LAB Research Ltd.

Address: 8200 Veszprém, Szabadságpuszta

Phone: +36 88 545 300

Fax: +36 88 545 301

FINAL REPORT

ACUTE IMMOBILISATION

TEST WITH

Ethylene Dibromide Industrial (EDB)

ON DAPHNIA *(Daphnia magna)*

Date of Final Report: 01 October 2010

STUDY CODE: 10/112-023DA

STATEMENT OF THE STUDY DIRECTOR

This study has been performed in accordance with the study plan the OECD Guidelines for Testing of Chemicals (No. 202, 13 April 2004) and Directive 92/69/EEC, Annex Part C, C.2 and the EPA Health Effects Test Guidelines (OPPTS 850.1010) and the Principles of Good Laboratory Practice Regulations as specified by national Hungarian GLP Regulations (9/2001.(III.30.) EüM-FVM joint decree of the Minister of Health and the Minister of Agriculture and Regional Development) which corresponds to the OECD GLP, ENV/MC/CHEM(98)17.

I the undersigned declare that this report constitutes a true record of the actions undertaken and the results obtained in the course of this study. By virtue of my dated signature I accept the responsibility for the validity of the data and the following conclusion drawn from them:

Under the conditions of this *Daphnia magna* acute immobilisation study, the calculated and observed endpoints for the effect of Ethylene Dibromide Industrial (EDB) were the followings:

The 24h EC_{50} value:	**15.71 mg/L** (95 % confidence limits: 12.80–19.27 mg/L)
The 48h EC_{50} value:	**11.61 mg/L** (95 % confidence limits: 9.49–14.21 mg/L)
The 48h EC_{100} value:	**22.26 mg/L**

The 48h No-Observed Effect Concentration (NOEC): 5.24 mg/L

The 48h Lowest Observed Effect Concentration (LOEC): 9.44 mg/L

Signature: _____ Date: 01 October 2010

István Ágh M.Sc.
Study Director

STATEMENT OF THE MANAGEMENT

According to the conditions of the research and development agreement between CHEMTURA CORPORATION (as Sponsor) and LAB Research Ltd. (as Testing Facility) "Acute immobilisation test with Ethylene Dibromide Industrial (EDB) on *Daphnia* (*Daphnia magna*)" has been performed in compliance with the study plan and the Principles of Good Laboratory Practice.

Signature: _____ Date: 01 Oct. 2010

Christopher Banks, DABT.
Managing Director

QUALITY ASSURANCE STATEMENT

Study Code: 10/112-023DA

Study Title: Acute immobilisation test with Ethylene Dibromide Industrial (EDB) on *Daphnia* (*Daphnia magna*)

Test Item: Ethylene Dibromide Industrial (EDB)

This study has been inspected, and this report audited by the Quality Assurance Unit in compliance with the Principles of Good Laboratory Practice. As far as it can be reasonably established the methods described and the results incorporated in this report accurately reflect the raw data produced during this study.

All inspections, data reviews and the report audit were reported in written form to the study director and to management. The dates of such inspections and of the report audit are given below:

Date of Inspection	Phase(s) Inspected/Audited	Date of report to	
		Management	Study Director
27 May 2010	Study Plan	27 May 2010	31 May 2010
17 August 2010	Test Item Formulation	18 August 2010	17 August 2010
06 September 2010	Analytical Report	06 September 2010	06 September 2010
21 September 2010	Draft Report	21 September 2010	21 September 2010
01 October 2010	Final Report	01 October 2010	01 October 2010

Signature: Fábián Makovi Éva

Éva Makovi-Fábián B.Sc.
On behalf of QAU

Date: 01 October 2010

GENERAL INFORMATION

STUDY TITLE	:	Acute immobilisation test with Ethylene Dibromide Industrial (EDB) on *Daphnia* (*Daphnia magna*)
TEST ITEM	:	Ethylene Dibromide Industrial (EDB)
SPONSOR	:	CHEMTURA CORPORATION Address: 199, Benson Road, Middlebury, Connecticut 06749 USA
TEST FACILITY	:	LAB Research Ltd. Address: H-8200 Veszprém, Szabadságpuszta Phone: 36 88 545-300 Fax: 36 88 545-301
STUDY DIRECTOR	:	István Ágh M.Sc.
QUALITY ASSURANCE	:	Ramóna Heiderné Grób B.Sc. Éva Makovi-Fábián B.Sc.
RESPONSIBLE PERSON FOR ANALYTICAL MEASUREMENTS	:	Zsolt Sárvári M.Sc.
TECHNICAL STAFF	:	Ecotoxicological staff
STUDY PLAN	:	31 May 2010
EXPERIMENTAL PERIOD I.	:	17 - 19 August 2010
EXPERIMENTAL PERIOD II.	:	25 - 27 August 2010
DRAFT REPORT	:	21 September 2010

BASIS OF STUDY:

- OECD Guidelines for Testing of Chemicals, No.202, "Daphnia sp., Acute Immobilisation Test" (Adopted: 13 April 2004)
- EPA Health Effects Test Guidelines, OPPTS 850.1010
- Commission Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restrictions of Chemicals (REACH), Annex Part C, C.2 (published in the Official Journal of the European Union L 142 of 31 May 2008)

CONTENTS

1. SUMMARY

Acute toxicity of Ethylene Dibromide Industrial (EDB) was assessed with Acute Immobilisation Test on *Daphnia magna*, over an exposure period of 48 hours in a static system.

Because significant toxic response was observed during the preliminary range-finding test, six test concentrations in a geometric series (factor 1.8) and one control was used in the main test.
As no significant toxic effect was observed during the main test (Experiment I.) four additional concentrations were tested at higher levels in a complementary experiment (Experiment II.) in order to determine the EC_{50}, NOEC and LOEC values.

The nominal concentrations of the test item were: 4.8, 8.6, 15.4, 27.8, 50, 90, 100, 150, 225 and 338 mg/L. The corresponding calculated concentrations (based on the analytical measurements) were: 0.50, 0.90, 1.62, 2.91, 5.24, 9.44, 22.26, 33.39, 50.09 and 84.67 mg/L

As the measured concentrations deviated more than 20 per cent from the nominal values during the exposure, the biological results are based on the calculated test item concentrations.

The test design included four replicates at each test group (test item and control). Each group comprised twenty *Daphnia*, five in each of the four replicate vessels, each containing ~ 40 mL test dilution.

All validity criteria were met during this study.

Under the conditions of this *Daphnia magna* acute immobilisation study, the calculated and observed endpoints for the effect of Ethylene Dibromide Industrial (EDB) were the followings:

The 24h EC_{50} value: **15.71 mg/L**
(95 % confidence limits: 12.80–19.27 mg/L)

The 48h EC_{50} value: **11.61 mg/L**
(95 % confidence limits: 9.49–14.21 mg/L)

The 48h EC_{100} value: **22.26 mg/L**

The 48h No-Observed Effect Concentration (NOEC): **5.24 mg/L**

The 48h Lowest Observed Effect Concentration (LOEC): **9.44 mg/L**

2. INTRODUCTION

The objective of this study was to determine the effect of Ethylene Dibromide Industrial (EDB) on *Daphnia magna*. Young *Daphnia* were exposed in a static test to aqueous test medium containing the test item at different concentrations. The percentage of *Daphnia* being no longer capable of swimming at the end of the test or being dead was recorded for each test concentration.

The test method of application and the test species *Daphnia magna* are recommended by the test guidelines.

3. MATERIALS AND METHODS

3.1. TEST ITEM AND CONTROLS AND VEHICLE

3.1.1. Test Item

Name:	Ethylene Dibromide Industrial (EDB)
Chemical name:	1,2-Dibromoethane
Batch No.:	510100003
Active component:	>99.94 % 1,2-Dibromoethane (CAS 106-93-4)
Description:	clear to amber liquid
Manufacture date:	February 2010
Expiry date:	February 2011
Storage:	room temperature; 15-25°C (humidity 50 % ± 20); protect from light
Safety Precautions:	see Safety Data Sheet
Manufacturer:	Chemtura Manufacturing UK Limited
	Address: Tenax Road, Trafford Park Manchester United Kingdom M17 1WT

The test item of a suitable chemical purity was supplied by the Sponsor. All precautions required in the handling and disposal of the test item were outlined by the Sponsor. These documents are part of the raw data. Identification of the test item was performed in the Central Dispensary of LAB Research Ltd. on the basis of the information provided by the Sponsor (name, batch number, appearance and colour).

3.1.2. Dilution and Preparation of Testing Solutions

Because the test item could not be directly dissolved in the test medium (ISO medium), the test item solutions used in the experiment were prepared as follows:

Experiment I.

A stock solution (nominally 90 mg/L) was prepared by mechanical dispersion one day before the start of the test. This solution was shaken for about 24 hours. After shaking the test solutions were prepared by appropriate diluting of the stock solution and distributed into the appropriate test vessels prior to introduction of the test animals. There were four replicates for each test item concentration and four for the untreated control.

Table 1.: Preparation of test solutions from stock solution

Nominal concentration (mg/L)	**Amount of stock solution** (mL)	**Amount of ISO Medium** (mL)
90	300	**--**
50	167	q.s. ad 300
27.8	93	q.s. ad 300
15.4	51	q.s. ad 300
8.6	29	q.s. ad 300
4.8	16	q.s. ad 300

q.s. ad = quantum sufficiat ad (a sufficient quantity to make)

Experiment II. (Complementary Experiment)

Individual solutions with nominal concentrations of 100, 150, 225 and 338 mg/L were prepared by mechanical dispersion one day before the start of the test and then were shaken for about 24 hours. After shaking the test solutions were distributed into the appropriate test vessels prior to introduction of the test animals. There were four replicates for each test item concentration and four for the untreated control.

3.1.3. Untreated Control

The dilution water (ISO-medium) was used without of addition of the test item.

3.1.4. Reference Control

For the evaluation of the quality of the *Daphnia* clone and the experimental conditions, Potassium dichromate is tested at least twice a year to demonstrate satisfactory test conditions.

The date of the last study (Study Code: 10/157-023DA) with reference item Potassium dichromate (batch no.: 0769128) is: 23 - 24 June 2010.

The 24h EC_{50}: 1.77 mg/L, (95 % confidence limits: 1.46 – 2.15 mg/L)

This value is within the range of laboratory ring test data (see ISO Guideline No. 6341).

3.1.5. Dilution Water

Reconstituted water (ISO medium, according to OECD 202) was used as dilution water for both the range finding and definitive tests. The same composition of reconstituted water was used for the tests and for breeding the test animals.

The composition of the reconstituted water and the chemicals used are given in Appendix 4.

3.2. EXPERIMENTAL ANIMALS

Species and strain:	*Daphnia magna*
Source:	Supplied by National Institute of Public Health. Cultured under standardised conditions at the Ecotoxicological Laboratory of LAB Research Ltd..
Justification of strain:	*Daphnia magna* is the standard species of the acute immobilisation test.
Number of animals:	There were 20 animals in test group and control group, divided into 4 replicates (5 animals / replicate).
Age of the animals:	They were less than 24 h old at the beginning of the test.
Acclimatization:	There was no acclimatization because the water used was similar to the culture water.
Food and feeding:	Before the test the *Daphnia* culture was fed with concentrated algal suspension of *Pseudokirchneriella subcapitata*. The test animals were not fed during the test.

3.3. EQUIPMENTS

Normal laboratory equipment and the following apparatus were necessary for the determination of test parameters:

- pH meter
- thermometer
- oxygenmeter
- balance
- light-meter
- climate chamber
- orbital shaker

As test vessels, laboratory glass beakers (volume: ~50 mL) were used.

3.4. TEST CONDITIONS
(During Experiment I. and II.)

Temperature:	The water temperature was measured at the start of the tests and 24-hour intervals thereafter in each test vessel. The test temperature was in the range of 20.9 – 21.1 °C measured in the test vessels. The additionally measured temperature in the climate chamber was between 20.7 and 21.4 °C.
Oxygen concentration:	The dissolved oxygen concentration was measured in each test vessel at the start and at the end of the tests and was in the range of 6.7 – 6.8 mg/L.
pH:	The pH of the test solution was not adjusted and not varied by more than 1.5 units in any one test. The pH was measured at the start and at the end of the tests in each test vessel and was in the range of 7.65 – 7.99.
Light:	The test was carried out in 16-hour light and 8-hour dark cycle.
Hardness:	The reconstituted water (ISO medium) had an approximate theoretical total hardness of 249 mg/L (as $CaCO_3$).

Data of test conditions are detailed in Appendix 1.

3.5. DESCRIPTION OF THE TEST PROCEDURE

The test duration was 48 hours. Twenty animals, divided into four groups (glass beaker) of five animals each (at least 4 mL test solution/animal) were used at the test concentrations and for the controls. The animals were not fed during the test.

The choice of the test concentrations was done on the basis of the results of a preliminary range-finding test.

3.5.1. Preliminary Range Finding Test

A concentration range-finding test was conducted to determine the approximate toxicity of the test item so that appropriate test concentrations can be selected for use in the definitive test. Ten daphnids (divided into two replicates) in each test concentration and control were exposed for 48 hours.

During the formulation procedure the test solutions were prepared by the method described above.

The concentration levels used and results (48 h) of the preliminary range-finding test are summarised in the following table.

Table 2: Results of the Preliminary Range-Finding Test

Nominal concentrations [mg/L]	Untreated control	0.01	0.1	1	10	100
Number of treated / immobilised animals	10 / 0	10 / 0	10 / 0	10 / 0	10 / 0	10 / 10

3.5.2. Concentration Levels Investigated in the Main Test

Because significant toxic response was observed during the preliminary range-finding test, six test concentrations in a geometric series (factor 1.8) and one control was used in the main test.
As no significant toxic effect was observed during the main test (Experiment I.) four additional concentrations were tested at higher levels in a complementary experiment (Experiment II.) in order to determine the EC_{50}, NOEC and LOEC values.

The nominal and corresponding calculated concentrations used in the main experiments are summarized in the following table:

Table 3: Concentrations tested in the main experiments

Experiment	**Nominal concentration** (mg/L)	**Calculated concentration** (mg/L)
Experiment I.	4.8	0.50
	8.6	0.90
	15.4	1.62
	27.8	2.91
	50	5.24
	90	9.44
Experiment II.	100	22.26
	150	33.39
	225	50.09
	338	84.67

As the measured concentrations deviated more than 20 % during the experiment, the biologically results are based on the calculated test item concentrations (based on the analytical measurements).

The analytically measured test item concentrations are detailed in Appendix 5 (Table 3).

3.6. OBSERVATIONS

The immobility or mortality of the *Daphnia* was determined by visual observation 24 and 48 hours after the start of the test. Those animals not able to swim within 15 seconds after gentle agitation of the test beaker are considered to be immobile.

The number of immobilised animals and the percentage of immobility were determined at 24 and 48 hours.

The water temperature, oxygen concentrations and pH of the test solutions and the control were measured at the beginning and at the end of the test.

3.7. STATISTICAL EVALUATION

The EC_{50} values were calculated by Probit analysis with 95 % confidence limits using TOXSTAT software.

The EC_{100}, Noec and Loec values are determined directly from the raw data

3.8. ANALYTICAL MEASUREMENTS

Concentration of Ethylene Dibromide Industrial (EDB) in the test solutions was determined at the beginning and at the end of the study.

On the first part of the study between August 17 and 19 the concentration of the samples was 4.8, 8.6, 15.4, 27.8, 50 and 90 mg/L. At the start of the test five samples were taken from the test solution. At the end of the test four samples were taken from each of the test vessels. Both occasions one sample was taken from the control solution.

On the second part of the study between August 25 and 27 the concentration was 100, 150, 225 and 338 mg/L. Four samples were taken from the test solutions and one sample was taken from the control solution.

The samples were analysed by an HPLC-UV method.

Methods and results of test item concentration analysis are described in Appendix 5.

3.9. ARCHIVES

The study documents:

- study plan and amendments,
- all raw data,
- sample of test item,
- study report, and any amendments,
- correspondence

are stored according to the Hungarian GLP and to the LAB Research Ltd. SOP-s in the archives of LAB Research Ltd. 8200 Veszprém, Szabadságpuszta, Hungary.

After the retention time has elapsed all the archived materials listed above will be returned to the Sponsor or retained for a further period if agreed by a contract. Otherwise the materials will be discarded.

3.10. CONDITIONS FOR THE VALIDITY OF THE TEST

- in the control, including the control containing the solubilising agent, not more than 10 per cent of the *Daphnia* should be immobilised or trapped at the surface of the water.
- the dissolved oxygen concentration at the end of the test in control and test vessels should be ≥ 3 mg/L of the air saturation value at the temperature used.

3.11. DEVIATION FROM THE STUDY PLAN

Concerning:	Storage of Test Item
According to the Study Plan:	room temperature; 15-25°C (humidity 50 % ± 20);
Deviation:	room temperature; 15-25°C (humidity 50 % ± 20); protect from light
Reason for this change:	Typing error
Presumed Effect on the Study:	None
Concerning:	Dilution and Preparation of Testing Solutions
According to the Study Plan:	stock solution of 100 mg/L (nominal) will be prepared
Deviation:	stock solution of 90 mg/L (nominal) was prepared
Reason for this change:	Typing error
Presumed Effect on the Study:	None
Concerning:	Test Item concentrations (in the Experiment I.)
According to the Study Plan:	8.6, 15.4, 27.8, 50, and 90 mg/L
Deviation:	4.8, 8.6, 15.4, 27.8, 50, and 90 mg/L
Reason for this change:	Toxic effect at lower concentrations was expected based on the results of the acute fish test on EDB (study code: 10/112-009H; 10/112-009HR)
Presumed Effect on the Study:	None

Concerning:	STUDY SCHEDULE
According to the Study Plan:	Start of experiment: 26 July 2010 Draft Report: 01 September 2010
Deviation:	Start of experiment: 17 August 2010 Draft Report: 21 September 2010
Reason for this change:	Technical
Presumed Effect on the Study:	None

3.12. DISTRIBUTION OF THE FINAL REPORT

Sponsor: 1x copy, bound
1x copy, unbound
1x PDF file

Archive: 1x original, bound

3.13. REFERENCES

1. OECD Principles of Good Laboratory Practice, adopted by Council on 26th November 1997 [C(97)186/Final], Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998.
2. Hungarian Good Laboratory Practice Regulations: 9/2001 (III. 30) EÜM-FVM joint decree of the Minister of Health and the Minister of Agriculture and Regional Development which corresponds to the OECD GLP, 1997
3. Commission Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restrictions of Chemicals (REACh), Annex Part C, C.2 (published in the Official Journal of the European Union L 142 of 31 May 2008)
4. EPA Guideline 712-C-96-114: OPPTS 850.1010, "Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids" April 1996
5. OECD Guideline for Testing of Chemicals, Section 2, No. 202, "*Daphnia sp.*, Acute Immobilisation Test", updated 13th April 2004.
6. Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures, section 3.1.2 Media preparation methods, Direct addition. OECD Series on Testing and Assessment No. 23, Paris September 2000.
7. Guidelines for studies on the new chemical substance as required by the Law Concerning the Evaluation of Chemical Substances and Regulation of their Manufacture, etc (Chemical Substance Control Law) 1973, amended 2009 under the reference of YAKUSHOKHATSU No. 1121002, SEIKYOKU No.2 and KANPOKIHATSU No. 021121002 and partially amended 2006 as the joint ordinance of The Japanese Ministry of Economy Trade and Industry (METI), Ministry of Health, Labour and Welfare (MHLW) and Ministry of the Environment (MOE)

4. RESULTS

4.1. VALIDITY

There were no immobilized animal in the control group and the dissolved oxygen concentration at the end of the test in control and test vessels was more than 3 mg/L. All validity criteria were within acceptable limits and therefore the study can be considered as valid.

4.2. TEST ITEM CONCENTRATIONS

For determination of the test item concentration, samples were taken for analytical measurements from each concentration level at the start and at the end of the experiment. The measured concentrations are summarized in Table 4 below.

At the end of the tests the measured concentrations of the test item solutions were below the limit of quantification in most cases.

In order to calculate the mean exposure concentrations, geometric mean was calculated from the start and end concentrations in the cases where the concentrations were analysable at the end of the test. In the cases where the concentrations were below the limit of quantification at the end of the experiment, the exposure concentrations were extrapolated from the closest upper geometric mean value using the factor between the nominal concentrations.

The biological results of the test are based on the calculated exposure concentrations.

The measured concentrations were significantly lower than the nominal at the start (after ~24h shaking) and particularly at the end of the test.

The possible reason for the quick decline of the concentrations could be that the test material is likely to be volatile. This could be supported by the results of the stability test in aqueous solutions at room temperature (see: “Validation of the Analytical Method for the Determination of Ethylene Dibromide Industrial (EDB)”; LAB code: 10/112-316AN) where the concentration was constant during the storage period in a well capped glass flask with minimal headspace.

The concentration decrease may be additionally explained by hydrolysis, because the hydrolysis of the test substance can increase at higher or lower pH from neutral (see: “Determination of the Hydrolysis of Ethylene Dibromide Industrial (EDB) as a Function of pH”; LAB code: 10/112-336AN). The pH of the present test was in the range of 7.65 – 7.99 during the exposure (see Appendix 1; Table 9 and 10).

Table 4: Concentration Levels in the main tests

Nominal concentrations (mg/mL)		**Control**	**4.8**	**8.6**	**15.4**	**27.8**	**5**	**90**	**100**	**150**	**225**	**338**
Measured concentrations (mg/L)	**Start**	n.d.	0.76	1.40	2.80	5.55	11.8	35.2	50.8	77.8	123	214
	End	n.d.	BQL	BQL	BQL	BQL	BQL	2.53	BQL	BQL	20.4	33.5
Calculated concentrations (mg/L)		**0.00**	**E**					**G**	**E**		**G**	
			0.50	**0.90**	**1.62**	**2.91**	**5.24**	**9.44**	**22.26**	**33.39**	**50.09**	**84.67**

n.d.:– not detectable

BQL: below quantification limit

G: geometric mean

E: extrapolated value

Methods and results of test item concentration analysis are described in Appendix 4.

4.3. IMMOBILISATION

The number of immobilised animals and the percentage of immobility were determined at the 24th and 48th hour (Table 3).

Data of immobility in each test vessel are detailed in Appendix 2.

Table 5: Number and percentage of immobilised animals

Concentrations [mg/L]		**Number of treated animals**	**Immobilised animals**			
			24 hours		**48 hours**	
nominal	**calculated**		**number**	**percent**	**number**	**percent**
Control	0.00	40	0	0	0	0
4.8	0.50	20	0	0	0	0
8.6	0.90	20	0	0	0	0
15.4	1.62	20	1*	5	1*	5
27.8	2.91	20	0	0	0	0
50	5.24	20	0	0	0	0
90	9.44	20	0	0	3	15
100	22.26	20	14	70	20	100
150	33.39	20	20	100	20	100
225	50.09	20	20	100	20	100
338	84.67	20	20	100	20	100

* A single daphnid was observed to be immobile after 24 and 48h exposure at 1.62 mg/L. This was considered to be due to natural causes rather than a toxic effect given that no immobilisation was detected at the concentrations of 2.91 and 9.44 mg/L.

5. CONCLUSION

Acute toxicity of Ethylene Dibromide Industrial (EDB) was assessed with Acute Immobilisation Test on *Daphnia magna*, over an exposure period of 48 hours in a static system.

Under the conditions of this *Daphnia magna* acute immobilisation study, the calculated and observed endpoints for the effect of Ethylene Dibromide Industrial (EDB) were the followings:

The 24h EC_{50} value: **15.71 mg/L**
(95 % confidence limits: 12.80–19.27 mg/L)

The 48h EC_{50} value: **11.61 mg/L**
(95 % confidence limits: 9.49–14.21 mg/L)

The 48h EC_{100} value: **22.26 mg/L**

The 48h No-Observed Effect Concentration (NOEC): **5.24 mg/L**

The 48h Lowest Observed Effect Concentration (LOEC): **9.44 mg/L**

A P P E N D I C E S

APPENDIX 1

DATA OF TEST CONDITIONS

Table 6: Temperature measured in the test vessels [°C] (Experiment I.)

Concentrations [mg/L]		**Replicate**	**Measuring**		
nominal	**calculated**		**0 h**	**24 h**	**48 h**
Control	**0.00**	1	21.1	21.0	21.1
		2	21.0	21.0	21.1
		3	21.0	21.0	21.1
		4	21.0	21.0	21.1
4.8	**0.50**	1	21.0	21.0	21.1
		2	21.1	21.1	21.1
		3	21.1	21.0	21.1
		4	21.0	21.0	21.1
8.6	**0.90**	1	21.0	21.0	21.1
		2	21.0	21.0	21.1
		3	21.0	21.0	21.1
		4	21.0	21.0	21.1
15.4	**1.62**	1	21.0	21.0	21.0
		2	21.0	21.0	21.0
		3	21.0	21.0	21.1
		4	21.0	21.1	21.1
27.8	**2.91**	1	21.0	21.1	21.0
		2	21.0	21.0	21.1
		3	21.1	21.1	21.1
		4	21.1	21.0	21.1
50	**5.24**	1	21.1	21.0	21.1
		2	21.0	21.0	20.9
		3	21.0	21.0	21.0
		4	21.0	21.1	21.0
90	**9.44**	1	21.0	21.0	21.1
		2	21.0	21.1	21.1
		3	21.0	21.1	21.1
		4	21.0	21.1	21.1

Table 7: Temperature measured in the test vessels [°C] (Experiment II.)

Concentrations [mg/L]		**Replicate**	**Measuring**		
nominal	**calculated**		**0 h**	**24 h**	**48 h**
Control	**0.00**	1	21.1	21.0	21.1
		2	21.1	21.0	21.1
		3	21.1	21.0	20.9
		4	21.1	21.0	21.0
100	**22.26**	1	21.1	21.0	21.1
		2	21.1	21.0	21.1
		3	21.0	21.0	21.0
		4	21.0	21.0	21.1
150	**33.39**	1	21.0	21.0	21.1
		2	21.1	21.0	21.1
		3	21.1	21.0	21.1
		4	21.1	20.9	21.1
225	**50.09**	1	21.1	21.0	21.1
		2	21.0	20.9	21.1
		3	21.1	20.9	21.1
		4	21.1	21.0	21.1
338	**84.64**	1	21.1	21.0	21.1
		2	21.1	20.9	21.1
		3	21.1	21.0	21.1
		4	21.1	21.0	21.1

Table 8: Oxygen concentration measured in the test vessels [mg/L] (Experiment I.)

Concentrations [mg/L]		**Replicate**	**Measuring**	
nominal	**calculated**		**0 h**	**48 h**
Control	**0.00**	1	6.7	6.7
		2	6.7	6.8
		3	6.7	6.8
		4	6.7	6.8
4.8	**0.50**	1	6.8	6.7
		2	6.7	6.7
		3	6.8	6.7
		4	6.7	6.7
8.6	**0.90**	1	6.7	6.7
		2	6.7	6.7
		3	6.7	6.8
		4	6.7	6.8
15.4	**1.62**	1	6.7	6.7
		2	6.8	6.7
		3	6.8	6.7
		4	6.7	6.7
27.8	**2.91**	1	6.7	6.7
		2	6.7	6.8
		3	6.7	6.7
		4	6.7	6.7
50	**5.24**	1	6.7	6.7
		2	6.7	6.7
		3	6.8	6.7
		4	6.7	6.7
90	**9.44**	1	6.8	6.7
		2	6.8	6.7
		3	6.7	6.7
		4	6.7	6.7

Table 9: Oxygen concentration measured in the test vessels [mg/L] (Experiment II.)

Concentrations [mg/L]		**Replicate**	**Measuring**	
nominal	**calculated**		**0 h**	**48 h**
Control	**0.00**	1	6.7	6.8
		2	6.7	6.7
		3	6.7	6.7
		4	6.7	6.7
100	**22.26**	1	6.7	6.7
		2	6.7	6.8
		3	6.7	6.8
		4	6.7	6.8
150	**33.39**	1	6.8	6.7
		2	6.7	6.8
		3	6.7	6.7
		4	6.7	6.8
225	**50.09**	1	6.8	6.7
		2	6.8	6.7
		3	6.7	6.7
		4	6.7	6.8
338	**84.64**	1	6.7	6.7
		2	6.7	6.7
		3	6.7	6.7
		4	6.7	6.7

Table 10: pH measured in the test vessels (Experiment I.)

Concentrations [mg/L]		**Replicate**	**Measuring**	
nominal	**calculated**		**0 h**	**48 h**
Control	**0.00**	1	7.70	7.82
		2	7.68	7.80
		3	7.71	7.89
		4	7.77	7.86
4.8	**0.50**	1	7.72	7.92
		2	7.73	7.91
		3	7.72	7.84
		4	7.68	7.81
8.6	**0.90**	1	7.71	7.90
		2	7.72	7.79
		3	7.72	7.88
		4	7.76	7.92
15.4	**1.62**	1	7.66	7.99
		2	7.70	7.87
		3	7.71	7.83
		4	7.72	7.88
27.8	**2.91**	1	7.74	7.84
		2	7.70	7.90
		3	7.72	7.92
		4	7.71	7.86
50	**5.24**	1	7.67	7.79
		2	7.75	7.88
		3	7.74	7.84
		4	7.76	7.81
90	**9.44**	1	7.70	7.79
		2	7.65	7.70
		3	7.69	7.82
		4	7.70	7.81

Table 11: pH measured in the test vessels (Experiment II.)

Concentrations [mg/L]		**Replicate**	**Measuring**	
nominal	**calculated**		**0 h**	**48 h**
Control	**0.00**	1	7.76	7.70
		2	7.70	7.75
		3	7.74	7.77
		4	7.69	7.72
100	**22.26**	1	7.77	7.80
		2	7.77	7.70
		3	7.80	7.76
		4	7.72	7.82
150	**33.39**	1	7.75	7.80
		2	7.74	7.84
		3	7.75	7.77
		4	7.80	7.84
225	**50.09**	1	7.78	7.86
		2	7.82	7.84
		3	7.77	7.89
		4	7.73	7.80
338	**84.64**	1	7.79	7.88
		2	7.84	7.92
		3	7.86	7.96
		4	7.80	7.83

APPENDIX 2

DATA OF IMMOBILISATION

Table 12: Immobilization of the test animals (Experiment I.)

Concentrations [mg/L]		Replicate	Number of treated animals	Number of immobilised animals	
nominal	calculated			24 h	48 h
Control	**0.00**	1	5	0	0
		2	5	0	0
		3	5	0	0
		4	5	0	0
4.8	**0.50**	1	5	0	0
		2	5	0	0
		3	5	0	0
		4	5	0	0
8.6	**0.90**	1	5	0	0
		2	5	0	0
		3	5	0	0
		4	5	0	0
15.4	**1.62**	1	5	0	0
		2	5	0	0
		3	5	1	1
		4	5	0	0
27.8	**2.91**	1	5	0	0
		2	5	0	0
		3	5	0	0
		4	5	0	0
50	**5.24**	1	5	0	0
		2	5	0	0
		3	5	0	0
		4	5	0	0
90	**9.44**	1	5	0	1
		2	5	0	0
		3	5	0	2
		4	5	0	0

Table 13: Immobilization of the test animals (Experiment II.)

Concentrations [mg/L]		**Replicate**	**Number of treated animals**	**Number of immobilised animals**	
nominal	**calculated**			**24 h**	**48 h**
Control	**0.00**	1	5	0	0
		2	5	0	0
		3	5	0	0
		4	5	0	0
100	**22.26**	1	5	3	5
		2	5	4	5
		3	5	4	5
		4	5	3	5
150	**33.39**	1	5	5	5
		2	5	5	5
		3	5	5	5
		4	5	5	5
225	**50.09**	1	5	5	5
		2	5	5	5
		3	5	5	5
		4	5	5	5
338	**84.64**	1	5	5	5
		2	5	5	5
		3	5	5	5
		4	5	5	5

APPENDIX 3

CONCENTRATION / RESPONSE CURVE (48 h)

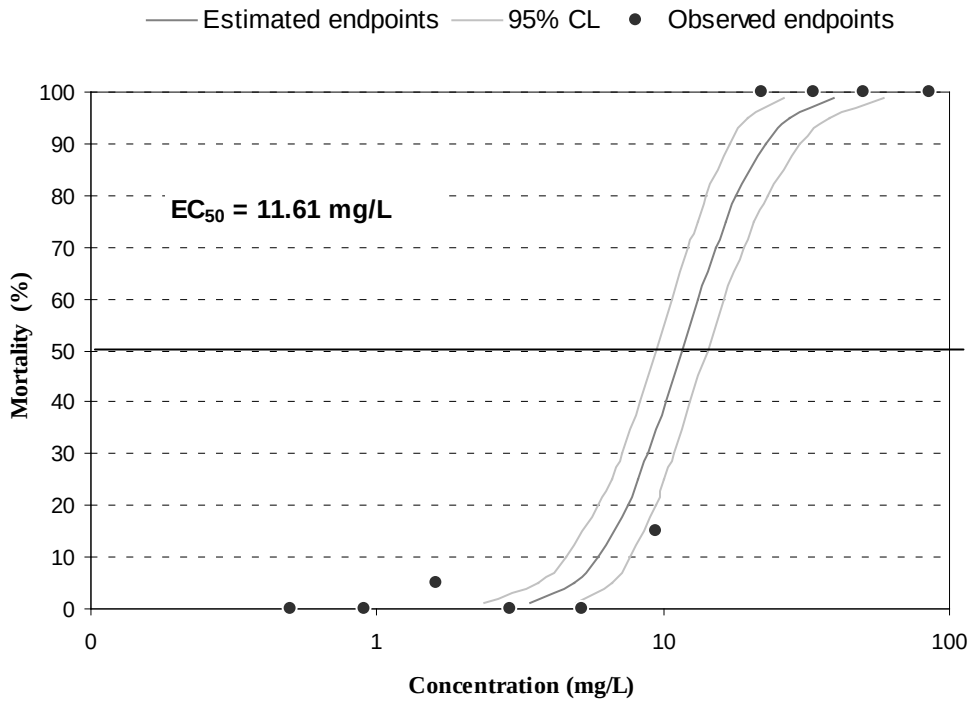

Figure 1: Effect of Ethylene Dibromide Industrial (EDB) on immobilisation

APPENDIX 4

COMPOSITION OF ISO MEDIUM

Separate stock solutions of individual trace elements were first prepared in deionised water. The medium was prepared of these different stock solutions, so it contained all trace elements (combined solution).

Stock solutions (single substance)	Amount added to deionised water	Stock solutions added to deionised water to prepare media
	(g/L)	(ml/L)
$CaCl_2 \times 2\ H_2O$	11.76	25
$MgSO_4 \times 7\ H_2O$	4.93	25
KCl	0.23	25
$NaHCO_3$	2.59	25

APPENDIX 5

ANALYTICAL INVESTIGATIONS OF MAIN STUDY (METHODS AND RESULTS)

1. Principle of the Analytical Method

Concentration of Ethylene Dibromide Industrial (EDB) in the test solutions was determined at the beginning and at the end of the study.

On the first part of the study between August 17 and 19 the concentration of the samples was 4.8, 8.6, 15.4, 27.8, 50 and 90 mg/L. At the start of the test five samples were taken from the test solution. At the end of the test four samples were taken from each of the test vessels. Both occasions one sample was taken from the control solution.

On the second part of the study between August 25 and 27 the concentration was 100, 150, 225 and 338 mg/L. Four samples were taken from the test solutions and one sample was taken from the control solution.

The samples were analysed by an HPLC-UV method.

2. Equipment and Chemicals

2.1. Apparatus

HPLC system:	Merck-Hitachi LaChrom HPLC system: D-7000 Interface, No.: 1442-122 L-7100 HPLC pump, No. : 1516-030 L-7200 Autosampler, No.: 1406-005 L-7400 UV Detector, No.: 1502-017 L-7360 Column Oven, No.: 00107295 L 7614 Degasser, No.: 14412YA0500
Balance:	BP221S Sartorius, No.: 11809117
Ultrasonic bath:	Elmasonic S300H, ELMA, No.: 010890105
Water purification system:	MILLIPORE, DIRECT Q3, FOMNO 7334I
Refrigerator:	Zanussi, No.: ZLKI-262

2.2. Materials

Name:	Ethylene Dibromide Industrial (EDB)
Chemical name:	1,2-Dibromoethane
Batch No.:	510100003
Active component:	>99.94 % 1,2-Dibromoethane (CAS 106-93-4)
Description:	clear to amber liquid
Manufacture date:	February 2010
Expiry date:	February 2011
Storage:	room temperature, protected from light
Safety Precautions:	see Safety Data Sheet
Manufacturer:	Chemtura Manufacturing UK Limited
Address:	Tenax Road, Trafford Park Manchester United Kingdom M17 1WT

Other materials:

Ultra pure water (ASTM Type I):	prepared by Direct-Q 3 system, Millipore
Acetonitrile:	HPLC grade, Fisher Chemical Batch: 0959537

3. Method for the analysis of Ethylene Dibromide Industrial (EDB) content

Samples were analysed by the HPLC-UV method detailed below.

Column:	ACE 5 C18 150 x 4.6 mm, 5µm No.: A78425
Mobil Phase:	ACN : water = 1:1
Flow:	1.0 ml/min.
Injection volume:	20 µl
Temperature:	25 °C
Detector:	UV at 207 nm
Retention time:	5.5 min ± 0.5 min

4. Results of the Method Validation

Table 1: Method validation results (Study code: 10/112-316AN)

Selectivity	No interfering component was observed
Reinjection repeatability (9 injections)	CV% < 2 %
Linear range	0.5 – 50 µg/ml
Limit of Quantification	0.5 µg/ml
Limit of Detection	0.2 µg/ml
Recovery (2 and 100 mg/l)	62 and 83 %
Accuracy	17 and 38 %
Precision	2.0 and 8.4 %
Stability of the samples	At least 51 hours in the autosampler
Stock solution stability	At least 14 days at 5 ± 3 °C
Stability of the test item in Daphnia Test Medium at room temperature (2 and 100 mg/l)	102 and 111 % after two days

5. Measured data

5.1. Calibration

Table 2: Data of the regression lines at the starts and at the ends of the study

Date of measurement	Analytical occasion	Constant	X Coefficient	R. Squared
17 August 2010	Start of the study	56	3924	0.9999
19 August 2010	End of the study	383	3740	0.9990
25 August 2010	Start of the study	-316	3774	0.9995
27 August 2010	End of the study	-247	3787	0.9992

5.2. Results of the Analysis

Table 3: Measured concentrations with the 95% confidence intervals

Sample code	Measured concentrations at the start		Measured concentrations at the end	
	mg/L	Percentage of the nominal	mg/L	Percentage of the nominal
Control	Not detected	-	Not detected	-
4.8 mg/L	0.76 ± 0.07	16	BQL	-
8.6 mg/L	1.40 ± 0.17	16	BQL	-
15.4 mg/L	2.80 ± 0.23	18	BQL	-
27.8 mg/L	5.55 ± 0.32	20	BQL	-
50 mg/L	11.8 ± 0.39	24	BQL	-
90 mg/L	35.2 ± 0.59	39	2.53 ± 0.56	3
100 mg/L	50.8 ± 1.5	51	BQL	-
150 mg/L	77.8 ± 0.6	52	BQL	-
225 mg/L	123 ± 3.6	55	< 20.4	-
338 mg/L	214 ± 6.9	63	< 33.5	-

BQL: below quantitation limit

Analyst

01 Oct. 2010

Date

APPENDIX 6

COPY OF THE GLP CERTIFICATE

ORSZÁGOS GYÓGYSZERÉSZETI INTÉZET
National Institute of Pharmacy

H-1051 Budapest, Zrínyi u. 3.
Mail: 1372 P.O. Box 450.
Phone: +36 1 8869-300
Fax: +36 1 8869-460
E-mail: ogyi@ogyi.hu

Budapest, 20th December 2008
No: 38625/48/2007
Our ref.: Szilvia Karsai
Subject: GLP Certificate

GOOD LABORATORY PRACTICE (GLP) CERTIFICATE

Based on the Inspection report and the discussion of follow up activities it is hereby certified that the test facility

LAB Research Ltd.
H-8201 Veszprém, Szabadságpuszta, Hungary

is able to carry out **Physical-chemical testing, Toxicity studies, Mutagenicity studies, Environmental toxicity studies on aquatic and terrestrial organisms, Studies on behaviour in water, soil and air; bioaccumulation, Bioanalytical, Analytical and clinical chemistry testing** compliance with the Principles of GLP (Good Laboratory Practice).

Date of the inspection: **13-22 October 2008.**

This GLP Certificate is valid for 2 years.

Zsuzsanna Szepezdi, Ph. D.
Director-General