8EHQ-0309-17441B
DCN: 89090000214

lyondellbasell

RECEIVED
09 APR -6 AM 10: 10

CERTIFIED MAIL

March 30, 2009

Document Processing Center (7407M)
EPA EAST - Room 6428
Office of Pollution Prevention and Toxics
Environmental Protection Agency
1200 Pennsylvania Avenue, NW
Washington DC 20460-0001

8EHQ-09-17441

Attention: TSCA 8(e) Coordinator

RE: 8EHQ Number - 8EHQ-09-17441
Final Report - 21 Day Daphnia magna Reproduction Study for 2,4,4-Trimethyl Pentene (CASRN: 25167-70-8)

Dear Sir or Madam:

In February 2009, Lyondell Chemical Company (Lyondell), a LyondellBasell Industries company, submitted information to EPA in accordance with Section 8(e) of the Toxic Substances Control Act (TSCA) and EPA's 1991 Section 8(e) Reporting Guide because it included findings that EPA may consider reportable. In that correspondence, Lyondell reported preliminary results for 2,4,4-Trimethyl Pentene (TMP), CASRN: 25167-70-8, from a 21-day Daphnia magna reproduction test (OECD 211). That submission was assigned 8EHQ Number 8EHQ-09-17441. At that time, the final report for this study was not available. Lyondell has now received the final report and is hereby providing a copy to EPA.

Lyondell has not made a determination as to whether a significant risk of injury to health or the environment is actually presented by the findings.

A copy of the study final report is attached.

This report is provided to EPA in accordance with Section 8(e) of the Toxic Substances Control Act (TSCA) and EPA's 1991 Section 8(e) Reporting Guide to meet Lyondell's obligations under TSCA 8(e). Submission of this report to EPA does not confer unto other third parties the right to use this report for product registration or other purposes without written permission from Lyondell Chemical Company.

Should you have any questions or require additional details, please do not hesitate to call me at 713-309-7791. I may also be reached by facsimile at 713-951-1574 or by e-mail at betsy.duncan@lyondellbasell.com.

Sincerely,

Contains No CBI

Betsy Duncan

Betsy Duncan
Business Consultant – Chemical Control / Corporate TSCA Coordinator

89090000214

Lyondell Chemical Company
A LyondellBasell Industries Company
One Houston Center, Suite 700
1221 McKinney Street
Houston, Texas 77010

Phone: 713.309.7791
Fax: 713.951.1574
www.lyondellbasell.com

MR 318330

harlan™

RECEIVED
09 APR -6 AM 10: 10

EPA 8EHQ Number:
8EHQ-09-17441

2,4,4-Trimethylpentene:

DAPHNIA MAGNA REPRODUCTION TEST

PROJECT NUMBER: 2197/0038

AUTHORS: T J Goodband
D M Mullee

Date of Completion:13 February 2009

STUDY SPONSOR:

LyondellBasell Industries
One Houston Center
Suite 700, 1221 McKinney Street
Houston TX 77010
USA

TEST FACILITY:

Harlan Laboratories Ltd
Shardlow Business Park
Shardlow
Derbyshire
DE72 2GD
UK

Telephone: +44 (0) 1332 792896

Facsimile: +44 (0) 1332 799018

2197-0038.doc/CS

This report has been provided to EPA in accordance with Section 8(e) of the Toxic Substances Control Act (TSCA) and EPA's 1991 Section 8(e) Reporting Guide to meet Lyondell's obligations under TSCA 8(e). Submission of this report to EPA does not confer unto other third parties the right to use this report for product registration or other purposes without written permission from Lyondell Chemical Company, a LyondellBasell Industries company.

QUALITY ASSURANCE REPORT

This study type is classed as short-term. The standard test method for this study type ("General Study Plan" in OECD terminology) was reviewed for compliance once only on initial production. Inspection of the routine and repetitive procedures that constitute the study is carried out as a continuous process designed to encompass the major phases at or about the time this study was in progress. In addition, inspection of general facilities not specifically related to this study are done monthly or annually in accordance with QA Standard Procedure.

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

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

	15 December 2006	Standard Test Method Compliance Audit
	14 November 2008	Test Material Preparation
	21 November 2008	Test System Preparation
	05 November 2008	Exposure
	19 November 2008	Assessment of Response
	06 November 2008	Chemical Analysis
§	02 January 2009	Draft Report Audit
§	Date of QA Signature	Final Report Audit

§ Evaluation specific to this study

..... G. Wren ..... DATE: 04 MAR 2009 .....

For the Quality Assurance Unit*

***Authorised QA Signatures:**
Deputy Head of Department: JM Crowther MIScT MRQA
Senior Audit Staff: G Wren ONC MRQA

GLP COMPLIANCE STATEMENT

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

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

..... Date: 13 FEB 2009

T J Goodband BSc
Study Director

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

..... Date: 13 FEB 2009

D M Mullee CChem MRSC
Director of Analytical Services

This report may be presented in final form as a digital (pdf) document. Such documents are prepared by scanning the paper original, and are considered of equivalent integrity and authenticity to versions produced by optical photocopy. However, in all cases the hand-signed paper original, held in secure archives, is the definitive document.

CONTENTS

QUALITY ASSURANCE REPORT		**2**
GLP COMPLIANCE STATEMENT		**3**
CONTENTS		**4**
SUMMARY		**6**
1.	**INTRODUCTION**	**8**
2.	**TEST MATERIAL**	**8**
2.1	Description, Identification and Storage Conditions	8
3.	**METHODS**	**9**
3.1	Test Species	9
3.2	Test Water	9
3.3	Procedure	9
4.	**ARCHIVES**	**16**
5.	**RESULTS**	**17**
5.1	Pre-study Media Preparation Trial	17
5.2	Range-finding Test	17
5.3	Dosing Trial	18
5.4	Definitive Test	18
6.	**CONCLUSION**	**22**
7.	**REFERENCES**	**22**
Table 1	Cumulative Immobilisation Data in the Range-finding Test	23
Table 2	Summary of Findings Following the Exposure of *Daphnia magna* for 21 Days	24
Table 3	Summary of Observations of the Control Group	25
Table 4	Summary of Observations of the Solvent Control Group	26
Table 5	Summary of Observations of the 0.0030 mg/l Test Group	27
Table 6	Summary of Observations of the 0.0085 mg/l Test Group	28
Table 7	Summary of Observations of the 0.034 mg/l Test Group	29
Table 8	Summary of Observations of the 0.16 mg/l Test Group	30
Table 9	Summary of Observations of the 0.70 mg/l Test Group	31
Table 10	Total Cumulative Production of Live Young	32
Table 11	Number of Live Young Produced per Adult (Non-Cumulative)	33
Figure 1	Total Number of Offspring per Adult *Daphnia* Alive at the End of the Test	34
Appendix 1	Typical Water Quality Characteristics	35
Appendix 2	Verification of Test Concentrations	38
Appendix 3	Statistical Analysis	53
Appendix 4	Daily Observations of the Control Group	57
Appendix 5	Daily Observations of the Solvent Control Group	59
Appendix 6	Daily Observations of the 0.0030 mg/l Test Group	61
Appendix 7	Daily Observations of the 0.0085 mg/l Test Group	63
Appendix 8	Daily Observations of the 0.034 mg/l Test Group	65
Appendix 9	Daily Observations of the 0.16 mg/l Test Group	67
Appendix 10	Daily Observations of the 0.70 mg/l Test Group	69

Appendix 11 Physico-Chemical Measurements (Recorded in One Replicate Test Vessel) of Oxygen (mg O_2/l), Temperature (°C) and pH 71
Appendix 12 Light Intensity Measurements 77
Appendix 13 Water Hardness Measurements 78
Appendix 14 The Length of Each Surviving Adult After 21 Days 79
Appendix 15 Protocol and Amendments 80
Appendix 16 Certificate of Analysis 95
Appendix 17 Statement of GLP Compliance in Accordance with Directive 2004/9/EC 96

2,4,4-Trimethylpentene:

DAPHNIA MAGNA REPRODUCTION TEST

SUMMARY

Introduction. A study was performed to assess the effect of the test material on the reproduction of *Daphnia magna* over a 21-day period. The method followed that described in the OECD Guidelines for Testing of Chemicals No 211 (1998) "*Daphnia magna*, Reproduction Test", referenced as Method C.20 of Commission Directive 2001/59/EC (which constitutes Annex V of Council Directive 67/548/EEC).

Methods. Information supplied by the Sponsor indicated that the test material had a low solubility in water and that use of high shear mixing and ultrasonication were not to be used to aid dissolution of the test material. Based on this information the test material fell into the category of a 'difficult substance' as defined by the OECD Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures (OECD 2000). Therefore a media preparation trial was conducted in order to determine the solubility of the test material under test conditions.

The pre-study media preparation trial indicated that the use of preliminary solution in auxiliary solvent to spike the test medium followed by filtration was the most appropriate method of preparation for the test material. From the results of the pre-study media preparation trial; target measured concentrations were determined for the preliminary range-finding test.

Based on the results of a preliminary range-finding test, *Daphnia magna* were exposed (10 replicates of a single daphnid per group) to an aqueous solution of the test material over a range of time-weighted mean measured test concentrations of 0.0030, 0.0085, 0.034, 0.16 and 0.70 mg/l for a period of 21 days using a semi-static test regime. The numbers of live and dead adult *Daphnia* and young daphnids (live and dead) were determined daily. The *Daphnia* were fed daily with an algal suspension.

Results. Analysis of the freshly prepared media showed measured concentrations to range from 0.00167 to 1.46 mg/l and analysis of the old media showed measured concentrations to range from 0.00158 to 1.25 mg/l. The analytical results were variable throughout the trial with the highest test concentration showing measured concentrations of 0.395 to 1.46 mg/l for the fresh media and 0.327 to 1.25 mg/l for the old media. In

addition, a general decline in measured concentration was observed over the 24 hour dosing period. This variation and general decline in measured concentration was considered to be due to the highly volatile nature of the test material despite every effort being made to reduce losses through volatility.

Given this variation it was considered justifiable to base the results on the time-weighted mean measured test concentrations.

The 21-Day EC_{50} (immobilisation) values based on the time-weighted mean measured test concentrations for the parental *Daphnia* generation (P_1) was calculated to be 0.45 mg/l with 95% confidence limits of 0.29 - 0.71 mg/l.

The 21-Day EC_{50} (reproduction) value based on the time-weighted mean measured test concentrations was calculated to be 0.33 mg/l with 95% confidence limits of 0.16 - 0.70 mg/l.

The "Lowest Observed Effect Concentration" was considered to be 0.70 mg/l on the basis that at this test concentration significantly fewer live young per adult ($P<0.05$) were produced when compared to the solvent control.

The "No Observed Effect Concentration" was considered to be 0.16 mg/l on the basis that at this test concentration there were no significant mortalities (immobilisation) observed in the parental generation (P_1) and that there was no significant decrease in the numbers of live young produced per adult by Day 21 ($P\geq0.05$) between the solvent control and the 0.16 mg/l test group.

2,4,4-Trimethylpentene:

***DAPHNIA MAGNA* REPRODUCTION TEST**

1. INTRODUCTION

On the 9th November 2008 Safepharm Laboratories Ltd. changed its name to Harlan Laboratories Ltd. Therefore, this study has been reported and archived under the name Harlan Laboratories Ltd., and not Safepharm Laboratories Ltd. as stated in the protocol. This amendment was considered not to affect the purpose or integrity of the study as the facilities and the management structure remain the same.

This report contains a description of the methods used and the results obtained during a study to investigate the effect of the test material on the reproduction of *Daphnia magna*. The method followed the recommendations of the OECD Guidelines for Testing of Chemicals (1998) No 211 "*Daphnia magna*, Reproduction Test", referenced as Method C.20 of Commission Directive 2001/59/EC (which constitutes Annex V of Council Directive 67/548/EEC).

Daphnia magna is a freshwater invertebrate representative of a wide variety of natural habitats and can therefore be considered as an important non-target organism in freshwater ecosystems.

The study was conducted between 15 September 2008 and 4 December 2008.

2. TEST MATERIAL

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	2,4,4-Trimethylpentene
Description	:	clear, colourless liquid
CAS number	:	25167-70-8
Batch number	:	2381195
Purity	:	93.0%
Date received	:	30 July 2008
Storage conditions	:	room temperature in the dark

The integrity of supplied data relating to the identity, purity and stability of the test material is the responsibility of the Sponsor. A Certificate of Analysis is included in Appendix 16.

3. METHODS

3.1 Test Species

The test was carried out using 1st instar *Daphnia magna* derived from in-house laboratory cultures.

Adult *Daphnia* were maintained in polypropylene vessels containing approximately 2 litres of dechlorinated tap water at a temperature of approximately 20°C. The lighting cycle was controlled to give a 16 hours light and 8 hours darkness cycle with 20 minute dawn and dusk transition periods at a light intensity preferably not exceeding 600 lux. Each culture was fed daily with a suspension of algae (*Chlorella* sp.). Culture conditions ensured that reproduction was by parthenogenesis. Gravid adults were isolated the day before initiation of the test, such that the young daphnids produced overnight were less than 24 hours old. These young were removed from the cultures and used for testing. The diet and diluent water are considered not to contain any contaminant that would affect the integrity or outcome of the study.

3.2 Test Water

The test water used for the range-finding and definitive tests was the same as that used to maintain the stock animals.

Laboratory tap water was dechlorinated by passage through an activated carbon filter (Purite Series 500) and partly softened (Elga Nimbus 1248D Duplex Water Softener) giving water with a total hardeness of approximately by 140 mg/l as $CaCO_3$. After dechlorination and softening the water was passed through a series of computer controlled plate heat exchangers to achieve the required temperature. Typical water quality characteristics for the tap water as supplied, prior to dechlorination and softening are given in Appendix 1.

3.3 Procedure

3.3.1 Pre-study media preparation trial

Information supplied by the Sponsor indicated that the test material had a low solubility in water and that use of high shear mixing and ultrasonication were not to be used to aid

dissolution of the test material. Pre-study solubility work showed that the highest attainable test concentration (by visual inspection) that could be prepared was 10 mg/l with an aliquot (100 µl/l) of a solvent stock solution prepared in dimethylformamide. At higher test concentrations precipitation of the test material was observed on addition of the test material solvent stock solution to water.

Based on this information the test material fell into the category of a 'difficult substance' as defined by the OECD Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures (OECD 2000). Therefore a media preparation trial was conducted in order to determine the solubility of the test material under test conditions.

3.3.1.2 Saturated solution preparation

An amount of test material (1100 mg) was dispersed in 11 litres of dechlorinated tap water with the aid of propeller stirring at approximately 1500 rpm at a temperature of approximately 21°C. After the stirring period samples were taken for chemical analysis after the following pre-treatments:

- Untreated
- Centrifugation at 10000 *g* for 30 minutes
- Centrifugation at 40000 *g* for 30 minutes
- Filtration through a 0.2 µm Gelman AcroCap filter (initial approximate 100 ml discarded)
- Filtration through a 0.2 µm Gelman AcroCap filter (initial approximate 500 ml discarded)

The above was prepared in duplicate with samples being taken after stirring for 24 hours and 48 hours.

3.3.1.2 Solvent spike preparations

An amount of test material (1000 mg) was dissolved in dimethylformamide and the volume adjusted to 10 ml to give a 1000 mg/10 ml solvent stock solution. An aliquot (1000 µl) of this solvent stock solution was dispersed in 10 litres of dechlorinated tap water with the aid of magnetic stirring for approximately 10 minutes to give a 10 mg/l test concentration.

Samples were taken for chemical analysis after the following pre-treatments:

- Untreated
- Centrifugation at 10000 *g* for 30 minutes
- Centrifugation at 40000 *g* for 30 minutes
- Filtration through a 0.2 µm Gelman AcroCap filter (initial approximate 100 ml discarded)
- Filtration through a 0.2 µm Gelman AcroCap filter (initial approximate 500 ml discarded)

Following sampling, the remainder of the test concentration was stirred using a magnetic stirrer with samples being taken after stirring for 24 hours and 48 hours.

3.3.2 Range-finding Test

The test concentrations to be used in the definitive test were determined by a preliminary range-finding test.

The results of the media preparation trial indicated that a test concentration of 0.70 mg/l could be attained using a solvent spike method of preparation. Therefore, in the range-finding test *Daphnia magna* were exposed to a series of target test concentrations of 0.0070, 0.070 and 0.70 mg/l. The test material was prepared using a preliminary solution in dimethylformamide.

An amount of test material (1000 mg) was dissolved in dimethylformamide and the volume adjusted to 10 ml to give a 1000 mg/10 ml solvent stock solution. An aliquot (100 µl) was dispersed in dechlorinated tap water and the volume adjusted to 1 litre to give a 10 mg/l test material dispersion. This dispersion was then filtered through a 0.2 µm Gelman AcroCap filter (first approximate 100 ml discarded to pre-condition the filter) to give the test concentration of 0.70 mg/l. Serial dilutions in dechlorinated tap water were prepared from the 0.70 mg/l test concentration to give the remaining test concentrations of 0.070 and 0.0070 mg/l.

Each stock solution and prepared concentration was inverted several times to ensure adequate mixing and homogeneity.

In the range-finding test 10 daphnids were placed in each test and control vessel and maintained in a temperature controlled room at 21°C to 22°C with a photoperiod of 16 hours light and 8 hours darkness for a period of 48 hours with 20 minute dawn and dusk transition periods. Some of the temperatures during the range-finding test were

observed to be slightly in excess of the range given in the protocol of 20 ± 1°C. This deviation was considered not to have affected the outcome or the validity of the test as no adverse effects of exposure were observed in the control daphnids throughout the duration of the test and that the temperature range was within guideline specification.

Due to the volatile nature of the test material the test was conducted using 300 ml completely filled and sealed glass stoppered conical flasks. After 24 and 48 hours the number of immobilised *Daphnia magna* were recorded.

The control and solvent control groups were maintained under identical conditions but not exposed to the test material. The solvent control group was exposed to 100 µl/l of dimethylformamide.

3.3.3 Dosing Trial

A dosing trial was conducted to determine the amounts of test material present under test conditions.

An amount of test material (1000 mg) was dissolved in 10 ml of dimethylformamide to give a 1000 mg/10 ml solvent stock solution. This stock solution was filtered through a 0.2 µm Gelman Acrocap filter with the first 100 ml being discarded to give a 0.70 mg/l test concentration. Aliquots (20 and 200 ml) were diluted to a final volume (2 litres) in dechlorinated tap water to give 0.0070 and 0.070 mg/l test concentrations which were dispensed into 100 ml completely filled and sealed glass stoppered conical flasks. Samples were analysed immediately and then after 24 and 48 hours.

3.3.4 Definitive Test

Based on the results of a preliminary range-finding test the following test concentrations were assigned to the definitive test: 0.0070, 0.022, 0.070, 0.22 and 0.70 mg/l.

Chemical analysis of the test preparations at (see Appendix 2) showed measured concentrations to be variable through out the duration of the test and also a general decline in measured concentrations over the 24 hour dosing periods. These effects were considered to be due to losses through volatility as the test material was shown to be highly volatile.

Based on these results it was considered appropriate to express the exposure concentrations in terms of the time-weighted mean measured concentrations which were determined to be 0.0030, 0.0085, 0.034, 0.16 and 0.70 mg/l.

3.3.4.1 Experimental preparation

The test material was prepared using a preliminary solution in dimethylformamide.

An amount of test material (1000 mg) was dissolved in dimethylformamide and the volume adjusted to 10 ml to give a 1000 mg/10 ml solvent stock solution. An aliquot (500 µl) was dispersed in dechlorinated tap water and the volume adjusted to 5 litres to give a 10 mg/l test material dispersion. This dispersion was then filtered through a 0.2 µm Gelman AcroCap filter (first approximate 100 ml discarded to pre-condition the filter) to give the target test concentration of 0.70 mg/l. Aliquots (20, 63, 200 and 629 ml) of the 0.70 mg/l test concentration were each separately dispersed in a final volume of 2 litres of dechlorinated tap water to give the target test series of 0.0070, 0.022, 0.070 and 0.22 mg/l respectively.

Each stock solution/dispersion and prepared concentration was inverted several times to ensure adequate mixing and homogeneity.

The concentration and stability of the test material in the test preparations were verified by chemical analysis on Days 0, 4, 7, 11, 14, 18, 20 (fresh media) and 1, 5, 8, 12, 15, 19 and 21 (old media) (see Appendix 2).

3.3.4.2 Exposure conditions

For each concentration a single daphnid was placed in approximately 100 ml of the test preparation in 100 ml completely filled and sealed glass stoppered conical flask to reduce losses through volatility. For each test and control group ten replicate test vessels were prepared. The flasks were maintained at approximately 20°C with a photoperiod of 16 hours light (512 to 557 lux) and 8 hours darkness with 20 minute dawn and dusk transition periods for 21 days. Each vessel was randomly assigned to a position in the laboratory. The test vessels were not aerated.

The control and the solvent control groups were maintained under identical conditions but not exposed to the test material. The solvent control group was exposed to 100 µl/l of dimethylformamide.

The test preparations were renewed daily. The adult *Daphnia* were transferred to fresh media by wide-bore pipette before the contents of each vessel were passed through a fine mesh. Young daphnids (live and dead) and any unhatched eggs were collected on the mesh and counted using a stereo microscope before being discarded.

Each daphnid received approximately 5 to 10 µl of a unicellular algal culture (*Chlorella* sp.), daily. Feeding was at a level of approximately 0.1 to 0.2 mg carbon/daphnid/day, dependent on the age and size of the animals. Equal amounts of food were given to each daphnid.

On a daily basis the numbers of live and dead of the "Parental" (P_1) generation, the numbers of live and dead "Filial" (F_1) *Daphnia* and the number of discarded unhatched eggs were counted. An assessment was also made of the general condition and size of the parental *Daphnia* as compared with the controls.

The number of *Daphnia* with eggs or young in the brood pouch was determined daily. Young daphnids were considered to be dead if no sign of movement was apparent during microscopic examination. Adult *Daphnia* which were unable to swim for approximately 15 seconds after gentle agitation (ie. immobile), were considered to be dead. An immobilisation criterion for the young daphnids was considered to be inappropriate due to the large numbers of off-spring produced in the flasks.

At the end of the test, the length of each surviving parent animal was determined.

3.3.4.3 Physico-chemical measurements

Temperature of the test preparations and light intensity were recorded daily throughout the test. Dissolved oxygen concentrations, pH and temperature were recorded before and after each test media renewal. The pH and dissolved oxygen concentration were measured using a WTW pH/Oxi 340I pH and dissolved oxygen meter and the temperature was measured using a Hanna Instruments HI 93510 digital thermometer. Measurements were made on one replicate for each test concentration. The water hardness of the solvent control and the highest surviving test concentration in the fresh and old media was measured once per week.

3.3.4.4 Verification of test concentrations

Water samples were taken from the solvent control and each surviving test group (replicates pooled) for quantitative analysis. Samples of the fresh test preparations were taken on Days 0, 4, 7, 11, 14, 18 and 20 and of the expired test preparations on Days 1, 5, 8, 12, 15, 19 and 21.

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

3.3.4.5 Evaluation of data

The EC_{50} (immobilisation) values up to Day 21 of the test were estimated by inspection of the data.

The EC_{50} (immobilisation) value and associated confidence limits at Day 21 were calculated by the trimmed Spearman-Karber method (Hamilton *et al* 1977) using the ToxCalc computer software package (ToxCalc 1999).

When only one partial response is shown the trimmed Spearman-Karber method is appropriate.

The observed mortalities in the parental (P_1) generation of the 0.0030 and 0.0085 mg/l test groups were compared to the solvent control group using the corrected chi-squared statistic (Breslow and Day 1980).

The EC_{50} (reproduction) value after 21 days was calculated by the geometric mean method as follows:

$$EC_{50}\text{value} = \sqrt{C_1 \times C_2}$$

where:

C_1 = concentration showing 0% suppression of reproduction
C_2 = concentration showing 100% suppression of reproduction.

If there is no suppression of reproduction between 0% and 100% suppression of reproduction, then the geometric mean of the highest test concentration showing no

suppression of reproduction and the lowest test concentration showing 100% suppression of reproduction is calculated. The concentrations resulting in 0% and 100% suppression of reproduction will be the 95% confidence limits.

For the estimation of the "Lowest Observed Effect Concentration" (LOEC) and the "No Observed Effect Concentration" (NOEC) the numbers of live young produced per adult over the duration of the test for the control, solvent control and each test group were compared using one way analysis of variance incorporating Bartlett's test for homogeneity of variance (Sokal and Rohlf 1981) and Dunnett's multiple comparison procedure for comparing several treatments with a control (Dunnett 1955) (see Appendix 3). Results from the control, solvent control and each test group *Daphnia* length data, determined for the surviving daphnids on termination of the test, were compared using one way analysis of variance incorporating Bartlett's test for homogeneity of variance (Sokal and Rohlf 1981) and Dunnett's multiple comparison procedure for comparing several treatments with a control (Dunnett 1955) (see Appendix 3). All statistical analyses were performed using the SAS computer software package (SAS 1999 - 2001).

The time-weighted mean measured test concentrations were calculated as follows:

$$\text{TWM} = \frac{\text{Total Area}}{\text{Total Number of Days of the Test}}$$

$$\text{where Total area} = \frac{C_0 - C_1}{\ln(C_0) - \ln(C_1)} \times \text{Days}$$

TWM = time-weighted mean measured test concentration (mg/l)
C_0 = measured concentration at the start of each renewal period (mg/l)
C_1 = measured concentration at the end of each renewal period (mg/l)
Days = number of days in the renewal period

4. ARCHIVES

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

5. RESULTS

5.1 Pre-study Media Preparation Trial

Analysis of the samples prepared from a saturated solution method of preparation showed measured concentrations of less than the limit of quantitation. It was considered that these results were possibly due to loss of the test material through volatility as a result of the prolonged, aggressive stirring used to prepare the saturated solutions. It was therefore considered that the use of a saturated solution method of preparation was unsuitable for the test material.

The results of the centrifuged samples prepared from a solvent spike method of preparation showed lower measured concentrations compared to the filtered samples. This was considered to be possible due to losses through volatility during the centrifugation process. Prolonged stirring of the solvent spike preparation resulted in a decrease in measured concentration.

It was therefore considered that the most appropriate method of preparation for the test material was as a solvent spike at an initial 10 mg/l followed by filtration to remove all excess undissolved test material.

5.2 Range-finding Test

Cumulative immobilisation data from the exposure of *Daphnia magna* to the test material during the range-finding test are given in Table 1.

No immobilisation was observed at the target test concentrations of 0.0070, 0.070 and 0.70 mg/l.

Based on the results of a preliminary range-finding test the following target test concentrations were assigned to the definitive test: 0.0070, 0.022, 0.070, 0.22 and 0.70 mg/l.

Chemical analysis of the test preparations (see Appendix 2) showed measured concentrations to be variable throughout the duration of the test and also a general decline in measured concentrations over the 24 hour dosing periods. These effects were considered to be due to losses through volatility as the test material was shown to be highly volatile.

Based on these results it was considered appropriate to express the exposure concentrations in terms of the time-weighted mean measured concentrations which were determined to be 0.0030, 0.0085, 0.034, 0.16 and 0.70 mg/l.

5.3 Dosing Trial

The results of the dosing trial (see Appendix 2) indicated that the test material was stable under test conditions. The results from the definitive test showed more variable results than the dosing trial. This was considered to be due to losses through volatility due to the longer preparation required during the definitive test to media change all the test vessels.

5.4 Definitive Test

The observations for each test and control group are summarised in Tables 2 to 9. The total cumulative production of live young is given in Table 10 and the number of live young produced per adult is shown in Table 11. The total number of offspring per parent daphnia (for each replicate) alive at the end of the test is shown in Figure 1. Data for each individual replicate flask are given in Appendices 4 to 10.

5.4.1 Verification of Test Concentrations

Analysis of the freshly prepared media (see Appendix 2) showed measured concentrations to range from 0.00167 to 1.46 mg/l and analysis of the old media showed measured concentrations to range from 0.00158 to 1.25 mg/l. The analytical results were variable throughout the trial with the highest test concentration showing measured concentrations of 0.395 to 1.46 mg/l for the fresh media and 0.327 to 1.25 mg/l for the old media. In addition, a general decline in measured concentration was observed over the 24 hour dosing period. This variation and general decline in measured concentration was considered to be due to the highly volatile nature of the test material despite every effort being made to reduce losses through volatility.

Given this variation it was considered justifiable to base the results on the time-weighted mean measured test concentrations. The time-weighted mean measured test concentrations of the test media were determined to be 0.0030, 0.0085, 0.034, 0.16 and 0.70 mg/l.

5.4.2 Physico-chemical Measurements

The results of the physico-chemical measurements are given in Appendix 11. Temperature was maintained at approximately 20°C throughout the test, while there were no treatment related differences for oxygen concentration or pH.

Throughout the test the light intensity was observed to be in the range 512 to 557 lux (see Appendix 12).

The water hardness was observed to be in the range 112 to 150 mg/l as $CaCO_3$ in the solvent control and the highest surviving test group throughout the test. The results of the water hardness measurements are given in Appendix 13.

5.4.3 Observations on Test Material Solubility

The freshly prepared test media were observed to be clear, colourless solutions whereas the old test media were observed to be green tinged due to the presence of algal cells used as feed for the daphnids.

5.4.4 Validation Criteria

The following validation criteria were achieved during the test:

		Required	Actual
a	Solvent control mortality	≤ 20%	0%
b	Dissolved oxygen	> 3 mg O_2/l	≥ 8.5 mg O_2/l
c	pH (solvent control group)	deviation ≤ 1.5	0.4
d	Mean number of live young per surviving adult (solvent control group)	≥ 60 after 21 days	83
e	Coefficient of variation for solvent control group	≤ 25%	9%

5.4.5 Lethal Effects on the Parental Generation (P_1)

Mortality (immobilisation) occurred predominantly at the highest test concentration of 0.70 mg/l resulting in 70% mortality by Day 21.

Mortality was also observed at the test concentrations of 0.0030 mg/l on Day 19 and 0.0085 mg/l on day 5. However, statistical analysis of the mortality data using the

corrected chi-squared statistic (Breslow and Day 1980) showed that the observed mortalities in the 0.0030 and 0.0085 mg/l test groups were not significantly different ($P \geq 0.05$) when compared to the solvent control group.

No mortalities occurred at the 0.034 and 0.16 mg/l test concentrations throughout the test.

The following EC_{50} (immobilisation) values based on time-weighted mean measured test concentrations were estimated by inspection of the immobilisation data at 24, 48 and 96 hours and Days 7 and 14 and calculated by the trimmed Spearman-Karber method (Hamilton *et al* 1977) at 21 days:

Time	EC_{50} (mg/l)	95% Confidence Limits (mg/l)
24 hours	>0.70	-
48 hours	>0.70	-
96 hours	>0.70	-
7 days	>0.70	-
14 days	approximately 0.70*	-
21 days	0.45	0.29 - 0.71

5.4.6 Sub-lethal Effects on the Parental Generation (P_1)

There was a significant effect on size and colour of the daphnids in that 100% of the surviving daphnids from Day 4 onwards at the test concentration of 0.70 mg/l were markedly smaller and paler in colour than the solvent control animals.

The daphnids at the remaining test concentrations were observed to be the same size and colour as the solvent control animals.

After 21 days there were no statistically significant decreases between the solvent control and the 0.0030, 0.0085, 0.034 and 0.16 mg/l test groups in terms of the number of live young produced per adult. The 0.70 mg/l test group showed a statistically significant difference from the solvent control and the remaining test groups after 21 days in terms of producing fewer numbers of live young per adult (see Appendix 3).

---

* Inspection of the immobilisation data showed an EC_{50} value of approximately 0.70 mg/l. It was not possible to calculate an EC_{50} value at this time point due to the unsuitable nature of the data.

The EC_{50} (reproduction) value calculated by the geometric mean method on Day 21, based on time-weighted mean measured test concentrations was 0.33 mg/l with 95% confidence limits of 0.16 - 0.70 mg/l.

After 21 days the length of each surviving adult was determined, the results of which are given in Appendix 14. The results showed that there were no statistically significant differences ($P \geq 0.05$) between the solvent control and the 0.0030, 0.0085, 0.034 and 0.16 mg/l test groups in terms of length of the daphnids after 21 days exposure to the test material (see Appendix 3). The surviving daphnids from the 0.70 mg/l were shown to be significantly smaller ($P<0.05$) compared to the solvent control in terms of daphnid length.

5.4.7 Effects on the Filial Generation (F_1)

Information on the effects of the test material on the F_1 generation is limited, since, by study design, the young are removed soon after liberation from the brood pouch. However, an assessment made at each media renewal showed the "filial" daphnids produced by all the test groups were in the same general condition as the young produced by the solvent controls over the duration of the test.

Young were first produced in the solvent control test group on Day 7 of the test.

Numbers of unhatched eggs and dead young were low in all control and treatment groups surviving to maturation.

5.4.8 Lowest Observed Effect Concentration

The "Lowest Observed Effect Concentration" (LOEC) was 0.70 mg/l as this test group produced significantly fewer live young per adult ($P<0.05$) than the solvent control group.

5.4.9 No Observed Effect Concentration

The "No Observed Effect Concentration" (NOEC) was 0.16 mg/l as there were no significant mortalities (immobilisation) observed in the parental generation (P_1) and there was not a significant decrease ($P \geq 0.05$) in terms of the number of live young produced per adult when compared to the solvent control after 21 days.

6. CONCLUSION

Exposure of *Daphnia magna* to the test material resulted in significant mortalities at the test concentration of 0.70 mg/l (based on the time-weighted mean measured test concentrations) resulting in 70% mortalities by Day 21.

The 21-Day EC_{50} (immobilisation) value, based on time-weighted mean measured test concentrations, for the parental *Daphnia* generation (P_1) was calculated to be 0.45 mg/l with 95% confidence limits of 0.29 - 0.71 mg/l.

The 21-Day EC_{50} (reproduction) based on time-weighted mean measured test concentrations was 0.33 mg/l with 95% confidence limits of 0.16 - 0.70 mg/l.

The "Lowest Observed Effect Concentration" (LOEC) and the "No Observed Effect Concentration" (NOEC) based on time-weighted mean measured test concentrations were 0.70 and 0.16 mg/l respectively.

7. REFERENCES

Breslow, N E and Day, N E (1980) Statistical Methods in Cancer Research. Volume 1 - The Analysis of Case-Control Studies. Lyon : International Agency for Research on Cancer.

Dunnett, C W (1955) A Multiple Comparison Procedure for Comparing Several Treatments With a Control. *J Am Stat Assoc* **50**, 1096-1121.

Hamilton, M A, Russo, R C and Thurston, R V (1977) Trimmed Spearman-Karber Method for Estimating Median Lethal Concentration in Toxicity Bioassays. *Environ Sci Technol* **11**, 714-719.

SAS/STAT Proprietary Software Release 8.02 (1999 - 2001), SAS Institute Inc, Cary, NC, USA.

Sokal, R R and Rohlf, F J (1981) Biometry. New York : W H Freeman and Company.

ToxCalc Version 5.0.23C (1999), Tidepool Scientific Software, McKinleyville, CA 95519, USA.

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Table 1 Cumulative Immobilisation Data in the Range-finding Test

Target Concentration (mg/l)	Cumulative Immobilised *Daphnia* (Initial Population: 10 Per Replicate)	
	24 Hours	48 Hours
Control	0	0
Solvent Control	0	0
0.0070	0	0
0.070	0	0
0.70	0	0

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Table 2 Summary of Findings Following the Exposure of *Daphnia magna* for 21 Days

Time-Weighted Mean Measured Concentration (mg/l)	% Survival of P_1	Number of Live Young		Number of Dead Young		Number of Unhatched Eggs	
		Total	Per Female (cumulative)	Total	Per Female (cumulative)	Total	Per Female (cumulative)
Control	90	784	87	0	0	0	0
Solvent Control	100	834	83	0	0	0	0
0.0030	90	790	88	0	0	0	0
0.0085	90	724	81	0	0	0	0
0.034	100	898	90	0	0	0	0
0.160	100	1114	111	0	0	1	<1
0.70	30	0	0	0	0	0	0

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Table 3 Summary of Observations of the Control Group

Day	Adults Surviving	Number of Adults with Eggs in Brood Pouch	Live Young	Dead Young	Unhatched Eggs
1	10	0	0	0	0
2	10	0	0	0	0
3	10	0	0	0	0
4	10	0	0	0	0
5	10	9	0	0	0
6	10	10	0	0	0
7	10	10	12	0	0
8	10	10	70	0	0
9	10	10	24	0	0
10	10	10	9	0	0
11	10	10	124	0	0
12	10	10	64	0	0
13	9	9	0	0	0
14	9	9	46	0	0
15	9	9	148	0	0
16	9	9	0	0	0
17	9	9	39	0	0
18	9	9	115	0	0
19	9	9	0	0	0
20	9	9	0	0	0
21	9	9	167	0	0
		TOTALS	818	0	0

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Table 4 Summary of Observations of the Solvent Control Group

Day	Adults Surviving	Number of Adults with Eggs in Brood Pouch	Live Young	Dead Young	Unhatched Eggs
1	10	0	0	0	0
2	10	0	0	0	0
3	10	0	0	0	0
4	10	0	0	0	0
5	10	9	0	0	0
6	10	10	0	0	0
7	10	10	10	0	0
8	10	10	28	0	0
9	10	10	25	0	0
10	10	10	49	0	0
11	10	10	68	0	0
12	10	10	42	0	0
13	10	10	89	0	0
14	10	10	81	0	0
15	10	10	49	0	0
16	10	10	72	0	0
17	10	10	99	0	0
18	10	10	34	0	0
19	10	10	55	0	0
20	10	10	65	0	0
21	10	10	68	0	0
		TOTALS	834	0	0

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Table 5 Summary of Observations of the 0.0030 mg/l Test Group

Day	Adults Surviving	Number of Adults with Eggs in Brood Pouch	Live Young	Dead Young	Unhatched Eggs
1	10	0	0	0	0
2	10	0	0	0	0
3	10	0	0	0	0
4	10	0	0	0	0
5	10	10	0	0	0
6	10	10	0	0	0
7	10	10	0	0	0
8	10	10	41	0	0
9	10	10	21	0	0
10	10	10	9	0	0
11	10	10	134	0	0
12	10	10	43	0	0
13	10	10	19	0	0
14	10	10	119	0	0
15	10	10	107	0	0
16	10	10	0	0	0
17	10	10	61	0	0
18	10	10	102	0	0
19	9	9	0	0	0
20	9	9	50	0	0
21	9	9	138	0	0
		TOTALS	844	0	0

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Table 6 Summary of Observations of the 0.0085 mg/l Test Group

Day	Adults Surviving	Number of Adults with Eggs in Brood Pouch	Live Young	Dead Young	Unhatched Eggs
1	10	0	0	0	0
2	10	0	0	0	0
3	10	0	0	0	0
4	10	0	0	0	0
5	9	8	0	0	0
6	9	8	0	0	0
7	9	9	0	0	0
8	9	9	45	0	0
9	9	9	12	0	0
10	9	9	0	0	0
11	9	9	110	0	0
12	9	9	0	0	0
13	9	9	40	0	0
14	9	9	134	0	0
15	9	9	21	0	0
16	9	9	51	0	0
17	9	9	90	0	0
18	9	9	48	0	0
19	9	9	37	0	0
20	9	9	55	0	0
21	9	9	81	0	0
		TOTALS	724	0	0

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Table 7 Summary of Observations of the 0.034 mg/l Test Group

Day	Adults Surviving	Number of Adults with Eggs in Brood Pouch	Live Young	Dead Young	Unhatched Eggs
1	10	0	0	0	0
2	10	0	0	0	0
3	10	0	0	0	0
4	10	0	0	0	0
5	10	7	0	0	0
6	10	10	0	0	0
7	10	10	0	0	0
8	10	10	44	0	0
9	10	10	7	0	0
10	10	10	0	0	0
11	10	10	122	0	0
12	10	10	0	0	0
13	10	10	0	0	0
14	10	10	228	0	0
15	10	10	46	0	0
16	10	10	0	0	0
17	10	10	109	0	0
18	10	10	98	0	0
19	10	10	0	0	0
20	10	10	95	0	0
21	10	10	149	0	0
		TOTALS	898	0	0

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Table 8 Summary of Observations of the 0.16 mg/l Test Group

Day	Adults Surviving	Number of Adults with Eggs in Brood Pouch	Live Young	Dead Young	Unhatched Eggs
1	10	0	0	0	0
2	10	0	0	0	0
3	10	0	0	0	0
4	10	0	0	0	0
5	10	9	0	0	0
6	10	10	0	0	0
7	10	10	0	0	0
8	10	10	69	0	0
9	10	10	0	0	0
10	10	10	0	0	0
11	10	10	181	0	0
12	10	10	0	0	0
13	10	10	0	0	0
14	10	10	309	0	0
15	10	10	0	0	0
16	10	10	0	0	0
17	10	10	220	0	0
18	10	10	52	0	0
19	10	10	0	0	0
20	10	10	126	0	1
21	10	10	157	0	0
		TOTALS	1114	0	1

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Table 9 Summary of Observations of the 0.70 mg/l Test Group

Day	Adults Surviving	Number of Adults with Eggs in Brood Pouch	Live Young	Dead Young	Unhatched Eggs
1	9	0	0	0	0
2	9	0	0	0	0
3	9	0	0	0	0
4	9	0	0	0	0
5	9	0	0	0	0
6	7	0	0	0	0
7	6	0	0	0	0
8	6	0	0	0	0
9	6	0	0	0	0
10	6	0	0	0	0
11	6	0	0	0	0
12	6	2	0	0	0
13	5	1	0	0	0
14	5	1	0	0	0
15	5	1	0	0	0
16	4	0	0	0	0
17	4	0	0	0	0
18	4	0	0	0	0
19	3	2	0	0	0
20	3	2	0	0	0
21	3	2	0	0	0
		TOTALS	0	0	0

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Table 10 Total Cumulative Production of Live Young

Time-Weighted Mean Measured Concentration (mg/l)	Day									
	1	2	3	4	5	6	7	8	9	10
Control	0	0	0	0	0	0	12	82	106	115
Solvent Control	0	0	0	0	0	0	10	38	63	112
0.0030	0	0	0	0	0	0	0	41	62	71
0.0085	0	0	0	0	0	0	0	45	57	57
0.034	0	0	0	0	0	0	0	44	51	51
0.160	0	0	0	0	0	0	0	69	69	69
0.70	0	0	0	0	0	0	0	0	0	0

Time-Weighted Mean Measured Concentration (mg/l)	Day										
	11	12	13	14	15	16	17	18	19	20	21
Control	239	303	303	349	497	497	536	651	651	651	818
Solvent Control	180	222	311	392	441	513	612	646	701	766	834
0.0030	205	248	267	386	493	493	554	656	656	706	844
0.0085	167	167	207	341	362	413	503	551	588	643	724
0.034	173	173	173	401	447	447	556	654	654	749	898
0.160	250	250	250	559	559	559	779	831	831	957	1114
0.70	0	0	0	0	0	0	0	0	0	0	0

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

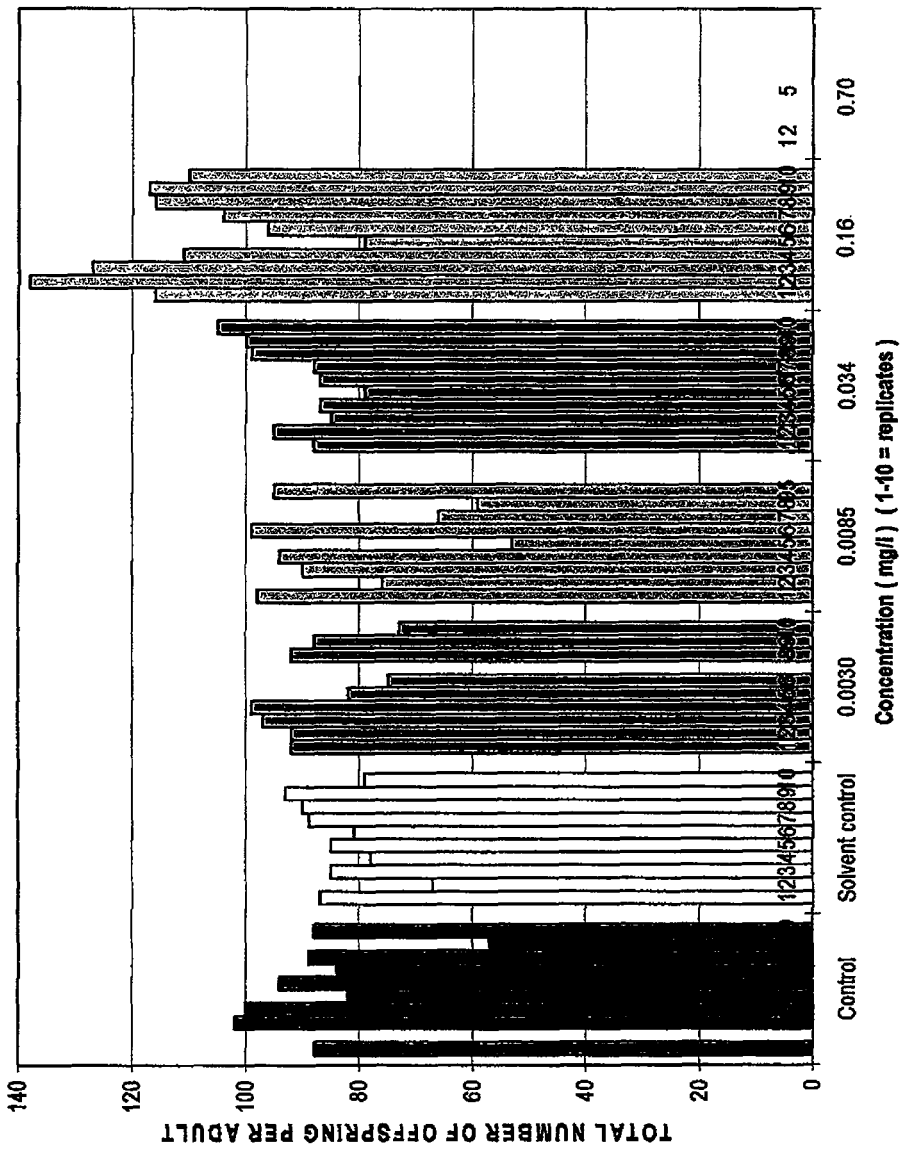
Table 11 Number of Live Young Produced per Adult (Non-Cumulative)

Time-Weighted Mean Measured Concentration (mg/l)	Day									
	1	2	3	4	5	6	7	8	9	10
Control	0	0	0	0	0	0	1	7	2	1
Solvent Control	0	0	0	0	0	0	1	3	3	5
0.0030	0	0	0	0	0	0	0	4	2	1
0.0085	0	0	0	0	0	0	0	5	1	0
0.034	0	0	0	0	0	0	0	4	1	0
0.160	0	0	0	0	0	0	0	7	0	0
0.70	0	0	0	0	0	0	0	0	0	0

Time-Weighted Mean Measured Concentration (mg/l)	Day										
	11	12	13	14	15	16	17	18	19	20	21
Control	12	6	0	5	16	0	4	13	0	0	19
Solvent Control	7	4	9	8	5	7	10	3	6	7	7
0.0030	13	4	2	12	11	0	6	10	0	6	15
0.0085	12	0	4	15	2	6	10	5	4	6	9
0.034	12	0	0	23	5	0	11	10	0	10	15
0.160	18	0	0	31	0	0	22	5	0	13	16
0.70	0	0	0	0	0	0	0	0	0	0	0

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Figure 1 **Total Number of Offspring per Adult *Daphnia* Alive at the End of the Test**



2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 1 Typical Water Quality Characteristics

WATER SUPPLY ZONE ZDB13 ALVASTON								
REPORTING PERIOD: 01/01/2007 – 31/12/2007								
PARAMETER	ANALYSIS UNITS	NO. SAMPLES TAKEN	CONCENTRATION OR VALUE (ALL SAMPLES)			PCV*	SAMPLES CONTRAVENING PCV	
			MINIMUM	AVERAGE	MAXIMUM		NUMBER	PERCENT
1,2 Dichloroethane	µg/l	8	<0.080	<0.080	<0.080	3	0	0.00
2, 3, 6 – TBA	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
2, 4 – DB	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
2, 4, 5, - TCPA	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
2,4-D	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Aldrin	µg/l	8	<0.001	<0.001	<0.001	0.03	0	0.00
Alpha-HCH	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
Aluminium	µg/l	52	<11.000	<12.692	29.000	200	0	0.00
Ametryn	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Antimony	µg/l	8	<0.120	<0.529	1.200	5	0	0.00
Arsenic	µg/l	8	<0.370	<0.370	<0.370	10	0	0.00
Atrazine	µg/l	8	<0.001	<0.001	0.001	0.1	0	0.00
Benazolin	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Bentazone	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Benzene	µg/l	8	<0.060	<0.060	<0.060	1	0	0.00
Benzo 3,4 Pyrene	µg/l	8	<0.001	<0.001	<0.001	0.01	0	0.00
Beta – HCH	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
Boron	mg/l	8	0.021	0.034	0.052	1	0	0.00
Bromacil	µg/l	8	<0.012	<0.012	<0.012	0.1	0	0.00
Bromate	µg/l	8	<0.600	<0.600	<0.600	10	0	0.00
Bromoxynil	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Cadmium	µg/l	8	<0.060	<0.111	0.150	5	0	0.00
Captan	µg/l	8	<0.007	<0.007	<0.007	0.1	0	0.00
Carbenazim	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Carbetamide	µg/l	8	<0.002	<0.003	<0.006	0.1	0	0.00
Chlordane A	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Chlorothalonil	µg/l	8	<0.003	<0.003	<0.003	0.1	0	0.00
Chlorotoluron	µg/l	8	<0.002	<0.002	<0.004	0.1	0	0.00
Chromium	µg/l	8	<0.700	<0.700	<0.700	50	0	0.00
Cis – Permethrin	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Clopyralid	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
Colour	mg/l Pt/Co	26	<0.400	<0.408	0.600	20	0	0.00
Copper	mg/l	8	<0.003	<0.008	0.027	2	0	0.00
Cyanazine	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Cyanide	µg/l	8	<0.400	<0.625	0.700	50	0	0.00
Cyfluthrin	µg/l	8	<0.003	<0.003	<0.003	0.1	0	0.00
Cypermethrin	µg/l	8	<0.004	<0.004	<0.004	0.1	0	0.00
D.D.E – Ortho Para	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
D.D.E – Para Para	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
D.D.T – Ortho Para	µg/l	8	<0.003	<0.003	<0.003	0.1	0	0.00
D.D.T – Para Para	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
Delta – HCH	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Deltamethrin	µg/l	8	<0.005	<0.005	<0.005	0.1	0	0.00
Dicamba	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Dichlobenil	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
Dichloprop	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Dieldrin	µg/l	8	<0.002	<0.002	<0.002	0.03	0	0.00
Diflufenican	µg/l	8	<0.004	<0.004	<0.004	0.1	0	0.00
Diuron	µg/l	8	<0.003	<0.003	<0.003	0.1	0	0.00
E. coli	No. / 100 ml	180	0.000	0.000	0.000	0	0	0.00
Endosulfan A	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Endosulfan B	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Endrin	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Enterococci	No. / 100 ml	8	0.000	0.000	0.000	0	0	0.00
EPTC	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
Ethofumesate	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Fenoprop	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Fenpropidin	µg/l	8	<0.003	<0.003	<0.003	0.1	0	0.00

*PCV Prescribed concentration or value for mandatory parameters and specified limit or value for non-mandatory parameters (indicators and disinfectants).

*1 No numerical limit applies.

*2 Guidance levels only. Monitored for the calculation of Total Indicative Dose, which is the regulatory parameter. No samples exceeded the TID in 2007.

*3 The formula for the calculated Nitrate/Nitrite level is [Nitrate]/50 + [Nitrite]/3. This number has no units.

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 1 (continued) Typical Water Quality Characteristics

WATER SUPPLY ZONE ZDB13 ALVASTON

REPORTING PERIOD: 01/01/2007 – 31/12/2007

PARAMETER	ANALYSIS UNITS	NO. SAMPLES TAKEN	CONCENTRATION OR VALUE (ALL SAMPLES)			PCV*	SAMPLES CONTRAVENING PCV	
			MINIMUM	AVERAGE	MAXIMUM		NUMBER	PERCENT
Fenpropimorph	µg/l	8	<0.006	<0.006	<0.006	0.1	0	0.00
Fenvalerate	µg/l	8	<0.003	<0.003	<0.003	0.1	0	0.00
Fluoride	mg/l	8	0.310	0.351	0.415	1.5	0	0.00
Fluroxypyr	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Flutriafol	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Gamma – HCH	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
Heptachlor	µg/l	8	<0.001	<0.001	<0.001	0.03	0	0.00
Heptachlor epoxide	µg/l	8	<0.002	<0.002	<0.002	0.03	0	0.00
Hexachlorobenzene	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
Hexachlorobutadiene	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
Ioxynil	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
Iron	µg/l	52	<7.000	<13.481	82.000	200	0	0.00
Isodrin	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
Isoprofuron	µg/l	8	<0.002	<0.004	<0.004	0.1	0	0.00
Lead	µg/l	8	<0.500	<1.138	5.100	25	0	0.00
Linuron	µg/l	8	<0.003	<0.003	<0.005	0.1	0	0.00
Manganese	µg/l	52	<1.500	<1.546	2.600	50	0	0.00
MCPA	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
MCPB	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Mecoprop	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Mercury	µg/l	8	<0.012	<0.012	<0.012	1	0	0.00
Metamitron	µg/l	8	<0.002	<0.002	<0.003	0.1	0	0.00
Metazachlor	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Methabenzthiazuron	µg/l	8	<0.002	<0.002	<0.003	0.1	0	0.00
Methoxychlor	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
Monolinuron	µg/l	8	<0.002	<0.002	<0.004	0.1	0	0.00
Nickel	µg/l	8	<0.900	<1.788	2.500	20	0	0.00
Nitrate	mg/l	8	10.200	14.775	17.900	50	0	0.00
Nitrite	mg/l	8	<0.002	<0.003	0.004	0.5	0	0.00
Nitrite/Nitrate Calculated	-*3	8	0.200	0.295	0.360	<1*3	0	0.00
Odour	Dilution Number	26	0.000	0.000	0.000	3 at 25°C	0	0.00
PAH	µg/l	8	0.000	0.000	0.001	0.1	0	0.00
PCB – Arochlor 1254	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Pendimethalin	µg/l	8	<0.003	<0.003	<0.003	0.1	0	0.00
pH	pH value	52	7.260	7.719	8.240	Max 9.5, Min 6.5	0	0.00
Pirimicarb	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
Prometryn	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Propachlor	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Propazine	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
Propiconazole	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Propyzamide	µg/k	8	<0.002	<0.002	<0.002	0.1	0	0.00
Selenium	µg/l	8	0.420	0.670	1.200	10	0	0.00
Simazine	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
Sodium	mg/l	8	21.000	27.250	35.000	200	0	0.00
T.D.E – Ortho Para	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
T.D.E – Para Para	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Taste	Dilution Number	26	0.000	0.000	0.000	3 at 25°C	0	0.00
Tecnazene	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
Terbutryn	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Tetrachloroethene and Trichloroethene	µg/l	8	0.000	0.000	0.000	10	0	0.00
Tetrachloromethane	µg/l	8	<0.020	<0.030	<0.040	3	0	0.00
Total Pesticides	µg/l	8	0.000	0.000	0.001	0.5	0	0.00
Total Trihalomethanes	µg/l	8	22.200	34.138	50.600	100	0	0.00
Trans - Permethrin	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Triadimefon	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Triallate	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
Trichlorobenzene	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00

*PCV Prescribed concentration or value for mandatory parameters and specified limit or value for non-mandatory parameters (indicators and disinfectants).

*1 No numerical limit applies.

*2 Guidance levels only. Monitored for the calculation of Total Indicative Dose, which is the regulatory parameter. No samples exceeded the TID in 2007.

*3 The formula for the calculated Nitrate/Nitrite level is [Nitrate]/50 + [Nitrite]/3. This number has no units.

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 1 (continued) Typical Water Quality Characteristics

WATER SUPPLY ZONE ZDB13 ALVASTON

REPORTING PERIOD: 01/01/2007 – 31/12/2007

PARAMETER	ANALYSIS UNITS	NO. SAMPLES TAKEN	CONCENTRATION OR VALUE (ALL SAMPLES)			PCV*	SAMPLES CONTRAVENING PCV	
			MINIMUM	AVERAGE	MAXIMUM		NUMBER	PERCENT
Triclopyr	µg/l	8	<0.003	<0.003	<0.003	0.1	0	0.00
Trietazine	µg/l	8	<0.001	<0.001	<0.001	0.1	0	0.00
Trifluralin	µg/l	8	<0.002	<0.002	<0.002	0.1	0	0.00
Turbidity	NTU	52	<0.050	<0.066	0.190	4	0	0.00
Free Chlorine	mg/l	180	0.030	0.231	0.550	-*1	-	-
Total Chlorine	mg/l	180	0.080	0.297	0.610	-*1	-	-
Ammonium	mg/l	52	<0.009	<0.012	0.021	0.5	0	0.00
Chloride	mg/l	8	17.600	24.788	32.500	250	0	0.00
Clostridium perfringens	No. / 100 ml	26	0.000	0.000	0.000	0	0	0.00
Coliform Bacteria	No. / 100 ml	180	0.000	0.000	0.000	0	0	0.00
Colony Counts after 48 hours at 37°C	No. / 100 ml	52	0.000	3.096	63.000	-*1	-	-
Colony Count after 72 hours at 22°C	No. / 100 ml	52	0.000	7.654	142.000	-*1	-	-
Conductivity	µS/cm at 20°C	26	251.000	421.385	542.000	2500	0	0.00
Gross Alpha Activity	Bq/l	8	0.063	0.087	0.125	0.1*2	-	-
Gross Beta Activity	Bq/l	8	0.012	0.042	0.071	1*2	-	-
Sulphate	mg/l	8	52.100	69.763	85.800	250	0	0.00
Total Organic Carbon	mg/l	8	0.810	1.041	1.380	-*1	-	-

*PCV Prescribed concentration or value for mandatory parameters and specified limit or value for non-mandatory parameters (indicators and disinfectants).

*1 No numerical limit applies.

*2 Guidance levels only. Monitored for the calculation of Total Indicative Dose, which is the regulatory parameter. No samples exceeded the TID in 2007.

*3 The formula for the calculated Nitrate/Nitrite level is [Nitrate]/50 + [Nitrite]/3. This number has no units.

Appendix 2 Verification of Test Concentrations

1. METHOD OF ANALYSIS

1.1 Introduction

The test material concentration in the test samples was determined by gas chromatography (GC) using an external standard prepared from the test material (see Section 1.3). The test material gave a chromatographic profile consisting of two peaks. The results have been calculated using the total peak area associated with the test material.

The method was developed by the Department of Analytical Services, Harlan Laboratories Ltd, Shardlow, UK.

1.2 Sample Preparation

Sodium chloride was added to a volume of test sample (5.0 g/100 ml) which was then extracted with a mixture of diethyl ether and hexane (10/90v/v). The extract was then analysed directly to give final theoretical concentrations of 0.63 to 31.5 mg/l.

1.3 Standards

Standard solutions of test material were prepared in a mixture of diethyl ether and hexane (10/90v/v) at a nominal concentration of 10 mg/l.

1.4 Procedure

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

GC System	:	Agilent Technologies 5890, incorporating autosampler and workstation
Column	:	ZB-624 (30m x 0.32 mm id, 1.8 µm film)
Oven temperature program	:	initial 45°C for 6 minutes rate 1: 25°C/minute final 150°C
Injector temperature	:	240°C
Detector temperature	:	290°C

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 2 (continued) Verification of Test Concentrations

Carrier gas and pressure	:	nitrogen at 15 psi
Injection volume	:	1 µl
Injection mode	:	splitless purge on at 1 minutes
Detector	:	flame ionisation detector (FID)
Retention time	:	approximately 7-8 minutes

2. PRE-STUDY MEDIA PREPARATION TRIAL

2.1 Saturated Solution Preparation

An amount of test material (1100 mg) was dispersed, in duplicate, in 11 litres of diluent to give initial test material dispersions of 100 mg/l. These were stirred using a propeller stirrer at approximately 1500 rpm at approximately 21°C for periods of 24 and 48 hours.

Samples were taken for analysis following removal of any undissolved test material by centrifugation at 10000 or 40000 *g* for 30 minutes or following filtration through 0.2 µm Gelman Acrocap filters with the first 100 or 500 ml being discarded.

Stirring Period and Treatment	Concentration Found (mg/l)
24 Hours Control	<LOQ
24 Hours Centrifuged 10000 *g*	<LOQ
24 Hours Centrifuged 40000 *g*	<LOQ
24 Hours Filtered 100 ml discarded	<LOQ
24 Hours Filtered 500 ml discarded	<LOQ
48 Hours Control	<LOQ
48 Hours Centrifuged 10000 *g*	<LOQ
48 Hours Centrifuged 40000 *g*	<LOQ
48 Hours Filtered 100 ml discarded	<LOQ
48 Hours Filtered 500 ml discarded	<LOQ

LOQ = Limit of quantitation

Appendix 2 (continued) Verification of Test Concentrations

2.2 Solvent Spike Preparation

An amount of test material (1000 mg) was dissolved in dimethylformamide and the volume adjusted to 10 ml to give a 1000 mg/10 ml solvent stock solution. An aliquot (1000 µl) of this solvent stock solution was dispersed in 10 litres of dechlorinated tap water with the aid of magnetic stirring for approximately 10 minutes to give a 10 mg/l test concentration. This test concentration was then stirred by magnetic stirrer at 21°C with samples taken for analysis initially and after stirring for periods of 24 or 48 hours.

Samples were analysed untreated, following centrifugation at 10000 or 40000 *g* for 30 minutes or following filtration through 0.2 µm Gelman Acrocap filters with the first 100 or 500 ml being discarded.

Stirring Period and Treatment	Nominal Concentration (mg/l)	Concentration Found (mg/l)	Expressed as a Percent of the Nominal Concentration (%)
0 Hours Solvent Control	-	<LOQ	-
0 Hours Untreated	10	1.85	18
0 Hours Centrifuged 10000 *g*	10	0.169	2
0 Hours Centrifuged 40000 *g*	10	0.180	2
0 Hours Filtered 100 ml discarded	10	0.670	7
0 Hours Filtered 500 ml discarded	10	0.737	7
24 Hours Solvent Control	-	<LOQ	-
24 Hours Untreated	10	0.324	3
24 Hours Centrifuged 10000 *g*	10	<LOQ	-
24 Hours Centrifuged 40000 *g*	10	<LOQ	-
24 Hours Filtered 100 ml discarded	10	0.311	3
24 Hours Filtered 500 ml discarded	10	0.328	3
48 Hours Solvent Control	-	<LOQ	-
48 Hours Untreated	10	0.124	1
48 Hours Centrifuged 10000 *g*	10	<LOQ	-
48 Hours Centrifuged 40000 *g*	10	<LOQ	-
48 Hours Filtered 100 ml discarded	10	0.105	1
48 Hours Filtered 500 ml discarded	10	0.113	1

LOQ = Limit of quantitation

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 2 (continued) Verification of Test Concentrations

2.3 Dosing Trial

An amount of test material (1000 mg) was dissolved in 10 ml of dimethylformamide to give a 1000 mg/10 ml solvent stock solution. This stock solution was filtered through a 0.2 µm Gelman Acrocap filter with the first 100 ml being discarded to give a 0.70 mg/l test concentration. Aliquots (20 and 200 ml) were diluted to a final volume (2 litres) in dechlorinated tap water to give 0.0070 and 0.070 mg/l test concentrations which were analysed immediately and then after 24 and 48 hours.

Target Concentration (mg/l)	0 hours		24 hours		48 hours	
	Measured Concentration (mg/l)	% of nominal	Measured Concentration (mg/l)	% of nominal	Measured Concentration (mg/l)	% of nominal
Solvent control	<LOQ	-	<LOQ	-	<LOQ	-
0.0070	0.00625	89	0.00504	72	0.00443	63
0.070	0.0550	79	0.0493	70	0.0446	64
0.70	0.636	91	0.506	72	0.463	66

3. VALIDATION

3.1 Linearity

A range of standard solutions covering 0.095 to 47.3 mg/l (exceeding the range of the working sample concentrations) was analysed.

Linearity was confirmed (R^2 = 9995) in the range 0 to 47.3 mg/l.

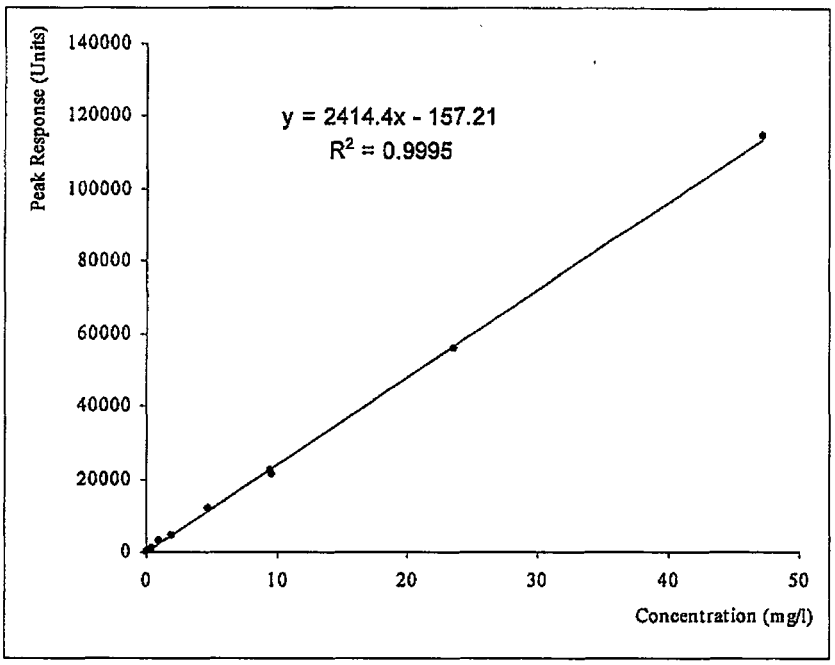
The results are presented graphically on page 42.

LOQ = Limit of quantitation

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 2 (continued) Verification of Test Concentrations

Linearity of Detector Response



Appendix 2 (continued) Verification of Test Concentrations

3.2 Recoveries

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

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

Fortification (mg/l)	Recoveries		
	(mg/l)	(%)	Mean %
0.00724	0.00766	106	104
0.00724	0.00739	102	
0.0724	0.0711	98	98
0.0724	0.0705	97	
0.724	0.676	93	96
0.724	0.712	98	

The method has been considered to be sufficiently accurate for the purposes of this test. The test sample results have not been corrected for recovery.

3.3 Limit of Quantitation

The limit of quantitation has been assessed down to 0.0011 mg/l.

4. STABILITY

A range of preliminary test samples was prepared, analysed initially and then after storage in sealed glass vessels at ambient temperature in light and dark conditions for approximately 48 and 72 hours. In addition a test sample was tested for stability without prior mixing (sonication) of the test sample bottle to assess for losses due to adsorption and/or insolubility.

A further portion of sample was tested following the addition of algal cells.

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 2 (continued) Verification of Test Concentrations

Nominal concentration (mg/l)	0.0070	0.070	0.70
Concentration found initially (mg/l)	0.00753	0.0708	0.694
Concentration found after storage in light conditions (mg/l) 48 hours	0.00253	0.0278	0.169
Expressed as a percent of the initial concentration	34	39	24
Concentration found after storage in dark conditions (mg/l) 48 hours	0.00158	0.0289	0.234
Expressed as a percent of the initial concentration	21	41	34
Concentration found after storage in dark conditions (mg/l) – unsonicated sample 48 hours	0.00245	N/A	0.245
Expressed as a percent of the initial concentration	33	-	35
Concentration found after storage in dark conditions (mg/l) plus algae sample 48 hours	0.00160	N/A	0.191
Expressed as a percent of the initial concentration	21	-	27
Concentration found after storage in light conditions (mg/l) 72 hours	0.00499	0.0342	0.358
Expressed as a percent of the initial concentration	66	48	52
Concentration found after storage in dark conditions (mg/l) 72 hours	0.00399	0.0394	0.404
Expressed as a percent of the initial concentration	53	56	58
Concentration found after storage in dark conditions (mg/l) – unsonicated sample 72 hours	0.00529	N/A	0.460
Expressed as a percent of the initial concentration	70	-	66
Concentration found after storage in dark conditions (mg/l) plus algae sample 72 hours	0.00649	N/A	0.376
Expressed as a percent of the initial concentration	86	-	54

The test samples have been shown to be unstable in the test medium in light and dark conditions.

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

NA = Not applicable

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 2 (continued) Verification of Test Concentrations

The addition of algal cells showed no effect.

5. RESULTS

Sample	Target Concentration (mg/l)	Time-Weighted Mean Measured Concentration (mg/l)	Concentration Found (mg/l)
Day 0	Solvent Control	Solvent Control	<LOQ
Fresh Media	0.0070	0.0030	0.00231
	0.022	0.0085	0.0151
	0.070	0.034	0.0785
	0.22	0.16	0.363
	0.70	0.70	1.46
Day 1	Solvent Control	Solvent Control	<LOQ
Old Media	0.0070	0.0030	0.00158
	0.022	0.0085	0.0109
	0.070	0.034	0.0568
	0.22	0.16	0.225
	0.70	0.70	1.25
Day 4	Solvent Control	Solvent Control	<LOQ
Fresh Media	0.0070	0.0030	0.00200
	0.022	0.0085	0.00913
	0.070	0.034	0.0437
	0.22	0.16	0.255
	0.70	0.70	1.10
Day 5	Solvent Control	Solvent Control	<LOQ
Old Media	0.0070	0.0030	0.00159
	0.022	0.0085	0.00698
	0.070	0.034	0.0305
	0.22	0.16	0.168
	0.70	0.70	0.522

LOQ = Limit of quantitation

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 2 (continued) Verification of Test Concentrations

Sample	Target Concentration (mg/l)	Time-Weighted Mean Measured Concentration (mg/l)	Concentration Found (mg/l)
Day 7	Solvent Control	Solvent Control	<LOQ
Fresh Media	0.0070	0.0030	0.00294
	0.022	0.0085	0.00973
	0.070	0.034	0.0216
	0.22	0.16	0.180
	0.70	0.70	0.692
Day 8	Solvent Control	Solvent Control	<LOQ
Old Media	0.0070	0.0030	0.00213
	0.022	0.0085	0.00730
	0.070	0.034	0.0271
	0.22	0.16	0.111
	0.70	0.70	0.503
Day 11	Solvent Control	Solvent Control	<LOQ
Fresh Media	0.0070	0.0030	0.00331
	0.022	0.0085	0.00948
	0.070	0.034	0.0389
	0.22	0.16	0.175
	0.70	0.70	0.779
Day 12	Solvent Control	Solvent Control	<LOQ
Old Media	0.0070	0.0030	0.00306
	0.022	0.0085	0.00946
	0.070	0.034	0.0284
	0.22	0.16	0.122
	0.70	0.70	0.606

LOQ = Limit of quantitation

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 2 (continued) Verification of Test Concentrations

Sample	Target Concentration (mg/l)	Time-Weighted Mean Measured Concentration (mg/l)	Concentration Found (mg/l)
Day 14	Solvent Control	Solvent Control	<LOQ
Fresh Media	0.0070	0.0030	0.00167
	0.022	0.0085	0.00591
	0.070	0.034	0.0219
	0.22	0.16	0.0929
	0.70	0.70	0.395
Day 15	Solvent Control	Solvent Control	<LOQ
Old Media	0.0070	0.0030	0.00239
	0.022	0.0085	0.00386
	0.070	0.034	0.0178
	0.22	0.16	0.0626
	0.70	0.70	0.327
Day 18	Solvent Control	Solvent Control	<LOQ
Fresh Media	0.0070	0.0030	0.00268
	0.022	0.0085	0.0105
	0.070	0.034	0.0373
	0.22	0.16	0.166
	0.70	0.70	0.768
Day 19	Solvent Control	Solvent Control	<LOQ
Old Media	0.0070	0.0030	0.00198
	0.022	0.0085	0.00738
	0.070	0.034	0.0275
	0.22	0.16	0.107
	0.70	0.70	0.562

LOQ = Limit of quantitation

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 2 (continued) Verification of Test Concentrations

Sample	Target Concentration (mg/l)	Time-Weighted Mean Measured Concentration (mg/l)	Concentration Found (mg/l)
Day 20	Solvent Control	Solvent Control	<LOQ
Fresh Media	0.0070	0.0030	0.00199
	0.022	0.0085	0.00897
	0.070	0.034	0.0353
	0.22	0.16	0.127
	0.70	0.70	0.583
Day 21	Solvent Control	Solvent Control	<LOQ
Old Media	0.0070	0.0030	0.00204
	0.022	0.0085	0.00580
	0.070	0.034	0.0213
	0.22	0.16	0.0877
	0.70	0.70	0.398

6. DISCUSSION

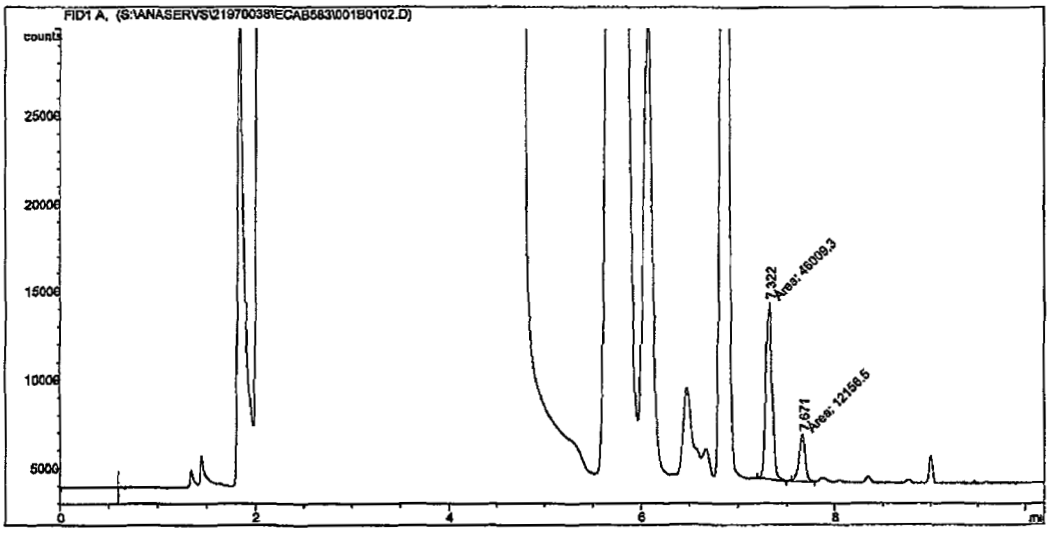
The detection system was found to have acceptable linearity. The analytical procedure had acceptable recoveries of test material in test medium. A method of analysis was validated and proven to be suitable for use.

LOQ = Limit of quantitation

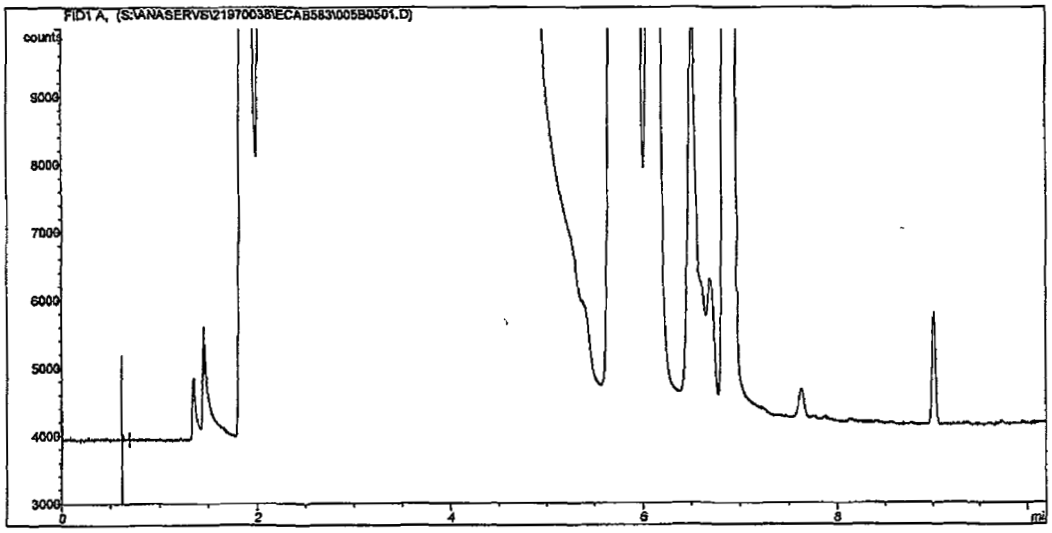
2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 2 (continued) Verification of Test Concentrations

7. TYPICAL CHROMATOGRAPHY



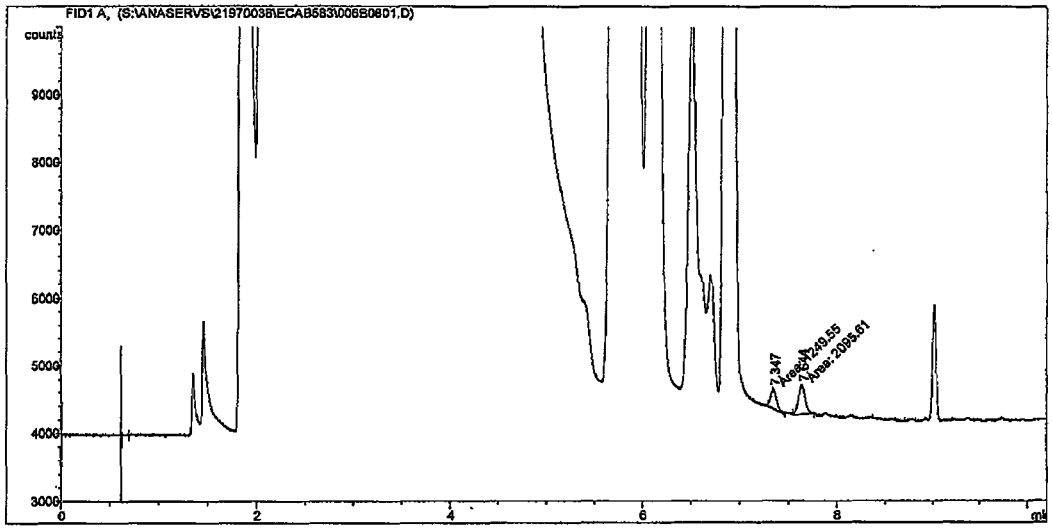
Standard 10 mg/l



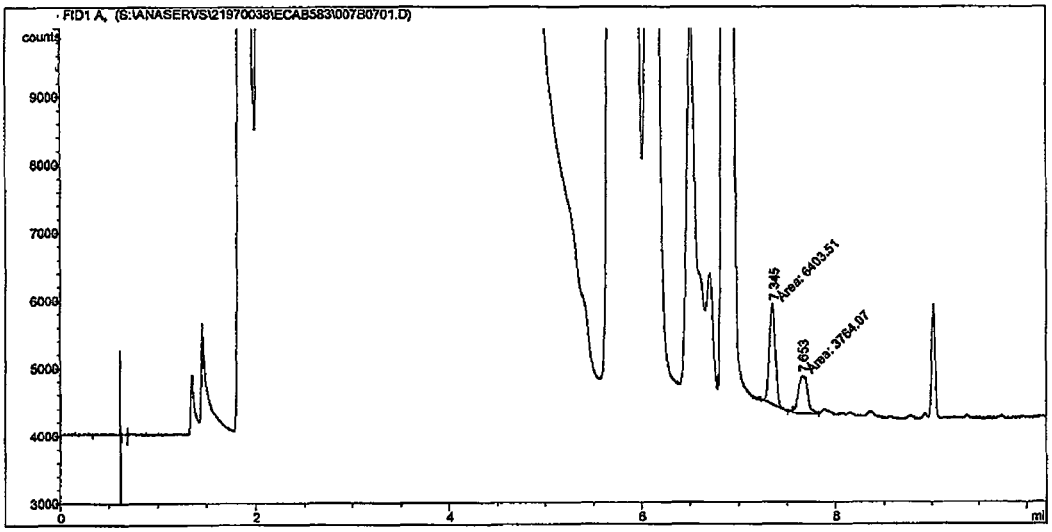
Control Sample

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 2 (continued) Verification of Test Concentrations



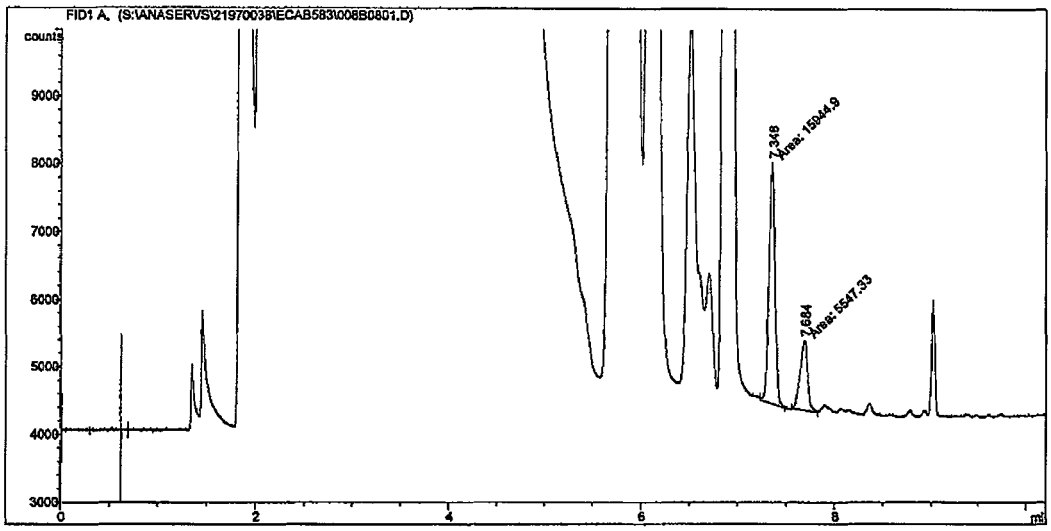
Test Sample 0.0030 mg/l



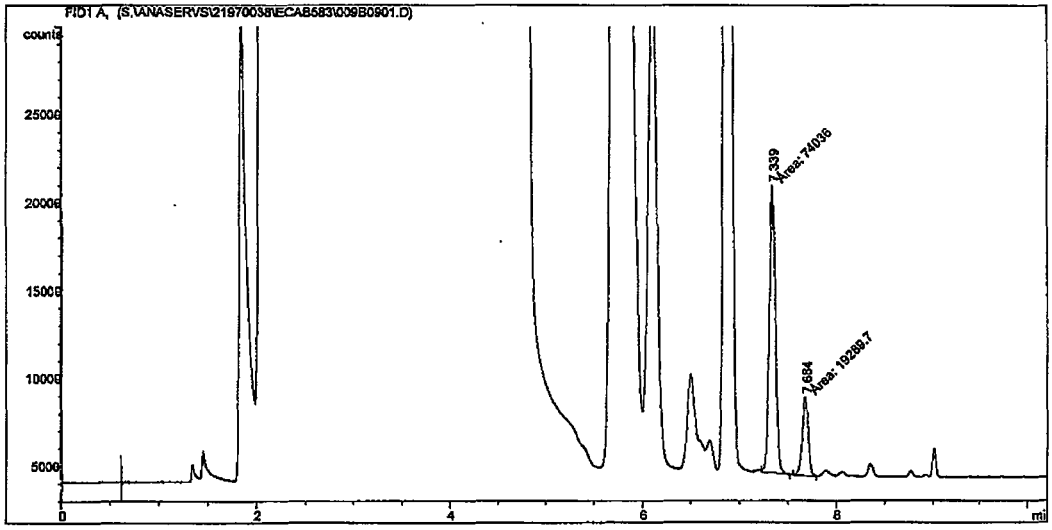
Test Sample 0.0085 mg/l

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 2 (continued) Verification of Test Concentrations



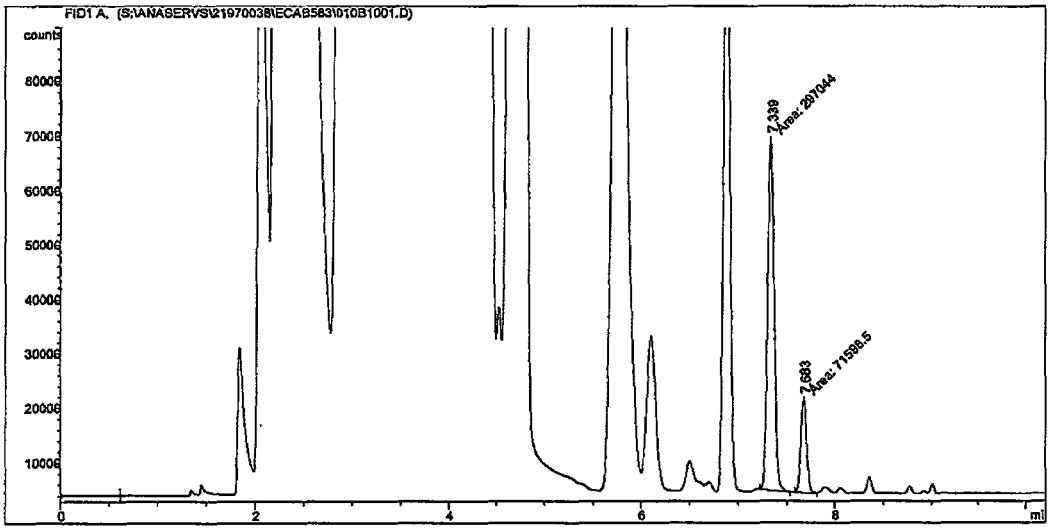
Test Sample 0.034 mg/l



Test Sample 0.16 mg/l

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 2 (continued) Verification of Test Concentrations



Test Sample 0.70 mg/l

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 3 Statistical Analysis

Analysis of Numbers of Live Young Produced per Adult

Results from the control, solvent control and all test groups were compared using one way analysis of variance incorporating Bartlett's test for homogeneity of variance (Sokal and Rohlf 1981) and Dunnett's multiple comparison procedure for comparing several treatments with a control (Dunnett 1955).

Mean and standard deviation values derived from the number of live young produced per surviving adult in the control, solvent control and all the test groups were used for this analysis and are given in the table below:

Time-Weighted Mean Measured Concentration (mg/l)		Number of Live Young Produced per Adult by Day 21
Control	Mean Standard Deviation	87 13
Solvent Control	Mean Standard Deviation	83 7.5
0.0030	Mean Standard Deviation	84 14
0.0085	Mean Standard Deviation	73 31
0.034	Mean Standard Deviation	91 8.1
0.16	Mean Standard Deviation	111 16
0.70	Mean Standard Deviation	0* 0

No significant differences ($P \geq 0.05$) were found between the solvent control and 0.0030, 0.0085, 0.034 and 0.16 mg/l test groups in terms of the number of live young produced per adult by Day 21 using the above methods of statistical analysis.

However, significant differences ($P < 0.05$) were found between the solvent control and the 0.70 mg/l test group in terms of fewer numbers of live young produced per adult by Day 21.

* Significant differences

Appendix 3 (continued) Statistical Analysis

Analysis of Mortalities in the Parental (P_1) Generation

A corrected chi-squared statistic (Breslow and Day 1980) was performed to show whether the mortalities in the 0.0030 and 0.0085 mg/l test groups observed during the test, were statistically significant when compared to the solvent control group.

The data is expressed in the form of a 2 x 2 table as follows:

	Treated	Control	Total
Mortalities	a	b	n_1
No Mortalities	c	d	n_0
Total	m_1	m_0	N

In order to show whether the proportion of mortalities in the treated group (a/m_1) differs from that in the control group (b/m_0) the corrected chi-squared statistic was used as follows:

$$\text{chi squared} = \frac{[(ad - bc) - \tfrac{1}{2}N]^2 (N-1)}{n_0 n_1 m_0 m_1}$$

The following results were obtained for the 0.0030 and 0.0085 mg/l test groups:

	Treated	Solvent Control	Total
Mortalities	1	0	1
No Mortalities	9	10	19
Total	10	10	20

Appendix 3 (continued) Statistical Analysis

Therefore using the corrected chi-squared statistic:

$$\text{chi-squared} = \frac{[(1(10) - 0(9)) - ½(20)]^2(20-1)}{19(1)10(10)} = 0$$

The chi-squared table value for one degree of freedom is 3.84 at the 0.05 probability level.

Therefore, there was no significant difference ($P \geq 0.05$) between the observed mortalities in the solvent control and the 0.0030 and 0.0085 mg/l test groups.

Analysis of the *Daphnia* Length Data

Results from the control, solvent control and all test groups *Daphnia* length data, determined for the surviving daphnids on termination of the test were compared using one way analysis of variance incorporating Bartlett's test for homogeneity of variance (Sokal and Rohlf 1981) and Dunnett's multiple comparison procedure for comparing several treatments with a control (Dunnett 1955).

Mean and standard deviation values derived from the length of the surviving daphnids of the parental (P_1) generation in the control, solvent control and all the test groups were used for this analysis and are given in the table overleaf:

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 3 (continued) Statistical Analysis

Time-Weighted Mean Measured Concentration (mg/l)		*Daphnia* Lengths at Day 21 (mm)
Control	Mean Standard Deviation	4.8 0.1
Solvent Control	Mean Standard Deviation	4.8 0.1
0.0033	Mean Standard Deviation	4.8 0.1
0.0085	Mean Standard Deviation	4.8 0.1
0.034	Mean Standard Deviation	4.8 0.1
0.16	Mean Standard Deviation	4.8 0.3
0.70	Mean Standard Deviation	3.1* 0.1

No statistically significant differences ($P \geq 0.05$) were found between the control, solvent control and the 0.0030, 0.0085, 0.034 and 0.16 mg/l test groups in terms of length of the surviving parental daphnids on Day 21 of the test, using the above methods of statistical analyses.

However, significant differences ($P < 0.05$) were found between the solvent control and the 0.70 mg/l test group in terms of the daphnids being smaller on Day 21.

* Significant differences

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 4 Daily Observations of the Control Group

Vessel Number	Observation	Day									
		1	2	3	4	5	6	7	8	9	10
1	Live young	0	0	0	0	0	0	0	0	11	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
2	Live young	0	0	0	0	0	0	12	3	0	9
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
3	Live young	0	0	0	0	0	0	0	13	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
4	Live young	0	0	0	0	0	0	0	15	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
5	Live young	0	0	0	0	0	0	0	7	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
6	Live young	0	0	0	0	0	0	0	12	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
7	Live young	0	0	0	0	0	0	0	0	13	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
8	Live young	0	0	0	0	0	0	0	8	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
9	Live young	0	0	0	0	0	0	0	7	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
10	Live young	0	0	0	0	0	0	0	5	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
Total	Live young	0	0	0	0	0	0	12	70	24	9
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
	Gravid adults	0	0	0	0	9	10	10	10	10	10
	Surviving adults	10	10	10	10	10	10	10	10	10	10

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 4 (continued) Daily Observations of the Control Group

Vessel Number	Observation	Day										
		11	12	13	14	15	16	17	18	19	20	21
1	Live young	0	16	0	0	27	0	0	15	0	0	19
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
2	Live young	10	0	0	-	-	-	-	-	-	-	-
	Dead Young	0	0	0	-	-	-	-	-	-	-	-
	Unhatched eggs	0	0	0	-	-	-	-	-	-	-	-
3	Live young	21	0	0	0	28	0	3	17	0	0	20
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
4	Live young	22	0	0	0	21	0	0	19	0	0	23
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0.	0	0	0	0	0	0	0	0	0	0
5	Live young	0	21	0	0	27	0	0	16	0	0	11
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
6	Live young	14	8	0	0	23	0	0	18	0	0	19
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
7	Live young	0	19	0	0	22	0	0	14	0	0	16
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
8	Live young	25	0	0	17	0	0	1	16	0	0	22
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
9	Live young	12	0	0	8	0	0	14	0	0	0	16
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
10	Live young	20	0	0	21	0	0	21	0	0	0	21
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
Total	Live young	124	64	0	46	148	0	39	115	0	0	167
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
	Gravid adults	10	10	9	9	9	9	9	9	9	9	9
	Surviving adults	10	10	9	9	9	9	9	9	9	9	9

- Indicates no further observations made due to adult mortality

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 5 Daily Observations of the Solvent Control Group

Vessel Number	Observation	Day									
		1	2	3	4	5	6	7	8	9	10
1	Live young	0	0	0	0	0	0	0	0	0	17
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
2	Live young	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
3	Live young	0	0	0	0	0	0	0	8	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
4	Live young	0	0	0	0	0	0	0	3	12	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
5	Live young	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
6	Live young	0	0	0	0	0	0	10	0	0	11
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
7	Live young	0	0	0	0	0	0	0	4	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
8	Live young	0	0	0	0	0	0	0	13	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
9	Live young	0	0	0	0	0	0	0	0	0	21
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
10	Live young	0	0	0	0	0	0	0	0	13	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
Total	Live young	0	0	0	0	0	0	10	28	25	49
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
	Gravid adults	0	0	0	0	9	10	10	10	10	10
	Surviving adults	10	10	10	10	10	10	10	10	10	10

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 5 (continued) Daily Observations of the Solvent Control Test Group

Vessel Number	Observation	Day										
		11	12	13	14	15	16	17	18	19	20	21
1	Live young	0	0	19	0	0	25	0	0	26	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
2	Live young	14	0	0	22	0	0	24	0	0	0	7
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
3	Live young	14	0	0	18	0	0	21	0	0	0	24
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
4	Live young	0	26	0	0	21	0	0	16	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
5	Live young	18	0	0	17	0	0	23	0	0	0	27
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
6	Live young	0	0	21	0	3	0	11	0	0	25	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
7	Live young	13	0	0	24	0	0	20	0	0	27	1
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
8	Live young	9	16	0	0	25	0	0	18	0	0	9
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
9	Live young	0	0	24	0	0	22	0	0	13	13	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
10	Live young	0	0	25	0	0	25	0	0	16	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
Total	Live young	68	42	89	81	49	72	99	34	55	65	68
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
	Gravid adults	10	10	10	10	10	10	10	10	10	10	10
	Surviving adults	10	10	10	10	10	10	10	10	10	10	10

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 6 Daily Observations of the 0.0030 mg/l Test Group

Vessel Number	Observation	Day									
		1	2	3	4	5	6	7	8	9	10
1	Live young	0	0	0	0	0	0	0	11	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
2	Live young	0	0	0	0	0	0	0	3	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
3	Live young	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
4	Live young	0	0	0	0	0	0	0	4	4	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
5	Live young	0	0	0	0	0	0	0	8	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
6	Live young	0	0	0	0	0	0	0	0	11	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
7	Live young	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
8	Live young	0	0	0	0	0	0	0	0	6	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
9	Live young	0	0	0	0	0	0	0	15	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
10	Live young	0	0	0	0	0	0	0	0	0	9
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
Total	Live young	0	0	0	0	0	0	0	41	21	9
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
	Gravid adults	0	0	0	0	10	10	10	10	10	10
	Surviving adults	10	10	10	10	10	10	10	10	10	10

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 6 (continued) Daily Observations of the 0.0030 mg/l Test Group

Vessel Number	Observation	Day										
		11	12	13	14	15	16	17	18	19	20	21
1	Live young	19	0	0	0	30	0	0	17	0	0	15
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
2	Live young	14	0	0	34	0	0	18	0	0	23	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
3	Live young	16	0	0	31	0	0	22	0	0	0	28
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
4	Live young	20	1	0	0	28	0	0	17	0	0	25
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
5	Live young	21	1	0	22	0	0	0	11	0	0	19
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
6	Live young	0	24	0	0	22	0	0	16	0	0	2
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
7	Live young	19	0	0	16	0	0	3	16	0	-	-
	Dead Young	0	0	0	0	0	0	0	0	0	-	-
	Unhatched eggs	0	0	0	0	0	0	0	0	0	-	-
8	Live young	0	17	0	0	27	0	0	13	0	0	29
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
9	Live young	25	0	0	16	0	0	0	12	0	0	20
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
10	Live young	0	0	19	0	0	0	18	0	0	27	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
Total	Live young	134	43	19	119	107	0	61	102	0	50	138
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
	Gravid adults	10	10	10	10	10	10	10	10	9	9	9
	Surviving adults	10	10	10	10	10	10	10	10	9	9	9

- Indicates no further observations made due to adult mortality

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 7 Daily Observations of the 0.0085 mg/l Test Group

Vessel Number	Observation	Day									
		1	2	3	4	5	6	7	8	9	10
1	Live young	0	0	0	0	0	0	0	13	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
2	Live young	0	0	0	0	0	0	0	0	0	6
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
3	Live young	0	0	0	0	0	0	0	2	12	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
4	Live young	0	0	0	0	0	0	0	10	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
5	Live young	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
6	Live young	0	0	0	0	0	0	0	7	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
7	Live young	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
8	Live young	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
9	Live young	0	0	0	0	0	0	0	13	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
10	Live young	0	0	0	0	0	-	-	-	-	-
	Dead Young	0	0	0	0	0	-	-	-	-	-
	Unhatched eggs	0	0	0	0	0	-	-	-	-	-
Total	Live young	0	0	0	0	0	0	0	45	12	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
	Gravid adults	0	0	0	0	8	8	9	9	9	9
	Surviving adults	10	10	10	10	9	9	9	9	9	9

- Indicates no further observations made due to adult mortality

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 7 (continued) Daily Observations of the 0.0085 mg/l Test Group

Vessel Number	Observation	Day.										
		11	12	13	14	15	16	17	18	19	20	21
1	Live young	20	0	0	9	20	0	0	18	0	0	18
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
2	Live young	0	0	19	0	0	27	0	0	23	0	1
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
3	Live young	12	0	0	26	0	0	20	0	0	0	18
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
4	Live young	23	0	0	22	0	0	0	14	0	0	25
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
5	Live young	5	0	0	8	0	0	20	0	0	5	15
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
6	Live young	17	0	0	27	0	0	27	0	0	21	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
7	Live young	8	0	0	18	0	0	23	0	0	17	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
8	Live young	0	0	21	0	0	24	0	0	14	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
9	Live young	25	0	0	24	1	0	0	16	0	12	4
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
10	Live young	-	-	-	-	-	-	-	-	-	-	-
	Dead Young	-	-	-	-	-	-	-	-	-	-	-
	Unhatched eggs	-	-	-	-	-	-	-	-	-	-	-
Total	Live young	110	0	40	134	21	51	90	48	37	55	81
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
	Gravid adults	9	9	9	9	9	9	9	9	9	9	9
	Surviving adults	9	9	9	9	9	9	9	9	9	9	9

- Indicates no further observations made due to adult mortality

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 8 Daily Observations of the 0.034 mg/l Test Group

Vessel Number	Observation	Day									
		1	2	3	4	5	6	7	8	9	10
1	Live young	0	0	0	0	0	0	0	13	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
2	Live young	0	0	0	0	0	0	0	2	7	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
3	Live young	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
4	Live young	0	0	0	0	0	0	0	2	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
5	Live young	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
6	Live young	0	0	0	0	0	0	0	3	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
7	Live young	0	0	0	0	0	0	0	7	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
8	Live young	0	0	0	0	0	0	0	2	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
9	Live young	0	0	0	0	0	0	0	4	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
10	Live young	0	0	0	0	0	0	0	11	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
Total	Live young	0	0	0	0	0	0	0	44	7	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
	Gravid adults	0	0	0	0	7	10	10	10	10	10
	Surviving adults	10	10	10	10	10	10	10	10	10	10

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 8 (continued) Daily Observations of the 0.034 mg/l Test Group

Vessel Number	Observation	Day										
		11	12	13	14	15	16	17	18	19	20	21
1	Live young	12	0	0	28	0	0	15	0	0	0	20
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
2	Live young	1	15	0	0	31	0	0	13	0	0	26
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
3	Live young	12	0	0	25	0	0	20	0	0	28	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
4	Live young	12	0	0	27	0	0	21	0	0	25	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
5	Live young	12	0	0	26	0	0	0	19	0	0	22
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
6	Live young	5	0	0	27	0	0	29	0	0	23	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
7	Live young	17	0	0	5	15	0	0	22	0	0	22
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
8	Live young	12	0	0	31	0	0	24	0	0	19	11
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
9	Live young	19	0	0	28	0	0	0	22	0	0	27
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
10	Live young	20	0	0	31	0	0	0	22	0	0	21
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
Total	Live young	122	0	0	228	46	0	109	98	0	95	149
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
	Gravid adults	10	10	10	10	10	10	10	10	10	10	10
	Surviving adults	10	10	10	10	10	10	10	10	10	10	10

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 9 Daily Observations of the 0.16 mg/l Test Group

Vessel Number	Observation	Day									
		1	2	3	4	5	6	7	8	9	10
1	Live young	0	0	0	0	0	0	0	8	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
2	Live young	0	0	0	0	0	0	0	7	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
3	Live young	0	0	0	0	0	0	0	6	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
4	Live young	0	0	0	0	0	0	0	9	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
5	Live young	0	0	0	0	0	0	0	2	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
6	Live young	0	0	0	0	0	0	0	6	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
7	Live young	0	0	0	0	0	0	0	7	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
8	Live young	0	0	0	0	0	0	0	9	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
9	Live young	0	0	0	0	0	0	0	9	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
10	Live young	0	0	0	0	0	0	0	6	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
Total	Live young	0	0	0	0	0	0	0	69	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
	Gravid adults	0	0	0	0	9	10	10	10	10	10
	Surviving adults	10	10	10	10	10	10	10	10	10	10

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 9 (continued) Daily Observations of the 0.16 mg/l Test Group

Vessel Number	Observation	Day										
		11	12	13	14	15	16	17	18	19	20	21
1	Live young	11	0	0	29	0	0	30	0	0	0	38
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
2	Live young	24	0	0	39	0	0	33	0	0	0	35
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
3	Live young	21	0	0	32	0	0	5	25	0	0	38
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
4	Live young	19	0	0	30	0	0	25	0	0	28	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
5	Live young	8	0	0	27	0	0	19	0	0	0	23
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
6	Live young	17	0	0	28	0	0	22	0	0	23	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
7	Live young	14	0	0	33	0	0	0	27	0	0	23
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
8	Live young	24	0	0	33	0	0	25	0	0	25	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
9	Live young	24	0	0	30	0	0	30	0	0	24	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
10	Live young	19	0	0	28	0	0	31	0	0	26	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	1	0
Total	Live young	181	0	0	309	0	0	220	52	0	126	157
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
	Gravid adults	10	10	10	10	10	10	10	10	10	10	10
	Surviving adults	10	10	10	10	10	10	10	10	10	10	10

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 10 Daily Observations of the 0.70 mg/l Test Group

Vessel Number	Observation	Day									
		1	2	3	4	5	6	7	8	9	10
1	Live young	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
2	Live young	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
3	Live young	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
4	Live young	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
5	Live young	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
6	Live young	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
7	Live young	0	-	-	-	-	-	-	-	-	-
	Dead Young	0	-	-	-	-	-	-	-	-	-
	Unhatched eggs	0	-	-	-	-	-	-	-	-	-
8	Live young	0	0	0	0	0	0	0	-	-	-
	Dead Young	0	0	0	0	0	0	0	-	-	-
	Unhatched eggs	0	0	0	0	0	0	0	-	-	-
9	Live young	0	0	0	0	0	0	-	-	-	-
	Dead Young	0	0	0	0	0	0	-	-	-	-
	Unhatched eggs	0	0	0	0	0	0	-	-	-	-
10	Live young	0	0	0	0	0	0	-	-	-	-
	Dead Young	0	0	0	0	0	0	-	-	-	-
	Unhatched eggs	0	0	0	0	0	0	-	-	-	-
Total	Live young	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0
	Gravid adults	0	0	0	0	0	0	0	0	0	0
	Surviving adults	9	9	9	9	9	7	6	6	6	6

- Indicates no further observations made due to adult mortality

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 10 (continued) Daily Observations of the 0.70 mg/l Test Group

Vessel Number	Observation	Day										
		11	12	13	14	15	16	17	18	19	20	21
1	Live young	0	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
2	Live young	0	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
3	Live young	0	0	0	-	-	-	-	-	-	-	-
	Dead Young	0	0	0	-	-	-	-	-	-	-	-
	Unhatched eggs	0	0	0	-	-	-	-	-	-	-	-
4	Live young	0	0	0	0	0	0	0	0	0	-	-
	Dead Young	0	0	0	0	0	0	0	0	0	-	-
	Unhatched eggs	0	0	0	0	0	0	0	0	0	-	-
5	Live young	0	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
6	Live young	0	0	0	0	0	0	-	-	-	-	-
	Dead Young	0	0	0	0	0	0	-	-	-	-	-
	Unhatched eggs	0	0	0	0	0	0	-	-	-	-	-
7	Live young	-	-	-	-	-	-	-	-	-	-	-
	Dead Young	-	-	-	-	-	-	-	-	-	-	-
	Unhatched eggs	-	-	-	-	-	-	-	-	-	-	-
8	Live young	-	-	-	-	-	-	-	-	-	-	-
	Dead Young	-	-	-	-	-	-	-	-	-	-	-
	Unhatched eggs	-	-	-	-	-	-	-	-	-	-	-
9	Live young	-	-	-	-	-	-	-	-	-	-	-
	Dead Young	-	-	-	-	-	-	-	-	-	-	-
	Unhatched eggs	-	-	-	-	-	-	-	-	-	-	-
10	Live young	-	-	-	-	-	-	-	-	-	-	-
	Dead Young	-	-	-	-	-	-	-	-	-	-	-
	Unhatched eggs	-	-	-	-	-	-	-	-	-	-	-
Total	Live young	0	0	0	0	0	0	0	0	0	0	0
	Dead Young	0	0	0	0	0	0	0	0	0	0	0
	Unhatched eggs	0	0	0	0	0	0	0	0	0	0	0
	Gravid adults	0	2	1	1	1	0	0	0	2	2	2
	Surviving adults	6	6	5	5	5	4	4	4	3	3	3

- Indicates no further observations made due to adult mortality

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 11 Physico-Chemical Measurements (Recorded in One Replicate Test Vessel) of Oxygen (mg O_2/l), Temperature (°C) and pH

Time-Weighted Mean Measured Concentration (mg/l)		Day							
		0	1*	1+	2*	2+	3*	3+	4*
Control	O_2	9.3	9.6	9.5	9.5	9.5	9.1	9.5	9.5
	%ASV$^{\Delta}$	104	108	107	107	107	102	107	107
	pH	7.6	7.9	7.7	7.9	7.6	7.9	7.7	7.8
	T°C	21	21	21	21	21	21	21	21
Solvent Control	O_2	9.2	9.6	9.1	9.6	9.3	9.2	9.4	9.8
	%ASV$^{\Delta}$	103	108	102	108	104	103	106	110
	pH	7.6	7.7	7.5	7.6	7.6	7.8	7.7	7.8
	T°C	21	21	21	21	21	21	21	21
0.0030	O_2	9.4	9.6	9.2	9.5	9.3	9.2	9.4	9.5
	%ASV$^{\Delta}$	106	108	103	107	104	103	106	107
	pH	7.6	7.9	7.7	7.8	7.6	7.9	7.7	7.8
	T°C	21	21	21	21	21	21	21	21
0.0085	O_2	9.4	9.6	9.5	9.5	9.3	9.2	9.3	9.3
	%ASV$^{\Delta}$	106	108	107	107	104	103	104	104
	pH	7.7	7.8	7.6	7.9	7.6	7.8	7.6	7.8
	T°C	21	21	21	21	21	21	21	21
0.034	O_2	9.4	9.6	9.4	9.5	9.4	9.1	9.4	9.5
	%ASV$^{\Delta}$	106	108	106	107	106	102	106	107
	pH	7.7	7.9	7.6	7.9	7.6	7.9	7.6	7.7
	T°C	21	21	21	21	21	21	21	21
0.16	O_2	9.2	9.6	9.5	9.6	9.3	9.1	9.3	9.5
	%ASV$^{\Delta}$	103	108	107	108	104	102	104	107
	pH	7.7	7.8	7.7	7.9	7.6	7.9	7.6	7.8
	T°C	21	21	21	21	21	21	21	21
0.70	O_2	9.4	9.6	9.4	9.5	9.3	9.3	9.3	9.5
	%ASV$^{\Delta}$	106	108	106	107	104	104	104	107
	pH	7.6	7.9	7.6	7.9	7.6	7.9	7.6	7.9
	T°C	21	21	21	21	21	21	21	21

* Before renewal
\+ After renewal
$^{\Delta}$ASV = Dissolved oxygen concentration expressed as a percentage of Air Saturation Value

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 11 (continued) Physico-Chemical Measurements (Recorded in One Replicate Test Vessel) of Oxygen (mg O_2/l), Temperature (°C) and pH

Time-Weighted Mean Measured Concentration (mg/l)		Day							
		4+	5*	5+	6*	6+	7*	7+	8*
Control	O_2	9.1	9.5	9.5	9.4	9.4	9.5	9.3	9.6
	%ASV[Δ]	102	107	107	106	106	107	104	108
	pH	7.9	7.8	7.8	8.0	7.8	7.7	7.8	7.9
	T°C	21	21	21	21	21	21	21	21
Solvent Control	O_2	9.4	9.5	9.3	9.2	9.2	9.2	9.3	9.1
	%ASV[Δ]	106	107	104	103	103	103	104	102
	pH	7.7	7.8	7.8	7.9	7.8	7.9	7.8	7.8
	T°C	21	21	21	21	21	21	21	21
0.0030	O_2	9.4	9.4	9.3	9.3	9.3	8.9	9.3	8.9
	%ASV[Δ]	106	106	104	104	104	100	104	100
	pH	7.8	7.9	7.7	7.8	7.7	7.8	7.7	7.8
	T°C	21	21	21	21	21	21	21	21
0.0085	O_2	9.4	9.2	9.5	9.1	9.4	9.1	9.4	9.1
	%ASV[Δ]	106	103	107	102	106	102	106	102
	pH	7.7	7.8	7.7	7.8	7.8	7.8	7.7	7.9
	T°C	21	21	21	21	21	21	21	21
0.034	O_2	9.5	9.5	9.5	8.9	9.4	9.2	9.4	9.0
	%ASV[Δ]	107	107	107	100	106	103	106	101
	pH	7.7	7.9	7.7	7.7	7.7	7.8	7.8	7.8
	T°C	21	21	21	21	21	21	21	21
0.16	O_2	9.4	9.5	9.5	9.5	9.4	9.0	9.3	9.2
	%ASV[Δ]	106	107	107	107	106	101	104	103
	pH	7.7	7.8	7.9	7.8	7.8	7.8	7.7	7.8
	T°C	21	21	21	21	21	21	21	21
0.70	O_2	9.4	9.2	9.5	9.4	9.4	9.5	9.0	9.5
	%ASV[Δ]	106	103	107	106	106	107	101	107
	pH	7.7	7.8	7.9	7.9	7.7	8.0	7.8	8.1
	T°C	21	21	21	21	21	21	21	21

* Before renewal
\+ After renewal
[Δ]ASV = Dissolved oxygen concentration expressed as a percentage of Air Saturation Value

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 11 (continued) Physico-Chemical Measurements (Recorded in One Replicate Test Vessel) of Oxygen (mg O_2/l), Temperature (°C) and pH

Time-Weighted Mean Measured Concentration (mg/l)		Day							
		8+	9*	9+	10*	10+	11*	11+	12*
Control	O_2	9.5	9.2	9.2	9.4	9.3	8.9	9.5	9.1
	%ASV[Δ]	107	103	103	103	104	100	107	100
	pH	7.9	7.8	7.9	8.0	7.9	7.8	7.9	7.8
	T°C	21	21	21	20	21	21	21	20
Solvent Control	O_2	9.4	9.0	9.3	8.9	9.2	9.0	9.4	9.0
	%ASV[Δ]	106	101	104	98	103	101	106	99
	pH	7.8	7.8	7.7	7.9	7.9	7.8	7.8	7.9
	T°C	21	21	21	20	21	21	21	20
0.0030	O_2	9.5	9.0	9.5	9.2	9.3	8.6	9.1	8.8
	%ASV[Δ]	107	101	107	101	104	97	102	97
	pH	7.8	7.8	7.8	7.9	7.9	7.7	7.7	7.8
	T°C	21	21	21	20	21	21	21	20
0.0085	O_2	9.4	9.3	9.5	8.9	9.4	9.0	9.2	8.9
	%ASV[Δ]	106	104	107	98	106	101	103	100
	pH	7.8	7.8	7.8	7.9	7.9	7.8	7.7	7.7
	T°C	21	21	21	20	21	21	21	21
0.034	O_2	9.4	9.1	9.5	9.1	9.3	8.7	9.3	8.8
	%ASV[Δ]	106	102	107	100	104	98	104	97
	pH	7.8	7.9	7.7	7.9	7.9	7.8	7.7	7.8
	T°C	21	21	21	20	21	21	21	20
0.16	O_2	9.5	9.4	9.4	9.4	9.2	9.1	9.4	8.8
	%ASV[Δ]	107	106	106	103	103	102	106	97
	pH	7.8	7.8	7.8	8.0	7.9	7.8	7.8	7.8
	T°C	21	21	21	20	21	21	21	20
0.70	O_2	9.5	9.6	9.2	9.6	8.9	9.6	9.2	9.5
	%ASV[Δ]	107	108	103	105	100	107	103	107
	pH	7.7	8.0	7.8	8.0	7.9	7.9	7.8	7.9
	T°C	21	21	21	20	21	21	21	21

* Before renewal
\+ After renewal
[Δ]ASV = Dissolved oxygen concentration expressed as a percentage of Air Saturation Value

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 11 (continued) Physico-Chemical Measurements (Recorded in One Replicate Test Vessel) of Oxygen (mg O_2/l), Temperature (°C) and pH

Time-Weighted Mean Measured Concentration (mg/l)		Day							
		12+	13*	13+	14*	14+	15*	15+	16*
Control	O_2	9.5	9.0	9.5	9.4	9.6	9.4	9.5	9.0
	%ASV[Δ]	107	101	107	106	108	106	107	101
	pH	7.9	7.7	7.8	7.8	7.8	7.8	7.7	7.8
	T°C	21	21	21	21	21	21	21	21
Solvent Control	O_2	9.4	8.8	9.6	9.3	9.5	9.3	9.2	8.7
	%ASV[Δ]	106	99	108	104	107	104	103	98
	pH	7.8	7.7	7.7	7.8	7.9	7.7	7.6	7.7
	T°C	21	21	21	21	21	21	21	21
0.0030	O_2	9.5	9.0	9.6	8.9	9.5	8.5	9.2	8.6
	%ASV[Δ]	107	101	108	100	107	96	103	97
	pH	7.8	7.7	7.8	7.8	7.8	7.7	7.8	7.7
	T°C	21	21	21	21	21	21	21	21
0.0085	O_2	9.5	9.1	9.6	8.9	9.6	8.6	9.4	8.8
	%ASV[Δ]	107	102	108	100	108	97	106	99
	pH	7.7	7.6	7.8	7.8	7.8	7.6	7.6	7.7
	T°C	21	21	21	21	21	21	21	21
0.034	O_2	9.5	9.0	9.6	8.9	9.5	9.1	9.5	8.9
	%ASV[Δ]	107	101	108	100	107	102	107	100
	pH	7.8	7.7	7.7	7.7	7.8	7.6	7.6	7.7
	T°C	21	21	21	21	21	21	21	21
0.16	O_2	9.6	9.4	9.5	9.1	9.5	9.4	9.5	8.8
	%ASV[Δ]	108	106	107	102	107	106	107	99
	pH	7.7	7.7	7.7	7.7	7.8	7.8	7.5	7.6
	T°C	21	21	21	21	21	21	21	21
0.70	O_2	9.4	9.5	9.1	9.4	9.1	9.5	9.4	9.6
	%ASV[Δ]	106	107	102	106	102	107	106	108
	pH	7.8	7.8	7.7	7.8	7.8	7.8	7.6	7.8
	T°C	21	21	21	21	21	21	21	21

* Before renewal
\+ After renewal
[Δ]ASV = Dissolved oxygen concentration expressed as a percentage of Air Saturation Value

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 11 (continued) Physico-Chemical Measurements (Recorded in One Replicate Test Vessel) of Oxygen (mg O_2/l), Temperature (°C) and pH

Time-Weighted Mean Measured Concentration (mg/l)		Day							
		16+	17*	17+	18*	18+	19*	19+	20*
Control	O_2	9.6	8.9	9.6	9.0	9.6	9.2	9.6	9.2
	%ASV$^{\Delta}$	108	98	108	99	108	101	108	101
	pH	7.8	7.8	7.7	7.9	7.8	7.7	7.7	7.7
	T°C	21	20	21	20	21	20	21	20
Solvent Control	O_2	9.6	9.2	9.6	9.1	9.6	9.0	9.6	9.4
	%ASV$^{\Delta}$	108	101	108	100	108	99	108	103
	pH	7.8	7.8	7.7	7.8	7.8	7.6	7.7	7.7
	T°C	21	20	21	20	21	20	21	20
0.0030	O_2	9.5	9.0	9.6	9.0	9.4	8.7	9.5	8.8
	%ASV$^{\Delta}$	107	99	108	99	106	96	107	97
	pH	7.7	7.7	7.7	7.9	7.7	7.7	7.6	7.7
	T°C	21	20	21	20	21	20	21	20
0.0085	O_2	9.5	8.9	9.6	8.9	9.6	9.1	9.5	8.8
	%ASV$^{\Delta}$	107	98	108	98	108	100	107	97
	pH	7.7	7.7	7.7	7.7	7.7	7.7	7.8	7.7
	T°C	21	20	21	20	21	20	21	20
0.034	O_2	9.6	8.7	9.6	9.3	9.5	8.8	9.6	8.9
	%ASV$^{\Delta}$	108	96	108	102	107	97	108	98
	pH	7.6	7.7	7.6	7.8	7.8	7.7	7.7	8.7
	T°C	21	20	21	20	21	20	21	20
0.16	O_2	9.5	8.8	9.4	9.1	9.4	8.9	9.6	8.8
	%ASV$^{\Delta}$	107	97	106	100	106	98	108	97
	pH	7.6	7.7	7.6	7.7	7.7	7.7	7.6	7.7
	T°C	21	20	21	20	21	20	21	20
0.70	O_2	9.4	9.6	9.3	9.4	9.3	9.5	9.5	9.6
	%ASV$^{\Delta}$	106	105	104	103	104	107	107	105
	pH	7.6	7.9	7.6	7.9	7.7	7.7	7.7	7.8
	T°C	21	20	21	20	21	21	21	20

* Before renewal
\+ After renewal
$^{\Delta}$ASV = Dissolved oxygen concentration expressed as a percentage of Air Saturation Value

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 11 (continued) Physico-Chemical Measurements (Recorded in One Replicate Test Vessel) of Oxygen (mg O_2/l), Temperature (°C) and pH

Time-Weighted Mean Measured Concentration (mg/l)		Day	
		20+	21
Control	O_2	9.6	9.1
	%ASV[Δ]	105	100
	pH	7.8	7.8
	T°C	20	20
Solvent Control	O_2	9.6	9.1
	%ASV[Δ]	105	100
	pH	7.7	7.8
	T°C	20	20
0.0030	O_2	9.6	8.8
	%ASV[Δ]	108	97
	pH	7.8	7.8
	T°C	21	20
0.0085	O_2	9.6	8.8
	%ASV[Δ]	108	97
	pH	7.7	7.7
	T°C	21	21
0.034	O_2	9.6	8.7
	%ASV[Δ]	108	96
	pH	7.7	7.7
	T°C	21	20
0.16	O_2	9.4	9.1
	%ASV[Δ]	103	100
	pH	7.8	7.8
	T°C	20	20
0.70	O_2	9.6	9.1
	%ASV[Δ]	108	100
	pH	7.6	7.9
	T°C	21	20

+ After renewal
[Δ]ASV = Dissolved oxygen concentration expressed as a percentage of Air Saturation Value

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 12 Light Intensity Measurements

Day	Light Intensity (Lux)
0	535
1	526
2	531
3	529
4	544
5	537
6	541
7	535
8	542
9	557
10	551
11	549
12	553
13	548
14	552
15	549
16	521
17	512
18	517
19	520
20	517
21	523

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 13 Water Hardness Measurements

Day	Water Hardness (mg/l as $CaCO_3$)	
	Solvent Control	Highest Surviving Test Concentration
0 (Fresh Media)	132	132
1 (Old Media)	150	138
4 (Fresh Media)	128	126
5 (Old Media)	138	132
11 (Fresh Media)	118	114
12 (Old Media)	116	112
18 (Fresh Media)	130	130
19 (Old Media)	126	128

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 14 The Length of Each Surviving Adult After 21 Days

Time-Weighted Mean Measured Concentration (mg/l)	Individual *Daphnia* Lengths (mm)									
	1	2	3	4	5	6	7	8	9	10
Control	4.8	*	4.9	4.6	4.8	4.9	4.9	4.8	4.8	5.0
Solvent Control	4.6	4.8	5.0	4.7	4.8	4.8	4.7	4.9	4.8	5.0
0.0030	4.8	5.0	4.8	4.8	4.7	4.9	*	4.9	4.6	4.9
0.0085	4.9.	4.8	4.6	4.9	4.7	4.9	4.7	4.9	4.8	*
0.034	4.9	4.8	4.8	4.9	4.5	4.8	4.9	5.0	5.0	4.8
0.16	5.1	5.0	5.0	4.9	4.1	4.9	4.8	4.9	4.8	4.7
0.70	3.0	3.0	*	*	3.2	*	*	*	*	*

* Indicates no observations made due to adult mortality

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 15 Protocol and Amendments

SafePharm Laboratories

PROTOCOL

TEST MATERIAL	:	2,4,4-Trimethylpentene
STUDY TYPE	:	*Daphnia magna* Reproduction Test (OECD (1998) 211, EEC C.20)
TEST METHOD	:	901.18
PROJECT NUMBER	:	2197/0038
PROPOSED START DATE	:	August 2008
PROPOSED COMPLETION DATE	:	October 2008
TARGET (DRAFT) REPORT DATE	:	October 2008
SPONSOR	:	LyondellBasell Industries One Houston Center Suite 700, 1221 McKinney Street Houston TX 77010 USA
STUDY MONITOR	:	Sarah Gaul Bootman Chemical Safety Ltd. Diss Business Centre Diss Norfolk IP21 4HD UNITED KINGDOM
SPECIAL CONDITIONS	:	None

APPROVED FOR SPONSOR BY: S. Gaul DATE: 23/07/2008

AUTHORISED BY: [signature] DATE: 28 JUL 2008

T J Goodband BSc
STUDY DIRECTOR

This protocol is issued without signature by the Study Director to enable changes to be made if necessary prior to authorisation. Sponsors should sign and return the document to indicate approval and GLP authorisation will be confirmed by the Study Director's signature prior to the start of the study.

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 15 (continued) Protocol and Amendments

DAPHNIA MAGNA REPRODUCTION TEST

1. INTRODUCTION AND OBJECTIVES

This test method details a study designed to comply with the following regulatory guidelines:

i) The OECD Guidelines for Testing of Chemicals (1998) No 211 "*Daphnia magna*, Reproduction Test", referenced as Method C.20 of Commission Directive 2001/59/EC (which constitutes Annex V of Council Directive 67/548/EEC).

The purpose of this study is to assess the chronic toxicity of the test material to the freshwater invertebrate *Daphnia magna*. The toxicity will be expressed by the Lowest Observed Effect Concentration (LOEC) or Lowest Observed Effect Loading rate (LOEL) for reproduction and the No Observed Effect Concentration (NOEC) or No Observed Effect Loading rate (NOEL) for reproduction and where appropriate, the EC_{50} or EL_{50} for reproduction and associated 95% confidence limits. The Lowest Observed Effect Concentration (LOEC) or Lowest Observed Effect Loading rate (LOEL) and the No Observed Effect Concentration (NOEC) or No Observed Effect Loading rate (NOEL) for the parent animals will also be determined where appropriate.

Daphnia magna is a freshwater invertebrate representative of a wide variety of natural habitats, and can therefore be considered as an important non-target organism in freshwater ecosystems. The dispersal of the test material in the surrounding medium is considered to represent the most probable route of exposure in the environment.

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

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

Appendix 15 (continued) Protocol and Amendments

2. TEST FACILITY

Safepharm Laboratories Ltd
Shardlow Business Park
Shardlow
Derbyshire
DE72 2GD

3. ANIMALS

Specification

Daphnia magna of the 1st instar derived from in-house laboratory cultures.

Justification

Daphnia magna has been selected following the recommendations of OECD Guideline No 211 and EEC Directive 92/69/EEC.

4. ANIMAL HUSBANDRY

Environment

Water Temperature:	20 ± 1°C
Lighting:	Sixteen hours of continuous artificial light and eight hours continuous darkness with a 20 minute dawn and dusk transition period.

Housing

Animals will be maintained in 2-3 litre polypropylene beakers, stocked at a density of 30-80 daphnids per vessel initially.

Appendix 15 (continued) Protocol and Amendments

Water and Diet

The animals will be maintained in a reconstituted water as defined in Appendix 1. The daphnids will be fed an algal suspension, details of which will be documented in the data. Any change in species of algae fed to the daphnids will be documented in the data. Culture conditions ensure that reproduction will be by parthenogenesis. Cultures containing gravid adults will have any young daphnids removed at a time during the day prior to initiation of the test such that the young daphnids produced overnight will be equal to or less than 24 hours old. The diet and diluent water are considered not to contain any contaminant that would affect the integrity or outcome of the study.

5. PRE-TEST PROCEDURES

Identification

The animals will not be individually identified.

Allocation

Animals will be selected at random from the total number collected. No other method of randomisation will be used. At the start of the study the 1st instar daphnids should be less than 24 hours old but not first brood progeny.

6. TEST MATERIAL AND EXPERIMENTAL PREPARATION

Identification

Supplied by the study Sponsor with the details of hazardous properties if known. The integrity of supplied data relating to the identity, purity and stability of the test material will be the responsibility of the Sponsor.

Storage

Room temperature, in the dark, unless otherwise specified by the Sponsor.

Appendix 15 (continued) Protocol and Amendments

Preparation

The test material will be dissolved/dispersed in the test water once at the start of the study and at each test media renewal. If auxiliary solvents or surfactants are used to aid in the dispersion, they will be used at a maximum concentration of 100 µl/l (final volume). Other methods such as prolonged mixing, ultrasonication, high shear force stirring or the production of Water Accommodated Fractions (WAFs) or saturated solutions may be used depending on the nature of the test material. Details of the method of preparation will be documented in the data.

Analysis

Details of identification of the test material will be supplied by the study Sponsor. The test material formulations will be analysed for concentration and stability by Safepharm Analytical Services.

Absorption

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

7. STUDY DESIGN

Administration

The test material will be dissolved/dispersed in the test water at periods throughout the study. The study duration will be 21 days. The actual study dates will be documented in the data.

Test vessels

Glass flasks (150 ml) containing approximately 100 ml of test solution, covered with a plastic lid to reduce evaporation. Larger test volumes may be necessary to meet the requirements of the analytical method used to determine the test material concentration in the test media. The volumes used will be documented in the data and stated in the report. The test vessels will not be aerated during the test.

The test vessels will be allocated to a position within the test vessel holding frame in a random fashion.

Appendix 15 (continued) Protocol and Amendments

Loading

One 1st instar daphnid per vessel. Ten animals individually held for each test concentration and control series.

Test Concentrations

Five, logarithmically spaced by a factor preferably not exceeding 3.2, test groups plus 1 control and 1 solvent control if appropriate, each of ten replicates, will be employed in the test. The actual test concentrations are assigned according to the results of an acute toxicity or range-finding test, and will be documented in the data prior to the start of the main test.

The test media will be renewed three times per week (ie Mondays, Wednesdays and Fridays). Alternative renewal periods may be utilised dependent on the chemical or physical stability of the test material or specific requests by the Sponsor and will be documented in the data prior to the start of the study. The adult *Daphnia* will be transferred to fresh media by wide-bore pipette before the contents of each vessel are passed through a fine mesh. Young daphnids (live and dead) and any unhatched eggs are collected on the mesh and counted using a stereo microscope before being discarded.

The control group will be exposed to the test water alone.

A positive control study will not be conducted.

Environment and Feeding

The environmental parameters will be as used to maintain the stock animals. No auxiliary aeration will be supplied to the test vessels. The oxygen concentration should remain >3 mg O_2/l throughout the study. However, during normal working practice oxygen concentrations are maintained at ≥60% Air Saturation Value (≥5.3 mg O_2/l). All test and control vessels will be fed daily with a concentrated algal suspension details of which will be documented in the data. Any change in species of algae fed to the daphnids will be documented in the data. The volume of concentrated algal suspension given will be dependent on a range of factors eg density of the algal culture and carbon content. Sufficient food will be given in concentrated form to produce a very faint colouration of the water typically equivalent to 0.1 - 0.2 mg carbon per daphnid per day. The lighting regime will be 16 hours light:8 hours dark with a 20 minute dawn and dusk transition period at a light intensity preferably not exceeding 1000 lux.

Appendix 15 (continued) Protocol and Amendments

Detailed information on feeding, including amount (mg C/daphnid/day) and schedule (eg type of food including specific name of the algal strain and the culture conditions), will be documented in the data.

Verification of Test Concentrations

Analysis of test concentrations will be carried out for each surviving test group and the control (or solvent control where appropriate) at each medium renewal. Where possible duplicate samples will be taken at each occasion and stored at approximately -20°C, or other methods appropriate to the particular test material. These samples may also be analysed should the need arise. Samples at additional time points may also be taken and stored for future analysis. Samples will be taken from the old or expired media at each media renewal except on Day 0 where the fresh media will be sampled. For each control and test group the sample for analysis will be taken from a pooled sample of all ten replicate vessels. Stability analysis will also be performed to determine the stability of the test material in water at the concentrations employed in the test.

Stock solutions may also be analysed, if necessary, for example if the test concentrations are below the limit of quantitation of the analytical method or for reasons of stability.

Final details of the sample size, etc, will be documented in the data.

Where it is known or determined during the stability analysis that the test material is unstable in water and the measured concentrations are not expected to fall within ± 20% of nominal then analysis will be performed on fresh and old media at each renewal period on all concentrations. The results from the analyses will be expressed in terms of the time-weighted mean.

8. OBSERVATIONS

The following observations are to be made daily:

Any mortalities observed in the parental (P_1) generation for the control and test groups. The general size and condition of the parental generation in the test groups are assessed by comparison with the controls.

The appearance of eggs or young in the brood pouch is recorded.

The number of offspring produced by each parent animal are removed and counted each day from the appearance of the first brood, to prevent them using food intended for the adults. The general condition of the young in the test groups are assessed by comparison with the controls and the number of aborted eggs counted.

Appendix 15 (continued) Protocol and Amendments

Observations of the test material preparations.

Temperature and light intensity are recorded.

The following observations are to be made three times per week (ie Monday, Wednesday and Friday) at each test media renewal period:

pH and dissolved oxygen of the control and test vessels are measured (both before and after renewal).

At the end of the study, the length of each parent animal will be determined (ie body length excluding the anal spine).

Once per week the water hardness will be determined in the controls and highest surviving test concentration in the fresh and old media.

9. EVALUATION OF DATA

The purpose of this test is to determine the effect of the test material on the total number of living offspring produced per parent animal alive at the end of the test. The total number of offspring per parent animal should be calculated for each test vessel (i.e. replicate). If, in any replicate the parent animal dies during the test or turns out to be male, then the replicate is excluded from the analysis. The analysis will then be based on a reduced number of replicates.

The Lowest Observed Effect Concentration (LOEC) or Lowest Observed Effect Loading rate (LOEL) is the lowest test concentration (or loading rate) where a statistically significant effect on reproduction is observed.

The No Observed Effect Concentration (NOEC) or No Observed Effect Loading rate (NOEL) is the test concentration (or loading rate) immediately below the LOEC (or LOEL) which when compared to the control has no statistically significant effect.

For the estimation of the Lowest Observed Effect Concentration (LOEC), or Lowest Observed Effect Loading rate (LOEL) and hence the No Observed Effect Concentration (NOEC), or No Observed Effect Loading rate (NOEL) for effects of the chemical on reproductive output, it is necessary to calculate the mean reproductive output across replicates for each concentration and the pooled residual standard deviation, and this can be done using eg. analysis of variance (ANOVA) incorporating Bartlett's test for homogeneity of variance. The mean for each concentration must then be compared with the control mean using an appropriate statistical method which will be documented in the data.

Appendix 15 (continued) Protocol and Amendments

Where appropriate, an estimation of the EC_{50} or (EL_{50}) for reproduction and immobilisation will be made, using the trimmed Spearman-Karber method of Hamilton *et al* (1977) *Environ. Sci. Technol* 11, 714-719, the Probit method of Finney (1971) Statistical Method in Biological Assay. London : Griffin and Company Ltd or any other appropriate method. The EC_{50} or (EL_{50}), rounded to two significant figures, will be calculated together with the associated 95% confidence limits.

The Lowest Observed Effect Concentration (LOEC) or Lowest Observed Effect Loading rate (LOEL) for the parent animals is the lowest test concentration (or loading rate) where significant immobilisation or sub-lethal effects of exposure are observed.

The No Observed Effect Concentration (NOEC) or No Observed Effect Loading rate (NOEL) is the test concentration (or loading rate) immediately below the LOEC (or LOEL) where no significant immobilisation or sub-lethal effects of exposure are observed.

10. VALIDATION CRITERIA

The test is considered valid if the following criteria are achieved:

Mortality in the controls should not exceed 20% at the end of the test.

Dissolved oxygen should remain >3 mg O_2/l throughout the test.

pH of the controls should be within the range 6 – 9 pH units and not vary by >1.5 units throughout the test.

The mean number of live young produced per adult surviving in the controls at the end of the test must be ≥60.

The coefficient of variation around the mean number of living offspring produced per parent animal in the control should be ≤25%.

NOTE: For the purpose of this study, parental *Daphnia* which are unable to swim for approximately 15 seconds after gentle agitation (ie immobile) are considered to be dead. Filial *Daphnia* (1st instars) are considered to be dead if no signs of movement are observed during microscopic examination.

Appendix 15 (continued) Protocol and Amendments

11. QUALITY ASSURANCE

This standard test method will be reviewed for GLP compliance and the final report will be audited by Safepharm Quality Assurance Unit. This type of study is subject to process-based QA inspection designed to encompass the major phases once per month.

12. PROTOCOL AMENDMENTS

Amendments to protocol will be made only by completion of an Amendment to Protocol form authorised by the Study Director.

13. REPORT

The Sponsor will be informed immediately of all relevant findings. A full report will be prepared including the following information:

A description of the test material including batch and/or lot numbers, purity, etc. (when available).

A description of the test species.

Details of the experimental procedures used.

A summary of observations and environmental parameters.

A description of the culture medium used.

A description and amount of the food given to the test organisms.

The number of live young produced by each parent animal and time to production of first brood.

The number of mortalities observed amongst the parent daphnids and on which day they occurred.

The coefficient of variation for control fecundity (based on the total number of living offspring per parent daphnid alive at the end of the test).

A graphical presentation of the total number of offspring per parent daphnid (for each replicate) alive at the end of the test vs concentration of the test media.

The LOEC (or LOEL) and NOEC (or NOEL) and a description of the statistical procedures used.

Appendix 15 (continued) Protocol and Amendments

Where appropriate the EC_{50} (or EC_x) (or EL_{50} (or EL_x)) for reproduction and associated 95% limits. a graph of the fitted model used for its calculation, the slope of the dose-response curve and its standard error.

Details of the analytical method and solution analysis will be given as an Appendix.

A draft report audited by the Quality Assurance Unit will be sent to the Sponsor for review and comments before issue of the final report.

14. ARCHIVE

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

Appendix 15 (continued) Protocol and Amendments

Appendix 1 Reconstituted Water

Stock Solutions

a)	$CaCl_2.2H_2O$	11.76 g/l
b)	$MgSO_4.7H_2O$	4.93 g/l
c)	$NaHCO_3$	2.59 g/l
d)	KCl	0.23 g/l

Preparation

All chemicals must be of analytical grade. The water will be deionised water with a conductivity of $< 5\mu S\ cm^{-1}$.

Mix 25 ml of each of the four stock solutions and make up to 1 litre with water. The pH should be 7.8 ± 0.2. If necessary adjust the pH with NaOH or HCl. Aerate until the dissolved oxygen concentration is approximately the air-saturation value.

The reconstituted water has an approximate theoretical total hardness of 250 mg/l as $CaCO_3$.

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 15 (continued) Protocol and Amendments

SAFEPHARM LABORATORIES LIMITED

AMENDMENT TO PROTOCOL

AMENDMENT NUMBER:	1
PROTOCOL TITLE:	*Daphnia magna* Reproduction Test (OECD (1998) 211, EEC C.20)
TEST MATERIAL:	2,4,4-Trimethylpentene
PROJECT NUMBER:	2197/0038
TEST METHOD:	901.18
SPONSOR:	LyondellBasell Industries One Houston Center Suite 700, 1221 McKinney Street Houston TX 77010 USA
STUDY MONITOR:	Sarah Gaul Bootman Chemical Safety Ltd. Diss Business Park Diss Norfolk IP21 4HD UNITED KINGDOM
AMENDMENT:	The study will be conducted using dechlorinated tap water and young produced from cultures maintained in this diluent. This is due to a shift in reproduction timing of the cultures maintained in reconstituted water which would result in the test validation criteria not being met.

AUTHORISED FOR SAFEPHARM LABORATORIES LIMITED BY: ..... DATE: 15 SEP 2008

T J Goodband BSc
STUDY DIRECTOR

C:\Documents and Settings\tgoodband.HARLANUK\My Documents\REPRO PROTOCOL AMENDMENT.doc

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 15 (continued) Protocol and Amendments

SAFEPHARM LABORATORIES LIMITED

AMENDMENT TO PROTOCOL

AMENDMENT NUMBER:	2
PROTOCOL TITLE:	*Daphnia magna* Reproduction Test (OECD (1998) 211, EEC C.20)
TEST MATERIAL:	2,4,4-Trimethylpentene
PROJECT NUMBER:	2197/0038
TEST METHOD:	901.18
SPONSOR:	LyondellBasell Industries One Houston Center Suite 700, 1221 McKinney Street Houston TX 77010 USA
STUDY MONITOR:	Sarah Gaul Bootman Chemical Safety Ltd. Diss Business Park Diss Norfolk IP21 4HD UNITED KINGDOM
AMENDMENT:	The test material is volatile therefore the test will be conducted using ~100 ml completely filled and sealed glass stoppered conical flasks. The test media will be replaced daily and frozen duplicate samples will not be taken.

AUTHORISED FOR SAFEPHARM LABORATORIES LIMITED BY: ..... DATE: 14 OCT 2008

T J Goodband BSc
STUDY DIRECTOR

C:\Documents and Settings\tgoodband.HARLANUK\My Documents\REPRO PROTOCOL AMENDMENT.doc

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 15 (continued) Protocol and Amendments

HARLAN LABORATORIES LIMITED

AMENDMENT TO PROTOCOL

AMENDMENT NUMBER:	3
PROTOCOL TITLE:	*Daphnia magna* Reproduction Test (OECD (1998) 211, EEC C.20)
TEST MATERIAL:	2,4,4-Trimethylpentene
PROJECT NUMBER:	2197/0038
TEST METHOD:	901.18
SPONSOR:	LyondellBasell Industries One Houston Center Suite 700, 1221 Mckinney Street Houston TX 77010 USA
STUDY MONITOR	Sarah Gaul Bootman Chemical Safety Ltd. Diss Business Park Diss Norfolk IP21 4HD UNITED KINGDOM
AMENDMENT:	On the 9th November 2008 Safepharm Laboratories Ltd. changed its name to Harlan Laboratories Ltd. Therefore, the study will be reported and archived under the name Harlan Laboratories Ltd., and not Safepharm Laboratories Ltd. as stated in the protocol. This amendment was considered not to affect the purpose or integrity of the study.

AUTHORISED FOR HARLAN LABORATORIES LIMITED BY: in absence of TG DATE: 12 NOV 2008

T J Goodband BSc
STUDY DIRECTOR 28.11.08

G:\ALL\HarlanDocs\PROTOCOL AMENDMENT - Name Change.dot

2,4,4-Trimethylpentene: *DAPHNIA MAGNA* REPRODUCTION TEST

Appendix 16 Certificate of Analysis

Information for Analytical Certification Provided by LyondellBasell Industries

Date Prélevement	Numéro Mach1	Numéro Lot
02/05/2008 08:00	2381195	\Q01218R02

Property	Method	Unit	Spec Min	Spec Max	Value
GLC of DIB					
Purity	BMS 0182	%w	90		93.02
Light Ends	BMS 0182	%w			0.13
DiIsoPropylEther	BMS 0182	%w			0
DiIsoButylen1 (2,4,4-Trimethylpent-1-ene, CAS-No.: 107-39-1))	BMS 0182	%w			76.38
DiIsoButylen2 (2,4,4-Trimethylpent-2-ene (CAS-No.: 107-40-4))	BMS 0182	%w			16.64
Others C8	BMS 0182	%w			4.17
TriIsoButylene	BMS 0182	%w			0
Dimethyl hexène	BMS 0182	%w			2.67
Heavy Ends	BMS 0182	%w			0
GLC Tertiary Butyl Alcohol + Secondary Butyl Alcohol					
Tertiary Butyl Alcohol	BMS 0194	mg/kg			57
Secondary Butyl Alcohol	BMS 0194	mg/kg			172
Tertiary Butyl Alcohol + Secondary Butyl Alcohol	BMS 0194	mg/kg		300	229
Distillation					
Distillation, Initial Boiling Point	ASTM D1078	°C	101	103	101.5
Distillation, 5%w distilled Point	ASTM D1078	°C			101.9
Distillation, 95%w distilled Point	ASTM D1078	°C			102.5
Distillation, Dew Point	ASTM D1078	°C		107	104
Density @ 20°C	ASTM D4052	kg/l	0.715	0.721	0.718
Peroxides	BMS 0112	mg/kg		5	2
Acidity (equivalent as H2SO4)	ASTM D1613	mg/kg		15	6
Organic Sulphur content	BMS 0193	mg/kg		5	1
Inhibitor (Topanol)		mg/kg			73
Color Pt-Co (Hazen)	ASTM D1209			10	5
Appearance	ASTM D4176		Clear & Free from Suspended Matter		

Appendix 17 Statement of GLP Compliance in Accordance with Directive 2004/9/EC

Department of Health

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

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

TEST FACILITY	TEST TYPE
SafePharm Laboratories Ltd. **Shardlow Business Park** **Shardlow** **Derbyshire** **DE72 2GD**	**Analytical Chemistry** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Phys/Chem testing** **Toxicology**

DATE OF INSPECTION

21st August 2007

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

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

15/10/07

Dr. Andrew J. Gray
Head, UK GLP Monitoring Authority

MHRA

On 9th November 2008, Safepharm Laboratories Ltd changed its name to Harlan Laboratories Ltd. Therefore, until a new GLP Certificate is issued from the UK GLP monitoring authority the certificate in this report still states the name as Safepharm Laboratories Ltd.