Work Report

Pre-studies with pentadecanol ($C_{15}OH$) for the preparation of a study plan for a *Daphnia magna* reproduction test (OECD 211)

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1 General objective of the work

The objective of this work is to assess the chronic toxicity of fatty alcohols to *Daphnia magna* according to OECD Guideline 211 with a chain length of more than 10 C. To accomplish this we were have taken a two-step-approach. The first step comprises studies with C12- and C15-alcohols (dodecanol and pentadecanol). Because toxicity due to narcosis increases with the chain length, but at the same time solubility decreases, it is anticipated that a parabolic structure-activity-relationship exists in the range of C10 to greater than C15. The second set of studies will depend on the results of the first two studies. It either will comprise studies with intermediate and lower chain lengths (C14 and C10), if pentadecanol was found not to be toxic at concentrations below the water solubility, or it will comprise studies with higher chain lengths (C16 and C18) if pentadecanol was found to be still toxic.

The challenge is to reproducibly produce test item concentrations close to the water solubility and to maintain concentrations in water despite the fast and ready degradability of the test item. We chose the following approach:

- A saturated solution daily generated by stirring the test substance in test media under slow stir conditions under sterile conditions, served as highest concentration and was serially diluted to prepare the other concentrations.
- Performance in tightly closed 200 mL - vessels (single exposure according to OECD 211).
- The test media exchange and food added daily.
- Daily observations of immobility and counts of offspring
- Analysis for the test item concentration: Saturated solution, all freshly prepared test solutions and pooled aged solutions. The analytical details are described in the appendix.
- Prior to the OECD 211 studies, several pre-studies including a non-GLP Daphnia reproduction pilot study with dodecanol and pentadcanol were performed to demonstrate the feasibility of (parts of) the study plan.

The pre-studies on dodecanol were performed prior to the pentadecanol studies.

2 Sequence and objectives of pre-studies with pentadecanol

After the completion of the dodecanol pilot study and some further pre-tests with dodecanol concerning the kinetics of dissipation and the contribution of feeding algae,

1) the pilot study with pentadecanol was performed with two main objectives,

   a) to ensure a sufficiently constant preparation of test solutions, the highest being close to water solubility, and to show the maintenance of test concentrations,

   b) to screen the sensitivities of the Daphnia reproduction at the concentrations achieved and the test conditions.

   The very low and highly variable concentrations achieved provoked a discussion about the filtration procedure used when preparing the "saturated" solution. Thus,

2) a preparation without filtration at the same loading was performed. This resulted in considerably higher concentrations. In consequence, a test solution peparation study without filtration was performed

   a) to look at the effects of different loadings on the final test substance concentrations,

   b) to look at reproducibility of the approach by the same and different technicians.

   The preparations without filtration provided considerably higher concentrations than the pilot study and model predictions. This observation raised the question concerning the relationship between the water soluble and non-water soluble test material which were analytically detected in the test medium but could not be distinguished. The other question was whether these high concentrations might affect toxicity to Daphnia. Thus,

3) acute non-GLP toxicity tests were performed at higher loadings without filtration.

3 Pentadecanol Daphnia reproduction pilot study

Because the pre-studies with dodecanol showed rapid dissipation of the test substance by bacteria and algae to be used as daphnid food, we decided to perform the pentadecanol pilot study under sterile conditions with all working steps conducted in a clean bench, and feeding daphnia with autoclaved algae (axenic culture of *Desmodesmus subspicatus*).

3.1 Setup

The preparation of saturated solutions by stirring the test substance in test media under slow stir conditions under sterile conditions was conducted with one loading (corresponding to theoretical concentrations in the preparation vessels of 2.0 mg/L), introducing the ethanolic stock solution as one spot with evaporation by a gentle flow of nitrogen. For more details see the non-GLP study plan, annex 1. Due to a defect at the evaporation apparatus, from Dec 14 on, the ethanolic stock solution was added directly 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.

After 21 h of gentle stirring at 100 rpm and 2 h of settling, the preparation was filtered through an apolar 0.2 µm filter (Millex FG, 50 mm, Millipore), which was wetted with methanol prior to use to allow the passage of the aqueous solution. Of each flask, the first 100 mL (from Dec 14 on: 200 mL) were discarded. 1.5 L of the following fraction were taken for the preparation of the test vessels. The undiluted preparation, a tenfold dilution and dilution water only were tested in 250 mL flasks with 5 replicates, each containing a single daphnid. Oxygen saturation and pH were measured daily, and a measurement of bacterial contamination was performed after 24 h by using dip-sticks being incubated for 24 h.

3.2 Results

The results of the exposure concentrations (summary data given in Table 1 and Figure 1) can be summarized as follows:

- The measured concentrations of the saturation preparations after filtration were lower than the water solubility predicted from different physical-chemical models (the true value for pentadecanol seems to be in the very conservative range of 50 to 200 µg/L, realistically 50 to 100 µg/L) and varied considerably. After changing the method of adding the test substance to the test media, the concentrations increased by a factor of four to five.
- The factor between measured concentrations of the undiluted and tenfold diluted preparation was more or less satisfactory.

- Higher test substance concentrations resulted in lower dissolved oxygen concentrations after 24 h.
- Detected bacteria concentrations after 24 h up to 1000/mL seem not to be correlated with test substance losses, whereas higher titers do. (This is consistent with the dodecanol results.)
- Bacteria concentrations after 24 h of higher than 1000/mL were not found until day 5.
- 50 – 70 % losses seem to be caused by adsorption to the glassware.

Table 1: Test concentrations and dissolved oxygen concentrations in the *Daphnia magna* reproduction pilot study with pentadecanol

preparation on	Mean measured concentrations (1-4 measurements) [µg/L] 0 h undiluted	0 h 1:10 diluted	24 h undiluted	24 h 1:10 diluted	Dissolved oxgen [mg/L] 24 h
Dec 1	3.29	0.41	12.59*	0.15	7.8-8.3
Dec 2	3.32	0.25	1.03	0.27	8.0-8.5
Dec 3	1.02	0.15	0.53	0.22	8.0-8.2
Dec 4	1.18	0.32	0.50	0.14	7.1-8.0
Dec 5	0.42	0.27	0.09	< LOQ	4.7-7.6
Dec 6	1.93	0.25	0.12	0.02	6.8-8.0
Dec 7	2.66	0.21	0.23	0.06	6.6-7.3
Dec 8	4.51	0.45	0.30	0.16	6.7-7.1
Dec 9	0.62	0.06	0.11	< LOQ	5.0-6.6
Dec 10	0.67	0.23	0.05	< LOQ	4.8-7.1
Dec 11	2.83	0.29	0.12	< LOQ	6.1
Dec 12	1.04	0.08	0.02	< LOQ	5.1-6.4
Dec 13	2.71	0.23	0.10	< LOQ	4.5-5.8
Dec 14	14.47	1.12	2.85	0.08	3.1-6.0 '
Dec 15	8.99	1.18	0.50	0.06	2.7-4.6 #
Dec 16	9.25	0.88	1.85	0.25	3.4-6.0 '
Dec 17	6.50	3.24	0.33	0.07	3.0-5.8 '
Dec 18	7.14	2.10	0.41	0.03	2.0-6.3 #
Dec 19	6.07	1.07	0.95	0.18	4.3-7.7
Dec 20	3.33*	0.63	2.51	0.02	2.1-5.0 #
Dec 21	7.92	0.77	0.07	0.04	3.9-5.5 '
Mean till Dec 13	2.01	0.24	0.26	0.08	
Standard dev.	1.29	0.11	0.29	0.09	
Standard dev. (%)	64.2	45.9	111.0	109.2	
Mean Dec 14-21	8.62	1.37	1.18	0.09	
Standard dev.	2.84	0.88	1.07	0.08	
Standard dev. (%)	33.0	63.8	90.7	89.9	

* not consistent when regading 1:10 and undiluted after 24 h (should be 2-3 times higher)
' low oxygen level, but above guideline quality riterion
partially below guideline quality criterion of 3 mg/L

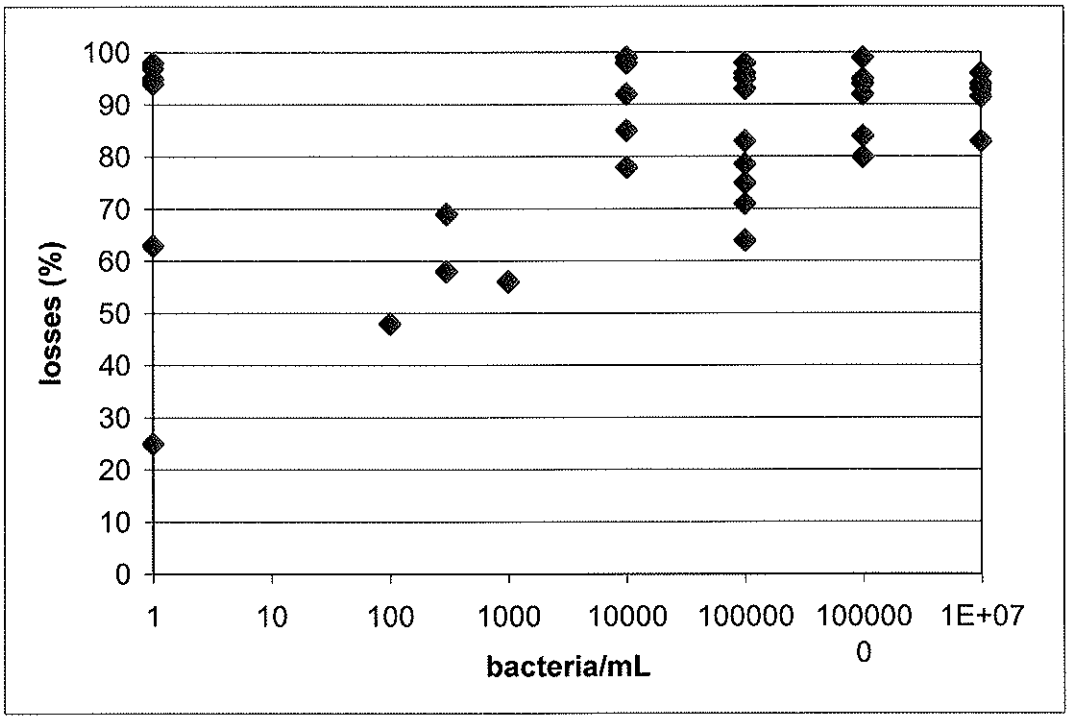

Figure 1: Relationship between test substance losses and bacteria concentrations after 24 h

The effect results can be summarized as follows:

- All daphnids survived in the control vessels. However, the reproductive output was very low (mean of 11 per female) and clearly did not fulfill the quality criteria of OECD 211 (60 per female).
- In the unfiltered preparation, all daphnids died (80 % during the last week when concentrations increased: mean measured concentration of 3.2 µg/L).
- In the tenfold diluted preparation, 40 % died during the last week (mean measured concentration of 0.35 µg/L). The offspring numbers of the survivors were slightly higher than of the controls (17 per female).
- All daphnids seemed to be in bad condition, indicated by weak pigmentation and very small sizes. The time until the first brood exceeded the quality criterion of OECD 211 of 9 days for all surviving daphnids (mean time needed: 13.3 days).

3.3 Conclusions

- Autoclaved algae are not appropriate for feeding of daphnids during the performance of a valid OECD 211 test.
- When regarding the stressing study conditions (daily renewal, rinsing procedure, closed vessels), the seasonal reproductivity of daphnids should be taken into account to achieve sufficient offspring. In our experience, daphnid reproduction is lower in December and January compared to other months of the year.
- The filtration procedure causes high losses of the test substance by adsorption to the filter and high variability of resulting test concentrations. A sufficient saturation of the filter is very difficult when regarding the necessity of daily renewal.
- The different test substance addition techniques combined with the filtration procedure yield different test concentrations.
- Bacterial densities increase in the test vessels especially from the fourth day onward. Transfer of bacteria likely occurs when transferring daphnids to fresh test solutions in spite of thorough rinsing. With increasing size of the daphnids the amount of transferred bacteria probably also increases.
- During the second week, less bacteria seem to be able to cause the same losses. This would be consistent with an bacterial acclimation hypothesis that results in enhanced rates of degradation.
- Test substance concentrations above 6 µg/L seem to cause critical oxygen depletion when subjected to biodegradation. Delivering oxygen to exposure vessels might be necessary in defintive tests..
- Due to the inappropriate study conditions, the results, although concentration-dependent, should not be over-interpreted.

4 Pentadecanol saturated solution preparations without filtration

4.1 Specific objectives and problem formulation

The objective of this study was the development of a preparation method which enables at the same time:

a) Daily preparation

b) Reproducible concentrations

c) Concentrations as near the water solubility as possible

d) Solutions excluding undissolved material / dispersion / emulsion as far as possible

The fixed elements and general parts of method in order to meet a) are

- 2 L glass vessels for the preparation in analogy to the WAF method (slow stir conditions in closed vessels, removal of the saturated solution through a drain port at the bottom),
- stock solution of the alcohol in ethanol,
- introduction of the ethanolic solution in the glass vessels, coating an area as big as possible (not the bottom, avoid interaction with later stirring), evaporation of ethanol, adding sterilized dilution water,
- stirring at 100 rpm for 21 h at 20 °C,
- settling period of 2 h,
- for use of the preparation as test solution: opening outlet at the bottom, discarding the fraction at the bottom, sampling the main preparation body, discarding the top fraction.

Because filtration seems to be optimal for d), but increase efforts for a) and causes difficulties with b) and c) due to filter saturation problems (especially when regarding a), a pentadecanol preparation should be prepared without filtration by using a loading of the test substance resulting in a concentration near solubility. The following problems had to be accounted for:

- The real solubility is unclear (different results by modelling and different experimental determinations). It should be approached under test conditions.
- The loss by adsorption to the glass is unclear. Thus, a higher loading should be used to meet c), but carefully dosed to account for d).
- The reproducibility (b) should be demonstrated for different loadings.

4.2 Setup

The preparation of saturated solutions by stirring the test substance in test media under slow stir conditions under sterile conditions was conducted

- with three loadings (corresponding to theoretical concentrations in the preparation vessels of 15, 50, and 150 µg/L),
- by using 3 replicates per concentration, thus 3 * 3 replicates with an ethanolic stock solution being widely distributed on the walls,
- with a fourth vessel per loading introducing the ethanol solution as one spot.

The preparation of 12 vessels as mentioned above was independently repeated by a different technician.

Of each flask, the first 200 mL were collected as bottom fraction. Three samples of the saturated preparation of 400 mL were taken. The bottom and the top fraction were combined. At last, the emptied flask was extracted by using hexane. Thus, of each flask, 5 samples were analyzed for pentadecanol.

With five samples each of 12 flasks, performed two times and hexane extractions of glassware blanks added, the study comprised 122 measurements.

4.3 Results

The results (summary data given in Table 2 and 3) can be summarized as follows:

- After preparation the reproducibility of concentrations without filtration was as least as good as it was when including a filtration step. When excluding single values regarded as outliers reproducibility was even better.
- Inreased loading decreased variability.
- There were no clear differences in concentrations following the two ways of test substance addition. The data after wall coating seem to be more consistent and less variable than after spot addition followed by evaporation.
- There were no differences between the main fractions and the combined bottom and top layer of the preparations.
- There were no significant differences between the mean concentrations generated by the two technicians. The differences became lower with increasing loadings.
- At a loading of 15 µg/L, the losses by adsorption to the glass seemed to be more dominant than at the higher loadings.
- There was no indication of a saturation.

Table 2: Preparation of saturated solutions without filtration, upper: three main fractions. The parameters of measured values are given for the replicate injections.

Loading [µg/L]	Replicate No.	Technician 1, 27.01.2005 measured conc. [µg/L] mean	sd	sd %	Recovery mean	Technician 2, 02.02.2005 measured conc. [µg/L] mean	s	s %	Recovery mean
Test item addition as ethanolic solution: coated walls									
15	1	*1.18*	*0.77*	*65.5%*	*7.8%*	1.35	1.06	78.8%	9.0%
	2	8.13	0.72	8.9%	54.2%	2.25	0.08	3.3%	15.0%
	3	5.73	0.61	10.6%	38.2%	1.13	0.00	0.0%	7.5%
	mean 1-3	**5.01**	**3.12**	**62.2%**	**33.4%**	**1.58**	**0.74**	**47.1%**	**10.5%**
50	1	33.50	4.88	14.6%	67.0%	21.40	7.68	35.9%	42.8%
	2	33.45	4.86	14.5%	66.9%	18.30	1.82	9.9%	36.6%
	3	36.68	2.45	6.7%	73.4%	24.60	2.03	8.2%	49.2%
	mean 1-3	**34.54**	**3.99**	**11.5%**	**69.1%**	**21.43**	**4.90**	**22.9%**	**42.9%**
150	1	90.58	9.43	10.4%	60.4%	104.28	9.56	9.2%	69.5%
	2	94.55	8.82	9.3%	63.0%	119.35	10.38	8.7%	79.6%
	3	92.25	8.29	9.0%	61.5%	*50.40*	*1.64*	*3.3%*	*33.6%*
	mean 1-3	**92.46**	**7.86**	**8.5%**	**61.6%**	**91.34**	**32.19**	**35.2%**	**60.9%**
Test item addition as ethanolic solution: spot application and nitrogen evaporation									
15	1	8.40	1.57	18.7%	56.0%	3.83	2.99	78.1%	25.5%
50	1	38.53	11.75	30.5%	77.1%	6.63	0.35	5.3%	13.3%
150	1	102.03	8.47	8.3%	68.0%	91.48	16.63	18.2%	61.0%

Table 3: Preparation of saturated solutions without filtration, combined bottom and top layers. The parameters of measured values are given for the replicate injections.

Loading [µg/L]	Replicate No.	Technician 1, 27.01.2005 measured conc. [µg/L] mean	s	Recovery mean	Technician 2, 02.02.2005 measured conc. [µg/L] mean	s	Recovery mean
Test item addition as ethanolic solution: coated walls							
15	1	*1.53*	*0.30*	*10.2%*	4.17	0.13	27.8%
	2	12.63	0.30	84.2%	2.67	0.13	17.8%
	3	9.72	0.25	64.8%	6.87	0.04	45.8%
	mean 1-3	**7.96**	**5.15**	**53.1%**	**4.57**	**1.91**	**30.5%**
50	1	42.87	1.57	85.7%	30.42	2.46	60.8%
	2	38.76	0.68	77.5%	27.72	1.27	55.4%
	3	42.63	2.42	85.3%	22.68	0.08	45.4%
	mean 1-3	**41.42**	**2.45**	**82.8%**	**26.94**	**3.73**	**53.9%**
150	1	103.71	2.16	69.1%	92.34	12.05	61.6%
	2	103.08	-	68.7%	125.37	4.88	83.6%
	3	84.39	0.30	56.3%	52.35	1.32	34.9%
	mean 1-3	**95.86**	**10.53**	**63.9%**	**90.02**	**33.22**	**60.0%**
Test item addition as ethanolic solution: spot application and nitrogen evaporation							
15	1	6.15	2.50	41.0%	4.20	0.17	28.0%
50	1	34.35	11.50	68.7%	7.68	0.25	15.4%
150	1	97.17	2.33	64.8%	115.23	3.69	76.8%

- The concentrations were considerably higher (up to one order of magnitude) compared to the preparation including filtration.

Since the concentrations were clearly higher than the solubility predicted by different models based on phys.-chem. properties and since there was no clear indication of a trend to saturation it was concluded that the measured concentrations consisted of a fraction of water soluble alcohol and a finely-divided dispersed fraction of alcohol.

5 Pentadecanol acute *Daphnia magna* toxicity test with higher loadings

5.1 Specific objectives and problem formulation

Aquatic toxicity tests should be performed up to a concentration as close as possible to the water solubility limit. Narcotic QSARs models predict acute daphnid toxicity EC50s in the range of the measured concentrations. Thus, combined studies of higher loadings and acute *Daphnia magna* toxicity were conducted

- to investigate whether saturation might be approached by higher loadings
- to look for immobilization related to measured concentrations

If immobilization occurred it would be challenging to differentiate between chemical toxicity due to narcotic action and physical toxicity due to interactions of particles or fine droplets with gill surfaces. A concentration-response relationship should be less clear when due to physical effects only, but cannot be excluded when dispersed material is homogeneously distributed. However, when regarding the realistic exposure in receiving waters, the differentiation between chemical and physical effects is arbitrary.

5.2 Setup

The preparation of saturated solutions by stirring the test substance in test media under slow stir conditions under sterile conditions was conducted

- with three loadings corresponding to theoretical concentrations in the preparation vessels of 250 and 500 µg/L in a first step and 750 µg/L in a second step,
- by using 3 replicates per concentration, thus 3 * 3 replicates with an ethanolic stock solution being widely distributed on the walls.

- The preparations of 250 µg/L (first step) and 750 µg/L (second step) were used as highest concentrations in the acute toxicity test, being prepared two times (daily renewal in the 48h test) and diluted by a factor of 2 to achive 4 and 2 further test concentrations, respectively.
- The test vessels were closed with autoclaved cellulose stoppers instead of gas-tight glass stoppers.

Of each flask, the first 200 mL were collected as bottom fraction. Three samples of the saturated preparation of 400 mL were taken. The bottom and the top fraction were combined. At last, the emptied flask was extracted by using hexane.

In the first step (loading of 250 µg/L), the first replicate was taken for the toxicity test, the other replicates were measured. All toxicity test concentrations were analysed after 24 h.

In the second step (loading of 750 µg/L), three samples of the main fraction of 500 mL were taken. Two samples were measured for the test substance concentrations. One sample of all replicates were combined for use in the toxicity test. All toxicity test concentrations were analysed before distribution to the test vessels and after 24 h.

With 15 flasks, 4 samples measured per flask and 12 additional samples per tocoty test step, the study comprised 84 measurements.

5.3 Results

The results (summary data given in Table 4 and Table 5) can be summarized as follows:

- The recovery of the test substance was around 100 % at all loadings.
- The fraction adsorbed to the glass walls varied considerably between replicates, indicating the importance of comparable glass ware quality.
- The variability of replicates was low (standard deviation between 1 and 12%).
- There were again no differences between the main fractions and the combined bottom and top layer of the preparations.
- There was again no indication of a saturation.
- There was no immobility at any concentration tested (up to maximum initial concentration of 500 µg/L and a mean measured concentration of 370 µg/L).
- A gas-tight sealing of test vessels as used in the pilot study does not ensure more sterile conditions; autoclaved cellulose stoppers are sufficient.

Table 4: Preparation of saturated solutions without filtration. Loading: 250-750 µg/L.
The parameters of measured values are given for the replicate injections.

Loading [µg/L] Date	Repli-cate No.	Main fraction (3 samples) measured conc. [µg/L] mean	s	s %	Recovery Mean	Bottom and top fraction measured conc. [µg/L] mean	s	Recovery mean	container walls Mass Recovery	Total Recovery[1]
250	1	-	-		-	192.8	0.21	77.1%	35.1%	112.2%
Feb 17	2	207.5	8.74	4.2%	83.0%	210.3	2.97	84.1%	28.3%	111.7%
2005	3	184.0	7.20	3.9%	73.6%	189.7	3.89	75.9%	34.8%	109.3%
	mean	**195.7**	**14.7**	**7.5%**	**78.3%**	**197.6**	**10.2**	**79.0%**	**32.7%**	**111.1%**
250	1	-	-		-	156.8	11.6	62.7%	25.3%	88.0%
Feb 18	2	244.8	27.0	11.0%	97.9%	235.4	6.79	94.2%	36.0%	132.4%
2005	3	227.8	15.2	6.7%	91.1%	229.8	15.3	91.9%	18.6%	110.0%
	mean	**236.3**	**21.7**	**9.2%**	**94.5%**	**207.3**	**40.3**	**82.9%**	**26.6%**	**110.2%**
500	1	107.8	9.00	8.3%	21.6%	123.8	3.46	24.8%	73.5%	96.3%
Feb 17	2	384.5	27.7	7.2%	76.9%	357.2	10.7	71.4%	28.6%	103.3%
2005	3	364.6	5.22	1.4%	72.9%	335.3	26.0	67.1%	20.3%	90.9%
	mean	**285.7**	**134.5**	**47.1%**	**57.1%**	**272.1**	**116.0**	**54.4%**	**40.8%**	**96.8%**
750	1	151.9	17.8	11.7%	20.3%	174.9	7.35	23.3%	78.9%	100.4%
Mar 2	2	322.3	28.3	8.8%	43.0%	385.0	7.85	51.3%	66.0%	112.3%
2005	3	489.1	6.45	1.3%	65.2%	490.2	4.31	65.4%	37.3%	102.6%
	mean	**321.1**	**151.5**	**47.2%**	**42.8%**	**350.0**	**143.7**	**46.7%**	**60.7%**	**105.1%**
750	1	478.3	31.6	6.6%	63.8%	577.0	17.1	76.9%	24.3%	93.4%
Mar 3	2	570.6	42.9	7.5%	76.1%	524.0	4.31	69.9%	32.0%	105.6%
2005	3	439.8	2.56	0.6%	58.6%	472.6	3.82	63.0%	44.4%	104.8%
	mean	**496.2**	**64.7**	**13.0%**	**66.2%**	**524.5**	**47.4**	**69.9%**	**33.6%**	**101.2%**

[1]((1.2*main fraction+0.8*bottom and top fraction)/2)+container walls

The results indicate that recovery of test substance in the water was between 60 to 70% with loadings of 50 to 750 ug/L. New glassware was used to minimize the adsorption to glass. No acute toxicity to Daphnia magna was observed even at the highest test concentrations. Thus,

- Water solubility seems to be surpassed without reaching a concentration critical fo acute toxicity
- The increase of the concentration seems to be merely due to non-dissolved material
- A preparation at a loading of up to 750 µg/L does not cause physical acute effects

Table 5: Concentrations of the acute toxicity test with *Daphnia magna* (no effect at all)

Dilutions of preparations	Nominal conc. [µg/L]	mean measured concentration [µg/L] 0h	% of nominal	24h	% of nominal	% left after 24 h
	0 (control)			14.52*	-	
1st step	**15.6**			13.68	87.7%	
250 µg/L	**31.3**			24.5	78.2%	
(replicate 1)	**62.5**			48.2	77.2%	
Feb 17, 05	**125**			84.8	67.9%	
	250			192.8	77.1%	
	0 (control)			0.84	-	
1st step	**15.6**			8.88	56.9%	
250 µg/L	**31.3**			14.0	44.9%	
(replicate 1)	**62.5**			35.4	56.6%	
Feb 18, 05	**125**			75.7	60.6%	
	250			175.3	70.1%	
2nd step	**0 (control)**	0.40	-	3.8	-	
750 µg/L	**187.5**	89.7	47.8%	32.8	17.5%	36.6%
(mix replicates 1-3)	**375**	181.3	48.3%	82.4	22.0%	45.4%
Mar 2, 05	**750**	321.1	42.8%	227.8	30.4%	70.9%
2nd step	**0 (control)**	2.80	-	0.8	-	-
750 µg/L	**187.5**	149.0	79.5%	92.2	49.2%	61.9%
(mix replicates 1-3)	**375**	297.2	79.3%	215.4	57.4%	72.5%
Mar 3, 05	**750**	496.2	66.2%	462.5	61.7%	93.2%

* high value (validated by repeated measurements), most probably due to a contamination of glassware. it could not be clarified whether the contamination happened to the vessels before exposure or to the sample equipment after exposure.

6 Resulting considerations for the study plan

The following considerations for the study plan of the definitive *Daphnia* reproduction test can be derived:

- The study should be performed between March and November.
- Daphnids will have to be fed with an axenic culture of live green algae (i.e., *Desmodesmus subspicatus*).
- The study should be performed under clean bench conditions.
- The study should be performed without filtration of preparations.
- The addition of the test substance to the preparation vessels should be performed by coating as large areas of the glass walls as possible.

- The pentadecanol study should be performed in new vessels, closed by autoclaved cellulose stoppers.
- Additional aeration with filtered air should be implemented
- The study will be performed in 100 mL flasks instead of 250 mL flasks. At a given concentration of live feeding algae and test substance, the absolute amount of both is reduced. Due to the feeding by the daphnid, the algae concentration at renewal of the test liquid after 24 h is more reduced. This should result in less biodegradation by algae and less oxygen consumption. Nevertheless, additional aeration should be delivered.
- The test concentrations will be daily and individually prepared by stirring the test substance in test media under slow stir conditions under sterile conditions at loadings of 30, 65, 139 and 300 µg/L, aiming at an initial top concentration of approximately 200 µg/L.

Annex 1: **Non-GLP pilot study plan** for *Daphnia magna*, reproduction test in closed vessels, OECD No. 211

Test item: **Pentadecanol**

GLP-Code of the definitive study: **SDA-002/4-21**

Test procedure, reproduction test

Daphnia magna less than 24 h old will be exposed to two concentrations of the test item for a period of 21 days. The test solution will be filled in the test vessels, the daphnids will be added and the vessels will be closed directly afterwards by a gas-tight stopper. The water will be renewed daily under clean-bench conditions by transferring the daphnids to new beakers with freshly prepared test solutions with the initial test concentrations. At each transfer, the daphnids will be carefully rinsed with dilution water before being placed in the new beakers.

For the two test concentrations and for the control 5x1 animals will be used. Each daphnid will be exposed separately in a completely filled and numbered vessel containing 250 mL of test medium.

General test conditions
The daphnids will be fed at each renewal with autoclaved suspensions of unicellular green algae. 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. 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 gas-tight 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.

Observation and measurements
The number of immobile daphnids will be visually determined daily. Vessels with immobile daphnids will be removed. Any abnormalities in appearance and behaviour will be recorded.

The newborn daphnids per vessels 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 daphnids
- Time to the first brood
- Number of live offspring per surviving female

Preparation of test media

The preparation of the highest test item concentration will be performed according to the CONCAWE test protocol and to the ASTM standard D6081-97 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:

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 generation of the sterile saturated solution procedure
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 saturated solution under a clean bench.

Test media preparation
For the highest test concentration, a saturated solution will be prepared by stirring the test substance in test media under slow stir conditions under sterile conditions by mixing the test item with the water in clean 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 saturated solution. The volume of the mixing vessels will be 2 L. To provide sufficient volume of the saturated solution for glassware rinsing, exposure and for analysis, two mixing vessels will be used for the preparation of the highest test concentration. The second concentration will be prepared by diluting the saturated solution by a factor of 10.

To prepare 2 L saturated solutions of the test item, measured amounts of sterilized dilution water will be added to measured volumes of the test item. The test item corresponding to a loading rate of 2 mg/L (1 mL of a stock solution of 4 g test item/L ethanol) will be added directly into the bottom of the 2 L mixing vessel. The ethanol solution must not moisten the inner area of the flask where a star-shaped magnetic stirrer bar will be placed later.

The ethanol will be evaporated gently with filtered nitrogen and the autoclaved dilution water (amount determined by weighing) and the sterilized stirring bar will be added. The vessels containing the medium and the test item will be sealed leaving only a small headspace. The contents of the vessels will be stirred at 100 rpm for approximately 21 h. Care will be taken to avoid even short time exceeding of the stirring speed at start. The vessels will be kept at room temperature (21 ± 1 °C).

After stirring the contents of the vessels were left to settle for 2 h. Then the saturated aqueous phase will be taken out of the drain port and filtered by suction through a 0.2 µm apolar filter (Millex FG, 50 mm, Millipore), which will be wetted with methanol prior to use to allow the passage of the aqueous solution. The first fraction 0-100 mL will be withdrawn. The fraction between 100 and 1500 mL will be filled into the test flasks for toxicity testing, the gas-tight glassware for preparing the lower test item concentrations by dilution, and in the flasks for analytic measurements. Care will be taken to ensure that any visible not dissolved material will be not transferred into the test vessels.

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 sealed immediately and only opened to introduce the test organisms and again at the renewals of the test media.

Chemical analysis of test media

Before filling the test vessels at study start and each renewal of the test liquids, the test concentrations will be analysed. At each renewal, the old test liquids will be pooled and analysed.

The method refers to the determination of C10-, C12-, C14-, C15-, C16- and C18-fatty alcohols (not of possible metabolites) in Daphnia magna test medium in concentrations above 1 µg/L. It is based on liquid-liquid extraction of samples of the analytes by n-hexane, derivatization by MSTFA (n-methyl-n-(trimethylsilyl)trifluoroacetamid; Fluka) and determination by GC-MS with deuterated C12-, C14- and C16-fatty alcohol internal standards.

GC-MS

Gas chromatograph:	5890 Serie II plus, Hewlett Packard		
Autosampler:	type 7673, Hewlett Packard		
Column:	BPX-5, 50 m, ID 0.32mm, film 0.25 µm		
Oven:	step:	1	2
	temperature [°C]:	60	280
	time [min]:	3	10
	rate [°C/min]:	10	
SSL-injector temperature:	280 °C		
Mass spectrometer:	MSD 5972, SIM Mode, Hewlett Packard		
Multiplier voltage:	1824 eV		
Source temperature:	170°C		

Validation
Validation of the analytical method will be performed according to guideline SANCO/825/00 rev. 6 and SANCO/3029/99 ver. 4 [reference list]. 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).

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

Linearity
The linearity of the detector response will be shown by the chromatography of calibration solutions. Calibration will not be based on the recovery of the internal standards but on their actual concentrations in the aqueous samples.

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

Accuracy
The accuracy of the method will be determined by recovery experiments with fortified samples. The recovery data will be reported for 2 fortification levels per matrix appropriate to the proposed LOQ (0.02 µg/L) and an appropriate higher concentration. Mean recoveries for each level should be in the range 70-110%, ideally with the mean in the range 80-100%.

Lower recoveries may be acceptable for difficult analytes, providing precision data are acceptable. Two replicates will be processed.

Identity
The identity of the analyte and the deuterated reference item will be taken as approved by the Certificate of Analysis. In addition the interpretation of the mass fragments and their relation obtained by mass spectrometric detection will be used for structure verification. 3 ions (ideally with an m/z ratio of >100) will be used for identification/quantification by mass spectrometry.

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

Limit of Quantification (LOQ)
The method will be validated at the LOQ of 0.02 µg/L test medium.

Sample clean-up
500 mL of each aqueous sample will be extracted by by liquid-liquid partitioning with 5 mL of n-hexane. After shaking for about 10 min and settling 100 µL of the supernatant (n-hexane) will be taken and 50 µL of the internal standard solution will be added. After derivatization by 100 µL of MSTFA the solutions will be measured by GC-MS in SIM mode using internal standard calibration with deuterated C_{15} fatty alcohol as internal standard.

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, D.-holding and breeding
V4 - 503/02	Daphnia test, acute tox., repro-test, prep. of test solutions
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
V7 - 220/02	Workup- and extraction procedures, organic pollutants
G3 - 002/02	Zentrifuge HERAEUS Minifuge, handling
G3 - 004/02	Scales, Calibration
G3 - 005/02	Checking of volumetric apparatus
G3 - 006/03	Checking of piston-operated pipetters
G3 - 007/02	Checking of thermometers
G3 - 008/02	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
G7 - 025/02	Rotavapor, handling
G7 - 032/02	Kältethermostat COMPACT RMS 6, handling
G7 - 170/02	Pure water extractor UHQ-PS, handling
G7 - 183/02	Washing machine Miele with Aquapurifikator, handling
G7 - 192/02	Vakuum pump VAN DER HEYDEN-AQUASTOP, handling
G7 - 226/02	Eppendorf Table Centrifuge 5415C
G7 - 227/02	Van der Heijden PARALAQUA, for rotavapor

Schedule

Presumed test start:	December 01, 2004
Presumed test end:	December 22, 2004

References

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

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

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

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

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