Fraunhofer Institut
Molekularbiologie und
Angewandte Oekologie

Study Report

Alga, Growth Inhibition Test

Effect of **hexanol** on the growth of the green alga *Pseudokirchneriella subcapitata*

(under consideration of proposal for updating OECD 201 of April 2004)

GLP-Code of Testing Facility: SDA-003/4-30

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Confidentiality Statement

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Summary

Study design:

A study was performed at the Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Germany, to determine the toxicity of the test item hexanol on the growth of the green alga *Pseudokirchneriella subcapitata* according to the OECD guideline 201 (1) under static conditions considering also the proposals for updating of April 2004.
The test item was dissolved in sterilized test medium. All work was performed under sterile conditions. For the assessment of algal growth, three replicates of each test concentration and six replicates of the control were exposed. For the assessment of the stability of the test item without algae (blanks), 2 replicates were prepared for each test concentration. The exposure concentrations in the algae cultures and in the blanks were chemically analyzed at test start, after 24 hours and at test end.

Results:

Test item concentrations: The measured concentrations deviated < 20 % from the initial measured concentrations in all concentration plots after 24 hours. After 72 hours, deviations of the measured initial concentrations were < 20 % in three concentration plots and > 20 % in two concentration plots. The test item was regarded stable during the test and there was no influence of the algae on the test concentration. Therefore, the effect concentrations were calculated based on the mean measured concentrations of the algae cultures.
Nominal test concentrations were: 7.7, 17, 37, 82 and 180 mg hexanol/L
Mean measured concentrations were: 4.72, 11.3, 31.2, 50.1 and 111.2 mg hexanol/L

Effect on algal growth: A concentration-effect relationship was observed and statistically analyzed to obtain effect concentrations. The effect concentrations for the inhibition of growth rate are summarized in Table 1 for the test item hexanol.

Table 1: Effective concentrations of the test item hexanol on the growth of *Pseudokirchneriella subcapitata* over 72 h

Parameter		EC_{50}	EC_{10}	LOEC	NOEC
Growth rate (r)	**Value [mg/L]**	**79.7**	**19.8**	**31.2**	**11.3**
	lower 95%-cl	62.8	8.04		
	upper 95%-cl	114	29.4		

Statement of GLP-compliance

Title of the study:	Alga, Growth Inhibition Test (OECD 201)
Test item:	hexanol
Study-Code:	SDA-003/4-30

The study was conducted in compliance with Good Laboratory Practice regulations (GLP) (2, 3).

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

Date: March 10, 2005

Dr. Andrea Wenzel
(Study Director)

Date: March 10, 2005

Dr. Josef Müller
(Chemical Investigator)

Date: March 11, 2005

Prof. Dr. Andreas Schäffer
(Test facility manager)

Statement of the Quality assurance unit

Title of the study: Alga, Growth Inhibition Test (OECD 201)
(under consideration of proposal for updating OECD 201 of April 2004)
Test item: hexanol
Study-Code: SDA-003/4-30

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

Dates of QAU inspections:	Study plan	December 6, 2004
	Alga, Growth Inhibition Test, Determination of cell density	January 28, 2005
	Study report	March 8, 2005

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

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

Date: March 10, 2005

U. Fritsche

Dr. Ulrich Fritsche
(QAU-Officer)

1 Study identification

1.1 Test

Algae Growth Inhibition Test with *Pseudokirchneriella subcapitata* following OECD Guideline 201 (1) (under consideration of proposal for updating OECD 201 of April 2004)

Test item: hexanol
GLP-Code: SDA-003/4-30

1.2 Sponsor

The Soap and Detergent Association
1500 K Street, N.W., Suite 300
Washington, D.C., 20005, USA

Kathleen Stanton
Assistant Director, Scientific Affairs

Study Monitor: Dr. Hans Sanderson
Director Environmental Safety

1.3 Testing facility

Fraunhofer-Institute for
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57377 Schmallenberg, Germany

Testing facility management: Prof. Dr. Andreas Schäffer

Study director: Dr. Andrea Wenzel
Deputy: Dr. Christoph Schäfers
Chemical investigator: Dr. Josef Müller

Technical staff, Biology: Elisabeth Hardebusch
Chemistry Heinrich Jürling

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

Subcontractor

The study was performed without subcontracting

1.4 Study dates

Initiation:	December 6, 2004
Performance of the pre-test:	January 10-13, 2005
Performance of the main test:	January 25-28, 2005
Study completion:	March 10, 2005

2 GLP

The test was performed in accordance with the Principles of Good Laboratory Practice (2, 3).

3 Information on test item and analytical standard

3.1 Test item specification

3.1.1	Product name	Hexyl alcohol
3.1.2	Product number	W256706
3.1.3	Chemical name	1-hexanol
3.1.4	CAS-number	111-27-3
3.1.5	Batch/Lot number	452734/1 3160 4133
3.1.6	State of matter and appearance	colorless liquid
3.1.7	Purity (w/w)	> 98 %
3.1.8	Water solubility	5.9 g/L (20 °C) (MERCK)
3.1.9	Vapour pressure	1 hPa (20 °C)
3.1.10	Specific density	0.82 g/cm³ (20 °C)
3.1.11	Log Kow	2.03 (experimental)
3.1.12	Chemical stability (Water/Light)	stable, avoid strong oxidizing agents and strong acids
3.1.13	Storage conditions	store tightly closed. storage temperature: no restrictions
3.1.14	Expiry date	November 30, 2006 (own classification)
3.1.15	Material Safety Data Sheet	yes (Risk phrases R22, Safety phrases S24/25)
3.1.16	Certificate of Analysis:	yes
3.1.17	Origin of the test item	FLUKA-SIGMA-ALDRICH Group

With his signature under the study plan the sponsor confirms his agreement with the fact that chemical identity and purity of the test item are not examined by the contractor.

3.2 Analytical reference standard

3.2.1	Name:	n-hexanol-d13
3.2.2	CAS number unlabeled:	111-27-3
3.2.3	Molecular weight:	115.3
3.2.4	Formula:	CD3(CD2)5OH
3.2.5	Chemical Purity:	99.9 %
3.2.6	Isotopic purity:	98 at % D
3.2.7	Lot No.:	PR-11895
3.2.8	Manufacturer:	Cambridge Isotope Laboratories, Inc.
3.2.9	50 Frontage Road, Andover, Massachusetts, USA	
3.2.10	Manufacturer catalog no.:	DLM-691-0
3.2.11	Origin of the test item:	Chemotrade Chemie Handelsgesellschaft mbH Brahestraße 27, 94347 Leipzig, Germany, supplier article no.: DV4102G
3.2.12	Storage:	room temperature away from light and moisture
3.2.13	Expiry:	two years after receipt (if stored at room temperature away from light and moisture)
3.2.14	Receipt:	22.10.2004
3.2.15	Material Safety data sheet:	yes
3.2.16	Certificate of Analysis:	yes

4 Test objective

The objective of this study (72 h EC_{50} growth inhibition test) was the assessment of the toxicity of the test item on the green alga *Pseudokirchneriella subcapitata.* The algae were exposed to various concentrations of the test item over several generations under static conditions over a period of 72 h.

The cell culture density was determined daily. The test was conducted following the "Alga, Growth Inhibition Test" Guideline OECD No. 201 (1) considering also the proposals for updating the testing guideline 201 of April 2004.

Growth and growth inhibition were quantified from measurements of the algal biomass density (cell counts) as a function of time.

The test endpoint was inhibition of growth, expressed as logarithmic algal biomass increase (average growth rate, r), yield expressed as cell number increase and algal biomass integral (area under the growth curve, b) during the exposure period. From average growth rates, cell numbers and biomass integrals recorded in a series of test solutions and the respective test item concentrations EC_{10} and EC_{50} values were determined. In addition, LOEC and NOEC were determined.

Definitions:

Growth rate (r)	logarithmic algal biomass increase (average growth rate) during the exposure period.
Yield (y)	(recommended in April 2004 proposal) algal biomass (cell counts/mL) at the end of the exposure period minus the algal biomass at the start of the exposure. Since the starting biomass is extremely small relative to the final biomass, the final biomass and the yield are very nearly equal and final cell counts/mL is used as yield in this study.
Biomass (b)	logarithmic biomass integral (area under the growth curve)
LOEC	(lowest observed effect concentration) is the lowest concentration tested at which the measured parameter shows significant inhibition relative to the control.
NOEC	(no observed effect concentration) is the highest concentration tested at which the measured parameter shows no significant inhibition relative to the control.
$EC_{10/20/50}$	(effective concentration) is the concentration of the test item, which results in a 10, 20 or 50 per cent reduction in the measured parameter relative to the control. OECD 201 (1984) requests EC_{50}.

5 Study setup and test conditions

5.1 Test organism

5.1.1 Justification for the use of the test organism

The unicellular green alga *Pseudokirchneriella subcapitata* was chosen by experts (1) as test organism representing freshwater primary producers.

5.1.2 Specification

Species: *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum)*, Chlorophycea, Chlorophyta.

Origin: SAG, Culture Collection of Algae at Pflanzenphysiologisches Institut of the University at Göttingen, Albrecht von Haller Institut, Untere Klarspüle 2, D-37073 Göttingen, Catalog No 61.81.

5.2 Primary standard

In order to confirm the reproducibility of the study and the sensitivity of the test organism growth inhibition tests with zinc sulfate ($ZnSO_4$ x 7 H_2O) are performed twice a year. The results are in agreement with published EC_{50}-values regarding biomass increase related to the Zn^{2+}-ion ranging between 45.0 and 65.4 µg/L for biomass (4, 5).

EC_{50} of Zn^{2+} for inhibition of biomass increase: between 13.5 and 46.9 µg/L (n=5)
EC_{50} of Zn^{2+} for inhibition of growth rate: between 34.7 and 68.3 µg/L (n=5)

5.3 Cultivation and pre-culture

The stock cultures were maintained fulfilling the criteria of the OECD guidelines (1). Prior to testing a pre-culture was established in test medium to obtain exponentially growing algae for the test.

5.4 Growth medium

A sterilized synthetic growth medium (OECD medium) according to OECD 201 was used for culture and preparation of the test medium for testing.

Table 2: OECD medium: Algal medium according to OECD 201

	mg/L		mg/L
$NaHCO_3$	50	H_3BO_3	0.185
NH_4Cl	15	$MnCl_2$ x 4 H_2O	0.415
K_2HPO_4	1.6	$ZnCl_2$	0.003
$MgSO_4$ x 7 H_2O	15	$CoCl_2$ x 6 H_2O	0.0015
$MgCl_2$ x 6 H_2O	12	$CuCl_2$ x 2 H_2O	0.00001
$CaCl_2$ x 2 H_2O	18	Na_2MoO_4 x 2 H_2O	0.007
$FeCl_3$ x 6 H_2O	0.064	Na_2EDTA x 2 H_2O	0.1
		pH, at test start	7.5 - 8.0

5.5 Test medium

A sterilized synthetic growth medium according to OECD 201 (1) was used for culture.

For the preparation of a homogenous test item solution a respective amount of the test item (180 mg) to obtain the highest test concentration was given in a sterilized bottle under a flow-box, mixed with sterilized test medium and adjusted to 1 L. The solution was stirred for 24 hours. The highest test concentration was diluted with sterilized growth medium to obtain the other four nominal test concentrations under sterile conditions: 7.7, 17, 37, 82 and 180 mg hexanol/L.

5.6 Test vessels

Test vessels are 250 mL conical glass flasks covered with silicone-sponge caps. The vessels and caps were sterilized prior to use (autoclaving or heating).

5.7 Temperature

During the exposure, the incubation temperature was measured with a calibrated thermometer in an additionally prepared control vessel which was continuously incubated once a day.

5.8 Light intensity

The light intensity was measured daily using an illuminance meter LI-189 (LI-COR, Lincoln, USA with radiation sensor) with a cosine (2π) receptor in lux.

5.9 Cell concentration

Cell concentrations were determined using an electronic particle counter (CASY 1 Model TT, Schärfe System, Reutlingen, Germany). The correctness of the electronic counts was checked by microscopically counting following internal standard operation procedures.

5.10 Replicates

For the assessment of algal growth, three replicates of each test concentration and six replicates of the control were prepared. For the assessment of the test concentration without algae (blanks), 2 replicates were prepared for each test concentration and 1 for the control. Samples for the determination of cell counts (1 mL) will be taken from the same test vessels after 24, 48 and 72 h. Samples for analyses of test item concentrations were taken from the same test vessels after 24 and 72 hours (1 subsample per replicate).

Sampling scheme for chemical analyses:

	Number of replicates		Number of replicates to be investigated					
			0 h		24 h		72 h	
Measured conc.	Algae cultures	Blanks	Algae cultures	Blanks	Algae cultures	Blanks	Algae cultures	Blanks
mg/L			chem. anal.	chem. anal.	chem. anal.	chem. anal.	chem. anal.	chem. anal.
Control	6	1	1#	1	1	1	6	1
4.72	3	2	1#	1	3	2	3	2
11.3	3	2	1#	1	3	2	3	2
31.2	3	2	1#	1	3	2	3	2
50.1	3	2	1#	1	3	2	3	2
111.2	3	2	1#	1	3	2	3	2

samples taken from test solution prior to the addition of algae

6 Test procedure

6.1 Alga, growth inhibition test

The initial cell concentration in the test cultures was around 10,000 cells/mL and the total volume of the test cultures was 110 mL. The test cultures were prepared under sterile conditions.
1.4 mL of the pre-culture (cell density 8.116 x 10^5 cells /mL) were added to the test vessels to achieve the initial cell concentration of 10,000 cells/mL.

At test start, the initial cell concentration was calculated based on the cell number of the pre-culture. During the test, the cell concentrations were determined after 24, 48 and 72 h in samples taken directly from the test vessels.

The culture vessels were incubated at 22 ± 1 °C with a light intensity adjusted to approximately 8000 Lux prior to the test and during the test. The cultures were resuspended continuously by shaking on a laboratory shaker (Incubation Shaker Multitron ®, INFORS, Switzerland).

The pH values were measured in an additionally prepared replicate at test start and directly in the test vessels at the end of the test. During the exposure the incubation temperature was measured once a day in an additionally prepared control vessel, which was continuously incubated.

6.2 Data evaluation

- The evaluation of the concentration-effect-relationships and the calculations of effect concentrations were based on the mean measured concentrations of the test item in the algae cultures.
- The mean value of the cell counts for each concentration plot was used for plotting growth curves.
- Calculation of the percent inhibition of growth rate [r], yield [y] and biomass integral [b] was performed according to the guidelines (1) and listed in a table.
- The percent inhibition values were plotted as a function of the test item concentration.
- The test results were statistically analyzed to determine EC_{10} and EC_{50} values together with 95 % confidence intervals using Probit-analysis (6) assuming log-normal distribution of the values by using the computer program ToxRat (7).
- The NOEC and LOEC values for yield and biomass were determined by the Welch t-test (8).

6.3 Chemical analysis of the test solutions

The content of n-hexanol in the test samples was analyzed using the analytical method described in Annex 1 (chapter 13).
According to (9, 10) the analytical method was validated in respect to specificity, linearity, accuracy, precision, identity and limit of quantification (LOQ). Details are shown in Annex 2 (chapter 14).

Outline of the method: The analyte was extracted from the algae test samples by liquid-liquid partitioning with n-hexane. After shaking and settling the n-hexane extract was removed and the analyte derivatized using MSTFA. Measurement was performed by GC-MS in SIM mode using internal standard calibration with deuterated n-hexanol as internal standard.

7 Results

7.1 Test item concentrations

The effect of the test item on the growth of *Pseudokirchneriella subcapitata* was tested with five concentrations arranged in a geometric series, spaced by a factor of 2.2, based on the results of the range-finding test. Table 3 shows the results of the analytical determinations.

Table 3: Results of the analytical determinations in mg/L

Nominal conc. mg/L	Sample	Repl. No	Measured					Mean measured (arithmetic mean of d0, d1, d3)
			day 0	day 1	day 1 % of initial	day 3	day 3 % of initial	
7.7	with algae	1	4.38	5.40	123.3	3.74	85.4	
		2		5.50	125.6	3.73	85.2	
		3		5.97	136.3	5.05	115.3	
		mean		5.62	128.4	4.17	95.3	4.72
	without algae	4	5.34	5.45	102.1	5.20	97.4	
		5		5.96	111.6	5.68	106.4	
		mean		5.71	106.8	5.4	101.9	5.48
17	with algae* (= without algae)	1	13.6	11.88	87.5	7.85	57.8	
		2		12.92	95.2	8.72	64.3	
		3		13.55	99.9	7.53	55.5	
		mean		12.78	94.2	7.53	55.5	11.3
	without algae	4	13.6	13.27	97.8	7.98	58.8	
		5		16.81	123.9	8.62	63.5	
		mean		15.04	110.8	8.3	61.2	12.3
37	with algae** (= without algae)	1	32.5	39.04	120.2	24.13	74.3	
		2		24.41	75.2	30.52	94.0	
		3		40.40	124.4	26.49	81.6	
		mean		34.62	106.6	26.49	81.6	31.2
	without algae	4	32.5	26.26	80.9	25.71	79.2	
		5		39.40	121.3	19.38	59.7	
		mean		32.83	101.1	22.5	69.4	29.3
82	with algae	1	54.7	50.50	92.3	28.70	52.5	
		2		62.20	113.7	31.50	57.6	
		3		71.10	130.0	34.20	62.5	
		mean		61.27	112.0	34.20	62.5	50.1
	without algae	4	52.5	78.70	149.9	42.90	81.7	
		5		64.60	123.0	38.40	73.1	
		mean		71.65	136.5	40.7	77.4	54.9
180	with algae	1	112.9	120.90	107.1	104.3	92.4	
		2		86.50	76.6	113.6	100.6	
		3		84.90	75.2	152.2	134.8	
		mean		97.43	86.3	123.4	109.3	111.2
	without algae	4	120.0	70.60	58.8	121.9	101.6	
		5		75.60	63.0	124.7	103.9	
		mean		73.10	60.9	123.3	102.8	105.5

* day 0 measured: 32.0 mg/L. Regarded as outlier, omitted from evaluation

** day 0 measured: 13.5 mg/L. Regarded as outlier, omitted from evaluation

7.2 Test conditions

Light intensity, temperature, and pH-values of the cultures were in accordance with the guideline (1).
Light intensity was between 7899 and 8135 lux and temperature was between 22.0 °C and 22.2 °C. The data are listed in Annex 3. The pH of the controls changed from 8.58 to maximum 8.52 during the test. In the test cultures the initial pH ranged between 8.08 and 8.40 and did not deviate by more than 0.4 units during the test. The pH-values at test start and test end are compiled in Table 7 of Annex 3.

7.3 Growth curves

The cell numbers dependent on the concentration of the test item are listed in Table 8 in Annex 3. The growth curves are shown in Figure 1. There was a concentration dependent inhibition of the algal growth.
Microscopic observation revealed normal appearances of the algae despite an increase in cell debris in the cultures with increasing growth inhibition.

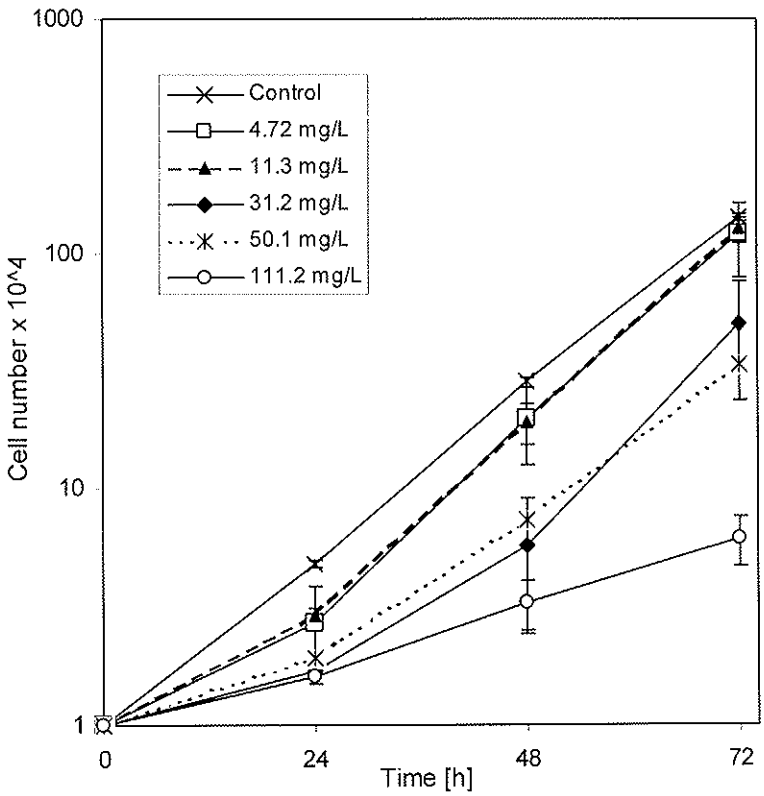

Figure 1: Cell number of *Pseudokirchneriella subcapitata* dependent on nominal concentrations of the test item.

7.4 Effect concentrations

The test results showed a clear dose response relationship. They were statistically analyzed to determine 72 hour EC_{50}-values together with 95% confidence intervals and an EC_{10}-value using Probit-analysis (6) assuming log-normal distribution of the values. The NOEC and LOEC were determined using Welch t-test (8).

The percent inhibition of growth rate, yield and biomass depending on the test item concentrations are listed in Table 4.
Details on the test results are shown in Annex 3 (Detailed test data) and Annex 4 (Evaluation).

Table 4: Percent inhibition of growth rate, biomass integral and yield by the test item.

Test item [mg/L]	% Inhibition of growth rate	% Inhibition of biomass integral	% Inhibition of yield
	0-72 h	0-72 h	0-72 h
4.72	4.3	20.9	15.1
11.3	2.5	18.7	11.3
31.2	23.1	70.7	65.2
50.1	29.1	76.9	76.4
111.2	63.6	94.6	95.7

The concentration-effect curves showing the influence of the test item on growth rate, biomass integral and yield and of *Pseudokirchneriella subcapitata* are shown in Figure 2, Figure 3 and Figure 4, respectively.

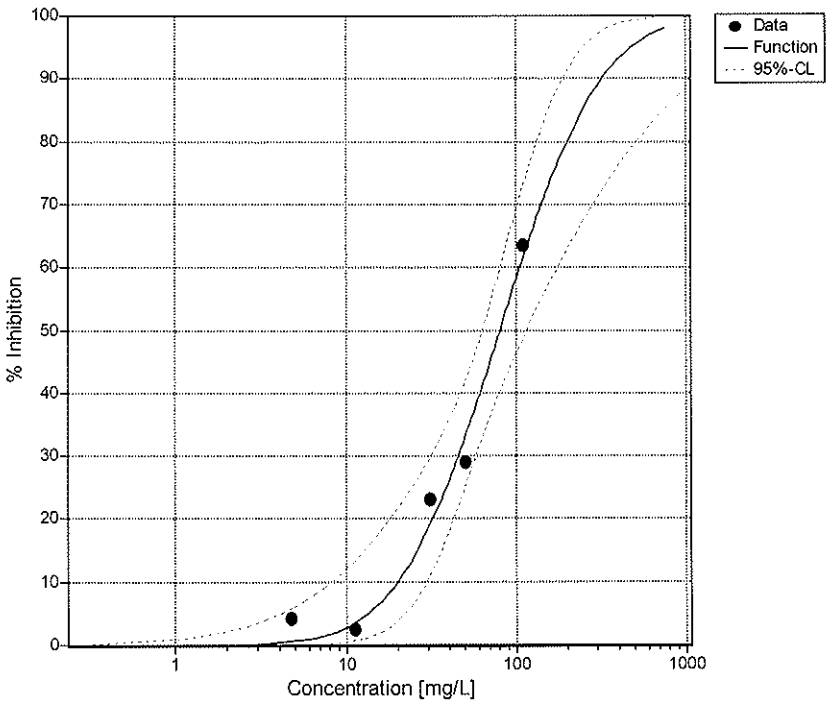

Figure 2:
Growth rate - Concentration-effect curve showing the influence of the nominal concentration of the test item on % inhibition as observed after 72 h.

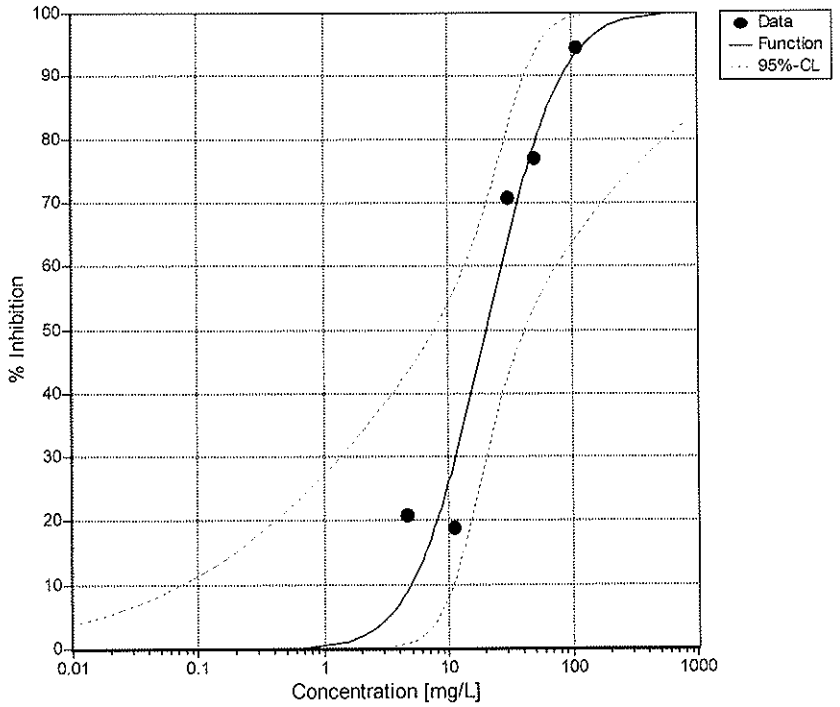

Figure 3:
Biomass integral - Concentration-effect curve showing the influence of the nominal concentration of the test item on % decrease as observed after 72 h.

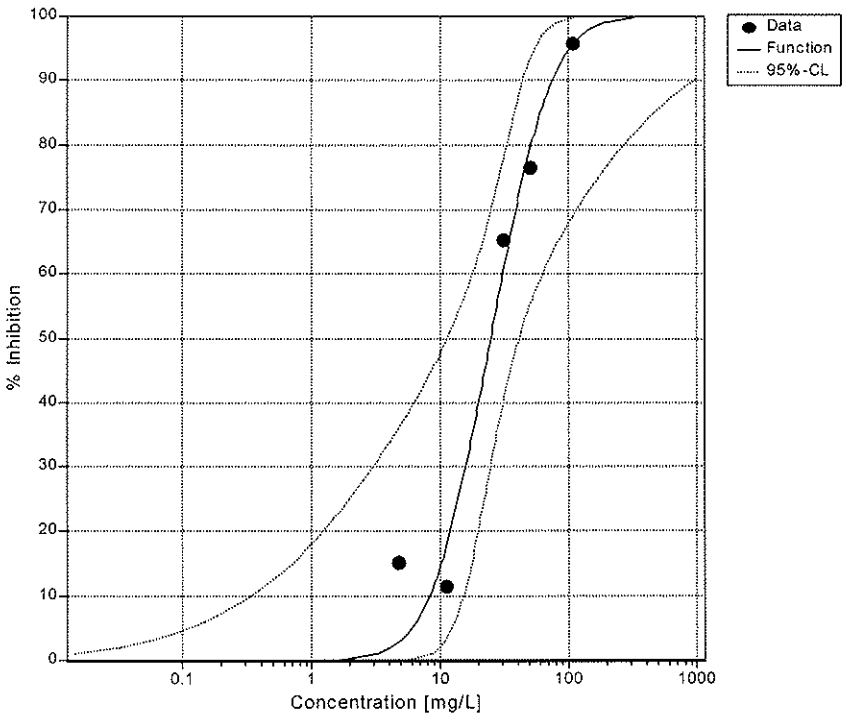

Figure 4:
Yield (cell number) - Concentration-effect curve showing the influence of the nominal concentration of the test item on % inhibition as observed after 72 h.

To obtain the effective concentrations Probit analysis was performed. The results are compiled in Table 5 for the test item.

Table 5: Summary of test results. Effective concentrations ($EC_{10, 50}$) of the test item hexanol and their 95 %-confidence limits (cl), and threshold concentrations based on mean measured concentrations.

		Effective concentrations of test item [mg/L]		
Critical Conc. [mg/L]		**0-24 h**	**0-48 h**	**0-72 h**
Growth rate				
	E_rC_{10}	0.26	4.92	19.81
95%-CL	lower	n.d.	n.d.	8.04
	upper	n.d.	15.9	29.36
	E_rC_{50}	**18.2**	**54.1**	**79.7**
95%-CL	lower	n.d.	18.3	62.8
	upper	n.d.	n.d.	114
	LOEC	<4.72	11.3	31.2
	NOEC	<4.72	4.72	11.3
Biomass integral				
	E_bC_{10}	0.11	1.34	4.97
95%-CL	lower	n.d.	0.00	0.07
	upper	1.49	4.78	10.9
	E_bC_{50}	**5.04**	**11.8**	**20.5**
95%-CL	lower	n.d.	1.32	7.73
	upper	14.37	28.9	41.5
	LOEC	<4.72	<4.72	<4.72
	NOEC	<4.72	<4.72	<4.72
Yield (Cell number)				
	E_yC_{10}	0.06	1.92	8.15
95%-CL	lower	n.d.	0.00	0.34
	upper	1.19	5.98	15.2
	E_yC_{50}	**14.7**	**14.6**	**24.8**
95%-CL	lower	0.01	3.03	11.3
	upper	50.8	35.2	41.2
	LOEC	<4.72	<4.72	31.2
	NOEC	<4.72	<4.72	11.3

n.d.: not determined due to mathematical reasons

8 Conclusion

As the relevant test endpoint the inhibition of growth, expressed as the logarithmic increase in cell number (average specific growth rate) during the exposure period was selected. This is justified since
- growth rate is the relevant test endpoint according to the revised Draft OECD guideline 201 (April 2004)
- growth rate is the most ecologically relevant endpoint
- concentration -effect dependency of growth rate shows least statistically variance of the evaluated parameters (r^2 of probit analysis for growth rate was found to be 0.948 compared to 0.865 for biomass integral and 0.869 for yield).

In conclusion, in the algal growth inhibition test with hexanol the relevant test endpoint, the E_rC_{50} for growth rate over 72 h, was estimated to be 79.7 mg/L.

The EC_{50} for biomass integral was found to be 20.5 mg/L and for yield 24.8 mg/L

The EC_{10} was determined to be 19.8 mg/L for growth rate,8.15 mg test item/L for yield and 4.97 mg test item/L for biomass integral.

For the growth rate over 72 h the NOEC was determined to be 11.3 mg test item/L, for yield 11.3 mg test item/L and for biomass integral < 4.72 mg test item/L.

Because significant effects were observed for biomass integral at the lowest test concentration, the E_bC_{10} of 4.97 mg/L is suggested to represent a more reliable threshold concentration than the NOE_bC < 4.72 mg/L.

9 Validity of the test

The alga growth inhibition test fulfills the validity criteria of the OECD guideline 201 as proposed for updating in April 2004:

- The cell number in the control cultures increased by a factor of 143 within the test period of 72 h (validity criterion: > 16).
- The pH of the control cultures did not deviate by more than 0.2 units during the test (validity criterion ≤ 1.5 pH units).
- Evaluation of the sectional growth rates: The observed rates were: 0 -24 h: 1.563; 24 -48 h: 1.786; 48 -72 h: 1.616 [1/d]. Arithmetic mean: 1.655; standard deviation: 0.116 [1/d]. The coefficient of variation of the sectional growth rate in the control was 7 % (validity criterion ≤ 35 %).

- The coefficient of variation of average specific growth rate during the whole test period in replicate control cultures was 5.7 % (validity criterion ≤ 7 %).

10 Archiving

An aliquot of the test item, the test protocols, all raw data and all records necessary to reconstruct the study were archived in the GLP-archive of the Fraunhofer Institute for Molecular Biology and Applied Ecology, 57392 Schmallenberg, Germany, following internal SOP's according to (3).

List of archived records:
- data specifying the test item
- data concerning the test organisms (origin, culture conditions)
- important correspondence between study director and monitor
- records of storage and storage conditions of test item
- original raw data of test (cell number/mL, test conditions, i.e. pH-values, temperature, method for chemical analysis, measured concentrations)
- records of statistical evaluation
- original study plan including all amendments
- original final report

11 List of SOPs that were used in the study

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

SOP No.	Title (translated)
0-017/01	Computer use
V4-204/03	Alga, Growth inhibition test
V4-209/02	Alga, Growth inhibition test, medium und stock cultures
V4-910/02	Internal standardization of ecotoxicity tests
V7-208/02	Mass spectrometry
G3-004/02	Scales, calibration
G3-005/02	Checking of volumetric apparatus
G3-006/03	Checking of piston-operated pipettes
G3-008/03	Refrigerator/freezer, control
G3 - 009/02	shaker
G4-005/02	Clean-bench, operation
G4-030/02	Particle counter CASY 1 TT
G4-043/01	pH-Meter, WTW 526
G4-210/02	Illuminance meter LI-189 with radiation sensor, LI-COR
G4-303/02	WTW pH-Meter pH 196
G5-109/02	Safety clean bench, Haeraeus
G5-134/02	Autoclave Varioklav
G5-201/02	Multitron-Incubation-Shaker
G7-170/02	Pure water preparation unit UHQ/PS, manual.
G7-183/02	Cleaner Miele with Aquapurificator, manual.
G7 - 177/02	Use of MIELE-cleaning machine G7783
G7-199/02	GC-Autosampler HP 7673, Helwett Packard
G7-203/04	GC/MS HP 5972 MSD
G7-226/02	Eppendorf benchtop centrifuge 5415 C

12 References

1 OECD Guideline for Testing of Chemicals, Sect. 2: Effects on Biotic Systems, No. 201 "Alga, Growth Inhibition Test". Adopted June 1984. Paris: OECD (1981). under consideration of
Draft revised guideline 201 "Freshwater Alga and Cyanobacteria, Growth Inhibition Test", April 2004.

2 OECD (Organisation for Economic Cooperation and Development): OECD Principles of Good Laboratory Practice (as revised in 1997), Paris, 1998

3 Grundsätze der Guten Laborpraxis (Principles of Good Laboratory Practice, GLP) Gesetz zum Schutz vor gefährlichen Stoffen (ChemG), published in: Bundesgesetzblatt 2001, Part I No. 21 from 14.05.2001 ,p. 843.

4 Blaise, C., R. Legault, N. Bermingham, R. v. Coillie and P. Vasseur (1986). A simple microplate algal assay technique for Aquatic Toxicity assessment. Toxic. Assess. 1, 261-281.

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6 Finney, D.J (1984): Statistical Method in Biological Assay. 2nd ed., London.

7 ToxRat® Professional 2.09. ToxRat® Solutions GmbH, Naheweg 15, D-52477 Alsdorf (http://www.toxrat-solutions.de).

8 Welch, B.L. (1937). The significance of the difference between two means when the population variances are unequal. Biometrika 29, 350-362.

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.

13 Annex 1: Analytical Report

Analytical method for the determination of n-hexanol in water

Principle of method and method summary

The method refers to the determination of n-hexanol (C_6-fatty alcohol) in aqueous test samples in the concentration range from 0.25 to 25 mg/L. Samples with higher concentrations have to be diluted before analysis to meet the concentration range mentioned. The analyte is extracted from the algae test samples by liquid-liquid partitioning with n-hexane and derivatized using MSTFA. Measurement is performed by GC-MS in SIM mode using internal standard calibration with deuterated n-hexanol as internal standard.

Equipment and chromatographic conditions

GC/MSD system

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

GC-MS Parameter

column:	SGE BPX-5, 50 m * 0.32 mm, Film 0.25 mm
transfer line temp.:	280 °C
injector:	Split-splitless
injection volume:	1 µL
pressure:	20 kPa, 60 °C
carrier gas:	Helium, 1 mL/min
MS-mode:	SIM (m/z 159,172)

Temperature program

temp 1:	60 °C
time 1:	1 min
rate 1:	10 °C/min
temp 2:	180 °C
time 2:	1 min

Reagents

n-hexane:	95-99.5 %, Baker
MSTFA:	N-tert.-butyldimethylsilyl-N-methyl-trifluoroacetamide, 97% Fluka
methanol:	HPLC grade, Baker
test-item:	n-hexanol; GC 98 %, Fluka 52840
internal standard:	n-hexanol-d_{13}; 98 At. %D, deuterated standard, Chemotrade

Solutions

Stock solutions n-hexanol and n-hexanol-d_{13} (IS)

S1:	25 mg	n-hexanol in 25 mL n-hexane =	1 mg/mL
S2:	500 mg	n-hexanol-d_{13} in 100 mL n-hexane =	5 mg/mL
S3:	50 mg	n-hexanol in 50 mL methanol =	1 mg/mL
IS:	0.1 mL	stock solution 2 in 10 mL n-hexane =	50 mg/L

Calibration solutions

C1:	0.10 mL S1 in 1 mL n-hexane =	100 mg/L
C2:	0.10 mL C1 in 1 mL n-hexane =	10 mg/L
C3:	0.10 mL C2 in 1 mL n-hexane =	1 mg/L
C4:	0.50 mL C1 in 1 mL n-hexane =	50 mg/L
C5:	0.50 mL C2 in 1 mL n-hexane =	5 mg/L
C6:	0.25 mL C1 in 1 mL n-hexane =	25 mg/L
C7:	0.25 mL C2 in 1 mL n-hexane =	2.5 mg/L
C8:	0.50 mL C3 in 1 mL n-hexane =	0.5 mg/L
C9:	0.25 mL C3 in 1 mL n-hexane =	0.25 mg/L

Preparation of silylation products

C10:	100 µL C2 + 50 µL IS solution + 50 µL MSTFA
C11:	100 µL C3 + 50 µL IS solution + 50 µL MSTFA
C12:	100 µL C4 + 50 µL IS solution + 50 µL MSTFA
C13:	100 µL C5 + 50 µL IS solution + 50 µL MSTFA
C14:	100 µL C6 + 50 µL IS solution + 50 µL MSTFA
C15:	100 µL C7 + 50 µL IS solution + 50 µL MSTFA
C16:	100 µL C8 + 50 µL IS solution + 50 µL MSTFA
C17:	100 µL C9 + 50 µL IS solution + 50 µL MSTFA

Fortification solutions (for recovery experiments)

S4:	0.1 mL S3 in 1 mL water =	100 mg/L
S5:	0.1 mL S4 in 1 mL water =	10 mg/L

Calibration

Calibration of the method is performed by chromatography of the calibration solutions C10, C11 and C13 to C17. Using the concentration/peak area data of the test item and the internal standard the calibration line is calculated by linear regression using internal standard method.

Sample preparation

1 mL of the aqueous test sample (algae culture) is shaken with 1 mL n-hexane for 10 min (shaking machine). After phase separation 100 µL of the upper n-hexane layer is removed and pipetted into a 1.5 mL vial. 50 µL MSTFA and 50 µL IS solution are added and mixed by shaking for some seconds. Then the extract is transferred into a

300 µL vial insert. Measurement is performed by GC-MS under the conditions given above.

Recovery

The following recovery experiments were performed:
900 µL water + 100 µL stock solution 4 (S4) (-> 10 µg/mL, five replicates)
900 µL water + 100 µL stock solution 5 (S5) (-> 1 µg/mL, five replicates)
The stock solutions were added into the water phase by a pipette. After mixing (Vortex) for 1 min the samples were processed like real samples (see 'Sample preparation').

Typical calibration line and chromatograms

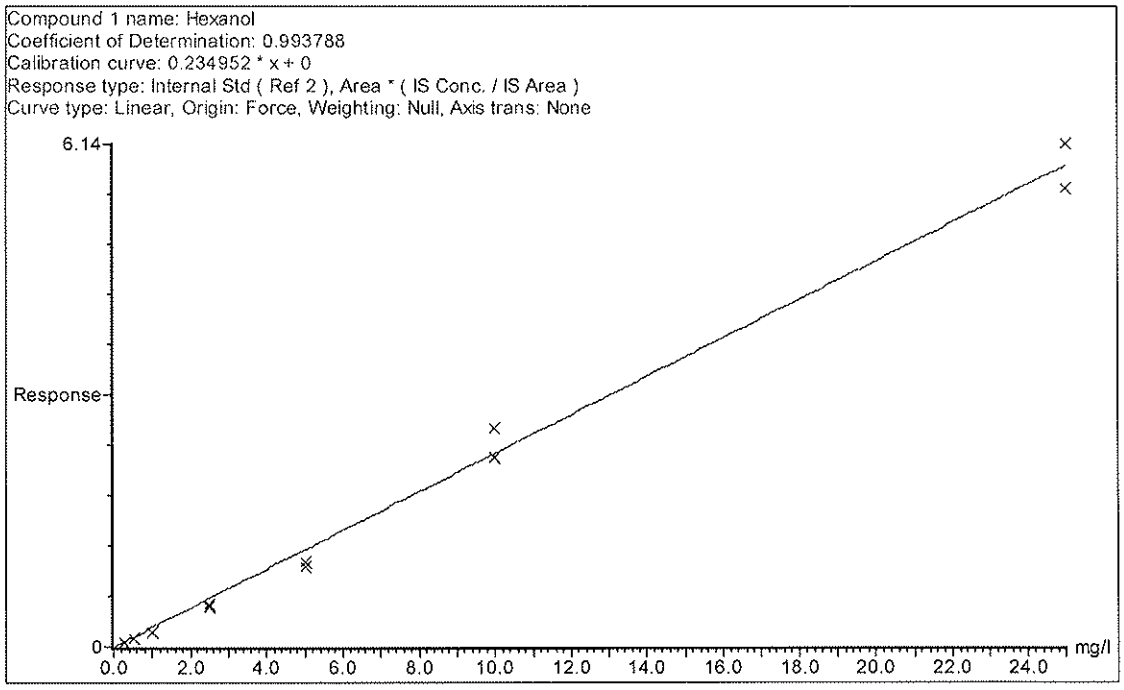

Figure 5: Typical calibration line of n-hexanol

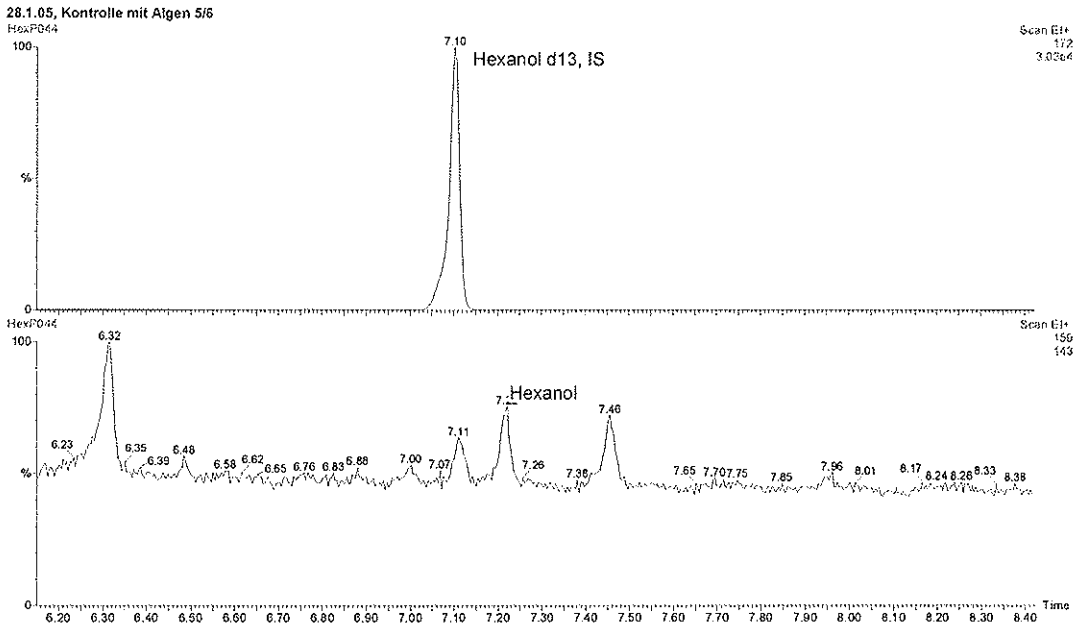

Figure 6: Analysis of n-hexanol: Typical chromatogram of blank with algae, upper: internal standard n-hexanol d_{13} , lower: n-hexanol, area corresponding to 0.03 mg/L (-> < LOQ)

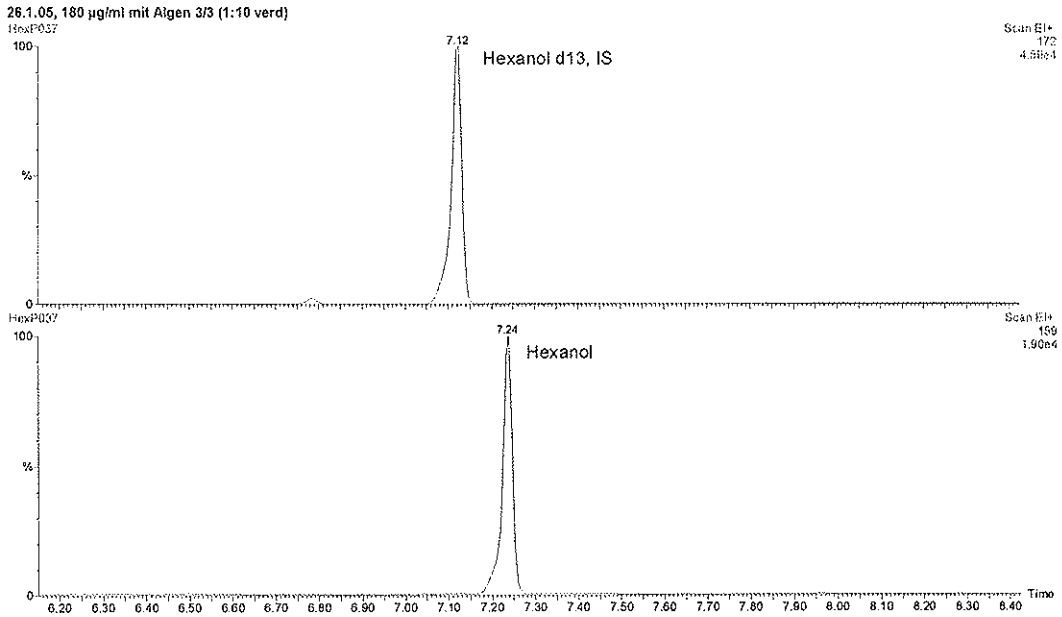

Figure 7: Analysis of n-hexanol: Typical chromatogram, upper: internal standard n-hexanol d_{13} , lower: n-hexanol, 8.5 mg/L

14 Annex 2: Validation of the analytical method for the determination of n-hexanol in water

The validation of the analytical method was based on the guidelines SANCO/825/00 rev. 6 and SANCO/3029/99 ver. 4 (9, 10). The guidelines describe the pesticide pre- and post-registration data requirements. According to this guidelines the analytical method was validated in respect to specificity, linearity, accuracy, precision, identity and limit of quantification (LOQ).

Specificity
The method was found to be sufficiently specific for the determination of n-hexanol in water in the range from 0.25 to 25 mg/L. The blanks with and without algae as well as the test samples showed no interfering peaks. A typical chromatogram is shown in Annex 2 (chapter 13).

Linearity/Calibration
The method was calibrated in the range from 0.25 to 25 mg/L using 7 calibration levels. Linear regressions of the peak responses and the concentrations were found resulting in typical correlation coefficients of $r > 0.99$. An example calibration line is shown in Annex 2 (Figure 5).

Accuracy
The results are shown in Table 6.
- The recoveries from water fortified with n-hexanol at 1 mg/L ranged from 71 % to 74 %. The mean recovery of five replicates was 73 %.
- The recoveries from water fortified with n-hexanol at 10 mg/L ranged from 68 % to 73 %. The mean recovery of five replicates was 69.8 %.
- The overall recovery values from water fortified at two levels was 71.4 %.

Precision
The precision of the method is reported as the repeatability of recovery at each fortification level. The results are shown in Table 6.
- The relative standard deviation of recoveries from water fortified with n-hexanol at 1 mg/L was 1.9 %.
- The relative standard deviation of recoveries from water fortified with n-hexanol at 10 mg/L was 2.8 %.
- The overall relative standard deviation of recoveries from water fortified with n-hexanol at two levels was 3.2 %.
- The precisions were below 20 % for every concentration level.

Table 6: Summary of recovery data – n-hexanol in water

Fortification level [mg/L]	Recovery [%]	Mean Recovery [%]	Recovery RSD [%]
0 (with algae)	-	-	-
0 (without algae)	-	-	-
1	71	73.0	1.9
1	74		
1	74		
1	72		
1	74		
10	70	69.8	2.8
10	69		
10	68		
10	73		
10	69		
	Overall values	**71.4**	**3.2**

Identity
The identity of the test item was approved by the interpretation of the mass fragments (m/z ratio 159 and 172) and their relation obtained by mass spectrometric detection.

Limit of Quantification (LOQ)
The limit of quantification - as the lowest calibration level - was 0.25 mg/L for n-hexanol. Blank values with and without algae were always below the LOQ.

15 Annex 3: Detailed test data

15.1 Light intensity

Light intensity was 7927 lux at test start and 8135, 8119 and 7899 lux after 24, 48 and 72 h, respectively.

15.2 Temperature

Temperature was 22.2 °C at test start and 22.0, 22.1 and 22.2 °C after 24, 48 and 72 h, respectively.

15.3 PH-values

Table 7: pH-values at test start and end of test

Test item [mg/L]	Test start	Test termination				
		algae cultures			blanks without algae	
		Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5
control	8.58	8.46 8.42*	8.52 8.40*	8.46 8.44*		
4.72	8.40	8.07	8.00	8.00	7.98	7.98
11.3	8.34	8.05	8.02	8.00	7.97	8.00
31.2	8.20	8.02	8.06	8.08	8.10	8.09
50.1	8.13	8.08	8.13	8.18	8.18	8.18
111.2	8.08	8.18	8.22	8.22	8.18	8.18

* replicates 0/4-0/6 of controls

15.4 Cell number

Table 8: Cell number (x 10^4) dependent on mean measured concentrations of the test item and time. (Mean: arithmetic mean; Std. Dev.: standard deviation; n: number of replicates; CV: coefficient of variation). Cell number at test start: 10,000 cells/mL.

Conc. [mg/L]	Control	4.72	11.3	31.2	50.1	111.2
24 h	5.0	3.7	3.0	1.6	1.8	1.6
	4.5	3.1	3.0	1.7	2.0	1.7
	4.6	1.4	2.6	2.0	1.9	1.5
	4.9					
	4.6					
	5.1					
Mean:	4.8	2.7	2.9	1.7	1.9	1.6
Std.Dev.:	0.2	1.2	0.2	0.2	0.1	0.1
n:	6	3	3	3	3	3
CV:	5.1	42.3	8.1	10.9	6.2	4.7
48 h	27.0	26.2	23.8	2.2	7.4	4.0
	27.0	21.6	17.5	6.5	7.7	2.4
	29.3	12.1	16.6	8.8	7.1	3.5
	30.2					
	29.7					
	27.8					
Mean:	28.5	20.0	19.3	5.8	7.4	3.3
Std.Dev.:	1.4	7.2	3.9	3.4	0.3	0.8
n:	6	3	3	3	3	3
CV:	5.0	36.0	20.2	57.6	4.3	24.1
72 h	136.3	154.5	145.7	78.5	32.6	7.8
	138.6	137.8	118.5	43.5	36.0	4.8
	148.6	72.4	117.0	27.5	32.6	6.0
	148.1					
	140.4					
	147.7					
Mean:	143.3	121.6	127.1	49.8	33.8	6.2
Std.Dev.:	5.5	43.4	16.2	26.1	2.0	1.5
n:	6	3	3	3	3	3
CV:	3.8	35.7	12.7	52.4	5.9	23.7

15.5 Growth rate

Table 9: Growth rates (1/d) dependent on mean measured concentrations of the test item and time. (Mean: arithmetic mean; Std. Dev.: standard deviation; n: number of replicates; CV: coefficient of variation).

Conc. [mg/L]	Control	4.72	11.3	31.2	50.1	111.2
24 h	1.617	1.296	1.111	0.461	0.560	0.453
	1.500	1.128	1.103	0.518	0.684	0.510
	1.524	0.362	0.963	0.668	0.638	0.417
	1.581					
	1.535					
	1.621					
Mean:	1.563	0.929	1.059	0.549	0.627	0.460
Std.Dev.:	0.051	0.498	0.083	0.107	0.063	0.047
n:	6	3	3	3	3	3
CV:	3.3	53.6	7.9	19.5	10.0	10.1
48 h	1.648	1.633	1.584	0.396	1.003	0.692
	1.648	1.536	1.430	0.933	1.020	0.445
	1.688	1.247	1.406	1.090	0.977	0.625
	1.704					
	1.696					
	1.662					
Mean:	1.674	1.472	1.473	0.806	1.000	0.587
Std.Dev.:	0.025	0.201	0.097	0.364	0.022	0.128
n:	6	3	3	3	3	3
CV:	1.5	13.7	6.6	45.2	2.2	21.8
72 h	1.638	1.680	1.661	1.454	1.162	0.683
	1.644	1.642	1.592	1.258	1.195	0.526
	1.667	1.428	1.587	1.104	1.161	0.599
	1.666					
	1.648					
	1.665					
Mean:	1.655	1.583	1.613	1.272	1.173	0.603
Std.Dev.:	0.013	0.136	0.041	0.175	0.019	0.079
n:	6	3	3	3	3	3
CV:	0.8	8.6	2.5	13.8	1.6	13.1

15.6 Biomass integral

Table 10: Biomass integral dependent on mean measured concentrations of the test item and time. (Mean: arithmetic mean; Std. Dev.: standard deviation; n: number of replicates; CV: coefficient of variation).

Conc. [mg/L]	Control	4.72	11.3	31.2	50.1	111.2
24 h	2.0	1.3	1.0	0.3	0.4	0.3
	1.7	1.0	1.0	0.3	0.5	0.3
	1.8	0.2	0.8	0.5	0.4	0.3
	1.9					
	1.8					
	2.0					
Mean:	1.9	0.9	0.9	0.4	0.4	0.3
Std.Dev.:	0.1	0.6	0.1	0.1	0.1	0.0
n:	6.0	3.0	3.0	3.0	3.0	3.0
CV:	6.4	66.8	12.4	25.7	13.3	12.7
48 h	17.0	15.3	13.4	1.2	4.0	2.1
	16.5	12.4	10.2	3.4	4.3	1.4
	17.7	6.0	9.4	4.9	3.9	1.8
	18.5					
	18.0					
	17.4					
Mean:	17.5	11.2	11.0	3.2	4.1	1.7
Std.Dev.:	0.7	4.8	2.1	1.9	0.2	0.3
n:	6.0	3.0	3.0	3.0	3.0	3.0
CV:	4.0	42.3	19.1	58.8	5.4	19.8
72 h	97.7	104.6	97.1	40.5	23.0	6.9
	98.3	91.1	77.2	27.4	25.2	4.0
	105.7	47.3	75.3	22.0	22.7	5.5
	106.6					
	102.0					
	104.2					
Mean:	102.4	81.0	83.2	30.0	23.6	5.5
Std.Dev.:	3.8	30.0	12.1	9.5	1.4	1.5
n:	6.0	3.0	3.0	3.0	3.0	3.0
CV:	3.7	37.0	14.6	31.7	5.7	26.6

16 Annex 4: Evaluation

16.1 Results on growth rate

16.1.1 Inhibition of growth rate

Table 11: Growth rate (G) and its inhibition relative to control (%I) as computed from the raw data for test intervals selected.

Treatment	0-24 h		0-48 h		0-72 h	
[mg/L]	G	%I	G	%I	G	%I
Control	1.563	0.0	1.674	0.0	1.655	0.0
4.7	0.929	40.6	1.472	12.1	1.583	4.3
11.3	1.059	32.2	1.473	12.0	1.613	2.5
31.2	0.549	64.9	0.806	51.8	1.272	23.1
50.1	0.627	59.9	1.000	40.3	1.173	29.1
111.2	0.460	70.6	0.587	64.9	0.603	63.6

16.1.2 Effective concentrations (ECx) with growth rate at 72 h

Probit analysis using linear max. likelihood regression

Table 12: Probit analysis using linear max. likelihood regression (growth rate at 72 h)

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

Treatm. [mg/L]	Log(x)	% Inhibition	n	Emp. Probit	Weight	Reg. Probit
Control		0.10	6			excluded
4.7	0.674	4.32	3	3.286	0.001	2.399
11.3	1.053	2.51	3	3.042	0.040	3.204
31.2	1.494	23.13	3	4.268	0.475	4.140
50.1	1.700	29.13	3	4.453	0.833	4.576
111.2	2.046	63.59	3	5.345	0.911	5.304

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

Table 13: Parameters of the probit analysis: Results of the regression analysis (growth rate at 72 h)

Parameter	Value
Computation runs:	5
Slope b:	2.1170
Intercept a:	0.9744
Variance of b:	7.6312
Goodness of Fit	
Chi^2:	0.0321
Degrees of freedom:	3
p(Chi^2):	0.9985
Log EC50:	1.9016
s Log EC50:	1.2153
g-Criterion:	0.1845
Residual Variance (Chi^2/df):	0.0107
r^2:	0.9480
F:	54.866
p(F) (df: 1;3):	0.0050

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).

Results of the probit analysis

Table 14: Results of the probit analysis: Selected effective concentrations (ECx) of the test item and their 95%- and 99%-confidence limits (growth rate at 72 h)

Parameter	EC_{10}	EC_{20}	EC_{50}
Value [mg/L]	19.81	32.00	79.72
lower 95%-cl	8.04	18.02	62.80
upper 95%-cl	29.36	42.50	114.36
lower 99%-cl	4.68	12.59	48.89
upper 99%-cl	50.42	60.83	146.91

n.d.: not determined due to mathematical reasons

Computation of variances and confidence limits was adjusted to metric data (Christensen & Nyholm 1984). Inhibitions lower equal 0% or greater equal 100 % were replaced by 0.00 and 99.900, respectively. Slope function after Litchfield and Wilcoxon: 2.967.

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

16.1.3 NOEC Determination

Welch-t test for Inhomogeneous Variances with Bonferroni Adjustment

Multiple sequentially rejective comparisons after Welch of treatments with "Control" by the t test procedure. Significance was Alpha = 0.05, one-sided smaller; Mean: arithmetic mean; n: sample size; s^2: variance; %MDD: minimum detectable difference to Control (in percent of Control); t: sample t; p(t): probability of sample t for Ho: $\mu 1 = \mu 2$; Alpha(i): adjusted significance levels; the differences are significant in case p(i) <= Alpha(i) ; dfm: modified degrees of freedom due to heteroscedascity. (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Table 15: Comparison of treatments with "Control" by the t test procedure after Welch, growth rate

Treatm. [mg/L]	Mean	s^2	df	%MDD	t	p(t)	Alpha(i)	Sign.
Control	1.655	0.008						
4.72	1.583	0.008	582	-7.38	-1.15	0.125	0.025	-
11.3	1.613	0.008	58	-6.278	-0.67	0.253	0.050	-
31.0	1.272	0.008	964	-8.009	-6.16	< 0.001	0.017	+
50.1	1.173	0.008	17	-9.241	-7.75	< 0.001	0.013	+
111.2	0.603	0.008	198	-8.816	-16.93	< 0.001	0.010	+

+: significant; -: non-significant

A NOEC of 11.3 mg/L is suggested by the program.

16.2 Results on biomass integral

16.2.1 Inhibition of biomass integral

Table 16: Biomass integral (BI) and its inhibition relative to control (%I) as computed from the raw data for test intervals selected.

Treatment	0-24 h		0-48 h		0-72 h	
[mg/L]	BI	%I	BI	%I	BI	%I
Control	1.9	0.0	17.5	0.0	102.4	0.0
4.72	0.9	54.3	11.2	36.0	81.0	20.9
11.3	0.9	50.0	11.0	37.0	83.2	18.7
31.0	0.4	80.5	3.2	82.0	30.0	70.7
50.1	0.4	76.8	4.1	76.8	23.6	76.9
111.2	0.3	84.5	1.7	90.1	5.5	94.6

16.2.2 Probit analysis using linear max. likelihood regression

Table 17: Probit analysis using linear max. likelihood regression (biomass integral at 72 h)

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

Treatm. [mg/L]	Log(x)	% Inhibition	n	Emp. Probit	Weight	Reg. Probit
Control		0.10	6			excluded
4.72	0.674	20.91	3	4.193	0.172	3.675
11.3	1.053	18.75	3	4.115	0.748	4.464
31.2	1.494	70.72	3	5.543	0.867	5.375
50.	1.700	76.91	3	5.733	0.523	5.802
111.2	2.046	94.63	3	6.610	0.098	6.523

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

Table 18: Parameters of the probit analysis: Results of the regression analysis (yield at 72 h)

Parameter	Value
Computation runs:	7
Slope b:	2.0770
Intercept a:	2.2739
Variance of b:	3.6536
Goodness of Fit	
Chi^2:	0.1843
Degrees of freedom:	3
$p(Chi^2)$:	0.9801
Log EC50:	1.3125
s Log EC50:	0.7948
g-Criterion:	0.5268
Residual Variance (Chi^2/df):	0.0614
r^2:	0.8650
F:	19.220
p(F) (df: 1;3):	0.0220

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).

Results of the probit analysis

Table 19: Results of the probit analysis: Selected effective concentrations (ECx) of the test item and their 95%- and 99%-confidence limits (yield at 72 h)

Parameter	EC_{10}	EC_{20}	EC_{50}
Value [mg/L]	4.97	8.10	20.53
lower 95%-cl	0.07	0.41	7.73
upper 95%-cl	10.90	15.34	41.51
lower 99%-cl	0.01	0.09	3.83
upper 99%-cl	88.26	69.65	83.76

n.d.: not determined due to mathematical reasons

Computation of variances and confidence limits was adjusted to metric data (Christensen & Nyholm 1984). Inhibitions lower equal 0% or greater equal 100 % were replaced by 0.00 and 99.900, respectively. Slope function after Litchfield and Wilcoxon: 3.030

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

16.2.3 NOEC Determination

Welch-t test for Inhomogeneous Variances with Bonferroni Adjustment

Multiple sequentially rejective comparisons after Welch of treatments with "Control" by the t test procedure. Significance was Alpha = 0.05, one-sided smaller; Mean: arithmetic mean; n: sample size; s^2: variance; %MDD: minimum detectable difference to Control (in percent of Control); t: sample t; p(t): probability of sample t for Ho: $\mu 1 = \mu 2$; Alpha(i): adjusted significance levels; the differences are significant in case p(i) <= Alpha(i) ; dfm: modified degrees of freedom due to heteroscedascity. (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Table 20: Comparison of treatments with "Control" by the t test procedure after Welch, biomass integral

Treatm. [mg/L]	Mean	s^2	df	%MDD	t	p(t)	Alpha(i)	Sign.
Control	102.4	156.7						
4.72	81.0	156.7	329	-17	-2.42	0.008	0.025	+
11.30	83.2	156.7	58	-14.4	-2.17	0.017	0.050	+
31.20	30.0	156.7	38	-20.2	-8.18	< 0.001	0.013	+
50.10	23.6	156.7	16	-20.1	-8.9	< 0.001	0.017	+
111.20	5.5	156.7	14	-22.7	-10.95	< 0.001	0.010	+

+: significant; -: non-significant

A NOEC of 4.72 mg/L is suggested by the program.

16.3 Results on yield

16.3.1 Inhibition of yield (cell number)

Table 21: Yield expressed as cell numbers (G) and its inhibition relative to control (%I) as computed from the raw data for test intervals selected.

Treatment	0-24 h		0-48 h		0-72 h	
[mg/L]	G	%I	G	%I	G	%I
Control	4.8	0.0	28.5	0.0	143.3	0.0
4.72	2.7	42.9	20.0	29.9	121.6	15.1
11.3	2.9	39.5	19.3	32.3	127.1	11.3
31.2	1.7	63.6	5.8	79.5	49.8	65.2
50.1	1.9	60.8	7.4	74.1	33.8	76.4
111.2	1.6	66.8	3.3	88.4	6.2	95.7

16.3.2 Probit analysis using linear max. likelihood regression

Table 22: Probit analysis using linear max. likelihood regression (yield at 72 h)

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

Treatm. [mg/L]	Log(x)	% Inhibition	n	Emp. Probit	Weight	Reg. Probit
Control		0.10	6			excluded
4.72	0.674	15.15	3	3.972	0.026	3.092
11.3	1.053	11.32	3	3.792	0.442	4.099
31.2	1.494	65.24	3	5.389	0.932	5.263
50.1	1.700	76.44	3	5.718	0.519	5.808
111.2	2.046	95.67	3	6.713	0.051	6.728

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

Table 23: Parameters of the probit analysis: Results of the regression analysis (yield at 72 h)

Parameter	Value
Computation runs:	8
Slope b:	2.6501
Intercept a:	1.3057
Variance of b:	7.2694
Goodness of Fit	
Chi²:	0.1451
Degrees of freedom:	3
p(Chi²):	0.9859
Log EC50:	1.3940
s Log EC50:	0.0946
g-Criterion:	0.5068
Residual Variance (Chi²/df):	0.0484
r²:	0.8690
F:	19.977
p(F) (df: 1;3):	0.0210

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).

Results of the probit analysis

Table 24: Results of the probit analysis: Selected effective concentrations (ECx) of the test item and their 95%- and 99%-confidence limits (yield at 72 h)

Parameter	EC_{10}	EC_{20}	EC_{50}
Value [mg/L]	8.15	11.95	24.78
lower 95%-cl	0.34	1.22	11.30
upper 95%-cl	15.16	19.77	41.20
lower 99%-cl	0.07	0.38	6.58
upper 99%-cl	74.38	63.24	70.73

n.d.: not determined due to mathematical reasons

Computation of variances and confidence limits was adjusted to metric data (Christensen & Nyholm 1984). Inhibitions lower equal 0% or greater equal 100 % were replaced by 0.00 and 99.900, respectively. Slope function after Litchfield and Wilcoxon: 2.384.

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

16.3.3 NOEC Determination

Welch-t test for Inhomogeneous Variances with Bonferroni Adjustment

Multiple sequentially rejective comparisons after Welch of treatments with "Control" by the t test procedure. Significance was Alpha = 0.05, one-sided smaller; Mean: arithmetic mean; n: sample size; s^2: variance; %MDD: minimum detectable difference to Control (in percent of Control); t: sample t; p(t): probability of sample t for Ho: $\mu 1 = \mu 2$; Alpha(i): adjusted significance levels; the differences are significant in case p(i) <= Alpha(i) ; dfm: modified degrees of freedom due to heteroscedascity. (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Table 25: Comparison of treatments with "Control" by the t test procedure after Welch, yield

Treatm. [mg/L]	Mean	s^2	df	%MDD	t	p(t)	Alpha(i)	Sign.
Control	143.3	387.0						
4.72	121.6	387.0	324	-19.1	-1.56	0.060	0.025	-
11.3	127.1	387.0	50	-16.3	-1.17	0.125	0.050	-
31.0	49.8	387.0	121	-22	-6.72	< 0.001	0.013	+
50.1	33.8	387.0	15	-22.8	-7.87	< 0.001	0.017	+
111.2	6.2	387.0	23	-24.3	-9.85	< 0.001	0.010	+

+: significant; -: non-significant

A NOEC of 11.3 mg/L is suggested by the program.

17 Annex 5: Certificate of analysis of the test item

SIGMA-ALDRICH

Certificate of Analysis

Product Name	1-Hexanol, purum ≥98.0% (GC)
Product Number	52840
Product Brand	FLUKA
CAS Number	111-27-3
Molecular Formula	$C_6H_{14}O$
Molecular Weight	102.17

TEST	LOT 452734/1 RESULTS
APPEARANCE (COLOUR)	COLOURLESS
APPEARANCE (FORM)	CLEAR LIQUID
ASSAY (GC AREA %)	98.9 % REL
DENSITY D20/4	0.819
REFRACTIVE INDEX N20/D	1.418
INFRARED SPECTRUM	CORRESPONDS
DATE OF QC-RELEASE	25/JUN/03

Fluka guarantees the 'Sales-Specification' values only, non-specified tests may be included as additional information. The current 'Sales-Specifications' sheet is available on request. For further inquiries, please contact our Technical Service. Fluka warrants, that its products conform to the information contained in this and other Fluka publications. Purchaser must determine the suitability of the product for its particular use. See reverse side of invoice for additional terms and conditions of sale. The values given on the 'Certificate of Analysis' are the results determined at the time of analysis.

Dr. Gert van Look, Manager
Quality Control
Buchs Switzerland

18 Annex 6: Certificate of analysis of the analytical standard

Cambridge Isotope Laboratories, Inc.

50 Frontage Road, Andover, MA 01810-5413 USA
800.322.1174 (N.AMERICA) 978.749.8000 (INTERNATIONAL)
www.isotope.com

CERTIFICATE OF ANALYSIS

Product Name: N-HEXANOL
(Isotopic Label & Enrichment Specification) (D13, 98%)

Lot Number: PR-11895

Catalog Number: DLM-691-0

Product Information

Chemical Purity Specification: ≥98%
Labeled CAS Number: NA
Unlabeled CAS Number: 111-27-3
Molecular Weight: 115.3
Chemical Formula: $CD_3(CD_2)_5OH$
Storage: Store at room temperature away from light and moisture
Stability: Two years after receipt of order if stored as above. Re-QC after two years.

Certification

Cambridge Isotope Laboratories, Inc. guarantees that this material meets or exceeds the specifications stated. Absolute identity as well as chemical and isotopic purities are assured by the use of unambiguous synthetic routes and multiple chemical analyses whenever possible.

Approved by: Deborah E. Costa
Deborah E. Costa, Quality Assurance

Quality Control Tests and Results

GC/FID for Chemical Purity	99.9%
GC/MS for Chemical Purity	Pass
Karl Fischer Titration for Total Water Content	2713 ppm
^1H NMR for Isotopic Enrichment	98%

Chemotrade
Chemiehandelsgesellschaft mbH Leipzig
Brahestraße 27
D - 04347 Leipzig
Tel.: 0341 / 244 49 24
Fax: 0341 / 244 49 22

19 Annex 7: GLP Certificate (2 pages)

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

Postanschrift: 40190 Düsseldorf

Aktenzeichen: VI-3- 31.11.79.05

GLP-Bescheinigung

Bescheinigung	Certificate
Hiermit wird bestätigt, dass die Prüfeinrichtung	It is hereby certified that the test facility
in D-57392 Schmallenberg, Auf dem Aberg 1 (Ort, Anschrift)	in D-57392 Schmallenberg, Auf dem Aberg 1 (location, address)
Fraunhofer Institut für Molekularbiologie und Angewandte Oekologie (IME) (Firma)	Of Fraunhofer Institut für Molekularbiologie und Angewandte Oekologie (IME) (company name)
vom 11. November- 13. November 2002 (Datum)	on 11 until 13 November 2002 (date)
von der für die Überwachung zuständigen Behörde über die Einhaltung der Grundsätze der Guten Laborpraxis inspiziert worden ist.	was (were) inspected by the competent authority regarding compliance with the Principles of Good Laboratory Practice.
Es wird hiermit bestätigt, dass folgende Prüfungen in dieser Prüfeinrichtung nach den Grundsätzen der Guten Laborpraxis durchgeführt werden.	It is hereby certified that following studies in this test facility are conducted in compliance with the Principles of Good Laboratory Practice.

GLP-Certificate continued

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

Düsseldorf, 19. Februar 2003

Im Auftrag

(Prof. Dr. Heinrich David)

Dienstsiegel
(official-seal)