Report: Biodegradability in the CO_2-evolution test according to OECD 301 B (July 1992)

Standard operating procedure:	**SOP V 6.5, edition 6 from 20.08.2008**
Sponsor:	**Sasol Germany GmbH**
Test item:	**NACOL 22-98**
Internal number of the test item:	**11/4206**

Study Director: 3.5.12 _____
(date, Felicitas Flach)

Test Facility Management: 3.5.12 _____
(date, Dr. Stefan Gartiser)

Quality Assurance: 3.5.12 _____
(date, Ismene Jäger)

Statement of Compliance

The study described in this report was conducted in compliance with the OECD Principles of 'Good Laboratory Practice' Standards. No foreseen circumstances were observed which might have affected the validity of the data or the quality and integrity of the study.

Study director: 3.5.12

(Date, F. Flach)

Quality Assurance Statement of Compliance

The Quality Assurance Unit (QAU) hereby declares that the study was inspected on the following dates and the findings of each audit were reported to the study director and the management.

The results reported in this study were checked on the basis of our current QAU-SOPs. This statement confirms that the final report reflects the raw data.

	Date of Quality Inspection	Date of report to the study director:	Date of report to the facility management:
Facility based inspection	December 22nd, 2011	December 22nd, 2011	January 18th, 2012
Study based inspection	March 15th, 2012	March 19th, 2012	March 19th, 2012
Inspection of study plan and draft report	April 23rd, 2012	April 23rd, 2012	May 3rd, 2012
Inspection of final report	May 3rd, 2012	May 3rd, 2012	May 3rd, 2012

Quality assurance: 3.5.12

(Date, Ismene Jäger)

QUALITÄTSGESICHERT durch HYDROTOX GmbH anerkanntes GLP-Labor

Summary

Test:	Biodegradability, CO_2-evolution test according to OECD 301 B (July 1992)
Sponsor:	Sasol Germany GmbH Paul-Baumann-Str. 1 D-45764 Marl
Test item:	NACOL 22-98
Chemical name:	1-docosanol (information from the sponsor)
Lot No. of test item:	03941/MA (information from the sponsor)
Purity:	99.1% (information from the sponsor)
Organic Carbon:	80.5% (measured by ASG Analytik)
Density:	Approx. 0.8 g/cm³ at 80°C (information from the sponsor)
Application of test item:	A stock solution of 10.355 g/L in trichlormethane was prepared
Inoculum:	Activated sludge, municipal wastewater treatment plant Breisgauer Bucht, 30 mg dry solids per litre
Study initial date:	January 23rd, 2012
Experimental starting date, 1st test:	January 24th, 2012
Experimental starting date, 2nd test:	March 15th, 2012
End of degradation phase:	April 12th, 2012 (28 d)
Last measurement:	April 13th, 2012 (second absorber)
Study completion date:	May 3rd, 2012 (date of final report)
Test duration:	29 d
Temperature:	21 – 22°C

Results: **The degradation of the test item was 87.5% within 28 days (after acidification). The test item reached the criterion for ready biodegradability (60% $ThCO_2$ and 10 d-window).**

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1 Descriptions of test system, test item and reference item

1.1 Descriptive title

Biodegradability, CO_2-evolution test according to OECD 301 B (July 1992)

1.2 Description of the test item

Name:	NACOL 22-98
Chemical name:	1-docosanol (information from the sponsor)
EG-No.:	211-546-6 (information from the sponsor)
CAS-No.:	661-19-8 (information from the sponsor)
Lot No. of test item:	03941/MA (information from the sponsor)
Hydrotox No.:	11/4206
Origin:	Sasol Germany GmbH
Certificate of analysis:	available from January 09th, 2011

1.3 Characterisation of the test item

Purity:	99.1% (information from the sponsor)
Appearance:	White solid (information from the sponsor)
Solubility in water:	<0.1 mg/L (information from the sponsor)
Volatility:	Vapour pressure <0.1 Pa (38°C) (information from the sponsor)
TOC-content:	80.5% (measured by ASG Analytik)
Density:	Approx. 0.8 g/cm³ at 80°C (information from the sponsor)
Stability:	Stable under recommended storage conditions
Storage-conditions:	Storage temperature 5 – 30°C, protection from moisture and sunlight (information from the sponsor)
Durability:	January 2015 (information from the sponsor)
Safety directions:	This substance is not classified as dangerous

1.4 Date of receipt of the test item

December 20th, 2011

Storage at room temperature

1.5 Test of identity of the test item

The identity was confirmed visually. The test item is a white solid (pellets). The designation on the flask of the test item was confirmed.

1.6 Reference item

Benzoic acid, sodium salt (=Sodium benzoate)
Roth, Lot: 14895425
Sum formula: $C_7H_5O_2Na$
Molecular weight: 144.1 g/mol
Storage-conditions: room temperature
Durability: 30.11.2013
Solubility in water: soluble
C-content: 0.583 mg/mg (calculation by Hydrotox)
ThCO2: 2.137 mg/mg (calculation by Hydrotox)

1.7 Solvent

Trichlormethane (= chloroform)
Roth, Lot: 461174259
Sum formula: $CHCl_3$
Molecular weight: 119.38 g/mol
Purity: ≥ 99%
Storage conditions: room temperature
Durability: 18.01.2019

2 Specification of the sponsor and the test facility

2.1 Name and address of the sponsor

Sasol Germany GmbH
Dr. Birte Dreeßen
Paul-Baumann-Str. 1
D-45764 Marl

2.2 Name and address of the test facility and the test site

Hydrotox GmbH
Bötzingerstr. 29
D-79111 Freiburg

2.3 Name and address of the study director

Felicitas Flach
Hydrotox GmbH
Bötzingerstr. 29
D-79111 Freiburg

2.4 Name and address of technical assistance

Petra Hönicke-Brugger
Hydrotox GmbH
Bötzingerstr. 29
D-79111 Freiburg

2.5 Name and address of cooperating laboratories

ASG Analytik-Service GmbH
Trentiner Ring 30
D-86356 Neusäss
(Accreditation DIN EN ISO/ IEC 17025:2005)

3 Dates

Date of receipt: December 20th, 2011
Study initial date: January 23rd, 2012
1st test:
Experimental starting date: January 24th, 2012
2nd test:
Sampling of activated sludge inoculum: March 14th, 2012
Start of incubation of the test item: March 15th, 2012

Sampling for IC-measurement: March 15th, 2012; March 19th, 2012; March 22nd, 2012; March 26th, 2012; March 29th, 2012; April 5th, 2012; April 12th, 2012 and April 13th, 2012 (day 0, 4, 7, 11, 14, 21, 28 and second absorber)

Experimental completion date: April 13th, 2012

4 Materials and Methods

4.1 Materials

All sources Germany if not stated otherwise

- Compressor NO10.AN 18, KNF Neuberger, Freiburg
- 1000 mL gas wash bottles with Teflon-sealing, Thoma, Freiburg
- Magnetic stirrer, 'MONO direct' with stir bars 2 cm, H+P Labortechnik AG, Oberschleißheim
- Row of air-tubes (air distributor) with two input and 22 output channels, Thoma, Freiburg
- Perforated plugs with PE-tubes (2.8/2.0 mm), Thoma, Freiburg
- 2000 mL gas wash flasks with GL14 hole-caps and frit pipes (reactors), Gerätebau Ochs, Bovenden-Lenglern
- Two 250 mL gas wash bottles connected in series with GL14 hole caps for each channel and frit pipes (CO_2-absorber flask), Gerätebau Ochs, Bovenden-Lenglern
- Total carbon analyser TOC-5050A with autosampler ASI 5000A, Shimadzu Deutschland, Duisburg
- Sealing Parafilm „M", Pechiney Plastic Packaging, Chicago, USA
- Analytical balance BP 221S, Sartorius AG Göttingen
- Precision balance LP 6200S, Sartorius AG Göttingen
- Thermometer with min/max-display
- Adjustable micropipettes 5 mL, 1 mL, Eppendorf, Wesseling/Berzdorf
- Syringe for sampling, Braun Injekt 5 mL, 2 mL, Melsungen
- Needle for sampling, Sterican Braun 21 G, Melsungen
- Drying oven (105°C), Memmert, Schwabach

4.2 Method

The CO_2-evolution test determines the ready biodegradability of test items according to the Modified Sturm test. A solution or suspension of the test item in a mineral medium,

corresponding to 10-20 mg TOC/L, is inoculated with activated sludge (30 mg d.s./L). The test vessels are aerated by the passage of carbon dioxide-free air and are incubated under aerobic conditions in diffuse light for 28 days. Degradation is followed by determining the carbon dioxide produced and absorbed to sodium hydroxide via IC-measurement (IC= inorganic carbon). The amount of carbon dioxide produced from the test item less the amount derived from the blank inoculum is expressed as a percentage of $ThCO_2$ (theoretical amount of CO_2). The pass level for ready biodegradability is 60% of $ThCO_2$ and must be reached within a 10-d window. The 10-d window begins when the degree of biodegradation reaches 10%. The test method is suitable for water soluble and non-soluble test items which are non-volatile and not toxic under the test conditions.
The test was performed without (1st test) and with solvent control (2nd test).

4.3 Stock solutions

4.3.1 Mineral medium

A:	Potassium dihydrogenphosphate	KH_2PO_4	8.50 g
	Dipotassium hydrogenphosphate	K_2HPO_4	21.75 g
	Disodium hydrogenphosphate dihydrate	$Na_2HPO_4 * 2\ H_2O$	33.40 g
	Ammonium chloride	NH_4Cl	0.50 g
	are dissolved in demineralised water and made up to 1 litre.		
B:	Calcium chloride dihydrate	$CaCl_2 * 2H_2O$	36.4 g
	is dissolved in demineralised water and made up to 1 litre.		
C:	Magnesium sulfate heptahydrate	$MgSO_4 * 7H_2O$	22.5 g
	is dissolved in demineralised water and made up to 1 litre.		
D:	Iron (III) chloride hexahydrate	$FeCl_3 * 6H_2O$	0.25 g
	is dissolved in demineralised water, stabilised with one drop of concentrated HCl and made up to 1 litre.		

For preparation of the mineral medium 10 mL of solution (A) is mixed with 800 mL demineralised water, 1 mL each of solutions (B), (C) and (D) are added and the volume is made up to 1 litre.

4.4 CO_2-absorption medium

44.15 g NaOH was dissolved in 5.5 L deionised water in closed recipients (0.2 M NaOH). The inorganic carbon concentration of the 0.2 M NaOH was determined.

4.5 Test item

Due to the low water solubility of the test item the stock solution was prepared with trichlormethane. A stock solution of 10.355 g/L was prepared by dissolving 207.1 mg of the test item in 20 mL trichlormethane. 3.6 mL of the stock solution were added into the test vessels and the solvent was evaporated to dryness under the fume hood by slewing the vessels. This corresponds to a test concentration of 20 mg/L organic carbon.

4.6 Reference substance

A stock solution of 10 g/L sodium benzoate in water was prepared. 5.15 mL of this stock solution were added into the reference vessels corresponding to a concentration of 20 mg/L organic carbon.

4.7 Solvent control

The solvent control was prepared in the same way as the test vessels.
Therefore 3.7 mL of the solvent trichlormethane was added into the solvent control vessel. The vessel was slewed under the fume hood until the solvent had evaporated completely.

4.8 Inoculum

Activated sludge from the municipal wastewater treatment plant Breisgauer Bucht was used as inoculum with a concentration corresponding to 30 mg dry solids per litre. The treatment plant clarifies predominantly domestic wastewater and has a capacity of 600.000 inhabitant equivalents. Sampling date of activated sludge was on March 14th, 2012. Dry solid of the activated sludge was 6.53 g/L by weight measurements after 3.0 h drying at 110°C (mean of triplicate measurements). The activated sludge was washed twice by settling the sludge, decanting the supernatant and re-suspending the sludge in tap water.

4.9 Experimental set up

The CO_2-free air production system consists of an air compressor, three 1000 mL gas wash

bottles filled with dry soda lime in series followed by one bottle filled with 0.1 M NaOH (sodium hydroxide). At the end of the system was one gas wash bottle filled with demineralised water, followed by an empty one to catch any drops of condensation water. A colour change of the soda lime from white to blue indicates that the CO_2 absorption capacity is depleted. The CO_2-free air is passed on to an air distributor with two input and 22 output channels and through PE-tubes.

In toto three reactors containing the test item, three reactors containing only inoculum (blank), three reactors containing the reference compound and one reactor containing the solvent control were set up.

Gas wash bottles (2000 mL volume) with lateral connecting pieces for butyl rubber septums were used as reactors. The liquid volume was fixed as 1500 mL each. Mixing was performed by a magnetic stirrer with 2 cm stir bars. A stock solution of 10.1 g/L test item was prepared and 3.7 mL of the stock solution were added into the three test vessels (corresponding to a TOC concentration of 20 mg/L). The reference compound (5.15 mL of a 10 g/L stock solution) was added to the reference vessels. 3.7 mL of the solvent was added into the solvent control vessel.

The CO_2 produced in the reactors was absorbed in two 250 mL gas wash bottles in series each filled with 200 mL 0.2 M NaOH. Sampling was performed through the lateral connecting pieces through the butyl rubber septum using 5 mL PE syringes.

4.10 Procedure

The TOC-content of the test item used for the calculation is 80.5% (measured by ASG Analytik).

The activated sludge and the mineral medium were aerated with CO_2-free air in separate containers overnight. On the next day, the test item, the reference compound and the solvent were added into the vessels. After complete evaporation of the solvent under the fume hood 6.9 mL activated sludge was filled up to 1500 mL with 1493.1 mL mineral medium and added to each vessel (corresponding to 30 mg/L dry solids). The absorber wash bottles were filled with 0.2 M NaOH. The reactors were kept mixed with magnetic stirrers and the aeration rate

was kept at a rate of 30 - 100 mL / min (1.6 - 5.5 bubbles / second). The aeration rate was and determined visually daily on working days. The determination by counting the gas bubbles over a defined period using a stop watch was made at day 1 and 26. The CO_2-free air production system, the air-tightness of the whole experimental set-up, the aeration of the absorber flasks and the magnetic stirrers were controlled daily on working days.

At the beginning of the study the IC concentration of the 0.2 M NaOH used for the CO_2-absorption flasks was determined as 4.12 ppm. The IC in the reactor at the beginning of the test showed insignificant amounts of IC (0.1 mg/L). On the 4th, 7th, 11th, 14th, 21st and 28th day 4 mL NaOH from the first of two CO_2-absorber flasks connected in line was sampled and the IC's were determined. The vials were immediately closed with sealing film in order to avoid CO_2 uptake from the air. On the 28th day 2 mL of 4M hydrochloric acid (HCl) was added into each reactor to release the CO_2 dissolved in water. On day 29 the IC was determined in both CO_2-absorber flasks in line.

IC measurement was performed with a total carbon analyser (TOC-5050A Shimadzu with an autosampler ASI-5000A) by purging the inorganic carbon with H_3PO_4 (25%) using a non dispersive infrared (NDIR) detector.

4.11 Evaluation of data

The theoretical CO_2 amount of the test item ($ThCO_2$) is calculated as follows:
$ThCO_2$ [mg] = weight of added test item [mg] * carbon-content [mg/mg] * 44/12

The amount of CO_2 released from the reactors is calculated through IC-measurements in the CO_2-absorber flasks as follows:

CO_2 [mg/1500 mL] = IC [mg/L]* $Volume_{absorber\ flask}$ [L] *44/12

Hereby the volume of the absorber flask at the beginning of the test was 0.2 litre (200 mL) and the volume was reduced through each sampling by 4 mL. The factor 44/12 corresponds the molar weight-ratio of CO_2 and C.

The amount of CO_2 removed for IC-measurement is considered by adding up the CO_2 content of each sampling to the current CO_2 content of each absorber flask:

CO_2 total (i, x) = CO_2 absorber flask (i, x) + Σ CO_2 sampling (i, x-1)

with

CO_2 total (i, x) = Total amount of CO_2 [mg] absorbed in the i^{th} absorber flask at the x^{th} sampling including the amount removed by sampling

CO_2 absorber flask (i, x) = CO_2 [mg] absorbed in the i^{th} absorber flask derived from IC-measurement at the x^{th} sampling.

Σ CO_2 sampling (i, x-1) = Sum of CO_2 [mg] removed from the i^{th} absorber flask with the 1^{th} to $x\text{-}1^{th}$sampling (sampling volume 4 mL each ➔ CO_2 removed = IC [mg/L] * 0.004 [L]*44/12]).

The percentage biodegradation of the **reference substance** is calculated from:

$Biodegradation_{CO2}$ [%] = 100*($CO_{2\ Reference\ reactor}$ [mg] - $CO_{2\ Blank\ reactor}$ [mg])/$ThCO_2$ [mg]

$CO_{2\ Reference\ reactor}$ = Total CO_2 evolution in the reference reactor [mg]

$CO_{2\ Blank\ reactor}$ = Total CO_2 evolution in the blank reactor [mg] (mean of three vessels)

The percentage biodegradation of the **test item** is calculated in two ways:

<u>With consideration of the solvent control:</u>

$Biodegradation_{CO2}$ [%] = 100*($CO_{2\ Test\ reactor}$ [mg] - $CO_{2\ Solvent\ control\ reactor}$ [mg])/$ThCO_2$ [mg]

$CO_{2\ Test\ reactor}$ = Total CO_2 evolution in the test reactor [mg]

$CO_{2\ Solvent\ control\ reactor}$ = Total CO_2 evolution in the solvent control reactor [mg]

<u>With reference to the blanks</u>:

$Biodegradation_{CO2}$ [%] = 100*($CO_{2\ Test\ reactor}$ [mg] - $CO_{2\ Blank\ reactor}$ [mg])/$ThCO_2$ [mg]

$CO_{2\ Test\ reactor}$ = Total CO_2 evolution in the test reactor [mg]

$CO_{2\ Blank\ reactor}$ = Total CO_2 evolution in the blank reactor [mg] (mean of three vessels)

4.12 Reference to the test-guideline

The ready biodegradability was determined according to OECD 301 B (July 1992).

4.12.1 Validity of the study

For a study to be valid the following conditions should be fulfilled:

- IC content of the test item reactor at the beginning of the test must be less than 5% of the TOC introduced with the test item.
- CO_2 evolution in the inoculum blank at the end of the test should not exceed 40 mg/L and must be below 70 mg/L.
- The difference of extremes of replicate values of the removal of the test item at the end of the test or at the end of the 10 day window should be less than 20%.
- The percentage degradation of the reference compound must have reached the pass level of 60% $ThCO_2$ by day 14.

4.12.2 Deviation from the guideline

None

4.13 Study-plan amendment and deviations

In an additional solvent control vessel 3.7 mL of the solvent (trichlormethane) were added and the solvent was evaporated by slewing the vessel under the fume hood. The solvent control was prepared in the same way as the vessels with the test item.

4.14 Study-plan deviation

None

5 Results

A first test was carried out without the addition of a solvent control. It could not be excluded that the solvent has any influence on the CO_2-evolution and on the degradation values of the test item. The test was therefore repeated with an additional solvent control. In both tests the test item was readily biodegradable. The mean degradation extent of the test item was lower in the 2nd test (1st test: 111.1%, 2nd test: 87.5%). The results of the 1st test are not reported in detail.

5.1 Summary of the results

Test item:

With consideration of the solvent control the degradation extent of the test item was 87.5% within 28 days (mean of three replicates, see table 1). On day 2 the mean degradation extent was for the first time higher than 10%. Therefore the end of the 10 d- window is on day 12. On day 11 the mean degradation extent reached 75.6% (mean value).

With reference to the blanks the degradation extent of the test item was 101.5% within 28 days (mean of three replicates, see table 5). The mean degradation extent reached 60% on day 7.

The degradation extents referred to the solvent control are considered more reliable.

In both cases the test item reached the pass level for ready biodegradability (60% ThCO$_2$ and 10 d-window, see table 1 and 5 and fig. 1 and 2).

Reference item:

The reference compound sodium benzoate reached the pass levels for ready biodegradability within 4 days (see table 1).

Blank:

The highest mean CO_2-evolution of the blank flasks was 27.2 mg/L within 28 days (see table 3).

Solvent control:

The highest mean CO_2-evolution of the solvent control flask was 39.5 mg/L within 28 days (see table 4). The CO_2-evolution was considerably higher than in the blanks. Thus the degradation extents were referred to the solvent control.

Before adding the test item, the IC in the reactor was determined, but only insignificant amounts of IC (0.1 mg/L) were found. The IC-concentration of the NaOH in the second CO_2-absorber flasks in line, used as protective flasks, was below 4 ppm and was not considered in the data processing, because CO_2 absorption from room air was its source.

The temperature was 21 - 22 °C throughout the whole study. The aeration rate was in the tolerated range of 1.6 – 5.5 bubbles/second (counted bubbles: 2.4 – 4.4 bubbles/second).

5.2 Criteria met for the validity of the study

- The IC content in the test vessel was less than 5% of the TOC introduced with the test item.
- The CO_2 evolution in the inoculum blank at the end of the test was below 40 mg/L.
- The difference of extremes of replicate values at the end of the 10-d-window and at the end of the test was less than 20%.
- The biodegradation of the reference compound reached the pass level of 60% $ThCO_2$ by day 4.

Conclusion: the test is valid.

6 Storage in archives

The depository of the study plan, the raw data and the original of the final report is the archive. Depository time: until April 2027.

A retention sample of the test item is stored in the material archive of Hydrotox GmbH, Freiburg. Depository time: until December 20th, 2016.

7 Appendix I: Data

Table 1: Ultimate biodegradation after x days [% of $ThCO_2$] with consideration of the solvent control

reactor	day	0	4	7	11	14	24	28	29
10	Test flasks	0	31,3	62,7	77,7	81,6	88,2	86,6	88,4
11		0	22,5	54,9	73,6	80,8	86,2	84,8	88,7
12		0	16,0	46,1	68,1	74,5	87,4	82,1	85,4
4	reference flasks	0	65,3	75,9	85,7	81,4	86,9	86,1	84,2
5		0	64,3	76,1	86,4	84,0	86,2	88,6	86,6
6		0	69,1	78,8	85,3	86,2	87,6	84,9	86,7

Table 2: Interpolated ultimate degradation extents [% of $ThCO_2$]

reactor	day	0	1	2	3	4	11
7	Test flask 1	0	*7,8*	*15,7*	*23,5*	31,3	77,7
8	Test flask 2	0	*5,6*	*11,3*	*16,9*	22,5	73,6
9	Test flask 3	0	*4,0*	*8,0*	*12,0*	16,0	68,1
mean degradation extent		**0,0**	***6,7***	***13,5***	***20,2***	**26,9**	**75,6**

interpolated values

Table 3: Mean CO_2-evolution of blank flasks after x days

Day		0	4	7	11	14	24	28	29
CO_2-evolution [mg/l]		0	8,6	12,8	16,9	19,0	22,4	27,2	24,8

Table 4: CO_2-evolution of solvent control after x days

Day		0	4	7	11	14	24	28	29
CO_2-evolution [mg/l]		0	16,3	21,7	27,0	28,7	32,2	39,5	35,1

Table 5: Ultimate biodegradation after x days [% of $ThCO_2$] with reference to the blanks

reactor	day	0	4	7	11	14	24	28	29
10	Test flasks	0	41,8	74,8	91,4	94,8	101,5	103,3	102,4
11		0	33,0	67,0	87,3	94,0	99,5	101,5	102,7
12		0	26,5	58,2	81,9	87,7	100,7	98,8	99,4
4	reference flasks	0	65,3	75,9	85,7	81,4	86,9	86,1	84,2
5		0	64,3	76,1	86,4	84,0	86,2	88,6	86,6
6		0	69,1	78,8	85,3	86,2	87,6	84,9	86,7

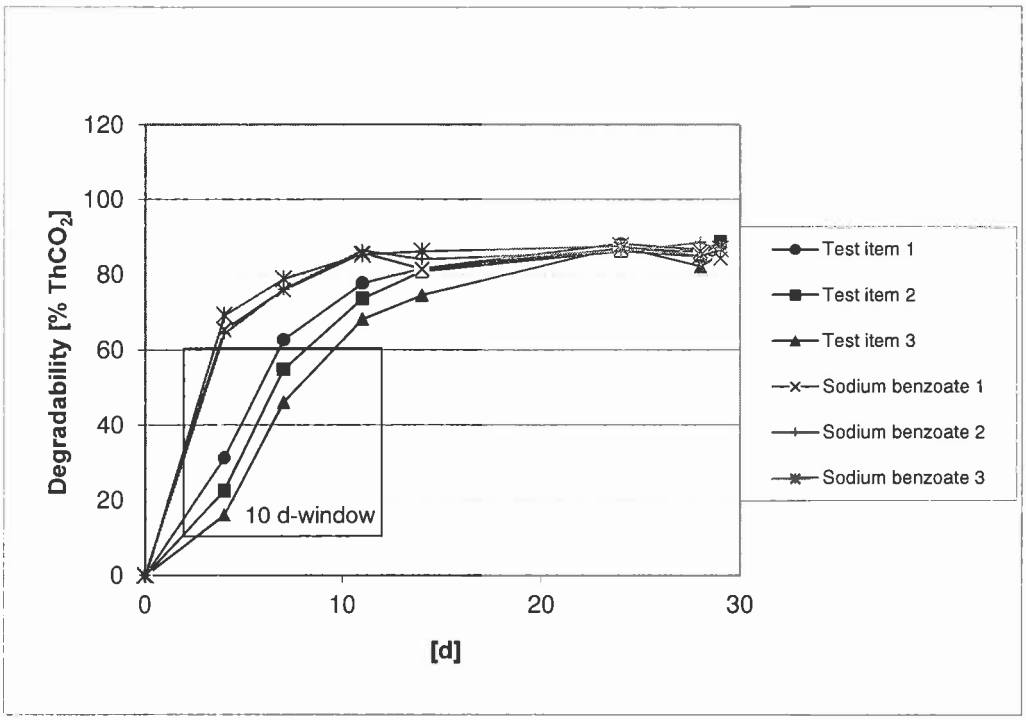

Figure 1: Biodegradability of the test item in the CO_2-evolution test (with consideration of the solvent control)

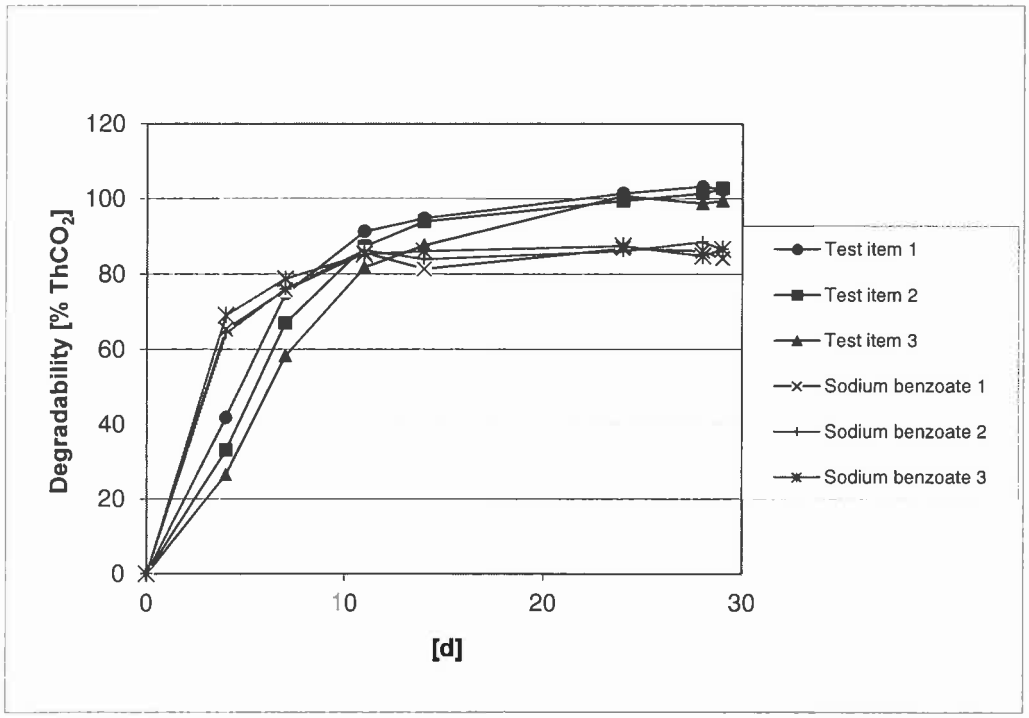

Figure 2: Biodegradability of the test item in the CO_2-evolution test (with reference to the blanks)

Hydrotox
Labor für
Ökotoxikologie und
Gewässerschutz GmbH

Data with consideration of the solvent control:

Test item: NACOL 22-98
Study No.: 738
Start: 15.03.2012 **Volume of reactor flasks** 1500 ml
IC-measurements from 19.03.12 (day 4) **Volume of absorber flasks** 200 ml
Inoculum: Activated sludge 45 mg/1500 ml

Flask No		Einwaage [mg/1500 ml]	C-Content %	$ThCO_2$ [mg]	IC NaOH [mg/l]	CO_2 absorb. absorber flask [mg]	Sum CO_2 absorb. Sampling [mg]	CO_2 absorb. Total [mg]	CO_2-CO_{2Blank} [mg]	Biodegradation [%]
1	Blank				16,1	11,8	0,00	11,8		
2					18,2	13,4	0,00	13,4		
3					18,4	13,5	0,00	13,5		
10	Test flasks	37,28	80,5	110,0	80,3	58,9	0,00	58,9	34,5	31,3
11		37,28	80,5	110,0	67,2	49,2	0,00	49,2	24,8	22,5
12		37,28	80,5	110,0	57,4	42,1	0,00	42,1	17,6	16,0
4	reference flasks	51,5	58,3	110,1	115,6	84,8	0,00	84,8	71,9	65,3
5		51,5	58,3	110,1	114,1	83,7	0,00	83,7	70,8	64,3
6	Sodium benzoate	51,5	58,3	110,1	121,3	89,0	0,00	89,0	76,1	69,1
13	Solvent control				33,3	24,4	0,00	24,4		

Test item: NACOL 22-98
Study No.: 738
Start: 15.03.2012 **Volume of reactor flasks** 1500 ml
IC-measurements from 21.05.03 (day 7) **Volume of absorber flasks** 196 ml
Inoculum: Activated sludge 45 mg/1500 ml

Flask No		Einwaage [mg/1500 ml]	C-Content %	$ThCO_2$ [mg]	IC NaOH [mg/l]	CO_2 absorb. absorber flask [mg]	Sum CO_2 absorb. Sampling [mg]	CO_2 absorb. Total [mg]	CO_2-CO_{2Blank} [mg]	Biodegradation [%]
1	Blank				25,2	18,1	0,24	18,3		
2					27,6	19,8	0,27	20,1		
3					26,7	19,2	0,27	19,4		
10	Test flasks	37,28	80,5	110,0	139,7	100,4	1,18	101,6	69,0	62,7
11		37,28	80,5	110,0	128,0	92,0	0,98	93,0	60,4	54,9
12		37,28	80,5	110,0	114,8	82,5	0,84	83,3	50,7	46,1
4	reference flasks	51,5	58,3	110,1	140,7	101,1	1,70	102,8	83,5	75,9
5		51,5	58,3	110,1	141,0	101,3	1,67	103,0	83,7	76,1
6	Sodium benzoate	51,5	58,3	110,1	145,0	104,2	1,78	106,0	86,7	78,8
13	Solvent control				44,7	32,1	0,49	32,6		

Test item: NACOL 22-98
Study No.: 738
Start: 15.03.2012 **Volume of reactor flasks** 1500 ml
IC-measurements from 26.03.12 (day 11) **Volume of absorber flasks** 192 ml
Inoculum: Activated sludge 45 mg/1500 ml

Flask No		Einwaage [mg/1500 ml]	C-Content %	$ThCO_2$ [mg]	IC NaOH [mg/l]	CO_2 absorb. absorber flask [mg]	Sum CO_2 absorb. Sampling [mg]	CO_2 absorb. Total [mg]	CO_2-CO_{2Blank} [mg]	Biodegradation [%]
1	Blank				33,3	23,4	0,61	24,0		
2					34,5	24,3	0,67	25,0		
3					37,6	26,4	0,66	27,1		
10	Test flasks	37,28	80,5	110,0	174,3	122,7	3,23	125,9	85,5	77,7
11		37,28	80,5	110,0	168,5	118,6	2,86	121,5	81,0	73,6
12		37,28	80,5	110,0	160,4	112,9	2,53	115,4	75,0	68,1
4	reference flasks	51,5	58,3	110,1	164,7	115,9	3,76	119,7	94,3	85,7
5		51,5	58,3	110,1	165,8	116,7	3,74	120,5	95,1	86,4
6	Sodium benzoate	51,5	58,3	110,1	163,9	115,4	3,91	119,3	93,9	85,3
13	Solvent control				55,9	39,3	1,14	40,5		

Test item: NACOL 22-98
Study No.: 738
Start: 15.03.2012 **Volume of reactor flasks** 1500 ml
IC-measurements from 29.03.12 (day 14) **Volume of absorber flasks** 188 ml
Inoculum: Activated sludge 45 mg/1500 ml

Flask No		Einwaage [mg/1500 ml]	C-Content %	$ThCO_2$ [mg]	IC NaOH [mg/l]	CO_2 absorb. absorber flask [mg]	Sum CO_2 absorb. Sampling [mg]	CO_2 absorb. Total [mg]	CO_2-CO_{2Blank} [mg]	Biodegradation [%]
1	Blank				38,2	26,3	1,09	27,4		
2					39,0	26,9	1,18	28,1		
3					41,9	28,9	1,21	30,1		
10	Test flasks	37,28	80,5	110,0	184,3	127,0	5,78	132,8	89,8	81.6
11		37,28	80,5	110,0	183,7	126,6	5,33	132,0	88,9	80.8
12		37,28	80,5	110,0	174,3	120,2	4,88	125,0	82,0	74.5
4	reference flasks	51,5	58,3	110,1	162,4	111,9	6,17	118,1	89,6	81.4
5		51,5	58,3	110,1	166,5	114,8	6,17	120,9	92,4	84.0
6	Sodium benzoate	51,5	58,3	110,1	169,8	117,0	6,31	123,4	94,8	86,2
13	Solvent control				59,6	41,1	1,96	43,0		

Test item: NACOL 22-98
Study No.: 738
Start: 15.03.2012 **Volume of reactor flasks** 1500 ml
IC-measurements from 05.04.12 (day 21) **Volume of absorber flasks** 184 ml
Inoculum: Activated sludge 45 mg/1500 ml

Flask No		Einwaage [mg/1500 ml]	C-Content %	$ThCO_2$ [mg]	IC NaOH [mg/l]	CO_2 absorb. absorber flask [mg]	Sum CO_2 absorb. Sampling [mg]	CO_2 absorb. Total [mg]	CO_2-CO_{2Blank} [mg]	Biodegradation [%]
1	Blank				46,0	31,1	1,65	32,7		
2					46,2	31,2	1,75	32,9		
3					49,6	33,5	1,83	35,3		
10	Test flasks	37,28	80,5	110,0	202,8	136,8	8,49	145,3	97,0	88.2
11		37,28	80,5	110,0	200,2	135,1	8,03	143,1	94,8	86.2
12		37,28	80,5	110,0	203,1	137,0	7,43	144,5	96,2	87.4
4	reference flasks	51,5	58,3	110,1	178,9	120,7	8,56	129,3	95,6	86.9
5		51,5	58,3	110,1	177,7	119,9	8,62	128,5	94,9	86.2
6	Sodium benzoate	51,5	58,3	110,1	179,7	121,2	8,80	130,0	96,4	87.6
13	Solvent control				67,4	45,4	2,84	48,3		

Test item: NACOL 22-98
Study No.: 738
Start: 15.03.2012 **Volume of reactor flasks** 1500 ml
IC-measurements from 12.04.12 (day 28) **Volume of absorber flasks** 180 ml
Inoculum: Activated sludge 45 mg/1500 ml

Flask No		Einwaage [mg/1500 ml]	C-Content %	$ThCO_2$ [mg]	IC NaOH [mg/l]	CO_2 absorb. absorber flask [mg]	Sum CO_2 absorb. Sampling [mg]	CO_2 absorb. Total [mg]	CO_2-CO_{2Blank} [mg]	Biodegradation [%]
1	Blank				56,7	37,4	2,33	39,7		
2					57,4	37,9	2,43	40,3		
3					60,5	40,0	2,55	42,5		
10	Test flasks	37,28	80,5	110,0	216,7	143,0	11,46	154,5	95,3	86.6
11		37,28	80,5	110,0	214,5	141,6	10,96	152,5	93,3	84.8
12		37,28	80,5	110,0	210,8	139,1	10,41	149,5	90,3	82.1
4	reference flasks	51,5	58,3	110,1	188,6	124,5	11,18	135,7	94,8	86.1
5		51,5	58,3	110,1	192,6	127,1	11,22	138,3	97,5	88.6
6	Sodium benzoate	51,5	58,3	110,1	186,2	122,9	11,44	134,3	93,5	84.9
13	Solvent control				83,9	55,4	3,83	59,2		

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Test item: NACOL 22-98
Study No.: 738
Start: 15.03.2012 **Volume of reactor flasks** 1500 ml
IC-measurements from 23.10.02 (day 29) after HCl addition **Volume of absorber flasks** 176 ml
Inoculum: Activated sludge 45 mg/1500 ml

Flask No		Einwaage [mg/1500 ml]	C-Content %	$ThCO_2$ [mg]	IC NaOH [mg/l]	CO_2 absorb. absorber flask [mg]	Sum CO_2 absorb Sampling [mg]	CO_2 absorb. Total [mg]	CO_2-CO_{2Blank} [mg]	Biodegradation [%]
1	Blank				51,5	33,2	3,16	36,4		
2					50,7	32,7	3,27	36,0		
3					55,6	35,9	3,44	39,3		
10	Test flasks	37,28	80,5	110,0	209,6	135,3	14,64	149,9	97,2	88.4
11		37,28	80,5	110,0	211,0	136,2	14,11	150,3	97,6	88.7
12		37,28	80,5	110,0	206,3	133,1	13,50	146,6	94,0	85.4
4	reference flasks	51,5	58,3	110,1	179,7	116,0	13,95	129,9	92,7	84,2
5		51,5	58,3	110,1	183,6	118,5	14,05	132,5	95,3	86.6
6	Sodium benzoate	51,5	58,3	110,1	183,6	118,5	14,17	132,6	95,4	86.7
13	Solvent control				73,8	47,6	5,06	52,7		

Data with reference to the blanks:

Test item: NACOL 22-98
Study No.: 738
Start: 15.03.2012 **Volume of reactor flasks** 1500 ml
IC-measurements from 19.03.12 (day 4) **Volume of absorber flasks** 200 ml
Inoculum: Activated sludge 45 mg/1500 ml

Flask No		Einwaage [mg/1500 ml]	C-Content %	$ThCO_2$ [mg]	IC NaOH [mg/l]	CO_2 absorb absorber flask [mg]	Sum CO_2 absorb Sampling [mg]	CO_2 absorb Total [mg]	CO_2-CO_{2Blank} [mg]	Biodegradation [%]
1	Blank				16,1	11,8	0,00	11,8		
2					18,2	13,4	0,00	13,4		
3					18,4	13,5	0,00	13,5		
10	Test flasks	37,28	80,5	110,0	80,3	58,9	0,00	58,9	46,0	41.8
11		37,28	80,5	110,0	67,2	49,2	0,00	49,2	36,4	33.0
12		37,28	80,5	110,0	57,4	42,1	0,00	42,1	29,2	26.5
4	reference flasks	51,5	58,3	110,1	115,6	84,8	0,00	84,8	71,9	65.3
5		51,5	58,3	110,1	114,1	83,7	0,00	83,7	70,8	64.3
6	Sodium benzoate	51,5	58,3	110,1	121,3	89,0	0,00	89,0	76,1	69.1
13	Solvent control				33,3	24,4	0,00	24,4		

Test item: NACOL 22-98
Study No.: 738
Start: 15.03.2012 **Volume of reactor flasks** 1500 ml
IC-measurements from 21.05.03 (day 7) **Volume of absorber flasks** 196 ml
Inoculum: Activated sludge 45 mg/1500 ml

Flask No		Einwaage [mg/1500 ml]	C-Content %	$ThCO_2$ [mg]	IC NaOH [mg/l]	CO_2 absorb absorber flask [mg]	Sum CO_2 absorb. Sampling [mg]	CO_2 absorb. Total [mg]	CO_2-CO_{2Blank} [mg]	Biodegradation [%]
1	Blank				25,2	18,1	0,24	18,3		
2					27,6	19,8	0,27	20,1		
3					26,7	19,2	0,27	19,4		
10	Test flasks	37,28	80,5	110,0	139,7	100,4	1,18	101,6	82,3	74,8
11		37,28	80,5	110,0	128,0	92,0	0,98	93,0	73,7	67.0
12		37,28	80,5	110,0	114,8	82,5	0,84	83,3	64,1	58.2
4	reference flasks	51,5	58,3	110,1	140,7	101,1	1,70	102,8	83,5	75.9
5		51,5	58,3	110,1	141,0	101,3	1,67	103,0	83,7	76.1
6	Sodium benzoate	51,5	58,3	110,1	145,0	104,2	1,78	106,0	86,7	78.8
13	Solvent control				44,7	32,1	0,49	32,6		

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Test item: NACOL 22-98
Study No.: 738
Start: 15.03.2012 **Volume of reactor flasks** 1500 ml
IC-measurements from 26.03.12 (day 11) **Volume of absorber flasks** 192 ml
Inoculum: Activated sludge 45 mg/1500 ml

Flask No.		Einwaage [mg/1500 ml]	C-Content %	$ThCO_2$ [mg]	IC NaOH [mg/l]	CO_2 absorb. absorber flask [mg]	Sum CO_2 absorb Sampling [mg]	CO_2 absorb. Total [mg]	CO_2-CO_{2Blank} [mg]	Biodegradation [%]
1	Blank				33,3	23,4	0,61	24,0		
2					34,5	24,3	0,67	25,0		
3					37,6	26,4	0,66	27,1		
10	Test flasks	37,28	80,5	110,0	174,3	122,7	3,23	125,9	100,6	91,4
11		37,28	80,5	110,0	168,5	118,6	2,86	121,5	96,1	87,3
12		37,28	80,5	110,0	160,4	112,9	2,53	115,4	90,1	81,9
4	reference flasks	51,5	58,3	110,1	164,7	115,9	3,76	119,7	94,3	85.7
5		51,5	58,3	110,1	165,8	116,7	3,74	120,5	95,1	86.4
6	Sodium benzoate	51,5	58,3	110,1	163,9	115,4	3,91	119,3	93,9	85.3
13	Solvent control				55,9	39,3	1,14	40,5		

Test item: NACOL 22-98
Study No.: 738
Start: 15.03.2012 **Volume of reactor flasks** 1500 ml
IC-measurements from 29.03.12 (day 14) **Volume of absorber flasks** 188 ml
Inoculum: Activated sludge 45 mg/1500 ml

Flask No		Einwaage [mg/1500 ml]	C-Content %	$ThCO_2$ [mg]	IC NaOH [mg/l]	CO_2 absorb. absorber flask [mg]	Sum CO_2 absorb Sampling [mg]	CO_2 absorb. Total [mg]	CO_2-CO_{2Blank} [mg]	Biodegradation [%]
1	Blank				38,2	26,3	1,09	27,4		
2					39,0	26,9	1,18	28,1		
3					41,9	28,9	1,21	30,1		
10	Test flasks	37,28	80,5	110,0	184,3	127,0	5,78	132,8	104,3	94.8
11		37,28	80,5	110,0	183,7	126,6	5,33	132,0	103,5	94,0
12		37,28	80,5	110,0	174,3	120,2	4,88	125,0	96,5	87.7
4	reference flasks	51,5	58,3	110,1	162,4	111,9	6,17	118,1	89,6	81.4
5		51,5	58,3	110,1	166,5	114,8	6,17	120,9	92,4	84.0
6	Sodium benzoate	51,5	58,3	110,1	169,8	117,0	6,31	123,4	94,8	86.2
13	Solvent control				59,6	41,1	1,96	43,0		

Test item: NACOL 22-98
Study No.: 738
Start: 15.03.2012 **Volume of reactor flasks** 1500 ml
IC-measurements from 05.04.12 (day 21) **Volume of absorber flasks** 184 ml
Inoculum: Activated sludge 45 mg/1500 ml

Flask No		Einwaage [mg/1500 ml]	C-Content %	$ThCO_2$ [mg]	IC NaOH [mg/l]	CO_2 absorb. absorber flask [mg]	Sum CO_2 absorb Sampling [mg]	CO_2 absorb Total [mg]	CO_2-CO_{2Blank} [mg]	Biodegradation [%]
1	Blank				46,0	31,1	1,65	32,7		
2					46,2	31,2	1,75	32,9		
3					49,6	33,5	1,83	35,3		
10	Test flasks	37,28	80,5	110,0	202,8	136,8	8,49	145,3	111,7	101.5
11		37,28	80,5	110,0	200,2	135,1	8,03	143,1	109,5	99.5
12		37,28	80,5	110,0	203,1	137,0	7,43	144,5	110,8	100,7
4	reference flasks	51,5	58,3	110,1	178,9	120,7	8,56	129,3	95,6	86.9
5		51,5	58,3	110,1	177,7	119,9	8,62	128,5	94,9	86.2
6	Sodium benzoate	51,5	58,3	110,1	179,7	121,2	8,80	130,0	96,4	87.6
13	Solvent control				67,4	45,4	2,84	48,3		

Test item: NACOL 22-98
Study No.: 738
Start: 15.03.2012 **Volume of reactor flasks** 1500 ml
IC-measurements from 12.04.12 (day 28) **Volume of absorber flasks** 180 ml
Inoculum: Activated sludge 45 mg/1500 ml

Flask No		Einwaage [mg/1500 ml]	C-Content %	$ThCO_2$ [mg]	IC NaOH [mg/l]	CO_2 absorb. absorber flask [mg]	Sum CO_2 absorb. Sampling [mg]	CO_2 absorb. Total [mg]	CO_2-CO_{2blank} [mg]	Biodegradation [%]
1	Blank				56,7	37,4	2,33	39,7		
2					57,4	37,9	2,43	40,3		
3					60,5	40,0	2,55	42,5		
10	Test flasks	37,28	80,5	110,0	216,7	143,0	11,46	154,5	113,6	103.3
11		37,28	80,5	110,0	214,5	141,6	10,96	152,5	111,7	101.5
12		37,28	80,5	110,0	210,8	139,1	10,41	149,5	108,7	98.8
4	reference flasks	51,5	58,3	110,1	188,6	124,5	11,18	135,7	94,8	86.1
5		51,5	58,3	110,1	192,6	127,1	11,22	138,3	97,5	88.6
6	Sodium benzoate	51,5	58,3	110,1	186,2	122,9	11,44	134,3	93,5	84.9
13	Solvent control				83,9	55,4	3,83	59,2		

Test item: NACOL 22-98
Study No.: 738
Start: 15.03.2012 **Volume of reactor flasks** 1500 ml
IC-measurements from 23.10.02 (day 29) after HCl addition **Volume of absorber flasks** 176 ml
Inoculum: Activated sludge 45 mg/1500 ml

Flask No		Einwaage [mg/1500 ml]	C-Content %	$ThCO_2$ [mg]	IC NaOH [mg/l]	CO_2 absorb. absorber flask [mg]	Sum CO_2 absorb. Sampling [mg]	CO_2 absorb. Total [mg]	CO_2-CO_{2blank} [mg]	Biodegradation [%]
1	Blank				51,5	33,2	3,16	36,4		
2					50,7	32,7	3,27	36,0		
3					55,6	35,9	3,44	39,3		
10	Test flasks	37,28	80,5	110,0	209,6	135,3	14,64	149,9	112,7	102.4
11		37,28	80,5	110,0	211,0	136,2	14,11	150,3	113,1	102.7
12		37,28	80,5	110,0	206,3	133,1	13,50	146,6	109,4	99.4
4	reference flasks	51,5	58,3	110,1	179,7	116,0	13,95	129,9	92,7	84,2
5		51,5	58,3	110,1	183,6	118,5	14,05	132,5	95,3	86.6
6	Sodium benzoate	51,5	58,3	110,1	183,6	118,5	14,17	132,6	95,4	86.7
13	Solvent control				73,8	47,6	5,06	52,7		

8 Appendix II: Certificate of Analysis

A N A L Y S E N Z E R T I F I K A T

Produkt:	N A C O L 22-98	(2298502)
Lotnummer:	03941/MA	
Herstellungsdatum:	9/01/2011	
Haltbarkeitsdatum:	01/2015	

Test	Einheit	Ergebnis
Gehalt an n-C 22-OH	[Gew. %]	99,1
Farbzahl	[Hazen]	6,
Esterzahl	[mg KOH/g]	0,02
Saeurezahl	[mg KOH/g]	0,00
Wasser	[%]	0,02
Jodzahl	[mg I/100mg]	0,10

Sasol Germany GmbH
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25534 Brunsbüttel
Tel: 04852/392-0 Fax: 04852/3285

Mit freundlichem Gruß
Abnahmebeauftragter
M. Sprung

Die Übersendung dieses Analysenzertifikats erfolgt lediglich zur Information und stellt keine Beschaffenheits- oder Haltbarkeitsgarantie dar. Die Übersendung erfolgt auf der Grundlage und im Rahmen der Durchführung einer ordnungsgemäßen Warenausgangskontrolle. Dieses Analysenzertifikat begründet keine Ansprüche Dritter an die es nicht gerichtet wird. Es gelten die unsere Allgemeinen Geschäftsbedingungen in der jeweils aktuellen Fassung.

This certificate of analysis is for information only and does not guarantee any particular product properties. It does not free the recipient of the obligation to carry out a product receiving inspection. This certificate of analysis does not create claims of third parties to whom it is passed on. All transactions are subject to our General Business Conditions as amended from time to time.

Sasol Germany GmbH

9 Appendix III: Statement of GLP Compliance

Baden-Württemberg
LANDESANSTALT FÜR UMWELT, MESSUNGEN UND NATURSCHUTZ BADEN-WÜRTTEMBERG

Gute Laborpraxis /Good Laboratory Practice

GLP-Bescheinigung / Statement of GLP Compliance

(gemäß / according to § 19 b Chemikaliengesetz)

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

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

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

Hydrotox GmbH
Bötzinger Str. 29
79111 Freiburg

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and adress)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according ChemVwV-GLP Nr. 5.3/OECD guidance)

3 **Prüfungen zur Bestimmung der erbgutverändernden Eigenschaften (in vitro, in vivo)**
4 **Ökotoxikologische Prüfungen zur Bestimmung der Auswirkung auf aquatische und terrestische Organismen**
5 **Prüfungen zum Verhalten im Boden, im Wasser und in der Luft; Prüfungen zur Bioakkumulation und zur Metabolisierung**

Datum der Inspektion/Date of inspection
(Tag, Monat, Jahr/day, month, year)

27.07.2009

Die/Der genannte Prüfeinrichtung/Prüfstandort befindet sich im nationalen GLP Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.
Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung/diesem Prüfstandort die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können

The above mentioned test facility/test site is included in the national GLP Compliance Programme and is inspected on a regular basis
Based on the inspection report it can be confirmed, that this test facility/test site is able to conduct the aforementioned studies in compliance with the Principles of GLP

Unterschrift, Datum/Signature, Date

Dr. Volker Giraud
Leiter der Abteilung Technischer Arbeits- und Umweltschutz
(Name und Funktion der verantwortlichen Person/Name and function of responsible person)

Karlsruhe, den 04.10.2009

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