Huntingdon Life Sciences

[redacted]

ACUTE TOXICITY TO FISH

Company Sanitized. Does not contain TSCA CBI

[redacted]

ACUTE TOXICITY TO FISH

Sponsor

DuPont Speciality Chemicals,
Jackson Laboratory,
Chambers Works,
Deepwater,
NJ 08023,
USA.

Research Laboratory

Huntingdon Life Sciences Ltd.,
Eye,
Suffolk IP23 7PX,
ENGLAND.

Draft Report Issued 3 February 1999
Final Report Issued 22 March 1999

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COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

The study described in this report was conducted in compliance with the following Good Laboratory Practice Standards and I consider the data generated to be valid.

The UK Good Laboratory Practice Regulations 1997 (Statutory Instrument No. 654).

EC Council Directive 87/18/EEC of 18 December 1986 (Official Journal No. L 15/29).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

Eileen Daly

Eileen C. Daly, Nat. Diploma, NCEA Eire,
Study Director,
Huntingdon Life Sciences Ltd.

22nd March 1999

Date

QUALITY ASSURANCE STATEMENT

The following have been inspected or audited in relation to this study

Study Phases Inspected	Date of Inspection	Date of Reporting
Protocol Audit	04 August 1998	04 August 1998
Process Based Inspections		
Fish observations	28 August 1998	02 September 1998
Test medium renewal	09 September 1998	09 September 1998
Formulation of test medium	02 October 1998	02 October 1998
Sampling of test medium	02 October 1998	02 October 1998
Experimental set-up	26 October 1998	26 October 1998
Report Audit	24 February 1999	24 February 1999

Protocol Audit: An audit of the protocol for this study was conducted and reported to the Study Director and Company Management as indicated above.

Process based inspections: At or about the time this study was in progress inspections of routine and repetitive procedures employed on this type of study were carried out. These were conducted and reported to appropriate Company Management as indicated above.

Report Audit: This report has been audited by the Quality Assurance Department. This audit was conducted and reported to the Study Director and Company Management as indicated above.

The methods, procedures and observations were found to be accurately described and the reported results to reflect the raw data.

Helen Comb

18 March 1999

Helen Comb, B.Sc.(Hons.),
Principal Auditor,
Department of Quality Assurance,
Huntingdon Life Sciences Ltd.

Date

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CONTRIBUTING SCIENTISTS

STUDY MANAGEMENT

Eileen C. Daly, Nat. Diploma, NCEA Eire
Study Director

Rosalyn Mazey, B.Sc.(Hons.)
Study Scientist

Philip S. Manson, B.Sc.(Hons.), M.Sc.
Study Scientist

Ben Smith, B.Sc.(Hons.), M.Sc., C.Chem., M.R.S.C.
Chief Chemist

Andrew Robertson, B.Sc.(Hons.)
Senior Chemist

Richard Cubberley, B.Sc.(Hons.)
Study Analyst

Martin Nash, B.Sc.(Hons.)
Scientific Officer

SUMMARY

A study was performed to assess the acute toxicity of [redacted] to rainbow trout (*Oncorhynchus mykiss*) under semi-static exposure conditions with 24-hourly renewal of the test medium. Throughout the report, the exposure concentration and test results have been expressed in terms of the active ingredient (a.i.) [redacted]

The study was conducted in accordance with EEC Methods for Determination of Ecotoxicity Annex to Directive 92/69/EEC (O.J. No. L383A, 29.12.92) Part C, Method 1 "Acute Toxicity for Fish" and the OECD Guideline for Testing of Chemicals No. 203 "Fish, Acute Toxicity Test".

A group of ten juvenile fish was exposed to [redacted] dissolved in water at a nominal concentration of 100 mg a.i./l; to aid dissolution, ultrasound treatment was employed.

The measured concentrations of [redacted] ranged between 91 and 94% of the nominal value in unfiltered samples of freshly prepared medium and between 101 and 109% of its starting value in samples of expired medium. The overall mean measured level of [redacted] was 94.1 mg a.i./l.

At 96 hours, one fish exposed to [redacted] at 94.1 mg a.i./l had died. Sub-lethal effects comprising hyperventilation and darkened pigmentation were also observed.

The 96-hour LC_{50} was not identified but must be >94.1 mg a.i./l.

Because sub-lethal effects were exhibited by fish exposed to [redacted] the "no-observed effect concentration" (NOEC) was not identified in the limit test; however, based on the results of an earlier rangefinding test, the NOEC was considered to be 1 mg a.i/l (nominal concentration).

Under the EC General Classification and Labelling Requirements for Dangerous Substances and Preparations, [redacted] is not considered to require classification as the 96-hour LC_{50} is considered to be greater than the highest nominal concentration, 100 mg a.i./l (mean measured level of 94.1 mg a.i./l).

INTRODUCTION

This study was designed to assess the acute toxicity of [redacted] to rainbow trout (*Oncorhynchus mykiss*) under semi-static exposure conditions.

The study was conducted in accordance with EEC Methods for Determination of Ecotoxicity Annex to Directive 92/69/EEC (O.J. No. L383A, 29.12.92) Part C, Method 1 "Acute Toxicity for Fish" and the OECD Guideline for Testing of Chemicals No. 203 "Fish, Acute Toxicity Test".

The protocol was approved by Huntingdon Life Sciences Management on 7 July 1998, by the Sponsor on 17 July 1998, and by the Study Director on 3 August 1998.

The experimental phase of the study was conducted between 28 September and 9 October 1998 and the results of chemical analysis were issued by 12 October 1998.

Information provided by the Sponsor indicated that the solubility of [redacted] in water was [redacted] by weight at 35 - 40°C and its purity was 25%. Throughout this report, the exposure concentration and test results have been expressed in terms of the active ingredient. Also, the Sponsor indicated that at room temperature the test substance was a suspension in water and upon standing it would separate out into its component phases; accordingly, at the recommendation of the Sponsor, the suspension was warmed to 35 - 40°C (in a water bath) with gentle stirring to obtain a homogenous composition before use.

TEST SUBSTANCE

Identity:	[redacted]
Chemical name:	[redacted]
Appearance:	[redacted]
Storage conditions:	Room temperature
Lot number:	[redacted]
Expiry date:	2 years from date of receipt
Purity:	[redacted]
Sample received:	23 June 1998

EXPERIMENTAL PROCEDURE

TEST SPECIES

Name

Rainbow trout (*Oncorhynchus mykiss*).

Source

The fish were supplied by Parkwood Trout Farm, Kent, UK. They were reared at Bowerchalk Trout Farm, Salisbury, UK from South African eggs which hatched in June 1998.

Acclimatisation

The stock of fish was obtained from the supplier on 28 July 1998 and they were held in an aerated supply of diluent water under flow-through conditions until use. During the 14-day period immediately before the definitive test, temperatures remained within the range 13.6 to 13.9°C, pH values within the range 7.8 to 7.9, dissolved oxygen concentrations within the range 98 to 100% air saturation value (ASV) and total hardness within the range 194 to 200 mg/l as $CaCO_3$.

The fish were fed daily with commercial fish food (TROUW (UK) Ltd; Nutra Fry 02) an amount equivalent to 2% of the total wet-weight of fish in the holding tank. No food was given during the 27-hour period immediately before exposure or during the exposure period itself. No medication was given during the holding period, and mortalities were recorded as 3% in the 14 days before the definitive test.

The size of the fish used in the definitive study was determined by weighing and measuring a sample of ten fish taken at random from the holding tank on 29 September 1998; their mean fork length was 5.4 cm and their mean wet weight was 1.6 g.

DILUENT WATER

The water used to hold the fish and for the study was laboratory tap water, dechlorinated and softened by passage through an Elga® water purification system. It was passed through a high grade activated carbon filter to remove chlorine and any organic contaminants. A proportion of the supply then passed through a water softener before final reverse osmosis treatment to produce a highly purified water supply. The two grades of dechlorinated water were then remixed to give a supply with the desired water hardness. This water was then held in an intermediate tank where it was equilibrated to the test temperature and gently aerated before being supplied to the holding and test areas. Typical water quality characteristics of the diluent supply are given in Appendix 1.

TEST SUBSTANCE PREPARATION

Method of preparation

Based on information provided by the Sponsor, the test substance was warmed (to *c.*38°C) in a water bath and gently swirled to ensure a homgeneous mixture before weighing.

The test substance (6.4 g) was mixed with diluent water (250 ml) before being poured into a volumetric flask (2 l) containing diluent water (1.5 l). The contents of the flask were treated by ultrasound for twenty minutes before being poured into a test vessel (glass aquarium). The volume was then adjusted to 16 litres with diluent water.

Stability of the test concentration

The test concentration of [redacted] was measured using an HPLC method of chemical analysis (Appendix 2). The stability of the test substance in dechlorinated tap water was determined under storage conditions of 4°C and room temperature before the start of the study (Table 2, Appendix 2).

Four, mid-vessel samples (100 ml) of medium were taken from the control and test vessels at 0 and 72 hours (fresh media) and at 24 and 96 hours (expired media) and reserved for analysis. They were stored in a refrigerator before two of the samples from each set were transferred to the Huntingdon Research Centre of Huntingdon Life Sciences Ltd, Cambridgeshire, for analysis; the other two samples remained in storage at the Eye Research Centre in case further analysis was required.

EXPOSURE CONDITIONS

Experimental design

A rangefinding test was followed by a definitive (limit) test with one test concentration plus one diluent water control.

In the definitive test, ten fish were placed at random into each glass aquarium containing the prepared test or control media. Each vessel contained 16 litres of medium to a depth of 16 cm. This provided an initial static loading of 0.99 g bodyweight/litre.

Test concentrations

The rangefinding study was conducted with test concentrations of 1, 10 and 100 mg/l. Based on the results of this test, the definitive (limit) test employed a nominal concentration of 100 mg/l.

Medium renewal

The fish were exposed to the control or test conditions for a period of 96 hours with daily batchwise renewal of the media to ensure the maintenance of satisfactory environmental conditions and to maintain a stable exposure level.

Environmental conditions

Treatment and control groups were maintained at 15 ± 2°C throughout the exposure period and constant to within ± 1°C during the study. The temperature of the water in the control vessel was continuously monitored during the study.

Supplementary aeration was provided via narrow bore glass tubes. A photoperiod of 16 hours light : 8 hours dark was maintained, with periods of subdued lighting at the beginning and end of each light phase. Daily records of temperature, pH and dissolved oxygen were kept for each control and test vessel together with measurements of total hardness for selected vessels at 0 hours. The fish were not fed during the 96 hour exposure period.

CRITERIA OF EFFECT

The criteria of death employed in this study were (i) absence of respiratory movement and (ii) absence of response to physical stimulation of the caudal peduncle.

In addition to observations on mortality at 15 minutes, 2, 4, 24, 48, 72 and 96 hours, subjective assessments were also made on the incidence and type of any sub-lethal effects compared with control fish.

EVALUATION OF DATA

The "no-observed-effect concentration" (NOEC) was derived by direct inspection of the data on the treatment-related-effects. An incidence rate of more than one affected fish out of ten is considered to be significant.

PROTOCOL DEVIATIONS

None.

MAINTENANCE OF RECORDS

All specimens, raw data and study related documents generated during the course of the study at Huntingdon Life Sciences, together with a copy of the final report will be lodged in the Huntingdon Life Sciences Archive.

Such specimens and records will be retained for a minimum period of five years from the date of issue of the final report. At the end of the five year retention period the Sponsor will be contacted and advice sought on the future requirements. Under no circumstances will any item be discarded without the Sponsor's knowledge.

RESULTS

Chemical analysis

The results of chemical analysis are given in Table 1 and an example chromatogram is illustrated in Figure 1. Concentrations are expressed in terms of the measured levels of the active ingredient [redacted].

Results for unfiltered samples of [redacted] indicated that the intended exposure concentration was adequately achieved (between 91 and 100% of the nominal value) and maintained during the test (between 101 and 109% of the starting value). The overall mean measured concentration of [redacted] was 94.1 mg a.i./l.

Mortality and observations

One fish had died (10% mortality) at 94.1 mg a.i./l at 96 hours.

The 96-hour LC_{50} could not be calculated but must be >94.1 mg a.i./l.

A chronological record of sub-lethal effects is given in Table 2. They comprised hyperventilation, darkened pigmentation, aggression and aggregation. At 24 and 48 hours, nine fish and one fish were affected respectively, and at 72 hours all of the fish were affected. At 96 hours, when one fish had died, the remaining fish were adversely affected although their symptoms may have been caused by a fish which showed aggressive behaviour. Aggregation was also exhibited by nine fish in the control group at 24 and 72 hours and is also attributed to the presence of an aggressive fish.

The "no-observed-effect" concentration (NOEC) could not be identified in the limit test; based on the rangefinding test, the NOEC was considered to be 1 mg a.i./l. (nominal concentration).

Under the EC General Classification and Labelling Requirements for Dangerous Substances and Preparations, [redacted] is not considered to require classification as the 96-hour LC_{50} is considered to be greater than the highest nominal concentration, 100 mg a.i./l (mean measured level of 94.1 mg a.i./l).

Environmental parameters

The measurements of water quality (temperature, pH, concentrations of dissolved oxygen and total hardness) are summarised in Table 3; they remained within acceptable limits throughout the study.

The test medium was clear and colourless.

CONCLUSIONS

[redacted] was not found to be toxic to rainbow trout when dissolved in water at 94.1 mg active ingredient/l.

The "no-observed-effect concentration", based on the results of the rangefinding test, was considered to be 1 mg a.i./l (nominal concentration).

REFERENCE

Official Journal of the European Communities Commission Directive (1 March 1991). Annex VI General classification and labelling requirements for dangerous substances and preparations. Part II "*Classification on the Basis of Environmental Effect*" p.62 – 64.

FIGURE 1

Typical sample chromatogram - 400 mg/l (100 mg a.i./l) taken on Day 1 of the test

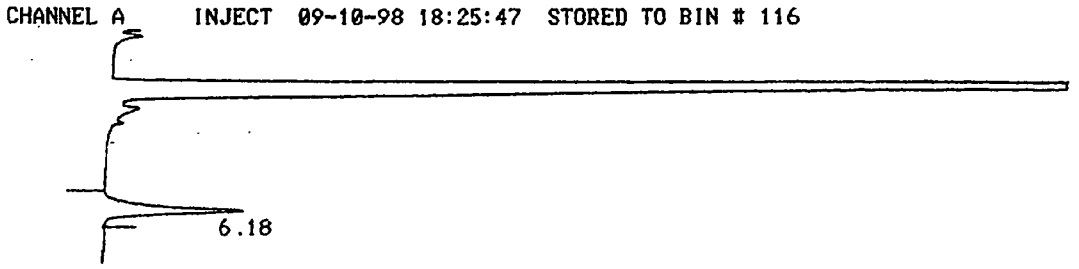


TABLE 1

Measured concentrations [redacted]

Nominal conc.,[s] mg/l	Measured [redacted] concentrations, mg/l								Overall mean
	0 hours		24 hours		% ti	72 hours		96 hours		% ti	
0	nd	nd	nd	nd	-	nd	nd	nd	nd	-	-
100	91.2	91.7	92.5	99.8	105	90.8	93.7	98.4	94.4	104	94.1
	[92]		[96]			[92]		[96]			(94)

[s] in terms of the active ingredient [redacted]

nd none detected (< 2.5 mg a.i./l).

% ti mean measured concentration after 24 or 96 hours expressed as a percentage of the mean starting concentration (0 and 72 hours).

[ ] mean measured concentration expressed as a percentage of the nominal concentration.

( ) overall mean measured concentration expressed as a percentage of the nominal concentration.

TABLE 2

Sub-lethal effects

[redacted] (mg/l)		Abnormality	Initial population = 10 fish / concentration						
nominal[s]	measured[s]		0.25h	2 h	4 h	24 h	48 h	72 h	96 h
Control	nd	Aggression	-	-	-	1/10	-	1/10	-
		Aggregation	-	-	-	9/10	-	9/10	-
100	94.1	Aggression	-	-	-	1/10	-	-	-
		Aggregation	-	-	-	8/10	-	-	-
		Darkened pigmentation (eye orbit only)	-	-	-	1/10	-	3/10	1/9
		Darkened pigmentation (eye and body)	-	-	-	-	1/10	3/10	3/9
		Hyperventilation	-	-	-	-	-	10/10	9/9

x/y number of fish affected / number of fish surviving.
nd none detected (< 2.5 mg a.i./l).
s as active ingredient, [redacted]

TABLE 3

Environmental parameters
temperature, pH, dissolved oxygen and total hardness

[redacted] (mg/l)		Temperature °C		pH		Dissolved oxygen (% ASV)		Total hardness mg/l as $CaCO_3$
nominal[s]	measured[s]	min	max	min	max	min	max	0 hours
Control	nd	14.0	15.1	7.6	7.9	81	103	202
100	94.1	14.1	15.2	7.3	7.8	79	103	204

s as active ingredient [redacted].
ASV air saturation value.
nd none detected (<2.5 mg a.i./l).

Continuous monitoring of control vessel media temperature = 13.8 to 15.3 °C.

APPENDIX 1

TYPICAL WATER QUALITY CHARACTERISTICS OF THE DILUENT SUPPLY

Diluent water comprised filtered dechlorinated tap water blended with tap water that had been softened and subsequently treated by reverse osmosis to a hardness of approx. 200 mg/l as $CaCO_3$. Analysis in March 1998 gave the following results:

Colony count 37°C	0
Coliform organisms MPN per 100 ml	nil
E. coli type 1 MPN per 100 ml	nil
	µg/l
Organochlorine pesticides	<0.02
Polychlorinated biphenyls	<0.02
Organophosphorus pesticides	<0.03
	mg/l
Aluminium	<0.01
Ammonia	<0.03
Arsenic	<0.0015
Boron	<0.05
Cadmium	<0.0004
Calcium	76
Carbon (total organic)	1
Chloride	25
Chlorine (free)	0.06
Chlorine (total)	0.08
Chromium	<0.001
C.O.D.	<15
Cobalt	<0.001
Copper	0.02
Fluoride	0.2
Iron	<0.01
Lead	<0.0019
Magnesium	4.1
Mercury	<0.0001
Nitrate (as NO_3)	6.46
Nitrite (as NO_2)	<0.01
Nickel	0.003
Phosphorus	<0.2
Potassium	1.78
Silver	<0.001
Sodium	13.4
Tin	nd
Total suspended solids	<2
Zinc	0.0125
conductivity	419 µS/cm
turbidity	<0.16 FTU

MPN most probable number
nd not determined.

APPENDIX 2

THE DETERMINATION OF [redacted] IN AQUEOUS MEDIA

SAMPLE ANALYSIS

The aqueous samples were diluted with sodium hydroxide and acetonitrile to bring the expected [redacted] concentrations within the calibration range. Determination of [redacted] was by high performance liquid chromatography (HPLC) using a conductivity detector[1].

CHROMATOGRAPHY INSTRUMENTATION AND CONDITIONS

A high performance liquid chromatography system comprising autosampler, pump, conductivity detector, anion suppressor and data collection system was used.

Column	
Type:	PLRP-S supplied by Polymer Laboratories
Dimensions (l × id):	250 × 4.6 mm
Temperature:	Ambient
Mobile phase	
Composition:	Acetonitrile : aqueous buffer solution (25 : 75% v/v)
Flow rate:	1.0 ml/min
Suppresser type:	
Regenerant composition:	50 mN sulphuric acid
Flow rate:	2.5 ml/min
Injection volume:	100 µl

Aqueous buffer solution: 2mM ammonium hydroxide/1mM sodium carbonate (made up in ultra high purity water) and filtered through 0.2 micron cellulose nitrate filter paper.

Under the above conditions [redacted] chromatographed as a single peak (see Figure 3).

[1] A method contained in a fax dated 23 July 1998 from Kavsy D Dastur, Dupont Specialty Chemicals was modified to comply with Huntingdon Life Sciences standard operating procedures and instrumentation.

CALIBRATION SOLUTIONS

Calibration solutions were prepared with the same batch of [redacted] used in the preparation of the toxicity test solutions.

Trout and Algae Studies

Working calibration solutions in the nominal range 40 to 500 mg/l (equal to 10 to 125 mg a.i./l) were prepared by volumetric dilution with acetonitrile: 100 mM sodium hydroxide (25:75% v/v) of a primary standard prepared in ultra high purity water.

***Daphnia* Study**

Working calibration solutions in the nominal range 4.0 to 50.0 mg/l (equal to 1.0 to 12.5 mg a.i./l) were prepared by volumetric dilution with acetonitrile : 100 mM sodium hydroxide (25: 75% v/v) of a primary standard prepared in ultra high purity water.

CALCULATIONS

[redacted] concentrations were determined using mean bracketing standards.

The mean peak height responses were calculated for [redacted] in bracketing standard chromatograms. The [redacted] concentration in each sample was then calculated using the following equation:

$$\text{Concentration [redacted] (mg/l)} = \frac{Y \times A}{Z} \times F$$

Where
- Y = Detector response to [redacted]
- Z = Mean detector response to bracketing standard.
- A = Concentration of bracketing standard (mg/l).
- F = Factor to take into account sample processing.

The purity (active ingredient) of the test substance is given in the Test Substance Data Sheet as [redacted] Nominal and fortified concentrations are reported as [redacted] as supplied and in terms of the active ingredient.

VALIDATION OF THE ANALYTICAL PROCEDURE

The analytical procedure was validated by determining the linearity of response of the analytical system, specificity of chromatographic analysis, the limit of detection, and the method's accuracy and precision.

During the course of the study the performance of the method was monitored by the analysis of quality control samples.

TABLE 1

Validation recoveries of [redacted] from fortified samples of dilution media

Medium	Fortification level (mg/l)		Recovery as a % of fortification level
	As supplied	As active ingredient	
Dechlorinated tap water	Control	Control	ND
	387.9	96.98	95.8
	387.9	96.98	104
Algal	Control	Control	ND
	535.5	133.9	92.0
	530.0	132.5	101
	214.2	53.55	92.6
	212.0	53.00	95.5
Elendt M4	Control	Control	ND
	9.93	2.483	98.5
	9.93	2.483	95.9
	496.5	124.1	103
	496.5	124.1	103
Overall mean (RSD)			98.1 (4.2)

Active ingredient content of [redacted]
RSD: relative standard deviation.
ND: none detected; less than the limit of detection (trout and algal studies : 2.5 mg a.i./l; *Daphnia* study: 0.5 mg a.i./l).
The limit of detection is defined as the analyte concentration in a processed sample which would give a peak equal to 3 × local base-line noise.

TABLE 2

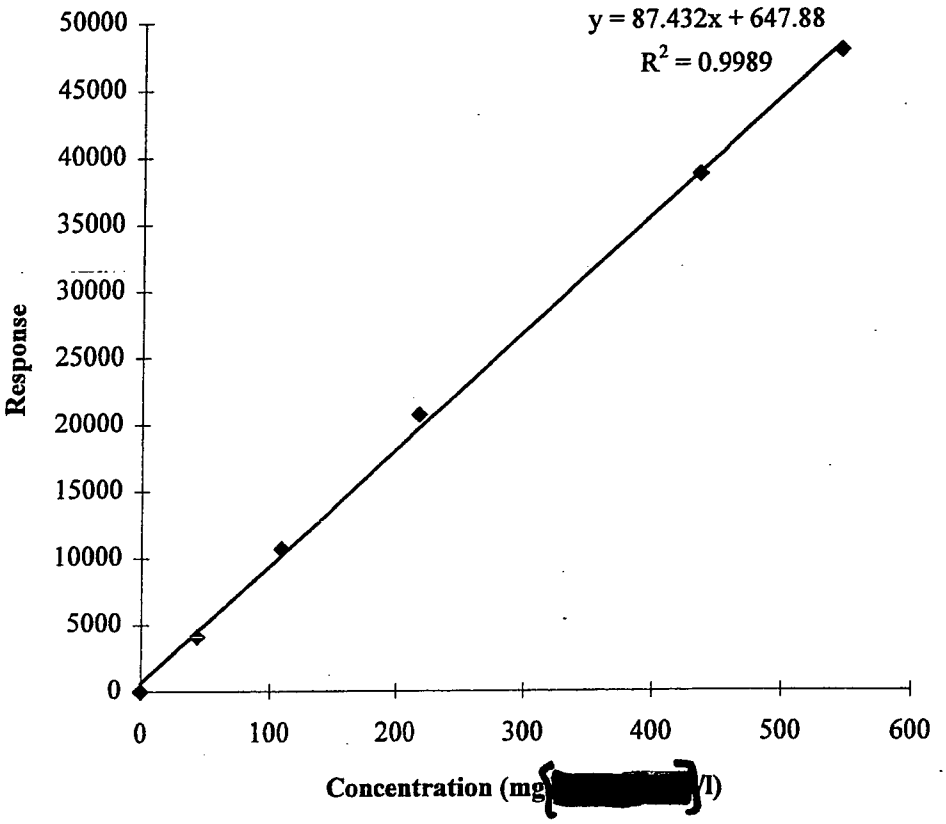
Stability of [redacted] in dechlorinated tap water

Storage conditions	Time-point	
	0 hours	20 hours
Procedural recovery [1]	110	-
Procedural recovery [1]	112	-
Light, sealed, room temperature [1]	-	103
Light, sealed, room temperature [1]	-	106
Dark, sealed, 4°C [1]	-	109
Dark, sealed, 4°C [1]	-	93.1
Procedural recovery [2]	-	109
Procedural recovery [2]	-	98.9

[1] Fortification level: 11.20 mg/l as [redacted]
[2] Fortification level: 11.65 mg/l as [redacted]
Results are given as percentage recoveries of [redacted] after storage for the indicated time.

FIGURE 1

Standard Calibration for [redacted]
(*ca* 40 - 500 mg/l)



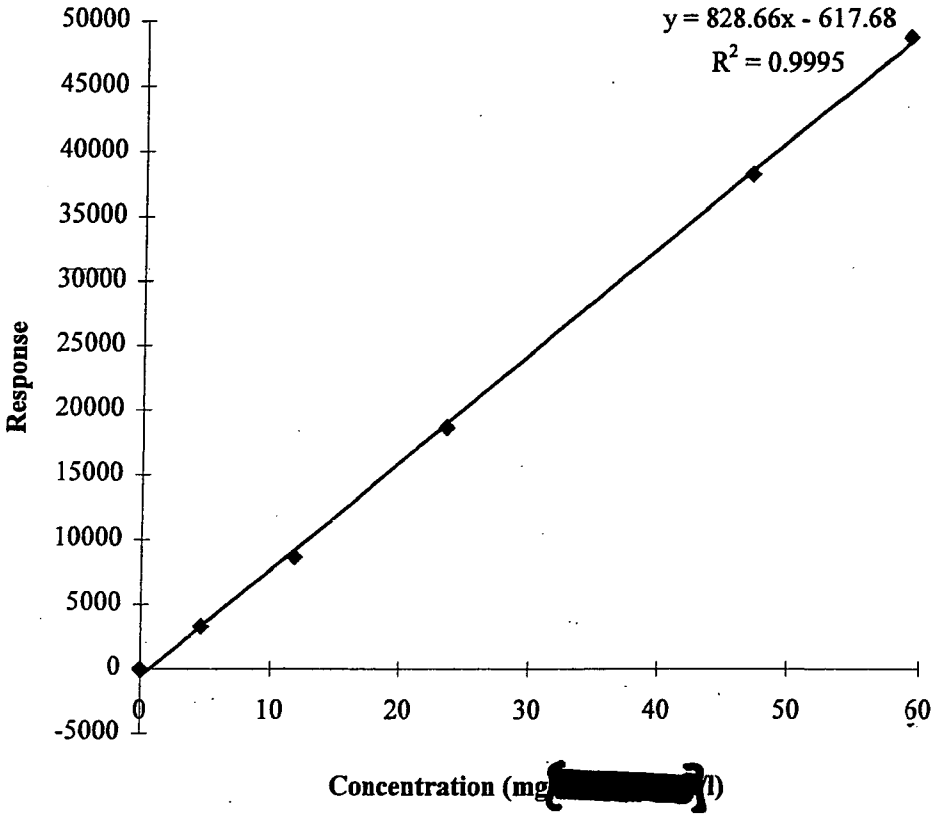
Run no.:DPT/439/013

Standard concentration (mg/l)	Peak height
0.0	NOP
43.57	4075
108.9	10668
217.9	20734
435.7	38644
544.7	47864

NOP: no observable peak.

FIGURE 2

Standard Calibration for [redacted]
(*ca* 4 to 50 mg/l)



Run no.:DPT/440/002

Standard concentration (mg/l)	Peak height
0.0	NOP
4.720	3295
11.80	8657
23.60	18669
47.20	38224
59.00	48699

NOP: no observable peak.

FIGURE 3

Typical calibration chromatography - 217.9 mg/l (= 54.47 mg/l as a.i.)

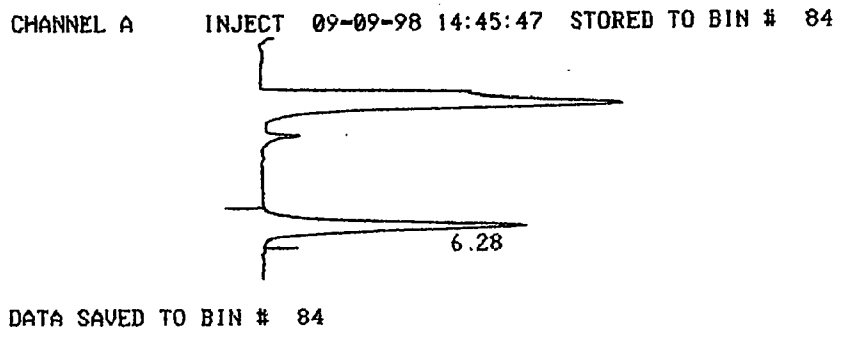


FIGURE 4

Typical chromatography - Unfortified algal medium

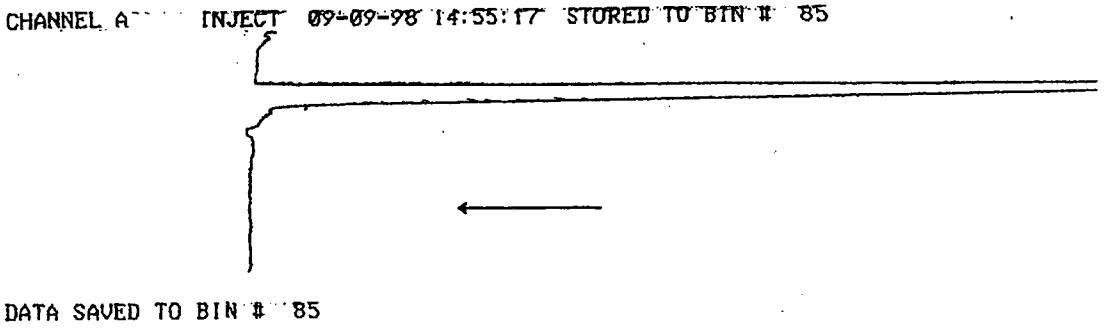


FIGURE 5

Typical chromatography
Sample of algal medium fortified at 535.5 mg/l (= 133.9 mg/l as a.i.)