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5.

TNO-report R 91/323

EFFECT OF CORROSION INHIBITOR A 251 ON THE GROWTH OF THE MARINE ALGA *SKELETONEMA COSTATUM* (ISO/DIS 10253)

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Date : April 23, 1992

Order no. : 51292

Sponsor:
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Approved by: Dr R.J. Dortland

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CONTENTS

	page
TITLE	1
CONTENTS	2
CONFIDENTIALITY STATEMENT	3
GLP COMPLIANCE STATEMENT	4
CONTRIBUTING PERSONNEL	5
QUALITY ASSURANCE STATEMENT	6
SUMMARY AND CONCLUSION	7
1. INTRODUCTION	8
2. MATERIALS AND METHODS	9
2.1 Test substance	9
2.2 Test organism	9
2.3 Test medium	9
2.4 Test methods	10
2.4.1 Preparation of test solutions	10
2.4.2 Range-finding test	10
2.4.3 Growth inhibition test	11
2.4.4 Calculation of EC values	11
2.4.5 Determination of the NOEC	12
3. RESULTS AND DISCUSSION	13
3.1 Range-finding test	13
3.2 Growth inhibition test	13
4. REFERENCES	16
5. RETENTION OF RECORDS AND SAMPLES	17
6. DEVIATIONS FROM THE PROTOCOL	17
ANNEX A SUMMARY OF THE CALCULATION METHOD	18
ANNEX B RESULTS OF THE RANGE-FINDING TEST	19
ANNEX C RESULTS OF THE GROWTH INHIBITION TEST	20
ANNEX D COMPOSITION OF ALGAL MEDIUM	22

CONFIDENTIALITY STATEMENT

This report contains confidential and proprietary information of Pumptech N.V. (and Dowell Schlumberger) which must not be disclosed to anyone except the employees of Pumptech N.V. (or Dowell Schlumberger) or to the authorities dealing with the matter without the express and written approval of Pumptech N.V. (or Dowell Schlumberger).

GLP COMPLIANCE STATEMENT

'I, the undersigned, hereby declare that the work to which this report refers was performed under my supervision according to the procedure herein described. To the best of my knowledge this report provides an accurate record of the results obtained. The study was carried out in compliance with the OECD code of Good Laboratory Practice. Characterization and verification of the test substance identity and properties is, however, the responsibility of the sponsor.'

Drs A.O. Hanstveit
Study Director

Date: April 23, 1992

CONTRIBUTING PERSONNEL

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Date: April 23, 1992

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Date: April 23, 1992

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Report no.: R91/323
Study no. : IMW-91-0090-01

QUALITY ASSURANCE STATEMENT

STUDY TITLE: Effect of Corrosion inhibitor A 251 on the growth of the marine alga skeletonema costatum (ISO/DIS 10253)

REPORT DATE: April 23, 1992

The following inspections relevant to this study have been carried out by the Quality Assurance Unit of the TNO Institute of Environmental Sciences (IMW), P. O. Box 6011, 2600 JA Delft, the Netherlands.

Type of inspection	Date and number of inspections	Date of report to Study Director
protocol:	October 2, 1991 (1)	October 2, 1991
experimental phase:	November 4, 1991 (1)	November 4, 1991
experimental phase of equivalent* studies:	October 7, 1991 (1)	October 7, 1991
	November 11, 1991 (1)	November 11, 1991
	November 18, 1991 (1)	November 18, 1991
	November 20, 1991 (1)	November 21, 1991
	November 26, 1991 (1)	November 26, 1991
report audit:	April 16, 1992 (1)	April 16, 1992

Any serious deviations were reported to management at the same time as the report to the study director; any other, less serious deviations were reported to management upon receipt of the reply from the Study Director.

* This type of study is carried out regularly by the personnel responsible for the present study; these dates refer to inspections of such equivalent studies with other test substances.

I, the undersigned, hereby declare that to the best of my knowledge this report provides an accurate record of the results obtained in this study.

M. A. Bikker
Quality Assurance Officer

Date: April 24, 1992

SUMMARY AND CONCLUSION

The toxicity of Corrosion inhibitor A 251 to the marine alga *Skeletonema costatum* was determined in a growth inhibition test according to ISO/DIS 10253: 'Water quality – Marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*' and the OECD principles of Good Laboratory Practice. Concentrations of 0, 10, 18, 32, 56, 100 and 180 $\mu g.l^{-1}$ were tested and the algal growth was determined by electronic particle counting. The effect values were calculated using a parametric model assuming an error constant per measurement.

The EC50 with respect to growth rate during logistic growth (E_rC50) was found to be 105 $\mu g.l^{-1}$, with a 95% confidence interval of 99-111 $\mu g.l^{-1}$. The corresponding EC10 and EC90 values were 54 $\mu g.l^{-1}$ and 204 $\mu g.l^{-1}$ respectively. The EC50 with respect to the area under the growth curve (E_bC50) was found to be 74 $\mu g.l^{-1}$ (in the range 56- 100 $\mu g.l^{-1}$). The corresponding E_bC10 and E_bC90 values were 26 $\mu g.l^{-1}$ (in the range 18-32 $\mu g.l^{-1}$) and 130 $\mu g.l^{-1}$ (in the range 100-180 $\mu g.l^{-1}$) respectively.

The no-observed-effect-concentration (NOEC) with respect to the effects referred to above was estimated to be 18 $\mu g.l^{-1}$.

The concentrations given are nominal concentrations of the test substance Corrosion inhibitor A 251.

1. INTRODUCTION

The toxicity of Corrosion inhibitor A 251 to the marine alga *Skeletonema costatum* was determined at sponsor's request.
The objective of the study was to determine the EC50; i.e. the concentration of Corrosion inhibitor A 251 inhibiting the growth of a logistically growing culture of algae by 50%.
The determination was conducted in accordance with ISO/DIS 10253 (1) and in compliance with the OECD principles of Good Laboratory Practice (2).

Relevant dates for the study were:
Protocol signed by the Study Director: October 2, 1991.
Period of the range-finding test: October 25-28, 1991.
Period of the final growth inhibition test: November 4-7, 1991.

2. MATERIALS AND METHOD

2.1 Test substance

The substance investigated was Corrosion inhibitor A 251, batch no. GT 901129, an amber liquid. A screw-capped 100 ml plastic flask labelled: "Sample A 251; Lot: GT 901129 from O.I.E.S. Moerdijk", with 100 ml sample was received from sponsor on October 11, 1991.
The sample was stored in a refrigerator.
Information on composition and properties of Corrosion inhibitor A 251 was provided by the sponsor; the solubility in water was stated to be 100%.

2.2 Test organism

The marine alga *Skeletonema costatum* (ISTPM P4), which belongs to the order of *Bacillariophyceae*, was used as the test organism.
The culture was supplied by Mr Cabridenc, IRCHA, BP 1, 91350 Vert-le-Petit, France.
A preculture of algae in the exponential growth phase was prepared as detailed in ISO/DIS 10253, using the test medium described in section 2.3. The organism forms chains containing several cells. As the electronic particle counter counts chains and not individual cells, the inoculum particle density was lower than presented in the guideline.

2.3 Test medium

The medium (1, see Annex D) was prepared in natural seawater with a salinity of approximately 32.8-33.2‰ and was sterilized by micropore filtration.

2.4 Test methods

2.4.1 Preparation of test solutions

Stock solutions were prepared by dissolving 500.4 mg or 62.0 mg in 250 ml (range-finding test) or 192.3 mg in 500 ml algal medium (growth inhibition test). From these stock solutions, appropriate dilutions were prepared in algal medium to yield final concentrations of 0, 0.01, 0.12, 1.2, 12.4, 124 and 1001 $mg.l^{-1}$ (range-finding test) or 0, 10, 18, 32, 56, 100 and 180 $\mu g.l^{-1}$ (growth inhibition test).

2.4.2 Range-finding test

The range-finding test was conducted as a simplified version of that described in ISO/DIS 10253 (1).

Suitable numbers of 200 ml conical test flasks were covered with silicone sponge caps, autoclaved and coded.
A suspension of algae in the algae medium containing 2×10^3 $particles.ml^{-1}$ was prepared by dilution of a preculture (section 2.2) containing 2.9×10^5 $particles.ml^{-1}$. Addition of 50 ml of this algal suspension to 50 ml of the appropriate solutions of the test substance (section 2.4.1) in the test flasks, yielded a mean inoculum particle density measured in the control cultures of 2.0×10^3 $particles.ml^{-1}$.

The test was carried out in duplicate with two controls with algae only and a single background series containing test substance without algae.
All flasks were incubated at 20 ± 1°C and shaken (100 rpm) in a Gallenkamp orbital shaker. The light intensity radiated by the fluorescent lamps was within the standard range of 60-120 $\mu mol.s^{-1}.m^{-2}$ (measured with a Bottemanne Weather Instruments Photosynthetic Radiometer RA200Q).
After 3 days of incubation one sample was taken from each flask, and the number of particles per ml in the samples was determined with the aid of a Coulter Counter model TAII.

2.4.3 Growth inhibition test

The final growth inhibition test was conducted as detailed in the protocol. This protocol was developed on the basis of ISO/DIS 10253 (1).
Test flasks, test solutions, and algal medium were prepared as detailed in sections 2.4.1 and 2.4.2.

A suspension of algae in the algal medium containing 2×10^3 particles.ml^{-1} was prepared by dilution of a preculture (section 2.2) containing 3.1×10^5 particles.ml^{-1}. Addition of 50 ml of this algal suspension to 50 ml of the appropriate solutions of the test substance (section 2.4.1) in the test flasks, yielded a mean measured inoculum particle density in the control cultures of 0.6×10^3 particles.ml^{-1}.

The test was carried out in duplicate with four controls with algae only and a single background series containing test substance without algae.
All flasks were incubated as described in section 2.4.1. One sample was taken from each flask after 0, 22.5, 45.5 and 69 hours, and the number of algal particles per ml in the samples was determined (section 2.4.2).
The pH was measured at the start (medium without algae) and after 69 h in selected cultures. The morphology of the algae was examined visually with the aid of a microscope at the start and the end of the test.

2.4.4 Calculation of EC values

The algal particle density in the duplicate test series was obtained by subtraction of the number of particles in the background control series (without algae) from the number of particles in the test series. The mean values calculated were used for further calculations.
The effect of a test substance on the growth of algae is expressed by quantities denoted as EC10, EC50 or EC90 i.e. the concentration of test substance that reduces the growth rate, the yield or the viability of the inoculum cells by 10%, 50% or 90% respectively.
In this study the EC values with respect to the growth rate during logistic growth (E_rC values), assuming an error constant per measurement, were calculated by means of a parametric model developed by Kooijman et al. (3); a summary of the method is given in Annex A.

EC values with respect to the area under the growth curve (E_bC values) were calculated by the method given in the ISO/DIS 10253 (1). The values were calculated by a linear interpolation of a plot of the percentage reduction in growth (I_A; derived from the area under the growth curve) against the log concentration of the test substance.

2.4.5 Determination of the NOEC

The 'no-observed-effect-concentration' (NOEC) was estimated by visual comparison of both the measured and calculated growth curves of the treated algal suspensions with those of the controls.

3. RESULTS AND DISCUSSION

3.1 Range-finding test

The calculated particle counts (and the background particle counts) in the various suspensions are given in Annex B, Table B1.
The range-finding test revealed that inhibiting effects could be expected at concentrations higher than 0.01 $mg.l^{-1}$.

3.2 Growth inhibition test

The variation with time of the algal particle counts (corrected for background particle counts) in the various suspensions is shown in Annex C, Tables C1-C2, as are the mean values in Annex C, Table C3. The particle counts in the background series are listed in Annex C, Table C4.
The results of the model calculations for effect on growth rate during logistic growth (E_rC values) are given in Annex C, Table C5.
The growth curves for the various concentrations of the test substance are shown in Figure 1, and the concentration-effect curves in Figure 2. The data points shown in these figures represent the mean of the particle counts in the flasks containing one concentration of test substance. The curves were obtained by parametric model calculations.
The EC50 was found to be 105 $\mu g.l^{-1}$, with a 95% confidence interval of 99-111 $\mu g.l^{-1}$.
The other parameters calculated from the data are given in Table 1.

Table 1 *The model parameters calculated from the results of the growth inhibition test.*

Parameter	Dimension	Value (95% confidence limit)	
inoculum	10^3 particles.ml^{-1}	0.55	(0.10-1.0)
yield	10^3 particles.ml^{-1}	182	(171-193)
growth rate	h^{-1}	0.12	(0.10-0.14)
EC50	µg.l^{-1}	105	(99-111)
gradient	dimensionless	3.3	(2.7-3.9)
EC10	µg.l^{-1}	54	
EC90	µg.l^{-1}	204	
E_bC10	µg.l^{-1}	26	(18-32) a)
E_bC50	µg.l^{-1}	74	(56-100) a)
E_bC90	µg.l^{-1}	130	(100-180) a)

a) range between tested concentrations

The area under the growth curves was calculated using the mean values given in Annex C, Table C3, and the results are given in Annex C, Table C6 together with the percentage reduction in growth (I_A). The E_bC values are shown in Table 1. As expected these effect values were lower than the effect values with respect to the growth rate, the latter being a biomass independent effect.

By visual comparison of the measured and the calculated growth curves of algal suspensions exposed to the test substance with those of the algal controls (see Fig. 1), the no-observed-effect-concentration (NOEC) of Corrosion inhibitor A 251 was estimated to be 18 µg.l^{-1}.

Microscopic examination of the cells at the start and end of the incubation period revealed no abnormalities. Only in an algal culture exposed to 100 µg.l^{-1} test substance, some cells contained chloroplasts coloured lighter than those in the control cultures at the end of the test.

The pH of the algal medium at the start of the test was 7.8. The medium containing different test substance concentrations remained constant during the test (pH 8.0-8.1). In the presence of algae, however, the pH of the test solutions was found to increase with algal cell density to pH 8.1-8.9.

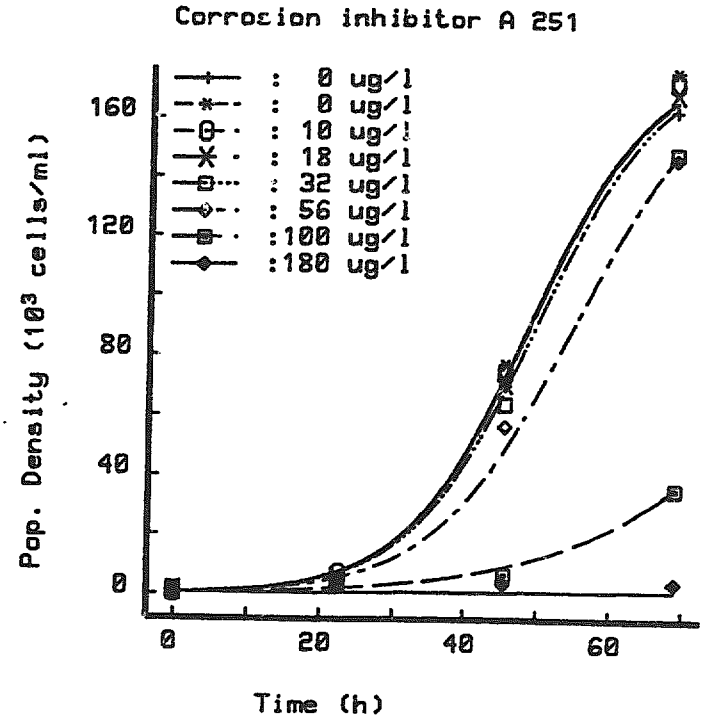

Figure 1 *Growth curves for Skeletonema costatum exposed to a range of concentrations of Corrosion inhibitor A 251.*

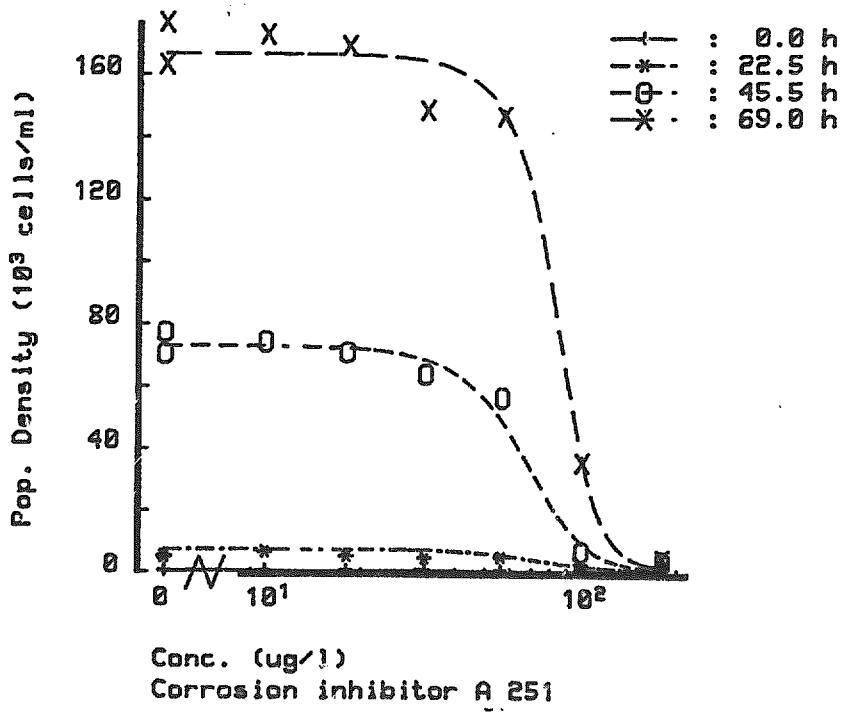

Figure 2 *Concentration-effect curves for Skeletonema costatum exposed to a range of concentrations of Corrosion inhibitor A 251.*

4. REFERENCES

1) ISO/DIS 10253: Water quality. Marine algal inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*.
Document no.: ISO/TC/147/SC5/WG5/N120.
Nederlands Normalisatie-instituut, Delft, March 1991.

2) Good Laboratory Practice in the testing of Chemicals.
OECD, Organization for Economic Cooperation and Development, Paris (1982).

3) Kooijman, S.A.L.M., A.O. Hanstveit and H. Oldersma.
Parametric analysis of population growth in bioassays.
Water Research 17, 527-538 (1983).

5. RETENTION OF RECORDS AND SAMPLES

All records concerning the observations, counts, analyses and all other information relevant to the quality and integrity of the study have been filed under the study reference IMW-91-0090-01 in the archives of the TNO Institute of Environmental Sciences, Schoemakerstraat 97, 2628 VK Delft, The Netherlands. These records will be retained for a period of at least ten years after the cover date of this report.
A sample of the test substance has been deposited under the sample reference IMW-91-0090-A in the sample archives of the TNO Institute of Environmental Sciences at the same address; this sample will be stored for a period of at least ten years.

6. DEVIATIONS FROM THE PROTOCOL

There were no deviations from the protocol.

ANNEX A SUMMARY OF THE CALCULATION METHOD

The model assumes that:

1. The number of cells in each culture increases as a logistic function of time;
2. The growth rate or growth yield decreases as a logistic function of the natural logarithm of concentration of the test substance.

The following equations were used:

$$N(t,c) = EY/[E + (Y-E) \exp\{-tR(c)\}]$$

where

$N(t,c)$ = number of cells.ml^{-1} at time t and concentration c of the test substance.
E = inoculum; number of cells.ml^{-1} in the culture time t = 0.
Y = yield; the ultimate number of cells.ml^{-1} obtained in the cultures.
$R(c)$ = the population growth rate at concentration c of the test substance.

In addition,

$$R(c) = Rb\,[1 + \exp\{Rg\,(\ln c - Re)\}]^{-1}$$

where

R_b = growth rate of the untreated cells.
R_e = natural logarithm of the EC50 value.
R_g = gradient of the growth rate function.

For cultures growing exponentially, the above equation for N(t,c) reduces to:

$$N(t,c) = E \exp\{tR(c)\}$$

The parameters E, Y, R_b, R_e, and R_g were calculated by a weighted least square fitting of the model to the results.

Calculations and curve plottings were performed by computer programs, on a HP 320 computer.

ANNEX B RESULTS OF THE RANGE-FINDING TEST

Table B1 Particle counts (10^3 particles.ml^{-1}) after 3 days incubation [a].

	Concentration of Corrosion Inhibitor A 251 ($mg.l^{-1}$)						
	0	0.01	0.12	1.2	12.4	124	1001
Set 1	172.8	195.2	7.7	1.6	11.9	3.2	2.5
Set 2	156.5	158.3	17.0	76.7	- 14.4	1.5	2.1
Mean	164.6	176.7	12.3	39.1	–	2.4	2.3
Back-ground	11.1	6.8	10.9	4.6	30.9	4.8	1.9

a) corrected for background counts

ANNEX C RESULTS OF THE GROWTH INHIBITION TEST

Table C1 *First set of particle counts (10^3 particles.ml^{-1}) a).*

Time (h)	Concentration of Corrosion Inhibitor A 251 (µg.l^{-1})							
	0	0	10	18	32	56	100	180
0.0	0.4	0.7	1.0	1.2	1.5	1.0	2.0	1.1
22.5	4.5	5.2	8.1	5.7	4.8	5.3	2.9	2.5
45.5	70.5	75.9	78.2	71.9	62.7	49.2	6.4	3.8
69.0	166.5	179.4	177.0	164.1	153.8	133.6	33.5	4.0

Table C2 *Second set of particle counts (10^3 particles.ml^{-1}) a).*

Time (h)	Concentration of Corrosion Inhibitor A 251 (µg.l^{-1})							
	0	0	10	18	32	56	100	180
0.0	0.5	0.9	0.9	1.0	0.8	1.0	0.9	1.0
22.5	5.3	5.6	5.5	5.0	4.6	5.0	2.8	2.5
45.5	70.4	78.9	70.7	70.3	65.2	64.0	7.4	4.0
69.0	160.0	173.6	168.9	174.7	143.8	160.4	37.9	5.0

Table C3 *Mean values of the particle counts (10^3 particles.ml^{-1}) a).*

Time (h)	Concentration of Corrosion Inhibitor A 251 (µg.l^{-1})							
	0	0	10	18	32	56	100	180
0.0	0.4	0.8	0.9	1.1	1.1	1.0	1.4	1.1
22.5	4.9	5.4	6.8	5.4	4.7	5.2	2.8	2.5
45.5	70.5	77.4	74.5	71.1	64.0	56.6	6.9	3.9
69.0	163.2	176.5	172.9	169.4	148.8	147.0	35.7	4.5

a) corrected for background counts

Table C4 *Background particle counts (10^3 particles.ml^{-1}).*

Time (h)	Concentration of Corrosion Inhibitor A 251 (µg.l^{-1})							
	0	0	10	18	32	56	100	180
0.0	0.5	0.3	0.1	0.1	0.3	0.1	0.1	0.1
22.5	0.5	0.1	0.1	0.1	0.2	0.1	0.1	0.1
45.5	1.7	2.0	2.3	1.8	2.5	2.6	3.1	2.1
69.0	3.6	2.5	2.3	2.3	2.9	3.4	2.8	2.3

Table C5 *Calculated values (10^3 particles.ml^{-1}).*

Time (h)	Concentration of Corrosion Inhibitor A 251 (µg.l^{-1})							
	0	0	10	18	32	56	100	180
0.0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
22.5	7.6	7.6	7.6	7.5	7.2	5.7	2.3	0.8
45.5	73.1	73.1	73.0	72.4	68.5	48.9	9.6	1.2
69.0	166.7	166.7	166.6	166.3	164.3	148.1	36.4	1.8

Table C6 *Mean area under the growth curve (A) and the percentage reduction in growth (I_A).*

Para-meter	Concentration of Corrosion Inhibitor A 251 (µg.l^{-1})							
	0	0	10	18	32	56	100	180
A	3644	3951	3865	3701	3278	3102	562	139
I_A	0		-2	3	14	18	85	96

ANNEX D COMPOSITION OF ALGAL MEDIUM

Nutrient stock solutions.

Nutrient	Concentration in stock solution	Final concentrations in test solution
Stock solution 1		
$K_3PO_4.H_2O$	3.25 $g.l^{-1}$	3.2 $mg.l^{-1}$
$NaNO_3$	50.0 $g.l^{-1}$	50.0 $mg.l^{-1}$
Stock solution 2		
$Na_2SiO_3.9H_2O$	28.4 $g.l^{-1}$	14.9 $mg.l^{-1}$
Stock solution 3		
$C_6H_8O_7Fe.3H_2O$	0.075 $g.l^{-1}$	140 $\mu g.l^{-1}$ (Fe)
$MnCl_2.4H_2O$	0.216 $g.l^{-1}$	605 $\mu g.l^{-1}$ (Mn)
$ZnSO_4.7H_2O$	2.35 $mg.l^{-1}$	150 $\mu g.l^{-1}$ (Zn)
$CuSO_4.5H_2O$	0.236 $mg.l^{-1}$	0.6 $\mu g.l^{-1}$ (Cu)
$CoCl_2.6H_2O$	0.606 $mg.l^{-1}$	1.5 $\mu g.l^{-1}$ (Co)
H_3BO_3	1.71 $g.l^{-1}$	17.1 $mg.l^{-1}$
Na_2EDTA	1.5 $g.l^{-1}$	15.0 $mg.l^{-1}$
Stock solution 4		
Thiamin hydrochloride	25 $mg.l^{-1}$	25 $\mu g.l^{-1}$
Biotin	0.005 $mg.l^{-1}$	0.005 $\mu g.l^{-1}$
B_{12}	0.05 $mg.l^{-1}$	0.05 $\mu g.l^{-1}$

The medium is prepared by making 1 ml of stock solution 1, 0.52 ml of stock solution 2, 10 ml of stock solution 3, and 1 ml of stock solution 4 up to one litre with natural seawater. The pH of this medium is pH 8.0 ± 0.2 after equilibration.

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REPORT

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ACARTIA TONSA OF

INHIBITOR AID A179

NOTOX Project 113118
NOTOX Substance 36702

STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report was conducted in compliance with the most recent edition of:

The OECD Principles of Good Laboratory Practice

which are essentially in conformity with:

The United States Food and Drug Administration. Title 21 Code of Federal Regulations Part 58.

The United States Environmental Protection Agency (FIFRA). Title 40 Code of Federal Regulations Part 160.

The United States Environmental Protection Agency (TSCA). Title 40 Code of Federal Regulations Part 792.

Study Director

Ing. M.J.E. Koopmans

M.J.E. Koopmans

Date: December 24, 1993

QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands.

Study procedures were subject to periodic inspections and general non study specific processes were also inspected at periodic intervals.

This report was audited by the NOTOX Quality Assurance Unit and the methods and results accurately reflect the raw data.

DATES OF QAU INSPECTIONS/ AUDITS	REPORTING DATES
19-11-1993	19-11-1993
08-12-1993	08-12-1993
23-12-1993	23-12-1993

Quality Assurance Manager

C.J. Mitchell B.Sc.
i.a.

Date: December 28, 1993

REPORT APPROVAL

STUDY DIRECTOR:

Ing. M.J.E. Koopmans

.....

Date: December 27, 1993

MANAGEMENT:

Ing. E.J. van de Waart
(Section Head, Genetic &
Ecotoxicology)

.....

Date: 27/12/1993

PREFACE

Sponsor	Dowell Schlumberger c/o. P.O. Box 20 4780 AA MOERDIJK The Netherlands
Study Monitor	Mr. H. Romijn
Testing Facility	NOTOX B.V. Hambakenwetering 3 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Ing. M.J.E. Koopmans
Study Plan	Start : November 30, 1993 Completed : December 09, 1993

TEST SUBSTANCE

Identification	Inhibitor aid A179
Description	Blue-green crystals
Batch	RM 930319
Purity	100%
Instructions for test substance storage	At room temperature in the dark
Stability under storage conditions	Stable
Expiry date	November 01, 1995
Stable for at least 96 hours in vehicle	Water : yes

PURPOSE

The purpose of the study is to evaluate the test substance for its ability to generate acute toxic effects in Acartia tonsa during an exposure period of 48 hours and, if possible, to determine the 48h-LC50.

GUIDELINES

The study procedure described in this protocol is based on the PARCOM Ring test protocol for the Determination of acute lethal toxicity to marine copepods, November 1990, including the alterations produced by Ms. E. Bjørnestad, January 1991.

ARCHIVING

NOTOX B.V. will archive the following data for at least 10 years: protocol, report, test substance reference sample, all specimens and raw data.

DEFINITIONS

Copepods are considered to be dead when they do not start to swim within 10 seconds after gently shaking of the test vessel. Additionally, an immobile copepod will be gently touched, if it is not sure that the organism is dead.

The LC50 is the concentration estimated to kill 50% of the copepods after a defined period of exposure.

TEST SYSTEM

Species	Acartia tonsa (Copepoda, Crustacea) (Dana)
Stage	Stage 5 or adults (ca. 3 weeks old)
Reason for selection	This system has been selected as a marine crustacean sensitive to toxic substances in the marine environment. It was one of the species tested in the PARCOM Ring test.

BREEDING / HOLDING

Breeding	The parental populations are held in natural seawater, filtered over 0.2 µm filter, at 20 ± 2°C. Oil-free air is gently bubbled in so that no turbulence occurs in the water.
Measurements	Temperature: every day. At the start of each culture: oxygen concentration.
Feeding	Acartia were fed on a diet of Rhodomonas baltica and/or Skeletonema costatum.
Eggs	Every week eggs are collected, put apart in a new vessel with natural seawater and further treated in the same way as their parents. After 3 weeks following hatching they will have developed to stage 5 or adults and can be used for the test.
Control of sensitivity	A reference test with potassium dichromate ($K_2Cr_2O_7$) is carried out every month. The results of the most recent test are appended.

RANGE-FINDING TEST

A range-finding test was performed to provide information about the range of concentrations to be used in the final test. A. tonsa were exposed for 48 hours to a concentration range of 0.1 to 100 mg/l forming a geometric progression with a factor of 10.

TEST PROCEDURE AND CONDITIONS

Test duration	48 hours.
Test vessels	50 ml, polystyrene.
Medium	Natural sea water, filtered through a 0.2 µm filter, with a salinity of 36 per mille.
Test concentrations	Based on the results of the range-finding test: 0.032, 0.056, 0.1, 0.18, 0.32, 0.56 and 1 mg/l.
Control	Medium without test substance or other additives (0 mg/l).
Number of copepods	20 per concentration, 30 in the control.
Loading	Five per vessel containing 25 ml test solution.
Illumination	16 hours photoperiod daily.
Feeding	No feeding.
Aeration	No aeration of the test solution.
Introduction of A. tonsa	The copepods were introduced directly after preparation of the test media.

PREPARATION OF TEST MEDIA

The test media were prepared using a stock solution of 100 mg/l by adding quantitatively 19.9 mg of test substance to 200 ml natural seawater. Subsequently stock solutions of 10 and 1 mg/l were prepared. An amount of test substance stock solution (1 mg/l) corresponding to the test concentrations was then added in the test medium.
The final test solutions were all clear without precipitation.

MEASUREMENTS AND RECORDINGS

Mortality	At 24 and 48 hours following the start of exposure. Dead copepods were not removed when observed.
pH and Oxygen	Daily, beginning at the start of the test, in one extra vessel without copepods at 0 mg/l and the highest test concentration.
Temperature	Daily in a vessel with water in the same room, beginning at the start of the test.

ACCEPTABILITY OF THE TEST

a) The mortality in the blank and, if relevant, in the control containing the vehicle should not exceed 10%.
b) Dissolved oxygen concentration at the end of the test should be ≥ 4 mg/l.

DATA HANDLING

Calculation of LC50: The LC50-value was calculated at 48 hours of exposure from the probits of the percentages of affected copepods and the logarithms of the corresponding nominal concentrations using the maximum likelihood estimation method (Finney, D.J., 1971: Probit analysis, Cambridge University Press, Cambridge, U.K., 3rd edition).

RESULTS

Range-finding test

In the range finding 100% mortality of A. tonsa was recorded at 1 mg/l and higher. At 0.1 mg/l 40% mortality was recorded.

Final study: mortality of copepods

The mortality data of the final test are presented in Table 1.

After 24 hours of exposure 25% mortality was recorded at 0.32 and 0.56 mg/l and 35% at 1 mg/l. No significant (>10%) mortality of A. tonsa was seen at concentrations of 0.032, 0.1 and 0.18 mg/l.
At 0.056 mg/l 15% of the copepods were immobile and did not start to swim within 10 seconds after gently shaking of the test vessel, however only 10% mortality was recorded after 48 hours. Therefore the response at 0.056 mg/l was considered as not significant.

After 48 hours of exposure mortality of all copepods exposed was seen at 1 mg/l. At 0.32 mg/l the total incidence of mortality had increased to 40% and at 0.56 mg/l to 65%. No significant (>10%) mortality of A. tonsa was seen at concentrations of 0.18 and lower.

Final study: experimental conditions

The results of measurement of pH and oxygen concentrations are presented in Table 2.
The temperature of the test medium measured in the blank control varied from 20.5 to 21°C.
The salinity (Δ2 $^{o}/_{oo}$) was slightly out of the range mentioned in the protocol, but was considered to have no effect on the integrity of this study.

Final study: acceptability of the test

In the control, not more than 10% of the copepods died or became trapped at the surface of the water. Further, all test conditions remained within the ranges prescribed by the protocol.

Determination of LC50-value

The 48h-LC50 was calculated to be nominally 0.40 mg/l with 95% confidence interval ranging from 0.33 to 0.48 mg/l (regression line: log10(conc.)= -0.40+(probit-4.99)/4.50, Table 3 and Figure 1).

CONCLUSION

Under the conditions of the present study INHIBITOR AID A179 did not induce acute mortality of Acartia tonsa at or below 0.18 mg/l after 48 hours of exposure (LCO) with a 48h-LC50 of 0.40 mg/l (95% confidence interval between 0.33 and 0.48 mg/l).

TABLE 1: Acute mortality of A. tonsa after 24 and 48 hours in the final test.

Nominal concentration (mg/l)	Vessel code	number exposed	number dead at 24 h	number dead at 48 h	Total mortality at the end of the test (%)
0	A	5	0	0	7
	B	5	0	0	
	C	5	0	0	
	D	5	0	1	
	E	5	0	1	
	F	5	0	0	
0.032	A	5	0	0	5
	B	5	0	1	
	C	5	0	0	
	D	5	0	0	
0.056	A	5	0	0	10
	B	5	2	2	
	C	5	0	0	
	D	5	1[1]	0	
0.1	A	5	2	2	10
	B	5	0	0	
	C	5	0	0	
	D	5	0	0	
0.18	A	5	0	0	5
	B	5	0	0	
	C	5	0	1	
	D	5	0	0	
0.32	A	5	1	2	40
	B	5	2	2	
	C	5	0	1	
	D	5	2	3	
0.56	A	5	2	4	65
	B	5	0	3	
	C	5	1	3	
	D	5	2	3	
1	A	5	1	5	100
	B	5	2	5	
	C	5	2	5	
	D	5	2	5	

[1] Probably not dead.

TABLE 2: pH[1] and oxygen concentrations[1] during the final test.

Nominal Conc. (mg/l)	Start (Day 0) pH	DO(mg O_2/l)	Day 1 pH	DO(mg O_2/l)	Day 2 pH	DO(mg O_2/l)
0	8.1	7.0	8.3	7.4	8.2	7.1
1	8.1	7.2	8.3	7.2	8.2	7.0

[1] Measured in an extra vessel without copepods present in the test solution.
DO = dissolved oxygen

TABLE 3: Determination of the 48h-LC50 value for INHIBITOR AID A179.

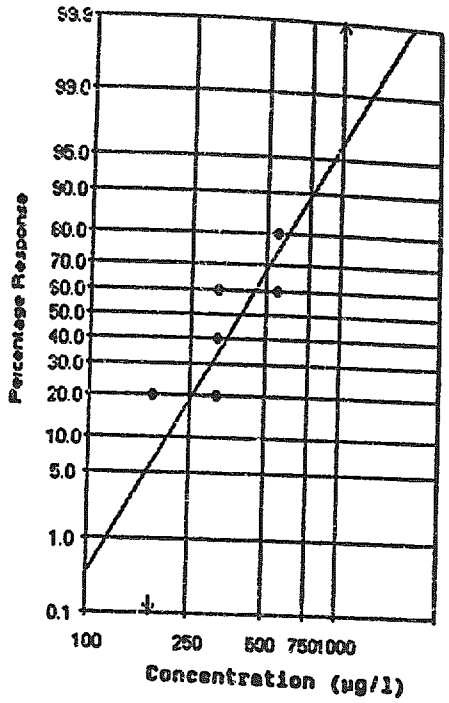
48h-LC50 = 0.40 mg/l
95% fiducial limits: 0.33 - 0.48 mg/l

index of regression significance: g=0.12
chi-squared=7.72, with 14 degrees of freedom

regression line: log10(conc.)=-0.40+(probit-4.99)/4.50

conc. mg/l	group size	response	corrected fraction	expected fraction	chi2
0.18	5	0	0.00	0.05	0.28
0.18	5	0	0.00	0.05	0.28
0.18	5	1	0.20	0.05	2.13
0.18	5	0	0.00	0.05	0.28
0.32	5	2	0.40	0.34	0.08
0.32	5	2	0.40	0.34	0.08
0.32	5	1	0.20	0.34	0.43
0.32	5	3	0.60	0.34	1.52
0.56	5	4	0.80	0.75	0.06
0.56	5	3	0.60	0.75	0.61
0.56	5	3	0.60	0.75	0.61
0.56	5	3	0.60	0.75	0.61
1	5	5	1.00	0.96	0.18
1	5	5	1.00	0.96	0.18
1	5	5	1.00	0.96	0.18
1	5	5	1.00	0.96	0.18
					7.72

FIGURE 1: Percentage response (=mortality) of Acartia tonsa as function of the log concentration of INHIBITOR AID A179 at 48h.



REFERENCE TEST

NOTOX PCAA-12 (BATCH: A)

Acute toxicity study in the A. tonsa with potassium dichromate.

From 09-11-1993 to 11-11-1993

This reference test was carried out to check the sensitivity of the test system as used by NOTOX. The reference substance potassium dichromate was tested under similar conditions as the final test with the test substance, using copepods from the same culture.

The positive control substance, potassium dichromate ($K_2Cr_2O_7$, art. 4864) was obtained from Merck, Darmstadt, Germany.

Concentrations: 1.0, 3.0, 6.0, 10, 20, 30 and 60 mg/l in natural seawater.
Control: Natural seawater without test substance.

Acute mortality of A. tonsa after 24 and 54½ hours in the reference test with potassium dichromate:

Concentration mg/l	total number exposed	total number dead at 24h[1]	at 54½h
0.0	30	3[2]	3
1.0	10	0	2
3.0	10	1	1
6.0	10	1	3
10	10	1	4
20	10	2	6
30	10	5	8
60	10	7	10

[1] At t=24h the vessel was gently touched instead of the copepods, to make sure that the organism was dead.
[2] In two vessels two copepods (one in each vessel) were probably not dead at t=24h, but immobile. In these two vessels no dead copepods were scored at t=54½h. This has no influence on the 24h-LC50 value.

The relation between test concentration and response is in agreement with what should be expected.

The 24h-LC50 was 35.44 mg/l (95% fiducial limits: 23.38 - 82.96 mg/l).
The 54½h-LC50 was 11.94 mg/l (95% fiducial limits: 7.69 - 17.95 mg/l).

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