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March 7, 1995

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Mr. Terry R. O'Bryan

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In response to your request for additional information, please find a copy of the final report, "Vectomer E2200 - Acute Toxicity to *Daphnia Magna*".

Information reflecting the results of the test is being added to MSDSs for the material and for mixtures containing the material. The information will be placed in section 12, *Ecological Information* (as per the ANSI MSDS standard - Z400.1).

Daniel Levine

Daniel Levine
Director-Product Safety & Integrity



8EHQ-95-13308
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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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OFFICE OF
PREVENTION, PESTICIDES AND
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EPA acknowledges the receipt of information submitted by your organization under Section 8(e) of the Toxic Substances Control Act (TSCA). For your reference, copies of the first page(s) of your submission(s) are enclosed and display the TSCA §8(e) Document Control Number (e.g., 8EHQ-00-0000) assigned by EPA to your submission(s). Please cite the assigned 8(e) number when submitting follow-up or supplemental information, and refer to the reverse side of this page for "EPA Information Requests".

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EPA looks forward to continued cooperation with your organization in its ongoing efforts to evaluate and manage potential risks posed by chemicals to health and the environment.

Sincerely,

Terry R. O'Bryan
Risk Analysis Branch

Enclosure

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4. A description of all voluntary actions taken by your company in response to the findings indicated in your submission.
5. A complete copy of the current and/or revised Material Safety Data Sheets and labels for the following chemical(s) listed in your submission:

6.

Please direct questions regarding these requests to Mr. Terry O'Bryan (202-260-3483) or Mr. John Myers (202-260-3543) of the OPPT Risk Analysis Branch.

HRC Report

VECTOMER E2200
ACUTE TOXICITY
TO *DAPHNIA MAGNA*

SET 19 10 7:36

REPORT

Huntingdon Research Centre



CONFIDENTIAL

ALS 27/941056
Sponsor Protocol Number 93853/TOX-50B

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VECTOMER E2200
ACUTE TOXICITY TO *DAPHNIA MAGNA*

Sponsor

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Report issued: 1 November 1994

Page 1 of 43

TOX Computer Entry No.
TOX-50B-93-7 11/8/94

CONTENTS

	Page
TITLE PAGE	1
CONTENTS	2
COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS	4
QUALITY ASSURANCE STATEMENT	5
RESPONSIBLE PERSONNEL	6
SUMMARY	7
INTRODUCTION	8
TEST SUBSTANCE	9
EXPERIMENTAL PROCEDURE	10
RESULTS	13
CONCLUSION	13
TABLES	
1. Cumulative immobilisation data for <i>Daphnia magna</i> exposed for 48 hours to Vectomer E2200	14
2. Environmental parameters. Mean values for pH, temperature and dissolved oxygen	15
3. Measured concentrations. Mean values and percentages of nominal	16

Page

FIGURE

1.	Concentration-response curve for <i>Daphnia magna</i> exposed for 24 hours to Vectomer E2200	17
2.	Concentration-response curve for <i>Daphnia magna</i> exposed for 48 hours to Vectomer E2200	18

APPENDICES

1.	Environmental measurements. Individual records of pH, temperature and dissolved oxygen	19
2.	Verification of test concentrations	20
3.	Elendt M7 medium	27
4.	Protocol	28

COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards and I consider the data generated to be valid.

Good Laboratory Practice, The United Kingdom Compliance Programme, Department of Health & Social Security 1986 and subsequent revision, Department of Health 1989.

EC Council Directive, 87/18 EEC of 18 December 1986, (No. L 15/29).

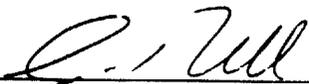
Good Laboratory Practice in the testing of Chemicals OECD, ISBN 92-64-12367-9, Paris 1982, subsequently republished OECD Environment Monograph No. 45, 1992.

United States Environmental Protection Agency, (TSCA), Title 40 Code of Federal Regulations Part 792, Federal Register, 29 November 1983 and subsequent amendment Federal Register 17 August 1989.

Japan Ministry of International Trade and Industry, Directive 31 March 1984 (Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85 MITI).

United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Federal Register, 22 December 1978, and subsequent amendments.

Japan Ministry of Health and Welfare, Notification No. Yakuhatu 313 Pharmaceutical Affairs Bureau, 31 March 1982 and subsequent amendment Notification No. Yakuhatu 870, Pharmaceutical Affairs Bureau, 5 October 1988.



Graeme Bell, B.Sc., M.Sc.,
Study Director,
Huntingdon Research Centre Ltd.

1 November 1996
Date

QUALITY ASSURANCE STATEMENT

Certain studies such as that described in this report, are conducted at HRC in a setting which involves frequent repetition of similar or identical procedures. At or about the time the study described in this report was in progress, 'process-based' inspections were made by the Quality Assurance Department of critical procedures relevant to this study type. The findings of these inspections were reported promptly to the Study Director and to HRC Management.

This report has been audited by the Huntingdon Research Centre Quality Assurance Department. The methods, practices and procedures reported herein are an accurate description of those employed at HRC during the course of the study. Observations and results presented in this final report form a true and accurate representation of the raw data generated during the conduct of the study at HRC.

Date(s) of inspection 8 - 15 March 1994

Date(s) of reporting inspection findings
to the Study Director and HRC Management 18 March 1994

Date of reporting audit findings to the
Study Director and HRC Management 15 July 1994



P. Watson,
Systems Compliance Auditor,
Department of Quality Assurance,
Huntingdon Research Centre Ltd.

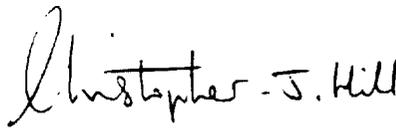
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RESPONSIBLE PERSONNEL

We the undersigned, hereby declare that the work was performed under our supervision according to the procedures herein described, and that this report provides a correct and faithful record of the results obtained.



Graeme Bell, M.Sc.,
Study Director,
Department of Aquatic Toxicology.



Christopher J. Hill, B.Sc(Hons).,
Scientific Officer,
Department of Aquatic Toxicology.



Ben Smith, M.R.S.C., C.Chem., M.Sc.,
Section Head, Ecotoxicology Analysis,
Department of Environmental Analysis.

SUMMARY

A study was performed to assess the acute toxicity of Vectomer E2200 to *Daphnia magna*.

The study was conducted in accordance with EEC Methods for Determination of Ecotoxicity Annex to Directive 92/69/EEC (O.J.No. L383A.29.12.92) Part C, Method 2 "Acute toxicity for *Daphnia*".

Groups of twenty, 1st instar *Daphnia* (less than 24 hours old) were exposed for 48 hours to seven concentrations (0.1 - 10mg/l, nominal concentration) of Vectomer E2200 dispersed in Elendt M7 medium. The incidence of immobilisation was recorded for each test and control group at 24 hours and at 48 hours and the following values determined:

Time (h)	EC ₅₀ (mg/l)	95% confidence limits (mg/l)
24	1.8	0.99 - 3.9
48	0.28	0.20 - 0.39

Highest test concentration resulting in 0% immobilisation at 48 hours: < 0.027 mg/l
Lowest test concentration resulting in 100% immobilisation at 48 hours: 3.6 mg/l

All results are expressed in terms of mean measured concentration. Measured concentrations ranged from 70 - 105% of nominal at 0 hours and 0 - 68% of nominal at 48 hours.

INTRODUCTION

This study was designed to assess the acute toxicity of Vectomer E2200 to *Daphnia magna*.

The study was conducted in accordance with EEC Methods for Determination of Ecotoxicity Annex to Directive 92/69/EEC (O.J.No. L383A.29.12.92) Part C, Method 2 "Acute toxicity for Daphnia".

The protocol was approved by the Study Director and HRC Management on 12 August 1993 and by the Sponsor on 30 September 1993.

The experimental phase of the study was conducted between 23 and 25 February 1994.

TEST SUBSTANCE

Identity:	Vectomer E2200
Chemical name:	1,3-Benzene dicarboxylic acid, bis(4-ethenoxy)butylester
Sample number:	373-93A
Expiry:	4 August 1994
Purity:	> 92%
Appearance:	Colourless to light straw liquid
Storage conditions:	In darkness at room temperature
Date received:	23 August 1993
Amount received:	500 ml

EXPERIMENTAL PROCEDURE

TEST SPECIES

Name

Daphnia magna (Straus).

Source

Laboratory culture originating from a strain supplied by the Institute National de Recherche Chimique Appliquée (IRChA), France.

Culture

Brood stocks of *Daphnia magna* were cultured under a 16 h light : 8 h dark photoperiod at $20 \pm 2^\circ\text{C}$ in polypropylene vessels containing two litres of Elendt M7 medium. Cultures were fed daily with a suspension of mixed algae (predominantly *Scenedesmus* and *Selenastrum* spp.). Culture conditions ensure that reproduction is by parthenogenesis.

Gravid adults were isolated 24 hours prior to initiation of the test. Young daphnids produced overnight were used for testing.

TEST WATER

Elendt M7 medium was prepared using analytical grade reagents and reverse osmosis purified water (see Appendix 3).

TEST SUBSTANCE PREPARATION

Method of preparation

The test substance was dissolved in an auxillary solvent (20% tween 80-acetone) to give an initial stock solution of 100 mg/ml. Serial dilutions of this stock solution were prepared with the auxillary solvent and aliquots added to test water (with ultrasonic disruption) to give the desired series of exposure levels.

Stability of test concentrations

Test concentrations were verified by chemical analysis. Water samples (15 ml) were taken directly from the solvent control and each exposure level (replicates pooled) at 0 hours and 48 hours by the Department of Environmental Analysis (see Appendix 2), additional samples (50 ml) were taken from

the solvent control and each exposure level (replicates pooled) at 0 hours and 48 hours and stored at +4°C by the HRC Department of Environmental Analysis for further analysis, if required.

EXPOSURE CONDITIONS

Experimental design

Seven test concentrations plus one control and one solvent control (100 µl auxiliary solvent per litre) each in duplicate (20 animals per test group, 10 per replicate).

Ten 1st instar *Daphnia* were placed at random in each glass jar containing 200 ml of prepared test medium, test water only or test water plus 100 µl auxiliary solvent per litre, as appropriate, to give a loading of 20 ml test solution per organism. The jars were loosely covered with aluminium foil to minimise evaporation losses.

Test concentrations

Nominal test concentrations: 0.10, 0.22, 0.46, 1.0, 2.2, 4.6, & 10 mg/l
Measured test concentrations: 0.027, 0.066, 0.17, 0.33, 1.0, 3.6, & 8.0 mg/l
(Geometric means of fresh and expired media)

Measured test concentrations used for calculation of all EC₅₀ information.

Medium renewal

Daphnia were exposed to the test or control conditions for a period of 48 hours without renewal of test media.

Environmental conditions

Cultures were maintained at 21 ± 1°C under a photoperiod of 16 hours light : 8 hours dark and without supplementary aeration or feeding during the 48 hour exposure period.

The temperature in each vessel was measured daily and the pH and dissolved oxygen levels recorded at the start and at the end of the study (see Table 2 and Appendix 1).

Criterion of effect

Daphnia were considered to be immobilised if they were unable to swim for approximately 15 seconds after gentle agitation.

EVALUATION OF DATA

EC₅₀ values were calculated using a logistic model (Berkson, 1944) for which 95% confidence limits were estimated by the likelihood ratio method (Williams, 1986).

REFERENCES

Berkson, J. (1944) Application of the logistic function to bioassay.
J. Amer. Statist. Assoc. **39**, 357-365.

Williams, D.A. (1986) Interval estimation of the median Lethal Dose.
Biometrics, **42**, 641-645.

ARCHIVES

All specimens, raw data and study related documents generated during the course of the study at HRC, together with a copy of the final report will be lodged in the Huntingdon Research Centre, Archive.

Such specimens and records will be retained for a a minimum period of five years from the date of issue of the final report. At the end of the five year retention period the client will be contacted and advice sought on the future requirements. Under no circumstances will any item be discarded without the client's knowledge.

RESULTS

Cumulative immobilisation data are given in Table 1 and the relationships between percentage immobilisation and concentration at 24 h and 48 h are given in Figures 1 and 2. All results are expressed in terms of mean measured concentration (see Table 3 and Appendix 2). Measured concentrations ranged from 70 - 105% of nominal at 0 hours and 0 - 68% of nominal at 48 hours.

The low percentage nominal values obtained in all test concentrations at study termination (48 hours) were not unexpected given the known instability of Vectomer E2200 in water (see Appendix 2, Table C3).

Analysis of the immobility data gave the following results:

Time (h)	EC ₅₀ (mg/l)	95% confidence limits (mg/l)
24	1.8	0.99 - 3.9
48	0.28	0.20 - 0.39

Highest test concentration resulting in 0% immobilisation at 48 hours: <0.027 mg/l

Lowest test concentration resulting in 100% immobilisation at 48 hours: 3.6 mg/l

Individual pH, temperature and dissolved oxygen values remained within acceptable limits throughout the duration of the study (see Appendix 1). A summary table giving mean values for each control and test group is given in Table 2.

CONCLUSION

The 48 h EC₅₀ (immobilisation) value for Vectomer E2200 with *Daphnia magna* is 0.28 mg/l.

TABLE 1

Cumulative immobilisation data for *Daphnia magna*
 exposed for 48 hours to Vectomer E2200

Nominal concentration of Vectomer E2200 mg/l	Mean measured concentration mg/l	Cumulative immobilised <i>Daphnia magna</i> (initial population: 10 per replicate)							
		24 hours				48 hours			
		R ₁	R ₂	Total	%	R ₁	R ₂	Total	%
Control	Control	0	0	0	0	0	0	0	0
Solvent control	Solvent control	0	0	0	0	0	0	0	0
0.10	0.027	0	1	1	5	0	1	1	5
0.22	0.066	1	1	2	10	1	1	2	10
0.46	0.17	2	1	3	15	3	2	5	25
1.0	0.33	3	2	5	25	5	5	10	50
2.2	1.0	4	4	8	40	10	9	19	95
4.6	3.6	6	6	12	60	10	10	20	100
10	8.0	7	8	15	75	10	10	20	100

R₁ Replicate 1
 R₂ Replicate 2

TABLE 2

Environmental parameters
Mean values for pH, temperature and dissolved oxygen

Nominal concentration of Vectomer E2200 mg/l	Mean measured concentration mg/l	Mean values		
		pH	mgO ₂ /l	T°C
Control	Control	7.4	7.4	21
Solvent control	Solvent control	7.4	7.4	21
0.10	0.027	7.4	7.4	21
0.22	0.066	7.4	7.3	21
0.46	0.17	7.4	7.3	21
1.0	0.33	7.4	7.3	21
2.2	1.0	7.4	7.3	21
4.6	3.6	7.4	7.3	21
10	8.0	7.4	7.2	21

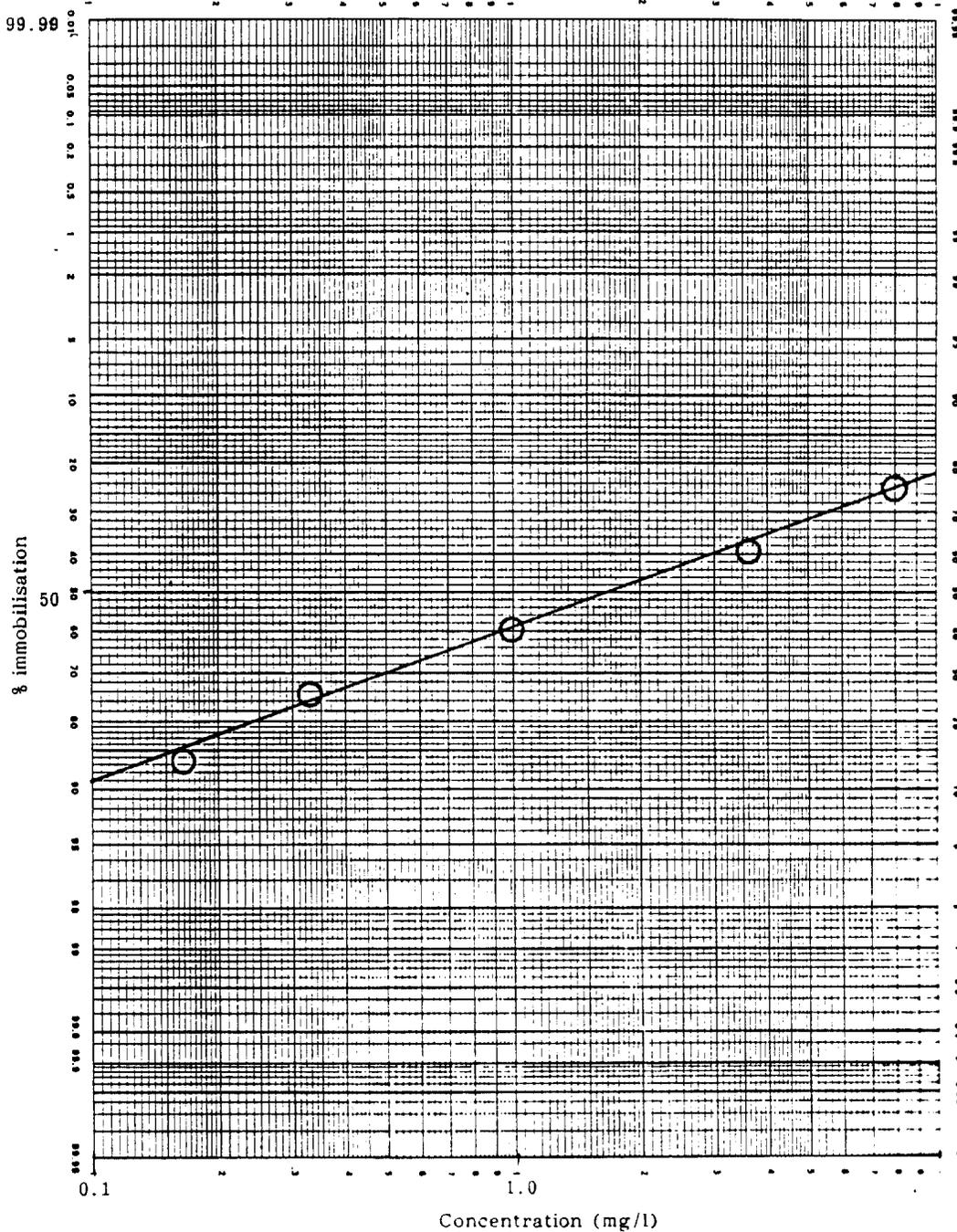
TABLE 3
Measured concentrations
Mean values and percentages of nominal

Nominal concentration of Vectomer E2200 mg/l	Number of samples analysed	Mean measured concentration* mg/l	% Nominal
Control	2	-	-
0.10	2	0.0271	27
0.22	2	0.0659	30
0.46	2	0.1704	37
1.0	2	0.3256	33
2.2	2	1.045	47
4.6	2	3.595	78
10	2	7.961	80

* Geometric mean of fresh and expired samples

FIGURE 1

Concentration-response curve for
Daphnia magna exposed for 24 hours to Vectomer E2200

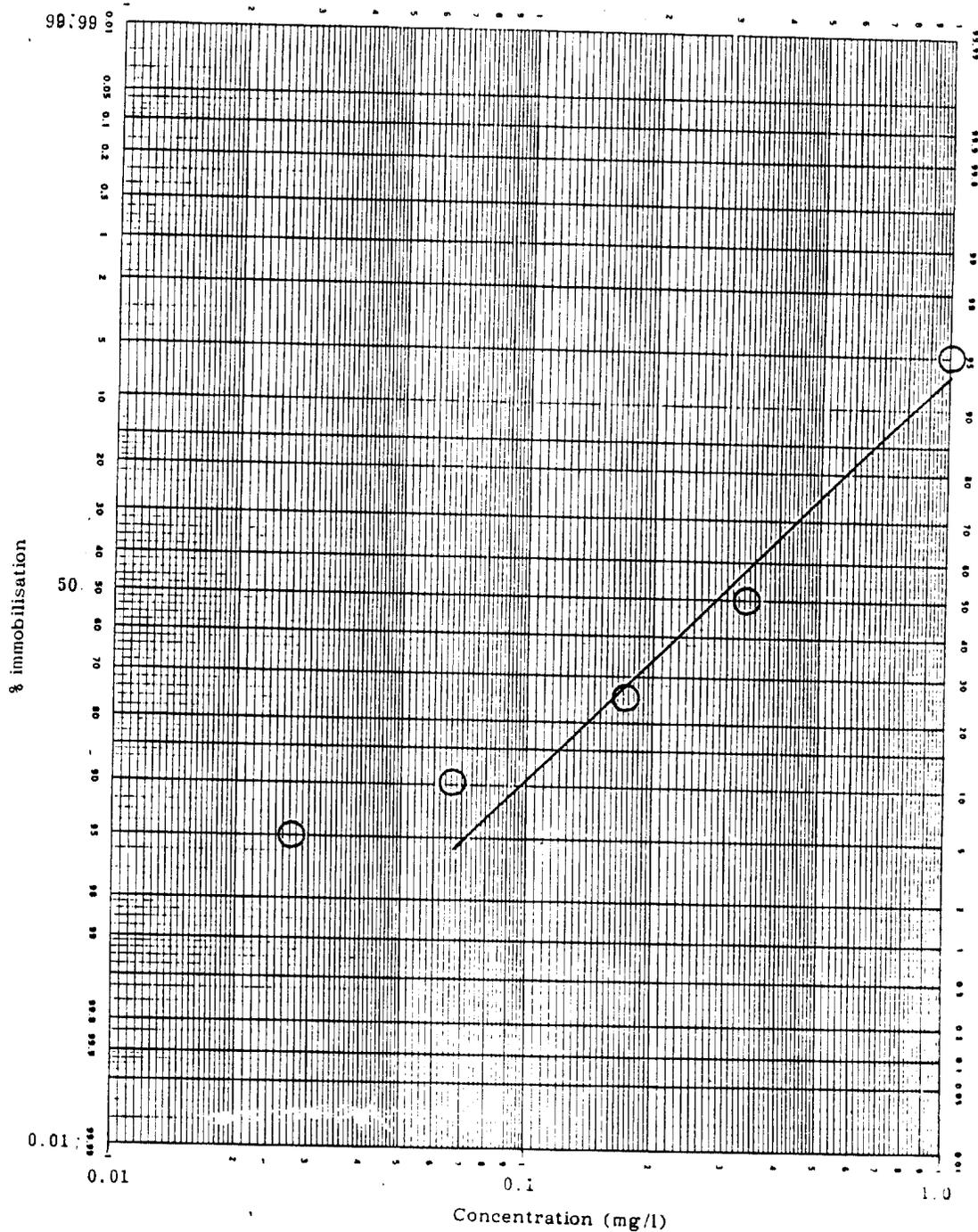


Charting
Graph Data Ref. 1074

Log 2 Cycle Probability

FIGURE 2

Concentration-response curve for
Daphnia magna exposed for 48 hours to Vectomer E2200



APPENDIX I

Environmental measurements
 Individual records of pH, temperature and dissolved oxygen

Nominal concentration of Vectomer E2200 mg/l		0 hours			24 hours	48 hours		
		pH	mgO ₂ /l	T°C	T°C	pH	mgO ₂ /l	T°C
Control	R ₁	7.4	8.1	21	21	7.4	6.6	20
	R ₂	7.4	8.1	21	21	7.4	6.6	20
Solvent control	R ₁	7.4	8.1	21	21	7.4	6.6	20
	R ₂	7.4	8.1	21	21	7.4	6.6	20
0.10	R ₁	7.3	8.1	21	21	7.4	6.6	20
	R ₂	7.3	8.1	21	21	7.4	6.6	20
0.22	R ₁	7.4	8.0	21	21	7.4	6.6	20
	R ₂	7.4	8.0	21	21	7.4	6.6	20
0.46	R ₁	7.4	8.0	21	21	7.4	6.5	20
	R ₂	7.4	8.0	21	21	7.4	6.5	20
1.0	R ₁	7.4	8.0	21	21	7.4	6.5	20
	R ₂	7.4	8.0	21	21	7.4	6.5	20
2.2	R ₁	7.4	8.0	21	21	7.4	6.5	20
	R ₂	7.4	8.0	21	21	7.4	6.5	20
4.6	R ₁	7.4	8.0	21	21	7.4	6.5	20
	R ₂	7.4	8.0	21	21	7.4	6.5	20
10	R ₁	7.4	8.0	21	21	7.4	6.4	20
	R ₂	7.4	8.0	21	21	7.4	6.4	20

R₁ Replicate 1

R₂ Replicate 2

pH meter: Sentron 1001

Dissolved oxygen meter: YSI Model 57

APPENDIX 2

Verification of test concentrations

SUMMARY

Samples of test medium received from the Department of Aquatic Toxicology were analysed for Vectomer E2200 by a method developed at HRC using information supplied by the Sponsor.

The analytical method was proven satisfactory with regard to linearity, accuracy and precision with a mean validation recovery of 94.6%; coefficient of variation 5.5% (Table C2).

A static test system was used by the Department of Aquatic Toxicology from which one set of test solutions, consisting of fresh and expired samples, was analysed during the two days of the study. The results obtained are presented in Table C1: the mean measured test concentrations were 91.5% of nominal (fresh media) and 28.6% of nominal (expired).

Typical chromatography from the analysis of test solutions is reproduced in Figure C1.

METHOD OF ANALYSIS

A sample from each test vessel was taken on two occasions (23 and 25 February 1994) and transferred to a volumetric flask (20.0 ml) (a duplicate sample was taken at the same time and stored at 4°C in case a check analysis was required).

Each flask was diluted to volume with acetonitrile and diluted as required with acetonitrile : water (25 : 75 v/v) (see table). A subsample was then transferred to an autosampler vial and, using a Gilson 233 XL sample processor, an aliquot (400 µl) was loaded (at 1.5 ml/minute) onto a trace enrichment column which had been previously conditioned with acetonitrile (800 µl) and acetonitrile : water (25 : 75 v/v, 800 µl). The trace enrichment column was then switched in-line with the mobile phase allowing the retained compound to be flushed onto a high performance liquid chromatography column for analysis against prepared standards of Vectomer E2200.

Duplicate procedural recoveries and a blank were analysed concurrently with the samples.

APPENDIX 2

(continued)

Nominal concentration (mg/l)	Sample volume (ml)	Initial dilution (ml)	Final dilution (ml)
Control	15.0	15.0 - 20.0	-
0.10	15.0	15.0 - 20.0	-
0.22	15.0	15.0 - 20.0	-
0.46	15.0	15.0 - 20.0	-
1.0	15.0	15.0 - 20.0	5.0 - 10.0
2.2	15.0	15.0 - 20.0	5.0 - 25.0
4.6	15.0	15.0 - 20.0	5.0 - 50.0
10	15.0	15.0 - 20.0	5.0 - 100

CALIBRATION STANDARDS

A primary standard was prepared by dissolving approximately 50 mg Vectomer E2200 (accurately known) in acetonitrile (50 ml).

Working calibration solutions (range: 0.1473 to 2.455 mg/l) were prepared by diluting the primary standard with acetonitrile : water (50 : 50 v/v).

BLANK

Elendt M7 medium (15 ml).

RECOVERIES

Validation

An aliquot (75 μ l) of a solution of Vectomer E2200 in acetonitrile (9.490 mg/l) was added to Elendt M7 medium (15 ml) to give an aqueous concentration of 0.04745 mg/l.

An aliquot (150 μ l) of a solution of Vectomer E2200 in acetonitrile (94.90 mg/l) was added to Elendt M7 medium (15 ml) to give an aqueous concentration of 0.9490 mg/l.

An aliquot (230 μ l) of a solution of Vectomer E2200 in acetonitrile (949.0 mg/l) was added to Elendt M7 medium (15 ml) to give an aqueous concentration of 14.55 mg/l.

Mean validation results were 94.6% with a coefficient of variation of 5.5%.

Procedural

An aliquot (150 μ l) of a solution of Vectomer E2200 in acetonitrile (94.90 mg/l) was added to Elendt M7 medium (15 ml) to give an aqueous concentration of 0.9490 mg/l.

APPENDIX 2

(continued)

Mean procedural recoveries were 92.5% with a coefficient of variation of 5.3%.

MATERIALS

Vectomer E2200

Sample number:

373-93A.

CALCULATIONS

The concentrations of Vectomer E2200 in each sample was calculated as follows:

$$\text{Concentration (mg/l)} = \frac{Y - A}{B} \times \frac{C}{F} \times D \times \frac{E}{G}$$

Where Y = Peak area of sample

A = Intercept derived from linear regression of calibration data

B = Slope derived from linear regression of calibration data

C = Initial dilution ratio

D = Final dilution ratio

E = Injection volume of standard (μ l)

F = Sample volume (ml)

G = Injection volume of sample (μ l)

The limit of detection for the study was calculated as follows:

$$\text{Limit of detection (mg/l)} = 3 \times F \times I \times \frac{100}{J}$$

Where F = Concentration Vectomer E2200 equivalent to baseline noise (mg/l)

I = Concentration factor¹ (lowest level validation recovery)

J = Lowest level validation recovery (%)

Limit of detection: 0.007 mg/l Vectomer E2200.

ND (none detected): Peak response less than limit of detection.

APPENDIX 2

(continued)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Instrument:

Sample processor:	Gilson 233 XL.
Pump:	Spectra-Physics 8800.
Detector:	Perkin Elmer LC 90 BIO.
Integrator:	Spectra-Physics 4270.

Analytical column:

Dimensions:	25 cm × 4.6 mm.
Description:	YMC-Pack ODS-AQ.

Trace enrichment column:

Dimensions:	1 cm × 4.0 mm.
Description:	Perisorb A RP-18.

Mobile phase:	Acetonitrile : water (80 : 20 v/v).
---------------	-------------------------------------

Flow rate:	1.0 ml/minute.
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Injection volume:

Standards:	80 μ l.
Samples:	400 μ l.

Analytical wavelength:	230 nm.
------------------------	---------

Attenuation:	32.
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Chart speed:	0.5 cm/minute.
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Under these conditions Vectomer E2200 chromatographed as a single peak with a retention time of approximately ten minutes.

Vectomer E2200 was quantified by integrated peak area.

APPENDIX 2

(continued)

TABLE C1

Summary of analytical results; acute toxicity of Vectomer E2200 to *Daphnia magna* study

HRC reference	Nominal concentration (mg/l)	Measured concentration (mg/l)	Corrected ¹ concentration (mg/l)	Concentration expressed as % of nominal
0 hours				
G94/0515	Control	ND	-	-
G94/0516	0.10	0.9739	0.1053	105
G94/0517	0.22	0.1859	0.2010	91.4
G94/0518	0.46	0.3998	0.4322	94.0
G94/0519	1.0	0.8440	0.9124	91.2
G94/0520	2.2	1.423	1.538	69.9
G94/0521	4.6	3.820	4.130	89.8
G94/0522	10	9.152	9.894	98.9
48 hours				
G94/0537	Control	ND	-	-
G94/0538	0.10	ND	-	-
G94/0539	0.22	0.01999	0.02161	9.82
G94/0540	0.46	0.06213	0.06717	14.6
G94/0541	1.0	0.1075	0.1162	11.6
G94/0542	2.2	0.6567	0.7099	32.3
G94/0543	4.6	2.895	3.130	68.0
G94/0544	10	5.926	6.406	64.1

ND None detected (limit of detection: 0.007 mg/l)

¹ Correction for overall mean recovery value (92.5%)

Correction factor = 100/92.5

(continued)

TABLE C2

Analytical results; recoveries of Vectomer E2200 from test medium

Recovery	Fortification level (mg/l)	Recovery (%)
Validation	0.04745	92.0, 97.2
	0.9490	91.1, 104
	14.55	92.5, 90.6
Mean		94.6
Coefficient of variation		5.5
Procedural		
0 hours	0.9490	92.2, 89.1
48 hours	0.9490	90.4, 86.3
Overall mean		92.5
Coefficient of variation		5.3

The method was validated prior to the study by the analysis of fortified samples of test medium. Performance of the method during the study was checked by procedural recoveries (samples of test medium fortified and then analysed along with that days' samples).

TABLE C3

Analytical results; stability of Vectomer E2200 in solution

Fortification (mg/l)	% Recovered
0.9936	(a) 79.3, 100
0.9936	(b) 78.6, 79.4
0.9936	(c) 63.3, 64.3

Solutions of Vectomer E2200 in dechlorinated water were stored for 24 hours at ambient temperature under the following conditions:

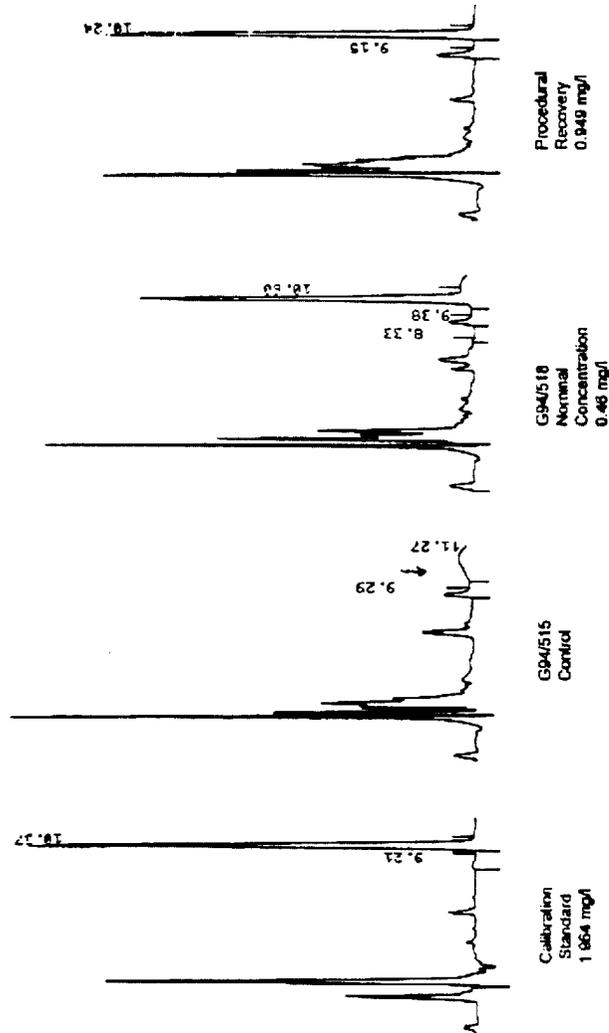
- (a) In a sealed glass vessel in the dark
- (b) In a sealed glass vessel in ambient light
- (c) In an open glass vessel in ambient light

APPENDIX 2

(continued)

FIGURE C1

Typical chromatography; acute *Daphnia magna* study - 0 hours



APPENDIX 3

Elendt M7 media

1. Trace elements	mg/l
H ₃ BO ₃	0.71
MnCl ₂ ·4H ₂ O	0.090
LiCl	0.077
RbCl	0.018
SrCl ₂ ·6H ₂ O	0.038
NaBr	0.0040
Na ₂ MoO ₄ ·2H ₂ O	0.016
CuCl ₂ ·2H ₂ O	0.0042
ZnCl ₂	0.013
CoCl ₂ ·6H ₂ O	0.010
KI	0.0033
Na ₂ SeO ₃	0.0022
NH ₄ VO ₃	0.00058
Fe-EDTA solution	1.7
2. Macro nutrients	mg/l
CaCl ₂ ·2H ₂ O	294
MgSO ₄ ·7H ₂ O	123
KCL	5.80
NaHCO ₃	64.8
Na ₂ SiO ₃ ·5H ₂ O	6.83
NaNO ₃	0.274
KH ₂ PO ₄	0.143
K ₂ HPO ₄	0.184
3. Vitamins	mg/l
Thiamine hydrochloride	0.075
Cyanocobalamine (B12)	0.0010
Biotine	0.00075

The above analytical grade reagents are dissolved in reverse osmosis purified water.

APPENDIX 4
PROTOCOL



CONFIDENTIAL

Study Design Reference:

FT/022/EEC/0593

ACUTE TOXICITY FOR *DAPHNIA*

PROTOCOL

Test Substance: Vectomer 4010

Sponsor:

Allied Signal Inc
Engineered Materials Sector
P O Box 1139
Morristown
NJ 07962-1139
USA

Issued by:

Department of Aquatic Toxicology
Huntingdon Research Centre Ltd
Huntingdon
Cambridgeshire
PE18 6ES

Date: 12 August 1993

APPENDIX 4

(continued)

FT/022/EEC/0593
Page 1 of 10

ACUTE TOXICITY FOR *DAPHNIA*

1 INTRODUCTION

1.1 Study objective

To determine the acute toxicity (EC_{50} immobilisation) of the test substance when dissolved or dispersed in water.

1.2 Test species

Daphnia magna Straus

1.3 Purpose

In order to aid in the assessment of the toxic effect of the test substance in the aquatic environment, this study has been prepared following the EEC Methods for Determination of Ecotoxicity Annex to Directive 92/69/EEC (O.J. No. L383A, 29.12.92) Part C, Method 2 "Acute toxicity for *Daphnia*". The study also follows (with minor exceptions as indicated in bold type) the OECD Guideline for Testing of Chemicals No. 202, Part 1 "*Daphnia*, Acute Immobilisation Test".

1.4 Selection of species

Daphnia magna has been selected following recommendation in Official Journal No. L383A Part C.2 and OECD Guideline No. 202.

1.5 Route of administration

The solution or dispersion of the test substance in the surrounding medium is considered to represent the most probable route of exposure in the environment.

1.6 Duration of study

48 hours

1.7 Dates of study

Actual dates to be advised by Protocol Amendment.

APPENDIX 4

(continued)

FT/022/EEC/0593
Page 2 of 10

2 TEST SUBSTANCE

2.1 Preparation

The test substance will be dissolved or dispersed in the test water either directly or via an aqueous or solvent stock solution (ultrasonic disruption may also be employed to facilitate preparation). If auxiliary solvents (e.g. acetone, ethanol, dimethylformamide, tetrahydrofuran) and/or surfactants (e.g. Tween 80) are used to aid preparation of the exposure media, all test groups and an additional control group will be exposed to the same amount of auxiliary agent up to a maximum of 100 µl/l.

No adjustment of pH will be carried out on stock solutions or prepared exposure media unless specifically requested by the Sponsor.

2.2 Storage

The test substance will be stored at room temperature in darkness unless otherwise requested by the Sponsor.

2.3 Absorption

Absorption is via the membranes exposed to the surrounding water. Specific determination of absorption will not be made in this study.

2.4 Stability

Data concerning the stability, homogeneity and analytical purity of the test substance, as provided, are the responsibility of the Sponsor.

3 TEST ORGANISMS

3.1 Species

Daphnia magna Straus

3.2 Source

Derived from a laboratory strain originating from the Institute National de Recherche Chimique Appliquée (I R C H A), France.

3.3 Number

Approximately 300

3.4 Life stage

1st instar *Daphnia*, between 6 and 24 hours old and produced from a parthenogenetically reproducing population of brood females.

APPENDIX 4

(continued)

FT/022/EEC/0593
Page 3 of 10

4 ACCLIMATISATION/CULTURE

4.1 Duration

A parthenogenetically reproducing population is maintained in the laboratory, under conditions equivalent to those to be provided during the test.

4.2 Vessels

2-3 litre glass or polypropylene beakers stocked at approximately 20 daphnids per vessel.

4.3 Test water

Reconstituted water (Elendt M7) as specified in "10.1 Test water".

4.4 Photoperiod

Ambient laboratory lighting under a 16 h light : 8 h dark regime.

4.5 Temperature

The temperature will be maintained within the range 18-22°C during the culture period and will remain constant within $\pm 1^\circ\text{C}$ for at least 24 h prior to and during the test.

4.6 Aeration

The culture and acclimatisation vessels are not bubble-aerated at any stage.

4.7 Feeding

Cultures are fed daily with a suspension of mixed algal species as available (e.g. *Selenastrum* sp., *Scenedesmus* sp., *Chlorella* sp.).

Sufficient feed is given to impart a faint colouration to the water only. Cell densities and feed volumes cannot be specified as the amount required will depend on the clearance rate of each culture and the density and type of algal suspension available.

4.8 Collection of 1st instars

Brood females are removed by wide bore pipette or by gentle sieving to a separate culture vessel the day before the test. The following day, the 1st instars produced overnight are used for testing. Regular separation of brood females from their offspring ensures that all brood *Daphnia* are of the same age.

APPENDIX 4

(continued)

FT/022/EEC/0593
Page 4 of 10

5 EXPOSURE

5.1 Duration

48 hours

5.2 Vessels

250-300 ml glass beakers containing 200 ml of test solution covered with aluminium foil to reduce evaporation.

5.3 Loading

10 1st instars per vessel. Volatile chemicals will be tested in completely filled and stoppered vessels with a loading of no more than 1 daphnid per 50 ml (typically 5 daphnids per 250 ml vessel).

5.4 Animals per test concentration and control group

20 instars (2 groups of 10) will be employed as standard in this study for both EC_{50} tests and "limit" tests. The *Daphnia* will only be divided into 4 groups of 5 (OECD recommendation) at the specific request of the Sponsor.

5.5 Test concentrations

7 test concentrations, logarithmically spaced by a factor not exceeding 2.2, plus 1 control and, if appropriate, 1 solvent control (see "5.6 Negative control"). A spacing factor of 2.2 will be employed as standard in this study. A maximum factor of 2 (OECD recommendation) will only be employed if specifically requested by the Sponsor.

The actual test concentrations are assigned according to the results from a preliminary range finding test using approximately 10 *Daphnia* at each concentration, in a series spanning several orders of magnitude.

Alternatively, a "limit test" may be performed at:

- i 100-150 mg a.i./l (to ensure an achieved measured concentration \geq 100 mg a.i./l) or
- ii a concentration equal to the solubility of the substance in the dilution water or
- iii the maximum concentration forming a stable dispersion.

A maximum concentration of 1000 mg/l (OECD recommendation) will not be tested unless specifically requested by the Sponsor.

The decision on which test design is to be followed will be made by the Study Director. The limit test will be conducted using 20 *Daphnia* per test group and 20 *Daphnia* per control group(s). If immobilisations occur, a full EC_{50} test will be conducted.

Details of the final study design will be advised by Protocol Amendment.

APPENDIX 4

(continued)

FT/022/EEC/0593
Page 5 of 10

5.6 Negative control

Water with no test substance included. Where auxiliary solvents/surfactants are employed, additional control *Daphnia* will be exposed to a concentration of the auxiliary agent equal to that employed in the test series.

The test will be considered invalid if more than 2 *Daphnia* are immobilised in any control group or if the control *Daphnia* become trapped at the surface of the water.

The pH of the controls should not vary more than 1 unit during the test.

5.7 Positive control

A positive control is not included in this study.

5.8 Verification of test concentrations

Test concentrations achieved during the study will be verified by chemical analysis (unless requested otherwise by the Sponsor). Samples will be collected from each test vessel and from the control or solvent control vessel, as appropriate, at 0 and 48 hours and sent to the HRC Department of Environmental Analysis. Duplicate samples will be pooled except in the case of a limit test where samples from the duplicate test vessels will be analysed individually.

The samples will be collected from mid-water with minimum disturbance of any settled material by decanting to a collection vessel. The samples will not be filtered prior to analysis although any surviving *Daphnia* will be removed by passing the sample through a coarse sieve.

5.9 Frequency of exposure

The frequency of exposure is considered to be continuous for the duration of the study.

5.10 Renewal of test media

The test is conducted under static conditions without renewal of the test media. Ideally, test concentrations will remain at or above 80% of the initial concentration throughout the duration of the test. Under static conditions, however, this may not be possible to achieve for unstable substances.

5.11 Test water

Reconstituted water (Elendt M7) as specified in "10.1 Test water".

5.12 Photoperiod

Ambient laboratory lighting under a 16 h light : 8 h dark regime.

Substances which are unstable in light will be tested in darkness.

5.13 Temperature

The temperature will be maintained at $\pm 1^\circ\text{C}$ within the range 18-22°C during the exposure period.

APPENDIX 4

(continued)

FT/022/EEC/0593
Page 6 of 10

5.14 Aeration

The vessels are not bubble-aerated during the test. The oxygen concentration should, however, remain above 3 mgO₂/l air saturation value at the end of the test and must not fall below 2 mgO₂/l. In order to comply with OECD Guideline No. 202, Part I the dissolved oxygen concentration should remain \geq 60% air saturation value during the test. This validation criterion will only be ensured if specifically requested by the Sponsor.

5.15 Feeding

The *Daphnia* are not fed during test period.

5.16 Identification

The *Daphnia* are not individually identified.

5.17 Randomisation

Daphnia are selected from the total number available and assigned to the test vessels without conscious bias.

6 OBSERVATIONS

- 6.1 The numbers of *Daphnia* which are unable to swim within 15 seconds after gentle agitation of each vessel are recorded at 24 h and 48 h. Temperature is recorded daily and pH and dissolved oxygen measurements are made for each vessel at the start and at the end of the study.

7 RESULTS

- 7.1 The 24 h and 48 h EC₅₀ (immobilisation) values and associated 95% confidence limits are calculated using established procedures suitable for the data e.g. Finney, D J (1971) Probit Analysis (3rd Edition) Cambridge University Press; Litchfield, J T and Wilcoxon, F (1949) J Pharmac. Exp. Ther. 96: 99. Thompson, W R and Weil, C S (1952) Biometrics 8: 51-54; Berkson, J (1944) J. Amer. Statist. Ass. 39: 357-65; Williams, D A (1986) Biometrics 42: 641-5.
- 7.2 Wherever possible, the results will be based on the mean measured concentrations achieved at 0 h and 48 h. In the case of tests with unstable substances, measured concentrations will be either (i) those values obtained at 48 hours only or (ii) the geometric means of "fresh" and "expired" samples, depending on the data obtained.

APPENDIX 4

(continued)

FT/022/EEC/0593
Page 7 of 10

8 REPORTS

- 8.1 An advance copy of the report will be issued for comment by the Sponsor prior to printing the final report. Unless otherwise requested, 6 bound copies of the report will be issued. The report will include information on the following:

the test substance;
the test species;
the test water;
details of the experimental procedures followed;
a summary of observations and environmental parameters;
the maximum concentration causing no immobilisation after 48 hours;
the minimum concentration causing 100% immobilisation after 48 hours;
the calculated EC_{50} values and 95% confidence limits at 24 and 48 hours;
the concentration-immobilisation curve at 24 and 48 hours;
any available information on the concentration of test substance in the exposure media.

9 GOOD LABORATORY PRACTICE

9.1 Regulations

The study will be conducted in compliance with the Principles of Good Laboratory Practice as set forth in:

Good Laboratory Practice. The United Kingdom Compliance Programme. Department of Health and Social Security 1986 and subsequent revision, Department of Health 1989.

United States Food and Drug Administration. Title 21 Code of Federal Regulations Part 58. Federal Register, 22 December, 1978 and subsequent Amendments.

United States Environmental Protection Agency. (FIFRA) Title 40 Code of Federal Regulations Part 160. Federal Register, 29 November, 1983 and subsequent amendment Federal Register 17 August, 1989.

United States Environmental Protection Agency. (TSCA) Title 40 Code of Federal Regulations Part 792, Federal Register, 29 November, 1983 and subsequent amendment Federal Register 17 August, 1989.

Japan Ministry of Health and Welfare Notification No. Yakuhatsu 313 Pharmaceutical Affairs Bureau, 31 March 1982 and subsequent amendment Notification No. Yakuhatsu 870. Pharmaceutical Affairs Bureau, 5 October, 1988.

Japan Ministry of Agriculture, Forestry and Fisheries, 59 NohSan, Notification No. 3850. Agricultural Production Bureau, 10 August, 1984.

Japan Ministry of International Trade and Industry, Directive 31 March 1984 (Kanpogyo No 39 Environmental Agency, Kikyoku No. 85 MITI).

Organisation for Economic Co-operation and Development, ISBN 92-64-12367-9, Paris 1982

APPENDIX 4

(continued)

FT/022/EEC/0593
Page 8 of 10

9.2 Amendments to protocol

The Study Director will normally seek approval of the Sponsor before any amendment to protocol is made. However, in the event of difficulty in contacting the Sponsor, and for reasons of animal welfare or protection of scientific integrity, the Study Director reserves the right to act without the prior approval of the Sponsor.

9.3 Quality Assurance Department review

The Quality Assurance Department will:

- a conduct inspections of various phases of the study itself and/or of repetitive procedures relevant to the study design, and
- b audit the final report.

9.4 Maintenance of records

All specimens, raw data and study related documents generated during the course of the study at HRC, together with a copy of the final report will be lodged in the Huntingdon Research Centre Archive.

Such specimens and records will be retained for a minimum period of five years from the date of issue of the final report. At the end of the five year retention period the client will be contacted and advice sought on the future requirements. Under no circumstances will any item be discarded without the client's knowledge.

APPENDIX 4

(continued)

FT/022/EEC/0593
 Page 9 of 10

10 APPENDIX

10.1 Test water

Trace element stock solutions

Initially, individual stock solutions (I) are made up in deionised water to the concentrations indicated. A combined trace element stock solution (II) is then prepared from these individual solutions.

Stock solutions	Concentration (mg/l)	Volume (ml) of stock solutions (I) added to 1 litre deionised water to give combined stock solution (II)
H ₃ BO ₃	57190	0.25
MnCl ₂ ·4H ₂ O	7210	0.25
LiCl	6120	0.25
RbCl	1420	0.25
SrCl ₂ ·6H ₂ O	3040	0.25
NaBr	320	0.25
Na ₂ MnO ₄ ·2H ₂ O	1260	0.25
CuCl ₂ ·2H ₂ O	335	0.25
ZnCl ₂	260	1.0
CoCl ₂ ·6H ₂ O	200	1.0
KI	65	1.0
Na ₂ SeO ₃	43.8	1.0
NH ₄ VO ₃	11.5	1.0
Fe-EDTA soln.	*	5.0

* The Fe-EDTA solution is prepared from the following 2 solutions:

Na ₂ EDTA·2H ₂ O - 5000 mg/l	}	The solutions are poured together and autoclaved immediately. EDTA-containing solutions are not exposed to sunlight or UV radiation as they are photodegradable.
FeSO ₄ ·7H ₂ O - 1991 mg/l		

APPENDIX 4

(continued)

FT/022/EEC/0593
 Page 10 of 10

10.2 Preparation of the M7 medium

The M7 medium is made up from the combined trace element solution (II), a combined vitamin solution and individual macro-nutrient stock solutions as shown below:

Stock solutions	Concentration (mg/l)	Volume (ml) of stock solutions added to 1 litre deionised water to give M7 medium
Combined trace element (II)		50
Combined vitamin solution**		0.1
Macro nutrients:		
CaCl ₂ .2H ₂ O	293800	1.0
MgSO ₄ .7H ₂ O	246600	0.5
KCl	58000	0.1
NaHCO ₃	64800	1.0
Na ₂ SiO ₃ .5H ₂ O	34150	0.2
NaNO ₃	2740	0.1
KH ₂ PO ₄	1430	0.1
K ₂ HPO ₄	1840	0.1

** A combined vitamin stock solution is prepared by adding the following 3 vitamins to 1 litre deionised water:

Vitamin	mg added to 1 litre deionised water
Thiamine hydrochloride	750
Cyanocobalamine (B12)	10
Biotine	7.5

The vitamin stock solution is stored frozen in small aliquots. Vitamins are added to the medium shortly before use.

NB To avoid precipitation of salts when preparing the whole medium, aliquots of stock solutions are added to about 500-800 ml deionised water and the volume made up to 1 litre.

APPENDIX 4

(continued)



STUDY DETAILS

Study schedule number:	To be advised ALS 27
Test substance:	Vectomer 4040 E2200
a) Identity	To be advised
b) Batch number	To be advised
c) Purity	To be advised
Description:	To be advised
Special storage conditions:	To be advised
Sponsor:	Allied-Signal Inc., Engineered Materials Sector, P O Box 1139, NJ 07962-1139, USA
Sponsor's contact:	Mr G Roy
Study Director:	Robert W S Halls, M.Sc.
Location of study:	Department of Aquatic Toxicology, Building V13
Starting date:	To be advised
Completion date:	To be advised
Reporting date:	To be advised
Study Design Reference:	FT/022 EEC/0593
Modifications to Study Design:	None

APPENDIX 4

(continued)



PROTOCOL APPROVAL

Study Schedule: ALS 27 Study Design Reference: FT/022/EEC/0593

Quotation: 35509

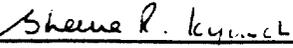
Test Substance: Vectomer ~~4010~~ E 2700

Study: Acute toxicity for *Daphnia*



Study Director
Robert W S Halls

Date: 12 August 1993



HRC Management

Date: 12 August 1993



Sponsor*
Allied Signal Inc

Date: 30 September 1993

*After signature, please return this page to HRC. The corresponding page should also be signed, and is for your retention with the protocol.

APPENDIX 4

(continued)

HUNTINGDON RESEARCH CENTRE LTD
PROTOCOL AMENDMENT

HRC Schedule No.: ALS 27

Amendment No.: 1
Date of issue of protocol: 12 August 1993
Test material: Vectomer E2200
Abbreviated study title:
Acute toxicity for *Daphnia magna*
To: G Roy From: C J Hill

Corrections to protocol:
Corrections to amendment (No.:):
Addition to protocol:
Other: Study details

YES*	NO*
✓	

Signature of Study Director: *G. Roy* 28.2.94
Signature of Sponsor: *[Signature]* 28.3.94

Copies: Client (2), QA, MTD.

Reason for Amendment

To provide specific study details

Amendment (Where appropriate put both original and revised statements)

Study Title: Acute toxicity for *Daphnia magna* Sponsor: Mr G Roy
Allied Signal Inc
Engineered Materials Sector
P O Box 1139
NJ 07962-1139
USA

Protocol Reference: FT/022/EEC/0593

Study Schedule No.: ALS 27

Test Substance:

Name : Vectomer E2200 Batch no.: 309A
Identity : Industrial chemical Hazard classification no.: 3
Purity : > 92% Expiry: 4 August 1994
Description : Colourless to light straw liquid
Storage : In darkness at room temperature
Preparation : Preliminary solution in acetone 20% Tween 80
Frequency : Once at the start of the study
Test Concentrations : 0.10, 0.22, 0.46, 1.0, 2.2, 4.6 and 10 mg/l in duplicate

*Tick where appropriate

APPENDIX 4

(continued)

HRC Schedule No.: ALS 27

Test Media Renewal : None

Diluent : ~~Dechlorinated tap water~~/Reconstituted water (1)* (2)*

Verification of test concentrations : Required* (see below)/Not-required

Dates of study : Arrival of test organisms:	Stock	Start of test:	23.2.94
Start of acclimatisation:	N A	End of test:	25.2.94
Issue of draft report:	April 1994		

Additional information*/Amendment to standard protocol*

- (1) Stability of test solutions: Verified by chemical analysis. Sample (= 15 ml) taken directly from control and exposure levels (Replicates 1 and 2 pooled) at 0 and 48 hours for analysis by the HRC Department of Environmental Analysis. Additional sample (= 50 ml) taken from control and exposure levels (Replicates 1 and 2 pooled) at 0 and 48 hours and stored at +4°C for further analysis, if required.
- (2) Name of test substance: Vectomer E2200
(previously Vectomer 4010)

Empirical Formula: N A

Molecular Weight: N A

Testing Facility : Department of Aquatic Toxicology, HRC Ltd., Building V13

*Delete as required

APPENDIX 4

(continued)

Huntingdon Research Centre Ltd

PROTOCOL AMENDMENT

Amendment No: 2 HRC Schedule No: ALS 27
Date of issue of Protocol: 12 August 1993
Test Material: Vectomer E2200

Corrections to protocol:	x
Corrections to Amendment:	x
Addition to Protocol:	x
Other:	✓

Abbreviated study title:

Acute toxicity for *Daphnia magna*

Signature for HRC Management: *Sheena P. Kynoch* 22/4/94

To: G Roy

Signature of Study Director: *G. T. U.* 22/4/94

From: S.R.Kynoch/G.Bell

Signature of Sponsor: *G. T. U.* 5/20/94

Copies: Client(2), QA, MTD, GB.

Reason for Amendment

Change of Study Director (original Study Director transferred from the Department of Aquatic Toxicology)

Amendment (Where appropriate put both original and revised statements)

1 Study Director : Graeme Bell, M.Sc.
(previously: Robert W S Halls, M.Sc.)

2 Effective date of change : 22 April 1994