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AR 226 - 1334

MR 267555

3M

Certified Mail

June 10, 2003

8EHQ - 0603 - 00373

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Office of Pollution Prevention and Toxics
US EPA
1200 Pennsylvania Avenue NW
Washington DC 20460-0001

8EHQ - 80 - 373

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RE: TSCA 8(E) SUPPLEMENTAL SUBMISSION:
Docket No. 8EHQ-0602-00373

Dear Docket Coordinators:

On May 16, 2003, 3M provided EPA with preliminary results from three ecotoxicity studies conducted on triphenylbenzyl phosphonium cation/N-methyl perfluorooctanesulfonamide salt (TPBP C8 amide). The final reports for these studies contain results that are consistent with the previously reported information.

Enclosed please find the following three final reports:

- 96-Hour Acute Toxicity Study in Zebra-fish
- Acute Toxicity Study in *Daphnia Magna*
- 96-Hour Fresh Water Algal Growth Inhibition Test

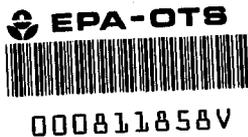
Please contact Susan Beach (651-778-7452) if you have any questions or if we can provide additional information.

Sincerely,

Larry R. Johnson
Director, Corporate Toxicology and Regulatory Services

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REPORT

96-HOUR ACUTE TOXICITY STUDY IN ZEBRA-FISH

WITH

T-4127

(STATIC)

NOTOX Project 334338
NOTOX Substance 113607

- Page 1 of 38 -

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STATEMENT OF GLP COMPLIANCE

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

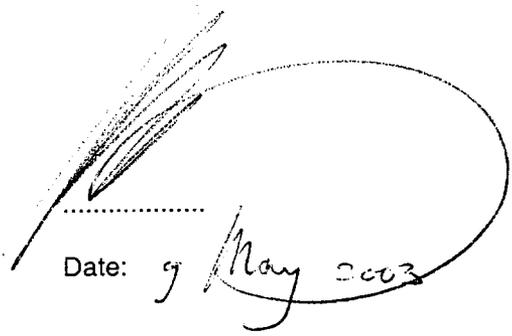
which are essentially in conformity with:

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
Drs. M. Bogers


Date: 9 May 2003

Management:
Ing. E.J. van de Waart, M.Sc.
Head of Genetic & Ecotoxicology


Date: 12/05/2003

QUALITY ASSURANCE STATEMENT

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) (Process)	
May 13-31, 2002 (Analytical Support)	June 04, 2002
July 08-15, 2002 (Ecotoxicology)	July 17, 2002
protocol inspection(s) (Study)	
December 06, 2001	December 06, 2001
report audit(s) (Study)	
April 28-29, 2003	April 29, 2003

Head of Quality Assurance

C.J. Mitchell B.Sc.

pt

A. Morgan

 Date: *13 May, 2003*

SUMMARY

96-Hour Acute Toxicity Study in Zebra-fish with T-4127.

The study procedures described in this report were based on the EEC directive 92/69; Part C: methods for the determination of ecotoxicity, Publication No. L383, December 1992, C.1. "Acute toxicity for fish", and the OECD guideline No. 203: "Fish Acute Toxicity Test", Adopted 17 July, 1992.

T-4127 is a salt consisting of Perfluorooctanesulfonylmethylamide (anionic part) and Triphenylbenzylphosphonium (cationic part). Based on the response of the anionic part of the test substance water solubility of T-4127 was determined to be between 0.6 and 0.9 mg/l at $19.5 \pm 0.6^\circ\text{C}$.

The study started with a limit test exposing seven fish per concentration to a filtered and an unfiltered solution, both prepared at loading rates of 100 mg/l and both magnetically stirred for three days. A blank-control was also included. Analysis of the samples taken during the limit test showed that the average exposure concentration calculated over the first 24 hours was 0.76 mg/l in the filtered solution and 0.28 mg/l in the unfiltered solution. The average exposure concentration calculated over the 96-hour test period was 0.51 mg/l in the filtered solution and 0.16 mg/l in the unfiltered solution. During the first 24 hours of exposure all fish died in the filtered solution, while only 1 out of seven died in the unfiltered solution and no more fish died during the remaining test period. Hence the biological effects confirmed a higher actual concentration in the filtered solution.

A final test was performed exposing seven fish per concentration to 4.5, 10, 20, 45 and 100% of a Water Accommodated Fraction (WAF) prepared at a loading rate of 100 mg/l and a blank control for a maximum of 96 hours. All glassware used in the final test was pre-treated with dichlorodimethylsilane. Although vapour pressure was relatively low, based on the Henry constant evaporation was still a possible cause for decrease of test concentrations. Therefore the study was performed in air-tight vessels. Samples taken during the final test were added directly to isopropanol at a ratio of 1:5 to prevent any additional loss.

The analytical program based on measurement of the anionic part showed that the actual concentrations could not be maintained at more than 80 % of the initial concentration, in spite of all precautions taken to prevent adsorption to glass and volatilization. The measured concentrations could not be quantified for the whole test period with the exception of the two highest concentrations which are relevant for determination of the LC_{50} .

In the control no mortality was observed. Further, all test conditions (pH, oxygen concentration and temperature) remained within the ranges prescribed by the protocol.

A water phase of T-4127 originating from a loading rate of 100 mg/l, induced 100% mortality in fish. This corresponded with 0.76 mg/l for 24 hours (limit test) and 0.44 mg/l for 48 hours (final test) when based on the anionic part in the water phase. No mortality or less than 50% mortality occurred at average concentrations of 0.095 mg/l (final test) and 0.16 mg/l (limit test). Hence, the 96h- LC_{50} of zebra-fish exposed to T-4127 was < 1 mg/l (between 0.16 and 0.44 mg/l) based on quantification of the anionic part.

Limiting the analytical support on only the anionic part of T-4127, validity of the test could not be ensured. There is a realistic possibility that the actual concentrations of the cationic part were higher and more stable than those of the anionic part. However, the sponsor did not agree with further research and decided to accept the highly toxic labelling (R50) based on the current results.

PREFACE

Sponsor	3M Corporate Toxicology 3M Center, Building 220-2E-02 P.O. Box 33220 ST. PAUL, MINNESOTA 55133-3220 U.S.A.
Study Coordinator	Mrs. M. Mitchell
Study Monitor	Mrs. Dr. S. Beach 3M Environmental Technology and Safety Services 935 Bush Avenue, Building 2-3E-09 ST. PAUL, MINNESOTA 55144 U.S.A.
Testing Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Aquatic Toxicology: Study Director	Drs. M. Bogers
Technical coordinator	Ing. E.J.H. Mutsaards
Analytical Chemistry: Principal Scientist	Ir. M.J.C. Brekelmans
Study Plan	Start of project: December 05, 2001 Start of first exposure: July 01, 2002 Completion of last exposure: November 15, 2002 Completion of analysis: November 15, 2002 Completion of project: May 09, 2003

TEST SUBSTANCE

Identification	T-4127
Description	Dark amber waxy solid
Batch	D-2491 lot 2
Composition	95 – 99% Fluoroelastomer curative <2% N-Methyl Perfluorooctanesulphonamide <1% Methyl Alcohol <1% Ethyl Alcohol <1% Isopropyl Alcohol
Test substance storage	At room temperature in the dark
Stability under storage conditions	Stable
Expiry date	31 March 2003
Stability in water	Not indicated

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

PURPOSE

The purpose of the study is to evaluate the test substance for its ability to generate acute toxic effects in *Danio rerio* during an exposure period of 96 hours and, if possible, to determine the LC₅₀ at all observation times.

GUIDELINES

The study procedures described in this report were based on the EEC directive 92/69; Part C: methods for the determination of ecotoxicity, Publication No. L383, December 1992, C.1. "Acute toxicity for fish", and the OECD guideline No. 203: "Fish Acute Toxicity Test", Adopted 17 July, 1992.

DEFINITIONS

Fish were considered to be dead when no reaction was observed after touching the caudal peduncle and visible breathing movements are absent.

The LC₅₀ is the concentration killing 50% of the fish after a defined period of exposure.

TEST SYSTEM

Species	Zebra-fish (<i>Danio rerio</i> , Teleostei, Cyprinidae).
Source	Ornamental Fish Hatchery Atlanta, Hellevoetsluis, the Netherlands.
Mean length	Limit test: 2.5 ± 0.2 cm Final test: 2.7 ± 0.2 cm
Mean weight	Limit test: 0.24 ± 0.06 g Final test: 0.36 ± 0.12 g
Characteristics	Healthy fish supplied with a health certificate.
Reason for selection	This system has been selected as an internationally accepted species.
Total fish used	63

HOLDING

Quarantine/Acclimatisation	At least 12 days after delivery.
Medium	ISO-medium, formulated using Milli-Ro water (tap-water purified by reverse osmosis; Millipore Corp., Bedford, Mass., USA) with the following composition: Ca ²⁺ 80 mg/l Mg ²⁺ 12 mg/l Na ⁺ 15 mg/l K ⁺ 3 mg/l Cl ⁻ 145 mg/l SO ₄ ²⁻ 49 mg/l HCO ₃ ⁻ 47 mg/l Hardness is 250 mg CaCO ₃ /l
Measurements	pH, nitrate and nitrite concentration and ammonia concentration: once a week. Temperature: every day.
Feeding	Daily with Trouvit.
Control of sensitivity	The results of the most recent performed test are appended to the report.
Validity of batch	In the batch of fish used for the test, mortality during the seven days prior to the start of the test was less than 5%.

PREPARATION OF TEST SOLUTIONS

The standard test procedures required generation of test solutions, which contain completely dissolved test substance concentrations or stable and homogeneous mixtures or dispersions. The testing of concentrations that disturbed the test system were prevented (e.g. film of the test substance on the water surface).

T-4127 is a salt consisting of Perfluorooctanesulfonylmethylamide (anionic part) and Triphenylbenzylphosphonium (cationic part). Based on the response of the anionic part of the test substance water solubility of T-4127 was determined to be between 0.6 and 0.9 mg/l at 19.5 ± 0.6°C (NOTOX Project 340842, using the column elution method).

Limit test

Two stock solutions were prepared by weighing 500 mg of T-4127 on a glass surface (twice). After placing these carriers in measuring flasks 5 litre of test medium was added to each flask to obtain a loading rate of 100 mg/l. Both mixtures were magnetically stirred for three days. After the stirring period the mixtures were clear but contained test substance particles, which settled at the bottom of the flasks. One of the mixtures was used as such while the second mixture was filtered through a paper filter (Schleicher and Schuell 604) to remove the larger undissolved test substance particles (ca. > 5µm). The final test solution was clear and colourless.

Final test

All glassware used in the final test was pre-treated with dichlorodimethylsilane to prevent loss of test substance due to adsorption to glass surfaces. A stock solution was prepared by weighing 500 mg of T-4127 on a glass surface. After placing the carrier in a measuring flask 5 litre of test medium was added to obtain a loading rate of 100 mg/l. Simultaneously, another measuring flask was prepared in the same way but without adding test substance (blank-control). After the stirring period, the solution prepared at a loading rate of 100 mg/l was transferred to a separation funnel and left to stabilise for 2¼ hours. Subsequently the Water Accommodated Fraction (WAF) was separated from the centre of the separation funnel. Lower test concentrations were prepared by dilution of the WAF. All final test solutions were clear and colourless. Part of the test solutions prepared were used for testing with *Daphnia magna* (NOTOX Project 334349).

LIMIT TEST

A limit test was performed exposing seven fish per concentration to a filtered and an unfiltered solution prepared at a loading rate of 100 mg/l and a blank-control.

Sampling: Frequency	at t=0 h, t=24 h and t=96 h.
Volume	10 ml from the approximate centre of the test vessel.
Storage	Not applicable, all samples were analysed on the day of sampling.

FINAL TEST:**TEST CONCENTRATIONS**

T-4127	4.5, 10, 20, 45 and 100% of a WAF prepared at a loading rate of 100 mg/l.
Control	Test medium without test substance or other additives (Blank-control).

TEST PROCEDURE AND CONDITIONS

Test duration	96 hours
Test type	Static without interim renewal of test solutions.
Test vessels	10 litres, all-glass. Pre-treated with dichlorodimethylsilane. Although vapour pressure was relatively low, based on the Henry constant evaporation was still a possible cause for decrease of test concentrations. Therefore the study was performed in air-tight vessels.
Test medium	ISO-medium, aerated until the dissolved oxygen concentration had reached saturation and the pH had stabilised. After aeration the hardness was 250 mg CaCO ₃ per litre and the pH was 8.0.

Number of fish	7 fish per concentration and control.
Loading	0.28 g fish/litre, i.e. 7 fish per 9 litres of test medium.
Illumination	16 hours photoperiod daily
Aeration	The test media were not aerated during the test.
Feeding	No feeding from 48 hours prior to the test and during the total test period.
Introduction of fish	Within 1½ hours after preparation of the test media.
Euthanasia	At the end of the test the surviving fish were rapidly killed by exposing them to ca. 1.2% ethylene glycol monophenylether in water.

SAMPLING FOR ANALYSIS OF TEST CONCENTRATIONS

Duplicate samples were taken from the stock solution directly after preparation of the WAF as a specification of the origin of samples taken during the test. During the final test duplicate samples were taken from all concentrations and the blank-control at the start and after 48 hours of exposure. At the end of the test period duplicate samples were taken from all dilutions (4.5, 10, 20 and 45%) of the WAF and the blank-control for analysis. The method of analysis is described in the appended Analytical Report.

Sampling: Volume	5 ml from the approximate centre of the test vessels.
Treatment	Samples were added directly to isopropanol at a ratio of 1:5 to prevent any additional loss.
Storage	Not applicable, samples were analysed on the day of sampling.

MEASUREMENTS AND RECORDINGS

Mortality and other effects	At 2½, 24, 48, 72 and 96 hours following the start of exposure. Dead fish were removed when observed.
Fish length and weight	Ten fish of the batch used for the test, were weighed and measured prior to the start of the test.
Dissolved oxygen content, pH and temperature.	Daily in all vessels, beginning at the start of the test (day 0).

DATA HANDLING

Determination of the average exposure concentrations

The average exposure concentrations over the first 24 or 48 hours of exposure were calculated as $\sqrt{C_{t=0} \times C_{t=x}}$, being the geometric means of the concentrations of T-4127 measured in the samples taken at the start ($C_{t=0}$) and after 24 or 48 hours of exposure ($C_{t=x}$).

The average exposure concentrations over the 96-hour test period were calculated as:

$$\frac{24 \times \sqrt{C_{t=0} \times C_{t=24}} + 72 \times \sqrt{C_{t=24} \times C_{t=96}}}{96} \quad \text{or} \quad \frac{48 \times \sqrt{C_{t=0} \times C_{t=48}} + 48 \times \sqrt{C_{t=48} \times C_{t=96}}}{96}$$

Being the geometric means of the concentrations of T-4127 measured in the samples taken at the start ($C_{t=0}$), after 24 or 48 hours ($C_{t=24}$ or $C_{t=48}$) and the end of the test ($C_{t=96}$).

RESULTS

Limit test:

Measured concentrations

The limit test included samples prepared at a nominal loading rate of 100 mg/l either filtered through 5 μm or unfiltered. Samples were diluted with methanol in 1:1 (v:v) ratio and analyzed using the LCQduo LCMSMS (injection volume 100 μl). Concentrations in the filtered sample decreased from 1.0 mg/l to 0.33 mg/l during the test whereas concentrations in the unfiltered sample decreased from 0.45 mg/l to 0.08 mg/l, see also Table 7 of the appended Analytical Report. The average exposure concentration calculated over the first 24 hours was 0.76 mg/l in the filtered solution and 0.28 mg/l in the unfiltered solution. The average exposure concentration calculated over the 96-hour test period was 0.51 mg/l in the filtered solution and 0.16 mg/l in the unfiltered solution.

Mortality and other effects

The mortality data are presented in Table 1. During the first 24 hours of exposure all fish died in the filtered solution, while only 1 out of seven died in the unfiltered solution and no more fish died during the remaining test period. Hence the biological effects confirmed a higher actual concentration in the filtered solution.

Table 1: Incidence of mortality and total mortality during the limit test.

Loading rate T-4127 (mg/l)	Initial number of fish	Cumulative mortality					Total Mortality (%)
		3½h	24h	48h	72h	96h	
Blank-control	7	0	0	0	0	0	0
100; filtered	7	0 ¹	7	7	7	7	100
100; unfiltered	7	0 ²	1 ³	1 ⁴	1 ⁵	1 ⁵	100

¹ 2 fish were slow and snapping at the surface, while the other 5 were slow and swimming at the bottom.

² 1 fish was immobile

³ All surviving fish were slow and 1 fish was also swimming at the surface.

⁴ 4 fish were swimming at the surface.

⁵ All fish were discoloured.

Experimental conditions

The results of measurement of pH and oxygen concentrations are presented in Table 2. The results of measurement of the temperature in the various test solutions is presented in Table 3.

Table 2: pH-values and dissolved oxygen concentrations (mg/l) during the limit test.

Loading rate T-4127 (mg/l)	Day 0		Day 1		Day 2		Day 3		Day 4	
	pH	O ₂								
Blank-control	8.0	8.8	7.8	8.3	7.7	7.9	7.7	7.8	7.7	7.5
100; filtered	8.1	8.4	7.7	6.4	7.3	-	-	-	-	-
100; unfiltered	7.9	8.1	7.6	6.8	7.3	6.5	7.5	6.7	7.5	6.4

Table 3: Temperatures (°C) measured during the limit test.

Loading rate T-4127 (mg/l)	Day 0	Day 1	Day 2	Day 3	Day 4
Blank-control	22.9	21.8	21.7	21.8	21.8
100; filtered	22.8	21.9	21.9	-	-
100; unfiltered	23.1	21.8	21.8	21.9	21.8

Final test:

Measured concentrations

The results of analysis of the samples taken during the study are described in Table 8 of the appended Analytical Report.

During the final test the exposure consisted of a range of dilutions of a water fraction prepared at 100 mg/l containing 4.5 to 100% of this water fraction. Samples of 5 ml taken from these dilutions were added to 25 ml isopropanol and analyzed using the API300 LCMSMS (injection volume 10 μ l). Recovery samples were prepared in ISO-medium at 0.01 mg/l, 0.1 mg/l and 0.6 mg/l. The 0.01 mg/l concentration proved to be below LOD. Recoveries at 0.1 mg/l and 0.6 mg/l were relatively high (117% and 111% respectively).

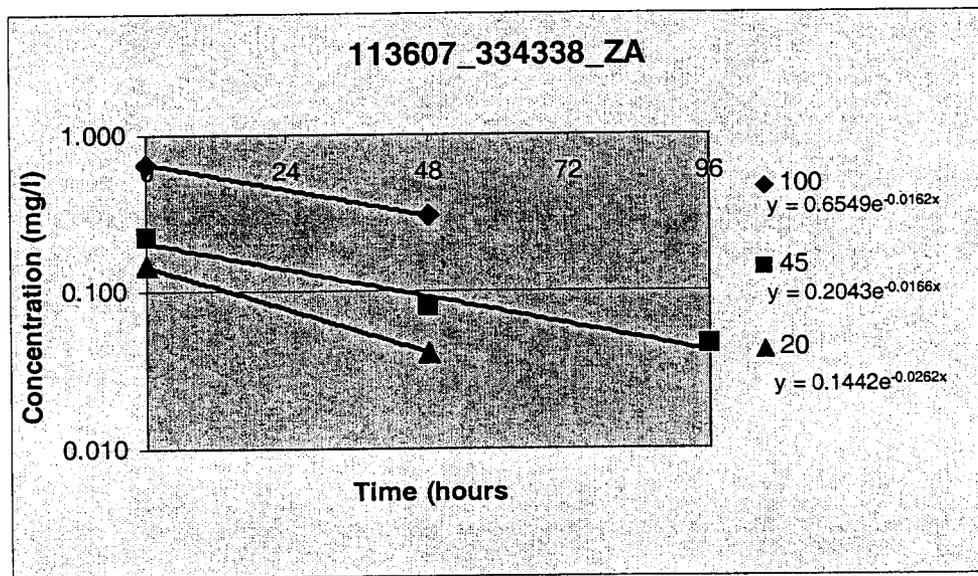
Samples were taken in duplicate from the test solutions and each pretreated sample was injected in triplicate. The results are listed in Table 4. Note that the decrease in concentration was more than 20% in spite of all precautions and measures taken.

Table 4: Mean of duplicate samples and their deviation.

T-4127 % of a WAF (100 mg/l)	Measured concentration (mg/l)		Mean concentration (mg/l)	Deviation
	0 h			
4.5	< LOD	0.024	0.024	
10	0.064	0.029	0.046	55
20	0.16	0.13	0.144	12
45	0.24	0.20	0.222	12
100	0.56	0.75	0.655	20
	48 h		Mean	Deviation
4.5	< LOD	< LOD		
10	< LOD	< LOD		
20	0.044	0.038	0.041	11
45	0.073	0.083	0.078	9
100	0.30	0.31	0.301	3
	96 h		Mean	Deviation
4.5	< LOD	< LOD		
10	< LOD	< LOD		
20	< LOD	< LOD		
45	0.049	0.040	0.045	14

Duplicate samples showed a significant difference in concentration at t=0. At t=48 hours and at t=96 hours, concentrations of duplicate samples were similar. Figure 1 includes the curves for the exponential decrease of the test concentrations with identical slopes for the concentrations measured in 45 and 100% of the water fraction.

Figure 1: Concentration curves for the three highest test concentrations.



Mortality and other effects

The mortality data are presented in Table 5. Table 6 specifies the clinical effects observed at different test concentrations.

All fish died at the highest treatment corresponding with an average concentration of 0.44 mg/l during the first 48 hours, whereas no fish died in the test solution containing 45% of the water fraction corresponding with an average concentration of 0.095 mg/l.

Table 5: Incidence of mortality and total mortality during the final test.

T-4127 % of a WAF (100 mg/l)	Initial number of fish	Cumulative mortality					Total Mortality (%)
		2½h	24h	48h	72h	96h	
Blank-control	7	0	0	0	0	0	0
4.5	7	0	0	0	0	0	0
10	7	0	0	0	0	0	0
20	7	0	0	0	0	0	0
45	7	0	0	0	0	0	0
100	7	0	5	7	7	7	100

Table 6: Clinical effects observed during the final test.

T-4127 % of a WAF (100 mg/l)	Time of recording (hours)	Specification of effects	Relative number
10	24 and 72	Swimming at the surface	7/7
45	24	Slow swimming	2/7
		Swimming at the surface	7/7
	48	Slow swimming and swimming at the surface	7/7
	72 and 96	Slow swimming	7/7
100	24	Immobile	2/2

Experimental conditions

The results of measurement of pH and oxygen concentrations are presented in Table 7. The results of measurement of the temperature in the various test solutions is presented in Table 8.

Table 7: pH-values and dissolved oxygen concentrations (mg/l) during the final test.

T-4127 % of a WAF (100 mg/l)	Day 0		Day 1		Day 2		Day 3		Day 4	
	pH	O ₂								
Blank-control	8.0	8.7	7.4	6.6	7.5	6.6	7.6	6.5	7.5	6.7
4.5	8.0	8.4	7.5	7.6	7.5	7.5	7.6	7.3	7.5	7.2
10	7.9	8.4	7.5	7.7	7.5	7.4	7.6	7.3	7.5	7.1
20	7.8	8.4	7.4	7.4	7.5	7.1	7.5	6.6	7.5	6.2
45	7.9	8.2	7.3	6.3	7.2	5.7	7.4	5.5	7.4	5.4
100	7.8	7.6	7.2	6.0	-	-	-	-	-	-

Table 8: Temperatures (°C) measured during the final test.

T-4127 % of a WAF (100 mg/l)	Day 0	Day 1	Day 2	Day 3	Day 4
Blank-control	22.0	22.3	22.3	22.2	22.5
4.5	21.8	22.4	22.3	22.3	22.6
10	21.8	22.4	22.3	22.2	22.5
20	21.9	22.3	22.3	22.1	22.4
45	22.1	22.3	22.1	22.1	22.3
100	21.8	22.3	-	-	-

ACCEPTABILITY OF THE TEST

1. No mortality was observed in the control group.
2. All test conditions (pH, oxygen concentration and temperature) remained within the ranges prescribed by the protocol.
3. The analytical program based on measurement of the anionic part showed that the actual concentrations could not be maintained at more than 80 % of the initial concentration, in spite of all precautions taken to prevent adsorption to glass and volatilization. The measured concentrations could not be quantified for the whole test period with the exception of the two highest concentrations which are relevant for determination of the LC₅₀.

CONCLUSIONS

Under the conditions of the present tests a water phase of T-4127 originating from a loading rate of 100 mg/l, induced 100% mortality in fish. This corresponded with 0.76 mg/l for 24 hours (limit test) and 0.44 mg/l for 48 hours (final test) when based on the anionic part in the water phase. No mortality or less than 50% mortality occurred at average concentrations of 0.095 mg/l (final test) and 0.16 mg/l (limit test). Hence, the 96h-LC₅₀ of zebra-fish exposed to T-4127 was < 1 mg/l (between 0.16 and 0.44 mg/l) based on quantification of the anionic part.

Limiting the analytical support on only the anionic part of T-4127, validity of the test could not be ensured. There is a realistic possibility that the actual concentrations of the cationic part were higher and more stable than those of the anionic part. However, the sponsor did not agree with further research and decided to accept the highly toxic labelling (R50) based on the current results.

REFERENCE TEST

96-hour acute toxicity study in the zebra-fish with PCP; NOTOX Project 352441 (Batch 02-01 ZA)

The study procedures described in this report were based on the EEC directive 92/69, Part C.1. "Acute toxicity for fish"; and the OECD guideline No. 203: "Fish Acute Toxicity Test", Adopted 17 July, 1992.

Start: 17-06-2002

End: 21-06-2002

This reference test was carried out to check the sensitivity of the test system as used by NOTOX. The reference substance was PENTACHLOROPHENOL (PCP, SIGMA, Art. P-9441, Batch 103H3488).

Concentrations: 0.06, 0.10, 0.15, 0.22 and 0.33 mg/l in ISO-medium.

Control: ISO-medium without test substance.

Incidence of mortality observed in the reference study:

Concentration PCP (mg/l) Nominal	Initial Number Of fish	Cumulative number of dead fish recorded at various time points after start of exposure					Total Mortality (%)
		3h	24h	48h	72h	96h	
Control	5	0	0	0	0	0	0
0.06	5	0	0	0	0	0	0
0.10	5	0	0	0	0	0	0
0.15	5	0	0	0	0	0	0
0.22	5	0	0	0	0	0	0
0.33	5	0	2	4	4	4	80

During the test the pH, oxygen concentration and the temperature of the medium were within the optimal ranges for fish.

Under the conditions of the present test PENTACHLOROPHENOL induced no lethal effects in zebra-fish at or below 0.22 mg/l. The 96h-LC₅₀ for zebra-fish exposed to PCP was 0.29 mg/l with 0% mortality at 0.22 mg/l and 80% mortality at 0.33 mg/l. The 24h-LC₅₀ was 0.35 mg/l with 0% mortality at 0.22 mg/l and 40% mortality at 0.33 mg/l. The range of the 96h-LC₅₀ for zebra-fish is generally between 0.18 mg/l and 0.30 mg/l (with a 95% confidence interval between 0.28 and 0.39 mg/l) based on historical data of reference tests performed by NOTOX. The response observed in zebra-fish originating from the present batch falls within this range.

The raw data and report from this study are kept in the NOTOX archives. The test described above was performed under GLP-conditions with a QA-check.

ANALYTICAL REPORT

96 HOUR ACUTE TOXICITY STUDY IN ZEBRA-FISH

WITH

T-4127

(STATIC);

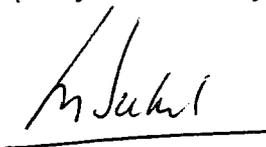
DETERMINATION OF THE CONCENTRATIONS

**NOTOX Project 334338
NOTOX Substance 113607**

REPORT APPROVAL

PRINCIPAL SCIENTIST:

Ir. M.J.C. Brekelmans
(Analytical Chemistry)



A handwritten signature in black ink, appearing to read 'M.J.C. Brekelmans', is written over a solid horizontal line.

Date: *May 08, 2003*

SAMPLE PRETREATMENT PROCEDURE

Analyses in July 2002

Each sample (10 ml) was quantitatively transferred to a 20 ml volumetric flask. A volume of 200 μ l internal standard solution was added to the samples taken at t=24 and t=96 hours, after which the flasks were filled up to the mark using methanol (internal standard concentration 120 μ g/l). No internal standard was added to the flasks containing the t=0 samples.

Analyses in November 2002

In order to prevent from adsorption, it was decided to change sample pre-treatment. Based on additional information supplied by the sponsor, each sample (5 ml) was transferred quantitatively into a 50 ml volumetric flask and 25 ml of isopropanol containing internal standard (internal standard concentration 164 μ g/l) was added.

ANALYTICAL METHODS

T-4127 is a salt consisting of Perfluorooctanesulfonylmethylamide (anionic part) and Triphenylbenzylphosphonium (cationic part). Upon request of the sponsor, quantitative analyses were only based on the anionic part of T-4127 using High Performance Liquid Chromatography with Mass Spectrometric detection (LCMSMS). The analytical method used in July 2002 was not sensitive enough to measure concentrations in pretreated samples from the final test. Therefore, a second method was used in November 2002.

Conditions for the analytical method used in July 2002

Column	Betasil C18, 50 x 2.0 mm; $d_p = 5 \mu$ m (Thermo Hypersil Keystone)																					
Mobile phase	Gradient Eluents A: 2.0 mM ammonium acetate Eluents B: 100 % methanol																					
	<table border="1"> <thead> <tr> <th>Time (min)</th> <th>Eluents A (%)</th> <th>Eluents B (%)</th> </tr> </thead> <tbody> <tr> <td>0.00</td> <td>90</td> <td>10</td> </tr> <tr> <td>1.00</td> <td>90</td> <td>10</td> </tr> <tr> <td>5.50</td> <td>5</td> <td>95</td> </tr> <tr> <td>10.00</td> <td>5</td> <td>95</td> </tr> <tr> <td>11.00</td> <td>90</td> <td>10</td> </tr> <tr> <td>13.00</td> <td>90</td> <td>10</td> </tr> </tbody> </table>	Time (min)	Eluents A (%)	Eluents B (%)	0.00	90	10	1.00	90	10	5.50	5	95	10.00	5	95	11.00	90	10	13.00	90	10
Time (min)	Eluents A (%)	Eluents B (%)																				
0.00	90	10																				
1.00	90	10																				
5.50	5	95																				
10.00	5	95																				
11.00	90	10																				
13.00	90	10																				
Flow	300 μ l/min																					
Column temperature	ambient temperature																					
Autosampler temperature	ambient temperature																					
Injection volume	100 μ l																					
Detection	LCQ Duo mass spectrometer (Thermo Finnigan, San Jose, CA, USA)																					
T-4127, anionic part	ESI negative mass-mass detection Position 3 Collision energy: 30% Isolation width: 1.5 M/z 512.0 \rightarrow 388.2, 419.0																					
Internal standard	ESI negative mass detection Position 3 M/z 499.2																					

Conditions for the analytical method used in November 2002

Column Betasil C18, 50 x 2.0 mm; $d_p=5 \mu\text{m}$ (Thermo Hypersil, Keystone)
 Column temperature 25°C

Mobile phase

Time (minutes)	2.0 mM Ammonium acetate (%)	Methanol (%)
0	90	10
1	90	10
5.5	5	95
7.5	5	95
8.0	90	10
11	90	10

Flow 300 $\mu\text{l}/\text{min}$
 Column temperature 25 °C
 Autosampler temperature 4 °C
 Injection volume 10 μl
 Detection SCIEX MSMS system API-300 mass spectrometer (Applied Biosystems, Toronto, Canada)
 Interface Turbo ionspray at 450°C; N_2 flow rate of 7000 ml/min.; operated in negative ion mode
 Monitored masses MRM test substance m/z 512.1 → 168.8
 MRM internal standard m/z 499.0 → 99.0

Standard solutions

Standard solutions of T-4127 were prepared in methanol.

Calibration solutions used in July 2002

On each day of analysis, calibration solutions in 50/50 (v/v) methanol/ISO-medium were made up from two standard solutions. Internal standard solution was added to a final concentration of 120 $\mu\text{g}/\text{l}$.

Calibration solutions used in November 2002

On each day of analysis, calibration solutions in 1/5 (v/v) ISO-medium/isopropanol containing internal standard (internal standard concentration 164 $\mu\text{g}/\text{l}$) were made up from two standard solutions.

VALIDATION OF THE ANALYTICAL METHOD

The LCMSMS methods used were only partly validated. Upon request of the sponsor, validation was not completed. The parameters validated are given below.

Validation of the analytical method used in July 2002Specificity

Blank ISO-medium was pretreated as specified in 'sample pretreatment procedure' ¹ and subsequently injected in duplicate into the HPLC system. The resulting chromatograms were critically evaluated for interfering peaks by comparison with chromatograms of a test substance solution in the same medium. Interfering peaks are required to be $\leq 30\%$ of the LOQ.

Linearity

From two standard solutions (1020 and 1060 mg/l), eight dilutions were prepared in 50/50 (v/v) methanol/ISO-medium. This resulted in a concentration range of 0.0509 – 1.00 mg/l ¹. Each of these solutions was injected in triplicate. Responses were plotted against the concentrations. A linear regression program was used to calculate the regression line from the responses and concentrations. The correlation coefficient is required to be at least 0.99.

Stability of the solutions

Solutions of 0.0509 mg/l, 0.106 mg/l and 1.00 mg/l T-4127 ¹ were injected two times (in triplicate) over a 9.6, 8.9 and 6.1-hour time period, respectively. The maximum deviation of the responses was calculated for each concentration.

Validation of the analytical method used in November 2002Specificity

Blank ISO-medium was pretreated as specified in 'sample pretreatment procedure' ² and subsequently injected in duplicate into the HPLC system. The resulting chromatograms were critically evaluated for interfering peaks by comparison with chromatograms of a test substance solution in the same medium. Interfering peaks are required to be $\leq 30\%$ of the LOQ.

Linearity

From two standard solutions (782 and 874 mg/l), eight dilutions were prepared in 1/5 (v/v) ISO-medium/isopropanol containing internal standard. This resulted in a concentration range of 0.00500 – 0.100 mg/l ². Each of these solutions was injected in duplicate. Responses were plotted against the concentrations. A linear regression program was used to calculate the regression line from the responses and concentrations. The correlation coefficient is required to be at least 0.99.

Accuracy and Precision - repeatability

Blank ISO-medium (5 ml) was spiked using 50 μ l of a 1.00 mg/l, 10.0 mg/l or 60.1 mg/l solution of the test substance in methanol, yielding concentration levels of 0.00991 mg/l, 0.0991 mg/l and 0.595 mg/l test substance. At all concentration levels, six samples were prepared. These samples were treated as described in 'sample pretreatment procedure' and analysed in triplicate. The recovery was calculated for each sample. The mean recovery (n=6 in triplicate) at each concentration level is required to be in the range 70-110%.

¹ Internal standard was added to a final concentration 120 μ g/l.

² Internal standard was added to a final concentration 164 μ g/l.

Limit of quantitation (LOQ)

The LOQ was determined as the lowest concentration at which the mean recovery is in the range 70-110% and the coefficient of variation of the recovery is $\leq 20\%$.

Limit of detection (LOD)

A 0.00500 mg/l solution of the test substance in 1/5 (v/v) ISO-medium/isopropanol containing internal standard ² was injected in duplicate. In each chromatogram, the test substance peak height was measured as well as the noise level of the system (both in cps). The LOD was calculated from the mean peak height and the mean noise level.

DATA HANDLING –VALIDATION OF THE ANALYTICAL METHOD

Response:
$$R = \frac{\text{Peak area test substance}}{\text{Peak area internal standard}}$$

Mean:
$$\bar{x} = \frac{1}{n} \sum_{i=1}^n x_i$$

where:

x_i : measured value

n : number of measurements

Standard deviation:
$$s_{n-1} = \sqrt{\frac{\sum_{i=1}^n (x_i - \bar{x})^2}{(n-1)}}$$

Coefficient of variation: (standard deviation / mean value) * 100%

Maximum deviation: $[(\text{highest} - \text{lowest})/\text{mean}] * 100\%$
where 'mean' is the mean value of the highest and the lowest value.

Linearity A regression program was used to calculate the regression line or curve from the responses and concentrations. Regression analysis was performed using the least squares method. A (1/concentration) weighting factor was used in November 2002.

Validation of analytical method (July 2002) Regression curve: $Y = f X^2 + g X + h$

² Internal standard was added to a final concentration 164 $\mu\text{g/l}$.

Validation of analytical method
(November 2002)

Regression line: $Y = a X + b$

where:

Y: response

X: concentration

a: slope

b: intercept

f, g, h: regression constants

Recovery:

$(\text{Concentration analysed}^3 / \text{Concentration prepared}) * 100\%$

Limit of quantification (LOQ):

The lowest concentration of T-4127 tested with an acceptable recovery and coefficient of variation.

Limit of detection (LOD):

The limit of detection is defined as the concentration of T-4127 with a signal (peak height) of three times the noise level (S/N=3).

Limit of detection = $((3 * \text{noise level}) / \text{signal}) * \text{conc.}$

where:

noise level (N) : height of the noise [cps]

signal (S): height of the test substance peak [cps]

conc. : concentration of test substance [mg/l]

DATA HANDLING – SAMPLE ANALYSIS

Calibration

Response:

$$R = \frac{\text{Peak area test substance}}{\text{Peak area internal standard}}$$

Calibration curve:
(July 2002)

A regression program was used to calculate the regression curve from the responses and concentrations. Regression analysis was performed using the least squares method. If necessary, a $(1/\text{concentration})$ or $(1/\text{concentration}^2)$ weighting factor was used.

$$R = a * C + b$$

$$R = f * C^2 + g * C + h$$

R : response calibration solution

C : concentration of test substance in calibration solution [mg/l]

a, b, f, g, h: regression coefficients

On each day of analysis, a calibration curve or line was constructed using eight concentrations injected in triplicate. The coefficient of correlation was > 0.99 .

³ See 'DATA HANDLING – SAMPLE ANALYSIS'.

Calibration curve:
(November 2002)

The response was correlated with the concentration of test substance, using linear regression analysis (least squares method).

$$R = a * C + b$$

R = response calibration solution
C = concentration in the calibration solution [mg/l]
a = slope [l/mg]
b = intercept

On each day of analysis, two calibration solutions were used for quantification. Both calibration solutions were injected (in duplicate) before and after a maximum of six samples. Using the four responses, a calibration curve was constructed.

Samples

Recovery of recovery samples:

$$\frac{\text{Concentration analysed}}{\text{Concentration prepared}} * 100 \text{ [%]}$$

Concentration relative to nominal:

$$\frac{\text{Concentration analysed}}{\text{Concentration nominal}} * 100 \text{ [%]}$$

Concentration analysed:
(Quadratic regression July 2002)

$$C = d * \frac{-g + \sqrt{g^2 - 4 * f * (h - R)}}{2 * f} \text{ [mg/l]}$$

Concentration analysed :
(Linear regression July 2002)

$$C = d * \frac{R - b}{a} \text{ [mg/l]}$$

R : response sample [units]
d : dilution factor
a, b, f, g, h : regression constants

Concentration analysed:
(November 2002)

$$C = d * \frac{R - b}{a} \text{ [mg/l]}$$

R : response sample [units]
d : dilution factor
a : slope [units*l/mg]
b : intercept [units]

RESULTS – VALIDATION OF THE ANALYTICAL METHOD

The calculations for the validation tests were performed using not-rounded concentrations and responses. Therefore, some differences might be observed when calculating the statistical parameters using the values as mentioned in the tables.

Validation of the analytical method in July 2002

Specificity

Figures 1 and 2 show chromatograms of a blank solution (50/50 (v/v) methanol/ISO-medium containing internal standard) and of a 1.00 mg/l T-4127 solution, respectively. It was clear that blank chromatograms did not contain any interfering peaks at the position of the test substance.

Linearity

The results are summarized in Table 1. The regression line is shown in Figure 3.

Table 1 Linearity.

Concentration [mg/l]	Response ¹ [units]
0.0509	0.0109 / 0.0102 / 0.0098
0.0714	0.0141 / 0.0149 / 0.0148
0.106	0.0215 / 0.0207 / 0.0226
0.204	0.0408 / 0.0407 / 0.0444
0.403	0.0701 / 0.0726 / 0.0697
0.612	0.0979 / 0.1038 / 0.1030
0.806	0.125 / 0.130 / 0.118
1.00	0.146 / 0.153 / 0.156

¹ Triplicate measurements.

These results show that there is a relationship ($Y = 0.00244 + 0.1846 X - 0.03718 X^2$; $R^2 = 0.9959$) between response and concentration in the concentration range of 0.059 – 1.00 mg/l though a deviation of more than 10% of the calibration points from the calculated line was observed at various concentrations.

Stability of the solutions

The results are summarised in Tables 2-4.

Table 2 Stability of a 0.0509 mg/l T-4127 solution.

Elapsed time [hours]	Response	Maximum deviation [%]
0.0	0.0109	14.2
0.2	0.0102	
0.5	0.0098	
9.1	0.0107	
9.4	0.0105	
9.6	0.0095	

Table 3 Stability of a 0.106 mg/l T-4127 solution.

Elapsed time [hours]	Response	Maximum deviation [%]
0.0	0.0215	11.0
0.2	0.0207	
0.5	0.0226	
8.4	0.0217	
8.7	0.0228	
8.9	0.0204	

Table 4 Stability of a 1.00 mg/l T-4127 solution.

Elapsed time [hours]	Response	Maximum deviation [%]
0.0	0.1456	10.3
0.2	0.1530	
0.5	0.1559	
5.6	0.1583	
5.9	0.1426	
6.1	0.1573	

These results show that the solutions were stable (maximum deviation <20%) over at least a 9.6-hour time interval at a concentration of 0.0509 mg/l, at least a 8.9-hour time interval at a concentration of 0.106 mg/l and at least a 6.1-hour time interval at a concentration of 1.00 mg/l.

Validation of the analytical method in November 2002

Specificity

Figures 4 and 5 show chromatograms of a blank solution (1/5 (v/v) ISO-medium/isopropanol containing internal standard) and of a 0.500 mg/l T-4127 solution, respectively. It was clear that blank chromatograms did not contain any interfering peaks at the position of the test substance.

Linearity

The results are summarized in Table 5. The regression line is shown in Figure 6.

Table 5 Linearity.

Concentration [mg/l]	Response ¹ [units]
0.00500	0.0742 / 0.0727
0.00699	0.101 / 0.0944
0.0100	0.127 / 0.126
0.0199	0.272 / 0.260
0.0400	0.563 / 0.558 ²
0.0600	0.780 / 0.803
0.0782	0.891 / 0.952
0.100	1.23 / 1.16
Slope	12.1
Intercept with Y-axis	0.0134
Weighting factor	1/concentration
R	0.9979

¹ Duplicate measurements.

² Outliers; not used in calculation of the calibration curve.

From these results, it was concluded that there is a linear relationship between response and concentration in the concentration range of 0.00500 – 0.100 mg/l if a (1/concentration) weighting factor is used.

Accuracy and Precision - repeatability

ISO-medium spiked with T-4127 to concentrations of 0.00991 mg/l, 0.0991 and 0.595 mg/l (and 164 µg/l internal standard), was pretreated as described in 'sample pretreatment procedure'. Chromatograms of pretreated 0.00991 mg/l, 0.0991 and 0.595 mg/l samples are shown in Figures 7, 8 and 9. The results are summarised in Table 6.

Table 6 Accuracy and Precision.

Date of Preparation (dd-mm-yy)	Date of Analysis (dd-mm-yy)	Conc. prepared [mg/l]	Conc. analysed [mg/l]	Recovery ¹ [%]	Mean Recovery [%]	Coefficient of variation [%]
15-Nov-02	15-Nov-02	0.00991	<LOD <LOD <LOD <LOD <LOD <LOD	n.a. n.a. n.a. n.a. n.a. n.a.	n.a.	n.a.
15-Nov-02	15-Nov-02	0.0991	0.114 0.111 0.118 0.117 0.118 0.118	115 112 119 118 119 119	117	2.4
15-Nov-02	15-Nov-02	0.595	0.644 ² 0.641 ² 0.668 ² 0.670 ² 0.658 ² 0.662 ²	108 108 112 113 111 111	111	1.8

¹ Triplicate measurements. Maximum deviation of triplicate measurements was <20%

² Calculated by extrapolation. Responses were ≤110% of the high calibration level.

Though mean recoveries were all (slightly) higher than 110%, the analytical method was considered acceptable for samples in ISO-medium in the concentration range of 0.0991 – 0.595 mg/l because the coefficient of variation was <<20%.

Limit of quantification (LOQ)

The LOQ for the samples in ISO-medium is reported to be 0.0991 mg/l though recovery was 117%.

Limit of detection (LOD)

From two chromatograms of a 0.00500 mg/l solution of the test substance, the mean noise level (N) was determined to be 22.5 cps. From the same chromatograms, the test substance signal (S), i.e. the mean height of the test substance peak, was determined to be 87.5 cps. Using these values, the limit of detection (S/N=3) was calculated to be 0.0039 mg/l at an injection volume of 10 µl. Because samples from the ecotoxicological study were diluted by a factor of 6, the limit of detection for these samples is 0.023 mg/l.

RESULTS - SAMPLE ANALYSIS**Samples July 2002**

Table 7 shows the analytical results for the samples from the test performed in July 2002*.

Table 7 Concentrations of T-4127 in test medium in July 2002 (Full test 1).

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [dd-mm-yy]	Concentration		
			Nominal [mg/l]	Analysed ¹ [mg/l]	Relative to nominal [%]
0	01-Jul-02	02-Jul-02 ⁴	0	n.d.	n.a.
			100 ²	1.030	1.030
			100	0.447	0.447
24	02-Jul-02	02-Jul-02	0	n.d.	n.a.
			100 ²	0.562	0.562
			100	0.178	0.178
96	05-Jul-02	05-Jul-02	0	n.d.	n.a.
			100 ²	0.332	0.332
			100	0.082 ³	0.082

¹ Mean of triplicate analysis. The maximum deviation between the responses calculated for each sample was < 20%.

² 5 µm filtered solution prepared at a nominal concentration of 100 mg/l.

³ Calculated by extrapolation. Responses were ≥80% of the lowest calibration level.

⁴ Samples were pretreated on 01-Jul-02 and had to be stored overnight in a refrigerator together with the calibration solutions due to analytical problems. Inadvertently no internal standard was added to these samples and calibration solutions.

* All relative values were calculated using not-rounded concentrations. Therefore, some differences might be observed when calculating the relative values using the concentrations as mentioned in the table.

Samples November 2002

Table 8 shows the analytical results for the samples from the test performed in November 2002.

Table 8 Concentrations of T-4127 in test medium in November 2002 (final test).

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [dd-mm-yy]	Concentration		
			Nominal [mg/l]	Analysed ¹ [mg/l]	Relative to nominal [%]
0 ²	11-Nov-02	11-Nov-02	100	0.770	
			100	0.750	
0 ²	11-Nov-02	11-Nov-02	0	n.d.	n.a.
			0	n.d.	n.a.
			0.034	0.021 ³	61
			0.034	0.024 ^{3,4}	69
			0.076	0.064	84
			0.076	0.029 ³	38
			0.15	0.16	103
			0.15	0.13	87
			0.34	0.24	70
			0.34	0.20	59
			0.76	0.56	74
			0.76	0.75	98
			48	13-Nov-02	13-Nov-02
0	n.d.	n.a.			
0.034	< LOD	-			
0.034	< LOD	-			
0.076	0.022 ³	29			
0.076	0.021 ^{3,4}	27			
0.15	0.044	29			
0.15	0.038	25			
0.34	0.073	21			
0.34	0.083	24			
0.76	0.30	39			
0.76	0.31	40			

(Table 8 Continued)

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [dd-mm-yy]	Concentration		
			Nominal [mg/l]	Analysed ¹ [mg/l]	Relative to nominal [%]
96	15-Nov-02	15-Nov-02	0	n.d.	n.a.
			0	n.d.	n.a.
			0.034	< LOD	-
			0.034	< LOD	-
			0.076	< LOD	-
			0.076	< LOD	-
			0.15	0.021 ³	14
			0.15	0.019 ^{3,4}	13
			0.34	0.049 ⁴	14
			0.34	0.040 ⁴	12

¹ Mean of triplicate analysis. The maximum deviation between the responses calculated for each sample was < 20% unless otherwise indicated.

² Combined with NOTOX project 334349.

³ Calculated by extrapolation. Responses were between 50 and 100% of the lowest calibration level. These concentrations were not reported as < LOD because they were only slightly below the limit of detection.

⁴ Maximum deviation between responses was 23%, 36%, 32%, 22% and 22%, respectively.

n.d. Not detected. The limit of detection (LOD) was 0.023 mg/l.

n.a. Not applicable.

Samples July 2002

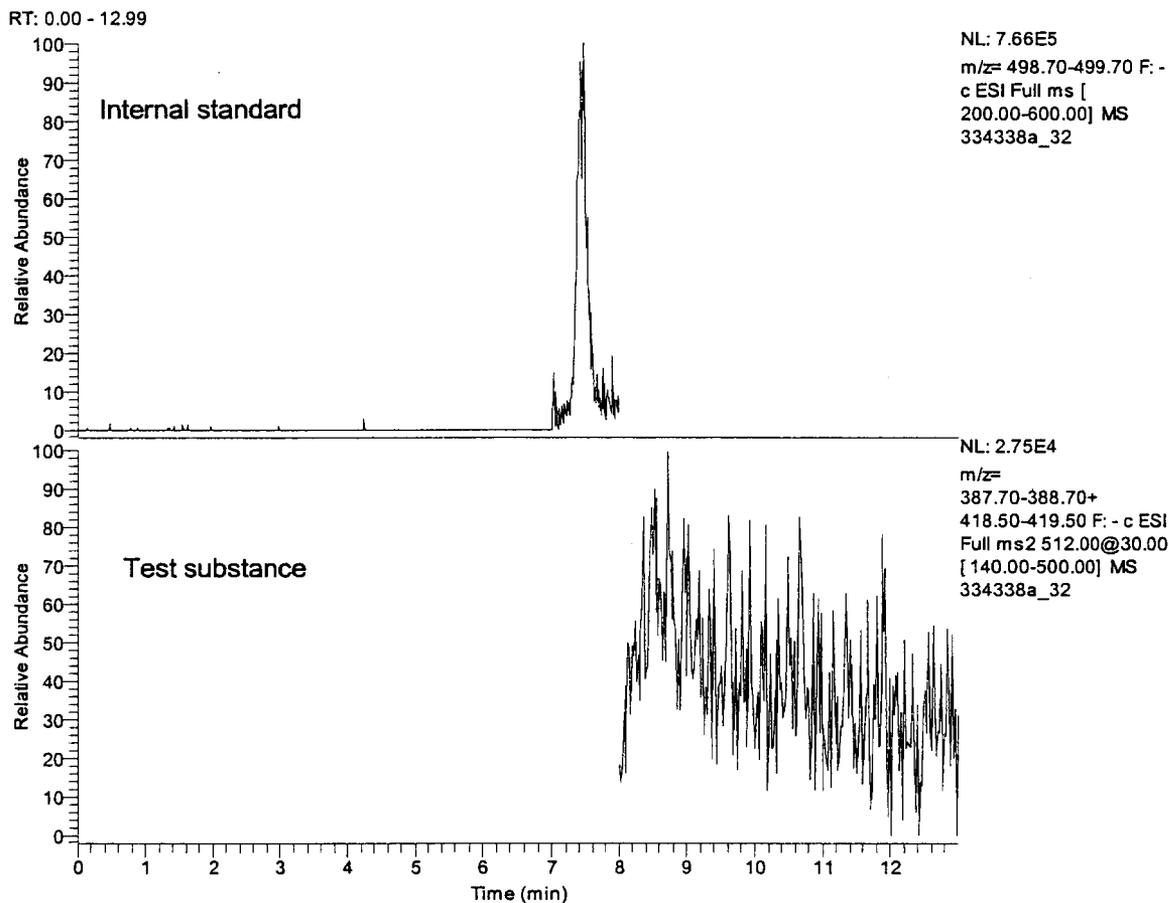


Figure 1 HPLC chromatogram of a blank (50/50 (v/v) methanol/ISO-medium containing internal standard).

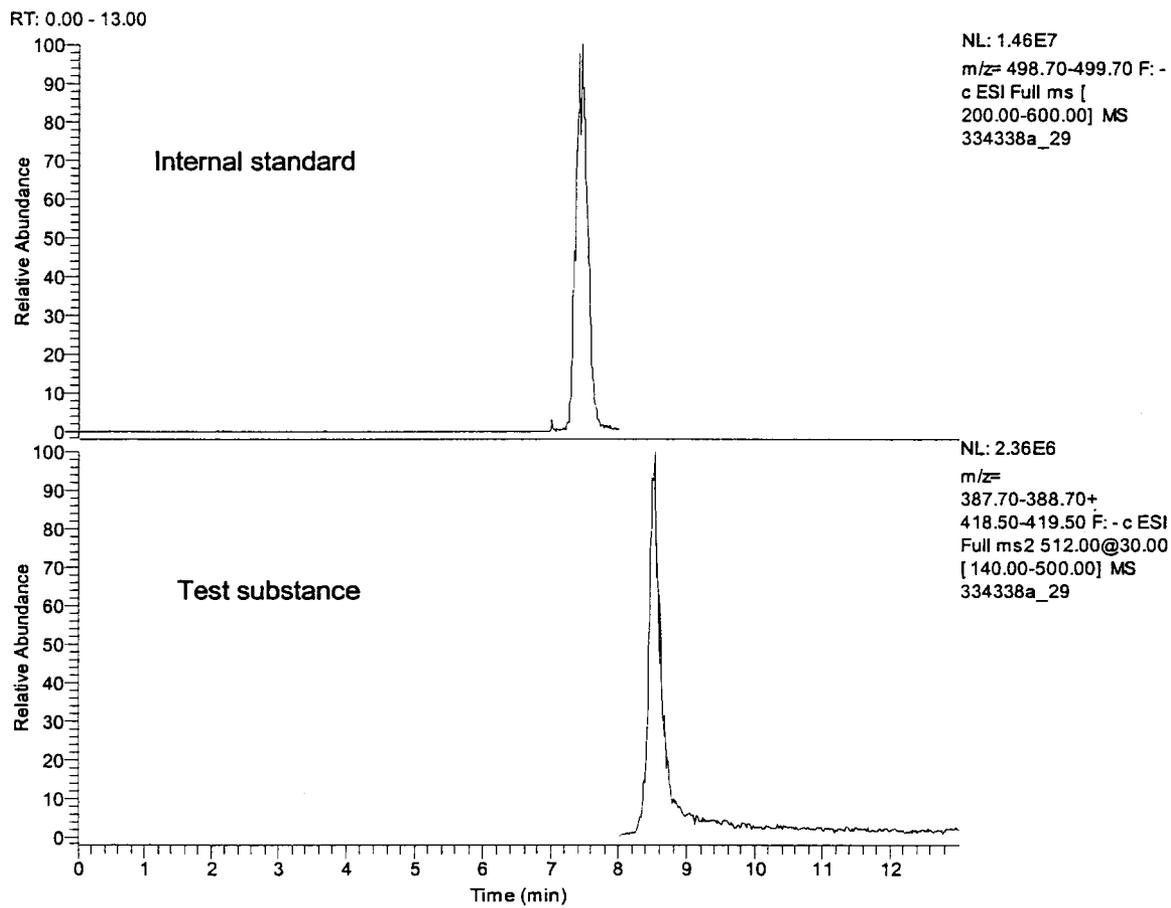


Figure 2 HPLC chromatogram of 1.00 mg/l T-4127 in 50/50 (v/v) methanol/ISO-medium containing internal standard.

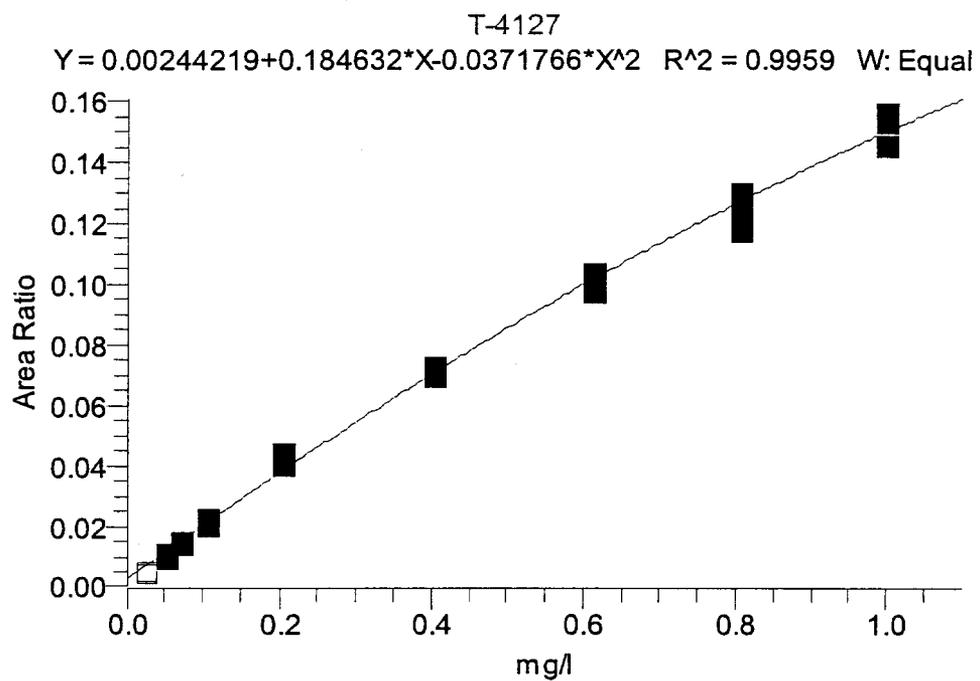


Figure 3 Regression line for solutions in 50/50 (v/v) methanol/ISO-medium containing internal standard: Responses against concentrations.

Samples November 2002

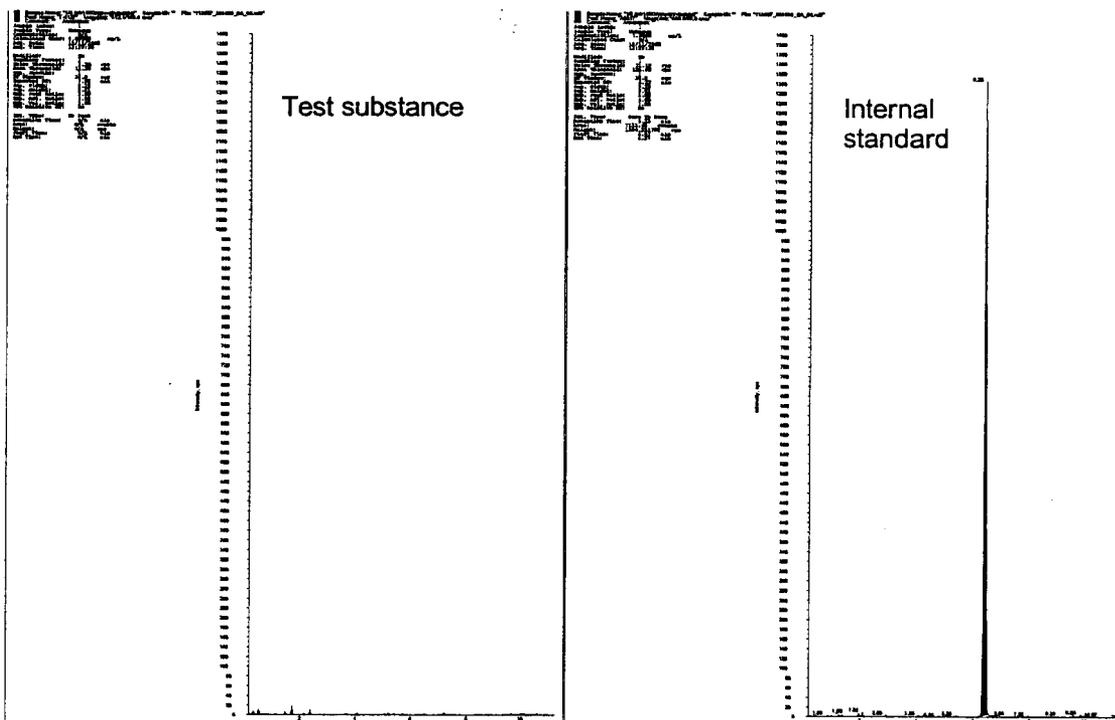


Figure 4 HPLC-chromatogram of a blank (1/5 (v/v) ISO-medium/isopropanol containing internal standard).

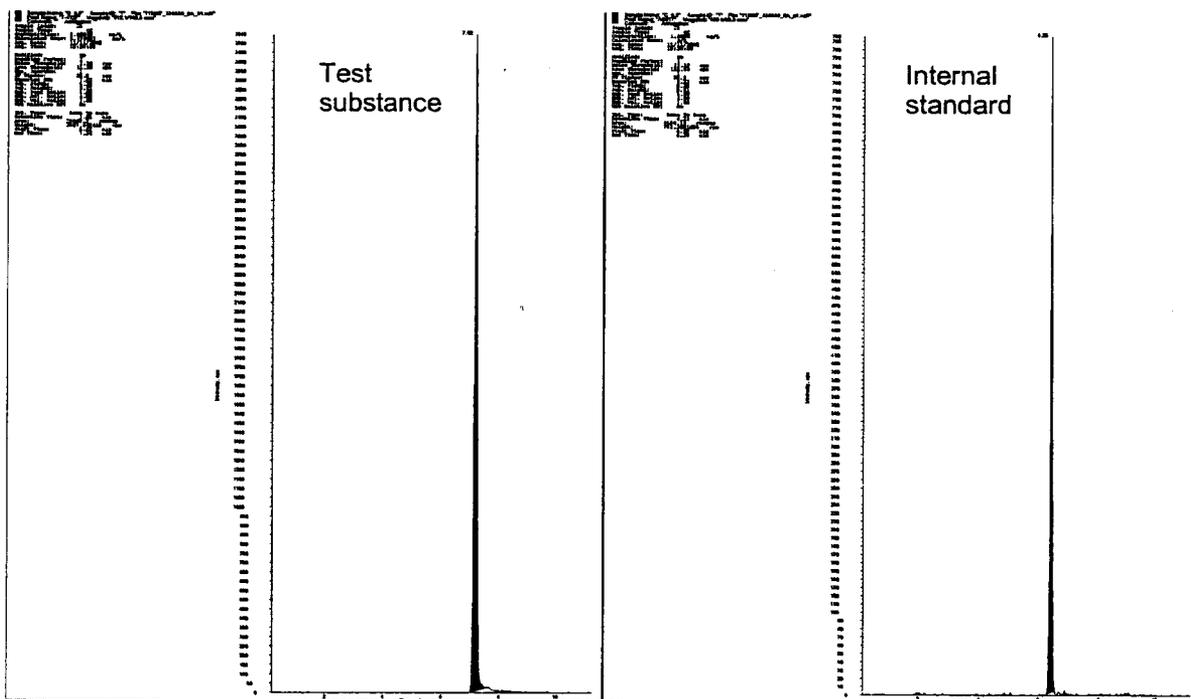


Figure 5 HPLC-chromatogram of 0.500 mg/l T-4127 in 1/5 (v/v) ISO-medium/isopropanol containing internal standard.

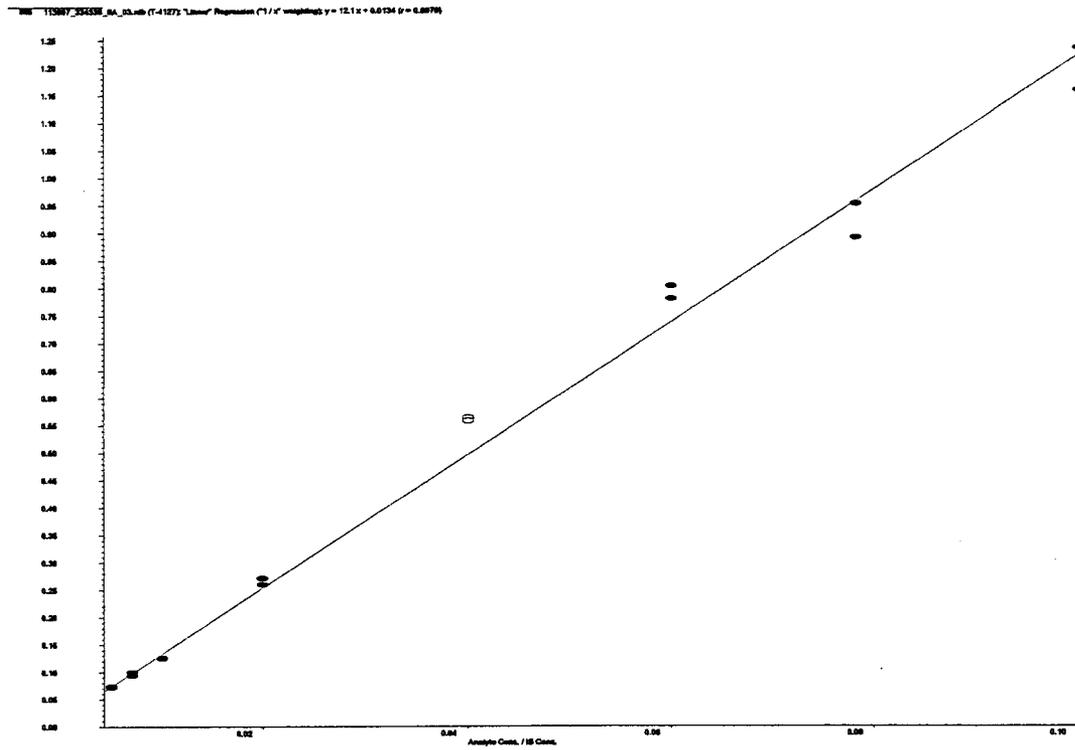


Figure 6 Regression line for solutions in 1/5 (v/v) ISO-medium/isopropanol containing internal standard: Responses against concentrations.

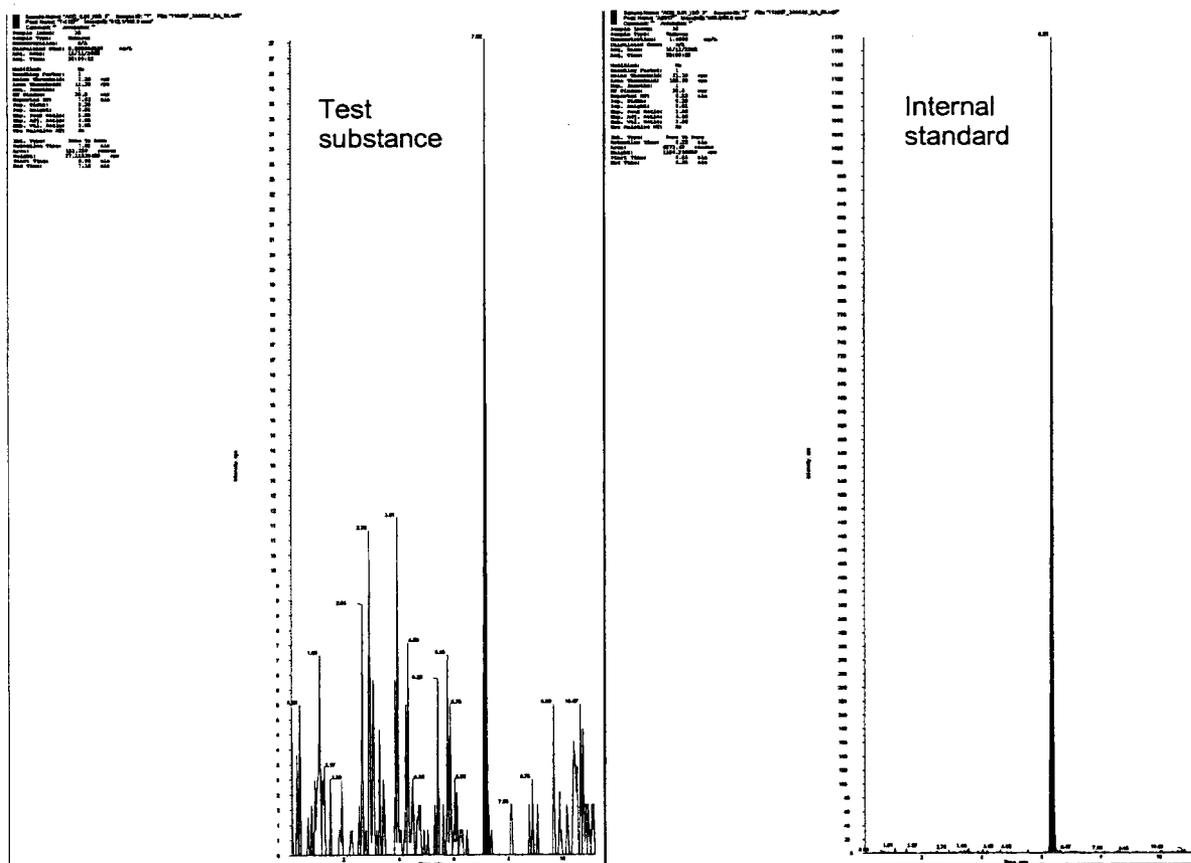


Figure 7 HPLC-chromatogram of 0.00991 mg/l T-4127 in 1/5 (v/v) ISO-medium/isopropanol containing internal standard.

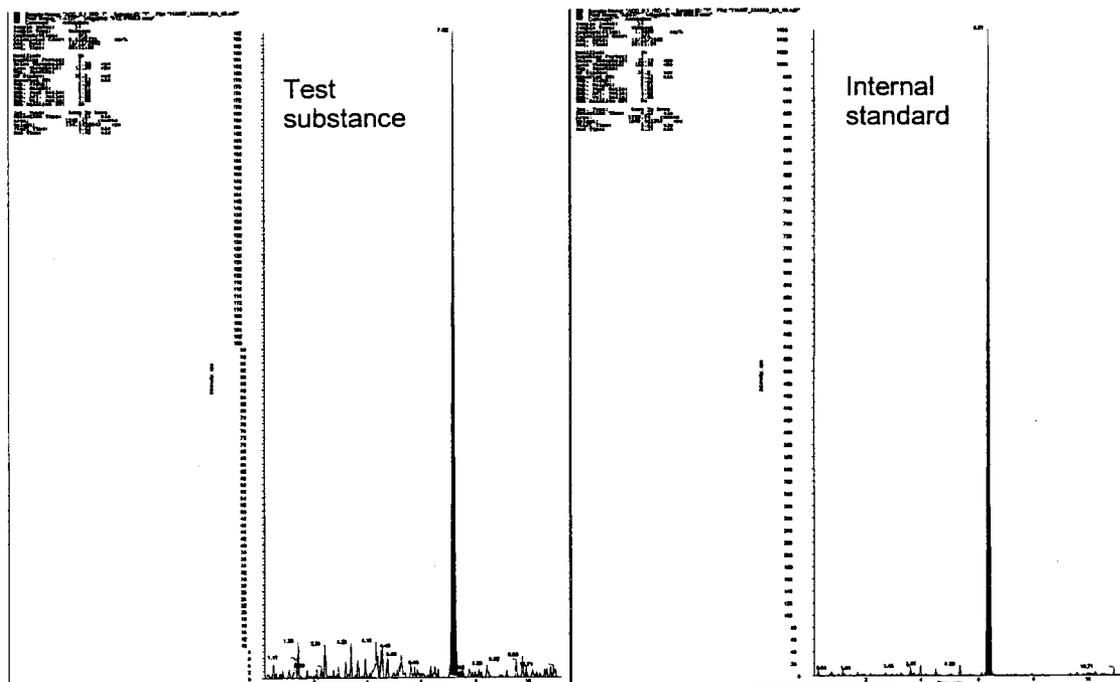


Figure 8 HPLC-chromatogram of 0.0991 mg/l T-4127 in 1/5 (v/v) ISO-medium/isopropanol containing internal standard.

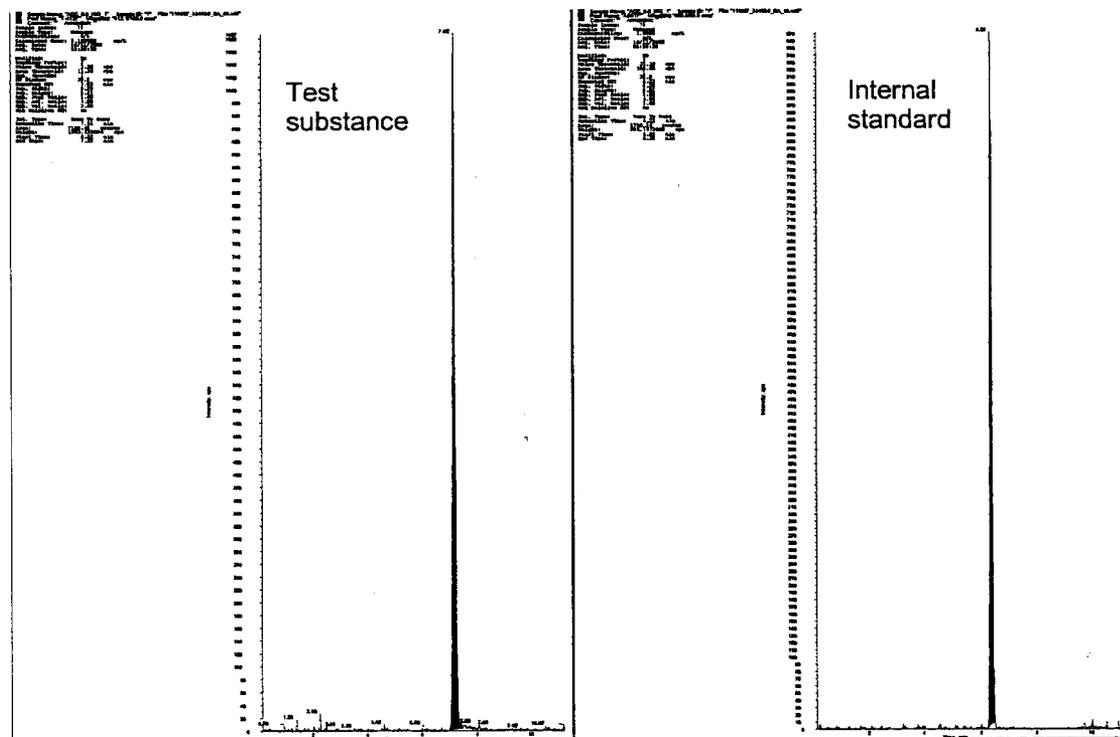


Figure 9 HPLC-chromatogram of 0.595 mg/l T-4127 in 1/5 (v/v) ISO-medium/isopropanol containing internal standard.

AR 226-1336

MR 267555

REPORT

ACUTE TOXICITY STUDY IN *DAPHNIA MAGNA*

WITH

T-4127

NOTOX Project 334349
NOTOX Substance 113607

- Page 1 of 21 -

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STATEMENT OF GLP COMPLIANCE

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

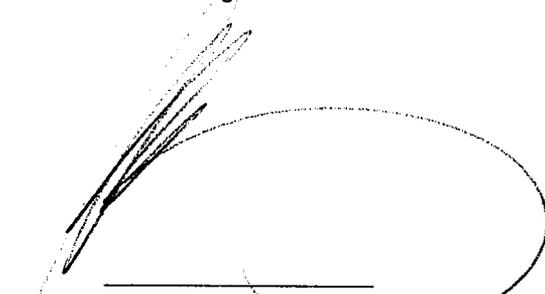
which are essentially in conformity with:

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.

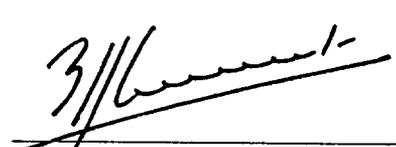
The United States Environmental Protection Agency (TSCA). Title 40 Code of Federal Regulations Part 792.

Study Director
Drs. M. Bogers



Date: 9 May 2003

Management:
Ing. E.J. van de Waart M.Sc.
Head of Genetic & Ecotoxicology



Date: 12/05/2003

QUALITY ASSURANCE STATEMENT

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) (Process)	
May 13-31, 2002 (Analytical Support)	June 04, 2002
July 08-15, 2002 (Ecotoxicology)	July 17, 2002
protocol inspection(s) (Study)	
January 31, 2002	January 31, 2002
report audit(s) (Study)	
May 01, 2003	May 01, 2003

Head of Quality Assurance

C.J. Mitchell B.Sc.



Date: 12 - 5 - 03

SUMMARY

Acute Toxicity Study in *Daphnia magna* with T-4127.

The study procedures described in this report were based on the ISO International Standard 6341: "Water quality – Determination of the inhibition of the mobility of *Daphnia magna* Straus – Acute toxicity test, Third edition, 1996-04-01. In addition, the procedures were designed to meet the test methods and validity criteria of the EEC directive 92/69, Part C: Methods for the determination of ecotoxicity, Publication No. L383, December 1992, C.2. "Acute Toxicity for *Daphnia*", and the OECD guideline No. 202 Part I: "*Daphnia* sp., Acute Immobilisation Test", Adopted April 4, 1984.

T-4127 is a salt consisting of Perfluorooctanesulfonylmethylamide (anionic part) and Triphenylbenzylphosphonium (cationic part). Based on the response of the anionic part of the test substance water solubility of T-4127 was determined to be between 0.6 and 0.9 mg/l at $19.5 \pm 0.6^\circ\text{C}$.

The study started with a limit test exposing twenty daphnids per concentration to a filtered and an unfiltered solution, prepared at a loading rate of 100 mg/l and magnetically stirred for four days. A blank-control was also included. After 24 hours of exposure all organisms exposed to the filtered and the unfiltered solutions prepared at a loading rate of 100 mg/l became immobilised.

A final test was performed exposing twenty daphnids per concentration to 4.5, 10, 20, 45 and 100% of a Water Accommodated Fraction (WAF) prepared at a loading rate of 100 mg/l and a blank control for a maximum of 48 hours. All glassware used in the final test was pre-treated with dichlorodimethylsilane. Although vapour pressure was relatively low, based on the Henry constant evaporation was still a possible cause for decrease of test concentrations. Therefore the study was performed in air-tight vessels. Samples taken during the final test were added directly to isopropanol at a ratio of 1:5 to prevent any additional loss.

The analytical program based on the anionic part showed that the actual concentrations could not be maintained at more than 80 % of the initial concentration, in spite of all precautions taken to prevent adsorption to glass and volatilization.

In the control no mortality was observed. Further, all test conditions (pH, oxygen concentration and temperature) remained within the ranges prescribed by the protocol.

Dilutions of a water phase of T-4127 originating from a loading rate of 100 mg/l induced 100% immobility of *Daphnia magna*. Chemical analysis of the anionic part of T-4127 showed that this corresponded with initial T-4127 concentrations down to 0.02 mg/l. Hence, the 48h-EC₅₀ of *Daphnia magna* exposed to T-4127 was < 1 mg/l (< 0.023 mg/l) based on quantification of the anionic part and was already reached within 24 hours of exposure.

Limiting the analytical support on only the anionic part of T-4127, validity of the test could not be ensured. There is a realistic possibility that the actual concentrations of the cationic part were higher and more stable than those of the anionic part. However, the sponsor did not agree with further research and decided to accept the highly toxic labeling (R50) based on the current results.

PREFACE

Sponsor	3M Corporate Toxicology 3M Center, Building 220-2E-02 P.O. Box 33220 ST. PAUL, MINNESOTA 55133-3220 U.S.A.
Study Monitor	S. A. Beach 3M Environmental Technology and Safety Services Building 2-3E-09 935 Bush Avenue St. Paul, MN 55144 (Address to which all communication is to be sent)
Testing Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Aquatic Toxicology: Study Director Technical co-ordinator Analytical Chemistry: Principal Scientist	Drs. M. Bogers Ing. B. van Wees Ir. M.J.C. Brekelmans
Study Plan	Start Project: January 30, 2002 Start first exposure: July 01, 2002 Completion last exposure: November 13, 2002 Completion Analysis: November 13, 2002 Completion project: May 09, 2003

TEST SUBSTANCE

Identification	T-4127
Description	Dark amber waxy solid
Batch	D-2491 lot 2
Composition	95 – 99% Fluoroelastomer curative <2% N-Methyl Perfluorooctanesulphonamide <1% Methyl Alcohol <1% Ethyl Alcohol <1% Isopropyl Alcohol
Test substance storage	At room temperature in the dark
Stability under storage conditions	Stable
Expiry date	31 March 2003
Stability in water	Not indicated

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

PURPOSE

The purpose of the toxicity test was to evaluate the influence of T-4127 on the mobility of *Daphnia magna*.

GUIDELINES

The study procedures described in this report were based on the ISO International Standard 6341: "Water quality - Determination of the inhibition of the mobility of *Daphnia magna* Straus - Acute toxicity test, Third edition, 1996-04-01.

In addition, the procedures were designed to meet the test methods and validity of the following guidelines:

- European Economic Community (EEC), EEC directive 92/69, Part C: Methods for the determination of ecotoxicity, Publication No. L383, December 1992, C.2. "Acute Toxicity for *Daphnia*".
- Organization for Economic Co-operation and Development (OECD), OECD guidelines for Testing of Chemicals, guideline No. 202 Part I: "*Daphnia sp.*, Acute Immobilisation Test", Adopted April 4, 1984.

ARCHIVING

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

DEFINITIONS

Immobilisation: those animals not able to swim within 15 seconds after gentle agitation of the test vessel are considered to be immobile.

EC₅₀: the concentration estimated to immobilise 50% of the daphnia after a defined period of exposure.

No Observed Effect Concentration (NOEC): the highest tested concentration at which no effect (i.e. immobilisation) is recorded.

TEST SYSTEM

Species	<i>Daphnia magna</i> (Crustacea, Cladocera) (Straus, 1820)
Reason for selection	This system has been selected as an internationally accepted species.
Validity of batch	Daphnids originated from a healthy stock, 2 nd to 5 th brood, showing no signs of stress such as mortality >20%, presence of males, ephippia or discoloured animals and there was no delay in the production of the first brood.
Characteristics	For the test selection of young daphnia with an age of < 24 hours.

BREEDING

Start of each batch	With new-born animals, i.e. less than 3 days old, by placing about 250 of them into 10 litres of medium in an all-glass culture vessel.
Maximum age of the cultures	4 weeks
Renewal of the cultures	After 7 days of cultivation half of the medium twice a week.
Temperature of medium	18-22°C, constant within $\pm 1^\circ\text{C}$
Feeding	Daily, a suspension of fresh water algae.
Medium	M7, as prescribed by Dr. Elendt-Schneider (Elendt, B.-P., 1990: Selenium deficiency in Crustacea. An ultrastructural approach to antennal damage in <i>Daphnia magna</i> Straus. Protoplasma 154, 25-33).

Composition of medium M7:

The following chemicals (analytical grade) are dissolved in tap water purified by reverse osmosis (milli-RO):

Macro salts (mg/l):	CaCl ₂ ·2H ₂ O	293.8
	MgSO ₄ ·7H ₂ O	123.3
	NaHCO ₃	64.8
	KCl	5.8
Trace elements (mg/l):	B	0.125
	Fe	0.05
	Mn	0.025
	Li, Rb and Sr	0.0125
	Mo	0.0063
	Br	0.0025
	Cu	0.0016
	Zn	0.0063
	Co and I	0.0025
	Se	0.0010
	V	0.0003
		Na ₂ EDTA·2H ₂ O
Macro nutrients (mg/l):	Na ₂ SiO ₃ ·9H ₂ O	10.0
	NaNO ₃	0.27
	KH ₂ PO ₄	0.14
	K ₂ HPO ₄	0.18
Vitamins (μg/l):	Thiamine	75.0
	B ₁₂	1.0
	Biotin	0.75

The hardness: 250 mg/l expressed as CaCO₃ and the pH: 8.0 \pm 0.2 after aeration.

REFERENCE SUBSTANCE

This report includes the results of a reference test with potassium dichromate.

PREPARATION OF TEST SOLUTIONS

The standard test procedures required generation of test solutions, which should contain completely dissolved test substance concentrations or stable and homogeneous mixtures or dispersions. The testing of concentrations that disturb the test system should be prevented (e.g. film of the test substance on the water surface).

T-4127 is a salt consisting of Perfluorooctanesulfonylmethylamide (anionic part) and Triphenylbenzylphosphonium (cationic part). Based on the response of the anionic part of the test substance water solubility of T-4127 was determined to be between 0.6 and 0.9 mg/l at $19.5 \pm 0.6^\circ\text{C}$ (NOTOX Project 340842, using the column elution method).

Limit test

A stock solution was prepared by weighing 100 mg of T-4127 on a glass surface. After placing the carrier in a measuring flask 1 litre of test medium was added to obtain a loading rate of 100 mg/l. The mixture was then magnetically stirred for four days. After the stirring period the mixture was clear but contained test substance particles, which settled at the bottom of the flask. Part of the mixture was used as such while another part was filtered through a paper filter (Schleicher and Schuell 604) to remove the larger undissolved test substance particles (ca. $> 5\mu\text{m}$). The final test solution was clear and colourless.

Final test

All glassware used in the final test was pre-treated with dichlorodimethylsilane to prevent loss of test substance due to adsorption to glass surfaces. A stock solution was prepared by weighing 500 mg of T-4127 on a glass surface. After placing the carrier in a measuring flask 5 litre of test medium was added to obtain a loading rate of 100 mg/l. Simultaneously, another measuring flask was prepared in the same way but without adding test substance (blank-control). After the stirring period, the solution prepared at a loading rate of 100 mg/l was transferred to a separation funnel and left to stabilise for 2 $\frac{3}{4}$ hours. Subsequently the Water Accommodated Fraction (WAF) was separated from the centre of the separation funnel. Lower test concentrations were prepared by dilution of the WAF. All final test solutions were clear and colourless. The test solutions originated from those used for the acute fish toxicity test (NOTOX Project 334338).

LIMIT TEST

A limit test was performed exposing twenty daphnids per concentration to a filtered and an unfiltered solution prepared at a loading rate of 100 mg/l and a blank-control. No samples were taken during the limit test.

FINAL TEST:

TEST CONCENTRATIONS

T-4127

4.5, 10, 20, 45 and 100% of a WAF prepared at a loading rate of 100 mg/l.

Control Test medium without test substance or other additives (Blank-control).

TEST PROCEDURE AND CONDITIONS

Test type	Static
Test duration	48 hours
Test vessels	100 ml, all-glass. Pre-treated with dichlorodimethylsilane. Although vapour pressure was relatively low, based on the Henry constant evaporation was still a possible cause for decrease of test concentrations. Therefore the study was performed in air-tight vessels.
Medium	ISO, prepared in milli-RO water
Number of daphnia	20 per concentration
Loading	10 per vessel containing 80 ml medium
Light	16 hours photoperiod daily
Feeding	No feeding
Aeration	No aeration of the test solutions.
Introduction of daphnia	Within 2 hours after preparation of the test solutions.

SAMPLING FOR ANALYSIS OF TEST CONCENTRATIONS

Duplicate samples were taken from the stock solution directly after preparation of the WAF as a specification of the origin of samples taken during the test. During the final test duplicate samples were taken from all concentrations and the blank-control at the start of the test, from the 4.5 and 10% dilutions after 24 hours of exposure and from the 4.5% dilution at the end of the test period. The method of analysis is described in the appended Analytical Report.

Sampling: Volume	5 ml from the approximate centre of the test vessels.
Treatment	Samples were added directly to isopropanol at a ratio of 1:5 to prevent any additional loss.
Storage	Not applicable, samples were analysed on the day of sampling.

MEASUREMENTS AND RECORDINGS

Immobility (including mortality)	At 24 hours and 48 hours.
pH and dissolved oxygen	At the beginning and at the end of the test, for all concentrations and the control(s).

Temperature of medium

Continuously in a temperature control vessel,
beginning at the start of the test.**RESULTS**Limit test:

Immobility

Table 1 shows the responses recorded during the limit test. After 24 hours of exposure all organisms exposed to the filtered and the unfiltered solutions prepared at a loading rate of 100 mg/l became immobilised.

Table 1: Incidence of immobility in the limit test:

Loading rate T-4127 (mg/l)	Vessel number	Number Daphnia exposed	Response at 24 h		Response at 48 h	
			number	Total %	number	Total %
Blank-control	A	10	0	0	0	0
	B	10	0		0	
100; filtered	A	10	10	100	10	100
	B	10	10		10	
100; unfiltered	A	10	10	100	10	100
	B	10	10		10	

Experimental conditions

The results of measurement of pH and oxygen concentrations (mg/l) are presented in Table 2. The temperature of the test medium measured in the temperature control vessel varied from 19.2 to 19.9 °C.

Table 2: pH and oxygen concentrations during the limit test.

Loading rate T-4127 (mg/l)	Start (t=0 h)		End (t=48 h)	
	pH	O ₂	pH	O ₂
Blank-control	8.0	9.1	7.8	9.0
100; filtered	8.0	9.0	7.8	8.9
100; unfiltered	8.0	9.0	7.8	8.8

Final test:

Measured concentrations

The results of analysis of the samples taken during the study are described in Table 1 of the appended Analytical Report.

During the final test the exposure consisted of a range of dilutions of a water fraction prepared at 100 mg/l containing 4.5 to 100% of this water fraction. Samples of 5 ml taken from these dilutions were added to 25 ml isopropanol and analyzed using the API300 LCMSMS (injection volume 10 µl). Recovery samples were prepared in ISO-medium at 0.01 mg/l, 0.1 mg/l and 0.6 mg/l. The 0.01 mg/l concentration proved to be below LOD. Recoveries at 0.1 mg/l and 0.6 mg/l were relatively high (117% and 111% respectively).

Samples were taken in duplicate from the test solutions and each pretreated sample was injected in triplicate. The initial concentration in the water phase was 0.76 mg/l which corresponds with the water solubility of 0.6 – 0.9 mg/l (anionic part). Only the lowest treatment was sampled and analyzed during the whole exposure period. At the start of the test, the concentration of the lowest treatment was 0.023 mg/l, i.e. just above the limit of detection (LOD). After 48 hours it was below the LOD.

Immobility

Table 3 shows the responses recorded during the final test. After 24 hours 55% immobilization of daphnids was reached at the lowest treatment, while 100% effect was recorded in the 10% treatment and higher. After 48 hours also all daphnids at the lowest treatment were immobile. An acceptable 10% effect was recorded in the control replicates.

Table 3: Acute immobilisation of daphnia after 24 and 48 hours in the final test.

T-4127 % of a WAF (100 mg/l)	Vessel number	Number Daphnia exposed	Response at 24 h		Response at 48 h	
			number	Total %	number	Total %
Blank-control	A	10	0 (1)	0	1 (2)	10
	B	10	0		1	
4.5	A	10	5 (1)	55	10	100
	B	10	6 (1)		10	
10	A	10	10 (1)	100	10	100
	B	10	10		10	
20	A	10	10 (5)	100	10	100
	B	10	10 (1)		10	
45	A	10	10	100	10	100
	B	10	10		10	
100	A	10	10 (1)	100	10	100
	B	10	10		10	

Between brackets: number of daphnids observed trapped at the surface. These organisms were reimmersed into the respective solutions before recording of mobility.

Experimental conditions

The results of measurement of pH and oxygen concentrations (mg/l) are presented in Table 4. The temperature of the test medium measured in the temperature control vessel varied from 20.2 to 20.9 °C.

Table 4: pH and oxygen concentrations during the final test.

T-4127 % of a WAF (100 mg/l)	Start (t=0 h)		End (t=48 h)	
	pH	O ₂	pH	O ₂
Blank-control	8.0	8.7	7.9	8.5
4.5	8.0	8.4	7.9	8.3
10	7.9	8.4	7.9	8.6
20	7.8	8.4	7.9	8.6
45	7.9	8.2	7.9	8.6
100	7.8	7.6	7.9	8.6

ACCEPTABILITY OF THE TEST

1. In the controls, not more than 10% of the daphnids became immobilised or trapped at the surface of the water.
2. All test conditions (pH, oxygen concentration and temperature) remained within the ranges prescribed by the protocol.
3. The analytical program based on the anionic part showed that the actual concentrations could not be maintained at more than 80 % of the initial concentration, in spite of all precautions taken to prevent adsorption to glass and volatilization.

CONCLUSION

Under the conditions of the present acute tests, dilutions of a water phase of T-4127 originating from a loading rate of 100 mg/l induced 100% immobility of *Daphnia magna*. Chemical analysis of the anionic part of T-4127 showed that this corresponded with initial T-4127 concentrations down to 0.02 mg/l. Hence, the 48h-EC₅₀ of *Daphnia magna* exposed to T-4127 was < 1 mg/l (< 0.023 mg/l) based on quantification of the anionic part and was already reached within 24 hours of exposure.

Limiting the analytical support on only the anionic part of T-4127, validity of the test could not be ensured. There is a realistic possibility that the actual concentrations of the cationic part were higher and more stable than those of the anionic part. However, the sponsor did not agree with further research and decided to accept the highly toxic labelling (R50) based on the current results.

REFERENCE TEST

Start: November 04, 2002
End : November 06, 2002

48-hour Acute Toxicity Study in *Daphnia magna* with $K_2Cr_2O_7$ (NOTOX Project 356669).

The study procedures described in this report were based on the ISO International Standard 6341, the EEC directive 92/69, Part C.2. "Acute toxicity for *Daphnia*" and the OECD guideline No. 202: "Daphnia sp., Acute Immobilisation Test", Adopted April 4, 1984.

The reference test was carried out to check the sensitivity of the test system as used by NOTOX. *Daphnia* were exposed for a maximum of 48 hours to $K_2Cr_2O_7$ concentrations of 0.10, 0.18, 0.32, 0.56, 1.0 and 1.8 mg/l and to a blank control. Ten daphnia were exposed per concentration.

The reference substance, potassium dichromate ($K_2Cr_2O_7$, art. 4864, batch no. K28974764) was obtained from Merck, Darmstadt, Germany.

Acute immobilization of daphnia after 24 and 48 hours in the reference test with potassium dichromate:

Concentration $K_2Cr_2O_7$ (mg/l)	Number exposed	% immobile		Expected response (%) after 48 hours ¹	
		24h	48h	Minimal	Maximal
Blank-control	10	0	0	0	10 ²
0.10	10	0	0 ³	0	10
0.18	10	0	0 ³	0	10
0.32	10	0	0 ⁴	0	30
0.56	10	0	20	0	100
1.0	10	60	100	40	100
1.8	10	100	100	100	100

¹ Based on historical data of the previous years (n>60).

² A maximum response of 10% does not invalidate the results of the test.

³ Two daphnids were observed trapped at the surface of the test solutions. These daphnids were reimmersed in the respective solutions before scoring of mobility.

⁴ Slight precipitation and a floating layer were observed.

The actual responses in this reference test with $K_2Cr_2O_7$ are within the ranges of the expected responses at the different concentrations. Hence, the sensitivity of this batch of *Daphnia magna* was in agreement with the historical data collected at NOTOX.

The 24h-EC₅₀ was 0.93 mg/l with 95% fiducial limits of 0.82 – 1.2 mg/l.

The 48h-EC₅₀ was estimated to be 0.63 mg/l with 20% immobility at 0.56 mg/l and 100% immobility at 1.0 mg/l.

The protocol, raw data and report of this study are kept in the NOTOX archives. The test described above was performed under GLP conditions with a QA-check.

ANALYTICAL REPORT

ACUTE TOXICITY STUDY IN *DAPHNIA MAGNA*

WITH

T-4127;

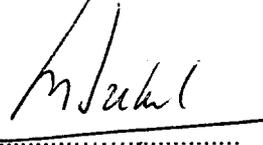
DETERMINATION OF THE CONCENTRATIONS

**NOTOX Project 334349
NOTOX Substance 113607**

REPORT APPROVAL

PRINCIPAL SCIENTIST:

Ir. M.J.C. Brekelmans
(Analytical Chemistry)



A handwritten signature in cursive script, appearing to read 'M.J.C. Brekelmans', is written over a solid horizontal line. Below this line is a dotted horizontal line.

Date: *May 08, 2003*

PREFACE

Analytical study	Start:	11 November 2002
	Completed:	13 November 2002

PURPOSE

The purpose of the analytical study was to determine the test concentrations and to validate the analytical method used.

REAGENTS

Milli-Q water	Tap water purified by reversed osmosis and subsequently passed over activated carbon and ion-exchange cartridges; Millipore, Bedford, MA, USA
Methanol	P.a., Merck, Darmstadt, Germany
ISO-medium	see main report
Ammonium acetate	Fractopur, Merck, Darmstadt, Germany
2.0 mM ammonium acetate	154 mg ammonium acetate in 1 litre of Milli-Q water
Isopropanol (2-propanol)	p.a., Merck, Darmstadt, Germany
Isopropanol containing internal standard	140 µl of a solution of 2816 mg/l perfluorooctane sulfonate in methanol filled up to 2000 ml with isopropanol.

INTERNAL STANDARD

Identification number	AS517
Name	Perfluorooctane sulfonate (FC-95)
Description	White solid (determined at NOTOX)
Batch number	Lot 217
Purity	90.49%
Expiry Date	21 February 2003 (determined at NOTOX)
Certified	Yes
Storage conditions	At room temperature in the dark
Supplier	3M

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

SAMPLE PRETREATMENT PROCEDURE

Each sample (5 ml) was transferred quantitatively into a 50 ml volumetric flask and 25 ml of isopropanol containing internal standard was added (internal standard concentration 164 µg/l).

ANALYTICAL METHOD

T-4127 is a salt consisting of Perfluorooctanesulfonylmethylamide (anionic part) and Triphenylbenzylphosphonium (cationic part). Upon request of the sponsor, quantitative analyses were only based on the anionic part of T-4127 using High Performance Liquid Chromatography with Mass Spectrometric detection (LCMSMS).

Analytical conditions

Column	Betasil C18, 50 x 2.0 mm; $d_p = 5 \mu\text{m}$ (Thermo Hypersil, Keystone)																					
Column temperature	25°C																					
Mobile phase																						
	<table border="1"> <thead> <tr> <th>Time (minutes)</th> <th>2.0 mM Ammonium acetate (%)</th> <th>Methanol (%)</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>90</td> <td>10</td> </tr> <tr> <td>1</td> <td>90</td> <td>10</td> </tr> <tr> <td>5.5</td> <td>5</td> <td>95</td> </tr> <tr> <td>7.5</td> <td>5</td> <td>95</td> </tr> <tr> <td>8.0</td> <td>90</td> <td>10</td> </tr> <tr> <td>11</td> <td>90</td> <td>10</td> </tr> </tbody> </table>	Time (minutes)	2.0 mM Ammonium acetate (%)	Methanol (%)	0	90	10	1	90	10	5.5	5	95	7.5	5	95	8.0	90	10	11	90	10
Time (minutes)	2.0 mM Ammonium acetate (%)	Methanol (%)																				
0	90	10																				
1	90	10																				
5.5	5	95																				
7.5	5	95																				
8.0	90	10																				
11	90	10																				
Flow	300 $\mu\text{l}/\text{min}$																					
Column temperature	25 °C																					
Autosampler temperature	4 °C																					
Injection volume	10 μl																					
Detection	SCIEX MSMS system API-300 mass spectrometer (Applied Biosystems, Toronto, Canada)																					
Interface	Turbo ionspray at 450°C; N_2 flow rate of 7000 ml/min.; operated in negative ion mode																					
Monitored masses	MRM test substance m/z 512.1 --> 168.8 MRM internal standard m/z 499.0 --> 99.0																					

Standard and calibration solutions

Standard solutions of T-4127 were prepared in methanol.

On each day of analysis, calibration solutions in 1/5 (v/v) ISO-medium/isopropanol containing internal standard (internal standard concentration 164 $\mu\text{g}/\text{l}$) were made up from two standard solutions.

VALIDATION OF THE ANALYTICAL METHOD

The LCMSMS method used was only partly validated during NOTOX project 334338. Upon request of the sponsor, validation was not completed.

DATA HANDLING – SAMPLE ANALYSIS

Calibration

Response (R):

$$R = \frac{\text{Peak area test substance}}{\text{Peak area internal standard}}$$

Calibration curve: The response was correlated with the concentration of test substance, using linear regression analysis (least squares method) and a (1/concentration) weighting factor.

$$R = a * C + b$$

R = response calibration solution
 C = concentration in the calibration solution [mg/l]
 a = slope [l/mg]
 b = intercept

On each day of analysis, two calibration solutions were used for quantification. Both calibration solutions were injected (in duplicate) before and after a maximum of six samples. Using the four responses, a calibration curve was constructed.

Samples

Concentration analysed:

$$C = d * \frac{R - b}{a} \text{ [mg/l]}$$

R = response calibration solution
 C = concentration in the calibration solution [mg/l]
 a = slope [l/mg]
 b = intercept

Recovery of recovery samples:

$$\frac{\text{Concentration analysed}}{\text{Concentration prepared}} * 100 \text{ [%]}$$

Concentration relative to nominal:

$$\frac{\text{Concentration analysed}}{\text{Concentration nominal}} * 100 \text{ [%]}$$

RESULTS – VALIDATION OF THE ANALYTICAL METHOD

See NOTOX project 334338 “96-hour acute toxicity study in zebra-fish with T-4127 (static)”.

RESULTS - SAMPLE ANALYSIS

Table 1 shows the analytical results for the samples from the ecotoxicity study*.

Table 1 Concentrations of T-4127 in test medium (Full test 1).

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [dd-mm-yy]	Concentration			
			Percentage of filtrate ¹ [%]	Expected ² [mg/l]	Analysed ³ [mg/l]	Relative to expected [%]
0 ²	11-Nov-02	11-Nov-02	100		0.770	
			100		0.750	
0 ²	11-Nov-02	11-Nov-02	100	0.76	0.56	74
			100	0.76	0.75	98
			45	0.34	0.24	70
			45	0.34	0.20	59
			20	0.15	0.16	103
			20	0.15	0.13	87
			10	0.076	0.029 ⁵	38
			10	0.076	0.064	84
			4.5	0.034	0.021 ⁵	61
			4.5	0.034	0.024 ^{5,6}	69
			0	0	n.d.	n.a.
0	0	n.d.	n.a.			
24	12-Nov-02	12-Nov-02	10	0.076	0.027 ⁶	36
			10	0.076	0.024	32
			4.5	0.034	< LOD	-
			4.5	0.034	< LOD	-
48	13-Nov-02	13-Nov-02	4.5	0.034	< LOD	-
			4.5	0.034	< LOD	-

¹ Percentage of a 5 µm filtered solution prepared at nominal concentration of 100 mg/l.

² Based on the measured concentration (i.e. 0.760 mg/l) in the undiluted filtrate without algae.

³ Mean of triplicate analysis. The maximum deviation between the responses calculated for each sample was < 20% unless otherwise indicated.

⁴ Combined with NOTOX project 334338.

⁵ Calculated by extrapolation. Responses were >70% of the lowest calibration level.

⁶ Maximum deviation between responses was 23% and 27%, respectively.

n.d. Not detected. The limit of detection (LOD) was 0.023 mg/l.

n.a. Not applicable.

* All relative values were calculated using not-rounded concentrations. Therefore, some differences might be observed when calculating the relative values using the concentrations as mentioned in the table.

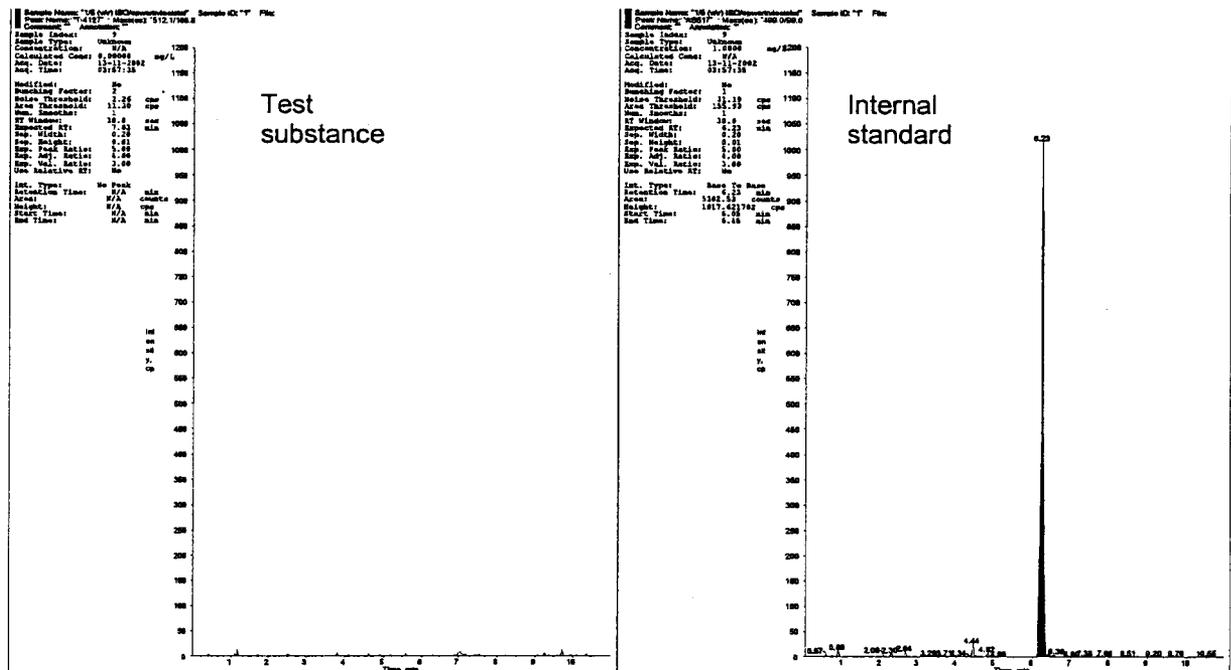


Figure 1 HPLC-chromatogram of a blank (1/5 (v/v) ISO-medium/isopropanol containing internal standard).

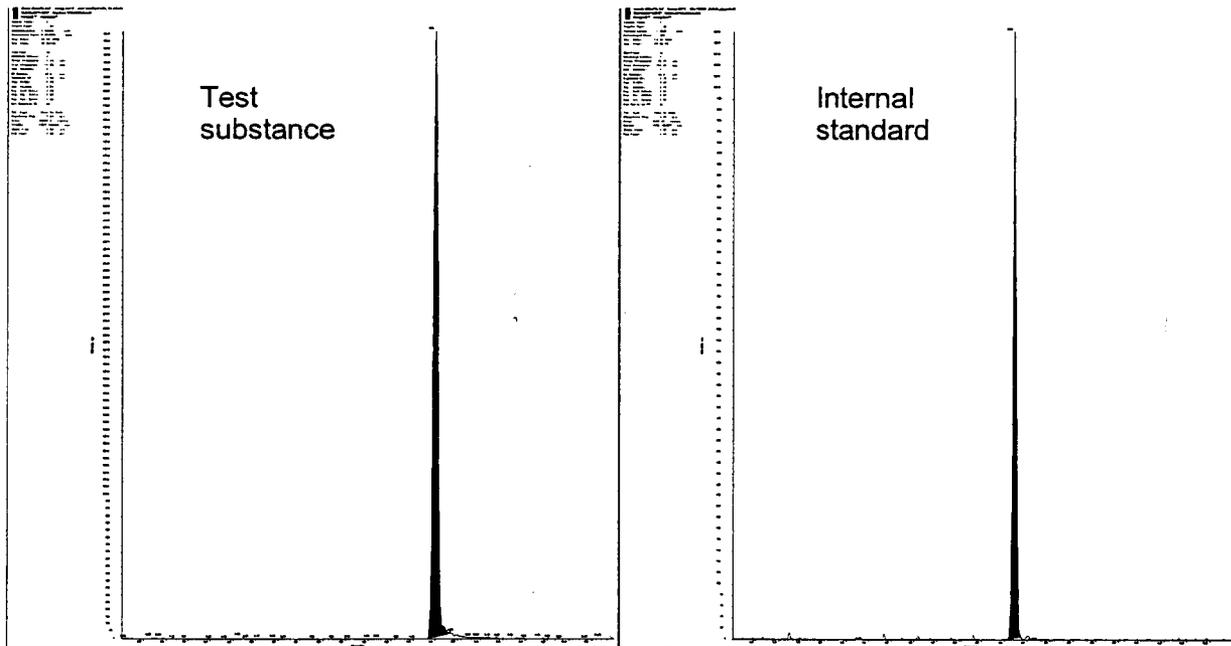


Figure 2 HPLC-chromatogram of 0.500 mg/l T-4127 in 1/5 (v/v) ISO-medium/isopropanol containing internal standard.

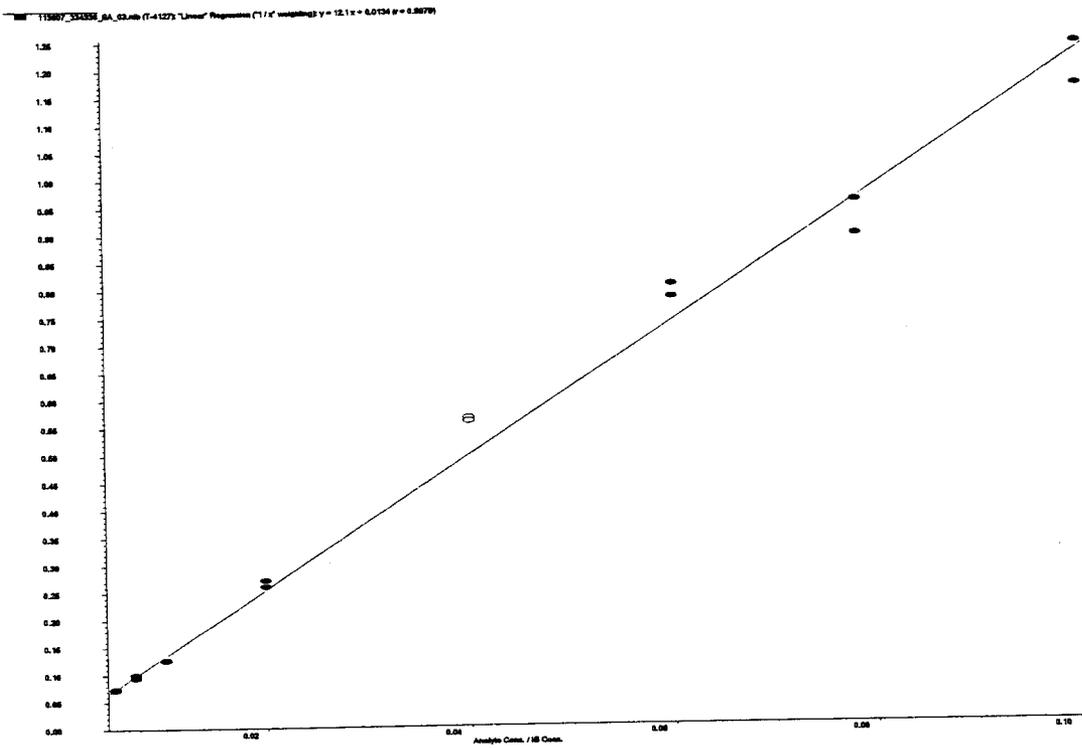


Figure 3 Regression line for solutions in 1/5 (v/v) ISO-medium/isopropanol containing internal standard: Responses against concentrations.

AR 226-1337

MR 267555

REPORT

96-HOUR FRESH WATER ALGAL GROWTH INHIBITION TEST

WITH

T-4127

RECEIVED
OPT NCIC
2003 JUN 25 PM 2:15

NOTOX Project 334351
NOTOX Substance 113607

RECEIVED
OPT NCIC
03 JUN 16 AM 11:14

STATEMENT OF GLP COMPLIANCE

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

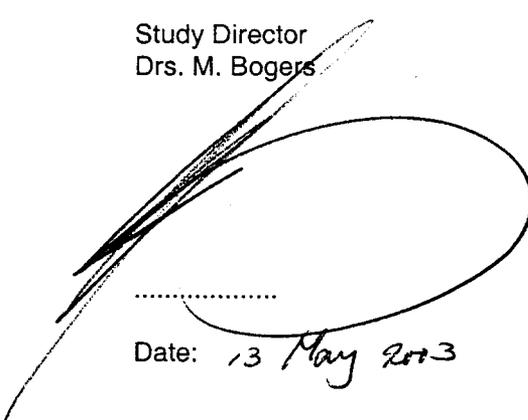
which are essentially in conformity with:

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
Drs. M. Bogers


.....
Date: 13 May 2003

Management:
Ing. E.J. van de Waart M.Sc.
Head of Genetic & Ecotoxicology


.....
Date: 13/05/2003

QUALITY ASSURANCE STATEMENT

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) (Process)	
October 07-17, 2002 (Ecotoxicology)	October 21, 2002
November 05-15, 2002 (Analytical Support)	November 19, 2002
protocol inspection(s) (Study)	
December 06, 2001	December 06, 2001
report audit(s) (Study)	
April 29, 2003	April 29, 2003

Head of Quality Assurance

C.J. Mitchell B.Sc.



Date: 14-5-03

SUMMARY

Selenastrum capricornutum, 96-Hour Fresh Water Algal Growth Inhibition Test with T-4127.

The study procedures described in this report were based on the EEC Directive 92/69, Publication No. L383 Part C-3 adopted December, 1992; OECD guideline No. 201, Adopted June 7, 1984; and ISO Standard 8692, First edition, 15 November 1989.

T-4127 is a salt consisting of Perfluorooctanesulfonylmethylamide (anionic part) and Triphenylbenzylphosphonium (cationic part). Based on the response of the anionic part of the test substance water solubility of T-4127 was determined to be between 0.6 and 0.9 mg/l at $19.5 \pm 0.6^\circ\text{C}$.

The study started with a limit test exposing exponentially growing algae to a filtered solution prepared at a loading rate of 100 mg/l, which was magnetically stirred for three days before filtration. A blank-control was also included. Concentrations in the filtered solution decreased from 1.2 mg/l to below the limit of detection during the test. The biological results showed that approximately total inhibition of cell growth and reduction of growth rate occurred at the limit concentration.

A final test was performed exposing exponentially growing algae to 4.5, 10, 20, 45 and 100% of a Water Accommodated Fraction (WAF) prepared at a loading rate of 100 mg/l and a blank control for a maximum of 96 hours. All glassware used in the final test was pre-treated with dichlorodimethylsilane. Although vapour pressure was relatively low, based on the Henry constant evaporation was still a possible cause for decrease of test concentrations. Therefore the study was performed in air-tight vessels. Samples taken during the final test were added directly to isopropanol at a ratio of 1:5 to prevent any additional loss.

Since 100% effect was recorded in the lowest treatment throughout the test only samples taken from this level were analyzed. Results showed that the measured anion concentration varied between 77 and 87%, relative to the initial concentration.

In the controls, cell density increased by an average factor of > 16 within 3 days. Further, temperature conditions remained within the ranges prescribed by the protocol. However, pH increased by more than 1.5 unit in the blank-control, but this was related to the air tightly capped vessels. Hence, this deviation did not affect the validity criterion for algal growth.

T-4127 induced total inhibition of cell growth and total reduction of growth rate of *Selenastrum capricornutum* at concentrations < 0.05 mg/l. Hence, the 96h-EC₅₀ of this fresh water algae species exposed to T-4127 was < 1 mg/l (< 0.05 mg/l).

Limiting the analytical support on only the anionic part of T-4127, validity of the test could not be ensured. There is a realistic possibility that the actual concentrations of the cationic part were higher and more stable than those of the anionic part. However, the sponsor did not agree with further research and decided to accept the highly toxic labelling (R50) based on the current results.

PREFACE

Sponsor	3M Corporate Toxicology 3M Center, Building 220-2E-02 P.O. Box 33220 ST. PAUL, MINNESOTA 55133-3220 U.S.A.
Study Coordinator	Mrs. M. Mitchell
Study Monitor	Mrs. Dr. S. Beach 3M Environmental Technology and Safety Services 935 Bush Avenue, Building 2-3E-09 ST. PAUL, MINNESOTA 55144 U.S.A.
Testing Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Aquatic Toxicology: Study Director Technical Coordinator Analytical Chemistry: Principal Scientist	Drs. M. Bogers Mrs. W. Koolen Ir. M.J.C. Brekelmans
Study Plan	Start Project: December 05, 2001 Start of first exposure: July 01, 2002 Completion last exposure: November 15, 2002 Completion Analysis: November 15, 2002 Completion project: May 13, 2003

TEST SUBSTANCE

Identification	T-4127
Description	Dark amber waxy solid
Batch	D-2491 lot 2
Composition	95 – 99% Fluoroelastomer curative <2% N-Methyl Perfluorooctanesulphonamide <1% Methyl Alcohol <1% Ethyl Alcohol <1% Isopropyl Alcohol
Test substance storage	At room temperature in the dark
Stability under storage conditions	Stable
Expiry date	31 March 2003
Stability in water	Not indicated

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

PURPOSE

The purpose of the study was to evaluate the test substance for its ability to inhibit the growth of green algae in a short-term experiment.

GUIDELINES

The study procedures described in this report were based on the ISO International Standard 8692: "Water quality - Fresh water algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum*", First edition, 15 November 1989.

In addition, the procedures were designed to meet the test methods and validity criteria prescribed by the following guidelines:

- European Economic Community (EEC), EEC Directive 92/69, Part C: Methods for the determination of ecotoxicity, Publication No. L383, C-3: "Algal Inhibition Test" adopted December, 1992.
- Organization for Economic Co-operation and Development (OECD), OECD guideline for Testing of Chemicals, guideline No. 201: "Algae, Growth Inhibition Test", Adopted June 7, 1984.

ARCHIVING

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

DEFINITIONS

- Cell density is the number of cells per millilitre.
- Growth is the increase in cell density over the test period.
- Growth rate is the increase in cell density per unit time. It is derived from the slope under the growth curve in a logarithmic plot. Following from the mathematical nature of exponential growth, the measure of the specific growth rate is preferable over biomass. The $E_R C_{50}$ is the concentration of test substance that results in a 50% reduction in growth rate relative to the control.
- Total growth or biomass is defined as the increase in total cell density over the test period. It is derived from the area under the growth curve in a linear plot. The $E_B C_{50}$ is the concentration of test substance that results in a 50% inhibition of total cell growth relative to the control.
- No Observed Effect Concentration (NOEC) is the highest tested concentration at which the measured parameter(s) show(s) no statistically significant effect on algal growth relative to control values.

TEST SYSTEM

Species	<i>Selenastrum capricornutum</i> , strain: NIVA CHL 1.
Reason for selection	This system is an unicellular algal species sensitive to toxic substances in the aquatic ecosystem and has been selected as an internationally accepted species.
Control of sensitivity	The results of the most recent reference test with potassium dichromate (Merck, Art. 4864) are appended to this report.

FRESH WATER ALGAE CULTURE

Stock culture	Algae stock cultures were started by inoculating growth medium with algal cells from a pure culture on agar. The suspensions were continuously aerated and exposed to light (4000-9000 lux) in a climate room at a temperature of $23 \pm 2^\circ\text{C}$.																																							
Stock culture medium	M1; formulated using Milli-RO water and with the following composition: <table> <tr> <td>NaNO₃</td> <td>500</td> <td>mg/l</td> </tr> <tr> <td>K₂HPO₄</td> <td>40</td> <td>mg/l</td> </tr> <tr> <td>MgSO₄.7H₂O</td> <td>76</td> <td>mg/l</td> </tr> <tr> <td>Na₂CO₃.10H₂O</td> <td>54</td> <td>mg/l</td> </tr> <tr> <td>C₈H₈O₇.H₂O</td> <td>6</td> <td>mg/l</td> </tr> <tr> <td>NH₄NO₃</td> <td>330</td> <td>mg/l</td> </tr> <tr> <td>CaCl₂.H₂O</td> <td>36</td> <td>mg/l</td> </tr> <tr> <td>C₈H₅FeO₇.xH₂O</td> <td>6</td> <td>mg/l</td> </tr> <tr> <td>H₃BO₃</td> <td>2.9</td> <td>mg/l</td> </tr> <tr> <td>MnCl₂.4H₂O</td> <td>1.81</td> <td>mg/l</td> </tr> <tr> <td>ZnCl₂</td> <td>0.11</td> <td>mg/l</td> </tr> <tr> <td>CuSO₄.5H₂O</td> <td>0.08</td> <td>mg/l</td> </tr> <tr> <td>(NH₄)₆Mo₇O₂₄.4H₂O</td> <td>0.018</td> <td>mg/l</td> </tr> </table>	NaNO ₃	500	mg/l	K ₂ HPO ₄	40	mg/l	MgSO ₄ .7H ₂ O	76	mg/l	Na ₂ CO ₃ .10H ₂ O	54	mg/l	C ₈ H ₈ O ₇ .H ₂ O	6	mg/l	NH ₄ NO ₃	330	mg/l	CaCl ₂ .H ₂ O	36	mg/l	C ₈ H ₅ FeO ₇ .xH ₂ O	6	mg/l	H ₃ BO ₃	2.9	mg/l	MnCl ₂ .4H ₂ O	1.81	mg/l	ZnCl ₂	0.11	mg/l	CuSO ₄ .5H ₂ O	0.08	mg/l	(NH ₄) ₆ Mo ₇ O ₂₄ .4H ₂ O	0.018	mg/l
NaNO ₃	500	mg/l																																						
K ₂ HPO ₄	40	mg/l																																						
MgSO ₄ .7H ₂ O	76	mg/l																																						
Na ₂ CO ₃ .10H ₂ O	54	mg/l																																						
C ₈ H ₈ O ₇ .H ₂ O	6	mg/l																																						
NH ₄ NO ₃	330	mg/l																																						
CaCl ₂ .H ₂ O	36	mg/l																																						
C ₈ H ₅ FeO ₇ .xH ₂ O	6	mg/l																																						
H ₃ BO ₃	2.9	mg/l																																						
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(NH ₄) ₆ Mo ₇ O ₂₄ .4H ₂ O	0.018	mg/l																																						
Pre-culture	3 or 4 days before the start of the test, cells from the algal stock culture were inoculated in culture medium at a cell density of $2 \cdot 10^4$ cells/ml. The pre-culture was maintained under the same conditions as used in the test. The cell density was measured immediately before use.																																							
Pre-culture medium	M2; according to the ISO-Standard "Algal growth inhibition test" Nov. 1989; formulated using Milli-Q water preventing precipitation and with the following composition: <table> <tr> <td>NH₄Cl</td> <td>15</td> <td>mg/l</td> </tr> <tr> <td>MgCl₂.6H₂O</td> <td>12</td> <td>mg/l</td> </tr> <tr> <td>CaCl₂.2H₂O</td> <td>18</td> <td>mg/l</td> </tr> <tr> <td>MgSO₄.7H₂O</td> <td>15</td> <td>mg/l</td> </tr> <tr> <td>KH₂PO₄</td> <td>1.6</td> <td>mg/l</td> </tr> <tr> <td>FeCl₃.6H₂O</td> <td>80</td> <td>μg/l</td> </tr> <tr> <td>Na₂EDTA.2H₂O</td> <td>100</td> <td>μg/l</td> </tr> </table>	NH ₄ Cl	15	mg/l	MgCl ₂ .6H ₂ O	12	mg/l	CaCl ₂ .2H ₂ O	18	mg/l	MgSO ₄ .7H ₂ O	15	mg/l	KH ₂ PO ₄	1.6	mg/l	FeCl ₃ .6H ₂ O	80	μg/l	Na ₂ EDTA.2H ₂ O	100	μg/l																		
NH ₄ Cl	15	mg/l																																						
MgCl ₂ .6H ₂ O	12	mg/l																																						
CaCl ₂ .2H ₂ O	18	mg/l																																						
MgSO ₄ .7H ₂ O	15	mg/l																																						
KH ₂ PO ₄	1.6	mg/l																																						
FeCl ₃ .6H ₂ O	80	μg/l																																						
Na ₂ EDTA.2H ₂ O	100	μg/l																																						

H ₃ BO ₃	185	μg/l
MnCl ₂ ·4H ₂ O	415	μg/l
ZnCl ₂	3	μg/l
CoCl ₂ ·6H ₂ O	1.5	μg/l
CuCl ₂ ·2H ₂ O	0.01	μg/l
Na ₂ MoO ₄ ·2H ₂ O	7	μg/l
NaHCO ₃	150	mg/l
Hardness (Ca+Mg)	0.24	mmol/l (24 mg CaCO ₃ /l)
pH	8.3 ± 0.2	

PREPARATION OF TEST SOLUTIONS

The standard test procedures required generation of test solutions, which contained completely dissolved test substance concentrations or stable and homogeneous mixtures or dispersions. The testing of concentrations that would disturb the test system were prevented as much as possible (e.g. film of the test substance on the water surface).

T-4127 is a salt consisting of Perfluorooctanesulfonylmethylamide (anionic part) and Triphenylbenzylphosphonium (cationic part). Based on the response of the anionic part of the test substance water solubility of T-4127 was determined to be between 0.6 and 0.9 mg/l at 19.5 ± 0.6°C (NOTOX Project 340842, using the column elution method).

Limit test

A stock solution was prepared by weighing 100 mg of T-4127 on a glass surface. After placing the carrier in a measuring flask 1 litre of test medium was added to obtain a loading rate of 100 mg/l. The mixture was then magnetically stirred for three days. After the stirring period the mixture was clear but contained test substance particles, which settled at the bottom of the flask. The mixture was filtered through a paper filter (Schleicher and Schuell 604) to remove the larger undissolved test substance particles (ca. > 5 μm). The final test solution was clear and colourless. Note that the blank-control received the same treatment. After preparation, volumes of 50 ml were added to each replicate of the respective test concentration. Subsequently, adequate volumes of an algal suspension were added to each replicate providing a cell density of 10⁴ cells/ml.

Final test

All glassware used in the final test was pre-treated with dichlorodimethylsilane to prevent loss of test substance due to adsorption to glass surfaces. A stock solution was prepared by weighing 200 mg of T-4127 on a glass surface. After placing the carrier in a measuring flask 2 litre of test medium was added to obtain a loading rate of 100 mg/l. After the stirring period, the stock solution was transferred to a separation funnel and left to stabilise for 4 hours. Subsequently the Water Accommodated Fraction (WAF) was separated from the centre of the separation funnel. Lower test concentrations were prepared by dilution of the WAF. All final test solutions were clear and colourless. Note that the blank-control received the same treatment. After preparation, volumes of 80 ml were added to each replicate of the respective test concentration to reduce the headspace as much as possible. The algal medium contained extra NaHCO₃ (150 mg/l instead of 50 mg/l) to prevent depletion of CO₂. Subsequently, adequate volumes of an algal suspension were added to each replicate providing a cell density of 10⁴ cells/ml.

LIMIT TEST

A limit test was performed exposing exponentially growing algae to a filtered solution prepared at a loading rate of 100 mg/l and a blank-control for a period of 72 hours.

Sampling: Frequency	at t=0 h, t=24 h and t=72 h.
Volume	10 ml from the approximate centre of the test vessel.
Storage	Not applicable, all samples were analysed on the day of sampling.

FINAL TEST:**TEST CONCENTRATIONS**

T-4127	4.5, 10, 20, 45 and 100% of a WAF prepared at a loading rate of 100 mg/l.
Controls	Test medium without test substance or other additives (Blank-control).
Replicates	3 replicates of each test concentration. 6 replicates of the blank-control. 1 replicate of each test concentration and the blank-control for sampling purposes. 2 replicates of the highest test concentration without algae, filled to the rim and protected from light for the course of the test.

TEST PROCEDURE AND CONDITIONS

Test type	Static
Test vessels	100 ml, all-glass Although vapour pressure was relatively low, based on the Henry constant evaporation was still a possible cause for decrease of test concentrations. Therefore the study was performed in air-tight vessels.
Medium	M2-medium
Cell density	An initial cell density of 1×10^4 cells/ml.
Test duration	96 hours
Illumination	Continuously using TLD-lamps of the type 'Cool-white' of 30 Watt, with a light intensity within the range of 73 to $102 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.
Incubation	During incubation the algal cells were kept in suspension by continuous shaking.

SAMPLING FOR ANALYSIS OF TEST CONCENTRATIONS

Duplicate samples were taken from all dilutions and the blank-control directly after preparation, from separate vessels of the 4.5% dilution and the blank-control after 48 and 96 hours. The method of analysis is described in the appended Analytical Report.

Sampling: Volume	5 ml.
Treatment	Samples were added directly to isopropanol at a ratio of 1:5 to prevent any additional loss.
Storage	Not applicable, samples were analysed on the day of sampling.

MEASUREMENTS

pH	At the beginning and at the end of the test. The pH of the solutions should preferably not deviate by more than 1.5 units during the test.
Temperature of medium	Continuously in a temperature-control vessel.

RECORDING OF CELL DENSITIES

At the beginning of the test, cells were counted by microscope, using a counting chamber. Thereafter cell densities were determined by spectrophotometric measurement of samples at 720 nm using a Varian Cary 50 single beam spectrophotometer with immersion probe (pathlength =20 mm). Varian Nederland BV., Houten, The Netherlands. Algal medium was used as blank.

DATA HANDLING

Calibration curve:

Quantification of cell densities was based on a calibration curve. Cell density was plotted versus extinction using spectrophotometric measurements of a minimum of six dilutions of an algal suspension with different cell densities. The calibration curve was composed using linear regression. The equation of this curve was then used to calculate the cell densities of the various test solutions at different points in time during the test period.

Comparison of areas under the growth curves:

The area below the growth curve was calculated using the formula:

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

Where: A = area

N_0 = nominal number of cells/ml at the start of the test

N_1 = measured number of cells/ml at t_1

N_n = measured number of cells/ml at t_n

t_1 = time of first measurements after beginning of the test

t_n = time of n^{th} measurement after beginning of the test

The percentage inhibition of cell growth at each test concentration (I_T) was calculated using the following formula:

$$I_T = \frac{A_C - A_T}{A_C} \times 100$$

Where: A_C = area below the growth curve obtained in the control
 A_T = area below the growth curve at each test substance concentration

Growth inhibition was calculated for the total period of 96h.

Comparison of growth rates:

The average specific growth rate (μ) for exponentially growing cultures was calculated as:

$$\mu = \frac{\ln N_n - \ln N_1}{t_n - t_1}$$

The average growth rate at each test substance concentration was then compared to the control value and the percentage reduction in growth rate was calculated.

RESULTS

Limit test:

The first test included samples prepared at a nominal loading rate of 100 mg/l filtered through a paper filter (ca. 5 μ m). Samples were diluted with methanol in 1:1 (v:v) ratio and analyzed using the LCQduo LCMSMS (injection volume 100 μ l). Concentrations in the filtered sample decreased from 1.2 mg/l to below the limit of detection during the test, see also Table 6 of the appended Analytical Report. The mean cell densities measured during the limit test are presented in Table 1. Table 2 presents the percentages growth inhibition and growth rate reduction per concentration. The results showed that approximately total inhibition of cell growth and reduction of growth rate occurred at the limit concentration.

Table 1: Mean cell densities ($\times 10^4$ cells/ml) during the limit test

Loading rate T-4127 (mg/l)	Exposure time (hours)			
	0	24	48	72
Blank-control	1.0	5.8	34.0	129.6
100; filtered	1.0	1.5	3.7	1.7

Table 2: Percentage reduction of growth rate and inhibition of total growth during the limit test

Loading rate T-4127 (mg/l)	Cell growth (0-72 hrs)		Mean growth rate	
	Mean area (A)	Inhibition (%)	μ (0-72 hrs)	Reduction (%)
Blank-control	2448.16		0.06744	
100; filtered	85.36	96.5	0.00721	89.3

Experimental conditions

Table 3 shows the pH recorded at the beginning and the end of the test. The temperature of the test medium was 23.2°C at the start of the test. During the exposure period the temperature measured in the incubator was maintained between 23.0 and 23.5°C.

Table 3: pH levels recorded during the limit test.

Loading rate T-4127 (mg/l)	Exposure time (hours)	
	0	96
Blank-control	8.0	9.0
100; filtered	8.0	8.0

Final test:

Measured test substance concentrations

The results of analysis of the samples taken during the study are described in Table 7 of the appended Analytical Report.

During the final test, sampling procedure was identical to that applied in the fish test. Because concentrations were expected to become below the limit of detection at the end of the test, it was decided to change sample pretreatment during the test and to test different injection volumes. Samples were taken in duplicate and each pretreated sample was injected in triplicate. The initial concentration in the water phase was 1.67 mg/l and thus higher than the water solubility limit of 0.6 – 0.9 mg/l. The concentrations measured at the start of the test are in Table 4.

Table 4: Mean concentrations of duplicate samples taken at the start of the test

T-4127 % of a WAF (100 mg/l)	Concentration T-4127 at the start of the test in mg/l		
	Sample 1	Sample 2	Mean
4.5	0.0671	0.0564	0.0617
10	0.152	0.160	0.1560
20	0.328	0.330	0.3292
45	0.754	0.742	0.7479
100	1.69	1.64	1.6670

Mean cell densities

Algal density was determined every 24 hours for a total of 96 hours and no algal growth was recorded in any of the treatments, while algae cells grew exponentially during the first 72 hours in the control group. Between 72 and 96 hours growth rate decreased, resulting in a sigmoid curve (see Figure 1).

Since 100% effect was recorded in the lowest treatment throughout the test only samples taken from this level were analyzed. The actual concentration remained rather stable (77-87% of initial), see Table 5.

Table 5: Mean concentration in the lowest treatment during the test

T-4127 % of a WAF (100 mg/l)	Concentration T-4127		
	0	48	96
4.5	0.0617	0.0474	0.0535

Table 6 shows mean cell densities measured at 24-hour intervals at the different concentrations of T-4127. The respective growth curves are shown in Figure 1 (see the Appendix I for the cell densities per replicate).

Table 6: Mean cell densities ($\times 10^4$ cells/ml) during the final test

T-4127 % of a WAF (100 mg/l)	Exposure time (hours)				
	0	24	48	72	96
Blank-control	1.0	1.5	17.1	53.3	62.1
4.5	1.0	1.0	1.0	1.0	1.0
10	1.0	1.0	1.0	1.0	1.0
20	1.0	1.0	1.0	1.0	1.0
45	1.0	1.0	1.0	1.0	1.0
100	1.0	1.0	1.0	1.0	1.0

Experimental conditions

Table 7 shows the pH recorded at the beginning and the end of the test. In spite of the addition of extra NaHCO_3 to the M2-medium pH increased more than 1.5 units in the blank-control during the test period. This was related to the air tightly capped vessels as a result of the limited amount of NaHCO_3 in the solutions and the limited CO_2 exchange from the headspace.

The temperature of the test medium was 23.1°C at the start of the test. During the exposure period the temperature measured in the incubator was maintained between 22.6 and 23.8°C .

Table 7: pH levels recorded during the final test.

T-4127 % of a WAF (100 mg/l)	Exposure time (hours)	
	0	96
Blank-control	8.4	10.4
4.5	8.4	8.6
10	8.4	8.3
20	8.4	8.2
45	8.5	8.1
100	8.6	8.2

ACCEPTABILITY OF THE TEST

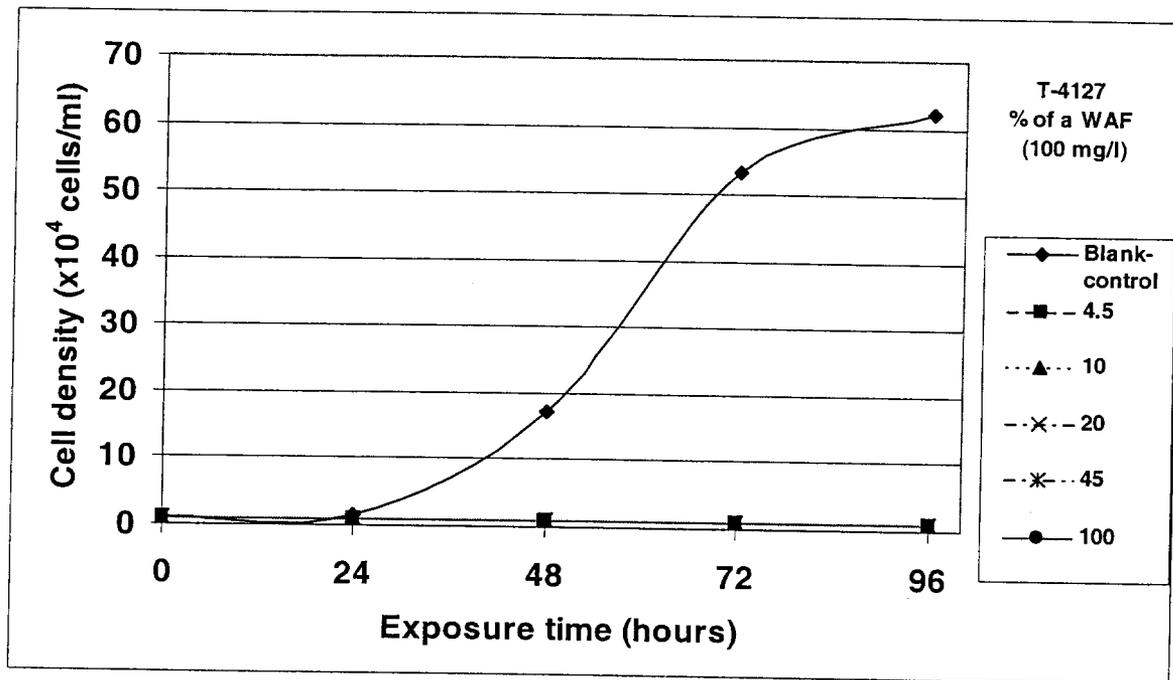
1. In the controls, cell density increased by an average factor of > 16 within 3 days.
2. Temperature conditions remained within the ranges prescribed by the protocol. However, pH increased by more than 1.5 unit in the blank-control, but this was related to the air tightly capped vessels. Hence, this deviation did not affect the validity criterion for algal growth.
3. Analysis of samples taken from the lowest treatment showed that the measured anion concentration varied between 77 and 87%, relative to the initial concentration. However, this concentration remained far more stable than similar concentrations in the fish and daphnia tests (NOTOX Projects 334338 and 334349). This was probably due to the difference in composition of test medium.

CONCLUSION

Under the conditions of the present study with *Selenastrum capricornutum*, T-4127 induced total inhibition of cell growth and total reduction of growth rate of this fresh water algae species at concentrations < 0.05 mg/l. Hence, the 96h-EC₅₀ of *Selenastrum capricornutum* exposed to T-4127 was < 1 mg/l (< 0.05 mg/l).

Limiting the analytical support on only the anionic part of T-4127, validity of the test could not be ensured. There is a realistic possibility that the actual concentrations of the cationic part were higher and more stable than those of the anionic part. However, the sponsor did not agree with further research and decided to accept the highly toxic labelling (R50) based on the current results.

Figure 1: Growth curves at different concentrations of T-4127.



REFERENCE TEST

Selenastrum capricornutum, fresh water algal growth inhibition test with potassium dichromate (NOTOX Project 356647).

Start of first exposure: September 30, 2002
 Completion last exposure: October 03, 2002

The study procedures described in this report were based on the EEC Directive 92/69, Publication No. L383 Part C-3 adopted December, 1992; OECD guideline No. 201, Adopted June 7, 1984; and ISO Standard 8692, First edition, 15 November 1989.

This reference test was carried out to check the sensitivity of the test system used by NOTOX to POTASSIUM DICHROMATE (Merck, Art. 4864, Batch K28974764).

Algae were exposed for a period of 72 hours to $K_2Cr_2O_7$ concentrations of 0.18, 0.32, 0.56, 1.0, 1.8 and 3.2 mg/l and to a blank-control. The initial cell density was 1.0×10^4 cells/ml.

Results:

Calculation of % reduction in growth rate in the reference test:

Concentration $K_2Cr_2O_7$ (mg/l)	Total cell growth:		Growth rate:	
	Mean Area for 0-72h	Inhibition %	Interval 0-72h	Reduction %
0	2000.16		0.06480	
0.18	1872.93	6.4	0.06427	0.8
0.32	2068.42	-3.4	0.06564	-1.3
0.56	1696.29	15.2	0.06266	3.3
1.0	1222.95	38.9	0.05723	11.7
1.8	355.99	82.2	0.03315	48.8
3.2	147.24	92.6	0.01067	83.5

Under the conditions of the reference study with *Selenastrum capricornutum*, potassium dichromate inhibited cell growth of this fresh water algae species at nominal concentrations of 0.56 mg/l and higher and reduced growth rate at 1.0 mg/l and higher.

The EC_{50} for cell growth inhibition ($E_B C_{50}$: 0-72h) was 1.1 mg/l with a 95 % confidence interval ranging from 0.78 to 1.6 mg/l. The historical ranges of the 72h EC_{50} for growth inhibition lie between 0.49 and 1.4 mg/l. Hence, the $E_B C_{50}$: 0-72h for the present batch corresponds with this range.

The EC_{50} for growth rate reduction ($E_R C_{50}$: 0-72h) was 1.9 mg/l with a 95 % confidence interval ranging from 1.6 to 2.2 mg/l. The historical ranges for growth rate reduction lie between 0.82 and 2.3 mg/l. Hence, the $E_R C_{50}$: 0-72h for the present batch corresponds with this range.

The protocol, raw data and report of this study are kept in the NOTOX archives. The test described above was performed under GLP conditions with a QA-check.

APPENDIX I

WORKSHEET DATA

Table I1: Cell densities calculated from the individual extinction values

Number of inoculated cells at t=0: 1×10^4 cells/ml						
T-4127 % of a WAF (100 mg/l)	Vessel number	Exposure time (hours)				
		0	24	48	72	96
Blank-control	1	1.00	1.81	15.67	50.75	63.03
	2	1.00	1.47	17.33	57.00	60.42
	3	1.00	1.97	19.49	55.29	62.50
	4	1.00	1.44	17.64	53.42	60.64
	5	1.00	1.05	16.87	52.00	64.73
	6	1.00	1.00	15.54	51.59	61.37
4.5	1	1.00	1.00	1.00	1.00	1.00
	2	1.00	1.00	1.00	1.00	1.00
	3	1.00	1.00	1.00	1.00	1.00
10	1	1.00	1.00	1.00	1.00	1.00
	2	1.00	1.00	1.00	1.00	1.00
	3	1.00	1.00	1.00	1.00	1.00
20	1	1.00	1.00	1.00	1.00	1.00
	2	1.00	1.00	1.00	1.00	1.00
	3	1.00	1.00	1.00	1.00	1.00
45	1	1.00	1.00	1.00	1.00	1.00
	2	1.00	1.00	1.00	1.00	1.00
	3	1.00	1.00	1.00	1.00	1.00
100	1	1.00	1.00	1.00	1.00	1.00
	2	1.00	1.00	1.00	1.00	1.00
	3	1.00	1.00	1.00	1.00	1.00

ANALYTICAL REPORT

96 HOUR FRESH WATER ALGAL GROWTH INHIBITION TEST

WITH

T-4127;

DETERMINATION OF THE CONCENTRATIONS

**NOTOX Project 334351
NOTOX Substance 113607**

REPORT APPROVAL

PRINCIPAL SCIENTIST:

Ir. M.J.C. Brekelmans
(Analytical Chemistry)



M. J. C. Brekelmans

Date: *May 13, 2003*

SAMPLE PRETREATMENT PROCEDURE**Analyses in July 2002**

Each sample (10 ml) was quantitatively transferred to a 20 ml volumetric flask. A volume of 200 μ l internal standard solution was added to the samples taken at 24, 48 and 72 hours, after which the flasks were filled up to the mark using methanol (internal standard concentration 120 μ g/l). No internal standard was added to the flasks containing the t=0 samples.

Analyses in November 2002

In order to prevent from adsorption, it was decided to change sample pre-treatment. Based on additional information supplied by the sponsor, each sample (5 ml) was quantitatively transferred to a 50 ml volumetric flask. A volume of 25 ml isopropanol containing internal standard was added to the 50 ml-flasks (internal standard concentration 164 μ g/l).

ANALYTICAL METHOD

T-4127 is a salt consisting of Perfluorooctanesulfonylmethylamide (anionic part) and Triphenylbenzylphosphonium (cationic part). Upon request of the sponsor, quantitative analyses were only based on the anionic part of T-4127 using High Performance Liquid Chromatography with Mass Spectrometric detection (LCMSMS). The analytical method used in July 2002 was not sensitive enough to measure concentrations in pretreated samples from the final test. Therefore, a second method was used in November 2002.

Analytical conditions for the analyses in July 2002

Column	Betasil C18, 50 x 2.0 mm; d_p = 5 μ m (Thermo Hypersil Keystone)																					
Mobile phase	Gradient Eluents A: 2.0 mM ammonium acetate Eluents B: 100 % methanol																					
	<table border="1"> <thead> <tr> <th>Time (min)</th> <th>Eluents A (%)</th> <th>Eluents B (%)</th> </tr> </thead> <tbody> <tr> <td>0.00</td> <td>90</td> <td>10</td> </tr> <tr> <td>1.00</td> <td>90</td> <td>10</td> </tr> <tr> <td>5.50</td> <td>5</td> <td>95</td> </tr> <tr> <td>10.00</td> <td>5</td> <td>95</td> </tr> <tr> <td>11.00</td> <td>90</td> <td>10</td> </tr> <tr> <td>13.00</td> <td>90</td> <td>10</td> </tr> </tbody> </table>	Time (min)	Eluents A (%)	Eluents B (%)	0.00	90	10	1.00	90	10	5.50	5	95	10.00	5	95	11.00	90	10	13.00	90	10
Time (min)	Eluents A (%)	Eluents B (%)																				
0.00	90	10																				
1.00	90	10																				
5.50	5	95																				
10.00	5	95																				
11.00	90	10																				
13.00	90	10																				
Flow	300 μ l/min																					
Column temperature	ambient temperature																					
Autosampler temperature	ambient temperature																					
Injection volume	100 μ l																					
Detection	LCQ Duo mass spectrometer (Thermo Finnigan, San Jose, CA, USA)																					
T-4127, anionic part	ESI negative mass-mass detection Position 3 Collision energy: 30% Isolation width: 1.5 M/z 512.0 \rightarrow 388.2, 419.0																					
Internal standard	ESI negative mass detection Position 3 M/z 499.2																					

Analytical conditions for the analyses in November 2002

Column	Betasil C18, 50 x 2.0 mm; $d_p = 5 \mu\text{m}$ (Thermo Hypersil Keystone)																					
Mobile phase	Gradient Eluents A: 2.0 mM ammonium acetate Eluents B: 100 % methanol																					
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Time (min)	Eluents A (%)	Eluents B (%)																				
0.00	90	10																				
1.00	90	10																				
5.50	5	95																				
7.50	5	95																				
8.00	90	10																				
11.00	90	10																				
Flow	300 $\mu\text{l}/\text{min}$																					
Column temperature	25 °C																					
Autosampler temperature	4 °C																					
Injection volume	10 μl ¹																					
Detection	SCIEX MSMS system API-300 mass spectrometer (Applied Biosystems, Toronto, Canada)																					
Interface	Turbo ionspray at 450°C; N_2 flow rate of 7000 ml/min.; operated in negative ion mode																					
Monitored masses	MRM test substance m/z 512.1 \rightarrow 168.8 MRM internal standard m/z 499.0 \rightarrow 99.0																					

Standard solutions

Standard solutions of T-4127 were prepared in methanol.

Calibration solutions for the analyses in July 2002

On each day of analysis, calibration solutions in 50/50 (v/v) methanol/M2-medium were made up from two standard solutions. Internal standard was added to a final concentration of 120 $\mu\text{g}/\text{l}$ except for samples taken at $t=0$ hours. Inadvertently, no internal standard was added to these samples.

Calibration solutions for the analyses in November 2002

On the day of analysis, calibration solutions in 1/5 (v/v) M2-medium/isopropanol containing internal standard (internal standard concentration 164 $\mu\text{g}/\text{l}$) were made up from two standard solutions.

VALIDATION OF THE ANALYTICAL METHOD

The LCMSMS methods used were only partly validated. Upon request of the sponsor, validation was not completed. The parameters validated are given below.

¹ Injection volumes of 20, 50 and 100 μl were tested but accuracy and precision was not improved and therefore it was decided to use 10 μl injection for analysis of samples.

Validation of the analytical method used in July 2002Specificity

Blank M2-medium¹ was pretreated as specified in 'sample pretreatment procedure' and subsequently injected in triplicate into the HPLC system. The resulting chromatograms were critically evaluated for interfering peaks by comparison with chromatograms of a test substance solution in the same medium. Interfering peaks are required to be $\leq 30\%$ of the LOQ.

Linearity

From two standard solutions (1020 and 1060 mg/l), eight dilutions were prepared in 50/50 (v/v) methanol/ M2-medium. This resulted in a concentration range of 0.0510 – 0.996 mg/l¹ internal standard. Each of these solutions was injected in triplicate. Responses were plotted against the concentrations. A linear regression program was used to calculate the regression line from the responses and concentrations. The correlation coefficient is required to be at least 0.99.

Stability of solutions

Solutions¹ of 0.0510 mg/l, 0.102 mg/l and 0.996 mg/l T-4127 were injected two times (in triplicate) over a 8.9, 8.4 and 5.4-hour time period, respectively. The maximum deviation of the responses was calculated for each concentration.

Limit of detection (LOD)

A 0.051 mg/l solution of the test substance in 50/50 (v/v) methanol/ M2-medium¹ was injected in triplicate. In each chromatogram, the test substance peak height was measured as well as the noise level of the system (both in mV). The LOD was calculated from the mean peak height and the mean noise level.

Validation of the analytical method used in November 2002Specificity

Blank M2-medium² was pretreated as specified in 'sample pretreatment procedure' and subsequently injected in triplicate into the HPLC system. The resulting chromatograms were critically evaluated for interfering peaks by comparison with chromatograms of a test substance solution in the same medium. Interfering peaks are required to be $\leq 30\%$ of the LOQ.

Linearity

From two standard solutions (782 and 874 mg/l), six dilutions were prepared in 1/5 (v/v) M2-medium/isopropanol containing internal standard. This resulted in a concentration range of 0.00501– 0.0996 mg/l². Each of these solutions was injected in duplicate. Responses were plotted against the concentrations. A linear regression program was used to calculate the regression line from the responses and concentrations. The correlation coefficient is required to be at least 0.99.

¹ Internal standard was added to a final concentration 120 $\mu\text{g/l}$.

² Internal standard was added to a final concentration of 164 $\mu\text{g/l}$.

DATA HANDLING – VALIDATION OF THE ANALYTICAL METHOD

Response:	$R = \frac{\text{Peak area test substance}}{\text{Peak area internal standard}}$
Mean:	$\bar{x} = \frac{1}{n} \sum_{i=1}^n x_i$
	where: x_i : measured value n : number of measurements
Standard deviation:	$s_{n-1} = \sqrt{\frac{\sum_{i=1}^n (x_i - \bar{x})^2}{(n-1)}}$
Coefficient of variation:	(standard deviation / mean value) * 100%
Maximum deviation:	[(highest - lowest)/mean] * 100% where 'mean' is the mean value of the highest and the lowest value.
Linearity	A regression program was used to calculate the regression line or curve from the responses and concentrations. Regression analysis was performed using the least squares method. If necessary a (1/concentration) weighting factor was used.
Validation of the analytical method (July 2002)	Regression curve: $Y = f X^2 + g X + h$
Validation of the analytical method (November 2002)	Regression line: $Y = a X + b$ where: Y : response X : concentration a : slope b : intercept f, g, h : regression constants
Recovery:	(Concentration analysed ¹ / Concentration prepared) * 100%
Limit of detection (LOD):	The limit of detection is defined as the concentration of T-4127 with a signal (peak height) of three times the noise level (S/N=3).

¹ See 'DATA HANDLING – SAMPLE ANALYSIS'.

Limit of detection= ((3 * noise level)/ signal) * conc.

where:

noise level (N) : height of the noise [%]

signal (S) : height of the test substance peak [%]

conc. : concentration of test substance [mg/l]

DATA HANDLING – SAMPLE ANALYSIS

Calibration

Response:

$$R = \frac{\text{Peak area test substance}}{\text{Peak area internal standard}}$$

Calibration curve:
(July 2002)

A regression program was used to calculate the regression curve from the responses and concentrations. Regression analysis was performed using the least squares method. If necessary a (1/concentration) weighting factor was used.

$$R = a * C + b$$

$$R = f * C^2 + g * C + h$$

R : response calibration solution

C : concentration of test substance in calibration solution [mg/l]

a, b, f, g, h: regression coefficients

An each day of analysis, a calibration curve or line was constructed using eight concentrations injected in triplicate. The coefficient of correlation was > 0.99.

Calibration curve:
(November 2002)

The response was correlated with the concentration of test substance, using linear regression analysis (least squares method) and a (1/concentration) weighting factor.

$$R = a * C + b$$

R = response calibration solution

C = concentration in the calibration solution [mg/l]

a = slope [l/mg]

b = intercept

On each day of analysis, two calibration solutions were used for quantification. Both calibration solution were injected (in duplicate) before and after a maximum of seven samples. Using the four responses, a calibration curve was constructed.

Samples

Recovery of recovery samples:

$$\frac{\text{Concentration analysed}}{\text{Concentration prepared}} * 100 [\%]$$

Concentration relative to nominal:

$$\frac{\text{Concentration analysed}}{\text{Concentration nominal}} * 100 [\%]$$

Concentration analysed:
(Quadratic regression July 2002)

$$C = d * \frac{-g + \sqrt{g^2 - 4 * f * (h - R)}}{2 * f} \quad [\text{mg/l}]$$

Concentration analysed :
(Linear regression July 2002)

$$C = \frac{(R-b) * d}{a} \quad [\text{mg/l}]$$

R : response sample [units]
d : dilution factor
a, b, f, g, h : regression constants

Concentration analysed:
(November 2002)

$$C = \frac{(R-b) * d}{a} \quad [\text{mg/l}]$$

R : response sample [units]
d : dilution factor
a : slope [units*l/mg]
b : intercept [units]

RESULTS –VALIDATION OF THE ANALYTICAL METHOD

The calculations for the validation tests were performed using not-rounded concentrations and responses. Therefore, some differences might be observed when calculating the statistical parameters using the values as mentioned in the tables.

Validation of the analytical method used in July 2002

Specificity

Figures 1 and 2 show chromatograms of a blank solution (50/50 (v/v) methanol/M2-medium) and of a 0.996 mg/l T-4127 solution, respectively. It was clear that blank chromatograms did not contain any interfering peaks at the position of the test substance.

Linearity

A quadratic regression was performed. The results are summarized in Table 1. The regression curve is shown in Figure 3.

Table 1 Quadratic regression curve.

Concentration [mg/l]	Response ¹ [units]
0.0510	0.0123 / 0.0117 / 0.0120
0.0700	0.0152 / 0.0144 / 0.0143
0.102	0.0213 / 0.0214 / 0.0224
0.199	0.0416 / 0.0418 / 0.0391
0.400	0.0804 / 0.0776 / 0.0774
0.602	0.1086 / 0.1088 / 0.1100
0.800	0.1325 / 0.1333 / 0.1384
0.996	0.1637 / 0.1553 / 0.1466

¹ Triplicate measurements.

From these results, it was concluded that there is a quadratic relationship ($Y = 6.07669 \cdot 10^{-5} + 0.220598 \cdot X - 0.065148 \cdot X^2$; $R^2 = 0.9973$) between response and concentration in the concentration range of 0.0510 – 0.996 mg/l though a deviation of more than 10% of the calibration points from the calculated line was observed at some concentrations.

Stability of the solutions

The results are summarised in Table 2.

Table 2 Stability of a 0.0510 mg/l T-4127 solution.

Elapsed time [hours]	Response	Maximum deviation [%]
0.0	0.0123	9.8
0.2	0.0117	
0.5	0.0120	
8.4	0.0113	
8.7	0.0111	
8.9	0.0114	

Table 3 Stability of a 0.102 mg/l T-4127 solution.

Elapsed time [hours]	Response	Maximum deviation [%]
0.0	0.0213	11.1
0.2	0.0214	
0.5	0.0224	
8.0	0.0215	
8.2	0.0217	
8.4	0.0200	

Table 4 Stability of a 0.996 mg/l T-4127 solution.

Elapsed time [hours]	Response	Maximum deviation [%]
0.0	0.1637	13.2
0.2	0.1553	
0.5	0.1466	
4.9	0.1617	
5.2	0.1675	
5.4	0.1542	

From these results, it was concluded that the solutions were stable (maximum deviation <20%) over at least a 8.9-hour time interval at a concentration of 0.0510 mg/l, at least a 8.4-hour time interval at a concentration of 0.102 mg/l and at least a 5.4-hour time interval at a concentration of 0.996 mg/l.

Limit of detection (LOD)

From three chromatograms of a 0.0510 mg/l solution of the test substance, the mean noise level (N) was determined to be 11.8%. From the same chromatograms, the test substance signal (S), i.e. the mean height of the test substance peak, was determined to be 100%. Using these values, the limit of detection (S/N=3) was calculated to be 0.0181 mg/l at an injection volume of 100 μ l. Because samples from the ecotoxicological study were diluted by a factor of 2, the limit of detection for these samples is 0.0362 mg/l.

Validation of the analytical method used in November 2002Specificity

Figures 4 and 5 show chromatograms of a blank solution (1/5 (v/v) M2-medium/isopropanol containing internal standard) and of a 0.100 mg/l T-4127 solution, respectively. It was clear that blank chromatograms did not contain any interfering peaks at the position of the test substance.

Linearity

The results are summarized in Table 5. The regression line is shown in Figure 6.

Table 5 Linearity.

Concentration [mg/l]	Response ¹ [units]
0.00501	0.0867 / 0.0764
0.00699	0.111 / 0.0964
0.0100	0.120 / 0.122
0.0199	0.273 / 0.248
0.0559	0.661 / 0.751
0.0996	1.43 / 1.14
Slope	12.6
Intercept with Y-axis	0.0122
Weighting factor	1/concentration
R	0.993

¹ Duplicate measurements.

Though duplicate injections showed a relatively large spread, it was concluded that there is a linear relationship between response and concentration in the concentration range of 0.00501 – 0.0996 mg/l if a (1/concentration) weighting factor is used.

For quantitation of samples, calibration curves were constructed using two concentrations over only part of the linearity range tested. The calibration solutions are injected before and after the samples.

RESULTS - SAMPLE ANALYSIS

Samples July 2002

Table 6 shows the analytical results for the samples from the test performed in July 2002*. Inadvertently no internal standard was added to the samples pretreated on 01-Jul-02 and the calibration solutions.

Table 6 Concentrations of T-4127 in test medium (Full test 1).

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [dd-mm-yy]	Concentration		
			Nominal [mg/l]	Analysed ¹ [mg/l]	Relative to nominal [%]
0	01-Jul-02 ⁵	02-Jul-02	0	n.d.	n.a.
			100	1.209	1.209
			100 ²	1.537	1.537
24	02-Jul-02	02-Jul-02	0	n.d.	n.a.
			100	0.252	0.252
			100 ²	0.0624 ³	0.0624
72	04-Jul-02	04-Jul-02	100	<0.0362 ⁴	<0.0362
			100 ²	<0.0362 ⁴	<0.0362

¹ Mean of triplicate analysis. The maximum deviation between the responses calculated for each sample and was < 20%.

² Without algae.

³ Calculated by extrapolation.

⁴ Concentrations below the limit of detection.

⁵ Inadvertently no internal standard was added to these samples and calibration solutions .

n.d. Not detected.

n.a. Not applicable.

* All relative values were calculated using not-rounded concentrations. Therefore, some differences might be observed when calculating the relative values using the concentrations as mentioned in the table.

Samples November 2002

Table 7 shows the analytical results for the samples from the test performed in November 2002.

Table 7 Concentrations of T-4127 in test medium (Full test 2).

Time of sampling [hours]	Date of sampling [dd-mm-yy]	Date of analysis [dd-mm-yy]	Concentration		
			Nominal [mg/l]	Analysed ¹ [mg/l]	Relative to nominal [%]
0	11-Nov-02	11-Nov-02	100	1.59	
			100	1.62	
0	11-Nov-02	11-Nov-02	0	n.d.	n.a.
			0	n.d.	n.a.
			0.0721	0.0671	93
			0.0721	0.0564	78
			0.160	0.152	95
			0.160	0.160	100
			0.320	0.328	102
			0.320	0.330	103
			0.721	0.754	105
			0.721	0.742	103
			1.60	1.69	105
			1.60	1.64	103
48	13-Nov-02	13-Nov-02	0	n.d.	n.a.
			0	n.d.	n.a.
			0.0721	0.0471	65
			0.0721	0.0477	66
96	15-Nov-02	15-Nov-02	0	n.d.	n.a.
			0	n.d.	n.a.
			0.0721	0.0526	73
			0.0721	0.0543	75

¹ Mean of triplicate analysis. The maximum deviation between the responses calculated for each sample was < 20%.
n.d. Not detected.
n.a. Not applicable.

Samples July 2002

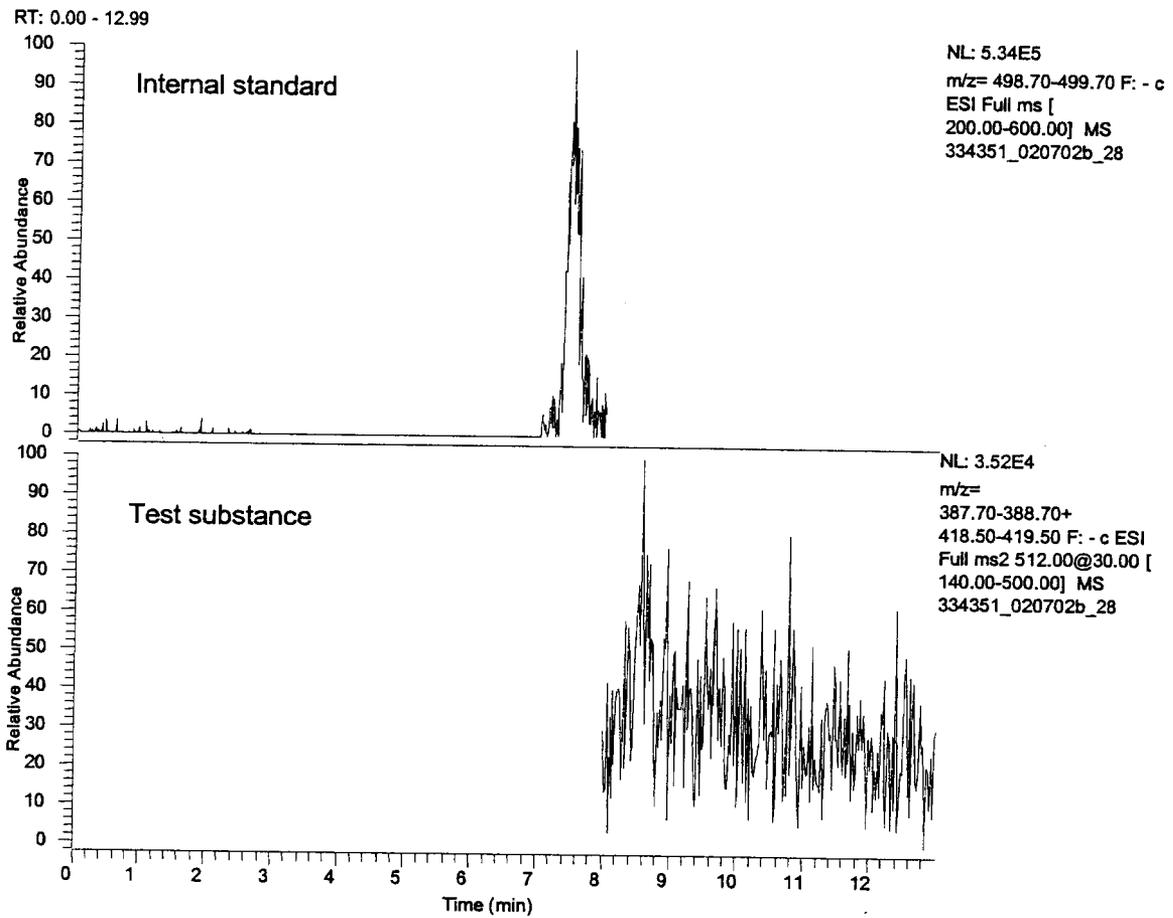


Figure 1 HPLC chromatogram of a blank (50/50 (v/v) methanol/M2-medium containing internal standard).

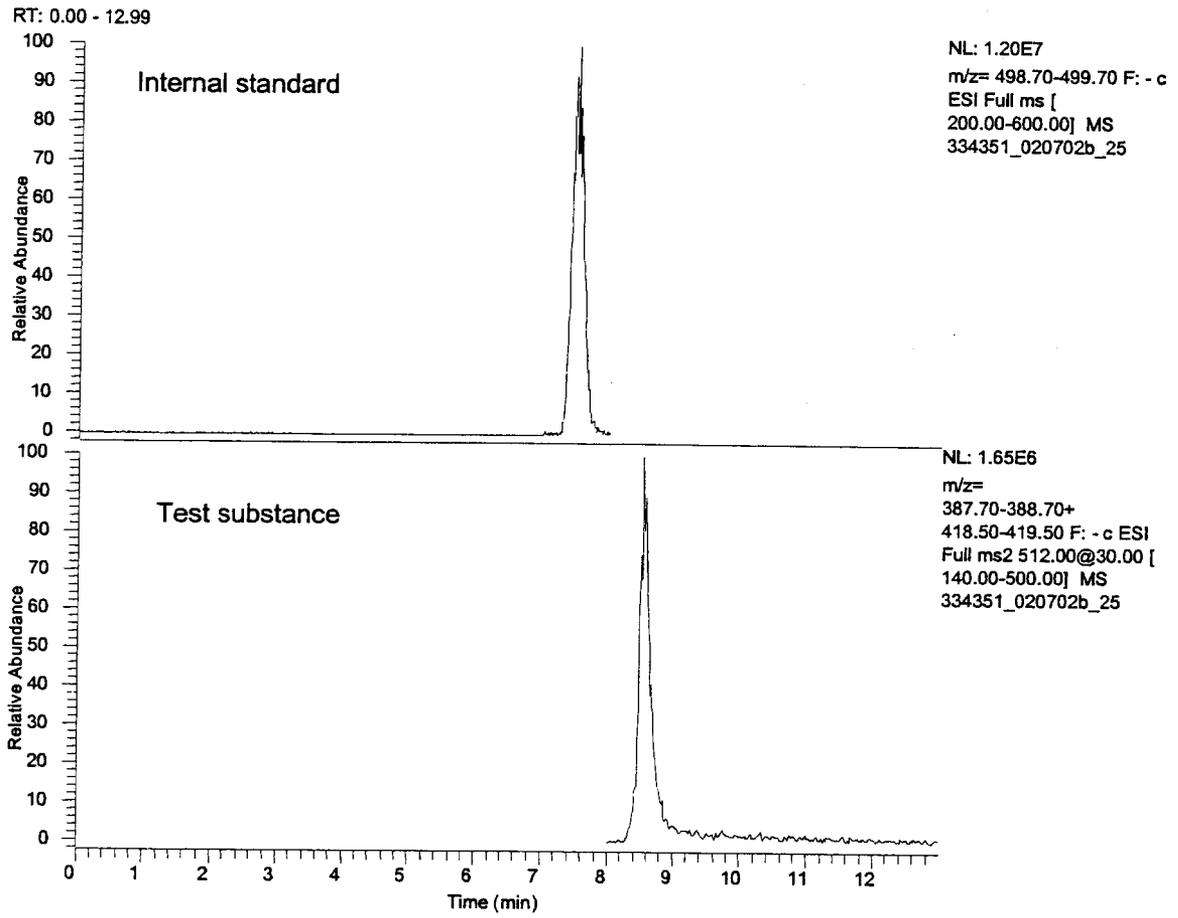


Figure 2 HPLC chromatogram of 0.996 mg/l T-4127 in 50/50 (v/v) methanol/M2-medium containing internal standard.

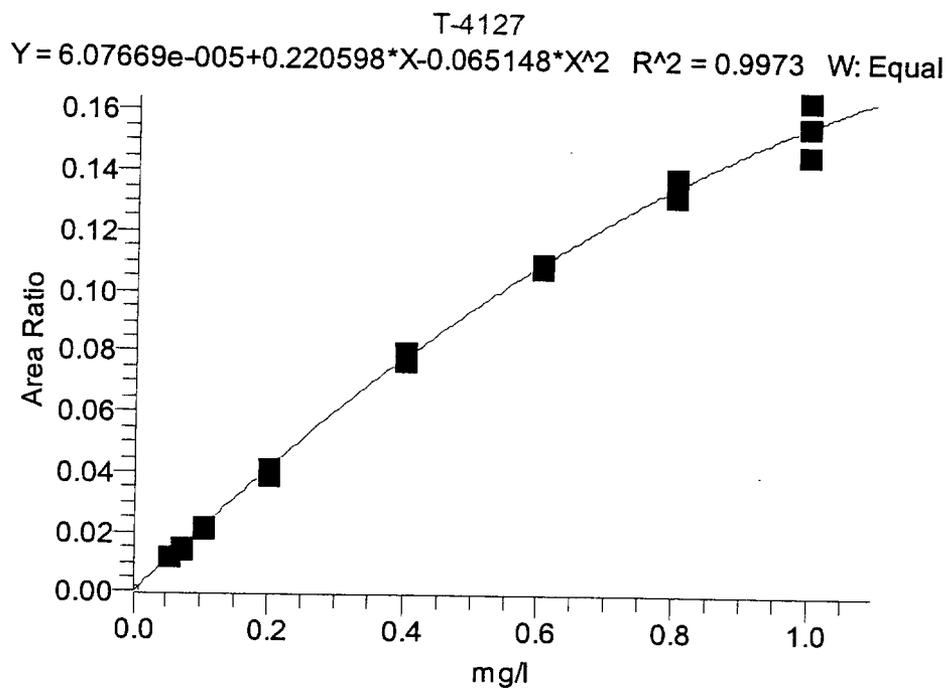


Figure 3 Regression line for solutions in 50/50 (v/v) methanol/M2-medium containing internal standard: Responses against concentrations.

Samples November 2002

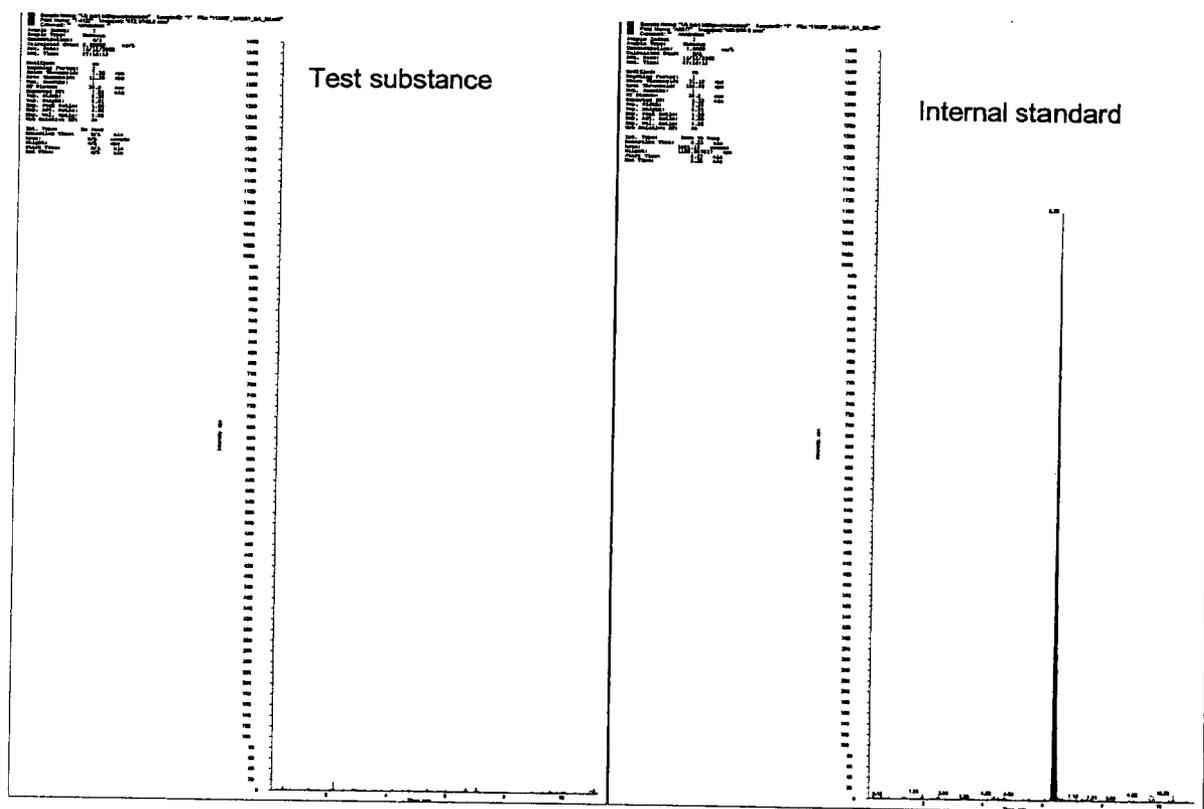


Figure 4 HPLC-chromatogram of a blank (1/5 (v/v) M2-medium/isopropanol containing internal standard).

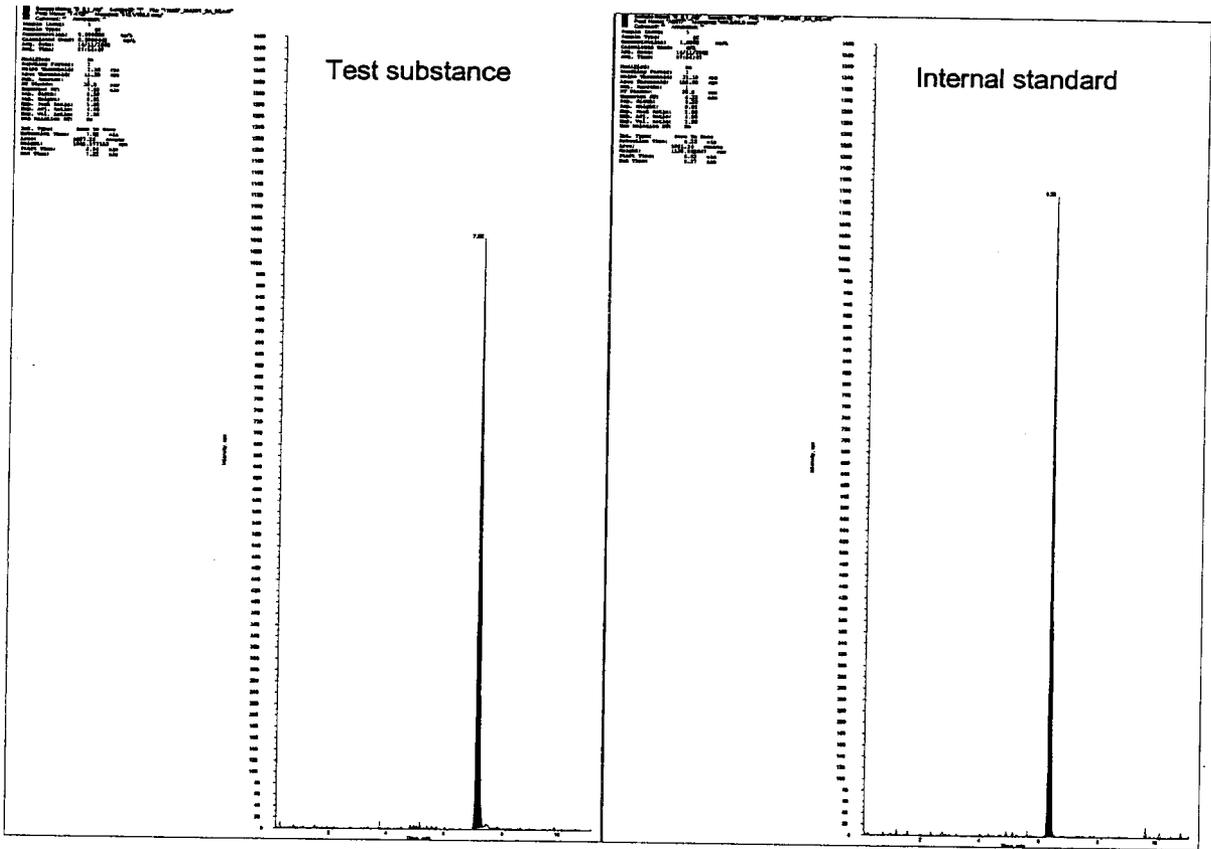


Figure 5 LC-MSMS chromatogram of 0.100 mg/l T-4127 in 1/5 (v/v) M2-medium/isopropanol containing internal standard.

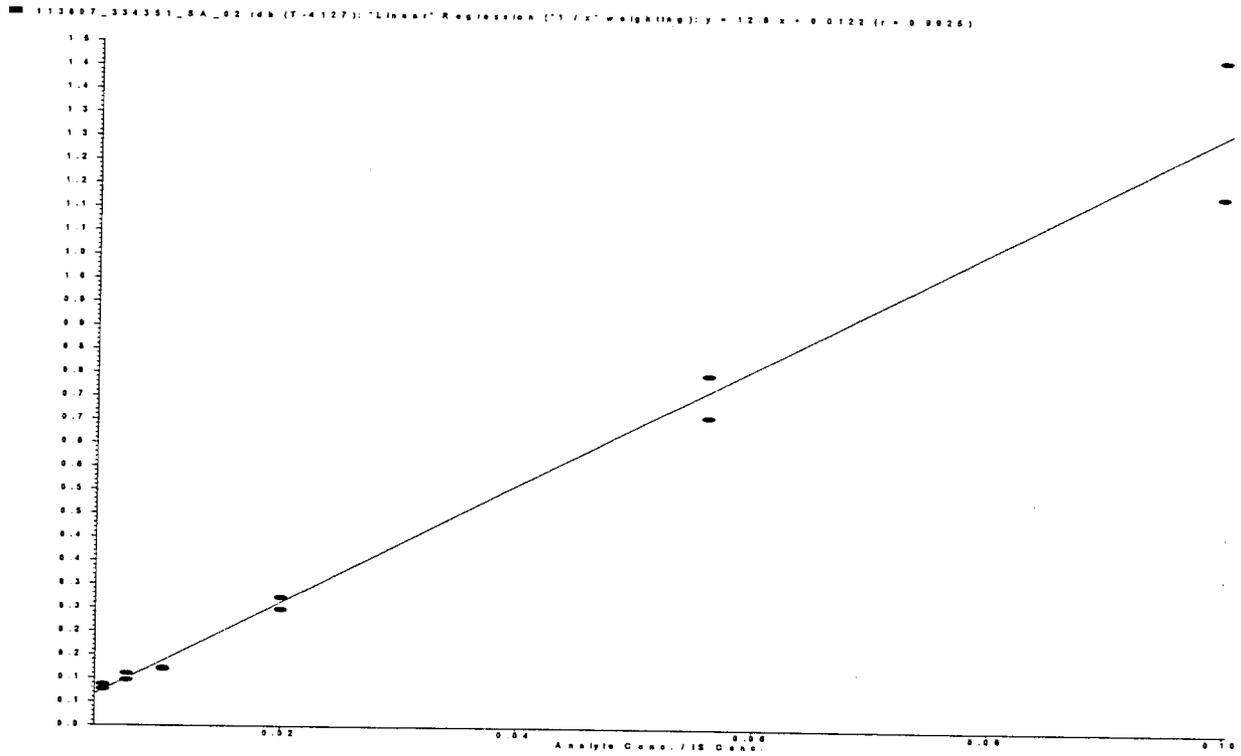


Figure 6 Regression line for solutions in 1/5 (v/v) M2-medium/isopropanol containing internal standard: Responses against concentrations.