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FINAL REPORT

GROWTH INHIBITION TEST WITH

Ethylene Dibromide Industrial (EDB)

ON ALGAE *(Pseudokirchneriella subcapitata)*

Date of Final Report: 01 October 2010

STUDY CODE: 10/112-022AL

STATEMENT OF THE STUDY DIRECTOR

This study has been performed in accordance with the study plan the OECD Guidelines for Testing of Chemicals (No. 201, 23 March, 2006) and Directive 92/69/EEC, Annex Part C, C.3 and the EPA Health Effects Test Guidelines (OPPTS 850.5400) 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 algal growth inhibition test the calculated endpoints for the effect of Ethylene Dibromide Industrial (EDB) were the following:

Parameter (0-72 h)		Growth rate (r) [mg/L]	Yield (y) [mg/L]	Biomass (b) [mg/L]
EC_{50}	Observed values*	**> 4.48**	**> 4.48**	**> 4.48**
	Theoretically, calculated values**	25.94	6.90	6.87
	95 % conf. limits**	7.20 – 93.44	5.18 – 9.19	5.11 – 9.23
NOEC*		**1.79**	**1.79**	**1.33**
LOEC*		**2.48**	**2.48**	**1.79**

* determined directly from the raw data

** calculated using Probit analysis by TOXSTAT software

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) „Growth inhibition test with Ethylene Dibromide Industrial (EDB) on Algae (*Pseudokirchneriella subcapitata*)" 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: 10/112-022AL

Study Title: Growth inhibition test with Ethylene Dibromide Industrial (EDB) on Algae (*Pseudokirchneriella subcapitata*)

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
26 July 2010	Treatment	26 July 2010	26 July 2010
06 September 2010	Analytical Report	06 September 2010	06 September 2010
08 September 2010	Draft Report	08 September 2010	08 September 2010
01 October 2010	Final Report	01 October 2010	01 October 2010

Signature: Fábián Makovi Éva Date: 01 October 2010

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

GENERAL INFORMATION

STUDY TITLE	:	Growth inhibition test with Ethylene Dibromide Industrial (EDB) on Algae (*Pseudokirchneriella subcapitata*)
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
START OF EXPERIMENT	:	26 July 2010
END OF EXPERIMENT	:	29 July 2010
DRAFT REPORT	:	13 September 2010

BASIS OF STUDY:

- OECD Guideline for Testing of Chemicals, Section 2, No. 201: "Freshwater Alga and Cyanobacteria, Growth Inhibition Test", adopted 23rd March, 2006.
- EPA Ecological Effects Test Guidelines, OPPTS 850.5400, Algal Toxicity, Tiers I and II, EPA 712-C-96-164, Adopted April 1996
- 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 the of the Council on the Registration, Evaluation, Authorisation and Restrictions of Chemicals (REACH), Annex Part C, C.3 (published in the Official Journal of the European Union L 142 of 31 May 2008)

CONTENTS

1. SUMMARY

The effect of Ethylene Dibromide Industrial (EDB) test item was assessed on algal growth using the unicellular green alga *Pseudokirchneriella subcapitata* (*Selenastrum capricornutum)*, over an exposure period of 72 hours.

Based upon the results from the preliminary experiment, nominal concentrations of 9.5 17.1, 30.9, 55.6, 100, and 180 mg/L were examined in the definitive test; the corresponding calculated test item concentrations were: 0.97, 1.33, 1.79, 2.48, 3.26 and 4.48 mg/L.

Because the analysed concentrations deviated more than 20 percent from the nominal concentration throughout the test, the biologically results are based on the geometric mean concentrations calculated from the results of the analytical measurements.

Statistical comparisons of biomass, average specific growth rates and yield in control and in treated groups were carried out using analysis of variance (ANOVA) and Bonferroni t-Test ($\alpha = 0.05$) by TOXSTAT software.

The E_rC_{50}, E_bC_{50} and E_yC_{50} values of the test item and their confidence limits were calculated using Probit analysis by TOXSTAT software (based on the calculated geometric mean concentrations).

The test design included three replicates at each test concentration and six for the untreated control.

The test concentrations were obtained by an appropriate dilution of the stock solution which was continuously shaken for approx. 24h before the start of the test in order to dissolve the test substance in the test medium.

The alga cell concentration was 10^4 cells/mL in all of the test cultures, at the start of the test.

Glass flasks with total capacity of 250 mL were used as test vessels. The volume of the test liquid in the vessels was 100 mL.

The alga cell concentration was determined by microscope in each testing flask during the 72-hour test at 24-hour intervals.

With respect to the inhibitory effect of the test item, the 0-72 h average specific growth rates and yield were significantly different from that of the control group in the concentration range of 2.48 – 4.48 mg/L; the 0-72 h areas were significantly different from that of the control in the concentration range of 1.79 – 4.48 .
The overall NOEC was determined as 1.33 mg/L.

Table 1: Influence of Ethylene Dibromide Industrial (EDB)on the Growth of *Pseudokirchneriella subcapitata*

Parameter (0-72 h)		Growth rate (r) [mg/L]	Yield (y) [mg/L]	Biomass (b) [mg/L]
EC_{50}	Observed values*	**> 4.48**	**> 4.48**	**> 4.48**
	Theoretically, calculated values**	25.94	6.90	6.87
	95 % conf. limits**	7.20 – 93.44	5.18 – 9.19	5.11 – 9.23
NOEC*		**1.79**	**1.79**	**1.33**
LOEC*		**2.48**	**2.48**	**1.79**

* determined directly from the raw data

** calculated using Probit analysis by TOXSTAT software

2. INTRODUCTION

The purpose of the study was to determine the effect of the test item Ethylene Dibromide Industrial (EDB) on the growth of the unicellular green algal species *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum).*

Exponentially growing cultures of *Pseudokirchneriella subcapitata* were exposed to various concentrations of the test item over several generations under defined conditions.

The algal growth in relation to a control culture was determined over a fixed period of 72 hours. The method of application and the test species *Pseudokirchneriella subcapitata* was 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 (OECD medium), the test item solutions used in the experiment were prepared as follows:

A stock solution (nominally 180 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 (see Table 2) and distributed into the appropriate test vessels prior to introduction of algae. There were three replicates for each test item concentration and six for the untreated control.

Table 2: Preparation of test solutions from stock solution

Nominal concentration (mg/L)	**Amount of stock solution** (mL)	**Amount of OECD Medium** (mL)
180	400	--
100	222	q.s. ad 400
55.6	123	q.s. ad 400
30.9	69	q.s. ad 400
17.1	38	q.s. ad 400
9.5	21	q.s. ad 400

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

3.1.3. Untreated Control

Algal growth medium was inoculated with algal cells (without test item) and was examined in parallel to the test item concentrations.

3.1.4. Reference Control

For the evaluation of the quality of the algae and validation of the experimental conditions, Potassium dichromate (Batch Number: 0769128) is tested at least twice a year to demonstrate satisfactory test conditions.

The date of the last study (Study Code: 10/157-022AL) with the reference item Potassium dichromate is: 22 – 25 June 2010.

The E_rC_{50} : 1.00 mg/L, (95 % confidence limits: 0.89 – 1.13 mg/L)
The E_bC_{50} : 0.61 mg/L, (95 % confidence limits: 0.54 – 0.68 mg/L)
The E_yC_{50} : 0.47 mg/L, (95 % confidence limits: 0.43 – 0.52 mg/L)

These values are within the range of laboratory ring test data (see ISO Guideline No. 8692).

3.1.5. Dilution Water

Reconstituted algal growth medium (OECD medium, according to OECD 201) was used as dilution water for both the range finding and definitive tests.

The composition of the growth medium and the chemicals used are given in Appendix 5.

3.2. EXPERIMENTAL ORGANISMS

Species:	*Pseudokirchneriella subcapitata* (Printz-Starr) (formerly known as *Selenastrum capricornutum*)
Strain number:	61.81 SAG (identical strains: CCAP 278/4; UTEX 1648; ATCC 22662) [strain number by isolator: NIVA CHL 1 (O.M. Skulberg, 1959)]
Justification of species:	The species of *Pseudokirchneriella subcapitata* used, being a fast-growing species, is convenient for culturing and testing and is a recommended species by relevant guidelines.
Initial cell number:	The initial cell number in the test cultures was 10^4 cells/mL.
Pre-culturing:	The pre-culture was intended to give an amount of alga suspension suitable for the inoculation of test cultures. The pre-culture was incubated under the conditions of the study in an aerated Algal Growth Medium and used when still exponentially growing (after an incubation period of 3 days). The cell count of above culture was determined by microscopic method and this cell suspension was diluted with Algal Growth Medium to 10^7 cells/mL.

3.3. TEST CONDITIONS

3.3.1. Parameters in the Study

3.3.1.1. Temperature

Culture temperature was checked at the beginning of the study and every 24 hours in a flask filled with water, in the climatic chamber. In addition, water temperature was continuously measured (with a min/max thermometer) within the climate chamber. The temperature was in the range of 22.8 – 23.2 °C measured in the flask and between 22.5 and 23.4 °C measured within the climate chamber.

3.3.1.2. pH

The pH was checked at the beginning and at the end of the study, in the control and each concentration. The pH of the control medium was not increased by more than 1.5 units during the test. The range of the pH was 7.47 – 8.00 at the start and 7.77 – 8.35 at the end of the study.

3.3.1.3. Light Intensity

The algal culture flasks were continuously illuminated. The light intensity at the position occupied by algal culture flasks during the test was about 110 $\mu E/m^2/s$, which was ensured with fluorescent lamps (with a spectral range of 400-700 nm) and it is checked periodically.

The data of test conditions are detailed in Appendix 1.

3.3.2. Equipment and Test Vessels

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

- pH meter
- thermometer
- light-meter
- microscope with counting chamber
- climate chamber
- orbital shaker
- balance

For test vessels all-glass flasks with total capacity of about 250 mL were used. The volume of the test liquid in the vessels: 100 mL.

3.4. DESCRIPTION OF THE TEST PROCEDURE

The exposure time was 72 hours. The test was started (0 hours) by inoculation of a biomass of approximately 10^4 algal cells per mL test medium.

The test was performed with three replicates per test concentration and six replicates in the control group. Volumes of 100 mL algal suspension per replicate in 250 mL Erlenmeyer flasks were continuously shaken by a laboratory orbital shaker to keep algae in suspension. The flasks were covered with air-permeable stoppers.

3.4.1. 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. Algal cells were exposed to each concentration of the test item plus a control, for 72 hours. The test was performed with two replicates per each test concentration and three in the control group.

During the formulation procedure the test solutions were prepared by the method described above* (section 3.1.2.).

* except the concentration of the stock solution (100 mg/L nominal in the range finding test)

The concentration levels used and results (72 h) of the preliminary range-finding test are summarised in the following table.

Table 1: Results of the Preliminary Range-Finding Test

Nominal concentrations [mg/L]	Untreated control	0.01	0.1	1	10	100
Average of cell number at 72 hours (x 10^4cell/mL)	71.00	68.00	68.50	70.00	68.00	54.5

3.4.2. Concentration Levels Investigated in the Main Test

Six concentrations arranged in a geometric series (factor 1.8) and one control group was tested in the main test. The choice of the test item concentrations was based on the results of a preliminary range-finding test.

The nominal concentrations of test item were: 9.5 17.1, 30.9, 55.6, 100, and 180 mg/L.

The corresponding calculated geometric mean exposure concentrations (based on the analytical measurements) were: 0.97, 1.33, 1.79, 2.48, 3.26 and 4.48 mg/L.

The test results are based on the calculated test item concentrations. The analytically measured test item concentrations are detailed in Appendix 4.

3.5. OBSERVATIONS

The cell numbers were determined at 24, 48 and 72 hours after starting the test by manual cell counting using a microscopic method with a counting chamber.

Microscopic observation of the algal cells in each concentration and in the control was performed (at 24h, 48h and 72h) to detect any abnormal appearance of the algae.

3.6. ANALYTICAL MEASUREMENTS

Analytical measurements were performed at the control and at the applied test concentration levels at the start and at the end of the test. Three samples were taken from the test solutions and one sample was taken from the control solution. All samples were analysed directly after sampling.

The samples were analysed by an HPLC-UV method.

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

3.7. RESULTS EVALUATION

3.7.1. Definitions

Cell Density:	the number of cells per mL
Growth:	the increase of cell density over the test period
Biomass (b):	the actual number of cells per volume of medium (cells/mL) calculated as the area under the growth curve (A)
Yield (y):	the cell density at the end of the test minus the starting cell density
Average Specific Growth Rate (μ):	the increase in cell density per time unit
E_bC_{50}:	the calculated concentration of test item which results in a 50 % reduction of biomass (b) relative to the control
E_rC_{50}:	the calculated concentration of test item which results in a 50 % reduction of growth rate (μ) relative to the control
E_yC_{50}:	the calculated concentration of test item which results in a 50 % reduction of yield (y) relative to the control
NOEC:	(No Observed Effect Concentration) the highest test concentration at which no significant inhibition of growth is observed relative to the control
LOEC:	(Lowest Observed Effect Concentration) the lowest test concentration at which a significant inhibition of growth is observed relative to the control

3.7.2. Calculation of Average Specific Growth Rate

Concentration-effect relationship was calculated by comparing growth rates in control, test cultures in the following way.

The average specific growth rate (μ) for individual cultures are calculated from the following relationship:

$$\mu = \frac{\ln(N_n) - \ln(N_0)}{t_n - t_0}$$

Where $\ln(N_n)$ = natural logarithm of measured number of cells/mL at time t_n
$\ln(N_0)$ = natural logarithm of measured number of cells/mL at time t_0
t_0 = time (hour) of the beginning of the test
t_n = time (hour) of n^{th} measurements after the beginning of the test

The percentage inhibition of growth rate (% I_μ):

$$\%\, I_\mu = \frac{\mu_c - \mu_t}{\mu_c} \cdot 100\ \%$$

Where $\%\, I_\mu$ = percent inhibition in average specific growth rate
μ_c = mean growth rate of the control
μ_t = mean growth rate of test concentration t

3.7.3. Calculation of Area Under the Growth Curve

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

Where N_0 = nominal number of cells/mL at time t_0 (start of the test)
N_1 = mean measured number of cells/mL at t_1 (24 hours)
N_2 = mean measured number of cells/mL at t_2 (48 hours)
N_n = mean measured number of cells/mL at t_n
t_1 = time of first measurement after start of the test
t_2 = time of second measurement after start of the test
t_n = time of n^{th} measurement after start of the test

The percentage inhibition of area (% I_A):

$$\%\ I_A = \frac{A_c - A_t}{A_c} \cdot 100\ \%$$

Where $\%\, I_A$ = percent inhibition in area under the growth curve
A_c = mean area of the control
A_t = mean area of test concentration t

3.7.4. Calculation of Yield

Yield is calculated as the biomass at the end of the test minus the starting biomass for each single vessel of controls and treatments. For each test concentration and control, mean yield values were calculated.

Percent inhibition in yield (% I_y):

$$I_y = \frac{y_c - y_i}{y_c} \cdot 100\ \%$$

where: y_c = mean value for yield in the control group
y_i = mean value for yield for the test concentration

Area under the growth curve (biomass), average specific growth rate and yield were calculated for each test flask. Then the mean area under the growth curve, the growth rate and mean yield were determined as arithmetic mean value over all test flasks per treatment.

3.7.5. Statistical Analysis

The section-by-section specific growth rates in the control cultures were assessed (calculated as the specific growth rates for each day during the course of the test (days 0-1, 1-2 and 2-3) and to demonstrate exponential growth for the entire study period.

The inhibition of alga growth was determined from the biomass (area under the growth curves, A), the average specific growth rate (r) and from the yield (y). Mean values and standard deviations were calculated for each concentration at the start, and at the end of the test using Excel for Windows software (Microsoft Co./One Microsoft Way/Redmond, WA 98052-6399).

The E_rC_{50}, E_bC_{50} and E_yC_{50} values of the test item and their confidence limits were calculated using Probit analysis by TOXSTAT software (based on the calculated geometric mean concentrations).

Statistical comparisons of biomass, average specific growth rates and yield in controls and in the treated groups were carried out using analysis of variance (ANOVA) and Bonferroni t-Test ($\alpha = 0.05$) by TOXSTAT software.

For the determination of the LOEC and NOEC, the calculated mean biomass, growth rates and yield at the test concentrations were tested on significant differences to the control values by Bonferroni t-Test.

3.8. ARCHIVES

The study documents:

- study plan and amendment,
- all raw data,
- sample of test item,
- study report and any amendment
- 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.

3.9. DEVIATION FROM THE STUDY PLAN

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 180 mg/L (nominal) was prepared
Reason for this change:	Typing error
Presumed Effect on the Study:	None

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:	Date of Draft Report
According to the Study Plan:	02 September 2010
Deviation:	13 September 2010
Reason for this change:	Unscheduled delay
Presumed Effect on the Study:	None

3.10. VALIDITY CRITERIA OF THE STUDY

The cell density in the control cultures, increased by a factor of more than 16 within 72 hours.

The mean coefficient of variation for section-by-section specific growth rates in the control cultures did not exceed 35 % during the course of the test (days 0-1; 1-2; 2-3).

The section-by-section growth rates remained nearly constant indicating the exponential growth for the entire study period.

The coefficient of variation of average specific growth rates during the whole test period in the control cultures did not exceed 7 %.

All validity criteria were met, therefore the study can be considered as valid.

3.11. REFERENCES

1. OECD Guideline for Testing of Chemicals, Section 2, No. 201: "Freshwater Alga and Cyanobacteria, Growth Inhibition Test", adopted 23rd March, 2006.
2. 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)
3. EPA Ecological Effects Test Guidelines, OPPTS 850.5400, Algal Toxicity, Tiers I and II, EPA 712-C-96-164, Adopted April 1996
4. Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004
5. OECD Principles of Good Laboratory Practice, adopted by Council on 26th November 1997 [C(97)186/Final], Environment Directorate, Organisation for Economic Cooperation and Development, ENV/MC/CHEM(98)17, Paris 1998
6. 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 ENV/MC/CHEM (98) 17
7. 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.

4. RESULTS

4.1. CONCENTRATIONS OF THE TEST ITEM

The nominal concentrations of test item were: 9.5 17.1, 30.9, 55.6, 100, and 180 mg/L.

The analytically measured concentrations at the start of the test were: 4.68, 8.87, 16.1, 30.7, 53.3 and 100.5 mg/L.

The measured concentrations were below the Limit of Detection (LOD = 0.2 mg/L) at each tested concentration level at the end of the experiment.

In order to calculate the mean exposure concentrations, the final concentrations below the detectable range were taken as the limit of detection (according to OECD 23).

The calculated mean exposure concentrations (based on the analytical measurements) were: 0.97, 1.33, 1.79, 2.48, 3.26 and 4.48 mg/L.

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.

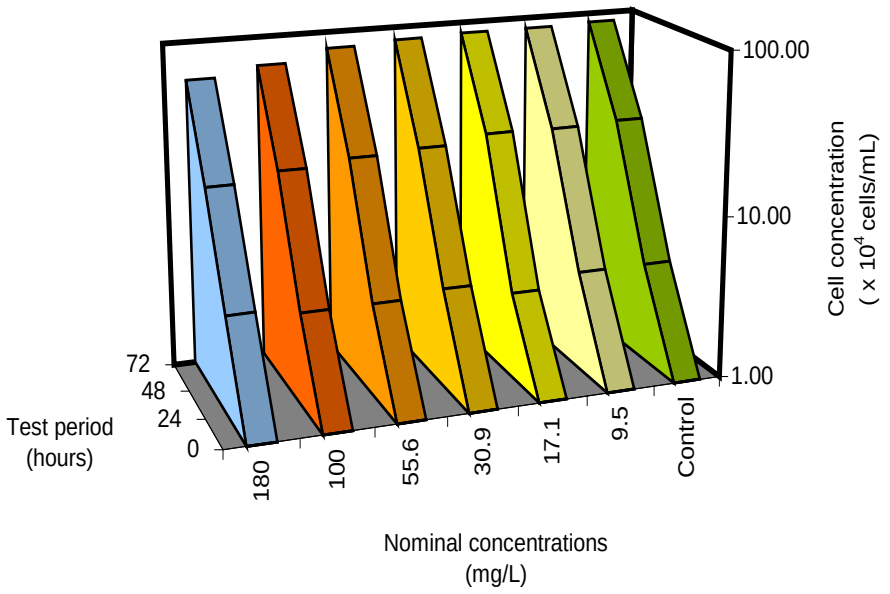
The concentration decrease may be additionally explained by hydrolysis, because the hydrolysis of the test substance can increase at higher or lower pH from neutral (see: “Determination of the Hydrolysis of Ethylene Dibromide Industrial (EDB) as a Function of pH”; LAB code: 10/112-336AN). The range of the pH of the present test was 7.47 – 8.00 at the start and 7.77 – 8.35 at the end of the study (see Appendix 1; Table 8).

Full details of the analytical method and results are given in Appendix 4.

4.2. CELL NUMBERS

The cell number in each flask was determined at the 24th, 48th, 72nd hours. The results of determinations are listed in Appendix 2.

Figure 1: Mean Values of Cell Numbers Plotted against Time



4.3. AVERAGE SPECIFIC GROWTH RATES

Table 4: Growth Rates (μ) and Percentage Inhibition of μ during the Test Period

Concentration		**Growth rate μ and % inhibition of μ**					
Nominal	**Calculated**	**0–24 h**		**0–48 h**		**0–72 h**	
mg/L	mg/L	**μ**	**%**	**μ**	**%**	**μ**	**%**
Control	**0.0**	0.0569	–	0.0672	–	0.0622	–
9.5	**0.97**	0.0569	0.0	0.0665	1.0	0.0618	0.6
17.1	**1.33**	0.0498	12.5	0.0668	0.6	0.0618	0.6
30.9	**1.79**	0.0569	0.0	0.0644*	4.2	0.0614	1.2
55.6	**2.48**	0.0538	5.5	0.0634*	5.6	0.0607*	2.3
100	**3.26**	0.0529	7.0	0.0617*	8.2	0.0584*	6.1
180	**4.48**	0.0569	0.0	0.0590*	12.2	0.0565*+	9.0

* : statistically significantly different compared to the control values (Bonferroni t-Test; α = 0.05)

+ : at these values the rounding of the EXCEL and TOXSTAT software was different. The table contains the values calculated with EXCEL. The TOXSTAT rounding was: 0.0566.

The results of the statistical evaluation (based on Bonferroni t-Test; α=0.05) show that the 0-72 h average specific growth rates were statistically significantly different from the untreated control value in the concentration range of 2.48 – 4.48 mg/L. The 72 hours No-Observed Effect Concentration related to specific growth rates was determined as 1.79 mg/L.

4.4. AREAS UNDER THE GROWTH CURVES

Table 5: Area under the Growth Curves (A) and Percentage Inhibition of A during the Test Period

Concentration		Areas under the Growth Curves (A) and % inhibition of A					
Nominal	Calculated	0–24 h		0–48 h		0–72 h	
mg/L	mg/L	μ	%	μ	%	μ	%
Control	**0.0**	36.0	–	362.0	–	1694.0	–
9.5	**0.97**	36.0	0.0	352.0	2.8	1648.0	2.7
17.1	**1.33**	28.0	22.2	340.0	6.1	1640.0	3.2
30.9	**1.79**	36.0	0.0	324.0	10.5	1564.0	7.7*
55.6	**2.48**	32.0	11.1	304.0	16.0*	1484.0	12.4*
100	**3.26**	32.0	11.1	284.0	21.5*	1296.0	23.5*
180	**4.48**	36.0	0.0	264.0	27.1*	1148.0	32.2*

* : statistically significantly different compared to the control values (Bonferroni t-Test; $\alpha = 0.05$)

The areas under the growth curves were used to represent biomass. The results of the statistical evaluation (based on Bonferroni t-Test; α=0.05) show that the 0-72 h areas were statistically significantly different from the untreated control value in the concentration range of 1.79 – 4.48 mg/L. The 72 hours No-Observed Effect Concentration related to biomass was determined as 1.33 mg/L.

4.5. YIELD

Table 6: Yield (Y) and Percentage Inhibition of Y during the Test Period

Concentration		Yield (Y) and % inhibition of Y	
Nominal	Calculated	0–72 h	
mg/L	mg/L	Y	%
Control	**0.0**	86.8	–
9.5	**0.97**	84.7	2.5
17.1	**1.33**	84.7	2.5
30.9	**1.79**	82.3	5.2
55.6	**2.48**	78.3	9.8*
100	**3.26**	66.0	24.0*
180	**4.48**	57.7	31.9*

* : statistically significantly different compared to the control values (Bonferroni t-Test; $\alpha = 0.05$)

The results of the statistical evaluation (based on Bonferroni t-Test; α=0.05) show that the 0-72 h yield was statistically significantly different from the untreated control value in the concentration range of 2.48 – 4.48 mg/L. The 72 hours No-Observed Effect Concentration related to the yield was determined as 1.79 mg/L.

5. DISCUSSION AND CONCLUSION

The effect of Ethylene Dibromide Industrial (EDB) test item was assessed on algal growth using the unicellular green alga *Pseudokirchneriella subcapitata* (*Selenastrum capricornutum)*, over an exposure period of 72 hours.

Based upon the results from the preliminary experiment, nominal concentrations of 9.5 17.1, 30.9, 55.6, 100, and 180 mg/L were examined in the definitive test; the corresponding calculated test item concentrations were: 0.97, 1.33, 1.79, 2.48, 3.26 and 4.48 mg/L.

Because the analysed concentrations deviated more than 20 percent from the nominal concentration throughout the test, the biologically results are based on the concentrations calculated from the results of the analytical measurements.

Statistical comparisons of biomass, average specific growth rates and yield in control and in treated groups were carried out using analysis of variance (ANOVA) and Bonferroni t-Test ($\alpha = 0.05$) by TOXSTAT software.

The E_rC_{50}, E_bC_{50} and E_yC_{50} values of the test item and their confidence limits were calculated using Probit analysis by TOXSTAT software (based on the calculated geometric mean concentrations).

With respect to the inhibitory effect of the test item, the 0-72 h average specific growth rates and yield were significantly different from that of the control group in the concentration range of 2.48 – 4.48 mg/L; the 0-72 h areas were significantly different from that of the control in the concentration range of 1.79 – 4.48 .
The overall NOEC was determined as 1.33 mg/L.

Table 7: Influence of Ethylene Dibromide Industrial (EDB)on the Growth of *Pseudokirchneriella subcapitata*

Parameter (0-72 h)		Growth rate (r) [mg/L]	Yield (y) [mg/L]	Biomass (b) [mg/L]
EC_{50}	Observed values*	**> 4.48**	**> 4.48**	**> 4.48**
	Theoretically, calculated values**	25.94	6.90	6.87
	95 % conf. limits**	7.20 – 93.44	5.18 – 9.19	5.11 – 9.23
NOEC*		**1.79**	**1.79**	**1.33**
LOEC*		**2.48**	**2.48**	**1.79**

* determined directly from the raw data

** calculated using Probit analysis by TOXSTAT software

A P P E N D I C E S

APPENDIX 1

TEST CONDITIONS

Table 8: Test Conditions measured during the Main Experiment

Parameter	Test group	Measurement (hours) 0	24	48	72
pH	Control	8.00			8.32
					8.20
					8.35
					8.30
					8.34
					8.17
	9.5	7.70			8.19
					8.20
					8.12
	17.1	7.58			7.95
					8.10
					7.89
	30.9	7.59			7.98
					8.07
					8.12
	55.6	7.56			7.90
					7.82
					8.05
	100	7.51			7.77
					7.91
					8.02
	180	7.47			7.88
					7.99
					7.90
Temperature (ºC) *		22.8	22.9	23.1	23.2
Min / Max temperature (ºC) of the climate chambers		22.5 / 23.1	22.6 / 23.0	22.5 / 23.4	22.7 / 23.4

* : temperature in the measuring flask (same as every test flask)

APPENDIX 2

CELL NUMBER

Table 9: Cell Number (x 10^4 cell/mL) determined in the Main Experiment

Concentration			**Number of cells**			
Nominal mg/L	**Calculated** mg/L		**0 h**	**24 h**	**48 h**	**72 h**
Control	**0.0**		1	4	25	86
			1	3	24	91
			1	5	26	88
			1	5	26	85
			1	3	25	90
			1	4	25	87
		Mean	**1.00**	**4.00**	**25.17**	**87.83**
		SD	0.0	0.9	0.8	2.3
9.5	**0.97**		1	4	25	86
			1	5	24	83
			1	3	24	88
		Mean	**1.00**	**4.00**	**24.33**	**85.67**
		SD	0.0	1.0	0.6	2.5
17.1	**1.33**		1	4	24	85
			1	3	26	85
			1	3	24	87
		Mean	**1.00**	**3.33**	**24.67**	**85.67**
		SD	0.0	0.6	1.2	1.2
30.9	**1.79**		1	5	22	83
			1	4	23	86
			1	3	21	81
		Mean	**1.00**	**4.00**	**22.00**	**83.33**
		SD	0.0	1.0	1.0	2.5
55.6	**2.48**		1	3	21	82
			1	4	22	76
			1	4	20	80
		Mean	**1.00**	**3.67**	**21.00**	**79.33**
		SD	0.0	0.6	1.0	3.1
100	**3.26**		1	5	19	64
			1	3	20	72
			1	3	19	65
		Mean	**1.00**	**3.67**	**19.33**	**67.00**
		SD	0.0	1.2	0.6	4.4
180	**4.48**		1	4	17	61
			1	5	16	58
			1	3	18	57
		Mean	**1.00**	**4.00**	**17.00**	**58.67**
		SD	0.0	1.0	1.0	2.1

APPENDIX 3

CONCENTRATION /EFFECT RELATIONSHIP GRAPHS OF THE TEST ITEM

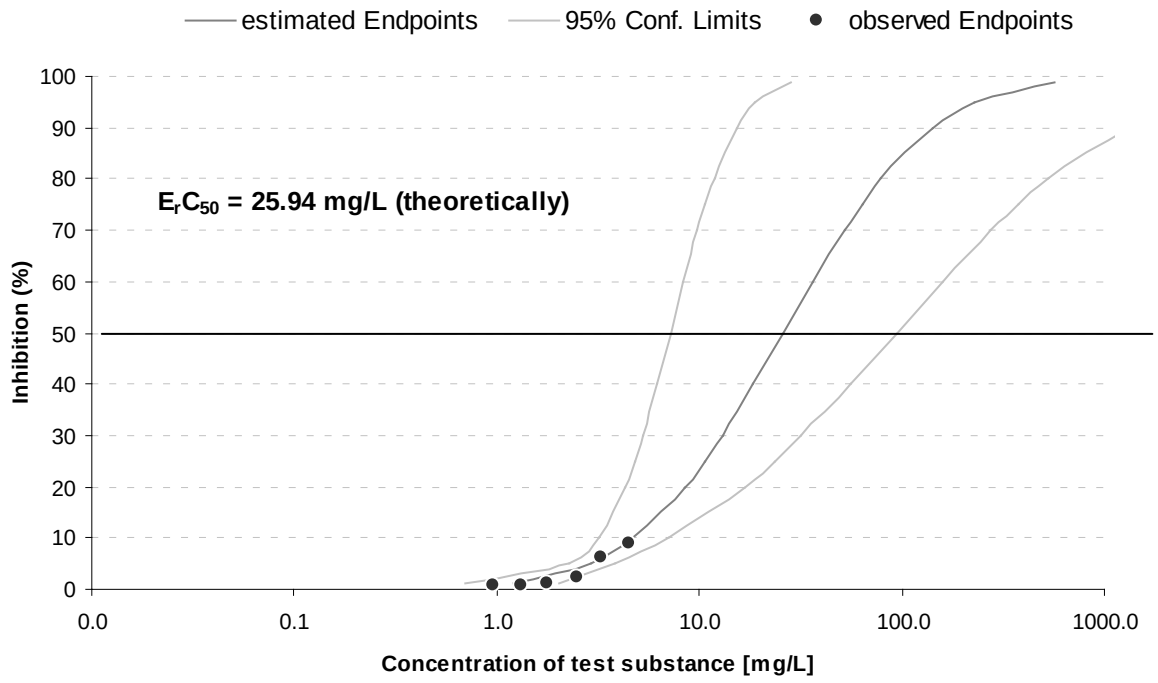


Figure 2: Effect of Ethylene Dibromide Industrial (EDB) on Average Specific Growth Rates

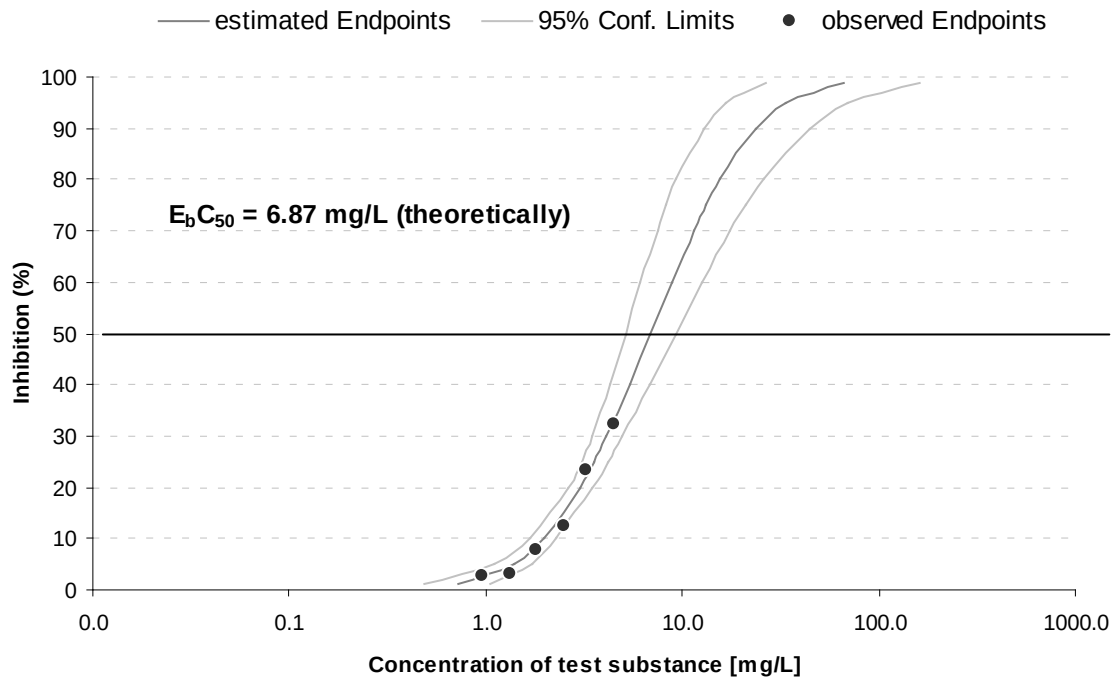


Figure 3: Effect of Ethylene Dibromide Industrial (EDB) on Biomass

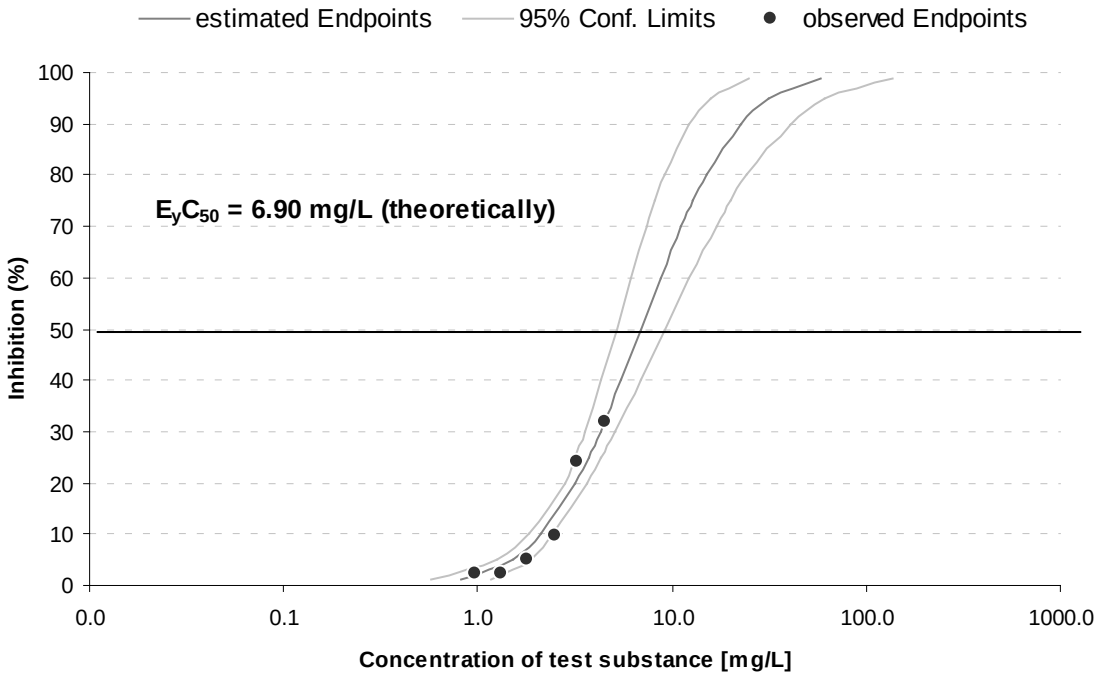


Figure 4: Effect of Ethylene Dibromide Industrial (EDB) on Yield

APPENDIX 4

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.

Three 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 Detection	0.2 µg/ml
Limit of Quantification	0.5 µg/ml
Recovery (2 and 100 mg/l)	75 and 88 %
Accuracy	12 and 25 %
Precision	3.1 and 4.8 %
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 Alga Test Medium at room temperature (2 and 100 mg/l)	117 and 109 % after three days

5. Measured data

5.1. Calibration

Table 2: Data of the regression lines at the start and at the end of the study

Date of measurement	Analytical occasion	Constant	X Coefficient	R. Squared
26 July 2010	Start of the study	411	3367	0.9994
29 July 2010	End of the study	-139	3801	0.9999

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	-
9.5 mg/L	4.68±0.48	49	Not detected	-
17.1 mg/L	8.87 ± 0.89	52	Not detected	-
30.9 mg/L	16.1 ± 2.04	52	Not detected	-
55.6 mg/L	30.7 ± 4.92	55	Not detected	-
100 mg/L	53.3 ± 13.77	53	Not detected	-
180 mg/L	100.5 ± 1.72	56	Not detected	-

Analyst

01 Oct. 2010

Date

APPENDIX 5

COMPOSITION OF OECD MEDIUM

Separate stock solutions were first prepared in deionised water. The growth medium was prepared by adding an appropriate volume of these different stock solutions to deionised water in order to achieve the final concentrations.

Stock solution	Substance	Final concentration in the prepared growth medium
Stock solution 1 (macro nutrients)	NH_4Cl	15.0 mg/L
	$MgCl_2 \times 6\ H_2O$	12.0 mg/L
	$CaCl_2 \times 2\ H_2O$	18.0 mg/L
	$MgSO_4 \times 7\ H_2O$	15.0 mg/L
	KH_2PO_4	1.6 mg/L
Stock solution 2 (iron)	$FeCl_3 \times 6\ H_2O$	64.0 µg/L
	$Na_2EDTA \times 2H_2O$	100.0 µg/L
Stock solution 3 (trace elements)	H_3BO_3	185.0 µg/L
	$MnCl_2 \times 4\ H_2O$	415.0 µg/L
	$ZnCl_2$	3.0 µg/L
	$CoCl_2 \times 6\ H_2O$	1.5 µg/L
	$CuCl_2 \times 2\ H_2O$	0.01 µg/L
	$Na_2MoO_4 \times 2\ H_2O$	7.0 µg/L
Stock solution 4 (bicarbonate)	$NaHCO_3$	50.0 mg/L

APPENDIX 6

COPY OF THE GLP CERTIFICATE

ORSZÁGOS GYÓGYSZERÉSZETI INTÉZET
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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