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October 11, 2013

Document Control Office (DCO) (7407M)
Office of Pollution Prevention and Toxics (OPPT)
US EPA
1200 Pennsylvania Ave., NW
Washington, DC 20460-0001

Attention: Mr. John Schaeffer
TSCA Section 4
40 CFR 799.5089
Docket ID Number EPA-HQ-OPPT-2009-0112

RE: Testing of Certain High Production Volume Chemicals; Third Group of Chemicals
2-Oxiranemethanamine, N-[4-(2-oxiranylmethoxy)phenyl]-N-(2-oxiranylmethyl)- (CAS No. 5026-74-4)

Dear Mr. Schaeffer:

Enclosed please find the final reports for the following studies on 2-Oxiranemethanamine, N-[4-(2-oxiranylmethoxy)phenyl]-N-(2-oxiranylmethyl)- (CAS No. 5026-74-4):

Determination of Physico-Chemical Properties
Ready Biodegradability (OECD 301 B)
Fresh Water Algal Growth Inhibition Test (OECD 201)
Daphnia magna, Reproduction Test (OECD 211)
96-hour Acute Toxicity Study in Carp (OECD 203)
Pseudomonas Cell Multiplication Inhibition Test

Huntsman previously committed to performing both a Reproduction/Developmental Toxicity Screening Test and a Repeated Dose 28-Day Oral Toxicity Study in rodents, however, because preliminary results from the Repeated Dose 28-Day Oral Toxicity Study in rodents on a surrogate, (m-(2,3-epoxypropoxy)-N-N-bis(2,3-epoxypropyl)aniline, CAS # 71604-74-5, resulted in a No Observed Adverse Effect Level (NOAEL) of 50 mg/kg/day based on atrophy of the female reproductive organs observed at 150 mg/kg/day, Huntsman determined the Reproduction/Developmental Toxicity Screening Test was not needed. The data generated from the 28-day study is being used as read across for the test rule substance, 5026-74-4. The final report will be sent once received. Enclosed is a letter sent to the TSCA 8(e) Coordinator for your review.

Please do not hesitate to contact me at the number listed below if additional information is required.

Sincerely,

Lynne Oreschnigg
Regulatory Compliance Specialist
Huntsman Corporation
Phone: 281-719-3021
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lynne_oreschnigg@huntsman.com

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April 30, 2013

Via Overnight Courier

TSCA Confidential Business Information Center (7407M)
EPA East – Room 6428
US Environmental Protection Agency
1201 Constitution Ave, NW
Washington, DC 20004-3302
Attention: TSCA Section 8(e) Coordinator

Draft Final Report of a Repeated Dose 28 Day Oral Toxicity Study with m-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl) aniline, CAS# 71604-74-5.

Dear Sir or Madam:

The European office of Huntsman Advanced Materials (Huntsman) has received a draft final report from a Repeated Dose 28 Day Oral Toxicity Study with m-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline, CAS# 71604-74-5. This study was conducted using a standard OECD 407 protocol by WIL Research Europe B.V. in the Netherlands. The results of this repeated dose study have established a No Adverse Effect Level (NOAEL) at 50 mg/kg/day, based on observed atrophy of female reproductive organs at the next highest dose level, 150 mg/kg/day.

Huntsman is submitting this information pursuant to Section 8(e) of the Toxic Substances Control Act (TSCA). Huntsman has not made a determination as to whether a significant risk of injury to human health or the environment is actually presented by these findings.

Study Design:
The test substance was administered, by gavage, to groups of 5 male and 5 female Wistar rats over 28 consecutive days. Dose levels used in this study, formulated in propylene glycol, were 0, 50, 150, and 450 mg/kg/day, with the untreated control group receiving only propylene glycol. The following study parameters were evaluated: daily clinical signs; functional observation battery at study week 4, body weights and food consumption determined weekly; clinical pathology and macroscopic evaluation at study termination, and organ weights and histopathology evaluations on specified tissues.

Study Findings:
- No mortality occurred during this study. However, a poor health status was noted for all animals at the highest dose level of 450 mg/kg/day. Although the functional observation battery (FOB) revealed no effects on hearing, pupil reflex, righting reflex and grip

strength, the motor activity measurement showed lower activity (total movements and ambulations) at 450 mg/kg/day.

- Animals administered 450 mg/kg/day showed lower body weight gains and reduced body weights compared to control animals. As a result of the poor health status of the animals at 450 mg/kg/day, changes were observed in hematology, clinical biochemistry, organ weights and histopathology. These findings are considered to be a secondary effect unrelated to any specific systemic toxicity of the test substance.
- Atrophy of the uterus, cervix and vagina were observed at 150 mg/kg/day accompanied by the macroscopically visible reduced size of ovaries and uterus at 450 mg/kg/day. In males at 450 mg/kg/day, the prostate gland was considered to be small but with normal histology. At this level, the contents in the seminal vesicles and coagulating glands were considered to be reduced. A lower absolute and/or relative prostate and seminal vesicle weight was noted at the lowest dose level of 50 mg/kg/day, but these observations were not supported by any histological findings, and are not considered to be toxicologically relevant.
- The effects on liver and kidney were noted at 450 mg/kg/day: centrilobular hypertrophy in the liver, discoloration of kidney and adrenals and enlarged adrenal glands in females, with microscopically observed vacuolization of the zona fasciculata in the adrenal glands.
- Effects were observed in the gastrointestinal tract and associated lymphatic system, but these effects are considered to be secondary findings related to the physical properties of the test material and not due to any specific systemic toxicity of the test substance.

Study Conclusion:
Based on the results of this study, the No Observed Adverse Effect Level (NOAEL) for Wistar rats exposed to 2-Propanol, 1-[bis[3-(dimethylamino)propyl]amino]- over 28 consecutive days is considered to be 50 mg/kg/day, based on atrophy of the female reproductive organs observed at 150 mg/kg/day.

As always, if I can provide any additional information on the above study, please call me at (281) 719-3017, or contact me via e-mail at: Ray_Papciak@Huntsman.com.

Regards,

Raymond J. Papciak
Manager, Product Safety

FINAL REPORT

Study Title

DETERMINATION OF PHYSICO-CHEMICAL PROPERTIES OF P-(2,3-EPOXYPROPOXY)-N,N-BIS(2,3-EPOXYPROPYL)ANILINE

- ***Validation of an analytical method***
- **Melting and boiling temperature**
- **Density**
- **Vapour pressure**
- **Water solubility**
- **Flash-point**
- ***Auto-ignition temperature***
- **Hydrolysis as a function of pH**
- **Oxidizing properties**
- **Adsorption coefficient**
- **Dissociation constant**
- **Viscosity**

Author

Dr. Ir. E. Baltussen

Test Facility

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Laboratory Project Identification

NOTOX Project 496981
NOTOX Substance 203277/A

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2. STATEMENT OF GLP COMPLIANCE

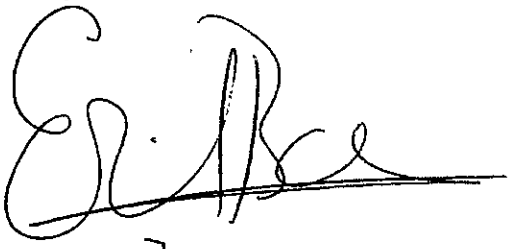
NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Co-operation and Development (OECD), OECD Principles on Good Laboratory Practice (GLP) (as revised in 1997), ENV/MC/CHEM (98) 17.

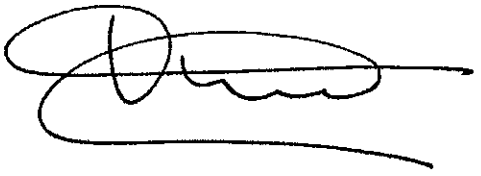
The sponsor is responsible for Good Laboratory Practice (GLP) compliance for all test substance information unless determined by NOTOX.

Dr. Ir. E. Baltussen
Study Director



Date: 19 January 2012

Dr. Ir. T.H.M. Noij
Head of Chemistry



Date: 18 January 2012

3. QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the NOTOX Quality Assurance Unit (QAU) to confirm that the methods and results accurately and completely reflect the raw data.

During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

Type of Inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date
Study	Protocol	19-Aug-11	19-Aug-11	19-Aug-11
	Protocol Amendment 01	15-Nov-11	15-Nov-11	15-Nov-11
	Analysis	17-Nov-11	17-Nov-11	17-Nov-11
	Observations /measurements chemical properties	17-Nov-11	17-Nov-11	17-Nov-11
	Report	17-Dec-11	18-Dec-11	19-Dec-11
	Protocol Amendment 02	11-Jan-12	11-Jan-12	11-Jan-12
Process	**Analytical and physical chemistry** Test Substance Handling Observations/Measurements	08-Aug-11	16-Aug-11	16-Aug-11
	Analytical and physical chemistry Test Substance Handling Observations/Measurements	31-Oct-11	10-Nov-11	10-Nov-11

NOTOX B.V.

C.J. Mitchell B.Sc.
Head of Quality Assurance

Date: 19-Jan-2012

4. SUMMARY

The results of the physico-chemical properties of the test substance are given below.

Parameter	Guideline(s)	Result	Comment
Analytical method	Sanco 3029	successfully validated	
Melting temperature	EC A.1 OECD 102	-39°C (234K)	Glass transition
Boiling temperature	EC A.2 OECD 103	no boiling temperature	reaction and/or decomposition of the test substance at > 250°C (> 523K) was observed
Density	EC A.3 OECD 109	D_4^{20} : 1.22	
Vapour pressure	EC A.4 OECD 104	20°C 5.5×10^{-7} Pa = 4.1×10^{-9} mm Hg 25°C 1.3×10^{-6} Pa = 9.9×10^{-9} mm Hg	isothermal TGA effusion method
Water solubility	EC A.6 OECD 105	3.34 g/l	at 20°C by the flask method
Flash-point	EC A.9 UN p. 32.4.1 ISO 2719 ASTM D 93	228°C	Pensky-Martens closed cup method
Auto-ignition temperature	EC A.15 DIN 51794 IEC 79-4	410°C	at 1012.6 – 1013.9 hPa
Oxidizing properties	EC A.21 UN O.2	not oxidizing	statement
Hydrolysis as a function of pH	EC C.7 OECD 111	pH 4: $t_{½}$ = 2.2 days pH 7: $t_{½}$ = 2.3 days pH 9: $t_{½}$ = 2.6 days	at 25°C

Adsorption coefficient	EC C.19 OECD 121	log K_{oc} : 1.93	HPLC method based on soil-adsorption-reference data
Dissociation constant	OECD 112	pK_a value : 3.25 (basic)	spectrophotometric method
Viscosity	OECD 114 ISO 3104 ASTM D 445-09	20°C: 840.6 mm^2/s 40°C: 138.9 mm^2/s	

5. INTRODUCTION

5.1. Preface

Sponsor	Huntsman Advanced Materials GmbH Klybeckstrasse 200 4057 BASEL Switzerland
Study Monitor	Dr. H. Wilbers
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Dr. Ir. E. Baltussen
Study Plan	Start : 24 August 2011 Completion : 01 December 2011

5.2. Aim of the study

The aim of the study was to determine the following physico-chemical properties for p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline:

- Validation of an analytical method
- Melting and boiling temperature
- Density
- Vapour pressure
- Water solubility
- Flash-point
- Auto-ignition temperature
- Hydrolysis as a function of pH
- Oxidizing properties
- Adsorption coefficient
- Dissociation constant
- Viscosity

5.3. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report are retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. NOTOX will retain information concerning decisions made.

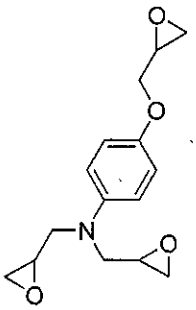
Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

6. MATERIALS

6.1. Test substance

6.1.1. Test substance information

Identification	p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline
Structure	
Molecular formula	$C_{15}H_{19}NO_4$
Molecular weight	277.3
CAS Number	5026-74-4
EC Number	225-716-2
Description	Yellow liquid
Batch	AA00373400
Purity	94.33%
Test substance storage	In refrigerator (2-8°C) in the dark
Stability under storage conditions	Stable
Expiry date	28 February 2013

6.1.2. Study specific test substance information

There was no study specific test substance information necessary for this study.

6.2. Electronic data capture

System control, data acquisition and data processing were performed using the following programmes:
- Cary WinUV pharma version 3.1 (Varian, Mulgrave, Australia)
- Empower version 7.00 (Waters, Milford, MA, USA)
- Advantage Integrity Software version 3.0 (TA Instruments, New Castle, DE, USA)

Temperature and/or relative humidity and/or pressure during sample storage and/or performance of the studies were monitored continuously using the following programme:
- REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA)

6.3. List of deviations

6.3.1. List of protocol deviations

1. *Chapter 13 "Hydrolysis as a function of pH", paragraph 13.4 "Performance of the study"*: During the main studies - Tier 2 at all pH values at 20°C, the temperature was controlled within ± 0.6°C (criterion was ± 0.5°C).

   Evaluation: The temperature ranges were slightly higher than the criterion. This was considered not to be of significantly influence on the determined half-life time of the test substance at the temperatures.

The study integrity was not adversely affected by the deviations.

6.3.2. List of standard operating procedures deviations

Any deviations from standard operating procedures (SOPs) were evaluated and filed in the study file. There were no deviations from SOPs that affected the integrity of the study.

7. VALIDATION OF AN ANALYTICAL METHOD

7.1. Guideline

The study was based on the following guideline:

European Commission: Guidance for Generating and Reporting Methods of Analysis in Support of Pre-registration Data Requirements for Annex II (Part A, section 4) and Annex III (Part A, section 5) of Directive 91/414, SANCO/3029/99 rev. 4 (11/07/00).

7.2. Reagents

Water	Tap water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA)
Acetonitrile	Biosolve, Valkenswaard, The Netherlands.

All reagents were of analytical grade, unless specified otherwise.

7.3. Performance of the study

A high performance liquid chromatographic method with spectrophotometric detection (HPLC-UV) for the quantitative analysis of the test substance in water was developed. Validation of the analytical method was performed for the following parameters:

Specificity
A test substance solution and blank accuracy sample were analysed by single injection. The analytical method was found to be specific if the blank chromatogram showed no response for the test substance or a response of < 30% of the limit of quantification.

Linearity
Calibration solutions were analysed in duplicate. The response of the calibration solutions was correlated with concentration using regression analysis with a 1/concentration^2 weighting factor. A calibration curve with a coefficient of correlation (r) of > 0.99 and back calculated accuracies of the calibration solutions in the range 85-115% was accepted.

Accuracy and repeatability
Accuracy samples were analysed by single injection into the analytical system. The analytical method was considered applicable for the determination of the test substance if the mean accuracy was in the range 70-110% and the coefficient of variation was ≤ 20%.

Limit of quantification
The limit of quantification (LOQ) is defined as the lowest concentration level at which an accuracy in the range 70-110% and a repeatability of ≤ 20% is demonstrated. The LOQ was obtained from the data of the accuracy- and repeatability test.

Stability of the analytical system and end solutions
Calibration solutions were injected throughout the validation sequence including the beginning and end. The analytical system and/or end solutions were found to be stable if the coefficient of variation on the responses of the solutions was ≤ 20%.

Stability of stock solutions
Stock solutions of the test substance were stored at room temperature for at least 12 hours. Additional calibration solutions were prepared and analysed by single injection. The stock solutions were found to be stable if the coefficient of variation on the response factors of the calibration solutions prepared with fresh and stored stock solutions was ≤ 10%.

Storage stability of samples
Additional accuracy samples were prepared and stored in the freezer for at least 16 hours. On the day of analysis, the samples were defrosted at room temperature and treated identically as freshly analysed accuracy samples. The stored samples were found to be stable if the mean accuracy was in the range 70-110%.

7.4. Analytical method

7.4.1. Analytical conditions

Instrument	Alliance Separation Module 2695 (Waters, Milford, MA, USA)
Detector	Dual λ Absorbance Detector 2487 (Waters)
Column	Symmetry Shield RP-18, 100 mm × 4.6 mm i.d., dp = 3.5 µm (Waters)
Column temperature	40°C ± 1°C
Injection volume	100 µl
Mobile phase	30/70 (v/v) acetonitrile/water
Flow	1.0 ml/min
UV detection	253 nm

7.4.2. Preparation of solutions

Stock and spiking solutions
Stock solutions of the test substance were prepared in acetonitrile at concentrations of 1712 - 2420 mg/l.

Spiking solutions were made up from a stock solution and/or dilutions of this solution. The solvent of the spiking solutions was acetonitrile.

Calibration solutions
Six calibration solutions in the concentration range of 0.04 – 2 mg/l were prepared from two stock solutions. The end solution of the calibration solutions was 30/70 (v/v) acetonitrile/water.

Accuracy samples
2 ml water was spiked with the test substance at a target concentration of 0.1 or 100 mg/l. The accuracy samples were diluted in a 1:1 (v:v) ratio with 60/40 (v/v) acetonitrile/water and analysed. If necessary, the samples were further diluted with 30/70 (v/v) acetonitrile/water to obtain concentrations within the calibration range.

The blank accuracy sample was prepared and treated similar to the accuracy samples.

7.5. Formulas

Response (R): Peak area of the test substance [units]

Response factor (R_f): $$R_f = \frac{R}{C_N}$$

where:
C_N = nominal concentration [mg/l]

Calibration curve: $$R = a\,C_N + b$$

where:
a = slope [units × l/mg]
b = intercept [units]

Analysed concentration (C_A) $$C_A = \frac{(R - b)}{a} \times d \ [mg/l]$$

where:
d = dilution factor

Accuracy $$\frac{C_A}{C_N} \times 100 \ [\%]$$

7.6. Results

7.6.1. Specificity

Chromatograms of a test substance solution and the blank accuracy sample are shown in Figure 1 and Figure 2, respectively.

Several small peaks and one large peak were observed in the chromatogram of the test substance solution. It was assumed that the large peak derives from the major component of the test substance, whereas the small peaks derive from impurities. The peak area of the major component was used as response in the calculations.

The chromatogram of the blank sample showed no peak at the retention time of the test substance. Since no interferences were detected, the specificity requirements were met and the analytical method was found to be specific for the test substance.

7.6.2. Linearity

The calibration line was constructed using all data points. Figure 3 illustrates the calibration curve and Table 1 shows the statistical parameters. There was a linear relationship between response and test substance concentration in the range of 0.0401 – 2.00 mg/l (in end solution). Since the coefficient of correlation (r) was > 0.99 and the back calculated accuracies of the data points were in the range 85-115% the calibration line was accepted.

Table 1 Statistical parameters of the calibration curve

Slope	2.03×10^5
Intercept	-1.75×10^3
Weighting factor	$1/concentration^2$
r	0.997

7.6.3. Accuracy and repeatability

The results of the accuracy samples are given in Table 2. Since the mean accuracy at each concentration level fell in the criterion 85-115% and the coefficient of variation was ≤ 20% the analytical method was accepted for the analysis of the test substance in water in the target concentration range of 0.1 - 100 mg/l.

Table 2 Accuracy samples

Target concentration [mg/l]	Nominal concentration [mg/l]	Analysed concentration [mg/l]	Accuracy [%]	Mean accuracy [%]	Coefficient of variation [%]
0.1	0.0995	0.109	109	107	2.4
	0.0995	0.104	104		
	0.0995	0.103	104		
	0.0995	0.108	108		
	0.0995	0.107	108		
100	99.5	105	106	101	3.3
	99.5	98.7	99		
	99.5	97.7	98		
	99.5	104	104		
	99.5	99.4	100		

7.6.4. Limit of quantification

The limit of quantification (LOQ) was assessed at 0.1 mg/l in water.

7.6.5. Stability of the analytical system and end solutions

The results of the stability of the analytical system and end solutions are given in Table 3. Since the coefficient of variation at both concentration levels was ≤ 20% the analytical system and end solutions were stable over at least a 26.7 hour time interval.

Table 3 Stability of the analytical system and end solutions

Nominal concentration [mg/l]	Elapsed time [hours]	Coefficient of variation [%]
0.0401	28.3	17 (n=6)
0.0901	28.1	14 (n=6)
2.00	26.7	6.2 (n=6)

7.6.6. Stability of stock solutions

The coefficient of variation on the response factors of the calibration solutions prepared with fresh and stored stock solutions was 2.2%. Since the value was ≤ 10% the stock solutions were stable when stored at room temperature for at least 16 days.

7.6.7. Storage stability of samples

The results of the storage stability of the test substance in the freezer are given in Table 4. Since the mean accuracy of the frozen samples fell in the criterion 70-110% the samples were stable when stored in the freezer.

Table 4 Stored accuracy samples

Target concentration [mg/l]	Nominal concentration [mg/l]	Analysed concentration [mg/l]	Accuracy [%]	Mean accuracy [%]
0.1	0.0995	0.0766	77	77
	0.0995	0.0761	76	
100	99.5	80.8	81	80
	99.5	78.3	79	

7.7. Conclusion

The analytical method was validated for the following parameters:

Specificity	specific
Linearity	r = 0.997
Accuracy	101 and 107%
Repeatability	2.4 and 3.3%
Limit of quantification	0.1 mg/l
Stability analytical system and end solutions	stable
Stability stock solutions	stable
Storage stability	stable

7.8. Figures

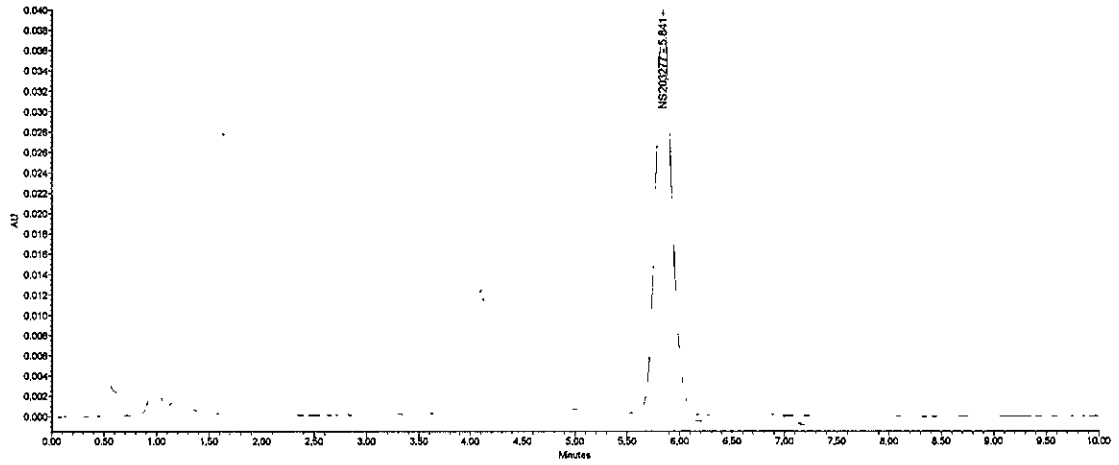


Figure 1 HPLC-UV chromatogram of a 2.00 mg/l test substance solution [res. id. 1509].

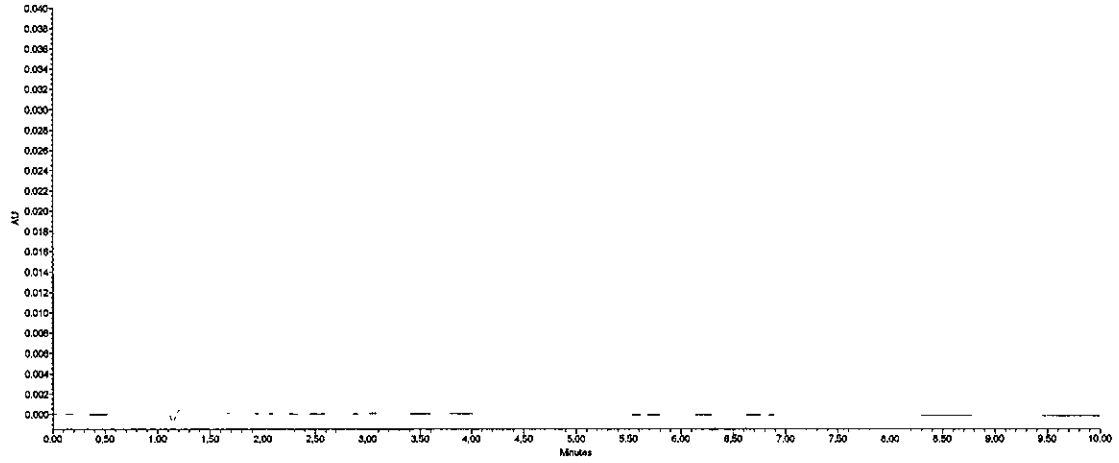


Figure 2 HPLC-UV chromatogram of the blank accuracy sample [res. id. 1529].

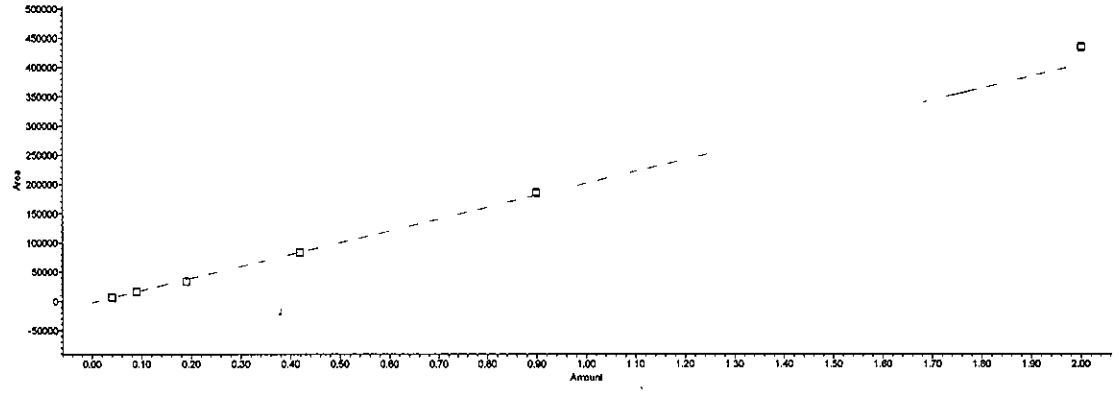


Figure 3 Regression line: response of the test substance as a function of concentration [cal. curve id. 1497].

8. MELTING AND BOILING TEMPERATURE

8.1. *Guidelines*

Organization for Economic Co-operation and Development (OECD), OECD Guidelines for the Testing of Chemicals no. 102: "Melting Point / Melting Range", July 27, 1995.

European Community (EC), EC no. 440/2008, Part A: Methods for the Determination of Physico-Chemical Properties, Guideline A.1: "Melting/Freezing Temperature", Official Journal of the European Union no. L142, May 31, 2008.

Organization for Economic Co-operation and Development (OECD), OECD Guidelines for the Testing of Chemicals no. 103: "Boiling Point", July 27, 1995.

European Community (EC), EC no. 440/2008, Part A: Methods for the Determination of Physico-Chemical Properties, Guideline A.2: "Boiling Temperature", Official Journal of the European Union no. L142, May 31, 2008.

8.2. Performance of the test

The melting and boiling temperature of the test substance were determined using differential scanning calorimetry (DSC). This technique records the difference in heat flow to two crucibles, one filled with test substance and the other filled with an inert reference material, while these crucibles are subjected to a controlled temperature programme. When the test substance undergoes a transition, such as melting or evaporation, it is indicated by a deviation from the base line of the heat-flow record. Because melting and evaporation are processes in which heat is consumed, for these processes the deviation from the base line is in the endothermic direction. The melting temperature or boiling temperature (if any) of the test substance is evaluated from the DSC curve as the extrapolated onset temperature of the endothermic melting or evaporation peak.

As a safety precaution for the DSC cell a preliminary test was performed using a thermogravimetric analyzer (TGA).

Preliminary test
6.69 mg test substance was heated with a rate of 20°C/minute from 25°C to 432°C. At this point 70% weight loss of the test substance was observed. After the experiment, the sample was cooled to 50°C and the consistency of the test substance was determined.

Main study
An overview with the experimental conditions of the main study is given below.

Experiment	First	Second
Sample amount (mg)	6.97	5.97
Initial temperature (°C)	25	25
Rate (°C/minute)	5	5
Temperature 2 (°C)	-90	-90
Rate (°C/minute)	20	20
End temperature (°C)	345	50

The experiments were performed under a flow of nitrogen and at atmospheric pressure.

8.3. Analytical method

Instrument	Q100 differential scanning calorimeter (TA Instruments, New Castle, DE, USA)
Sample container	aluminum; closed with a lid in which a small hole was drilled
Reference	empty sample container

Preliminary test

Instrument	Q50 thermogravimetric analyzer (TGA) (TA Instruments)
Sample container	aluminum; closed with a lid in which a small hole was drilled

8.4. Interpretation

The melting temperature is defined as the temperature at which the phase transition from solid to liquid state takes place at normal atmospheric pressure. This temperature ideally corresponds to the solidification or freezing temperature.

The standard boiling temperature is defined as the temperature at which the vapour pressure of a liquid is the same as the standard pressure (1013.25 hPa).

8.5. Results

8.5.1. Preliminary test

The TGA-curve of the test substance is shown in Figure 4. From 325°C upwards the weight of the sample decreased significantly. At 345°C the sample weight had decreased by 25%.

After the experiment, black residue was observed on the lid of the sample container and a black molten residue remained in the sample container (original colour: yellow). The change of the colour indicated reaction and/or decomposition of the test substance.

8.5.2. Main study

The DSC-curve obtained with the first experiment is shown in Figure 5.

During cooling, a glass transition was observed between -40°C and -60°C (results are archived in the raw data).

During heating, a glass transition between -50°C and -25°C was observed (see Figure 5). The inflection point of the glass transition (melting point) of the test substance was -39.42°C. An exothermic peak was observed from 250°C. The effect was due to reaction and/or decomposition of the test substance. After the experiment, dark brown residue was observed on the lid of the sample container and a dark brown molten residue remained in the sample container. The change of the colour indicated reaction and/or decomposition of the test substance.

The second experiment was performed to determine the duplicate glass transition temperature of the test substance. Figure 6 shows the DSC-curve obtained. Similar results to the first experiment were obtained. The inflection point of the glass transition was -39.53°C. After the experiment, the sample appeared to have been unchanged, i.e. no decomposition and/or evaporation were observed.

8.6. Conclusion

The melting and boiling temperature of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline were determined using DSC.

The melting temperature (glass transition) of the test substance was -39°C (234K).

Reaction and/or decomposition of the test substance was observed at temperatures of > 250°C (> 523K). Boiling of the test substance was not observed below the temperature at which reaction and/or decomposition started. Based on this, the test substance has no boiling temperature.

8.7. Figures

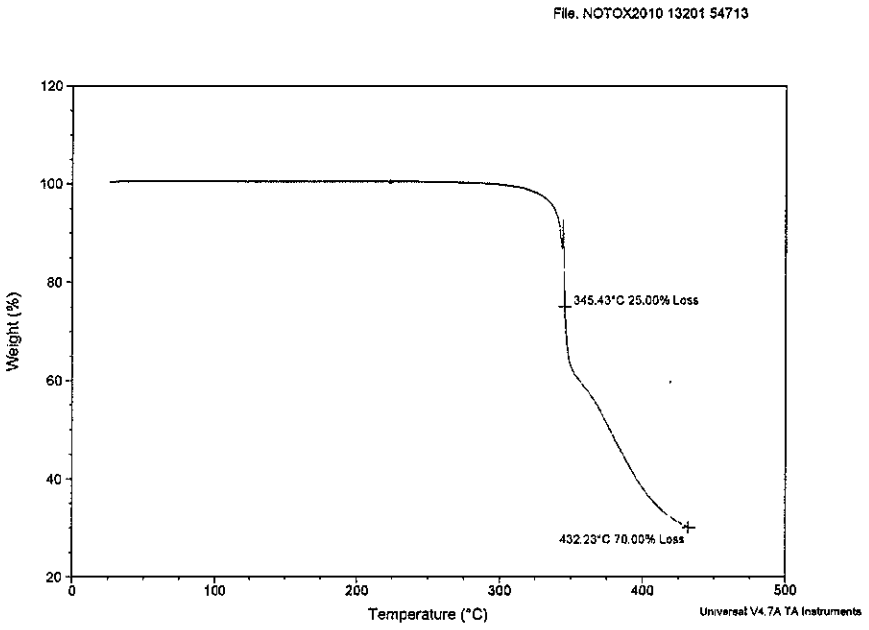


Figure 4 TGA curve of the test substance.

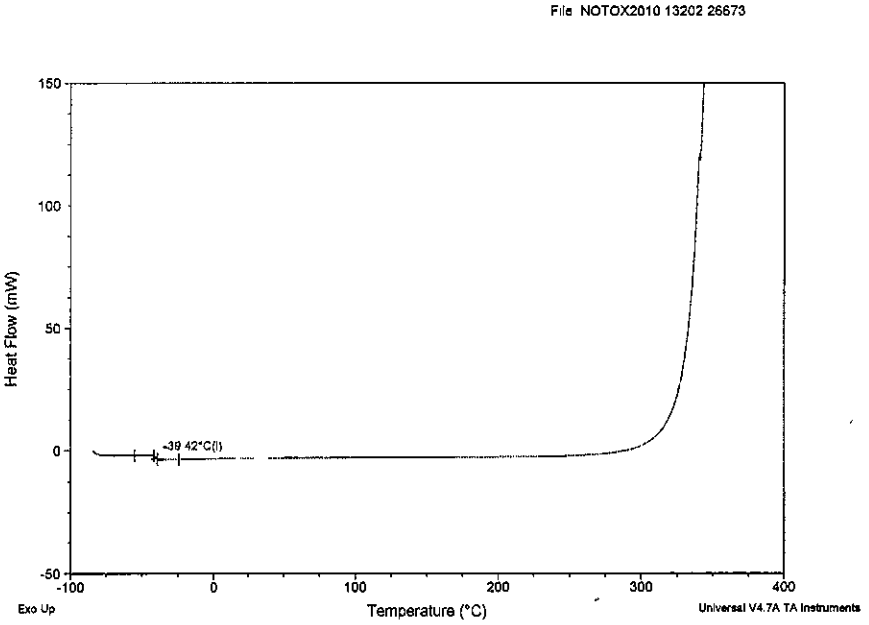


Figure 5 DSC curve of the test substance obtained with the first experiment.

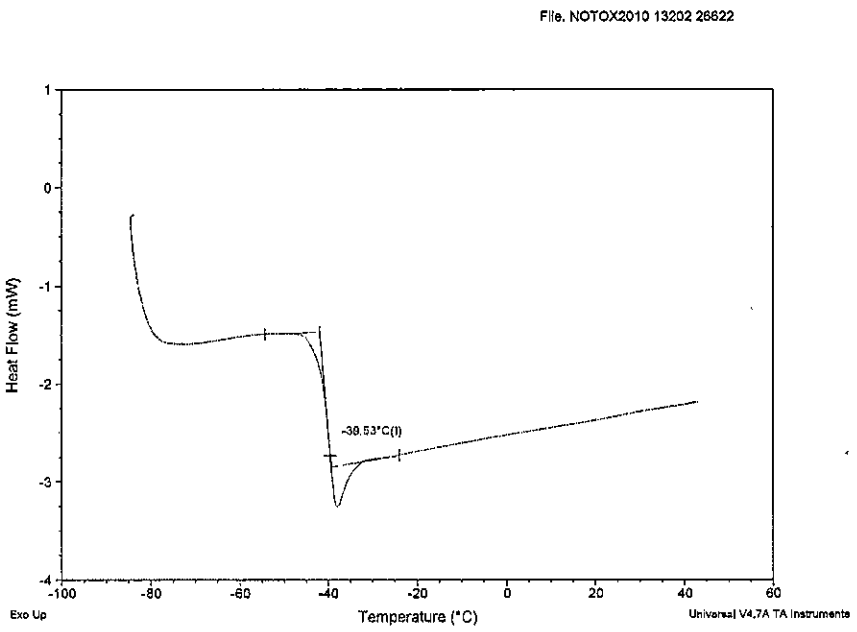


Figure 6 DSC curve of the test substance obtained with the second experiment.

9. DENSITY

9.1. Guidelines

Organization for Economic Co-operation and Development (OECD), OECD Guideline for the Testing of Chemicals no. 109: "Density of Liquids and Solids", July 27, 1995.

European Community (EC), EC no. 440/2008, Part A: Methods for the Determination of Physico-Chemical Properties, Guideline A.3: "Relative Density", Official Journal of the European Union no. L142, May 31, 2008.

9.2. Reagents

Water	Tap water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA).

9.3. Performance of the study

The density (ρ) and relative density (D_4^{20}) of the test substance were determined using a pycnometer with a volume of 10 ml. With this method the density of the test substance is calculated from the difference in weight between the full and empty pycnometer and its volume.

The volume of the pycnometer was determined first. The pycnometer was weighed empty. Then it was filled with water, dried on the outside and weighed again. The temperature of the water was measured. The procedure was performed in duplicate. The mean volume of the pycnometer was calculated.

The pycnometer was filled with test substance, dried on the outside and weighed. The temperature of the test substance was measured. The test was performed in duplicate. The mean density and relative density of the test substance were determined.

Handlings were performed at 20.0 ± 0.5°C. Weighings were performed on an analytical balance with an accuracy of 0.1 mg.

9.4. Interpretation

The density (ρ) of a substance is the quotient of its mass and its volume and is expressed in SI units of kg/m^3.

The relative density (D_4^{20}) is the ratio between the mass of a volume of a substance to be examined, determined at 20°C, and the mass of the same volume of water, determined at 4°C ($\rho_{H_2O}^4$ = 1000 kg/m^3). The relative density has no dimension.

Volume of the pycnometer (V)

$$V = \frac{B - A}{\rho_{H_2O}^{20}} \quad [cm^3]$$

where:
A = weight of the empty pycnometer [g]
B = weight of the pycnometer with water [g]
$\rho_{H_2O}^{20}$ = density of water at 20°C i.e. 0.9982 g/cm^3 (CRC Handbook of Chemistry and Physics, 1981)

Density (ρ)

$$\rho = \frac{C - A_{mean}}{V_{mean}} \quad [g/cm^3]$$

where:
C = weight of the pycnometer with test substance [g]

9.5. Results

The results of the tests on the determination of the density of the test substance are given in Table 5.

The individual measurements were obtained with an accuracy of < 0.01 g/cm^3. According to the guideline, the density of the test substance is given as the mean value.

Table 5 Density of the test substance

	Test 1	Test 2	Mean
Mass empty pycnometer (A) Mass pycnometer with water (B)	30.9325 g 40.6660 g	30.9310 g 40.6585 g	30.93175 g
Volume of the pycnometer	9.7511 cm^3	9.7450 cm^3	9.7481 cm^3
Mass pycnometer with test substance (C)	42.7754 g	42.7787 g	
Density	1.215 g/cm^3	1.215 g/cm^3	1.22 g/cm^3

9.6. Conclusion

A pycnometer was used for the determination of the density and relative density of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline.

The density of the test substance at 20°C was 1.22 g/cm^3 (1.22 × 10^3 kg/m^3).

The relative density was 1.22.

10. VAPOUR PRESSURE

10.1. Guidelines

European Community (EC), EC no. 761/2009, Part A: Methods for the Determination of Physico-Chemical Properties, Guideline A.4: "Vapour Pressure", Official Journal of the European Union no. L220, August 24, 2009.

Organization for Economic Co-operation and Development (OECD), OECD Guidelines for the Testing of Chemicals no.104: "Vapour Pressure", March 23, 2006.

10.2. Performance of the test

The vapour pressure of the test substance (P_T) was determined by the isothermal thermogravimetric effusion method. The method is validated in the range 10^{-6} - 10^{3} Pa using a set of five reference substances with known vapour pressures (see paragraph 10.4). The validity of the method was verified maximum one month before this study using hexachlorobenzene (>99%, Fluka Chemie, Buchs, Switzerland) as reference control substance. The logarithm of the evaporation rate at 20°C (log $v_{T, 20}$) deviated < 10% from the average value obtained during the validation test. From this, it was possible to apply the constants obtained with the validation test for the determination of the vapour pressure of the test substance.

Approximately 8.24 – 13.3 mg of the test substance was applied to the surface of a roughened glass plate as a homogeneous layer. The weight loss of the test substance was measured continuously as a function of time. The following temperature program was used.

Start temperature	100°C
Isothermal intervals	10 minutes
Increment steps	10°C
End temperature	240°C

The weight loss of the test substance was measured continuously as a function of time at defined isothermal temperatures. The evaporation rate of the test substance (v_T) was calculated from the weight loss of the compound. The P_T values at the defined isothermal temperatures were determined using the vapour pressure equation with the constants *c* and *d* specific for the experimental arrangement. Linear regression analysis of the log P_T values and 1/T was performed using a least squares method. The P_T values of the test substance at 20°C and 25°C were determined using the vapour pressure regression curve.

10.3. Analytical method

Instrument	Q50 thermogravimetric analyzer (TGA) (TA Instruments, New Castle, DE, USA)
Surface of the glass plate	0.50 cm^2

The experiments were performed under a flow of nitrogen and at atmospheric pressure.

10.4. Reference substances

Validation of the method is performed at least once every year (NOTOX project 495910).

Each references substance was measured in duplicate by TGA using a temperature program that was specific for the substance. Plots of log v_T obtained at elevated temperatures and 1/T were inter- or extrapolated to determine the log v_T values at 20°C (log $v_{T,20}$). The log $v_{T,20}$ values were plotted against the logarithm of the vapour pressure at 20°C in Pascal units (log $P_{T,20}$). Linear regression analysis using the least squares method yielded an equation of log $P_{T,20}$ = 1.16 × log $v_{T,20}$ + 4.34. The coefficient of correlation (r) was > 0.99. The constants *c* and *d* specific for the experimental arrangement were 1.16 and 4.34, respectively.

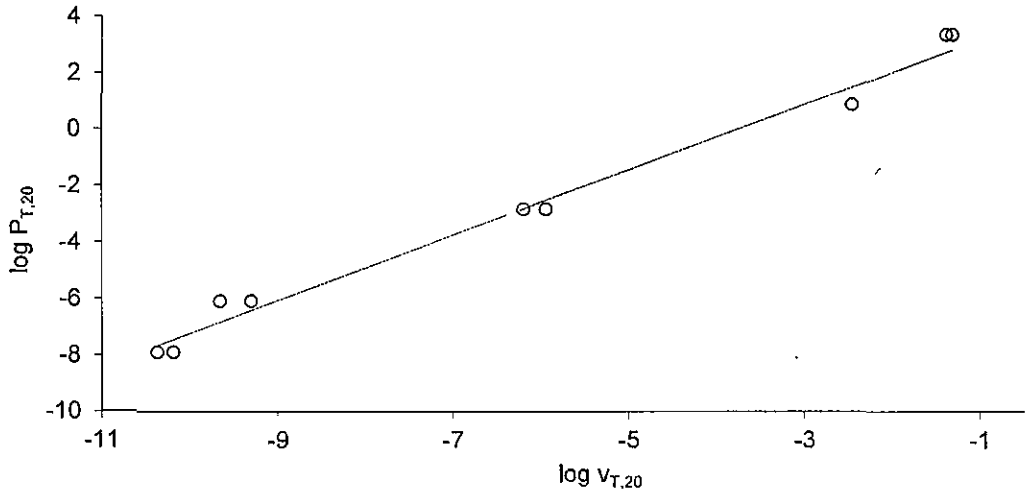
Reference substance	Reference P_T values 20°C [Pa]	[mm Hg]	Corresponding P_T values 25°C [Pa]	[mm Hg]	Literature
Benzo(ghi)perylene	1.33×10^{-8}	1.00×10^{-10}	6.46×10^{-9} #	4.85×10^{-10} #	1
Chrysene	8.40×10^{-7}	6.30×10^{-9}	5.76×10^{-7} #	4.32×10^{-9} #	2
Hexachlorobenzene	1.47×10^{-3}	1.10×10^{-5}	4.21×10^{-3} #	3.16×10^{-5} #	1
Naphthalene	7.56×10^{0}	5.67×10^{-2}	1.15×10^{1}	8.61×10^{-2}	3
Water	2.34×10^{3}	1.75×10^{1}	3.17×10^{3}	2.38×10^{1}	3

\# Determined by the vapour pressure regression curve of the validation study. No literature value was available.

1 Verschueren, K., Handbook of environmental data on organic chemicals, 4th edition, Wiley Chemistry

2. Borges, H T , Formal toxicity summary for Chrysene, The Risk Assessment Information System (RAIS), 1994

3. Lide, D R , CRC Handbook of Chemistry and Physics, 79th edition, CRC Press LLC., Boca Raton, FL, USA, 1998



10.5. Interpretation

10.5.1. Specifications

The vapour pressure is a function of the temperature and is specified in Pascal (Pa) or in mm Hg.

10.5.2. Formulas

Evaporation rate (v_T)

$$v_T = \frac{\Delta m}{F \times t} \quad [g/cm^2/h]$$

where:
Δm = weight loss of the test substance [g]
F = surface of the sample plate [cm^2]
t = elapsed time for the weight loss [h]

Vapour pressure equation

$$\log P_T = c \log v_T + d$$

where:
c = constant specific for the experimental arrangement
d = constant specific for the experimental arrangement

Vapour pressure regression curve

$$\log P_T = a\ 1/T + b$$

where:
T = temperature [K]
a = slope [K]
b = intercept

10.6. Results

A representative weight loss curve of the test substance is shown in Figure 7. The results of the isothermal TGA analysis for the test substance and the vapour pressure at 20°C and 25°C are given in Table 6 and Table 7.

The plot of the log P_T of the test substance as function of the reciprocal temperatures is shown in Figure 8. The equation of the curve was: log $P_{T,20}$ = -6573 × 1/T + 16.16 (r = 0.997, n = 8).

Table 6 Results of the isothermal TGA analysis

Temperature [°C]	Weight loss [µg/min]	v_T [g/cm^2/h]	log v_T	log P_T	P_T [Pa]
150	5.003	5.97×10^{-4}	- 3.22	0.61	4.1
	5.111	6.10×10^{-4}	- 3.21	0.62	4.2
160	10.42	1.24×10^{-3}	- 2.91	0.98	9.6
	11.37	1.36×10^{-3}	- 2.87	1.03	11
170	20.46	2.44×10^{-3}	- 2.61	1.32	21
	22.58	2.70×10^{-3}	- 2.57	1.37	23
180	36.74	4.39×10^{-3}	- 2.36	1.61	41
	41.75	4.80×10^{-3}	- 2.30	1.68	48

Table 7 Vapour pressure of the test substance

Temperature [°C]	log P_T	P_T [Pa]	P_T [mm Hg]
20	- 6.26	5.5×10^{-7}	4.1×10^{-9}
25	- 5.88	1.3×10^{-6}	9.9×10^{-9}

10.7. Conclusion

The isothermal TGA effusion method was applied for the determination of the vapour pressure of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline.

The vapour pressure of the test substance at 20°C (293K) and 25°C (298K) was:

	20°C		25°C	
	[Pa]	[mm Hg]	[Pa]	[mm Hg]
Test substance	5.5×10^{-7}	4.1×10^{-9}	1.3×10^{-6}	9.9×10^{-9}

10.8. Figures

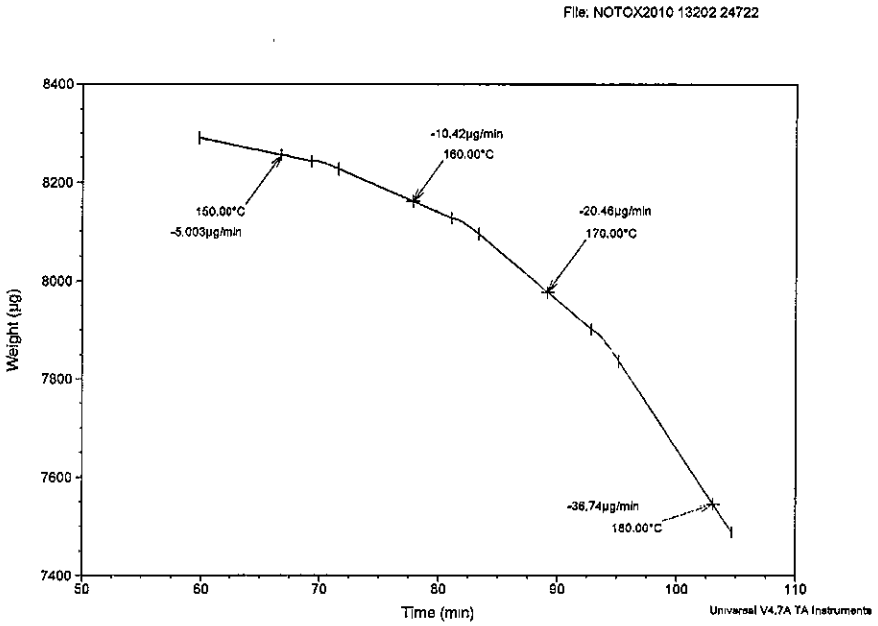


Figure 7 Weight loss curve of the test substance.

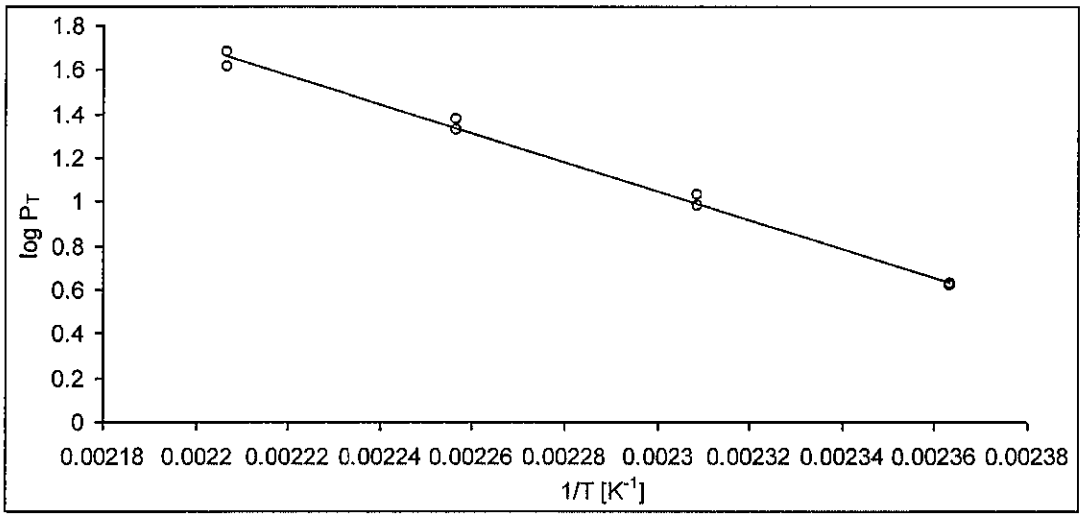


Figure 8 Plot of log P_T of the test substance *versus* 1/T.

11. WATER SOLUBILITY

11.1. Guidelines

Organization for Economic Co-operation and Development (OECD), OECD Guidelines for the Testing of Chemicals no. 105: "Water Solubility", July 27, 1995.

European Community (EC), EC no. 440/2008, Part A: Methods for the Determination of Physico-Chemical Properties, Guideline A.6: "Water Solubility", Official Journal of the European Union no. L142, May 31, 2008.

11.2. Reagents

Water	Tap water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA)
Double distilled water	Waldeck, Münster-Roxel, Germany
Acetonitrile	VWR International, Leuven, Belgium

All reagents were of analytical grade, unless specified otherwise.

11.3. Performance of the study

Two methods are available for the determination of the water solubility of a test substance:

1. Column elution method
The method is based on the elution of the test substance with water from a column which is charged with an inert support material, such as bare silica particles, coated with an excess of the test substance. It can be applied to test substances with a water solubility of $< 10^{-2}$ g/l.

2. Flask method
The mass concentration of the test substance in aqueous solutions saturated with the test substance will be determined after stirring or shaking at a specific temperature for a specific period of time. The method can be used for test substances with a water solubility of $\geq 10^{-2}$ g/l.

A preliminary test was carried out as a range-finding test prior to performance of the column elution method or flask method.

Preliminary test
Test substance was stirred with double distilled water at a nominal concentration of 2.02 g/l at 20.2 ± 0.6°C for 23 hours (1 day). After stirring, the water phase was centrifuged, treated and analysed.

The centrifugation method was checked and was found to be sufficient to remove all undissolved test substance.

In the preliminary test the water solubility of the test substance was $\geq 10^{-2}$ g/l. Therefore, the flask method was chosen for the main study.

Main study
Three aliquots between 1.44 and 1.48 mg of the test substance were weighed into glass vessels. To each vessel, 100 ml double distilled water was added. The vessels were placed on a magnetic stirring device in a climate room at 19.9 ± 0.7°C where the content of each vessel was stirred for 24, 48 or 72 hours. As the blank, 100 ml double distilled water was stirred for 72 hours.

After the stirring period, the water phases were two-times centrifuged at 16421 g and 20°C for 5 minutes. The aqueous phases were diluted in a 7:3 (v:v) ratio with acetonitrile and analysed. If necessary, the samples were further diluted with 30/70 (v/v) acetonitrile/water to obtain concentrations

within the calibration range. The pH of each of the water mixtures (except for the blank) was determined after removal of undissolved test substance.

11.4. Analytical method

11.4.1. Analytical conditions

Quantitative analysis was performed according to the analytical method as validated in chapter 7.

11.4.2. Preparation of the calibration solutions

Stock solutions
Stock solutions of the test substance were prepared in acetonitrile at concentrations of 1712 - 2236 mg/l.

Calibration solutions
Calibration solutions in the concentration range of 0.0401 – 2.00 mg/l (preliminary study) or 0.0401 – 59.9 mg/l (main study) were prepared from two stock solutions. The end solution of the calibration solutions was 30/70 (v/v) acetonitrile/water.

11.4.3. Sample injections

Calibration solutions were injected in duplicate. Test samples were analysed by single injection.

11.4.4. Calibration curves

Calibration curves were constructed using five concentrations. For each concentration, two responses were used. Linear regression analysis was performed using the least squares method with a 1/concentration^2 weighting factor. The coefficient of correlation (r) was > 0.99 for each curve.

11.5. Interpretation

11.5.1. Specifications

The water solubility of a test substance is specified by the saturation mass concentration of the test substance in water at a given temperature and is expressed in g/l.

11.5.2. Formulas

Response (R): Peak area of the test substance [units]

Maximum difference: $\frac{\text{highest value - lowest value}}{\text{mean value}} \times 100\%$

where:
'mean value' is the mean of the highest and lowest value.

Calibration curve: $R = a\,C_N + b$

where:
C_N = nominal concentration [mg/l]
a = slope [units × l/mg]
b = intercept [units]

Analysed concentration (C_A): $C_A = \frac{(R-b)}{a} \times d \times 10^{-3}$ [g/l]

where:
d = dilution factor

11.6. Results

11.6.1. Preliminary test

The content of test substance dissolved in the water sample was 2.93 g/l.

11.6.2. Main study

The results for the samples taken at 24, 48 and 72 hours are given in Table 8.

The maximum difference (MD) of the concentrations determined in the 24, 48 and 72 hour test samples was ≤ 15%. Based on this, the water solubility of the test substance is given as the mean value of the analysed concentrations.

No test substance was detected in the pretreated sample from the blank water mixture.

Table 8 Main study - water solubility of the test substance

Stirring time [hours]	Analysed concentration [g/l]	Mean [g/l]	MD [%]	pH
24	3.27	3.34	3.1	9.5
48	3.36			9.1
72	3.38			8.3

11.7. Conclusion

The flask method was applied for the determination of the water solubility of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline.

The water solubility of the test substance at 20°C was 3.34 g/l.

The pH of the aqueous samples was 8.3 – 9.5.

12. FLASH-POINT

12.1. Guidelines

European Community (EC), EC no. 440/2008, Part A: Methods for the Determination of Physico-Chemical Properties, Guideline A.9: "Flash-point", Official Journal of the European Union no. L142, May 31, 2008.

United Nations (UN), UN no. ST/SG/AC.10/11/Rev.5: Recommendations on the Transport of Dangerous Goods, Part III: Classification Procedures, Test Methods and Criteria Relating to Class 2, Class 3, Class 4, Division 5.1, Class 8 and Class 9, Paragraph 32.4.1: "Non-Viscous Flammable Liquids", 2009.

The International Organization for Standardization (ISO), ISO Guide 2719: "Determination of Flash Point – Pensky Martens Closed Cup method", 2002.

ASTM International, ASTM D 93: "Standard Test Methods for Flash Point by Pensky-Martens Closed Cup Tester", December 10, 2002.

12.2. Performance of the test

The flash-point of the test substance was determined using a model APM-7 Pensky-Martens closed cup automatic flash-point tester (Tanaka Scientific Limited, Tokyo, Japan).

12.2.1. Preliminary test

The test cup was filled with approximately 70 ml test substance and placed in the heating block of the flash-point tester. The lid with a thermocouple for the determination of the flash-point and a resistance thermometer for measuring the temperature was placed on the cup. The test cup was heated with a rate of approximately 5°C/minute. The sample was stirred during heating. The flame igniter was applied so that it was lowered into the vapour space of the cup in 0.5 seconds, left in its lowered position for 1 second, and quickly raised to its high position again. The sample was not stirred when the igniter was applied.

The temperature of the test substance at the start of the test was 16.2°C. Starting at 20°C, an attempt was made to ignite the vapour of the test substance for every 2°C temperature rise.

12.2.2. Main study

Two tests were performed. The temperature of the test substance at the start of the test was 19.3°C and 22.1°C, respectively. Starting at 206°C, the flame igniter was applied for every 2°C temperature rise.

The atmospheric pressure was 1014 hPa.

12.3. Interpretation

The flash-point of the test substance is the lowest temperature, corrected to a barometric pressure of 1013 hPa, at which the test substance evolves vapours in such amount that a flammable vapour/air mixture is produced in the test vessel.

Flash point (T_c) $T_c = T_0 + 0.025(1013 - p)$

where:
T_0 = flash point of the test substance at ambient atmospheric pressure [°C]
P = ambient atmospheric pressure [hPa]

12.4. Results

12.4.1. Preliminary test

The estimated flash-point of the test substance was 228°C.

12.4.2. Main study

The results of the main study on the determination of the flash-point of the test substance are given in *Table 9*.

The difference between the results obtained with the two tests was ≤ 6°C.

Table 9 Main study - flash-point of the test substance

Test	Flash-point [°C]	Lowest flash-point [°C]
1	228	228
2	228	

12.5. Conclusion

The Pensky-Martens closed cup method was applied for the determination of the flash-point of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline.

The flash-point of the test substance was 228°C.

13. AUTO-IGNITION TEMPERATURE

13.1. Guidelines

European Community (EC), EC no. 440/2008, Part A: Methods for the Determination of Physico-Chemical Properties, Guideline A.15: "Auto-Ignition Temperature (Liquids and Gases)", Official Journal of the European Union no. L142, May 31, 2008.

Deutsches Institut für Normung (DIN), DIN Guide 51794: "Determining the Ignition Temperature of Petroleum Products", May 2003.

International Electrotechnical Commission (IEC), IEC standard 79-4: "Electrical Apparatus for Explosive Gas Atmospheres, Part 4, Method of Test for Ignition Temperature", 1975.

13.2. Performance of the test

The auto-ignition temperature of the test substance was determined using a commercially available auto-ignition temperature apparatus (Chilworth Technology, Southampton, UK). The method is applicable to test substances with an auto-ignition temperature in the range 75°C -650°C.

In every ignition test, liquefied test substance was introduced into a preheated test vessel using a volumetric pipette. It was observed visually whether a reaction (clearly perceptible flame and/or explosion) of the sample within five minutes after introduction took place at the prevailing temperature. After each ignition test, any vaporizable components left in the flask were blown out with air (Air Products, Amsterdam, The Netherlands).

13.2.1. Preliminary test

Starting at 200°C, for every 20°C temperature rise, 70 µl test substance was introduced into the test vessel until ignition of the test substance was first observed (i.e. at 440°C).

Starting at 455°C, for every 5°C decrease, 70 µl test substance was tested until no ignition of the test substance had been observed. The temperature at which ignition was last observed is the estimated auto-ignition temperature.

13.2.2. Main study

Three tests were performed. The sample volumes applied and temperature range of each test are given below. Starting at the "high temperature", for every 2°C decrease, the prevailing volume of test substance was tested until no ignition of the test substance had been observed. The temperature at which ignition was last observed is the auto-ignition temperature at the sample volume applied. This procedure was repeated with the other volumes until a minimum auto-ignition temperature for each test was obtained. With these results the auto-ignition temperature of the test substance was determined.

The atmospheric pressure during the performance of the main study was between 1012.6 and 1013.9 hPa.

Test	Volume [µl]	Temperature (°C) start	end
Test 1	70	432	420
	250	410	404
	650	425	417
Test 2	85	428	410
	175	427	405
	350	423	415
Test 3	60	431	419
	200	420	408
	450	430	416

13.3. Interpretation

The degree of auto-ignitability is expressed in terms of the auto-ignition temperature. The auto-ignition temperature is the lowest temperature at which the test substance ignites when mixed with air under the conditions defined in the test method.

13.4. Results

13.4.1. Preliminary test

The estimated auto-ignition temperature was 425°C.

13.4.2. Main study

The results of the main study on the determination of the auto-ignition temperature of the test substance are given in Table 10.

Ignition of the test substance within 5 minutes after introduction was observed with each sample volume and with each test a minimum auto-ignition temperature was obtained. The maximum deviation between the three values was < 20°C. According to the guidelines, no further testing was required.

The lowest temperature at which ignition of the test substance occurred was 410°C. This temperature was a multiple of 5°C and therefore given as the auto-ignition temperature of the test substance.

Table 10 Main study – auto-ignition temperature of the test substance

Test	Volume [µl]	Auto-ignition temperature [°C]	Ignition delay [s]	Colour of the flame	Minimum auto-ignition temperature [°C]
1	70	426	11	Orange	410
	250	410	18	Orange	
	650	423	13	Orange	
2	85	416	19	Orange	411
	175	411	21	Orange	
	350	421	27	Orange	
3	60	425	25	Orange	414
	200	414	16	Orange	
	450	422	21	Orange	

13.5. Conclusion

Commercially available auto-ignition temperature apparatus was used for the determination of the degree of auto-ignitability of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline.

The test substance is auto-ignitable with an auto-ignition temperature of 410°C at 1012.6 – 1013.9 hPa.

14. OXIDIZING PROPERTIES

14.1. Guideline

European Community (EC), EC no. 440/2008, Part A: Methods for the Determination of Physico-Chemical Properties, Guideline A.21: "Oxidizing Properties (Liquids)", Official Journal of the European Union no. L142, May 31, 2008.

United Nations (UN), UN no. ST/SG/AC.10/11/Rev.5: Recommendations on the Transport of Dangerous Goods, Part III: Classification Procedures, Test Methods and Criteria Relating to Class 2, Class 3, Class 4, Division 5.1, Class 8 and Class 9, Test O.2: "Test for Oxidizing Liquids", 2009.

United Nations (UN), UN no. ST/SG/AC.10/11/Rev.5: Recommendations on the Transport of Dangerous Goods, Appendix 6: "Screening Procedures", 2009.

14.2. Statement

A test substance is considered an oxidizing substance when a mixture of the substance and cellulose in a 1:1 ratio (by mass) spontaneously ignites or the mean pressure rise time of the mixture is less than or equal to mean pressure rise time of a 1:1 reference mixture (by mass) of 65% aqueous nitric acid and cellulose.

The molecular structure of the test substance is shown in paragraph 6.1. The test substance does not contain groups that act as an oxidizing agent. The oxygen atoms that are present in the molecular structure of the test substance are chemically bonded to carbon or hydrogen. The molecular structures of impurities at ≤ 6% were not taken into account but these are not expected to be of influence on the oxidizing properties of the test substance.

In conclusion, p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline has no oxidizing properties.

15. HYDROLYSIS AS A FUNCTION OF PH

15.1. Guideline

European Community (EC), EC no. 440/2008, Part C: Methods for the Determination of Ecotoxicity, Guideline C.7: "Degradation - Abiotic Degradation: Hydrolysis as a Function of pH", Official Journal of the European Union no. L142, May 31, 2008.

Organization for Economic Co-operation and Development (OECD), OECD Guidelines for the Testing of Chemicals no. 111: "Hydrolysis as a Function of pH", April 13, 2004.

15.2. Reagents

Water	Tap water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA)
Acetonitrile	Biosolve, Valkenswaard, The Netherlands
Sodium acetate	Merck, Darmstadt, Germany
Acetic acid, 100%	Merck
Potassium *di*-hydrogenphosphate	Merck
Boric acid	Merck
Potassium chloride	Merck
Sodium azide	Merck
Sodium hydroxide	Boom, Meppel, The Netherlands

All reagents were of analytical grade, unless specified otherwise.

15.3. Buffer solutions

Acetate buffer pH 4, 0.1 M	solution of 16.6% 0.1 M sodium acetate and 83.4% 0.1 M acetic acid. The buffer contains 0.0009% (w/v) sodium azide.
Phosphate buffer pH 7, 0.1 M	solution of 0.1 M potassium dihydrogenphosphate adjusted to pH 7 using 10 N sodium hydroxide. The buffer contains 0.0009% (w/v) sodium azide.
Borate buffer pH 9, 0.1 M	solution of 0.1 M boric acid and 0.1 M potassium chloride adjusted to pH 9 using 10 N sodium hydroxide. The buffer contains 0.0009% (w/v) sodium azide.

15.4. Performance of the study

The rate of hydrolysis of the test substance as a function of pH was determined at pH values normally found in the environment (pH 4-9).

Preliminary test - Tier 1
Test substance solutions were prepared in the buffer solutions at a target concentration of 1000 mg/l, after stirring for at least 60 minutes the test substance was not completely dissolved. Each solution was filter-sterilised through a 0.2 µm FP 30/0.2 CA-S filter (Whatman, Dassel, Germany) and transferred into a sterile vessel. To exclude oxygen, nitrogen gas was purged through the solution for 5 minutes. For each sampling time, duplicate sterile vessels under vacuum were filled with 6 ml test solution and placed in the dark in a temperature controlled environment at 49.9°C ± 0.1°C.

The concentration of the test substance in the test samples was determined immediately after preparation (t=0) and after 5 days. The samples taken at t=5 days were cooled to room temperature using running tap water. The samples were diluted in a 1:1 (v:v) ratio with 60/40 (v/v) acetonitrile/buffer solution (the same buffer as where test substance solution in was prepared) and analysed. If necessary, the samples were further diluted with 30/70 (v/v) acetonitrile/buffer solution to obtain concentrations within the calibration range.

Blank buffer solutions were treated similarly as the test samples and analysed at t=0.

The pH of each of the test solutions (except for the blanks) was determined at each sampling time.

Main study - Tier 2

The buffer solutions were filter-sterilised through a 0.2 µm FP 30/0.2 CA-S filter (Whatman, Dassel, Germany) and transferred into a sterile vessel. To exclude oxygen, nitrogen gas was purged through the solution for 5 minutes. The test substance was spiked to the solutions at a target concentration of 500 mg/l using a spiking solution in acetonitrile. The obtained solution was filtered again through a 0.2 µm FP 30/0.2 CA-S filter to remove possibly present undissolved test substance. For each sampling time, duplicate sterile vessels under vacuum were filled with 6 ml test solution and placed in the dark in a temperature controlled environment.

Note: the spiking volume was < 1% of the sample volume. Nominal concentrations were not corrected for the spiking volume.

The concentrations of the test substance were determined immediately after preparation (t=0) and at several sampling points after t=0. The concentration of the bulk solution was also determined.
The samples taken after t=0 were cooled to room temperature using running tap water (except the samples stored at 20°C). The samples were diluted to 30/70 (v/v) acetonitrile/borate buffer pH 9 as test substance stability was found to be optimal at pH 9.

Blank buffer solutions containing a similar content of blank spiking solution were treated similarly as the test samples and analysed at t=0.

The pH of each of the test solutions (except for the blanks) was determined at least at the beginning and at the end of the test.

The study was performed at the following temperatures:

pH code	Temperature I	Temperature II	Temperature III
pH 4	20.2°C ± 0.6°C	40.0°C ± 0.1°C	49.9°C ± 0.1°C
pH 7	20.2°C ± 0.6°C	40.0°C ± 0.1°C	49.9°C ± 0.1°C
pH 9	20.2°C ± 0.6°C	40.0°C ± 0.1°C	49.8°C ± 0.2°C

Identification of hydrolysis products – Tier 3

Research to investigate the identity or nature and rates of formation and decline of hydrolysis products was not required since the studies were conducted according to the testing guidelines provided in Annex VIII (volume 10-100 tons/year).

15.5. Analytical method

15.5.1. Analytical conditions

Quantitative analysis was performed according to the analytical method as validated in chapter 7.

15.5.2. Preparation of the calibration solutions

Stock solutions
Stock solutions of the test substance were prepared in acetontrile at concentrations of 2000 – 4385 mg/l.

Calibration solutions
Calibration solutions in the concentration range 0.04 – 2 mg/l were prepared from two stock solutions. The end solution of the calibration solution was:

pH code	Preliminary test	Main test
pH 4	30/70 (v/v) acetonitrile/acetate buffer pH4	30/70 (v/v) acetonitrile/borate buffer pH9
pH 7	30/70 (v/v) acetonitrile/phosphate buffer pH7	30/70 (v/v) acetonitrile/borate buffer pH9
pH 9	30/70 (v/v) acetonitrile/borate buffer pH9	30/70 (v/v) acetonitrile/borate buffer pH9

15.5.3. Sample injections

Calibration solutions were injected in duplicate. Test samples were analysed by single injection.

15.5.4. Calibration curves

Calibration curves were constructed using at least four concentrations. For each concentration, two responses were used. Linear regression analysis was performed using the least squares method with a 1/concentration^2 weighting factor. If necessary, the calibration line was excluding one data point since the back calculated accuracy was > 15% from the nominal concentration. The coefficient of correlation (r) was > 0.99 for each curve.

15.6. Interpretation

Response (R): Peak area of the test substance [units]

Calibration curve: $R = a\,C_N + b$

where:
C_N = nominal concentration [mg/l]
a = slope [units × l/mg]
b = intercept [units]

Analysed concentration (C_A): $C_A = \frac{(R-b)}{a} \times d$ [mg/l]

where:
d = dilution factor

Recovery: $\frac{C_A}{C_N} \times 100\%$

where:
C_N = nominal concentration [mg/l]

Degree of hydrolysis

$$\frac{\text{mean}C_0 - C_t}{\text{mean}C_0} \times 100\%$$

where:
C_0 = concentration at t=0
C_t = concentration at t=5 days

Relative concentration (C_r)

$$C_r = \frac{C_t}{\text{mean}C_0} \times 100\%$$

where:
C_0 = concentration at t=0
C_t = concentration at t=x hours

Pseudo-first order curve

$$^{10}\log C_r = -a \times t + b$$

where:
t = time [hours]
a = slope [$hours^{-1}$]
b = intercept

Rate constant (k_{obs})

$$k_{obs} = -a \times 2.303 \ [hours^{-1}]$$

where:
2.303 is the conversion factor between natural and base 10 logarithms

Arrhenius equation

$$\ln k_{obs} = \frac{-E}{R \times T} + \ln A$$

where:
A plot of ln k_{obs} *versus* 1/T gives a linear relationship with a slope of – E/R and intercept ln A

Half-life time ($t_{½}$)

$$t_{½} = \frac{\ln 2}{k_{obs}} \ [hours]$$

15.7. Results

15.7.1. Preliminary test - Tier 1

The analytical results of the preliminary test are given in Table 11 and Table 12.

At pH 4, pH 7 and pH 9 a degree of hydrolysis of ≥ 10% after 5 days was observed. According to the guideline, the higher Tier test was required to determine the half-life time of the test substance.

No test substance was detected in the blank buffer solutions pH 4 and pH 7.
In the blank buffer solutions of pH9 a small response was observed at the test substance retention time, the contribution base on area to the samples at t=0 was 0.09%, this was negligible.

The mean recoveries of the buffer solutions fell within the criterion range of 70-110%. It demonstrated that the analytical method was adequate to support the hydrolysis study on the test substance.

Table 11 Preliminary test – hydrolysis of the test substance at pH 4, pH 7 and pH 9

pH code	Sampling time	Analysed concentration [mg/l]	Degree of hydrolysis [%]		Actual pH
			Individual	Mean	
pH 4	0 hours	786 782			4.0 4.0
	5 days	0.0776 [1] 0.0616 [1]	100 100	100	4.0 4.0
pH 7	0 hours	876 891			7.1 7.1
	5 days	0.0412 [1] 0.115	100 100	100	7.1 7.1
pH 9	0 hours	978 956			9.0 9.0
	5 days	0.248 n.d.	100 100	100	9.0 9.0

n.d. not detected
[1] Extrapolated value

Table 12 Recoveries

pH code	Nominal concentration [mg/l]	Analysed concentration [mg/l]	Recovery [%]	Mean recovery [%]
pH4	1122 1122	786 782	70 70	70
pH7	994 994	876 891	88 90	89
pH9	1055 1055	978 956	93 91	92

15.7.2. Main study - Tier 2

pH 4
The analytical results of the main study are given in Table 13 - Table 16.

No test substance was detected in the blank buffer solutions.

The mean recoveries of the buffer solutions relative to the stock solution fell within the criterion range of 90-110%. It demonstrated that the analytical method was adequate to support the hydrolysis study on the test substance.

Table 13 Main test – hydrolysis of the test substance at 20°C

Sampling time [hours]	Analysed concentration [mg/l]	Relative concentration [%]	Logarithm relative concentration	Actual pH
0	462	100	2.00	4.0
0	461	100	2.00	4.0
47	318	69	1.84	4.0
47	323	70	1.85	4.0
71	275	60	1.78	4.0
71	276	60	1.78	4.0
120.5	186	40	1.61	4.0
120.5	189	41	1.61	4.0
144	135	29	1.47	4.0
144	136	29	1.47	4.0
167.5	117	25	1.40	4.0
167.5	117	25	1.40	4.0
191	98.0	21	1.33	4.0
191	97.7	21	1.33	4.0
215	85.7	19	1.27	4.0
215	87.1	19	1.28	4.0
294.5	56.2	12	1.09	3.9
294.5	56.2	12	1.09	3.9
359	32.3	7.0	0.85	4.0
359	31.4	6.8	0.83	4.0

Table 14 Main test – hydrolysis of the test substance at 40°C

Sampling time [hours]	Analysed concentration [mg/l]	Relative concentration [%]	Logarithm relative concentration	Actual pH
0	126	100	2.00	4.0
0	126	100	2.00	4.0
2	109	86	1.93	4.0
2	109	86	1.94	4.0
4.5	91.7	73	1.86	4.0
4.5	90.7	72	1.86	4.0
7	73.0	58	1.76	4.0
7	75.5	60	1.78	4.0
7.6	73.1	58	1.76	4.0
7.6	73.3	58	1.76	4.0
22.75	25.5	20	1.31	4.0
22.75	25.2	20	1.30	4.0
25.5	20.0	16	1.20	4.0
25.5	20.4	16	1.21	4.0
28.25	17.0	13	1.13	4.0
28.25	17.3	14	1.14	4.0
31	14.9	12	1.07	4.0
31	14.3	11	1.05	4.0
47.5	4.82	3.8	0.58	4.0
47.5	4.84	3.8	0.58	4.0

Table 15 Main test – hydrolysis of the test substance at 50°C

Sampling time [hours]	Analysed concentration [mg/l]	Relative concentration [%]	Logarithm relative concentration	Actual pH
0	474	102	2.01	4.0
0	459	98	1.99	4.0
1	385	83	1.92	4.0
1	396	85	1.93	4.0
2.25	310	66	1.82	4.1
2.25	305	65	1.82	4.0
3	269	58	1.76	4.0
3	274	59	1.77	4.1
4	228	49	1.69	4.1
4	228	49	1.69	4.1
5	197	42	1.63	4.1
5	192	41	1.61	4.1
6.25	151	32	1.51	4.1
6.25	152	33	1.51	4.1
7	133	28	1.45	4.1
7	132	28	1.45	4.1
23.5	7.56	1.6	0.21	4.1
23.5	7.26	1.6	0.19	4.1

Table 16 Recoveries

Temperature (°C)	Bulk solution concentration [mg/l]	Analysed concentration [mg/l]	Recovery [1] [%]	Mean recovery [%]
20	453	462 461	102 102	102
40	122	126 126	103 104	103
50	455	474 459	104 101	102

[1] Recovery was determined relative to the concentration of the prepared bulk solution. A relatively high spiking concentration had to be used to allow dilution of the samples to the end solution (30/70 (v/v) acetonitrile/borate buffer pH 9) to ensure sample stability during chromatographic analysis and sufficient analytical sensitivity.

For testing of pseudo-first order kinetics the mean logarithms of the relative concentrations between 10% and 90% were plotted against time. At all temperatures linear relationships were obtained. The half-life times of the test substance were determined according to the model for *pseudo*-first order reactions.

All logarithms of the relative concentrations were correlated with time using linear regression analysis. Figure 9 illustrates the regression curves and Table 17 shows the statistical parameters.

Table 17 Statistical parameters of the regression curves

Temperature (°C)	Slope [hours^{-1}]	Intercept	Coefficient of correlation
20	-3.21×10^{-3}	1.98	0.996
40	-3.00×10^{-2}	1.99	0.9997
50	-7.65×10^{-2}	2.00	0.99991

The rate constant (k_{obs}) and half-life time of the test substance at each temperature was obtained and the Arrhenius equation was used to determine the rate constant and half-life time at 25°C (see Table 18).

Table 18 Rate constants (k_{obs}) and half-life time ($t_{½}$)

Temperature [°C]	k_{obs} [hours^{-1}]	$t_{½}$
20	7.39×10^{-3}	3.9 days
25	1.33×10^{-2}	2.2 days
40	6.90×10^{-2}	10 hours
50	1.76×10^{-1}	3.9 hours

pH 7
The analytical results of the main study are given in Table 19 - Table 22.

No test substance was detected in the blank buffer solutions.

The mean recoveries of the buffer solutions relative to the stock solution fell within the criterion range of 90-110%. It demonstrated that the analytical method was adequate to support the hydrolysis study on the test substance.

Table 19 Main test – hydrolysis of the test substance at 20°C

Sampling time [hours]	Analysed concentration [mg/l]	Relative concentration [%]	Logarithm relative concentration	Actual pH
0	253	100	2.00	7.1
0	252	100	2.00	7.1
7	249	99	1.99	7.1
7	256	101	2.01	7.1
71	156	62	1.79	7.1
71	155	61	1.79	7.1
95	133	53	1.72	7.1
95	134	53	1.73	7.1
144.5	94.5	37	1.57	7.1
144.5	94.2	37	1.57	7.1
168	68.5	27	1.43	7.1
168	69.5	28	1.44	7.1
191.5	59.8	24	1.37	7.1
191.5	59.9	24	1.38	7.1
215	50.5	20	1.30	7.1
215	50.5	20	1.30	7.1
239	44.3	18	1.24	7.1
239	44.9	18	1.25	7.1
318.5	29.6	12	1.07	7.1
318.5	30.1	12	1.08	7.1
383	17.5	6.9	0.84	7.1
383	17.5	6.9	0.84	7.1

Table 20 Main test – hydrolysis of the test substance at 40°C

Sampling time [hours]	Analysed concentration [mg/l]	Relative concentration [%]	Logarithm relative concentration	Actual pH
0	328	100	2.00	7.1
0	326	100	2.00	7.1
2.75	301	92	1.96	7.1
2.75	306	93	1.97	7.1
5	257	79	1.90	7.1
5	261	80	1.90	7.1
6.5	250	76	1.88	7.1
6.5	245	75	1.87	7.1
23.25	69.2	21	1.33	7.1
23.25	68.9	21	1.32	7.1
25.75	57.3	18	1.24	7.1
25.75	58.3	18	1.25	7.1
28.25	49.4	15	1.18	7.1
28.25	50.6	15	1.19	7.1
30.75	41.9	13	1.11	7.1
30.75	41.3	13	1.10	7.1
47.25	15.4	4.7	0.67	7.1
47.25	15.4	4.7	0.67	7.1

Table 21 Main test – hydrolysis of the test substance at 50°C

Sampling time [hours]	Analysed concentration [mg/l]	Relative concentration [%]	Logarithm relative concentration	Actual pH
0	433	100	2.00	7.1
0	433	100	2.00	7.1
1	373	86	1.93	7.1
1	386	89	1.95	7.1
2.25	328	76	1.88	7.1
2.25	323	75	1.87	7.1
3	280	65	1.81	7.1
3	287	66	1.82	7.1
4.25	233	54	1.73	7.1
4.25	236	54	1.74	7.1
5.25	195	45	1.65	7.1
5.25	196	45	1.66	7.1
6.25	162	37	1.57	7.1
6.25	165	38	1.58	7.1
7.25	138	32	1.50	7.1
7.25	139	32	1.51	7.1
24.75	8.08	1.9	0.27	7.1
24.75	8.05	1.9	0.27	7.1

Table 22 Recoveries

Temperature (°C)	Nominal concentration [mg/l]	Analysed concentration [mg/l]	Recovery [1] [%]	Mean recovery [%]
20	263	253 252	96 96	96
40	342	328 326	96 95	95
50	442	433 433	98 98	98

[1] Recovery was determined relative to the concentration of the prepared bulk solution. A relatively high spiking concentration had to be used to allow dilution of the samples to the end solution (30/70 (v/v) acetonitrile/borate buffer pH 9) to ensure sample stability during chromatographic analysis and sufficient analytical sensitivity.

For testing of pseudo-first order kinetics the mean logarithms of the relative concentrations between 10% and 90% were plotted against time. At all temperatures linear relationships were obtained. The half-life times of the test substance were determined according to the model for *pseudo*-first order reactions.

All logarithms of the relative concentrations were correlated with time using linear regression analysis. Figure 10 illustrates the regression curves and Table 23 shows the statistical parameters.

Table 23 Statistical parameters of the regression curves

Temperature (°C)	Slope [hours^{-1}]	Intercept	Coefficient of correlation
20	-3.06×10^{-3}	2.00	0.996
40	-2.95×10^{-2}	2.03	0.998
50	-7.06×10^{-2}	2.02	0.9997

The rate constant (k_{obs}) and half-life time of the test substance at each temperature was obtained and the Arrhenius equation was used to determine the rate constant and half-life time at 25°C (see Table 24).

Table 24 Rate constants (k_{obs}) and half-life time ($t_{½}$)

Temperature [°C]	k_{obs} [hours^{-1}]	$t_{½}$
20	7.05×10^{-3}	4.1 days
25	1.27×10^{-2}	2.3 days
40	6.79×10^{-2}	10 hours
50	1.63×10^{-1}	4.3 hours

pH 9
The analytical results of the main study are given in Table 25 - Table 28.

No test substance was detected in the blank buffer solutions.

The mean recoveries of the buffer solutions relative to the stock solution fell within the criterion range of 90-110%. It demonstrated that the analytical method was adequate to support the hydrolysis study on the test substance.

Table 25 Main test – hydrolysis of the test substance at 20°C

Sampling time [hours]	*Analysed* concentration [mg/l]	*Relative* concentration [%]	*Logarithm relative* concentration	*Actual pH*
0	403	100	2.00	9.1
0	403	100	2.00	9.1
7	415	103	2.01	9.0
7	411	102	2.01	9.0
24	394	98	1.99	9.0
24	397	99	1.99	9.0
95	236	59	1.77	9.0
95	231	57	1.76	9.0
168.5	144	36	1.55	9.0
168.5	145	36	1.56	9.1
192	108	27	1.43	9.1
192	109	27	1.43	9.1
215	95.2	24	1.37	9.0
215	95.0	24	1.37	9.0
239	79.0	20	1.29	9.0
239	79.8	20	1.30	9.0
263	74.8	19	1.27	9.0
263	76.6	19	1.28	9.0
288	58.2	14	1.16	9.1
288	59.0	15	1.17	9.1
342	54.2	13	1.13	9.0
342	54.7	14	1.13	9.0
407	36.6	9.1	0.96	9.0
407	36.7	9.1	0.96	9.0

Table 26 Main test – hydrolysis of the test substance at 40°C

Sampling time [hours]	Analysed concentration [mg/l]	Relative concentration [%]	Logarithm relative concentration	Actual pH
0	462	99	2.00	9.0
0	471	101	2.00	9.0
3	435	93	1.97	9.0
3	425	91	1.96	9.0
5.25	377	81	1.91	9.0
5.25	373	80	1.90	9.0
7	338	73	1.86	9.0
7	344	74	1.87	9.0
23.5	121	26	1.41	9.0
23.5	123	26	1.42	9.0
26	105	23	1.35	9.0
26	106	23	1.36	9.0
28.5	94.1	20	1.31	9.0
28.5	94.9	20	1.31	9.0
31	82.1	18	1.25	9.0
31	80.6	17	1.24	9.0
47.5	37.9	8.1	0.91	9.0
47.5	37.9	8.1	0.91	9.0

Table 27 Main test – hydrolysis of the test substance at 50°C

Sampling time [hours]	Analysed concentration [mg/l]	Relative concentration [%]	Logarithm relative concentration	Actual pH
0	377	100	2.00	9.1
0	381	100	2.00	9.1
1	341	90	1.95	9.1
1	338	89	1.95	9.1
2	300	79	1.90	9.1
2	303	80	1.90	9.1
3	271	72	1.85	9.1
3	267	71	1.85	9.1
4	240	63	1.80	9.1
4	244	65	1.81	9.1
5	214	56	1.75	9.1
5	218	58	1.76	9.1
6	187	49	1.69	9.1
6	190	50	1.70	9.1
7	168	44	1.65	9.1
7	167	44	1.65	9.1
24.5	19.1	5.0	0.70	9.1
24.5	19.4	5.1	0.71	9.1

Table 28 Recoveries

Temperature (°C)	Nominal concentration [mg/l]	Analysed concentration [mg/l]	Recovery [1] [%]	Mean recovery [%]
20	410	403 403	98 98	98
40	514	462 471	90 92	91
50	387	377 381	97 98	98

[1] Recovery was determined relative to the concentration of the prepared bulk solution. A relatively high spiking concentration had to be used to allow dilution of the samples to the end solution (30/70 (v/v) acetonitrile/borate buffer pH 9) to ensure sample stability during chromatographic analysis and sufficient analytical sensitivity.

For testing of pseudo-first order kinetics the mean logarithms of the relative concentrations between 10% and 90% were plotted against time. At all temperatures linear relationships were obtained. The half-life times of the test substance were determined according to the model for *pseudo*-first order reactions.

All logarithms of the relative concentrations were correlated with time using linear regression analysis. Figure 11 illustrates the regression curves and Table 29 shows the statistical parameters.

Table 29 Statistical parameters of the regression curves

Temperature (°C)	Slope [hours^{-1}]	Intercept	Coefficient of correlation
20	-2.74×10^{-3}	2.01	0.992
40	-2.42×10^{-2}	2.01	0.997
50	-5.30×10^{-2}	2.01	0.9998

The rate constant (k_{obs}) and half-life time of the test substance at each temperature was obtained and the Arrhenius equation was used to determine the rate constant and half-life time at 25°C (see Table 30).

Table 30 Rate constants (k_{obs}) and half-life time ($t_{½}$)

Temperature [°C]	k_{obs} [hours^{-1}]	$t_{½}$
20	6.30×10^{-3}	4.6 days
25	1.11×10^{-2}	2.6 days
40	5.58×10^{-2}	12 hours
50	1.22×10^{-1}	5.7 hours

15.8. Conclusion

The preliminary test (Tier 1) and main study (Tier 2) were performed for the determination of the rate of hydrolysis of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline at pH values normally found in the environment (pH 4-9).

The half-life times of the test substance were:

pH 4		pH 7		pH 9	
Temperature [°C]	$t_{½}$	Temperature [°C]	$t_{½}$	Temperature [°C]	$t_{½}$
20	3.9 days	20	4.1 days	20	4.6 days
25	2.2 days	25	2.3 days	25	2.6 days
40	10 hours	40	10 hours	40	12 hours
50	3.9 hours	50	4.3 hours	50	5.7 hours

15.9. Figures

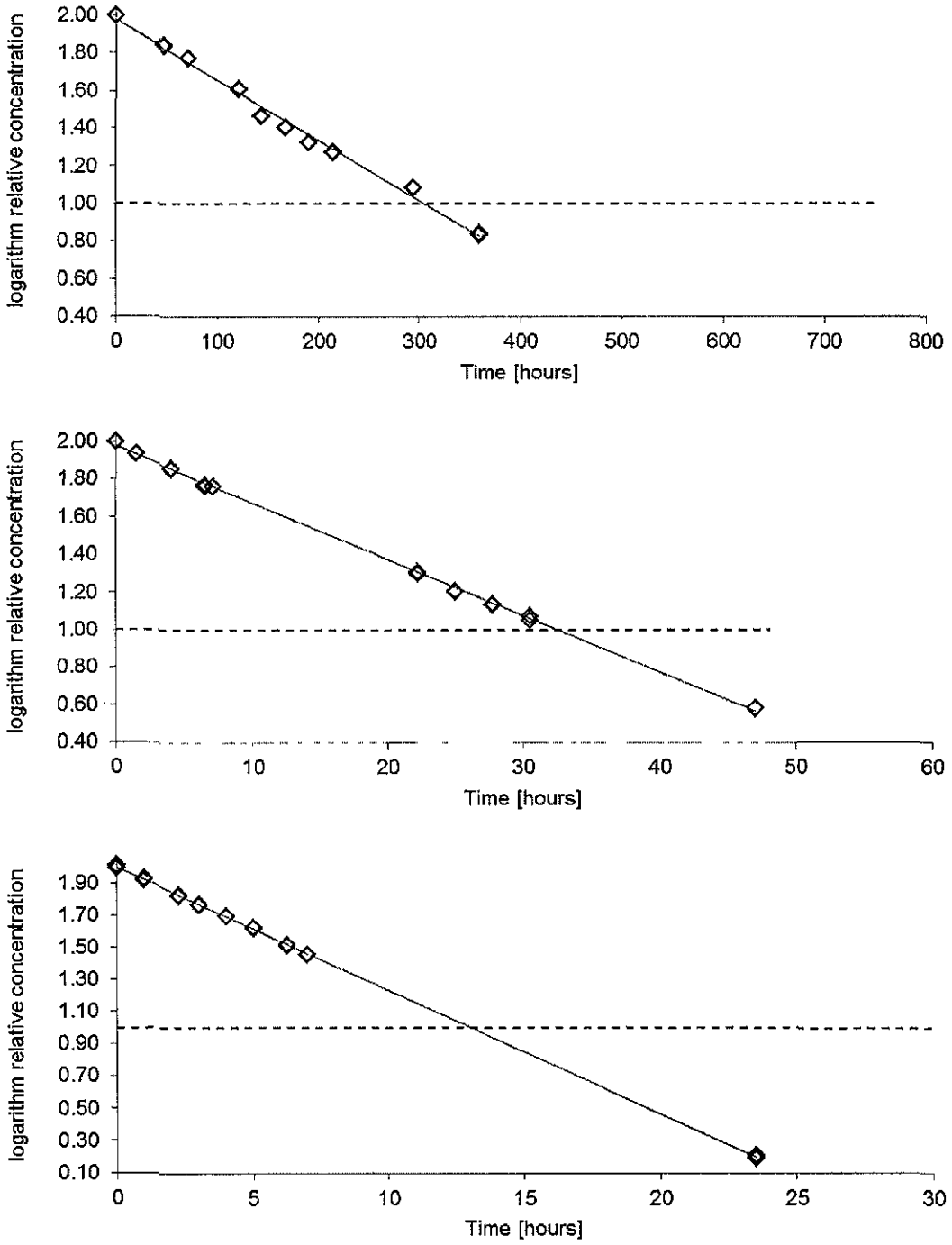


Figure 9 Plot of the logarithms of relative concentration against time at 20°C [top], 40°C [middle] and 50°C [bottom] at pH4.

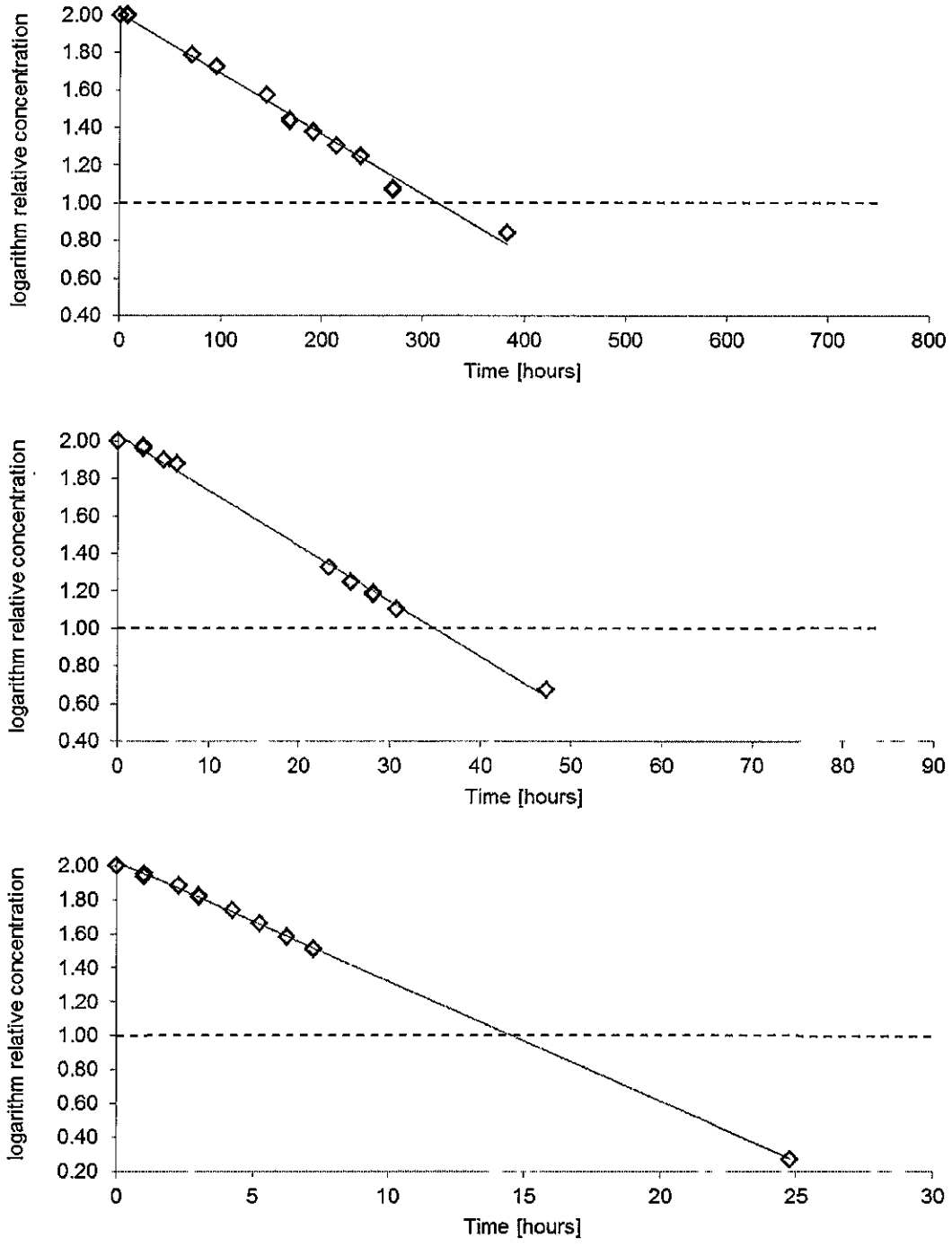


Figure 10 Plot of the logarithms of relative concentration against time at 20°C [top], 40°C [middle] and 50°C [bottom] at pH7.

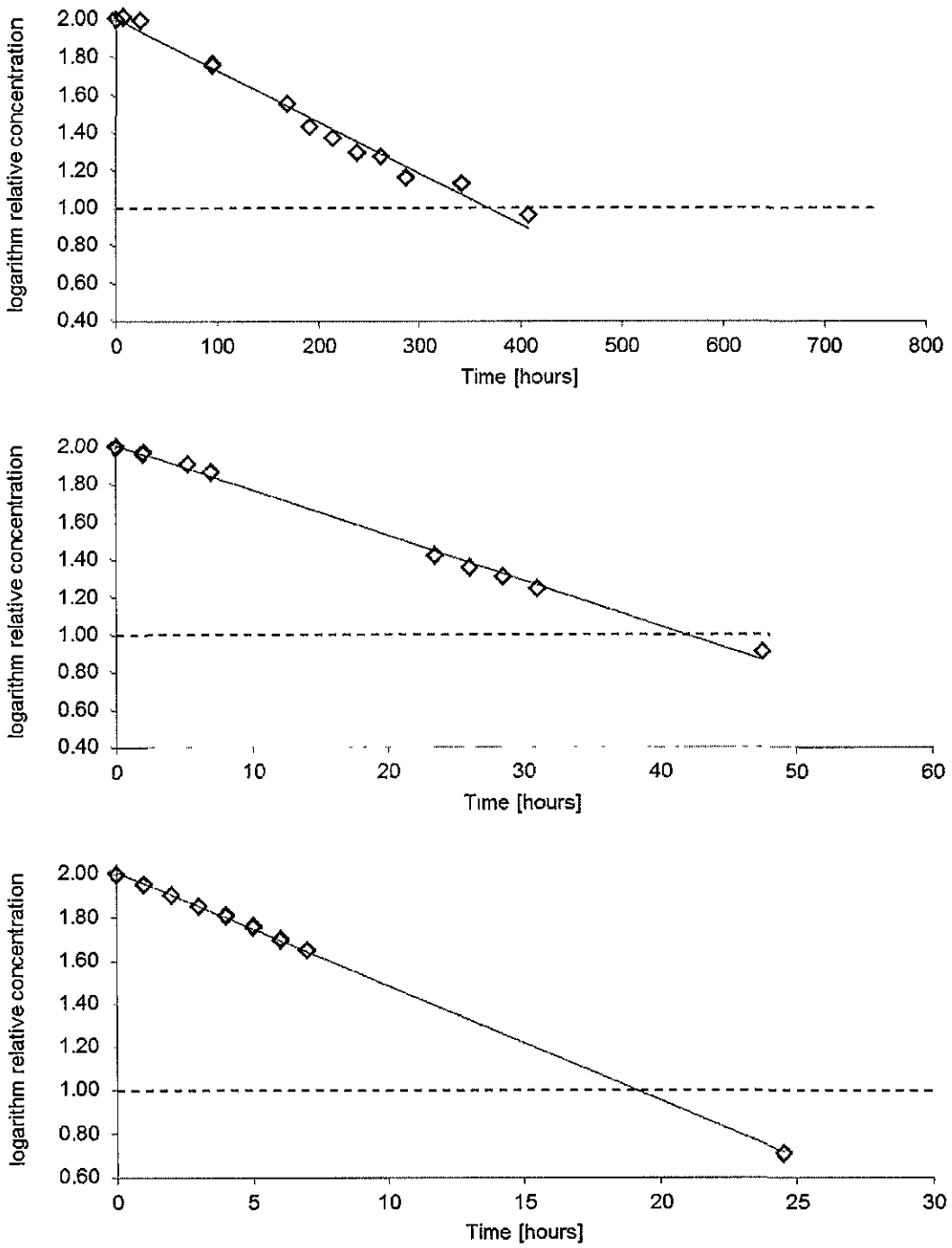


Figure 11 Plot of the logarithms of relative concentration against time at 20°C [top], 40°C [middle] and 50°C [bottom] at pH9.

16. ADSORPTION COEFFICIENT

16.1. Guidelines

Organization for Economic Co-operation and Development (OECD), OECD Guideline for the Testing of Chemicals no. 121: "Estimation of the Adsorption Coefficient (K_{oc}) on Soil and on Sewage Sludge using High Performance Liquid Chromatography (HPLC)", January 22, 2001.

European Community (EC), EC no. 440/2008, Part C: Methods for the Determination of Ecotoxicity, Guideline C.19: "Estimation of the Adsorption Coefficient (K_{oc}) on Soil and on Sewage Sludge using High Performance Liquid Chromatography (HPLC)", Official Journal of the European Union no. L142, May 31, 2008.

16.2. Reagents

Water	Tap water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA)
Methanol	VWR International, Leuven, Belgium

All reagents were of analytical grade, unless specified otherwise.

16.3. Performance of the study

The principle of the test method is similar to that of the OECD guideline no. 117: "Partition coefficient (n-octanol/water), high performance liquid chromatography (HPLC) method". While passing through the column along with the mobile phase the test substance interacts with the stationary phase. As a result of partitioning between mobile and stationary phases, the test substance is retarded. The dual composition of a cyanopropyl stationary phase, having polar and non-polar sites allows for interaction of polar and non-polar groups of a molecule in a similar way as is the case for organic matter in soil or sewage sludge matrices. This enables the relationship between the retention time on the column and the K_{oc} on organic matter to be established.

According to the guidelines, the determination of the K_{oc} for test substances that are ionized for at least 10% within pH 5.5 to 7.5 should be performed with both the ionized and non-ionized form. Therefore, the pK_a values of the test substance were calculated using the Perrin calculation method (pKalc 5.0, module in Pallas 3.0, CompuDrug International San Francisco, CA, USA). Based on the calculations, the HPLC analysis was performed at neutral pH.

Solutions of reference substances with known log K_{oc} values based on soil adsorption data and the test substance were analysed. The capacity factor (k') of each compound was calculated from its retention time. The log k' values of the references substances were plotted against the known log K_{oc} values. A linear regression program was used to calculate the calibration curve. Linear regression analysis was performed using the least squares method. The coefficient of correlation (r) was calculated. The log K_{oc} value for the test substance was calculated by substituting its mean log k' in the calibration curve. The value of log K_{oc} obtained from duplicate measurements was within ± 0.25 log units.

16.4. Analytical method

16.4.1. Analytical conditions

Instrument	Alliance Separation Module 2695 (Waters, Milford, MA, USA)
Detector	Dual λ Absorbance Detector 2487 (Waters)
Column	Hypersil BDS-CN, 150 mm × 4.6 mm i.d., dp = 5 µm (Thermo Fisher Scientific, Waltham, MA, USA)
Column temperature	35°C ± 1°C
Mobile phase	30/70 (v/v) methanol/water
Flow	1 ml/min
Injection volume	10 µl
UV detection	210 nm

16.4.2. Preparation of the solutions

Solution of the unretained compound
A 4.81 g/l stock solution of formamide (99.8%, [75-12-7], Acros Organics, Geel, Belgium) in methanol was used. 500µl of the stock solution + 667 µl water was diluted in a 5 ml volumetric flask. The volumetric flask was filled up to the mark with 30/70 (v/v) methanol/ water.

The formamide blank solution was 30/70 (v/v) methanol/water.

Reference substance solutions
Stock solutions of the reference substances at concentrations of approximately 1 g/l in methanol were used. The stock solutions were diluted. Two reference substance solutions were made. Reference solution containing Acetanilide, Atrazine, Benzoic acid phenylester and Phenanthrene was made as followed: 50 µl of each reference substance + 267 µl water were diluted in a 5 ml volumetric flask. The volumetric flask was filled up to the mark with 30/70 (v/v) methanol/ water. The second reference substance solution containing Monuron, 2,5-Dichloroaniline and Fenthion was made as followed: 50 µl of each reference substance + 200 µl water were diluted in a 5 ml volumetric flask. The volumetric flask was filled up to the mark with 30/70 (v/v) methanol/ water.

The blank solution for the mixture of reference substances was 70/30 (v/v) methanol/water.

Reference substance	Purity	CAS number	Supplier	log K_{oc} #
Acetanilide	> 99.9%	103-84-4	Sigma-Aldrich	1.26
Atrazine	97.5%	1912-24-9	Sigma-Aldrich	1.81
Monuron	99.9%	150-68-5	Sigma-Aldrich	1.99
2,5-Dichloroaniline	99.8%	95-82-9	Sigma-Aldrich	2.55
Benzoic acid phenylester	99.9%	93-99-2	Sigma-Aldrich	2.87
Fenthion	98.3%	55-38-9	Sigma-Aldrich	3.31
Phenanthrene	98.1%	85-01-8	Acros Organics	4.09

values according to the OECD 121 guideline based on soil adsorption data
Acros Organics, Geel, Belgium
Sigma-Aldrich, Steinheim, Germany

Test solution
A 1445 mg/l stock solution of the test substance was prepared in methanol. The stock solution was diluted to obtain an end solution of 30/70 (v/v) methanol/water. The final concentration of the test substance solution was 14.5 mg/l.

The test substance blank solution was 30/70 (v/v) methanol/water.

16.4.3. Injections

The reference substance and test substance solutions were injected in duplicate. Blank solutions were analysed by single injection.

16.5. Formulas

Capacity factor (k') $$k' = \frac{(t_r - t_0)}{t_0}$$

where:
t_r = retention time
t_0 = mean column dead time

Calibration curve $$\log k' = a \log K_{oc} + b$$

where:
a = slope
b = intercept

16.6. Results

16.6.1. Calculation of pK_a values

The following pK_a values in the logarithm range of 1 - 14 for acidic and basic groups in the molecular structure of the test substance were calculated using the Perrin calculation method:

Acidic	Basic	
none	RARN(R1R2)	pK_a 4.21
	ROR	pK_a -3.28
	ROR	pK_a -3.88
	ROR	pK_a -4.37

Based on the calculated pK_a values the study was performed at neutral pH.

16.6.2. Determination of the K_{oc}

HPLC chromatograms of the test substance solution and corresponding blank are shown in Figure 12.

In the chromatogram of the test solution, one major peak and several small test substance peaks were observed.

The results of the HPLC method are given in the Table 31. Figure 13 shows the calibration curve of the log k' of the reference substances as function of log K_{oc}. The equation of the regression line was: log k' = 0.502 × log K_{oc} – 0.930 (r = 0.96, n = 14).

Table 31 K_{oc} of the test substance

Substance	$t_{r,1}$ [min]	$t_{r,2}$ [min]	mean t_r (n=2)	log K_{oc}	K_{oc}	Area %
Formamide (t_0)	1.997	1.995	1.996			
Acetanilide	2.853	2.853		1.26		
Atrazine	4.262	4.257		1.81		
Monuron	4.148	4.147		1.99		
2,5-Dichloroaniline	5.380	5.382		2.55		
Benzoic acid phenylester	9.522	9.529		2.87		
Fenthion	21.314	21.347		3.31		
Phenanthrene	19.634	19.691		4.09		
Impurity I	1.969	1.968	1.969	*	*	0.28
Impurity II	2.946	2.939	2.943	1.21	1.6×10^1	0.67
Impurity III	3.672	3.648	3.660	1.70	5.0×10^1	1.1
Test substance	4.169	4.163	4.166	1.93	8.4×10^1	96
Impurity IV	4.766	4.760	4.763	2.14	1.4×10^2	0.91
Impurity V	5.005	5.004	5.005	2.21	1.6×10^2	0.77
Impurity VI	5.173	5.169	5.171	2.26	1.8×10^2	0.52

* Since the retention time of Impurity I was less than that of formamide, a K_{oc} and log K_{oc} value could not be calculated.

16.7. Conclusion

The HPLC method using soil-adsorption-reference data was applied for the determination of the adsorption coefficient (K_{oc}) of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline.

The K_{oc} and log K_{oc} value of the test substance and impurities with peak areas > 1% at neutral pH was:

	K_{oc}	log K_{oc}	Area %
Test substance	8.4×10^1	1.93	96
Impurity III	5.0×10^1	1.70	1.1

16.8. Figures

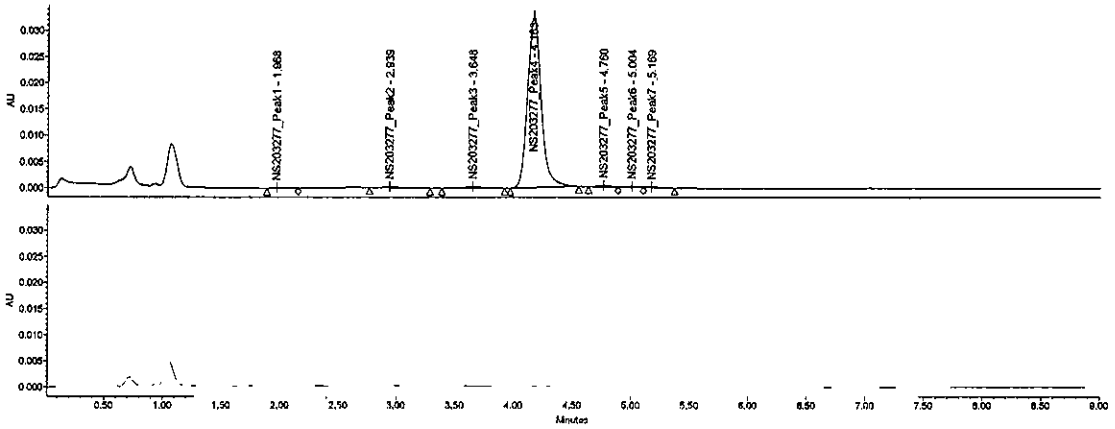


Figure 12 HPLC chromatograms of the 14.5 mg/l test solution [top; res. id. 1571] and corresponding blank [bottom; res. id. 1572].

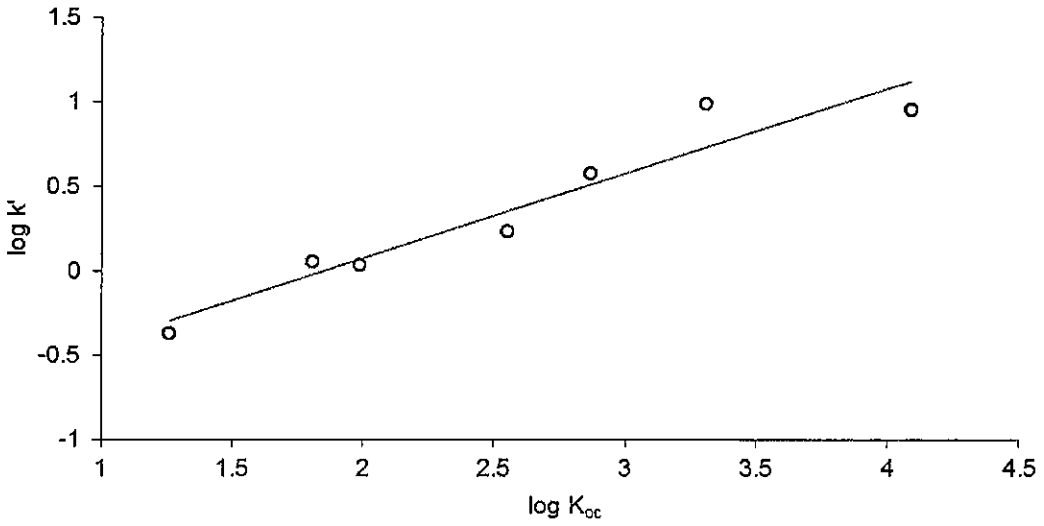


Figure 13 The regression line of the reference substances: log k' *versus* log K_{oc}.

17. DISSOCIATION CONSTANT

17.1. Guidelines

Organization for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals, Guideline no. 112: "Dissociation Constants in Water" Adopted May, 12 1981.

17.2. Reagents

Citric acid monohydrate	Merck, Darmstadt, Germany
Sodium chloride (NaCl)	Merck
Triethylamine	Merck
Acetic acid	Merck
Disodium tetraborate decahydrate ($Na_2B_4O_7*10H_2O$)	Merck
Hydrochloric acid (HCl)	37% Merck
Hydrochloric acid (HCl)	1M, Merck
Sodium hydroxide (NaOH)	pellets, Merck
Sodium hydroxide	1M, Merck
Potassium dihydrogen phosphate (KH_2PO_4)	Merck
Disodium hydrogenphosphate dodehydrate ($Na_2HPO_4*12H_2O$)	Merck
Potassium chloride	Merck
Acetonitrile	Biosolve, Valkenswaard, The Netherlands
Water	Tap water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA).

All reagents were of analytical grade, unless specified otherwise.

17.3. Buffers

All buffers were prepared in water and adjusted to the required pH with the chemical written after the sign "/".

Buffer pH 0: 1M HCl

Buffer pH 1: 0.488 g HCl + 0.372 g KCl in 1000 ml water / adjusted to pH 1 with 1 M HCl

Buffer pH 2: 3.17 g Citric acid monohydrate + 0.158 g HCl + 1.80 g NaCl in 1000 ml water / adjusted to pH 2 with 1 M HCl

Buffer pH 3: 4.21 g Citric acid monohydrate + 0.445 g NaOH + 1.81 g NaCl in 1000 ml water

Buffer pH 4: 5.86 g Citric acid monohydrate + 1.35 g NaOH + 1.30 g NaCl in 1000 ml water / adjusted to pH 4 with 1 M NaOH

Buffer pH 5: 0.6 g Acetic acid in 1000 ml water / adjusted to pH 5 with 1 M NaOH

Buffer pH 6: 4.21 g Citric acid monohydrate + 2.02 g NaOH in 1000 ml water / adjusted to pH 6 with 1 M NaOH

Buffer pH 7: 3.56 g KH_2PO_4 + 14.68 g $Na_2HPO_4*12H_2O$ in 1000 ml water / adjusted to pH 7 with 1 M HCl

Buffer pH 8: 4.96 g $Na_2B_4O_7*10H_2O$ + 0.78 g HCl in 1000 ml water / adjusted to pH 8 with 1 M HCl

Buffer pH 9: 4.95 g $Na_2B_4O_7*10H_2O$ + 0.16 g HCl in 1000 ml water / adjusted to pH 9 with 1 M HCl

Buffer pH 10: 4.96 g $Na_2B_4O_7*10H_2O$ + 0.76 g NaOH in 1000 ml water / adjusted to pH 10 with 1 M HCl

Buffer pH 11: 1.02 g Triethylamine in 1000 ml water / adjusted to pH 11 with 1 M HCl

Buffer pH 12: 0.39 g NaOH in 1000 ml water / adjusted to pH 12 with 1 M NaOH

Buffer pH 13: 4.05 g NaOH in 1000 ml water / adjusted to pH 13 with 1 M NaOH

Buffer pH 2.6: 100 ml buffer pH 3 adjusted to pH 2.6 with 1 M HCl

Buffer pH 2.8: 100 ml buffer pH 3 adjusted to pH 2.8 with 1 M HCl

Buffer pH 3.0: 100 ml buffer pH 3 adjusted to pH 3.0 with 1 M HCl

Buffer pH 3.2: 100 ml buffer pH 3 adjusted to pH 3.2 with 1 M NaOH

Buffer pH 3.4: 100 ml buffer pH 3 adjusted to pH 3.4 with 1 M NaOH

Buffer pH 3.6: 100 ml buffer pH 3 adjusted to pH 3.6 with 1 M NaOH

Buffer pH 3.8: 100 ml buffer pH 3 adjusted to pH 3.8 with 1 M NaOH

17.4. Performance of the study

Three methods are available for the determination of the dissociation constant(s) (pK_a) of the test substance. The principle of each method is described below:

1. Titration method
A test solution of the test substance is titrated with standard acid or base, as appropriate. The pK_a values are determined based on the titration curve obtained. The method is not suitable for low solubility compounds.

2. Spectrophotometric method
The spectrophotometric method involves the determination of the relative concentrations of the ionised and unionised forms of the test substance and their pH dependence. The method is only applicable to compounds having appreciably different UV/Vis adsorption spectra for the ionised and unionised forms. This method may be suitable for low solubility compounds.

3. Calculation method
Calculation of the pK_a values from the structural formula is performed using the Perrin calculation method (pKalc 5.0, module in Pallas 3.0, CompuDrug International, San Francisco, CA, USA).

The spectrophotometric method was selected for the determination of the pK_a values of the test substance. The calculation method was used for a preliminary estimation of the P_{ow} of the test substance.

17.5. Spectrophotometric method

17.5.1. Test system

Spectrophotometer	Single-beam, Varian Cary 50 UV/VIS, Varian, Mulgrave, Victoria, Australia
Slit	1.5 nm
Scan speed	600 nm/min
Probe	Ultra mini immersion probe with titanium tube and stainless steel handle. Prism made from quartz. pathlength = 10 mm
Type of cuvette	Quartz
Background-correction	Reference solution

17.6. Performance of the test

17.6.1. Search for spectra of the pure molecule and the pure anion

The –N group in the test substance can dissociate. The pK_a value to be determined is thus for a basic group.

A 4385 mg/l solution of the test substance was prepared in acetonitrile and further diluted in acetonitrile prior to use. Then, fourteen test solutions (all 20 mg/l) were prepared by diluting the stock solution with various buffers (pH 0, pH 1, pH 2, pH 3, pH 4, pH 5, pH 6, pH 7, pH 8, pH 9, pH 10, pH 11, pH 12 and pH 13).

Reference solutions were prepared by diluting acetonitrile the same as the test solution with each of the above mentioned buffers.

From each of the test solutions, an absorption spectrum between 200 and 900 nm was recorded with the appropriate reference solution as reference. The pH of each solution (test and reference solution) was measured using a pH meter.

17.6.2. Choice of an analytical wavelength

The analytical wavelength was chosen from the absorption spectra recorded from the test substance between pH 0 and pH 13.

17.6.3. Search for an approximate pK_a value

The optical absorbances at the analytical wavelength were obtained from the absorption spectra recorded from 20 mg/l solutions between pH 0 and pH 7. From these results, an approximate pK_a value was calculated.

17.6.4. Exact determination of pK_a

Three different stock solutions of the test substance in acetonitrile; 3770 mg/l, 4385 mg/l and 15376 mg/l were prepared and diluted with acetonitrile prior to use. Nine test solutions (all 20 mg/l) were prepared by diluting each stock solution with buffers pH 0, pH 2.6, pH 2.8, pH 3, pH 3.2, pH 3.4, pH 3.6, pH 3.8 and pH 6.

Reference solutions were prepared by diluting acetonitrile the same as the test solution with each of the above mentioned buffers.

The optical absorbances at the analytical wavelength of all nine test solutions were measured after zero-adjustment with the appropriate reference solution. The pH of each solution (test and reference) was measured.

17.7. Formulas

For an basic group, the following equation is used if Ai > Am:

$$pKa = pH + \log \frac{A - Am}{Ai - A}$$

If Am > Ai:

$$pKa = pH + \log \frac{Am - A}{A - Ai}$$

Where Am is the optical absorbance of the neutral molecule. Ai is the optical absorbance of the ion and A is the optical absorbance of the mixture of ion and neutral molecule. pH is the actual pH.

17.8. Results

17.8.1. Calculation method

The following pK_a values in the logarithm range of 1 - 14 for acidic and basic groups in the molecular structure of the test substance were calculated using the Perrin calculation method:

Acidic	Basic	
none	RARN(R1R2)	pK_a 4.21
	ROR	pK_a -3.28
	ROR	pK_a -3.88
	ROR	pK_a -4.37

17.8.2. Search for spectra of the pure molecule and the pure anion

The –N group in the test substance can dissociate. The pK_a value to be determined is thus for an basic group.

The spectra recorded at pH 0 and pH 1 were comparable and so were the spectra recorded at pH 5 till pH 13. The spectra recorded at pH 2, pH 3 and pH 4 had a form somewhere between those at pH 1 and pH 5 and therefore called intermediate spectra. It was concluded that spectra recorded at pH $\leq$ 1 are of the ionized test substance, whereas spectra recorded at pH $\geq$ 5 are of the non-ionized test substance. The intermediate spectra are of a combination of both species. Absorption spectra at pH 0 to 13 are shown in Figure 14 to Figure 27.

17.8.3. Choice of an analytical wavelength

From the spectra of the ionized substance with the spectra of the non-ionized substance, it was concluded that the difference in optical absorbance is maximal at 249 nm. Hence 249 nm was chosen as the analytical wavelength.

17.8.4. Search for an approximate pK_a value

The optical absorbances at 249 nm were determined. As well as the actual pH values of the test solutions and actual pH values of the reference solutions. From these absorbances and the actual pH value, the approximate pK_a value was calculated. All results are summarized in Table 32.

Table 32 Actual pH values and optical absorbances at 249 nm.

Concentration stock [mg/l]	actual pH values of the reference solutions	actual pH values of the test solutions	Absorbance for the test solution [units]	pK_a value
4385	0.39	0.36	0.0138	
	0.97	1.01	0.0179	3.25
	1.97	1.99	0.0824	2.97
	3.02	3.04	0.3550	3.08
	4.12	4.12	0.6398	3.29
	5.08	5.08	0.7211	3.32
	6.10	6.11	0.7334	
	7.12	7.11	0.7284	
	7.99	8.02		
	9.02	9.04		
	9.97	10.01		
	10.78	10.8		
	11.63	11.65		
	12.68	12.71		

The approximate pK_a value was calculated to be 3.18.

17.8.5. Exact determination of the pK_a value

The actual pH values of the reference solutions were 0.30, 2.62, 2.81, 3.03, 3.24, 3.45, 3.64, 3.84 and 5.70.

The optical absorbances at 249 nm of all nine test solutions and the seven calculated pKa values are summarized in Table 33.

Table 33 Optical absorbances at 249 nm.

Concentration stock [mg/l]	Actual pH	Absorbance for the test solution [units]	pK_a value
4385	0.30	0.0170	
	2.62	0.1662	3.23
	2.81	0.2328	3.21
	3.04	0.3137	3.23
	3.24	0.3945	3.24
	3.45	0.4783	3.26
	3.64	0.5488	3.27
	3.84	0.6134	3.27
	5.70	0.7732	
3770	0.29	0.0230	
	2.62	0.1719	3.24
	2.82	0.2353	3.24
	3.04	0.3229	3.23
	3.24	0.4024	3.25
	3.45	0.4925	3.25
	3.64	0.5680	3.25
	3.84	0.6304	3.26
	5.75	0.7896	
15376	0.29	0.0230	
	2.62	0.1844	3.22
	2.82	0.2465	3.23
	3.04	0.3316	3.24
	3.24	0.4144	3.26
	3.46	0.5073	3.28
	3.64	0.5806	3.28
	3.84	0.6486	3.29
	5.78	0.8240	

The twenty one pK_a values were averaged, resulting in the pK_a value of 3.25. The standard deviation is 0.02.

The test was performed in a room where the temperature (20.4 ± 0.6°C) was measured continuously using a validated computerised system (Rees Scientific, NJ, USA).

17.9. Conclusion

The pK_a value for p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline was determined to be 3.25 using the spectrophotometric method. This pK_a value corresponds to dissociation of a basic group in the molecule.

17.10. Figures

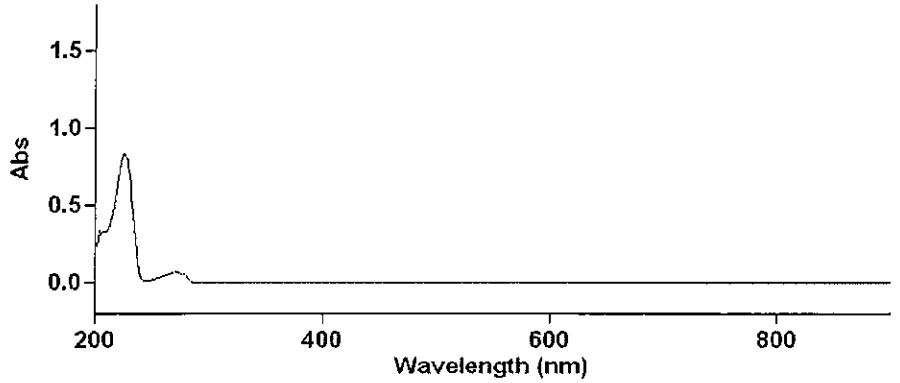


Figure 14 Absorption spectrum of a 20 mg/l solution of test substance in buffer pH 0.

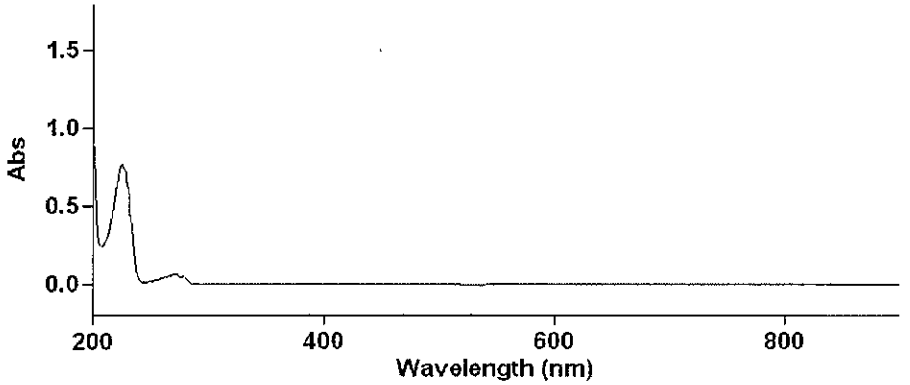


Figure 15 Absorption spectrum of a 20 mg/l solution of test substance in buffer pH 1.

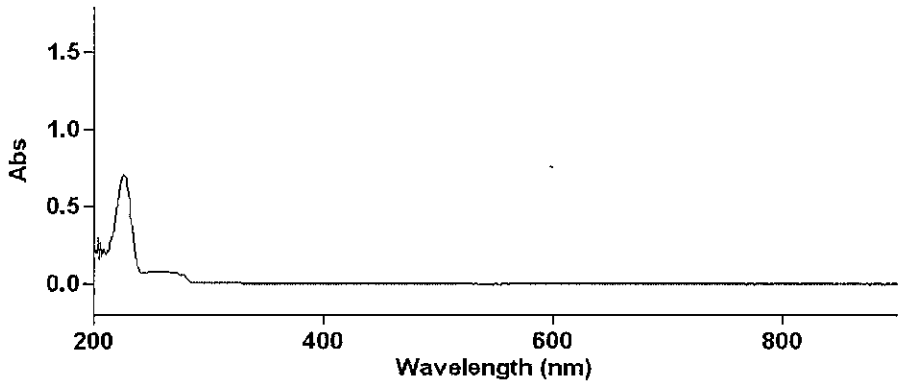


Figure 16 Absorption spectrum of a 20 mg/l solution of test substance in buffer pH 2.

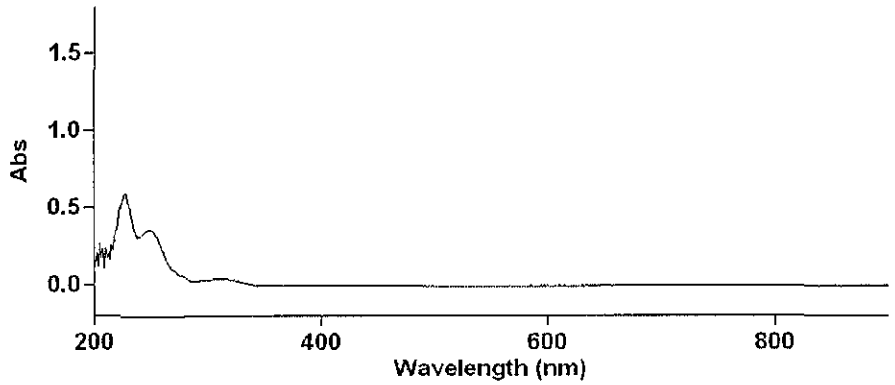


Figure 17 Absorption spectrum of a 20 mg/l solution of test substance in buffer pH 3.

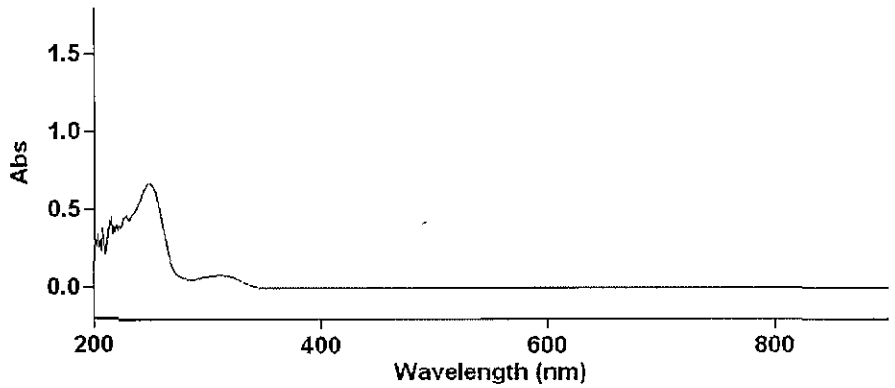


Figure 18 Absorption spectrum of a 20 mg/l solution of test substance in buffer pH 4.

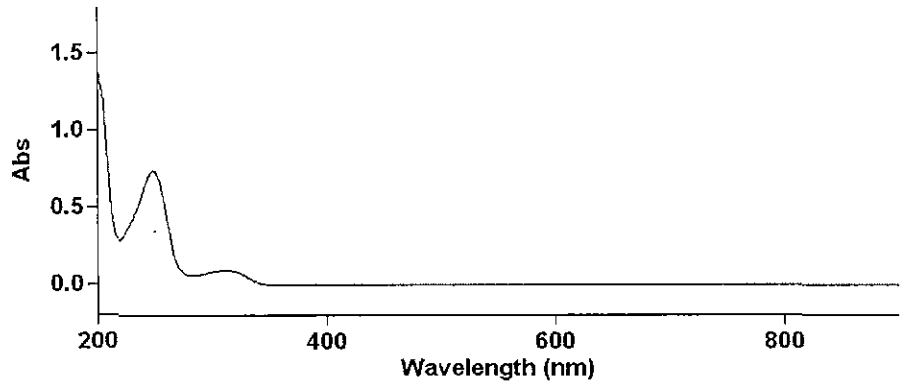


Figure 19 Absorption spectrum of a 20 mg/l solution of test substance in buffer pH 5.

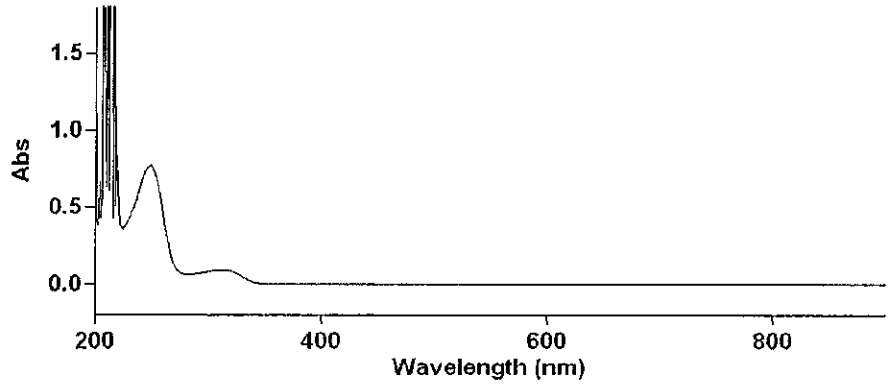


Figure 20 Absorption spectrum of a 20 mg/l solution of test substance in buffer pH 6.

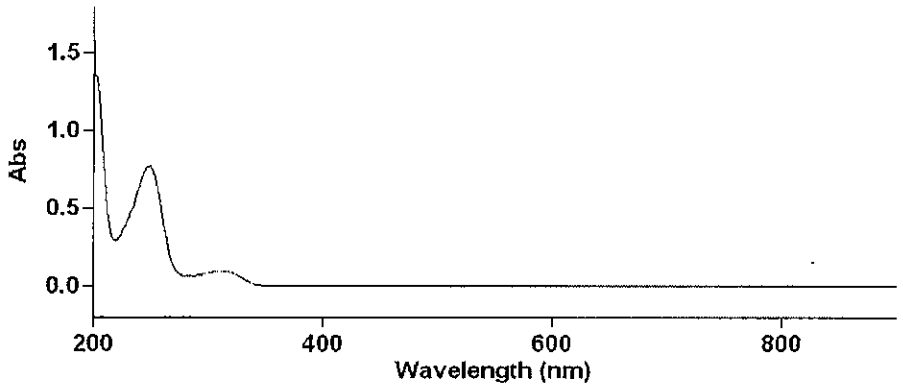


Figure 21 Absorption spectrum of a 20 mg/l solution of test substance in buffer pH 7.

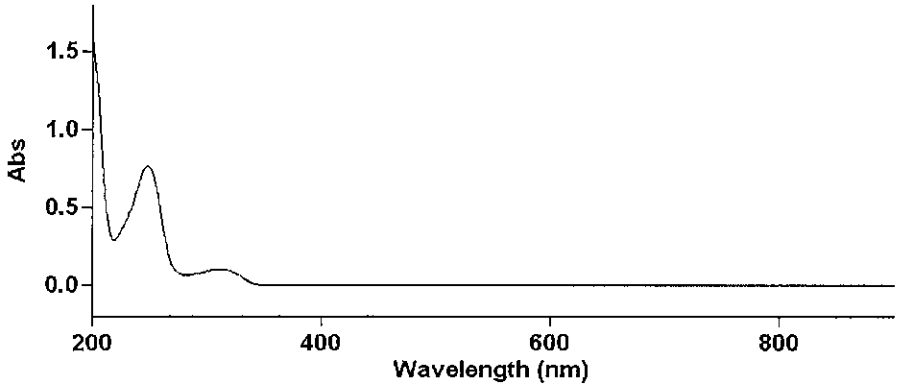


Figure 22 Absorption spectrum of a 20 mg/l solution of test substance in buffer pH 8.

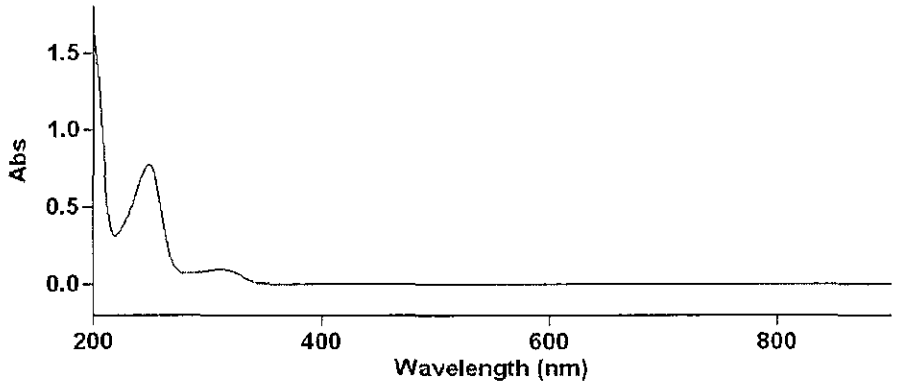


Figure 23 Absorption spectrum of a 20 mg/l solution of test substance in buffer pH 9.

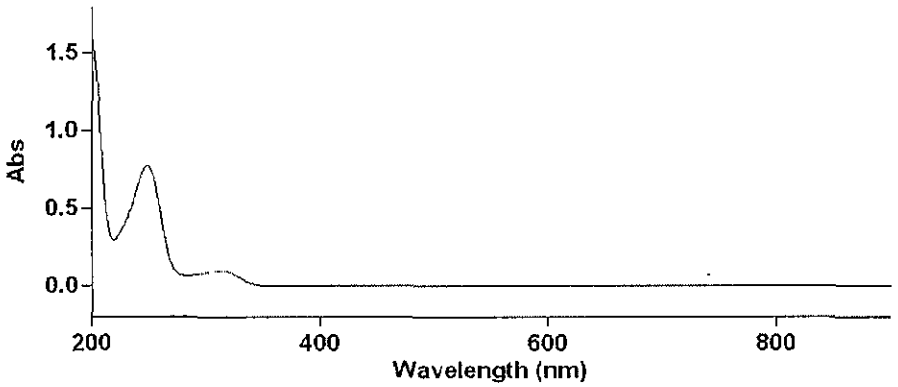


Figure 24 Absorption spectrum of a 20 mg/l solution of test substance in buffer pH 10.

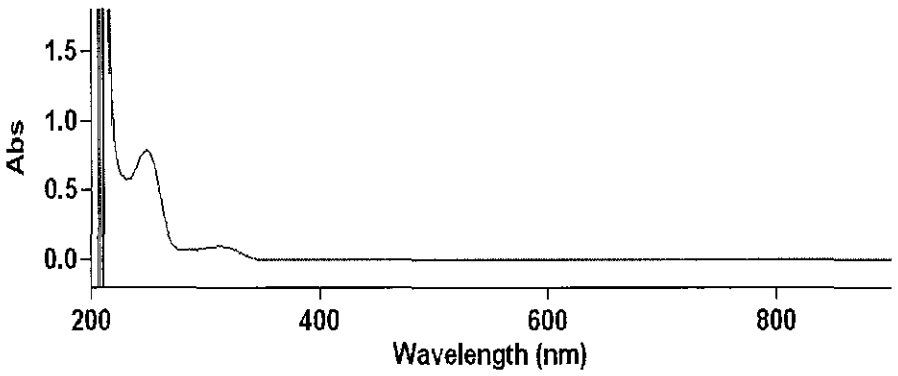


Figure 25 Absorption spectrum of a 20 mg/l solution of test substance in buffer pH 11.

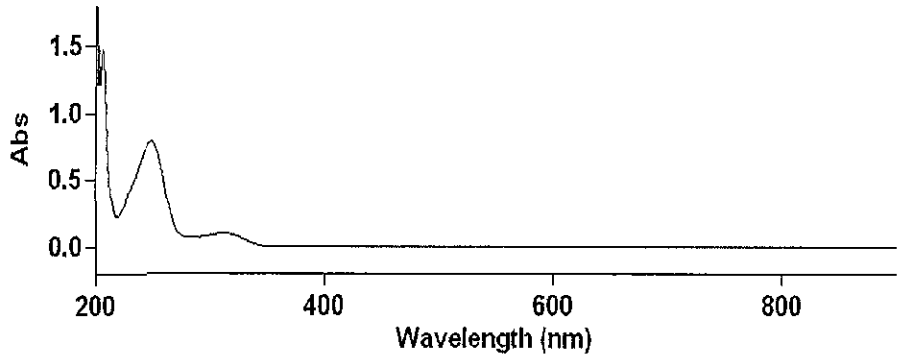


Figure 26 Absorption spectrum of a 20 mg/l solution of test substance in buffer pH 12.

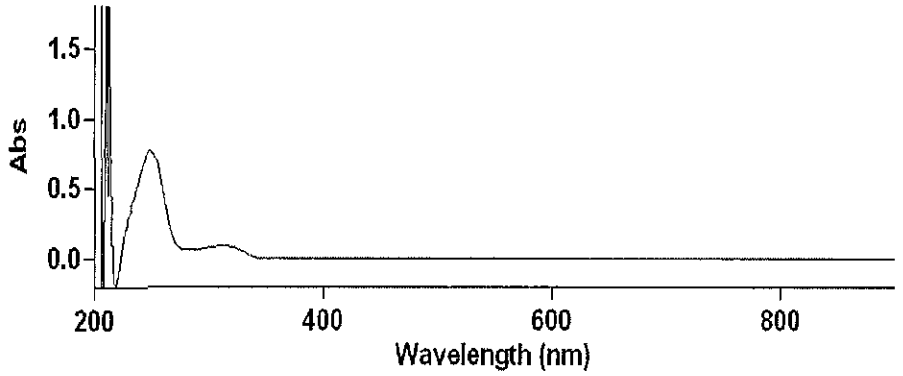


Figure 27 Absorption spectrum of a 20 mg/l solution of test substance in buffer pH 13.

18. VISCOSITY

18.1. Guidelines

Organization for Economic Co-operation and Development (OECD), OECD Guidelines for the Testing of Chemicals no. 114: "Viscosity of Liquids", May 12, 1981.

International Organization for Standardization (ISO), ISO 3104: "Petroleum Products - Transparent and Opaque Liquids - Determination of Kinematic Viscosity and Calculation of Dynamic Viscosity", 1994.

ASTM International, ASTM D 445-09: "Standard Test Method for Kinematic Viscosity of Transparent and Opaque Liquids (and Calculation of Dynamic Viscosity)", July 1, 2009.

18.2. Performance of the test

The viscosity of the test substance was determined at 20°C and 40°C using a glass capillary viscometer (Tamson Instruments, Bleiswijk, The Netherlands).

Based on appearance of the test substance, the Ubbelohde viscometers with constants 0.4655 and 0.4685 were selected. The viscometer was charged with the test substance by tilting it about 30° from the vertical position. When the viscometer was returned to the vertical the meniscus of the test substance was between the filling marks of the reservoir bulb.

The viscometer was placed in the thermostatic water bath and remained in the bath for at least 30 minutes to reach the test temperature.

The test substance was drawn by vacuum through the timing tube to about 5 mm above the upper timing mark. The vacuum was released. The time required for the meniscus to pass from the upper to the lower timing mark was measured and the kinematic viscosity of the test substance was determined.

The kinematic viscosity of the test substance was determined at 20 ± 0.1°C and 40 ± 0.1°C. Each test was performed in duplicate.

18.3. Interpretation

18.3.1. Specifications

The kinematic viscosity is defined as the resistance to flow of a fluid under gravity. The kinematic viscosity is expressed in mm^2/s.

18.3.2. Formulas

Kinematic viscosity $\upsilon = C \times t$

where:
C = calibration constant of the viscometer [$(mm^2/s)/s$]
t = flow time [s]

18.4. Results

The results for the kinematic viscosity of the test substance at 20°C and 40°C are given in Table 34.

Table 34 Viscosity of the test substance

Temperature [°C]	Test	Calibration constant [(mm²/s)/s]	Flow time [s]	Kinematic viscosity [mm²/s]	Mean [mm²/s]
20	I	0.4685	1790.9	839.1	840.6
	II	0.4655	1809.1	842.2	
40	I	0.4655	294.5	137.1	138.9
	II	0.4685	300.4	140.7	

18.5. Conclusion

The viscosity of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline was determined using a glass capillary viscometer.

The kinematic viscosity of the test substance was:

	Kinematic viscosity [mm²/s]	
	20°C	40°C
Test substance	840.6	138.9

Appendix 1 GLP certificate

ENDORSEMENT OF COMPLIANCE

WITH THE OECD PRINCIPLES OF GOOD LABORATORY PRACTICE

Pursuant to the Netherlands GLP Compliance Monitoring Programme and according to Directive 2004/9/EC, the conformity with the OECD Principles of GLP was assessed on 21 – 25 March, 2011 at

NOTOX B.V.
Hambakenwetering 7
5231 DD 's Hertogenbosch

It is herewith confirmed that the afore-mentioned test facility is currently operating in compliance with the OECD Principles of Good Laboratory Practice in the following area of expertise: Physical-chemical testing, Toxicity studies; Mutagenicity studies; Environmental toxicity studies on aquatic and terrestrial organisms; Studies on behaviour in water, soil, and air, bioaccumulation; Residue studies; Analytical and clinical chemistry testing; Kinetic and metabolism studies, Safety Pharmacology.

Den Haag, 19 May 2011

Dr. R. Jaspers
Manager GLP Compliance Monitoring Program

Food and Consumer Product Safety Authority (VWA)
Catharijnesingel 59
3511 GG Utrecht, The Netherlands

REPORT

DETERMINATION OF 'READY' BIODEGRADABILITY:

CARBON DIOXIDE (CO_2) EVOLUTION TEST (MODIFIED STURM TEST)

WITH

TK 12759, ARALDITE MY 0510

NOTOX Project 169875
NOTOX Substance 59652
Ciba Project 964625

TK 12759, ARALDITE MY 0510

NOTOX Project 169875
Ciba Project 964625

REPORT APPROVAL

STUDY DIRECTOR: Ing. M.J.E. Desmares-Koopmans

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Date: March 27, 1996

MANAGEMENT: Ing. E.J. van de Waart
Section Head, Genetic &
Eco-Toxicology

i.a. Dr. Ilona C. Enninga
Technical Director

.....

Date: 29/03/1996

SUMMARY

TK 12759, ARALDITE MY 0510 was tested for its ready biodegradability in the Carbon Dioxide (CO_2) Evolution Test (Modified Sturm Test) at ca. 39 mg per 2 litres, corresponding to ca. 12 mg TOC/l.

The study procedure was based on EEC directive 92/69, C.4-C, December 1992, and OECD guideline No. 301 B July 17, 1992.

The Theoretical CO_2 production ($ThCO_2$) of TK 12759, ARALDITE MY 0510 (MF: $C_{15}H_{19}NO_4$, MW: 277 g) was calculated to be 2.383 mg CO_2/mg.

The relative degradation values calculated from the measurements performed during the test period revealed no significant degradation of TK 12759, ARALDITE MY 0510 in test bottle A (significant: >10%).
In test bottle B 30% degradation was measured on day 29. This result was not comparable to the results of test bottle A and the toxicity control, and it was assumed that the produced CO_2 in test bottle B was not test substance related, but the result of a technical failure. Therefore, the degradation was based on the data of day 27 and considered as not significant.

Since all further acceptability criteria were met, this study was considered to be valid.

In conclusion, TK 12759, ARALDITE MY 0510 was not readily biodegradable under the conditions of the modified Sturm test presently performed.

PREFACE

Sponsor	Ciba-Geigy Ltd. Polymers Division R-1006.520 CH-4002 BASEL Switzerland
Study Monitor	Dr. E.G. Semadeni
Testing Facility	NOTOX B.V. Hambakenwetering 3 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Ing. M.J.E. Desmares-Koopmans
Technical Coordinator	Mrs. C.J. van Leeuwen-van Drunen
Study Plan	Start: February 14, 1996 Completion: March 14, 1996

TEST SUBSTANCE

Identification	TK 12759, ARALDITE MY 0510
Description	Clear yellow liquid (Determined at NOTOX)
Batch	50008M
Purity	> 95%
Test substance storage	In freezer in the dark
Stability under storage conditions	Stable
Expiry date	May 01, 1998
Stability in water	At least 96 h

The sponsor is responsible for the completeness and GLP Compliance of all test substance data.

PURPOSE

The purpose of the study is to evaluate a non-volatile test substance for its ready biodegradability in an aerobic aqueous medium with microbial activity introduced by inoculation of activated sludge.

GUIDELINES

The study procedure described in this report was based on the following guidelines:

European Economic Community (EEC), EEC directive 92/69, Part C: Methods for the determination of ecotoxicity, Publication No. L383, December 1992, C.4. "Biodegradation: determination of the 'ready' biodegradability, C.4-C: Carbon dioxide (CO_2) evolution test (Modified Sturm Test).

Organisation for Economic Co-operation and Development (OECD), OECD guidelines for Testing of Chemicals, Section 3, Degradation and Accumulation, guideline No. 301 B: "Ready Biodegradability: CO_2 Evolution Test" adopted July 17, 1992.

DEFINITIONS

Readily biodegradable: Test substances giving a result of at least 60% yield of CO_2 (within 28 days). This pass level must be reached within 10 days of biodegradation exceeding 10%.

$ThCO_2$: Theoretical carbon dioxide (mg) is the quantity of carbon dioxide calculated to be produced from the known or measured carbon content of the test substance when fully mineralized; also expressed as mg carbon dioxide evolved per mg test substance.

TOC : Total organic carbon of a sample is the sum of the organic carbon in solution and in suspension.

PREPARATION OF TEST SOLUTIONS

After addition of milli-Q water to each weighing bottle containing TK 12759, ARALDITE MY 0510 (A: 38.7 mg, B: 39.5 and toxicity control: 39.0 mg), followed by ultra sonication (15 min.), the resulting mixture was quantitatively added to the test media. The test solutions were continuously stirred during the test.

TEST SYSTEM

Source	The source of test organisms was activated sludge freshly obtained from a municipal sewage treatment plant: 'Waterschap de Maaskant', 's-Hertogenbosch, the Netherlands.
Treatment	The sludge was kept under continuous aeration until further treatment. The concentration of suspended solids was 3.2 g/l in the concentrated sludge. Before use, the sludge was allowed to settle for at least 30 minutes and the liquid decanted for use as inoculum at the amount of 10 ml/l of mineral medium.
Reason for selection	The test has been accepted internationally (EEC, OECD) for determining the 'ready' biodegradability of test substances under aerobic conditions.

TEST PROCEDURE AND CONDITIONS

Test duration	28 days (last CO_2-measurement on the 29th day).
Test vessels	2 litre all-glass brown coloured bottles.
Milli-Q water	Tap-water purified by reverse osmosis and subsequently passed over activated carbon and ion-exchange cartridges (Millipore Corp., Bedford, Mass., USA).
Stock solutions of mineral components	A) 8.50 g KH_2PO_4 21.75 g K_2HPO_4 67.20 g $Na_2HPO_4.12H_2O$ 0.50 g NH_4Cl dissolved in 1 l Milli-Q water, pH 7.4±0.2 B) 22.50 g $MgSO_4.7H_2O$ dissolved in 1 l Milli-Q water. C) 36.40 g $CaCl_2.2H_2O$ dissolved in 1 l Milli-Q water. D) 0.25 g $FeCl_3.6H_2O$ dissolved in 1 l Milli-Q water.

Mineral medium	1 l mineral medium contains: 10 ml of solution (a), 1 ml of solutions (b) to (d) and Milli-Q water.
Barium hydroxide, 0.0125 M	4 g $Ba(OH)_2.8H_2O$ per litre Milli-Q water was filtered through filter paper and stored in a sealed vessel to prevent absorption of CO_2 from the air.
CO_2-free air	A mixture of oxygen (21%) and nitrogen (79%) was led through a bottle, containing 0.5 - 1 litre 0.0125 M $Ba(OH)_2$ solution to trap CO_2 which might be present in small amounts. The CO_2-free air was sparged through the scrubbing solutions at a constant rate.
Test concentration	The test substance was tested in duplicate at ca. 39 mg per 2 litres, corresponding to ca. 12 mg TOC/l. The organic carbon content was based on the molecular formula.
Preparation of bottles:	
Pre-incubation medium	Mineral components, Milli-Q water (ca. 80% total volume) and inoculum (1% final volume) were added to each bottle. This mixture was aerated with CO_2-free air overnight to purge the system of CO_2.
Type and number of bottles	Test suspension: containing test substance and inoculum (2 bottles). Inoculum blank: containing only inoculum (2 bottles) Positive control: containing reference substance (ca. 40 mg/l sodium acetate (Merck art. 6268, batch 049 TA933768), TOC= 12 mg/l) and inoculum (1 bottle). Toxicity control: containing test substance, reference substance and inoculum (1 bottle).
Preparation	The test substance and positive control were added to the bottles. The volumes of suspensions were made up in all bottles by the addition of Milli-Q water previously aerated with CO_2-free air, resulting in the mineral medium described before. Three CO_2-absorbers (bottles filled with 100 ml 0.0125 M $Ba(OH)_2$) were connected in series to the exit air line of each test bottle.
Start of the incubation	The test was started by bubbling CO_2-free air through the solution at a rate of approximately 1-2 bubbles per second (ca. 30-100 ml/min).

DETERMINATION OF CO_2

Experimental CO_2 production	The CO_2 produced in each test bottle reacted with the barium hydroxide in the gas scrubbing bottle and precipitated out as barium carbonate. The amount of CO_2 produced was determined by titrating the remaining $Ba(OH)_2$ with 0.05 M standardized HCl.
Measurements	Titrations were made every second or third day during the first 10 days, and thereafter at least every fifth day until the 28th day. Each time the CO_2-absorber nearest to the test bottle was removed for titration; each of the remaining two absorbers was moved one position in the direction of the test bottle. A new CO_2-absorber was placed at the far end of the series. Phenolphthalein was used as pH-indicator. On the 28th day, the pH of the test suspensions was measured and 1 ml of concentrated HCl was added to each bottle. The bottles were aerated overnight to drive off CO_2 present in the test suspension. The final titration was made on day 29.
Theoretical CO_2 production	The theoretical CO_2 production was calculated from the molecular formula.

DATA EVALUATION

$ThCO_2$, expressed as mg CO_2/mg test substance, that can be generated by a test substance was calculated as follows:

$$ThCO_2 = \frac{\text{No. of carbon in test substance} \times MW\ CO_2}{MW\ \text{test substance}}$$

The first step in calculating the amount of CO_2 produced is to correct for background (endogeneous) CO_2 production. Thus the amount of CO_2 produced by a test material is determined by the difference (in ml of titrant) between the experimental and blank $Ba(OH)_2$ traps.

The amount of 0.05 N HCl titrated is converted into mg of CO_2 produced:

$$\text{mg } CO_2 = \frac{(0.05 \times \text{ml HCl titrated})}{2} \times 44 = 1.1 \times \text{ml of HCl titrated.}$$

Calculations were based on the actual normality without rounding off. Relative degradation values was calculated from the cumulative CO_2 production relative to the total expected CO_2 production based on the total carbon content of the amount of test material present in the test bottles. They were plotted versus time together with the relative degradation of the positive control.

ACCEPTABILITY OF THE TEST

The results of the biodegradation test were considered to be valid when:

- the total CO_2 evolution in the inoculum blank at the end of the test did not normally exceed 40 mg/l. If values greater than 70 mg CO_2/l are obtained, the data and experimental technique should be examined critically.

- the difference of duplicate values for the %-degradation of the test substance at the plateau, at the end of the test or at the end of the 10-day window, as appropriate, was less than 20.

- the percentage degradation of the reference substance reached the level for ready biodegradability (60%) by 14 days.

Because of the stringency of the method, low values do not necessarily mean that the test substance is not biodegradable under environmental conditions, but indicates more work will be necessary to establish biodegradability.

Toxicity control: if less than 35% degradation (based on DOC) or less than 25% degradation (based on $ThCO_2$) occurred in 14 days, the test substance can be assumed to be inhibitory.

RESULTS

Theoretical CO_2 production

The Theoretical CO_2 production ($ThCO_2$) of TK 12759, ARALDITE MY 0510 (MF: $C_{15}H_{19}NO_4$, MW: 277 g) was calculated to be 2.383 mg CO_2/mg.

The concentration was 38.7 (A) and 39.5 mg (B) TK 12759, ARALDITE MY 0510 in 2 litres test medium. Hence, the theoretical CO_2 production following complete degradation was 92.2 mg for A and 94.1 mg per 2 litres for B.

The positive control contained 80.1 mg sodium acetate ($ThCO_2$= 1.073 mg CO_2/mg) resulting in a theoretical CO_2 production following complete degradation of 85.9 mg per 2 litres.

The toxicity control contained 80.1 mg sodium acetate and 39.0 mg TK 12759, ARALDITE MY 0510 in 2 litres of test medium. Hence, the theoretical CO_2 production following complete degradation of TK 12759, ARALDITE MY 0510 plus sodium acetate was 178.9 mg per 2 litres.

Biodegradation

The results are summarized in Tables 1-4, and Figure 1.

The relative degradation values calculated from the measurements performed during the test period revealed no significant degradation of TK 12759, ARALDITE MY 0510 in test bottle A (significant: >10%).
In test bottle B 30% degradation was measured on day 29. This result was not comparable to the results of test bottle A and the toxicity control, and it was assumed that the produced CO_2 in test bottle B was not test substance related, but the result of a technical failure. Therefore, the degradation was based on the data of day 27 and considered as not significant.

In the toxicity control more than 25% degradation occurred in 14 days (based on $ThCO_2$). Therefore, the test substance was assumed to be not inhibitory.

Monitoring of temperature and pH

The temperature recorded during the study varied between 20 and 23°C.

The pH values of the different test media were:

	Just before the start of the test:	On day 28:
Blank control (A)	7.5	7.5
Blank control (B)	7.5	7.5
Positive control	7.5	7.8
TK 12759, ARALDITE MY 0510 (A)	7.6	7.7
TK 12759, ARALDITE MY 0510 (B)	7.6	7.6
Toxicity control	7.6	7.9

Acceptability of the test

The positive control substance was degraded 79% in 14 days (see Table 1 and Figure 1).

The total CO_2 release in the blank reached a total value of 20 mg CO_2 per 2 litres of medium (see Table 4).

Except for day 29, the difference of duplicate values for %-degradation of TK 12759, ARALDITE MY 0510 was always less than 20. Since the degradation on day 29 in test bottle B was considered to be due to a technical failure, and the final judgement for this bottle was based on the data of day 27, the difference of duplicate values on day 29 had no effect on the outcome of this study.

TK 12759, ARALDITE MY 0510 was found to be not inhibitory.

CONCLUSION

TK 12759, ARALDITE MY 0510 was not readily biodegradable under the conditions of the modified Sturm test presently performed.

Table 1: CO_2 evolution and percentage of biodegradation of the positive control substance.

Day	ml titrant Blank (mean)	ml titrant CO_2 trap	Produced CO_2 in ml HCl	Normality HCl	Produced mg CO_2	Cumulative mg CO_2	Degradation %
2	49.07	40.27	8.80	0.048	9.29	9.29	10.8
5	48.90	22.88	26.02	0.048	27.48	36.77	42.8
7	47.94	33.57	14.37	0.048	15.17	51.94	60.5
9	48.88	40.73	8.15	0.048	8.61	60.55	70.5
14	47.23	40.36	6.87	0.048	7.25	67.80	78.9
19	47.90	41.48	6.42	0.049	6.92	74.72	87.0
23	48.51	41.59	6.92	0.049	7.46	82.18	95.7
27	45.60	38.15	7.45	0.049	8.03	90.21	105.0
29	43.95	42.12	1.83	0.049	1.97	92.18	107.3
29	45.20	44.80	0.40	0.049	0.43	92.61	107.8
29	48.26	48.50	0.00	0.049	0.00	92.61	107.8

Theoretical CO_2 production sodium acetate: 85.9 mg CO_2/2l

Table 2a: CO_2 evolution and percentage of biodegradation of the test substance (bottle A).

Day	ml titrant Blank (mean)	ml titrant CO_2 trap	Produced CO_2 ml HCl	Normality N	Produced mg CO_2	Cumulative mg CO_2	Degradation %
2	49.07	48.52	0.55	0.048	0.58	0.58	0.6
5	48.90	48.70	0.20	0.048	0.21	0.79	0.9
7	47.94	48.71	0.00	0.048	0.00	0.79	0.9
9	48.88	48.74	0.14	0.048	0.15	0.94	1.0
14	47.23	47.84	0.00	0.048	0.00	0.94	1.0
19	47.90	47.96	0.00	0.049	0.00	0.94	1.0
23	48.51	47.89	0.62	0.049	0.67	1.61	1.7
27	45.60	45.26	0.34	0.049	0.37	1.98	2.1
29	43.95	45.77	0.00	0.049	0.00	1.98	2.1
29	45.20	44.15	1.05	0.049	1.13	3.11	3.4
29	48.26	48.62	0.00	0.049	0.00	3.11	3.4

Theoretical CO_2 production test substance: 92.2 mg CO_2/2l

Table 2b: CO_2 evolution and percentage of biodegradation of the test substance (bottle B).

Day	ml titrant Blank (mean)	ml titrant CO_2 trap	Produced CO_2 ml HCl	Normality N	Produced mg CO_2	Cumulative mg CO_2	Degradation %
2	49.07	48.69	0.38	0.048	0.40	0.40	0.4
5	48.90	49.00	0.00	0.048	0.00	0.40	0.4
7	47.94	48.49	0.00	0.048	0.00	0.40	0.4
9	48.88	47.35	1.53	0.048	1.62	2.02	2.1
14	47.23	48.63	0.00	0.048	0.00	2.02	2.1
19	47.90	48.26	0.00	0.049	0.00	2.02	2.1
23	48.51	48.37	0.14	0.049	0.15	2.17	2.3
27	45.60	45.28	0.32	0.049	0.34	2.51	2.7
29	43.95	37.69	6.26	0.049	6.75	9.26	9.8
29	45.20	29.47	15.73	0.049	16.96	26.22	27.9
29	48.26	46.35	1.91	0.049	2.06	28.28	30.1

Theoretical CO_2 production test substance: 94.1 mg CO_2/2l

Table 3: CO_2 evolution and percentage of biodegradation of the toxicity control.

Day	ml titrant Blank (mean)	ml titrant CO_2 trap	Produced CO_2 ml HCl	Normality N	Produced mg CO_2	Cumulative mg CO_2	Degradation %
2	49.07	35.01	14.06	0.048	14.85	14.85	8.3
5	48.90	26.70	22.20	0.048	23.44	38.29	21.4
7	47.94	38.77	9.17	0.048	9.68	47.97	26.8
9	48.88	44.58	4.30	0.048	4.54	52.51	29.4
14	47.23	42.48	4.75	0.048	5.02	57.53	32.2
19	47.90	42.96	4.94	0.049	5.33	62.86	35.1
23	48.51	41.76	6.75	0.049	7.28	70.14	39.2
27	45.60	32.36	13.24	0.049	14.27	84.41	47.2
29	43.95	41.82	2.13	0.049	2.30	86.71	48.5
29	45.20	44.61	0.59	0.049	0.64	87.35	48.8
29	48.26	48.42	0.00	0.049	0.00	87.35	48.8

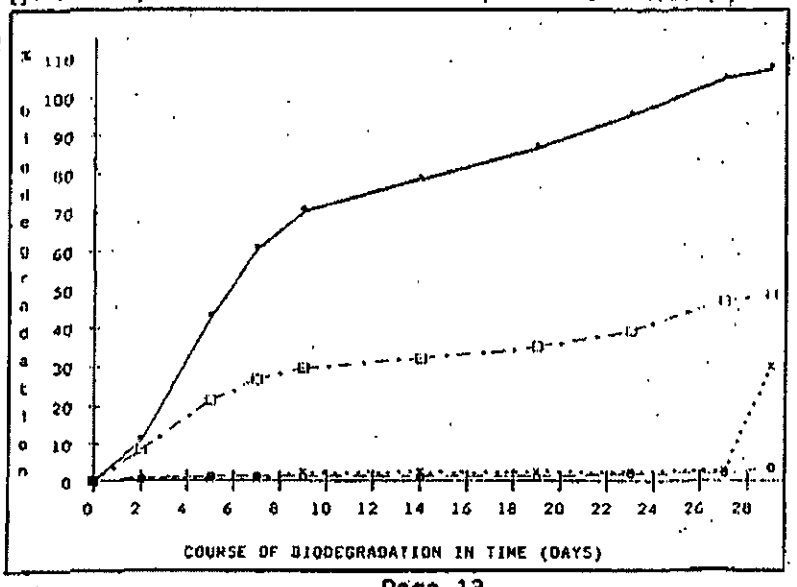
Theoretical CO_2 production test substance: 92.9 mg CO_2/2l
Theoretical CO_2 production sodium acetate: 85.9 mg CO_2/2l
Theoretical CO_2 production test substance plus sodium acetate: 178.9 mg CO_2/2l

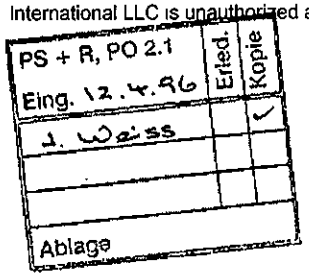
Note: Since calculations were performed without rounding off a deviation in the last figure of the theoretical CO_2 production per 2 litres may be observed.

Table 4: CO_2 evolution in the blank.

Day	Stand. Ba(OH)2 ml HCl * 5	ml titrant Blank (mean value)	Produced CO_2 ml HCl	Normality N	Produced mg CO_2	Cumulative mg CO_2
2	9.90	49.07	0.43	0.048	0.45	0.45
5	9.81	48.90	0.15	0.048	0.16	0.61
7	9.81	47.94	1.11	0.048	1.17	1.78
9	9.83	48.88	0.27	0.048	0.29	2.07
14	9.81	47.23	1.82	0.048	1.92	3.99
19	9.84	47.90	1.30	0.049	1.40	5.39
23	9.84	48.51	0.69	0.049	0.74	6.13
27	9.85	45.60	3.55	0.049	3.83	9.96
29	9.80	43.95	5.05	0.049	5.44	15.40
29	9.80	45.20	3.80	0.049	4.10	19.50
29	9.80	48.26	0.74	0.049	0.80	20.30

Figure 1: Biodegradation of TK 12759, ARALDITE MY 0510 in the modified Sturm test:
* : Positive control
[]: Toxicity control
o : TK 12759, ARALDITE MY 0510 (A)
x : TK 12759, ARALDITE MY 0510 (B)





FINAL REPORT

Study Title

FRESH WATER ALGAL GROWTH INHIBITION TEST WITH P-(2,3-EPOXYPROPOXY)-N,N-BIS(2,3-EPOXYPROPYL)ANILINE

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Laboratory Project Identification

NOTOX Project 496987
NOTOX Substance 203277/A

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2. STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (GLP) (as revised in 1997) ENV/MC/CHEM (98) 17.

The sponsor is responsible for Good Laboratory Practice (GLP) compliance for all test substance information unless determined by NOTOX.

NOTOX B.V.

Ir. L.M. Bouwman
Study Director

Date: 01 December 2011

Ing. M.H.J. Migchielsen
Section head Environmental Toxicology

Date: 01 December 2011

3. QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the NOTOX Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

Type of Inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date
Study	Protocol	02-Aug-11	02-Aug-11	02-Aug-11
	Protocol Amendment 01	09-Aug-11	09-Aug-11	09-Aug-11
	Report	24-Nov-11	24-Nov-11	24-Nov-11
Process	**Environmental Toxicology** Test Substance Handling Exposure Observations/Measurements	18-Jul-11	22-Jul-11	22-Jul-11
	Analytical and physical chemistry Test Substance Handling Observations/Measurements	08-Aug-11	16-Aug-11	16-Aug-11

NOTOX B.V.

C.J. Mitchell B.Sc.
Head of Quality Assurance

A. Morgan
A. MORGAN

Date: 21 December, 2011

4. SUMMARY

Pseudokirchneriella subcapitata, Fresh Water Algal Growth Inhibition Test with p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline.

The study procedures described in this report were based on the OECD guideline No. 201, 2006. In addition, the procedures were designed to meet the test methods of the Commission Regulation (EC) No 440/2008, Part C.3, 2008; Amended by EC No. 761/2009, the ISO International Standard 8692, 2004 and the OECD series on testing and assessment number 23, 2000.

The batch of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline tested was a yellow liquid with a purity of 94.33% and the substance was not completely soluble in test medium at loading rates of 1.0 mg/l and higher.

A final test was performed based on the results of a preceding combined limit/range-finding test. All test solutions were prepared separately applying two days of magnetic stirring to reach maximum solubility of the test substance in the test medium. The resulting aqueous mixtures were left to stabilize for 2 hours where after the Water Accommodated Fractions (WAFs) were siphoned off and used for testing. The final test solutions were all clear and colourless.

Three replicates of exponentially growing algal cultures were exposed to WAFs prepared at loading rates of 4.6, 10, 22, 46 and 100 mg/l. Simultaneously, six replicates were exposed in a control group. The initial cell density was 10^4 cells/ml and the total test period was 72 hours. Samples for analytical confirmation of actual exposure concentrations were taken at the start, after 24 hours of exposure and at the end of the test.

At the start of the test, the actual test concentrations were 3.2, 3.3, 5.5, 33 and 34 mg/l in the WAFs prepared at loading rates of 4.6, 10, 22, 46 and 100 mg/l, respectively. These concentrations remained stable during the first 24 hours of exposure (82-87% of initial) but decreased to 59-66% of initial at the end of the test period. Note that actual concentrations being almost similar is related to medium solubility and the preparation of WAFs. Based on these results, the average exposure concentrations were calculated to be: 2.5, 2.6, 4.2, 26 and 26 mg/l.

The study met the acceptability criteria prescribed by the protocol and was considered valid.

p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline reduced growth rate of this fresh water algae species significantly at an average exposure concentration of 26 mg/l.

The EC_{50} for growth rate reduction (E_RC_{50}: 0-72h) was 13 mg/l with a 95% confidence interval ranging from 8.3 to 19 mg/l.

The EC_{50} for yield inhibition (E_YC_{50}: 0-72h) was 7.0 mg/l with a 95% confidence interval ranging from 5.3 to 9.3 mg/l.

The NOEC for growth rate reduction was 4.2 mg/l, while the NOEC for yield inhibition could only be estimated to be <2.5 mg/l.

5. INTRODUCTION

5.1. Preface

Sponsor	Huntsman Advanced Materials GmbH Klybeckstrasse 200 4057 BASEL Switzerland
Study Monitor (Tox and ecotox)	Dr. P. Dollenmeier
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Ir. L.M. Bouwman
Technical Coordinator	R.W.A.M. Coolen
Principal Scientist	Dr. Ir. E. Baltussen
Study Plan	Start : 19 September 2011 Completion : 13 October 2011

5.2. Aim of the study

The purpose of the study was to evaluate the test substance for its ability to generate toxic effects in *Pseudokirchneriella subcapitata* during an exposure period of at least 48 and at most 96 hours and, if possible, to determine the EC_{50} for both reduction of growth rate and inhibition of yield.

5.3. Guidelines

The study procedures described in this report were based on the Organization for Economic Co-operation and Development (OECD), OECD guidelines for Testing of Chemicals, guideline No. 201: "Freshwater Alga and Cyanobacteria, Growth Inhibition Test", Adopted March 23, 2006.

In addition, the procedures were designed to meet the test methods prescribed by the following guidelines and guidance document:

- Commission regulation (EC) No. 440/2008 of 30 May 2008, Part C: Methods for the determination of ecotoxicity, Publication No. L142, C3: "Algal Inhibition Test"; Amended by EC No. 761/2009 of 23 July 2009, Publication No. L220.
- ISO International Standard 8692: "Water quality - Freshwater algal growth inhibition test with unicellular green algae", Second edition, 01 October 2004.
- Guidance document on aquatic toxicity testing of difficult substances and mixtures, OECD series on testing and assessment number 23, December 14, 2000.

5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report are retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. NOTOX will retain information concerning decisions made.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

5.5. Definitions

Cell density is the number of cells per millilitre.

Growth rate is the increase in cell density per unit time. It is derived from the slope of the growth curve in a logarithmic plot. Following from the mathematical nature of exponential growth, the measure of the specific growth rate is preferable over biomass or yield. The E_RC_{50} is the concentration of test substance that results in a 50% reduction in growth rate relative to the control.

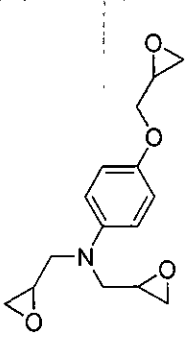
Yield is defined as the biomass at the end of the exposure period minus the biomass at the start of the exposure period. The E_yC_{50} is the concentration of test substance that results in a 50% inhibition of yield relative to the control.

No Observed Effect Concentration (NOEC) is the highest concentration tested at which the measured parameter(s) show(s) no significant effect on algal growth relative to control values.

6. MATERIALS AND METHODS

6.1. Test Substance

6.1.1. Test substance information

Identification	p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline
Structure	
Molecular formula	$C_{15}H_{19}NO_4$
Molecular weight	277.3
CAS Number	5026-74-4
EC Number	225-716-2
Description	Yellow liquid
Batch	*AA00373400*
Purity	94.33%
Test substance storage	In refrigerator (2-8°C) in the dark
Stability under storage conditions	Stable
Expiry date	28 February 2013

6.1.2. Study specific test substance information

Volatile	No
Stability in water	No
Solubility in water	No

6.1.3. Reference substance

This report includes the results of the most recent reference test with potassium dichromate (APPENDIX 5).

6.2. Test System

Species	*Pseudokirchneriella subcapitata*, strain: NIVA CHL 1
Source	In-house laboratory culture.
Reason for selection	This system is an unicellular algal species sensitive to toxic substances in the aquatic ecosystem and has been selected as an internationally accepted species.

6.3. Fresh water algae culture

Stock culture	Algae stock cultures were started by inoculating growth medium with algal cells from a pure culture on agar. The suspensions were continuously aerated and exposed to light in a climate room at a temperature of 21-24°C.
Light intensity	60 to 120 µE/m^2/s when measured in the photosynthetically effective wavelength range of 400 to 700 nm.
Stock culture medium	M1; according to the NPR 6505 ("Nederlandse Praktijk Richtlijn no. 6505") formulated using Milli-RO water (tap-water purified by reverse osmosis; Millipore Corp., Bedford, Mass., USA) with the following composition:

$NaNO_3$	500	mg/l
$K_2HPO_4.3H_2O$	52	mg/l
$MgSO_4.7H_2O$	75	mg/l
$Na_2CO_3.10H_2O$	54	mg/l
$C_6H_8O_7.H_2O$	6	mg/l
NH_4NO_3	330	mg/l
$CaCl_2.2H_2O$	35	mg/l
$C_6H_6FeO_7.xH_2O$	6	mg/l
H_3BO_3	2.9	mg/l
$MnCl_2.4H_2O$	1.81	mg/l
$ZnCl_2$	0.11	mg/l
$CuSO_4.5H_2O$	0.08	mg/l
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.018	mg/l

Pre-culture	3 days before the start of the test, cells from the algal stock culture were inoculated in culture medium at a cell density of 1 x 10^4 cells/ml. The pre-culture was maintained under the same conditions as used in the test. The cell density was measured immediately before use.
Pre-culture medium	M2; according to the OECD 201 Guideline, formulated using Milli-Q water (tap water purified by reverse osmosis (Milli-RO) and subsequently passed over activated carbon and ion-exchange cartridges: Milli-Q water; Millipore Corp., Bedford, Mass., USA) preventing precipitation and with the following composition:

NH_4Cl	15	mg/l
$MgCl_2.6H_2O$	12	mg/l
$CaCl_2\ 2H_2O$	18	mg/l
$MgSO_4.7H_2O$	15	mg/l
KH_2PO_4	1.6	mg/l
$FeCl_3.6H_2O$	64	µg/l
$Na_2EDTA.2H_2O$	100	µg/l
H_3BO_3	185	µg/l

$MnCl_2.4H_2O$	415	µg/l
$ZnCl_2$	3	µg/l
$CoCl_2.6H_2O$	1.5	µg/l
$CuCl_2.2H_2O$	0.01	µg/l
$Na_2MoO_4.2H_2O$	7	µg/l
$NaHCO_3$	50	mg/l
Hardness (Ca+Mg)	0.24	mmol/l (24 mg $CaCO_3$/l)
pH	8.1 ± 0.2	

6.4. Preparation of test solutions

The standard test procedures required generation of test solutions, which contained completely dissolved test substance concentrations or stable and homogeneous mixtures or dispersions. The testing of concentrations that would disturb the test system was prevented as much as possible (e.g. *film of the test substance on the water surface*).

The batch of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline tested was a yellow liquid with a purity of 94.33% and the substance was not completely soluble in test medium at loading rates of 1.0 mg/l and higher.

All test solutions with loading rates of 1.0 mg/l and higher were prepared separately applying two days of magnetic stirring to reach maximum solubility of the test substance in the test medium. The resulting aqueous mixtures were left to stabilize for 1-2 hours where after the Water Accommodated Fractions (WAFs) were siphoned off and used for testing. One lower test concentration for the combined limit/range-finding test was prepared by subsequent dilution of the 1.0 mg/l WAF in test medium. The final test solutions were all clear and colourless.

After preparation, volumes of 50 ml were added to each replicate of the respective test concentration. Subsequently, 1 ml of an algal suspension was added to each replicate providing a cell density of 10^4 cells/ml.

6.5. Combined limit/range-finding test

The study started with a combined limit/range-finding test. Six replicates of exponentially growing algae were exposed to a control and a WAF prepared at a loading rate of 100 mg/l. Test procedure and conditions were similar to those applied in the final test with the following exceptions:

- Three replicates per concentration were exposed to WAFs prepared at 1.0 and 10 mg/l and to a 10% dilution of the WAF prepared at 1.0 mg/l;
- One extra test vessel per concentration without algae was used as background for the determination of the algal cell density at each time interval;
- pH was only measured in the control and the highest test concentration.

6.6. Final test

6.6.1. Test concentrations

p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline	WAFs prepared at loading rates of 4.6, 10, 22, 46 and 100 mg/l
Controls	Test medium without test substance or other additives
Replicates	3 replicates of each test concentration; 6 replicates of the control; 1 extra replicate of the control and each test concentration for sampling purposes; 2 replicates of the highest concentration without algae.

6.6.2. Test procedures and conditions

Test duration	72 hours

Test type	Static
Test vessels	100 ml, all-glass, containing 50 ml of test solution
Medium	M2
Cell density	An initial cell density of 1 x 10^4 cells/ml.
Illumination	Continuously using TLD-lamps of the type 'Cool-white' of 30 Watt, with a light intensity within the range of 77 to 90 $\mu E.m^{-2}.s^{-1}$.
Incubation	Capped vessels were distributed at random in the incubator and as such were daily repositioned. During incubation the algal cells were kept in suspension by continuous shaking.

6.6.3. Sampling for analysis of test concentrations

Samples for possible analysis were taken from all test concentrations and the control according to the schedule below. The method of analysis is described in the appended Analytical Report (APPENDIX 6).

Frequency	at t=0 h, t=24 h and t=72 h
Volume	2 ml
Storage	Samples were stored in a freezer until analysis.

At the end of the exposure period, the replicates with algae were pooled at each concentration before sampling.

Compliance with the Quality criteria regarding maintenance of actual concentrations was demonstrated by running a test vessel at the highest substance concentration but without algae and samples for analysis were taken at the start, after 24 hours of exposure and at the end of the test period.

Additionally, reserve samples of 2 ml were taken from all test solutions for possible analysis. If not already used, these samples were stored in a freezer for a maximum of three months after delivery of the draft report, pending on the decision of the sponsor for additional analysis.

6.6.4. Measurements

pH	At the beginning and at the end of the test. The pH of the solutions should preferably not deviate by more than 1.5 units during the test.
Temperature of medium	Continuously in a temperature control vessel.

6.6.5. Recording of cell densities

At the beginning of the test, cells were counted using a microscope and a counting chamber. Thereafter cell densities were determined by spectrophotometric measurement of samples at 720 nm using a spectrophotometer with immersion probe (pathlength =20 mm). Algal medium was used as blank.

6.7. Electronic data capture

Observations/measurements in the study were recorded electronically using the following programme(s):

- Shimadzu Spectrophotometer UV-1800 including UVProbe 2.33 software (Shimadzu, Kyoto, Japan): Algal cell density.

- REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA): Temperature.

6.8. Interpretation

6.8.1. Data handling

Calibration curve
Quantification of cell densities was based on a calibration curve. Cell density was plotted versus extinction using spectrophotometric measurements of a minimum of six dilutions of an algal suspension with different cell densities. The calibration curve was composed using linear regression. The software automatically calculates the cell densities based on this curve for the spectrophotometric measurements at the various points in time during the test period.

Comparison of average growth rates
The average specific growth rate for a specific period is calculated as the logarithmic increase in the biomass from the equation for each single vessel of controls and treatments:

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i} (\text{day}^{-1})$$

Where: μ_{i-j} = the average specific growth rate from time i to j
X_i = the biomass at time i
X_j = the biomass at time j

The average growth rate at each test substance concentration is then compared with the control value and the percentage reduction in growth rate is calculated:

$$\%I_r = \frac{\mu_C - \mu_T}{\mu_C} \times 100$$

Where: $\%I_r$ = percent inhibition in average specific growth rate
μ_C = mean value for average specific growth rate in the control group
μ_T = average specific growth rate for the treatment replicate

Yield
The percent inhibition in yield is calculated for each treatment replicate as follows:

$$\%I_y = \frac{Y_C - Y_T}{Y_C} \times 100$$

Where: $\%I_y$ = percent inhibition of yield
Y_C = mean value for yield in the control group
Y_T = value for yield for the treatment replicate

Determination of the average exposure concentrations
The average exposure concentrations were calculated as: $\frac{24 \times \sqrt{C_{t=0} \times C_{t=24}} + 48 \times \sqrt{C_{t=24} \times C_{t=72}}}{72}$,
being the Time Weight Average (TWA) of the concentrations of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline measured in the samples taken at the start ($C_{t=0}$), after 24 hours ($C_{t=24}$) and the end of the test ($C_{t=72}$).

Determination of the NOEC and calculation of the EC_{50}
For determination of the NOEC and the EC_{50} the approaches recommended in the OECD guideline 201 were used. An effect was considered to be significant if statistical analysis of the data obtained for the test concentrations compared with those obtained in the negative control revealed significant

reduction of growth rate or inhibition of yield (ANOVA, Bonferroni t-Test, TOXSTAT Release 3.5, 1996, D.D. Gulley, A.M. Boelter, H.L. Bergman). Additionally, the EC_{10} was determined to meet the recommendations as put down in "A Review of Statistical Data Analysis and Experimental Design in OECD Aquatic Toxicology Test Guidelines" by S. Pack, August 1993. Calculation of the EC_{50} and EC_{10} values was based on log-linear regression analysis of the percentages of growth rate reduction and the percentages of yield inhibition versus the logarithms of the corresponding average exposure concentrations of the test substance.

6.8.2. Acceptability of the test

1. In the control, cell density increased by an average factor of >16 within two days.
2. The mean coefficient of variation for section-by-section specific growth rates in the control cultures did not exceed 35%.
3. The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures did not exceed 7%.

6.9. List of deviations

6.9.1. List of protocol deviations

1. Combined limit range/finding test: After 48 hours of exposure one out of the six replicate vessels for the highest test concentration broke and could therefore no longer be used for measurement of algal cell density.
   Evaluation: The combined limit/range-finding test could still be used as a range-finding finding test to determine the range of concentrations used in the final test. Results were based on five instead of six replicates.
2. Final test: After 72 hours of exposure one out of the six replicate vessels for the control group broke and could therefore not be used for measurement of algal cell density at the end of the test.
   Evaluation: Results were based on five instead of six replicates. According to the OECD guideline the number of replicate vessels for the control group should be at least three. Therefore, the test can still be used to determine the effect parameters (EC_{50} and NOEC).
3. Final test: No microscopic observations of the algal cells were made at the end of the test.
   Evaluation: The study could still be used to determine EC_{50} values, which was the main goal.

The study integrity was not adversely affected by the deviations.

6.9.2. List of standard operating procedures deviations

Any deviations from standard operating procedures were evaluated and filed in the study file. There were no deviations from standard operating procedures that affected the integrity of the study.

7. RESULTS

7.1. Combined limit/range-finding test

The mean cell densities measured during the combined limit/range-finding test are presented in Table 1. Table 2 presents the percentages growth rate reduction and yield inhibition per concentration. Based on these results samples taken from the WAFs prepared at 1.0 and 100 mg/l were analysed. The initial concentrations were 0.72 and 60 mg/l. These concentrations remained stable during the first 24 hours of exposure (80-81% of initial) but decreased to 45-62% of initial at the end of the test period (see also Table 2 of the appended Analytical Report). The expected EC_{50} for both growth rate reduction and yield inhibition was between concentrations obtained in WAFs prepared at 10 and 100 mg/l.

All test conditions were maintained within the limits prescribed by the protocol.

Table 1 Mean cell densities (x10^4 cells/ml) during the combined limit/range-finding test

Loading rate[1] Test substance[2] (mg/l)	Exposure time (hours)			
	0	24	48	72
control	1.0	11.0	54.0	227.2
10% of 1.0	1.0	11.2	59.2	239.5
1.0	1.0	10.6	53.3	219.2
10	1.0	10.9	48.0	197.7
100	1.0	5.7	8.5	6.4

[1] WAF prepared at the given loading rate
[2] The test substance is p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline

Table 2 Percentage reduction of growth rate and inhibition of yield during the combined limit/range-finding test

Loading rate[1] Test substance[2] (mg/l)	Mean growth rate		Yield (0-72 h)	
	μ (0-72 h)	Reduction (%)	x10^4 cells/ml	Inhibition (%)
control	0.07532		226.19	
10% of 1.0	0.07608	-1.0	238.50	-5.4
1.0	0.07482	0.7	218.24	3.5
10	0.07342	2.5	196.70	13.0
100	0.02576	65.8	5.41	97.6

[1] WAF prepared at the given loading rate
[2] The test substance is p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline

7.2. Final test

7.2.1. Measured test substance concentrations

The results of analysis of the samples taken during the final test are described in Table 3 of the appended Analytical Report.

At the start of the test, the actual test concentrations were 3.2, 3.3, 5.5, 33 and 34 mg/l in the WAFs prepared at loading rates of 4.6, 10, 22, 46 and 100 mg/l, respectively. These concentrations remained stable during the first 24 hours of exposure (82-87% of initial) but decreased to 59-66% of initial at the end of the test period. Note that actual concentrations being almost similar is related to medium solubility and the preparation of WAFs. Based on these results, the average exposure concentrations were calculated to be: 2.5, 2.6, 4.2, 26 and 26 mg/l.

7.2.2. Mean cell densities

Table 3 shows mean cell densities measured at 24-hour intervals at the different concentrations of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline. The respective growth curves are shown in Figure 1 (see APPENDIX 1 for the cell densities per replicate). Note that the mean cell density of the control was based on 5 replicates at the end of the test due to the fact that one vessel fall and broke.

Table 3 Mean cell densities (x 10^4 cells/ml) during the final test

Loading rate[1] Test substance[2] (mg/l)	Exposure time (hours)			
	0	24	48	72
control	1.0	8.7	38.4	158.5
4.6 (2.5)	1.0	8.2	34.2	131.7
10 (2.6)	1.0	9.0	36.6	145.6
22 (4.2)	1.0	8.7	31.5	108.7
46 (26)	1.0	6.2	7.1	4.2
100 (26)	1.0	5.5	7.6	3.5

[1] WAF prepared at the given loading rate
[2] The test substance is p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline
( ) Average exposure concentration

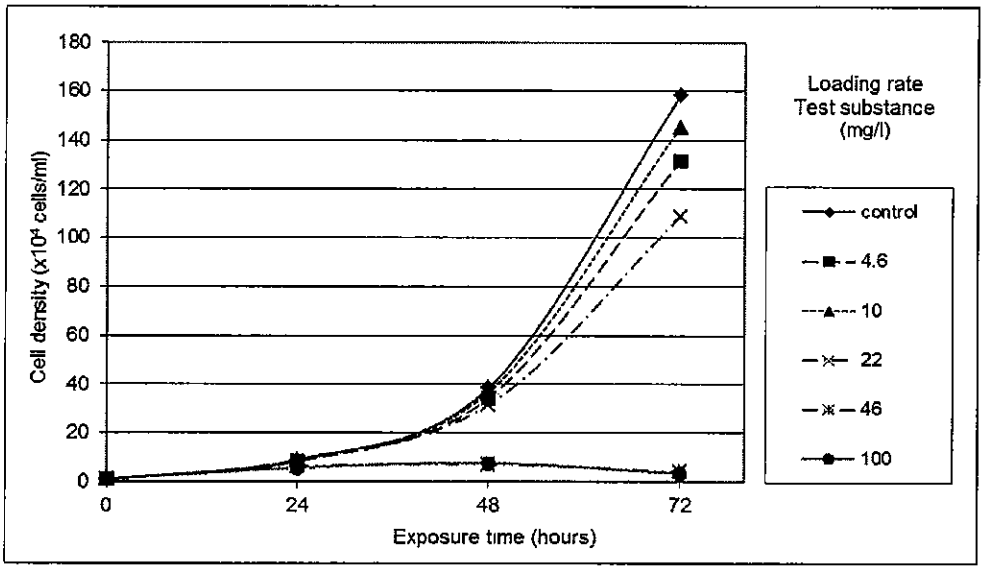


Figure 1 Growth curves at different concentrations of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline

7.2.3. Reduction of growth rate and inhibition of yield

Table 4 shows the calculation of the percentages of growth rate reduction (total test period) and the percentages of yield inhibition. Table 5 shows the calculation of the percentages of growth rate reduction at different time intervals (see APPENDIX 1 for the values of growth rate and yield per replicate). Statistical analysis of the data is shown in APPENDIX 2 and APPENDIX 3.

Growth rates were in the range of the control at 2.5/2.6 mg/l during the 72-hour test period, whereas the growth rate of algae exposed to 4.2 and 26 mg/l were reduced. Statistically significant reduction of growth rate was found at a test concentration of 26 mg/l (Bonferroni t-Test, $\alpha = 0.05$), that was measured in WAFs prepared at both 46 and 100 mg/l.

Inhibition of yield increased with increasing concentration of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline from 2.5/2.6 mg/l upwards resulting in almost complete inhibition at 26 mg/l. Statistically significant inhibition of yield was found at all test concentrations, except for 2.6 mg/l measured in the WAF prepared at 10 mg/l (Bonferroni t-Test, $\alpha = 0.05$). No explanation can be given for the relatively high inhibition at the lowest loading rate. Statistical analysis does not give a clear output on the NOEC for yield inhibition.

Table 4 Percentage reduction of growth rate (total test period) and percentage inhibition of yield during the final test

Loading rate[1] Test substance[2]	Mean growth rate		Yield (0-72 h)	
(mg/l)	μ (0-72 h)	Reduction (%)	x10^4 cells/ml	Inhibition (%)
control	0.07030		157.50	
4.6 (2.5)	0.06774	3.6	130.66	17.0
10 (2.6)	0.06917	1.6	144.61	8.2
22 (4.2)	0.06512	7.4	107.69	31.6
46 (26)	0.01977	71.9	3.24	97.9
100 (26)	0.01683	76.1	2.50	98.4

[1] WAF prepared at the given loading rate
[2] The test substance is p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline
( ) Average exposure concentration

Table 5 Percentage reduction of growth rate at different time intervals during the final test

Loading rate[1] Test substance[2]	Mean growth rate					
(mg/l)	μ (0-24 h)	Reduction (%)	μ (24-48 h)	Reduction (%)	μ (48-72 h)	Reduction (%)
control	0.09011		0.06165		0.05898	
4.6 (2.5)	0.08753	2.9	0.05961	3.3	0.05609	4.9
10 (2.6)	0.09126	-1.3	0.05875	4.7	0.05751	2.5
22 (4.2)	0.09029	-0.2	0.05339	13.4	0.05167	12.4
46 (26)	0.07573	16.0	0.00535	91.3	0.00000	100.0
100 (26)	0.07138	20.8	0.01280	79.2	0.00000	100.0

[1] WAF prepared at the given loading rate
[2] The test substance is p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline
( ) Average exposure concentration

7.2.4. Determination of effect concentrations

Table 6 shows the effect parameters based on average exposure concentrations, see also APPENDIX 4.

Table 6 Effect parameters

Parameter	Concentration Test substance[1] (mg/l)	95%-confidence interval
NOE_RC	4.2	
72h-E_RC_{10}	3.6	2.3 - 5.4
72h-E_RC_{50}	13	8.3 - 19
NOE_YC	<2.5	
72h-E_YC_{10}	2.4	1.8 - 3.2
72h-E_YC_{50}	7.0	5.3 - 9.3

[1] The test substance is p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline

7.2.5. Experimental conditions

Table 7 shows the pH recorded at the beginning and the end of the test. The pH was within the limits prescribed by the protocol (6.0-9.0, preferably not varying by more than 1.5 unit). The temperature of the test medium was 22.1°C at the start of the test. During the exposure period the temperature measured in the incubator was maintained between 22.4 and 23.5°C. Temperature remained within the limits prescribed by the protocol (21-24°C, constant within 2°C).

Table 7 pH levels recorded during the final test

Loading rate[1] Test substance[2] (mg/l)	Exposure time (hours)	
	0	72
control	8.1	7.9
4.6 (2.5)	8.2	7.9
10 (2.6)	8.1	7.9
22 (4.2)	8.2	7.8
46 (26)	8.1	7.9
100 (26)	8.2	7.9

[1] WAF prepared at the given loading rate
[2] The test substance is p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline
( ) Average exposure concentration

8. CONCLUSION

Under the conditions of the present study with *Pseudokirchneriella subcapitata*, p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline reduced growth rate of this fresh water algae species significantly at an average exposure concentration of 26 mg/l.

The EC_{50} for growth rate reduction (E_RC_{50}: 0-72h) was 13 mg/l with a 95% confidence interval ranging from 8.3 to 19 mg/l.

The EC_{50} for yield inhibition (E_YC_{50}: 0-72h) was 7.0 mg/l with a 95% confidence interval ranging from 5.3 to 9.3 mg/l.

The NOEC for growth rate reduction was 4.2 mg/l, while the NOEC for yield inhibition could only be estimated to be <2.5 mg/l.

APPENDIX 1 WORKSHEET DATA

Table 8 Individual cell densities

Number of inoculated cells at t=0: $1x10^4$ cells/ml					
Loading rate[1] Test substance[2] (mg/l)	Vessel number	Exposure time (hours)			
		0	24	48	72
control	1	1.00	8.95	39.79	165.54
	2	1.00	8.00	37.48	[3]
	3	1.00	8.74	41.73	169.66
	4	1.00	8.18	30.46	135.09
	5	1.00	9.80	43 13	173.60
	6	1.00	8.61	37.88	148.60
4.6 (2.5)	1	1.00	7.83	31.65	122.04
	2	1.00	8.40	34.50	144.65
	3	1.00	8.29	36.54	128.28
10 (2.6)	1	1.00	8 66	35.69	145 46
	2	1.00	9.63	38.97	150.05
	3	1.00	8.56	35.27	141.31
22 (4.2)	1	1.00	8.85	31.60	109.20
	2	1.00	8.68	31.40	107.03
	3	1.00	8.67	31.35	109.86
46 (26)	1	1.00	5.66	5.68	3.86
	2	1.00	6.36	7.47	5.44
	3	1.00	6.48	8.09	3.42
100 (26)	1	1 00	5.43	6.62	2.20
	2	1.00	5.72	7.69	4.17
	3	1.00	5.49	8 42	4.13

[1] WAF prepared at the given loading rate
[2] The test substance is p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline
[3] No measurement of algal cell density at the end of the test; vessel was broken
( ) Average exposure concentration

APPENDIX 1 WORKSHEET DATA – continued –

Table 9 Calculation of growth rate and yield

Loading rate[1] Test substance[2] (mg/l)	Vessel number	Growth rate (μ) 0-72 h	Yield (x10^4 cells/ml) 0-72 h	Growth rate red. (%) 0-72 h	Yield inhib. (%) 0-72 h
control	1	0.07096	164.54		
	2	[3]	[3]		
	3	0.07130	168 66		
	4	0.06814	134.09		
	5	0.07162	172.60		
	6	0.06946	147.60		
	mean	0.07030	157 50		
	CV	2%			
4 6 (2.5)	1	0.06673	121.04	5	23
	2	0.06909	143.65	2	9
	3	0.06742	127.28	4	19
10 (2.6)	1	0.06916	144 46	2	8
	2	0.06960	149.05	1	5
	3	0.06876	140.31	2	11
22 (4 2)	1	0.06518	108.20	7	31
	2	0.06490	106.03	8	33
	3	0.06527	108.86	7	31
46 (26)	1	0.01874	2.86	73	98
	2	0.02351	4.44	67	97
	3	0.01706	2.42	76	98
100 (26)	1	0.01098	1.20	84	99
	2	0.01983	3.17	72	98
	3	0.01968	3.13	72	98

[1] WAF prepared at the given loading rate
[2] The test substance is p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline
[3] No measurement of algal cell density at the end of the test; vessel was broken
( ) Average exposure concentration

APPENDIX 1 WORKSHEET DATA – continued –

Table 10 Calculation of growth rate (section-by-section)

Loading rate[1] Test substance[2] (mg/l)	Vessel number	Growth rate (μ)			Growth rate reduction (%)		
		0-24 h	24-48 h	48-72 h	0-24 h	24-48 h	48-72 h
control	1	0.09131	0 06217	0.05940			
	2	0.08666	0 06433	[3]			
	3	0.09032	0.06515	0.05844			
	4	0.08758	0.05478	0.06206			
	5	0.09511	0.06173	0.05803			
	6	0.08969	0.06174	0.05696			
	mean	0.09011	0.06165	0.05898			
	CV	3%	6%	3%			
	The mean CV for section-by-section specific growth rate was:				21%		
4.6 (2.5)	1	0.08576	0 05819	0.05623	5	6	5
	2	0.08869	0.05884	0.05973	2	5	-1
	3	0 08814	0.06179	0.05232	2	0	11
10 (2.6)	1	0.08994	0.05901	0.05854	0	4	1
	2	0.09439	0.05823	0.05617	-5	6	5
	3	0.08946	0.05900	0.05783	1	4	2
22 (4.2)	1	0.09083	0 05305	0.05166	-1	14	12
	2	0.09004	0.05357	0.05109	0	13	13
	3	0.09001	0.05354	0.05225	0	13	11
46 (26)	1	0.07220	0.00016	0.00000	20	100	100
	2	0 07710	0.00666	0.00000	14	89	100
	3	0.07789	0.00923	0.00000	14	85	100
100 (26)	1	0 07052	0.00823	0.00000	22	87	100
	2	0.07266	0.01232	0.00000	19	80	100
	3	0.07095	0.01784	0.00000	21	71	100

[1] WAF prepared at the given loading rate
[2] The test substance is p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline
[3] No measurement of algal cell density at the end of the test; vessel was broken
( ) Average exposure concentration

APPENDIX 2 STATISTICS: GROWTH RATE (0-72 HOURS)

Chi-Square Test for Normality

Actual and Expected Frequencies

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	1.3400	4.8400	7.6400	4.8400	1.3400
OBSERVED	0	7	5	8	0

Chi-Square = 6.6194 (p-value = 0.1574)
Critical Chi-Square = 13.277 (alpha = 0.01 , df = 4)
= 9.488 (alpha = 0.05 , df = 4)

Data PASS normality test (alpha = 0.01). Continue analysis.

Levene's Test for Homogeneity of Variance

ANOVA Table

SOURCE	DF	SS	MS	F
Between	5	0.0000	0.0000	0.7627
Within (Error)	14	0.0001	0.0000	
Total	19	0.0001		

(p-value = 0.5914)

Critical F = 4.6950 (alpha = 0.01, df = 5,14)
= 2.9582 (alpha = 0.05, df = 5,14)
Since F < Critical F FAIL TO REJECT Ho: All equal (alpha = 0.01)

ANOVA Table

SOURCE	DF	SS	MS	F
Between	5	0.0106	0.0021	346.7543
Within (Error)	14	0.0001	0.0000	
Total	19	0.0107		

(p-value = 0.0000)

Critical F = 4.6950 (alpha = 0.01, df = 5,14)
= 2.9582 (alpha = 0.05, df = 5,14)
Since F > Critical F REJECT Ho: All equal (alpha = 0.05)

Bonferroni t-Test - TABLE 1 OF 2 Ho: Control=Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	t STAT	SIG 0.05
1	control	0.0703	0.0703		
2	4.6	0.0677	0.0677	1.4113	
3	10	0.0692	0.0692	0.6215	
4	22	0.0651	0.0651	2.8672	
5	46	0.0198	0.0198	27.9700	*
6	100	0.0168	0.0168	29.5975	*

Bonferroni t critical value = 2.9768 (2 Tailed, alpha = 0.05, df = 5,14)

Bonferroni t-Test - TABLE 2 OF 2 Ho: Control=Treatment

GROUP	IDENTIFICATION	NUM OF REPS	MIN SIG DIFF (IN ORIG. UNITS)	% OF CONTROL	DIFFERENCE FROM CONTROL
1	control	5			
2	4.6	3	0.0054	7.6	0.0025
3	10	3	0.0054	7.6	0.0011
4	22	3	0.0054	7.6	0.0052
5	46	3	0.0054	7.6	0.0505
6	100	3	0.0054	7.6	0.0535

APPENDIX 3 STATISTICS: YIELD (0-72 HOURS)

Chi-Square Test for Normality

Actual and Expected Frequencies

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	1.3400	4.8400	7.6400	4.8400	1.3400
OBSERVED	0	7	5	8	0

Chi-Square = 6.6194 (p-value = 0.1574)
Critical Chi-Square = 13.277 (alpha = 0.01 , df = 4)
= 9.488 (alpha = 0.05 , df = 4)

Data PASS normality test (alpha = 0.01). Continue analysis.

Levene's Test for Homogeneity of Variance

ANOVA Table

SOURCE	DF	SS	MS	F
Between	5	433.2516	86.6503	1.6350
Within (Error)	14	741.9633	52.9974	
Total	19	1175.2148		

(p-value = 0.2150)

Critical F = 4.6950 (alpha = 0.01, df = 5,14)
= 2.9582 (alpha = 0.05, df = 5,14)
Since F < Critical F FAIL TO REJECT Ho: All equal (alpha = 0.01)

ANOVA Table

SOURCE	DF	SS	MS	F
Between	5	81995.1222	16399.0244	167.7935
Within (Error)	14	1368.2673	97.7334	
Total	19	83363.3895		

(p-value = 0.0000)

Critical F = 4.6950 (alpha = 0.01, df = 5,14)
= 2.9582 (alpha = 0.05, df = 5,14)
Since F > Critical F REJECT Ho: All equal (alpha = 0.05)

Bonferroni t-Test - TABLE 1 OF 2 Ho: Control=Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	t STAT	SIG 0.05
1	control	157.4980	157.4980		
2	4.6	130.6567	130.6567	3.7178	*
3	10	144.6067	144.6067	1.7856	
4	22	107.6967	107.6967	6.8980	*
5	46	3.2400	3.2400	21.3662	*
6	100	2.5000	2.5000	21.4687	*

Bonferroni t critical value = 2.9768 (2 Tailed, alpha = 0.05, df = 5,14)

Bonferroni t-Test - TABLE 2 OF 2 Ho: Control=Treatment

GROUP	IDENTIFICATION	NUM OF REPS	MIN SIG DIFF (IN ORIG. UNITS)	% OF CONTROL	DIFFERENCE FROM CONTROL
1	control	5			
2	4.6	3	21.4920	13.6	26.8413
3	10	3	21.4920	13.6	12.8913
4	22	3	21.4920	13.6	49.8013
5	46	3	21.4920	13.6	154.2580
6	100	3	21.4920	13.6	154.9980

APPENDIX 4 EC-VALUES

Table 11 EC-values for growth rate reduction

Concentration (mg/l)	X Log conc. (mg/l)	Y Reduction (%)
2.5	0.398	5.1
2.5	0.398	1.7
2.5	0.398	4.1
2.6	0.415	1.6
2.6	0.415	1.0
2.6	0.415	2.2
4.2	0.623	7.3
4.2	0.623	7.7
4.2	0.623	7.2
26	1.415	73.3
26	1.415	66.6
26	1.415	75.7
26	1.415	84.4
26	1.415	71.8
26	1.415	72.0

Slope:	72.9723
Intercept:	-30.1554
Multiple R:	0.9872
n = number of observations:	15

Regression line: Y= 72.97X - 30.16

Prediction of X values based on known Y values

Known Y Reduction (%)	10^{Xreg} (mg/l)	$10^{X95\%-}$ (mg/l)	$10^{X95\%+}$ (mg/l)
10	3.55	2.33	5.40
20	4.87	3.21	7.38
50	12.54	8.26	19 06

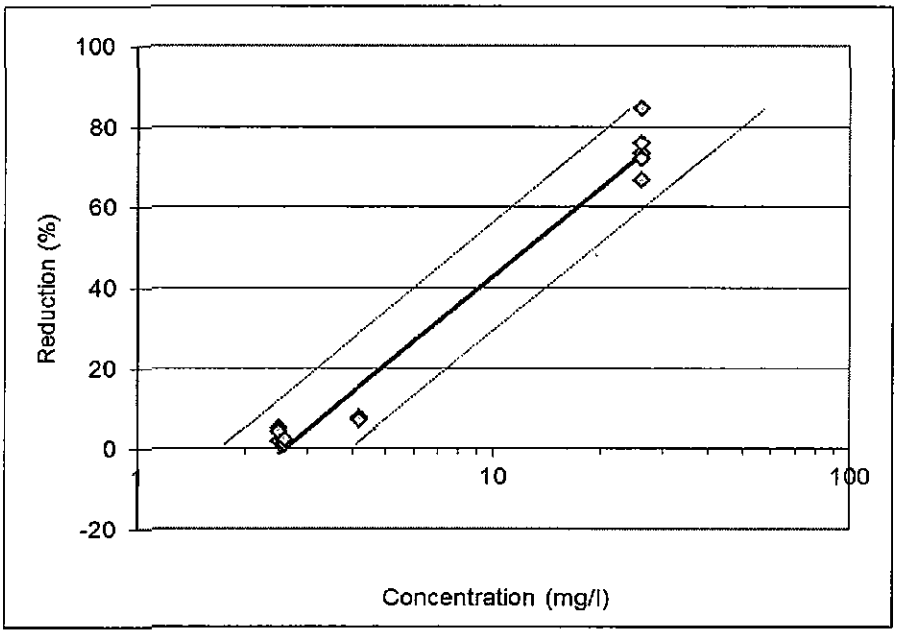


Figure 2 Percentage reduction of growth rate as function of the log concentration (mg/l) of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline.

Dashed curves represent the 95% confidence limits.

APPENDIX 4 EC-VALUES – continued –

Table 12 EC-values for yield inhibition

Concentration (mg/l)	X Log conc. (mg/l)	Y Inhibition (%)
2.5	0.398	23 2
2.5	0.398	8.8
2.5	0.398	19.2
2 6	0.415	8.3
2.6	0.415	5.4
2.6	0.415	10.9
4 2	0.623	31.3
4.2	0.623	32.7
4.2	0.623	30 9
26	1.415	98.2
26	1 415	97.2
26	1.415	98.5
26	1.415	99.2
26	1.415	98.0
26	1.415	98.0

Slope:	84.6297
Intercept.	-21.5651
Multiple R:	0.9938
n = number of observations:	15

Regression line: Y= 84.63X - 21.57

Prediction of X values based on known Y values

Known Y Inhibition (%)	10^{Xreg} (mg/l)	$10^{X95\%-}$ (mg/l)	$10^{X95\%+}$ (mg/l)
10	2.36	1.76	3 17
20	3.10	2.31	4.15
50	7.01	5.26	9.33

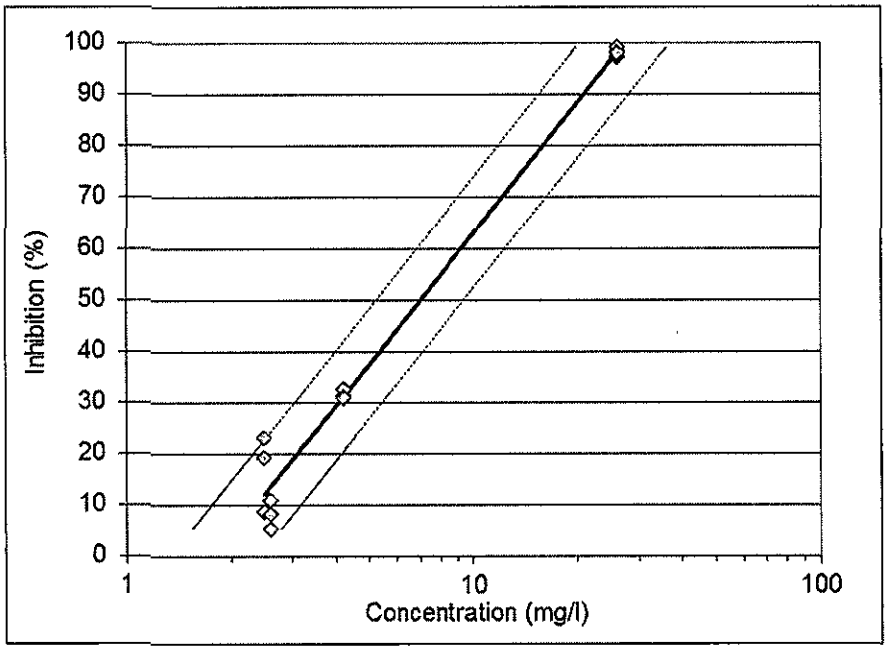


Figure 3 Percentage inhibition of yield as function of the log concentration (mg/l) of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline.

Dashed curves represent the 95% confidence limits.

APPENDIX 5 REFERENCE TEST

Pseudokirchneriella subcapitata, strain: NIVA CHL-1. Fresh water algal growth inhibition test with potassium dichromate (NOTOX Project 498136).

Start of first exposure: 10 October 2011
Completion last exposure: 13 October 2011

The study procedures described in this report were based on the OECD guideline No. 201, Adopted March 23, 2006 and ISO Standard 8692, Second edition, 01 October 2004.

This reference test was carried out to check the sensitivity of the test system used by NOTOX to Potassium dichromate (Merck, Art. 1.04864, Batch K34869764 607).

Algae were exposed for a period of 72 hours to $K_2Cr_2O_7$ (Potassium dichromate) concentrations of 0.18, 0.32, 0.56, 1.0, 1.8 and 3.2 mg/l and to a control. The initial cell density was 1.0×10^4 cells/ml.

Results:

Overview of % reduction of growth rate and % inhibition of yield in the reference test:

Nominal conc. $K_2Cr_2O_7$ (mg/l)	Mean growth rate		Yield (0-72 h)	
	μ (0-72 h)	Reduction (%)	$x10^4$ cells/ml	Inhibition (%)
Control	0.06997		153.26	
0.18	0.06933	0.9	146.59	4.4
0.32	0.06681	4.5	122.63	20.0
0.56	0.06004	14.2	74.56	51.4
1.0	0.04340	38.0	21.89	85.7
1.8	0.02860	59.1	6.86	95.5
3.2	0.02109	69.9	3.57	97.7

Potassium dichromate reduced growth rate of this fresh water algae species at nominal concentrations of 0.56 mg/l and higher.

The EC_{50} for growth rate reduction (E_RC_{50}: 0-72h) was 1.5 mg/l with a 95% confidence interval ranging from 1.1 to 2.1 mg/l. The historical ranges for growth rate reduction lie between 0.82 and 2.3 mg/l. Hence, the E_RC_{50}: 0-72h for the algal culture tested corresponds with this range.

The EC_{50} for yield inhibition (E_YC_{50}: 0-72h) was 0.52 mg/l with a 95% confidence interval ranging from 0.34 to 0.78 mg/l. The historical ranges of the 72h-EC_{50} for yield inhibition lie between 0.43 and 1.1 mg/l. Hence, the E_YC_{50}: 0-72h for the algal culture tested corresponds with this range.

The protocol, raw data and report of this study are kept in the NOTOX archives. The test described above was performed under GLP conditions with a QA-check.

APPENDIX 6 ANALYTICAL REPORT

DETERMINATION OF THE CONCENTRATIONS

Author

Dr. Ir. E. Baltussen

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2. REPORT APPROVAL

NOTOX B.V.

Principal Scientist
Analytical Chemistry

Dr. Ir. E. Baltussen

Date: 30 November 2011

3. INTRODUCTION

3.1. Preface

Study plan analytical phase	Start	:	30 September 2011
	Completion	:	22 October 2011

3.2. Aim of the study

The purpose of the analytical phase was to determine the actual concentrations in samples taken from the test solutions used during the ecotoxicity test.

4. MATERIALS AND METHODS

4.1. Reagents

Water	Tap water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA)
Acetonitrile	Biosolve, Valkenswaard, The Netherlands
M2-medium	see main report

All reagents were of analytical grade, unless specified otherwise.

4.2. Samples

The samples were stored in the freezer (≤ -15°C). Storage stability of samples under these conditions was demonstrated in NOTOX project 496981.

On the day of analysis, the samples were defrosted at room temperature. The test samples were diluted in a 1:1 (v:v) ratio with 60/40 (v/v) acetonitrile/M2-medium and analysed. If necessary, the samples were further diluted with 30/70 (v/v) acetonitrile/M2-medium to obtain concentrations within the calibration range.

4.3. Analytical method

4.3.1. Analytical conditions

Quantitative analysis was based on the analytical method validated for the test substance in NOTOX project 496981.

Instrument	Alliance Separation Module 2695 (Waters, Milford, MA, USA)
Detector	Dual λ Absorbance Detector 2487 (Waters)
Column	Symmetry Shield RP-18, 100 mm × 4.6 mm i.d., dp = 3.5 µm (Waters)
Column temperature	40°C ± 1°C
Injection volume	100 µl
Mobile phase	30/70 (v/v) acetonitrile/water
Flow	1.0 ml/min
UV detection	253 nm

4.3.2. Preparation of the calibration solutions

Stock and spiking solutions

Stock solutions of the test substance were prepared in acetonitrile at concentrations of 2415 - 3820 mg/l.

Spiking solutions were made up from a stock solution and/or dilutions of this solution. The solvent of the spiking solutions was acetonitrile.

Calibration solutions
Calibration solutions in the concentration range of 0.04 – 2 mg/l were prepared from two stock solutions. The end solution of the calibration solutions was 30/70 (v/v) acetonitrile/M2-medium.

Procedural recovery samples
2 ml blank medium was spiked with the test substance at a target concentration of 0.1 or 100 mg/l. The accuracy samples were treated similarly as the test samples (see paragraph 4.2 'Samples').

4.3.3. Sample injections

Calibration solutions were injected in duplicate. Test samples and procedural recovery samples were analysed by single injection.

4.4. Electronic data capture

System control, data acquisition and data processing were performed using the following programme:
- Empower version 7.00 (Waters, Milford, MA, USA).

Temperature and/or relative humidity during sample storage and/or performance of the studies were *monitored continuously using the following programme:*
- REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA).

4.5. Formulas

Response (R): Peak area test substance [units]

Calibration curve: $R = a\,C_N + b$

where:
C_N = nominal concentration [mg/l]
a = slope [units × l/mg]
b = intercept [units]

Analysed concentration (C_A): $C_A = \frac{(R-b)}{a} \times d$ [mg/l]

where:
d = dilution factor

Recovery: $\frac{C_A}{C_N} \times 100$ [%]

Relative to initial concentration: $\frac{C_A\,(t = x\,\text{hours})}{C_A\,(t = 0\,\text{hours})} \times 100$ [%]

5. RESULTS

5.1. Calibration curves

Calibration curves were constructed using five concentrations. For each concentration, two responses were used. Linear regression analysis was performed using the least squares method with a $1/concentration^2$ weighting factor. The coefficient of correlation (r) was > 0.99 for each curve.

5.2. Samples

5.2.1. Procedural recovery samples

The results for the procedural recovery samples are given in Table 1.

The mean recoveries of the procedural recovery samples fell within the criterion of 70-110%. It demonstrated that the analytical method was adequate for the determination of the test substance in the test samples.

5.2.2. Test samples

The results for the test samples are given in Table 2 and Table 3

6. TABLES

Table 1 Procedural recovery samples

Date of preparation [dd-mm-yy]	Date of analysis [dd-mm-yy]	Target concentration [mg/l]	Nominal concentration [mg/l]	Analysed concentration [mg/l]	Recovery [%]	Mean recovery [%]
30-09-11	30-09-11	0.1	0.100 0.100	0.0924 0.103	92 103	98
30-09-11	30-09-11	100	100 100	101 101	101 101	101
22-10-11	22-10-11	0.1	0.0993 0.0993	0.0867 0.0808	87 81	84
22-10-11	22-10-11	100	99.3 99.3	95.9 99.6	97 100	98

Table 2 Concentrations of the test substance in test medium – combined limit/range-finding test

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [1] [dd-mm-yy]	Loading rate [2] [mg/l]	Concentration analysed [mg/l]	Relative to initial [%]
0	19-09-11	30-09-11	1	0.728	
			100	60.0	
			100[3]	56.4	
24	20-09-11	30-09-11	1	0.580	80
			100	48.8	81
			100[3]	51.4	91
72	22-09-11	30-09-11	1	0.325	45
			100	37.0	62
			100[3]	33.7	60

[1] Samples were stored in the freezer (≤ -15°C) until the day of analysis.

[2] A water accommodated fraction (WAF) prepared at the loading rate.

[3] Without algae.

Table 3 Concentrations of the test substance in test medium - final test

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [1] [dd-mm-yy]	Loading rate [2] [mg/l]	Concentration analysed [mg/l]	Relative to initial [%]
0	10-10-11	22-10-11	0	n.d.	
			4.6	3.23	
			10	3.30	
			22	5.48	
			46	32.6	
			100	33.8	
			100[3]	36.4	
24	11-10-11	22-10-11	0	n.d.	n.a.
			4.6	2.82	87
			10	2.74	83
			22	4.59	84
			46	27.1	83
			100	27.6	82
			100[3]	28.5	78
72	13-10-11	22-10-11	0	n.d.	n.a.
			4.6	1.89	59
			10	2.03	61
			22	3.22	59
			46	21.4	66
			100	21.3	63
			100[3]	22.8	62

[1] Samples were stored in the freezer (≤ -15°C) until the day of analysis

[2] A water accommodated fraction (WAF) prepared at the loading rate.

[3] Without algae.

n.d. Not detected.

n.a. Not applicable.

FINAL REPORT

Study Title

DAPHNIA MAGNA, REPRODUCTION TEST WITH P-(2,3-EPOXYPROPOXY)-N,N-BIS(2,3-EPOXYPROPYL)ANILINE (SEMI-STATIC)

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Laboratory Project Identification

NOTOX Project 496989
NOTOX Substance 203277/A

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2. STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (GLP) (as revised in 1997) ENV/MC/CHEM (98) 17.

The sponsor is responsible for Good Laboratory Practice (GLP) compliance for all test substance information unless determined by NOTOX.

NOTOX B.V.

Ir. L.M. Bouwman
Study Director

Date: 13 March 2012

Ing. M.H.J. Migchielsen
Section head Environmental Toxicology

Date: 13 March 2012

3. QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the NOTOX Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

Type of Inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date
Study	Protocol	02-Aug-11	02-Aug-11	02-Aug-11
	Protocol Amendment 01	09-Aug-11	09-Aug-11	09-Aug-11
	Protocol Amendment 02	23-Dec-11	23-Dec-11	23-Dec-11
	Report	28-Feb-12	29-Feb-12	29-Feb-12
Process	**Environmental Toxicology** Test Substance Handling Exposure Observations/Measurements	17-Oct-11	21-Oct-11	21-Oct-11
	Analytical and physical chemistry Test Substance Handling Observations/Measurements	31-Oct-11	10-Nov-11	10-Nov-11

NOTOX B.V.

C.J. Mitchell B.Sc.
Head of Quality Assurance

Date: 13-Mar-2012

4. SUMMARY

Daphnia magna, 21-day reproduction study with
p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline.

The study procedures described in this report were based on the OECD guidelines for Testing of Chemicals: Guideline No. 211, 2008. In addition, the procedures were designed to meet the test methods and validity criteria of the ISO International Standard 10706, 2000, the Commission Regulation (EC) No 440/2008 Part C.20, 2008 and the OECD guidance document number 23, 2000.

The batch of test substance tested was a yellow liquid with a purity of 94.33%.

A reproduction test was performed based on the result of a preceding 7-day preliminary test. Preparation of test solutions started with a loading rate of 10 mg/l applying two days of magnetic stirring to reach maximum solubility of the test substance in the test medium. The resulting aqueous mixture was left to stabilize for 1-1.5 hours where after the Water Accommodated Fraction (WAF) was siphoned off and used as the highest test concentration. All lower test concentrations were prepared by subsequent dilutions of the WAF in test medium. The final test concentrations contained 1.0, 3.2, 10, 32 and 100% of the WAF prepared at a loading rate of 10 mg/l.

The reproduction test was performed in a semi-static system, included 10 vessels per test concentration and 20 vessels for an untreated control group. Each of the vessels contained one neonate (<24h old) *Daphnia magna* in 50 ml test medium. The study duration was 21 days and the test solutions were renewed every 48 hours. The daphnids were fed on a daily basis with a *Chlorella pyrenoidosa* suspension. During the test samples for analytical confirmation of actual exposure concentrations were taken at the start and the end of four intervals of 48 hours.

Analysis of the sample taken from the WAF prepared at 10 mg/l at the start of four renewal periods showed that the actual test concentration ranged between 4.2 and 6.6 mg/l. Test concentrations slightly decreased during the 48 hour renewal periods (to 70-82% of initial). Test concentrations containing 10 and 32% of the WAF proved to have been prepared properly and also slightly decreased during the 48 hour renewal periods (to 63-79% of initial). The test concentration containing 1.0% of the WAF was below the lowest calibration standard at all times, while the test concentration containing 3.2% of the WAF varied between being below or just above the lowest calibration standard. Given these results effect parameters were based on the average exposure concentrations: 0.040, 0.096, 0.42, 1.7 and 4.8 mg/l.

Three out of the twenty parental daphnids in the control group died during the test period. Mortality in the test concentrations was not statistically significant when compared to the control.

The average cumulative number of young per female in the control group after 21 days was 136 ± 16. The reproduction curves recorded at 0.040, 0.096 and 0.42 mg/l closely followed the curve of the control. The curve recorded at 1.7 mg/l stayed behind from the start and dapnids exposed to 4.8 mg/l did not reproduce at all. There were some recordings of immobile young and/or aborted eggs in the control and the three lowest test concentrations, while significant amounts of aborted eggs were recorded at the two highest test concentrations throughout the 21-day test period. Statistical analysis showed that the reproduction of the daphnids was significantly reduced at 1.7 mg/l and higher.

Mean parental body length was not significantly reduced at any of the test concentrations.

The study met the acceptability criteria prescribed by the protocol and was considered valid.

p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline did not affect reproduction of *Daphnia magna* at an average concentration of 0.42 mg/l after 21 days of exposure (NOEC).

Exposure to average concentrations of 1.7 mg/l and higher induced significant inhibition of the reproductive capacity of the parental daphnids (LOEC). The 21d-EC_{50} for reproduction was calculated to be 1.4 mg/l with a 95% confidence interval ranging from 0.91 to 2.2 mg/l.

5. INTRODUCTION

5.1. Preface

Sponsor	Huntsman Advanced Materials GmbH Klybeckstrasse 200 4057 BASEL Switzerland
Study Monitor	Dr. P. Dollenmeier
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Ir. L.M. Bouwman
Technical Coordinator	Ing. V.E. Carolus
Principal Scientist	Dr. Ir. E. Baltussen
Study Plan	Start : 20 September 2011 Completion : 29 December 2011

5.2. Aim of the study

The purpose of the test was to evaluate the effects of the test substance on the mobility, the growth and the reproductive capacity of *Daphnia magna*. For this purpose, test organisms were exposed to aqueous solutions containing the test substance at various concentrations. The time of the first production of young, the number of young born, immobility and other signs of intoxication observed were compared with corresponding parameters in the controls.

5.3. Guidelines

The study procedures described in this report were based on the Organization for Economic Co-operation and Development (OECD), OECD guidelines for Testing of Chemicals, guideline No. 211: "*Daphnia magna*, Reproduction Test", Adopted: October 2008.

In addition, the procedures were designed to meet the test methods prescribed by the following guidelines and guidance document:

- Commission Regulation (EC) No 440/2008 of 30 May 2008, Part C: Methods for the determination of ecotoxicity, Publication No. L142, C.20. "*Daphnia magna* Reproduction Test".
- ISO International Standard 10706: "Determination of long term toxicity of substances to *Daphnia magna* Straus (*Cladocera, Crustacea*)", 2000-03-30.
- Guidance document on aquatic toxicity testing of difficult substances and mixtures, OECD series on testing and assessment number 23, December 14, 2000.

5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report are retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. NOTOX will retain information concerning decisions made.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

5.5. Definitions

Parent animals are those female daphnids present at the start of the test and of which the reproductive output is the object of study.

Offspring are the young daphnids produced by the parent animals in the course of the test.

The Lowest Observed Effect Concentration (LOEC) is the lowest tested concentration at which the substance is observed to have a statistically significant effect on reproduction and parent mortality (at $p < 0.05$) when compared with the control, within a stated exposure period. However, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC. When these two conditions cannot be satisfied, a full explanation must be given for how the LOEC (and hence the NOEC) has been selected.

The No Observed Effect Concentration (NOEC) is the test concentration immediately below the LOEC, which when compared with the control, has no statistically significant effect ($p < 0.05$), within a stated exposure period.

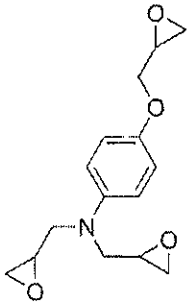
EC_X is the concentration of the test substance dissolved in water that results in a x per cent reduction in reproduction of *Daphnia magna* within a stated exposure period.

Mortality. An animal is recorded as dead when it is immobile, i.e. when it is not able to swim, or if there is no observed movement of appendages or postabdomen, within 15 seconds after gentle agitation of the test vessel.

6. MATERIALS AND METHODS

6.1. Test Substance

6.1.1. Test substance information

Identification	p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline
Structure	
Molecular formula	$C_{15}H_{19}NO_4$
Molecular weight	277.3
CAS Number	5026-74-4
EC Number	225-716-2
Description	Yellow liquid
Batch	AA00373400
Purity	94.33%
Test substance storage	In refrigerator (2-8°C) in the dark
Stability under storage conditions	Stable
Expiry date	28 February 2013

6.1.2. Study specific test substance information

Volatile	No
Stability in water	No
Solubility in water	No

6.2. Test system

Species	*Daphnia magna* (Crustacea, Cladocera) (Straus, 1820), at least third generation, obtained by acyclical parthenogenesis under specified breeding conditions.
Source	In-house laboratory culture with a known history.
Reason for selection	This system has been selected as an internationally accepted invertebrate species.
Validity of batch	Daphnids originated from a healthy stock, 2nd to 5th brood, showing no signs of stress such as mortality >20%, presence of males, ephippia or discoloured animals and there was no delay in the production of the first brood.
Characteristics	To initiate the test, young daphnids < 24 hours old were selected, from parental daphnids greater than two weeks old.

6.3. Breeding

Start of each batch	With newborn daphnids, i.e. less than 3 days old, by placing them individually in 50 ml M7-medium.
Maximum age of the cultures	4 weeks
Monitoring of the individual cultures	Three times a week the young are counted and the parental daphnids are transferred to new media.
Temperature of medium	18-22°C
Feeding	Daily, a suspension of fresh water algae
Validity of the cultures	Historical data on the reproductive capacity are based on the numbers of living young counted three times a week in the individual cultures and tested to meet the validity criteria for survival and reproduction.
Medium	M7, as prescribed by Dr. Elendt-Schneider (Elendt, B.-P., 1990: Selenium deficiency in Crustacea. An ultrastructural approach to antennal damage in *Daphnia magna* Straus. Protoplasma 154, 25-33).

Composition of medium M7

Adjusted ISO medium: the following chemicals (analytical grade) are dissolved in tap water purified by Reverse Osmosis (RO-water, GEON Waterbehandeling, Berkel-Enschot, The Netherlands)

Macro salts.	$CaCl_2.2H_2O$	211.5	mg/l
	$MgSO_4.7H_2O$	88.8	mg/l
	$NaHCO_3$	46.7	mg/l
	KCl	4.2	mg/l

Medium M7: trace elements, macro nutrients and vitamins are added to freshly prepared ISO medium to reach the following concentrations.

Trace elements:	B	0.125	mg/l
	Fe	0.05	mg/l
	Mn	0.025	mg/l
	Li, Rb and Sr	0.0125	mg/l
	Mo	0.0063	mg/l
	Br	0.0025	mg/l
	Cu	0.0016	mg/l
	Zn	0.0063	mg/l
	Co and I	0.0025	mg/l
	Se	0.0010	mg/l
	V	0.0003	mg/l
	$Na_2EDTA.2H_2O$	2.5	mg/l
Macro nutrients	$Na_2SiO_3.9H_2O$	10.0	mg/l
	$NaNO_3$	0.27	mg/l
	KH_2PO_4	0.14	mg/l
	K_2HPO_4	0.18	mg/l
Vitamins:	Thiamine	75.0	µg/l
	B_{12}	1.0	µg/l
	Biotin	0.75	µg/l

The hardness: 180 mg/l expressed as $CaCO_3$ and the pH: 7.7 ± 0.3.

6.4. Preparation of test solutions

The standard test procedures required generation of test solutions, which should contain completely dissolved test substance concentrations or stable and homogeneous mixtures or dispersions. The testing of concentrations that disturb the test system should be prevented (e.g. precipitate or a film of the test substance on the water surface).

The batch of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline tested was a yellow liquid with a purity of 94.33% and the substance was not completely soluble in test medium at loading rates of 1.0 mg/l and higher.

Preliminary test
All test solutions with loading rates of 1.0 mg/l and higher were prepared separately applying two days of magnetic stirring to reach maximum solubility of the test substance in the test medium. The resulting aqueous mixtures were left to stabilize for 1 hour where after the Water Accommodated Fractions (WAFs) were siphoned off and used for testing. One lower test concentration was prepared by subsequent dilution of the 1.0 mg/l WAF in test medium. The final test solutions were all clear and colourless.

Reproduction test
Preparation of test solutions started with a loading rate of 10 mg/l applying two days of magnetic stirring to reach maximum solubility of the test substance in the test medium. The resulting aqueous mixture was left to stabilize for 1-1.5 hours where after the Water Accommodated Fraction (WAF) was siphoned off and used as the highest test concentration. All lower test concentrations were prepared by subsequent dilutions of the WAF in test medium.

6.5. Preliminary test

A preliminary test was performed to define the effects of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline on mobility and mortality of *Daphnia magna* to be tested in the reproduction test. Test procedure and conditions were similar to those applied in the reproduction test with the following exceptions:

- Ten daphnids per concentration (two replicates, five daphnids per replicate) were exposed to a control, WAFs prepared at loading rates of 1.0, 10 and 100 mg/l and to a dilution containing 10% of the WAF prepared at a loading rate of 1.0 mg/l;
- Test duration was 7 days;
- Test solutions were renewed on days 1, 3 and 6;
- No body length was recorded at the end of the test;
- Samples for analysis were taken at days 0, 1 and 3 from the freshly prepared solutions and at days 1, 3 and 6 from the old solutions;
- Samples were taken from solutions containing the daphnia and food (algae), but also from an extra vessel incubated under the same conditions, but without daphnia and algae.

6.6. Reproduction test

6.6.1. Test concentrations

p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline	1.0, 3.2, 10, 32 and 100% of a WAF prepared at a loading rate of 10 mg/l
Controls	Test medium without test substance or other additives

6.6.2. Test procedure and conditions

Test duration	21 days
Test type	Semi-static
Frequency of renewal	Every 48 hours
Test vessels	Volume: 60 ml (6 x Ø 3.5 cm), all-glass covered with a Perspex plate.
Medium	M7[1]
Experimental design	At the start of the experiment (nominal day 0) 10 neonate daphnids, less than one day old, per group were divided over ten vessels each containing a minimum of 50 ml test medium. The control group consisted of 20 daphnids.
Light	16 h photoperiod daily; intensity at the start: 624-708 lux (8.8-10 $\mu E.m^{-2}.s^{-1}$) intensity at the end: 612-710 lux (8.8-9.9 $\mu E.m^{-2}.s^{-1}$)
Feeding	Daily, 0.5 ml of *Chlorella pyrenoidosa* suspension[2] containing 0.40-0.54 $x10^8$ cells/ml was added as feed for the daphnids. This daily ration corresponded to 0.2 mg C/Daphnia/day, which is the recommended value for daily feeding per daphnid in the reproduction test according to the OECD Guideline 211.

[1] Total (dissolved) organic carbon of the M7 medium used in the reproduction study was measured at the start of the test. Measurements were performed with a Shimadzu TOC-V_{CPH} total organic carbon analyzer combined with a Shimadzu ASI-V auto sampler (Shimadzu Kyoto, Japan). The sample volume was 40 ml and the number of repeats was at least 3.

[2] Algae suspensions were made by inoculating growth medium with *Chlorella pyrenoidosa* from a pure culture. The suspensions were continuously aerated and exposed to light (6000-10000 lux; 60-120 $\mu E.m^{-2}.s^{-1}$) in a climate room at a temperature of 23 ± 2°C for 2-3 weeks. After this period, the TOC concentration of the batch algal suspension was measured before use as feed in the reproduction study. The exact volume to be added per test vessel was then calculated.

6.6.3. Sampling for analysis of test concentrations

Samples for analysis were taken from all test concentrations and the control. The method of analysis is described in the appended Analytical Report (APPENDIX 7).

Sampling:	Frequency	At the beginning and at the end of four intervals of 48 hours (nominal days 0 and 2, 6 and 8, 12 and 14, 18 and 20)
	Volume	2 ml
	Storage	Samples were stored in a freezer until analysis.

At the end of the refreshment period, the replicates were pooled at each concentration before sampling.

Additionally, reserve samples of 2 ml were taken from all test solutions for possible analysis. If not already used, these samples were stored in a freezer for a maximum of three months after delivery of the draft report, pending on the decision of the sponsor for additional analysis.

6.6.4. Measurements and recordings

Parental daphnids	
Condition	Every workday and upon renewal on non-workdays, the number of living, immobile and dead parental daphnids was recorded. Dead daphnids were removed when observed.
Presence of eggs in the brood pouch	Every workday and upon renewal on non-workdays.
Body length	At the end of the test.
Offspring	
Appearance first brood	When observed.
Newborn daphnids	Every workday and upon renewal on non-workdays, the number of newborn young was counted and the condition of the young recorded. Thereafter the young were removed.
Presence of unhatched eggs	When observed.
Incidence of immobility	When observed.
Test medium	
Temperature, oxygen and pH	At the start of the test and just before and after each renewal in one of the vessels of each test group with surviving daphnids.
Hardness	Once a week in fresh and old media from the control and the highest test concentration
Light	At the start and the end of the test

6.7. Interpretation

6.7.1. Data handling

The values for reproduction observed at various concentrations of the test substance were expressed as mean number of living young per parent. The mean values for reproduction at each concentration were compared to those recorded in the control on the various days of recording. Further, the length of the parental daphnids (day 21) exposed to the test substance were compared to the control.

Exposure concentrations

The exposure concentrations were calculated as

$$\frac{\sqrt{C_{t=0,fresh} \times C_{t=2,old}} + \sqrt{C_{t=6,fresh} \times C_{t=8,old}} + \sqrt{C_{t=12,fresh} \times C_{t=14,old}} + \sqrt{C_{t=18,fresh} \times C_{t=20,old}}}{4}$$

being the mathematical means of the concentrations of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline measured in the samples taken from the freshly prepared solutions ($C_{t=0}$, $C_{t=6}$, $C_{t=12}$ and $C_{t=18}$) and from the 48-hour old solutions ($C_{t=2}$, $C_{t=8}$, $C_{t=14}$ and $C_{t=20}$).

In case concentrations measured were below the lowest calibration solution, the final exposure concentration(s) were taken as a factor of 2 below the lowest calibration solution. This procedure is based on the OECD "Guidance document on the use of the harmonised system for the classification of chemicals which are hazardous for the aquatic environment".

Statistical analysis

Parental survival on day 21 was statistically tested using Fisher's exact test (TOXSTAT Release 3.5, 1996, D.D. Gulley, A.M. Boelter, H.L. Bergman). Total reproduction on day 21 was tested for normality and for homogeneity of variance and statistically tested using an ANOVA test followed by a mean comparison test (Bonferroni t-Test, TOXSTAT Release 3.5, 1996). The respective threshold levels of effect (LOEC) and the respective NOECs were determined on basis of these statistics.

EC-values

The 21d-EC_{50} for reproduction was based on log-linear regression analysis of the percentages of reproductive reduction versus the logarithms of the corresponding average exposure concentrations of the test substance.

6.7.2. Electronic data capture

Observations/measurements in the study were recorded electronically using the following programme(s):

- REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA)
- Shimadzu TOC-Control V version 2.10 (Shimadzu, Kyoto, Japan): carbon analysis.

6.7.3. Acceptability of the test

1. The mortality of the parent animals (female *Daphnia*) did not exceed 20% at the end of the test (15%).
2. The average cumulative number of young per female in the controls after 21 days was ≥60 (136 ± 16).

6.8. List of deviations

6.8.1. List of protocol deviations

1. The condition of parental daphnids was not recorded on the first day of exposure.
   Evaluation: No parental daphnids died during the first refreshment period. In addition no adverse effect on mobility of the daphnids were observed.
2. Inadvertently, oxygen concentrations and pH were not measured in the old solutions on the second day of the reproduction test.
   Evaluation: No parental daphnids died during the first refreshment period. In addition no adverse effect on mobility of the daphnids were observed.

The study integrity was not adversely affected by these deviations.

6.8.2. List of standard operating procedures deviations

Any deviations from standard operating procedures were evaluated and filed in the study file. There were no deviations from standard operating procedures that affected the integrity of the study.

7. RESULTS

7.1. Preliminary test

Table 1 contains the daily observations on mortality and reproductive potency of surviving parental daphnids during the 7-day preliminary test. All daphnids exposed to the highest test concentration died within 24 hours of exposure. No (significant) mortality was observed at the three lowest test concentrations and the control. Note that a maximum of 20% mortality is acceptable for the control and therefore not considered significant. Almost all surviving daphnids in the control and the three lowest test concentrations carried eggs in their brood pouch on day 7 of exposure. Overall, no effects on survival or time of egg development were observed at the three lowest test concentrations when compared to the control.

Analysis of the samples taken from the WAFs prepared at 1.0 and 10 mg/l at the start of the three renewal periods showed that the actual test concentrations ranged between 0.65 and 0.97 mg/l for the 1.0 mg/l WAF and between 6.6 and 7.3 mg/l for the 10 mg/l WAF. These test concentrations remained (relatively) stable during the 24 and 48 hour renewal periods (79-90% of initial), but decreased to 61-68% of initial during the 72 hour renewal period. See also Table 2 of the appended Analytical Report.

Test conditions were maintained within the limits prescribed by the protocol.

Based on the results of this preliminary test, it was decided to perform a reproduction test with a WAF prepared at a loading rate of 10 mg/l as the highest test concentration.

Table 1 Mortality and reproductive potency during the preliminary test

Loading rate[1] Test substance[2] (mg/l)	Number Daphnia exposed	Cumulative number of dead parental daphnids and parents with eggs in the brood pouch between parentheses						
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
control	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (1)	0 (10)
10% of 1.0	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (1)	0 (9)
1.0	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (5)	1 (9)
10	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (2)	0 (10)
100	10	10 (0)	10	10	10	10	10	10

[1] WAF prepared at the given loading rate
[2] The test substance is p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline

7.2. Reproduction test

7.2.1. Measured concentrations

The results of analysis of the samples taken during the reproduction test are described in Table 3 of the appended Analytical Report.
Analysis of the sample taken from the WAF prepared at 10 mg/l at the start of four renewal periods showed that the actual test concentration ranged between 4.2 and 6.6 mg/l. Test concentrations slightly decreased during the 48 hour renewal periods (to 70-82% of initial). Test concentrations containing 10 and 32% of the WAF proved to have been prepared properly and also slightly decreased during the 48 hour renewal periods (to 63-79% of initial. The test concentration containing 1.0% of the WAF was below the lowest calibration standard at all times, while the test concentration containing 3.2% of the WAF varied between being below or just above the lowest calibration solution. Given these results effect parameters were based on the average exposure concentrations: 0.040, 0.096, 0.42, 1.7 and 4.8 mg/l.

7.2.2. Condition of parental daphnids

Three out of the twenty parental daphnids in the control died during the test period (see Table 2). Hence, parental mortality did not exceed 20% in the control. Mortality in all p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline concentrations tested was lower or slightly higher than in the control but not statistically significant (Fisher's exact test, α=0.05, see APPENDIX 3).

In the control group, the presence of eggs in the brood pouch was recorded for the first time on day 4-6 and the first brood appeared between day 7 and day 9. The first recording of the presence of eggs in the brood pouch was similar to the control in treated solutions up to and including 4.8 mg/l. Hence, no delay in appearance of the first brood was observed in the treatments when compared to the control.

Table 2 Cumulative mortality of parental daphnids during the 21-day exposure period.

Average conc. Test substance[1] (mg/l)	Number exposed	Cumulative number of dead parental daphnids on day:												Mortality %
		2	4	7	10	11	12	14	16	18	19	20	21	
control	20	0	0	0	0	0	0	1	1	2	2	3	3	15
0.040	10	0	0	0	0	0	0	0	0	0	1	1	1	10
0.096	10	0	0	0	0	0	0	0	0	0	0	0	0	0
0.42	10	0	0	0	0	1	1	1	1	1	1	1	1	10
1.7	10	0	0	0	2	2	2	2	2	2	3	3	3	30
4.8	10	0	0	0	1	1	1	1	1	1	1	1	1	10

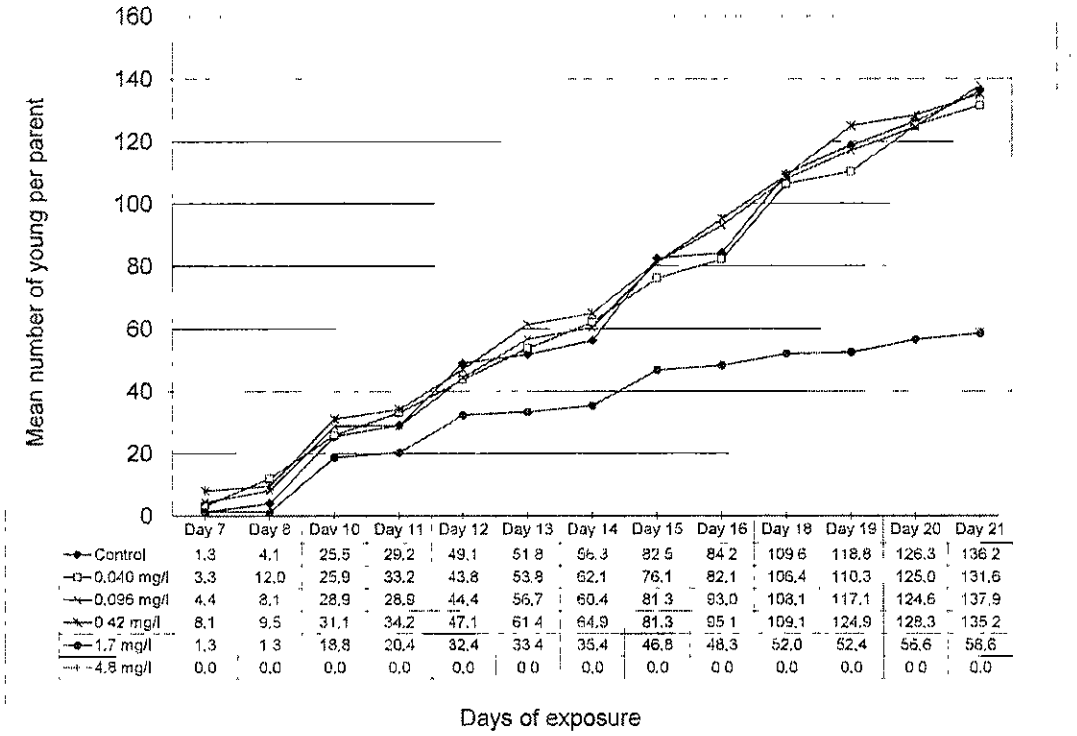
[1] The test substance is p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline

7.2.3. Reproduction

Records of cumulative mean number of living offspring produced per parent during the test period are summarised in Figure 1 with a data table. The data recorded per parent are presented in APPENDIX 1.

The average cumulative number of young per female in the control group after 21 days was 136 with a coefficient of variation of 11%. The reproduction curves recorded at 0.040, 0.096 and 0.42 mg/l closely followed the curve of the control. The curve recorded at 1.7 mg/l stayed behind from the start and daphnids exposed to 4.8 mg/l did not reproduce at all. There were some recordings of immobile young and/or aborted eggs in the control and the three lowest test concentrations, while significant amounts of aborted eggs were recorded at the two highest test concentrations throughout the 21-day test period.

Statistical analysis showed that the reproduction of the daphnids was significantly reduced at 1.7 mg/l and higher (Bonferroni t-Test, α=0.05, see APPENDIX 4).



	Day 7	Day 8	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 18	Day 19	Day 20	Day 21
Control	1.3	4.1	25.5	29.2	49.1	51.8	56.3	82.5	84.2	109.6	118.8	126.3	136.2
0.040 mg/l	3.3	12.0	25.9	33.2	43.8	53.9	62.1	76.1	82.1	106.4	110.3	125.0	131.6
0.096 mg/l	4.4	8.1	28.9	28.9	44.4	56.7	60.4	81.3	93.0	108.1	117.1	124.6	137.9
0.42 mg/l	8.1	9.5	31.1	34.2	47.1	61.4	64.9	81.3	95.1	109.1	124.9	128.3	135.2
1.7 mg/l	1.3	1.3	18.8	20.4	32.4	33.4	35.4	46.8	48.3	52.0	52.4	56.6	56.6
4.8 mg/l	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Figure 1 **Cumulative mean number of living young per parent at the various average concentrations of p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline during the 21-day test period**

7.2.4. Body length

The average body lengths of the surviving daphnids per concentration measured at the end of the test and the relative reduction of body lengths compared to the control are summarised in Table 3. See APPENDIX 2 for the individual body lengths of the surviving parental daphnids. Statistical analysis of the data was not needed as the % reduction recorded was not biologically significant (<10%).

Table 3 **Average body lengths (mm) of parental daphnids at the end of the test**

Concentration 4,4'-DDS (mg/l)	Average length (mm)	SD	% Reduction
control	4.32 (n=17)	0.12	
0.040	4.29 (n=9)	0.11	1
0.096	4.40 (n=10)	0.10	-2
0.42	4.39 (n=9)	0.10	-2
1.7	4.31 (n=7)	0.12	0
4.8	4.16 (n=9)	0.14	4

[1] The test substance is p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline
n= number of daphnids surviving the test period
SD= standard deviation

7.2.5. Determination of effect concentrations

Table 4 shows the effect parameters based on mean measured concentrations, see also APPENDIX 5.

Table 4 Effect parameters

Parameter	Concentration Test substance[1] (mg/l)	95%-confidence interval
21-day LC_{50} (parental mortality)	>4.8	
21-day NOEC for parental mortality[3]	4.8	
21-day EC_{50} for reproduction[2]	1.4	0.91 - 2.2
21-day LOEC for reproduction[4]	1.7	
21-day NOEC for reproduction[4]	0.42	
21-day LOEC for parental body length	>4.8	
21-day NOEC for parental body length	4.8	

[1] The test substance is p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline
[2] Log-linear regression analysis
[3] Fisher's exact test (Toxstat)
[4] Bonferroni t -Test (Toxstat)

7.2.6. Experimental conditions

The pH values recorded during the test are presented in APPENDIX 6, Table 12. The pH remained within the range of 7.5 to 8.4 throughout the test and thus was maintained within the limits prescribed by the protocol (6.0-9.0, constant within 1.5 units).

The dissolved oxygen concentrations measured during the study are presented in APPENDIX 6, Table 13. The oxygen concentration in all test solutions remained within the range of 8.2 to 9.7 mg/l during the exposure period and thus complied to the requirements as laid down in the protocol (>3 mg/l).

Temperatures recorded in the test media are shown in APPENDIX 6, Table 14. The temperatures in the test media varied between 19.4 and 20.5°C. The temperature continuously measured in a temperature control vessel varied between 19.3 and 20.3°C during the test, and complied with the requirements as laid down in the protocol (18-22°C, constant within 2°C).

The results of the measurements of total hardness are presented in APPENDIX 6, Table 15. Total hardness varied between 179 and 214 mg calcium carbonate per litre, and thus complied to the requirements as laid down in the protocol (>140 mg $CaCO_3$ per liter).

The total dissolved organic carbon content of the M7 medium was 0.42 mg/l. This complied with the requirements as laid down in the guidelines (TOC <2 mg/l).

8. CONCLUSION

Under the conditions of the present study p-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline did not affect reproduction of *Daphnia magna* at an average concentration of 0.42 mg/l after 21 days of exposure (NOEC).

Exposure to average concentrations of 1.7 mg/l and higher induced significant inhibition of the reproductive capacity of the parental daphnids (LOEC). The 21d-EC_{50} for reproduction was calculated to be 1.4 mg/l with a 95% confidence interval ranging from 0.91 to 2.2 mg/l.

APPENDIX 1 CUMULATIVE SCORES OF LIVING NEWBORN DAPHNIDS PER PARENT

Table 5 Cumulative scores of living newborn per parent in the control

Parent	Day 7	Day 8	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 18	Day 19	Day 20	Day 21
P1	0	2	7	31	31	34 (2i)	+						
P2	0	0	31	31	56	56	56	90	90	90	126	126	126
P3	25	29	63	63	63	87	87	87	119	119	161	161	161
P4	0	0	27	27	48	48	48	84	84	109	122	122	122
P5	0	0	20	20	45	45	45	81	81	117	117	117	140
P6	0	0	29	29	56	56	56	89	89	129	129	129	129
P7	0	0	33	33	52	52	52	84	84	+			
P8	0	0	26	26	66	66	66	101	101	141	141	141	168
P9	0	13	13	39	39	39	72	72	72	100	100	135	135
P10	0	0	19	19	41	41	41	68	68	89	89	89	105
P11	0	18	18	41	41	41	70	70	70	99	99	137	137
P12	0	0	22	22	48	48	48	82	82	82	117	117	117
P13	0	0	27	27	52	52	52	88	88	126	126	126	152
P14	0	0	25	25	55	55	55	91 (1i)	91	129	129	129	129
P15	0	0	29	29	62	62	62	93	93	93	133	133	133
P16	0	0	20	20	50	50	50	85 (1i)	85	121	121	121	147 (1i)
P17	0	0	24	24	53	53	53	85 (1i)	85	125	125	125	144
P18	0	19	30	30	30	58	63	63	63	92	92	136	136
P19	0	0	22	22	45	45	45	77 (1i)	77	109	109	+	
P20	0	0	25	25	48	48	48	77	77	103	103	103	134
Mean	1.3	4.1	25.5	29.2	49.1	51.8	56.3	82.5	84.2	109.6	118.8	126.3	136.2
SD	5.6	8.5	10.8	9.9	9.7	11.4	11.4	9.5	12.6	17.0	18.1	15.7	15.5
n	20	20	20	20	20	20	19	19	19	18	18	17	17

+ Parental daphnid was found dead
( ) Number of immobilised young (i) on the respective day (not cumulative)

Table 6 Cumulative scores of living newborn per parent at 0.040 mg/l

Parent	Day 7	Day 8	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 18	Day 19	Day 20	Day 21
P1	18	18	47	47	47	78	78	78	106	106	106	138	138
P2	0	0	12	12	39	39	39	78	78	106	106	107	107
P3	0	23	23	50	50	50	78	78	78	97	97	130	130
P4	15	15	23	23	23	63	63	63	95	95	124	124	124
P5	0	0	25	25	50	50	50	85	85	118	118	118	118
P6	0	24	24	46	46	46	68	68	68	100	+		
P7	0	16	39	39	39	68	72	72	72	102	102	139	139
P8	0	0	28	28	53	53	53	85 (1i)	85	124	124	124	154
P9	0	24	24	48	48	48	77	77	77	108	108	137	137
P10	0	0	14	14	43	43	43	77	77	108	108	108	137
Mean	3.3	12.0	25.9	33.2	43.8	53.8	62.1	76.1	82.1	106.4	110.3	125.0	131.6
SD	7.0	10.8	10.4	14.6	8.7	12.2	14.9	6.9	11.3	9.0	9.5	12.2	13.7
n	10	10	10	10	10	10	10	10	10	10	9	9	9

+ Parental daphnid was found dead
( ) Number of immobilised young (i) on the respective day (not cumulative)

APPENDIX 1 CUMULATIVE SCORES OF LIVING NEWBORN DAPHNIDS PER PARENT – continued –

Table 7 Cumulative scores of living newborn per parent at 0.096 mg/l

Parent	Day 7	Day 8	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 18	Day 19	Day 20	Day 21
P1	0	0	28	28	65	65	65	103	103	137	137	137	154
P2	0	0	25	25	49	49	50	85	85	85	113	113	113
P3	0	11	36	36	36	73	73	73	101	101	101	143	143
P4	16	16	37	37	37	62	62	62	88	88	88	121	121
P5	0	0	19	19	45	45	45	93	93	119	119	119	147
P6	20	20	49	49	49	82	82	82	114	115	149 (1e)	149	149
P7	8	8	26	26	26	54	54	54	84	84	112	112	112
P8	0	26	26	26	43	43	79	79	79	116	116	116	148
P9	0	0	20	20	45	45	45	90	91	124	124	124	152
P10	0	0	23	23	49	49	49	92	92	112	112	112	140
Mean	4.4	8.1	28.9	28.9	44.4	56.7	60.4	81.3	93.0	108.1	117.1	124 6	137 9
SD	7.6	9.8	9.2	9.2	10.3	13.3	13.9	14.9	10.4	18.0	17.1	13.6	16.2
n	10	10	10	10	10	10	10	10	10	10	10	10	10

( ) Number of unhatched eggs (e) on the respective day (not cumulative)

Table 8 Cumulative scores of living newborn per parent at 0.42 mg/l

Parent	Day 7	Day 8	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 18	Day 19	Day 20	Day 21
P1	0	0	29	+									
P2	0	0	24	24	56	56	56	91	91	129	129	129	159
P3	0	0	23	23	49	49	49	88	88	88	111	111	111
P4	0	0	26	26	57	57	57	99	99	135	135	135	135
P5	17	17	40	40	40	67	67	67	95	95	120	124	124
P6	18	18	39	39	39	72 (1i)	72	72	100	100	132 (2e)	132	132
P7	0	0	23	23	50	50	50	82	82	105	105	105	137
P8	21	21	47	47	47	80	80	80	120	120	149	149	149
P9	0	14	14	40	40	40	71	71	71	100	100	127	127
P10	25	25	46	46	46	82	82 (1e)	82	110	110	143 (1e)	143	143
Mean	8.1	9.5	31 1	34.2	47.1	61.4	64.9	81.3	95.1	109.1	124.9	128.3	135.2
SD	10.7	10.4	11.2	10.1	6.7	14.6	12.4	10.3	14.6	15.9	17.0	14.0	14.2
n	10	10	10	9	9	9	9	9	9	9	9	9	9

+ Parental daphnid was found dead

( ) Number of immobilised young (i) or unhatched eggs (e) on the respective day (not cumulative)

APPENDIX 1 CUMULATIVE SCORES OF LIVING NEWBORN DAPHNIDS PER PARENT – continued –

Table 9 ***Cumulative scores of living newborn per parent at 1.7 mg/l***

Parent	Day 7	Day 8	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 18	Day 19	Day 20	Day 21
P1	0	0	+										
P2	0	0	+										
P3	0	0	0	13	13	13	29	29	29	36	36	62(1e)	62
P4	13	13	15	15	15	22 (2i)	22	22	34 (4e)	34	34	37 (10i, 9e)	37
P5	0	0	26	26	48	49	49	68	68	68	68 (10e)	68 (1i)	68
P6	0	0	27	27	48	48	48	70	70	78 (7e)	78	78	85 (2i, 8e)
P7	0	0	23	23	27	27	27	49	49	51 (11e)	51	51	51(1e)
P8	0	0	28	28	40	40	40	48 (9i)	48	58	58 (7i, 3e)	58	58
P9	0	0	14	14	31	31	31	39 (6i, 4e)	39	42 (13e)	42	42	49 (3i, 7e)
P10	0	0	17	17	37	37	37	49	49	49 (10e)	+		
Mean	1.3	1.3	18 8	20.4	32.4	33.4	35.4	46.8	48.3	52.0	52.4	56.6	58.6
SD	4.1	4.1	9.4	6.3	13.5	12.6	9.8	16.9	14.8	15.4	16.6	14.4	15.3
n	10	10	8	8	8	8	8	8	8	8	7	7	7

\+ Parental daphnid was found dead
( ) Number of immobilised young (i) or unhatched eggs (e) on the respective day (not cumulative)

Table 10 **Cumulative scores of living newborn per parent at 4.8 mg/l**

Parent	Day 7	Day 8	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 18	Day 19	Day 20	Day 21
P1	0	0	0	0 (18e)	0 (3e)	0 (3e)	0 (1i, 21e)	0	0	0 (23e)	0	0 (1i, 27*)	0
P2	0	0	0	0	0 (9e)	0 (18e)	0	0	0 (34e)	0	0	0 (11i, 14e)	0
P3	0	0	0	0 (17e)	0	0	0 (19e)	0	0	0 (24e)	0 (2e)	0 (2i, 18e)	0
P4	0	0	0	0	0 (18e)	0	0 (4e)	0 (45e)	0	0 (31e)	0 (7e)	0 (1i)	0
P5	0	0	0	0	0 (22e)	0	0	0 (24e)	0	0 (34e)	0	0 (1e)	0 (11e)
P6	0 (17e)	0	0	0	0 (4e)	0 (23e)	0	0	0 (43e)	0	0 (8e)	0 (5i, 16e)	0
P7	0	0	0	0	0	0 (2i, 25e)	0	0	0 (23e)	0 (10e)	0 (32e)	0	0
P8	0	0	0	0 (22e)	0 (2e)	0 (2e)	0 (17e)	0	0	0 (33e)	0 (1e)	0 (2i, 23e)	0
P9	0	0	0	0 (12e)	0	0 (1e)	0 (1i, 16e)	0	0	0 (28e)	0	0 (28e)	0 (1e)
P10	0 (10e)	0	+										
Mean	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
n	10	10	9	9	9	9	9	9	9	9	9	9	9

\+ Parental daphnid was found dead
( ) Number of immobilised young (i) or unhatched eggs (e) on the respective day (not cumulative)

APPENDIX 2 PARENTAL BODY LENGTH

Table 11 Individual values for body length of the surviving parental daphnids

Parent	Control (mm)	0.040 mg/l (mm)	0.096 mg/l (mm)	0.42 mg/l (mm)	1.7 mg/l (mm)	4.8 mg/l (mm)
P1	+	4.45	4.30	+	+	4.38
P2	4.45	4.30	4.22	4.38	+	4.14
P3	4.38	4.38	4.45	4.22	4.06	4.30
P4	4.14	4.22	4.45	4.45	4 38	4.14
P5	4.22	4.14	4.45	4.45	4.45	3.91
P6	4.45	+	4.53	4.45	4.38	4.22
P7	+	4.14	4.38	4.30	4.30	4.06
P8	4 30	4.30	4.45	4.30	4.30	4.22
P9	4.06	4.30	4.30	4.45	4.30	4.06
P10	4.45	4.38	4.45	4.53	+	+
P11	4.45					
P12	4.22					
P13	4.22					
P14	4.30					
P15	4.38					
P16	4.30					
P17	4.45					
P18	4.30					
P19	+					
P20	4.38					
Mean	4.32	4.29	4.40	4.39	4.31	4.16
SD	0.12	0.11	0.10	0.10	0.12	0.14
n	17	9	10	9	7	9

+ Parental daphnid was found dead

APPENDIX 3 STATISTICS: PARENTAL MORTALITY

Summary of Fisher's Exact Tests

GROUP	IDENTIFICATION	NUMBER EXPOSED	NUMBER DEAD	SIG 0.05
	CONTROL	20	3	
1	0.040	10	1	
2	0.096	10	0	
3	0.42	10	1	
4	1.7	10	3	
5	4.8	10	1	

APPENDIX 4 STATISTICS: REPRODUCTION

Chi-Square Test for Normality

Actual and Expected Frequencies

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	4.0870	14.7620	23.3020	14.7620	4.0870
OBSERVED	5	10	29	12	5

Chi-Square = 3.8542 (p-value = 0.4261)
Critical Chi-Square = 13.277 (alpha = 0.01 , df = 4)
= 9.488 (alpha = 0.05 , df = 4)

Data PASS normality test (alpha = 0.01). Continue analysis.

Levene's Test for Homogeneity of Variance

ANOVA Table

SOURCE	DF	SS	MS	F
Between	5	954.9890	190.9978	2.0135
Within (Error)	55	5217.3388	94.8607	
Total	60	6172.3279		

(p-value = 0.0910)

Critical F = 3.3700 (alpha = 0.01, df = 5,55)
= 2.3828 (alpha = 0.05, df = 5,55)
Since F < Critical F FAIL TO REJECT Ho: All equal (alpha = 0.01)

ANOVA Table

SOURCE	DF	SS	MS	F
Between	5	156065.2029	31213.0406	159.8602
Within (Error)	55	10738.8627	195.2520	
Total	60	166804.0656		

(p-value = 0.0000)

Critical F = 3.3700 (alpha = 0.01, df = 5,55)
= 2.3828 (alpha = 0.05, df = 5,55)
Since F > Critical F REJECT Ho: All equal (alpha = 0.05)

Bonferroni t-Test - TABLE 1 OF 2 Ho: Control-Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	t STAT	SIG 0.05
1	control	136.1765	136.1765		
2	0.040	131.5556	131.5556	0.8022	
3	0.096	137.9000	137.9000	0.3095	
4	0.42	135.2222	135.2222	0.1657	
5	1.7	58.5714	58.5714	12.3669	*
6	4.8	0.0000	0.0000	23.6409	*

Bonferroni t critical value = 2.6682 (2 Tailed, alpha = 0.05, df = 5,55)

Bonferroni t-Test - TABLE 2 OF 2 Ho: Control=Treatment

GROUP	IDENTIFICATION	NUM OF REPS	MIN SIG DIFF (IN ORIG. UNITS)	% OF CONTROL	DIFFERENCE FROM CONTROL
1	control	17			
2	0.040	9	15.3695	11.3	4.6209
3	0.096	10	14.8585	10.9	1.7235
4	0.42	9	15.3695	11.3	0.9542
5	1.7	7	16.7437	12.3	77.6050
6	4.8	9	15.3695	11.3	136.1765

APPENDIX 5 EC-VALUES

Concentration (mg/l)	X Log conc. (mg/l)	Y Reduction (%)
0.040	*	-1.3
0.040	*	21.4
0.040	*	4.5
0.040	*	8.9
0.040	*	13.3
0.040	*	-2.1
0.040	*	-13.1
0.040	*	-0.6
0.040	*	-0.6
0.096	*	-13.1
0.096	*	17.0
0.096	*	-5.0
0.096	*	11.1
0.096	*	-7.9
0.096	*	-9.4
0.096	*	17.8
0.096	*	-8.7
0.096	*	-11.6
0.096	*	-2.8
0.42	-0.377	-16.8
0.42	-0.377	18.5
0.42	-0.377	0.9
0.42	-0.377	8.9
0.42	-0.377	3.1
0.42	-0.377	-0.6
0.42	-0.377	-9.4
0.42	-0.377	6.7
0.42	-0.377	-5.0
1.7	0.230	54.5
1.7	0.230	72.8
1.7	0.230	50.1
1.7	0.230	37.6
1.7	0.230	62.5
1.7	0.230	57.4
1.7	0.230	64.0
4.8	0.681	100.0
4.8	0.681	100.0
4.8	0.681	100.0
4.8	0.681	100.0
4.8	0.681	100.0
4.8	0.681	100.0
4.8	0.681	100.0
4.8	0.681	100.0

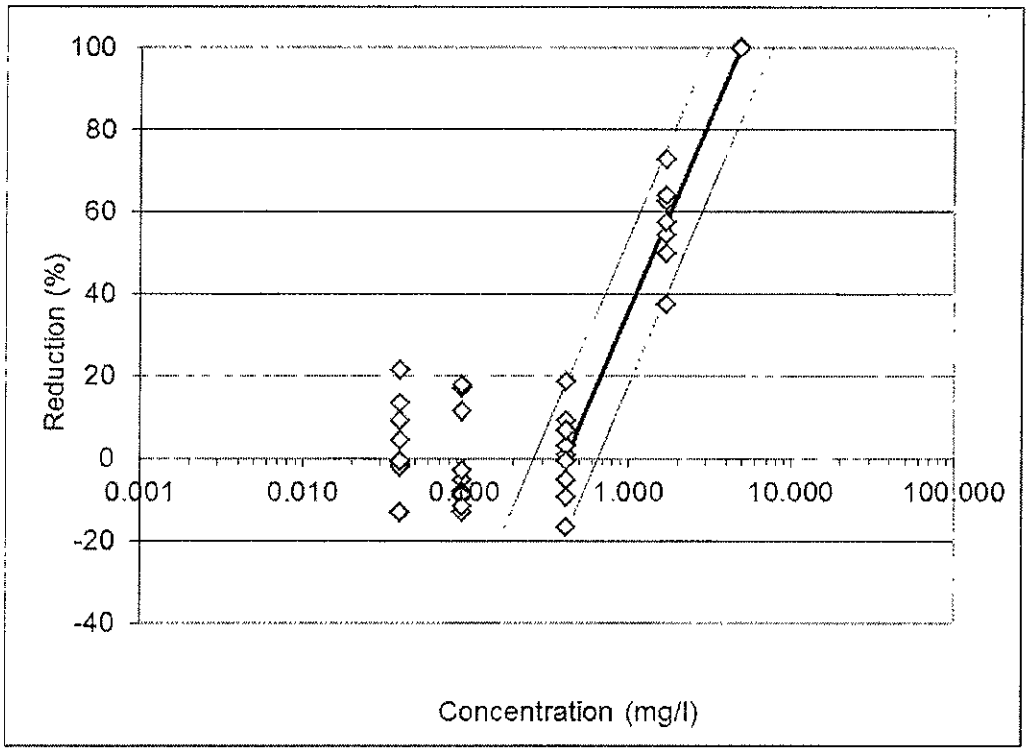
Slope:	93.8018
Intercept:	35.8742
Multiple R:	0.9822
n = number of observations	25

Regression line:
Y= 93.80 X + 35.87

Prediction of X values based on known Y values

Known Y Reduction (%)	10^{Xreg} (mg/l)	$10^{X95\%-}$ (mg/l)	$10^{X95\%+}$ (mg/l)
10	0.53	0.34	0.83
20	0.68	0.44	1.05
50	1.41	0.91	2.19

APPENDIX 5 EC-VALUES – continued –



APPENDIX 6 EXPERIMENTAL CONDITIONS

Table 12 pH values in freshly prepared media (F) and old media (O)

Nominal day	0	2	2	4	4	6	6	8	8	10	10	12	12
Group (mg/l)	F	O	F	O	F	O	F	O	F	O	F	O	F
control	8.0		7.9	8.1	7.9	8.0	7.9	7.9	8.0	7.8	8.0	7.9	7.9
0.040	8.0		7.9	8.1	7.9	8.1	7.9	7.9	8.0	7.9	8.0	7.9	7.9
0.096	8.0		7.9	8.2	7.9	8.1	7.9	7.9	8.0	7.8	8.0	7.9	7.9
0.42	8.1		7.9	8.1	7.9	8.0	7.9	7.9	8.0	7.8	7.9	7.9	7.9
1.7	8.1		7.9	8.1	7.9	8.0	7.9	7.9	8.0	7.8	7.9	7.9	7.9
4.8	8.1		7.9	8.1	7.9	8.1	7.9	7.9	8.0	8.0	7.9	8.0	8.0
Nominal day	14	14	16	16	18	18	20	20					
Group (mg/l)	O	F	O	F	O	F	O	F					
control	8.0	7.9	7.9	8.0	7.9	8.0	7.5	8.4					
0.040	8.0	8.0	7.8	7.9	7.8	8.0	7.5	8.4					
0.096	8.0	8.0	7.8	7.9	7.8	8.0	7.6	8.3					
0.42	8.0	8.0	7.7	8.0	7.8	8.0	7.6	8.3					
1.7	8.0	8.0	7.8	8.0	7.8	8.0	7.7	8.2					
4.8	8.1	8.0	7.8	8.0	7.8	8.0	7.8	8.2					

Table 13 Dissolved oxygen concentrations (mg/l) in freshly prepared media (F) and old media (O)

Nominal day	0	2	2	4	4	6	6	8	8	10	10	12	12
Group (mg/l)	F	O	F	O	F	O	F	O	F	O	F	O	F
control	9.1		8.8	9.2	9.0	9.2	9.4	8.7	9.0	8.4	9.1	8.6	9.2
0.040	9.0		8.9	9.2	9.0	9.4	9.3	8.6	9.0	8.6	9.1	8.8	9.1
0.096	9.0		8.8	9.4	9.0	9.5	9.3	8.6	9.0	8.7	9.1	8.8	9.1
0.42	8.9		8.9	9.1	8.9	9.4	9.3	8.6	9.0	8.5	9.1	8.8	9.0
1.7	8.8		8.9	9.0	9.0	9.4	9.3	8.7	9.0	8.5	9.0	8.9	9.1
4.8	8.7		9.0	9.0	9.0	9.7	9.3	8.7	9.0	9.0	9.0	9.1	9.0
Nominal day	14	14	16	16	18	18	20	20					
Group (mg/l)	O	F	O	F	O	F	O	F					
control	9.3	9.2	8.9	9.4	8.4	9.2	8.7	9.3					
0.040	9.2	9.3	8.4	9.4	8.3	9.2	8.5	9.3					
0.096	9.3	9.3	8.3	9.4	8.5	9.2	8.6	9.3					
0.42	9.3	9.2	8.5	9.4	8.3	9.2	8.7	9.2					
1.7	9.6	9.2	8.2	9.3	8.7	9.2	8.9	9.2					
4.8	9.7	9.1	8.5	9.2	8.6	9.1	9.0	9.1					

APPENDIX 6 EXPERIMENTAL CONDITIONS - continued -

Table 14 Temperature (°C) in freshly prepared media (F) and old media (O)

Nominal day	0	2	2	4	4	6	6	8	8	10	10	12	12
Group (mg/l)	F	O	F	O	F	O	F	O	F	O	F	O	F
control	19.8	19.6	19.7	19.8	19.6	20.0	19.9	19.9	19.9	19.9	19.7	19.5	19.8
0.040	19.9	19.8	19.8	19.6	19.5	20.0	19.9	19.9	19.9	19.9	19.8	19.8	19.9
0.096	19.9	19.8	19.8	19.5	19.4	20.0	19.9	19.9	19.9	19.9	19.8	19.8	19.9
0.42	20.0	19.8	19.8	19.5	19.4	20.0	19.9	20.0	19.9	19.9	19.9	19.9	19.8
1.7	20.0	19.9	20.0	19.5	19.5	20.0	20.0	20.0	19.9	19.9	20.0	19.8	20.0
4.8	20.2	20.0	20.5	19.5	19.6	20.0	20.1	20.0	20.0	19.9	20.1	19.9	20.2

Nominal day	14	14	16	16	18	18	20	20
Group (mg/l)	O	F	O	F	O	F	O	F
control	20.0	19.9	20.2	20.2	20.0	19.8	19.5	19.4
0.040	20.2	19.9	20.2	20.3	20.1	19.7	19.7	19.4
0.096	20.2	20.0	20.2	20.2	20.1	19.8	19.8	19.5
0.42	20.2	20.1	20.3	20.2	20.0	19.8	19.8	19.6
1.7	20.2	20.3	20.3	20.2	20.0	19.9	19.8	19.7
4.8	20.1	20.5	20.3	20.1	20.1	20.1	19.7	19.8

Table 15 Hardness (mg/l $CaCO_3$) in freshly prepared media (F) and old media (O)

Nominal day	4	4	12	12	20	20
Group (mg/l)	O	F	O	F	O	F
control	179	179	196	196	214	214
4.8	179	179	196	196	214	214

APPENDIX 7 ANALYTICAL REPORT

DETERMINATION OF THE CONCENTRATIONS

Author

Dr. Ir. E. Baltussen

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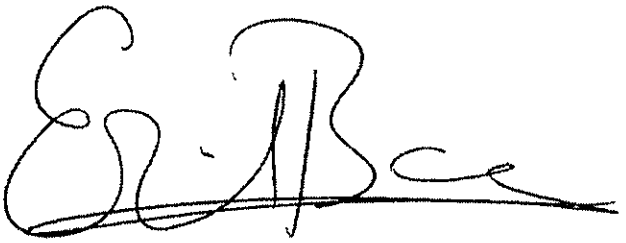
2. REPORT APPROVAL

NOTOX B.V.

Principal Scientist
Analytical Chemistry

E. Baltussen, PhD



Date: 12 March 2012

3. INTRODUCTION

3.1. Preface

Study plan analytical phase	Start	:	22 August 2011
	Completion	:	07 January 2012

3.2. Aim of the study

The purpose of the analytical phase was to determine the actual concentrations in samples taken from the test solutions used during the ecotoxicity test.

4. MATERIALS AND METHODS

4.1. Reagents

Water	Tap water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA)
Acetonitrile	Biosolve, Valkenswaard, The Netherlands
M7-medium	see main report

All reagents were of analytical grade, unless specified otherwise.

4.2. Samples

The samples were stored in the freezer (≤ -15°C). Storage stability of samples under these conditions was demonstrated in NOTOX project 496981.

On the day of analysis, the samples were defrosted at room temperature. The test samples were diluted in a 1:1 (v:v) ratio with 60/40 (v/v) acetonitrile/M7-medium and analysed. If necessary, the samples were further diluted with 30/70 (v/v) acetonitrile/M7-medium to obtain concentrations within the calibration range.

4.3. Analytical method

4.3.1. Analytical conditions

Quantitative analysis was based on the analytical method validated for the test substance in NOTOX project 496981.

Instrument	Alliance Separation Module 2695 (Waters, Milford, MA, USA)
Detector	Dual λ Absorbance Detector 2487 (Waters)
Column	Symmetry Shield RP-18, 100 mm × 4.6 mm i.d., dp = 3.5 µm (Waters)
Column temperature	40°C ± 1°C
Injection volume	100 µl
Mobile phase	30/70 (v/v) acetonitrile/water
Flow	1.0 ml/min
UV detection	253 nm

4.3.2. Preparation of the calibration solutions

Stock and spiking solutions
Stock solutions of the test substance were prepared in acetonitrile at concentrations of 2064 - 2572 mg/l.

Spiking solutions were made up from a stock solution and/or dilutions of this solution. The solvent of the spiking solutions was acetonitrile.

Calibration solutions
Calibration solutions in the concentration range of 0.0400 – 2.00 mg/l were prepared from two stock solutions. The end solution of the calibration solutions was 30/70 (v/v) acetonitrile/M7-medium.

Procedural recovery samples
2 ml blank medium was spiked with the test substance at a target concentration of 0.1 or 10 mg/l. The accuracy samples were treated similarly as the test samples (see paragraph 4.2 'Samples').

4.3.3. Sample injections

Calibration solutions were injected in duplicate. Test samples and procedural recovery samples were analysed by single injection.

4.4. Electronic data capture

System control, data acquisition and data processing were performed using the following programme:
- Empower version 7.00 (Waters, Milford, MA, USA).

Temperature and/or relative humidity during sample storage and/or performance of the studies were monitored continuously using the following programme:
- REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA).

4.5. Formulas

Response (R): Peak area test substance [units]

Calibration curve: $R = a\,C_N + b$

where:
C_N = nominal concentration [mg/l]
a = slope [units × l/mg]
b = intercept [units]

Analysed concentration (C_A): $C_A = \frac{(R-b)}{a} \times d$ [mg/l]

where:
d = dilution factor

Recovery: $\frac{C_A}{C_N} \times 100$ [%]

Relative to nominal concentration: $\frac{C_A}{C_N} \times 100$ [%]

Relative to initial concentration: $\frac{C_A\,(t = x\ \text{days})}{C_A\,(t = 0\ \text{days})} \times 100$ [%]

5. RESULTS

5.1. Calibration curves

Calibration curves were constructed using five concentrations. For each concentration, two responses were used. Linear regression analysis was performed using the least squares method with a 1/concentration^2 weighting factor. The coefficient of correlation (r) was > 0.99 for each curve.

5.2. Samples

5.2.1. Procedural recovery samples

The results for the procedural recovery samples are given in Table 1.

The mean recoveries of the procedural recovery samples fell within the criterion of 70-110% except for the high concentrations level on 29-09-11 where one of the duplicate results deviated above 110%. It was assumed that this was probably an outlier and since it occurred during the range-finding test it has no influence on the study. The procedural recovery samples demonstrated that the analytical method was adequate for the determination of the test substance in the test samples.

5.2.2. Test samples

The results for the test samples are given in Table 2 and Table 3.

6. TABLES

Table 1 Procedural recovery samples

Date of preparation [dd-mm-yy]	Date of analysis [dd-mm-yy]	Target concentration [mg/l]	Nominal concentration [mg/l]	Analysed concentration [mg/l]	Recovery [%]	Mean recovery [%]
29-09-11	29-09-11	0.1	0.100 0.100	0.102 0.105	102 105	104
29-09-11	29-09-11	10	10.0 10.0	12.1 10.9	121 109	115
17-12-11	17-12-11	0.1	0.100 0.100	0.0788 0.0820	79 82	80
17-12-11	17-12-11	10	10.0 10.0	8.44 7.98	84 80	82
06-01-12	06-01-12	0.1	0.101 0.101	0.0875 0.0480	86 47 [1]	n.a.
06-01-12	06-01-12	10	10.1 10.1	8.73 8.54	86 84	85

[1] This result was identified as an outlier based on a Dixon's Q-test.

Table 2 Concentrations of the test substance in test medium – preliminary test

Time of sampling [days]	Date of sampling [dd-mm-yy]	Date of analysis [1] [dd-mm-yy]	Percentage of WAF filtrate [2] [%]	Analysed concentration [mg/l]	Relative to initial [%]
0 (fresh)	20-09-11	29-09-11	1	0.811	
			10	7.34	
1 (old)	21-09-11	29-09-11	1	0.732	90
			10	6.33	86
			1 [3]	0.746	92
			10 [3]	6.55	89
1 (fresh)	21-09-11	29-09-11	1	0.646	
			10	6.60	
3 (old)	23-09-11	29-09-11	1	0.509	79
			10	5.21	79
			1 [3]	0.513	79
			10 [3]	5.28	80
3 (fresh)	23-09-11	29-09-11	1	0.966	
			10	7.16	
6 (old)	26-09-11	29-09-11	1	0.593	61
			10	4.85	68
			1 [3]	0.656	68
			10 [3]	5.14	72

[1] Samples were stored in the freezer (≤ -15°C) until the day of analysis.

[2] Percentage of a water accommodated fraction (WAF) prepared at a loading rate of 10 mg/l.

[3] *Without algae.*

Table 3 Concentrations of the test substance in test medium – reproduction test

Time of sampling [days]	Date of sampling [dd-mm-yy]	Date of analysis [1] [dd-mm-yy]	Percentage of WAF filtrate [2] [%]	Analysed concentration [3] [mg/l]	Relative to initial [%]
0 (fresh)	08-12-11	17-12-11	0	n.d.	
			1	< 0.0800	
			3.2	0.145	
			10	0.566	
			32	2.42	
			100	6.55	
2 (old)	10-12-11	17-12-11	0	n.d.	n.a.
			1	< 0.0800	n.a.
			3.2	0.122	84
			10	0.443	78
			32	1.92	79
			100	5.35	82
6 (fresh)	14-12-11	17-12-11	0	n.d.	
			1	< 0.0800	
			3.2	0.152	
			10	0.553	
			32	2.23	
			100	6.12	
8 (old)	16-12-11	17-12-11	0	n.d.	n.a.
			1	< 0.0800	n.a.
			3.2	0.110	72
			10	0.418	76
			32	1.74	78
			100	4.75	78
12 (fresh)	20-12-11	06-01-12	0	n.d.	
			1	< 0.0800	
			3.2	< 0.0800	
			10	0.390	
			32	1.45	
			100	4.16	
14 (old)	22-12-11	06-01-12	0	n.d.	n.a.
			1	< 0.0800	n.a.
			3.2	< 0.0800	n.a.
			10	0.244	63
			32	1.05	72
			100	2.89	70
18 (fresh)	26-12-11	06-01-12	0	n.d.	
			1	< 0.0800	
			3.2	0.172	
			10	0.502	
			32	1.91	
			100	5.23	

Table 3 Concentrations of the test substance in test medium – reproduction test (continued)

Time of sampling [days]	Date of sampling [dd-mm-yy]	Date of analysis [1] [dd-mm-yy]	Percentage of WAF filtrate [2] [%]	Analysed concentration [3] [mg/l]	Relative to initial [%]
20 (old)	28-12-11	06-01-12	0	n.d.	n.a.
			1	< 0.0800	n.a.
			3.2	< 0.0800	n.a.
			10	0.330	66
			32	1.27	67
			100	3.87	74

[1] Samples were stored in the freezer (≤ -15°C) until the day of analysis.

[2] Percentage of a water soluble fraction (WSF) prepared at a loading rate of 10 mg/l.

[3] In several samples test substance was detected at a concentration below the lowest calibration solution. These samples were reported as < 0.0800 mg/l (i.e. a concentration analysed lower than the lowest calibration standard taken the dilution factor of the samples (2) into account).

n.d. Not detected.

n.a. Not applicable.

REPORT

96-HOUR ACUTE TOXICITY STUDY IN CARP

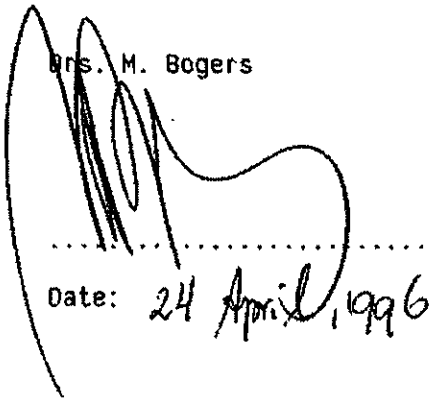
WITH

TK 12759, Araldite MY 0510

NOTOX Project 169548
CIBA test number 964623

REPORT APPROVAL

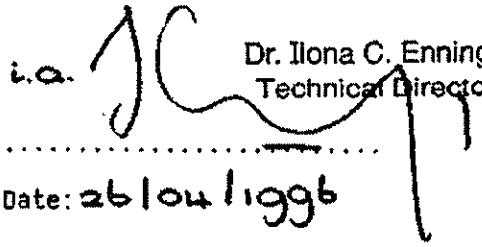
STUDY DIRECTOR: Drs. M. Bogers



Date: 24 April 1996

MANAGEMENT: Ing. E.J. van de Waart
Section Head, Genetic & Eco-Toxicology

i.a. Dr. Ilona C. Enninga
Technical Director



Date: 26/04/1996

SUMMARY

96-Hour Acute Toxicity Study in Carp with TK 12759, Araldite MY 0510.

The study procedures described in this report were based on the EEC directive 92/69, Part C.1. "Acute toxicity for fish"; and the OECD guideline No. 203: "Fish Acute Toxicity Test", Adopted 17 July, 1992.

The definitive test was performed with carp exposed to concentrations ranging from 1.0 to 10 mg/l in a static system. Acetone was used as a solvent in the stock solutions.

The 96h-LC50 for carp is 4.2 mg/l with 0 % mortality at 3.2 mg/l and 100 % mortality at 5.6 mg/l.

The BWZ of TK 12759, Araldite MY 0510 for acute toxicity in fish is 5.4.

PREFACE

Sponsor	Ciba-Geigy Ltd. Polymers Division R-1006.520 P.O. Box CH-4002 BASEL Switzerland
Study Monitor	Dr. E.G. Semadeni Additives Division R-1002.2.62
Testing Facility	NOTOX B.V. Hambakenwetering 3 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Drs. M. Bogers
Study Plan	Start : 25 March, 1996 Completion : 19 April, 1996

TEST SUBSTANCE (NOTOX Substance Code: 59652)

Identification	TK 12759, Araldite MY 0510
Description	Clear yellow liquid (Determined at NOTOX)
Batch	50008M
Purity	> 95%
Test substance storage	In freezer in the dark
Stability under storage conditions	Stable
Expiry date	May 01, 1998
Stability in vehicle	Water: at least 96 h Acetone: at least 96 h

PURPOSE

The purpose of the study was to evaluate the test substance for its ability to generate acute toxic effects in Cyprinus carpio during an exposure period of 96 hours.

GUIDELINES

The study procedures described in this report were based on the following guidelines:

European Economic Community (EEC), EEC directive 92/69, Part C: Methods for the determination of ecotoxicity, Publication No. L383, December 1992, C.1. "Acute toxicity for fish".

The OECD guidelines for Testing of Chemicals, guideline No. 203: "Fish Acute Toxicity Test", Adopted 17 July, 1992.

ARCHIVING

NOTOX B.V. will archive the following data for at least 10 years: report, and raw data.

DEFINITIONS

Fish were considered to be dead when no reaction was observed after touching the caudal peduncle and visible breathing movements were absent.

The LC50 is the concentration killing 50% of the fish after a defined period of exposure.

TEST SPECIES

Species	Carp (Cyprinus carpio, Teleostei, Cyprinidae) (Linnaeus, 1758)
Source	Zodiac, proefacc, "De Haar Vissen", L.U. Wageningen, the Netherlands.
Mean length	Range-finding test: 1.90 ± 0.27 cm Final test: 1.96 ± 0.10 cm
Mean weight	Range-finding test: 0.30 ± 0.07 g Final test: 0.21 ± 0.03 cm
Characteristics	F1 from a single parent-pair bred in UV-treated water.
Reason for selection	This system has been selected as an internationally accepted species.
Total fish used	79

HOLDING

Quarantine/Acclimation	At least 12 days after delivery.
Medium	ISO-medium, formulated using Milli-Ro water (tap-water purified by reverse osmosis; Millipore Corp., Bedford, Mass., USA) with the following composition: Ca^{2+} 80 mg/l Mg^{2+} 12 mg/l Na^{+} 15 mg/l K^{+} 3 mg/l Cl^{-} 145 mg/l SO_4^{2-} 49 mg/l HCO_3^{-} 47 mg/l Hardness is 250 mg $CaCO_3$/l
Measurements	Oxygen concentration, pH, nitrate and nitrite concentration and ammonia concentration: once a week. Temperature: every day.
Feeding	Daily with Trouvit.
Control of sensitivity	A reference test with pentachlorophenol (PCP, SIGMA) is carried out within a period of 3 months. The results of this test are appended to the report.
Validity of batch	In the batch of fish used for the test, mortality during the seven days prior to the start of the test was less than 5%.

RANGE-FINDING TEST

A range-finding test was performed to provide information about the range of concentrations to be used in the final test:
three fish per concentration were exposed to a concentration range of 0.1 to 10 mg/l with an increasing factor of 10, while seven fish were exposed to 100 mg/l and the blank and solvent control.

FINAL STUDY: TEST PROCEDURE AND CONDITIONS

Test duration	96 hours
Test type	Static
Test vessels	5 litres, all-glass.
Test medium	ISO-medium, aerated until the dissolved oxygen concentration had reached saturation and the pH had stabilized. After aeration the hardness was 250 mg $CaCO_3$ per litre and the pH was 8.0 ± 0.2.
Number of fish	7 fish per concentration and controls.
Loading	0.74 g fish/litre, i.e. 7 fish per 2 litres of test medium.
Illumination	16 hours photoperiod daily
Aeration	The test media were aerated continuously.
Feeding	No feeding from 24 hours prior to the test and during the total test period.
Introduction of fish	Directly after preparation of the test media.
Euthanasia	At the end of the test the surviving fish were rapidly killed by exposing them to ca. 1.2% ethylene glycol monophenylether in water.

PREPARATION OF TEST SOLUTIONS

Stock solutions were prepared in acetone at 100, 56, 32, 18 and 10 mg/ml. A volume of 0.2 ml was taken from each stock and mixed with 2 litres of test medium providing the test concentrations of 10, 5.6, 3.2, 1.8 and 1.0 mg/l. The final test solutions were all clear without precipitation.

MEASUREMENTS AND RECORDINGS

Mortality and other effects	At (2), 24, 48, 72 and 96 hours following the start of exposure. Dead fish were removed when observed.
Dissolved oxygen content, pH and Temperature	Daily, beginning at the start of the test.

RESULTS

Range-finding test:

Incidence and total mortality:

Concentration (mg/l)	Initial number of fish	Cumulative mortality				Total Mortality (%)
		24 h	48 h	72 h	96 h	
Blank-control	7	0	0	0	0	0
Solvent-control	7	0	0	0	0	0
0.1	3	0	0	0	0	0
1	3	0	0	0	0	0
10	3	0	3	3	3	100
100	7	7	7	7	7	100

Definitive study:

Incidence and total mortality:

Concentration (mg/l)	Initial number of fish	Cumulative mortality				Total Mortality (%)
		24 h	48 h	72 h	96 h	
Blank-control	7	0	0	0	0	0
Solvent-control	7	0	0	0	0	0
1.0	7	0	0	0	0	0
1.8	7	0	0	0	0	0
3.2	7	0	0	0	0	0
5.6	7	0	3 *	7	7	100
10	7	0	7	7	7	100

* Surviving fish showed severe disturbance of swimming behaviour.

pH, dissolved oxygen and temperature:

Concentration (mg/l)	Day 0		Day 1		Day 2		Day 3		Day 4	
	pH	DO	pH	DO	pH	DO	pH	DO	pH	DO
Blank-control	8.1	9.1	8.0	9.0	7.8	8.8	7.9	8.9	7.9	8.9
Solvent-control	8.1	9.0	8.0	8.8	7.8	8.7	7.9	8.8	7.8	8.8
1.0	8.1	9.0	8.0	8.8	7.8	8.8	7.8	8.9	7.8	8.6
1.8	8.0	9.2	8.0	8.8	7.8	9.0	7.8	8.9	7.7	8.8
3.2	8.0	9.1	7.9	8.7	7.8	8.8	7.9	8.9	7.6	8.7
5.6	8.0	9.1	8.0	8.9	7.8	8.9	8.0	8.9		
10	8.0	9.2	8.0	8.8						
Temperature	20.5		20.4		20.5		20.5		20.4	

CONCLUSION

TK 12759, Araldite MY 0510:

The 96h-LC50 for carp is 4.2 mg/l with 0 % mortality at 3.2 mg/l and 100 % mortality at 5.6 mg/l.

PS + R, PO 2.1 Eing. 6.5.96	Erled.	Kopie
J. Weiss		✓
Ablage		

REPORT

PSEUDOMONAS CELL MULTIPLICATION INHIBITION TEST

WITH

TK 12759, ARALDITE MY 0510

NOTOX Project 169706
Ciba Project 964624
NOTOX Substance 59652

TK 12759, ARALDITE MY 0510

NOTOX Project 169706
Ciba Project 964624

REPORT APPROVAL

STUDY DIRECTOR: Ir. Y.H.M. van Erp

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Date: May 22, 1996

MANAGEMENT: Ing. E.J. van de Waart
Section Head, Genetic &
Eco-Toxicology

.....

Date: 23/05/1996

SUMMARY

TK 12759, ARALDITE MY 0510 was investigated for its ability to inhibit the cell multiplication of the bacteria species Pseudomonas putida according to DIN 38412 Part L 8 (March 1991) and NEN-EN-ISO 10712 (1996).

Suspensions of Pseudomonas putida were exposed to concentrations ranging from 312.5 to 10,000 µg TK 12759, ARALDITE MY 0510 per litre. Acetone was used as vehicle.
After incubation the extinction values of the bacterial cell suspensions were measured. No toxicity was observed.

Based on the results of the test presently performed, the toxicity threshold value (EC10) of TK 12759, ARALDITE MY 0510 was over 10 mg/l. The "Bewertungszahl" (Assessment figure or value for bacterial toxicity) is therefore $\leq$ 4.9 (Bewertung wassergefährdender Stoffe, 1989).

The EC50 value for the reference substance, 3,5-dichlorophenol, tested during the same experiment was 20 mg/l. Since this was within the accepted range of 10-30 mg/l for the EC50 and the multiplication factor of the bacteria within the test was over 60, it was concluded that the test conditions were optimal and the results obtained were valid.

PREFACE

Sponsor	Ciba-Geigy Ltd. Polymers Division Bau R-1002.2.60 CH-4002 BASEL Switzerland
Study Monitor	Dr. E.G. Semadeni
Testing Facility	NOTOX B.V. Hambakenwetering 3 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Ir. Y.H.M. van Erp
Technical Coordinator	A.M.C. Bertens
Study Plan	Start : March 28, 1996 Completed : March 29, 1996

TEST SUBSTANCE

Identification	TK 12759, Araldite MY 0510
Description	Clear yellow liquid (Determined at NOTOX)
Batch	50008M
Purity	> 95%
Test substance storage	In freezer in the dark
Stability under storage conditions	Stable
Expiry date	May 01, 1998
Stability in vehicle	Water : at least 96 h Acetone: at least 96 h

GUIDELINES

The study procedure described in this report was based on the following guidelines:

DIN 38412 Part L 8, March 1991: Determination of the Inhibition Effect of Water Constituents on Bacteria (Pseudomonas Cell Multiplication Inhibition Test) with minor modifications.

NEN-EN-ISO 10712, 1996: Water quality - Pseudomonas putida growth inhibition test (Pseudomonas cell multiplication inhibition test).

OBJECTIVE

Purpose of the study

The objective of this study is to evaluate the test substance for its ability to inhibit the cell multiplication of the bacterial species Pseudomonas putida. Furthermore, the toxicity threshold value (EC10) and EC50 value (if possible) of the test substance for Pseudomonas putida is determined.

Pseudomonas putida, an aerobic gram-negative bacterial species from the genus Pseudomonas, is a natural inhabitant of surface waters. This bacterium is a model water organism involved in biological self-purification. The inhibition of multiplication of bacterial cells of Pseudomonas putida is the selected parameter for dissolved toxic water ingredients.

DEFINITIONS

Effective concentration (EC10, EC50): the concentration of test substance determined after a test period of 16 ± 1 hour from the concentration-effect relationship at which cell multiplication is inhibited by 10 % or 50 % compared to that of the control. EC is expressed in mg test substance per litre.

Formazine nephelometric unit (FNU): Formazine turbidity units. The optical density of a bacterial suspension at λ=436 nm, measured as FNU.

Biomass: culture of bacterial cells after a certain time, expressed as FNU.

"Bewertungszahl": Assessment figure or value for bacterial toxicity expressed as the negative logarithmus of the EC10.
Example: EC10: 150 mg/l = 150 ppm = $1.5 \cdot 10^{-4}$ = Bewertungszahl 3.8.

TEST SYSTEM

Test system	Pseudomonas putida.
Strain	MIGULA, Berlin 33/2 (DSM 50026).
Source	BGA, Berlin, Germany.
Rationale	Recognized by international guidelines.

Stock cultures of the bacteria are stored in liquid nitrogen (-196°C).

TEST SUBSTANCE PREPARATION

Since the test substance was poorly soluble in water and no homogeneous suspension in water could be obtained, a stock solution (A) of 100 g per litre in acetone was prepared. Thorough mixing was performed to enhance the dissolving of the test substance. Solution A was diluted 1:1 with acetone (stock B, 50 g/l). In the same way stock C (25 g/l) to stock F (3.1 mg/l) were prepared.

REFERENCE SUBSTANCE

3,5 - Dichlorophenol (97 %, Aldrich).
A stock solution of 100 mg 3,5-dichlorophenol per litre in Milli-Q water was prepared.

EXPERIMENTAL CONDITIONS AND SOLUTIONS

Vessels	100 ml Erlenmeyer flasks
Incubation temperature	23 ± 1°C
Incubation time	
- preliminary suspension cultures	6 ± 1 hours
- exposure	16 ± 2 hours
Stock solution I	10.0 g $NaNO_3$ 2.4 g K_2HPO_4 1.2 g KH_2PO_4 1.0 g yeast extract Dissolved in 500 ml Milli-Q water.
Stock solution II	10.0 g $NaNO_3$ 2.4 g K_2HPO_4 1.2 g KH_2PO_4 Dissolved in 500 ml Milli-Q water.
Stock solution III	40.0 g D(+)- glucose Dissolved in 500 ml Milli-Q water.
Stock solution IV	0.01 g Fe (III) citrate 4.0 g $MgSO_4 . 7H_2O$ Dissolved in 1 l Milli-Q water.
Culture medium (stock)	Nutrient medium: 18 g of agar dissolved in water, plus 50 ml of solution I, 125 ml of solution III and 100 ml of solution IV, made up to one litre with water.

PERFORMANCE OF THE TEST

Preliminary suspension cultures

A small amount of bacteria from a maximally 7-day old stock culture of Pseudomonas putida was inoculated in pre-culture medium (25 ml of stock solution I and III plus 50 ml of solution IV, made up to one litre with water) in Erlenmeyer flasks. After incubation the final turbidity value of the bacterial suspension was adjusted up to FNU/436 nm = 50.

Exposure

Three parallel dilution series in 100 ml Erlenmeyer flasks covered with aluminium caps were prepared from the formulated test substance stock solution as described below.

Conc. test subst. (µg/l)	Milli-Q° water (ml)	Test solution[1] (µl of stock)		Stock solution II (ml)	III (ml)	IV (ml)	Bacterial suspension FNU/436=50 (ml)	total volume (ml)
10,000	32	4	A	1	1	2	4	40[2]
5,000	32	4	B	1	1	2	4	40
2,500	32	4	C	1	1	2	4	40
1,250	32	4	D	1	1	2	4	40
625	32	4	E	1	1	2	4	40
312	32	4	F	1	1	2	4	40
0[3]	32	4	acetone	1	1	2	4	40

° = Tap water purified by reverse osmosis and subsequently passed over activated carbon and ion-exchange cartridges (Millipore Corp., Bedford, Mass., USA).
[1] = Final volume of acetone was 0.01%.
[2] = pH of one of the test vessels was 8.1.
[3] = 10 control flasks were prepared.

Six flasks were made up identically as above, only 4 ml of pre-culture medium was added instead of the bacterial suspension (turbidity control).

The series of the reference substance were prepared by direct dilutions from the stock solution as follows:

Conc. reference subst. (mg/l)	Milli-Q water (ml)	Reference stock sol. (ml)	Stock solution II (ml)	III (ml)	IV (ml)	Bacterial suspension FNU/436=50 (ml)	total volume (ml)
40	16.0	16.0	1	1	2	4	40
28	20.8	11.2	1	1	2	4	40
20	24.0	8.0	1	1	2	4	40
14	26.4	5.6	1	1	2	4	40
10	28.0	4.0	1	1	2	4	40
7	29.2	2.8	1	1	2	4	40

After an appropriate time in an incubator shaker the turbidity values of the cell suspensions (biomasses) were measured.

MEASUREMENTS

Equipment	UV/VIS Spectrophotometer, Lambda 5 (Perkin Elmer, the Netherlands), λ = 436 nm; 10 mm layer
Calibration solution	Formazin standard suspension (in Formazine Turbidity Units) : FNU/436 nm = 100 FNU/436 nm = 75 FNU/436 nm = 50 FNU/436 nm = 25 FNU/436 nm = 12
Turbidity value bacterial suspension	FNU/436 nm = 50

CALCULATIONS

The percentage of cell multiplication inhibition for each tested concentration was calculated as follows:

$$I = \frac{Bc - Bn}{Bc - Bo} * 100$$

I Cell multiplication inhibition in %
Bn Measured biomass (extinction - extinction of turbidity control) at the end of the test period for the n^{th} concentration of the test material
Bc Measured biomass (extinction) at the end of the test period in the control batch
Bo Measured biomass (extinction) at the start of the test (t=0) in the control batch.

The inhibition values calculated for each concentration were plotted against the corresponding concentration of the test substance.

ACCEPTABILITY OF THE TEST

The EC50 value of the reference substance 3,5-dichlorophenol should be in the accepted range of 10-30 mg/l.

The inoculum used in the control batch should be multiplied by a factor of at least 60 within the test period.

The preceding criteria are not absolute and other modifying factors might enter into the final evaluation decision.

RESULTS

Bacterial toxicity

After incubation extinction values were measured and the cell multiplication inhibition for each test concentration was determined (see Table 1).

Since no significant (significant: >10%) inhibition of cell multiplication was observed the toxicity threshold value of TK 12759, ARALDITE MY 0510 for Pseudomonas putida was over 10 mg/l.

Acceptability of the test

The EC50 value of 3,5-dichlorophenol determined for Pseudomonas putida as calculated by linear regression (Y = 6.8169 X -83.9824) was 20 mg/l (see Table 2).

The inoculum used in the control batch was multiplied by a factor 77 within the test period.

With respect to the accepted range of 10-30 mg/l for the EC50 of the reference substance and the multiplication factor of the bacteria within the test being over 60, it was concluded that the test conditions were optimal and the results obtained were valid.

CONCLUSION

Based on the results of the test presently performed, the toxicity threshold value (EC10) of TK 12759, ARALDITE MY 0510 was over 10,000 mg/l. The "Bewertungszahl" is therefore $\leq$ 4.9 (Bewertung wassergefährdender Stoffe, 1989).

TABLE 1

Extinction values of the inoculated dilution series I, II, III, the non-inoculated dilution series t (turbidity control) and the control flasks B.

CONCENTRATION of TK 12759, ARALDITE MY 0510 (µg/l)	EXTINCTION IN FNU 436 nm series I	series II	series III	MEAN I,II,II	t	CORRECTED MEAN	INHIBITION %
312.5	1.291	1.219	1.292	1.267	0.004	1.263	2.8
625	1.259	1.356	1.248	1.288	0.001	1.287	1.0
1250	1.231	1.201	1.173	1.202	0.001	1.201	7.7
2500	1.231	1.181	1.254	1.222	0.001	1.221	6.1
5000	1.291	1.394	1.294	1.326	0.001	1.325	-2.0
10000	1.172	1.283	1.222	1.226	0.002	1.224	5.9
CONTROL 1.320	1.175	1.348	1.201	1.349	1.251	1.410	1.376	1.297	1.265
MEAN 1.299									
BO 0.0168									

TABLE 2

Extinction values of the reference substance dilution series: 3,5-dichlorophenol.

CONCENTRATION 3,5-dichlorophenol (mg/l)	EXTINCTION IN FNU 436 nm series r	INHIBITION %
7	1.324	-1.9
10	1.264	2.7
14	1.269	2.4
20	0.424	68.2
28	0.015	100.1
40	0.016	100.0

PS + R, PO 2.1 Eing. 3.6.96	Erled.	Kopie
J. Weiss		✓
Ablage		