REPORT

Study Title:

**ACUTE TOXICITY OF [redacted]
TO ZEBRA FISH (*BRACHYDANIO RERIO*)
IN A 96-HOUR SEMI-STATIC TEST**

Data Requirements / Test Guidelines:

OECD No. 203
EU Commission Directive 92/69/EEC, C.1

Study Director:

Dr. Birgit Seyfried

Study Completion Date:

October 04, 2002

Test Facility:

RCC Ltd
Environmental Chemistry &
Pharmanalytics Division
CH–4452 Itingen / Switzerland

Sponsor:

AUSIMONT SpA
Viale Lombardia 20
20021 Bollate (MI) / Italy

RCC Study Number:

842902

RCC

GLP CERTIFICATE

The Swiss GLP Monitoring Authorities

Swiss Federal Office of Public Health

Swiss Agency for the Environment, Forests and Landscape

Intercantonal Office for the Control of Medicines

Statement of GLP Compliance

It is hereby confirmed that

during the period of: August 15 – 17, 2000; August 28 - 29 , 2001 and April 15 , 2002

the following Test Facilities of: **RCC Ltd, 4452 Itingen, Switzerland**

were inspected by the Federal Office of Public Health, the Swiss Agency for the Environment, Forests and Landscape and the Intercantonal Office for the Control of Medicines with respect to the compliance with the Swiss legislation on Good Laboratory Practice.

Test Facilities	areas of expertise*
- Toxicology Division	TOX, ACC, MUT
- Environmental Chemistry and Pharmanalytics Division	ACC, ECT, ENF, EMN, PCT,RES,OTH (Animal metabolism)
- Microbiological Diagnostics by Biotechnology & Animal Breeding Division	OTH (Microbiology)

The inspection was performed in agreement with the OECD Guidelines for National GLP Inspections and Audits. It was found that the aforementioned test facilities were operating in compliance with the Swiss Ordinance relating to Good Laboratory Practice [RS 813.016.5] at the time they were inspected.

Federal Office of Public Health
The Director

Bern, May 2002

Prof. Th. Zeltner

* TOX = Toxicology ; ACC = Analytical and Clinical Chemistry ; ECT = Environmental toxicity on aquatic and terrestrial organisms ; ENF = Behaviour in water, soil and air, Bioaccumulation ; EMN = Studies on effects on mesocosms and natural ecosystems; MUT = Mutagenicity; PCT = Physical-chemical testing ; RES = Residue studies ; OTH = Other, to be specified.

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE

RCC Study Number: 842902

Test Item: [redacted]

Study Director: Dr. Birgit Seyfried

Study Title: Acute toxicity of [redacted] to zebra fish (*Brachydanio rerio*) in a 96-hour semi-static test

This study (with the exception of the pre-experiments as mentioned in the report) has been performed in compliance with the Swiss Ordinance relating to Good Laboratory Practice, adopted February 2nd, 2000 [RS 813.016.5]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted November 26th, 1997 by decision of the OECD Council [C(97) 186/Final].

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

Study Director: Dr. Birgit Seyfried

B. Seyfried

Date: October 04, 2002

SIGNATURES

Study Director: Dr. Birgit Seyfried

B. Seyfried

Date: October 04, 2002

Management: Dr. Uwe Morgenroth

Date: October 04, 2002

QUALITY ASSURANCE UNIT

RCC Ltd, Environmental Chemistry & Pharmanalytics Division, CH-4452 Itingen / Switzerland

STATEMENT

RCC Study Number: 842902

Test Item: [redacted]

Study Director: Dr. Birgit Seyfried

Study Title: Acute toxicity of [redacted] to zebra fish (*Brachydanio rerio*) in a 96-hour semi-static test

The general facilities and activities are inspected periodically and the results are reported to the responsible person and the management.

Study procedures (with the exception of the pre-experiments as mentioned in the report) were periodically inspected. The study plan and this report were audited by the Quality Assurance Unit. The dates are given below.

Dates and Types of QAU Inspections		Dates of Reports to the Study Director and to the Management
June 24, 2002	Study plan	June 24, 2002
July 17, 2002	Preparation of test medium	July 22, 2002
September 16 & 18, 2002	Final report	September 18, 2002

This statement also confirms that this final report reflects the raw data.

Quality Assurance: for Mrs. Margot Richter-Auer

D. Bürn

Date: October 04, 2002

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PREFACE

GENERAL

Study Title:	Acute toxicity of [redacted] to zebra fish (*Brachydanio rerio*) in a 96-hour semi-static test
Sponsor:	AUSIMONT SpA Viale Lombardia 20 20021 Bollate (MI) / Italy
Monitoring Scientist:	Mrs. Ilaria Colombo
Test Facility:	RCC Ltd Environmental Chemistry & Pharmanalytics Division Zelgliweg 1 CH-4452 Itingen / Switzerland
Analytical Identification No.:	842903

RESPONSIBILITIES

Study Director:	Dr. Birgit Seyfried
Deputy Study Director:	Dr. Ulrich Memmert
Responsible for Analytics:	Mr. Tobias Schoop
Technical Coordinator:	Mrs. Giulia Oran
Head of RCC Quality Assurance:	Mrs. Iris Wüthrich

SCHEDULE

Experimental Starting Date[1]:	July 01, 2002
Experimental Completion Date:	July 27, 2002
Study Completion Date:	October 04, 2002

ARCHIVING

RCC Ltd, CH-4452 Itingen, Switzerland will retain the study plan, raw data, a sample of the test item and the final report of the present study for at least ten years.

No data will be discarded without the Sponsor's consent.

[1] Start of the first test. The test had to be repeated since the validity criteria were not met.

DATA REQUIREMENTS / TEST GUIDELINES

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

OECD Guideline for Testing of Chemicals, No. 203, Fish, Acute Toxicity Test, 1992

EU Commission Directive 92/69/EEC, Part C.1: Acute Toxicity for Fish, 1992.

SUMMARY OF STUDY PLAN AMENDMENTS

First Amendment to Study Plan

Concerning:	Alteration:	Reason:
Study Title	Adaptation of title to semi-static test design	The test design was changed from static to semi-static due to scientific reasons.
Purpose	Adaptation of purpose to semi-static test design	
2.5.1 Experimental Conditions	Description of semi-static test design	
2.5.2 Dosage and Concentrations	Additional test medium preparation prior to each test medium renewal	
2.6.1 Determination of the LC50, NOEC, LOEC, LC0 and LC100	Observation of test fish before each test medium renewal	
2.6.2 Water Quality	Description of measurement of water quality parameter in this semi-static test	
2.6.3 Analysis of the Test Item Concentrations	Description of sampling in this semi-static test	

SUMMARY

The acute toxicity of the test item [redacted] to zebra fish (*Brachydanio rerio*) was determined in a 96-hour semi-static test with a daily test medium renewal according to the EU Commission Directive 92/69/EEC, Part C.1 (1992), and the OECD Guideline for Testing of Chemicals No. 203, (1992).

The nominal test item concentrations tested were 4.6, 10, 22, 46, and 100 mg/L, and in parallel a control.

The analytically determined test item concentrations in the test media of the two highest test concentrations of nominal 46 and 100 mg/L varied in the range of 88 to 95% of the nominal values during the test period. Under the test conditions, the test item concentrations were sufficiently constant during the renewal periods of 24 hours. All reported results are related to the nominal concentrations of the test item.

The biological test results after 96 hours test duration:

- 96-hour LC50: > 100 mg/L
- 96-hour LC0: 46 mg/L
- 96-hour LC 100: > 100 mg/L
- 96-hour NOEC: 46 mg/L
- 96-hour LOEC: 100 mg/L

1 PURPOSE

The purpose of this 96-hour toxicity test was to evaluate the acute toxicity of the test item [redacted] to fish. For this purpose, zebra fish (*Brachydanio rerio*) were exposed in a 96-hour semi-static test with daily test medium renewal to aqueous test media containing the test item at various concentrations under defined conditions.

The recorded effects were mortality and visible abnormalities of the test fish.

The used method of application and the tested fish species zebra fish are recommended by the international test guidelines of the OECD and EEC.

2 MATERIALS AND METHODS

2.1 DEFINITIONS

LC0:	The highest test concentration without a significant number of dead test organisms.
LC50:	The median lethal concentration, i.e. the calculated concentration of test item which causes death in 50% of the test organisms.
LC100:	The lowest test concentration at which all test organisms are dead.
NOEC (No Observed Effect Concentration):	The highest test concentration at which no significant toxic effect on the test organisms is observed.
LOEC (Lowest Observed Effect Concentration):	The lowest test concentration at which a significant toxic effect on the test organisms is observed.

2.2 TEST ITEM

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

Identity:	[redacted]
Batch No.:	90391/27
Expiration date:	March, 2004
Purity:	>90% referred to dry salt
Formulation or composition:	Ammonium salt of chlorofluoropolyether 5%; water 95%
Concentration:	Aqueous dispersion: dry weight 5% (the highest obtainable)
Stability in water:	Stable
Solubility in water:	Miscible
pH in aqueous solution:	7 / 10 at concentration of 5%
Aggregate state/physical form at room temperature:	Liquid (emulsion)
Color:	Colorless
Storage conditions:	At room temperature at about 20 °C, away from direct sunlight

2.3 ANALYTICAL STANDARD

The test item was used as analytical standard.

2.4 TEST SYSTEM

The study was performed with zebra fish *(Brachydanio rerio)*. The test fish were obtained from Zoohaus Schaub, CH-4410 Liestal, Switzerland. In accordance with the test guidelines, the fish were held in the laboratories of RCC for about two weeks without any medication. Prior to the test start, they were acclimated for one week to the test water and temperature. During holding and acclimatization until one day before the start of the test, the fish were fed with a commercial fish diet (TETRA MIN Hauptfutter, supplied by TETRA-Werke, D-49304 Melle, Germany). During holding and acclimatization, the mortality rate in the test fish batch was lower than 5% and all fish were healthy.

From the acclimated test fish batch, 10 fish were measured at the start of the test: The mean body length of the fish was 2.9 ± 0.3 cm (Mean ± SD), the mean body wet weight was 0.18 ± 0.05 g (Mean ± SD).

2.5 STUDY DESIGN

2.5.1 Experimental Conditions

According to analytical results from pre-experiments (without GLP), the test item has the tendency to adsorb to surfaces. Therefore, a semi-static test procedure was chosen. The test media were renewed daily to keep the concentrations of [redacted] in the test media as constant as possible during the test period of 96 hours.

One glass aquarium with 5 liters test medium was used for each test concentration and the control. The test vessels were labeled with the RCC study number and all necessary additional information to ensure unmistakable identification.

At the start of the test, 7 fish were introduced into each aquarium in a random order. The loading rate was lower than 1 g fish wet weight per liter test medium. The test media and the control were slightly aerated during the test period. The fish were not fed during the test.

During this semi-static test, each day the surviving test fish were placed into a clean aquarium with freshly prepared test medium of the corresponding test concentration.

Test duration: 96 hours

Water temperature: 21–22 °C during the test period (Table 5)

Light conditions: Photoperiod of 16 hours light and 8 hours darkness (with a 30 minute transition period). Light intensity at light period approximately 50–500 Lux.

Test water: Reconstituted water: analytical grade salts were dissolved in deionized water to obtain the following nominal concentrations:

$CaCl_2 \times 2H_2O$	:	2.0 mmol/L	(= 294 mg/L)
$MgSO_4 \times 7H_2O$	:	0.5 mmol/L	(= 123 mg/L)
$NaHCO_3$	:	0.75 mmol/L	(= 65 mg/L)
KCl	:	0.075 mmol/L	(= 5.8 mg/L)
Water Hardness	:	2.5 mmol/L	(= 250 mg/L) as $CaCO_3$
Alkalinity	:	0.8 mmol/L	
Ratio of Ca : Mg	=	4 : 1	(based on molarity)
Na : K	=	10 : 1	(based on molarity)

The test water was aerated prior to the preparation of the test media until oxygen saturation was reached.

2.5.2 Dosage and Concentrations

The following concentrations of [redacted] were tested: nominal 4.6, 10, 22, 46, and 100 mg/L. Additionally, a control was tested in parallel (test water without test item).

The test medium of the highest test concentration of nominal 100 mg/L was freshly prepared before the start of the test and prior to each test medium renewal by dissolving 950-952 mg of the test item completely in 9.5 L test water by stirring for 10 minutes. Adequate volumes of this test medium were added to test water in the aquaria and were intensively mixed to prepare the test media with lower test item concentrations.

The test media were freshly prepared just before introduction of the fish (= start of the test).

The actual concentrations of the test item in the test media were analytically determined (see Section 2.6.3).

The test concentrations were based on results of a pre-experiment to the solubility of the test item, a range-finding test (both without GLP) and on results of a first main test. However, concentrations in excess of nominal 100 mg/L were not tested according to the guidelines.

2.6 EVALUATIONS

2.6.1 Determination of the LC50, LC0, LC100, NOEC and LOEC

The test fish were observed after approximately 4, 24, 48, 72, and 96 hours test duration for mortality and visible abnormalities.

Dead fish were removed at least once daily and discarded.

The LC50 values at the different observation times could not be calculated because up to the highest test concentration a mortality rate below 50% was observed.

The NOEC, LOEC, LC0 and LC100 were determined directly from the raw data.

2.6.2 Water Quality Criteria

The water temperature, pH-values, and oxygen concentrations were measured in all test media concentrations and the control at the start of the test and once every day during the test in the freshly prepared and old test media.

At the same dates the appearance of the test media was recorded.

2.6.3 Analyses of the Test Item Concentrations

For the analysis of the actual test item concentrations in this semi-static test with daily test medium renewal normally the following samples were taken

just before test start (Day 0) and on the last day of preparation (Day 3):

- duplicate samples from each test medium
- duplicate samples from the control

after the first test medium renewal (Day 1) and at the end of the last renewal period (Day 4) (stability samples):

- duplicate samples from each test medium
- duplicate samples from the control

All samples were taken from the approximate center of the aquaria without mixing of the test media, and were deep-frozen (at about -20 °C) immediately after sampling. Based on pre-experiments for investigation of the storage stability (without GLP), the test item is sufficiently stable in the test water under these storage conditions.

The concentrations of the test item [redacted] were analyzed in the test medium samples from the test concentrations of nominal 46 and 100 mg/L from all sampling times. Samples from the lower test concentrations of nominal 4.6, 10, and 22 mg/L were not analyzed since these concentrations were below the 96-hour NOEC determined in this test. From the control samples, only one of the duplicate samples was analyzed from the corresponding sampling times. The analytical procedure and the results are described in the attached analytical report.

3 RESULTS AND DISCUSSION

In the analyzed test medium samples from the start and end of the test medium renewal periods, the measured test item concentrations ranged between 88 and 95% of the nominal values (see analytical results and Table 2 in the attached analytical report). The test item concentrations in the test were stable during the test medium renewal period of 24 hours. Therefore, the reported biological results are related to the nominal concentrations of the test item.

The biological results (visible abnormalities observed at the test fish, mortalities, and the LC50-values) are listed in Table 1. In the control and at the test concentrations up to and including 46 mg/L, all fish survived until the end of the test and no visible abnormalities were observed at the test fish. At the highest test concentration of 100 mg/L, three of seven test fish died until the end of the test. Thus, the 96-hour NOEC (highest concentration tested without observable toxic effects after the exposure period of 96 hours) and the 96-hour LC0 of [redacted] to zebra fish were determined to be 46 mg/L. The 96-hour LOEC (lowest concentration tested with observable toxic effects) was 100 mg/L. The 96-hour LC50 and LC100 could not be determined because up to the highest test concentration the mortality rate was below 50%.

No remarkable observations were made concerning the appearance of the test media. All test media were clear solutions throughout the test medium renewal periods (Table 2).

The pH values in the test media and the control ranged from 7.9 to 8.0 (Table 3). The oxygen concentration was always 7.8 mg/L or higher (Table 4), and thus higher than 60% oxygen saturation. The water temperature ranged from 21 to 22 °C (Table 5).

4 TABLES

Table 1: Mortality and observed visible abnormalities

Abbreviations of visible abnormalities for which fish were observed during the study:

AK: Strongly extended gills
AP: Apathy
BA: Distended abdomen
BO: Fish mainly at the bottom of the aquarium
GA: Exophthalmus
KR: Convulsions
OB: Fish mainly at the water surface
SA: Mucus secretion
SR: Fish lying on side or back on the bottom
SV: Strong ventilation
TS: Tumbling during swimming
VF: Changed body color

Number of fish tested at each treatment: 7

Nominal test item concentration	Number of affected fish* / number of dead fish visible abnormalities				
	Observation time				
(mg/L)	4 h	24 h	48 h	72 h	96 h
Control	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
4.6	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
10	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
22	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
46	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
100	0 / 0	0 / 0	0 / 0	0 / 0	3 / 3
LC50	> 100 mg/L				

*: number of dead fish plus number of fish with visible abnormalities

Table 2: Appearance of the test media

Abbreviations:

0: no remarkable observations, clear test medium
1: homogeneous dispersion in the water, turbidity observable
2: noticeable turbidity caused by the test item
3: noticeable coloration caused by the test item
4: inhomogeneous dispersion of the test item
5: precipitation of the test item
6: test item at the surface
7: test item lying at the bottom of the aquarium

Nominal test item concentration (mg/L)	Exposure time							
	0 h	24 h		48 h		72 h		96 h
	new	old	new	old	new	old	new	old
4.6	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0
46	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0	0

Table 3: pH-values in the test media and the control

Nominal test item concentration (mg/L)	Exposure time							
	0 h	24 h		48 h		72 h		96 h
	new	old	new	old	new	old	new	old
Control	8.0	8.0	8.0	7.9	7.9	8.0	8.0	8.0
4.6	8.0	8.0	8.0	7.9	7.9	8.0	8.0	8.0
10	8.0	8.0	8.0	7.9	8.0	8.0	8.0	8.0
22	8.0	8.0	8.0	7.9	8.0	8.0	8.0	8.0
46	8.0	8.0	8.0	7.9	8.0	8.0	8.0	8.0
100	8.0	8.0	8.0	7.9	8.0	7.9	8.0	8.0

Table 4: Oxygen concentrations (mg/L) in the test media and the control

Nominal test item concentration (mg/L)	Exposure time							
	0 h	24 h		48 h		72 h		96 h
	new	old	new	old	new	old	new	old
Control	8.4	8.5	8.2	8.5	8.3	8.5	8.3	8.4
4.6	8.3	8.4	8.2	8.3	8.3	8.3	8.2	8.5
10	8.4	8.4	8.2	7.8	8.3	8.2	8.3	8.3
22	8.4	8.3	8.3	8.5	8.2	8.2	8.3	8.4
46	8.3	8.3	8.1	8.2	8.3	8.2	8.2	8.3
100	8.4	8.4	8.1	8.2	8.2	7.8	8.3	8.3

Table 5: Temperatures (°C) in the test media and the control

Nominal test item concentration (mg/L)	Exposure time							
	0 h	24 h		48 h		72 h		96 h
	new	old	new	old	new	old	new	old
Control	22	22	22	21	22	22	22	21
4.6	22	21	22	21	22	22	22	21
10	22	22	22	21	22	22	22	21
22	22	22	22	21	22	22	22	21
46	22	22	22	21	22	22	22	21
100	22	22	22	21	21	22	22	21

ATTACHMENT

ANALYTICAL REPORT

ANALYTICAL REPORT

Study Title:

ACUTE TOXICITY OF [redacted]
TO ZEBRA FISH (*BRACHYDANIO RERIO*)
IN A 96-HOUR SEMI-STATIC TEST

Subtitle of Analytical Report:

DETERMINATION OF THE CONCENTRATIONS OF
THE TEST ITEM IN TEST MEDIUM

Test Facility:

RCC Ltd
Environmental Chemistry &
Pharmanalytics Division
CH-4452 Itingen/Switzerland

RCC Study Number:

842902

Analytical Id. No.:

842903

CONTENTS

PREFACE

GENERAL

Study Title:	Acute toxicity of [redacted] to zebra fish (*Brachydanio rerio*) in a 96-hour semi-static test
Subtitle:	Determination of the concentrations of the test item in test medium
Sponsor:	AUSIMONT SpA Viale Lombardia 20 20021 Bollate (MI) / Italy
Monitoring Scientist:	Mrs. Ilaria Colombo
Test Facility:	RCC Ltd Environmental Chemistry & Pharmanalytics Division Zelgliweg 1 CH-4452 Itingen/Switzerland
Analytical Id. No.:	842903

RESPONSIBILITIES

Responsible for Analytics: Mr. T. Schoop

T. Schoop

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Date: October 04, 2002

Technical Coordinator:	I. Weber
Reporting:	M. Viererbe

SCHEDULE

Experimental starting date:	July 23, 2002
Experimental completion date:	July 27, 2002

1 PURPOSE

In this analytical report the results obtained for the concentrations of [redacted] in test medium are described.

The quantification of the test item was performed by HPLC analysis with MS-detection.

2 MATERIALS AND METHODS

2.1 TEST ITEM

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

2.2 ANALYTICAL PROCEDURE

2.2.1 Storage

The samples were stored deep-frozen and protected from light until analysis was performed.

2.2.2 Reagents and Solvents

Acetonitrile	Baker, no. 9017
Ammonium carbonate	Fluka, no. 09716
Purified water for HPLC	in-house prepared by a water purification system (Millipore)
Test water	as described in the biological part of this study

2.2.3 Standard Solutions used for Sample Quantification

Measurement of treatment samples

17.3 mg of the test item was dissolved in purified water and made up to the mark in a 100 mL volumetric flask to prepare a stock solution of 173 mg/L. Defined volumes of this stock solution were diluted with purified water to obtain standard solutions in the range of 0.173 to 13.0 mg/L of the test item.

Measurement of spiked test water samples[1]

45.56 mg of the test item was dissolved in purified water and made up to the mark in a 100 mL volumetric flask to prepare a stock solution of 456 mg/L. Defined volumes of this stock solution were diluted with purified water to obtain standard solutions in the range of 0.456 to 13.7 mg/L of the test item.

These solutions were used to calibrate the HPLC-system.

2.2.4 Preparation of Spiked Test Water Samples

To demonstrate the validity of the method, untreated test water was spiked with the test item[1].

The test item (143.26 mg) was dissolved in purified water and made up to the mark in a 100 mL volumetric flask to prepare a stock solution of 1433 mg/L. Defined volumes of this stock solution were diluted with test water to obtain spiked test water samples of the test item with concentrations of 10.7 and 107 mg/L. These solutions were subjected to the same treatment as a sample.

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

2.2.5 Analysis of Samples

The samples from the biological test were thawed at room temperature for 2 hours and shaken mechanically to obtain homogeneous sample solutions.

Defined volumes (1 mL) of the samples were diluted to 10 mL or 20 mL with purified water. This leads to dilution factors of 10 or 20.

Aliquots of the samples were analysed by HPLC/MS -detection.

For results obtained see Table 2.

[1] The stock solution, spiked samples as well as the measurements were processed under the study number 842905. Original raw data will be archived under the main study of 842905.

2.3 HPLC/MS CONDITIONS

Separation Parameters

Pump System: Merck L-6200
Autosampler: Merck AS 4000

Column: X-Terra(R) MS C18; 30 x 2.1 mm; 2.5 µm

Eluent A: 0.1 % ammonium carbonate in purified water
Eluent B: acetonitrile

Gradient:

minutes	% eluent A	% eluent B
0	90	10
0.5	90	10
10.5	10	90
14.5	10	90
15	90	10
17	90	10

Injection volume: 30 µL
Flow rate: 0.5 mL/min

Detection Parameters

Detection Unit: Finnigan LCQ
Ionization Mode: ESI Negative Centroid

MS Conditions:
Capillary Voltage: - 40 V
Spray Voltage: approx. 4.5 kV
Capillary Temp: 200° C
Sheath: 70 psi N_2
Auxiliary: 20 psi N_2

Scan Mode:
SIM 5 micro 200 ms
Product m/z: 367
Isolation Width: 1.0

2.4 EVALUATION OF RESULTS

Injected samples were quantified by peak areas 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 (samples from the biological test) or a potential function (spiked test water samples) given below (equation 1a or 1b). For results obtained see Table 1.
From this curve the concentration x of the test item in an injected sample was calculated by the following equation:

$$y = b \cdot x + a \qquad (1a)$$

or

$$y = a \cdot x^{b} \qquad (1b)$$

where

y = Peak area of test item in injected sample [counts]
x = Concentration of test item in injected sample [mg/L]
a = y-axis intercept
b = Slope

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

$$c = x \cdot D \qquad (2)$$

where

c = Concentration of test item in sample [mg/L]
x = Amount of test item in injected sample found by equation 1a or 1b [mg/L]
D = Dilution factor

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

$$R = \frac{c}{c_{nom}} \cdot 100\ \% \qquad (3)$$

where

R = Recovery [%]
c = Concentration of test item in sample found by equation 2 [mg/L]
c_{nom} = Nominal concentration of test item in sample [mg/L]

3 RESULTS AND DISCUSSION

The results obtained for the concentrations of [redacted] in test medium are presented in Table 2.

An example of the calibration data for test item-standards are given in Table 1. The R^2 fits were at least 0.9994 (optimum 1.0000). This reflects the linearity of the HPLC/MS-system within the given calibration ranges.

Typical HPLC/MS chromatograms are shown in the attached Figures 1 to 6.

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

Concurrent with the sample analysis, recoveries of spiked test water samples in the relevant concentrations (10.7 and 107 mg/L of the test item) were performed in duplicate. The average concentrations were found to be 94 % and 89 % of the spiked values, with an overall mean of 91 % (n = 4). Therefore, no correction for possible losses during the analytical procedure is necessary.

The average concentrations found in the treatment samples ranged from 88 % to 95 % of the nominal concentrations.

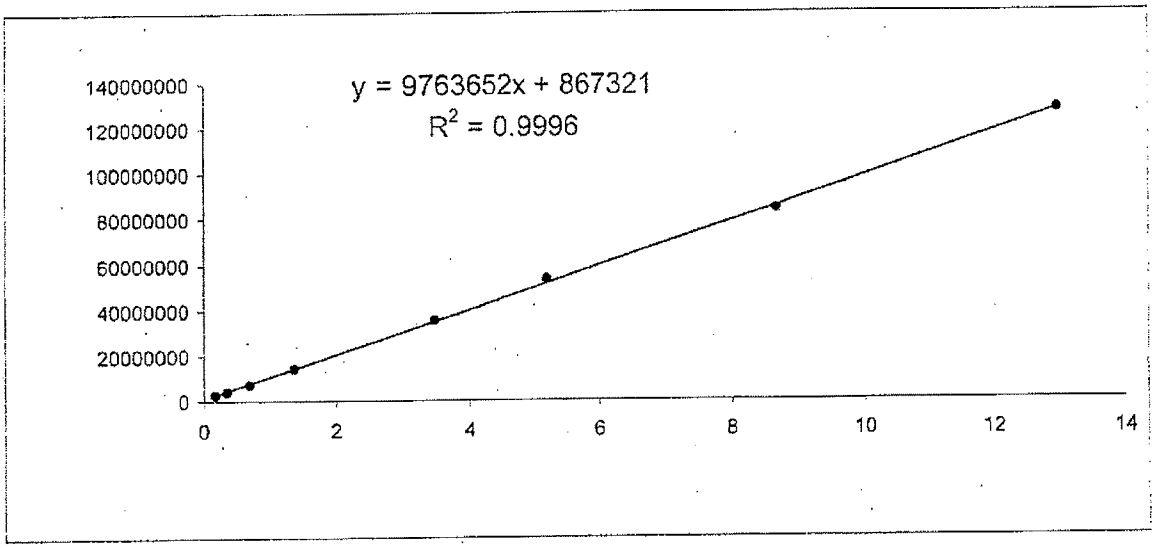
As can be seen from Table 2, [redacted] was stable during the performance of the biological test.

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

4 TABLES

Table 1: Typical calibration data of test item-standards

Standard [mg/L]	Peak area measured [counts]	Deviation from calculated value [%]
0.173	2344030	-12.6
0.346	4139301	-3.1
0.692	7144040	-7.1
1.38	14080491	-2.2
3.46	35081317	1.3
5.19	53404395	3.7
8.65	84122973	-1.4
13.0	127553256	0.0



where

y = Peak area of test item in injected solution [counts]

x = Concentration of test item in injected solution [mg/L]

Table 2: Results obtained for the concentrations of the test item in test medium

Nominal concentration of [redacted]	Sampling date	Age of sample	RCC sample code	[redacted] measured			
						average	
[mg/L]	[day]	[hours]		[mg/L]	[% of nominal]	[mg/L]	[% of nominal]
Treatment samples							
46	0	0	F-109	41.9	91		
	0	0	F-110	41.2	90	41.6	90
	1	24	F-121	42.6	93		
	1	24	F-122	40.3	88	41.4	90
	3	0	F-133	40.6	88		
	3	0	F-134	40.3	88	40.5	88
	4	24	F-145	41.7	91		
	4	24	F-146	41.7	91	41.7	91
				mean :		41.3	90
100	0	0	F-111	94.9	95		
	0	0	F-112	95.4	95	95	95
	1	24	F-123	89.6	90		
	1	24	F-124	94.1	94	92	92
	3	0	F-135	90.7	91		
	3	0	F-136	86.9	87	89	89
	4	24	F-147	91.1	91		
	4	24	F-148	91.2	91	91	91
				mean :		92	92
Biological control samples							
0	0	0	F-101	n.d.	n.a.	n.a.	n.a.
	1	24	F-114	n.d.	n.a.	n.a.	n.a.
	3	0	F-125	n.d.	n.a.	n.a.	n.a.
	4	24	F-138	n.d.	n.a.	n.a.	n.a.
Spiked test water samples							
10.7		0	FZ3	9.79	91		
		0	FZ4	10.3	96	10.1	94
107		0	FZ1	96.9	90		
		0	FZ2	94.0	87	95.5	89
				mean :			91
Analytical blank							
0		0	FZ5	n.d.	n.a.	n.a.	n.a.

n.d. = no test item detected
n.a. = not applicable

5 FIGURES

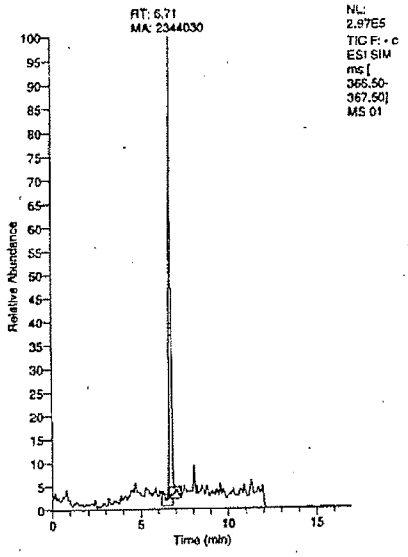


Figure 1: HPLC-chromatogram of standard solution (low-level)
Concentration: 0.173 mg/L of the test item

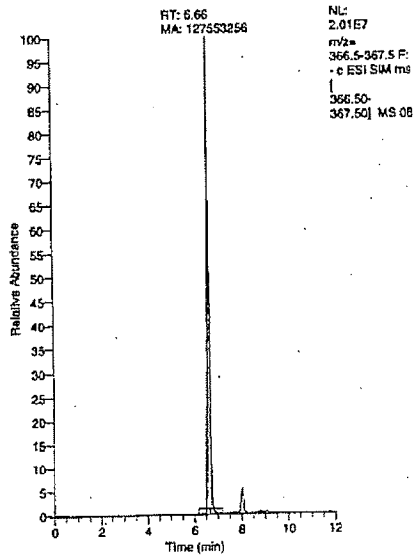


Figure 2: HPLC-chromatogram of standard solution (high-level)
Concentration: 13.0 mg/L of the test item

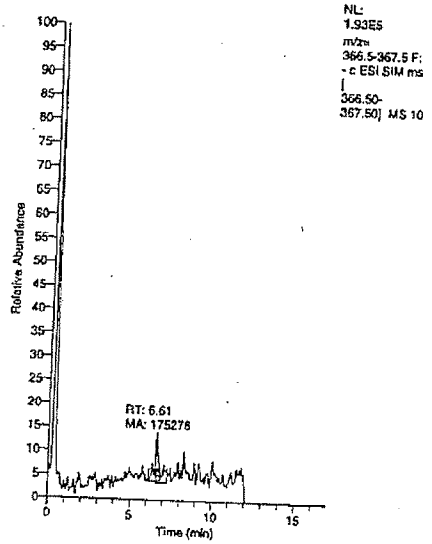


Figure 3: HPLC-chromatogram of biological control sample
Sample Id. No.: F-101
Sampling day 0; age of sample: 0 hours

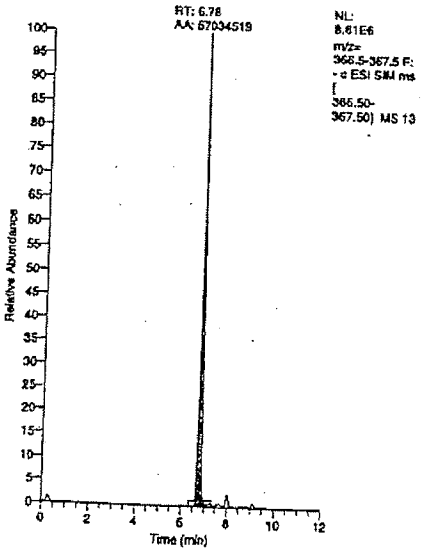


Figure 4: HPLC-chromatogram of spiked test water sample
Sample Id. No.: FZ4
(spiked with 10.7 mg/L of the test item)
Recovery: 96 % of the nominal concentration

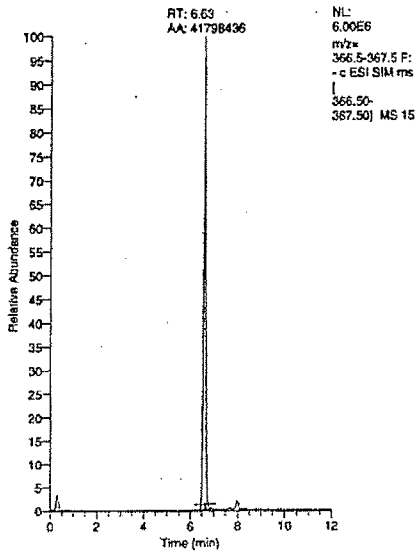


Figure 5: HPLC-chromatogram of non-aged treatment sample
Sample Id. No.: F-109
Sampling day 0; age of sample: 0 hours
(nominal concentration: 46 mg/L of the test item;
nominal concentration in injected sample: 4.6 mg/L)
Recovery: 91 % of the nominal concentration

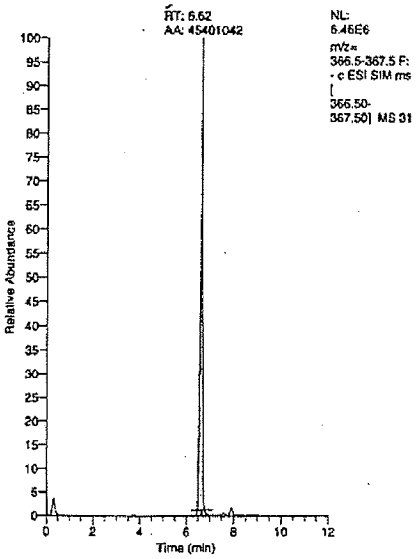


Figure 6: HPLC-chromatogram of aged treatment sample
Sample Id. No.: F-148
Sampling day 4; age of sample: 24 hours
(nominal concentration: 100 mg/L of the test item;
nominal concentration in injected sample: 5 mg/L)
Recovery: 91 % of the nominal concentration

REPORT

Study Title

ACUTE TOXICITY OF [redacted]
TO *DAPHNIA MAGNA*
IN A 48-HOUR IMMOBILIZATION TEST

Data Requirements / Test Guidelines:

OECD No. 202, Part I
EU Commission Directive 92/69/EEC, C.2

Study Director:

Dr. Birgit Seyfried

Study Completion Date:

October 04, 2002

Test Facility:

RCC Ltd
Environmental Chemistry &
Pharmanalytics Division
CH–4452 Itingen / Switzerland

Sponsor:

AUSIMONT SpA
Viale Lombardia 20
20021 Bollate (MI) / Italy

RCC Study Number:

842904

RCC

GLP CERTIFICATE

The Swiss GLP Monitoring Authorities

Swiss Federal Office of Public Health

Swiss Agency for the Environment, Forests and Landscape

Intercantonal Office for the Control of Medicines

Statement of GLP Compliance

It is hereby confirmed that

during the period of	August 15 – 17, 2000 August 28 - 29 , 2001 and April 15 , 2002
the following Test Facilities of	**RCC Ltd** **4452 Itingen** **Switzerland**

were inspected by the Federal Office of Public Health, the Swiss Agency for the Environment, Forests and Landscape and the Intercantonal Office for the Control of Medicines with respect to the compliance with the Swiss legislation on Good Laboratory Practice.

Test Facilities	areas of expertise*
- Toxicology Division	TOX, ACC, MUT
- Environmental Chemistry and Pharmanalytics Division	ACC, ECT, ENF, EMN, PCT,RES,OTH (Animal metabolism)
- Microbiological Diagnostics by Biotechnology & Animal Breeding Division	OTH (Microbiology)

The inspection was performed in agreement with the OECD Guidelines for National GLP Inspections and Audits. It was found that the aforementioned test facilities were operating in compliance with the Swiss Ordinance relating to Good Laboratory Practice [RS 813.016.5] at the time they were inspected.

Federal Office of Public Health
The Director

Bern, May 2002

Prof. Th. Zeltner

* TOX = Toxicology ; ACC = Analytical and Clinical Chemistry ; ECT = Environmental toxicity on aquatic and terrestrial organisms ; ENF = Behaviour in water, soil and air. Bioaccumulation ; EMN = Studies on effects on mesocosms and natural ecosystems; MUT = Mutagenicity; PCT = Physical-chemical testing ; RES = Residue studies ; OTH = Other, to be specified.

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE

RCC Study Number:	842904
Test Item:	[redacted]
Study Director:	Dr. Birgit Seyfried
Study Title:	Acute toxicity of [redacted] to *Daphnia magna* in a 48-hour immobilization test

This study (with the exception of the pre-experiments as mentioned in the report) has been performed in compliance with the Swiss Ordinance relating to Good Laboratory Practice, adopted February 2nd, 2000 [RS 813.016.5]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted November 26th, 1997 by decision of the OECD Council [C(97) 186/Final].

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

Study Director: Dr. Birgit Seyfried

B. Seyfried

Date: October 04, 2002

SIGNATURES

Study Director: Dr. Birgit Seyfried

B. Seyfried

Date: October 04, 2002

Management: Dr. Uwe Morgenroth

U. Morgenroth

Date: October 04, 2002

QUALITY ASSURANCE UNIT

RCC Ltd, Environmental Chemistry & Pharmanalytics Division, CH-4452 Itingen / Switzerland

STATEMENT

RCC Study Number: 842904

Test Item: [redacted]

Study Director: Dr. Birgit Seyfried

Study Title: Acute toxicity of [redacted] to *Daphnia magna* in a 48-hour immobilization test

The general facilities and activities are inspected periodically and the results are reported to the responsible person and the management.

Study procedures (with the exception of the pre-experiments as mentioned in the report) were periodically inspected. The study plan and this report were audited by the Quality Assurance Unit. The dates are given below.

Dates and Types of QAU Inspections		Dates of Reports to the Study Director and to the Management
June 24, 2002	Study plan	June 24, 2002
July 30, 2002	Process based	July 31, 2002
September 13 & 18, 2002	Final report	September 18, 2002

This statement also confirms that this final report reflects the raw data.

Quality Assurance: for Mrs. Margot Richter-Auer

D. Baur

Date: October 09, 2002

CONTENTS

CONTENTS CONTINUED (TABLES)

PREFACE

GENERAL

Study Title:	Acute toxicity of [redacted] to *Daphnia magna* in a 48-hour immobilization test
Sponsor:	AUSIMONT SpA Viale Lombardia 20 20021 Bollate (MI) / Italy
Monitoring Scientist:	Mrs. Ilaria Colombo
Test Facility:	RCC Ltd Environmental Chemistry & Pharmanalytics Division Zelgliweg 1 CH-4452 Itingen / Switzerland
Analytical Identification No.:	842905

RESPONSIBILITIES

Study Director:	Dr. Birgit Seyfried
Deputy Study Director:	Dr. Ulrich Memmert
Responsible for Analytics:	Mr. Tobias Schoop
Technical Coordinator:	Mrs. Géraldine Rappine
Head of RCC Quality Assurance:	Mrs. Iris Wüthrich

SCHEDULE

Experimental Starting Date:	July 09, 2002
Experimental Completion Date:	July 17, 2002
Study Completion Date:	October 04, 2002

ARCHIVING

RCC Ltd, CH-4452 Itingen, Switzerland will retain the study plan, raw data, a sample of the test item and the final report of the present study for at least ten years.

No data will be discarded without the Sponsor's consent.

DATA REQUIREMENTS / TEST GUIDELINES

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

OECD Guideline for Testing of Chemicals, No. 202, *Daphnia sp.*, Acute Immobilization Test and Reproduction Test, Part I, 1984.

EU Commission Directive 92/69/EEC, C.2, Acute Toxicity for Daphnia, 1992.

SUMMARY OF STUDY PLAN AMENDMENTS

There were no amendments to study plan.

SUMMARY

The acute toxicity of the test item [redacted] to *Daphnia magna* was determined in a 48-hour static test according to the EU Commission Directive 92/69/EEC, Part C.2 (1992), and the OECD Guideline for Testing of Chemicals, No. 202, Part I (1984).

The nominal test item concentrations tested were 0.32, 1.0, 3.2, 10, 32, and 100 mg/L, and in parallel a control.

At start and end of the test, test item concentrations in the test media were measured in the range from 83 to 105% of the nominal values. The test item concentrations were sufficiently constant during the test period of 48 hours. Therefore, all reported results are related to the nominal concentrations of the test item.

The biological test results:

– 24-hour EC50:	> 100	mg/L
– 24-hour EC0:	10	mg/L
– 24-hour EC100:	> 100	mg/L
– 48-hour EC50:	23	mg/L
95% confidence limits:	17 - 30	mg/L
– 48-hour EC0 and 48-hour NOEC:	10	mg/L
– 48-hour EC100:	100	mg/L

1 PURPOSE

The purpose of the 48-hour toxicity test was to evaluate the influence of the test item [redacted] on the mobility of *Daphnia magna*. For this purpose, a static test was performed, exposing juvenile daphnids for 48 hours to the test item, added to water at a range of concentrations. Under otherwise identical test conditions and an adequate range of test item concentrations, different concentrations of the test item result in different percentages of test organisms being no longer capable of swimming at the end of the test.

The used method of application and the test species *Daphnia magna* are recommended by the international test guidelines.

2 MATERIALS AND METHODS

2.1 DEFINITIONS

Immobilization:	those organisms which are not able to swim within 15 seconds after gentle agitation of the test container are considered to be immobile
EC0:	the highest test concentration without a significant number of immobilized test organisms
EC50:	the calculated concentration of test item which results in a 50% immobilization rate
EC100:	the lowest test concentration at which all test organisms are immobile
NOEC (No Observed Effect Concentration):	the highest test concentration at which no significant toxic effect on the test organisms is observed

2.2 TEST ITEM

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

Identity:	[redacted]
Batch No.:	90391/27
Expiration date:	March, 2004
Purity:	>90% referred to dry salt
Formulation or composition:	Ammonium salt of chlorofluoropolyether 5%; water 95%
Concentration:	Aqueous dispersion: dry weight 5% (the highest obtainable)
Stability in water:	Stable
Solubility in water:	Miscible
pH in aqueous solution:	7 / 10 at concentration of 5%
Aggregate state/physical form at room temperature:	Liquid (emulsion)
Color:	Colorless
Storage conditions:	At room temperature at about 20 °C, away from direct sunlight

2.3 ANALYTICAL STANDARD

The test item was used as analytical standard.

2.4 TEST SYSTEM

The study was performed with young daphnids of a clone of the species *Daphnia magna* Straus. A clone of this species was originally supplied by the University of Sheffield/UK in 1992, defined from the supplier as clone 5. Since this date the clone is bred in the laboratories of RCC in reconstituted water of the quality identical to the water quality used in the tests (regarding pH, main ions, and total hardness) and under temperature and light conditions identical to those of the tests (see below).

At the start of the test, the used test organisms were 6–24 hours old and were not first brood progeny.

2.5 STUDY DESIGN

2.5.1 Experimental Conditions

The test was performed in 100 mL glass beakers filled with 50 mL test medium. The beakers were covered with glass plates to reduce the loss of water and to avoid the entry of dust into the solutions. The test vessels were labeled with the RCC study number and all necessary additional information to ensure unmistakable identification.

At each test concentration and for the control, 20 daphnids were used divided into two replicates of ten daphnids each. The daphnids were randomly distributed to the test vessels at initiation of the test. The loading rate was lower than one daphnia per 2 mL test solution.

A static test was performed.

Water temperature: 19-21 °C during the test period (Table 3). The test was performed in a temperature-controlled room (room temperature continuously monitored)

Light conditions: A 16-hour light to 8-hour darkness photoperiod (with a 30 minute transition period). Light intensity at light period between 550 and 685 Lux.

Test duration: 48 hours

Test water: Reconstituted test water: analytical grade salts were dissolved in purified water to obtain the following nominal concentrations:

$CaCl_2 \times 2H_2O$	:	2.0	mmol/L (= 294.0 mg/L)
$MgSO_4 \times 7H_2O$	:	0.5	mmol/L (= 123.0 mg/L)
$NaHCO_3$	:	0.75	mmol/L (= 65.0 mg/L)
KCl	:	0.075	mmol/L (= 5.8 mg/L)

Water Hardness : 2.5 mmol/L (=250.0 mg/L) as $CaCO_3$
Alkalinity : 0.8 mmol/L

Ratio of Ca : Mg = 4 : 1 (based on molarity)
Na : K = 10 : 1 (based on molarity)

The test water was aerated prior to the start of the study until oxygen saturation was reached. During the test period, the test water was not aerated.

Feeding: None

Positive Control: For evaluation of the quality of the *Daphnia magna* clone and of experimental conditions, potassium dichromate is tested as a positive control at least once a year to demonstrate satisfactory test conditions.

2.5.2 Dosage and Concentrations

The following concentrations of [redacted] were tested: nominal 0.32, 1.0, 3.2, 10, 32, and 100 mg/L. Additionally, a control was tested in parallel (test water without test item).

The test medium of the highest test concentration of nominal 100 mg/L was prepared by dissolving 42 mg test item completely in 400 mL test water by intense stirring for 15 minutes at room temperature. Adequate volumes of this test medium were diluted with test water to prepare the test media with the lower test item concentrations.

The test media were prepared just before introduction of the daphnids (= start of the test).

The actual concentrations of the test item in the test media were analytically determined (see Section 2.6.3).

The test concentrations were based on the results of a range-finding test and on results of a pre-experiment to the solubility of the test item (without GLP). However, concentrations in excess of nominal 100 mg/L were not tested in compliance with EU Commission Directive 92/69/EEC.

The enlarged spacing factor of 3.2 between the test concentrations was chosen because according to the results of the range-finding test the concentration-effect relationship was rather flat and thus a large concentration range had to be tested.

2.6 EVALUATIONS

2.6.1 Determination of the Immobility, NOEC, EC0, EC50 and EC100

The immobility of the daphnids was determined by visual controls after 24 and 48 hours of exposure. Those organisms not able to swim within 15 seconds after gentle agitation of the test beaker were considered to be immobile.

The 48-hour EC50 and the 95% confidence limits were calculated Moving Average Interpolation (Ref. 1, 2). The 24-hour EC50 of the test item could not be calculated because an immobility below 50% was observed at the highest test concentration after 24 hours test duration. The NOEC, EC0, and EC100 were determined directly from the raw data.

2.6.2 Water Quality Criteria

At the start and at the end of the test, the pH values, the dissolved oxygen concentrations, and the water temperature were determined in each test concentration and the control.

The appearance of the test media was visually recorded at the start of the test and after 24 and 48 hours.

2.6.3 Analysis of the Test Item Concentrations

For the determination of the actual test item concentrations, the following samples were taken:

Just before test start: - duplicate samples from each test medium (without daphnids)
- duplicate samples from the control (without daphnids)

After 48 hours: (stability samples) - duplicate samples from each test medium
- duplicate samples from the control

For the 48-hour stability samples, the contents of the two respective test beakers were combined.

All samples were deep-frozen (at about -20 °C) immediately after sampling. Based on pre-experiments for investigation of the storage stability (without GLP), the test item is sufficiently stable in the test water under these storage conditions.

The concentrations of the test item [redacted] were measured in the duplicate test media samples from the test concentrations of nominal 10, 32, and 100 mg/L of both sampling times (0 and 48 hours). Samples of the test concentrations of nominal 0.32, 1.0, and 3.2 mg/L were not analyzed since these concentrations were below the 48-hour NOEC, determined in this test. From the control, only one of the duplicate samples was analyzed from each of both sampling times. The analytical procedure and the results are described in the attached analytical report.

3 RESULTS AND DISCUSSION

The analytically determined test item concentrations in the analyzed test media from the start and the end of the test varied in the range from 83 to 105% of the nominal values (see analytical results and Table 2 in the attached analytical report). The test item concentrations were sufficiently constant during the test period of 48 hours under the test conditions. Therefore, the reported biological results are based on the nominal concentration of the test item.

The biological results are listed in Table 1.

During the first 24 hours of the test, no immobility of the test organisms was determined in the control and up to and including the test item concentration of 10 mg/L. At the next higher concentration of 32 mg/L, 2 daphnids were found to be immobile (immobility rate of 10%). At the test concentration of 100 mg/L, 6 test organisms were found to be immobile at the observation after 24 hours (immobility rate of 30%). The 24-hour EC0 was 10 mg/L. The 24-hour EC50 and EC100 were determined to be above the highest test concentration of 100 mg/L.

After 48 hours of exposure, no immobility of the test organisms was observed in *Daphnia* exposed at concentrations of up to and including 10 mg/L. At the concentration of 32 mg/L, 16 daphnids were found to be immobile (immobility rate of 80%). At the highest test concentration of 100 mg/L, all daphnids were immobile.

The 48-hour EC50 was calculated to be 23 mg/L with 95% confidence limits from 17 to 30 mg/L. The 48-hour EC0 and the 48-hour NOEC (highest concentration tested without toxic effects after 48 hours) of [redacted] were both 10 mg/L since no immobilization was observed in the test organisms exposed to the test item at levels of up to and including this test concentration. The 48-hour EC100 was 100 mg/L.

No remarkable observations were made concerning the appearance of the test media. All test media were clear solutions throughout the entire test duration (Table 2).

At the beginning and the end of the test period, the dissolved oxygen concentrations in the test media and the control were at least 8.3 mg/L, the pH values ranged from 7.6 to 7.8 and water temperature was between 19 and 21 °C (Table 3).

4 TABLES

Table 1: Influence of [redacted] on the mobility of *Daphnia magna*

Nominal test item concentration (mg/L)	No. of daphnids tested	Immobilized daphnids after 24 hours		Immobilized daphnids after 48 hours	
		No.	%	No.	%
Control	20	0	0	0	0
0.32	20	0	0	0	0
1.0	20	0	0	0	0
3.2	20	0	0	0	0
10	20	0	0	0	0
32	20	2	10	16	80
100	20	6	30	200	100

Table 2: Appearance of the test medium during the test period

Abbreviations:

0: no remarkable observations, clear test medium
1: homogeneous dispersion in the water, turbidity observable
2: noticeable turbidity caused by the test item
3: noticeable coloration caused by the test item
4: inhomogeneous dispersion of the test item
5: precipitation of the test item
6: test item at the surface
7: test item lying at the bottom of the test beaker

Nominal test item concentration (mg/L)	Exposure time		
	0 hours	24 hours	48 hours
0.32	0	0	0
1.0	0	0	0
3.2	0	0	0
10	0	0	0
32	0	0	0
100	0	0	0

Table 3: Dissolved oxygen concentrations, pH values and temperature in the test media and the control

Nominal test item concentration (mg/L)	Start (0 hours)			End (48 hours)		
	pH	Oxygen (mg/L)	Temperature (°C)	pH	Oxygen (mg/L)	Temperature (°C)
Control	7.6	8.3	21	7.8	8.4	19
0.32	7.7	8.7	21	7.8	8.3	19
1.0	7.7	8.8	21	7.8	8.3	19
3.2	7.7	8.6	21	7.8	8.3	19
10	7.7	8.7	21	7.8	8.3	19
32	7.7	8.6	21	7.8	8.3	19
100	7.8	8.6	21	7.8	8.3	19

5 REFERENCES

1) THOMPSON, W.R., WEIL, C.S. (1952):
On the Construction of Tables for Moving Average Interpolation,
Biometrics 8, 51–54

2) FINNEY, D.J. (1978):
Statistical Methods in Biological Assay,
3rd Edition, Charles Griffin, London

REDACTED AS TO TRADE NAMES

ATTACHMENT

ANALYTICAL REPORT

REDACTED AS TO TRADE NAMES

ANALYTICAL REPORT

Study Title:

ACUTE TOXICITY OF [redacted]
TO *DAPHNIA MAGNA*
IN A 48-HOUR IMMOBILIZATION TEST

Subtitle of Analytical Report:

DETERMINATION OF THE CONCENTRATIONS OF
THE TEST ITEM IN TEST MEDIUM

Test Facility:

RCC Ltd
Environmental Chemistry &
Pharmanalytics Division
CH-4452 Itingen/Switzerland

RCC Study Number:

842904

Analytical Id. No.:

842905

CONTENTS

PREFACE

GENERAL

Study Title:	Acute toxicity of [redacted] to *Daphnia magna* in a 48-hour immobilization test
Subtitle:	Determination of the concentrations of the test item in test medium
Sponsor:	AUSIMONT SpA Viale Lombardia 20 20021 Bollate (MI) / Italy
Monitoring Scientist:	Mrs. Ilaria Colombo
Test Facility:	RCC Ltd Environmental Chemistry & Pharmanalytics Division Zelgliweg 1 CH-4452 Itingen/Switzerland
Analytical Id. No.:	842905

RESPONSIBILITIES

Responsible for Analytics:	Mr. T. Schoop

T. Schoop

Date: October 04, 2002

Technical Coordinator:	I. Wiedeking
Reporting:	M. Viererbe

SCHEDULE

Experimental starting date:	July 15, 2002
Experimental completion date:	July 17, 2002

1 PURPOSE

In this analytical report the results obtained for the concentrations of [redacted] in test medium are described.

The quantification of the test item was performed by HPLC analysis with MS-detection.

2 MATERIALS AND METHODS

The implementation of the analytical method was performed on basis of a method provided by the sponsor [1].

2.1 TEST ITEM

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

2.2 ANALYTICAL PROCEDURE

2.2.1 Storage

The samples were stored deep-frozen and protected from light until analysis was performed.

2.2.2 Reagents and Solvents

Acetonitrile	Baker, no. 9017
Ammonium carbonate	Fluka, no. 09716
Purified water for HPLC	in-house prepared by a water purification system (Millipore)
Test water	as described in the biological part of this study

[1] These experiments were not performed according to the regulations of GLP. However, the raw data or copies of the raw data will be archived under the study number of the main study.

2.2.3 Standard Solutions used for Sample Quantification

45.56 mg of the test item was dissolved in purified water and made up to the mark in a 100 mL volumetric flask to prepare a stock solution of 456 mg/L. Defined volumes of this stock solution were diluted with purified water to obtain standard solutions in the range of 0.456 to 13.7 mg/L of the test item.

These solutions were used to calibrate the HPLC-system.

2.2.4 Preparation of Spiked Test Water Samples

To demonstrate the validity of the method, untreated test water was spiked with the test item.

The test item (143.26 mg) was dissolved in purified water and made up to the mark in a 100 mL volumetric flask to prepare a stock solution of 1433 mg/L. Defined volumes of this stock solution were diluted with test water to obtain spiked test water samples of the test item with concentrations of 10.7 and 107 mg/L. These solutions were subjected to the same treatment as a sample.

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

2.2.5 Analysis of Samples

The samples from the biological test were thawed at room temperature for 2 hours and shaken mechanically to obtain homogeneous sample solutions.

High-level treatment samples (32 mg/L and 100 mg/L) and high-level spiked test water samples (107 mg/L)

Defined volumes (2.5 mL) of the samples were diluted to 25 mL with purified water. This leads to a dilution factor of 10.

The other samples were not diluted prior to analysis.

Aliquots of the samples were analysed by HPLC/MS -detection.

For results obtained see Table 2.

2.3 HPLC/MS CONDITIONS

Separation Parameters

Pump System: Merck L-6200
Autosampler: Merck AS 4000

Column: X-Terra(R) MS C18; 30 x 2.1 mm; 2.5 µm

Eluent A: 0.1 % ammonium carbonate in purified water
Eluent B: acetonitrile

Gradient:

minutes	% eluent A	% eluent B
0	90	10
0.5	90	10
10.5	10	90
14.5	10	90
15	90	10
17	90	10

Injection volume: 30 µL
Flow rate: 0.5 mL/min

Detection Parameters

Detection Unit: Finnigan LCQ
Ionization Mode: ESI Negative Centroid

MS Conditions:
Capillary Voltage: - 40 V
Spray Voltage: approx. 4.5 kV
Capillary Temp: 200° C
Sheath: 70 psi N_2
Auxiliary: 20 psi N_2

Scan Mode:
SIM 5 micro 200 ms
Product m/z: 367
Isolation Width: 1.0

2.4 EVALUATION OF RESULTS

Injected samples were quantified by peak areas 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 potential function given below (equation 1). For results obtained see Table 1.
From this curve the concentration x of the test item in an injected sample was calculated by the following equation:

$$y = a \cdot x^{b} \qquad (1)$$

where

y = Peak area of test item in injected sample [counts]
x = Concentration of test item in injected sample [mg/L]
a = y-axis intercept
b = Slope

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

$$c = x \cdot D \qquad (2)$$

where

c = Concentration of test item in sample [mg/L]
x = Amount of test item in injected sample found by equation 1 [mg/L]
D = Dilution factor

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

$$R = \frac{c}{c_{nom}} \cdot 100\ \% \qquad (3)$$

where

R = Recovery [%]
c = Concentration of test item in sample found by equation 2 [mg/L]
c_{nom} = Nominal concentration of test item in sample [mg/L]

3 RESULTS AND DISCUSSION

The results obtained for the concentrations of [redacted] in test medium are presented in Table 2.

The calibration data for test item-standards are given in Table 1. The R^2 fit was 0.9994 (optimum 1.0000). This reflects the linearity of the HPLC/MS-system within the calibration range of 0.456 – 13.7 mg/L of the test item.

Typical HPLC/MS chromatograms are shown in the attached Figures 1 to 6.

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

Concurrent with the sample analysis, recoveries of spiked test water samples in the relevant concentrations (10.7 and 107 mg/L of the test item) were performed in duplicate. The average concentrations were found to be 94 % and 89 % of the spiked values, with an overall mean of 91 % (n = 4). Therefore, no correction for possible losses during the analytical procedure is necessary.

The average concentrations found in the treatment samples ranged from 83 % to 105 % of the nominal concentrations.

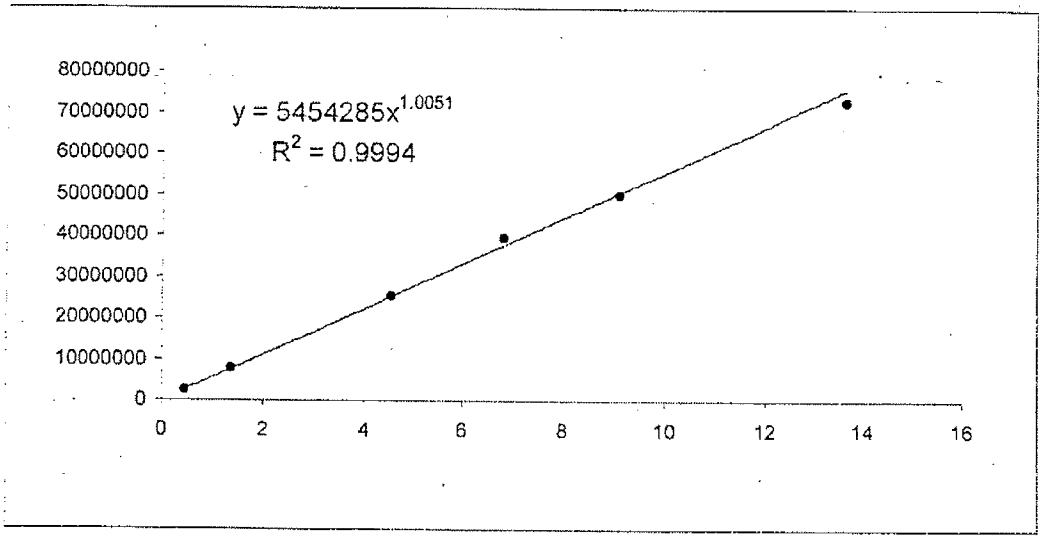
As can be seen from Table 2, [redacted] was stable during the performance of the biological test.

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

4 TABLES

Table 1: Calibration data of test item-standards

Standard [mg/L]	Peak area measured [counts]	Deviation from calculated value [%]
0.456	2417454	-2.3
1.37	7604605	1.8
4.56	25297722	1.0
6.83	39370242	4.6
9.11	49736915	-1.0
13.7	72653068	-3.8



where

y = Peak area of test item in injected solution [counts]

x = Concentration of test item in injected solution [mg/L]

Table 2: Results obtained for the concentrations of the test item in test medium

Nominal concentration of [redacted] [mg/L]	Sampling date [day]	Age of sample [hours]	RCC sample code	[redacted] measured [mg/L]	[% of nominal]	average [mg/L]	average [% of nominal]
Treatment samples							
10	0	0	D-9	8.30	83		
	0	0	D-10	8.91	89	8.60	86
	2	48	D-23	8.31	83		
	2	48	D-24	8.32	83	8.32	83
				mean :		8.46	85
32	0	0	D-11	31.3	98		
	0	0	D-12	32.1	100	31.7	99
	2	48	D-25	31.4	98		
	2	48	D-26	30.8	96	31.1	97
				mean :		31.4	98
100	0	0	D-13	102	102		
	0	0	D-14	108	108	105	105
	2	48	D-27	108	108		
	2	48	D-28	102	102	105	105
				mean :		105	105
Biological control samples							
0	0	0	D-1	n.d.	n.a.	n.a.	n.a.
	2	48	D-15	n.d.	n.a.	n.a.	n.a.
Spiked test water samples							
10.7		0	DZ3	9.79	91		
		0	DZ4	10.3	96	10.1	94
107		0	DZ1	96.9	90		
		0	DZ2	94.0	87	95.5	89
				mean :			91
Analytical blank							
0		0	DZ5	n.d.	n.a.	n.a.	n.a.

n.d. = no test item detected
n.a. = not applicable

5 FIGURES

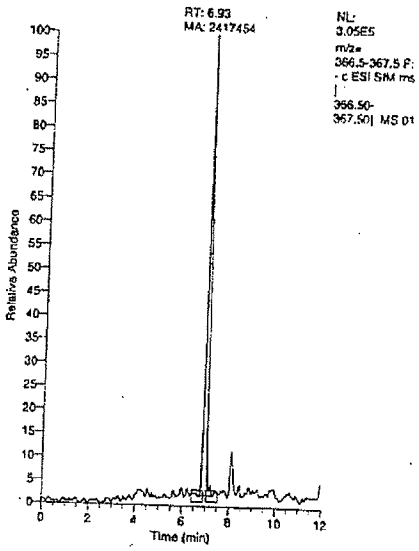


Figure 1: HPLC-chromatogram of standard solution (low-level)
Concentration: 0.456 mg/L of the test item

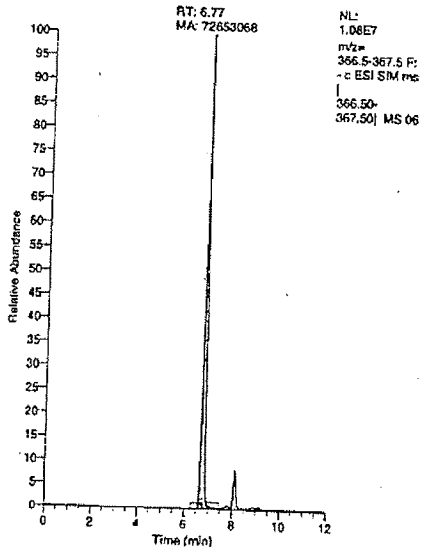


Figure 2: HPLC-chromatogram of standard solution (high-level)
Concentration: 13.7 mg/L of the test item

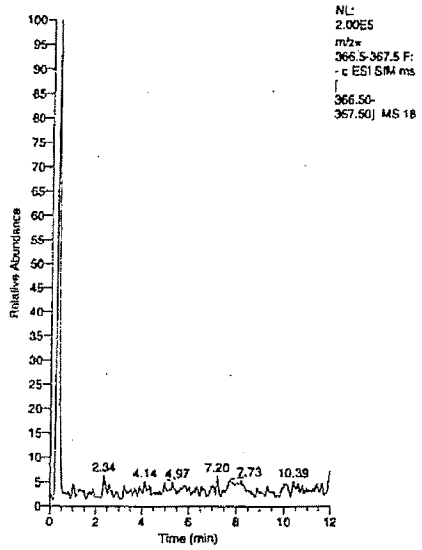


Figure 3: HPLC-chromatogram of biological control sample
Sample Id. No.: D-1
Sampling day 0; age of sample: 0 hours

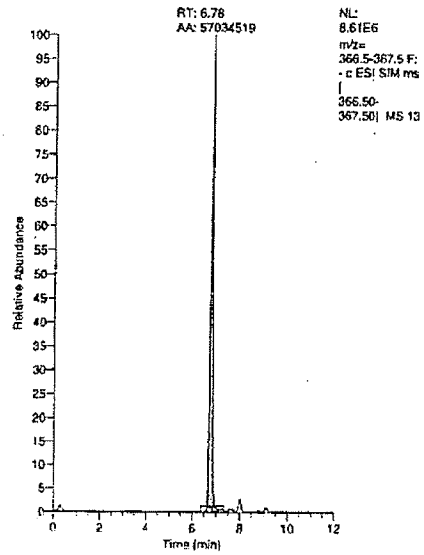


Figure 4: HPLC-chromatogram of spiked test water sample
Sample Id. No.: DZ4
(spiked with 10.7 mg/L of the test item)
Recovery: 96 % of the nominal concentration

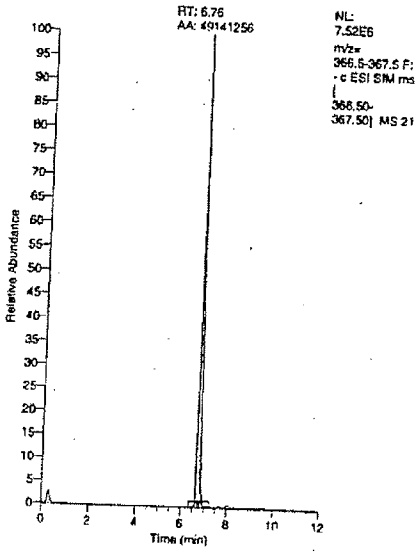


Figure 5: HPLC-chromatogram of non-aged treatment sample
Sample Id. No.: D-10
Sampling day 0; age of sample: 0 hours
(nominal concentration: 10 mg/L of the test item)
Recovery: 89 % of the nominal concentration

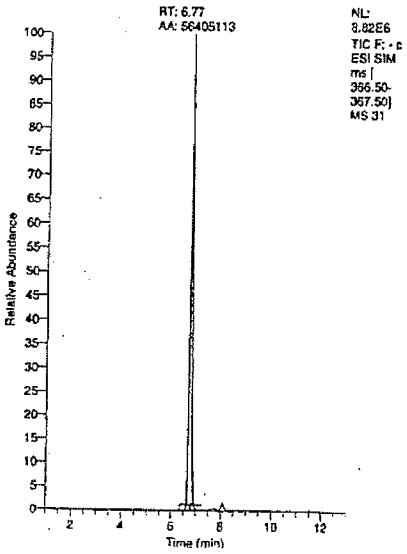


Figure 6: HPLC-chromatogram of aged treatment sample
Sample Id. No.: D-28
Sampling day 2; age of sample: 48 hours
(nominal concentration: 100 mg/L of the test item;
nominal concentration in injected sample: 10 mg/L)
Recovery: 102 % of the nominal concentration

REPORT

Study Title:

TOXICITY OF [redacted]
TO *SCENEDESMUS SUBSPICATUS*
IN A 72-HOUR ALGAL GROWTH INHIBITION TEST

Data Requirements / Test Guidelines:

OECD No. 201
EU Commission Directive 92/69/EEC, C.3

Study Director:

Dr. Birgit Seyfried

Study Completion Date:

October 04, 2002

Test Facility:

RCC Ltd
Environmental Chemistry &
Pharmanalytics Division
CH-4452 Itingen / Switzerland

Sponsor:

AUSIMONT SpA
Viale Lombardia 20
20021 Bollate (MI) / Italy

RCC Study Number:

842906

RCC

GLP CERTIFICATE

The Swiss GLP Monitoring Authorities

Swiss Federal Office of Public Health

Swiss Agency for the Environment, Forests and Landscape

Intercantonal Office for the Control of Medicines

Statement of GLP Compliance

It is hereby confirmed that

during the period of: August 15 – 17, 2000; August 28 - 29 , 2001 and April 15 , 2002

the following Test Facilities of: **RCC Ltd, 4452 Itingen, Switzerland**

were inspected by the Federal Office of Public Health, the Swiss Agency for the Environment, Forests and Landscape and the Intercantonal Office for the Control of Medicines with respect to the compliance with the Swiss legislation on Good Laboratory Practice.

Test Facilities	areas of expertise*
- Toxicology Division	TOX, ACC, MUT
- Environmental Chemistry and Pharmanalytics Division	ACC, ECT, ENF, EMN, PCT,RES,OTH (Animal metabolism)
- Microbiological Diagnostics by Biotechnology & Animal Breeding Division	OTH (Microbiology)

The inspection was performed in agreement with the OECD Guidelines for National GLP Inspections and Audits. It was found that the aforementioned test facilities were operating in compliance with the Swiss Ordinance relating to Good Laboratory Practice [RS 813.016.5] at the time they were inspected.

Federal Office of Public Health
The Director

Prof. Th. Zeltner

Bern, May 2002

* TOX = Toxicology ; ACC = Analytical and Clinical Chemistry ; ECT = Environmental toxicity on aquatic and terrestrial organisms ; ENF = Behaviour in water, soil and air. Bioaccumulation ; EMN = Studies on effects on mesocosms and natural ecosystems; MUT = Mutagenicity; PCT = Physical-chemical testing ; RES = Residue studies ; OTH = Other, to be specified.

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE

RCC Study Number:	842906
Test Item:	[redacted]
Study Director:	Dr. Birgit Seyfried
Study Title:	Toxicity of [redacted] to *Scenedesmus subspicatus* in a 72-hour algal growth inhibition test

This study (with the exception of the pre-experiments as mentioned in the report) has been performed in compliance with the Swiss Ordinance relating to Good Laboratory Practice, adopted February 2nd, 2000 [RS 813.016.5]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted November 26th, 1997 by decision of the OECD Council [C(97) 186/Final].

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

Study Director: Dr. Birgit Seyfried

B. Seyfried

Date: October 04, 2002

SIGNATURES

Study Director: Dr. Birgit Seyfried

B. Seyfried

Date: October 04, 2002

Management: Dr. Uwe Morgenroth

Date: October 04, 2002

QUALITY ASSURANCE UNIT

RCC Ltd, Environmental Chemistry & Pharmanalytics Division, CH-4452 Itingen / Switzerland

STATEMENT

RCC Study Number: 842906

Test Item: [redacted]

Study Director: Dr. Birgit Seyfried

Study Title: Toxicity of [redacted] to *Scenedesmus subspicatus* in a 72-hour algal growth inhibition test

The general facilities and activities are inspected periodically and the results are reported to the responsible person and the management.

Study procedures (with the exception of the pre-experiments as mentioned in the report) were periodically inspected. The study plan and this report were audited by the Quality Assurance Unit. The dates are given below.

Dates and Types of QAU Inspections		Dates of Reports to the Study Director and to the Management
June 24, 2002	Study Plan	June 24, 2002
July 16, 2002	Process based	July 22, 2002
September 11 & 18, 2002	Final Report	September 18, 2002

This statement also confirms that this final report reflects the raw data.

Quality Assurance: for Mrs. Margot Richter-Auer

D Burs

Date: October 04, 2002

CONTENTS

CONTENTS CONTINUED (TABLES & FIGURES)

PREFACE

GENERAL

Study Title:	Toxicity of [redacted] to *Scenedesmus subspicatus* in a 72-hour algal growth inhibition test
Sponsor:	AUSIMONT SpA Viale Lombardia 20 20021 Bollate (MI) / Italy
Monitoring Scientist:	Mrs. Ilaria Colombo
Test Facility:	RCC Ltd Environmental Chemistry & Pharmanalytics Division Zelgliweg 1 CH-4452 Itingen / Switzerland
Analytical Identification No.:	842907

RESPONSIBILITIES

Study Director:	Dr. Birgit Seyfried
Deputy Study Director:	Dr. Ulrich Memmert
Responsible for Analytics:	Mr. Tobias Schoop
Technical Coordinator:	Mrs. Nadja Jüstrich
Head of RCC Quality Assurance:	Mrs. Iris Wüthrich

SCHEDULE

Experimental Starting Date:	July 05, 2002
Experimental Completion Date:	July 18, 2002
Study Completion Date:	October 04, 2002

ARCHIVING

RCC Ltd, CH-4452 Itingen, Switzerland will retain the study plan, raw data, a sample of the test item and the final report of the present study for at least ten years.

No data will be discarded without the Sponsor's consent.

DATA REQUIREMENTS / TEST GUIDELINES

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

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

EU Commission Directive 92/69/EEC, C.3: Algal Inhibition Test, 1992.

SUMMARY OF STUDY PLAN AMENDMENTS

There were no amendments to the study plan.

SUMMARY

The influence of the test item [redacted] on the growth of the green algal species *Scenedesmus subspicatus* CHODAT was investigated in a 72-hour static test according to the EU Commission Directive 92/69/EEC, C.3, 1992, and the OECD Guideline No. 201, 1984.

The nominal test concentrations were 4.6, 10, 22, 46, and 100 mg/L in parallel with a control.

At the start and end of the test, the test item concentrations in the test media of the two highest test concentrations varied in the range from 84 to 90% of the nominal values. The test item concentrations were sufficiently constant during the test period of 72 hours. Therefore, all reported results are related to nominal concentrations of the test item.

The test item [redacted] had a statistically significant inhibition effect on the growth (biomass and growth rate) of *Scenedesmus subspicatus* after the exposure period of 72 hours first at the highest test concentration of 100 mg/L (= 72-hour LOEC: lowest concentration tested with toxic effects). The 72-hour NOEC (highest concentration tested without toxic effects) was determined to be 46 mg/L since up to this test concentration, both the mean biomass and the mean growth rate r of the algae were not statistically significantly lower than in the control.

The EC values were calculated for both parameters, the algal biomass (b) and the growth rate (r), after 72 hours test duration:

Results	
EbC50 = 100 mg/L	**ErC50** > 100 mg/L
EbC10 = 39 mg/L	**ErC10** = 74 mg/L
EbC90 > 100 mg/L	**ErC90** > 100 mg/L
NOECb = 46 mg/L	**NOECr** = 46 mg/L
LOECb = 100 mg/L	**LOECr** = 100 mg/L

The 95%-confidence limits c: could not be determined

1 PURPOSE

The purpose of this test was to determine the inhibitory effect of the test item [redacted] on the growth of the freshwater green algal species *Scenedesmus subspicatus*. Exponentially growing cultures of this algal species were exposed to various concentrations of the test item under defined conditions.

A static, non-renewal exposure system was used. The inhibition of growth in relation to control cultures was determined over a test-period of 72 hours and, thus, over several algal generations.

The method of test item application and the test system are recommended by the test guidelines.

2 MATERIALS AND METHODS

2.1 DEFINITIONS

Cell density:	the number of cells per mL
Growth:	the increase of cell density over the test period
Biomass (b) :	the actual number of cells per volume of medium (cells/mL) calculated as the area under the growth curve (AUC)
Growth rate (r):	the increase of cell density per unit time
E_bCx:	the calculated concentration of test item that results in an x% reduction of biomass b relative to the control
E_rCx:	the calculated concentration of test item that results in an x% reduction of growth rate r relative to the control
NOEC (No Observed Effect Concentration):	the highest test concentration at which no significant inhibition of growth is observed relative to the control
LOEC (Lowest Observed Effect Concentration):	the lowest test concentration at which a significant inhibition of growth is observed relative to the control

2.2 TEST ITEM

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

Identity:	[redacted]
Batch No.:	90391/27
Expiration date:	March, 2004
Purity:	>90% referred to dry salt
Formulation or composition:	Ammonium salt of chlorofluoropolyether 5%; water 95%
Concentration:	Aqueous dispersion: dry weight 5% (the highest obtainable)
Stability in water:	Stable
Solubility in water:	Miscible
pH in aqueous solution:	7 / 10 at concentration of 5%
Aggregate state/physical form at room temperature:	Liquid (emulsion)
Color:	Colorless
Storage conditions:	At room temperature at about 20 °C, away from direct sunlight

2.3 ANALYTICAL STANDARD

The test item was used as analytical standard.

2.4 TEST SYSTEM

The test organism used for the study was *Scenedesmus subspicatus* CHODAT, Strain No. 86.81 SAG, supplied by the Sammlung von Algenkulturen Göttingen (SAG, Experimentelle Phykologie und Sammlung von Algenkulturen, Albrecht-von-Haller-Institut für Pflanzenwissenschaften, Universität Göttingen, D-37073 Göttingen, Germany). The algae had been grown in the RCC laboratories under standardized conditions according to the test guidelines.

2.5 STUDY DESIGN

2.5.1 Experimental Conditions

The algae were cultivated and tested in synthetic test water, prepared according to the test guidelines. Analytical grade salts were dissolved in sterile purified water to obtain the following final nominal concentrations:

Macro-nutrients:

$NaHCO_3$	50.0 mg/L
$CaCl_2 \times 2\ H_2O$	18.0 mg/L
NH_4Cl	15.0 mg/L
$MgSO_4 \times 7\ H_2O$	15.0 mg/L
$MgCl_2 \times 6\ H_2O$	12.0 mg/L
KH_2PO_4	1.6 mg/L

Trace elements:

$Na_2EDTA \times 2\ H_2O$	100.0 µg/L
$FeCl_3 \times 6\ H_2O$	80.0 µg/L
$MnCl_2 \times 4\ H_2O$	415.0 µg/L
H_3BO_3	185.0 µg/L
$Na_2MoO_4 \times 2\ H_2O$	7.0 µg/L
$ZnCl_2$	3.0 µg/L
$CoCl_2 \times 6\ H_2O$	1.5 µg/L
$CuCl_2 \times 2\ H_2O$	0.01 µg/L

Calculated water hardness of the test water: 0.24 mmol/L (= 24 mg/L) as $CaCO_3$.

The test was started (0 hours) by inoculation of 10,000 algal cells per mL test medium. These cells were taken from an exponentially growing pre-culture which was set up 3 days prior to the test under the same conditions as in the test.

The test design included three replicates per test concentration and six replicates of the control. Volumes of 15 mL algal suspension for each replicate 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 at a temperature between 21 and 23 °C (Table 8), and continuously illuminated at a measured light intensity of about 8000 Lux (mean value), range: 7530 to 8320 Lux (minimum and maximum value of measurements at 9 places distributed over the experimental area at the surface of the test media). This illumination was achieved by fluorescent tubes (Philips TLD 36W/840), installed above the test flasks. The test vessels were labeled with the RCC study number and all necessary additional information to ensure unmistakable identification.

The test duration was 72 hours.

For evaluation of the algal quality and experimental conditions, potassium dichromate is tested as a positive control at least once per year to demonstrate satisfactory test conditions.

2.5.2 Dosage and Concentrations

The following nominal concentrations of [redacted] were tested: 4.6, 10, 22, 46, and 100 mg/L. Additionally, a control was tested in parallel (test water without test item).

The test medium of the highest test item concentration of nominal 100 mg/L was prepared by dissolving 40.2 mg of the test item completely in 400 mL test water by intense stirring for 15 minutes at room temperature. Adequate volumes of this intensively mixed test medium were diluted with test water to prepare the test media with the lower test item concentrations.

The test media were prepared just before addition of algae (= start of the test; see Section 2.5.1).

The actual concentration of the test item in the test media was analytically determined (see Section 2.6.4).

The test concentrations were based on the results of a range-finding test and the results of the pre-experiment to the solubility of the test item (without GLP). However, concentrations in excess of nominal 100 mg/L were not tested in compliance with EU Commission Directive 92/69/EEC.

2.6 EVALUATIONS

2.6.1 Counting and Examination of the Algal Cells

Small volumes of the test media and the control (1.0 mL) were taken out of all test flasks after 24, 48 and 72 hours exposure, and were not replaced. The algal cell densities in the samples were determined by counting with an electronic particle counter (Coulter Counter®, Model ZM), with at least two measurements per sample.

In addition, after 72 hours exposure, a sample was taken from the control and from a test concentration with reduced algal growth (nominal 100 mg/L) The shape of the algal cells was microscopically examined in these samples.

2.6.2 Determination of the Algal Growth Inhibition and the EC Values

Inhibition of algal growth was determined from:

a) the area under the growth curves AUC (= biomass, b)

b) the specific growth rates (r) for exponentially growing cultures

using the following equations:

a) Area under the growth curve (AUC):

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

where: N_0 = nominal number of cells/mL at time t_0 (start of the test)
N_1 = mean measured number of cells/mL at time t_1
N_2 = mean measured number of cells/mL at time t_2
N_n = mean measured number of cells/mL at time t_n
t_1 = time of first measurement after start of the test
t_2 = time of second measurement after start of the test
t_n = time of n^{th} measurement after start of the test

Percentage inhibition of AUC (I_{AUC}):

$$I_{AUC} = \frac{AUC_c - AUC_i}{AUC_c} \cdot 100\%$$

where: AUC_c = mean area under the control growth curve
AUC_i = mean area under the growth curve at test concentration i

b) **Growth rate (r):**

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

Percentage inhibition of growth rate (I_r):

$$I_r = \frac{r_c - r_i}{r_c} \cdot 100\%$$

where: r_c = mean growth rate of the control
r_i = mean growth rate of test concentration i

AUC and r were calculated for each test flask. Based on these values, the arithmetic mean area and the arithmetic mean growth rate were calculated for all test flasks per treatment. The tabulated values represent rounded results obtained by calculation using the exact raw data.

The E_bC10 and E_rC10 values (concentrations of the test item corresponding to 10% inhibition of algal biomass (b) and growth rate (r) compared to the control, respectively) were calculated by Probit Analysis (Ref. 1, 2). Their 95%-confidence limits could not be calculated.

The EC50 and the EC90 values of the test item could not be calculated because the inhibition of biomass and growth rate was only 49% and 17%, respectively, at the highest concentration tested.

For the determination of the LOEC and NOEC, the calculated mean biomass and the mean growth rate at the test concentrations were tested for significant differences compared to the control values by a Dunnett-test (Ref. 3, 4).

2.6.3 Water Quality Criteria

The pH was measured and recorded in each test concentration and the control at the start and at the end of the test. The water temperature was measured and recorded daily in an Erlenmeyer flask filled with water and incubated under the same conditions as the test flasks. The appearance of the test media was recorded daily.

2.6.4 Analysis of the Test Item Concentrations

For the determination of actual test item concentrations, the following samples were taken:

Just before the start of the test:	- duplicate samples from each test medium (without algae) - duplicate samples from the control (without algae)
After 72 hours: (stability samples)	- duplicate samples from each test medium (without algae) - duplicate samples from the control (without algae)

For the 72-hour stability samples additional flasks with adequate volumes of the freshly prepared test media of all test concentrations and the control were incubated under the same conditions as in the actual test but without algae.

All samples were deep-frozen (at about -20 °C) immediately after sampling. Based on pre-experiments (without GLP) for investigation of the storage stability the test item is sufficiently stable in the test water under these storage conditions.

The concentrations of the test item [redacted] were measured in the duplicate test media samples from the test concentrations of nominal 46 and 100 mg/L from both sampling times (0 and 72 hours). The samples from the test concentrations of nominal 4.6, 10, and 22 mg/L were not analyzed since these concentrations were below the determined 72-hour NOEC. From the control samples, only one of the duplicate samples was analyzed from the corresponding sampling times. The analytical procedure and results are described in the attached analytical report.

3 RESULTS AND DISCUSSION

The analytically determined test item concentrations in the test media of the two highest test concentrations varied in the range of 84 to 90% of the nominal values (see analytical results and Table 2 in the attached analytical report). The test item concentrations were sufficiently constant during the test period of 72 hours. Therefore, all reported results are related to nominal concentrations of the test item.

The influence of the test item [redacted] on the growth of *Scenedesmus subspicatus* is shown in Tables 1–5 and Figure 1. The test item had a statistically significant inhibitory effect on the growth (biomass and growth rate) of *Scenedesmus subspicatus* after the exposure period of 72 hours at the highest test concentration of 100 mg/L (results of Dunnett-tests, one-sided, α = 0.05, Tables 4 and 5). Thus, the test concentration of 100 mg/L was determined as the 72-hour LOEC (lowest concentration tested with toxic effects). The 72-hour NOEC (highest concentration tested without toxic effects after a test period of 72 hours) was determined to be 46 mg/L since up to and including this test concentration, both the mean biomass and the mean growth rates of the algae were not statistically significantly lower than in the control (Tables 4 and 5).

The EC values were calculated for both parameters, the algal biomass (b) and the growth rate (r), after 72 hours test duration:

Parameter (0-72 h)	Biomass b (mg/L)	Growth rate r (mg/L)
EC50	100 *	> 100
95%-confidence limits	n.d.	n.d.
EC10	39	74
95%-confidence limits	n.d.	n.d.
EC90	> 100	> 100
95%-confidence limits	n.d.	n.d.
NOEC	46	46
LOEC	100	100

n.d.: could not be determined
* the inhibition of b was around 50% at the highest test concentration

The microscopic examination of the algal cells after 72 hours exposure showed no difference between the algae growing in test media containing the test item at a nominal concentration of 100 mg/L and the algal cells in the controls. There were no obvious effects on the shape and size of the algal cells growing in test media containing the test item at up to and including this nominal concentration.

In the control the cell density increased from nominal $N = 1 \times 10^4$ cells/mL at the start of the test (0 hours) to $N = 93 \times 10^4$ cells/mL (mean value) after 72 hours (Table 1). Thus, the algal growth in the control was sufficiently high under the test conditions and the validity criterion of increase of cell density by at least a factor of 16 over the duration of the study was fulfilled.

No remarkable observations were made concerning the appearance of the test media. All test media were clear solutions throughout the test period (Table 6).

At the start of the test, the pH values in the test media and the control ranged from 7.8 to 7.9. At the end of the test, pH values between 8.3 and 8.5 were measured (Table 7). The water temperature ranged from 21 to 23 °C (Table 8).

4 TABLES

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

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

Nominal test item concentration (mg/L)	Flask No.	Density of algal cells (cell number x 10,000/mL) after					
		24 h		48 h		72 h	
Control	1	3.6	3.5	11.3	11.7	61.8	71.9
	2	4.3	4.7	16.0	16.1	97.9	97.8
	3	4.8	4.2	15.8	16.3	97.3	98.5
	4	4.1	4.4	15.9	15.7	95.3	97.0
	5	4.4	3.8	16.6	15.7	96.1	107.0
	6	4.1	3.9	15.0	15.6	93.4	103.5
	m	4.15		15.14		93.12	
	s	0.36		1.81		12.99	
	n	6		6		6	
4.6	1	4.5	4.6	18.0	17.3	95.3	98.2
	2	3.5	3.8	15.5	12.6	89.4	82.3
	3	4.3	4.3	15.7	16.8	101.3	103.8
	m	4.17		15.98		95.05	
	s	0.46		1.81		8.48	
	n	3		3		3	
10	1	4.1	4.1	17.0	17.3	94.3	92.0
	2	4.4	4.3	16.6	16.4	89.9	91.0
	3	3.9	4.1	15.4	14.9	83.0	80.0
	m	4.15		16.27		88.37	
	s	0.18		1.02		6.10	
	n	3		3		3	
22	1	3.4	3.7	15.0	12.7	77.7	89.8
	2	4.4	4.2	16.3	16.6	94.3	95.7
	3	4.3	4.4	17.6	16.1	92.2	92.1
	m	4.07		15.72		90.30	
	s	0.45		1.63		5.85	
	n	3		3		3	
46	1	3.9	4.2	16.7	16.6	89.6	87.7
	2	4.0	4.3	16.6	16.5	83.1	83.4
	3	4.1	4.2	12.7	13.8	77.9	77.2
	m	4.12		15.48		83.15	
	s	0.06		1.93		5.55	
	n	3		3		3	
100	1	3.0	3.2	9.8	9.8	40.2	45.1
	2	3.5	3.2	10.3	10.2	41.0	39.2
	3	3.9	3.6	11.4	12.0	41.0	47.0
	m	3.40		10.58		42.25	
	s	0.33		0.99		1.98	
	n	3		3		3	

m: mean value; s: standard deviation; n: number of flasks
At the start, 10,000 algal cells/mL were incubated.

Table 2: Areas under the growth curves (AUC) and percentage inhibition of AUC (I_{AUC}) during the test period

Nominal test item concentration (mg/L)	Areas under the growth curves (AUC) and % inhibition of AUC					
	0-24 h		0-48 h		0-72 h	
	AUC	I_{AUC} (%)	AUC	I_{AUC} (%)	AUC	I_{AUC} (%)
Control	38	0.0	245	0.0	1521	0.0
4.6	38	-0.5	256	-4.3	1564	-2.9
10	38	0.0	259	-5.5	1490	2.0
22	37	2.6	250	-2.0	1498	1.5
46	37	1.1	249	-1.3	1408	7.4
100	29	23.8	173	29.6	783	48.5

AUC x 10,000
- % inhibition: increase in growth relative to that of control

Table 3: Growth rates (r) and percentage inhibition of r (I_r) during the test period

Nominal test item concentration (mg/L)	Growth rate r and % inhibition of r					
	0-24 h		0-48 h		0-72 h	
	r (1/day)	I_r (%)	r (1/day)	I_r (%)	r (1/day)	I_r (%)
Control	1.42	0.0	1.36	0.0	1.51	0.0
4.6	1.42	-0.2	1.38	-2.1	1.52	-0.6
10	1.42	-0.2	1.39	-2.8	1.49	1.0
22	1.40	1.5	1.38	-1.5	1.50	0.5
46	1.41	0.3	1.37	-0.9	1.47	2.3
100	1.22	14.0	1.18	13.1	1.25	17.3

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

Table 4: Results of a Dunnett-test with the biomass (b, areas under the growth curves)

Nominal test item concentration (mg/L)	Calculated t-value		
	0-24 h	0-48 h	0-72 h
4.6	-0.07	-0.56	-0.42
10	0.00	-0.72	0.29
22	0.34	-0.26	0.21
46	0.14	-0.18	1.07
100	3.10 *	3.87 *	7.02 *
Tabulated t-value	2.44	2.44	2.44
Degrees of freedom	15	15	15

* mean value significantly different from the control ($\alpha = 0.05$, one-sided smaller)

Table 5: Results of a Dunnett-test with the growth rates (r)

Nominal test item concentration (mg/L)	Calculated t-value		
	0-24 h	0-48 h	0-72 h
4.6	-0.05	-0.70	-0.36
10	-0.04	-0.95	0.58
22	0.34	-0.50	0.30
46	0.08	-0.29	1.38
100	3.23 *	4.37 *	10.25 *
Tabulated t-value	2.44	2.44	2.44
Degrees of freedom	15	15	15

* mean value significantly different from the control ($\alpha = 0.05$, one-sided smaller)

Table 6: Appearance of the test media

Abbreviations:

0: no remarkable observations, clear test medium
1: homogeneous dispersion in the water, turbidity observable
2: noticeable turbidity caused by the test item
3: noticeable coloration caused by the test item
4: non-homogeneous dispersion of the test item
5: precipitation of the test item
6: test item at the surface
7: test item lying at the bottom of the flask

Nominal test item concentration (mg/L)	Exposure time			
	0 h	24 h	48 h	72 h
4.6	0	0	0	0
10	0	0	0	0
22	0	0	0	0
46	0	0	0	0
100	0	0	0	0

Table 7: pH values in the test media and in the control

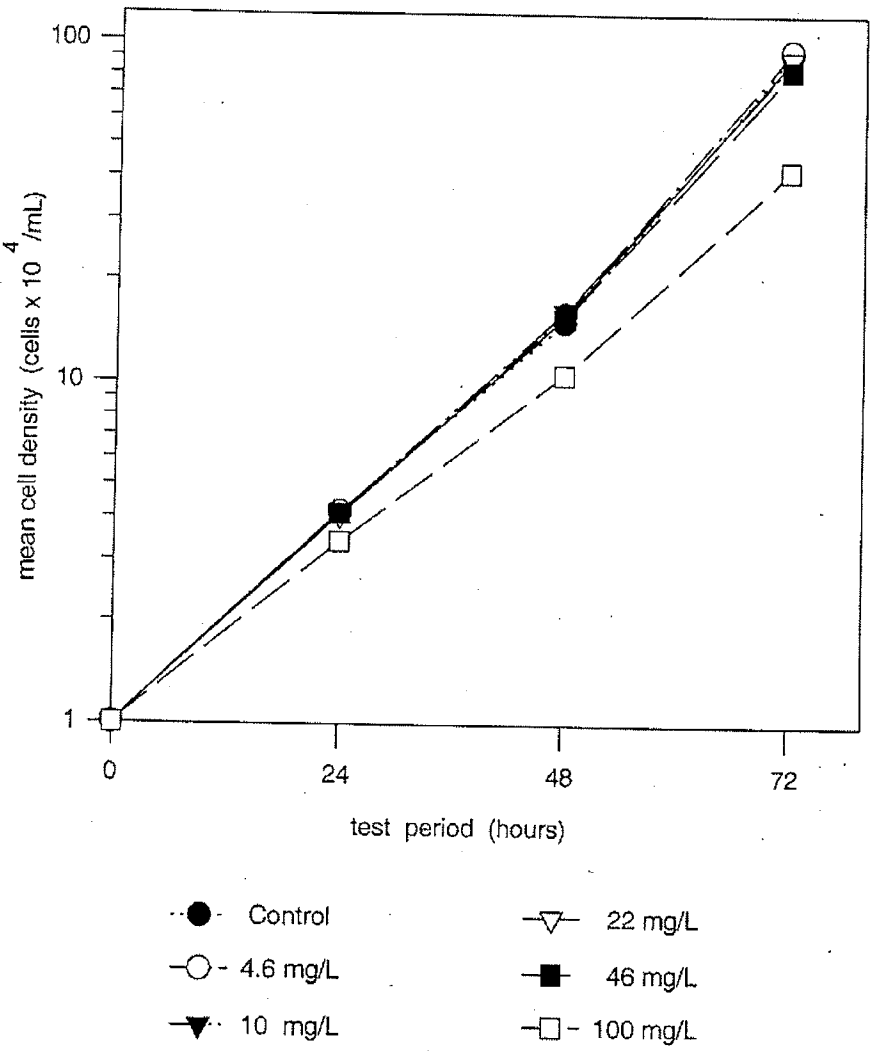
Nominal test item concentration (mg/L)	pH values	
	Start	End
Control	7.8	8.5
4.6	7.9	8.4
10	7.9	8.4
22	7.9	8.4
46	7.9	8.4
100	7.8	8.3

Table 8: Water temperature during the test period

	Temperature (°C)
Day 0 (Start)	21
Day 1	23
Day 2	22
Day 3 (End)	22

5 FIGURE

Figure 1: Growth curves of *Scenedesmus subspicatus*, incubated for 72 hours at different concentrations of the test item



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3 DUNNETT, C.W. (1955):
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4 DUNNETT, C.W. (1964):
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ATTACHMENT

ANALYTICAL REPORT

ANALYTICAL REPORT

Study Title:

TOXICITY OF [redacted]
TO *SCENEDESMUS SUBSPICATUS*
IN A 72-HOUR ALGAL GROWTH INHIBITION TEST

Subtitle of Analytical Report:

DETERMINATION OF THE CONCENTRATIONS OF
THE TEST ITEM IN TEST MEDIUM

Test Facility:

RCC Ltd
Environmental Chemistry &
Pharmanalytics Division
CH-4452 Itingen/Switzerland

RCC Study Number:

842906

Analytical Id. No.:

842907

CONTENTS

PREFACE

GENERAL

Study Title:	Toxicity of [redacted] to *Scenedesmus subspicatus* in a 72-hour algal growth inhibition test
Subtitle:	Determination of the concentrations of the test item in test medium
Sponsor:	AUSIMONT SpA Viale Lombardia 20 20021 Bollate (MI) / Italy
Monitoring Scientist:	Mrs. Ilaria Colombo
Test Facility:	RCC Ltd Environmental Chemistry & Pharmanalytics Division Zelgliweg 1 CH-4452 Itingen/Switzerland
Analytical Id. No.:	842907

RESPONSIBILITIES

Responsible for Analytics: Mr. T. Schoop

T. Schoop

Date: October 04, 2002

Technical Coordinator:	I. Wiedeking
Reporting:	M. Viererbe

SCHEDULE

Experimental starting date:	July 15, 2002
Experimental completion date:	July 18, 2002

1 PURPOSE

In this analytical report the results obtained for the concentrations of [redacted] in test medium are described.

The quantification of the test item was performed by HPLC analysis with MS-detection.

2 MATERIALS AND METHODS

The implementation of the analytical method was performed on basis of a method provided by the sponsor [1].

2.1 TEST ITEM

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

2.2 ANALYTICAL PROCEDURE

2.2.1 Storage

The samples were stored deep-frozen and protected from light until analysis was performed.

2.2.2 Reagents and Solvents

Acetonitrile	Baker, no. 9017
Ammonium carbonate	Fluka, no. 09716
Purified water for HPLC	in-house prepared by a water purification system (Millipore)
Test water	as described in the biological part of this study

[1] These experiments were not performed according to the regulations of GLP. However, the raw data or copies of the raw data will be archived under the study number of the main study.

2.2.3 Standard Solutions used for Sample Quantification

45.56 mg of the test item was dissolved in purified water and made up to the mark in a 100 mL volumetric flask to prepare a stock solution of 456 mg/L. Defined volumes of this stock solution were diluted with purified water to obtain standard solutions in the range of 0.456 to 13.7 mg/L of the test item.

These solutions were used to calibrate the HPLC-system.

2.2.4 Preparation of Spiked Test Water Samples

To demonstrate the validity of the method, untreated test water was spiked with the test item.

The test item (143.26 mg) was dissolved in purified water and made up to the mark in a 100 mL volumetric flask to prepare a stock solution of 1433 mg/L. Defined volumes of this stock solution were diluted with test water to obtain spiked test water samples of the test item with concentrations of 10.7 and 107 mg/L. These solutions were subjected to the same treatment as a sample.

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

2.2.5 Analysis of Samples

The samples from the biological test were thawed at room temperature for 2 hours and shaken mechanically to obtain homogeneous sample solutions.

Treatment samples and high-level spiked test water samples (107 mg/L of the test item)

Defined volumes (2.5 mL) of the samples were diluted to 25 mL with purified water. This leads to a dilution factor of 10.

Control samples and low-level spiked test water samples (10.7 mg/L of the test item)

The samples were not diluted prior to analysis.

Aliquots of the samples were analysed by HPLC/MS -detection.

For results obtained see Table 2.

2.3 HPLC/MS CONDITIONS

Separation Parameters

Pump System: Merck L-6200
Autosampler: Merck AS 4000

Column: X-Terra(R) MS C18; 30 x 2.1 mm; 2.5 µm

Eluent A: 0.1 % ammonium carbonate in purified water
Eluent B: acetonitrile

Gradient:

minutes	% eluent A	% eluent B
0	90	10
0.5	90	10
10.5	10	90
14.5	10	90
15	90	10
17	90	10

Injection volume: 30 µL
Flow rate: 0.5 mL/min

Detection Parameters

Detection Unit: Finnigan LCQ
Ionization Mode: ESI Negative Centroid

MS Conditions:
Capillary Voltage: - 40 V
Spray Voltage: approx. 4.5 kV
Capillary Temp: 200° C
Sheath: 70 psi N_2
Auxiliary: 20 psi N_2

Scan Mode:
SIM 5 micro 200 ms
Product m/z: 367
Isolation Width: 1.0

2.4 EVALUATION OF RESULTS

Injected samples were quantified by peak areas 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 potential function given below (equation 1). For results obtained see Table 1.
From this curve the concentration x of the test item in an injected sample was calculated by the following equation:

$$y = a \cdot x^{b} \qquad (1)$$

where

y = Peak area of test item in injected sample [counts]
x = Concentration of test item in injected sample [mg/L]
a = y-axis intercept
b = Slope

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

$$c = x \cdot D \qquad (2)$$

where

c = Concentration of test item in sample [mg/L]
x = Amount of test item in injected sample found by equation 1 [mg/L]
D = Dilution factor

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

$$R = \frac{c}{c_{nom}} \cdot 100\ \% \qquad (3)$$

where

R = Recovery [%]
c = Concentration of test item in sample found by equation 2 [mg/L]
c_{nom} = Nominal concentration of test item in sample [mg/L]

3 RESULTS AND DISCUSSION

The results obtained for the concentrations of [redacted] in test medium are presented in Table 2.

An example of the calibration data for test item-standards is given in Table 1. The R^2 fits were at least 0.9994 (optimum 1.0000). This reflects the linearity of the HPLC/MS-system within the calibration range of 0.456 – 13.7 mg/L of the test item.

Typical HPLC/MS chromatograms are shown in the attached Figures 1 to 6.

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

Concurrent with the sample analysis, recoveries of spiked test water samples in the relevant concentrations (10.7 and 107 mg/L of the test item) were performed. The average concentrations were found to be 100 %[2] and 98 % of the spiked values, with an overall mean of 99 % (n = 3). Therefore, no correction for possible losses during the analytical procedure is necessary.

The average concentrations found in the treatment samples ranged from 84 % to 90 % of the nominal concentrations.

As can be seen from Table 2, [redacted] was stable during the performance of the biological test.

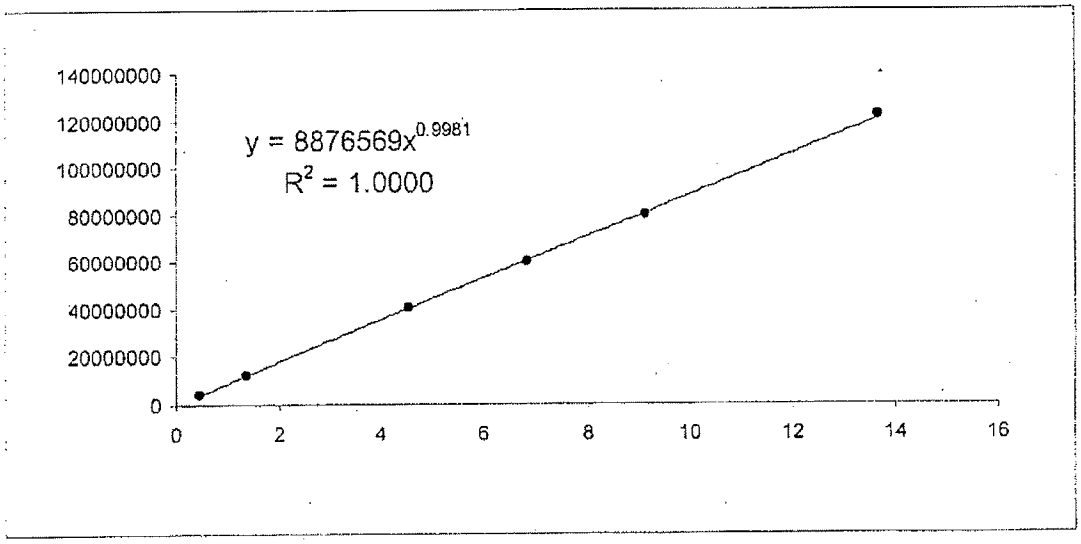
The tabulated values of the samples represent rounded results obtained by calculation using the exact raw data.

[2] single measurement

4 TABLES

Table 1: Example of calibration data of test item-standards

Standard [mg/L]	Peak area measured [counts]	Deviation from calculated value [%]
0.456	4081889	0.8
1.37	12015797	-0.9
4.56	40321982	0.0
6.83	60254534	-0.3
9.11	79882805	-0.8
13.7	122282536	1.3



where

y = Peak area of test item in injected solution [counts]

x = Concentration of test item in injected solution [mg/L]

Table 2: Results obtained for the concentrations of the test item in test medium

Nominal concentration of [redacted] [mg/L]	Sampling date [day]	Age of sample [hours]	RCC sample code	[redacted] measured [mg/L]	[% of nominal]	average [mg/L]	average [% of nominal]
Treatment samples							
46	0	0	A-9	42.0	91		
	0	0	A-10	40.9	89	41.5	90
	3	72	A-21	41.0	89		
	3	72	A-22	39.8	86	40.4	88
				mean :		40.9	89
100	0	0	A-11	92.2	92		
	0	0	A-12	88.6	89	90.4	90
	3	72	A-23	83.9	84		
	3	72	A-24	83.5	84	83.7	84
				mean :		87.1	87
Biological control samples							
0	0	0	A-1	n.d.	n.a.	n.a.	n.a.
	3	72	A-13	n.d.	n.a.	n.a.	n.a.
Spiked test water samples							
10.7		0	AZ3	10.8	100	10.8 *	100 *
107		0	AZ1	106	99		
		0	AZ2	104	97	105	98
				mean :			99
Analytical blank							
0		0	AZ5	n.d.	n.a.	n.a.	n.a.

n.d. = no test item detected
n.a. = not applicable
* = single measurement

5 FIGURES

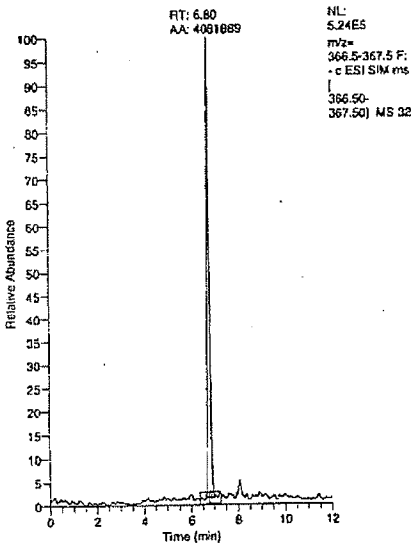


Figure 1: HPLC-chromatogram of standard solution (low-level)
Concentration: 0.456 mg/L of the test item

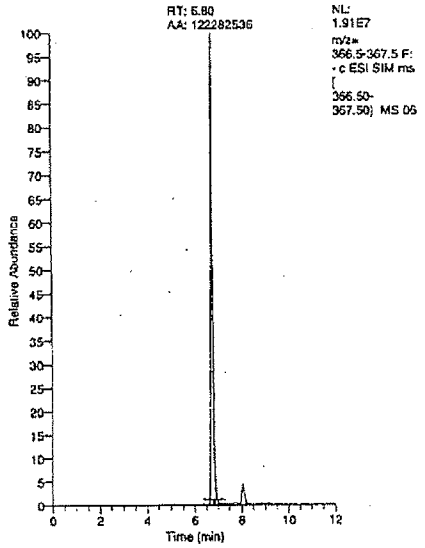


Figure 2: HPLC-chromatogram of standard solution (high-level)
Concentration: 13.7 mg/L of the test item

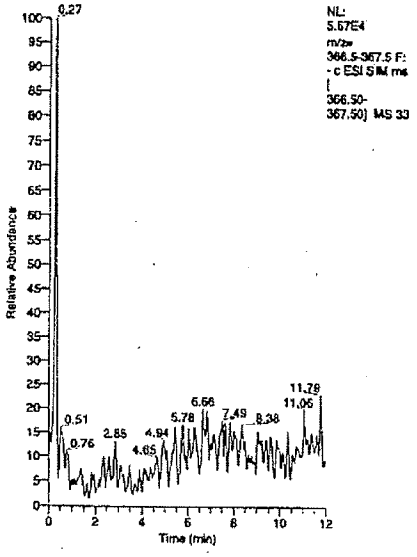


Figure 3: HPLC-chromatogram of biological control sample
Sample Id. No.: A-1
Sampling day 0; age of sample: 0 hours

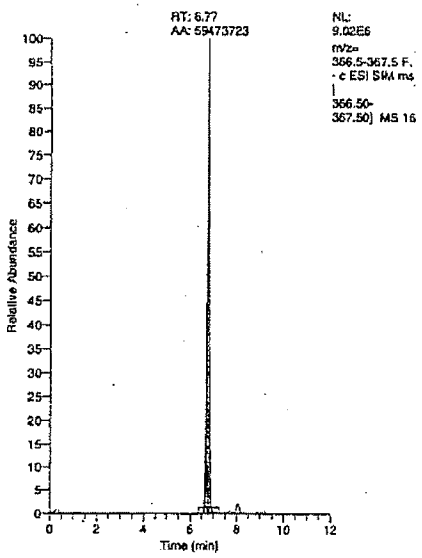


Figure 4: HPLC-chromatogram of spiked test water sample
Sample Id. No.: AZ3
(spiked with 10.7 mg/L of the test item)
Recovery: 100 % of the nominal concentration

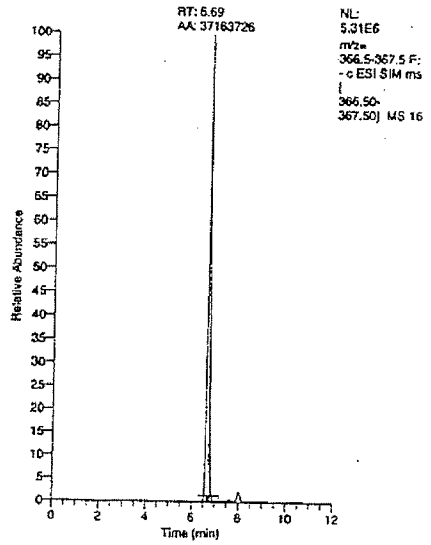


Figure 5: HPLC-chromatogram of non-aged treatment sample
Sample Id. No.: A-9
Sampling day 0; age of sample: 0 hours
(nominal concentration: 46 mg/L of the test item
nominal concentration in injected sample: 4.6 mg/L)
Recovery: 91 % of the nominal concentration

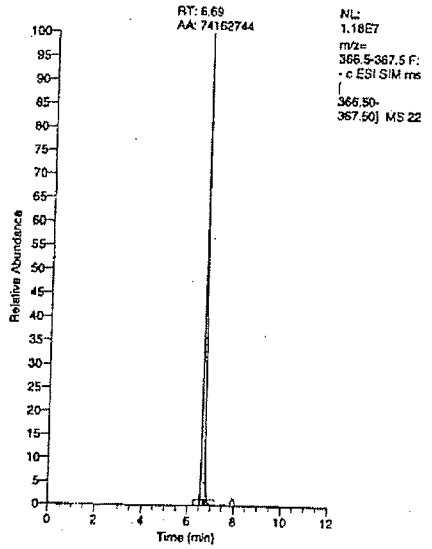


Figure 6: HPLC-chromatogram of aged treatment sample
Sample Id. No.: A-23
Sampling day 3; age of sample72 hours
(nominal concentration: 100 mg/L of the test item;
nominal concentration in injected sample: 10 mg/L)
Recovery: 84 % of the nominal concentration