1317 A/04 Al

Phthalsäure

ALGA, GROWTH INHIBITION TEST

Sponsor:

Bayer Chemicals AG
LXS-TS-HSEQ

D-51368 Leverkusen

Testing facility:

Bayer Industry Services
GmbH & Co. OHG
BIS-SUA Analytics
BIS-SUA-PUA I

D-51368 Leverkusen

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Test item : Phthalsäure
CAS number : 88-99-3

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GLP DECLARATION

This study was performed in accordance with the principles of Good Laboratory Practice (GLP) as specified in Annex I of Law on Hazardous Substances (German Chemicals Law - according to the respective valid issue).

Date / Signature

Study director

2004-10-04 E.

(Dr. Bruns / Prof. Dr. Caspers)

Study number : 1317 A/04 Al
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QUALITY ASSURANCE STATEMENT

STUDY NUMBER : 1317 A/04 Al
TEST ITEM : Phthalsäure

TITLE	:	**Alga, Growth Inhibition Test**

The Quality Assurance Unit inspected the performing of phases of this study and examined the study report. The head of the test facility was informed on the results of these inspections at the dates mentioned below.

Date of study plan inspection:	Date of report:
specification study plan September 20, 2004	----
Date of QA inspection:	Date of report:
process-based inspection SOP 00001 June 21, 2004	----
Date of report inspection:	Date of report:
2004-10-04	2004-10-04

The results of this study have been examined on the basis of the valid SOPs and we confirm that, to the best of our knowledge, the raw data have been stated correctly.

Date: 2004-10-04 **Quality Assurance Unit:**

A. Senic

(Senic / ~~Dr. Dörzbach-Lange~~ / ~~Dr. Wolf~~)

Study number : 1317 A/04 Al
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RESPONSIBLE PERSONNEL

Date / Signature

Dr. Bruns / Prof. Dr. Caspers
Study Director

2004-10-04

~~Dr. Longen / Dr. Kolloch~~ / Dr. Königer /
~~Dr. Foldhues~~
Analysis

2004-10-04

Senic / ~~Dr. Dörzbach-Lange / Dr. Wolf~~
Quality Assurance Unit

2004-10-04 A. Senic

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SUMMARY

A study was performed to assess adverse effects of Phthalsäure on the growth (= increase in cell density) and the growth rate (= rate of increase in cell density with time) of the planktonic freshwater algal species *Desmodesmus subspicatus* over several generations.

The study was conducted in accordance with EEC Methods for Determination of Ecotoxicity Annex to Directive 92/69/EEC (O.J. No. L383A, 29.12.92) Part C, Method 3 'Algal inhibition test' which is in most parts equivalent to the OECD Guideline for Testing of Chemicals No. 201 'Alga, Growth Inhibition Test'.

Exponentially growing algal cells were exposed for a period of 72 hours to a limit test concentration of nominally 100 mg/l of Phthalsäure dissolved in water. Auxiliaries used to prepare the test media were an ultrasonic bath and magnetic stirrer.
The cell densities were measured at 24 hour intervals. Inhibition of the algal population was measured as reduction in growth (index **b**) and growth rate (index **r**), relative to control cultures grown under identical conditions.The following values were determined:

Results [mg/l]:

No toxic effects against algae at 100 mg/l.

EC 0 : ≥ 100 mg/l

NOEC : ≥ 100 mg/l

All results are expressed in terms of nominal concentrations. Recovery rates ranged from 98.1 – 99.7% of nominal values at 0 hours, and from 98.3 – 100.6% of nominal values at 72 hours, respectively. For additional information compare „Comments" on page 23 of 30.

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INTRODUCTION

This report contains a description of the methods used and the results obtained during a study to investigate adverse effects of Phthalsäure on the growth and the growth rate of a population of the planktonic freshwater algal species *Desmodesmus subspicatus*. In the present test, adverse effects are expressed as those effective concentrations which reduce growth (index **b**) and growth rate (index **r**) by 50% (E_bC 50, E_rC 50) within a continuous 72 hour period of exposure. In those cases where the slope of the concentration/percentage response curve is too steep to permit calculation of the EC 50, this value is estimated graphically. In items of minor toxicity (or low aqueous solubility) a limit test is performed at 100 mg/l (or at the limit of water solubility) in order to demonstrate that the EC 50 is greater than this concentration.
In addition to the EC 50 value, NOEC/LOEC is calculated based on growth [b] and growth rate [r], respectively.

The study was commissioned by Bayer Chemicals AG, LXS-TS-HSEQ and undertaken between September 20, 2004 and September 23, 2004

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EXPERIMENTAL PROCEDURE

The method described in the Council Directive 92/69/EEC (O.J. No. L383A, 29.12.92) Part C, Method 3 'Algal inhibition test' assesses adverse effects (inhibition of growth and growth rate) of various concentrations of a test item to a unicellular planktonic freshwater algal species.

The purpose of this method is to determine those concentrations which cause a 50% adverse effect (= EC 50) or, if conducted as a limit test, to determine the adverse effects at a maximum test concentration of 100 mg/l or at the limit of water solubility, respectively. All effect data are expressed on the basis of growth **[b]** and growth rate **[r]**.

A range-finding test may precede the main test. It provides information about the range of concentrations to be used in the main test.

In the main test, the algae are exposed to the test item added to water at a range of concentrations for a period of 72 hours. Defined concentrations of the test item will lead to a certain inhibition of algal growth and growth rate at the end of the 72 hour study period. Cell densities are recorded at 24-hour intervals. The 72 hour EC 50 (based on both, growth **[b]** and growth rate **[r]**, is calculated or read from the concentration/percentage response curve. During the test a temperature range of 21 - 25°C is to be maintained in the test vessels. The pH is measured at the beginning of the test and at 72 hours.

The cell density in the control cultures should increase by a factor of at least 16 within 72 hours.

The concentration of the test item shall ideally be maintained to within 80% of the initial concentration throughout the study. The maintenance of concentrations is proved by analytical measurements.

In order to avoid an impairment of the test system, an additional replicate is used for analysis and pH measurement at the beginning of the test (control: replicate VII; test concentrations: replicate IV). Analysis and pH measurement at the end of the test are performed using replicate I.

In order to check whether or not significant amounts of the test item are incorporated into the algal biomass during the test period, a test flask at the highest test concentration **without algae** is run in parallel to the geometric series of test concentrations. The results of measured item concentrations in these test media without algae are indicated by the symbol $_{[al-]}$.

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MATERIALS AND METHODS

Test item

CAS number	:	88-99-3
CAS preferred name	:	----
Chemical name	:	Phthalsäure
Article number	:	----
Study number BIS-SUA Anlytics (O 13)	:	04/0069 LEV
Batch	:	A0163727
Empirical formula	:	$C_8H_6O_4$
Structural formula	:	
Molecular weight	:	166.13 g/mol
Purity	:	99.5 %
Major impurities	:	----
Water solubility	:	5.74 g/l (20 °C) (according to data of the sponsor)
Vapour pressure	:	----
Chemical stability in water and light	:	----
Stability of test concentration/s during exposure	:	Verified by chemical analysis (HPLC) at 0 and 72 hours.

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MATERIALS AND METHODS

Test species

Name	:	*Desmodesmus subspicatus*
Source	:	Non-axenic strain of the test species obtained from 'The Collection of Algal Cultures' of the Institute of Plant Physiology at the University of Göttingen (Germany)
Maintenance of stock cultures	:	Exponentially-growing stock cultures are maintained in the test facility under constant temperature conditions (23 +/- 2°C) at a light intensity in the range 60 – 120 µE. x m^{-2} x s^{-1} (measured in the range 400 to 700 nm using a spherical quantum flux meter). The nutrient medium (according to BRINGMANN & KÜHN (1977) is renewed once a week. Cell density measurements are made using a microcell counter.
Preparation of pre-cultures	:	Pre-cultures are set up three days before the start of a test. They are grown under identical exposure conditions as the stock cultures, except from the use of a different nutrient medium **(annex 1)**.
Test cultures	:	The algal inocula for a test are taken from an exponentially-growing pre-culture and are mixed with the nutrient medium **(annex 1)** to make up to a final cell density of about 10^4 cells per millilitre in the test medium.

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MATERIALS AND METHODS

Pretreatment of the test item

To produce the only test concentration 125.2 mg of the test item was added to 1 litre of dilution water and treated for 1 h in an ultrasonic bath and stirred for 1 hour on a magnetic stirrer.

Exposure conditions

Test vessels	:	300 ml Erlenmeyer flasks with cotton balls
Culturing apparatus	:	Light chamber in which a temperature in the range 21°C to 25°C can be maintained at +/- 2°C, and continuous uniform illumination is provided in the spectral range 400 to 700 nm.
Light intensity	:	At the average of the test solutions, a light intensity in the range 60 to 120 µE. x m^{-2} x s^{-1}, or an equivalent range of 4000 to 8000 lx, is recommended to use.
Cell density measurements	:	Cell densities are measured in a microcell counter or, alternatively, are determined by means of a microscopic counting chamber.
Experimental design	:	1 test concentration plus 1 control 3 replicates per concentration, 6 replicates per control initial cell density in the test cultures approximately 10^4 cells per millilitre additionally highest test concentration without algae
Test concentration/s (nominal)	:	100 mg/l and 100 mg/l without pH adjustment
Method of administration	:	direct weighing
Duration of exposure	:	72 hours
Criteria of effects	:	The criteria of adverse effects used in this study were the item-induced inhibition of growth **[b]** and growth rate **[r]**, respectively, of the algal population.

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MATERIALS AND METHODS

Chemical analysis

In this chapter the methods used to determine the concentrations of the test item are described. The quantification of the test item was performed by **HPLC** analysis and **UV/VIS**-detection.

Materials and Methods

Test Item

The test item as described in the biological part of this study was also used to prepare the spiked test water samples.

Analytical Standard

The test item as described in the biological part of this study was used for analytical purposes assumed as 100%.

Analytical Procedure – Storage

Routinely, the samples are analysed immediately. Only in exceptional cases, they are stored overnight deeply frozen and protected from light.

Analytical Procedure – Reagents and Solvents

KH_2PO_4 ; Na_2SO_4 ; Acetonitrile; Methanol; Milliporewater

Analytical Procedure – Standard Solutions used for Calibration

52.0 mg of the test item were dissolved in 20 ml Methanol and made up with Milliporewater to the mark in a 100 ml volumetric flask to prepare a stock solution of 520 mg/l. Defined volumes of this stock solution were diluted with Milliporewater to obtain standard solutions in the range of 0.013 to 5.20 mg/l.

These solutions were used to calibrate the HPLC-system.

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Analytical Procedure – Standard Solutions used for Verification of the Calibration and Blank Test

19.0 mg of the test item were dissolved in 20 ml Methanol and made up with Milliporewater to the mark in a 100 ml volumetric flask to prepare a verification solution with a nominal concentration of 190 mg/l. The daily verification of the calibration was performend with this Standard Solution of Calibration. This solution was diluted with Milliporewater to obtain a concentration of 1.90 mg/l.

The mean recovery rate has to be calculated.

In addition, water without the test item was analysed (analytical blank).

Analytical Procedure – Analysis of Samples

Aliquots of the samples from the biological test were directly analysed by HPLC and UV/VIS-detection (range of the injection volume: 1-100 µl, depending on the expected concentration).

HPLC Conditions

HPLC system:	e.g. Agilent Technologies 1100 system with DAD
Column:	4*4 mm + 4*125 mm stainless steel
Stationary phase:	Purospher Star RP18e, 3 µm (Merck)
Eluent A:	5 mM KH_2PO_4 + 25 mM Na_2SO_4
Eluent B:	Acetonitrile
Isocratic:	95 % A + 5 % B (v/v)
Temperature:	40 °C
Flow rate:	0.75 ml/min
Detection:	198 nm
Injection volume:	1 – 100 µl

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Analytical Procedure – Calculation

The calibration, verification, blank and sample solution were chromatographed using the conditions described in the paragraph above.

Calculation was done using an external standardization method and a calibration curve. The calibration curve was calculated according to equation (1)

(1) $y = a \cdot x^2 + b \cdot x + c$

where:

y = peak area of test item in injected sample (counts)
x = concentration of test item in injected sample (mg/l)
a = -6.5155
b = 1370.88
c = -0.17865

The correlation coefficient R^2 was

$R^2 = 1.00000$

To verify the calibration the recovery rate of the verification solution has to be calculated according to equation (2)

(2) $$\text{recovery } \% = \frac{\text{mean value of assay}}{\text{nominal concentration}} * 100$$

Results

The analytical results are presented in detail in the following tables, together with the biological effect data.

The recovery rate was 101.1 % (number of determinations : 3)

Control : at 0 and 72 hours
Test concentration/s : at 0 and 72 hours

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MATERIALS AND METHODS

Expression of biological results

- **Cell density measurements** in the test and control cultures are tabulated according to the concentration of test item and the time of measurement.
- **Growth curves** are plotted for each test concentration and control.
- **The area under the growth curve [b]** is calculated for each test culture using equation **[1]** in **annex 2**.
- **The growth rate [r]** is calculated for each test culture using equation **[3]** in **annex 2**.
- **The percentage inhibition** of both, growth [b] and growth rate [r], is calculated for each test concentration using equations **[2]** and **[4]** **(annex 2)**.
- If possible, **EC 50 values** for both, growth [b] and growth rate [r], are calculated by probit analysis. If a **limit test** is performed it has to be demonstrated that the EC 50 is greater than this concentration.
- If possible, **NOEC and LOEC** of both, growth [b] and growth rate [r], are determined by a multisample comparison (according to DUNNETT 1955, 1964) **(annex 3)**

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MATERIALS AND METHODS

Applied SOPs

SOP number	Function	Validity
2030-0000205-99 D	Balances	June 22, 1999
2030-6600401-96 D	Direct weighing	Nov. 29, 1996
2030-1110102-02 D	pH measurement	June 1, 2002
2030-7000806-01 D	Algal growth inhibition test	Sept. 1, 2001
2030-1080102-01 D	HPLC analysis	Nov. 15, 2001

Deviations from the SOPs: On demand of the sponsor the only test concentration was tested without neutralized medium. Additionally the test item concentration 100 mg/l was tested after pH adjustment.

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RESULTS

Nominal concentration [mg/l]: **Control**

Replicate	Cell density [cells/ml] (initial cell density = 10^4 cells/ml) 24 h	48 h	72 h	Growth (b) [integral of biomass]	Growth rate (r)
I	43333	313333	713333	688333	1.42
II	40000	320000	720000	695000	1.43
III	40000	333333	700000	698333	1.42
IV	43333	340000	720000	718333	1.43
V	40000	346667	703333	713333	1.42
VI	43333	350000	710000	723333	1.42
Ø	41667	333889	711111	706111	1.42

Growth (b)

Area under growth curve (Ø) : 706111
Percentage inhibition of cell growth : 0.0

Growth rate (r)

Specific growth rate (Ø) : 1.42
Percentage inhibition of cell growth rate : 0.0

Analysis	0 h	72 h
pH values	8.3	10.0
HPLC replicates [mg/l]	< 0.013 / < 0.013	< 0.013 / < 0.013
HPLC mean value [mg/l]	< 0.013	< 0.013

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RESULTS

Nominal concentration [mg/l]: **100**

Replicate	Cell density [cells/ml] (initial cell density = 10^4 cells/ml) 24 h	48 h	72 h	Growth (b) [integral of biomass]	Growth rate (r)
I	40000	326667	730000	706667	1.43
II	40000	340000	750000	730000	1.44
III	40000	316667	756667	710000	1.44
Ø	40000	327778	745556	715556	1.44

Growth (b)

Area under growth curve (Ø) : 715556
Percentage increase of cell growth : -1.3

Growth rate (r)

Specific growth rate (Ø) : 1.44
Percentage increase of cell growth rate : -1.1

Analysis	0 h	72 h
pH values	8.1	10.0
HPLC replicates [mg/l]	98.646 / 98.562	99.940 / 98.858
HPLC mean value [mg/l]	98.604	99.399

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RESULTS

Nominal concentration [mg/l]: **100 without pH adjustment**

Replicate	Cell density [cells/ml] (initial cell density = 10^4 cells/ml) 24 h	48 h	72 h	Growth (b) [integral of biomass]	Growth rate (r)
I	20000	26667	30000	36667	0.37
II	20000	26667	26667	35000	0.33
III	20000	26667	26667	35000	0.33
Ø	20000	26667	27778	35556	0.34

Growth (b)

Area under growth curve (Ø) : 35556
Percentage inhibition of cell growth : 95.0

Growth rate (r)

Specific growth rate (Ø) : 0.34
Percentage inhibition of cell growth rate : 76.0

Analysis	0 h	72 h
pH values	4.9	5.1
HPLC replicates [mg/l]	99.753 / 99.681	100.633 / 100.528
HPLC mean value [mg/l]	99.717	100.581

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RESULTS

Nominal concentration [mg/l]: **100 mg/l $_{[al-]}$ (without algal inoculum)**

Analysis	0 h	72 h
HPLC replicates [mg/l]	98.290 / 97.975	98.088 / 98.495
HPLC mean value [mg/l]	98.133	98.292

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SUMMARY OF RESULTS

Mean cell density during the test

Nominal concentration [mg/l]	Cell density [cells/ml] (initial cell density = 10^4 cells/ml)		
	24h	48h	72h
Control	41667	333889	711111
100	40000	327778	745556
100 without pH adjustment	20000	26667	27778

Mean growth (b) [integral of biomass]

Nominal concentration [mg/l]	Area under growth curve	Inhibition (+) / Increase (-) [%]
Control	706111	0.0
100	715556	-1.3
100 without pH adjustment	35556	95.0

Mean growth rate (r)

Nominal concentration [mg/l]	Growth rate [1/d]	Inhibition (+) / Increase (-) [%]
Control	1.42	0.0
100	1.44	-1.1
100 without pH adjustment	0.34	76.0

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RESULTS

Analysis of the growth and growth rate of the algal population within the 72 h exposure period give the following results:

Results [mg/l]:

No toxic effects against algae at 100 mg/l.

EC 0 : ≥100 mg/l

NOEC : ≥100 mg/l

All results are expressed in terms of nominal concentrations. Recovery rates ranged from 98.1 – 99.7% of nominal values at 0 hours, and from 98.3 – 100.6% of nominal values at 72 hours, respectively. For additional information compare „Comments" on page 23 of 30.

The growth curves for *Desmodesmus subspicatus* are given in **figure 1.**

The nutrient medium for pre-cultures and test cultures is given in **annex 1.** The equations used to calculate the algal growth and growth rate as well as their percentage inhibition are presented in **annex 2.**

COMMENTS

According to the recommendations of the respective guideline algae tests should normally be performed without pH-adjustment. As extreme pH-decreases were observed due to inherent properties of the test item addtional replicates of the only test item concentration were investigated after pH-adjustment. The results of the respective replicates clearly demonstrate that the inhibitory effects observed in this study were caused by "pH-effects". Under pH-adjusted conditions no inhibition of the algae growth has been observed at a nominal concentration of 100 mg/l. The presented results are based on the investigations using pH-adjusted test medium.

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ARCHIVATION OF DOCUMENTS AND TEST ARTICLES

Study plans, raw data and study reports are archived at the GLP archives of the test facility.

Test articles are archived at BIS-SUA Analytics.

The sponsor must ensure that information submitted about the test item (identity testing or information about physical and chemical properties) is complete and that it complies to the principles of Good Laboratory Practice.
The customer must also retain a sample of the test item used where sampling has not been performed by BIS-SUA Analytics.

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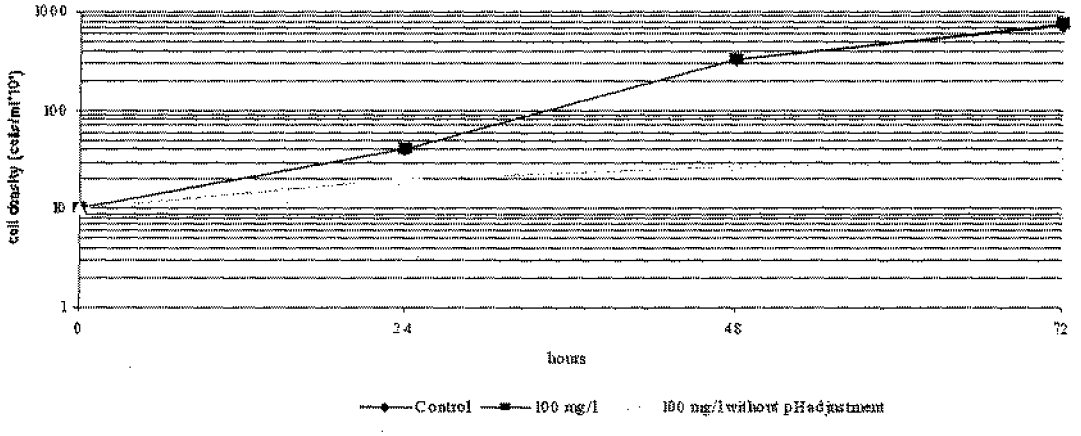
Figure 1:

Growth curves



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ANNEX 1

Nutrient medium of pre-cultures and test cultures

Nutrient	Concentration in stock solution	Final concentration in the solution of the pre-cultures and test cultures
Stock solution 1: macro-nutrients		
NH_4Cl	1.5 g/l	15 mg/l
$MgCl_2 \times 6\ H_2O$	1.2 g/l	12 mg/l
$CaCl_2 \times 2\ H_2O$	1.8 g/l	18 mg/l
$MgSO_4 \times 7\ H_2O$	1.5 g/l	15 mg/l
KH_2PO4	0.16 g/l	1.6 mg/l
Stock solution 2: Fe-EDTA		
$FeCl_3 \times 6\ H_2O$	80 mg/l	80 µg/l
$Na_2EDTA \times 2\ H_2O$	100 mg/l	100 µg/l
Stock solution 3: trace elements		
H_3BO_3	185 mg/l	185 µg/l
$MnCl_2 \times 4\ H_2O$	415 mg/l	415 µg/l
$ZnCl_2$	3 mg/l	3 µg/l
$CoCl_2 \times 6\ H_2O$	1.5 mg/l	1.5 µg/l
$CuCl_2 \times 2\ H_2O$	0.01 mg/l	0.01 µg/l
$Na_2MoO_4 \times 2\ H_2O$	7 mg/l	7 µg/l

Solid $NaHCO_3$ is added to the nutrient media to make up a final concentration of 50 mg/l in the solutions of the pre-cultures and test cultures.

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ANNEX 2/1

The area under the growth curve (A)

is calculated according to **Equation [1]**

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1) + \frac{N_2 + N_3 - 2N_0}{2} \times (t_3 - t_2)$$

where t_1 is the time of the first measurement after the beginning of the test **(24 h)**,
t_2 is the time of the second measurement after the beginning of the test **(48 h)**,
t_3 is the time of the last measurement at the end of the test **(72 h)**.
No is the nominal initial cell density,
N_1, N_2, N_3 is the measured cell density at time t_1, t_2, t_3.

From the mean values of A for each test concetration the percentage inhibition is calculated according to **Equation [2]**

$$I_{Ai} = \frac{A_c - A_i}{A_c} \times 100$$

where I_{Ai} is the percentage inhibition (area) for test concentration **i**,
A_i is the mean area for test concentration **i**,
A_c is the mean area for the control.

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ANNEX 2/2

The growth rate [r]
for each test concentration and for the control is calculated according to

Equation [3]

$$r = \frac{\ln N_3 - \ln N_0}{t_3}$$

From the mean values of **r** the percentage inhibition of growth rate for each test concentration is calculated according to

Equation [4]

$$Ir_i = \frac{r_c - r_i}{r_c} \times 100$$

where **Iri** is the percentage inhibition (growth rate) for test concentration **i**
r_i is the mean growth rate for test concentration **i**
r_c is the mean growth rate for the control.

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GLP CERTIFICATE

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

Postanschrift: 40190 Düsseldorf — Aktenzeichen: VI-5. 31.11.98.D8

GLP-Bescheinigung

Bescheinigung

Hiermit wird bestätigt, dass die Prüfeinrichtung

in D-51368 Leverkusen
(Ort, Anschrift)

der Bayer AG – Institut für Umweltanalyse und Bewertungen, Gebäude W 15
(Firma)

vom 28. - 29. November 2001
(Datum)

von der für die Überwachung zuständigen Behörde über die Einhaltung der Grundsätze der Guten Laborpraxis inspiziert worden ist.

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

Certificate

It is hereby certified that the test facility

in D-51368 Leverkusen,
(location, address)

of Bayer AG – Institut für Umweltanalyse und Bewertungen, Gebäude W 15
(company name)

on 28-29 of November 2001
(date)

was inspected by the competent authority regarding compliance with the Principles of Good Laboratory Practice.

It is hereby certified that following studies in this test facility are conducted in compliance with the Principles of Good Laboratory Practice.

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GLP CERTIFICATE

Kategorie 4

Ökotoxikologische Prüfungen zur Bestimmung der Auswirkungen auf aquatische und terrestrische Organismen

category 4

environmental toxicity studies on aquatic and terrestrial organisms

Kategorie 5

Prüfungen zum Verhalten im Boden, im Wasser und in der Luft; Prüfungen zur Bioakkumulation und zur Metabolisierung

category 5

studies on behaviour in water, soil and air; bioaccumulation

Düsseldorf, 3. Mai 2002

Im Auftrag

(Peter Kaltsch)