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Contractor	NO TOX B.V.		
Document Title	INITIAL SUBMISSION: LETTER FROM AKZO NOBEL, ELF ATOCHEM NA, AND AZTEC PEROXIDES INC TO USEPA REPORTING TOXICITY STUDIES OF PEROXIDES, WITH ATTACHMENTS AND DATED 4/28/2000		
Chemical Category	TRIGONOX 23 - C 75; TRIGONOX 44B; LUCIDOL		

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BEHQ-0500-14713



Polymer Chemicals

April 28, 2000

Document Control Officer  
Attn: Section 8(e) Coordinator  
TS-790  
EPA/OPPT  
401 M Street, SW  
Washington, DC 20460

MR 35253

RECEIVED  
OPPT/CBIC  
2000 MAY -1 AM 11:32

Re: TSCA Section 8(e) Notification

Dear Sir/Madam:

Akzo Nobel Polymer Chemicals LLC (PC LLC), Elf Atochem NA, and Aztec Peroxides Inc. are submitting this notification in accordance with TSCA Section 8(e) and EPA's Statement of Interpretation and Enforcement Policy 43, Fed. Reg. 1110 (March 16, 1978). The studies being submitted in this notification were conducted in Europe as a part of a voluntary testing consortium formed by the companies.

Four studies are being submitted in this notification, as follows:

- (1) NOTOX Project 273498 report (Contact hypersensitivity of CAS# 26748-41-4)
- (2) NOTOX Project 273511 report (Contact hypersensitivity of CAS# 37187-22-7)
- (3) NOTOX Project 273487 report (Dermal irritation of CAS# 26748-41-4)
- (4) Akzo Nobel Report# ICS-103 (Algal aquatic toxicity of CAS# 94-36-0)

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OPPT/CBIC  
2000 MAY 11 AM 10:20

Sincerely,

Contain NO CBI

Edwin C. Bisinger Jr., M.S., DABT  
Product Regulatory Manager  
Akzo Nobel Polymer Chemicals LLC  
Telephone: 312-906-7639



BEHQ-00-14713



88000000152

Akzo Nobel Polymer Chemicals LLC  
300 South Riverside Plaza  
Chicago, Illinois 60606-6697  
Tel: (312) 906 7500  
Fax: (312) 906 7681

A 04

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2000 MAY -1 AM 11: 02

## REPORT

**ASSESSMENT OF CONTACT HYPERSENSITIVITY TO**

**TRIGONOX 23 - C 75**

**IN THE ALBINO GUINEA PIG**

**(MAXIMISATION-TEST)**

CAS: 26748-41-4

**NOTOX Project 273498  
NOTOX Substance 94032**

STATEMENT OF GLP COMPLIANCE

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

The study described in this report has been correctly reported and was conducted in compliance with the most recent edition of:

*The OECD Principles of Good Laboratory Practice* which are essentially in conformity with:

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

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

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

Japanese Ministry of Agriculture, Forestry and Fisheries. 59 NohSan, Notifications No. 3850.

Japanese Ministry of International Trade and Industry. Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85.

Japanese Ministry of Health and Welfare. Ordinance No.21.

Study Director:  
Drs. A. van Huygevoort



Date: 9 February 2000

Management:  
Drs. W.J.A.M. Frieling



Date: 9 February 2000

QUALITY ASSURANCE STATEMENT

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

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

The dates of Quality Assurance inspections and audits are given below.  
During the on-site inspections procedures applicable to this type of study were inspected.

DATES OF QAU INSPECTIONS/AUDITS	REPORTING DATES
on-site inspection(s)	
04 January 2000	04 January 2000
protocol inspection(s)	
28 October 1999	28 October 1999
report audit(s)	
07 February 2000	07 February 2000

Head of Quality Assurance:

C.J. Mitchell B.Sc.



Date: 10-2-2000

**SUMMARY**

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**Assessment for Contact Hypersensitivity to TRIGONOX 23 - C 75 in the Albino Guinea Pig (Maximisation Test).**

The study was carried out based on the guidelines described in: EC Commission Directive 96/54/EC, Part B.6, "Skin Sensitisation" and OECD No. 406, "Skin Sensitisation", and based on the method described by Magnusson and Kligman, "Allergic Contact Dermatitis in the Guinea Pig - Identification of Contact Allergens".

Test substance concentrations selected for the main study were based on the results of a preliminary study.

In the main study, ten experimental animals were intradermally injected with and epidermally exposed to a 100% concentration. Five control animals were similarly treated, but with vehicle alone (water). Two weeks after the epidermal application all animals were challenged with a 100% test substance concentration and the vehicle.

In the challenge phase in response to the 100% test substance concentration, skin reactions varying between grades 1 and 4 were observed in all experimental animals and skin reactions of grade 1 were observed in all control animals.

Eschar formation was seen in the treated skin sites among the experimental animals.

Taking into account the intensity of the responses and comparing these with the skin reactions seen in the control animals, it was considered that hypersensitivity had been induced in nine (of the ten) experimental animals. The skin reactions as observed in the control animals were considered to be non-specific signs of irritation.

These results indicate a sensitisation rate of 90 per cent.

Based on these results and according to the EC criteria for classification and labelling requirements for dangerous substances and preparations (Guidelines in Commission Directive 93/21/EEC), TRIGONOX 23 - C 75 should be labelled as: may cause sensitisation by skin contact (R 43).

**PREFACE**

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<b>Sponsor</b>	Akzo Nobel BU BU Polymer Chemicals P.O. Box 9300 6800 SB ARNHEM The Netherlands
<b>Study Monitor</b>	Dr. C.L.J. Braun
<b>Testing Facility</b>	NOTOX B.V. Hambakenwetering 3 5231 DD 's-Hertogenbosch The Netherlands
<b>Study Director</b>	Drs. A. van Huygevoort
<b>Study Plan</b>	Start : 7 December 1999 End : 15 January 2000

**TEST SUBSTANCE**

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The sponsor is responsible for all test substance data unless determined by NOTOX.

<b>Identification</b>	TRIGONOX 23 - C 75
<b>Description</b>	Colourless liquid
<b>Batch</b>	04199094130293
<b>Purity</b>	74.7 %
<b>Test substance storage</b>	In deep freezer in the dark
<b>Stability under storage conditions</b>	Not indicated
<b>Expiry date</b>	04 October 2000 (Allocated by NOTOX, 1 year after receipt of the test substance)
<b>Stability in vehicle</b>	Not indicated
• Propylene glycol	
<b>Vehicle</b>	Propylene glycol
<b>Rationale</b>	The vehicle was selected based on a pretest performed at NOTOX.
<b>Preparation</b>	When required, the test substance formulations (w/w) were prepared within 4 hours prior to each treatment. No correction was made for the density of the vehicle. Homogeneity was obtained to visually acceptable levels.

## PURPOSE AND RATIONALE

The purpose of this study was to evaluate whether the test substance induces contact hypersensitivity in guinea pigs after intradermal and epidermal exposure of the animals under the conditions described in this report.

This study should provide a rational basis for risk assessment in man.

The Maximisation test is selected because it is regarded as the most sensitive and the preferred method with regard to testing for sensitisation potential.

## GUIDELINES

As required by the Dutch Act on Animal Experimentation, the study protocol was reviewed and agreed by the Article 14-functionary and the Ethical Committee of NOTOX. The study procedures described in this report were based on the following guidelines and test method:

European Community (EC), Council Directive 67/548/EEC, Annex V, Part B, Methods for the Determination of Toxicity, as last amended by Commission Directive 96/54/EC, Annex IV C, B.6: "Skin sensitisation", Official Journal of the European Communities No. L 248, 1996.

Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals, Section 4, Health Effects, No.406, "Skin Sensitisation", Paris Cedex, 1992.

"Allergic Contact Dermatitis in the Guinea-Pig: Identification of Contact Allergens" Magnusson B. Kligman A.M., 1970 published by C.C. Thomas, Springfield, Illinois, USA.

## ARCHIVING

NOTOX B.V. will archive for at least 10 years raw data, protocol, report and test substance reference sample. No data will be withdrawn without the sponsor's written consent.

## TEST SYSTEM

Species	Dunkin Hartley strain, albino guinea pig (SPF-quality) Recognised by international guidelines as the recommended test system (e.g. OECD, EC). Source: Charles River Deutschland, Kisslegg Germany.
Number of animals	Experimental group: 10 females. Control group: 5 females. (females were nulliparous and non-pregnant).
Age and body weight	Young adult animals (approx. 4 weeks old, individual body weights < 500 grams) were selected.
Identification	Ear tattoo.
Reliability check	The results of a reliability test performed not more than 6 months previously are summarised in the Appendix. Similar procedures were used in the reliability test and in this study.

## ANIMAL HUSBANDRY

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### Conditions

A controlled environment was maintained in the room with optimal conditions considered as being approximately 15 air changes per hour, a temperature of 21°C, a relative humidity of 30-70% and 12 hours artificial fluorescent light and 12 hours dark per day. Deviations from these optimal conditions were noted, but were considered not to have affected study integrity.

### Accommodation

Group housing of 5 animals per labelled metal cage with wire-mesh floors and equipped with an automatic drinking system (ITL, Bergen, The Netherlands). The acclimatisation period was at least 5 days before the start of treatment under laboratory conditions.

### Diet

Free access to standard guinea pig diet, including ascorbic acid (1000 mg/kg); (Charles River Breeding and Maintenance Diet for Guinea Pigs, Altromin, Lage, Germany). Certificates of analysis were examined and retained in the NOTOX archives. Hay (B.M.I., Helmond, The Netherlands) was provided once a week.

### Water

Free access to tap water. Certificates of quarterly analysis for tap-water were examined and retained in the NOTOX archives.

## PRELIMINARY IRRITATION STUDY

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A preliminary irritation study was conducted in order to select test substance concentrations to be used in the main Study. The selection of concentrations was based on the following criteria:

- The concentrations are well-tolerated by the animals.
- For the induction exposures: the highest possible concentration that produced mild to moderate irritation (grades 2 - 3).
- For challenge exposure: the maximum non-irritant concentration.

Series of test substance concentrations were tested. Practical feasibility of administration determined the highest starting-concentration for each route. The starting- and subsequent concentrations were taken from the series: 100% (undiluted), 50%, 20%, 10%, 5%, 2%, 1% and if needed, further lower concentrations using the same steps.

The test system and procedures were identical to those used during the main study, unless otherwise specified. The six animals selected were between 4 and 9 weeks old. No body weights were determined at termination.

### Intradermal injections:

A series of four test substance concentrations was used; the highest concentration being the maximum concentration that could technically be injected. Each of two animals received two different concentrations in duplicate (0.1 ml/site) in the clipped scapular region. The resulting dermal reactions were assessed 24 and 48 hours after treatment.

### Epidermal application:

A series of four test substance concentrations was used: the highest concentration being the maximum concentration that could technically be applied. Two different concentrations were applied (0.5 ml each) per animal to the clipped flank, using Metalline patches# (2x3 cm) mounted on Medical tape\*, which were held in place with Micropore tape\* and subsequently Coban elastic bandage\*. The initially used animals receiving intradermal injections were treated with the lowest concentrations and two further animals with the highest concentrations. After 24 hours, the dressing was removed and the skin cleaned of residual test substance.

The resulting dermal reactions were assessed for irritation 24 and 48 hours after exposure. Based on the results in the initially treated animals, two additional animals were treated in a similar manner with two lower concentrations at a later stage.

#. Suppliers: Lohmann GmbH, Neuwied, Germany (Coban) and 3M, St. Paul, Minnesota, U.S.A. (Metalline and Micropore)

## MAIN STUDY

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### INDUCTION - Experimental animals

Day 1 The scapular region was clipped and three pairs of intradermal injections (0.1 ml/site) were made in this area as follows:

- A) A 1:1 w/w mixture of Freund's Complete Adjuvant (Difco, Detroit, U.S.A.) with water for injection (Fresenius AG, Bad Homburg, Germany).
- B) The test substance at a 100% concentration.
- C) A 1:1 w/w mixture of the undiluted test substance and Freund's Complete Adjuvant.

Note: One of each pair was on each side of the midline and from cranial A) to caudal C).

Day 3 The dermal reactions caused by the intradermal injections were assessed for irritation.

Day 8 The scapular area was treated with 0.5 ml of a 100% test substance concentration using a Metalline patch (2x3 cm) mounted on Medical tape, which was held in place with Micropore tape and subsequently Coban elastic bandage.

The dressing was removed after 48 hours exposure, the skin cleaned of residual test substance and the dermal reactions caused by the epidermal exposure were assessed for irritation.

### INDUCTION - Control animals

The control animals were treated as described for the experimental animals except that, instead of the test substance, vehicle alone was administered.

### CHALLENGE - All animals

Day 21 One flank of all animals was clipped and treated by epidermal application of a 100% test substance concentration and the vehicle (0.15 ml each), using Patch Test Plasters (Leukotest®, Beiersdorf Medical, Almere, The Netherlands). The patches were held in place with Micropore tape and subsequently Coban elastic bandage.

The dressing was removed after 24 hours exposure and the skin cleaned of residual test substance and vehicle. The treated sites were assessed for challenge reactions 24 and 48 hours after removal of the dressing.

**OBSERVATIONS**

<b>Mortality/Viability</b>	Twice daily
<b>Toxicity</b>	At least once daily.
<b>Body weights</b>	Prior to start and at termination of the study.
<b>Necropsy</b>	The moribund animal was subjected to necropsy for gross macroscopic examination.
<b>Irritation</b>	Skin reactions were graded according to the following numerical scoring systems. Furthermore, a description of all other (local) effects was recorded. Whenever necessary, the treated skin-areas were clipped at least 3 hours before the next skin reading to facilitate scoring.

**Grading Irritation Reactions\* :****Erythema and eschar formation:**

No erythema.....	0
Slight erythema (barely perceptible) .....	1
Well-defined erythema.....	2
Moderate erythema .....	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth) .....	4

**Oedema formation:**

No oedema.....	0
Slight oedema (barely perceptible) .....	1
Well-defined oedema (edges of area well-defined by definite raising) .....	2
Moderate oedema (raised approximately 1 millimeter).....	3
Severe oedema (raised more than 1 millimeter and extending beyond the area of exposure) 4	

(\* Intradermal reactions were assessed for erythema only or, if necrosis is present, the diameter of necrosis.)

**Grading Challenge Reactions:**

No visible change .....	0
Discrete or patchy erythema.....	1
Moderate and confluent erythema .....	2
Moderate erythema and swelling .....	3
Intense erythema and swelling .....	4

After the end of the study all animals were killed by asphyxiation using an oxygen/carbon dioxide procedure.

**INTERPRETATION**

The results for the experimental animals at the challenge phase were compared with the results for the control animals.

Positive skin reactions (grade 1 or more) were considered signs of sensitisation, provided that such reactions were not observed or were less persistent in the control group.

A sensitisation rate (%) was calculated as follows: the number of sensitised animals as a proportion of the total number of animals in the experimental group.

The results were evaluated according to the EC criteria for classification and labelling requirements for dangerous substances and preparations (Guidelines in Commission Directive 93/21/EEC).

**RESULTS****PRELIMINARY IRRITATION STUDY**

The results of the intradermal injections and epidermal exposures for the selection of suitable test substance concentrations for the main study are described in Table 1.

The necrosis seen at intradermal injection of the concentrations was considered to be caused mainly by propylene glycol rather than the test substance.

Based on the results, the test substance concentrations selected for the Main Study were a 5% concentration for the intradermal induction and a 100% concentration for the epidermal induction exposure.

A 5% test substance concentration was selected for the challenge phase.

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effect

**MAIN STUDY****Induction phase**

The skin effects caused by the intradermal injections and epidermal exposure during the induction phase are given in Table 2. The signs of necrosis seen in the control animals after the intradermal injection with vehicle only were in consistency with the results in the preliminary irritation study after injection of propylene glycol.

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**Challenge phase**

Inadvertently, a 100% test substance concentration was used, instead of the selected 5% concentration. Since the results of the challenge clearly indicated sensitisation of experimental animals, this deviation was considered not to have affected the study integrity.

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In response to the 100% test substance concentration, skin reactions varying between grades 1 and 4 were observed in all experimental animals and skin reactions of grade 1 were observed in all control animals (see Table 3).

Eschar formation was seen in the treated skin sites among the experimental animals.

**Toxicity / Mortality**

No mortality occurred and no symptoms of systemic toxicity were observed in the animals of the main study.

**Body Weights**

Body weights and body weight gain of experimental animals remained in the same range as controls over the study period (see Table 4).

**CONCLUSION**

Taking into account the intensity of the responses and comparing these with the skin reactions seen in the control animals, it was considered that hypersensitivity had been induced in nine (of the ten) experimental animals. The skin reactions as observed in the control animals were considered to be non-specific signs of irritation.

These results indicate a sensitisation rate of 90 per cent.

Based on these results and according to the EC criteria for classification and labelling requirements for dangerous substances and preparations (Guidelines in Commission Directive 93/21/EEC), TRIGONOX 23 - C 75 should be labelled as: may cause sensitisation by skin contact (R 43).

TABLE 1: PRELIMINARY IRRITATION STUDY

## SKIN REACTIONS AFTER INTRADERMAL INJECTION

Animal number	Conc %	24 hours after injection		48 hours after injection	
		Erythema (grade)	Necrosis (mm)	Erythema (grade)	Necrosis (mm)
18	100		5		6
	50		6		6
19	20		6		6
	10		6		6

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## SKIN REACTIONS AFTER EPIDERMAL EXPOSURE

Animal number	Body weight (gram)	Conc. %	24 hours after exposure		48 hours after exposure	
			Erythema (grade)	Oedema (grade)	Erythema (grade)	Oedema (grade)
16	318	100	3 p	0	2 p	0
		50	3 p	0	2 p	0
17	324	100	2 p	0	2 p	0
		50	2 p	0	2 p	0
18	334	20	1 p	0	1 p	0
		10	1 p	0	1 p	0
19	321	20	1 p	0	1 p	0
		10	1 p	0	1 p	0
146	375	5	0 p	0	0 p	0
		2	0 p	0	0 p	0
147	360	5	0 p	0	0 p	0
		2	0 p	0	0 p	0

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## REPORT

**ASSESSMENT OF CONTACT HYPERSENSITIVITY TO**

**TRIGONOX 44B**

**IN THE ALBINO GUINEA PIG**

**(MAXIMISATION-TEST)**

CAS No.: 37187-22-7

NOTOX Project 273511  
NOTOX Substance 94041

STATEMENT OF GLP COMPLIANCE

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

The study described in this report has been correctly reported and was conducted in compliance with the most recent edition of:

*The OECD Principles of Good Laboratory Practice* which are essentially in conformity with:

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

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

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

Japanese Ministry of Agriculture, Forestry and Fisheries. 59 NohSan, Notifications No. 3850.

Japanese Ministry of International Trade and Industry. Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85.

Japanese Ministry of Health and Welfare. Ordinance No.21.

Study Director:  
Ers. A. van Huygevoort

Management:  
Drs. W.J.A.M. Frieling



Date: 9 February 2000



Date: 9 February 2000

QUALITY ASSURANCE STATEMENT

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

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

The dates of Quality Assurance inspections and audits are given below.  
During the on-site inspections procedures applicable to this type of study were inspected.

DATES OF QAU INSPECTIONS/AUDITS	REPORTING DATES
on-site inspection(s)	
22 November 1999	22 November 1999
04 January 2000	04 January 2000
protocol inspection(s)	
28 October 1999	28 October 1999
report audit(s)	
07 February 2000	07 February 2000

Head of Quality Assurance:

C.J. Mitchell B.Sc.



Date: 10-2-2000

## SUMMARY

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**Assessment for Contact Hypersensitivity to TRIGONOX 44B in the Albino Guinea Pig (Maximisation Test).**

The study was carried out based on the guidelines described in: EC Commission Directive 96/54/EC, Part B.6, "Skin Sensitisation" and OECD No. 406, "Skin Sensitisation", and based on the method described by Magnusson and Kligman, "Allergic Contact Dermatitis in the Guinea Pig - Identification of Contact Allergens".

Test substance concentrations selected for the main study were based on the results of a preliminary study.

In the main study, ten experimental animals were intradermally injected with a 5% concentration and epidermally exposed to a 100% concentration. Five control animals were similarly treated, but with vehicle alone (water). Approximately 24 hours before the epidermal induction exposure all animals were treated with 10% SDS.

Two weeks after the epidermal application the animals were challenged with a 100% test substance concentration and the vehicle.

In the challenge phase, skin reactions of grade 4 were observed in all (of nine) experimental animals in response to the 100% test substance concentration.

No skin reactions were evident in the control animals.

Eschar formation or scabs were seen in all treated skin sites among the experimental animals.

One experimental animal was removed from the study on day 22 following the observation of piloerection, emaciation, watery discharge from the eyes, rales, dirty teeth and the animal felt cold. Macroscopic post-mortem examination showed scabs and/or alopecia on back and abdomen and pelvic dilation of the left kidney.

It was considered that the death of this animal was incidental and that the study outcome, based on the healthy surviving animals, was not adversely affected.

No further mortality occurred and no further symptoms of systemic toxicity were observed in the remaining animals of the main study.

The skin reactions observed in response to a 100% test substance concentration in all (of the nine) experimental animals in the challenge phase were considered indicative of sensitisation, based on the absence of any response in the control animals.

These results indicate a sensitisation rate of 100 per cent.

Based on these results and according to the EC criteria for classification and labelling requirements for dangerous substances and preparations (Guidelines in Commission Directive 93/21/EEC), TRIGONOX 44B should be labelled as: may cause sensitisation by skin contact (R 43).

**PREFACE**

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<b>Sponsor</b>	Akzo Nobel BU BU Polymer Chemicals P.O. Box 9300 6800 SB ARNHEM The Netherlands
<b>Study Monitor</b>	Dr. C.L.J. Braun
<b>Testing Facility</b>	NOTOX B.V. Hambakenwetering 3 5231 DD 's-Hertogenbosch The Netherlands
<b>Study Director</b>	Drs. A. van Huygevoort
<b>Study Plan</b>	Start : 23 November 1999 End : 7 January 2000

**TEST SUBSTANCE**

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The sponsor is responsible for all test substance data unless determined by NOTOX.

<b>Identification</b>	TRIGONOX 44B
<b>Description</b>	Colourless liquid
<b>Batch</b>	0419906045251
<b>Purity</b>	4.1 % Active O <sub>2</sub>
<b>Test substance storage</b>	At room temperature in the dark
<b>Stability under storage conditions</b>	Not indicated
<b>Expiry date</b>	04 October 2000 (Allocated by NOTOX, 1 year after receipt of the test substance)
<b>Stability in vehicle</b>	
• Water	Not indicated
<b>Vehicle</b>	Water (Milli-U)
<b>Rationale</b>	The vehicle was selected based on a pretest performed at NOTOX.
<b>Preparation</b>	When required, the test substance formulations (w/w) were prepared within 4 hours prior to each treatment. Homogeneity was obtained to visually acceptable levels.

## PURPOSE AND RATIONALE

The purpose of this study was to evaluate whether the test substance induces contact hypersensitivity in guinea pigs after intradermal and epidermal exposure of the animals under the conditions described in this report.

This study should provide a rational basis for risk assessment in man.

The Maximisation test is selected because it is regarded as the most sensitive and the preferred method with regard to testing for sensitisation potential.

## GUIDELINES

As required by the Dutch Act on Animal Experimentation, the study protocol was reviewed and agreed by the Article 14-functionary and the Ethical Committee of NOTOX. The study procedures described in this report were based on the following guidelines and test method:

European Community (EC), Council Directive 67/548/EEC, Annex V, Part B, Methods for the Determination of Toxicity, as last amended by Commission Directive 96/54/EC, Annex IV C, B.6: "Skin sensitisation", Official Journal of the European Communities No. L 248, 1996.

Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals, Section 4, Health Effects, No.406, "Skin Sensitisation", Paris Cedex, 1992.

"Allergic Contact Dermatitis in the Guinea-Pig: Identification of Contact Allergens" Magnusson B. Kligman A.M., 1970 published by C.C. Thomas, Springfield, Illinois, USA.

## ARCHIVING

NOTOX B.V. will archive for at least 10 years raw data, protocol, report and test substance reference sample. No data will be withdrawn without the sponsor's written consent.

## TEST SYSTEM

Species	Himalayan strain, albino guinea pig (SPF-quality) Recognised by international guidelines as the recommended test system (e.g. OECD, EC). Source: BRL Ltd., Basel, Switzerland.
Number of animals	Experimental group: 10 females. Control group: 5 females. (females were nulliparous and non-pregnant).
Age and body weight	Young adult animals (approx. 4 weeks old, individual body weights < 500 grams) were selected.
Identification	Ear tattoo.
Reliability check	The results of a reliability test performed not more than 6 months previously are summarised in the Appendix. Similar procedures were used in the reliability test and in this study.

### ANIMAL HUSBANDRY

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#### Conditions

A controlled environment was maintained in the room with optimal conditions considered as being approximately 15 air changes per hour, a temperature of 21°C, a relative humidity of 30-70% and 12 hours artificial fluorescent light and 12 hours dark per day. Deviations from these optimal conditions were noted, but were considered not to have affected study integrity.

#### Accommodation

Group housing of 5 animals per labelled metal cage with wire-mesh floors and equipped with an automatic drinking system (ITL, Bergen, The Netherlands). The acclimatisation period was at least 5 days before the start of treatment under laboratory conditions.

#### Diet

Free access to standard guinea pig diet, including ascorbic acid (1000 mg/kg); (Charles River Breeding and Maintenance Diet for Guinea Pigs, Altromin, Lage, Germany). Certificates of analysis were examined and retained in the NOTOX archives. Hay (B.M.I., Helmond, The Netherlands) was provided once a week.

#### Water

Free access to tap water. Certificates of quarterly analysis for tap-water were examined and retained in the NOTOX archives.

### PRELIMINARY IRRITATION STUDY

---

A preliminary irritation study was conducted in order to select test substance concentrations to be used in the main Study. The selection of concentrations was based on the following criteria:

- The concentrations are well-tolerated by the animals.
- For the induction exposures: the highest possible concentration that produced mild to moderate irritation (grades 2 - 3).
- For challenge exposure: the maximum non-irritant concentration.

Series of test substance concentrations were tested. Practical feasibility of administration determined the highest starting-concentration for each route. The starting- and subsequent concentrations were taken from the series: 100% (undiluted), 50%, 20%, 10%, 5%, 2%, 1% and if needed, further lower concentrations using the same steps.

The test system and procedures were identical to those used during the main study, unless otherwise specified. The five animals selected were between 4 and 9 weeks old and the body weights of some animals did exceed 500 grams. No body weights were determined at termination.

#### Intradermal injections:

Initially, a series of four test substance concentrations was used; the highest concentration being the maximum concentration that could technically be injected. Each of two animals received two different concentrations in duplicate (0.1 ml/site) in the clipped scapular region. The resulting dermal reactions were assessed 24 and 48 hours after treatment. Based on the results in the initially treated animals, one additional animal was treated in a similar manner with two lower concentrations at a later stage.

#### Epidermal application:

A series of four test substance concentrations was used: the highest concentration being the maximum concentration that could technically be applied. Two different concentrations were applied (0.5 ml each) per animal to the clipped flank, using Metalline patches# (2x3 cm) mounted on Medical tape\*, which were held in place with Micropore tape\* and subsequently

Coban elastic bandage\*. The initially used animals receiving intradermal injections were treated with the lowest concentrations and two further animals with the highest concentrations. After 24 hours, the dressing was removed and the skin cleaned of residual test substance. The resulting dermal reactions were assessed for irritation 24 and 48 hours after exposure.

\* Suppliers: Lohmann GmbH, Neuwied, Germany (Coban) and 3M, St. Paul, Minnesota, U.S.A. (Metalline and Micropore)

## MAIN STUDY

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### INDUCTION - Experimental animals

**Day 1** The scapular region was clipped and three pairs of intradermal injections (0.1 ml/site) were made in this area as follows:

- A) A 1:1 w/w mixture of Freund's Complete Adjuvant (Difco, Detroit, U.S.A.) with water for injection (Fresenius AG, Bad Homburg, Germany).
- B) The test substance at a 5% concentration.
- C) A 1:1 w/w mixture of the test substance, at twice the concentration used in (B) and Freund's Complete Adjuvant.

Note: One of each pair was on each side of the midline and from cranial A) to caudal C).

**Day 3** The dermal reactions caused by the intradermal injections were assessed for irritation.

**Day 7** The scapular area between the injection sites was clipped and subsequently rubbed with 10% sodium-dodecyl-sulfate (SDS, Boom, Meppel, The Netherlands) in vaseline using a spatula. This concentration of SDS provokes a mild inflammatory reaction.

**Day 8** The 10% SDS treated area between the injection sites was treated with 0.5 ml of a 100% test substance concentration using a Metalline patch (2x3 cm) mounted on Medical tape, which was held in place with Micropore tape and subsequently Coban elastic bandage.

The dressing was removed after 48 hours exposure, the skin cleaned of residual test substance and the dermal reactions caused by the epidermal exposure were assessed for irritation.

### INDUCTION - Control animals

The control animals were treated as described for the experimental animals except that, instead of the test substance, vehicle alone was administered.

### CHALLENGE - All animals

**Day 22** One flank of all animals was clipped and treated by epidermal application of a 100% test substance concentration and the vehicle (0.15 ml each), using Patch Test Plasters (Leukotest®, Beiersdorf Medical, Almere, The Netherlands). The patches were held in place with Micropore tape and subsequently Coban elastic bandage.

The dressing was removed after 24 hours exposure and the skin cleaned of residual test substance and vehicle. The treated sites were assessed for challenge reactions 24 and 48 hours after removal of the dressing.

**OBSERVATIONS**

<b>Mortality/Viability</b>	Twice daily
<b>Toxicity</b>	At least once daily.
<b>Body weights</b>	Prior to start and at termination of the study.
<b>Necropsy</b>	The moribund animal was subjected to necropsy for gross macroscopic examination.
<b>Irritation</b>	Skin reactions were graded according to the following numerical scoring systems. Furthermore, a description of all other (local) effects was recorded. Whenever necessary, the treated skin-areas were clipped at least 3 hours before the next skin reading to facilitate scoring.

**Grading Irritation Reactions\* :**

<b>Erythema and eschar formation:</b>	
No erythema.....	0
Slight erythema (barely perceptible) .....	1
Well-defined erythema.....	2
Moderate erythema .....	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth) .....	4

<b>Oedema formation:</b>	
No oedema.....	0
Slight oedema (barely perceptible) .....	1
Well-defined oedema (edges of area well-defined by definite raising) .....	2
Moderate oedema (raised approximately 1 millimeter).....	3
Severe oedema (raised more than 1 millimeter and extending beyond the area of exposure) 4	

\*. Intradermal reactions were assessed for erythema only or, if necrosis is present, the diameter of necrosis.

**Grading Challenge Reactions:**

No visible change .....	0
Discrete or patchy erythema .....	1
Moderate and confluent erythema .....	2
Moderate erythema and swelling .....	3
Intense erythema and swelling .....	4

After the end of the study all animals were killed by asphyxiation using an oxygen/carbon dioxide procedure.

**INTERPRETATION**

The results for the experimental animals at the challenge phase were compared with the results for the control animals.

Positive skin reactions (grade 1 or more) were considered signs of sensitisation, provided that such reactions were not observed or were less persistent in the control group.

A sensitisation rate (%) was calculated as follows: the number of sensitised animals as a proportion of the total number of animals in the experimental group.

The results were evaluated according to the EC criteria for classification and labelling requirements for dangerous substances and preparations (Guidelines in Commission Directive 93/21/EEC).

## RESULTS

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### PRELIMINARY IRRITATION STUDY

The results of the intradermal injections and epidermal exposures for the selection of suitable test substance concentrations for the main study are described in Table 1.

Based on the results, the test substance concentrations selected for the Main Study were a 5% concentration for the intradermal induction and a 100% concentration for the epidermal induction exposure.

No signs of irritation were observed to the highest test substance concentration epidermal tested. Therefore, the test site of all animals was treated with 10% SDS approximately 24 hours before the epidermal induction in the main study, to provoke a mild inflammatory reaction. A 100% test substance concentration was selected for the challenge phase.

### MAIN STUDY

#### **Induction phase**

The skin effects caused by the intradermal injections and epidermal exposure during the induction phase are given in Table 2. The reactions noted in the experimental animals after the epidermal induction exposure were considered to be enhanced by the SDS treatment.

#### **Challenge phase**

Skin reactions of grade 4 were observed in all experimental animals in response to the 100% test substance concentration.

No skin reactions were evident in the control animals (see Table 3).

Eschar formation or scabs were seen in all treated skin sites among the experimental animals.

#### **Toxicity / Mortality**

One experimental animal was removed from the study on day 22 following the observation of piloerection, emaciation, watery discharge from the eyes, rales, dirty teeth and the animal felt cold. Macroscopic post-mortem examination showed scabs and/or alopecia on back and abdomen and pelvic dilation of the left kidney. It was considered that the death of this animal was incidental and that the study outcome, based on the healthy surviving animals, was not adversely affected. No further mortality occurred and no further symptoms of systemic toxicity were observed in the remaining animals of the main study.

#### **Body Weights**

Body weights and body weight gain of experimental animals remained in the same range as controls over the study period (see Table 4).

## CONCLUSION

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The skin reactions observed in response to a 100% test substance concentration in all (of the nine) experimental animals in the challenge phase were considered indicative of sensitisation, based on the absence of any response in the control animals.

These results indicate a sensitisation rate of 100 per cent.

Based on these results and according to the EC criteria for classification and labelling requirements for dangerous substances and preparations (Guidelines in Commission Directive 93/2 i/EEC), TRIGONOX 44B should be labelled as: may cause sensitisation by skin contact (R 43).

TABLE 1: PRELIMINARY IRRITATION STUDY

## SKIN REACTIONS AFTER INTRADERMAL INJECTION

Animal number	Conc %	24 hours after injection		48 hours after injection	
		Erythema (grade)	Necrosis (mm)	Erythema (grade)	Necrosis (mm)
23	100		10		11
	50		5		6
24	20		1		2
	10	4			1
25	5	2		2	
	2	1		1	

Body weight animal 25 = 511 gram.

## SKIN REACTIONS AFTER EPIDERMAL EXPOSURE

Animal number	Body weight (gram)	Conc. %	24 hours after exposure		48 hours after exposure	
			Erythema (grade)	Oedema (grade)	Erythema (grade)	Oedema (grade)
21	446	100	0	0	0	0
		50	0	0	0	0
22	474	100	0	0	0	0
		50	0	0	0	0
23	515	20	0	0	0	0
		10	0	0	0	0
24	439	20	0	0	0	0
		10	0	0	0	0

TABLE 2: INDUCTION READINGS

## SKIN REACTIONS AFTER INTRADERMAL INJECTION

Animal Number	Intraderma injection (DAY 3)			Epidermal exposure (DAY 10)	
	A	B	C	D	
<b>Control</b>				<b>Erythema</b>	<b>Oedema</b>
76	E2	E0	E4	0	0
77	E2	E0	E1	0	0
78	E2	E0	E3	0	0
79	E2	E0	E3	0	0
80	E2	E0	E1	0	0
<b>Experimental</b>					
81	E3	E2	N8	2	0
82	E3	E2	N2	2 a	0
83	E3	N2	N6	2 a	0
84	E3	N2	N8	2 a	0
85	E2	N2	N6	3 a	0
86	E3	E2	N2	2 a	0
87	E2	E2	N4	2 a	0
88	E2	N3	E4	3 a	0
89	E4	N7	N10	2 a	0
90	E3	E2	N2	3 a	0

A. 1:1 Mixture of FCA and water for injection.

B. A 5% test substance concentration (Experimental); vehicle (Control).

C. 1:1 Mixture of FCA and a 10% test substance concentration (Experimental) or vehicle (Control).

D. A 100% test substance concentration (Experimental); vehicle (Control).

a. Small scabs

Skin effects Intradermal injections:

E( . ) Erythema (grade)

N( . ) Signs of necrosis (mm in diameter)

TABLE 3: CHALLENGE READINGS

Animal number	Challenge readings				Comments
	DAY 24		DAY 25		
	100%#	Vehicle*	100%#	Vehicle*	
<b>Control</b>					
76	0	0	0	0	
77	0	0	0	0	
78	0	0	0	0	
79	0	0	0	0	
80	0	0	0	0	
<b>Experimental</b>					
81	4 k	0	4 k	0	sensitised
82	4 k	0	4 k	0	sensitised
83	4 k	0	4 k	0	sensitised
84	4 s	0	4 k	0	sensitised
85	4 s	0	4 k	0	sensitised
86	4 k	0	4 k	0	sensitised
87	-	-	-	-	
88	4 s	0	4 k	0	sensitised
89	4 s	0	4 k	0	sensitised
90	4 k	0	4 k	0	sensitised

#. Test substance concentration.

\*. Water

s. Eschar formation, k. Scabs

Note: Animal 87 was removed from the study on day 22 after showing signs of ill health.

TABLE 4: BODY WEIGHTS (GRAM)

GROUP / SEX	ANIMAL	DAY 1	DAY 25	
GROUP 1 / FEMALES (CONTROL)	76	295	394	
	77	324	431	
	78	290	444	
	79	311	448	
	80	292	430	
	MEAN	302	429	
	ST. DEV.	15	21	
	N	5	5	
	GROUP 2 / FEMALES (EXPERIMENTAL)	81	301	456
		82	295	441
83		317	433	
84		281	406	
85		313	433	
86		288	407	
87		273	---	
88		324	484	
89		286	402	
90		303	437	
MEAN		298	433	
ST. DEV.		17	26	
N		10	9	

Note: Animal 87 was removed from the study on day 22 after showing signs of ill health.

TABLE 2: INDUCTION READINGS

SKIN REACTIONS AFTER INTRADERMAL INJECTION

Animal Number	Intradermal injection (DAY 3)			Epidermal exposure (DAY 10)	
	A	B	C	D	
<b>Control</b>				<b>Erythema</b>	<b>Oedema</b>
181	E3	N3	N5	0	0
182	E2	N3	N5	0	0
183	E3	E4	N5	0	0
184	E3	N3	N5	0	0
185	E3	N4	N5	0	0
<b>Experimental</b>					
186	E3	N6	N3	3	0
187	E3	N3	N6	2	0
188	N1	N6	N5	2	0
139	E3	N2	N6	2	0
190	E3	N4	N7	4 s	0
191	E3	N4	N7	2	0
192	E3	N5	N8	3	0
193	E3	N6	N3	4 s	0
194	E3	N4	N6	3	0
195	E3	N5	N5	3	0

- A. 1:1 Mixture of FCA and water for injection.
- B. A 100% test substance concentration (Experimental); vehicle (Control).
- C. 1:1 Mixture of FCA and a 100% test substance concentration (Experimental) or vehicle (Control).
- D. A 100% test substance concentration (Experimental); vehicle (Control).
- s. Eschar formation.

Skin effects intradermal injections:  
 E( . ) Erythema (grade)  
 N( . ) Signs of necrosis (mm in diameter)

**TABLE 3: CHALLENGE READINGS**

Animal number	Challenge readings				Comments
	DAY 23		DAY 24		
	100%#	Vehicle*	100%#	Vehicle*	
<b>Control</b>					
181	1	0	1	0	
182	1	0	1	0	
183	1	0	1	0	
184	1	0	1	0	
185	1	0	1	0	
<b>Experimental</b>					
186	2	0	2	0	sensitised
187	2	0	2	0	sensitised
188	2	0	4 s	0	sensitised
189	2	0	4 s	0	sensitised
190	2	0	2	0	sensitised
191	1	0	1	0	not sensitised
192	2	0	1	0	sensitised
193	2	0	4 s	0	sensitised
194	2	0	4 s	0	sensitised
195	2	0	4 s	0	sensitised

#. Test substance concentration.

\*. Propylene glycol.

s. Eschar formation.

TABLE 4: BODY WEIGHTS (GRAM)

GROUP / SEX	ANIMAL	DAY 1	DAY 25
GROUP 1 / FEMALES (CONTROL)	181	340	488
	182	395	511
	183	372	497
	184	353	492
	185	363	496
	MEAN	365	497
	ST. DEV.	21	9
	N	5	5
GROUP 2 / FEMALES (EXPERIMENTAL)	186	342	446
	187	361	505
	188	342	493
	189	351	480
	190	364	509
	191	392	510
	192	346	469
	193	425	543
	194	379	532
	195	363	494
	MEAN	367	498
	ST. DEV.	26	29
	N	10	10

**APPENDIX**

**ASSESSMENT OF CONTACT HYPERSENSITIVITY TO  
ALPHA-HEXYLCINNAMIC ALDEHYDE, TECH. 85%  
IN THE ALBINO GUINEA PIG (MAXIMISATION-TEST),  
a Reliability Check.**

**NOTOX Project 275974**

SUMMARY

A reliability check is carried out at regular intervals to check the sensitivity of the test system and the reliability of the experimental techniques as used by NOTOX. In this study, performed in October/November 1999, females of the albino Dunkin Hartley guinea pig (from Charles River Deutschland, Kisslegg, Germany) were checked for the sensitivity to ALPHA-HEXYLCINNAMICALDEHYDE, TECH. 85%. The females were approx. 4 weeks old (individual body weights <500 grams) at commencement of the study. The study was based on the OECD Guideline No. 406, the EC Directive 96/54/EC, Part B.6 and on the method described in "Allergic Contact Dermatitis in the Guinea-Pig: Identification of Contact Allergens" Magnusson and Kligman, 1970. ALPHA-HEXYLCINNAMICALDEHYDE, TECH. 85% (CAS no. 101-86-0) was fabricated under lot no. 10021HF (Aldrich Chemicals Co., Germany).

Test substance concentrations selected for this study were:  
 Intradermal induction: A 5% solution in water (Milli-U, w/w).  
 Epidermal induction: undiluted.  
 Challenge: a 10% solution in water (w/w).

SKIN REACTIONS IN THE CHALLENGE PHASE (Number of animals with skin reactions)

	ALPHA-HEXYLCINNAMICALDEHYDE Concentration	
	10%	vehicle
	24/48*	24/48*
Experimental group (10 females)		
Score 2	3a/4a	0/0
Score 1	4a/5a	0/0
Score 0 with scaliness	1/1	0/0
No reactions	2/2	10/10
	a. All animals also showed scaliness.	
Control group (5 females)		
No reactions	5/5	5/5

\*. time (hours) after the challenge exposure.

CONCLUSION

The skin reactions in eight experimental animals observed in response to the 10% test substance concentration in the challenge phase were considered indicative of sensitisation, based on the absence of any response in the control animals. These results lead to a sensitisation rate of 80 per cent to the 10% concentration. From these results, it was concluded that the female guinea pig of the albino Dunkin Hartley strain is an appropriate animal model for the performance of studies designed to evaluate the sensitising potential of a substance in a Maximisation type of test.

The raw data, protocol and report from this study are kept in the NOTOX archives. The test described above was performed in accordance with NOTOX Standard Operating Procedures and the report was audited by the QA-unit.

**D. 01**

TRIGONOX 44B

NOTOX Project 273511

## **APPENDIX**

**ASSESSMENT OF CONTACT HYPERSENSITIVITY TO  
ALPHA-HEXYLCINNAMIC ALDEHYDE, TECH. 85%  
IN THE ALBINO GUINEA PIG (MAXIMISATION-TEST),  
a Reliability Check.**

**NOTOX Project 275963**

**D 02**

TRIGONOX 44B

NOTOX Project 273511

**SUMMARY**

A reliability check is carried out at regular intervals to check the sensitivity of the test system and the reliability of the experimental techniques as used by NOTOX. In this study, performed in October/November 1999, females of the albino Himalayan (from BRL Ltd, füllinsdorf, Basel, Switzerland) were checked for the sensitivity to ALPHA-HEXYLCINNAMICALDEHYDE, TECH. 85%. The females were approx. 7 weeks old (Group mean body weights <500 grams. Individually: ≤ 507 grams) at commencement of the study. The study was based on the OECD Guideline No. 406, the EC Directive 96/54/EC, Part B.6 and on the method described in "Allergic Contact Dermatitis in the Guinea-Pig: Identification of Contact Allergens" Magnusson and Kligman, 1970. ALPHA-HEXYLCINNAMICALDEHYDE, TECH. 85% (CAS no. 131-86-0) was fabricated under lot no. 10021HF (Aldrich Chemicals Co., Germany).

Test substance concentrations selected for this study were:

Intradermal induction: A 5% solution in water (Milli-U, w/w).

Epidermal induction: undiluted.

Challenge: a 10% solution in water (w/w).

**SKIN REACTIONS IN THE CHALLENGE PHASE (Number of animals with skin reactions)**

	ALPHA-HEXYLCINNAMICALDEHYDE Concentration	
	10% 24/48*	vehicle 24/48*
<b>Experimental group (10 females)</b>		
Score 2	3/3	0/0
Score 1	3/3	0/0
No reactions	4/4	10/10
<b>Control group (5 females)</b>		
No reactions	5/5	5/5

\*. time (hours) after the challenge exposure.

**CONCLUSION**

The skin reactions in the experimental animals observed in response to the 10% test substance concentration in the challenge phase were considered indicative of sensitisation, taking into account the intensity and persistence of the response in the control animals. These results lead to a sensitisation rate of 60 per cent to the 10% concentration. From these results, it was concluded that the female guinea pig of the albino Himalayan strain is an appropriate animal model for the performance of studies designed to evaluate the sensitising potential of a substance in a Maximisation type of test.

The raw data, protocol and report from this study are kept in the NOTOX archives. The test described above was performed in accordance with NOTOX Standard Operating Procedures and the report was audited by the QA-unit.

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## REPORT

**PRIMARY SKIN IRRITATION/CORROSION STUDY WITH  
TRIGONOX 23 - C 75  
IN THE RABBIT  
(4-HOUR SEMI-OCCLUSIVE APPLICATION)**

CAS : 26748-4-4

NOTOX Project 273487  
NOTOX Substance 94032

**E:02**

TRIGONOX 23 - C 75

NOTOX Project 273487

STATEMENT OF GLP COMPLIANCE

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

The study described in this report has been correctly reported and was conducted in compliance with the most recent edition of:

*The OECD Principles of Good Laboratory Practice* which are essentially in conformity with:

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

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

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

Japanese Ministry of Agriculture, Forestry and Fisheries. 59 NohSan, Notifications No. 3850.

Japanese Ministry of International Trade and Industry. Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85.

Japanese Ministry of Health and Welfare. Ordinance No.21.

Study Director:  
Drs. A.H.B.M. van Huygevoort

Management:  
Drs. W.J.A.M. Frieling



Date: 2 February 2000



Date: 7<sup>5</sup> February 2000

**QUALITY ASSURANCE STATEMENT**

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

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

The dates of Quality Assurance inspections and audits are given below.  
During the on-site inspections procedures applicable to this type of study were inspected.

<b>DATES OF QAU INSPECTIONS/ AUDITS</b>	<b>REPORTING DATES</b>
on-site inspection(s)	
22 November 1999	22 November 1999
protocol inspection(s)	
28 October 1999	28 October 1999
report audit(s)	
2 February 2000	2 February 2000

Head of Quality Assurance:

C.J. Mitchell B.Sc.



Date: 10-2-2000

**SUMMARY**

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Primary skin irritation/corrosion study with TRIGONOX 23 - C 75 in the rabbit (4-hour semi-occlusive application).

The study was carried out based on the guidelines described in: EC Commission Directive 92/69/EEC, B.4, "Acute Toxicity - Skin irritation" and OECD No.404, "Acute Dermal Irritation/Corrosion".

Three rabbits were exposed to 0.5 ml of TRIGONOX 23 - C 75, applied onto clipped skin for 4 hours using a semi-occlusive dressing. Observations were made 1, 24, 48 and 72 hours and 7, 14 and/or 21 days after exposure.

Exposure to TRIGONOX 23 - C 75 resulted in moderate to severe or severe erythema and moderate to severe oedema in the treated skin-areas of the three rabbits.

The skin irritation had resolved within 14 days after exposure in one animal and within 21 days in the two remaining animals.

Reduced flexibility and/or fissuring of the skin, scaliness and bald skin were noted among the animals between 48 hours and termination (after 14 or 21 days)

On the skin of all animals, sticky remnants of the test substance were present on day 1 and dry remnants were present from day 2 onwards until day 4.

No evidence of full thickness destruction of the skin or scar tissue was observed during the observation period, indicating that no corrosion of the skin had occurred by dermal application of the test substance to the intact rabbit skin.

Based on the results and according to the EC criteria for classification and labelling requirements for dangerous substances and preparations (Guidelines in Commission Directive 93/21/EEC), TRIGONOX 23 - C 75 should be labelled as: irritating to skin (R 38).

**PREFACE**

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<b>Sponsor</b>	Akzo Nobel BU BU Polymer Chemicals P.O. Box 9300 6800 SB ARNHEM The Netherlands
<b>Study Monitor</b>	Dr. C.L.J. Braun
<b>Testing Facility</b>	NOTOX B.V. Hambakenwetering 3 5231 DD 's-Hertogenbosch The Netherlands
<b>Study Director</b>	Drs. A.H.B.M. van Huygevoort
<b>Study Plan</b>	Start : 9 November 1999 End : 4 January 2000

**TEST SUBSTANCE**

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The sponsor is responsible for all test substance data unless determined by NOTOX.

<b>Identification</b>	TRIGONOX 23 - C 75
<b>Description</b>	Colourless liquid
<b>Batch</b>	04199094130293
<b>Purity</b>	74.7 %
<b>Test substance storage</b>	In deep freezer in the dark
<b>Stability under storage conditions</b>	Not indicated
<b>Expiry date</b>	04 October 2000 (Allocated by NOTOX, 1 year after receipt of the test substance)

**TEST SUBSTANCE PREPARATION**

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<b>Preparation</b>	The test substance was applied undiluted as delivered by the sponsor.
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**PURPOSE AND RATIONALE**

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The purpose of this primary skin irritation study was to assess the possible irritation or corrosion potential of a single dose of the test substance when administered to the intact skin of rabbits. This study should provide a rational basis for risk assessment in man. The absence of skin pigmentation in the albino rabbit facilitates the evaluation of induced skin reactions. The dermal route was selected because the test substance may accidentally come into contact with the skin during manufacture, handling and/or use.

**GUIDELINES**

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As required by the Dutch Act on Animal Experimentation, the study protocol was reviewed and agreed by the Article 14-functionary and the Ethical Committee of NOTOX. The study procedures described in this report were based on the following guidelines:

European Community (EC), Council Directive 67/548/EEC, Annex V, Part B, Methods for the Determination of Toxicity, as last amended by Commission Directive 92/69/EEC, B.4: "Acute Toxicity - Skin Irritation". Official Journal of the European Communities No. L 383, 1992

Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals, Section 4, Health Effects, No.404: "Acute Dermal Irritation / Corrosion", Paris Cedex, 1992.

**ARCHIVING**

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NOTOX B.V. will archive the following data for at least 10 years:  
raw data, protocol, report and test substance reference sample.  
No data will be withdrawn without the sponsor's written consent.

**TEST SYSTEM**

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Species	Albino Rabbit, New Zealand White, (SPF-Quality) Recognised by international guidelines as the recommended test system (e.g. EC, OECD) Source: Charles River UK Limited, Margate, Kent, England.
Number of animals	3 Animals of one sex.
Age and body weight	Animals used within the study were at least 6 weeks old and body weights were less than 3.5 kg.
Identification	Earmark.

**ANIMAL HUSBANDRY**

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**Conditions**

A controlled environment was maintained in the room with optimal conditions considered as being approximately 15 air changes per hour, a temperature of 21°C, a relative humidity of 30-70% and 12 hours artificial fluorescent light and 12 hours dark per day. Deviations from these optimal conditions were noted, but were considered not to have affected study integrity.

**Accommodation**

Individually housed in labelled cages with perforated floors (Scanbur, Denmark) and equipped with an automatic drinking system (ITL, Bergen, The Netherlands). Acclimatisation period was at least 5 days before start of treatment under laboratory conditions.

**Diet**

Standard laboratory rabbit diet (Charles River Breeding and Maintenance Diet for Rabbits, Altromin, Germany) approx. 100 g. per day. Certificates of analysis were examined and retained in the NOTOX archives. In addition, hay (BMI, Helmond, the Netherlands) was provided once a week.

**Water**

Free access to tap-water. Certificates of quarterly analysis were examined and retained in the NOTOX archives.

**TREATMENT**

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Approximately 24 hours before treatment, the dorsal fur was clipped with electric clippers, exposing an area of approximately 150 square centimeters (10x15 cm<sup>2</sup>). Whenever considered necessary the treated skin areas were re-clipped at least 3 hours before the observations, to facilitate scoring.

A health inspection was performed prior to the commencement of treatment, to ensure that the animals were in a good state of health. Special attention was paid to the skin to be treated, which was intact and free from abnormalities.

Each animal was treated by dermal application of 0.5 ml of the test substance. The test substance was applied to the skin of one flank, using a metalline patch<sup>#</sup> of 2x3 cm. The patch was mounted on Micropore tape<sup>\*</sup>, which was wrapped around the abdomen and secured with Coban elastic bandage<sup>\*</sup>.

Four hours after the application, the dressing was removed and the skin cleaned of residual test substance using water.

<sup>#</sup>. Supplier: Lohmann GmbH, Neuwied, Germany

<sup>\*</sup>. Supplier: 3M, St. Paul, Minnesota, U.S.A.

**OBSERVATIONS**

Mortality/Viability	Twice daily.
Toxicity	At least once daily.
Body Weight	Day of treatment (prior to application).
Irritation	The skin reactions were assessed at approximately 1, 24, 48 and 72 hours and 7 and 14 and/or 21 days after the removal of the dressings and test substance. The irritation scores and a description of all other (local) effects were recorded. Adjacent areas of the untreated skin of each animal served as controls.

The irritation was assessed according to the following numerical scoring system. At each observation, the highest scores given was recorded:

**ERYTHEMA AND ESCHAR FORMATION**

No erythema .....	0
Very slight erythema (barely perceptible) .....	1
Well defined erythema .....	2
Moderate to severe erythema .....	3
Severe erythema (beet redness)* .....	4

\* In cases where signs of necrosis or corrosion (injuries in depth) prevent erythema scoring, the maximum grade for erythema (= 4) is given.

**OEDEMA FORMATION**

No oedema .....	0
Very slight oedema (barely perceptible) .....	1
Slight oedema (edges of area well defined by definite raising).....	2
Moderate oedema (raised approximately 1 mm) .....	3
Severe oedema (raised more than 1 mm and extending beyond area of exposure) .....	4

**HISTOPATHOLOGY**

No histopathology was performed.

**INTERPRETATION**

The results were evaluated according to the EC criteria for classification and labelling requirements for dangerous substances and preparations (Guidelines in Commission Directive 93/21/EEC).

**RESULTS**

---

**Irritation**

Four hours exposure to 0.5 ml of TRIGONOX 23 - C 75 resulted in moderate to severe or severe erythema and moderate to severe oedema in the treated skin-areas of the three rabbits. The skin irritation had resolved within 14 days after exposure in one animal and within 21 days in the two remaining animals.

Reduced flexibility and/or fissuring of the skin, scaliness and bald skin were noted among the animals between 48 hours and termination (after 14 or 21 days)

**Corrosion**

There was no evidence of a corrosive effect on the skin.

**Colouration**

On the skin of all animals, sticky remnants of the test substance were present on day 1 and dry remnants were present from day 2 onwards until day 4.

**Toxicity / Mortality**

No symptoms of systemic toxicity were observed in the animals during the test period and no test substance related mortality occurred.

One animal was removed from the study after showing signs of a general decline in health (lethargy, pale skin, diarrhoea, piloerection, loss of appetite, emaciation, reduced faeces production and the animal felt cold), 7 days after treatment. Since skin reactions were still present, the results obtained from this animal were considered to be invalid and therefore the results were not used for the interpretation for the outcome of the study. Since this deviation affected the study integrity, the treatment of one additional animal was performed.

**CONCLUSION**

---

No evidence of full thickness destruction of the skin or scar tissue was observed during the observation period, indicating that no corrosion of the skin had occurred by dermal application of the test substance to the intact rabbit skin.

Based on the results and according to the EC criteria for classification and labelling requirements for dangerous substances and preparations (Guidelines in Commission Directive 93/21/EEC), TRIGONOX 23 - C 75 should be labelled as: irritating to skin (R 38).

TABLE 1

Following exposure, the treated skin-area remained sticky after removal of the test substance.

**INDIVIDUAL SKIN IRRITATION SCORES**

Animal no.#	655			693			767		
Time After exposure	Erythema	Oedema	Comments	Erythema	Oedema	Comments	Erythema	Oedema	Comments
1 hour	2	2	b	2	2	b	2	3	b
24 hours	2	2	b1	2	4	b1	2	3	b1
48 hours	3	2	b1	3	2	b1	3	3	b1f
72 hours	3	3	b1	4	3	b1	3	2	b1f
7 days	4	2	g	2	3	l	1	1	l
14 days	1	0	hl	1	0	h	0	0	-
21 days	0	0	h	0	0	h			

**Comments:**

- b. Sticky remnants of the test substance present on the edges of the application site.
- b1. Dry remnants of the test substance present on the edges of the application site.
- f. Reduced flexibility of the skin.
- g. Fissuring of the skin.
- h. Bald skin.
- l. Scaliness.

TABLE 2

MEAN VALUES OF SKIN IRRITATION SCORES (24, 48 and 72 h after exposure).

Animal no. #	Mean 24 - 72 hours	
	Erythema	Oedema
655	2.7	2.3
693	3.0	3.0
767	2.7	2.7

## #. Animal specifications:

Animal no.	Sex	At commencement of the study	
		Age (weeks)	Body weight (grams)
655	♂	9	1619
693	♂	7	1236
767	♂	8	1776

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### Final Research Report

RGL F99121 T 99009 AL

December 07, 1999

R.J. van Wijk, I.C.M. Garttener-Arends

**EFFECTS OF THE WATER-ACCOMODATED  
FRACTION OF LUCIDOL ON THE GROWTH OF THE  
FRESHWATER GREEN ALGA  
*PSEUDOKIRCHNERIELLA SUBCAPITATA***

ICS-103

**CONFIDENTIAL**

Page 1 of 23

**Project identification**

Client BU Polymer Chemicals  
Subclient

Client No  
Subclient No

Project No  
Task No 69886

**Drafted by department**

General Analytical and Environmental Chemistry



## ABSTRACT

In order to predict effects of chemicals in an aquatic environment, the toxicity to freshwater algae was assessed. The algal toxicity was determined in the Algal Growth Inhibition test in accordance with OECD, EEC and ISO test guidelines and with Monograph 26 (1996). The test was performed according to the OECD Principles of Good Laboratory Practice. The guidelines were slightly modified to ensure good growth and pH control of the cultures.

Lucidol dissolved in OECD medium at approximately 10 mg/l forms a solution with particles. Therefore water accommodated fraction's (WAF's) of Lucidol were tested, prepared from stirring a solution of 9.6 mg/l for 24 hours and then filtrated. From this solution the following dilutions were prepared and tested: 1:32 - 1:16 - 1:8 - 1:4 and 1:2. Chemical analyses were performed as NPOC (non-purgeable organic carbon). Based on the NPOC concentration determined at the beginning of the test after filtration, the test concentrations Lucidol were calculated as indicative values. Based on these indicative values the toxicity of these WAF's to exponentially growing *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*) was determined over an exposure period of 72 hours. The test was conducted in a mineral salts medium in a climatized illuminated orbital incubator. The maximum variation in pH in the test media was 1.1 pH unit.

The  $E_0C_{50}$  and  $E_1C_{50}$  (0-72 h) values based on the indicative concentrations of the test compound for *P. subcapitata* are 0.44 mg/l (0.31-0.62 mg/l 95% confidence limits) and 0.83 mg/l (0.59-1.13 mg/l 95% confidence limits), respectively. The indicative NOEC determined from the results is 0.12 mg/l, the LOEC is 0.23 mg/l.

**ALGAL GROWTH INHIBITION TEST WITH LUCIDOL**

**Sponsor** Akzo Nobel Chemicals  
P.O Box 247  
3800 AE Amersfoort

**Study monitor** Drs. C. L.J. Braun, M.D., reg.tox.  
AN-H

**STUDY ORGANISATION**

**Location** Akzo Nobel Chemicals Research Arnhem  
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**Study director** Dr. R.J. van Wijk

**Quality Assurance Unit** Ir. J.M. Plantinga

**Management, Head of Department RGL** Dr.Ir. C.J. Groenenboom

**Initiation date of the study** 1999-10-25  
**Completion date of the study** 1999-11-22

**ARCHIVING AND STORAGE**

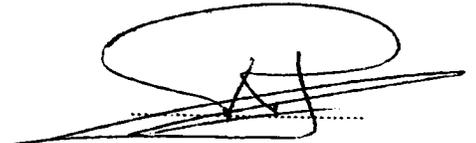
The project file including the final report, amendments to the final report, the study plan, amendments to the study plan, records of quality assurance inspections, all letters, memos and notes and raw data pertaining to the study will be retained in the archives of Akzo Nobel Chemicals Research Arnhem for a period of ten years. Other records including master schedule sheet, laboratory notebooks, logbooks, records of the maintenance and calibration of equipment, summary of training, curricula vitae and job descriptions of the personnel involved in the study, records related to location and storage of the test substance will also be kept in the Akzo Nobel Chemicals Research Arnhem archives for a period of ten years. Test material will be stored deepfrozen under the sample code 99009 for ten years or only as long as the quality of the test substance permits evaluation.



**GLP COMPLIANCE STATEMENT**

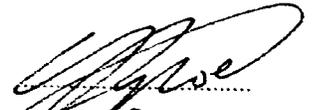
The study reported here was carried out according to the study plan in compliance with the OECD Principles of Good Laboratory Practice. The report contains an accurate description of the results.

**Study director**  
Dr. R.J. van Wijk



date 7-12-99

**Management, Head of Department RGL**  
Dr.ir. C.J. Groenenboom



date 12-12-99



**QUALITY ASSURANCE STATEMENT**

This report was audited by the Quality Assurance Unit of Akzo Nobel Chemicals Research Arnhem. It is considered to be an accurate presentation of the methods and procedures applied in the course of the study and an accurate reproduction of the data recorded.

Listed below are the dates of inspection of this study by the Quality Assurance Unit and the dates on which its findings were reported to Study Director and Management.

<b>Dates of inspection</b>	<b>Dates of reporting</b>
1999-10-26	1999-10-26
1999-11-10	1999-11-10
1999-11-30	1999-11-30

**Quality Assurance Unit**  
Ir. J.M. Plantinga

*J.M. Plantinga*  
date 9-12-1999

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## 1. INTRODUCTION

The objective of this algal growth inhibition test is to determine the effect of Lucidol on the growth of a freshwater unicellular green alga. In the present toxicity test exponentially growing cultures of *Pseudokirchneriella subcapitata* (Korshikov) Hindák (formerly known as *Selenastrum capricornutum*) were exposed to various concentrations of the test substance over several generations under defined conditions. The inhibition of growth in relation to control cultures was determined during 72 hours.

Toxicity to algae measured as growth inhibition can be expressed as  $EC_{n,70,50,30}$  (Effect Concentration) values. The  $EC_n$  values are the concentrations of the test substance showing n% reduction in either growth ( $E_nC_n$  refers to the increase in cell concentration (i.e. biomass) over the test period) or specific growth rate ( $E_nC_n$  refers to the rate of increase in cell concentration per unit time over the test period) relative to the controls. On the test results obtained, the LOEC (Lowest Observed Effect Concentration) and NOEC (No Observed Effect Concentration) can also be determined. The LOEC is defined as the lowest tested concentration at which growth is significantly inhibited as compared to the control. The NOEC is defined as the highest tested concentration at which growth shows no significant inhibition relative to the control values and the tested concentration next lower than the LOEC.

## 2. TEST GUIDELINES, MODIFICATIONS AND DEVIATIONS

The algal growth inhibition test with *P. subcapitata* was carried out in accordance with an OECD Test Guideline for testing of chemicals (9.1), which is basically identical to the EEC (9.2) and ISO Guidelines, (9.3) and with ECETOC Monograph 26 (1996) (9.4) with the following modifications and deviations:

- The  $\text{NaHCO}_3$  concentration of the test medium was 150 mg/l instead of 50 mg/l, as recommended by the OECD/EEC Guidelines, in order to maintain a more constant pH during the test.
- The pH should not deviate more than 1.5 units during the test (EEC).



### 3. MATERIALS

#### 3.1 Test substance

A sample of Lucidol (project sample code T 99009) was received on 1999-10-20. The following test substance data were submitted by the sponsor, who accepted full responsibility for the validity thereof.

Akzo Nobel trade name	Lucidol
Chemical name	Dibenzoyl peroxide
CAS-reg. No	94-36-0
Appearance	White powder
Storage until required	Refrigerator

All concentrations cited in this report refer to the sample of Lucidol (technical product) as received.

#### 3.2 Chemicals

All reagents used were of reagent grade quality and obtained from J.T. Baker Chemicals BV, Deventer, The Netherlands and Janssen Chimica, Tilburg, The Netherlands.

#### 3.3 Deionized water

The deionized water used had a conductivity of less than  $5 \mu\text{S}\cdot\text{cm}^{-1}$  and a TOC content of less than 2 mg/l. This water was produced from tap water in a water purification system (Spectrum-Eigastat, Breukelen, The Netherlands) according to Standard Operation Procedure K 10 (9.5).

#### 3.4 Test flasks

The test was performed in 100 ml erlenmeyers containing 40 ml of medium. The test flasks were closed with cotton-wool stoppers.

#### 3.5 Culturing cabinet and test conditions

The culturing apparatus was a temperature-controlled illuminated orbital incubator (Standard Operation Procedure K 11 (9.6)), in which the temperature was kept at  $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ , and a continuous uniform illumination was provided in the spectral range of 400 to 700 nm by using 30 W fluorescent lamps of the type 'universal white' (colour temperature of approximately 4000 K), at a distance of approximately 0.35 m from the algal cultures. The test vessels were rotated continuously at a speed sufficient to prevent sedimentation of the algae.



### 3.6 Apparatus

The pH was determined with the aid of a microcomputer pH meter (Standard Operation Procedure K 1 (9.7)). The temperature was measured with an electronic min-max thermometer (Standard Operation Procedure K 6 (9.8)). The light intensity was measured with a light intensity meter (Standard Operation Procedure K 16 (9.9)). The purity of the algae will be determined with a microscope (Standard Operation Procedure K 17 (9.10)).

### 3.7 Source and maintenance of algae

The test was carried out with the freshwater unicellular algae *P. subcapitata* (CCAP 278/4) obtained from the Culture Collection of Algae and Protozoa, The Ferry House, Cumbria, Ambleside, United Kingdom (ISBN 1 871105056). After purchasing this strain was cultured and maintained according to Standard Operation Procedure E 3 (9.11). Cultures on sloped agar tubes were stored at 4°C until required.

## 4. METHODS

### 4.1 Test principle and procedures

The test was carried out as a WAF (Water Accommodated Fraction). Adequate dilutions were prepared from the WAF obtained as described under 4.2 to which algae from an exponentially growing culture were added using a sterile pipette. In addition six control replicates were included. The extinction in each erlenmeyer was measured after 0, 24, 48 and 72 hours. Algal medium was used as a blank in the spectrophotometer.

### 4.2 Preparation of the test solutions

A solution of the test substance of 9.6 mg in 1 L algal medium was prepared in a stoppered flask. This solution was stirred for 24 h and subsequently filtrated (Sartolab, 0.2 µm). The undiluted WAF prepared as described was diluted as follows: 1:32 – 1:16 – 1:8 – 1:4 and 1:2 with filter sterilized (0.2µm) medium. Controls containing only test medium were included in the test.

### 4.3 Chemical analysis

Chemical analyses were performed using determination of NPOC according to SOP K 7 (9.12). Samples were taken from additional test vessels without algae from the undiluted fraction and from the control at the beginning and end of the test. Samples were either stored in a refrigerator when analyzed within 2 days or stored in a freezer (-≤10°C).



#### 4.4 Preparation of the inoculum

The initial stock culture was inoculated with *P. subcapitata* from a sloped agar tube and checked for purity by microscopic means. This algal stock culture (40 ml) of *P. subcapitata* was regularly transferred to fresh medium to act as inoculum for testing.

The extinction of an exponentially growing stock culture was measured. The cell density was determined using the calibration curve described below. From this algal culture a dilution was prepared to obtain an initial cell density of approximately  $1 \cdot 10^4$  cells/ml in the test medium.

#### 4.5 Preparation of the test medium

The test medium, described by EEC and OECD (9.1, 9.2) contained the following nutrients (in mg/l):

Macro-nutrients	
NH <sub>4</sub> Cl	15
KH <sub>2</sub> PO <sub>4</sub>	1.6
CaCl <sub>2</sub> (H <sub>2</sub> O) <sub>2</sub>	18
MgSO <sub>4</sub> (H <sub>2</sub> O) <sub>7</sub>	15
MgCl <sub>2</sub> (H <sub>2</sub> O) <sub>6</sub>	12
Fe-EDTA	
FeCl <sub>3</sub> (H <sub>2</sub> O) <sub>6</sub>	0.08
Na <sub>2</sub> H <sub>2</sub> EDTA(H <sub>2</sub> O) <sub>2</sub>	0.1
Trace elements	
H <sub>3</sub> BO <sub>3</sub>	0.185
ZnCl <sub>2</sub>	0.003
MnCl <sub>2</sub> (H <sub>2</sub> O) <sub>4</sub>	0.415
CoCl <sub>2</sub> (H <sub>2</sub> O) <sub>6</sub>	0.0015
CuCl <sub>2</sub> (H <sub>2</sub> O) <sub>2</sub>	1x10 <sup>-5</sup>
Na <sub>2</sub> MoO <sub>4</sub> (H <sub>2</sub> O) <sub>2</sub>	0.007
NaHCO <sub>3</sub>	
NaHCO <sub>3</sub>	150

The test medium was prepared from concentrated solutions of the mineral salts prepared in deionized water and stored at 4°C in the dark.



#### 4.6 Determination of cell concentrations

Cell concentrations were determined photometrically with a UV/VIS Spectrophotometer (Standard Operation Procedure K 3 (9.13)). Measurements were carried out at 436 nm in a cuvette with a light path of 4 cm. To establish the relation between extinction and cell density, a calibration curve was made which is checked yearly. The procedure of constructing the calibration curve as well as the control procedure are described in Standard Operation Procedure E 3 (9.11). From the relation between extinction (E) and counted cell number (N) the following calibration curve was determined using linear regression:

$$N = 2.648 \cdot 10^6 E + 0.0793 \cdot 10^6 \quad (r^2 = 0.9926)$$

The calibration curve was used to determine the cell density of the inoculum before and during the test when needed.

#### 4.7 Determination of pH, temperature, light and purity of algae

The pH of all samples and controls were measured at the beginning (t=0 h) and at the end (t=72 h) of the test. The temperature in the culturing apparatus was continuously measured and read out at the end of the test. The light intensity was measured at the beginning and at the end of the test. At the end of the test five random samples were microscopically checked for purity of the algal culture.

#### 4.8 Quality control of the algae

The sensitivity of the algae was checked by performing a growth inhibition test with a reference compound (potassium dichromate) twice a year, when the cultures are grafted from the sloped agar tubes. The sensitivity was tested for compliance with the Guidelines, which means that the EC<sub>50</sub> values are between 0.25 and 2.0 mg/l.

#### 4.9 Evaluation of data

The mean values of the extinction's for each test substance concentration were used to calculate the growth and specific growth rate. The inhibition of growth was calculated by determining the area under the growth curves according to the formula:

$$A = \frac{E_1 - E_0}{2} \cdot t_1 + \frac{E_1 + E_2 - 2E_0}{2} \cdot (t_2 - t_1) \\ + \frac{E_{n-1} + E_n - 2E_0}{2} \cdot (t_n - t_{n-1})$$



where:

- A area  
 $E_0$  extinction at  $t_0$   
 $E_n$  extinction at  $t_n$   
 $t_n$  time of  $n^{\text{th}}$  measurement after the beginning of the test

The percentage inhibition of the cell growth at each test substance concentration ( $I_A$ ) is calculated as the difference between the area under the control growth curve ( $A_c$ ) and the area under the growth curve at each test substance concentration ( $A_t$ ) as:

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

where:

- $I_A$  percentage inhibition  
 $A_c$  area under the growth curve of the control  
 $A_t$  area under the growth curve for a test substance

The average specific growth rate was derived from the slope of the regression line in a plot of  $\ln E$  versus time. The percentage inhibition of the specific growth rate at each concentration was calculated according to the formula

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

where:

- $I_{\mu}$  inhibition percentage of the specific growth rate at concentration  $t$   
 $\mu_c$  average specific growth rate of the control  
 $\mu_t$  average specific growth rate at test concentration  $t$

Where possible, the  $EC_{20,50,80}$  values were computed from the best fitted line (least-squares method) through the points given by the probit of the percentage of inhibition and the logarithm of the concentration of the test substance. The  $EC_{50}$  value calculated for the area under the growth curve is termed  $E_c C_{50}$  (0-72 h), whereas the  $EC_{50}$  value calculated for the specific growth rate is termed  $E_s C_{50}$ . The Lowest Observed Effect Concentration (LOEC) was determined by comparison of the growth at each concentration and the control using threshold values from the William's test (9.14). The No Observed Effect Concentration (NOEC) was derived from the results as the first concentration below the LOEC value, where growth shows no significant inhibition relative to the control values. Confidence limits were



computed on the basis of Fieller's theorem (9.15). All computations were performed using the TOXCALC™ version 5.0 programme, according to Standard Operation Procedure L 1 (9.16).

## 5. RESULTS

### 5.1 Toxicity

The results of the extinction measurements are presented in Table 1. The concentrations used in the calculations are based on NPOC measurements, which can only be used as indicative values (see chapter 6). Based on these indicative values the  $E_3C_{50}$  and  $E_7C_{50}$  (0-72 h) of Lucidol are 0.49 mg/l (0.31-0.62 95% confidence limits) and 0.83 mg/l (0.59-1.13 95% confidence limits), respectively. The NOEC determined from the results of the test compound is 0.12 mg/l, the LOEC is 0.23 mg/l. The most important test results and parameters are summarised in Table 2. The graphical presentations of the test results are given in Figures 1-4.

### 5.2 pH, temperature, light and purity of algae

The pH measurements in Table 3 show a maximum increase of 1.1 pH units per test vessel. The temperature varied from 22.8 to 23.0°C during the test, and the light intensity varied between approximately 108 and 109  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , which are both in accordance with the required conditions described in the study plan. The five random samples were all pure and not contaminated with bacteria.

## 6. CHEMICAL ANALYSES

Chemical analyses of the test concentrations were performed using NPOC (non-purgeable organic carbon) analyses according to SOP K7 (9.12). The results of the analyses are presented in Table 4. Since the NPOC analysis is not a specific analysis for the test compound, the results can only be used as an indication of the concentration. The results of the NPOC concentration of the control at 0 and 48 h also indicate that the data should be considered with care. The calculation of the Lucidol concentrations were based on the ratio of the Lucidol mol weight and carbon content (242.23/168) and the measured NPOC concentration at  $t_0$  corrected for the DSW control (4.66-2.08=2.56 mg/l), resulting in a concentration for the undiluted fraction of 3.73 mg/l Lucidol. From this value the dilutions were calculated. The values are included in Table 1 as indicative Lucidol concentrations.

## 7. QUALITY CRITERIA

The following quality criteria have been met in the present study:

- The cell density of the controls increased at least a factor 16 within 72 hours.

## 8. DEVIATIONS FROM THE STUDY PLAN

There was 1 study plan amendment specifying the lower range of dilutions tested, as described in 4.2.

## 9. REFERENCES

- 9.1 OECD, 1984. Algal, Growth Inhibition Test, Test Guideline 201, June 7<sup>th</sup>, 1984. Guidelines for testing of chemicals, Organisation for Economic Co-operation and Development, Paris.
- 9.2 EEC, 1992. Algal growth inhibition test. Off. J. Of the European communities, L383 A/179, 1992-12-29.
- 9.3 ISO 8692,1989. Water quality, freshwater algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum*.
- 9.4 ECETOC, Monograph 26 (1996)
- 9.5 Standard Operation Procedure K 10: Deionizer.
- 9.6 Standard Operation Procedure K 11: Illuminated orbital incubators
- 9.7 Standard Operation Procedure K 1: pH-meter.
- 9.8 Standard Operation Procedure K 6: Thermometers.
- 9.9 Standard Operation Procedure K 16: Light intensity meter.
- 9.10 Standard Operation Procedure K 17: Microscope and counting chamber.
- 9.11 Standard Operation Procedure E 3: Obtaining and culturing of algae for toxicity tests.
- 9.12 Standard Operation Procedure K 7: TOC analyses
- 9.13 Standard Operation Procedure K 3: Spectrophotometer.
- 9.14 William's, D.A., 1972. The comparison of seven dose levels with a zero-dose control. *Biometrics* 28, pp. 519-531.
- 9.15 Zerbe G.O., 1978. On Fieller's theorem and general linear model. *The American Statistician*, Vol. 32, 3, pp. 103-105.
- 9.16 Standard Operation Procedure L 1: Algal growth inhibition test (calculation of EC<sub>n</sub> values).



**Table 1**  
 Extinctions measured in the algal growth inhibition test with Lucidol (indicative concentrations based on NPOC are given in brackets in mg/l) and inhibition percentages calculated from the mean values.

Duration tested	Time (hours)				% inhibition based on	
	0	24	48	72	growth	Specific growth rate
Control	0.005	0.020	0.127	0.584		
	0.005	0.021	0.135	0.622		
	0.006	0.021	0.140	0.622		
	0.005	0.021	0.123	0.594		
	0.005	0.021	0.142	0.661		
	0.005	0.022	0.158	0.738		
Mean	0.005	0.021	0.138	0.637	0	0
1:32 (0.12)	0.009	0.023	0.112	0.537		
	0.009	0.024	0.132	0.613		
	0.009	0.026	0.145	0.685		
Mean	0.009	0.024	0.130	0.612	5.7	1.3
1:16 (0.23)	0.010	0.023	0.123	0.500		
	0.009	0.023	0.132	0.541		
	0.010	0.023	0.139	0.576		
Mean	0.010	0.023	0.131	0.539	13.9	2.9
1:8 (0.47)	0.010	0.019	0.072	0.326		
	0.010	0.017	0.064	0.300		
	0.011	0.020	0.066	0.341		
Mean	0.010	0.019	0.067	0.322	52.3	15.0
1:4 (0.93)	0.010	0.013	0.015	0.054		
	0.011	0.013	0.015	0.054		
	0.010	0.013	0.013	0.054		
Mean	0.010	0.013	0.014	0.054	93.9	57.8
1:2 (1.87)	0.010	0.016	0.007	0.012		
	0.010	0.017	0.007	0.020		
	0.011	0.021	0.008	0.015		
Mean	0.010	0.018	0.007	0.016	98.4	97.9

Table 2

Test parameters and summary of the results based on nominal concentrations.

Parameter	Name	Dimension	Value
$N_0$	Inoculum	Cells/ml	$1.0 \cdot 10^4$
$N_{72}$	Number of cells of the control at the end of the test <sup>1</sup>	Cells/ml	$1.338 \cdot 10^6$
$\mu$	growth rate (control)	$h^{-1}$	0.068
-	Increase factor of cell growth over 72 h	-	133.8
LOEC	lowest observed effect concentration	mg/l	0.23
NOEC	no observed effect concentration	mg/l	0.12
Toxicity endpoints			95% confidence limits
$E_b C_{20}$	0.27	mg/l	0.10 – 0.37
$E_b C_{50}$	0.44	mg/l	0.31 – 0.62
$E_b C_{80}$	0.70	mg/l	0.53 – 1.97
$E_r C_{20}$	0.53	mg/l	0.20 – 0.70
$E_r C_{50}$	0.83	mg/l	0.59 – 1.13
$E_r C_{80}$	1.28	mg/l	0.98 – 3.33

<sup>1</sup> = Calculation was based on the exponential growth of the algae according to the equation:  $N_t = N_0 \cdot e^{\mu t}$



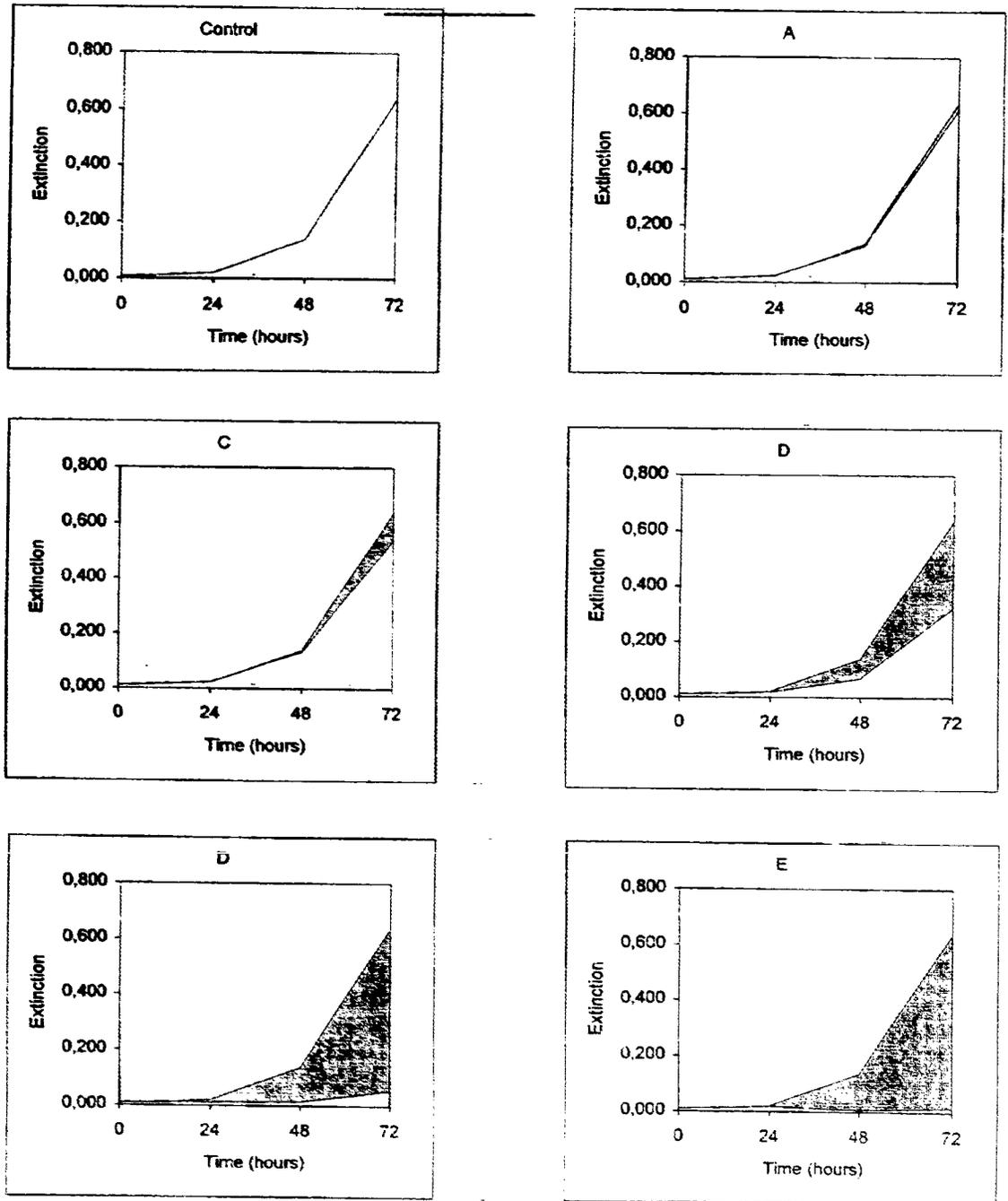
Table 3 Measurements of pH

Dilutions tested	Time(hours)	
	0	72
Control	8.1	8.7
	8.1	8.8
	8.1	8.8
	8.1	8.8
	8.1	9.0
	8.1	9.2
1:32	8.1	8.9
	8.1	9.0
	8.2	9.1
1:16	8.2	8.8
	8.2	8.9
	8.2	8.9
1:8	8.2	8.6
	8.2	8.6
	8.2	8.7
1:4	8.2	8.4
	8.2	8.4
	8.2	8.4
1:2	8.2	8.3
	8.2	8.4
	8.2	8.4



**Table -;**  
NPOC (non-purgeable organic carbon) measurements

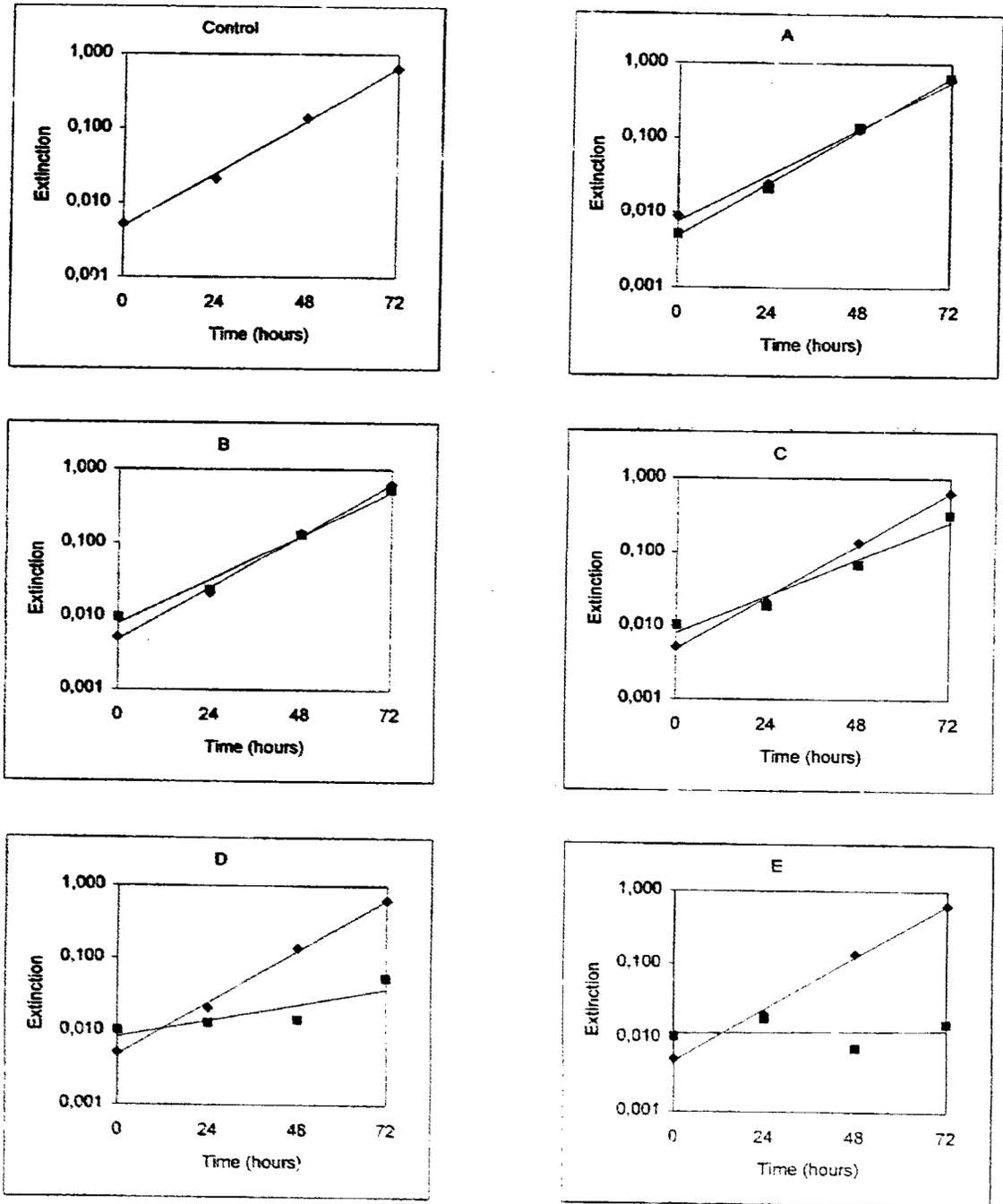
Sample	NPOC (mg/l) Average (n=3)	SD (mg/l)
Control t=0h	2.08	0.02
Control t=72h	2.41	0.02
Undiluted sample t=0h	4.66	0.06
Undiluted sample t=72h	4.99	0.01



**Figure 1.** Growth curves of *P. subcapitata* at various concentrations of Lucidol. The inhibition of the growth compared to the control is given by the hatched area. The indicative test concentrations were 0.12 (A), 0.23 (B), 0.47 (C), 0.93 (D) and 1.87 (E) mg/l.



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**Figure 2.** Specific growth rates of *P. subcapitata* at various concentrations of Lucidol. The indicative test concentrations were 0.12 (A), 0.23 (B), 0.47 (C), 0.93 (D) and 1.87 (E) mg/l.

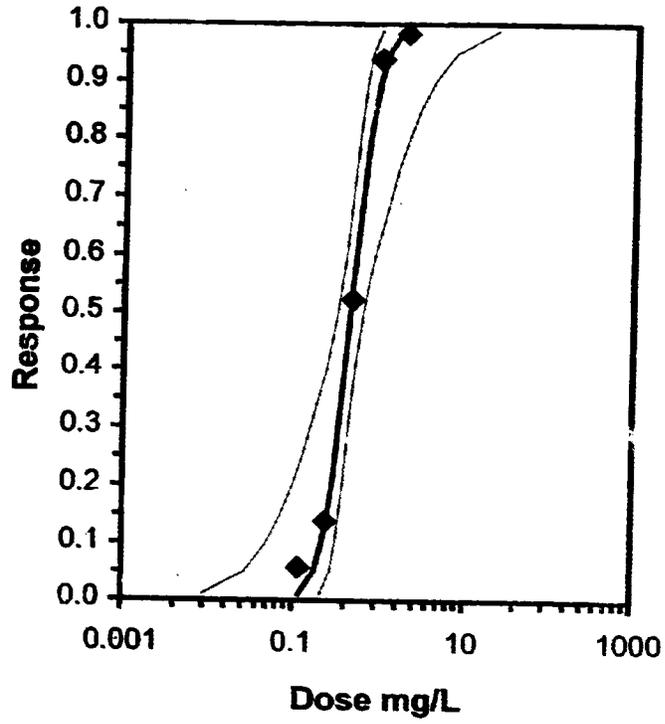
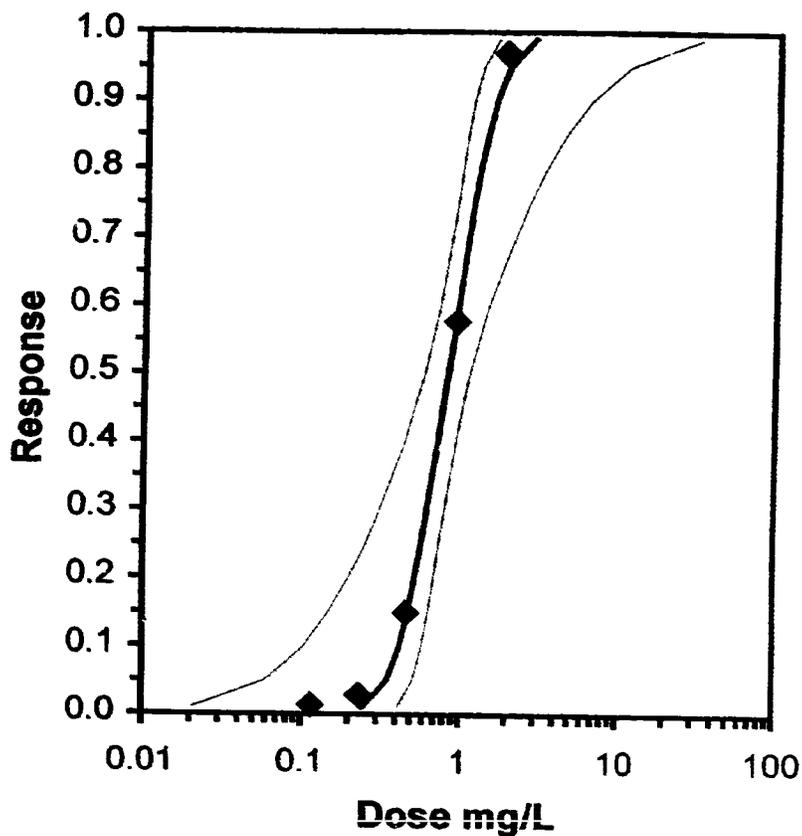


Figure 3. 'Dose/response' relationship of *P subcapitata* plotted as concentration/effect curve for Lucidol. The 'effect' is the growth inhibition as compared to the control (presented as fractions of 1), as determined at the various tested concentrations.



**Figure 4.** 'Dose/response' relationship of *P subcapitata* plotted as concentration/effect curve for Lucidol. The 'effect' is the inhibition of specific growth rates relative to the control (presented as fractions of 1), as determined at the various tested concentrations



**Distribution**

Client

ACH-Amersfoort Clous

Others

AN-H: C. Braun (2x + e-mail)

RGL/Groenenboom, Plantinga, archive

Van Wijk, GLP-archive

Central file Chemicals

**Abstract**

Technical Discipline Manager

RG/Verhelst