Final Report

Original 2 of 2

Determination of short term toxicity of
(9Z)-9-Octadecenoic acid - N-
cyclohexylcyclohexanamine (1:1)
against *Daphnia magna* STRAUS
according to OECD 202 resp. EU C.2

Study No.: 16020502G201

Sponsor:
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89077 Ulm
Germany

Monitor:
Dr. Ben Müller-Zermini

Test Facility:
LAUS GmbH
Auf der Schafweide 20
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Germany

Study Director:
Manfred Muckle

1 GLP-Compliance Statement

It is hereby declared that all tests were made in accordance with the „Revised OECD Principles of Good Laboratory Practice" (Paris, 1997) as stated in the following guidelines:

- OECD Principles of Good Laboratory Practice, adopted by Council on 26th November 1997; Environment Directorate, Organisation for Economic Cooperation and Development, Paris 1998
- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version)
- Chemicals Act of the Federal Republic of Germany (ChemG) §19a and §19b and annexes 1 and 2 from 28. Aug. 2013, published in Federal Law Gazette, Germany (BGBl) No. 55/2013 as of 06. Sep. 2013, and further revisions.

Responsibility for the accuracy of the information concerning the test item as well as for its authenticity rests with the sponsor.

I herewith accept responsibility for the data presented within this report.

There were no circumstances that may have affected the quality or integrity of the study.

Manfred Muckle
Study Director

22 JUL 2016
Date

Information on Study Organisation:

Deputy Study Director	Uli Bangert
Study Plan dated	19. May 2016
Experimental Starting Date	06. Jun. 2016
Experimental Completion Date	09. Jun. 2016

2 QUALITY ASSURANCE UNIT STATEMENT

This study has been inspected by the quality assurance unit according to the principles of Good Laboratory Practice. Study Plan and Final Report were checked at the dates given below, the Study Director and the management were informed with the corresponding report.

Also, the performance of the study was inspected, and findings were reported to Study Director and management. The inspection of short-term studies (duration less than four weeks) is carried out as audit of process concerning major technical phases of at least one similar test. Frequency is once or more a quarter.

The study was conducted and the reports were written in accordance with the Study Plan and the Standard Operating Procedures of the test facility.

Deviations from the Study Plan were acknowledged and assessed by the Study Director and included in the Final Report.

The reported results reflect the raw data of the study.

Verified Procedure	Inspected on	Findings reported on	Audit report no.
Study plan	13. May 2016	13. May 2016	160513-10
Performance of study	07. Jun. 2016	07. Jun. 2016	160607-03
Draft report	08. Jul. 2016	08. Jul. 2016	160708-08
Final report	22. Jul. 2016	22. Jul. 2016	160722-04

Revina-Rosa Resch
Quality Assurance

22 JUL 2016
Date

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3 SUMMARY

Title of Study: Determination of short term toxicity of (9Z)-9-Octadecenoic acid - N-cyclohexylcyclohexanamine (1:1) against *Daphnia magna* STRAUS according to OECD 202 resp. EU C.2

Findings and Results:

One valid experiment was performed.

The study was performed using 5 concentrations ranging from 4.6 to 100 mg/L. For each test concentration, 20 Daphnia were exposed to the test item for 48 hours in a static test system. After 24 and 48 hours, the immobilised Daphnia were counted.

Only the highest concentrated treatment 100 mg/L showed toxicity of 90% immobilisation. None of the animals were immobilised in the lower concentrated treatments and in the blank control.

Potassium dichromate $K_2Cr_2O_7$ (CAS No. 7778-50-9) was used as positive control in a current reference study. The 24h-EC50 value was determined as 1.6 mg/L, lying within the demanded range of 0.6 – 1.7 mg/L stated in the guideline.

At the beginning and at the end of the test, the content of the test item in the test solutions was determined using GC/FID-determination. The measured concentrations were in range of 82 - 114 % of the nominal concentration. Therefore, the determination of the biological results was based on the nominal concentrations.

The following results were determined for the test item (9Z)-9-Octadecenoic acid - N-cyclohexylcyclohexanamine (1:1) (species: *Daphnia magna*).

48h-NOEC = 46 mg/L
48h-LOEC = 100 mg/L
24h-EC50 > 100 mg/L
48h-EC50 = 85 mg/L

4 PURPOSE AND PRINCIPLE OF THE STUDY

This study was performed in order to evaluate the toxic potential of (9Z)-9-Octadecenoic acid - N-cyclohexylcyclohexanamine (1:1) towards freshwater flea, using the species *Daphnia magna* STRAUS, which was chosen in the guideline as a typical part of zooplankton.

Young Daphnia, aged less than 24 hours at the start of the test, were exposed to the test item at a range of concentrations for a period of 48 hours. Immobilisation was recorded after 24 hours and 48 hours and compared with control values. The results were analysed in order to calculate the EC50 at 48h and 24h.

A positive control was tested for EC50 determination as a means of assuring that the test conditions are reliable.

5 LITERATURE

The study was conducted in accordance with the following guidelines:

- OECD Guideline for Testing of Chemicals No. 202, adopted 13. Apr. 2004: ”Daphnia sp., Acute Immobilisation Test“
- Commission Regulation (EC) No. 440/2008, Method C.2. “Daphnia sp. Acute Immobilisation Test”, adopted 30. May 2008
- OECD guidance document no. 23, GUIDANCE DOCUMENT ON AQUATIC TOXICITY TESTING OF DIFFICULT SUBSTANCES AND MIXTURES, adopted 14. Dec. 2000
-

Corresponding SOP of LAUS GmbH:

- SOP 118 002 01 “Bestimmung der akuten Toxizität von Substanzen gegenüber *Daphnia magna* STRAUS“ version 12, valid from 04. Nov. 2013.

6 MATERIALS AND METHODS

6.1 Test Item

Designation in Test Facility:	16020502G
Date of Receipt:	05. Feb. 2016
Condition at Receipt	Room temperature, in proper conditions

6.1.1 Specification

The following information concerning identity and composition of the test item was provided by the sponsor.

Name	(9Z)-9-Octadecenoic acid - N-cyclohexylcyclohexanamine (1:1)
Batch no.	LV 16-026
Appearance	yellow fluid
Composition	100% (9Z)-9-Octadecenoic acid - N-cyclohexylcyclohexanamine (1:1)
CAS No.	22256-71-9
EINECS-No.	244-876-4
Molecular formula	$C_{12}H_{24}N$ $C_{18}H_{33}O_2$
Molecular weight	463.8 g/mol
Purity	100% (gas chromatography)
Homogeneity	homogeneous
Volatility	unknown
Stability	H_2O: unknown; EtOH: not stated; acetone: not stated; CH_3CN: not stated; DMSO: not stated
Solubility	H_2O: 0.1-1g/L; EtOH: unknown; acetone: unknown CH_3CN: unknown; DMSO: unknown
Production date	02. Feb. 2016
Expiry date	02. Feb. 2017
Storage	Room Temperature (20 ± 5°C)

6.1.2 Storage

The test item was stored in a closed vessel dark and dry at room temperature (15.2 – 23.3 °C.

6.1.3 Preparation

A saturated solution was prepared for the test. This was done by weighing the nominal load 100 mg/L (real load 100.8 mg/L) adding the corresponding amount of dilution water and shaking vigorously for 24 hours. The resulting solution was filtrated through 0.45 µm nylon filters.

6.2 Positive Control

Potassium dichromate $K_2Cr_2O_7$ (CAS No. 7778-50-9) was used as positive control in a current reference study (15102901R201). The 24h-EC50 value was determined as 1.6 mg/L, lying within the demanded range of 0.6 – 1.7 mg/L stated in the guideline.

6.3 Test System

6.3.1 Specification

Species	*Daphnia magna*
Variety	STRAUS
Strain	Berlin
Sex	female
Age	between 0 and 24 hours
Origin	Umweltbundesamt Berlin
In-house breeding since	27. September 2007

Selection of the test system was made following the proposal of the guidelines.

6.3.2 Animal Husbandry

Daphnia magna is bred in the LAUS GmbH throughout the year. The animals are kept for the use in toxicity tests. They multiply by parthenogenesis, thus being genetically identical. The husbandry is performed similar to the method described in the OECD guideline, following SOP 115 002 01 („Zucht und Hälterung von *Daphnia magna* STRAUS“), Version 12.

Vessels	preserving glasses, nominal volume 2 L
Medium	M4-Medium (recipe of ELENDT), composition see Annex 2: Composition of M4-Medium, page 19
Food	unicellular green algae (*Desmodesmus subspicatus*)
Medium renewal	twice a week
Photo period	16/8 hours, using neon tubes
Temperature	20 ± 2 °C

6.4 Dilution Water

The dilution water stated below was used as daphnia medium during the test.

Deionised water with an enrichment of certain minerals (as demanded in the guidelines) is used in the test.

Table 6.4-a Dilution water specification

Parameter	Concentration in mg/L
$CaCl_2*2H_2O$	293.80
$MgSO_4*7H_2O$	123.30
$NaHCO_3$	64.80
KCl	5.80
Resulting hardness in mmol/L:	2.502
Resulting hardness in mg $CaCO_3$/L:	250

The real weighted loads are stated in the raw data.
Deviations from the nominal weighted loads were less than 5%.

6.5 Test Vessels

Beakers, glass, nominal volume 100 mL.

6.6 Instruments and Devices

The following instruments and devices were used in the performance of the study:

- Data logger for temperature
- Analytical scales Mettler Toledo XS 205 DU
- Precision scales Mettler Toledo XS6001S
- Adjustable pipettes with one-way tips, Mettler Toledo, Rainin
- Automatic repeater pipettes with one-way tips, Mettler Toledo, Rainin
- Glass measuring cylinders
- Glass measuring flasks
- pH-meter 340i wtw
- Oxygen meter Oxical 340i wtw
- Orbital Shaker GFL
- GC hp 6890

Usage and, if applicable, calibration of all instruments followed the corresponding SOP in the current edition.

Standard laboratory equipment (glassware) was also used.

6.7 Analytical Method

A GC/FID-Method for the determination of (9Z)-9-Octadecenoic acid - N-cyclohexyl-cyclohexanamine (1:1) via determination dicyclohexylamine was validated. For the calibration, a dilution series in iso-hexane was prepared, using a stock solution. The GC-method was validated for the parameters specificity, stability, linearity, precision and limit of detection and quantification. Accuracy was tested in Daphnia test medium.

The method is fully described in validation report VB16020502G926. This document and the corresponding raw data is archived following GLP regulations.

Before the start of the test, a new calibration was performed. When measuring the samples from this study on day 2, validity of calibration was controlled by measuring of a QC sample at the concentration 30 mg/L. The measured concentration was 98 % of the nominal concentration.

All data are summarised in the following table:

Table 6.7-a Validation Parameters determined during method validation (LAUS study No. 16020502G926)

Parameter	Value	Unit
Linear Calibration Range (nominal)	5 – 70	mg/L
Slope	0.020879	1 / mg/L
Intercept y-axis	-0.016920	
Method variation coefficient (Precision)	1.51	%
Correlation coefficient r	0.999776	
Specificity	was given	-
Accuracy in Daphnia Test Medium (Conc. 4.5 mg/L)	75.9	%
Accuracy in Daphnia Test Medium (Conc. 46 mg/L)	85.0	%
Accuracy in Daphnia Test Medium (Conc. 100 mg/L)	95.8	%
Accuracy in Daphnia Test Medium (Conc. 4.5 - 46 mg/L)	80.5	%
Stability in Medium after 2 d (conc. 4.6 mg/L)	95.2	%
Stability in Medium after 2 d (conc. 100 mg/L)	94.6	%
LOD/LOQ	5.0	mg/L
LOD/LOQ*	0.5*	mg/L

*** taking into account the tenfold enrichment of the samples**

Table 6.7-b Parameters Calibration from 07. Jun. 2016

Parameter	Value	Unit
Linear Calibration Range (nominal)	5 – 70	mg/L
Slope	0.021835834	1 / mg/L
Intercept y-axis	-0.084049448	
Method variation coefficient (Precision)	1.98	%
Correlation coefficient r	0.999613587	

6.7.1 Sample Preparation (control, 4.6 mg/L and 10 mg/L nominal concentration)

To 100 mL test solution, 1 mL 2 M NaOH solution (for hydrolysis of ester) and 7 g NaCl was added, then, the solution was extracted two times with the solvent iso-hexane (2 * 4 mL), the organic phase was collected into a 10 mL flask, after drying with Na_2SO_4. The flask was filled up to 10 mL with iso-hexane after addition of 0.5 mL ISTD solution (1000 mg/L) and the solution was measured via GC/FID. Tenfold enrichment was achieved during the sample preparation.

6.7.2 Sample Preparation (22 mg/L and 46 mg/L nominal concentration)

To 50 mL demineralized water and 50 mL test solution, 1 mL 2 M NaOH solution (for hydrolysis of ester) and 7 g NaCl was added, then, the solution was extracted two times with the solvent iso-hexane (2 * 4 mL), the organic phase was collected into a 10 mL flask, after drying with Na_2SO_4. The flask was filled up to 10 mL with iso-hexane after addition of 0.5 mL ISTD solution (1000 mg/L) and the solution was measured via GC/FID. Fivefold enrichment was achieved during the sample preparation.

6.7.3 Sample Preparation (100 mg/L nominal concentration)

To 80 mL demineralized water and 20 mL test solution, 1 mL 2 M NaOH solution (for hydrolysis of ester) and 7 g NaCl was added, then, the solution was extracted two times with the solvent iso-hexane (2 * 4 mL), the organic phase was collected into a 10 mL flask, after drying with Na_2SO_4. The flask was filled up to 10 mL with iso-hexane after addition of 0.5 mL ISTD solution (1000 mg/L) and the solution was measured via GC/FID. Twofold enrichment was achieved during the sample preparation.

7 CONDUCT OF THE STUDY

7.1 Selection of Daphnia

19 hours before the start of the test, the adult animals were separated from the young. 0.5 hours before test start, the adults were caught with the help of a glass tube, and the newborn Daphnia, aged between 0 and 18 hours, were sieved from the medium and immediately placed into a 250 mL-beaker containing dilution water. After a settling-in period of 30 minutes, animals which showed no apparent damage were used for the test.

Switching from M4-medium (husbandry) to Dilution water (test) has been shown not to cause any detrimental effects for test Daphnia.

7.2 Study Performance

Using a glass tube, the Daphnia were caught and lifted from the beaker. They were put on a small sieve, and the medium surrounding the animals was sucked off using absorbent paper. Immediately after that, the animals were put into the respective test solution.

The test vessels were left to stand for 48 hours. After 24 and 48 hours, the immobilised Daphnia were counted. Daphnia are considered immobilised if they perform no movements or are only able to move their antennae when the beaker is gently agitated. Daphnia which are trapped at the surface of the test solution are also considered immobilised.

The pH, the concentration of dissolved oxygen and the content of the test item in the test vessels were measured at the beginning and at the end of the test.

7.3 Experimental Conditions

Date of performance	07. – 09. Jun. 2016
Treatments	100 / 46 / 22 / 10 / 4,6 mg/L
Temperature	20.3 – 21.8 °C
Duration	48 hours
Observation times	24 and 48 hours
Test vessels	glass beakers, nominal volume 100 mL, tall shape
Treatments	4 vessels, each containing 60 mL test solution and 5 Daphnia
Blank control	4 vessels, each containing 60 mL dilution water and 5 Daphnia

8 FINDINGS

8.1 Immobility

In the blank control, none of the Daphnia were immobilised (see table below).

Table 8.1-a Immobility

Nominal Concentration in mg/L	Immobility 24 hours					Immobility 48 hours				
	absolute				in %	absolute				in %
Blank control	0	0	0	0	0	0	0	0	0	0
4.6	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0
46	0	0	0	0	0	0	0	0	0	0
100	0	0	0	0	0	4	4	5	5	90

8.2 pH and O_2

The pH values in the test media and the blank control and the concentration of dissolved oxygen are given in the following tables:

Table 8.2-a pH and O_2-values

Nominal Concentration in mg/L	pH		O_2-Concentration in mg/L	
	0 h	48 h	0 h	48 h
Blank control	7.6	7.8	9.3	9.0
4.6	7.6	7.8	9.4	9.0
10	7.6	7.8	9.5	8.9
22	7.6	7.8	9.5	8.9
46	7.6	7.8	9.4	8.9
100	7.7	7.8	9.3	8.7

8.3 Analytical Determinations

The measured concentrations were in a range between 82 % and 114 % of the nominal concentration. Therefore, the determination of the biological results was based on the nominal concentrations.

The measured concentrations for treatment and control are given in the following table:

Table 8.3-a Measured Concentrations

Nominal Concentration Test Item	Measured Concentration t = 0 h	% of Nominal t = 0 h	Measured Concentration t = 48 h	% of Nominal t = 48 h
mg/L	mg/L	%	mg/L	%
Blank control	n. d.	--	n. d.	--
4.6	4.74	103	3.77	82
10	11.36	114	10.19	102
22	24.24	110	18.98	86
46	48.33	105	39.01	85
100	92.68	93	104.22	104

n. d. = not detectable

9 RESULTS AND STATISTICS

The estimation of the biological results was accomplished using the software ToxRat® Professional, version 3.2.1. The details of calculation are stated in the annex 5, page 25. Biological results are stated using two significant digits.

9.1 Biological Results Test Item

The biological results are presented in the following table:

Table 9.1-a Biological Results Test Item

Parameter	Value	95% confidence interval
24h EC50	> 100 mg/L	n. d.
48h EC50	85 mg/L	n. d.
48h NOEC	46 mg/L	--
48h LOEC	100 mg/L	--

10 VALIDITY

- Immobilisation in the controls may not exceed 10 %.
  Immobilisation in the controls was 0 %.
- The concentration of dissolved oxygen at the end of the test must be at least 3 mg/L.
  The lowest concentration of dissolved oxygen at the end of the test was 8.7 mg/L.
- The pH-value in the test solutions should not vary by more than 1.5 units during the test.
  The highest variation was 0.2 units.

11 DISCUSSION

The study was performed using 5 concentrations ranging from 4.6 to 100 mg/L. 20 Daphnia were exposed to the test item for the treatment for 48 hours in a static test system.

Only the highest concentrated treatment 100 mg/L showed toxicity of 90% immobilisation. None of the animals were immobilised in the lower concentrated treatments and in the blank control.

At the beginning and at the end of the test, the content of the test item in the test solutions was determined using GC/FID -determination. The measured concentrations were in range of 82 - 114 % of the nominal concentration. Therefore, the determination of the biological results was based on the nominal concentration.

Potassium dichromate $K_2Cr_2O_7$ (CAS No. 7778-50-9) was used as positive control in a current reference study. The 24h-EC50 value was determined as 1.6 mg/L, lying within the demanded range of 0.6 – 1.7 mg/L stated in the guideline.

No observations were made which might cause doubts concerning the validity of the study outcome. All validity criteria were met.

The result of the test is considered valid.

12 DEVIATIONS

12.1 Deviations from the Study Plan

The following deviation was documented:

- 100 mL glass beakers were used instead of 50 mL glass beakers. Theis deviation can be stated as meaningless.

The deviation was assessed and signed by the study director on 22. Jul. 2016.

12.2 Deviations from the Guideline

None.

13 RECORDING AND ARCHIVING

One original of study plan and final report, respectively, all raw data of the study and all documents mentioned or referred to in study plan or final report will be kept in the GLP Document Archive of the test facility for 15 years. After that, the sponsor's instructions will be applied (destruction of documentation) A retain sample of the test item will be kept in the GLP Substance Archive for 15 years; then, the retain sample will be discarded.

Number of originals which will be sent to the sponsor: 1

14 ANNEX 1: COPY OF GLP-CERTIFICATE

RheinlandPfalz
LANDESAMT FÜR UMWELT, WASSERWIRTSCHAFT UND GEWERBEAUFSICHT

GUTE LABORPRAXIS – GOOD LABORATORY PRACTICE

GLP-BESCHEINIGUNG
STATEMENT OF GLP COMPLIANCE

gemäß/according to § 19b Abs. 1 Chemikaliengesetz

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EC at:

Prüfeinrichtung / Test facility

LAUS GmbH
Auf der Schafweide 20
67489 Kirrweiler

Prüfung nach Kategorien / Areas of Expertise
(gemäß / according ChemVwV-GLP Nr. 5.3/OECD guidance)
1, 3, 4, 5, 6 , 8, 9 (toxikologische in Vitro Prüfungen an Säugerzellen und Bakterien / toxicological in vitro studies on mammalian cells and bacteria)

Datum der Inspektion / Date of Inspection
(Tag.Monat.Jahr / day.month.year)
13.und 14.10.2014

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

Eine erneute behördliche Überprüfung der Einhaltung der GLP-Grundsätze durch die Prüfeinrichtung ist spätestens drei Jahre nach der letzten Inspektion zu beantragen. Ohne diesen Antrag wird die Prüfeinrichtung nach Ablauf der Frist aus dem deutschen GLP-Überwachungsprogramm genommen und diese GLP-Bescheinigung verliert ihre Gültigkeit.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Based on the inspection report it can be con- firmed, that the test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Verification of the compliance of the test facility with the Principles of the GLP has to be applied for not later than three years after the last inspection. Elapsing this term, the test facility will be taken out of the German GLP-Monitoring Programme and this GLP Certificate becomes invalid.

Landesamt für Umwelt, Wasserwirtschaft und Gewerbeaufsicht 2 Rheinland-Pfalz

Unterschrift, Datum / Signature, Date

8.4.15

Dr.-Ing. Stefan Hill - Präsident -
(Name und Funktion der verantwortlichen Person / name and function of responsible person)

Landesamt für Umwelt, Wasserwirtschaft und Gewerbeaufsicht
Kaiser-Friedrich-Straße 7, 55116 Mainz
(Name und Adresse der GLP-Überwachungsbehörde / Name and adress of the GLP Monitoring Authority)

MESSEN BEWERTEN BERATEN

15 ANNEX 2: COMPOSITION OF M4-MEDIUM

Parameter	Concentration	
$CaCl_2*2H_2O$	293.80	mg/L
$MgSO_4*7H_2O$	123.30	mg/L
$NaHCO_3$	64.80	mg/L
KCl	5.80	mg/L
$NaNO_3$	274	µg/L
K_2HPO_4	184	µg/L
KH_2PO_4	143	µg/L
$Na_2SiO_3*9H_2O$	10	mg/L
H_3BO_3	2.8595	mg/L
$Na_2EDTA*2H_2O$	2.5	mg/L
$FeSO_4*7H_2O$	0.9955	mg/L
$MnCl_2*4H_2O$	0.3605	mg/L
LiCl	0.306	mg/L
$SrCl_2*6H_2O$	0.152	mg/L
RbCl	0.071	mg/L
$Na_2MoO_4*2H_2O$	0.0615	mg/L
$CuCl_2*2H_2O$	16.75	µg/L
NaBr	16	µg/L
$ZnCl_2$	13	µg/L
$CoCl_2*6H_2O$	10	µg/L
KI	3.25	µg/L
Na_2SeO_3	2.19	µg/L
NH_4VO_3	0.575	µg/L
ThiaminHCl	75	µg/L
Cyanocobalamin	1	µg/L
D+Biotin	0.75	µg/L

Real weighted loads are stated in the raw data.
Deviations from the nominal weighted loads were less than 5%.

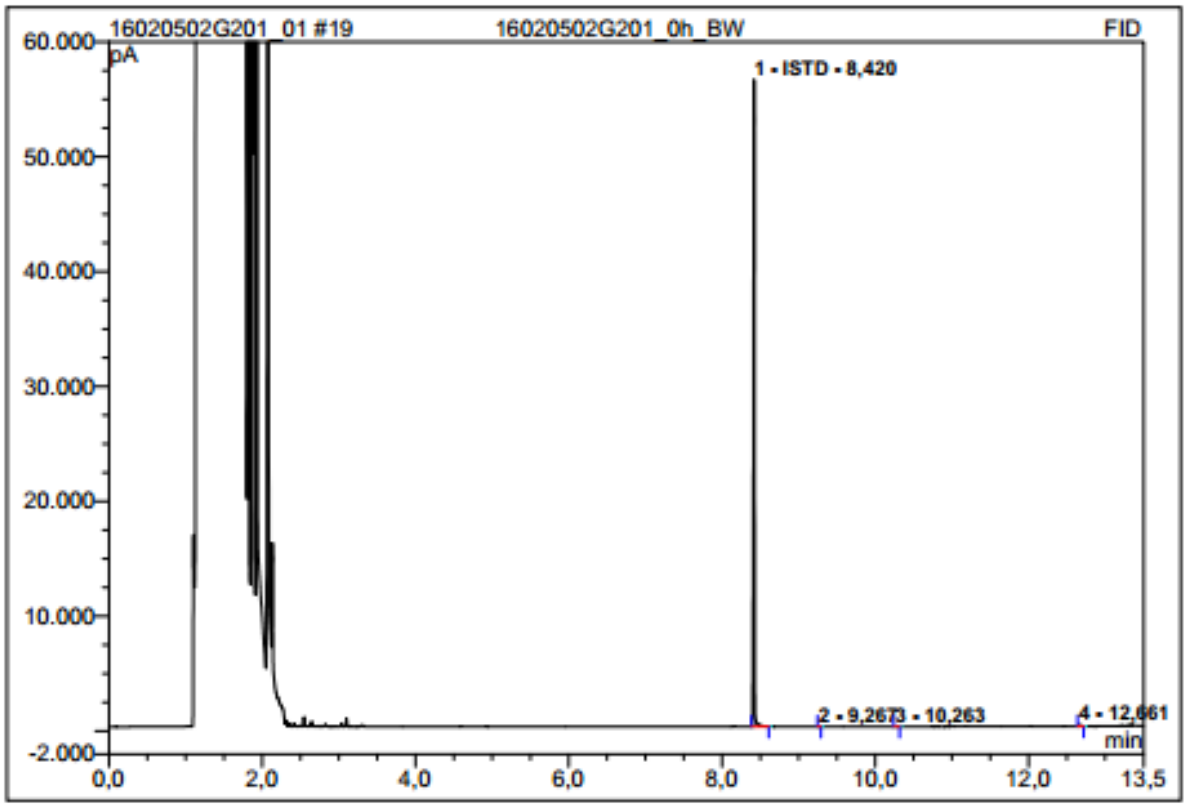
Deionised water is used as base of M4.

16 ANNEX 3: CHROMATOGRAMS

16.1 Blank control 0h

19 16020502G201_0h_BW

Sample Name:	**16020502G201_0h_BW**	*Injection Volume:*	**1,0**
Vial Number:	**10**	*Channel:*	**FID**
Sample Type:	**unknown**	*Wavelength:*	**n.a.**
Control Program:	**16020502G**	*Bandwidth:*	**n.a.**
Quantif. Method:	**16020502G**	*Dilution Factor:*	**1,0000**
Recording Time:	**7.6.2016 20:56**	*Sample Weight:*	**1,0000**
Run Time (min):	**13,50**	*Sample Amount:*	**1,0000**

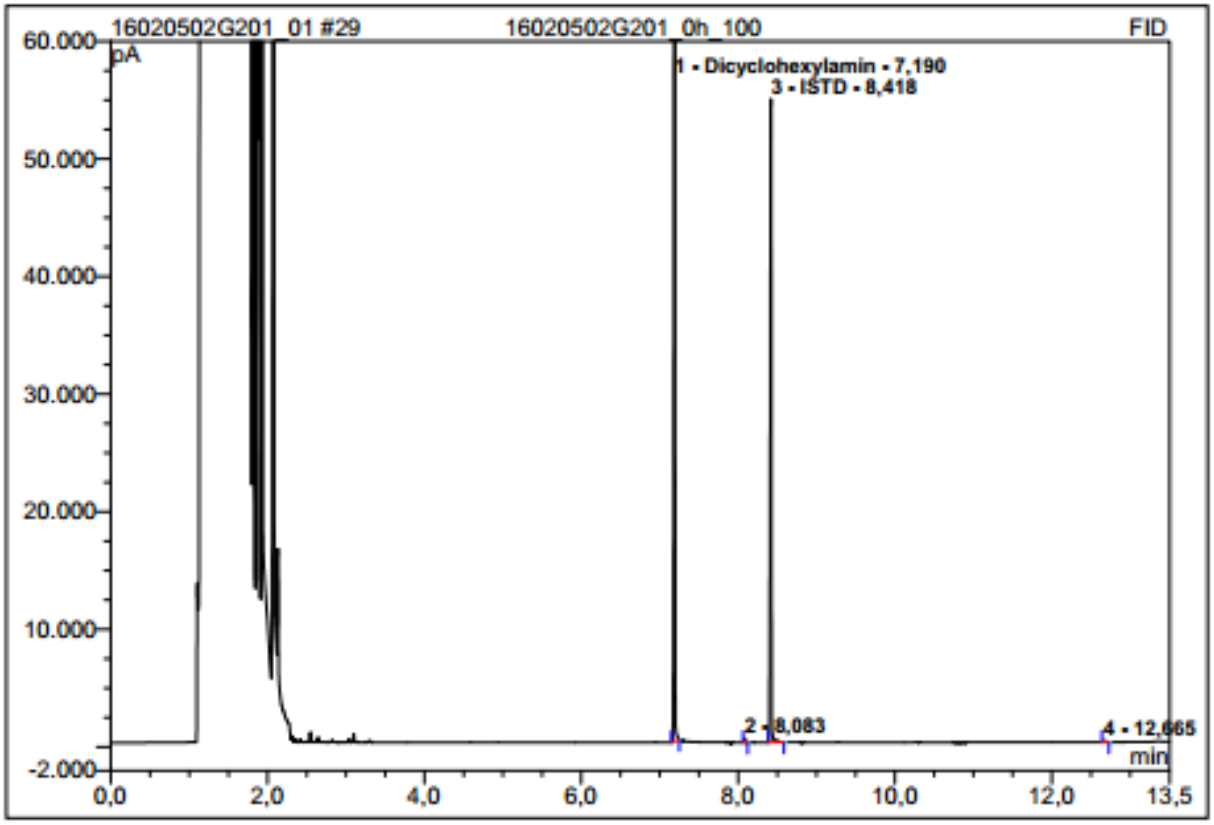


No.	Ret.Time min	Peak Name	Height pA	Area pA*s	Rel.Area %	Amount	Type
1	8,42	ISTD	56271,047	881,143	99,30	n.a.	BMB
Total:			56271,047	881,143	99,30	0,000	

16.2 Treatment 100 mg/L 0h

29 16020502G201_0h_100

Sample Name:	**16020502G201_0h_100**	*Injection Volume:*	**1,0**
Vial Number:	**15**	*Channel:*	**FID**
Sample Type:	**unknown**	*Wavelength:*	**n.a.**
Control Program:	**16020502G**	*Bandwidth:*	**n.a.**
Quantif. Method:	**16020502G**	*Dilution Factor:*	**1,0000**
Recording Time:	**8.6.2016 0:43**	*Sample Weight:*	**1,0000**
Run Time (min):	**13,50**	*Sample Amount:*	**1,0000**

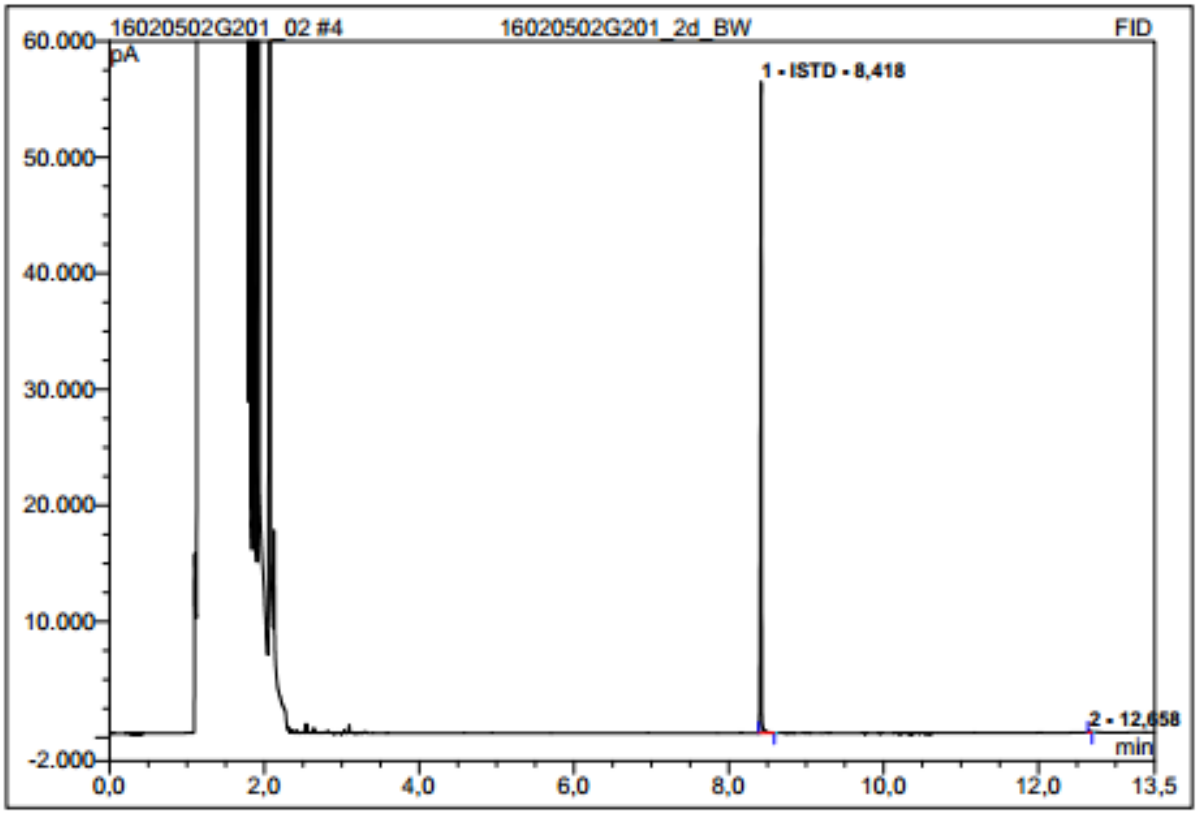


No.	Ret.Time min	Peak Name	Height pA	Area pA*s	Rel.Area %	Amount	Type
1	7,19	Dicyclohexylamin	80551,625	1238,388	59,00	n.a.	BMB
3	8,42	ISTD	54660,367	851,543	40,57	n.a.	BMB
Total:			135211,992	2089,931	99,57	0,000	

16.3 Blank control 48h

4 16020502G201_2d_BW

Sample Name:	**16020502G201_2d_BW**	*Injection Volume:*	**1,0**
Vial Number:	**8**	*Channel:*	**FID**
Sample Type:	**unknown**	*Wavelength:*	**n.a.**
Control Program:	**16020502G**	*Bandwidth:*	**n.a.**
Quantif. Method:	**16020502G**	*Dilution Factor:*	**1,0000**
Recording Time:	**9.6.2016 18:35**	*Sample Weight:*	**1,0000**
Run Time (min):	**13,50**	*Sample Amount:*	**1,0000**

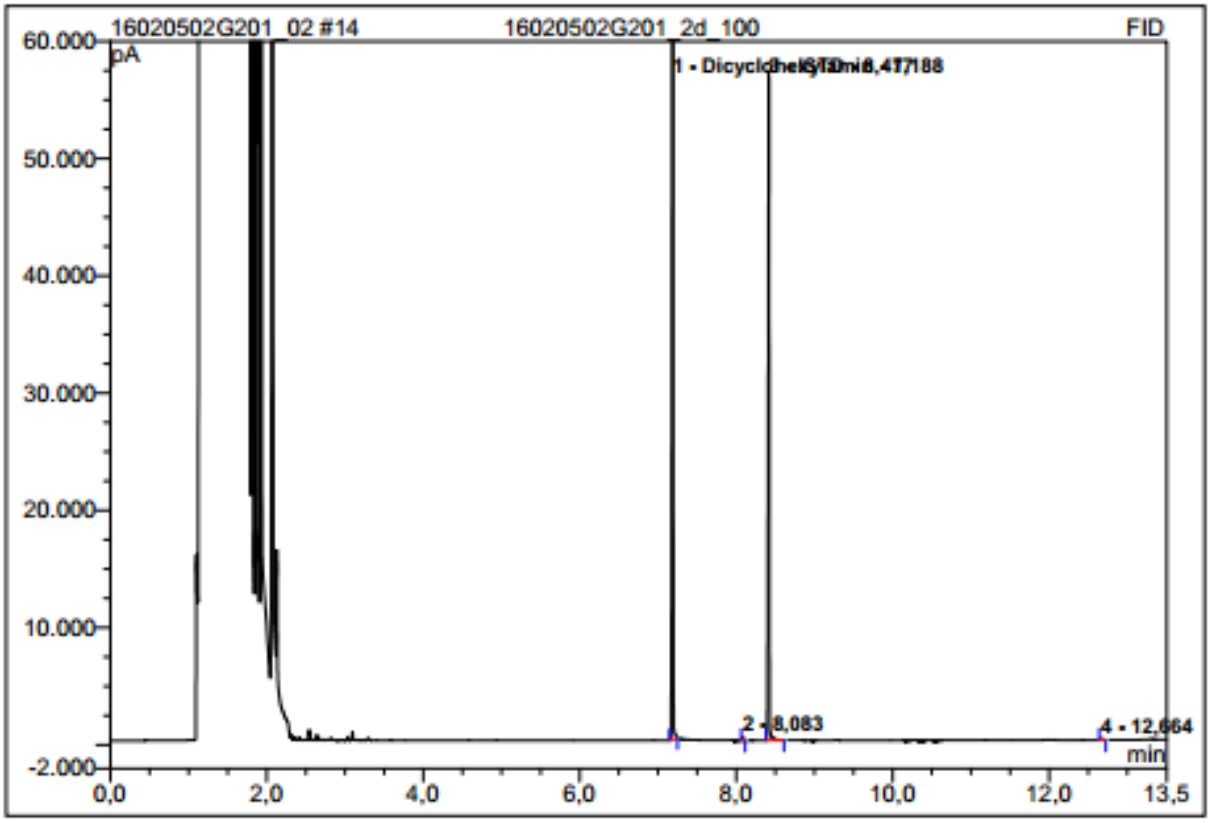


No.	Ret.Time min	Peak Name	Height pA	Area pA*s	Rel.Area %	Amount	Type
1	8,42	ISTD	56057,023	865,827	99,54	n.a.	BMB
Total:			56057,023	865,827	99,54	0,000	

16.4 Treatment 100 mg/L 48h

14 16020502G201_2d_100

Sample Name:	**16020502G201_2d_100**	*Injection Volume:*	**1,0**
Vial Number:	**13**	*Channel:*	**FID**
Sample Type:	**unknown**	*Wavelength:*	**n.a.**
Control Program:	**16020502G**	*Bandwidth:*	**n.a.**
Quantif. Method:	**16020502G**	*Dilution Factor:*	**1,0000**
Recording Time:	**9.6.2016 22:22**	*Sample Weight:*	**1,0000**
Run Time (min):	**13,50**	*Sample Amount:*	**1,0000**



No.	Ret.Time min	Peak Name	Height pA	Area pA*s	Rel.Area %	Amount	Type
1	7,19	Dicyclohexylamin	89373,977	1452,107	61,64	n.a.	BMB
3	8,42	ISTD	56736,820	894,765	37,98	n.a.	BMB
Total:			146110,797	2346,872	99,62	0,000	

18 ANNEX 4: ABBREVIATIONS

LOEC	Lowest concentration of the test item where more than 10 % of the daphnia were immobilised
NOEC	Highest concentration of the test item where less than 10 % of the daphnia were immobilised
24h-EC50	Concentration of the test item causing 50% immobility within 24 hours.
48h-EC50	Concentration of the test item causing 50% immobility within 48 hours.

19 ANNEX 5: STATISTICAL CALCULATION USING TOXRAT® PROFESSIONAL 3.2.1

Daphnia, Acute Immobilisation Test (OECD 202-2004): 16020502G201

General:

Test identification/project no.	16020502G201
Test item	
Unit of test item concentration	mg/L
Start of experiment on day	
Date and time of the evaluation	13.06.2016; 10:14:47
Raw data filename:	OECD202 Daphnia AcuteTest 20041.xls

Test design

Number of treatments (incl. control(s))	6
Duration of the test	48 h
Measurement interval	
Measurement variable	Immobility
Test system	Daphnia magna
Statistical design	Hypothesis testing (NOEC) and regression (LCx)

Validity of the test

To be a valid test, a maximum control mortality of 10% is allowed.
In the present test 0,0% of the introduced animals died.
Thus the test is valid.

16020502G201 / Test item: test item / Date: 13.06.2016; 10:15:38 h

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Relation of Daphnia magna Endpoints on Concentration

Summary of Results for all Endpoints at the End of Exposure Period

Tab. 1: Summary of Results for all Endpoints at the End of Exposure Period: Critical effect and threshold concentration as observed at end of experimental time; LC: Effective concentration for xx% reduction; 95%-CL: 95% Confidence limits; LOEC: Lowest observed effect concentration; NOEC: No observed effect concentration.

Critical Conc.s [mg/L]

Immobility		
	LC10	72,297
95%-CL	lower	n.d.
	upper	n.d.
	LC20	76,436
95%-CL	lower	n.d.
	upper	n.d.
	LC50	85,027
95%-CL	lower	n.d.
	upper	n.d.
Immobility	LOEC	100,000
	NOEC	46,000

n.d.: not determined due to mathematical reasons or inappropriate data

Immobility (Data)

Immobility of Daphnia magna as Dependent on Concentration and Time

Tab. 2: Immobility of Daphnia magna as dependent on concentration of the test item and time (from InputRawData)

Treatm. [mg/L]	Control	4,600	10,000	22,000	46,000	100,000
0 h	5	5	5	5	5	5
	5	5	5	5	5	5
	5	5	5	5	5	5
	5	5	5	5	5	5
Total Introduced	20	20	20	20	20	20
n:	4	4	4	4	4	4

Tab. 2 (continued): Immobility of Daphnia magna as dependent on concentration of the test item and time (from InputRawData)

24 h	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0
Total Immobile:	0	0	0	0	0	0
n:	4	4	4	4	4	4
48 h	0	0	0	0	0	4
	0	0	0	0	0	4
	0	0	0	0	0	5
	0	0	0	0	0	5
Total Immobile:	0	0	0	0	0	18
n:	4	4	4	4	4	4

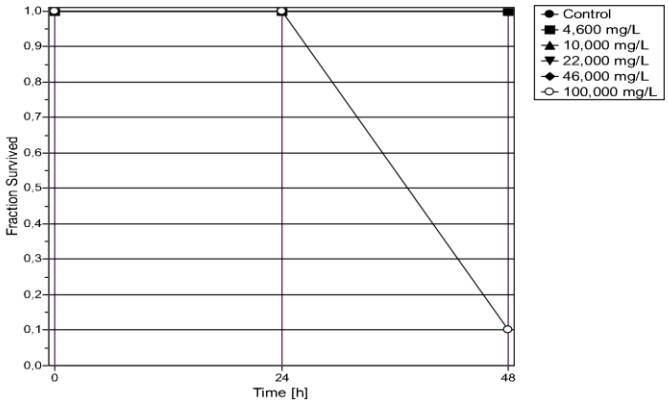

Fig. 1: Immobility of the introduced Daphnia magna as observed under presence of the test item.

Lethal Concentrations (LCx) for Immobility at 24 h

Overview Immobility

Tab. 3: Overview Immobility: Overview over the effects on immobility in Daphnia magna at 24 h

Treatm.[mg/L]	Total Introduced	Mobile	Immobile	% Immobility
Control	20	20	0	0,0
4,600	20	20	0	0,0
10,000	20	20	0	0,0

22,000	20	20	0	0,0
46,000	20	20	0	0,0
100,000	20	20	0	0,0

Because no change in immobility was to be observed, no further computations have been performed for 24 h.

Lethal Concentrations (LCx) for Immobility at 48 h

Overview Immobility

Tab. 4: Overview Immobility: Overview over the effects on immobility in Daphnia magna at 48 h

Treatm.[mg/L]	Total Introduced	Mobile	Immobile	% Immobility
Control	20	20	0	0,0
4,600	20	20	0	0,0
10,000	20	20	0	0,0
22,000	20	20	0	0,0
46,000	20	20	0	0,0
100,000	20	2	18	90,0

Probit analysis using linear max. likelihood regression

Tab. 5: Probit analysis using linear max. likelihood regression with immobility at 48 h: Determination of the concentration /response function; data is shown which entered the probit analysis; Log(x): logarithm of the concentration; n: number of organisms; Emp. Probit: empirical probit; Reg. Probit: calculated probit for the final function.

Treatm. [mg/L]	Log(x)	% Immobility	n	Emp. Probit	Weight	Reg. Probit
Control		0,0	20			excluded
4,600	0,663	0,0	20	-1,2533	0,000	-4,753
10,000	1,000	0,0	20	-1,2533	0,000	-4,753
22,000	1,342	0,0	20	-1,2533	0,000	-4,753
46,000	1,663	0,0	20	-1,2533	0,001	-4,638
100,000	2,000	90,0	20	1,0027	6,844	1,282

excluded: value not in line with the chosen function

Parameters of the probit analysis

Tab. 6: Parameters of the probit analysis with immobility at 48 h: Results of the regression analysis

Parameter	Value
Computation runs:	16
Slope b:	18,19318
Intercept a:	-35,10481
Variance of b:	10.701,04297
Goodness of Fit	
Chi²:	0,00001
Degrees of freedom:	3
p(Chi²):	1,000
Log LC50:	1,92956
SE Log LC50:	0,40085
g-Criterion:	124,19525
F:	8797,023
p(F) (df: 1;3):	<0.001

Chi² is a goodness of fit measure. If the probability, p(Chi²), is lower or equal than 0,100 data is much scattering

round the computed dose/response function. In this case and with quantal data, confidence limits are corrected for heterogeneity (extra-binomial variance).

A statistically significant concentration/response was found (p(F) <= 0.05; i.e. slope of the relationship is significantly different from zero).

Results of the probit analysis

Tab. 7: Results of the probit analysis with immobility at 48 h: Selected effective concentrations (LCx) of the test item and their 95%-confidence limits (according to Fieller`s theorem).

Toxicity Metric	LC10	LC20	LC50
Value [mg/L]	72,297	76,436	85,027
lower 95%-cl	n.d.	n.d.	n.d.
upper 95%-cl	n.d.	n.d.	n.d.

n.d.: not determined either due to mathematical reasons or value is beyond the tested concentrations by more than factor 1000.

Slope function after Litchfield and Wilcoxon: 1,135

(The slope function is derived from the slope, b, of the linearized probit function and computes as S = 10^(1/b); please note that small values refer to a steep concentration/response relation and large ones to a flat relation.)

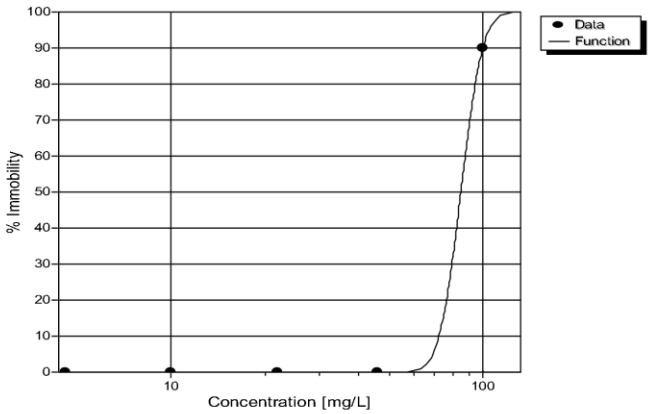

Fig. 2: Concentration-effect curve showing the influence of the test item on immobility of the introduced Daphnia magna as observed after 48 h

Overview over the LCs of the Test Item on Immobility

Increase in Immobility

Tab. 8: Immobility (M) and percent mortality (%M) as computed from the raw data for test intervals selected; LCxx with immobility: effect levels as selected; lower 95%-cl, upper 95%-cl: lower and upper 95%-confidence limits.; *: ; *: ; *: ; *pm: Probit analysis using linear max. likelihood regression.

Treatment		0-24 h		0-48 h
[mg/L]	M	%M	M	%M
Control	20,0	0,0	20,0	0,0
4,600	20,0	0,0	20,0	0,0
10,000	20,0	0,0	20,0	0,0

22,000	20,0	0,0	20,0	0,0
46,000	20,0	0,0	20,0	0,0
100,000	20,0	0,0	2,0	90,0

LC10	0,000	*	72,297	*pm
lower 95%-cl	0,000		n.d.	
upper 95%-cl	0,000		n.d.	
LC20	0,000	*	76,436	*pm
lower 95%-cl	0,000		n.d.	
upper 95%-cl	0,000		n.d.	
LC50	0,000	*	85,027	*pm
lower 95%-cl	0,000		n.d.	
upper 95%-cl	0,000		n.d.	

Threshold concentrations (NOEC) for Immobility at 24 h

To justify the use of the Step-down Cochran-Armitage test at first a trend analysis by contrasts using proportions was performed.

Qualitative Trend Analysis by Contrasts (Monotonicity of Concentration/Response)

Tab. 9: Qualitative trend analysis by contrasts (monotonicity of concentration/response) with immobility at 24 h: Psi: total of proportions weighted by contrasts; Var(psi): variance of psi; df: degrees of freedom; Chi²: Chi²-statistic; p(Chi²): probability that the trend is due to chance (Ho: Slope = 0). Hypothesis of monotonicity is accepted if at least the linear contrast is significant.

Trend	Psi	Var(psi)	df	Chi²	p(Chi²)
Linear	0,0000	0,0000	5	NAN	1,000
Quadratic	0,0000	0,0000	5	NAN	1,000

The linear trend is not significant (p > 0,05) The quadratic trend is not significant (p > 0,05)

The analysis of contrasts did not reveal a linear trend, thus the selected Step-down Cochran-Armitage test was replaced by the Bonferroni Fisher test.

Fisher`s Exact Binomial Test with Bonferroni Correction

Tab. 10: Fisher´s Exact Binomial Test with Bonferroni Correction with immobility at 24 h: Two-sample comparisons between treatment and control on the multiple significance level (Alpha is 0,050; one-sided greater). Two-sample comparisons are performed sequentially using the adjusted Alpha* (= alpha/(k-1); k: number of comparisons (after Holm, 1979); Ho (no effect) is accepted, if the probability p > Alpha*; p(exact) is the probability that the increase in category "Immobile" observed in the treatments is due to chance. Note that the step-down test terminates after the first non-significant treatment is encountered

Treatm.[mg/L]	Introduced	Mobile	Immobile	% Immobility	p	Alpha*	sign.
Control	20	20	0	0,0			
4,600	20	20	0	0,0	1,000	0,050	-
10,000	20	20	0	0,0	1,000	0,025	-
22,000	20	20	0	0,0	1,000	0,017	-
46,000	20	20	0	0,0	1,000	0,013	-
100,000	20	20	0	0,0	1,000	0,010	-

+: significant; -: non-significant

The NOEC appears to be higher than or equal 100,000 mg/L.

Threshold concentrations (NOEC) for Immobility at 48 h

To justify the use of the Step-down Cochran-Armitage test at first a trend analysis by contrasts using proportions was performed.

Qualitative Trend Analysis by Contrasts (Monotonicity of Concentration/Response)

Tab. 11: Qualitative trend analysis by contrasts (monotonicity of concentration/response) with immobility at 48 h: Psi: total of proportions weighted by contrasts; Var(psi): variance of psi; df: degrees of freedom; Chi²: Chi²-statistic; p(Chi²): probability that the trend is due to chance (Ho: Slope = 0). Hypothesis of monotonicity is accepted if at least the linear contrast is significant.

Trend	Psi	Var(psi)	df	Chi²	p(Chi²)
Linear	4,5000	0,1125	5	180,000	<0.001
Quadratic	4,5000	0,1125	5	180,000	<0.001

The linear trend is significant (p <= 0,05) The quadratic trend is significant (p <= 0,05)

The analysis of contrasts revealed a linear trend, thus the selected Step-down Cochran-Armitage test was performed.

Ahead of the Cochran-Armitage test Tarone´s test had to be performed to test for extra-binomial variance.

Tarone´s Test Procedure

Tab. 12: Tarone Test with immobility at 48 h: Treatment-wise testing the homgeneity of proportions (Alpha = 0,010). The statistic TZ has an asymptotic chi² distribution with one degree of freedom and measures the deviation from homogeneity. Ho (Phi = 0; i.e. homogeneity) is accepted, if the probability p(TZ) > Alpha; p(TZ) is the probability that the deviation from homogeneity observed in the treatment(s) is due to chance.

Treatm.[mg/L]	Introduced	Mobile	Immobile	TZp(TZ)	sign.
Control	20	20	0	2,5000,114	-
4,600	20	20	0	2,5000,114	-
10,000	20	20	0	2,5000,114	-
22,000	20	20	0	2,5000,114	-
46,000	20	20	0	2,5000,114	-
100,000	20	2	18	0,4940,482	-

+: significant; -: non-significant

In treatments no signs of extra-bionmial variance were found.

Step-down Cochran-Armitage Test Procedure

Tab. 13: Step-down Cochran-Armitage Test Procedure with immobility at 48 h: Step-down test to detect an increasing trend in responses (Alpha is 0,050; one-sided greater); Chi²(tot): total (Pearson) Chi²; z(trend): standardized one-sided deviation due to the linear upward trend; Chi²(err): unexplained component of Chi²(tot); p(tot|trend|err): probabilities that the observed results could be due to chance; Ho (no trend) is accepted, if p(trend) > Alpha. Note that the step-down test terminates after the first non-significant treatment is encountered

Treatm. [mg/L]	Total	Introduced	Immobile	% Immobility	Chi²(tot)	p(tot)	Chi²(err)	p(err)|z|(trend)	p(trend)	Sign.
Control	20	0	0,0							
4,600	20	0	0,0	0,000	1,000	0,000	<0.001	0,000	1,000	-
10,000	20	0	0,0	0,000	1,000	0,000	<0.001	0,000	1,000	-
22,000	20	0	0,0	0,000	1,000	0,000	<0.001	0,000	1,000	-
46,000	20	0	0,0	0,000	1,000	0,000	<0.001	0,000	1,000	-
100,000	20	18	90,0	105,882	<0.001	60,504	<0.001	6,736	<0.001	+

+: significant; -: non-significant

A NOEC of 46,000 mg/L is suggested by the program.

Overview over the Effect-Thresholds of the Test Item on Immobility

Overview over the LOEC and NOEC Determination

Tab. 14: Overview over the LOEC and NOEC Determination with immobility: Arithmetic means and significance marks as computed for immobility for all inspection intervals (top); bottom part: obtained LOEC and NOEC with indication of statistical test used; *bf: Fisher`s exact binomial test with Bonferroni correction; *casd: Step-down Cochran-Armitage test procedure, significance level was 0,050, one-sided greater.

Treatm. [mg/L]	0-24 h	0-48 h
4,600	0,0 -	0,0 -
10,000	0,0 -	0,0 -
22,000	0,0 -	0,0 -
46,000	0,0 -	0,0 -
100,000	0,0 -	90,0+

LOEC >100,000 *bf100,000 *casd
NOEC>=100,000 *bf 46,000 *casd

+: Significant difference to control (p <=0,050)

Summary of Results for all Endpoints

Tab. 15: Summary of Results for all Endpoints: Critical effect and threshold concentration as observed at end of experimental time; LC: Effective concentration for xx% reduction; 95%-CL: 95% Confidence limits; LOEC: Lowest observed effect concentration; NOEC: No observed effect concentration.

Critical Conc.s [mg/L]		0-24 h	0-48 h
Immobility			
	LC10	0,000	72,297
95%-CL	lower	0,000	n.d.
	upper	0,000	n.d.
	LC20	0,000	76,436
95%-CL	lower	0,000	n.d.
	upper	0,000	n.d.
	LC50	0,000	85,027
95%-CL	lower	0,000	n.d.
	upper	0,000	n.d.
Immobility	LOEC	>100,000	100,000
	NOEC	>=100,000	46,000

n.d.: not determined due to mathematical reasons or inappropriate data

Settings Table

Tab. 16:

Area	Item	Default Settings	User Settings
Global			
	Type of Exposure	Concentration	Concentration
	Extrapolation of LCx	By program	By program
	Show non-significant ECx	YES	YES
	Statistical design		NOEC/ECx
Variables			
Immobility	State	Selected for analysis	Selected for analysis

Data transformation	none	ArcSine Square Root(p)
Decimals data	1	1
Statistical pre-testing		
Extra-binomial variance	Tarone test	Deselected
Additional tests	None	None
Final testing (NOEC)		
Test procedures	SD Cochran-Armitage	Bonferroni Fisher
Who selected final test	Program	Program
Additional tests	None	None
Significance level	0,05	0,05
Test direction	one-sided greater	one-sided greater
LCx computation		
Selected LCx values	LC10, LC20, LC50	LC10, LC20, LC50
Selected method	Linear Regression	Linear Regression
Regress. type	Max. Likelihood	Max. Likelihood
Dose/response function	Probit (normal sigmoid)	Probit (normal sigmoid)
Sig. level goodness of fit	0,10	0,10
Data	Treatment mean/total	Treatment totals
Confidence limits	after Fieller	after Fieller
Control mortality	Not compensated	Not compensated