Fraunhofer Institut
Molekularbiologie und
Angewandte Oekologie

Study Report

Daphnia magna, reproduction test in closed vessels following OECD 211

C_{10} Fatty alcohol

GLP-Code of Testing Facility: SDA-005/4-21

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October 27, 2005

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Preface: introductory information generated by non-GLP pre-studies

This study is one of a series of studies performed to assess the chronic toxicity of fatty alcohols with a carbon chain length of ≥ 10 to *Daphnia magna*. The challenge was to reproducibly produce concentrations of selected fatty alcohols without addition of solubilisation agents at concentrations expected to be close to the water solubility limit; furthermore to maintain these concentrations in water despite the rapid degradability of these test items. This was approached by daily renewal of test solutions and preparation of individual test concentrations under sterile slow stir (21 h) conditions. The studies were performed in closed vessels on a clean bench. Non-GLP pre-studies were performed to develop and optimize this approach (ref. 13, 14). The study protocol is the result of these pre-studies and accounted for the following aspects:

- The test solutions of fatty alcohols were not filtered as it was shown that the filtration procedure caused highly variable and much lower test concentrations than the water solubility predicted from physico-chemical models (ref. 12). An important aspect was to ensure that these test items were tested up to their water solubility limit or as close as practically possible.
- When omitting a filtration procedure, the concentrations were clearly higher than the solubility predicted by different models based on physico-chemical properties and there was no clear indication of a trend to saturation. It was therefore concluded that the measured concentrations comprised a fraction of dissolved test substance and a fraction of finely dispersed material. It was considered technically impossible to avoid the presence of very fine undissolved particles or droplets at the higher test concentrations.
- It was shown that algae, the food source for *Daphnia magna,* are responsible for the degradation of approximately 60% of C12 fatty alcohol after 24 h (ref 13). It was subsequently demonstrated that feeding autoclaved algae is not appropriate for meeting the validity criteria for number of offspring produced.
- The daily transfer of *Daphnia magna,* although thoroughly rinsed, did result in re-introduction of associated bacteria, which increased in number with growth of carapace and resulted in more pronounced losses in the last two weeks of the reproduction studies.
- Due to the unavoidable biodegradation processes, which have to be accepted as an intrinsic and realistic feature of the test procedure, severe oxygen depletion occurred. In contradiction to the test guideline, cautious aeration with sterile filtered air was implemented to overcome this.
- Due to the seasonal differences in reproductive performance of *Daphnia magna,* i.e., a depression in the winter, the GLP studies were performed between March and November 2005.

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Summary

A study sponsored by The Soap and Detergent Association, Washington D.C., USA, was performed at the Fraunhofer Institute for Molecular Biology and Applied Ecology (IME) to determine the effects of a chronic exposure to C_{10} fatty alcohol on the reproduction of aquatic invertebrates, represented by *Daphnia magna*.

The test item solutions were prepared by slow stirring (21 h) of the test substance in test media. The test was carried out under sterile conditions, in closed vessels and with daily renewal of the test solutions. The study was conducted at nominal concentrations of 0, 152, 411, 1110, 3000 µg test item/L. Each treatment group consisted of 10 replicates each of one *Daphnia magna* (individual exposure). The *Daphnia magna* were rinsed with sterilized water before being transferred into new test media on each day. Effects on growth (adult length at test termination) and reproductive performance were investigated.

Freshly prepared test solutions, obtained before distribution to the replicates, and aged test solutions, obtained before the renewal on the next day of all test concentrations, were sampled three times a week and analysed by GC-MS for content of the test item at the LOQ of 1.0 µg/L. Pooled samples were taken at each concentration from vessels 1-5 and 6-10for the measurement of aged test solutions.

Results

The mean measured test item concentrations of the freshly prepared test solutions were 1.6, 122, 351, 962, 2800 µg/L, which were approximately 85 % of the nominal concentrations. At the beginning of the test, test item losses during 24 h were between 60% and 85% at the highest and lowest test item concentration, respectively. During the last week of the test, the losses exceeded 95% at all treatment levels. The losses were caused by adsorption and degradation by food algae and degradation by adapted bacteria introduced with transferred *Daphnia magna,* despite thorough rinsing. The effect concentrations were based on mean measured initial concentrations (representative for 21 peak concentration events) as well as on geometric means of mean measured initial and aged concentrations after 24 h hours, calculated to be <LOQ, 23.3, 107, 367 and 1227 µg/L.

During the test no parental mortality occurred, neither was any clinical signs observed, except for the highest treatment level where all animals died before reproduction. Adult body length showed no significant differences between treatments. There was no statistically significant deviation from controls with respect to the age at the first brood. Due to the low variability of the results and the high statistical power, the cumulative number of offspring

was significantly reduced by 14% at the highest treatment level without mortality. The intrinsic rate of increase, r, was also significantly reduced at this concentration, but by less than 10%. Thus, an EC10 could not be determined for r.

Conclusion

Based on mean measured concentrations, after 21 d the following (no observed) effect concentrations for the most sensitive endpoint, **cumulative number of offspring,** were determined:

Related to daily initial concentrations:

EC10 = 610 µg test item/L
EC20 = 1500 µg test item/L
LOEC = 960 µg test item/L
NOEC = 350 µg test item/L

Related to mean measured concentrations:

EC10 = 210 µg test item/L
EC20 = 670 µg test item/L
LOEC = 370 µg test item/L
NOEC = 110 µg test item/L

No *Daphnia magna* survived until reproduction at the highest test concentration. At the lower concentrations, no effects being relevant at the population level could be observed.

Approvals Page

Title of the study: *Daphnia magna*, reproduction test in closed vessels following OECD 211
Test item: C_{10} fatty alcohol
GLP code: SDA-005/4-21

Date: October 27, 2005

Dr. Christoph Schäfers
(Study Director)

Date: October 27, 2005

Dr. Josef Müller
(Chemical Investigator)

Date: October 27, 2005

Prof. Dr. Andreas Schäffer
(Management)

The study was performed without any subcontract.

Statement of GLP-Compliance

Title of the study: *Daphnia magna*, reproduction test in closed vessels following OECD 211
Test item: C_{10} fatty alcohol
GLP code: SDA-005/4-21

This study was conducted in compliance with Good Laboratory Practice Regulations (Grundsätze der Guten Laborpraxis (Principles of Good Laboratory Practice, GLP). Gesetz zum Schutz vor gefährlichen Stoffen (ChemG), Anhang 1, Version from 20.06.2002, published in: Bundesgesetzblatt 2002 Teil I Nr. 40 from 27. June 2002, 2090-2130).

We hereby attest to the authenticity of the study and guarantee that the data are correct, and that the study was performed by the procedures described. This report accurately reflects the raw data.

Schmallenberg,

Date: October 27, 2005

Dr. C. Schäfers
Study Director

Date: October 27, 2005

Prof. Dr. A. Schaeffer
Management

Statement of the Quality Assurance Unit

Title of the study: *Daphnia magna*, reproduction test in closed vessels following OECD 211
Test item: C_{10} fatty alcohol
GLP code: SDA-005/4-21

The Quality Assurance Unit of the testing facility inspected the study and audited the final report according to GLP-Regulations. Possible findings were reported to the study director and to the management.

Dates of audits:

July 29, 2005	(approval of the protocol)
August 11, 2005	(chemical analysis)
October 25, 2005	(audit of the final report)

Generally, inspections of 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.

Schmallenberg,

Date: October 27, 2005

G. Wasmus

Dr. G. Wasmus
QAU - Officer

1. Study Identification

Test

Daphnia magna, reproduction test in closed vessels following OECD 211
Test item: C_{10} fatty alcohol
GLP-Code: SDA-005/4-21

Sponsor

The Soap and Detergent Association
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Management:	Prof. Dr. A. Schaeffer
Study director:	Dr. C. Schäfers
Deputy:	Dr. A. Wenzel
Chemical Investigator:	Dr. J. Müller
Technical staff:	U. Boshof; H. Jürling
Quality Assurance Unit:	Dr. G. Wasmus, Dr. U. Wahle

Study dates

Initiation:	July 29, 2005
Experimental start:	August 3, 2005
Experimental termination:	August 24, 2005
Study completion:	October 27, 2005

2. GLP

The test was performed in accordance with the Principles of Good Laboratory Practice (ref. 3, 4).

An aliquot of the test item, the test protocol, all raw data and all records necessary to reconstruct the study are archived for 15 years following internal SOPs in the GLP-archive of the Fraunhofer-Institute for Molecular Biology and Applied Ecology, D-57377 Schmallenberg, according to ref. 4. Thereafter, the sponsor will be asked whether the data should be disposed, transferred to the sponsor or whether IME will be contracted for further archiving.

3. Test item

The test item and specification information were delivered by Dr. Ehrenstorfer, Augsburg, Germany. The identity and purity of the test item were proven by a certificate of analysis (see appendix). They were not confirmed analytically by the Fraunhofer Institute for Molecular Biology and Applied Ecology, Schmallenberg, Germany.

Chemical name:	1-Decanol
Molecular formula:	$C_{10}H_{22}O$
CAS-Number:	112-30-1
Molecular weight:	158.29
Log Kow	4.23
Vapour pressure:	0.0085 mm Hg at 25 °C
Water solubility:	37 mg/L at 25 °C (SRC PhysProp Database)
Melting point:	7 °C
Specific Gravity:	0.83 g/mL
State of matter and appearance:	colorless to light yellow, viscous refractive liquid.
Lot Number:	21011
Purity:	99.5 % ± 0.5 %
Expiry date:	October 1, 2008

4. Test objective

The objective of this study was the assessment of the effects of a chronic exposure to the test item on the reproduction of *Daphnia magna* according to OECD 211 (ref. 1). Ten *Daphnia magna* each were exposed to four concentrations of the test item and untreated dilution water in closed vessels with daily renewal of the test solution.

5. Details concerning the test

5.1 Test organism

Justification of use:	*Daphnia magna* (Crustacea, Phyllopoda, Cladocera) is accepted by the OECD (ref. 1) as test organism representing aquatic invertebrates.
Specification:	*Daphnia magna* STRAUS, Crustacea, Cladocera.
Age:	4 - 24 hours old
Origin:	Umweltbundesamt (German Federal Environment Agency) bred in the laboratory of the Fh-IME (testing facility).
Breeding:	Adult *Daphnia magna*, at least 3 weeks old, are separated from the stock population by sieving. Batches of 30 to 50 animals are held at room temperature in ca. 1800 mL dilution water. During the week the *Daphnia magna* are fed daily with an algal suspension (*Desmodesmus subspicatus*) and LiquizellR (HOBBY). Algae growing in the log-phase are centrifuged and the pellet is re-suspended in a few mL of medium. 30 mL of this suspension is given to 1 L *Daphnia* medium. The water is changed once per week. Newborn *Daphnia magna* are separated by sieving, the first generation is discarded.
Reference tests:	The sensitivity of the test organism is routinely checked following internal SOPs.

5.2 Holding- and dilution water

Purified drinking water was used. The purification included filtration with activated charcoal, passage through a limestone column and aeration until oxygen saturation. To avoid copper contamination, plastic water pipes were used for serving the test facility. Based on periodical measurements the dilution water was characterised as follows: conductivity 227 µS/cm, total hardness 0.7 mmol/L, alkalinity 1.8 mmol/L, calcium 0.6 mmol/L, magnesium 0.1 mmol/L, nitrate <0.5 mg/L, nitrite <0.002 mg/L, ammonium <0.01 mg/L, phosphate 0.56 mg/L, DOC 1.72 mg/L, copper 0.0031 mg/L,

iron 0.0066 mg/L, manganese 0.0003 mg/L and zinc 0.0075 mg/L. The dilution water was autoclaved before use.

5.3 Test procedure and conditions

Daphnia magna less than 24 h old were exposed to four concentrations of the test item and dilution water as control for a period of 21 days. The sterile test solutions were added to the sterilized test vessels, the *Daphnia magna* introduced and the vessels were closed directly afterwards by an autoclaved silicone stopper. The test solutions were renewed daily by transferring the *Daphnia magna* to new beakers with freshly prepared test solutions with the initial test concentrations. At each transfer, the *Daphnia magna* were carefully rinsed with sterilized dilution water before being placed in the new beakers. For each test concentration and for the control 10x1 animals were used. Each *Daphnia magna* was exposed separately in a numbered vessel containing 100 mL of test medium.

The *Daphnia magna* were fed at each renewal with suspensions of unicellular green algae. The suspensions of *Desmodesmus subspicatus* (daily prepared from axenic cultures) were analyzed for microbial contamination one and two weeks after test start by using "Cult-Dip combi® Dip Slides (Merck)". No bacterial contamination was detected. The content of food in the test suspensions, measured as turbidity at 758 nm, increased during the test from 7 mg C/L equivalents to 15 mg C/L equivalents. The pH value, oxygen concentration, and temperature were checked directly before adding the organisms, and before and after each renewal. The vessels were closed with autoclaved silicone stoppers and subjected to a light/dark cycle of 16/8 hours. The test temperature during the test was 21 ± 1°C, the light intensity did not exceed 1000 lux. The test was performed on a clean bench under sterile conditions. In contradiction to OECD 211, all test vessels were aerated with sterile filtrated synthetic air: the autoclaved silicone stoppers were fitted with fine glass capillaries connected to the aeration unit. The aeration was necessary to avoid severe oxygen depletion due to the increase of transferred bacteria with growing *Daphnia magna* as observed in pre-studies and the associated oxygen consumption by the degradation of the test substance. At each renewal, the glass capillaries were sterilized by washing with 70% ethanol. The recommendation of OECD 211 not to use aeration was supposed to be due to two reasons: to avoid evaporation of the test item and to minimize water movements and resulting stress for the planktonic filter feeders. The volatility of the test item is low and the test was run in closed vessels. To minimize movements of the test solution, the capillaries were introduced just below the surface of the test

solutions. It was verified by observation that the *Daphnia magna* were not influenced in their swimming behaviour.

5.4 Selection of test concentrations

The selection of the test concentrations of 152, 411, 1110 and 3000 µg/L was based on the following criteria:

- The highest nominal concentration was set above the acute *Daphnia magna* LC50 of 2300 µg/L (ref. 12), as it was supposed to decline in the daily courses.
- A spacing factor of 2.7 was supposed to be sufficient to guarantee a no effect concentration.

5.5 Preparation of the test media

Due to the difficulties in dissolving the test item, and its rapid biodegradation, the appropriate method of dosing the test item is not covered by the OECD methods. The preparation of the test solutions was performed according to the CONCAWE test protocol (ref. 8) and to the ASTM standard D6081-97 (ref. 9) with the modifications given below.

Glassware preparation

For first time of use, all glassware used in testing was 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.

The procedure for glassware preparation was:

a) Cleaning in a cleaning machine with detergent
b) Cleaning in a cleaning machine without detergent
c) Rinsing with acetone
d) Rinsing with water
e) Sterilization of glassware at 160 °C overnight

Sterilization of dilution water for the preparation procedure of the test solutions

To avoid microbiological degradation of the test item during the test media preparation, the dilution water was sterilised by heating in an autoclave at 121 °C for 20 min. The sterilised mixing vessels were then filled with this sterilised water for the preparation of the test solution on a clean bench.

Test media preparation

Test solutions were prepared daily by stirring the test substance in test media under slow stir conditions (21 h) in sterilized mixing vessels. The mixing vessels were cylindrical brown glass bottles with teflon covered screw caps, fitted with a drain port near the bottom for drawing off the test solution. The volume of the mixing vessels was 2 L.

One mixing vessel was used for the preparation of each test concentration. The loading of the mixing vessels corresponded to 152, 411, 1110 and 3000 µg test item/L. For each test concentration, an ethanolic stock solution was prepared. 1 mL of the appropriate ethanolic stock solution was pipetted into the 2 L mixing vessel taken from the sterilization process, the still warm mixing vessel slowly turned to cover a maximum area of the glass walls with the ethanolic solution. The movements were continued until the liquid was no longer visible. The ethanol solution did not moisten the inner area of the flask where a star-shaped magnetic stirring bar was placed later.

After the addition of the stirring bar the vessels were sealed leaving only a small headspace. The contents of the vessels were stirred at 100 rpm for approximately 21 h. Care was taken to avoid exceeding the stirring speed even for a short-time, at the start of the period. The control medium was prepared similarly with 1 mL of ethanol. The vessels were kept at room temperature (21 ± 1 °C).

After stirring, the contents of the vessels were left to settle for 2 h. The saturated aqueous phase was then taken out of the drain port. The first fraction 0-100 mL was withdrawn. The fraction between 100 and 1800 mL was used for rinsing (200 mL) and filling (1000 mL) the test flasks for toxicity testing and for analytical measurements (500 mL), if done. Rinsing of the test vessels was carried out to saturate the surfaces of the test vessels. After filling, the vessels were closed immediately by using autoclaved silicone stoppers and only opened to introduce the test organisms and again at the renewals of the test media. The test media were not stored for more than 1 - 2 hours prior to testing.

5.6 Sampling and chemical analysis of the test solutions

All the test concentrations were sampled for chemical analysis three times a week at renewal of the test media. A 500 mL aliquot of the fresh solutions was used for analysis. After 24 h, at the next renewal, the aged test liquids were pooled (vessels 1-5 and 6-10) and analysed. The analyte was extracted from the aqueous test samples by liquid-liquid partitioning with n-hexane. After derivatization of the analyte by MSTFA measurement was performed by GC-MS using n-dodecanol-d_{25} as internal

standard. The method was validated for the determination of the test item in *Daphnia* test medium in the concentration range of 1.0 - 100 µg/L (see Appendix).

5.7 Observations and effect data generation

The parent *Daphnia magna* were assessed visually daily for immobility and any other abnormalities in appearance and behaviour. At study termination, the length of the adults was measured by digital photography and image analysis and their statistics compared with those of the control animals.

The newborn *Daphnia magna* in each beaker were counted at each daily renewal of the test solutions, inspected for abnormalities in condition, and removed.

The following endpoints observed in the reproduction test were evaluated quantitatively:

- Mortality (immobility) of parental generation *Daphnia magna*
- Age at first brood
- Total number of offspring per replicate
- Cumulative Number of live offspring per surviving female at the time of recording
- Intrinsic rate of increase, r
- Individual length of adults

5.8 Data evaluation and statistical analysis

The evaluation of the concentration-effect-relationships and the calculations of effect concentrations were based on mean measured initial concentrations as multiple (21) peak concentrations, as well as on geometric means between mean measured initial and aged (24h) test concentrations. For each endpoint, the NOEC, LOEC, and, if possible, the EC50, EC20 and EC10 were determined. A LOEC and NOEC were calculated by ANOVA followed by Williams' test (ref. 5-6) or an appropriate non-parametric test suggested by the ToxRat (ref. 7) program. When the test results showed a concentration-response relationship, the data were analysed by regression using Probit-analysis assuming log-normal distribution of the values using the computer program ToxRat (ref. 7).

6. Test conditions

The temperature was in the range of 20.0 and 21.0 °C, the light intensity between 588 and 657 Lux (see appendix, Table 5). The oxygen saturation never fell below 70 % (5.7 mg/L), and the mean pH was 9.4 to 9.5 at all treatment levels (Table 1). Thus, all water quality criteria mentioned in the guideline (ref. 3) were met.

Table 1: pH and oxygen saturation

		Nominal concentration (µg/L)				
		Control	152	411	1110	3000
pH	Total mean	9.4	9.4	9.4	9.4	9.5
	Max	9.8	9.9	9.9	9.9	9.9
	Min	8.8	9.0	8.9	9.0	9.1
Oxygen saturation (%)	Mean fresh	95.5	96.4	96.0	96.3	96.9
	Mean old	105.5	105.1	104.8	100.7	103.4
	Total mean	100.4	100.6	100.3	98.5	100.1
	SD	9.3	9.5	9.3	7.8	8.0
	Max	125	125	125	115	120
	Min	70	74	74	74	75

SD = standard deviation
For individual data see appendix, Table 6, Table 7.

7. Results

7.1 Test item concentrations

The mean measured test item concentrations of the freshly prepared test solutions were < LOQ, 122, 333, 962 and 2800 µg/L. This is >80 % of the nominal concentrations (Table 2). At the beginning of the test, test item losses over 24 h were between 60% and 85% at the highest and lowest test item concentration, respectively. The losses were mainly caused by adsorption and degradation by food algae. During the last week of the test, the losses exceeded 95% at all treatment levels and were mainly due to degradation by adapted bacteria introduced with transferred *Daphnia magna,* despite thorough rinsing.

As a consequence of the losses the (no observed) effect concentrations were based on mean measured initial concentrations (representative for 21 peak concentration events) and on geometric means of mean measured initial and aged concentrations after 24 h hours. In the latter case these were calculated to be 23.3, 104, 367 and 1227 µg test item/L (Table 2).

Table 2: Mean measured test item concentrations (µg/L)
Fresh and old test solutions, geometric means of both; detailed data see appendix, Table 8

	Nominal concentration							
	152		411		1110		3000	
	Mean*	SD %	Mean*	SD %	Mean*	SD %	Mean*	SD %
Fresh	122	15	351	10	962	30	2800	10
Old	4.4	119	33	109	140	91	537	65
Mean°	23.3		107		367		1227	

* arithmetic mean of three weekly measurements
° geometric mean of fresh and old means

7.2 Effects

The mean values for the different test endpoint parameters for each treatment level are listed in Table 3. The NOEC, EC10, EC20 and EC50 values of the biological endpoints are summarised in Table 4 (details see appendix 10.6).

Survival and growth

During the entire test no parental mortality occurred up to and including the third treatment level (1110 µg/L nominal) and neither were any clinical symptoms observed. At the highest treatment level all animals died before they could reproduce. Therefore, the highest treatment level was not considered in the evaluation of body growth, reproduction and population growth. Adult body length (Figure 2) exhibited no significant differences between treatments.

Reproduction and population growth

The mean age at first brood was between 8.9 and 9.4 days for all test item concentrations. There was no statistically significant difference between treatments. No offspring mortality occurred.

The cumulative number of offspring was significantly reduced by 14% at a nominal test concentration of 1100 µg/L. The intrinsic rate of increase r was also significantly reduced at this concentration, but by clearly less than 10%. Thus, a reliable EC10 could not be interpolated.

Table 3: Survival, growth and reproduction data

SD = standard deviation. For raw data see appendix 10.5, Table 9, Table 10. Number of *D. magna* per concentration: n = 10.

Test item nominal conc. (µg/L)	Survival (%)	Growth (length) Mean ± SD (mm)	Age at first brood Mean± SD (days)	Cumulative offspring per female Mean ± SD (#)	Intrinsic rate of increase r Mean ± SD (1/d))
Control	100	5.41 ± 0.22	8.9 ± 0.74	68.1 ± 9.5	0.294 ± 0.017
152	100	5.52 ± 0.19	9.2 ± 0.79	68.0 ± 5.6	0.297 ± 0.018
411	100	5.43 ± 0.21	9.0 ± 0.82	62.9 ± 5.8	0.281 ± 0.011
1110	100	5.26 ± 0.38	9.4 ± 0.97	58.6 ± 7.7 *	0.277 ± 0.024 *
3000	0	n.d.	n.d.	n.d.	n.d

* significant difference to control according Williams-test (a = 0.05, one-sided smaller)

Validity of the test

All validity criteria for the performance of the controls were met despite the clearly higher stress compared to standard conditions: Survival (100%) was above 80 %, the mean parental *Daphnia magna* started to reproduce on average before day 9 and the mean number of offspring per female was above 60. The statistical power of the test was high due to low variability in reproduction (the coefficients of variation of cumulative offspring number ranged from 8.2% to 13.9%) and in the intrinsic rate of increase (r ranged from 3.9% (411 µg/L) to 8.5 % (1110 µg/L)) in the controls and treatments.

Table 4: Effect summary table. Effect concentrations of the test item (µg/L) based on mean measured concentrations

Endpoint	Survival	Length	Age at 1st brood	Offspring	Intrinsic rate r
EC50 initial	1500	n.d.	n.d.	n.d.	n.d.
all	620	n.d.	n.d.	n.d.	n.d.
EC20 initial	1100	n.d.	n.d.	1500	n.d.
all	410	n.d.	n.d.	670	n.d.
EC10 initial	930	n.d.	n.d.	610	2300
all	340	n.d.	n.d.	210	1100
NOEC initial	960	≥960	≥960	350	350
all	370	≥370	≥370	110	110

n.d. = not determined (far above concentration range tested or evaluated)
initial = mean measured concentrations at the start of daily exposure
all = geometric mean of initial and aged concentrations

8. Conclusion

Based on mean measured concentrations, after 21 d the following (no observed) effect concentrations were determined for the most sensitive endpoint cumulative number of offspring:

Related to daily initial concentrations:

EC10 = 610 µg test item/L
EC20 = 1500 µg test item/L
LOEC = 960 µg test item/L
NOEC = 350 µg test item/L

Related to mean measured concentrations:

EC10 = 210 µg test item/L
EC20 = 670 µg test item/L
LOEC = 370 µg test item/L
NOEC = 110 µg test item/L

The effects started one treatment level below the threshold level for lethal effects.

9. References

(1) OECD-Guideline for Testing of Chemicals 211. "Daphnia magna Reproduction Test" Adopted 21st September 1998. Organisation de coopération et de développement économiques, Paris.

(2) OECD-Guideline for Testing of Chemicals 202. "Daphnia sp., Acute Immobilization Test and Reproduction Test". Adopted April 4th 1984. Organisation de coopération et de développement économiques, Paris.

(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) Gesetz zum Schutz vor gefährlichen Stoffen (ChemG), Anhang 1, Version vom 27. Juni 2002 published in: Bundesgesetzblatt 2002, Part I No. 40 from June 27, 2002, p. 2090-2130.

(5) Williams, D.A. (1971): A test for differences between treatment means when several dose levels are compared with a zero dose control. Biometrics, 27, 103-117.

(6) Williams, D.A. (1972): The comparison of several dose levels with a zero dose control. Biometrics 28, 519-531.

(7) ToxRat® 2.09. ToxRat SOLUTIONS GmbH: Statistics and Programming in Research industry and Teaching. Dr. Monika M. Ratte, Naheweg 15, 52477 Alsdorf, Germany (http://www.toxrat-solutions.de).

(8) CONCAWE (1992): Ecotoxicological testing of petroleum products: test methodology. Report No. 92/56. CONCAWE, Madouplein 1, B-1210 Brussels.

(9) ASTM Standard D 6081 - 97: Aquatic Toxicity Testing of Lubricants: Sample Preparation and Results Interpretation, January 10, 1997.

(10) Preliminary Tests to an Algae Growth Inhibition Test of Water Accommodated Fractions (WAFs) of Fatty Alcohols, Intermediate Report for Cognis Deutschland GmbH, Düsseldorf, November 20, 2001.

(11) Alga, Growth Inhibition Test: Effects of Water Accommodated Fractions (WAF) of Fatty Alcohols on the growth of *Pseudokirchneriella subcapitata*, Report for Cognis Deutschland GmbH, Düsseldorf.

(12) Fisk, P. (2005): Annex XX, SIAR Long Chained (C6-C22) aliphatic alcohols

(13) Schäfers, C. (2005 a): Pre-studies with dodecanol ($C_{12}OH$) for the preparation of a study plan for a Daphnia magna reproduction test (OECD 211). Fraunhofer IME, Schmallenberg, Germany.

(14) Schäfers, C. (2005 b): Pre-studies with pentadecanol ($C_{15}OH$) for the preparation of a study plan for a Daphnia magna reproduction test (OECD 211). Fraunhofer IME, Schmallenberg, Germany.

10. Appendix

10.1 Test set-up

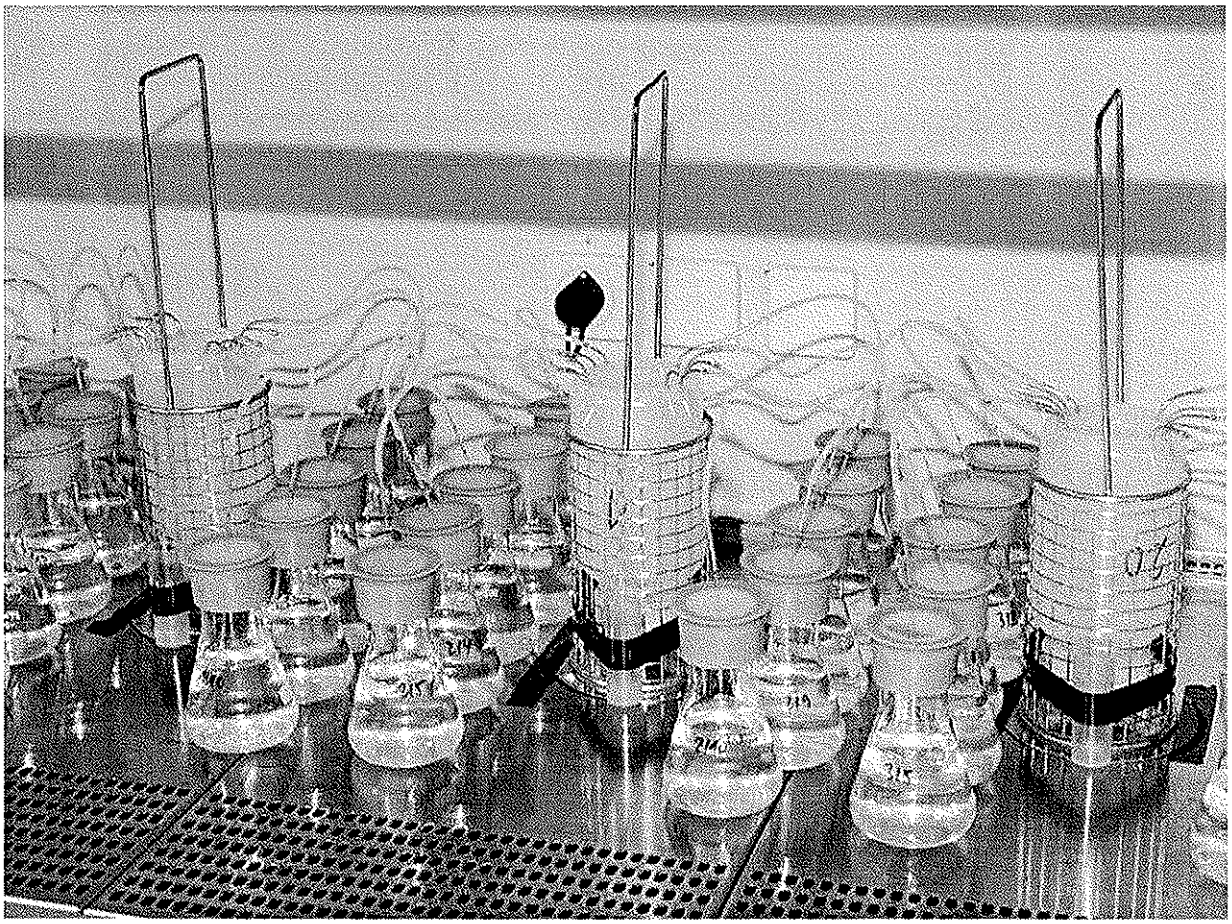


Figure 1: Photograph of the test set-up

10.2 Study plan (protocol) and amendments (18 pages)

Study Plan

Daphnia magna, reproduction test in closed vessels following OECD Guideline 211

Effect of 1-decanol

GLP-Code of Testing Facility: SDA-005/4-21

Sponsor
The Soap and Detergent Association
Technical & International Affairs
1500 K Street, N.W., Suite 300
Washington, D.C., 20005, USA
Dr. Hans Sanderson
Director Environmental Safety

Study Monitor
Scott Belanger, PhD
The Procter & Gamble Company
11810 East Miami River Rd
Cincinnati, Ohio, USA

Testing facility
Fraunhofer-Institute for Molecular
Biology and Applied Ecology (IME)
57377 Schmallenberg, Germany

Study director
Dr. Christoph Schäfers

July 29, 2005

Contents

Distribution list for study report

Sponsor:	1 original
GLP-archive:	1 original
Study director:	1 copy
QAU:	1 copy
Chemical investigator:	1 copy
Biological laboratory:	1 copy

STUDY PLAN

1 Test

Daphnia magna, reproduction test in closed vessels following OECD Guideline 211 (1)

Test item: C_{10} fatty alcohol
GLP-Code: SDA-005/4-21

2 Sponsor

The Soap and Detergent Association
Technical & International Affairs
Hans Sanderson, PhD
1500 K Street, NW, Suite 300
Washington, DC 20005, USA
P +1-202-662-2516

Study Monitor

Scott Belanger, PhD
The Procter & Gamble Company
11810 East Miami River Rd
Cincinnati, Ohio, USA
P: +1-513-627-1928

3 Testing facility

Fraunhofer-Institute for
Molecular Biology and Applied Ecology (IME)
P.O. 1260
57377 Schmallenberg, Germany

Test facility management:	Prof. Dr. A. Schäffer
Study director:	Dr. C. Schäfers P +49-2972-302-270
Deputy:	Dr. A. Wenzel P +49-2972-302-329
Chemical investigator:	Dr. J. Müller P +49-2972-302-216
Deputy	Dr. K. Derz P +49-2972-302-291

Technical staff	Chemistry:	H. Jüring
	Biology:	U. Boshof
		E. Hardebusch
		P. Schulte
Quality Assurance Unit:		Dr. U. Fritsche
		Dr. G. Wasmus

The study will be performed without subcontractors

4 Objective

In the reproduction test, the effects of the rapidly biodegradable test item on the mortality and the reproductive capacity and other signs of intoxication in *Daphnia magna* will be determined. In order to maintain exposure to the test item throughout the test period, the test will be performed in test item solutions prepared by stirring the test substance in test media under slow stir conditions under sterile conditions, in closed vessels and with daily renewal of the test solution. The test will be completely carried out on a clean bench.

5 Specification of the test item

The test item, specification information as well as the certificate of analyses will be obtained from Dr. Ehrenstorfer, Augsburg, Germany.
The purity of the test item will not be analytically checked by the testing facility.

3.1	Chemical name:	1-Decanol
3.2	Molecular formula:	$C_{10}H_{22}O$
3.3	CAS-Number:	112-30-1
3.4	Molecular weight:	158.29
3.5	Log K_{ow}	4.23
3.6	Vapour pressure:	0.0085 mm Hg at 25 °C
3.7	Water solubility:	37 mg/L at 25 °C (SRC PhysProp Database)
3.8	Specific Gravity:	0.83 g/mL
3.9	Melting point:	7 °C

3.10	State of matter and appearance:	colorless to light yellow, viscous refractive liquid.
3.11	Stability:	Stable. Combustible. Incompatible with strong oxidizing agents, acid chlorides, acid anhydrides.
3.12	Lot Number:	not yet available
3.13	Purity:	not yet available
3.14	Expiry date:	not yet available
3.15	Storage conditions:	cool location (refrigerator)
3.16	Safety data sheet:	from J.T. Baker SAF-T-DATA(TM)
3.17	Hazard properties (as defined in "Gefahrstoffverordnung' (2)):	R36 R37 R38 R41 R52 R53 see table 1
3.18	Waste disposal:	municipal waste, after dilution to non-toxic concentration

Table 1: **Hazard properties**

	Property	Negative	Positive
E	Explosive	X	
O	oxidizing	X	
F^+	extremely flammable	X	
F	highly flammable	X	
	flammable	X	
T^+	very toxic	X	
T	toxic	X	
Xn	Harmful	X	
C	corrosive	X	
Xi	irritant		X
	carcinogenic	X	
	mutagenic	X	
	teratogenic	X	
N	ecologically harmful		X

6 GLP

The test will be performed in accordance with the Principles of Good Laboratory Practice (3), (4).

7 Test principle

In the reproduction test, effects on the mortality, the reproductive capacity and other signs of intoxication in *Daphnia magna* will be determined to be used as indications of the toxicity of a substance dissolved in water. For this purpose, the test organisms will be exposed to dilution water containing the test item in four concentrations for a total period of at least 21 days in closed vessels. The reproduction test will consist of 10 replicates per treatment with 1 daphnid each (individual exposure). The test will be performed according to OECD 211 (1). The test media will be renewed daily. Observations of all relevant endpoints will be made at each renewal.

8 Details concerning the test

8.1 Test organism

8.1.1 Justification for the use of the test organism

Daphnia magna was chosen by OECD-experts (1) and the EEC (5) as test organism representing aquatic invertebrates.

8.1.2 Test organism

Specification:	*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 Fh-IME (testing facility).

Breeding and holding conditions:

Adult *Daphnia magna*, at least 3 weeks old, are separated from the stock population by sieving. Batches of 30 to 50 animals are held at room temperature in ca. 1800 mL dilution water. During the week *Daphnia magna* are fed daily with an algal suspension (*Scenedesmus subspicatus*) and HOBBY® LiquizellR (liquid starter feed for invertebrates, Dohse Aquaristik KG, Otto-Hahn-Str. 9, 53501 Grafschaft-Gelsdorf,

Germany). Algae growing in the log-phase, are centrifuged and the pellet is resuspended in a few mL of medium. 30 mL of this suspension is given to 1 L Daphnia medium. The water is changed once per week. Newborn *Daphnia magna* are separated by sieving, the first generation is discarded.

8.2 Holding- and dilution water

Purified drinking water will be used according to the OECD-Guideline (1). The purification includes filtration with activated charcoal, passage through a lime-stone column and aeration until oxygen saturation. Carbonate hardness of the water is nearly 90 mg/L $CaCO_3$, pH is in the range of 7.5-8.5. Conductivity, total hardness, alkalinity, as well as nitrate, nitrite, ammonium, phosphate, and DOC contents of the dilution water are measured regularly and will be reported. The dilution water will be autoclaved before use.

8.3 Test procedure, reproduction test

Daphnia magna less than 24 hours old will be exposed to four concentrations of the test item and dilution water as control for a period of at least 21 days. The test solution will be filled in the sterilized test vessels, the *Daphnia magna* will be added and the vessels will be closed directly afterwards by an autoclaved silicone stopper. The water will be renewed daily by transferring the *Daphnia magna* to new beakers with freshly prepared test solutions with the initial test concentrations. At each transfer, the *Daphnia magna* will be carefully rinsed with sterilized dilution water before being placed in the new beakers.
For each test concentration and for the control 10 x 1 animals will be used. Each daphnid will be exposed separately in a numbered vessel containing 100 mL of test medium.

Test concentrations
A control and 4 concentration plots will be established in a geometric progression. Following concentrations are agreed with the sponsor (spaced by factor of 2.7):
3.00 mg/L, 1.11 mg/L, 0.411 mg/L, and 0.152 mg/L 1-decanol.
Test item concentrations in the test media and blanks will be assessed by chemical analysis.

General test conditions
The animals will be fed at each renewal with suspensions of unicellular green algae. The algae suspension will be measured once weekly for quantification of the

microbial contamination. The content of food in the test suspensions, measured as turbidity at 758 nm, will increase during the test from 7 mg C/L equivalents to 15 mg C/L equivalents. The pH value, oxygen concentration, and temperature will be checked directly before adding the animals, and before and after each renewal. The vessels will be closed with autoclaved silicone stoppers and subjected to a light/dark cycle of 16/8 hours. The test temperature during the test will be 20 ± 2°C, the light intensity will not exceed 1000 lux. The test will be completely performed on a clean bench under sterile conditions. All test vessels with be aerated with sterile filtrated (0.2 µm) synthetic air. The autoclaved silicone stoppers will be penetrated with fine glass capillaries connected with the aeration unit. At each renewal, the glass capillaries will be sterilized by using 70 % ethanol. This will be necessary due to the increase of transferred bacteria with growing *Daphnia magna*.

Observation and measurements

The *Daphnia magna* will be evaluated visually each day. Mortality among the parent animals and any abnormalities in appearance and behavior will be recorded. At study termination, the lengths of the adults will be measured by digital photography and image analysis.

The newborn *Daphnia magna* per vessel will be counted at each renewal, and abnormalities in condition (including male sex) recorded. The presence of winter eggs will be checked and recorded. The following endpoints observed in the reproduction test will be evaluated quantitatively:

- Mortality (immobility) of parental generation Daphnia magna
- Time to the first brood
- Number of live offspring per surviving female
- Intrinsic rate of increase
- Individual length of adults

Statistical analysis

For each endpoint, the NOEC, LOEC, and, if possible, the EC_{50} will be determined: A LOEC will be calculated by using ANOVA followed by Williams' or Dunnett's test or an appropriate non-parametric test. When the test results show a concentration-response relationship, the data will be analyzed by regression to determine the EC_{50} including the 95% confidence interval as well as the EC_{10} using Probit-analysis (6) assuming log-normal distribution of the values.

8.4 Preparation of test media

Due to the rapid biodegradation of the test item and the low water solubility, the appropriate method of dosing the test item is not covered by the OECD methods. The preparation of the test solutions will be performed according to the CONCAWE test protocol (7) and to the ASTM standard D6081-97 (8) with the modifications given below.

Glassware Preparation

For first time use, all glassware used in testing will 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 will be omitted.

The procedure for glassware preparation will be:

- Cleaning in a cleaning machine with detergent
- Cleaning in a cleaning machine without detergent
- Rinsing with acetone
- Rinsing with water
- Sterilization of glassware at 180 °C overnight

Sterilization of dilution water for the preparation procedure of the test solutions

In order to avoid microbiological degradation of the test item the water will be sterilized by heating in an autoclave at 121 °C for 20 min. Then the water will be filled into the sterilized mixing vessels for the preparation of the test solution in a clean bench.

Test media preparation

Test solutions will be daily prepared by stirring the test substance in test media under slow stir conditions under sterile conditions in pre-sterilized mixing vessels. The mixing vessels will be cylindrical brown glass bottles with teflon covered screw caps, fitted with a drain port near the bottom for drawing off the test solution. The volume of the mixing vessels will be 2 L. One mixing vessel will be used for the preparation of each test concentration. The loading of the mixing vessels will correspond to the respective test concentrations (see p. 8.3). For each test concentration, an ethanolic stock solution will be prepared. 1 mL of the appropriate ethanolic stock solution will be pipetted into the 2 L mixing vessel taken from the sterilization process, the still warm mixing vessel slowly turned to cover a maximum area of the glass walls with the ethanolic solution. The movements will be continued until the ethanol has evaporated

and the liquid will no longer be visible. The ethanol solution must not moisten the inner area of the flask where a star-shaped magnetic stirring bar will be placed later. After the addition of the required volume of control media together with a stirring bar each vessel will be sealed leaving only a small headspace. The contents of the vessels will be stirred at 100 rpm (digital display) for approximately 21 hours. Care will be taken to avoid even short time exceeding of the stirring speed at start. The control will be prepared adequately with 1 mL of ethanol. The vessels will be kept at room temperature (21 ± 1 °C).
After stirring, the contents of the vessels will be left to settle for 2 hours. Then the saturated aqueous phase will be taken out of the drain port. The first fraction 0-100 mL will be withdrawn. The fraction between 100 and 1800 mL will be used for the rinsing (200 mL) and filling (1000 mL) of the test flasks for toxicity testing and for analytic measurements (500 mL), if done.
A first portion of the test media will be used to rinse the test vessels in order to saturate the surfaces. After filling the vessels will be closed immediately by using autoclaved silicone stoppers and only opened to introduce the test organisms and again at the renewals of the test media.
Prior to testing the test media will be not stored for more than 1 - 2 hours.

8.5 Chemical analysis of test media

At renewal of the test liquids, all freshly prepared test solutions will be sampled for chemical analysis three times a week. After 24 hours, the aged test liquids will be pooled (vessels 1-5 and 6-10) and analyzed.

The analyte (1-decanol) will be extracted from the daphnia test media by liquid-liquid partitioning with n-hexane. After shaking for 10 min and settling for 1 hour the n-hexane extract will be removed, an aliquot of the supernatant (n-hexane) will be taken and the internal standard solution (deuterated C_{12}-fatty alcohol) will be added. After derivatisation by MSTFA (N-methyl-N-trimethylsilyl-trifluoroacetamide) the solutions will be measured by GC-MS in SIM mode. Two replicates will be performed for every concentration.

Validation of the analytical method will be performed according to guideline SANCO/-825/00 rev. 6 and SANCO/3029/99 ver. 4 (9), (10). The guidelines describe the pesticide pre-registration data requirements in Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414 and the requirements for post-registration

monitoring and control. According to these guidelines the analytical method will be validated in respect to specificity, linearity, accuracy, precision, identity and limit of quantification (LOQ).

8.6 Validity criteria

The validity of the test conditions given by OECD 211 (1) is tested by controls in purified drinking water (dilution water); mortality must not exceed 20 %. The mean number of live offspring produced per parent animal surviving at the end of the test should be at least 60. The stressing study conditions due to the test design accounting for the test item properties may result in lower offspring numbers after 21 days. Thus, exposure will be continued until the control mean surpasses 60 newborns per parent.

9 Archiving

An aliquot of the test item, the original study plan including all amendments, all raw data and all records necessary to reconstruct the study and the original final report will be archived for 15 years following internal SOPs in the GLP-archive of the Fraunhofer-Institute for Molecular Biology and Applied Ecology, 57377 Schmallenberg, according to (4). Thereafter, the sponsor will be asked whether the data should be disposed, transferred to the sponsor or whether IME will be contracted for further archiving.

10 List of SOPs that will be used in the study

The Generalia-SOPs as well as the following SOPs will be used:

SOP No.	Title (partly translated)
V4 - 501/02	Daphnia test, acute tox., repro-test, dilution water
V4 - 502/02	Daphnia test, acute tox., repro-test, Daphnia-holding and breeding
V4 - 504/02	Daphnia test, acute tox. calculation of LC0, LC100, LC50
V4 - 505/02	Daphnia test, aquat. tox., dilution water
V4 - 507/02	Daphnia test, holding conditions
V4 - 510/02	Daphnia test, prolonged tox., solitary exposure conditions
V4 - 511/02	Daphnia test, prolonged tox., solitary exposure, sample identification
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 - 007/02	Checking of thermometers
G3 - 008/03	Checking of coolers and freezers
G3 - 009/02	Handling of shakers
G4 - 007/02	Illuminance Meter, Minolta, operation
G4 - 302/02	Aquatic Microcosms, Measurement of oxygen, OXI 196, WTW
G4 - 303/02	Aquatic Microcosms, Measurement of pH
G5 - 003/02	Benchtop cooling centrifuge Sigma 3K12, handling
G5 - 109/02	Safety clean bench, Haereus
G5 - 134/02	Autoclave Varioklav
G7 - 183/02	Washing machine Miele with Aquapurifikator, handling
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

11 Schedule

Presumed test start:	August 2, 2005
Presumed test end:	August 23, 2005
Draft report:	September 23, 2005

12 Reporting

The draft report will include but is not limited to the following:

- detailed description of preparation of test medium
- detailed description of chemical analysis and evaluation
- data concerning test organisms (age, origin, holding conditions)
- description of the test design (incl. vessels)
- test conditions (temperature, pH-values, concentrations of dissolved oxygen)
- results of the study
- explanation of study plan deviations, if any
- Certificate of Analysis
- GLP certificate of testing facility

13 Amendment procedures

Protocol amendments will be discussed beforehand with the sponsor, where practicable. Detailed descriptions of all amendments and reasons for each of them will be signed by the study director. The amendment will be effective at the time of the study director's signature. The sponsor will receive two copies: one should be signed and returned to the study director. The amendment will be added to all copies of the protocol. Protocol deviations will be explained in the report.

14 Approval of the protocol

Study director: July 29, 2005 — Date — for Dr. Christoph Schäfers

Chemical investigator: July 29, 2005 — Date — for Dr. Josef Müller

Quality Assurance Personnel: July 29, 2005 — Date — for Dr. Ulrich Fritsche

Management: July 29, 2005 — Date — for Prof. Dr. Andreas Schäffer

Sponsor's representative: August 10, 2005 — Date — Dr. Hans Sanderson

15 References

1) OECD-Guideline for Testing of Chemicals 211. *'Daphnia magna* Reproduction Test' Adopted 21st September 1998. Organisation de coopération et de développement économiques, Paris.

2) Verordnung zum Schutz vor gefährlichen Stoffen (GefStoffV) in der Fassung der Vierten Verordnung zur Änderung der Gefahrstoffverordnung vom 16. Oktober 1999 (BGBl I S. 2059) und den Änderungen vom 25. Mai 2000 (BGBl. I p. 747) und 26. Juni 2000 (BGBl I p. 932).

3) Grundsätze der Guten Laborpraxis (Principles of Good Laboratory Practice, GLP). In: Chemikaliengesetz der Bundesrepublik Deutschland (ChemG) §19, sowie die Anhänge 1 und 2 in der Fassung der Bekanntmachung vom 25. Juli 1994 (BGBl. I S. 1703) und den Änderungen vom 08. Mai 2001 (BGBl. S. 843).

4) „OECD Principles of Good Laboratory Practice, adopted by Council on 26th November 1997; Environment Directorate, Organisation for Economic Co-Operation and Development, Paris 1998"

5) Official Journal of the European Communities No. L383 A/172. C2: Acute Toxicity for Daphnia (1992).

6) Finney, D.J.: Statistical Method in Biological Assay. 2nd ed., London. 1964

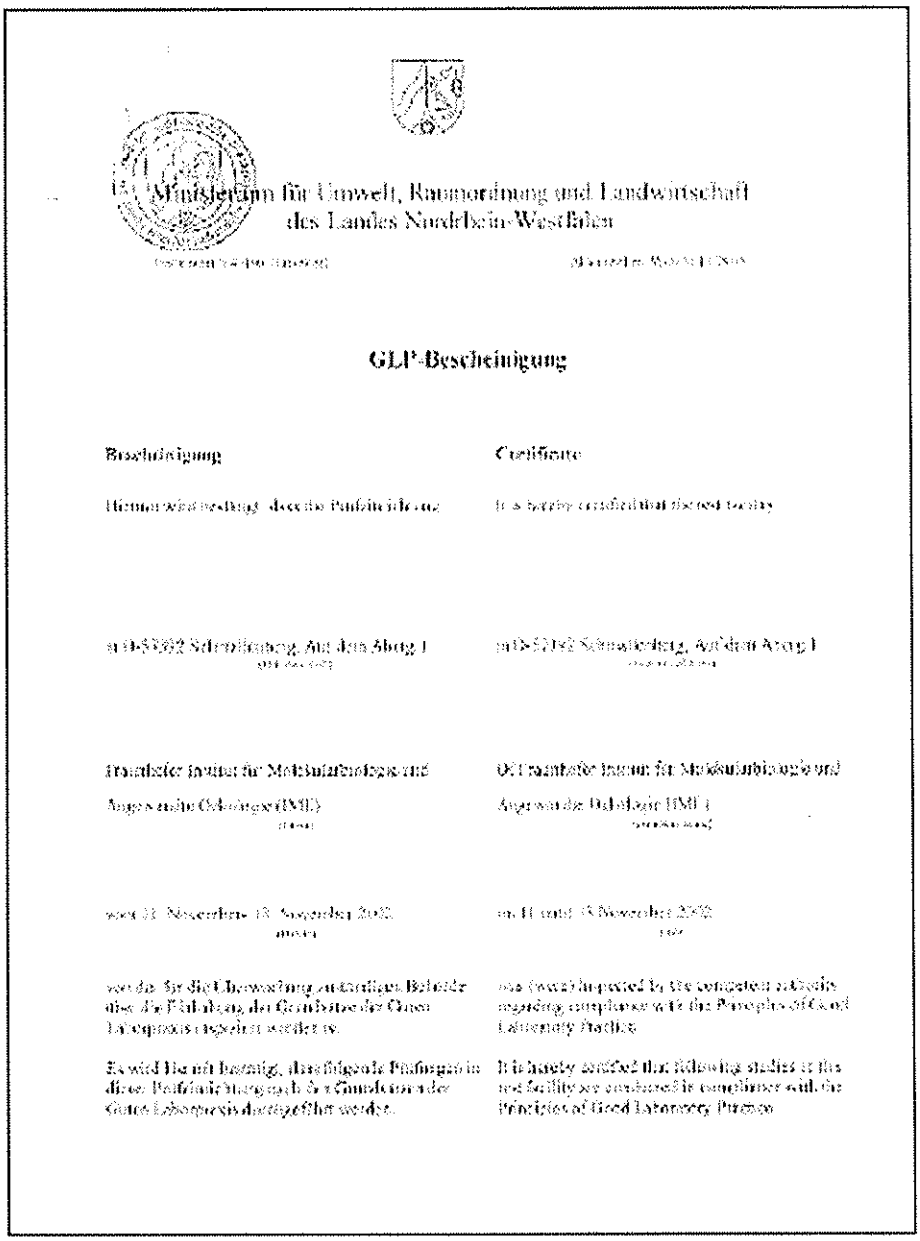
7) CONCAWE (1992). Ecotoxicological testing of petroleum products: test methodology. Report No. 92/56. CONCAWE, Madouplein 1, B-1210 Brussels.

8) ASTM Standard D 6081 - 97: Aquatic Toxicity Testing of Lubricants: Sample Preparation and Results Interpretation. January 10, 1997.

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

10) 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 A: GLP-Certificate of the test facility



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

GLP-Bescheinigung

Bescheinigung

Hiermit wird bestätigt, dass die Prüfeinrichtung

gemäß § 19 b Abs. 1 Satz 1 Chemikaliengesetz vom 25. Juli 1994 (BGBl. I S. 1703, 2005), zuletzt geändert durch Artikel 4 Abs. 3 des Gesetzes vom 21. Juli 2004 (BGBl. I S. 1857), in Verbindung mit § 1 Abs. 1 Satz 1 Nr. 1 der Verordnung zur Durchführung des Chemikaliengesetzes (Chemikalien-Verbotsverordnung) vom 14. Oktober 1993 (BGBl. I S. 1746), zuletzt geändert durch Artikel 1 der Verordnung vom 13. Juni 2002 (BGBl. I S. 2076)

Fraunhofer Institut für Molekularbiologie und Angewandte Oekologie (IME)
Schmallenberg

vom 11. November bis 18. November 2002
durch

geprüft wurde. Für die Überwachung zuständige Behörde ist die Bezirksregierung Arnsberg, Dienstsitze Olpe und Soest.

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

Certificate

It is hereby certified that the test facility

pursuant to § 19 b Abs. 1 Satz 1 Chemikaliengesetz vom 25. Juli 1994 (BGBl. I S. 1703, 2005), zuletzt geändert durch Artikel 4 Abs. 3 des Gesetzes vom 21. Juli 2004 (BGBl. I S. 1857), in Verbindung mit § 1 Abs. 1 Satz 1 Nr. 1 der Verordnung zur Durchführung des Chemikaliengesetzes (Chemikalien-Verbotsverordnung) vom 14. Oktober 1993 (BGBl. I S. 1746), zuletzt geändert durch Artikel 1 der Verordnung vom 13. Juni 2002 (BGBl. I S. 2076)

Fraunhofer Institute for Molecular Biology and Applied Ecology (IME)
Schmallenberg

on 11 and 18 November 2002
by

was 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.

STUDY PLAN:	*Daphnia magna*, reproduction test	
TEST ITEM:	C_{10} fatty alcohol	
GLP-CODE:	SDA-005/4-21	- page 17/17 -

GLP certificate continued

Kategorie 1	category 1
Prüfungen zur Bestimmung 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 Biotransformation und zur Metabolisierung	[illegible] soil and air. Biotransformation
Kategorie 6	category 6
Prüfungen zur Bestimmung von Rückständen	residue studies
Kategorie 7	category 7
Prüfungen zur Bestimmung der Auswirkungen auf Mikroorganismen und natürliche Ökosysteme	studies on effects on microorganisms and natural ecosystems
Kategorie 9	category 9
Modell- und Simulationsverfahren für das Verhalten von Stoffen in der Umwelt	mathematical modelling and simulation of the environmental fate of chemicals

Dortmund, 17. Februar 2004

Im Auftrag

(Prof. Dr. Heinrich Dorgerloh)

Dienstsiegel

10.3 Water parameter values throughout the test

Table 5: Water temperature (°C) and light intensity (Lux)

Day	Temperature (°C)	Light intensity (Lux)
0	21.0	618
1	20.8	603
2	20.7	648
3	20.4	619
4	20.2	598
5	20.0	647
6	20.4	621
7	20.1	657
8	20.8	588
9	20.6	613
10	20.5	623
11	20.5	647
12	20.2	608
13	20.2	597
14	20.9	634
15	20.8	646
16	20.5	608
17	20.4	641
18	20.3	619
19	20.5	630
20	20.7	619
21	20.7	634
Mean	**20.5**	**624**
SD	0.3	19

SD = standard deviation

Table 6: Oxygen saturation in percent (pooled samples) throughout the test.

Day		Test item concentration (µg/L)				
		control	152	411	1110	3000
0	fresh	94	97	96	97	97
1	old	103	102	103	103	103
	fresh	95	99	99	98	98
2	old	115	115	120	107	106
	fresh	99	99	99	99	99
3	old	125	125	125	111	108
	fresh	96	99	99	95	99
4	old	109	109	109	108	101
	fresh	95	95	94	95	94
5	old	106	106	105	103	102
	fresh	96	96	96	96	98
6	old	116	119	111	112	108
	fresh	94	96	96	96	96
7	old	113	113	110	105	109
	fresh	95	95	95	95	97
8	old	113	113	115	115	110
	fresh	97	97	98	98	100
9	old	112	113	113	111	120
	fresh	96	97	97	96	99
10	old	106	108	108	101	103
	fresh	98	99	93	94	94
11	old	103	103	102	104	107
	fresh	95	95	93	97	94
12	old	100	102	100	96	94
	fresh	97	94	94	94	95
13	old	105	110	110	105	90
	fresh	94	97	96	97	95
14	old	103	106	105	100	111
	fresh	98	98	98	98	97
15	old	104	107	107	90	107
	fresh	96	96	97	97	98
16	old	105	90	91	80	75
	fresh	91	93	93	94	All dead
17	old	99	90	91	96	All dead
	fresh	99	100	99	100	All dead
18	old	98	92	93	93	All dead
	fresh	86	89	92	92	All dead
19	old	70	74	74	74	All dead
	fresh	98	96	95	98	All dead

- Table 6, to be continued –

- Table 6, oxygen saturation (%), continued –

Day		Test item concentration (µg/L) control	152	411	1110	3000
20	old	88	92	95	96	All dead
	fresh	98	98	95	96	All dead
21	old	93	90	94	85	All dead
Mean fresh		95.5	96.4	96.0	96.3	96.9
Mean old		105.5	105.1	104.8	100.7	103.4
Total mean		100.4	100.6	100.3	98.5	100.1
SD		9.3	9.5	9.3	7.8	8.0
Max		125	125	125	115	120
Min		70	74	74	74	75

Table 7: pH (pooled samples) throughout the test.

Day		Test item concentration (µg/L) control	152	411	1110	3000
0	fresh	8.8	9.1	9.4	9.4	9.5
1	old	9.3	9.6	9.7	9.8	9.7
	fresh	9.4	9.4	9.5	9.4	9.4
2	old	9.7	9.8	9.8	9.7	9.6
	fresh	9.4	9.4	9.0	9.5	9.5
3	old	9.7	9.9	9.7	9.9	9.8
	fresh	9.4	9.5	9.5	9.5	9.5
4	old	9.7	9.8	9.8	9.8	9.6
	fresh	9.4	9.4	9.3	9.4	9.3
5	old	9.7	9.6	9.6	9.6	9.5
	fresh	9.3	9.3	9.3	9.5	9.4
6	old	9.7	9.8	9.8	9.8	9.8
	fresh	9.6	9.3	9.3	9.4	9.4
7	old	9.8	9.7	9.7	9.6	9.7
	fresh	9.4	9.2	9.3	9.0	9.1
8	old	9.7	9.7	9.8	9.7	9.6
	fresh	9.3	9.2	9.4	9.3	9.3
9	old	9.8	9.8	9.9	9.8	9.8
	fresh	9.2	9.2	9.1	9.2	9.4
10	old	9.7	9.8	9.8	9.8	9.9
	fresh	9.3	9.3	9.2	9.4	9.4
11	old	9.7	9.6	9.8	9.8	9.8
	fresh	9.5	9.4	9.2	9.3	9.3

- Table 7, to be continued –

- Table 7, pH, continued –

Day		Test item concentration (µg/L) control	152	411	1110	3000
12	old	9.5	9.7	9.6	9.6	9,6
	fresh	9.2	9.3	9.3	9.2	9,2
13	old	9.4	9.7	9.7	9.7	9,5
	fresh	9.2	9.2	9.2	9.2	9,3
14	old	9.5	9.8	9.8	9.7	9,7
	fresh	9.3	9.3	9.3	9.2	9,2
15	old	9.5	9.7	9.7	9.5	9,6
	fresh	9.1	9.2	9.2	9.4	9,5
16	old	9.4	9.5	9.5	9.5	9,6
	fresh	9.3	9.2	9.0	9.2	All dead
17	old	9.5	9.5	9.5	9.5	All dead
	fresh	9.1	9.2	9.1	9.2	All dead
18	old	9.5	9.6	9.6	9.5	All dead
	fresh	9.2	9.1	9.1	9.1	All dead
19	old	9.2	9.2	9.3	9.1	All dead
	fresh	9.1	9.0	8.9	9.0	All dead
20	old	9.1	9.3	9.3	9.2	All dead
	fresh	9.0	9.1	9.1	9.2	All dead
21	old	9.2	9.4	9.4	9.1	All dead
Mean fresh		9.3	9.3	9.2	9.3	9.4
Mean old		9.5	9.6	9.7	9.6	9.7
Total mean		9.4	9.4	9.4	9.4	9.5
Standard deviation		0.2	0.3	0.3	0.3	0.2
Max		9.8	9.9	9.9	9.9	9.9
Min		8.8	9.0	8.9	9.0	9.1

10.4 Detailed results of analytical measurements

Table 8: Test item concentrations (µg/L) in the test solutions.

New: Freshly prepared test solution directly before distribution to the replicates, samples A and B. Old: Aged test solutions taken from pooled replicates 1-5 and 6-10 after 24 h.

	Test item concentration (µg/L)									
nominal	control		152		411		1110		3000	
Day New	A	B	A	B	A	B	A	B	A	B
0	1	< LOQ	100	109	345	354	1352	1285	2467	2684
2	1	< LOQ	90	97	376	414	1213	1268	2914	2761
5	14	< LOQ	131	132	389	404	926	1035	3064	3052
7	5	< LOQ	115	117	341	347	1055	1101	2622	3027
9	< LOQ	< LOQ	126	135	353	370	912	1094	3192	3064
12	< LOQ	< LOQ	102	122	288	302	675	726	2285	2414
14	< LOQ	< LOQ	117	161	351	355	964	995	2659	2992
16	< LOQ	< LOQ	136	139	341	364	839	855	dead	Dead
20	< LOQ	< LOQ	131	135	302	322	929	98	dead	Dead
Mean	< LOQ		122		351		962		2800	
SD %	-		15		10		30		10	
% of nom.	-		80		85		87		93	
Day Old	1-5	6-10	1-5	6-10	1-5	6-10	1-5	6-10	1-5	6-10
1	< LOQ	< LOQ	17	16	90	102	304	337	1042	1039
3	< LOQ	< LOQ	10	8	79	86	269	227	343	465
6	< LOQ	< LOQ	3	5	26	35	138	156	438	-
8	< LOQ	< LOQ	6	6	47	47	258	275	513	-
10	< LOQ	< LOQ	2	3	34	33	276	189	741	-
13	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	1.0	18	39	11	-
15	< LOQ	< LOQ	< LOQ	< LOQ	2	3	29	1.0	244	-
17	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	-	-
21	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	-	-
Mean	< LOQ		4.4		33		140		537	
SD %	-		119		109		91		65	
Total mean	< LOQ		23.3		107		367		1227	

SD: standard deviation in %
LOQ: limit of quantification = 1.0 µg/L; values < LOQ are calculated with 0.5 * LOQ = 0.5 µg/L
Total mean: geometric mean of mean new and mean old

10.5 Toxicity data – Graphs

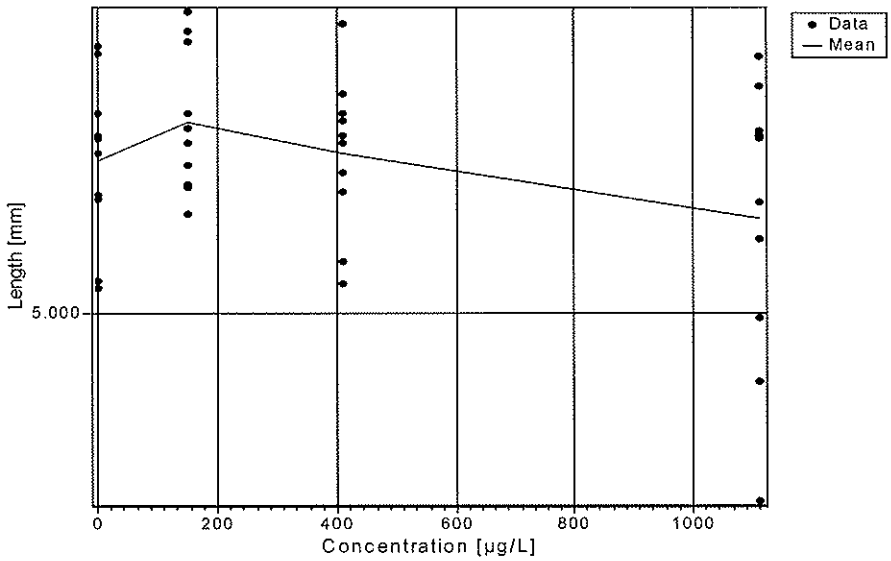


Figure 2: Length of Daphnia magna as observed under presence of the test item after 21 d.

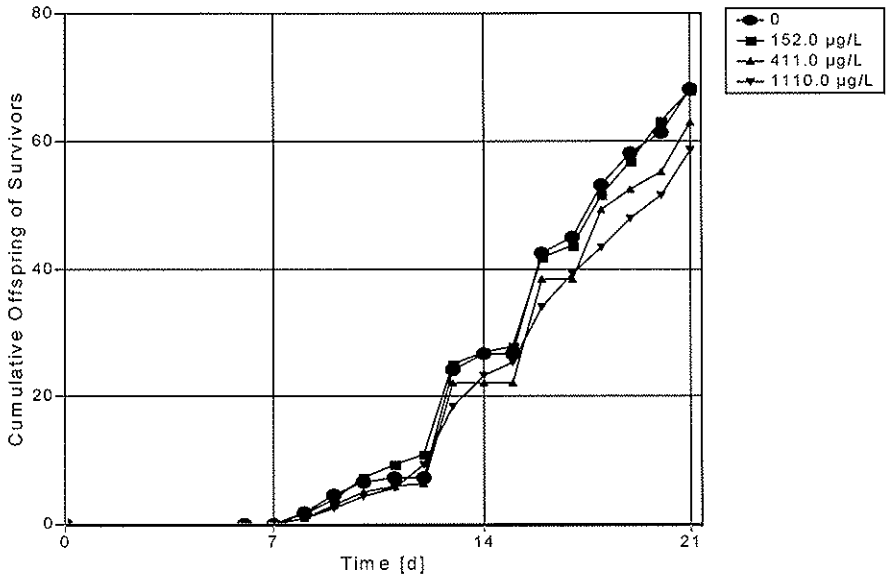


Figure 3: Cumulative offspring of surviving *Daphnia magna* as dependent on test item concentration and time.

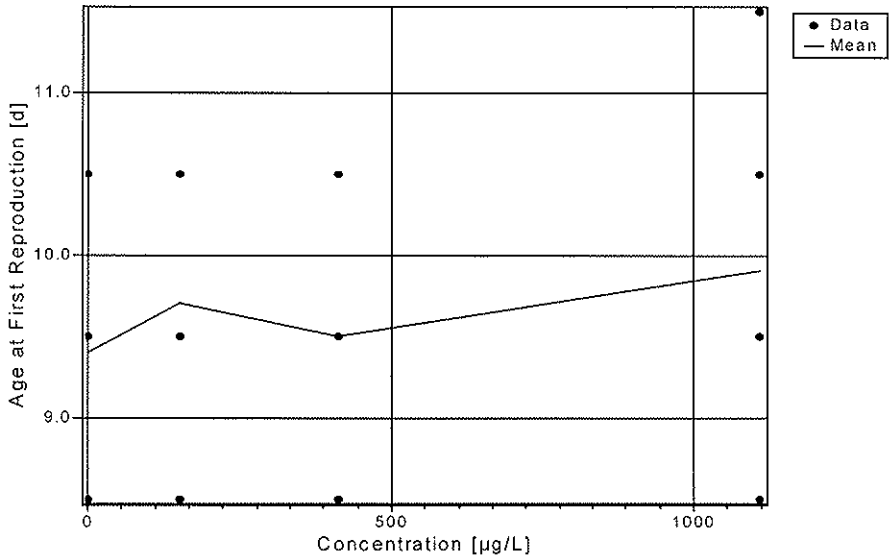


Figure 4: Age at first brood of *Daphnia magna* as observed under presence of the test item.

(ToxRat adds 0.5 days to compensate for the introductory age.)

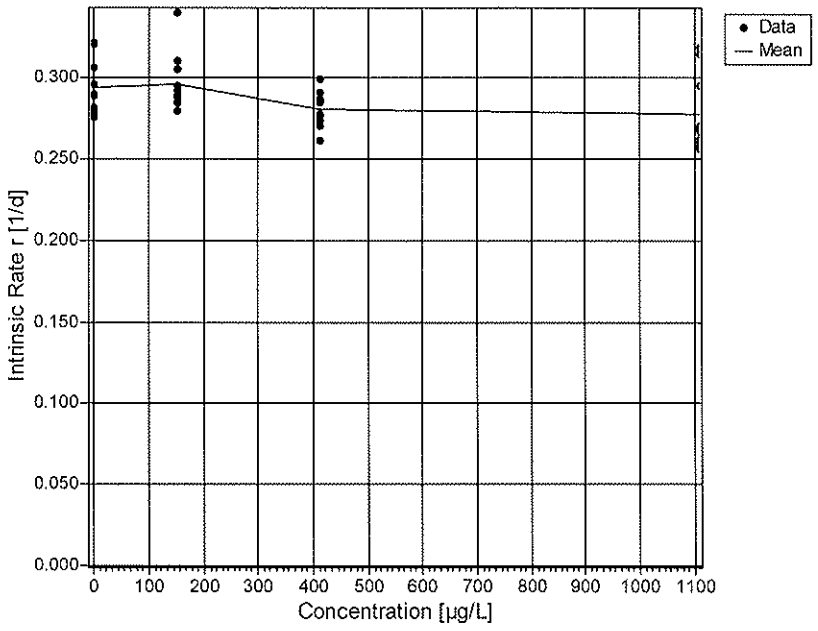


Figure 5: Intrinsic rate r of *Daphnia magna* as observed under presence of the test item after 21 d.

10.6 Raw data

Table 9: Parental *Daphnia magna* lengths at test end after 21 d (mm).

	Test item concentrations (µg/L)			
No	control	152	411	1110
1	5.43	5.50	5.78	5.69
2	5.72	5.81	5.38	5.48
3	5.32	5.54	5.08	5.49
4	5.70	5.35	5.33	5.61
5	5.07	5.34	5.59	4.50
6	5.47	5.46	5.52	5.20
7	5.54	5.27	5.46	4.82
8	5.48	5.76	5.48	4.99
9	5.31	5.73	5.14	5.47
10	5.09	5.40	5.54	5.30
mean	5.41	5.52	5.43	5.26
SD	0.22	0.19	0.21	0.38
Min	5.07	5.27	5.08	4.50
max	5.72	5.81	5.78	5.69

SD = standard deviation

Table 10: Offspring per replicate and day

	No. of replicate containing one parental *Daphnia magna*, controls									
Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
7	0	0	0	0	0	0	0	0	0	0
8	0	8	0	0	4	0	0	7	0	0
9	6	0	6	0	0	4	0	0	6	5
10	0	0	0	11	0	0	8	0	0	0
11	0	4	0	0	2	0	0	3	0	0
12	0	0	0	0	0	0	0	0	0	0
13	20	16	21	14	18	19	13	17	13	17
14	0	0	15	0	0	0	0	10	0	0
15	0	0	0	0	0	0	0	0	0	0
16	23	17	6	14	16	17	19	10	20	15
17	0	3	11	0	0	0	0	12	0	0
18	16	0	0	14	14	16	18	0	0	2
19	0	13	12	0	0	0	0	0	14	13
20	14	0	0	0	0	0	4	13	0	0
21	0	11	10	13	14	14	6	0	0	0
sum	79	72	81	66	68	70	68	72	53	52

	No. of replicate containing one parental *Daphnia magna*, 152 µg/L									
Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
7	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	7	0	8	0	0	0
9	7	0	6	0	0	4	0	0	6	0
10	0	9	0	8	0	0	2	10	0	7
11	0	0	0	0	6	0	9	4	0	0
12	0	0	0	0	0	16	0	0	0	0
13	17	18	17	15	12	0	14	12	19	18
14	0	0	0	0	0	4	0	13	0	0
15	0	0	0	0	0	11	0	0	0	0
16	18	22	15	16	16	2	20	0	16	14
17	0	0	0	0	0	0	0	17	0	0
18	14	14	13	0	0	15	0	0	14	10
19	0	0	0	16	19	0	18	0	0	0
20	13	16	13	0	0	0	0	16	0	5
21	0	0	0	9	0	15	0	0	16	9
sum	69	79	64	64	60	67	71	72	71	63

- Table 10, to be continued –

- Table 10, continued –

	No. of replicate containing one parental *Daphnia magna*, 411 µg/L									
Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
7	0	0	0	0	0	0	0	0	0	0
8	4	0	0	0	2	0	0	4	0	0
9	0	5	0	0	0	4	4	0	6	0
10	0	0	7	7	4	0	0	*0*	0	4
11	4	0	0	0	0	0	0	4	0	0
12	0	0	0	0	0	4	0	0	0	0
13	17	22	14	13	18	15	16	12	18	14
14	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0
16	18	20	18	15	16	14	13	15	16	16
17	0	0	0	0	0	0	0	0	0	0
18	15	0	8	12	13	8	15	15	12	12
19	0	16	9	0	0	6	0	0	0	0
20	16	0	0	9	0	0	0	0	3	0
21	0	6	10	4	13	6	11	9	8	10
sum	74	69	66	60	66	57	59	59	63	56

	No. of replicate containing one parental *Daphnia magna*, 1110 µg/L									
Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
7	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	2	0	0	0	6
9	7	0	0	4	0	0	6	0	0	0
10	0	2	0	0	4	0	0	8	4	0
11	0	0	3	0	0	3	0	0	0	7
12	0	0	0	10	0	2	22	3	0	0
13	19	17	9	0	4	8	0	0	18	16
14	0	0	17	4	18	0	0	9	0	0
15	0	0	0	0	0	0	13	7	0	0
16	15	14	0	13	2	12	0	0	16	15
17	4	0	18	0	12	0	12	8	0	0
18	0	9	0	10	0	14	0	8	0	0
19	9	0	0	0	0	0	12	0	12	12
20	9	12	12	0	3	0	0	0	0	0
21	0	0	0	10	8	11	0	10	14	18
sum	63	54	59	51	51	52	65	53	64	74

	No. of replicate containing one parental *Daphnia magna*, 3000 µg/L									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
	Parental Daphnia magna died before reproduction on									
	Day 3	Day 1	Day 5	Day 3	Day 3	Day 16	Day 6	Day 3	Day 7	Day 9

10.7 Statistical evaluation of effects: Parts of ToxRat reports

10.7.1 Mortality

Immobility of *Daphnia magna* as caused by the test item.

Treatment	Introduced	Mobile	Immobile	% Immobility
control	10	10	0	0.0
1	10	10	0	0.0
2	10	10	0	0.0
3	10	10	0	0.0
4	10	0	10	100.0

Probit analysis using linear max. likelihood regression: 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 probits; reg. probit: calculated probits for the final function.

Initial concentrations

Treatm. [µg/L]	Log(x)	% Immobility	n	Emp. Probit	Weight	Reg. Probit
0		0.10	10			excluded
122	2.086	0.10	10	1.9074	0.001	1.278
351	2.545	0.10	10	1.9074	0.549	2.544
962	2.983	0.10	10	1.9074	5.457	4.355
2800	3.447	99.90	10	8.0926	3.477	6.264

Total concentrations

Treatm. [µg/L]	Log(x)	% Immobility	n	Emp. Probit	Weight	Reg. Probit
0		0.10	10			excluded
23	1.367	0.10	10	1.9074	0.001	1.278
107	2.029	0.10	10	1.9074	0.791	2.721
367	2.565	0.10	10	1.9074	5.582	4.404
1227	3.089	99.90	10	8.0926	4.237	6.042

excluded: value not in line with the chosen function

Inhibitions lower equal 0% or greater equal 100% were replaced by 0.100 and 99.900, respectively.

Parameters of the probit analysis: Results of the regression analysis

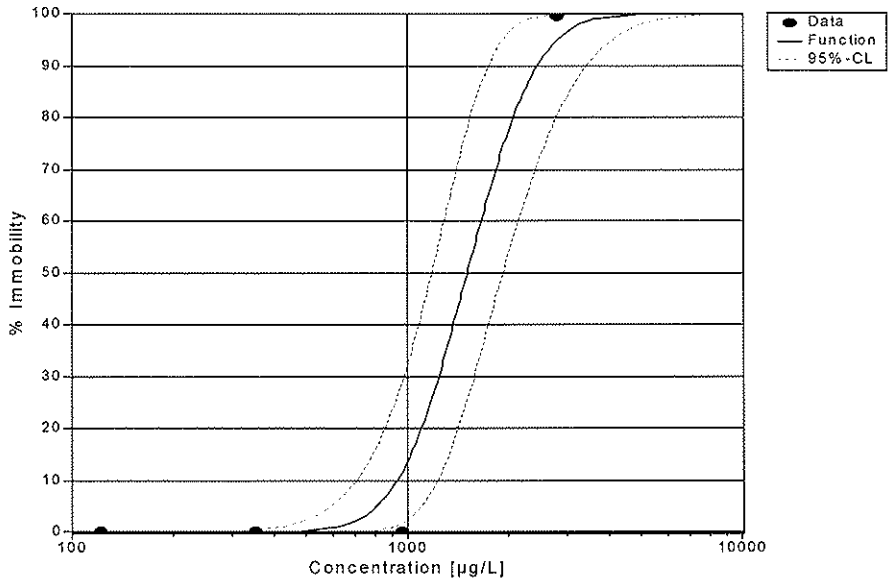
Initial concentrations

Parameter	Value
Parameter	**Value**
Computation runs:	1
Slope b:	6.12550
Intercept a:	-14.47426
Variance of b:	1.52348
Goodness of Fit	
Chi²:	2.72667
Degrees of freedom:	2
p(Chi²):	0.25581
Log EC50:	3.17921
s Log EC50:	12.58292
F:	18.065
p(F) (df: 1;2):	0.051

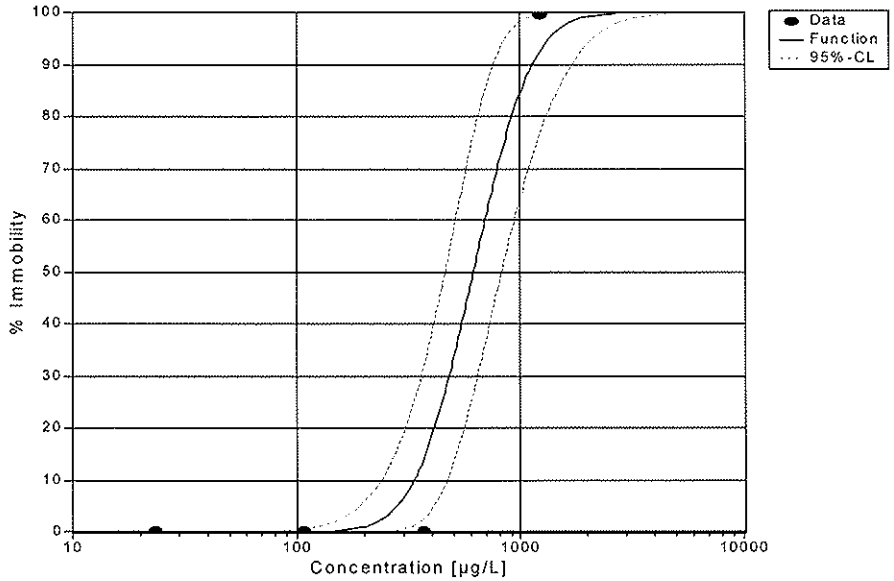
Total concentrations

Parameter	Value
Computation runs:	1
Slope b:	4.85109
Intercept a:	-8.52990
Variance of b:	0.91899
Goodness of Fit	
Chi²:	3.35084
Degrees of freedom:	2
p(Chi²):	0.18723
Log EC50:	2.78904
s Log EC50:	4.95848
F:	15.284
p(F) (df: 1;2):	0.060

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 (= are made wider; so, check whether these results are reasonable!).



Concentration-effect curve showing the influence of the initial test item concentrations on mobility of the introduced Daphnia magna as observed after 21 d.



Concentration-effect curve showing the influence of the total test item concentrations on mobility of the introduced Daphnia magna as observed after 21 d.

10.7.2 Length at 21 d

Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; sd: standard deviation; cv%: coefficient of variation; se: standard error; %se: %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatment No.	Mean	Med	Min	Max	n	sd	cv%	se	%se	95%l	95%u
Control	5.4	5.4	5.1	5.7	10	0.22	4.1	0.07	1.3	5.3	5.6
1	5.5	5.5	5.3	5.8	10	0.19	3.5	0.06	1.1	5.4	5.7
2	5.4	5.5	5.1	5.8	10	0.21	3.8	0.07	1.2	5.3	5.6
3	5.3	5.4	4.5	5.7	10	0.38	7.2	0.12	2.3	5.0	5.5

One-way Analysis of Variance: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	0.36	3	0.12	1.730	0.178
Residuals	2.46	36	0.07		
Total	2.82	39			

p(F) is greater than the selected significance level of 0.050; therefore, treatments are not significantly different.

10.7.3 Cumulative offspring of survivors after 21 d.

%Inhibition of cumulative offspring of survivors caused by the test item after 21 d.

Treatment		(µg/L)	Mean	sd	n	%Reduction
No.	initial	total				
Control	x	x	68.0	9.50	10	
Level 1	122	23	68.0	5.60	10	0.0
Level 2	351	107	63.0	5.80	10	7.4
Level 3	962	367	59.0	7.70	10	13.2
Level 4	2800	1227			0	

sd: standard deviation

Probit analyses using linear max. likelihood regression: Determination of the concentration/response function; data is shown which entered the probit analysis; Log(x): logarithm of the concentration; n: number of replicates; Emp. Probit: empirical probits; reg. probit: calculated probits for the final function.

Initial concentrations

Treatm. [µg/L]	Log(x)	% Offspring Reduction	n	Emp. Probit	Weight	Reg. Probit
0		0.10	10			excluded
122	2.086	0.15	10	2.0234	0.015	2.947
351	2.545	7.64	10	3.5710	0.092	3.454
962	2.983	13.95	10	3.9194	0.322	3.938

excluded: value not in line with the chosen function

Total concentrations

Treatm. [µg/L]	Log(x)	% Offspring Reduction	n	Emp. Probit	Weight	Reg. Probit
0		0.10	10			excluded
23	1.367	0.15	10	2.0234	0.012	2.900
107	2.029	7.64	10	3.5710	0.097	3.473
367	2.565	13.95	10	3.9194	0.322	3.937

Inhibitions lower equal 0% or greater equal 100% were replaced by 0.100 and 99.900, respectively.

Parameters of the probit analyses: Results of the regression analyses

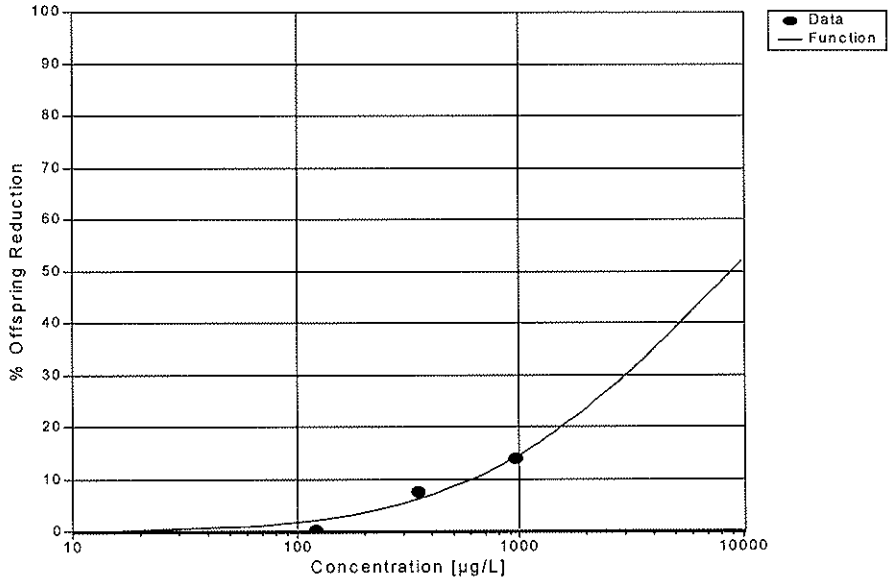
Initial concentrations

Parameter	Value
Computation runs:	8
Slope b:	1.10141
Intercept a:	0.65023
Variance of b:	43.76971
Goodness of Fit	
Chi²:	0.00377
Degrees of freedom:	1
p(Chi²):	0.95106
Log EC50:	3.94928
s Log EC50:	177.91754
g-Criterion:	21.94273
Residual Variance (Chi²/df):	0.00377
r²:	0.880
F:	7.357
p(F) (df: 1;1):	0.225

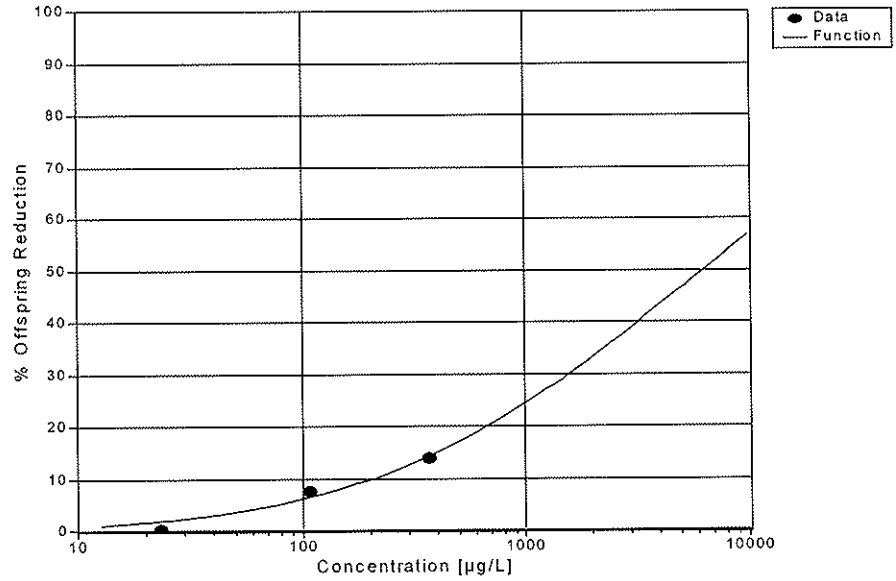
Total concentrations

Parameter	Value
Computation runs:	8
Slope b:	0.86392
Intercept a:	1.71940
Variance of b:	28.57065
Goodness of Fit	
Chi²:	0.00286
Degrees of freedom:	1
p(Chi²):	0.95736
Log EC50:	3.79733
s Log EC50:	21.38141
g-Criterion:	17.67048
Residual Variance (Chi²/df):	0.00286
r²:	0.901
F:	9.136
p(F) (df: 1;1):	0.203

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 (= are made wider; so, check whether these results are reasonable!). The coefficient of determination, r^2 ($0 <= r^2 <= 1$), gives the proportion of variance explained by the dose/response function.F-Test for regression: Ho: Slope = 0; hence, if p(F) <= alpha, the selected significance level, (e.g., alpha = 0.05) the regression revealed significant results (= slope is significantly different from zero).



Concentration-effect curve showing the influence of the initial test item concentrations on cumulative offspring of survivors of the introduced Daphnia magna as observed after 21 d.



Concentration-effect curve showing the influence of the mean measured test item concentration on cumulative offspring of survivors of the introduced Daphnia magna as observed after 21 d.

Threshold Concentrations (NOEC) with cumulative offspring after 21 d

Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; sd: standard deviation; cv%: coefficient of variation; se: standard error; %se: %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatment No.	Mean	Med	Min	Max	n	sd	cv%	se	%se	95%l	95%u
control	68.1	69.0	52.0	81.0	10	9.49	13.9	3.00	4.4	61.3	74.9
1	68.0	68.0	60.0	79.0	10	5.56	8.2	1.76	2.6	64.0	72.0
2	62.9	61.5	56.0	74.0	10	5.78	9.2	1.83	2.9	58.8	67.0
3	58.6	56.5	51.0	74.0	10	7.73	13.2	2.45	4.2	53.1	64.1

One-way Analysis of Variance: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	625.40	3	208.47	3.892	0.017
Residuals	1928.20	36	53.56		
Total	2553.60	39			

p(F) is smaller than or equal to the selected significance level of 0.050; therefore, treatments are significantly different. The first analysis revealed significant results: Pre-testing is continued.

Kolmogorov-Smirnov-test on Normal Distribution: Testing the normality hypothesis (goodness of fit; modification after Lilliefors 1967); significance level (Alpha = 0.05); Mean: arithmetic mean; sd: standard deviation; n: sample size; Diff: max. difference between observed and expected cumulative relative frequency distribution; Diff*: critical margin of H_o; the normality hypothesis (H_o) is rejected in case |Diff| > Diff* (+).

Treatment No.	Mean	sd	n	Diff	Diff*	Sign.
control	68.1	9.49	10	0.144	0.258	-
1	68.0	5.56	10	0.164	0.258	-
2	62.9	5.78	10	0.192	0.258	-
3	58.6	7.73	10	0.224	0.258	-

+: significant; -: non-significant (= in line with normal distribution)

Correspondence with normal distribution was seen in more than 50% of treatments. Cochran´s test is chosen for variance homogeneity testing.

Cochran´s Test Procedure on Variance Homogeneity: Homogeneity of variance is tested (Alpha = 0.05); var: variance; n: sample size; G*: critical limit for G_max; dfm: degrees of freedom for the multiple test procedure; homogeneity hypothesis is rejected if G_max > G*.

Treatment No.	var	n
control	90.1	10
1	30.9	10
2	33.4	10
3	59.8	10

G_max = 0.4; dfm = 9; G* = 0.50; G_max <= G*: homogeneity hypothesis is accepted.

Variance homogeneity check was passed.
Normal distribution and variance homogeneity requirements are fulfilled.
A parametric multiple test is advisable.

Williams Multiple Sequential t-test Procedure: Comparison of treatments with "0.2" by the t test procedure after Williams. Significance was Alpha = 0.05, one-sided smaller; Mean: arithmetic mean; n: sample size; sd: standard deviation; LhM: max. likelihood mean; %MDD: minimum detectable difference to 0.2 (in percent of 0.2); t: sample t; t*: critical t for H_o: $\mu 1 = \mu 2 = ... = \mu k$; the differences are significant in case $|t| > |t^*|$ (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatment No.	Mean	sd	df	LhM	%MDD	t	t*	Sign.
control	68.1	7.319						
1	68.0	7.319	36	68.0	8.1	-0.03	-1.69	-
2	62.9	7.319	36	62.9	8.5	-1.59	-1.77	-
3	58.6	7.319	36	58.6	8.6	-2.90	-1.79	+

+: significant; -: non-significant

A NOEC at treatment 2 (332.5 µg/L initial concentration, 104.0 µg/L total concentration) is suggested by the program.

10.7.4 Age at first reproduction

ToxRat adds 0.5 days to compensate for the introductory age.

Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; sd: standard deviation; cv%: coefficient of variation; se: standard error; %se: %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatment No.	Mean	Med	Min	Max	n	sd	cv%	se	%se	95%l	95%u
control	9.4	9.5	8.5	10.5	10	0.74	7.8	0.23	2.5	8.9	9.9
1	9.7	9.5	8.5	10.5	10	0.79	8.1	0.25	2.6	9.1	10.3
2	9.5	9.5	8.5	10.5	10	0.82	8.6	0.26	2.7	8.9	10.1
3	9.9	10.0	8.5	11.5	10	0.97	9.8	0.31	3.1	9.2	10.6

One-way Analysis of Variance: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	1.47	3	0.49	0.711	0.448
Residuals	24.90	36	0.69		
Total	26.38	39			

p(F) is greater than the selected significance level of 0.050; therefore, treatments are not significantly different.

10.7.5 Intrinsic rate of increase

Intrinsic rate of increase in *Daphnia magna* as dependent on concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV%: coefficient of variation (calculated from InputRawData)

Initial concentration (µg/L)	**1.6**	**122**	**351**	**962**	**2800**
Total concentration (µg/L)	**<LOQ**	**23.3**	**107**	**367**	**1227**
21.0 d	0.296	0.292	0.299	0.295	
	0.321	0.296	0.291	0.256	
	0.307	0.285	0.278	0.262	
	0.290	0.280	0.273	0.268	
	0.289	0.311	0.286	0.259	
	0.279	0.288	0.277	0.260	
	0.281	0.340	0.270	0.315	
	0.322	0.306	0.285	0.270	
	0.278	0.289	0.287	0.269	
	0.276	0.280	0.262	0.319	
Mean:	0.294	0.297	0.281	0.277	
Std.Dev.:	0.0173	0.0184	0.0110	0.0235	
n:	10	10	10	10	
CV:	5.9	6.2	3.9	8.5	

%Inhibition of intrinsic rate r caused by the test item after 21 d.

Treatment No.	**Mean**	**Std. Dev.**	**n**	**%Reduction**
control	0.294	0.0173	10	0.0
1	0.297	0.0184	10	-0.8
2	0.281	0.0110	10	4.5
3	0.277	0.0235	10	5.7

Initial concentrations

Probit analyses using linear max. likelihood regression: Determination of the concentration/response function; data is shown which entered the probit analysis; Log(x): logarithm of the concentration; n: number of replicates; Emp. Probit: empirical probits; reg. probit: calculated probits for the final function.

Treatm. [µg/L]	Log(x)	% Inhibition of r	n	Emp. Probit	Weight	Reg. Probit
0		0.10	10			excluded
122	2.086	0.10	10	1.9074	0.008	2.807
351	2.545	4.52	10	3.3066	0.031	3.138
962	2.983	5.69	10	3.4193	0.092	3.455

Results of the regression analyses

Parameter	Value
Computation runs:	9
Slope b:	0.71998
Intercept a:	1.30601
Variance of b:	108.53643
Goodness of Fit	
Chi²:	0.00240
Degrees of freedom:	1
p(Chi²):	0.96092
Log EC50:	5.13071
s Log EC50:	496.38782
g-Criterion:	81.17557
Residual Variance (Chi²/df):	0.00240
r²:	0.665
F:	1.989
p(F) (df: 1;1):	0.393

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 (= are made wider; so, check whether these results are reasonable!). The coefficient of determination, r^2 ($0 <= r^2 <= 1$), gives the proportion of variance explained by the dose/response function.F-Test for regression: Ho: Slope = 0; hence, if p(F) <= alpha, the selected significance level, (e.g., alpha = 0.05) the regression revealed significant results (= slope is significantly different from zero).

Total concentrations

Probit analyses using linear max. likelihood regression: Determination of the concentration/response function; data is shown which entered the probit analysis; Log(x): logarithm of the concentration; n: number of replicates; Emp. Probit: empirical probits; reg. probit: calculated probits for the final function.

Treatm. [µg/L]	Log(x)	% Inhibition of r	n	Emp. Probit	Weight	Reg. Probit
0		0.10	10			excluded
23	1.367	0.10	10	1.9074	0.007	2.779
107	2.029	4.52	10	3.3066	0.033	3.153
367	2.565	5.69	10	3.4193	0.092	3.455

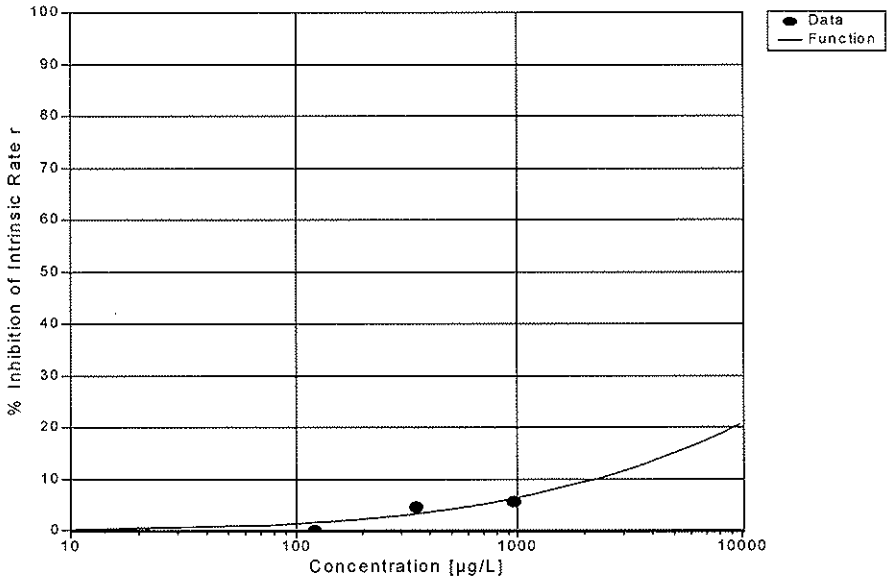
Inhibitions lower equal 0% or greater equal 100% were replaced by 0.100 and 99.900, respectively.

Parameters of the probit analyses:

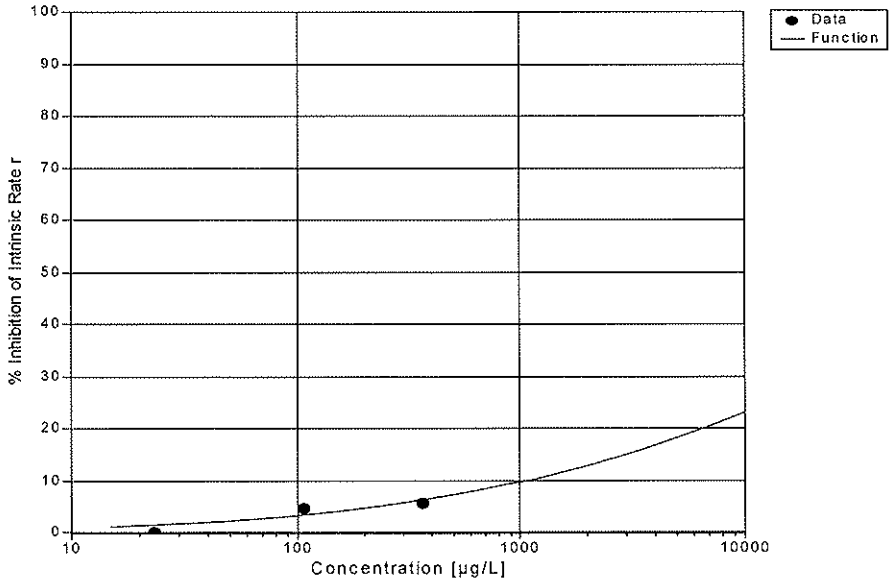
Results of the regression analyses

Parameter	Value
Computation runs:	10
Slope b:	0.56362
Intercept a:	2.00930
Variance of b:	68.57945
Goodness of Fit	
Chi^2:	0.00208
Degrees of freedom:	1
$p(Chi^2)$:	0.96365
Log EC50:	5.30624
s Log EC50:	51.19492
g-Criterion:	72.37442
Residual Variance (Chi^2/df):	0.00208
r^2:	0.690
F:	2.231
p(F) (df: 1;1):	0.376

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!). The coefficient of determination, r^2 ($0 <= r^2 <= 1$), gives the proportion of variance explained by the dose/response function.F-Test for regression: Ho: Slope = 0; hence, if p(F) <= alpha, the selected significance level, (e.g., alpha = 0.05) the regression revealed significant results (= slope is significantly different from zero).



Concentration-effect curve showing the influence of the initial concentration of the test item on intrinsic rate r of the introduced *Daphnia magna* as observed after 21 d.



Concentration-effect curve showing the influence of the mean measured total test item concentrations on intrinsic rate r of the introduced *Daphnia magna* as observed after 21 d.

Threshold Concentrations (NOEC) with Intrinsic Rate r at 21 d

Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; sd: standard deviation; cv%: coefficient of variation; se: standard error; %se: %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatment No.	Mean	Med	Min	Max	n	sd	cv%	se	%se	95%l	95%u
control	0.294	0.290	0.276	0.322	10	0.0173	5.9	0.0055	1.9	0.282	0.306
1	0.297	0.290	0.280	0.340	10	0.0184	6.2	0.0058	2.0	0.283	0.310
2	0.281	0.281	0.262	0.299	10	0.0110	3.9	0.0035	1.2	0.273	0.289
3	0.277	0.268	0.256	0.319	10	0.0235	8.5	0.0074	2.7	0.260	0.294

Kolmogorov-Smirnov-test on Normal Distribution: Testing the normality hypothesis (goodness of fit; modification after Lilliefors 1967); significance level (Alpha = 0.05); Mean: arithmetic mean; StdDev: standard deviation; n: sample size; Diff: max. difference between observed and expected cumulative relative frequency distribution; Diff*: critical margin of Ho; the normality hypothesis (Ho) is rejected in case |Diff| > Diff* (+).

Treatment No.	Mean	s	n	Diff	Diff*	Sign.
control	0.294	0.0173	10	0.184	0.258	-
1	0.297	0.0184	10	0.222	0.258	-
2	0.281	0.0110	10	0.108	0.258	-
3	0.277	0.0235	10	0.315	0.258	+

+: significant; -: non-significant (= in line with normal distribution)

Correspondence with normal distribution was seen in more than 50% of treatments. Cochran´s test is chosen for variance homogeneity testing.

Cochran´s Test Procedure on Variance Homogeneity: Homogeneity of variance is tested (Alpha = 0.05); var: variance; n: sample size; G*: critical limit for G_max; dfm: degrees of freedom for the multiple test procedure; homogeneity hypothesis is rejected if G_max > G*.

Treatment no.	var	n
control	0.000	10
1	0.000	10
2	0.000	10
3	0.001	10

G_max = 0.422; dfm = 9; G* = 0.50; G_max <= G*: homogeneity hypothesis is accepted.

Variance homogeneity check was passed. Normal distribution and variance homogeneity requirements are fulfilled. A parametric multiple test is advisable.

Williams Multiple Sequential t-test Procedure: Comparison of treatments with control by the t test procedure after Williams. Significance was Alpha = 0.05, one-sided smaller; Mean: arithmetic mean; n: sample size; sd: standard deviation; LhM: max. likelihood mean; %MDD: minimum detectable difference to 0.2 (in percent of 0.2); t: sample t; t*: critical t for Ho: μ1 = μ2 = ... = μk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatment no.	Mean	sd	df	LhM	%MDD	t	t*	Sign.
control	0.294	0.01810						
1	0.297	0.01810	36	0.297	4.648	0.30	-1.69	-
2	0.281	0.01810	36	0.281	4.860	-1.64	-1.77	-
3	0.277	0.01810	36	0.277	4.928	-2.07	-1.79	+

+: significant; -: non-significant

A NOEC at treatment 2 (332.5 µg/L initial concentration, 104.2 µg/L total concentration) is suggested by the program.

10.8 Analytical Report - details of method

10.8.1 Chemicals, solutions and equipment

Analyte:	n-Decanol, C_{10} fatty alcohol
Reference standards:	n-Decanol (Ehrenstorfer) 99.5 %, Lot. No. 21011
Internal standards:	n-Dodecanol-d_{25} (Larodan AB, Sweden) 95 At. D %
Internal standard solution:	n-Dodecanol-d_{25} in n-hexane: 50 mg/L
Calibration solutions: n-hexane:	0.01 to 25 µg/mL of the reference standard in n-hexane extra pure (Riedel-de Haën)
Test medium:	Purified drinking water
Derivatization reagent:	MSTFA (N-methyl-N-trimethylsilyl-trifluoroacetamide) puriss. for GC (Fluka)

GC-MS

Gas chromatograph:	5890 Series II plus, Hewlett Packard		
Autosampler:	type 7673, Hewlett Packard		
Column:	SGE BPX-5, 50 m, ID 0.32 mm, film 0.25 µm		
Oven	step:	1	2
	temperature [°C]:	60	280
	time [min]:	1	1
	rate [°C/min]:	10	
SSL-injector temperature:	280 °C		
Mass spectrometer:	MSD 5972, SIM Mode, Hewlett Packard		
Target ions:	m/z 215	C_{14}-fatty alcohol	
	m/z 266	C_{14}-fatty alcohol, deuterated	

10.8.2 Sample clean-up

The analyte was extracted from the aqueous test samples by liquid-liquid partitioning with n-hexane. Sample volume was 200 – 400 mL and the hexane volume 5 mL. After shaking for about 10 min and settling for 60 min, 100 µL of the supernatant (n-hexane) were taken and 50 µL each of the internal standard solution and the derivatizing agent (MSTFA) were added. Measurement was performed by GC-MS in

SIM mode using internal standard calibration with deuterated C_{12} fatty alcohol as internal standard.

10.8.3 Validation

Validation of the analytical method was performed according to guideline SANCO/825/00 rev. 6 and SANCO/3029/99 ver. 4 (ref 1, ref 2). The guidelines describe the pesticide pre-registration data requirements in Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414 and the requirements for post-registration monitoring and control. According to these guidelines the analytical method was validated in respect to specificity, linearity, accuracy, precision, identity and limit of quantification (LOQ).

Specificity

The specificity of the method was checked by the chromatography of unfortified matrix samples and the determination of possibly interfering peaks.

Linearity

The linearity of the detector response was shown by the chromatography of 8 calibration solutions in the range from 0.01 to 25 µg/mL. This corresponds to sample concentrations from 0.1 to 250 µg/L. Higher concentrated samples were diluted before processing. Calibration was not based on the recovery of the internal standards but on the actual concentrations in the aqueous samples.

To set up the calibration function, 50 µL of internal standard solution and 50 µL MSTFA (for derivatization) were added to 100 µL of each calibration solution. 1 µL of these mixtures were measured by GC-MS.

Accuracy and Precision

The accuracy of the method was determined by recovery experiments with fortified samples. The recovery data were reported for 2 fortification levels appropriate to the proposed LOQ and to 100 x LOQ (= 1.0 and 100 µg/L). The recoveries for each level were in the range of 91-112 % of nominal, with the mean in the range of 93-98 % (Table 11). Five replicates were processed.

2 control (unfortified) samples were analysed concurrently to determine any contamination by the analyte of interest or interferences.

100 µL of the spiking solutions containing the reference standard (C_{10}-fatty alcohol) in methanol were given to 500 mL tap water and processed as described under 'Sample clean-up'.

The precision of the method is defined as the repeatability of recovery at each fortification level and characterized by the overall relative standard deviation (RSD). The RSD was < 20 % for all levels (Table 11).

Table 11: Percent recovery of C_{10} fatty alcohol

Fortification	1.0 µg/L	100 µg/L
Replicate 1	93.0	112.1
Replicate 2	93.0	98.5
Replicate 3	92.2	93.0
Replicate 4	94.8	92.7
Replicate 5	91.3	93.4
Mean	92.9	97.9
SD	1.3	8.3
RSD	1.4	8.4

Identity

The identity of the analyte and the deuterated reference item were taken as approved by the Certificate of Analysis. In addition the interpretation of the mass fragments and their relation obtained by mass spectrometric detection were used for structure verification. For ions used for identification/quantification by mass spectrometry refer to GC-MS parameters.

No additional confirmatory analysis was required as the method was shown to be specific to the analyte in the matrices analysed.

Limit of Quantification (LOQ)

The lowest validated concentration was 1.0 µg/L (=LOQ). Blank values did not exceed 30% of the LOQ.

References

Ref 1 European Commission, Directorate General Health and Consumer Protection: SANCO/825/00 rev. 6 (20/06/2000), Guidance document on residue analytical methods

Ref 2 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

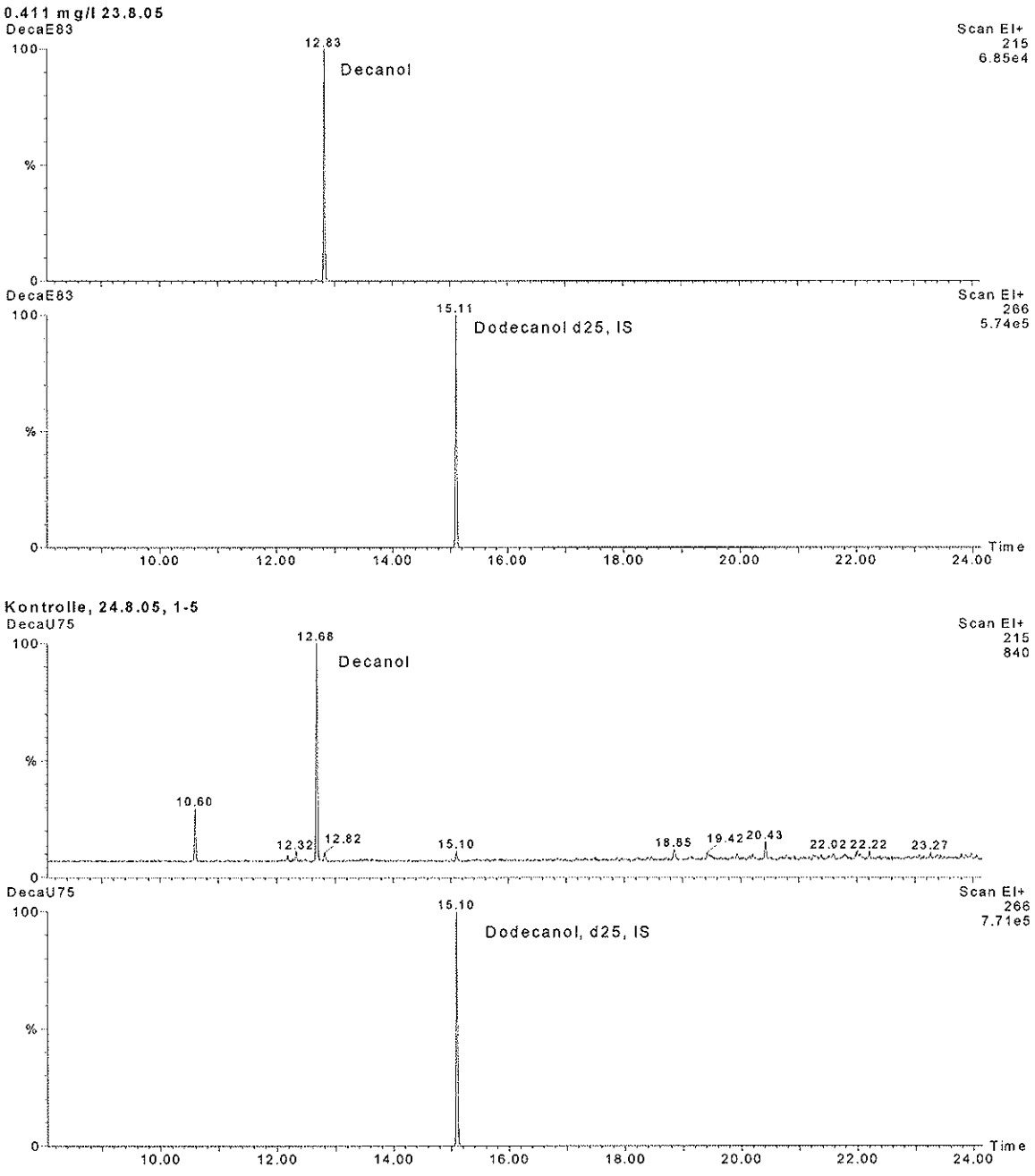


Figure 6: Representative chromatograms of the test item
upper: sample E83, 411 µg/L
lower: sample U75, blank (control)

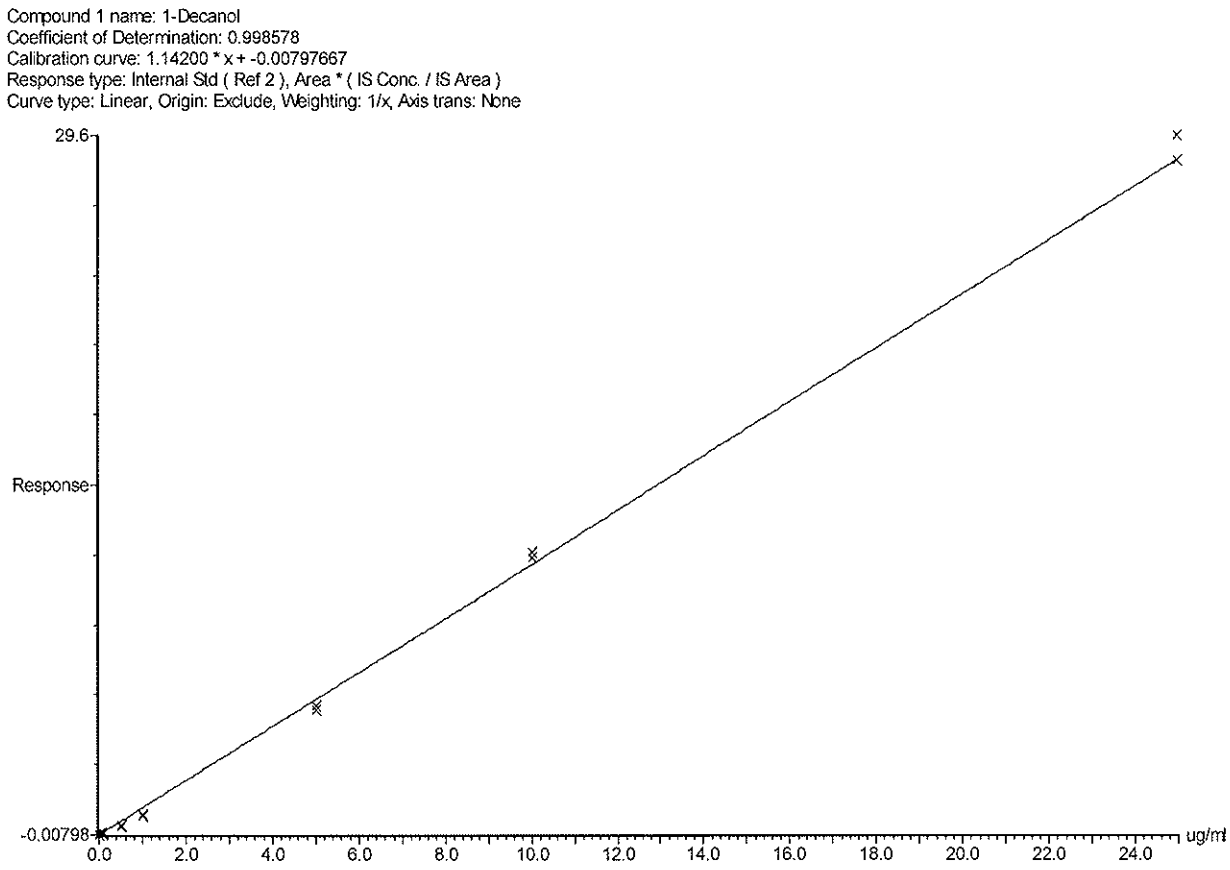


Figure 7: Representative calibration line of n-decanol

10.9 Certificate of Analysis of the test item

Certificate of Analysis

Dr. Ehrenstorfer
Reference Materials for Residue Analysis

Product Identification

12095200 1-Decanol
CA 1-Decanol
IUPAC 1-Decanol
Formula C10H22O
Mol.Weight 158.29
CAS No. 112-30-1

Expiry Date 01.10.2008
Lot Number 21011
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	Inj. Volume (µl)		RT 1	0.81
Column	3% OV 11 on Chromosorb W-HP	Inj. Temp.	260	Col. Temp.	180
Method 2		Inj. Volume (µl)		RT 2	
Column		Flow (ml/min)		Gradient	
Eluent A					
Eluent B					

Identity check
Comment

Water Content 0.2 % 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 14.10.2002
by Dr. J. Heidrich

A231

Labor Dr. Ehrenstorfer-Schäfers Bgm.-Schlosser-Str. 8 A · 86199 Augsburg · Germany
Phone +49 821 906060 Fax +49 821 9060686 info@analytical-standards.com

10.10 GLP-Certificate of the testing facility

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

Postanschrift 40190 Düsseldorf — Aktenzeichen VI-5- 51.11 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)
(Name)

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

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, 17. Februar 2003

Im Auftrag

(Prof. Dr. Heinrich David)

Dienstsiegel
(official-seal)