

8EHQ-0302-15092<sup>1</sup>

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March 11, 2002

Document Processing Center (TS-790)  
Attention: Section 8(e) Coordinator  
Office of Pollution Prevention and Toxics  
U.S. Environmental Protection Agency  
401 M Street, SW  
Washington, DC 20460

88020000078<sup>1</sup>  
8EHQ-02-15092<sup>1</sup>

Dear Coordinator:

[ ] submits this notice in accordance with Section 8(e) of the Toxic Substances Control Act.

This letter transmits preliminary results of toxicity test of Mortrace SB Concentrate ([ ] to *scenedesmus subspicatus* in an algal growth inhibition test.

In the study, a nominal concentration of 100 mg/l was continuously stirred for 24 hours. After 24 hours the stock suspension was filtered. The undiluted filtrate with the maximum concentration of dissolved or very fine dispersed test substance was used as the highest concentration of test medium. Additional dilutions of 1:100, 1:32, 1:10 and 1:3.2 were tested. All biological results were related to the mean measured test substance concentrations.

The 72-hour LOEC (lowest concentration tested with toxic effects after the exposure period of 72 hours) was determined at the mean measured concentration of 1.0 ug test substance/l. The 72-hour NOEC (highest concentration tested without toxic effects after the test period of 72 hours) was determined at 0.28 ug test substance/l, since up to and including this test concentration the mean growth rate of the algae was statistically not significantly lower than in the control. The 72-hour EC50 was calculated to be 0.90 ug test substance/l.

[ ] does consider the exact identity of this chemical to be Confidential Business Information (CBI). The confidentiality substantiation is included as Attachment II.

If you have any questions concerning this submittal, please do not hesitate to contact me at [ ]

Sincerely,

\_\_\_\_\_  
[ ]  
[ ]

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TOXICITY OF  
MORTRACE SB CONCENTRATE  
TO *SCENEDESMUS SUBSPICATUS*  
IN AN ALGAL GROWTH INHIBITION TEST

RCC PROJECT 600423

TEST REPORT

AUTHORS:

Dr. U. Memmert  
Dr. J. Schreitmüller

STUDY COMPLETION:

April 11, 1996

PERFORMING LABORATORY:

R C C Umweltchemie GmbH & Co. KG  
In den Leppsteinswiesen 19  
D-64380 Rossdorf, Germany

LABORATORY PROJECT ID:

RCC-D Project 520201

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# COPY OF GLP CERTIFICATE



HESSISCHES MINISTERIUM FÜR  
UMWELT, ENERGIE, JUGEND,  
FAMILIE UND GESUNDHEIT

## GLP-Bescheinigung

**Bescheinigung**

Hiermit wird bestätigt, daß die Prüferrichtung(en)  
RCC Umweltochemie GmbH & Co. KG  
in 64380 Roßdorf, In den Leppsteinswiesen 19  
(Ort, Anschrift)  
der RCC/CCR Holding Verwaltungs GmbH  
(Firma)  
am 05./06./07. April 1995  
(Datum)

von der für die Überwachung zuständigen Behörden über  
die Einhaltung der Grundsätze der Guten Laborpraxis  
inspiziert worden ist (sind).

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

Prüfkategorie nach § 19 d Abs. 3 Chemikaliengesetz in der Fassung vom 29. Juli 1994 (BGBl. I S. 1703),  
zuletzt geändert am 27. September 1994 (BGBl. I S. 2705) in Verbindung mit der Allgemeinen  
Verwaltungsvorschrift zum Verfahren der behördlichen Überwachung der Einhaltung der Grundsätze der Guten  
Laborpraxis vom 21. Oktober 1990 (BAnz. 204 a vom 31.10.1990):

Physikalisch-chemische Eigenschaften  
und Gehaltsbestimmung  
Ökotoxikologische Eigenschaften (aquatische Organismen)  
Rückstände

Prüfkategorie gemäß OECD Panel on Good Laboratory Practice (January 1992)

Prüfungen auf physikalisch-chemische Eigenschaften  
und Gehaltsbestimmung  
Umwelttoxikologische Prüfungen zu Auswirkungen  
auf aquatische Organismen  
Prüfungen auf Rückstände

**Certificate**

It is hereby certified that the test facility(ies)  
RCC Umweltochemie GmbH & Co. KG  
in 64380 Roßdorf, In den Leppsteinswiesen 19  
(location, address)  
of RCC/CCR Holding Verwaltungs GmbH  
(company name)  
on 05./06./07 April 1995  
(date)

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

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

Physical and chemical properties  
and determination of content  
Ecotoxicological properties (aquatic organisms)  
Residues

Physical and chemical properties  
and determination of content  
Environmental toxicity studies  
on aquatic organisms  
Residues

im Auftrag  
*Dr. Hecker*  
(Dr. Hecker) Wiesbaden, den ... August 1995



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ATTACHMENT: DETERMINATION OF THE CONCENTRATIONS OF MORTRACE SB CONCENTRATE IN TEST MEDIUM OF AN ALGAE TOXICITY TEST  
(15 pages)

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## PREFACE

### General Information

Sponsor:



Study Monitor:

C. G. Oger

Contracting Institute:

RCC Registration & Consulting Co. Ltd.  
Landstr. 33  
CH-4452 Itingen/Basel, Switzerland

RCC-CH Project No.:

600423

Testing Facility:

RCC Umweltchemie GmbH & Co. KG  
In den Leppsteinswiesen 19  
D-64380 Rossdorf, F.R.G.

RCC-D Project No.:

520201

Test Substance:

MORTRACE SB CONCENTRATE

Title of the Study:

Toxicity of MORTRACE SB CONCENTRATE  
to *Scenedesmus subspicatus*  
in an algal growth inhibition test

### Project Staff

Study Director:

Dr. U. Memmert

Management:

M. Arenz

Analytical Chemistry:

Dr. J. Schreitmüller

Quality Assurance Unit:

F. Hermann

Technical Coordinator:

A. Schmitt

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## Schedule

Date of Protocol:	January 12, 1996
(Date of German translation:	January 12, 1996)
Start of Experiment:	January 22, 1996
End of Experiment:	January 25, 1996
Date of Draft:	February 29, 1996
Date of Report:	April 11, 1996

## Quality Assurance

The study was performed in compliance with:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Anlage I ("Annex 1"), dated July 25, 1994 (BGBl. I 1994, p. 1703).

"OECD Principles of Good Laboratory Practice", Paris, 1981

## Guidelines

This study followed the procedures indicated by the following internationally accepted guidelines and recommendations:

Commission Directive 92/69/EEC, Annex Part C, C.3: "Algal Inhibition Test", Official Journal of the European Communities No. L 383 A, dated December 29, 1992.

OECD Guideline for Testing of Chemicals, No. 201: "Alga, Growth Inhibition Test", adopted June 7, 1984.

## Archiving

By order of RCC Umweltchemie GmbH & Co. KG the CCR Cytotest Cell Research GmbH & Co. KG, In den Leppsteinswiesen 19, D-64380 Rossdorf, F.R.G. will archive all raw data, the protocol and a copy of the report for thirty years, a sample of the test substance for at least two years following the date on which the report is audited by the Quality Assurance Unit.

No raw data or material relating to the study will be discarded without the sponsor's prior consent.

## Deviations to the Protocol

There were no deviations to the protocol.

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### Project Staff Signatures

Study Director

Dr. U. Memmert

*U. Memmert*

Date: *April 11, 1996*

Analytical Chemistry

*for* Dr. J. Schreitmüller

*J. Schreitmüller*

Date: *April 11, 1996*

Management

M. Arenz

*M. Arenz*

Date: *April 11, 1996*

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# STATEMENT OF COMPLIANCE

Project Number: 520201

Test Substance: MORTRACE SB CONCENTRATE

Study Director: Dr. U. Memmert

Title of the Study: Toxicity of MORTRACE SB CONCENTRATE  
to *Scenedesmus subspicatus*  
in an algal growth inhibition test

This study performed in the testing facility of RCC was conducted in compliance with Good Laboratory Practice Regulations according to:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Anlage 1 ("Annex 1"), dated July 25, 1994 (BGBl. I 1994, p. 1703).

"OECD Principles of Good Laboratory Practice", Paris, 1981

There were no circumstances that may have affected the integrity or quality of the study.

Study Director R C C  
Dr. U. Memmert

*U. Memmert*

Date: *April 12, 1996*

Responsible Scientist R C C  
for Analytical Chemistry Dr. J. Schreitmüller

*J. V. Schreitmüller*

Date: *April 12, 1996*

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## STATEMENT OF QUALITY ASSURANCE UNIT

C C R Cytotest Cell Research GmbH & Co. KG,  
 In den Leppsteinswiesen 19,  
 D-64380 Rossdorf, F.R.G.

Project Number: 520201  
 Test Substance: MORTRACE SB CONCENTRATE  
 Study Director: Dr. U. Memmert  
 Title of the Study: Toxicity of MORTRACE SB CONCENTRATE  
 to *Scenedesmus subspicatus*  
 in an algal growth inhibition test

This report was audited by the Quality Assurance Unit and the conduct of this study was inspected on the following dates:

Phases and Dates of QAU Inspections/Audits		Dates of Reports to the Study Director and to Management
Protocol Audit:	January 15, 1996	January 15, 1996
Draft Inspection		
- analytical part:	March 07, 1996	March 07, 1996
- biological part:	March 27, 1996	March 27, 1996

Head of  
 Quality Assurance Unit

F. Hermann

*i.V. U. Werner*

Date: *April 11, 1996*

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## SUMMARY

The influence of the test substance MORTRACE SB CONCENTRATE on the growth of the green algal species *Scenedesmus subspicatus* CHODAT was investigated in a 72-hour static test according to the Commission Directive 92/69/EEC, Annex Part C.3, dated December 29, 1992, and the OECD Guideline No. 201, adopted June 7, 1984. The test was performed in compliance with Good Laboratory Practice Regulations.

Due to the very low water solubility of the test substance a supersaturated stock suspension of the test substance with a nominal concentration of 100 mg/l was continuously stirred at room temperature in the dark over 24 hours. After 24 hours the stock suspension was filtered. The undiluted filtrate with the maximum concentration of dissolved respectively very fine dispersed test substance was used as the highest concentrated test medium. Additionally, the dilutions 1:100, 1:32, 1:10 and 1:3.2 were tested.

The analytically determined test substance concentrations in the freshly prepared test medium of the highest test concentration (the undiluted filtrate) amounted to 1.9 µg/l. At the lower concentrated test media lower test substance concentrations were measured according to the dilution steps down to 0.025 µg/l. However, the test substance was not stable under the test conditions during the test period. Therefore, all biological results are related to the mean measured test substance concentrations.

The 72-hour LOEC (lowest concentration tested with toxic effects after the exposure period of 72 hours) was determined at the mean measured concentration of 1.0 µg test substance/l. The 72-hour NOEC (highest concentration tested without toxic effects after the test period of 72 hours) was determined at 0.28 µg test substance/l, since up to and including this test concentration the mean growth rate of the algae was statistically not significantly lower than in the control.

The EC-values were calculated for both parameters the algal biomass  $b$  and the growth rate  $\mu$  after 72 hours test duration:

Parameter (0 - 72 h)	Biomass $b$ (µg/l)	Growth rate $\mu$ (µg/l)
EC 50	0.90	6.7 (extrapolated)
95% conf. limits	0.64 - 1.55	3.1 - 41.0
EC 10	0.25	0.88
95% conf. limits	0.13 - 0.36	0.63 - 1.48

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## INTRODUCTION

The purpose of this test was to determine toxic effects of the test substance on the growth of an unicellular green algae (*Scenedesmus subspicatus*) over several generations. Exponentially growing cultures of this green algal species were exposed to various concentrations of the test substance under defined conditions. The inhibition of growth in relation to control cultures was determined over a test period of 72 hours up to the highest test concentration of test substance, which could be dissolved respectively very fine dispersed in test water.

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## MATERIALS AND METHODS

### Test Substance

The test substance and the following information concerning the test substance were provided by the sponsor:

Name:	MORTRACE SB CONCENTRATE
Batch No.:	MR 26592 SBC
Expiration date:	June 01, 1996
Purity:	concentrate (not specified)
Solubility in water:	insoluble
Stability:	pure: see expiration date
	in water: not stable for 2 hours
	(UV-light can cause decomposition
	of the test substance in diluted solutions)
Aggregate state	
at room temperature:	thick liquid / tar
	(determined at RCC: solid)
Colour:	dark red brown
Vapour pressure:	not indicated by the sponsor
Storage:	at room temperature, in the dark

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## Test Organism

The test organism used for the study was *Scenedesmus subspicatus* CHODAT, Strain No. 86.81 SAG, supplied by the "Sammlung von Algenkulturen, Pflanzenphysiologisches Institut der Universität Göttingen", D-37073 Göttingen. The algae were grown in the laboratories of RCC under standardized conditions according to the test guidelines.

## Study Design

### Experimental conditions

The algae were cultivated and tested in synthetic test water, prepared according to the mentioned test guidelines: in deionized water with a conductivity lower than  $0.1 \mu\text{S cm}^{-1}$  (Milli-Q-water) analytical grade salts were added to following final nominal concentrations:

#### Macro-nutrients:

$\text{NaHCO}_3$		50.0 mg/l
$\text{CaCl}_2$	$\times 2 \text{ H}_2\text{O}$	18.0 mg/l
$\text{NH}_4\text{Cl}$		15.0 mg/l
$\text{MgSO}_4$	$\times 7 \text{ H}_2\text{O}$	15.0 mg/l
$\text{MgCl}_2$	$\times 6 \text{ H}_2\text{O}$	12.0 mg/l
$\text{KH}_2\text{PO}_4$		1.6 mg/l

#### Trace elements:

$\text{Na}_2\text{EDTA}$	$\times 2 \text{ H}_2\text{O}$	100.0 $\mu\text{g/l}$
$\text{FeCl}_3$	$\times 6 \text{ H}_2\text{O}$	80.0 $\mu\text{g/l}$
$\text{MnCl}_2$	$\times 4 \text{ H}_2\text{O}$	415.0 $\mu\text{g/l}$
$\text{H}_3\text{BO}_3$		185.0 $\mu\text{g/l}$
$\text{Na}_2\text{MoO}_4$	$\times 2 \text{ H}_2\text{O}$	7.0 $\mu\text{g/l}$
$\text{ZnCl}_2$		3.0 $\mu\text{g/l}$
$\text{CoCl}_2$	$\times 6 \text{ H}_2\text{O}$	1.5 $\mu\text{g/l}$
$\text{CuCl}_2$	$\times 2 \text{ H}_2\text{O}$	0.01 $\mu\text{g/l}$

Calculated water hardness of the test water: 0.24 mmol/l (= 24 mg/l) as  $\text{CaCO}_3$

The test was started (0 hours) by inoculation of a biomass of 10.000 algal cells per ml test medium. These cells were taken from an exponentially growing pre-culture, which was set up about 72 hours prior to the test at the same conditions as in the test.

The test design included three replicates per test concentration and six replicates in the control. Per replicate 50 ml algal suspension were continuously stirred by magnetic stirrers in 50 ml Erlenmeyer flasks. The flasks were covered with glass dishes. They were incubated in a temperature controlled water bath (temperatures see Table 7), and continuously illuminated at the measured light intensity of about 8500 Lux (mean value), range: 8000 to 8800 Lux (minimum and maximum value of measurements at 3 places distributed over the experimental area at the level of the test media). This illumination was achieved by fluorescent tubes (universal white L 25, 36 W), installed above the water bath in a distance of about 35 cm from the test flasks.

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## Dosage and concentrations

Just before use of the test substance it was homogenised by warming up in the test substance container to 60°C for 2 hours. Then the test substance was homogenised by intense mixing.

Due to the very low water solubility of the test substance the following dosage of the test substance was chosen:

A supersaturated stock suspension of the test substance with a nominal concentration of 100 mg/l was prepared by weighing 150 mg of the test substance into 1500 ml test water. No auxiliary solvent or emulsifier was used. This supersaturated stock suspension was stirred by a magnetic stirrer at room temperature in the dark over 72 hours to dissolve respectively disperse a maximum concentration of the test substance in the supersaturated stock suspension. The stirring period of 72 hours was chosen according to the results of a pre-test (without GLP), where the concentration of the test substance had slightly increased from 24 hours of stirring to 72 hours of stirring.

The supersaturated stock suspension of the test substance was filtered through a folded filter paper (Schleicher & Schuell, Type 1573  $\frac{1}{2}$ ) after the 72 hours stirring period just before the start of the test. The first 100 ml filtrate were discarded to avoid a loss of the test substance due to adsorption onto the filter. The undiluted filtrate of the supersaturated stock suspension was used as the highest concentrated test medium. Additionally, adequate volumes of the filtrate were diluted with test water for the preparation of the test media with lower test substance concentrations. No additional dilution step was inserted. In this way the following test concentrations were prepared: dilutions 1:3.2, 1:10, 1:32 and 1:100 of the filtrate (1 part of the filtrate added to 2.2, respectively 9, 31, and 99 parts of test water). The enlarged spacing factor of 3.2 between the test dilutions was chosen, since according to the results of the range-finding test a large concentration range had to be tested. The real concentration of the test substance in the test dilutions were analytically determined (see "Analyses of the test substance concentrations").

Additionally, a control was tested in parallel (test water without addition of the test substance).

The test concentrations were based on the results of a range-finding test. However, concentrations far above the water solubility limit of the test substance have not been tested according to the Commission Directive 92/69/EEC. The range-finding test was not performed in compliance with GLP-Regulations, but the raw data of the range-finding test will be archived under the RCC Project number of the present study.

Then, at the start of the test the algae were inoculated by adding an adequate volume from the pre-culture to the test media.

## Evaluations

### pH

The pH-values of the test media were measured in samples from all test concentrations and the control at the start and at the end of the test.

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## Temperature in the test media

During the test the temperature was measured daily in an Erlenmeyer flask, filled with water and incubated at the same conditions as the test flasks.

## Counting and examination of algal cells

Small volumes of the test media (1.0, respectively 0.1 ml) were taken out of all flasks after 24, 48 and 72 hours of exposure and not replaced. The algae cell densities in the samples were determined by counting with an electronic particle counter (AL CELLCOUNTER, Model 871, AL-Systeme, D-76149 Karlsruhe), three measurements per sample.

In addition, a sample was taken from the control and from the highest test concentration (the undiluted filtrate) after the test period of 72 hours. The shape of these treated algal cells was microscopically examined and compared with the cells in the control.

## Determination of the algal growth inhibition

Inhibition of algae growth was determined from:

- a) the area under the growth curves A (= biomass)
- b) the specific growth rates  $\mu$  for exponentially growing cultures using the following equations:

a) area under the growth curve (A):

$$A = \frac{(N_1 - N_0)}{2} \cdot t_1 + \frac{(N_1 + N_2) - 2N_0}{2} \cdot (t_2 - t_1) + \frac{(N_{n-1} + N_n) - 2N_0}{2} \cdot (t_n - t_{n-1})$$

- where:  $N_0$  = mean number of cells/ml at  $t_0$  (start of the test)  
 $N_1$  = mean number of cells/ml after  $t_1$  (24 hours)  
 $N_2$  = mean number of cells/ml after  $t_2$  (48 hours)  
 $N_n$  = mean number of cells/ml after  $t_n$   
 $t_1$  = time of first measurement after beginning of test  
 $t_2$  = time of second measurement after beginning of test  
 $t_n$  = time of  $n^{\text{th}}$  measurement after beginning of test

Percentage inhibition of area A ( $I_A$ ):

$$I_A = \frac{A_c - A_i}{A_c} \cdot 100$$

- where:  $A_c$  = mean area of the control  
 $A_i$  = mean area of test concentration  $i$

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b) growth rate ( $\mu$ ):

$$\mu = \frac{\ln N_n - \ln N_0}{t_n}$$

Percentage inhibition of growth rate  $\mu$  ( $I_\mu$ ):

$$I_\mu = \frac{\mu_c - \mu_i}{\mu_c} \cdot 100$$

where:  $\mu_c$  = mean growth rate of the control  
 $\mu_i$  = mean growth rate of test concentration i

The test concentrations corresponding to 10 and 50 % inhibition of the algal biomass b ( $E_rC$  10, -50), respectively of the growth rate  $\mu$  ( $E_rC$  10) and the 95 % confidence limits were calculated by the PROBIT ANALYSIS (Ref. 1, 2). The biological results were related to the mean measured test substance concentrations (see Results and Discussion).

For the determination of the LOEC and NOEC, the calculated mean biomass of algae (= areas under the growth curves) and mean growth rates  $\mu$  at the test concentrations were tested on significant differences to the control values by DUNNETT-TESTS (Ref. 3, 4).

### Analyses of the test substance concentrations

For the analytical measurements of the test substance concentrations, duplicate samples were taken at the start of the test from the freshly prepared test media (without algae) of all test concentrations, the undiluted filtrate and all test dilutions and from the control.

For the determination of the stability of the test substance under the test conditions, sufficient volumes of all freshly prepared test media and the control were incubated under the same conditions as in the test itself (but without algae) and sampled in duplicate at the end of the test (after 72 hours test period).

The concentrations of the test substance MORTRACE SB CONCENTRATE were analysed in all duplicate test media samples from both sampling dates (0 and 72 hours) immediately after sampling. From the control samples only one of the duplicate samples were analysed from each of both sampling dates (0 and 72 hours). The analytical procedure is described in the test report RCC Project 520202 (attached).

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## RESULTS AND DISCUSSION

### Analytical results (see also the Attachment)

The analytically determined test substance concentrations in the samples from the freshly prepared test medium of the highest test concentration (the undiluted filtrate of the supersaturated stock suspension) amounted to 1.9 µg/l. At the lower concentrated test media lower test substance concentrations were measured according to the dilution steps down to 0.025 µg/l at the dilution 1:100 (see analytical results, Attachment Table 1).

In all test media, incubated under the test conditions (but without algae), the concentrations of MORTRACE SB CONCENTRATE strongly decreased during the test period to values below the determination limit of the analytical method of 0.02 µg/l. Thus, the test substance was obviously not stable under the test conditions, possibly due to the high light intensity, which is necessary in this algal growth inhibition test. Therefore, all biological results are related to the mean measured test substance concentrations (calculated as the average over all measurements during the test period):

Dilution	mean measured test substance concentrations in the test media samples
1:100	0.012 µg/l
1:32	0.034 µg/l
1:10	0.11 µg/l
1:3.2	0.28 µg/l
undiluted filtrate	1.0 µg/l

### Biological results

The influence of the test substance MORTRACE SB CONCENTRATE on the growth of *Scenedesmus subspicatus* is shown in Tables 1 - 5 and Figure 1. The test substance had a statistically significantly inhibitory effect on the biomass of *Scenedesmus subspicatus* after the exposure period of 72 hours first at the mean measured concentration of 0.28 µg test substance/l (results of a Dunnett-test, one-sided,  $\alpha = 0.05$ , see Table 4). However, the mean growth rate of the algae after 72 hours was statistically significantly reduced first at the next higher test concentration of in the mean 1.0 µg/l (Table 5). Thus, this test concentration was determined as the 72-hour LOEC (lowest concentration tested with toxic effects after the exposure period of 72 hours).

The 72-hour NOEC (highest concentration tested without toxic effects after the test period of 72 hours) was determined at the concentration of 0.28 µg test substance/l, since up to and including this test concentration the mean growth rate of the algae after the 72 hours exposure period was statistically not significantly lower than in the control (Table 5).

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The EC-values were calculated for both parameters the algal biomass  $b$  and the growth rate  $\mu$  after 72 hours test duration:

Parameter (0 - 72 h)	Biomass $b$ ( $\mu\text{g/l}$ )	Growth rate $\mu$ ( $\mu\text{g/l}$ )
EC 50	0.90	6.7 (extrapolated)
95% conf. limits	0.64 - 1.55	3.1 - 41.0
EC 10	0.25	0.88
95% conf. limits	0.13 - 0.36	0.63 - 1.48

At the microscopic examination of the shape of the algal cells after 72 hours test period no difference was observed between the algae growing in the undiluted filtrate and the algal cells in the control. Thus, the shape of the algal cells growing up to this test concentration of  $1.0 \mu\text{g/l}$  was obviously not affected.

In the control the cell density has increased from nominal  $N = 1 \times 10^4$  cells/ml at the start of the test (0 hours) to  $N = 123.00 \times 10^4$  cells/ml (mean value) after 72 hours by a factor of 123 (Table 1). Thus, the algal growth in the control was sufficiently high under the test conditions.

At the start of the test, the pH-values in the test media ranged from pH 7.9 to 8.1, at the end of the test pH-values were measured between pH 10.0 and 10.3 (Table 6). This increase of the pH during the test was obviously caused by the  $\text{CO}_2$ -consumption of the algae due to their rapid growth respectively their high densities (although the test media have been intensively stirred).

No remarkable observations were made concerning the behaviour of the test substance in the filtrate and in all dilutions of the filtrate.

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## TABLES

Table 1: Algal cell densities during the test period of 72 hours

Dilution	Flask No.	Density of Algal Cells (cell numberx10000/ml)									
		24 h			48 h			72 h			
control	1	8.3	8.3	8.2	43.8	44.0	44.2	113.0	110.0	117.0	
	2	8.5	8.3	8.5	45.0	44.9	44.5	118.0	117.0	126.0	
	3	9.6	9.5	10.1	52.1	52.0	52.4	126.0	129.0	130.0	
	4	8.5	8.9	8.7	41.2	41.5	41.6	99.0	93.0	97.0	
	5	9.7	9.6	9.6	46.2	45.9	46.4	143.0	138.0	140.0	
	6	9.4	9.6	9.7	48.5	48.5	48.4	138.0	140.0	140.0	
	m		9.06			46.17			123.00		
s		0.66			3.75			16.78			
1:100 (0.012 µg/l)#	7	8.4	8.6	8.3	48.2	48.7	47.9	128.0	126.0	129.0	
	8	8.3	8.9	8.9	42.2	41.7	42.0	119.0	126.0	123.0	
	9	10.4	10.2	10.1	50.6	50.4	51.0	119.0	117.0	119.0	
	m		9.12			46.97			122.89		
	s		0.97			4.49			4.67		
1:32 (0.034 µg/l)#	10	10.0	10.0	9.8	47.9	48.1	47.9	133.0	135.0	133.0	
	11	9.5	9.8	9.6	56.6	54.9	55.2	133.0	129.0	132.0	
	12	10.4	10.0	10.3	52.7	52.6	53.0	140.0	143.0	139.0	
	m		9.93			52.10			135.22		
	s		0.30			3.84			4.86		
1:10 (0.11 µg/l)#	13	9.5	9.4	9.7	56.1	55.9	55.7	141.0	140.0	138.0	
	14	10.6	10.4	10.7	54.8	54.6	55.0	140.0	143.0	140.0	
	15	10.6	10.7	10.0	50.6	50.0	49.2	127.0	120.0	123.0	
	m		10.18			53.54			134.67		
	s		0.56			3.18			9.84		
1:3.2 (0.28 µg/l)#	16	9.0	9.2	9.0	33.7	33.4	33.9	92.0	92.0	93.0	
	17	8.3	8.5	8.5	40.7	40.2	39.9	105.0	102.0	103.0	
	18	8.1	8.2	8.6	37.7	38.0	38.2	121.0	120.0	119.0	
	m		8.60			37.30			105.22		
	s		0.41			3.35			13.93		
undiluted filtrate (1.0 µg/l)#	19	3.7	3.6	3.5	16.6	16.4	16.7	70.0	72.0	68.0	
	20	3.9	3.9	3.6	17.9	17.7	17.2	72.0	70.0	74.0	
	21	3.8	3.7	3.9	16.5	16.9	16.4	76.0	77.0	73.0	
	m		3.73			16.92			72.44		
	s		0.12			0.59			2.69		

m: arithmetic mean; s: standard deviation

Algal counts are divided by 10000; at the start, 10000 algal cells/ml were incubated.

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**Table 2:** Influence of the test substance on algal growth: areas under growth curves (A) and percentage inhibition of A

Dilution	Areas (A) under the Growth Curves and % Inhibition					
	24 h		48 h		72 h	
	A	%	A	%	A	%
control	97	0.0	735	0.0	2741	0.0
1:100 (0.012 µg/l)#	97	-0.8	747	-1.5	2761	-0.7
1:32 (0.034 µg/l)#	107	-10.9	828	-12.5	3051	-11.3
1:10 (0.11 µg/l)#	110	-13.9	851	-15.7	3085	-12.5
1:3.2 (0.28 µg/l)#	91	5.7	618	16.0	2304	15.9
undiluted (1.0 µg/l)#	33	66.1	257	65.1	1305	52.4

-% inhibition: growth promotion, increase in growth rate relative to that of control

# mean measured test substance concentration

**Table 3:** Influence of the test substance on algal growth: growth rates  $\mu$  and percentage inhibition of  $\mu$ 

Dilution	Growth Rate ( $\mu$ ) and % Inhibition					
	24 h		48 h		72 h	
	$\mu$	%	$\mu$	%	$\mu$	%
control	2.20	0.0	1.91	0.0	1.60	0.0
1:100 (0.012 µg/l)#	2.21	-0.3	1.92	-0.4	1.60	-0.1
1:32 (0.034 µg/l)#	2.30	-4.3	1.98	-3.2	1.64	-2.1
1:10 (0.11 µg/l)#	2.32	-5.4	1.99	-3.9	1.63	-2.0
1:3.2 (0.28 µg/l)#	2.15	2.3	1.81	5.6	1.55	3.2
undiluted (1.0 µg/l)#	1.32	40.2	1.41	26.1	1.43	10.9

-% inhibition: growth promotion, increase in growth rate relative to that of control

# mean measured test substance concentration

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**Table 4:** Results of a Dunnett-Test ( $\alpha = 0.05$ , one-sided) with the biomass  $b$  (= areas under the growth curves)

Dilution	Calculated t-Value		
	24 h	48 h	72 h
1:100 (0.012 $\mu\text{g/l}$ )#	-0.16	-0.33	-0.14
1:32 (0.034 $\mu\text{g/l}$ )#	-2.10	-2.70	-2.16
1:10 (0.11 $\mu\text{g/l}$ )#	-2.68	-3.38	-2.40
1:3.2 (0.28 $\mu\text{g/l}$ )#	1.09	3.43 *	3.05 *
undiluted (1.0 $\mu\text{g/l}$ )#	12.72 *	14.01 *	10.01 *
Tabled t-Value	2.60	2.60	2.60
Degrees of Freedom	15	15	15
total variance	50.417	2335.6	41183

Results which were significantly different from the control are marked with an asterisk.  
# mean measured test substance concentration

**Table 5:** Results of a Dunnett-Test ( $\alpha = 0.05$ , one-sided) with the growth rates  $\mu$ 

Dilution	Calculated t-Value		
	24 h	48 h	72 h
1:100 (0.012 $\mu\text{g/l}$ )#	-0.13	-0.31	-0.10
1:32 (0.034 $\mu\text{g/l}$ )#	-2.06	-2.24	-1.43
1:10 (0.11 $\mu\text{g/l}$ )#	-2.57	-2.75	-1.35
1:3.2 (0.28 $\mu\text{g/l}$ )#	1.09	3.92 *	2.14
undiluted (1.0 $\mu\text{g/l}$ )#	19.28 *	18.42 *	7.26 *
Tabled t-Value	2.60	2.60	2.60
Degrees of Freedom	15	15	15
total variance	4.2074	1.4784	1.1481

Results which were significantly different from the control are marked with an asterisk.  
# mean measured test substance concentration

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**Table 6:** pH-values in the test media at the start and at the end of the test

Dilution	pH-values	
	Start	End
control	8.0	10.3
1:100	8.0	10.3
1:32	8.0	10.3
1:10	7.9	10.3
1:3.2	7.9	10.3
undiluted	8.1	10.0

**Table 7:** Temperature in the test media during the test period

	temperature [°C]
Day 0 (start)	23.4
Day 1	23.2
Day 2	23.4
Day 3 (End)	23.4

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FIGURE

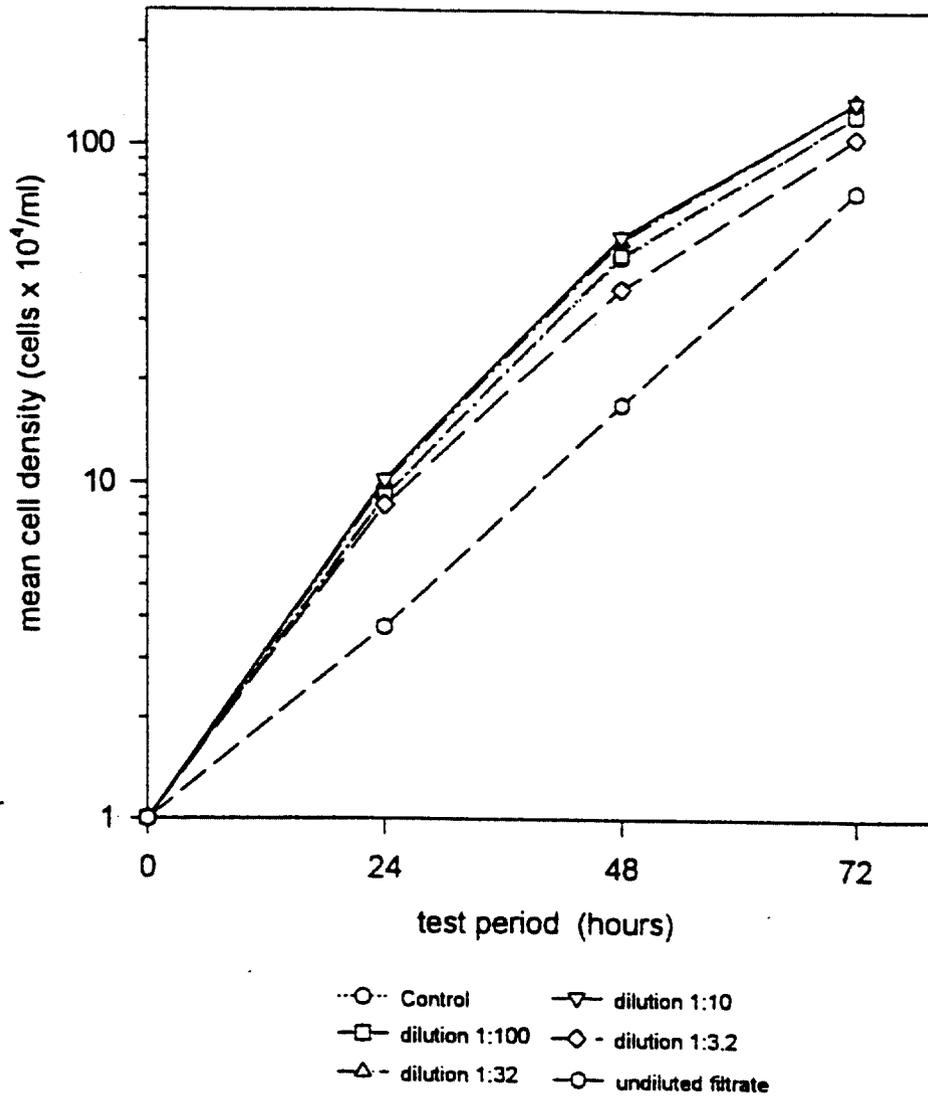


Figure 1: Growth curves of *Scenedesmus subspicatus*, incubated for 72 hours at different dilutions of a filtrate of a supersaturated test substance stock suspension (mean cell number per ml)

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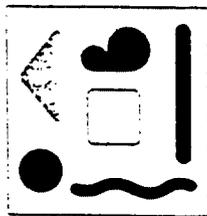
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**RCC PROJECT 600423**

TITLE:

**DETERMINATION OF THE CONCENTRATIONS OF  
MORTRACE SB CONCENTRATE  
IN TEST MEDIUM OF AN ALGAE TOXICITY TEST**

**REPORT**

AUTHOR:

Dr. Jörn Schreitmüller

STUDY COMPLETION:

April 11, 1996

PERFORMING LABORATORY:

**RCC Umweltchemie GmbH & Co. KG  
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LABORATORY PROJECT ID:

**RCC-D PROJECT 520202**

**ATTACHMENT TO RCC-D PROJECT 520201**

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## PREFACE

### PROJECT STAFF

Responsible Scientist for Analytical Chemistry:	Dr. Jörn Schreitmüller
Management:	Markus Arenz
Quality Assurance Unit:	Frauke Hermann
Technician:	Y. Crecelius

### SCHEDULE

Start of Experiments:	January 22, 1996
End of Experiments:	January 26, 1996
Date of Draft:	January 31, 1996
Date of Report:	April 11, 1996

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## OBJECTIVE

In this report the results obtained for the concentrations of Mortrace SB Concentrate in test medium are described.

The quantification of Mortrace SB Concentrate was performed by HPLC analysis and UV/VIS-detection.

## MATERIALS AND METHODS

### ANALYTICAL PROCEDURE

#### Storage

The samples were analysed directly after the sampling procedure.

#### Reagents and solvents

Methanol	Merck, no. 6007
Dichloromethane	Merck, no. 6054
Purified water for HPLC	in-house prepared by a Milli-Q water purification system (Millipore)
Test medium	as described in RCC-D report 520201

#### Test substance treatment

In order to homogenize the test substance completely, it was placed for a few hours in a water bath at 60°C. According to the information from the sponsor this procedure will not lead to degradation of the test substance.

#### Standard solutions used for sample quantification

15.77 mg of test substance were dissolved in 100 ml of a mixture of methanol / water (85:15, v:v) to prepare a stock solution of 157.7 mg test substance/l. Defined volumes of this stock solution were diluted with the a.m. mixture to obtain standard solutions in the range of 0.0237 to 15.77 mg test substance/l.

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### **Standard solutions used for spiked test medium samples**

To demonstrate the validity of the method untreated test medium samples were spiked with test substance.

#### Sampling day 0:

14.50 mg of test substance were dissolved in 100 ml of methanol to prepare a stock solution of 145.0 mg test substance/l. Defined volumes of this stock solution were diluted with methanol to obtain standard solutions of test substance with concentrations of 14.5 and 1.45 mg/l. 1 ml of the solution containing 14.5 mg/l was added to 200 ml of test medium to obtain spiked test medium samples with concentrations of 72.5 µg/l. 0.5 ml of the solution containing 1.45 mg/l was added to 1000 ml of test medium to obtain spiked test medium samples with concentrations of 0.725 µg/l.

#### Sampling day 3:

15.26 mg of test substance were dissolved in 100 ml of methanol to prepare a stock solution of 152.6 mg test substance/l. Defined volumes of this stock solution were diluted with methanol to obtain standard solutions of test substance with concentrations of 1.22 and 0.0122 mg/l.

1 ml of the solution containing 1.22 mg/l was added to 500 ml of test medium to obtain spiked test medium samples with concentrations of 2.44 µg/l. 4 ml of the solution containing 0.0122 mg/l was added to 1000 ml of test medium to obtain spiked test medium samples with concentrations of 0.0488 µg/l.

The spiked test medium samples were further handled like a sample.

In addition test medium without Mortrace SB Concentrate was analysed (analytical blank).

### **Analysis of treatment samples, control samples and spiked test medium samples**

The samples (200 - 1000 ml) were extracted twice with dichloromethane for 15 minutes, respectively, using a mechanical shaker. The organic phase was evaporated using a rotary evaporator and finally with a gentle stream of nitrogen. The residue was dissolved in a defined volume (1 ml or 2 ml) of a mixture of methanol / water (85:15, v:v). The samples were analysed by HPLC and UV/VIS-detection.

For results obtained see Table 1.

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**HPLC CONDITIONS**

Auto sampler:            Varian 9095  
Gradient pump:          Varian 2510  
Detector:                Varian 9050  
Workstation:            Varian LC 9020 STAR

Column:                 LiChrospher RP-8; 4 mm x 125 mm; 5  $\mu$ m

Eluent:                 methanol / water (85:15, v:v)

Flow rate:              1 ml/min

Injection volume:      200  $\mu$ l

Detection wave length: 394 nm

Retention time:        3.6 - 3.7 min

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## EVALUATION OF RESULTS

Injected samples were quantified by HPLC using UV/VIS-detection with reference to the respective calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentration in mg/l. The correlation was performed using a linear function given below (equation 1).

From this curve the concentration  $x$  of Mortrace SB Concentrate in a measured sample was calculated by the following equation:

$$Y = b \cdot x + a \quad (1)$$

where

- Y = Peak area of test substance [area counts] in measured sample
- x = Concentration of test substance [mg/l] in measured sample
- a = y-axis intercept
- b = Slope

The concentration of the test substance in a sample was calculated by equation 2:

$$C = X \cdot E \quad (2)$$

where

- C = Concentration of test substance [mg/l] in sample
- X = Amount of test substance [mg/l] in measured sample found by equation 1
- E = Enrichment factor

The recovery of the test substance in a sample was calculated by equation 3:

$$R = \frac{C}{C_{nom}} \cdot 100\% \quad (3)$$

where

- R = Recovery
- C = Concentration of test substance [mg/l] in sample found by equation 2
- $C_{nom}$  = Measured concentration of test substance [mg/l] in undiluted filtrate

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## RESULTS AND DISCUSSION

The results obtained for the concentrations of Mortrace SB Concentrate in test medium are presented in Table 1. For the preparation of the biological samples a stock suspension of the test substance in test medium was stirred for 72 hours in the dark. The suspension was filtered and the undiluted filtrate was taken as the treatment sample with the highest test substance concentration (see RCC-D report 520201).

An example of calibration data for Mortrace SB Concentrate is given in Table 2 and Figure 1.

Typical HPLC chromatograms are shown in the attached Figures 2 to 7.

The determination limit of the analytical method was derived from the concentration in the lowest standard solution (0.0237 mg/l). The height of the HPLC-peak of this solution corresponds to about ten times of the variation of the analytical noise, when the analyte concentration is zero. The extraction of 1000 ml of test water leads to a determination limit of 0.02 µg/l.

The biological control samples and an analysed analytical blank (test medium) did not affect the HPLC-chromatogram at the retention time of the test substance.

Concurrent with the sample analysis recoveries of spiked test medium samples in the relevant concentrations (72.5, 2.44, 0.725 and 0.0488 mg Mortrace SB Concentrate/l) were performed in duplicate. The mean concentrations were found to be in the range of 100 % to 111 % of the spiked values, with an average of 106 % (n = 8). Therefore, no correction for possible losses during the analytical procedure is necessary.

The mean concentrations found in the undiluted filtrate at the beginning of the test was 1.93 µg/l. The treatment samples with dilution factors of 3.2, 10 and 32 showed mean recoveries of 93 %, 117 % and 114 %, respectively, at the beginning of the test, if the undiluted filtrate is defined as the point of reference.

The 1:100 dilution of the filtrate with a measured concentration of 1.93 µg/l would lead to a concentration lower than the determination limit of 0.02 µg/l. However in one sample of this concentration level a recovery of 130% was measured. There is no obvious reason for this slightly enhanced value.

During the test period of 72 hours the concentration of the test substance in all of the treatment samples decreased to values below the determination limit. As it is known that Mortrace SB Concentrate degrades under the influence of UV-light, the losses could be explained by degradation of the test substance due to the intense irradiation of the treatment samples.

The tabulated values of the samples represent rounded results obtained by calculation using the exact raw data.

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**TABLES**

**Table 1: Results obtained for the concentration of the test substance in test medium.**

Date of analysis: January 22 to 26, 1996

Dilution factor of the undiluted filtrate	Sampling date	Age of sample	RCC sample code	Mortrace SB Concentrate found			
						mean	
				[µg/l]	[% of nominal]	[µg/l]	[% of nominal]
<b>Treatment samples</b>							
1:100	0	0	A-1	< 0.02	n.a.		
	0	0	A-2	0.0248	129 *	0.025	129 *
	3	72	A-11	< 0.02	n.a.		
	3	72	A-12	< 0.02	n.a.	< 0.02	n.a.
	total mean :						0.012
1:32	0	0	A-3	0.0662	110 *		
	0	0	A-4	0.0714	119 *	0.069	114 *
	3	72	A-13	< 0.02	n.a.		
	3	72	A-14	< 0.02	n.a.	< 0.02	n.a.
	total mean :						0.034
1:10	0	0	A-5	0.209	109 *		
	0	0	A-6	0.243	126 *	0.23	117 *
	3	72	A-15	< 0.02	n.a.		
	3	72	A-16	< 0.02	n.a.	< 0.02	n.a.
	total mean :						0.11
1:3.2	0	0	A-7	0.582	97 *		
	0	0	A-8	0.543	90 *	0.56	93 *
	3	72	A-17	< 0.02	n.a.		
	3	72	A-18	< 0.02	n.a.	< 0.02	n.a.
	total mean :						0.28
undiluted filtrate	0	0	A-9	1.87	n.a.		
	0	0	A-10	1.98	n.a.	1.93	n.a.
	3	72	A-19	< 0.02	n.a.		
	3	72	A-20	< 0.02	n.a.	< 0.02	n.a.
	total mean :						1.0

n.a. = not applicable

\* = the reference point for the recovery values is the measured mean value for the concentration of the undiluted filtrate at sampling day 0 (1.93 µg/l)

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**Table 1 cont.:** Results obtained for the concentration of the test substance in test medium.

Date of analysis: January 22 to 26, 1996

Nominal concentration of Mortrace SB Concentrate [µg/l]	Sampling date [day]	Age of sample [hours]	RCC sample code	Mortrace SB Concentrate found			
				[µg/l]	[% of nominal]	mean [µg/l] [% of nominal]	
<b>Biological control samples</b>							
0	0	0	K-1	< 0.02	n.a.	n.a.	n.a.
	3	72	K-3	< 0.02	n.a.	n.a.	n.a.
<b>Spiked test medium samples</b>							
72.5	0	0	AZ1	80.0	110	80.8	111
	0	0	AZ2	81.7	113		
0.725	0	0	AZ3	0.752	104	0.745	103
	0	0	AZ4	0.739	102		
2.44	3	0	AZ6	2.41	99	2.45	100
	3	0	AZ7	2.49	102		
0.0488	3	0	AZ8	0.0543	111	0.0532	109
	3	0	AZ9	0.0520	107		
total mean :							106
<b>Analytical blank</b>							
0	0	0	AZ5	< 0.02	n.a.	n.a.	n.a.
	3	0	AZ10	< 0.02	n.a.	n.a.	n.a.

n.a. = not applicable

\* = the reference point for the recovery values is the measured mean value for the concentration of the undiluted filtrate at sampling day 0 (1.93 µg/l)

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**Table 2:** Example of calibration data of test substance standards calculated on basis of test substance peak area.

For calibration curve see Figure 1.

Standard [mg/l]	Peak area observed	Peak area calculated	Deviation [%]
0.0237	3069	3128	-1.94
0.0631	7560	7956	-5.25
0.158	20751	19586	5.62
0.315	39952	38825	2.82
0.789	95769	96909	-1.19
1.58	191260	193838	-1.35
2.37	292527	290645	0.64

$$Y = 122540 \cdot x - 224$$

$$r = 0.9999$$

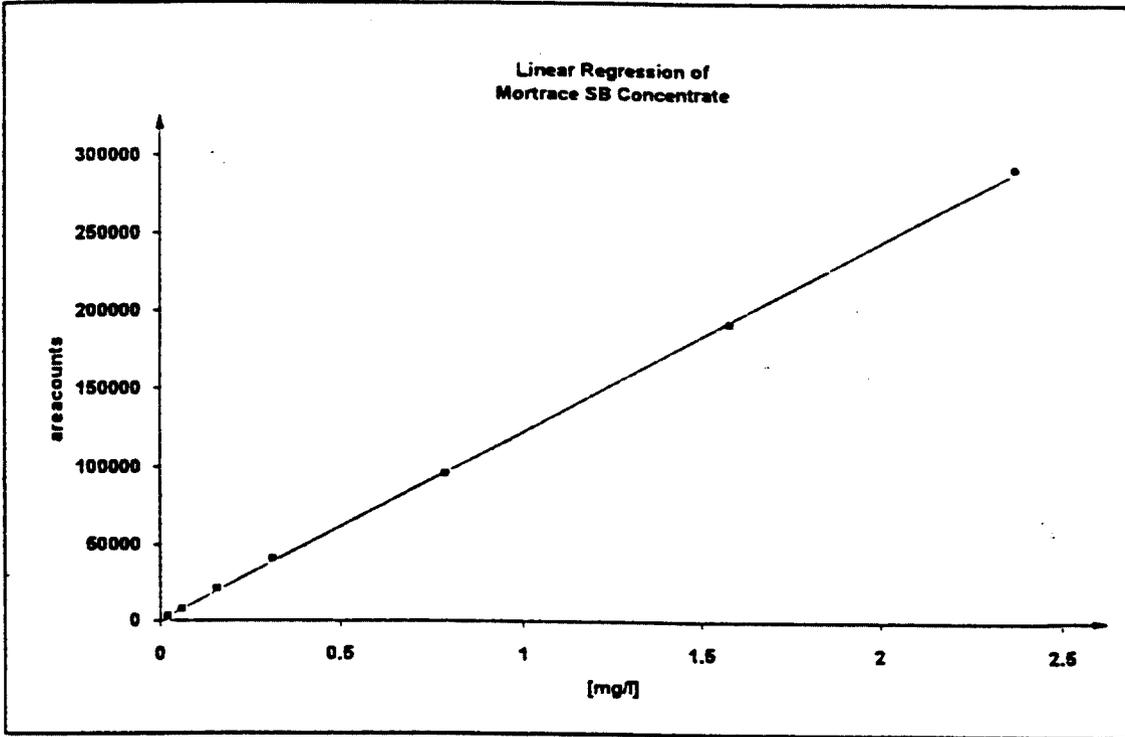
where

Y = Peak area of test substance [area counts] in injected sample

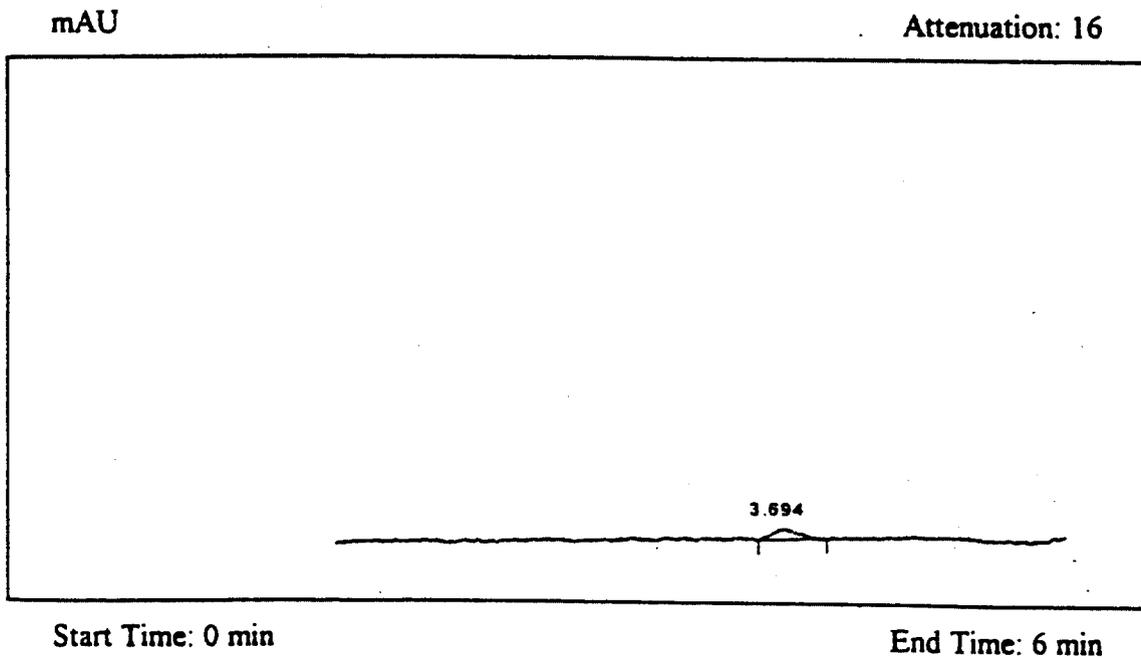
x = Concentration of test substance [mg/l] in injected sample

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**FIGURES**

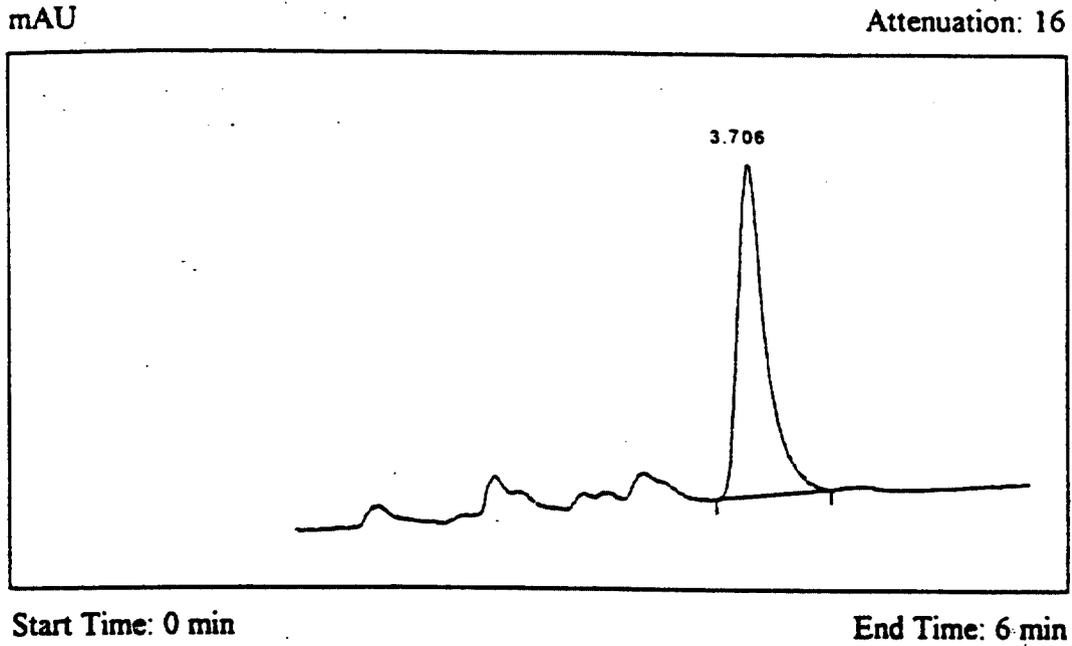


**Figure 1:** Example for a calibration curve of test substance

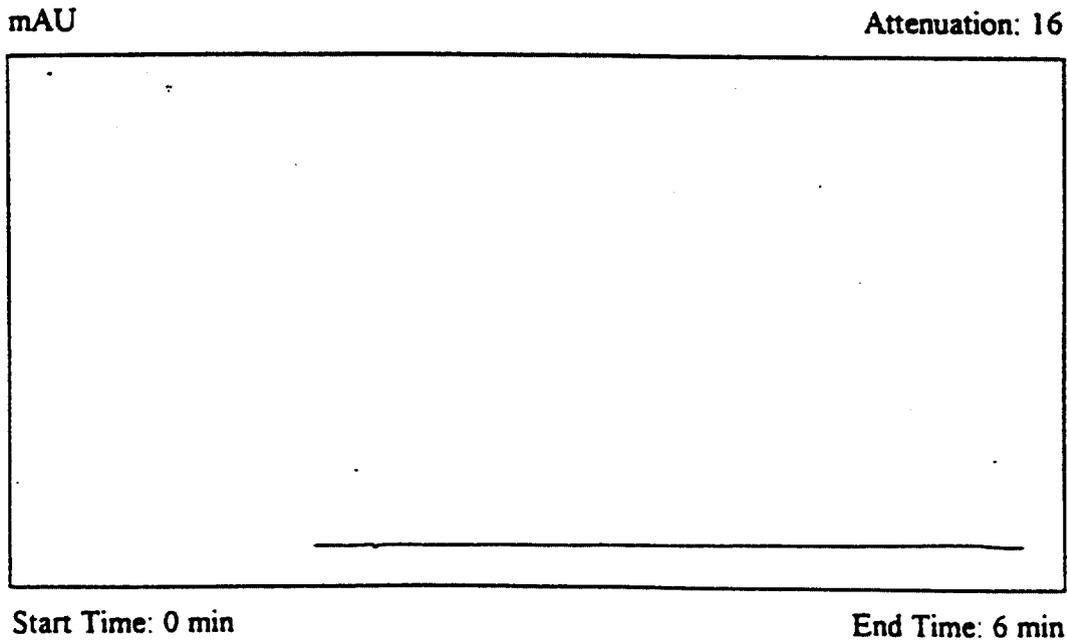


**Figure 2:** Standard solution (0.0237 mg test substance/l)  
Date of analysis: January 22, 1996

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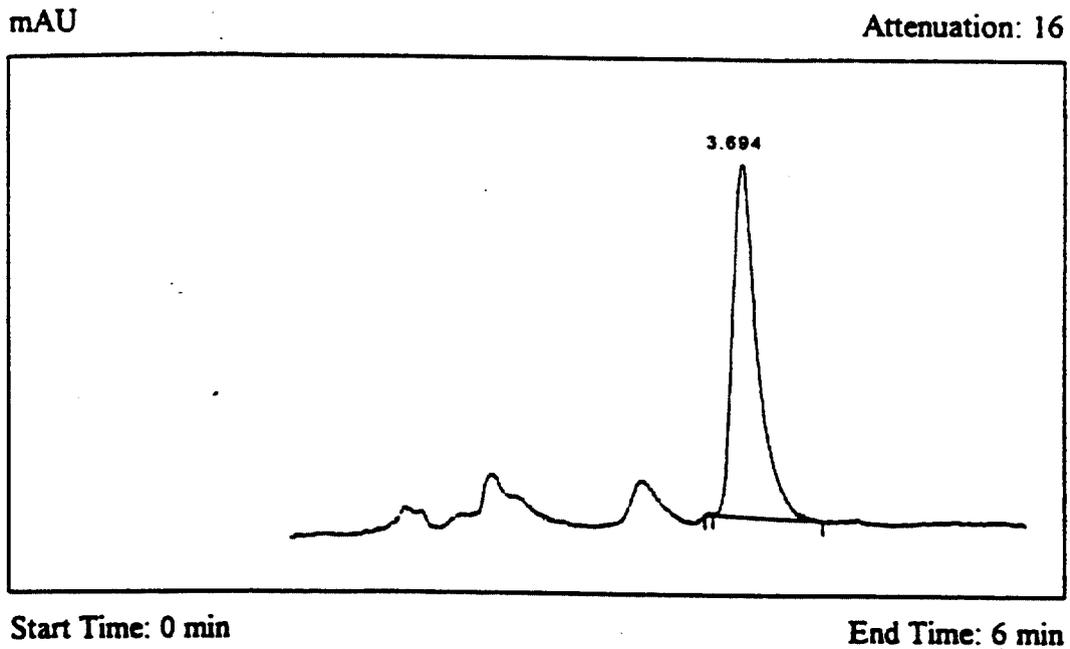


**Figure 3:** Standard solution (0.789 mg test substance/l)  
Date of analysis: January 22, 1996

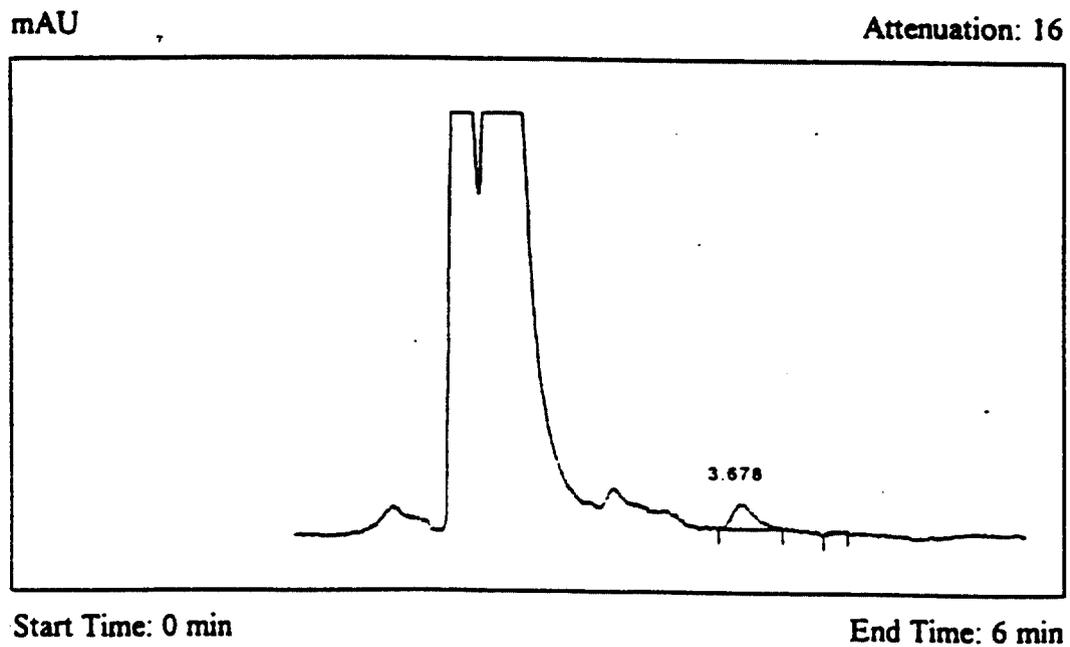


**Figure 4:** Biological control sample K3  
(sampling day 3; age of sample: 72 hours)  
Date of analysis: January 25, 1996

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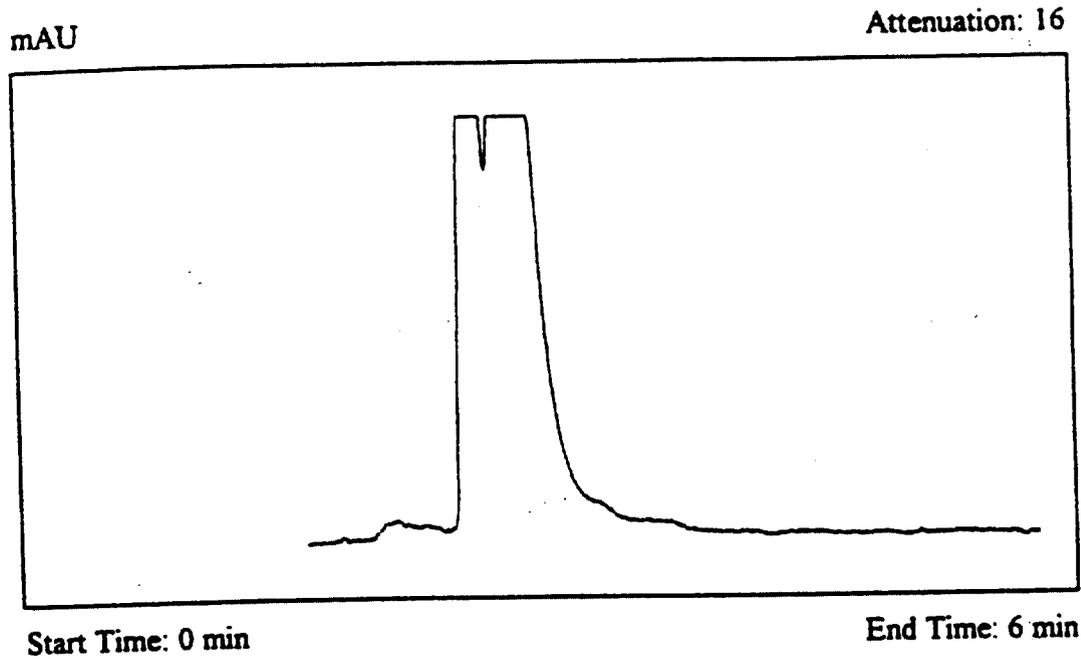


**Figure 5:** Spiked test medium sample AZ4  
(spiked with 0.725  $\mu\text{g}$  test substance/l; analytical sample: 0.725 mg/l);  
Recovery: 102 % of the nominal concentration;  
Date of analysis: January 22, 1996



**Figure 6:** Biological test sample A6  
(1:10-dilution of the undiluted filtrate;  
0.243  $\mu\text{g}$  test substance/l; analytical sample: 0.0485 mg/l;  
sampling day 0; age of sample: 0 hours)  
Date of analysis: January 22, 1996

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**Figure 7:**

**Biological test sample A16**  
(1:10-dilution of the undiluted filtrate;  
test substance below the determination limit;  
sampling day 3; age of sample: 72 hours)  
Date of analysis: January 25, 1996