Fraunhofer Institut
Molekularbiologie und
Angewandte Oekologie

Study Report

Daphnia, Acute Immobilization Test

Effect of 3-Phenoxybenzoic acid on the Immobilization of *Daphnia magna*

GLP-Code of Testing Facility: FEI-017/4-20

Sponsor
Irvita Plant Protection N.V.
Pos Cabai Office Park, Unit 13
P.O. Box 403
Curacao, Netherlands Antilles

Study Monitor:
Dr. R. Mendel-Kreusel
Feinchemie Schwebda GmbH
Eupener Straße 150
50933 Cologne, Germany

Testing facility
Fraunhofer-Institute for Molecular
Biology and Applied Ecology (IME)
57377 Schmallenberg
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Test facility management
Prof. Dr. A. Schäffer

Study director
Dr. C. Schäfers

August 10, 2006

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Abbreviations and definitions

$EC_{10/50}$	Effective concentration is the concentration of the test item, which results in a 10 or 50 per cent reduction in the measured parameter relative to the control.
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.
SOP	Standard operation procedure

Distribution list for study report

Sponsor:	1 original, 1 copy
GLP-archive:	1 original
Study director:	1 copy
Chemical investigator:	1 copy

Summary

A study was performed at the Fraunhofer Institute for Molecular Biology and Applied Ecology (IME) to evaluate the acute toxicity of the test item 3-Phenoxybenzoic acid to *Daphnia magna* according to the OECD guideline 202 (1) .

According to the results of a range-finding test and with regard to the solubility of the test item in water (16.9 mg/L; Meylan et al., 1996), the test item was investigated at nominal concentrations of 20.0, 10.0, 5.0, 2.5 and 1.25 mg test item/L under static conditions.

Measured concentrations of 3-Phenoybenzoic acid were between 94% and 111% of nominal concentrations. Thus, effect concentrations were based on nominal concentrations.

Neither immobilization nor any clinical signs of the daphnids were observed. Thus, the NOEC was determined to be at or higher than 20 mg /L based on the nominal initial test item concentrations. The EC_{50} was higher than 20 mg/L based on the nominal initial concentrations.

Fraunhofer Institut
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Angewandte Oekologie

Statement of GLP-compliance

Title of the study:	Daphnia , Acute Immobilization Test
Test item:	3-Phenoxybenzoic acid
Study-Code:	FEI-017/4-20

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

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

Date: August 10, 2006 for Dr. Christoph Schäfers
(Study Director)

Date: August 10, 2006 Dr. Helmut Klöppel
(Chemical Investigator)

Date: August 10, 2006 for Prof. Dr. Andreas Schäffer
(Test facility manager)

Fraunhofer Institut
Molekularbiologie und
Angewandte Oekologie

Quality assurance statement

Title of the study:	Daphnia, Acute Immobilization Test
Test item:	3-Phenoxybenzoic acid
Study-Code:	FEI-017/4-20

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

Dates of QAU inspections:	Study plan	June 30, 2006
	Daphnia, Acute Immobilization Test, test start	July 3, 2006
	Study report	August 3, 2006

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: August 10, 2006

G. Wasmus

Dr. Gerd Wasmus
(QAU-Officer)

1. Study identification

Test

Daphnia magna, Acute Immobilization Test, static conditions (OECD 202 (1))

Test item:	3-Phenoxybenzoic acid
GLP-Code:	FEI-017/4-20

Sponsor

Irvita Plant Protection N.V.
Pos Cabai Office Park, Unit 13
P.O. Box 403
Curacao, Netherlands Antilles

Study Monitor:
Dr. R. Mendel-Kreusel
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Testing facility

Fraunhofer-Institute for
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57377 Schmallenberg
Germany

Test facility management:	Prof. Dr. Andreas Schäffer
Study director:	Dr. Christoph Schäfers
Deputy:	Patrick Wellmann
Chemical investigator:	Dr. Helmut Klöppel
Deputy:	Dr. Kerstin Dertz
Technical staff, Biology:	Uwe Boshof
	Pamela Schulte
Technical staff, Chemistry:	Thomas Ludemann
Quality Assurance Unit:	Dr. Gerd Wasmus
	Dr. Ursula Wahle
	Karin Fink

Sub-contracting The study was performed without sub-contracting.

Study dates

Experimental start:	July 3, 2006
Experimental termination:	July 5, 2006

2. Objective

The objective of this study was the assessment of the acute effects (48 h EC50) of the test item to invertebrates, measured as immobilization of *Daphnia magna*.

3. Test item specification (Data supplied by Sigma Aldrich)

3.1	Product name	3-Phenoxybenzoic acid
3.2	Chemical name	3-Phenoxybenzoic acid
3.3	Empirical formula	$C_6H_5OC_6H_4CO_2H$
3.4	CAS-number	3739-38-6
3.5	Batch/Lot number	07121EY
3.6	Purity	98 %
3.7	Water solubility	16.9 mg/L (25°C) (Meylan, W.M. et al.; 1996)
3.8	Solubility in organic solvents	unknown
3.9	Vapor pressure	$5.45*10^{-6}$ mm Hg (25°C) (Neely, W.B. & Blau, G.E.; 1985)
3.10	Partition coefficient log P_{OW}	3.91, temperature not indicated (Hansch, C. et al.; 1995)
3.11	Specific density	unknown
3.12	Chemical stability	unknown
3.13	State of matter and appearance	shiny white fibres
3.14	Expiry date	4. 5. 2007
3.15	Material Safety Data Sheet	no
3.16	Origin of the test item	sponsor

3.17 Certificate of analyses was delivered by the sponsor before the start of the study. With his signature under the study plan, the sponsor confirmed his agreement with the fact that chemical identity and purity of the test item was not again examined by the contractor.

4. GLP

The test was performed in accordance with the Principles of Good Laboratory Practice (4, 5).

5. Test principle

Based on the results of a range-finding test and with regard to the water solubility of the test item, Daphnia magna was exposed to the test item under static conditions for a period of 48 h.
The number of immobile daphnids was determined after 24h and 48 h. The test was performed in accordance with the OECD guideline 202 (1).
The test concentrations were assessed by chemical analysis.

6. Materials and methods

6.1 Test organism

6.1.1 Justification for the use of the test organism
Daphnia magna was chosen by OECD-experts (1, 2) as test organism representing aquatic invertebrates.

6.1.2 Specification

Species:	Daphnia magna STRAUS, Crustacea, Cladocera.
Age:	4 - 24 hours old.
Origin:	Umweltbundesamt, Institut für Wasser-, Boden- und Lufthygiene, Berlin, Germany. Bred in the laboratory of the Fh-IME.

Breeding and holding conditions:

Adult Daphnia, at least 3 weeks old, were separated from the stock population by sieving. Batches of 30 to 50 animals were held at room temperature in ca. 1800 mL dilution water. During the week the daphnids were fed daily with an algal suspension (*Scenedesmus subspicatus*) and LiquizellR (HOBBY) according to the EEC-Guideline

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

6.2 Primary standard

The sensitivity of the test clone was checked by using $K_2Cr_2O_7$ as reference substance. In May 2006 the EC_{50} was 0.89 mg/L and meet the criteria of the OECD guideline [0.6 -2.1 mg/L] (1).

6.3 Holding and dilution water

Purified drinking water was used according to the OECD-Guideline (1). The purification included filtration with activated charcoal, passage through a lime-stone column and aeration. Carbonate hardness of the water was nearly 90 mg/L $CaCO_3$, pH was in the range of 7.5 - 8.5.

6.4 Range finding test

In order to determine the concentration range in which effects are likely to occur a non-GLP range-finding test was performed with concentrations of 0.1, 1.0 and 10 mg test item/L.
No significant immobility occurred at any of the test concentrations.
To obtain a possible dose response relationship and with respect to the solubility of the test item a range was chosen between 1.25 and 20 mg test item/L for the main test.

6.5 Test medium - preparation of the test item solution

After a non-GLP range finding test and with respect to the solubility of the test item in water the test organisms were exposed to five graded concentrations of 20, 10, 5, 2.5 and 1.25 mg test item/L for a period of 48 hours. A respective aliquot of the test item was stirred vigorously for 20 hours at room temperature. The test concentrations were obtained by dilution of the highest test concentration by a dilution factor of 2.
The test solutions were distributed into the test vessels (test medium volume 50 mL each) per test concentration.

6.6 Test procedure

For each test concentration 4 x 5 animals were exposed under static conditions. 60 mL glass beakers were used as test vessels. To each beaker 50 mL test solution and 5 daphnids, not older than 24 hours, were added. No feeding and no aeration occurred throughout the test. The controls were held under the same conditions in dilution water.

The temperature during the test was 20.1 °C. The beakers were covered with glass plates and subjected to a light/dark cycle of 16/8 h with light intensities of not more than 1000 Lux.

At test start before adding the daphnids and at test end, pH-values (WTW Microprocessor pH-Meter pH 196) and oxygen concentrations (WTW Micrprocessor Oximeter OXI 196) of pooled samples of the test solutions and control water were measured.

Immobility and abnormal behaviour were recorded after 24 h and 48 h. The animals were considered to be immobile if they are not able to swim within 15 seconds after gentle agitation of the test vessels.

6.7 Data evaluation

Numerical values in this report are frequently rounded to a smaller degree of precision (number of digits) than were used in the actual calculation. Minor differences in results obtained from calculations with such rounded values in comparison to those obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and thus of no practical concern.

Due to no immobility no effect concentrations were calculated

7. Test conditions

7.1 Water quality parameter values throughout the test

The oxygen saturation in all test concentration plots was between 97% and 99% (Table 1). The temperature was 20.1 °C, the light intensity was between 709 and 739 Lux.

At test start, the pH in the test vessels was between 7.96 and 8.04 (Table 1), after 48 h the pH was between 7.98 and 8.06.

Table 1: Oxygen saturation and pH throughout the test

Sample name	Oxygen saturation				pH	
	0 h		48 h			
	mg/L	%	mg/L	%	0 h	48 h
Control	8.5	99	8.3	98	8.09	8.36
1.25 mg/L	8.4	98	8.3	98	8.05	8.36
2.5 mg/L	8.5	99	8.3	98	8.04	8.35
5 mg/L	8.4	98	8.2	97	8.00	8.33
10 mg/L	8.5	99	8.3	98	7.93	8.23
20 mg /L	8.4	98	8.3	98	7.73	8.20

8. Results

8.1 Chemical analysis

The concentration of the test item in the test solution was analyzed at commencement and at the end of the 48 hour test period. At test start, prior to the addition of the daphnids, and at test end, every concentration plot was analyzed. A sample of 5 ml of each test solution was taken and a subsample of 1 mL was analyzed directly by UV/HPLC or diluted firstly with water 1:2 or 1: 4 and then analyzed by HPLC. Details of the method are shown in chapter 13 (Annex 1).

Table 2: Measured concentrations of the test item 3-Phenoxybenzoic acid

Nominal concentration	Measured 3-Phenoxybenzoic acid			
	0 h		48 h	
	Conc.	recovery	Conc.	recovery
controll	n.d		n.d.	
1.25 mg/L	1.19 mg/L	95.4 %	1.39 mg/L	111 %
2.5 mg/L	2.42 mg/L	96.8 %	2.69 mg/L	107 %
5.0 mg/L	4.81 mg/L	96.2 %	5.25 mg/L	105 %
10.0 mg/L	*	*	10.57 mg/L	106 %
20.0 mg/L	18.86 mg/L	94.3 %	21.49 mg/L	108 %

*no result due to measurement of the wrong sample (20.0 mg/L instead of 10.0 mg/L); as measured concentration after 48 h showed a recovery of 106% the correct preparation of the test item solution is assumed

Measured concentrations at test start and test end were between 94% and 111%, thus effect concentrations were based on nominal test item concentrations.

8.2 Immobilization of *Daphnia magna*

The effect (acute immobilization) of the test item on Daphnia magna was tested using five concentrations spaced by a factor of 2. The nominal test item concentrations were:

20.0, 10.0, 5.0, 2.5 and 1.25 mg test item/L

The test item did not cause any significant effects on mobility of the daphnids at the tested concentrations during the test period of 48 h (Table 3).

Table 3: Cumulative immobility during the test period of 48 h

Test item measured initial conc. [mg/L]	24 h beaker 1	beaker 2	beaker 3	beaker 4	48 h beaker 1	beaker 2	beaker 3	beaker 4	Sum (%)
Control	0	0	0	0	0	0	1	0	5
1.25	0	0	0	0	0	0	0	0	0
2.5	0	0	0	0	0	0	0	1	5
5.0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0

8.3 Effect concentrations

The NOEC was determined to be or higher than 20.0 mg test item /L and the LOEC >20.0 mg test item/L (Table 4). No EC_{50} could be calculated after 48 h.

Table 4: (No) effect concentrations (µg/L) of the test item after 48 h

Test duration	NOEC	LOEC	EC_{10}	EC_{50}	*C.I. of* EC_{50}
48 h	≥20.0	>20.0	>20.0	>20.0	-

C.I.: 95 % confidence limits

9. Validity of the test

The daphnia acute immobilization test fulfills the validity criteria of the OECD guideline 202 (1):

- Mortality did not exceed 10% in the controls
- The dissolved oxygen concentration at the end of the test was ≥ 3 mg/L in control and test vessels.

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 (4).

List of archived records:

- data specifying the test item
- data concerning the test organisms (origin, culture conditions)
- relevant correspondence between study director and monitor
- records of storage and storage conditions of test item
- original raw data of test ((% mortality, test conditions, i.e. pH-values, temperature, dissolved oxygen concentrations of test solutions, data of chemical analyses)
- original study plan
- 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 - 017/02	Computer use
V4 - 502/02	Daphnia test, acute tox., Repro-test, D.-holding and breeding
V4 - 503/02	Daphnia test, acute tox., Repro-test, prep. of test solutions
V4 - 505/02	Daphnia test, aquat. tox., dilution water
V4 - 507/02	Daphnia test, holding conditions
V4 - 509/02	Daphnia test, acute immobilization, procedure

G3 - 004/02	Scales, Calibration
G3 - 005/03	Checking of volumetric apparatus
G3 - 006/03	Checking of piston-operated pipettes
G3 - 007/02	Checking of thermometers
G3 - 008/03	Checking of coolers and freezers
G4 - 007/02	Illuminance Meter, Minolta, operation
G4 - 302/02	Aquatic Microcosms, Measurement of oxygen
G4 - 303/02	WTW pH-Meter pH 196, operation, calibration
G7 - 025/02	Rotavapor, use
G7 - 183/02	Washing machine Miele with Aquapurificator, handling
G3-009/02	Shaking machine
G7-189/01	HPLC gradient pump P 680LPG
G7-241/03	HPLC Diodearraydetector UVD-320S, 340S and 340U
G7-247/02	HPLC autosampler ASI 100, use

12. References

1) OECD Guideline for Testing of Chemicals, Sect. 2: Effects on Biotic Systems, No. 202 "*Daphnia sp.*, Acute Immobilization Test". Adopted April 13th 2004.

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

3) Verordnung zum Schutz vor gefährlichen Stoffen (Gefahrstoffverordnung-GefStoffV), vom 26.10.1993, (BGBl. I S. 1782), in der Fassung der Vierten Verordnung zur Änderung der Gefahrstoffverordnung vom 18. Oktober 1999 (BGBl. I S. 2059), zuletzt geändert durch die Verordnung vom 25. Mai 2000 (BGBl. I S. 747) und vom 26. Juni (BGBl- I S. 932), Carl Heymanns Verlag, Köln, 15. Auflage, 2000.

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

5) Grundsätze der Guten Laborpraxis (Principles of Good Laboratory Practice, GLP) in: Bundesgesetzblatt 2002 Teil I Nr. 40 from 27. June 2002, 2090-2130.

6) ISO 6341 (1996). Water Quality - Determination of the inhibition of the mobility of Daphnia magna Straus (Cladocera, Crustacea) - Acute toxicity test. Third edition, 1996.

13. Annex 1: Details of chemical analysis

13.1 Materials and instruments

HPLC conditions:

HPLC pump:	Dionex P580LPG
Injector	Dionex ASI-100
Detector	UVD 340S
Software	Dionex Chromeleon Vers. 6.30
Injection volume	25 µl, 100 µl
Column temperature	40°C
Column	ODS Hypersil, 150x2.0 mm, 5 µm, with Precolumn
Wavelength	244 nm
Eluent A:	0.1 % aqueous formic acid
Eluent B:	acetonitrile

Isocratic run with 60 % B and 40 % A

Retention time RT = 4.1 minutes

3-Phenoxybenzoic acid in the water samples was quantified by HPLC using external standards and comparing the integrated peak areas for the water samples with those obtained for the external calibration standards. For this purpose calibration standards of 3-Phenoxybenzoic acid in water/acetonitrile 7:3 (v/v) in the range from 10 ng/ml – 250 ng/ml and from 500 ng/ml – 10 µg/ml were prepared.

LOD: 0.003 mg/L
LOQ: 0.012 mg/L

Plots of calibration curves

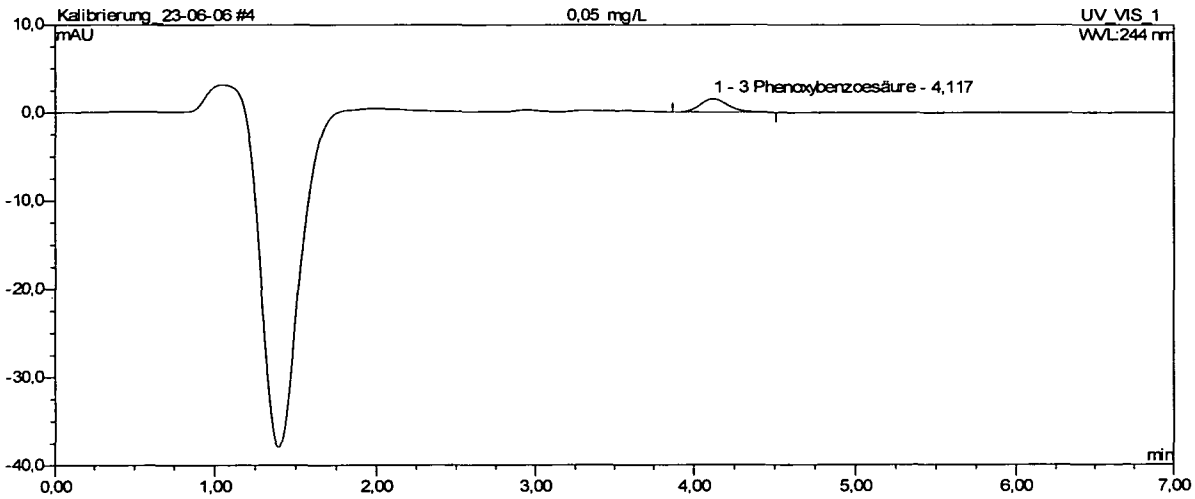

Figure 1: Calibration solution 0.05 mg/L

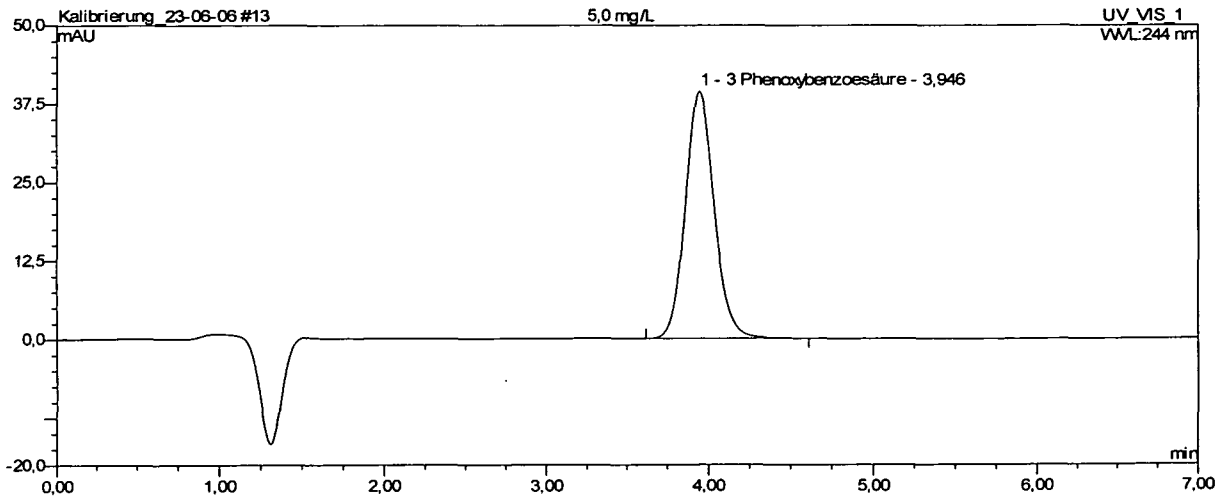

Figure 2: Calibration solution 5.0 mg/L

Representative chromatograms of the 3-Phenoxybenzoic acid analysis

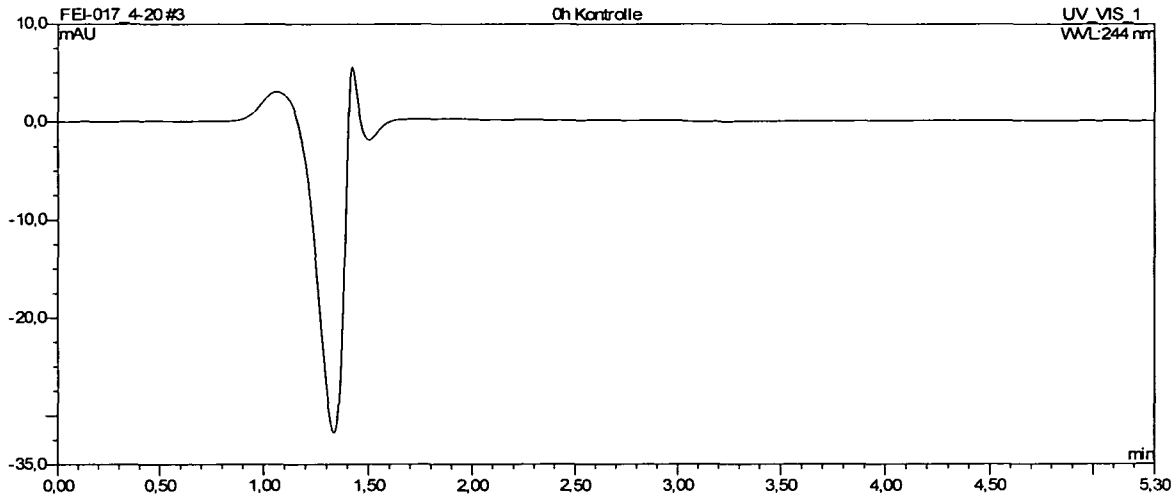

Figure 3: Control, start of test

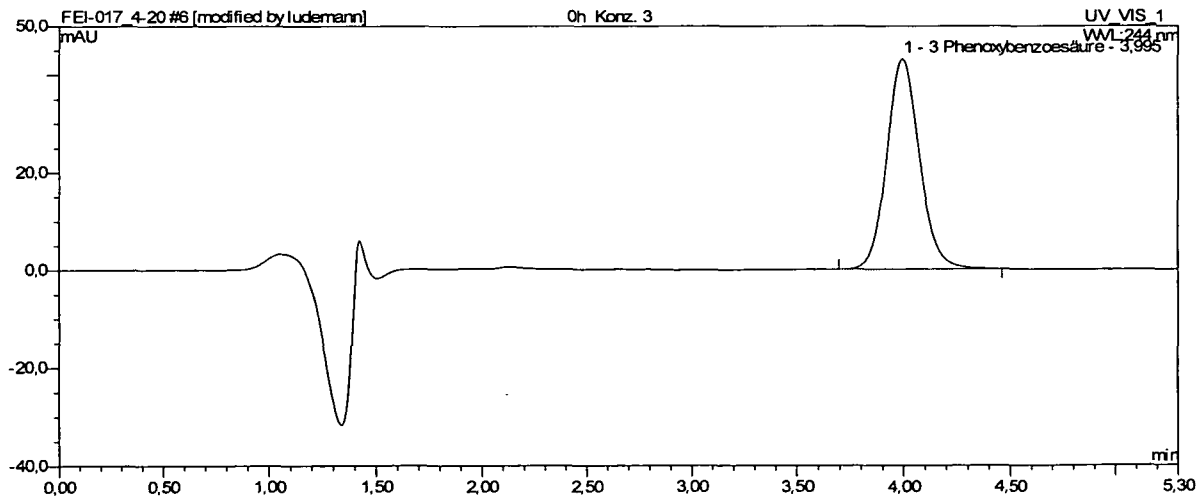

Figure 4: Test concentration 5 mg/L, start of test

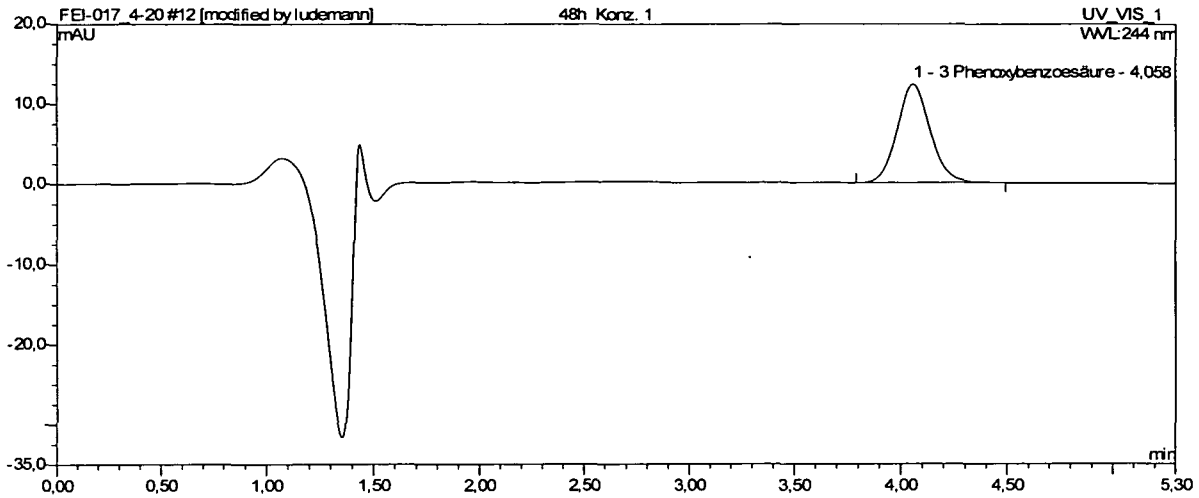

Figure 5: Test concentration 1.25 mg/L, end of test

14. Annex 2: Certificate of Analysis of the test item

Certificate Of Analysis Page 1 of 1

SIGMA-ALDRICH

Certificate of Analysis

Product Name	3-Phenoxybenzoic acid, 98%
Product Number	190276
Product Brand	Aldrich
CAS Number	3739-38-6
Molecular Formula	$C_6H_5OC_6H_4CO_2H$
Molecular Weight	214.22

TEST	SPECIFICATION	LOT 07121EY RESULTS
APPEARANCE	WHITE POWDER OR FIBERS AND/OR LUMPS	SHINY WHITE FIBERS
MELTING POINT		148-149 DEGREES CELSIUS
INFRARED SPECTRUM	CONFORMS TO STRUCTURE AND STANDARD AS ILLUSTRATED ON PAGE 237D OF EDITION I, VOLUME 2 OF "THE ALDRICH LIBRARY OF FT-IR SPECTRA".	CONFORMS TO STRUCTURE AND STANDARD AS ILLUSTRATED ON PAGE 237D OF EDITION I, VOLUME 2 OF "THE ALDRICH LIBRARY OF FT-IR SPECTRA".
TITRATION	97.5% - 102.5% (WITH NAOH)	100.3 % (WITH NAOH)
GAS LIQUID CHROMATOGRAPHY	97.5% (MINIMUM)	99.9 %

Ronnie J. Martin, Supervisor
Quality Control
Milwaukee, Wisconsin USA

15. Annex 3: 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)