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Part 1.
M-3

CONFIDENTIAL

LSR Schedule No : PSV031
LSR Report No : 89/PSV031/0195

PYRONIL 45:
ACUTE TOXICITY TO
DAPHNIA MAGNA

M-314

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: 13 April 1989
Final report: 10 July 1989

TR 89-6.
Part 2 of

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 Daphnia magna.
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-314. LSR Report No. 89/PSV031/0195. July 10, 1989.
For MSDS:	48-hour EC50 Daphnia magna: 0.27 mg/L, highly toxic.

Summary of Results: Daphnia magna were exposed to PYRONIL 45 in a static 48-hour acute toxicity test. Nominal test concentrations ranged from 0.063 to 1 mg/L. Acetone was used as a cosolvent. Measured 48-hour EC50 values were 0.27 mg/L for immobile and floating D. magna and 0.30 mg/L for immobile D. magna. These results indicate that PYRONIL 45 is toxic to Daphnia magna at concentrations below the estimated water solubility limit of 1 mg/L.

PREPARATION DATE: November 27, 1989

LIFE SCIENCE RESEARCH

PYRONIL 45: ACUTE TOXICITY TO *DAPHNIA MAGNA*

LSR Schedule No : PSV031

LSR Report No : 89/PSV031/0195

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. .....
(Study Director)

Date: 14-7-89..

LIFE SCIENCE RESEARCH

PYRONIL 45: ACUTE TOXICITY TO *DAPHNIA MAGNA*

LSR Schedule No : PSV/031
LSR Report No : 89/PSV031/0195

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 Studies)

Date: 10-7-89

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 10/7/89

LIFE SCIENCE RESEARCH

PYRONIL 45: THE ACUTE TOXICITY TO *DAPHNIA MAGNA*

LSR Schedule No : PSV031
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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	29.9.88	30.9.88	30.9.88
DATA			
Inspection of data generated on this type of study was made in accordance with LSR Standard Operating Procedure QAU/050	22.11.88		22.11.88
PROCEDURES			
Inspection of procedures on this type of study was made in accordance with LSR Standard Operating Procedure QAU/040	26.9.88 27.9.88 29.9.88 14.10.88 22.11.88		28.9.88 28.9.88 30.9.88 17.10.88 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: 7 July 1989

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

.....

Date: 10 July 1989

C O N T E N T S

TABLES

APPENDICES

1. SUMMARY

1.1 The acute toxicity of PYRONIL 45 to *Daphnia magna* has been assessed under static conditions over an exposure period of 48 hours.

1.2 In a preliminary test, groups of ten *Daphnia* were exposed to four nominal concentrations of the test material ranging from 1 to 1000 mg/l at a temperature of 19.7 ± 0.4°C in filtered, dechlorinated tap water (hardness 202 - 218 mg/l as $CaCO_3$, pH 7.3 - 8.0). Each dose level was individually prepared by shaking the required weight of test material in dilution water for two hours, and then treating this suspension with ultrasound for 15 minutes. At nominal concentrations of 1 and 10 mg/l the test dilutions were clear and colourless; at 100 and 1000 mg/l the test material was visible as oily droplets on the surface, and as globules on the bases of the preparation flasks. After 48 hours, all of the *Daphnia* exposed to the test material had been immobilised and, except for one *Daphnia* at the highest dose level, were floating on the surface of the test dilutions.

1.3 Concentrations of PYRONIL 45 determined by analysis of duplicate samples at the nominal concentration of 1 mg/l were 0.84 and 0.87 mg/l at the start of the test and 0.72 and 0.75 mg/l after 48 hours. At a nominal 1000 mg/l, measured concentrations were 256 and 159 mg/l at the start of the test and 203 and 141 mg/l after 48 hours. Test observations indicated that at levels above 10 mg/l, the aqueous solubility of the material had been exceeded. The variation in analytically determined values suggested that undissolved material was present in the samples taken for analysis.

1.4 In a second preliminary test, *Daphnia* were exposed to dilutions of the test material nominally containing 0.01, 0.1 and 1 mg/l. A solution of the test material was prepared in acetone (10 mg/ml) and diluted to give an aqueous stock at 1 mg/l; test solutions were prepared by further diluting this aqueous stock. The test was conducted at 19.5 ± 0.5°C in dechlorinated water (hardness 208 - 214 mg/l as $CaCO_3$) at pH 7.7 to 8.2. After 48 hours, all of the *Daphnia* at 1 mg/l had been immobilised and were floating on the surface of the test dilution; at 0.01 and 0.1 mg/l all of the *Daphnia* were mobile but 70% of those exposed at 0.1 mg/l were swimming at the surface.

1.5 In a definitive test, conducted at 19.7 ± 0.6°C in water of hardness 196 to 204 mg/l as $CaCO_3$ at pH 7.4 to 8.1, *Daphnia* were exposed to five concentrations of PYRONIL 45 in the range 0.063 to 1 mg/l. Test dilutions were prepared using the method employed in the second preliminary test. Each test and control group comprised twenty *Daphnia*: five in each of four replicate vessels. The numbers of mobile, immobile and floating *Daphnia* were counted 24 and 48 hours after the start of the test.

1.6 Mean concentrations of PYRONIL 45 determined in samples from each exposure level ranged between 77 and 88% of nominal values at the start of the test and between 64 and 124% of nominal after 48 hours. Analytical results and test observations suggest that the test material was soluble in the dilution water at the levels employed in the test.

1.7 The lowest nominal exposure concentration used in the test (0.063 mg/l) resulted in 5% immobility and 20% floating. The highest concentration (1 mg/l) resulted in 95% immobility, with 5% of the animals swimming at the surface of the test dilution.

1.8 The 48-hour median effect concentrations (EC50s), based on nominal and measured concentrations were:

	EC50 values, mg/l (95% confidence limits)	
	nominal	measured
Immobility	0.38 (0.31-0.47)	0.30 (0.25-0.37)
Immobility & Floating	0.34 (0.25-0.48)	0.27 (0.23-0.32)

2. INTRODUCTION

The objective of this study was to determine the 48-hour median effect concentration (EC50) of PYRONIL 45 on *Daphnia magna* for immobility and floating. The test was conducted in accordance with the OECD Guidelines for Testing of Chemicals, Procedure 202, adopted 4 April 1984 (1).

Three preliminary tests were conducted: only the results from two of these have been reported because the number of affected *Daphnia* in the control group in the other test exceeded the accepted level (10%).

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: 4-21 October 1988 Definitive test : 25-27 October 1988.
Data storage	:	The raw data and a copy of the final report will be stored in the archives of Life Science Research.

3. TEST MATERIAL

The test material, PYRONIL 45, was received from Pennwalt Corporation, Pennsylvania, USA on 17 August 1988. The substance was a pale-yellow, clear liquid contained in a translucent plastic container, labelled PYRONIL (TN) 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
Chemical structure	Halogenated phthalate ester
Other names	FR-45B
Specific gravity	1.545 (25°C/4°C)
Stability	unstable in strong acids/alkalis

4. METHODS

4.1 Test organism

The strain of *Daphnia magna* used in this study was obtained from the University of Sheffield where electrophoretic assay had confirmed genetic homogeneity. This strain originated from the National Institute for Applied Chemical Research (IRCHA), France.

Daphnia have been maintained in parthenogenetic culture at the Aquatic Studies Laboratories of Life Science Research since receipt.

4.1.1 *Daphnia* culture method

Daphnia were maintained in filtered dechlorinated tap water at a hardness of between 200 and 250 mg/l as $CaCO_3$. Control of hardness was achieved by mixing tap water with tap water which had been softened and subsequently treated by reverse osmosis.

The culture vessels were two-litre Pyrex glass beakers with loose-fitting clock glass covers.

The cultures were kept in a temperature-controlled laboratory maintained at 20 $\pm$ 3°C. The day length in the area was controlled giving a photoperiod of 16 hours light, supplied by overhead fluorescent tubes, and eight hours darkness. Dawn and dusk was simulated by a period of subdued lighting at the beginning and end of the light phase.

The water (1.5 litres) in each culture vessel was replaced at intervals of two weeks with fresh water of the correct temperature.

A maximum of twenty adult *Daphnia* were maintained in each culture vessel and the juveniles produced were removed at least twice each week.

4.1.2 Culture feeding regime

Daphnia cultures were fed at least five times each week with suspensions of the unicellular green alga *Chlorella vulgaris* and yeast.

Chlorella vulgaris, strain CCAP 211/12 obtained from the Culture Centre of Algae and Protozoa (CCAP, The Freshwater Biological Association, Cumbria, England), was cultured in a synthetic mineral salts medium in illuminated ten-litre glass fermenter vessels.

4.4 Apparatus

The test vessels were crystallising dishes of approximately 120 or 150 ml capacity. During the tests each vessel was covered with a watch-glass.

4.5 Preliminary tests

4.5.1 First preliminary test

Information supplied by the Sponsor indicated that the test material was poorly soluble in water; accordingly each test concentration was prepared separately. In each case the appropriate weight of material was rinsed into a flask and the volume adjusted to approximately 600 ml. Each preparation was placed on an orbital shaker for approximately two hours, then treated by ultrasound for 15 minutes, and finally the volume was adjusted to one litre with water. The test vessels were filled with approximately 100 ml of the appropriate dilution. Duplicate vessels were prepared at each exposure level except at 1 and 1000 mg/l, where additional vessels, without *Daphnia*, were established for water quality measurements and chemical analysis.

The test was carried out under static conditions. Groups of ten *Daphnia* were exposed to the test material at nominal concentrations of 1, 10, 100 and 1000 mg/l. A control group comprised ten *Daphnia* in dilution water alone.

Five juvenile *Daphnia* were pipetted from the holding vessel into each vessel containing the prepared test dilutions and into the control vessels. The order in which the groups of *Daphnia* were assigned to vessel was based on random numbers.

Test dilutions were not aerated during the test and their pH was not adjusted before the start of the test or controlled during the test.

Observations of the *Daphnia* were made 24 and 48 hours after the start of the test. They were not fed during the test.

4.5.2 Second preliminary test

Since all doses in the first preliminary test caused immobility, a second test was conducted using lower exposure levels.

Test dilutions were prepared from a 1 mg/l aqueous stock solution which was prepared from a concentrated solution of the test material dissolved in acetone (10 mg/ml).

Two groups of five *Daphnia* were exposed to the test material under static conditions at each of the following nominal concentrations; 0.01, 0.1 and 1 mg/l. In addition, control groups of *Daphnia* were exposed to dilution water alone and dilution water containing acetone (0.1 ml/l).

Five juvenile *Daphnia* were pipetted from the holding vessel into each vessel containing the prepared test dilution (100 ml) and into the control vessels. The order in which the groups of *Daphnia* were assigned to vessel was based on random numbers.

Test dilutions were not aerated during the test and their pH was not adjust before the start of the test or controlled during the test.

Observations of the *Daphnia* were made 1, 24 and 48 hours after the start of the test. They were not fed during the test.

4.6 Definitive test

4.6.1 Preparation of dilutions of the test material

Test dilutions were prepared as in the second preliminary test.

Four test vessels were established at each exposure concentration and for the two control groups, containing dilution water or the appropriate test dilution (100 ml). An additional vessel without *Daphnia* was established for water quality measurements and chemical analysis at each exposure concentration.

4.6.2 Test procedure

The test was carried out under static conditions with twenty *Daphnia* in each control and test group. The following nominal exposure concentrations were employed in the test:

0.063, 0.125, 0.25, 0.5 and 1 mg/l.

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

Daphnia were removed from the holding vessel using a pipette and allocated, in groups of five, to the test vessels according to random numbers. The test vessels were arranged in the test area in the same order as the *Daphnia* were added to the test vessels.

The test dilutions were not aerated during the test and their pH was not adjusted before the start of the test or controlled during the test.

Observations of the *Daphnia* were made 24 and 48 hours after the start of the test. The appearance of the test material in water was noted during the test.

4.7 Water quality analysis

The temperature, pH and concentration of dissolved oxygen (DO) of the dilution water and preparations of test material at each exposure concentration were measured at the start and end of the tests.

Temperature measurements were taken in the test vessels: pH and DO measurements were made using the test dilutions remaining in the preparation flasks after the test vessels were filled, from the pooled contents of the replicate vessels at each exposure level or, where all of the test dilutions were required for analysis, from the additional vessels.

The total hardness and alkalinity of the dilution water were measured at the start and end of the tests.

A sample of the dilution water was taken for determination of the concentrations of sodium, potassium, calcium and magnesium at the start of the definitive test.

4.8 Analysis of PYRONIL 45

The method used to determine the concentrations of the test material is described in Appendix 2.

4.8.1 First preliminary test

At the start of the test, samples (2 x 200 ml) were taken from the freshly-prepared dilutions of the test material remaining in the preparation flasks after the test vessels at 1 and 1000 mg/l were filled. After 48 hours the contents of the test vessels at each of these exposure levels were pooled and a further two samples (200 ml) were taken for analysis. Similar sized samples from the control vessels containing the dilution water served as the blank during analysis.

4.8.2 Definitive test

Samples (2 x 200 ml) were taken from the freshly-prepared dilutions of the test material remaining in the preparation flasks at the start of the test, and from the pooled contents of the replicate test vessels at each exposure level after test termination. Dilution water and acetone from the control vessels served as the blank during analysis.

4.9 Statistical Analysis

Wherever possible, median effect concentrations (EC50s) were calculated by an appropriate statistical method (binomial, moving average and/or probit) using the number of *Daphnia* exposed and the number immobile and/or floating at each nominal concentration. EC50 values were also computed using the mean of the concentrations measured in the test dilutions during the test.

5. RESULTS

5.1 Preliminary tests

5.1.1 First test

Measured concentrations of PYRONIL 45 at a nominal concentration of 1 mg/l were 0.84 and 0.87 mg/l at the start of the test, and 0.72 and 0.75 mg/l after 48 hours. At a nominal 1000 mg/l, measured concentrations were 256 and 159 mg/l at the start of the test and 203 and 141 mg/l after 48 hours.

Test dilutions at 1, 10 and 100 mg/l were clear and colourless; at 1000 mg/l it appeared hazy. At 100 and 1000 mg/l, the test material was present as colourless, oil-like droplets on the surfaces of the dilutions and as globules on the bases of the preparation flasks. After 48 hours, all test dilutions were clear and colourless; the oil-like droplets were still present on the surfaces of test dilutions at 100 and 1000 mg/l.

These results indicate that the aqueous solubility of the material had been exceeded and that the variation in determined values at 1000 mg/l was due to the presence of undissolved test material in samples taken for analysis.

Table 1 lists the effects observed during the test. After 48 hours, all *Daphnia* exposed to the test material had been immobilised and all except for one at 1000 mg/l floated on the surface of the test dilutions.

The air temperature of the test area varied between 18.5 and 20.5°C.

Water quality data for the test are shown below.

Parameter	Range
Temperature °C	19.3-20.1
pH	7.28-8.03
Concentration of dissolved oxygen % ASV*	94-98
Total hardness of the dilution water mg/l $CaCO_3$	202-218
Alkalinity of the dilution water mg/l $CaCO_3$	128-130

* = Air Saturation Value

5.1.2 Second test

Table 2 lists the effects observed during the test. After 48 hours, all of the *Daphnia* at 1 mg/l were immobile and floated on the surface of the test dilution; at 0.01 and 0.1 mg/l all of the *Daphnia* were mobile but 70% of those exposed at 0.01 mg/l were swimming at the surface.

All test dilutions were clear and colourless.

The air temperature of the test area varied between 18.5 and 21.0°C.

Water quality data for the test are shown below.

Parameter	Range
Temperature °C	19.0-20.0
pH	7.73-8.15
Concentration of dissolved oxygen % ASV	95-97
Total hardness of the dilution water mg/l $CaCO_3$	208-214
Alkalinity of the dilution water mg/l $CaCO_3$	140-143

5.2 Definitive test

5.2.1 Analysis

Analytical determinations of samples taken during the test are given in Table 3.

The mean concentrations of PYRONIL 45 measured in samples taken at the start of the test ranged between 77 and 88% of nominal values; after 48 hours they ranged between 64 and 124% of nominal values. These results indicate that the test material was soluble in water at the levels employed in the test.

5.2.2 Effects on *Daphnia*

Observations of the numbers of mobile, immobile and floating *Daphnia* are summarised in Table 4.

EC50 values of the test material at intervals during the test are given below. The values were calculated using the computer program of Stephan (2) and nominal and measured exposure concentrations.

Observation times	EC50 values mg/l (95% confidence limits) nominal	measured
24-hours		
immobility	0.84 (0.70-1.15)	0.68 (0.55-0.98)
immobility & floating	0.29 (0.24-0.37)	0.24 (0.20-0.30)
48-hours		
immobility	0.38 (0.31-0.47)	0.30 (0.25-0.37)
immobility & floating	0.34 (0.25-0.48)	0.27 (0.23-0.32)

The lowest nominal exposure concentration (0.063 mg/l) used in the test resulted in 5% immobility and 20% floating. The highest nominal exposure concentration (1 mg/l) caused 95% immobility after 48 hours.

5.2.3 Test environment and water quality data

Measurements of water quality taken from the test dilutions during the test are summarised below. The air temperature of the test area varied between 18.5 and 20.5°C.

Parameter	Range	
	0 hours	48 hours
Temperature °C	19.1 - 19.5	19.6 - 20.2
pH	7.40 - 7.59	7.95 - 8.06
Concentration of dissolved oxygen %ASV	95 - 96	94 - 95
Total hardness (dilution water) mg/l as $CaCO_3$	196	204
Alkalinity (dilution water) mg/l as $CaCO_3$	128	133
Sodium : potassium ratio	10:1	
Calcium : magnesium ratio	18.6:1	

6. DISCUSSION AND CONCLUSIONS

6.1 The 48-hour EC50 of PYRONIL 45 to *Daphnia magna* under static conditions, calculated using nominal exposure concentrations, was 0.38 mg/l (95% confidence limits 0.31 - 0.47 mg/l) based on the numbers of immobile *Daphnia*, and 0.34 mg/l (0.25 - 0.48 mg/l) based on the numbers immobile and/or floating.

6.2 The lowest nominal exposure concentration used in the test (0.063 mg/l) resulted in 5% immobility and 20% floating. The highest concentration (1 mg/l) resulted in 95% immobility.

6.3 Although analytical determinations of achieved concentrations were on occasions variable, overall these results indicated that exposure concentrations were acceptably close to nominal and adequately maintained during the definitive test.

6.4 The 48-hour EC50 value calculated using the mean of the measured concentrations was found to be 0.30 mg/l (95% confidence limits 0.25 - 0.37 mg/l) based on the number of immobile *Daphnia*, and 0.27 mg/l (95% confidence limits 0.23 - 0.32 mg/l) based on the numbers of immobile and floating *Daphnia*.

7. REFERENCES

1. OECD Guidelines for Testing of Chemicals. "*Daphnia*, sp., Acute Immobilisation Test and Reproduction Test, Part 1. Procedure 202, adopted 4 April 1984.

2. STEPHAN ET AL. A computer program for calculating an LC50. US Environmental Protection Agency.

TABLE 1

First preliminary test: numbers of mobile, immobile and floating *Daphnia*

Nominal PYRONIL 45 concentrations (mg/l) and observation times	Numbers of *Daphnia*			
	Mobile		Immobile	
	Submerged	Floating	Submerged	Floating
24-hour observations				
control	10	0	0	0
1	3	7	0	0
10	0	7	0	3
100	0	0	0	10
1000	0	0	0	10
48-hour observations				
control	9	1	0	0
1	0	0	0	10
10	0	0	0	10
100	0	0	0	10
1000	0	0	1	9

TABLE 2

Second preliminary test: numbers of mobile, immobile and floating *Daphnia*

Nominal PYRONIL 45 concentrations (mg/l) and observation times	Numbers of *Daphnia*			
	Mobile		Immobile	
	Submerged	Floating	Submerged	Floating
1-hour observations				
control	10	0	0	0
acetone control	10	0	0	0
0.01	10	0	0	0
0.1	10	0	0	0
1.0	4	6	0	0
24-hour observations				
control	10	0	0	0
acetone control	10	0	0	0
0.01	7	2	0	1
0.1	9	1	0	0
1.0	0	0	0	10
48-hour observations				
control	10	0	0	0
acetone control	10	0	0	0
0.01	3	7	0	0
0.1	10	0	0	0
1.0	0	0	0	10

TABLE 3

Definitive test: analytical determinations of PYRONIL 45

Nominal mg/l	Measured PYRONIL 45 values (mg/l) 0 hours	*mean*	48 hours	*mean*
control	0,0	*0*	0,0	*0*
0.063	0.071,0.026	*0.049*	0.062,0.093	*0.078*
0.125	0.119,0.090	*0.105*	0.078,0.133	*0.106*
0.25	0.218,0.226	*0.22*	0.174,0.158	*0.166*
0.5	0.393,0.470	*0.432*	0.270,0.366	*0.318*
1.0	0.730,0.803	*0.767*	1.09,0.707	*0.899*

TABLE 4

Definitive test: numbers of mobile, immobile and floating *Daphnia*

Nominal PYRONIL 45 concentrations (mg/l) and observation times	Numbers of *Daphnia*			
	Mobile		Immobile	
	Submerged	Floating	Submerged	Floating
24-hour observations				
control	20	0	0	0
acetone control	20	0	0	0
0.063	20	0	0	0
0.125	19	1	0	0
0.25	12	7	0	1
0.5	4	14	0	2
1.0	0	7	0	13
48-hour observations				
control	20	0	0	0
acetone control	20	0	0	0
0.063	16	3	0	1
0.125	20	0	0	0
0.25	16	1	0	3
0.5	3	1	0	16
1.0	0	1	1	18

APPENDIX 1

Chemical composition of test dilution water

Source: Filtered dechlorinated tap water blended with tap water that had been softened and subsequently treated by reverse osmosis to a hardness of 200 to 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)	1.9 - 4.0
Nitrite (as N)	< 0.01 - 0.03
Nickel	< 0.01
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 *Daphnia magna* 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 were 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 the absorbances of the acetonitrile 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 20 mg/l in the preliminary test, and 10, 20, 30, 40 and 50 mg/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 spectrophptometer
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:

$$y = a + bx$$

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