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REPORT

ACUTE TOXICITY STUDY IN *DAPHNIA MAGNA*

WITH

T-4127

NOTOX Project 334349
NOTOX Substance 113607

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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

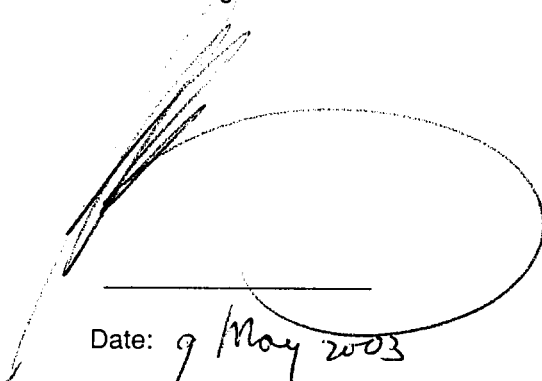
which are essentially in conformity with:

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

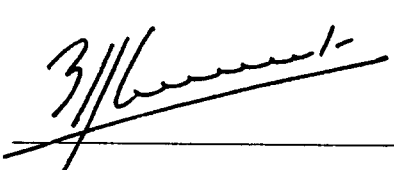
The United States Environmental Protection Agency (FIFRA). Title 40 Code of Federal Regulations Part 160.

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	2.5
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).
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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:

Immobiility

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 immobiility 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;

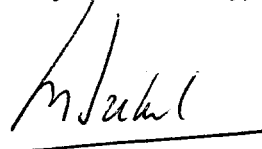
DETERMINATION OF THE CONCENTRATIONS

**NOTOX Project 334349
NOTOX Substance 113607**

REPORT APPROVAL

PRINCIPAL SCIENTIST:

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


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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 µm (Thermo Hypersil, Keystone)																							
Column temperature	25°C																							
Mobile phase	<table><tr><th>Time (minutes)</th><th>2.0 mM Ammonium acetate (%)</th><th>Methanol (%)</th></tr><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></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 µl/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 µg/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.

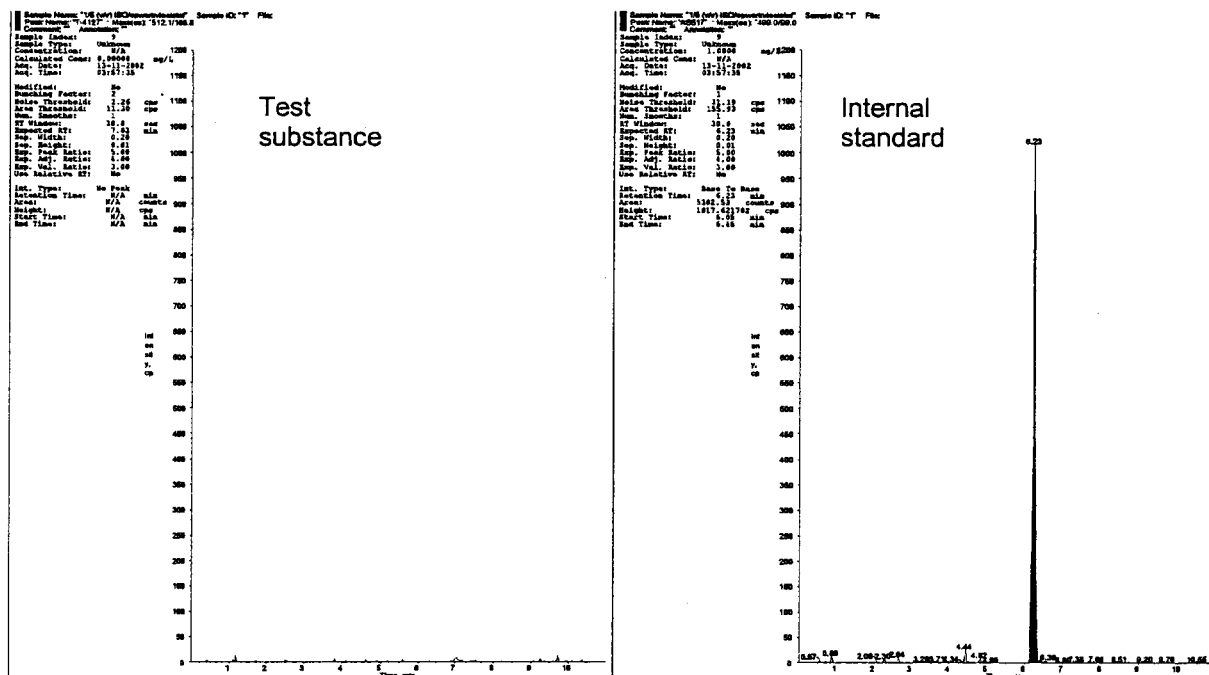


Figure 1 HPLC-chromatogram of a blank (1/5 (v/v) ISO-medium/isopropanol containing internal standard).

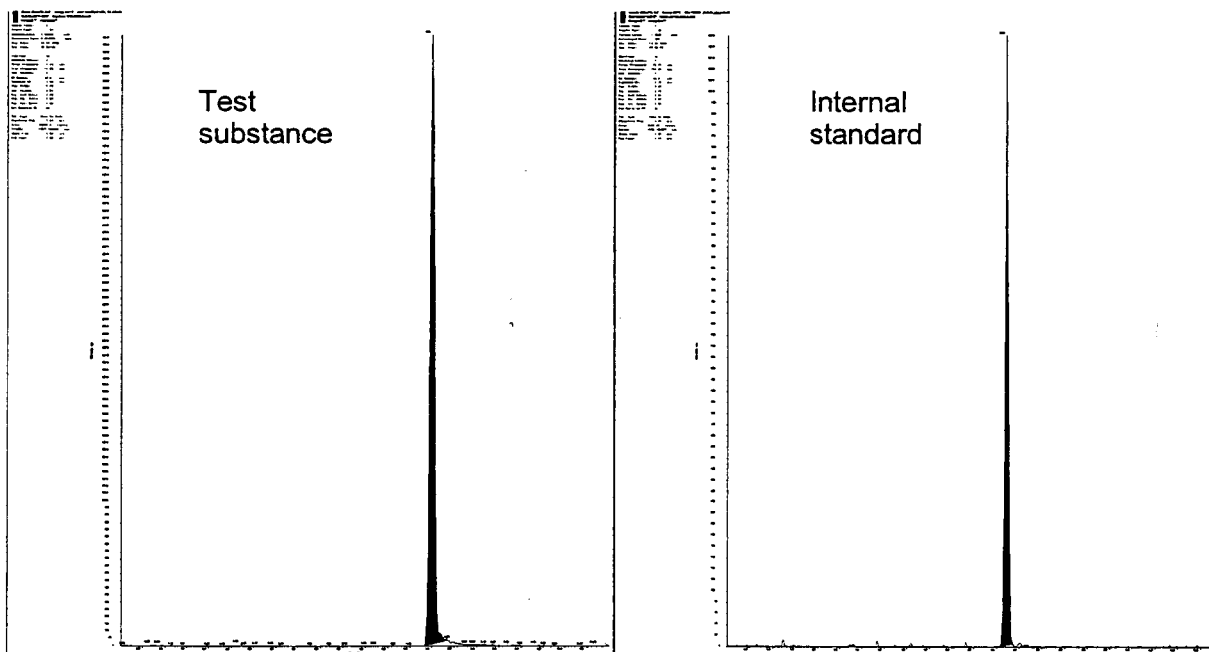


Figure 2 HPLC-chromatogram of 0.500 mg/l T-4127 in 1/5 (v/v) ISO-medium/isopropanol containing internal standard.

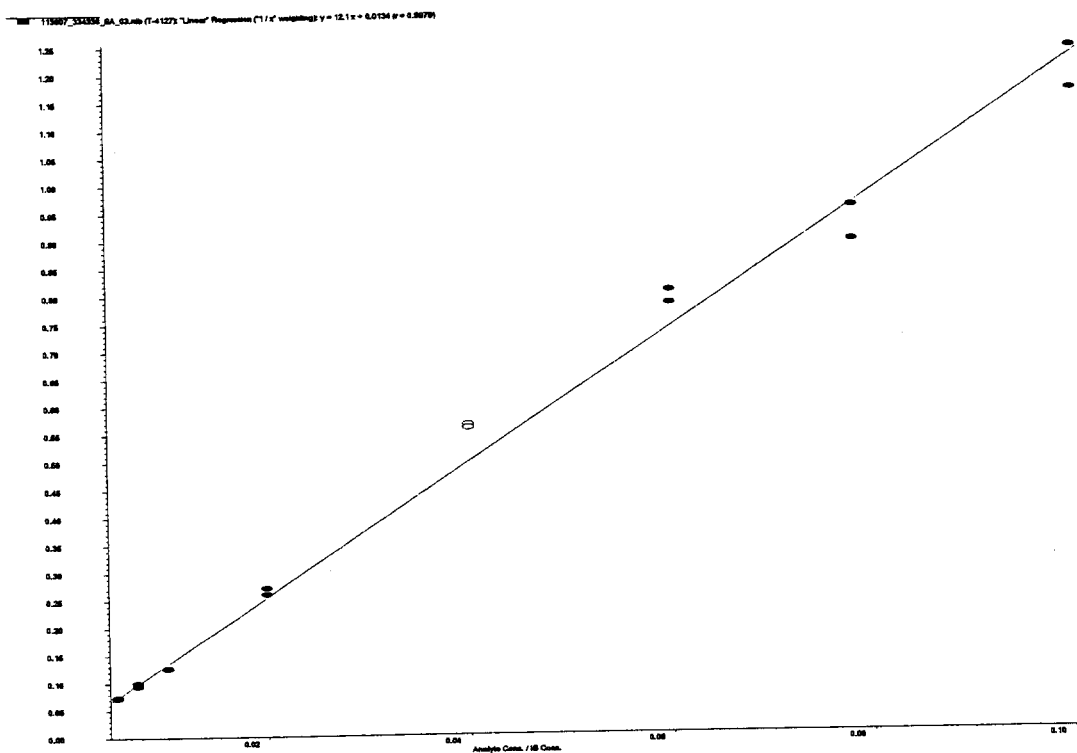


Figure 3 Regression line for solutions in 1/5 (v/v) ISO-medium/isopropanol containing internal standard: Responses against concentrations.