by the Maximum Likelihood-Probit Method)

95% confidence limits = 10 to 19mg/l

Graph 3

Estimation of 0 to 72 hours E_bC_{50} value



---------- Denotes 95% confidence limits

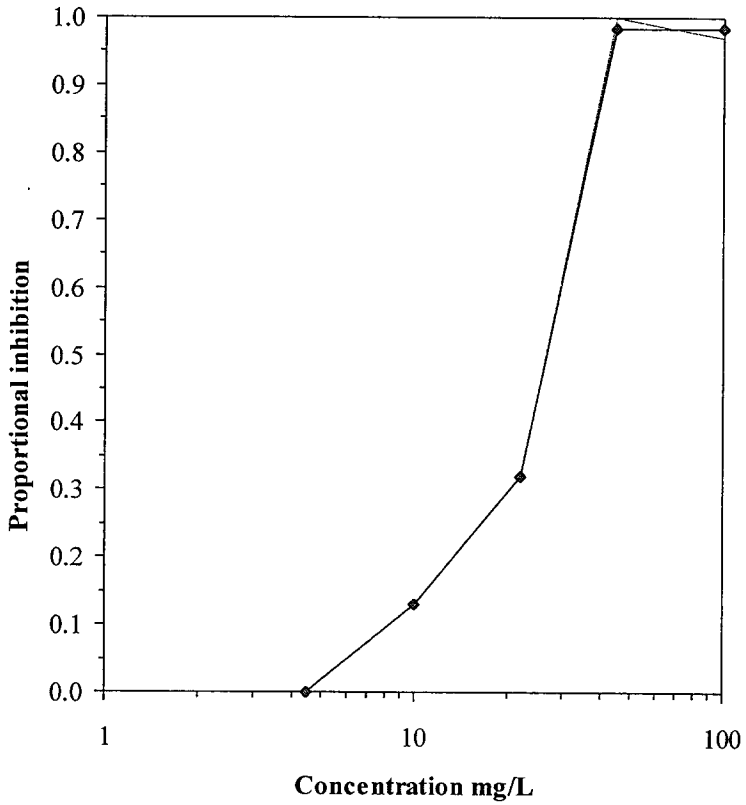
Statistical data

Estimated EC_{50} value = 13mg/l
(as determined by the Maximum Likelihood-Probit Method)

95% confidence limits = 10 to 17mg/l

Graph 4

Estimation of 0 to 48 hours E_rC_{50} value



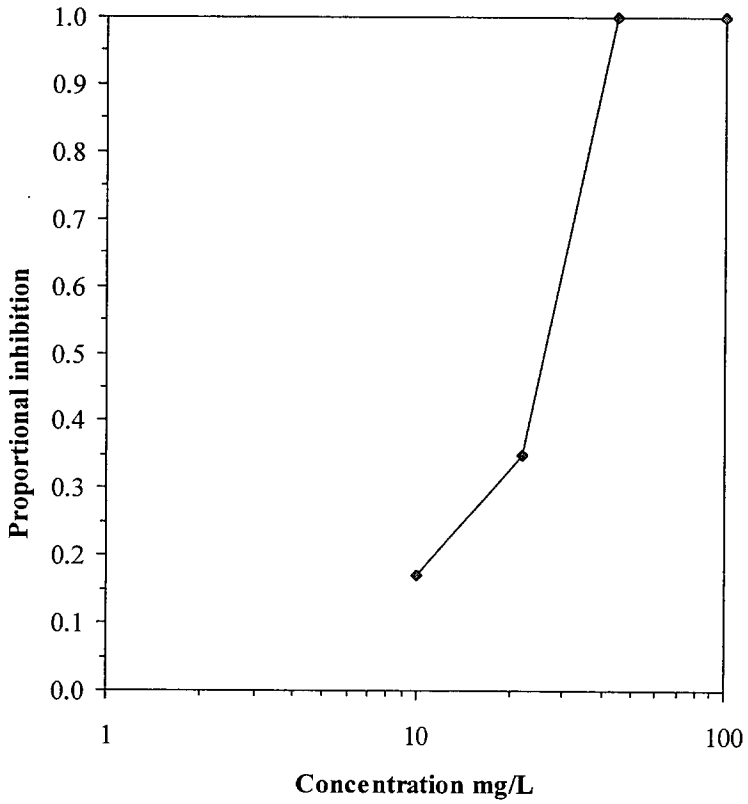
Statistical data

Estimated EC_{50} value = 23mg/l
(as determined by the Spearman-Karber Method)

95% confidence limits = 21 to 25mg/l

Graph 5

Estimation of 0 to 72 hour E_rC_{50} value



Statistical data

Estimated EC_{50} value = 24mg/l
(as determined by the Spearman-Karber Method)

95% confidence limits = 21 to 27mg/l

Appendix 1

Cell density measurements – test sample

Concentration (mg/l)	Replicate	Cell density measurements (cells/ml x 10^4)		
		24 hours	48 hours	72 hours
0	1	2.67	22.67	69.33
	2	6.33	21.67	62.33
	3	6.67	27.67	72.00
	4	4.00	24.00	56.67
	5	4.33	23.00	67.67
	6	8.33	22.67	73.33
	Mean	**5.39**	**23.61**	**66.89**
2.2	1	10.00	22.00	87.33
	2	5.33	24.67	71.00
	3	6.00	19.67	68.33
	Mean	**7.11**	**22.11**	**75.55**
4.5	1	4.67	29.33	66.67
	2	3.67	23.33	60.00
	3	6.67	20.33	64.33
	Mean	**5.00**	**24.33**	**63.67**
10	1	4.00	18.00	35.00
	2	2.00	13.00	32.67
	3	4.00	15.67	30.67
	Mean	**3.33**	**15.56**	**32.78**
22	1	0.00	5.33	14.33
	2	2.33	14.67	14.67
	3	3.00	8.00	16.33
	Mean	**1.78**	**9.33**	**15.11**
45	1	0.00	0.00	0.00
	2	1.33	0.00	0.00
	3	0.67	0.00	0.00
	Mean	**0.67**	**0.00**	**0.00**
100	1	0.00	0.00	0.00
	2	0.00	0.00	0.00
	3	0.00	1.33	0.00
	Mean	**0.00**	**0.44**	**0.00**

Mean initial cell density: 1 x 10^4 cells/ml.

Appendix 2

Reference material results

The test organism sensitivity was monitored during this study using the recommended reference substance 3,5 DCP. The algae were exposed to a 1.5mg/l concentration of 3,5 Dichlorophenol for 72 hours under test incubation conditions.

Percent inhibition by biomass integral and growth rate inhibition

Nominal concentration 3,5-DCP	Percent inhibition by biomass integral		Percent inhibition by growth rate	
	48 hours	72 hours	48 hours	72 hours
1.5mg/l	60	75	38	42

Cell density measurements

Concentration 3,5-DCP (mg/l)	Replicate	Cell density measurements (cells/ml x 10^4)		
		24 hours	48 hours	72 hours
0	1	2.67	22.67	69.33
	2	6.33	21.67	62.33
	3	6.67	27.67	72.00
	4	4.00	24.00	56.67
	5	4.33	23.00	67.67
	6	8.33	22.67	73.33
	Mean	**5.39**	**23.61**	**66.89**
1.5	1	6.00	9.67	17.33
	2	3.67	7.67	13.00
	3	2.33	5.00	7.00
	Mean	**4.00**	**7.45**	**12.44**

Appendix 3

Culture media

Culture medium: Prepared in natural seawater according to the ISO/DIS 10253 standard except that concentrations of iron and EDTA were at the levels recommended by VKI (Danish Water Quality Institute).

Seawater: Filtered, and sterilised by autoclaving at 120°C for 30 minutes.

Method of preparation: Sterile nutrient stock solutions were prepared and added to the seawater to obtain the culture medium.

Composition of culture medium:

Nutrient	Final concentration in test solution
$FeCl_3$ $6H_2O$	16.4µg/l (Fe)
$MnCl_2.4H_2O$	605µg/l (Mn)
$ZnSO_4.7$ H_2O	150µg/l (Zn)
$CuSO_4.5$ H_2O	0.6µg/l (Cu)
$CoCl_2.6$ H_2O	1.5µg/l (Co)
H_3 BO_3	17.1mg/l
Na_2 EDTA, $2H_2O$	100µg/l
Thiamin hydrochloride	25µg/l
Biotin	0.005µg/l
B_{12} (cyanocobalamin)	0.05µg/l
K_3 PO_4 H_2O	3.25mg/l
$NaNO_3$	50.0mg/l
$Na_2SiO_3.5H_2O$	14.9mg/l

This TSCA 8(e) substantiation applies to the following studies for materials that have never been commercialized in the U.S.: [***test substance***]

In addition to the chemical identities or formula of a proprietary mixture, 3M maintains its 3M proprietary ID #s and names of 3M personnel.

1. Yes, the CBI claim is on behalf of 3M Company, including its wholly-owned subsidiaries and affiliates.
2. The period of time for 3M's confidentiality claim is indefinite. 3M's claim is for the chemical identities of components of materials that have been commercialized in the U.S. 3M engaged in significant R&D efforts to develop this product. 3M has always claimed the components of the chemical identities in question as proprietary and continues to do so today. With this knowledge, competitors could do one or more of the following: (a) deduce (and potentially duplicate) 3M's process technology, process chemistry and process know-how; (b) develop products with components and/or functionalities identical or similar to 3M's current products; and (c) devise marketing strategies and tactics to promote their current products as compared to 3M's current products. In these ways, disclosure of 3M's proprietary information would deprive 3M of its intellectual property and its competitive advantage and would bestow a competitive advantage that 3M's competitors would not otherwise be able to achieve using their independent "know how". 3M would be deprived of research and development investment return as a result of such disclosure.
3. If a PMN was written, the PMN number is provided in the confidential copy of the Index to Studies Submitted. Disclosure would have been made to the U.S. EPA. Otherwise, no other disclosure was made.
4. The exact chemical identities or formula of the proprietary mixture has been kept confidential using general descriptions (e.g., experimental product) on all hazard communication (MSDS and labels). In addition, the measures taken to prevent undesired disclosure include strict R&D 3M security procedures with disclosure of the chemical identity of the substance within 3M on a need to know basis.
5. Contract employees, engineering and construction firms and other third parties with access to this information sign a confidentiality agreement or are otherwise bound through agency that prohibits disclosure of 3M business confidential information.
6. No, the chemical identities only appear as a generic name on the MSDS.
7. No.
8. Refer to response to Question #2.
9. No.
10. a) Yes b) Approximately 3 years. C) The mixture is a raw material used at a very small percentage in hard surface coating formulations. Competitors are not aware of the exact formulation of the mixture.
11. Determining the exact chemical identity of the substance would require the use of very sophisticated analytical equipment and methods by highly skilled chemists and would require an extended period of time.
12. a.) Yes, refer to response to Question #2. b.) Yes, refer to response to Question #2. c.) Yes, refer to response to Question #2.
13. No CAS number exists for the polymer in the product.
14. No.

3M Company
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