Fraunhofer Institut
Molekularbiologie und
Angewandte Oekologie

Study Report

Daphnia magna, Acute Immobilization Test

Effect of Linevol on the immobilization of *Daphnia magna* in closed vessels

Test guideline: OECD 202

GLP-Code of Testing Facility: SDA-004/4-20

Sponsor
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Study Monitor:
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Test facility management
Prof. Dr. A. Schäffer

Study director
Dr. Andrea Wenzel

September 23, 2005

STUDY No SDA-004/4-20

AMENDMENT No 1

TEST *Daphnia magna*, Acute Immobilization Test
TEST ITEM: Linevol 79

This amendment clarifies formal errors and omissions without influence on the integrity of the study.

Whole study report:
The expression "Water accommodated fraction (WAF)" should be replaced by the expression "solution".
Rationale: For the daphnia test, a stock solution of the test item was prepared by mixing the substance with water for 24 hours (see 6.3.3) and the concentration of the test item in the water phase was analyzed using chemical analysis. The stock solution was diluted to obtain the required test concentrations. Therefore, the expression "Water accommodated fraction (WAF)" for the solutions is incorrect.

Page 18: Point 7.2 - Test item concentrations throughout the test
A cross heading "Justification for the use of 1-nonanol for extrapolating Linevol 79 levels" was inserted and the justification was underlined with additional examples and references.

Page 31: Table 6 - Summary of recovery data
The recovery data were completed with data of 1-heptanol and 1-octanol.

Page 32-34: Tables 7-9 - Measured concentrations of 1-heptanol, 1-octanol or 1-nonanol
The structures of the tables were modified to include the column "% of initial" for the concentrations at the end of the exposure phases.

Fraunhofer Institut
Molekularbiologie und
Angewandte Oekologie

Date: September 23, 2005

Study director: (Dr. Andrea Wenzel)

Distribution list for the amendment:

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Enclosure:

Revised study report

STUDY REPORT:	**Daphnia, Acute Immobilization**	
TEST ITEM:	**Linevol 79**	
GLP-CODE:	**SDA-004/4-20**	

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Abbreviations and definitions

LOEC	(lowest observed effect concentration) is the lowest concentration tested at which the measured parameter shows significant inhibition relative to the control.
NOEC	(no observed effect concentration) is the highest concentration tested at which the measured parameter shows no significant inhibition relative to the control.
EC_{50}	(effective concentration) is the concentration of the test substance, which results in a 50 per cent reduction in the measured parameter relative to the control.
SOP	Standard operation procedure
RSD	relative standard deviation

Summary

A study was performed to evaluate the acute toxicity of the test item Linevol 79 to *Daphnia magna*. The daphnids were exposed under semi-static conditions for 48 hours according to the OECD guideline 202 (1).

Due to the relatively high vapor pressure of the rapidly biodegradable test item, the test was performed in gas-tight vessels under semi-static and sterile conditions with daily renewal of the test solution.
A stock solution of the test item was prepared by stirring 400 mg test item/L for 24 hours under sterile conditions. The stock solution was chemically analyzed and subsequently diluted with sterilized dilution water to obtain the required nominal test concentrations of 36.0, 14.4, 5.76, 2.30 and 0.92 mg test item/L
For assessing the test item concentrations, the C7-, C8 and C9 concentrations were analyzed and the respective Linevol 79 concentration was extrapolated from the C9-content of the test solutions and the stock solutions.

The concentrations of the test item were determined by chemical analyses at commencement and end of each 24 hours incubation period. The test item levels were found to be stable (82 % - 108 % of the initial concentrations at the end of the 24 h incubation periods) except for the lowest test concentration plot where the Linevol 79 level decreased to 76 % and 70 % during the two incubation periods, respectively.

The evaluation of the effects on daphnia was based on the mean measured concentrations of 41.3, 18.0, 6.28, 3.08 and 1.09 mg test item/L.

The **EC_{50}** of the test item was determined to be **5.91 mg test item/L.**

The highest concentration without immobilization, also determined as **NOEC**, was found to be **1.09 mg test item/L.**

Statement of GLP-compliance

Title of the study:	Daphnia, Acute Immobilization Test
Test item:	Linevol 79
Study-Code:	SDA-004/4-20

The study was conducted in compliance with Good Laboratory Practice regulations (GLP) (3, 4).

We hereby attest to the authenticity of the study and guarantee that the data are correct and accurate, and that the study was performed by the procedures described. There were no known circumstances which may have affected the quality or integrity of the study.

Date: September 23, 2005

Dr. Andrea Wenzel
(Study Director)

Date: September 23, 2005

Dr. Josef Müller
(Chemical Investigator)

Date: September 23, 2005

Prof. Dr. Andreas Schäffer
(Test facility manager)

Quality assurance statement

Title of the study: Daphnia, Acute Immobilization Test
Test item: Linevol 79
Study-Code: SDA-004/4-20

The Quality Assurance Unit of the testing facility inspected the study and audited the final report according to GLP-regulations.

Dates of QAU inspections:	Study plan	May 27, 2005
	Daphnia acute immobilization test, transfer of daphnids after 24 h	June 02, 2005
	Study report	July 21, 2005

Generally, the inspections of the GLP-laboratories were performed every three months.

The results reported in this study were checked on the basis of our current SOPs and to the best of our knowledge accurately reflect the raw data.

Date: September 27, 2005

for Dr. Ulrich Fritsche
(QAU-Officer)

1 Study identification

1.1 Test

Daphnia magna, Acute Immobilization Test,
OECD No. 202 (1)

Test item:	Linevol 79
GLP-Code:	SDA-004/4-20

1.2 Sponsor

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1.3 Testing facility

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Biology:	P. Schulte E. Hardebusch

Quality Assurance Unit: Dr. Ulrich Fritsche
Dr. Gerd Wasmus

Subcontractor The study was performed without subcontracting.

1.4 Study dates

Initiation:	May 27, 2005
Experimental start:	June 01, 2005
Experimental termination:	June 03, 2005

2 Objective

The objective of this study was the assessment of the acute effects (48 h EC_{50}) of the test item Linevol 79 to invertebrates, measured as immobilization of *Daphnia magna*. Due to the relatively high vapor pressure of the rapidly biodegradable test item, the test was performed in gas-tight vessels under semi-static and sterile conditions with daily renewal of the test solution.

3 Test item specification (Data supplied by the sponsor)

The test item as well as the certificate of analyses (Shell Chemicals U.K. Limited, Certificate No. 0000970, 09.03.2004) was delivered by the sponsor. The sponsor agreed by his signature that identity and purity of the test item were not analytically checked by the testing facility. Test item which was not needed for testing and for archiving will be returned to the sponsor.

3.1	Common name	Linevol 79
3.2	Substance formal name	C7-C9 primary alcohol
3.3	Product code	V9319
3.4	Batch/Lot number	T3608B AL69404F
3.5	CAS-number	68603-15-6

3.6	Content and carbon distribution	C-numbers	% (m/m)
		< C7	1
		C7	44
		C8	24
		C9	30
		> C9	1
3.7	Octanol/Water partition coefficient	log Pow: 2.5 -4.2	
3.8	Water solubility	800 mg/L	
3.9	Vapor pressure	72 Pa at 20 °C (estimated value)	
3.10	Specific density	0.832 g/cm³ at 20 °C	
3.11	Chemical stability	oxidizes on contact with air. stable up to 45 °C.	
3.12	Biological stability	readily biodegradable	
3.13	State of matter and appearance	liquid, colorless	
3.14	Expiry date	31.10.2005 (assessed by investigator)	
3.15	Origin of the test item	Shell Chemicals U.K. Limited	

4 Specification of reference items

4.1	Reference item 1	
	Common name	1-heptanol
	Substance formal name	C7 fatty alcohol
	Purity	99.5 % (GC)
	Origin	Dr. Ehrenstorfer GmbH, 86199 Augsburg Germany
	Lot number	30508
4.2	Reference item 2	
	Common name	1-octanol
	Substance formal name	C8 fatty alcohol
	Purity	99.5 % (GC)
	Origin	Dr. Ehrenstorfer GmbH, 86199 Augsburg Germany
	Lot number	20122

4.3 Reference item 3

Common name	1-nonanol
Substance formal name	C9 fatty alcohol
Purity	99.5 % (GC)
Origin	Dr. Ehrenstorfer GmbH, 86199 Augsburg Germany
Lot number	30403

5 GLP

The tests were performed in accordance with the Principles of Good Laboratory Practice (3, 4).

6 Materials and methods

6.1 Test organism

Daphnia magna (Crustacea, Phyllopoda, Cladocera) was chosen by OECD-experts (1) and EEC (2) as test organism representing aquatic invertebrates.

Specification

Species:	*Daphnia magna* STRAUS, Crustacea, Cladocera.
Age:	4 - 24 hours old.
Origin:	Umweltbundesamt (German Federal Environment Agency), Institut für Wasser-, Boden- und Lufthygiene, bred in the laboratory of the Fraunhofer-IME.

6.2 Breeding and holding conditions

Adult Daphnia, at least 3 weeks old, were separated from the stock population by sieving. Batches of 30 to 50 animals were held at room temperature in approx. 1800 mL purified drinking water. During the week the daphnids were fed daily with an algal suspension (*Scenedesmus subspicatus*) and HOBBY® LiquizellR (liquid starter feed for invertebrates, Dohse Aquaristik KG, 53501 Grafschaft-Gelsdorf, Germany)

according to the EEC-Guideline (2). Algae growing in the log-phase were centrifuged and the pellet was resuspended in a few mL of medium. 30 mL of this suspension was given to 1 L Daphnia medium. The water was changed once per week. Newborn daphnids were separated by sieving, the first generation was discarded.

Sensitivity
The sensitivity of the test clone was checked by using $K_2Cr_2O_7$ as reference substance. In January 2005 the EC_{50} was 0.8 mg/L.

6.3 Preparation of test media

6.3.1 Glassware Preparation

All glassware used in testing is to be given a detergent wash followed by a water rinse and an acid wash (10 % v/v HNO_3) followed by a rinse with reagent grade acetone and a final rinse with distilled water. For subsequent use, the acid wash with 10% v/v HNO_3 was omitted. Than, the procedure for glassware preparation was:
Cleaning in a cleaning machine with detergent
Cleaning in a cleaning machine without detergent
Rinsing with acetone
Rinsing with water
Sterilization of glassware at 160 °C overnight

6.3.2 Holding- and dilution water

Purified drinking water was used according to the OECD-Guideline (1). The purification includes filtration with charcoal, aeration and passage through a lime stone column. In order to avoid microbiological degradation of the hydrocarbons the water was sterilized by sterile filtration. Then the water was aerated up to oxygen saturation (using an air sterile filter) and filled into the sterilized mixing vessels for test media preparation under laminar flow conditions.

6.3.3 Test media-preparation

A stock solution was prepared with a nominal concentration of 400 mg test item/L, chemically analyzed and subsequently diluted with sterilized dilution water to obtain the required test concentrations. The test item concentrations of all test plots were assessed by chemical analyses at commencement and end of the incubation periods. The work was performed under sterile conditions using sterilized equipment.

Stock solution preparation
The mixing vessel was a cylindrical brown glass bottle to prevent photochemical degradation of dissolved components. The bottle was sealed with glass ground stoppers and was fitted with a drain port near the bottom for drawing off the solution. The volume of the mixing vessels was 2 L. A magnetic stirring bar was placed in the vessel and 2 L of the dilution water (6.3.2) was added. This is to use a maximum volume and to minimize head space whilst maintaining optimum surface contact between test item and the water. Then 961.5 µL of the test item corresponding to 400 mg/L were carefully added directly to the surface of the dilution water.
Mixing was initiated with the vortex in the center extending maximally around 10 % of vessel depth from the top to the bottom of the vessel. It was as low as possible to maintain mixing of the water phase. To avoid formation of fine droplets, care was taken not in draw a vortex of test material all the way to the bottom. The mixing period was 24 hours.
Following mixing the contents of the vessel was allowed to stand undisturbed for 1 hour to allow separation of the aqueous and undissolved phases, since some droplets had been observed on the surface of the solution. The stockt solution was then taken out of the drain port without filtration. A first portion was discarded (100 mL). Then a sample was taken from the following stock solution and chemically analyzed. The solutions were stored in the original bottle for 1.5 - 2 hours at room temperature until the chemical analysis was performed. Based on the measured 1-nonanol concentration, the stock solution was diluted with dilution water to obtain the required test concentrations and filled into the test vessels under laminar flow conditions for toxicity testing. After filling, the vessels were sealed immediately und only opened again to introduce the test organisms und again at the end of the incubation period.

6.3.4 Test vessels

Test vessels were 100 mL conical glass flasks with ground-in glass stoppers.

6.4 Test concentrations

The five concentrations to be tested are based on the findings of the range-finding test (measured concentrations) and agreed with the sponsor. They are spaced by a factor of 2.5. The nominal Linevol concentrations, based on the 1-nonanol content, were as follows:
36.0 mg, 14.40 mg, 5.76 mg, 2.30 mg and 0.92 mg Linevol/L

6.5 Test procedure

All work for the test preparation was performed under sterile conditions.

Daphnids (*Daphnia magna*), not older than 24 hours were exposed to 5 concentrations of the test item in 4 replicates each under semi-static conditions for a period of 48 hours. The numbered test vessels were completely filled with the test media, the test organisms were added and the vessels were closed with a gas-tight stopper directly afterwards by avoiding air bubbles. No feeding and no aeration occurred throughout the test. The controls were kept under the same conditions in dilution water.
The test media was renewed after 24 hours by transferring the test organisms to new vessels with freshly prepared test media under sterile conditions.
Immobility and abnormal behavior were recorded after 24 and 48 hours. Immobile animals were eliminated from the vessels as soon as they were discovered. The daphnids were considered to be immobile if they were not able to swim within 15 seconds after gentle agitation of the test vessels. The temperature during the test was adjusted to 21 ± 1 °C. The beakers were subjected to a light/dark cycle of 16/8 h with light intensities of less than 1000 Lux.
At test start before adding the daphnids and at test end, pH-values (WTW Microprocessor pH-Meter pH 196) and oxygen concentrations (WTW Microprocessor Oximeter OXI 196) of pooled samples for each concentration plot and the control water plot were measured.

6.6 Evaluation and statistics

Numerical values in this report are frequently rounded to a smaller degree of precision (number of digits) than were used in the actual calculation. Minor differences in results obtained from calculations with such rounded values in comparison to those obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and thus of no practical concern.
The evaluation was performed as follows:
The evaluation of the effects was based on mean measured test item concentrations, extrapolated from the measured 1-nonanol levels at commencement and end of each incubation period.
The percent immobile daphnids were listed in a table and the mean value of each plot was used for plotting an effect curve.

The data were analyzed by regression to determine the EC_{50} including the 95 % confidence interval as well as the EC_{10} using Probit-analysis (5) assuming log-normal distribution of the values. The NOEC and LOEC were determined using Fisher's Exact Binomial Test by using the computer program ToxRat (6).

6.7 Chemical analysis of the test solutions

The contents of C7-, C8- and C9- fatty alcohols in the test samples were analyzed in the freshly prepared test solutions at test start and at medium renewal as well as at the end of the 24 h exposure intervals (Table 1).

Table 1: Sampling

Time	Medium	Sampling	Number of samples
0 h	new medium 1	5 concentrations 1 control	1 sample per concentration = 6
24 h	aged medium 1	5 concentrations 1 control	pooled replicates per concentration and control = 6
24 h	new medium 2	5 concentrations 1 control	1 sample per concentration = 6
48 h	aged medium 2	5 concentrations 1 control	pooled replicates per concentration and control = 6

The method used is described in Annex 1.
According to (16, 17) the analytical method was validated in respect to specificity, linearity, accuracy, precision, identity and limit of quantification (LOQ). Details are shown in Annex 2.

Outline of the method

The analytes were extracted from the daphnia test media by liquid-liquid partitioning with n-hexane. After shaking and settling the n-hexane extract was removed and the analyte derivatized using MSTFA (n-Methyl-trimethylsilyl-trifluoroacetamid). Measurement was performed by GC-MS in SIM mode using internal standard calibration with deuterated 1-hexanol as internal standard.

7 Results

7.1 Water quality parameter values throughout the test

The oxygen saturation in all test concentration plots was between 76 % and 92 % (Table 2). The temperature was between 20.5 and 21.0 °C, the light intensity was between 815 and 789 Lux.
The pH was not influenced by the test item. At commencement of the incubation periods the pH in the control and test vessels was between 8.95 and 9.49 (Table 2); after 24 hours the pH was between 8.62 and 9.47.

Table 2: Oxygen saturation and pH throughout the test

Linevol concentration Mean measured (mg/L)	Oxygen saturation (%)		pH	
	1st incubation period			
	0 h	24 h	0 h	24 h
Control	85	88	9.32	9.24
1.09	87	90	9.28	8.62
3.08	87	88	9.16	9.26
6.28	82	90	8.95	8.94
18.0	87	92	8.98	9.32
41.3	87	90	9.10	8.91
	2nd incubation period			
	24 h	48 h	24 h	48 h
Control	78	87	9.33	9.28
1.09	79	86	9.34	9.29
3.08	78	84	9.49	9.47
6.28	76	88	9.35	9.21
18.0	83	87	9.41	9.32
41.3	82	89	9.38	9.26

7.2 Test item concentrations throughout the test

The stock solution (see chapter 6.3.3) for the preparation of test media was chemically analyzed and diluted to obtain the required nominal test concentrations spaced by a factor of 2.5. The chemical analysis of freshly prepared test media and of the aged solutions after 24 hours revealed test item losses between 24 and 30 %

only in the lowest test concentration. In the remaining four test concentrations losses of test item were maximum 18 %. The arithmetic means for the individual test intervals and the arithmetic means of these interval means (Table 3) demonstrate a sufficient spacing of the test item concentrations.
As Linevol 79 is a mixture containing 44 % C7-, 24 % C8-, and 30 % C9-alcohols and the method used for chemical analysis was optimized for rather apolar alcohols, the test item concentrations were extrapolated from the measured 1-nonanol concentrations (for recoveries and detailed results see annex 2 and annex 3).

Justification for the use of 1-nonanol for extrapolating Linevol 79 levels
The use of the C9-concentrations for the extrapolation of the Linevol 79 level is justified by the fact, that 1-nonanol is the most toxic component of the mixture consisting of C7, C8 and C9 alcohols.
Comparing experimental data on acute toxicity of the different alcohols to fathead minnow (*Pimephales promelas*) retrieved from the open literature (ECOTOX, US EPA), the LC_{50} values were found to be between 12.2-15 mg/L for 1-heptanol (7, 8), 34.5 and 37.9 mg/ for 1-octanol (9, 8) and 5,52 and 5.70 mg/L for 1-nonanol (8, 10) indicating highest toxicity of 1-nonanol. Modeling of the dependency of acute toxicity of pure linear long chain alcohols on lipophilicity (log K_{ow}) confirms increasing level of toxicity with increasing chain length. The LC_{50} for 96 hour acute fish toxicity were calculated to be 38, 13 and 5.5 mg/L for 1-heptanol, 1-octanol and 1-nonanol, respectively (Draft report 29/07/2005 on SDA, Peter Fisk Associates).

Experimental and modeled acute toxicity data of the effects of the different alcohols on *Daphnia magna* also show increasing toxicity with increasing molecular weight. Acute daphnia toxicity (24 hour EC_{50}) was experimentally found to be 94 mg/L for 1-heptanol and 47 mg/L for 1-octanol (11) and 48 hour EC_{50} values were 63 mg/L (12) and 31.8 mg/L (13), for branched heptanol and Octanol, respectively (experimental data on nonanol not available). Results of computer modeling using ECOSAR (14) show that these compounds cause acute toxicity at EC_{50} concentrations of 56 mg/L for heptanol, 22 mg/L for octanol and 7.5 mg/L for nonanol (14).

Data from fish and daphnia acute toxicity studies show a consistent trend of increasing toxicity as carbon number and molecular weight increases.
Therefore, the effect values obtained with the Linevol 79 concentrations extrapolated from measured 1-nonanol levels represent a worst case.

Table 3: Mean measured test item concentrations (mg/L). For details see annex 3

Nominal	Measured concentrations						
	0 h		24 h			0 - 24 h	
Linevol mg/L*	C9 mg/L	Linevol* mg/L	C9 mg/L	Linevol mg/L	% of initial	mean mg/L	
0	0.00	0	0	0	0	0	
0.92	0.35	1.17	0.27	0.89	76.2	1.03	
2.30	0.74	2.46	0.80	2.65	108	2.56	
5.76	1.63	5.42	1.76	5.87	108	5.65	
14.4	5.63	18.8	4.61	15.4	81.9	17.1	
36.0	12.6	41.8	11.6	38.6	92.3	40.2	
	24 h		48 h			24 - 48 h	0 – 48 h
Linevol mg/L*	C9 mg/L	Linevol* mg/L	C9 mg/L	Linevol mg/L	% of initial	mean mg/L	mean mg/L
0	0.00	0	0.00	0	0	0	
0.92	0.41	1.35	0.29	0.95	70.2	1.15	**1.09**
2.30	1.17	3.89	1.00	3.33	85.7	3.61	**3.08**
5.76	2.27	7.55	1.88	6.27	83.0	6.91	**6.28**
14.4	5.93	19.8	5.41	18.0	91.3	18.9	**18.0**
36.0	12.8	42.8	12.6	42.0	98.1	42.4	**41.3**

*extrapolated from C9 concentrations (C9 content of Linevol 79 = 30 %)

7.3 Results of the Daphnia test

The effect (acute immobilization) of the test item on *Daphnia magna* was tested using five concentrations arranged in a geometric series, spaced by a factor of 2.5. Based on the results of the range-finding test the selected concentration range was:

Nominal concentrations: 0.92, 2.30, 5.76, 14.4, and 36.0 mg Linevol/L
Mean measured concentrations: 1.09, 3.08, 6.28, 18.0, and 41.3 mg Linevol/L

The evaluation was based on the mean measured concentrations. The cumulative immobility of daphnids during the test period of 48 h (Table 4 and Figure 1) indicates a clear concentration-response relationship. The concentration of 1.09 mg/L did not

cause significant immobility. The NOEC was calculated to be 1.09 mg/L and the LOEC to be 3.08 mg test item/L.

After 48 hours the **EC_{50}** of the test item was determined to be **5.91 mg test item/L** (95 % confidence limits 44.3-71.5 µg/L)

Table 4: Cumulative immobility during the test period of 48 h

Linevol concentration mean measured (mg/L)	**24 h**				**48 h**				**48 h**
	beaker 1	beaker 2	beaker 3	beaker 4	beaker 1	beaker 2	beaker 3	beaker 4	**Sum (%)**
Control	0	0	0	0	0	0	0	0	0
1.09	0	0	0	0	1	0	0	0	5
3.08	0	2	0	2	0	3	2	3	40
6.28	0	0	1	2	1	2	2	2	35
18.0	4	3	4	4	4	4	5	4	85
41.3	5	5	5	5	5	5	5	5	100

Table 5: (No) effect concentrations (mg/L) of the test item.

	NOEC	**LOEC**	**EC_{10}**	**EC_{50}**	*C.I. of EC_{50}*
48 h	**1.09**	**3.08**	**1.52**	**5.91**	*4.27-8.20*

C.I.: 95 % confidence limits

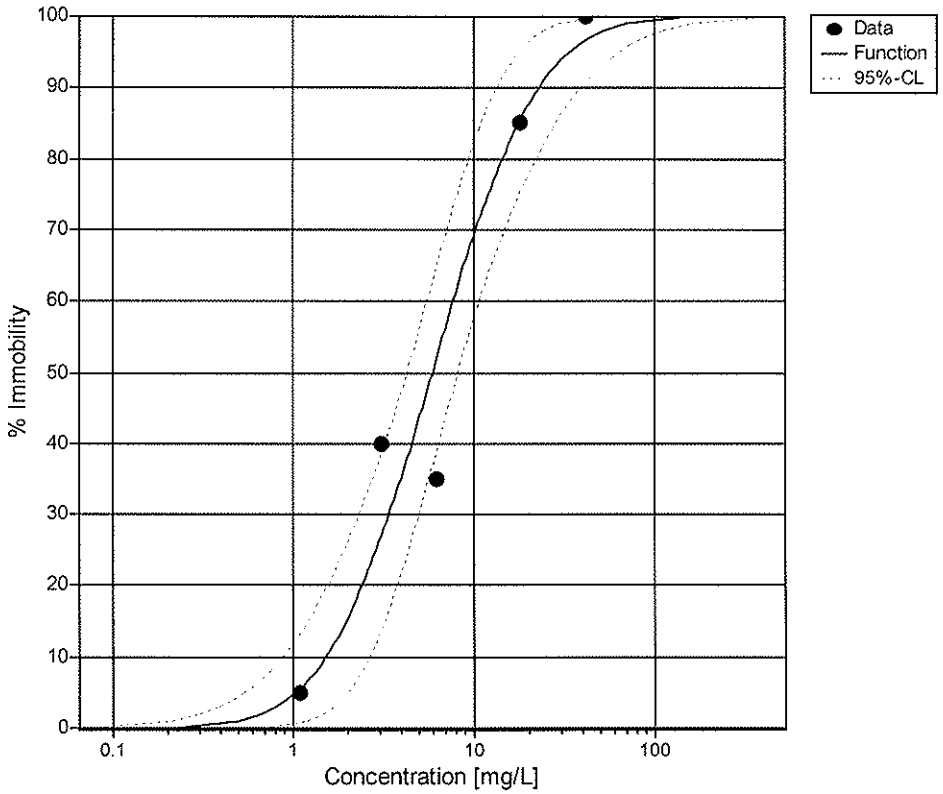


Figure 1: Concentration-effect relationship for the test item after 48 h

7.4 Validity of the test

The conditions of OECD guideline (1) for the validity of the test were adhered to: The immobility of controls in purified drinking water (dilution water) did not exceed 10 %.

8 Archiving

An aliquot of the test item, the test protocols, all raw data and all records necessary to reconstruct the study were archived in the GLP-archive of the Fraunhofer Institute for Molecular Biology and Applied Ecology, 57392 Schmallenberg, Germany, following internal SOP's according to (4).

List of archived records:

- data specifying the test item
- data concerning the test organisms (origin, culture conditions)

relevant correspondence between study director and monitor

- records of storage and storage conditions of test item
- original raw data of test (cell number/mL, test conditions, i.e. pH-values, temperature)
- records of chemical analyses
- records of statistical evaluation, if done
- original study plan including all amendments
- original final report

9 List of SOPs that were used in the study

The Generalia-SOPs as well as the following SOPs were used:

SOP No.	Title (translated)
0-017/02	Computer use
V4-502/02	Daphnia test, acute tox., Repro-test, D.-holding and breeding
V4-503/02	Daphnia test, acute tox., Repro-test, prep. of test solutions
V4-509/02	Daphnia test, acute immobilization, procedure
V4-910/02	Internal standardization of ecotoxicity tests
V7-208/02	Mass spectrometry
G3-004/02	Scales, calibration
G3-005/02	Checking of volumetric apparatus
G3-006/03	Checking of piston-operated pipettes
G3-008/03	Refrigerator/freezer, control
G3-009/02	Shaker
G4-005/02	Clean-bench, operation
G4-043/01	pH-Meter, WTW 526
G4-210/02	Light measurement: Illuminance meter LI-189 with radiation sensor, Fa. LI-COR
G4-303/02	WTW pH-Meter pH 196, operation, calibration
G5-109/02	Safety clean bench, Haereus
G5-134/02	Autoclave Varioklav
G7-170/02	Pure water preparation unit UHQ/PS, manual.
G7-183/02	Cleaner Miele with Aquapurificator. Manual.
G7-199/02	GC-Autosampler HP 7673, Hewlett Packard
G7-203/04	GC/MS HP 5972 MSD
G7-226/02	Eppendorf benchtop centrifuge 5415 C
G7-177/02	Use of MIELE-cleaning machine G7783

10 References

1. OECD Guideline for Testing of Chemicals, Sect. 2: Effects on Biotic Systems, No. 202 "*Daphnia sp.*, Acute Immobilization Test and Reproduction Test". Adopted April 4th 1984.
2. Official Journal of the European Communities No. L383 A/172. C2: Acute Toxicity for Daphnia (1992).
3. OECD (Organisation for Economic Cooperation and Development): OECD Principles of Good Laboratory Practice (as revised in 1997), Paris, 1998.
4. Grundsätze der Guten Laborpraxis (Principles of Good Laboratory Practice, GLP) in: Bundesgesetzblatt 2002 Teil I Nr. 40 from 27. June 2002, 2090-2130.
5. Finney. D.J.: Statistical Method in Biological Assay. 2nd ed., London. 1984.
6. ToxRat® Professional 2.09. ToxRat® Solutions GmbH, Naheweg 15, D-52477 Alsdorf (http://www.toxrat-solutions.de).
7. Geiger, D.L., D.J. Call, and L.T. Brooke (1988). Acute Toxicities of Organic Chemicals to Fathead Minnows (Pimephales promelas), Vol. 4, Center for Lake Superior Environmental Stud., Univ.of Wisconsin-Superior, Superior, WI I:355
8. Broderius, S., and M. Kahl (1985). Acute Toxicity of Organic Chemical Mixtures to the Fathead Minnow. Aquat.Toxicol. 6:302-322 (Author Communication Used)
9. Geiger, D.L., S.H. Poirier, L.T. Brooke, and D.J. Call (1986). Acute Toxicities of Organic Chemicals to Fathead Minnows (Pimephales promelas), Vol. 3. Center for Lake Superior Environmental Stud., Univ.of Wisconsin-Superior, Superior, WI I:328
10. Brooke, L.T., D.J. Call, D.L. Geiger, and C.E. Northcott (1984). Acute Toxicities of Organic Chemicals to Fathead Minnows (Pimephales promelas), Vol. 1. Center for Lake Superior Environmental Stud., Univ.of Wisconsin-Superior, Superior, WI :414
11. Bringmann, G. and R. Kühn (1977). The Effects of Water Pollutants on *Daphnia magna*. Wasser-Abwasser-Forsch. 10(5):161-166(English translation, german, English abstract).
12. Canton, J.H., and D.M.M. Adema. (1978). Reproducibility of Short-term and Reproduction Toxicity Experiments with Daphnia magna and Comparison of the Sensitivity of Daphnia magna with Daphnia pulex and Daphnia cucullata in Short-term Experiments. Hydrobiologica 59(2): 135-140.
13. Union Carbide Corp. (1980). "The Acute Toxicity of MRD-80-4 to the Water Flea (Daphnia magna Straus)." Unpublished report. Cited in http://www.epa.gov/chemrtk/alkal613/c13590rt.pdf
14. ECOSAR, aquatic toxicity modeling program, subroutine contained in EPIWIN model: EPIWIN (1999). Estimation Program Interface for Windows, version 3.02. Syracuse Research Corporation, Syracuse, NY, USA.

15. ExxonMobil Chemical Company (2003). High Production Volume (HPV) Chemicals Challenge Program. Test Plan for the alkyl alcohols C6-C13 category. Prepared by: ExxonMobil November 15,2001, (1st Revision February 5, 2002, 2nd Revision February 28, 2003), 201-15132A (http://www.epa.gov/chemrtk/alkal613/c13590rt.pdf, access on September 5, 2005).

16. European Commission, Directorate General Health and Consumer Protection: SANCO/825/00 rev. 6 (20/06/2000), Guidance document on residue analytical methods

17. European Commission, Directorate General Health and Consumer Protection: SANCO/3029/99 rev.4 (11/07/2000), Residues: 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.

Annex 1: Analytical method for the determination of Linevol 79 in water

Principle of method and method summary

The method refers to the determination of 1-heptanol, 1-octanol und 1-nonanol (C_7-, C_8- and C_9-fatty alcohol) in aqueous test samples in the concentration range from 1.25 to 625 µg/L for each alcohol. Based on 1-nonanol this corresponds to concentrations of Linevol 79 from about 4 to 2000 µg/L. Samples with higher concentrations were diluted before analysis to meet the concentration range mentioned. The analytes were extracted from the daphnia test media by liquid-liquid partitioning with n-hexane. After shaking and settling the n-hexane extract was removed and the analytes derivatized using MSTFA (n-Methyl-trimethylsilyl-trifluoracetamid). Measurement was performed by GC-MS in SIM mode using internal standard calibration with n-hexanol-d_{13} as internal standard.

Equipment and chromatographic conditions

GC/MSD system

Mass spectrometer:	MSD HP 5972 (Hewlett-Packard/Agilent)
Gas chromatograph:	HP 5890 (Hewlett-Packard/Agilent)
Autosampler:	HP 7673 (Hewlett-Packard/Agilent)

GC-MS Parameter

column:	SGE BPX-5, 50 m * 0.32 mm, Film 0.25 mm
transfer line temp.:	280 °C
injector:	Split-splitless
injection volume:	1 µL
pressure:	20 kPa, 60 °C
carrier gas:	Helium, 1 mL/min
MS-mode:	SIM (m/z 172, 173, 187, 201)
Temperature program	temp 1: 60 °C
	time 1: 1 min
	rate 1: 10 °C/min
	temp 2: 180 °C
	time 2: 1 min

Reagents

n-hexane:	95-99.5 %, Baker
MSTFA:	N-tert.-butyldimethylsilyl-N-methyl-trifluoroacetamide, 97% Fluka

methanol:	HPLC grade, Baker
reference items:	1-heptanol, GC 99.5 %, Ehrenstorfer 30508 1-octanol, GC 99.5 %, Ehrenstorfer 20122 1-nonanol, GC 99.5 %, Ehrenstorfer 30403
internal standard:	n-hexanol-d_{13}; 98 At. %D, deuterated standard, Chemotrade

Solutions

Stock solutions of the reference items and n-hexanol-d_{13} (IS)

1-heptanol, 1-octanol and 1-nonanol:	1 g/L each
solution of internal standard in n-hexane:	50 mg/L

Calibration solutions of the reference items: 0.1, 0.5, 1.0, 5.0, 10, 25 and 50 mg/L
corresponding to sample concentrations in the range from 1.25 µg/L to 625 µg/L

Fortification solutions (for recovery experiments): 100 and 1000 mg/L

Calibration

Calibration of the method was performed by chromatography of the calibration solutions after silylation. Using the concentration/peak area data of the reference items and the internal standard the calibration line was calculated by linear regression using internal standard method.

Sample preparation

400 mL of the aqueous test sample was shaken with 5 mL of n-hexane in a 1 L bottle for 20 min (shaking machine). Then the content was transferred into a 500 mL beaker. The bottle was washed with water and the beaker made up to 500 mL. After phase separation 100 µL of the upper n-hexane layer was removed and pipetted into a 1.5 mL vial. 50 µL MSTFA and 50 µL IS solution were added and mixed by shaking for some seconds. Then the extract was transferred into a 300 µL vial insert. Measurement was performed by GC-MS under the conditions given above. Backup samples were created by pipetting 200 µL of the n-hexane extracts in separate vials.

Recovery

The following recovery experiments were performed:
500 mL water + 100 µL fortification solution 1 (-> 20 µg/L, five replicates)
500 mL water + 100 µL fortification solution 2 (-> 200 µg/L, five replicates)
The fortification solutions were added into the water phase by a pipette. After mixing (Vortex) for 1 min the samples were processed like real samples (see 'Sample preparation').
2 blank samples (water without spikes) were processed in parallel.

Typical calibration line and chromatograms

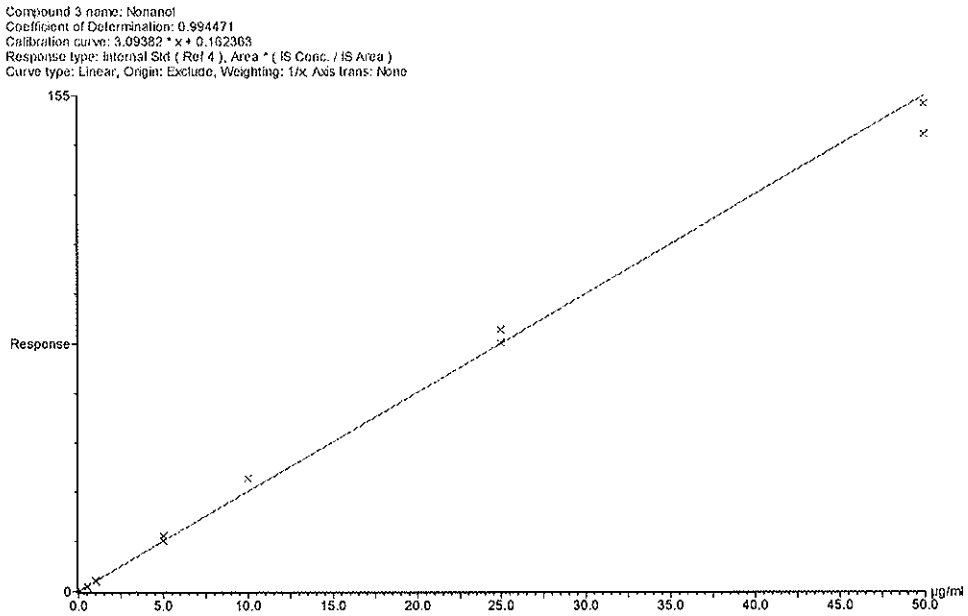


Figure 2: Typical calibration line of 1-nonanol

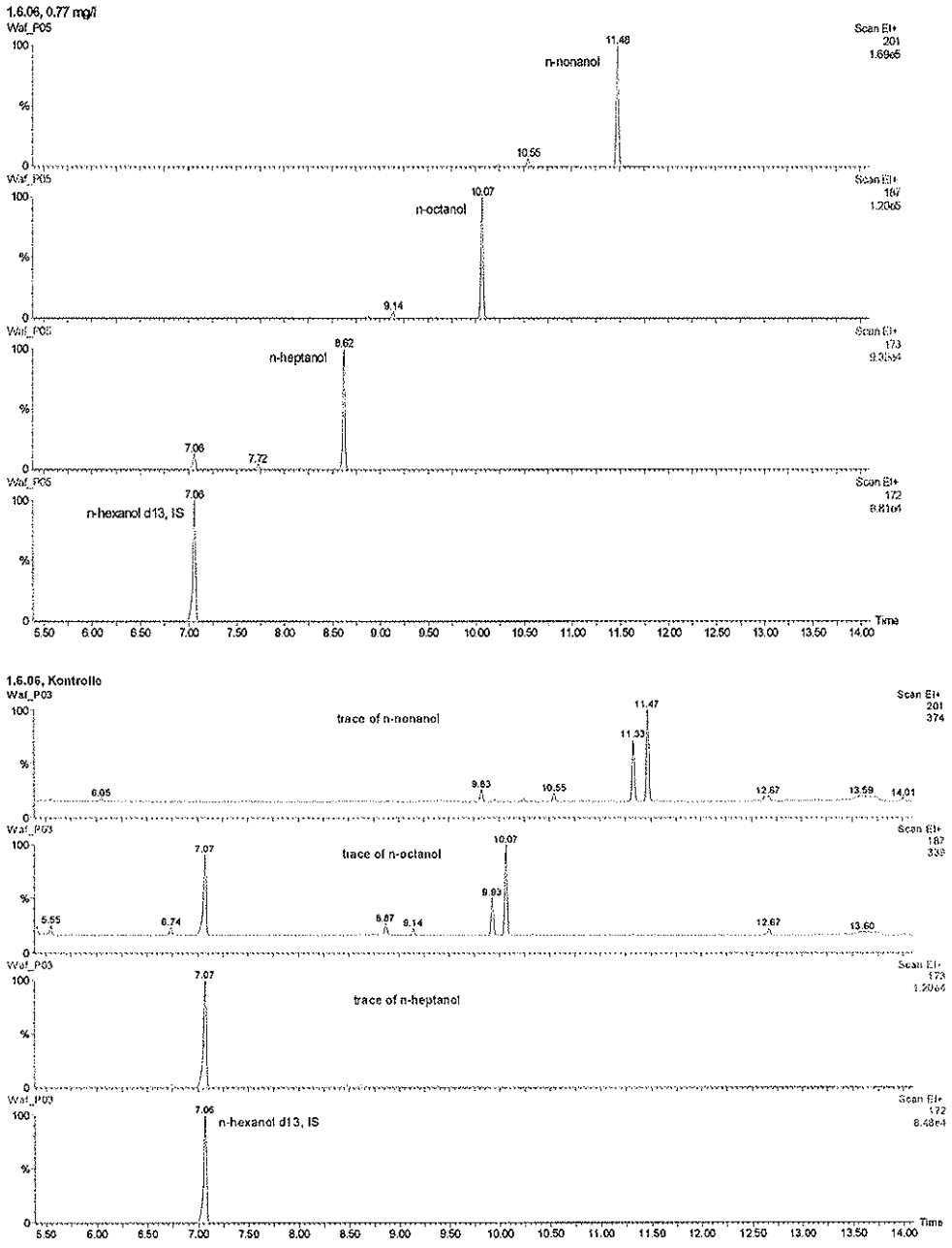


Figure 3: Typical chromatograms
upper: water sample, concentration of 1-nonanol = 350 µg/L
lower: control sample, concentration of 1-nonanol < 20 µg/L

Annex 2: Validation of the analytical method

The validation of the analytical method was based on the guidelines SANCO/825/00 rev. 6 and SANCO/3029/99 ver. 4 (16, 17). The guidelines describe the pesticide pre- and post-registration data requirements. According to these guidelines the analytical method was validated in respect to specificity, linearity, accuracy, precision, identity and limit of quantification (LOQ).
The validation of the method was performed for 1-nonanol as the results of the study were based on the 1-nonanol analytical results.

Specificity

The method was found to be sufficiently specific for the determination of Linevol 79. The blanks as well as the test samples showed no interfering peaks. A typical chromatogram of all compounds is shown in Figure 3.

Linearity/Calibration

The method was calibrated in the range from 0.1 to 50 mg/L for the reference items 1-heptanol, 1-octanol and 1-nonanol using 7 calibration levels. This corresponds to a range of 0.3 to about 170 mg/L for Linevol 79. Linear regressions of the peak responses and the concentrations were found resulting in typical correlation coefficients of $r > 0.99$. An example calibration line for 1-nonanol is shown in Figure 2.

Accuracy

The results are shown in Table 6.

- The recoveries from water fortified with 1-nonanol at 20 µg/L ranged from 71 % to 91 %. The mean recovery of five replicates was 83 %.
- The recoveries from water fortified with 1-nonanol at 200 µg/L ranged from 99 % to 126 %. The mean recovery of five replicates was 103 %.
- The overall recovery values from water fortified at two levels was 93 %.

Precision

The precision of the method is reported as the repeatability of recovery of 1-nonanol at each fortification level. The results are shown in Table 6.

- The relative standard deviation of recoveries from water fortified with 1-nonanol at 20 µg/L was 9.5 %.
- The relative standard deviation of recoveries from water fortified with 1-nonanol at 200 mg/L was 15.5 %.

- The overall relative standard deviation of recoveries from water fortified with 1-nonanol at two levels was 16.8 %.
- The precisions were below 20 % for every concentration level.

Table 6: Summary of recovery data

Compound	Fortification level [µg/L]	Recovery [%]	Mean Recovery [%]	Recovery RSD [%]
1-Heptanol	0	-	-	-
	0	-	-	-
	20	10.6	12.9	11.4
	20	12.9		
	20	13.2		
	20	13.3		
	20	14.7		
	200	15.1	15.5	9.5
	200	13.2		
	200	15.8		
	200	16.2		
	200	17.1		
		Overall values:	**14.2**	**13.6**
1-Octanol	0	-	-	-
	0	-	-	-
	20	41.3	44.8	8.1
	20	48.3		
	20	47.6		
	20	40.5		
	20	46.1		
	200	53.7	56.1	12.5
	200	46.2		
	200	55.7		
	200	59.9		
	200	65.0		
		Overall values:	**50.4**	**15.8**

Table 6: Summary of recovery data (continued)

Compound	Fortification level [µg/L]	Recovery [%]	Mean Recovery [%]	Recovery RSD [%]
1-Nonanol	0	-	-	-
	0	-	-	-
	20	83.9	83.3	9.5
	20	90.8		
	20	89.6		
	20	71.2		
	20	80.8		
	200	98.7	102.5	15.5
	200	81.4		
	200	101.0		
	200	105.8		
	200	125.8		
		Overall values:	**92.9**	**16.8**

Identity

The identity of the test item was approved by the interpretation of the mass fragments (m/z ratio 172, 173, 187 and 201) and their relation obtained by mass spectrometric detection.

Limit of Quantification (LOQ)

The limit of quantification - at the lowest calibration level - was 20 µg/L for 1-nonanol corresponding to 67 µg/L Linevol 79. Blank values were always below the LOQ.

Annex 3: Detailed results of chemical analyses

Table 7: Measured concentrations of 1-heptanol

Medium	time	Linevol	1-heptanol			Linevol*	% of
		expected	1	2	mean		initial
	h	mg/L	mg/L	mg/L	mg/L	mg/L	
1	0	0.00	< 0.02	< 0.02	-	-	-
		0.92	0.21	0.21	0.21	0.48	-
		2.30	0.47	0.44	0.45	1.03	-
		5.76	0.88	0.80	0.84	1.91	-
		14.4	2.36	2.67	2.51	5.71	-
		36.0	6.25	6.44	6.35	14.4	-
	24	0.00	< 0.02	< 0.02	-	-	-
		0.92	0.17	0.18	0.17	0.39	82.1
		2.30	0.45	0.47	0.46	1.05	102.0
		5.76	1.04	0.98	1.01	2.30	120.0
		14.4	2.69	2.59	2.64	6.00	105.1
		36.0	7.22	6.88	7.05	16.0	111.0
2	24	0.00	< 0.02	< 0.02	-	-	-
		0.92	0.19	0.18	0.18	0.41	-
		2.30	0.46	0.48	0.47	1.07	-
		5.76	1.16	0.96	1.06	2.41	-
		14.4	2.57	2.72	2.65	6.01	-
		36.0	6.53	5.77	6.15	14.0	-
	48	0.00	< 0.02	< 0.02	-	-	-
		0.92	0.16	0.16	0.16	0.36	87.0
		2.30	0.45	0.45	0.45	1.02	95.0
		5.76	0.96	0.89	0.92	2.09	87.0
		14.4	2.46	2.46	2.46	5.59	93.0
		36.0	6.33	5.90	6.11	13.9	99.4

Linevol expected mg/L	mean 0 - 24 h mg/L	mean 24 - 48 h mg/L	mean 0 - 48 h mg/L
0.00	-	-	-
0.92	0.43	0.39	0.41
2.30	1.04	1.05	1.04
5.76	2.10	2.25	2.18
14.4	5.86	5.80	5.83
36.0	15.2	13.9	14.6

* extrapolated from 1-heptanol concentrations (C7 content of Linevol 79 = 44 %)

Table 8: Measured concentrations of 1-octanol

Medium	time	Linevol	1-octanol			Linevol*	% of
		expected	1	2	mean		initial
	h	mg/L	mg/L	mg/L	mg/L	mg/L	
1	0	0.00	< 0.02	< 0.02	-	-	-
		0.92	0.19	0.19	0.19	0.80	-
		2.30	0.42	0.38	0.40	1.67	-
		5.76	0.87	0.79	0.83	3.47	-
		14.4	2.57	2.87	2.72	11.3	-
		36.0	6.11	6.19	6.15	25.6	-
	24	0.00	< 0.02	< 0.02	-	-	-
		0.92	0.15	0.16	0.16	0.65	81.2
		2.30	0.40	0.43	0.42	1.74	104.0
		5.76	0.96	0.90	0.93	3.88	112.0
		14.4	2.40	2.34	2.37	9.88	87.2
		36.0	6.24	5.90	6.07	25.3	98.7
2	24	0.00	< 0.02	< 0.02	-	-	-
		0.92	0.20	0.19	0.20	0.83	-
		2.30	0.49	0.51	0.50	2.09	-
		5.76	1.21	1.00	1.11	4.62	-
		14.4	2.75	2.86	2.81	11.7	-
		36.0	6.62	5.73	6.18	25.7	-
	48	0.00	< 0.02	< 0.02	-	-	-
		0.92	0.17	0.17	0.17	0.69	83.8
		2.30	0.47	0.49	0.48	2.00	95.9
		5.76	0.99	0.93	0.96	4.00	86.6
		14.4	2.59	2.61	2.60	10.8	92.7
		36.0	6.48	6.03	6.25	26.1	101.0

Linevol	mean	mean	mean
expected	0 - 24 h	24 - 48 h	0 - 48 h
mg/L	mg/L	mg/L	mg/L
0.00	-	-	-
0.92	0.73	0.76	0.74
2.30	1.71	2.04	1.87
5.76	3.68	4.31	3.99
14.4	10.6	11.3	10.9
36.0	25.5	25.9	25.7

* extrapolated from 1-octanol concentrations (C8 content of Linevol 79 = 24 %)

Table 9: Measured concentrations of 1-nonanol

Medium	time	Linevol	1-nonanol			Linevol*	% of
		expected	1	2	mean		initial
	h	mg/L	mg/L	mg/L	mg/L	mg/L	
1	0	0.00	< 0.02	< 0.02	-	-	-
		0.92	0.34	0.36	0.35	1.17	-
		2.30	0.76	0.71	0.74	2.46	-
		5.76	1.71	1.54	1.63	5.42	-
		14.4	5.32	5.95	5.63	18.8	-
		36.0	12.4	12.7	12.6	41.8	-
	24	0.00	< 0.02	< 0.02	-	-	-
		0.92	0.26	0.27	0.27	0.89	76.2
		2.30	0.78	0.81	0.80	2.65	108
		5.76	1.83	1.69	1.76	5.87	108
		14.4	4.71	4.52	4.61	15.4	81.9
		36.0	11.8	11.3	11.6	38.6	92.2
2	24	0.00	< 0.02	< 0.02	-	-	-
		0.92	0.42	0.39	0.41	1.35	-
		2.30	1.14	1.20	1.17	3.89	-
		5.76	2.48	2.05	2.27	7.55	-
		14.4	5.75	6.11	5.93	19.8	-
		36.0	13.6	12.1	12.8	42.8	-
	48	0.00	< 0.02	< 0.02	-	-	-
		0.92	0.28	0.29	0.29	0.95	70.2
		2.30	0.99	1.01	1.00	3.33	85.7
		5.76	1.92	1.84	1.88	6.27	83.0
		14.4	5.36	5.47	5.41	18.0	91.3
		36.0	13.0	12.1	12.6	42.0	98.1

Linevol expected mg/L	mean 0 - 24 h mg/L	mean 24 - 48 h mg/L	mean 0 - 48 h mg/L
0.00	-	-	-
0.92	1.03	1.15	1.09
2.30	2.56	3.61	3.08
5.76	5.65	6.91	6.28
14.4	17.1	18.9	18.0
36.0	40.2	42.4	41.3

* extrapolated from 1-nonanol concentrations (C9 content of Linevol 79 = 30 %)

Annex 4: Statistical analysis of the Daphnia test

4.1 Results of the Probit analysis (ToxRat® Report)

Effective Concentrations (ECx) with mobility at 48 h: Parameters of the Probit analysis

Table 10: Parameters of the probit analysis: Results of the regression analysis

Parameter	Value
Computation runs:	5
Slope b:	2.17217
Intercept a:	3.32331
Variance of b:	0.12711
Goodness of Fit	
Chi^2:	4.75994
Degrees of freedom:	3
$p(Chi^2)$:	0.19024
Log EC_{50}:	0.77190
s Log EC_{50}:	0.17837
F:	23.396
p(F) (df: 1;3):	0.017

Chi^2 is a goodness of fit measure. If the probability, $p(Chi^2)$, 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 (= are made wider; so, check whether these results are reasonable!).

Results of the probit analysis

Table 11: Results of the probit analysis: Selected effective concentrations (ECx) of the test item and their 95%- and 99%-confidence limits

Parameter	EC_{10}	EC_{20}	EC_{50}
Value [mg/L]	1.523	2.430	5.914
lower 95%-cl	0.874	1.561	4.265
upper 95%-cl	2.652	3.782	8.201
lower 99%-cl	0.734	1.359	3.849
upper 99%-cl	3.157	4.346	9.088

n.d.: not determined due to mathematical reasons

Inhibitions lower equal 0% or greater equal 100% were replaced by 0.100 and 99.900, respectively. Slope function after Litchfield and Wilcoxon: 2.886

(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.)

Threshold concentrations (NOEC) with Mobility at 48 h

Fisher`s Exact Binomial Test with Bonferroni Correction

Table 12: Fisher`s Exact Binomial Test with Bonferroni Correction.

Pair-wise comparisons between treatment and control on the multiple significance level (alpha is 0.05; one-sided greater). Pair-wise 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*.

Treatm.[mg/L]	Introduced	Mobile	Immobile	% Immobility	p	alpha*	sign.
Control	20	20	0	0.0			
1.09	20	19	1	5.0	1.000	0.050	-
3.09	20	12	8	40.0	0.003	0.017	+
6.28	20	13	7	35.0	0.008	0.025	+
17.99	20	3	17	85.0	<0.001	0.013	+
41.29	20	0	20	100.0	<0.001	0.010	+

+: significant; -: non-significant

A NOEC of 1.09 mg/L is suggested by the program.

Summary of Results for all Endpoints

Table 13: Summary of results for all endpoints.

Critical effect and threshold concentration as observed at end of experimental time; EC: Effective concentration for xx% reduction; 95%-CL: 95% Confidence limits; LOEC: Lowest observed effect concentration; NOEC: No observed effect concentration

Critical Concentrations [mg/L]		0-24 h	0-48 h
Mobility			
	EC_{10}	3.251	1.523
95%-CL	lower	2.100	0.874
	upper	5.032	2.652
	EC_{20}	4.720	2.430
95%-CL	lower	3.306	1.561
	upper	6.738	3.782
	EC_{50}	9.594	5.914
95%-CL	lower	7.196	4.265
	upper	12.792	8.201
	LOEC	17.990	3.090
	NOEC	6.280	1.090

n.d.: not determined due to mathematical reasons

Annex 5: Certificates of analysis (test and reference items)

Shell Chemicals
Shell Chemicals Limited

EINGEGANGEN
18. Okt. 2004
am .....

CERTIFICATE OF ANALYSIS

Certificate No: 00000970
Date: 09.03.2004

Ship to:

Shell References:

Customer References:

Order number:
Delivery note:

Y order num:
Transport Id:

Product name: LINEVOL 79
Batch Number: T3608B AL6940-F

Inspection number:

Property	Method	Units	Analysis
Colour	ASTM D1209-00	Pt-Co	<5
Carbon Distribution <C7	SMS 2914-02	% m/m	1
Carbon Distribution C7		% m/m	44
Carbon Distribution C8		% m/m	24
Carbon Distribution C9		% m/m	30
Carbon distribution >C9		% m/m	1
Normality		% m/m	83.6
Mean relative molecular mass		n/a	127
Carbonyl, (as C=O)	SMS 2097-02	mg/kg	10
Water Content	ASTM D1364-02 mod.	% m/m	0.03
Density @ 20 deg C	ASTM D4052-96	kg/l	0.825
Saponification Value	SMS 2916-02	mg KOH/g	<0.1
Hydrocarbons	SMS 2914-02	% m/m	0.11
Diols	UK 3704-03	% m/m	<0.01
Hydroxyl Value	SMS 2914-02	mg KOH/g	342

For Shell Chemicals U.K. Limited

Clive Chatterton

Shell Global Solutions UK, Stanlow Laboratory
(A UKAS Testing Laboratory No 1158)

Page 1 of 1

Certificate of analysis (reference item 1-heptanol)

Certificate of Analysis

Dr. Ehrenstorfer

Reference Materials for Residue Analysis

Product Identification

14127100 1-Heptanol
CA 1-Heptanol
IUPAC 1-Heptanol
Formula C7H16O
Mol.Weight 116.20
CAS No. 111-70-6

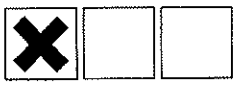
Expiry Date 01.05.2009
Lot Number 30508
Store at 20 °C

Please note: The expiry date is valid under recommended storage conditions only.

Physical Data

Phase liquid
Color colourless
Melting Range

Toxicological Data



R Code 36/37/38
S Code 26-36
LD50 (Rats female/male in mg/kg) N/A

Analytical Data

Method 1	GC/MSD	Inj. Volume (µl)	RT1 (min.) 8.70
Column	DB-5, 60 m, ID 0.25 mm	Inj. Temp. 200	Col. Temp. 40-200
Method 2		Inj. Volume (µl)	RT2 (min.)
Column		Flow (ml/min)	Gradient
Eluent A			
Eluent B			
Identity check	MS		
Comment			

Water Content 0.3 % Determined by Karl-Fischer Titration
Det. Purity 99.5 % Tolerance +/- 0.5 %

Please note: Results are based on a minimum of three determinations. Vapour pressure and solubility information according to literature.

Certified on 09.05.2003
by Dr. J. Heidrich

Labor Dr. Ehrenstorfer-Schäfers Bgmt.-Schlosser-Str. 6 A 86199 Augsburg · Germany
Phone +49 821 906080 Fax +49 821 9060888 info@analytical-standards.com

ISO 9001

Certificate of analysis (reference item 1-octanol)

Certificate of Analysis

Dr. Ehrenstorfer

Reference Materials for Residue Analysis

Product Identification

15711100 1-Octanol
CA 1-Octanol
IUPAC 1-Octanol
Formula C8H18O
Mol.Weight 130.23
CAS No. 111-87-5

Expiry Date 01.01.2008
Lot Number 20122
Store at 20 °C

Please note: The expiry date is valid under recommended storage conditions only.

Physical Data

Phase liquid
Color colourless
Melting Range

Vapour pressure N/A at °C
Solubility in water N/A at °C
Solubility in N/A N/A at °C

Toxicological Data

R Code 20/21/22-36/37/38
S Code 26-36
LD50 (Rats female/male in mg/kg) N/A

Analytical Data

Method 1 GC/FID
Column 3% OV 11 on Chromosorb W-HP
Inj. Volume (µl)
Inj. Temp. 260
RT 1 1,58
Col. Temp. 120

Method 2
Column
Eluent A
Eluent B
Inj. Volume (µl)
Flow (ml/min)
RT 2
Gradient

Comment

Water Content 0,1 % Determined by Karl-Fischer Titration
Det. Purity 99,5 % Tolerance +/- 0,5 %

Please note: Results are based on a minimum of three determinations. Vapour pressure and solubility information according to literature.

Certified on 25.01.2002
by Dr. J. Heidrich

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ISO 9001

Certificate of analysis (reference item 1-nonanol)

Certificate of Analysis

Dr. Ehrenstorfer

Reference Materials for Residue Analysis

Product Identification

15623200 1-Nonanol
CA 1-Nonanol
IUPAC 1-Nonanol
Formula C9H12O
Mol.Weight 144.26
CAS No. 143-08-8

Expiry Date 01.10.2008
Lot Number 21025
Store at 20 °C

Please note: The expiry date is valid under recommended storage conditions only

Physical Data

Phase liquid
Color colourless
Melting Range

Toxicological Data

R Code 23/24/25-37
S Code 45-36/37/39
LD50 (Rats female/male in mg/kg) N/A

Analytical Data

Method 1	GC/MSD	Inj. Volume (µl)		RT 1	16.45
Column	DB-5, 60 m, ID 0.25 mm	Inj. Temp.	200	Col. Temp.	40-200
Method 2		Inj. Volume (µl)		RT 2	
Column		Flow (ml/min)		Gradient	
Eluent A					
Eluent B					

Identity check MS
Comment

Water Content 0.1 % Determined by Karl-Fischer Titration
Det. Purity 99.6 % Tolerance +/- 0.5 %

Please note: Results are based on a minimum of three determinations. Vapour pressure and solubility information according to literature

Certified on 29.10.2002
by Dr. J. Heidrich

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ISO 9001

Annex 6: GLP Certificate

Ministerium für Umwelt, Raumordnung und Landwirtschaft
des Landes Nordrhein-Westfalen

Postanschrift: 40190 Düsseldorf

Aktenzeichen: VI-3- 31.11.79.05

GLP-Bescheinigung

Bescheinigung

Hiermit wird bestätigt, dass die Prüfeinrichtung

in D-57392 Schmallenberg, Auf dem Aberg 1
(Ort, Anschrift)

Fraunhofer Institut für Molekularbiologie und

Angewandte Oekologie (IME)
(Firma)

vom 11. November- 13. November 2002
(Datum)

von der für die Überwachung zuständigen Behörde über die Einhaltung der Grundsätze der Guten Laborpraxis inspiziert worden ist.

Es wird hiermit bestätigt, dass folgende Prüfungen in dieser Prüfeinrichtung nach den Grundsätzen der Guten Laborpraxis durchgeführt werden.

Certificate

It is hereby certified that the test facility

in D-57392 Schmallenberg, Auf dem Aberg 1
(location, address)

Of Fraunhofer Institut für Molekularbiologie und

Angewandte Oekologie (IME)
(company name)

on 11 until 13 November 2002
(date)

was (were) inspected by the competent authority regarding compliance with the Principles of Good Laboratory Practice.

It is hereby certified that following studies in this test facility are conducted in compliance with the Principles of Good Laboratory Practice.

GLP-Certificate continued

Kategorie 1	**category 1**
Prüfungen zur Bestimmung der physikalisch-chemischen Eigenschaften und Gehaltsbestimmungen	physical-chemical testing
Kategorie 4	**category 4**
Ökotoxikologische Prüfungen zur Bestimmung der Auswirkungen auf aquatische und terrestrische Organismen	environmental toxicity studies on aquatic and terrestrial organisms
Kategorie 5	**category 5**
Prüfungen zum Verhalten im Boden, im Wasser und in der Luft; Prüfungen zur Bioakkumulation und zur Metabolisierung	studies on behaviour in water, soil and air; bioaccumulation
Kategorie 6	**category 6**
Prüfungen zur Bestimmung von Rückständen	residue studies
Kategorie 7	**category7**
Prüfungen zur Bestimmung der Auswirkungen auf Mesokosmen und natürliche Ökosysteme	studies on effects on mesocosms and natural ecosystems
Kategorie 9	**category 9**
Modell- und Simulationsrechnungen für das Verhalten von Stoffen in der Umwelt	mathematical modelling and simulation of the environmental fate of chemicals

Düsseldorf, 19. Februar 2003

Im Auftrag

(Prof. Dr. Heinrich David)

Dienstsiegel
(official-seal)