Part 1 of 2

CONFIDENTIAL

LSR Schedule No : PSV/030
LSR Report No : 89/PSV030/0194

7-11-89

PYRONIL 45:

ACUTE TOXICITY TO RAINBOW TROUT

M-313

Study Director

C.A. Jenkins

To
Pennwalt Corporation
900, 1st Avenue
P.O. Box C
King of Prussia
Pennsylvania 19406-0018
USA

From
Life Science Research Limited
Eye
Suffolk, IP23 7PX
England

Draft report: 7 April 1989
Final report: 30 June 1989

TOXICOLOGY DATA
SUMMARY SHEET

Prepared by Pennwalt Corporation, Safety, Health & Environmental Department
900 First Avenue, King of Prussia, PA. 19406-0018

Test Material: PYRONIL™45

Sample Source: Pennwalt's Venture Research Laboratory, King of Prussia, PA

Study Type: Acute Toxicity to Rainbow Trout

Testing Laboratory: Life Science Research Limited
Eye
Suffolk, IP23 7PX
England

Storage: A report is filed in the Technical Records Center at King of Prussia under Master No. 22227. M-313. LSR Report No. 89/PSV030/0194.

For MSDS: Non-toxic to rainbow trout - 96-hour exposure.

Summary of Results: Rainbow trout were exposed to PYRONIL 45 in a static 96-hour acute toxicity test. Nominal concentrations ranged from 62.5 mg/L to 1,000 mg/L. Ethanol was used as a co-solvent. No mortality or adverse effects were observed. Analytical results showed that the material was poorly water soluble and not homogeneously dispersed in the test medium. Test concentrations achieved, however, represented levels equal to or greater than the solubility of PYRONIL 45 in water. PYRONIL 45 is not considered acutely toxic to rainbow trout at the limit of its water solubility.

PREPARATION DATE: November 27, 1989

PYRONIL 45: ACUTE TOXICITY TO RAINBOW TROUT

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LSR Report No : 89/PSV030/0194

I, the undersigned, hereby declare that the report following constitutes a true and faithful account of the procedures adopted and the results obtained in the performance of this study. The aspects of the study conducted by Life Science Research were performed in accordance with the principles of the following Good Laboratory Practice Standards or Guidelines relating to non-clinical studies:

Current OECD Good Laboratory Practice Principles
Current DHSS Principles of Good Laboratory Practice

I fulfilled the responsibilities of the Study Director required by these regulations.

pp.
C.A. Jenkins, B.Sc., C.Biol., M.I.Biol., M.I.F.M. CAJenkins.....
(Study Director)

Date: 29-6-89

PYRONIL 45: ACUTE TOXICITY TO RAINBOW TROUT

LSR Schedule No : PSV/030
LSR Report No : 89/PSV030/0194

I have reviewed this report, and concur with its contents.

W.R. Jenkins, B.Sc., C.Biol., M.I.Biol., M.I.F.M
(Chief Scientist, Aquatic Toxicology)

Date: 29-6-89

I, the undersigned, was responsible for the experimental work and reporting of the analytical chemistry conducted during this study.

J. O'Connor, B.Sc.
(Head, Environmental Chemistry)

Date 29 June 1989

PYRONIL 45: ACUTE TOXICITY TO RAINBOW TROUT

LSR Schedule No : PSV030
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QUALITY ASSURANCE INSPECTIONS

	Dates (Day/Month/Year)		
	Inspection	Report to Study Director	Report to Management
PROTOCOL			
Inspection of protocol was made in accordance with LSR Standard Operating Procedure QAU/020. Dates for inspection of any protocol amendments in accordance with this SOP, are not quoted	29.09.88	30.09.88	30.09.88
DATA			
Inspection of data generated on this type of study was made in accordance with LSR Standard Operating Procedure QAU/050	24.11.88		24.11.88
PROCEDURES			
Inspection of procedures on this type of study was made in accordance with LSR Standard Operating Procedure QAU/040	26.09.88 26.09.88 26.09.88 22.11.88		28.09.88 28.09.88 28.09.88 22.11.88

Other routine procedures used in this type of study, and facilities were inspected regularly and reports made in accordance with LSR Standard Operating Procedure QAU/040.

This report has been reviewed by the LSR Quality Assurance Unit employing methods laid down in LSR Standard Operating Procedure QAU/060. The reported methods and procedures were found to describe those used and the results to constitute an accurate representation of the data recorded.

This review was completed on: 26 June 1989

D.L.M. Weller, B.Sc.
(Head of Quality Assurance Unit)

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Date: 29 June 1989

C O N T E N T S

1. SUMMARY

1.1 The acute lethal toxicity of PYRONIL 45 to the rainbow trout has been assessed under static exposure conditions over an exposure period of 96 hours.

1.2 In a preliminary test, groups of five fish were exposed to four nominal concentrations of PYRONIL 45 ranging from 1 to 1000 mg/l. The test was conducted at a temperature of 13.9 ± 0.4°C in dechlorinated tap water (hardness 224 - 228 mg/l as $CaCO_3$) at pH 7.7 to 8.2. Test dilutions were individually prepared by adding the test material directly to the dilution water. No mortalities or adverse effects were observed during the test.

1.3 Analysis of PYRONIL 45 in duplicate mid-vessel samples taken at a nominal concentration of 1 mg/l gave measured values of 0.11 and 0.14 mg/l at the start of the test and 0.54 and 0.36 mg/l after 96 hours. At a nominal 1000 mg/l, measured concentrations were 1.06 and 0.58 mg/l at the start of the test and 33.6 and 42.2 mg/l after 96 hours. The surfaces of all test dilutions were covered by colourless oil-like droplets of the test material; test material was also visible on the bases of the test vessels at nominal levels of 100 and 1000 mg/l. These results indicate that the material was poorly soluble and was not homogeneously dispersed in the test medium.

1.4 Following the preliminary toxicity test, a trial was carried out to determine whether homogeneous test preparations could be achieved by using ultrasound treatment or solvents. Ultrasound treatment was not successful, and of the five solvents examined, ethanol was found to be the most effective at dispersing the test material.

1.5 In the definitive test, groups of ten fish were exposed to five nominal PYRONIL 45 concentrations between 62.5 and 1000 mg/l. Test dilutions were prepared individually by adding ethanol to the appropriate weights of test material and then adding these mixtures directly to the dilution water. The test was conducted at 14.5 ± 0.5°C in dechlorinated tap water with a hardness of 198 to 208 mg/l as $CaCO_3$ at pH 7.6 to 8.0. Observations of the fish were made after 2, 4 and 24 hours and then every 24 hours for the remainder of the test.

1.6 Analysis of mid-vessel samples from each dose level gave variable results which indicated that the test material was not homogeneously dispersed in the dilution water. Measured concentrations ranged from 2.21 to 41.1 mg/l at the start of the test and decreased to between 0.50 and 2.69 mg/l after 96 hours. This apparent decrease in dose levels during the test is presumed to reflect the presence of insoluble test material in samples taken at the start of the test. Again undissolved test material was observed at all dose levels. The surfaces of all test dilutions were initially covered by colourless oil-like droplets of test material which disappeared after 96 hours.

1.7 No mortalities or adverse effects were observed during the test. It is therefore concluded that PYRONIL 45 is not acutely toxic to rainbow trout under the test conditions, at nominal exposure concentrations up to 1000 mg/l. Although exposure concentrations were not achieved or maintained in the test, they nevertheless represent a condition of maximum attainable exposure.

2. INTRODUCTION

The objective of this study was to determine the acute lethal toxicity of PYRONIL 45 to the rainbow trout, *Salmo gairdneri*. The test was conducted in accordance with the OECD Guidelines for the Testing of Chemicals, Procedure 203, adopted 4 April 1984 (1).

The test material was known to be poorly soluble in water. Exposure concentrations greatly in excess of its known limit of aqueous solubility were employed under static test conditions to establish conditions of maximum attainable dose.

2.1 Study organisation

Location of study	:	Life Science Research Limited, Eye, Suffolk, IP23 7PX England
Chief Scientist, Aquatic Studies	:	W.R. Jenkins, B.Sc., C.Biol., M.I.Biol., M.I.F.M.
Study Director	:	C.A. Jenkins, B.Sc., C.Biol., M.I.Biol., M.I.F.M.
Responsible Scientist, Environmental Chemistry	:	J. O'Connor, B.Sc.
Study timing	:	Preliminary tests : 3-17 October 1988 Definitive test : 24-28 October 1988
Data storage	:	The raw data and a copy of the final report will be stored in the archives of Life Science Research, Eye, Suffolk, IP23 7PX.

3. TEST MATERIAL

The test material, PYRONIL 45, was received from Pennwalt Corporation, Pennsylvania, USA on 17 August 1988. It was a pale-yellow, clear liquid contained in a translucent plastic container, labelled PYRONIL™ 45, code 6605-57, expiry date August 1989.

The inclusive weight of the test material and container on receipt was 383.63 g.

The following information was supplied by the Sponsor:

Identity	PYRONIL 45
Other names	FR-45B
	RC9927
Batch number	6159-199-3
Chemical structure	Halogenated phthalate ester
Specific gravity	1.545 (25°C/4°C)
Stability	unstable in strong acids/alkalis
Purity	>95%

4. METHODS

4.1 Test organism

The fish used for the preliminary tests were obtained as fry from West Acre Trout Farm, Kings Lynn, Norfolk. The fry were reared from eggs imported from Denmark.

The fish used for the definitive test were obtained as fry from Padworth Fisheries, Mill House, Padworth, Berkshire. The fry were reared from eggs imported from USA and were transported to Life Science Research in aerated water taken from the farm, on 30 September 1988. The hardness of the water used to transport the fry, determined at the laboratory on the day of delivery, was 278 mg/l as $CaCO_3$.

At the laboratory, these fry were held in an aerated supply of filtered dechlorinated tap water under flow-through conditions until removed for testing. Control of hardness was achieved by mixing tap water with tap water which had been softened and subsequently treated by reverse osmosis.

Monitoring water quality during the holding period of fish used in the definitive test showed the following:

- temperatures ranged from 13.0 to 14.3°C
- pH values between 7.3 to 7.8
- dissolved oxygen concentrations (DO) ranged from 69 to 96% air saturation value (ASV)
- water hardness was from 188 to 228 mg/l as $CaCO_3$.

Each day during the holding period, the fish were fed with proprietary trout pellets (BP Nutrition Ltd., Mainstream Trout Fry 02), an amount equivalent to 1.5% of the total wet-weight of the fish in the holding tank.

Mortality during the 14-day period prior to the definitive test was less than 2.5%.

The fish were last fed 28 hours before the start of the definitive test.

The mean wet-weight of the fish used in the definitive test, based on a sample of ten fish taken at random from the holding tank on the 18 October 1988 was 1.45 g.

4.2 Test environment

The tests were carried out in a temperature-controlled area at 15 $\pm$ 1 °C. The day length in the test area was controlled giving a photoperiod of 16 hours light, supplied by overhead fluorescent tubes, and 8 hours darkness. Dawn and dusk were simulated by periods of subdued lighting at the beginning and end of each light phase.

4.3 Dilution water

The tests were carried out using dechlorinated tap water with hardness adjusted to between 200 and 250 mg/l as $CaCO_3$, taken from the same source as that used to maintain the test organisms (Appendix 1). Control of hardness was achieved by mixing treated and untreated tap water as previously described. The dilution water was delivered to an intermediate tank in which it was equilibrated to the test temperature and gently aerated before being supplied to the test area.

4.4 Apparatus

The test vessels were all-glass aquaria, with a total capacity of 6 and 15 litres for the preliminary tests and 25 litres for the definitive test. Aeration of the contents of each vessel was achieved through a Pasteur pipette connected to an oil-free supply of compressed air.

4.5 Preliminary tests

4.5.1 Preliminary toxicity test

Information supplied by the Sponsor indicated that the test material was poorly soluble in water; accordingly, test dilutions were prepared separately at each exposure concentration. The appropriate weight of PYRONIL 45 was rinsed into a volumetric flask and the volume adjusted to five litres with dilution water. The contents of the flask were poured into a test vessel, the flask was refilled with water and the rinsings were poured into the same vessel.

The test was carried out under static conditions using five fish in each control and test vessel. The fish were exposed to the following nominal exposure concentrations: 1, 10, 100 and 1000 mg/l and to the dilution water alone. The fish were allocated, in groups of five, to the vessels containing the prepared test or control dilutions.

Observations of the fish were made 1, 24, 48, 72 and 96 hours after the start of the test. During the test they were not fed.

The pH of the test dilutions was not controlled during the test.

4.5.2 Dispersion trials

Following the preliminary toxicity test, a series of trials was conducted to determine whether homogeneous test preparations could be achieved by the treatment of concentrated preparations with ultrasound, or by the use of solvents.

Two concentrated suspensions of PYRONIL 45 were prepared in test dilution water at nominal concentrations of 4 and 40 g/l. These dispersions were treated by ultrasound for 15 minutes and then further diluted with water to give nominal concentrations of 100 and 1000 mg/l.

Subsequently 100 mg and 1 g amounts of the test material were added to one millilitre of acetone, the solvent routinely employed as a solubilising agent in aquatic tests. The mixtures were vigorously shaken before being added to ten litres of dilution water.

Qualitative observations of these preparations indicated that homogeneous dispersions were not achieved by either method.

The toxicity of the preparations was examined by placing two fish in each for 48 hours. No mortality was observed in either case.

The effectiveness of the following solvents as solubilising agents for PYRONIL 45 was then examined using the methods employed in the acetone trial:

dimethyl formamide, triethylene glycol, methanol and ethanol.

A comparison of the appearance of each preparation suggested that ethanol gave the best dispersion with the least amount of undispersed test material present on the base of the vessel.

4.6 Definitive test

4.6.1 Preparation of dilutions of the test material

Test dilutions were prepared individually at each concentration by adding ethanol (2 ml) to the appropriate weights of the test material and then adding the mixtures directly to the dilution water (20 litres).

4.6.2 Test procedure

The test was carried out under static conditions. Groups of ten fish were exposed to the following nominal concentrations of the test material:

62.5, 125, 250, 500 and 1000 mg/l

Control groups of fish were exposed to dilution water alone or dilution water containing ethanol (0.1 ml/l).

Fourteen groups of five fish were removed from the holding tank and randomly placed into the test and control vessels until each contained ten fish.

The fish were not fed during the test.

Observations of the fish were made within 45 minutes of their addition to the test vessels and again after 2 and 4 hours. Thereafter, observations were made at 24, 48, 72 and 96 hours.

4.7 Water quality analysis

The temperature, pH and concentration of dissolved oxygen of the contents of each vessel was measured at the start of the tests and thereafter each day either immediately before or following the observations of fish behaviour. The total hardness of the dilution water control and selected test dilutions were determined at the start and end of the tests.

4.8 Analysis

The method used to determine the concentrations of the test material in test dilutions during the preliminary and definitive tests is described in Appendix 2.

4.8.1 Preliminary toxicity test

Before the fish were placed in the test vessels, two mid-vessel samples (100 ml) were taken, using a syringe, from those containing 1 and 1000 mg/l, and from the solvent control vessel. This sampling procedure was repeated after 96 hours when the volume of sample removed was increased to 200 ml.

4.8.2 Definitive test

Before the fish were placed in the test vessels, two mid-vessel samples (100 ml) were removed from each, including the control which was used as the blank during analysis.

4.9 Statistical analysis

Since no mortalities were observed during the tests, LC50 values could not be calculated.

5. RESULTS

5.1 Preliminary toxicity test

5.1.1 Analysis

Measured concentrations of PYRONIL 45 in duplicate samples at a nominal concentration of 1 mg/l were 0.11 and 0.14 mg/l at the start of the test and 0.54 and 0.36 mg/l after 96 hours. At a nominal 1000 mg/l, measured concentrations were 1.06 and 0.58 mg/l at the start of the test and 33.6 and 42.2 mg/l after 96 hours.

The surfaces of all test dilutions were covered by colourless oil-like droplets of test material and test material was visible on the bases of the test vessels at nominal concentrations of 100 and 1000 mg/l.

Analytical results and test observations indicated that the aqueous solubility of the material had been exceeded at all exposure levels and that the test preparations were not homogeneous.

5.1.2 Mortality

No mortalities or significant effects were observed during the test.

5.1.3 Test environment and water quality

The air temperature of the test area during the test varied between 14.5 and 15.5°C.

The measurements of temperature, pH, concentration of dissolved oxygen and total hardness taken during the test are summarised below.

Nominal exposure conc.mg/l	Temperature °C	pH	D.O. % ASV	Total hardness as mg/l CaCO3
Control	13.5-14.3	7.80-8.16	91-98	224-228
1	13.5-14.2	7.76-8.15	90-99	224-228
10	13.5-14.2	7.72-8.15	90-99	
100	13.6-14.2	7.70-8.14	91-98	
1000	13.7-14.3	7.67-8.14	86-97	226-228

5.2 Definitive test

5.2.1 Analysis

The results of analysis of samples taken during the test are summarised in Table 1.

Results again indicate that the aqueous solubility of the material had been exceeded at all exposure levels and that test preparations were not homogeneous. The variation observed in measured concentrations is presumed to reflect the presence of undissolved test material in samples taken for analysis.

Again undissolved material was observed in all dosed vessels. At 62.5 mg/l, the dilution water appeared hazy, but at 125 mg/l it was only slightly hazy. At 250 and 500 mg/l, test dilutions were white, hazy dispersions but at 1000 mg/l, the dilution water remained clear and colourless.

Large globules of the test material were visible on the bases of all the test vessels except at 62.5 mg/l where only traces were visible. The surfaces of all dilutions were initially covered by oily material which disappeared after 96 hours.

5.2.2 Mortality

No mortalities or adverse effects were observed during the test.

5.2.3 Test environment and water quality

The air temperature of the test area during the test varied between 15.0 and 15.5°C.

The measurements of temperature, pH, concentration of dissolved oxygen and total hardness taken during the test are summarised below.

Nominal exposure conc. mg/l	Temperature °C	pH	D.O. % ASV	Total hardness mg/l as CaCO3
Controls				
water	14.1-14.8	7.94-8.02	88-95	200-204
ethanol	14.0-14.9	8.00-8.04	91-96	
62.5	14.0-14.9	7.63-8.00	69-95	200-208
125	14.0-15.0	7.84-7.96	89-94	
250	14.0-14.9	7.88-7.94	89-93	
500	14.0-14.9	7.84-7.93	89-93	
1000	14.0-14.9	7.78-7.93	88-92	198-206

6. DISCUSSION AND CONCLUSIONS

6.1 No mortalities were observed during the tests. PYRONIL 45 was, therefore, not considered to be acutely toxic to rainbow trout at nominal concentrations up to 1000 mg/l, the highest level employed in the test.

6.2 Exposure levels intentionally exceeded the aqueous solubility of PYRONIL 45. Although the results of analysis showed that exposure levels were neither achieved or maintained and that test preparations were not homogeneous, they nevertheless represent conditions of maximum attainable exposure.

7. REFERENCE

1. OECD Guidelines for Testing of Chemicals. "Fish, Acute Toxicity Test". Procedure 203, adopted 4 April 1984.

TABLE 1

Definitive test: analytical determinations of PYRONIL 45 in test dilutions

Nominal conc. (mg/l)	0 hours measured values (mg/l)	mean (mg/l)	96 hours measured values (mg/l)	mean (mg/l)	*overall mean (mg/l)*
control	0,0	0	0,0	0	*0*
62.5	2.21,5.16	3.7	2.68,2.50	2.59	3.14
125	14.2,5.60	9.9	2.45,0.682	1.57	5.73
250	38.1,23.3	30.7	0.50,0.773	0.637	31.34
500	19.7,37.5	28.6	2.60,2.69	2.65	15.62
1000	41.1,19.7	30.4	1.17,0.673	0.922	15.66

APPENDIX 1

Chemical composition of the dilution water used in aquatic tests

Source : Filtered dechlorinated tap water blended with tap water that had been softened and subsequently treated by reverse osmosis to a hardness of 200 - 250 mg/l as $CaCO_3$.

Date : September 1987 to September 1988

Colony count 37°C 48 hours	40-80*
Coliform organisms MPN per 100 ml	nil
E. Coli type 1 MPN per 100 ml	nil
	ug/l
Organochlorine pesticides	< 0.1
Polychlorinated biphenyls	< 1.0
Organophosphorus pesticides	< 0.25
	mg/l
Ammoniacal nitrogen	<0.01
Cadmium	< 0.0005
Chloride	31-38
C.O.D.	ND-9.9
Calcium	68-107
Copper	<0.02-0.03
Fluoride	0.29-0.39
Lead	<0.05
Magnesium	3.2-4.2
Mercury	<0.0001
Nitrate (as N)	1.8-4.0
Nitrite (as N)	<0.01-0.03
Nickel	<0.01-0.1
Potassium	1.8-2.6
Sodium	12-37
Tin	<0.01
Zinc	0.26-1.3
pH	7.43-8.19
Total hardness	218-254 mg/l as $CaCO_3$
Alkalinity	158-170 mg/l as $CaCO_3$

* Markedly higher counts (but no coliforms or E.coli type 1) were recorded in a sample taken on 14.1.88.

APPENDIX 2

Analytical procedure for the determination of Pyronil 45 in test solutions

1. Introduction

Samples taken during tests to determine the acute toxicity of Pyronil 45 to Rainbow trout (*Salmo gairdneri*) were analysed to determine the concentration of Pyronil 45 achieved in the aqueous test solutions.

2. Principle of method

A method supplied by Pennwalt Corporation (received February 1989) was modified to suit Life Science Research standard operating procedures and instrumentation, and is based on HPLC employing UV detection for the definitive study. A method was developed for the preliminary study employing ultra-violet spectrophotometry.

3. Materials

3.1 Standard

Pyronil 45 from the batch used in the toxicity studies. Results are consequently expressed in terms of the test material as supplied.

3.2 Reagents

Acetonitrile (HPLC grade)
Hexane (AR grade)

4. Procedure

4.1 Preparation of samples for analysis

The test samples were extracted with hexane (2 x 10 ml) and the combined extracts evaporated to dryness, under nitrogen at 40°C. The extract residue was re-dissolved in an appropriate volume of acetonitrile to give a nominal concentration of Pyronil 45 in the calibration range.

In the preliminary test, either the acetonitrile solutions were chromatographed or the absorbances of the solutions were read at λ_{max} using an ultra-violet spectrophotometer. In the definitive test the solutions were chromatographed.

4.2 Chemical standards

Accurate standard solutions, with nominal Pyronil 45 concentrations of 4, 8, 12, 16 and 20mg/l in the preliminary test, and 10, 20, 30, 40 and 50mg/l in the definitive test, were prepared and analysed to demonstrate linearity of calibration. A chemical standard of intermediate concentration was repeated at intervals throughout the chromatographic run to monitor any variation in performance, and to update the response factor for computation (where appropriate).

4.3 Recovery

Spike solutions of Pyronil 45 in control water were prepared spanning the range of Pyronil 45 concentration in the test samples. The recovery (expressed as percentage) was used to calculate the actual Pyronil 45 concentration in the test samples.

5. Analysis conditions for high performance liquid chromatography

The following conditions have been found suitable. Minor modifications to these conditions may have been applied in order to improve sensitivity or resolution from any interfering substances.

HPLC instrument	: Varian 8500
Column	: Spherisorb 5μ ODS (25 cm x 4.6 mm i.d.)
Mobile phase	: 97:3 methanol/water
Flow rate	: 2 ml/min
Temperature	: ambient
Injection volume	: 10 μl
Retention volume	: 6 ml (approx)
Detector	: UV 220 nm

Analysis conditions for ultra-violet spectrophotometry

Instrument	: Philips Scientific PU8820 UV-Visible spectrophotometer
Cell type	: quartz
Cell path length	: 1 cm
Wavelength	: 225 nm
Slit width	: 1 nm
Absorbance span	: 2A

6. Calculations

A graph of standard concentration of Pyronil 45 (mg/l) versus absorbance or peak height was plotted and the data linearly regressed.

The concentration of Pyronil 45 in aqueous samples was calculated from the regression equation:

$$y = a + b\,x$$

or from standards introduced before and after samples (bracketting standards).

Sample concentration was corrected for recovery from spikes at equivalent levels to the nominal Pyronil 45 concentration.

7. Assessment of analytical results

7.1 Validity

The limit of detection, defined as the concentration of Pyronil 45 in injection solution required to produce a peak twice the height of baseline uncertainty, was approximately 0.1 mg/l.

The limit of assay is normally set at five times the limit of detection and as such was 0.5 mg/l.

7.2 Linearity

The detector calibration was found to be linear over the range 0 to 50 mg/l, using the conditions described above, with a regression coefficient better than 0.99

LSR Schedule No :
LSR Enquiry No : 2651C
No. of pages : 10

PYRONIL 45: DETERMINATION OF ITS 96-HOUR LC_{50} TO A COLD WATER SPECIES OF FISH (15°C) UNDER STATIC TEST CONDITIONS

Protocol prepared for

Pennwalt Corporation

by

Life Science Research Limited
Eye, Suffolk, IP23 7PX
England

27 June 1988

MANAGEMENT OF STUDY

Study Director : C A Jenkins, B.Sc., M.I.Biol., M.I.F.M.

Quality Assurance Manager : David J. Ford, Ph.D.

Sponsor : Pennwalt Corporation
900, 1st Avenue
P.O. Box C
King of Prussia
Pennsylvania 19406-0018
USA

Monitor : Dr J A Seckar

TEST MATERIAL IDENTITY : Pyronil 45

METHODS

The study will be carried out, under UK legislation (1986) Project Licence 'Aquatic Toxicicology' (No. 70/00762), according to the Standard Protocol ATId attached.

SCHEDULED TIME-PLAN

The study will be performed and reported to a time schedule designed to minimise delays. Due to the short duration of the test, no detailed time-plan will be issued unless specifically requested.

PROTOCOL APPROVAL

For LIFE SCIENCE RESEARCH LIMITED

Issued by : D. C Hodge ..... Date : 27/6/88

Released by : C B Jenkins ..... Date : 28/6/88

For PENNWALT CORPORATION

Approved by : Joel A. Sechan ..... Date : 7/7/88

In short studies using standardised methods, protocol alterations or revisions are not normally required. If changes to this protocol are necessary please contact LSR. Please note that the study cannot begin unless Life Science Research Limited is in receipt of a signed protocol.

DETERMINATION OF THE 96-HOUR LC50 OF TEST MATERIALS TO COLD WATER SPECIES OF FISH (15°C) UNDER STATIC TEST CONDITIONS

Standard Protocol ATId

of

Life Science Research Limited
Eye, Suffolk, IP23 7PX
England

March 1988

1. INTRODUCTION AND OBJECTIVES

This test has been designed to determine the acute toxicity of test materials to cold water species of fish at 15°C. The fish, rainbow trout, will be exposed, for 96 hours, to the test material prepared in treated tap water. The test solutions will not be replaced during the test period.

The study will comprise a preliminary (range-finding) test, followed by a definitive study to determine, where possible, the 96-hour median lethal concentration (LC_{50}) of the test material, with 95% confidence limits. Mortalities will be recorded at 24-hour intervals during the tests.

The study is appropriate for the determination of the effects of insoluble test materials and water soluble materials known to be stable in aqueous solution for greater than 96 hours. It is not appropriate for volatile materials. Test materials may include effluents and formulated products containing more than one substance.

The study will meet the requirements of Procedure 203 of the 'Guidelines for Testing of Chemicals' of the OECD: "Fish, Acute Toxicity" adopted 4 April 1984.

2. TEST ORGANISM

Rainbow trout, *Salmo gairdneri* (Richardson), are usually selected as the test organism because of their known sensitivity to changes in water quality, availability throughout the year and ease of maintenance in the laboratory. They are obtained either as eyed eggs or fry from trout farms in the UK.

3. DILUTION MEDIUM

The dilution water will be filtered de-chlorinated tap water to which has been added de-chlorinated tap water that has been softened and treated by reverse-osmosis to maintain constant hardness. The proportion of treated and un-treated tap water will be adjusted to give a final total hardness of 200-250 mg/l as $CaCO_3$.

4. PRE-EXPOSURE PERIOD

Eggs will be held in hatching troughs supplied with a continuous flow of aerated water. After hatching and resorption of the yolk sac, the fry will be transferred to a holding tank supplied with a continuous flow of aerated water.

Feeding fry obtained from trout farms will be kept in holding tanks supplied with water as above for a minimum of 14 days before being used for testing.

During the holding period the tanks will be inspected at least three times each week and any debris, or unhealthy or dead fish removed.

The temperature of water supplied to the holding tanks will be $14 \pm 2°C$. A photoperiod of 16 hours light, provided by overhead flourescent tubes and 8 hours dark will be maintained. Dawn and dusk will be simulated by the provision of a period of subdued lighting.

The temperature, pH and concentration of dissolved oxygen will be measured at least three times each week and the hardness of the water determined weekly, in each holding tank.

The fish will be fed at least three times each week during the holding period on proprietary trout pellets and feeding records maintained.

Fish will not be used in tests if the mortality of the batch exceeds 10% during the 14-day period prior to starting a test. They will be held without food at the test temperature for approximately 24 hours before being placed into the test vessels.

Fork length will be between 4 and 6 cm and an estimate of the wet-weight of the animals to be used will be made before the start of the definitive test.

5. PREPARATION OF THE TEST MATERIAL

The identity, stability and purity of the test material, and the amount and nature of any other components present are the responsibility of the Sponsor.

Where a material is known to be of low solubility in water a preliminary study can be included, at additional cost, to determine the most appropriate method of preparing dilutions of the test material in the dilution medium used.

Where the test material is poorly soluble and forms a particulate suspension in water, preliminary tests to determine the settling rate and particle size can be carried out at additional cost.

For compliance with international GLP regulations it may be necessary to analyse a sample of test material solution as used in testing, to confirm the concentration, homogeneity and stability of the test material. Also, the test guidelines require evidence that exposure concentrations have been maintained over the test period (within 80% of the nominal value throughout the test). Tests for stability and homgeneity of the test material and for verification of the concentrations of the stock and test dilutions can be conducted by LSR, at the request of the Sponsor, at additional cost.

A concentrated stock solution or dispersion of the test material will be prepared as appropriate in the dilution water. Dilutions of this will then be made to achieve the required test concentrations.

If problems of solubility or homogeneity prevent the use of a concentrated solution or dispersion, appropriate weights of the test material will be added directly to water in test vessels.

The dilution water will be obtained from the same source as that used to maintain the test system during the 14-day holding period preceding the test.

If there is a marked change in the pH of the dilution water after the addition of the test material (outside the range 6 to 8.5), the pH of the stock will be adjusted by the addition of hydrochloric acid and/or sodium hydroxide.

Where a test material is known to adsorb onto the surfaces of test vessels, the vessels will be conditioned to the appropriate concentration of the test material for a period of up to 48 hours before the test starts. This will attract an additional cost.

6. TEST PROCEDURE

The study which will be carried out under static conditions will comprise a preliminary test, to provide an estimate of the toxicity of the material, followed by a definitive test to define the relationship between concentration and effect.

In the preliminary test, fish will be exposed to concentrations of the test material selected from the following range unless otherwise specified by the Sponsor:

1, 3.2, 10, 32, 100, 320 and 1000 mg/l

The range of concentrations used in the definitive test will be based on the results of the preliminary test and will provide at least five exposure groups, spaced by a constant factor not exceeding 2, and a control group.

Where requested by the Sponsor, the concentration of the test material in the concentrated stock will be verified by chemical analysis in the preliminary and definitive tests. In addition the exposure concentrations in appropriate vessels will be determined at the start and end of the definitive test.

When an intermediate vehicle is used to facilitate the preparation of aqueous solutions or dispersions of the test material, an additional control group will be included containing the vehicle in the dilution water at a concentration comparable to that present at the highest exposure concentration (not exceeding 100 mg/l).

The temperature of the test solutions will be maintained between 13 and 17°C but constant to within $\pm$ 1°C during each test. The photoperiod will be the same as that in the holding area.

The prepared test dilutions will be placed into the test vessels (glass aquaria) and gently aerated using a Pasteur pipette during the test. The aquaria will be of an adequate size to meet the guideline criterion of a maximum loading of 1 g fish per litre of test solution.

Groups of five fish will be randomly assigned to the test vessels containing test dilutions, until each contains the required number of animals. In the preliminary test five fish will be exposed to each concentration; in the definitive test a minimum of ten will be used at each exposure level.

The fish will not be fed during the tests.

The fish will be observed for mortalities and visible abnormalities 15 minutes after their addition to the vessels and then after 24, 48, 72 and 96 hours. During the definitive test additional observations will be made 2 and 4 hours after the start of the test.

The temperature, pH and concentration of dissolved oxygen of the dilution water and all test solutions will be measured every 24 hours. The total hardness of the dilution water and of solutions of the test material at low and high exposure concentrations will be measured at the start and end of the tests.

For the test to be valid, the mortality in the control group must not exceed 10% at the end of the test, the concentration of dissolved oxygen must be > 60% of the air saturation value during the test and exposure concentrations must be maintained throughout the test (within 80% of the nominal concentrations).

8. QUALITY ASSURANCE

This study will be conducted in accordance with the precepts of currently recognised international Good Laboratory Practice (including the recommendations of the OECD, 1981) and will be subjected to the following quality assurance procedures.

- the protocol will be inspected for compliance
- procedures and data as used and produced on this type of study are periodically inspected
- the final report will be reviewed to ensure that it accurately describes the methods and relevant Standard Operating Procedures and that the results are in accord with the primary data.

Periodic reports on these activities are made to Management and the Study Director.

All raw data pertaining to the study will be available for inspection by the study monitor (for scientific monitoring) or the Quality Assurance Unit of the Sponsor (compliance monitoring). In addition, specified scientists designated by the Sponsor may, upon appointment, examine any set of data.

9. LOCATION OF STUDY : Life Science Research Limited
Eye
Suffolk IP23 7PX
England

10. RECORDS KEPT

All raw data, test material information, QA records and reports and other records pertaining to the study will be retained in the Archives of LSR.

The raw data will comprise: operational and observations sheets covering every aspect of the study.

11. REPORTING

The following information and data will be included in the final report.

- Name and address of the facility performing the study and the initiation and termination dates.

6.1 Amendments to the standard procedure

The following amendments to the standard test design are available at additional cost:

Where the Sponsor requires a study of comparative toxicity of a number of test materials, single tests can be carried out in which a wide range of exposure concentrations can be used and the test terminated after 48 hours.

The test can be carried out :

- without verification of exposure concentrations (this test may not fulfil regulatory requirements),
- using an alternative dilution medium eg. low-hardness water,
- at low levels of dissolved oxygen,
- at pH levels outside the range 6 to 8.5,
- in the presence of suspended solids,
- using an alternative species of fish.

At the Sponsor's request internal and/or external examinations of the fish can be carried out and the body tissues either analysed at the testing facility or returned to the Sponsor for histological analysis. Where appropriate, quantitative analysis of gill damage can be performed.

7. APPRAISAL OF DATA

The lowest concentration producing 100% mortality/effect and the highest concentration producing no mortality/effect will be stated.

Where possible the median lethal concentrations (LC_{50}s), and their 95% confidence limits, will be calculated by either the moving average, probit or binomial methods at 24-hour intervals.

Water quality data will be expressed as minimum and maximum values measured during the tests.

Results of analysis of the test material will be given as found values together with the estimated values after adjustment for recovery if appropriate.

Any calculations made will be included in the written report, together with advice on the significance of the results.

8. QUALITY ASSURANCE

This study will be conducted in accordance with the precepts of currently recognised international Good Laboratory Practice (including the recommendations of the OECD, 1981) and will be subjected to the following quality assurance procedures.

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The raw data will comprise: operational and observations sheets covering every aspect of the study.

11. REPORTING

The following information and data will be included in the final report.

- Name and address of the facility performing the study and the initiation and termination dates.

- Objectives and procedures stated in the approved study plan or protocol amendment, including any changes subsequently made.

- Raw data generated while conducting the study including any transformations, calculations or operations performed on the data. Tabulated mean and range values where appropriate.

- Statistical methods employed for analysing the data.

- The test material identified by name and/or code number, strength, stability and purity, as instructed by the Sponsor.

- The Sponsor's information regarding stability of the test material under the conditions of the test.

- Methods and procedures used.

- Concentrations tested.

- Frequency and modes of observation. Observations recorded.

- Any unforeseen circumstances which may have affected the quality or integrity of the study.

- The name of the Study Director.

- A summary of the data, an analysis of the data and a statement of the conclusions drawn from the analysis.

- The dated signature of the Study Director.

- The location where all raw data and the final report are stored.

- A statement by the Quality Assurance Unit.

Corrections or additions to a final report will be in the form of an amendment by the Study Director. The amendment will clearly identify that part of the final report that is being added to or corrected and the reasons for the correction or addition, and will be signed and dated by the person responsible.

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