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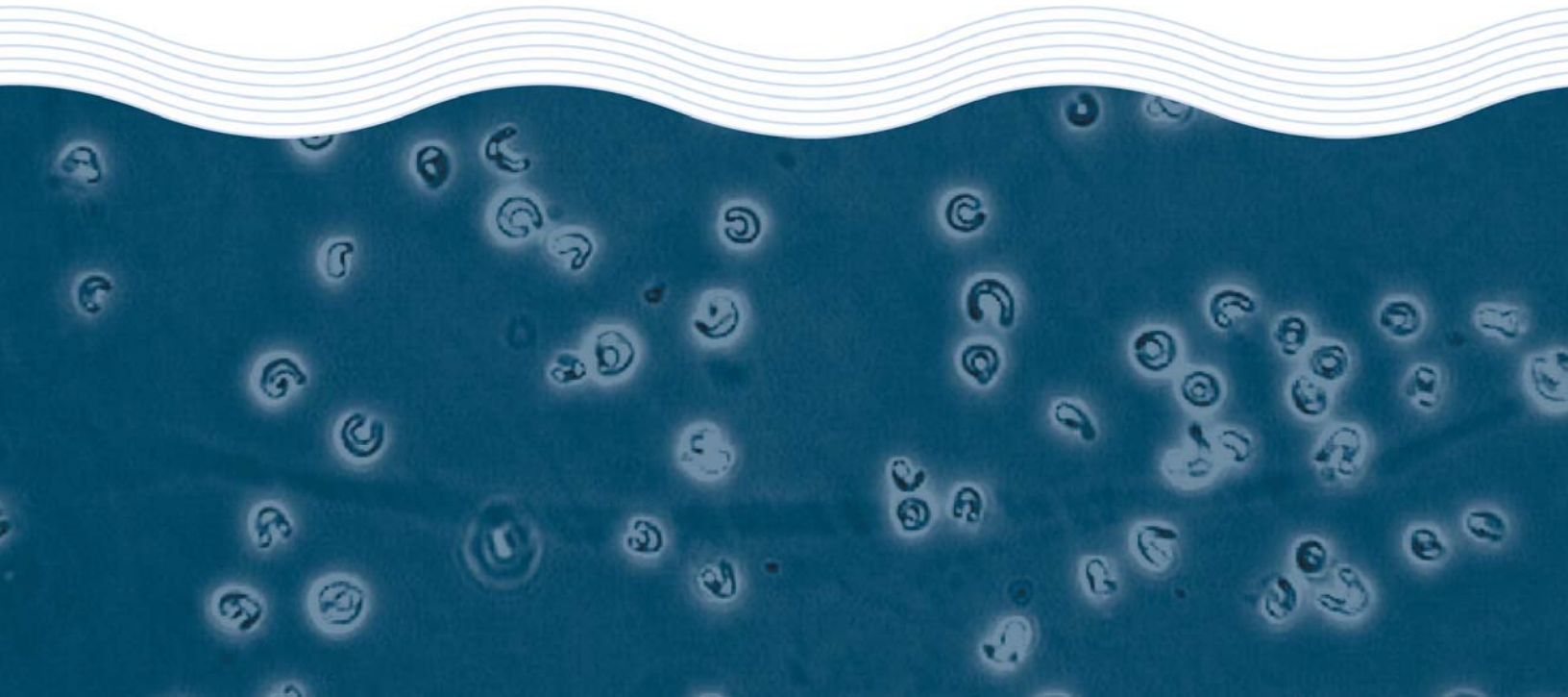
Toxicity Assessment of Granulated Phoslock to the Cladoceran *Ceriodaphnia dubia*

Phoslock Water Solutions Ltd

Test Report

July 2008



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ECOTOX Services Australasia Pty Ltd ABN>45 094 714 904
unit 27/2 chaplin drive lane cove nsw 2066 T>61 2 9420 9481 F>61 2 9420 9484 W>www.ecotox.com.au

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1 Executive Summary

Ecotox Services Australasia Pty Ltd (ESA) was commissioned by Phoslock Water Solutions Ltd to undertake the following two freshwater ecotoxicity tests on granulated Phoslock in Fitzroy Falls Reservoir water as diluent:

- The 48 h acute (immobilisation) toxicity test using the freshwater cladoceran *Ceriodaphnia dubia,* based on USEPA Method (2002a)
- The 7-day, 3-brood partial life-cycle test using the freshwater cladoceran *Ceriodaphnia dubia,* based on USEPA Method 1002.0 (USEPA 2002b)

This document describes the methodology used in undertaking these ecotoxicity tests and the results obtained. Statistical printouts of each of the analyses are provided in **Appendices A and B**. A guidance document provided by Phoslock Water Solutions Ltd for the preparation of test solutions is given in **Appendix C**. A copy of the Certificate of Analysis for the batch of granulated Phoslock used for the testing is provided in **Appendix D**.

The test concentrations for the 48-h acute test were 50, 20, 2, 1, 0.75, 0.5 and 0.25 mg Phoslock/L The test concentrations for the 7-d reproductive impairment test were 1, 0.75, 0.5 and 0.25 mg Phoslock/L. The test results are summarised as follows:

- ***48-h acute toxicity:*** The 24- and 48-h EC50 (with 95% confidence limits) of granular Phoslock to *C. dubia* survival was estimated to be >50mg/L. No significant mortalities were observed at any of the treatments tested, and consequently the NOEC and LOEC estimates were 50 and >50 mg/L, respectively.

- ***Survival over 7-d:*** The granular Phoslock sample did not exhibit toxicity to the 7-d chronic (partial life-cycle) toxicity test with the freshwater cladoceran *Ceriodaphnia dubia,* with respect to survival. The 7-d EC50, NOEC and LOEC estimates for survival were >100, 100 and >100%, respectively.

- ***Reproductive impairment over 7-d:*** The granular Phoslock sample did not exhibit toxicity to the 7-d chronic (partial life-cycle) toxicity tests with the freshwater cladoceran *Ceriodaphnia dubia,* with respect to reproduction. The 7-d IC50, NOEC and LOEC estimates for reproduction were >100, 100 and >100%, respectively.

- All toxicity tests performed for this study met test acceptability criteria.

2 Sample Preparation and Methodology

2.1 Preparation of Test Material

Phoslock Water Solutions Ltd prepared a guidance document to help facilitate the toxicity testing program, and included details of the methods to be used for the preparation of test solutions. A copy of this guidance document is given in **Appendix C**. As the granulated Phoslock material readily formed a slurry on mild agitation in water, the technique of gentle stirring of solutions using a magnetic stirrer was selected for the preparation of test solutions.

A clear 'zip-lock' polyethylene bag containing approximately 500g of granulated Phoslock (refer to Certificate of Analysis provided in **Appendix D**) was shipped to ESA by Phoslock Water Solutions Ltd personnel on 1 July 2008 and received the same day. The test sample consisted of irregularly shaped pale brown granules, mostly of between 2 and 5mm in size, although the sample also contained significant fine-powdery material of similar appearance (except with respect to size) to the larger granules.

In addition to the granulated Phoslock, 20L of water from the Fitzroy Falls Reservoir was also collected Phoslock Water Solutions Ltd personnel and delivered to the ESA laboratory on 1 July 2008. This water was to be used for the preparation of the Phoslock test solutions. This water was not filtered prior to use as the water was of similar clarity to tap-water. The Fitzroy Reservoir water was refrigerated overnight at 4^{o}C until required for testing on the following day. The granulated phoslock was held at room temperature in a laboratory cupboard until required for testing on the following day.

Approximately 3 hours prior to initiating the toxicity test, a stock solution was prepared by first making a 100g/L slurry of phoslock using a volumetric 1L flask. The diluent used for the preparation of the slurry, stock solutions and all test solutions was the un-filtered Fitzroy Falls Reservoir water. The slurry was mixed for 10-minutes using a magnetic stirrer. Following mixing, it was noted that the final volume had fallen below the 1L mark, and additional diluent was added to return the volume to 1L.

Once the slurry had been prepared, a stock solution of 500mg/L was prepared by adding 5mL of the homogenous 100g/L slurry to a fresh 1L volumetric flask, and made up to the 1000mL mark with diluent. This solution was gently mixed for 5-minutes using a magnetic stirrer. This stock solution was then used to prepare the individual test treatments described below.

The acute toxicity tests were run as a static, non-renewal system, meaning that the test organisms were subject to the same solution throughout the 48-h exposure period. However the 7-d chronic tests were subject to a complete renewal (ie static-renewal) on each day for the duration of the test. Sufficient test solutions were prepared on Day 0 to provide for the test solution renewals on Days 1, 2 and 3. On Day 4, a fresh slurry, stock and test solutions were prepared, and used to provide renewal solutions on Days 4, 5 and 6. The test was terminated on Day 7.

2.2 48-h Acute Toxicity Test with *Ceriodaphnia dubia*

Acute toxicity tests with the freshwater cladoceran *Ceriodaphnia dubia* were undertaken in accordance with the NATA endorsed ESA Standard Operating Procedure 101. This procedure is based on methods described by the USEPA (2002a) and adapted for use with the locally collected *C. dubia* (referred to as the Sydney Clone) by Bailey *et al.* (2000a). The conditions for the test are summarised in **Table 2.1**.

Dilution Water

Dilute Mineral Water (DMW) is typically used as the diluent for the toxicity tests and as the culture medium for the culturing of the test organisms, however for this study the Fitzroy Falls Reservoir water was used as the diluent. However DMW was used for the culturing of the test organisms, as diluent for the positive control (concurrent reference toxicant test) and as an additional control treatment. DMW was prepared 24-48 h prior to use in accordance with the procedure described by the USEPA (2002a). DMW was prepared by diluting Perrier mineral water to a concentration of 20 % (vol/vol) with deionised water. A vitamin B12 and selenium supplement was added to the DMW to give final concentrations of 10 and 2 μg/L, respectively. The DMW was prepared in a 5 L Schott bottle and aerated using an aquarium aerator. The conductivity and pH of the water was checked prior to use.

Test Organisms

Neonates (<24 h) of *C. dubia* were obtained from cultures maintained at the ESA laboratory. All neonates were removed from the cladoceran mass cultures no more than 24 h prior to test initiation to ensure that only neonates less than 24 h old were used in the toxicity tests. The neonates to be used in the toxicity tests were isolated approximately 2 h prior to test initiation in a 250 mL beaker containing 200 mL of DMW.

Table 2.1 Summary of test conditions for the *Ceriodaphnia dubia* acute toxicity test

Test Type	Static, non-renewal
Test duration	48 h
Temperature	25 ± 1°C
Light quality	cool-white fluorescent tube lighting
Light intensity	800 ± 160 Lux
Photoperiod	16hr light : 8hr dark
Test Chamber size	20mL
Test solution volume	18mL
Age of test organisms	Less than 24 hrs old
No. of organisms per replicate	5
No. of replicates per treatment	4
No. of organisms per treatment	20
Feeding regime	Fed *Selenastrum* and freeze-dried *Dunalliella* while holding prior to test. Newly-released neonates should have food available at least 2 hr prior to testing
Dilution water	Fitzroy Falls Water (un-filtered) used as diluent. Dilute Mineral Water (DMW) was used as diluent for reference toxicant and for control treatment , prepared by mixing 20% Perrier mineral water with deionised water, with vitamin B12 and selenium supplements
Test concentrations	7 concentrations plus a DMW control and Fitzroy Falls Reservoir Water Control
Dilution series	50, 20, 2, 1, 0.75, 0.5 and 0.25 mg/L
Endpoint	Immobilisation
Test acceptability criterion	≥ 90% survival in controls. Reference toxicant EC50 within Cusum chart control limits

Test Procedures

The test concentrations were 50, 20, 2, 1, 0.75, 0.5 and 0.25 mg/L, and were prepared in 250 mL beakers by diluting with Fitzroy Falls Reservoir Water. After homogenising the test solutions, the conductivity, pH, and dissolved oxygen saturation of the test treatments were measured. The conductivity was measured using a WTW LF330 salinity/conductivity meter with a WTW Tetracon 325 probe. The pH and temperature were measured using a WTW pH330 meter, with a WTW SenTix 41 electrode. Dissolved oxygen was measured using a WTW Oxi 330 Oximeter, with a WTW CellOx 325 probe. Eighteen millilitres of each test concentration were dispensed into 20 mL glass scintillation vials, with four replicates being prepared for each test concentration and control. The test was initiated on 3 July 2008 at 1800h.

Five *C. dubia* neonates were randomly selected and introduced into each test vial using a Pasteur pipette. The test vials were then incubated in a constant temperature chamber at 25 ± 1 ºC. A 16:8 h light/dark cycle was provided. The condition of the test

animals was assessed at 24 h and again at 48 h, and the number of immobilised animals was recorded. Tests were terminated after 48 h of exposure.

Statistical analyses

The 48-h EC50 estimates (with 95% confidence limits) could not be determined given that there were no significant mortalities in any of the treatments tested. The concentration of Phoslock causing no significant toxicity (NOEC) and the lowest concentration of Phoslock causing significant toxicity (LOEC) were determined by performing an Analysis of Variance followed by Dunnetts test for parametric data, or Steels Many-One Rank test for non-parametric data. All analyses were performed using ToxCalc v5.0.23 (Tidepool Scientific Software).

Quality Assurance

A dilution water only control (negative control) was run at each testing period for quality assurance purposes. For the test to be considered valid, the % of immobilised *C. dubia* in the DMW control must be $\leq$10 %. In addition, to test the relative sensitivity of the test organisms and the proficiency of the Laboratory Technicians, a separate positive (toxic) control test was conducted using potassium chloride. This test was performed in the same manner and run concurrently with the test with granulated Phoslock. The results of this reference toxicant test were compared with the results from previous testing using a control (cusum) chart.

2.3 7-d reproductive Impairment Test with *Ceriodaphnia dubia*

The 7-d chronic (partial life-cycle) toxicity tests with the freshwater cladoceran *Ceriodaphnia dubia* was undertaken in accordance with the NATA endorsed ESA Standard Operating Procedure 102. This procedure was based on methods described by the USEPA (2002b) and adapted for use with the locally collected *C. dubia* (referred to as the Sydney Clone) by Bailey *et al.* (2000a). The conditions for the chronic test are summarised in **Table 2.2**.

Dilution Water

Dilute Mineral Water (DMW) is typically used as the diluent for the toxicity tests and as the culture medium for the culturing of the test organisms, however for this study the Fitzroy Falls Reservoir water was used as the diluent. However DMW was used for the culturing of the test organisms, as diluent for the positive control (concurrent reference toxicant test) and as an additional control treatment. DMW was prepared 24-48 h prior to use in accordance with the procedure described by the USEPA (2002a). DMW was prepared by diluting Perrier mineral water to a concentration of 20 % (vol/vol) with deionised water. A vitamin B12 and selenium supplement was added to the DMW to give final concentrations of 10 and 2 μg/L, respectively. The DMW was prepared in a 5 L Schott bottle and aerated using an aquarium aerator. The conductivity and pH of the water was checked prior to use.

Test Organisms

Neonates (<24 h) of *C. dubia* were obtained from mass cultures maintained at the ESA laboratory. All neonates were removed from the cladoceran mass cultures no more than 24 h prior to test initiation to ensure that only neonates less than 24 h old were used in the toxicity tests. The neonates to be used in the toxicity tests were isolated approximately 2 h prior to test initiation in a 250 mL beaker containing 200 mL of DMW.

Test Procedures

The test concentrations were 1, 0.75, 0.5 and 0.25 mg/L, and were prepared in 250 mL beakers by diluting with Fitzroy Falls Reservoir Water. After homogenising the test solutions, and following the addition live *Selenastrum* and freeze-dried *Dunalliella* feed, the conductivity, pH, and dissolved oxygen saturation of the test treatments were measured. The conductivity was measured using a WTW LF330 salinity/conductivity meter with a WTW Tetracon 325 probe. The pH and temperature were measured using a WTW pH330 meter, with a WTW SenTix 41 electrode. Dissolved oxygen was measured using a WTW Oxi 330 Oximeter, with a WTW CellOx 325 probe. Eighteen millilitres of each test concentration were dispensed into 20 mL glass scintillation vials, with ten replicates being prepared for each test concentration and control treatments. The test was initiated on 3 July 2008 at 1900h.

A single *C. dubia* neonate was randomly selected and introduced into each test vial using a Pasteur pipette. The test vials were incubated in a constant temperature chamber at 25 $\pm$ 1 ºC, with a 16:8 h light/dark cycle. At approximately 24-h intervals, the test solutions were renewed by holding the test organisms in a clean Pasteur pipette while the 24-h old test solution was poured off to labelled 250mL beakers (for subsequent physico-chemical analysis), and replaced with fresh solution. The test organism was then returned to the vial. This procedure was repeated daily. Fresh slurry, stock and test solutions were prepared on Day 0 (and used for renewals on Days 1, 2 and 3) and Day 4 (used for renewals on Days 4, 5 and 6). These test solutions were stored in 2L Schott bottles at 4^{o}C. Once the test organisms had started to reproduce, the number of neonates were counted and recorded prior to renewing the test solution. The test was terminated once the surviving control organisms reached an average of 15 young, which occurred on Day 7.

Sub-samples of each test treatment were taken on each day for the determination of lanthanum, phosphate and Hardness. The sub-samples taken on Days 2, 5 and 7 were of 'old solutions', ie the discard following the renewal process. The sub-samples taken on Days 0 and 6 were of 'fresh solutions', ie those that were used for that days renewals. Samples were collected in prepared test bottles supplied by Envirolab Services Pty Ltd. The bottles containing the sub-samples from each days renewals were held in at 4^{o}C in the dark until the test had terminated, and then shipped as a single batch by same-day express courier to Envirolab Services Pty Ltd. The results of the chemical determinations were provided directly to Phoslock Water Solutions Ltd, and are not reported herein.

Statistical Analyses

The 7-d EC50 estimates (with 95% confidence limits) for survival over the 7-d exposure could not be determined given that there were no significant mortalities in any of the treatments tested. The concentration of effluent causing no significant mortalities over the 7-day exposure period (NOEC 7-d survival) and the lowest concentration of effluent causing significant mortalities (LOEC 7-d survival) were determined by performing Fisher's Exact Test (1-tailed, P=0.05) for non-parametric data.

The 7-day IC50 estimate for reproduction was determined by Linear Interpolation (with 200 resamples) using the DMW dilution water as the control. The concentration of effluent causing no significant reduction in the production of young over the 7-day exposure period (NOEC) and the lowest concentration of effluent causing significant reduction in young production (LOEC) were determined by performing Steels Many-

One Rank Test (1-tailed, P=0.05) for non-parametric data. All analyses were performed using ToxCalc v5.0.23 (Tidepool Scientific Software).

Table 2.2 Summary of test conditions for the *Ceriodaphnia dubia* 7-d chronic toxicity test

Test Type	Static, 24-h renewal of test solutions
Test duration	7-d
Temperature	25 ± 1°C
Light quality	cool-white fluorescent tube lighting
Light intensity	800 ± 160 Lux
Photoperiod	16hr light : 8hr dark
Test Chamber size	20mL
Test solution volume	18mL
Age of test organisms	Less than 24 hrs old
No. of organisms per replicate	1
No. of replicates per treatment	10
No. of organisms per treatment	10
Feeding regime	Fed *Selenastrum* and freeze-dried *Dunalliella* while holding prior to test. Fed daily.
Dilution water	Fitzroy Falls Water (un-filtered) used as diluent. Dilute Mineral Water (DMW) was used as diluent for reference toxicant and for control treatment , prepared by mixing 20% Perrier mineral water with deionised water, with vitamin B12 and selenium supplements
Test concentrations	4 concentrations plus a DMW control and Fitzroy Falls Reservoir Water Control
Dilution series	1, 0.75, 0.5 and 0.25 mg/L
Endpoint	Immobilisation and number young produced
Test acceptability criterion	≥ 80% survival in controls. Minimum average of 15 young produced in surviving control animals. Reference toxicant IC estimate within Cusum chart control limits

Quality Assurance

A dilution water only control (negative control) was run for quality assurance purposes. For the 48-h acute and 7-d chronic tests to be considered valid, the % of immobilised *C. dubia* in the DMW control must be ≤10 and ≤20 %, respectively. In addition for the 7-d chronic assay, the average number of young from the surviving organisms in the DMW control treatment must be at least 15.

To test the relative sensitivity of the test organisms and the proficiency of the Laboratory Technicians, a separate positive (toxic) control test was conducted with the 7-d chronic assays using potassium chloride. This test was performed in the same manner and run concurrently with the effluent sample. The results of the reference

toxicant test were compared with the results from previous testing using control (cusum) charts.

3 Results and Discussion

3.1 48-h Acute Toxicity Test with *Ceriodaphnia dubia*

The percent survival of *C.dubia* in each of the test treatments is given in **Table 3.1.** Statistical print-outs for the toxicity test are provided in **Appendix A**. The 24- and 48-h EC50 (with 95% confidence limits) estimates of granular Phoslock to *C. dubia* survival was >50mg/L. No significant mortalities were observed at any of the treatments tested, and consequently the NOEC and LOEC estimates were 50 and >50 mg/L, respectively.

Table 3.1. The percent survival of *C. dubia* after 24- and 48-h exposure to granular Phoslock

Concentration (mg/L)	Percent survival at 24 h (Mean ± SD)	Percent survival at 48 h (Mean ± SD)
0 (Fitzroy Falls control)	100 ± 0.0	100 ± 0.0
DMW control	100 ± 0.0	100 ± 0.0
0.25	100 ± 0.0	100 ± 0.0
0.5	100 ± 0.0	100 ± 0.0
0.75	100 ± 0.0	100 ± 0.0
1	100 ± 0.0	100 ± 0.0
2	100 ± 0.0	100 ± 0.0
20	100 ± 0.0	100 ± 0.0
50	100 ± 0.0	95.0 ± 10.0

Quality Assurance

The toxicity test performed with the granular Phoslock sample met all quality assurance criteria. The percent survival in the control treatment was 100%, exceeding the minimum criteria of 90%. The physico-chemical parameters were also within the specified limits for the test. In addition, to test the relative sensitivity of the test organisms and the proficiency of the Laboratory Technicians, a separate positive (toxic) control test was conducted using potassium chloride. This test was performed in the same manner and run concurrently with the effluent sample. The 48-h EC50 for potassium chloride was 204.9 mg/L, which fell within the control chart control limits of 175.4 and 245.5mg/L. This indicated that all toxicity tests were within the expected range with respect to performance and sensitivity.

Table 3.2 The Quality Assurance limits for the 48-h *Ceriodaphnia dubia* acute toxicity test.

QA Measure	QA Limit	Observed value	Within Limits?
Control % survival	>90	100	Yes
Test temperature Limits	25±1°C	25.0-25.5°C	Yes
Reference toxicant within Cusum limits	175.4-245.5 mg KCl/L	204.9 mg KCl/L	Yes

3.2 7-d reproductive Impairment Test with *Ceriodaphnia dubia*

The results for the 7-d chronic (partial life-cycle) toxicity tests with the freshwater cladoceran *Ceriodaphnia dubia* are summarised in **Tables 3.3 to 3.4** below. Statistical print-outs for the toxicity test are provided in **Appendix B**.

The granular Phoslock sample did not exhibit toxicity to the 7-d chronic (partial life-cycle) toxicity tests with the freshwater cladoceran *Ceriodaphnia dubia,* with respect to either survival or reproduction (**Tables 3.3 and 3.4**). The 7-d EC50, NOEC and LOEC estimates for survival were >100, 100 and >100%, respectively. The 7-d IC50, NOEC and LOEC estimates for reproduction were >100, 100 and >100%, respectively.

Table 3.3. The percent survival and the number of young produced by *C. dubia* in the 7-d chronic toxicity test with the granular Phoslock.

Concentration (%)	Percent survival at 7-d	Number of Young (mean ± SD)
0 (Fitzroy Falls control)	100	15.8 ± 4.8
DMW control	100	15.3 ± 2.1
0.25	100	17.3 ± 4.6
0.5	100	14.1 ± 4.3
0.75	100	15.4 ± 4.5
1	100	16.0 ± 3.0

Table 3.5. The 7-d EC50 (survival), NOEC (survival) and LOEC (survival) for the granulated Phoslock sample with the *Ceriodaphnia dubia* 7-d chronic toxicity test

Sample	7-d EC50 (survival) (%)	NOEC (survival) (%)	LOEC (survival) (%)
Granulated Phoslock	>100	100	>100

* No significant reduction in survival at 7-d compared with the control treatment (Fisher's Exact Test, P=0.05, 1-tailed)

Table 3.6. The 7-d IC50 (reproduction), NOEC (reproduction) and LOEC (reproduction) for the granualted sample with the *Ceriodaphnia dubia* 7-d chronic toxicity test

Sample	7-d EC50 (reproduction) (%)	NOEC (reproduction) (%)	LOEC (reproduction) (%)
Granulated Phoslock	>100	100	>100

*No significant reduction in the production of young compared with the control treatment (Dunnett's Test, P=0.05, 1-tailed)

Quality Assurance

The 7-d chronic toxicity test performed with the granulated Phoslock sample met all quality assurance criteria. The percent survival in the control treatment was 100%, exceeding the minimum criteria of 80%. The number of young produced in DMW control treatment in the 7-d chronic test was 15.8, which exceeded the minimum criteria of 15. The physico-chemical parameters were also within the specified limits for the test. In addition, to test the relative sensitivity of the test organisms and the proficiency of the Laboratory Technicians, a separate positive (toxic) control test was conducted using potassium chloride. This test was performed in the same manner and run concurrently with the test with granular Phoslock. The 7-d IC50 estimate was 71.3 mg/L, which fell within the control limits of 69.2 and 338.9 mg/L.

Table 3.7 The Quality Assurance limits for the 7-d *Ceriodaphnia dubia* chronic toxicity test.

QA Measure	QA Limit	Observed value	Within Limits?
Control % survival	>90	100	Yes
Test temperature Limits	25±1°C	24.5-25.5.0°C	Yes
Reference toxicant within Cusum limits	69.2-338.9 mg KCl/L	71.3 mg KCl/L	Yes

4 References

Bailey, H.C., Krassoi, R., Elphick, J.R., Mulhall, A.M., Hunt, P., Tedmanson, L. and Lovell, A. (2000a) Application of *Ceriodaphnia dubia* for whole effluent toxicity tests in the Hawkesbury-Nepean watershed, New South Wales, Australia: Method development and application. Environmental Toxicology and Chemistry 19:88-93.

Bailey, H.C., Krassoi, R., Elphick, J.R., Mulhall, A.M., Hunt, P., Tedmanson, L. and Lovell, A. (2000b) Whole effluent toxicity of sewage treatment plants in the Hawkesbury-Nepean watershed, New South Wales, Australia, to *Ceriodaphnia dubia* and *Selenastrum capricornutum*. Environmental Toxicology and Chemistry 19:72-81.

USEPA (2002a) Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. Fifth Edition. United States Environmental Protection Agency, Office of Research and Development, Washington DC, EPA-821-R-02-102

USEPA (2002b) Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. Fourth Edition. United States Environmental Protection Agency, Office of Water, Washington DC, EPA-821-R-02-013.

Appendix A: Statistical analyses for *Ceriodaphnia dubia* acute toxicity test

Ceriodaphnia Survival and Reproduction Test-48 Hr Survival

Start Date:	3/07/2008 18:00	Test ID:	PR391/1	Sample ID:	Phoslock
End Date:	5/07/2008 18:00	Lab ID:	2763	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	101-ESA SOP101	Test Species:	CD-Ceriodaphnia dubia
Comments:					

Conc-mg/L	1	2	3	4
DMW-Control	1.0000	1.0000	1.0000	1.0000
Diln-Control	1.0000	1.0000	1.0000	1.0000
0.25	1.0000	1.0000	1.0000	1.0000
0.5	1.0000	1.0000	1.0000	1.0000
0.75	1.0000	1.0000	1.0000	1.0000
1	1.0000	1.0000	1.0000	1.0000
2	1.0000	1.0000	1.0000	1.0000
20	1.0000	1.0000	1.0000	1.0000
50	0.8000	1.0000	1.0000	1.0000

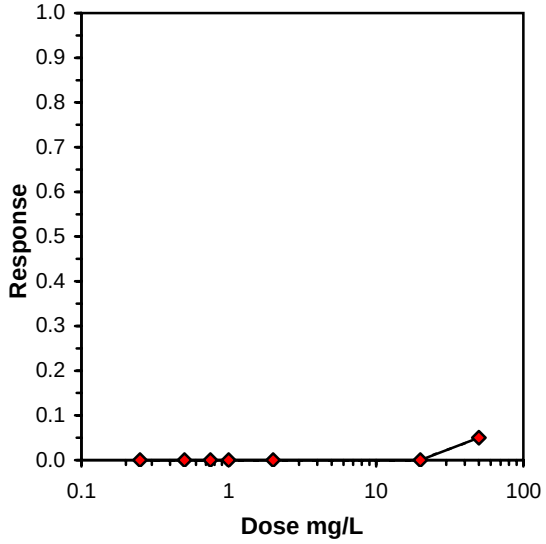
			Transform: Arcsin Square Root					1-Tailed			Isot
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Mean
DMW-Control	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4				
Diln-Control	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4				1.0000
0.25	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	0.000	2.480	0.0738	1.0000
0.5	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	0.000	2.480	0.0738	1.0000
0.75	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	0.000	2.480	0.0738	1.0000
1	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	0.000	2.480	0.0738	1.0000
2	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	0.000	2.480	0.0738	1.0000
20	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	0.000	2.480	0.0738	1.0000
50	0.9500	0.9500	1.2857	1.1071	1.3453	9.261	4	2.000	2.480	0.0738	0.9500

Auxiliary Tests	Statistic	Critical	Skew
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.01)	0.40745	0.904	-3.42885
Equality of variance cannot be confirmed			
The control means are not significantly different (p = 1.00)	0	2.446912	

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob
Dunnett's Test	50	>50			0.036957	0.038902	0.001772	0.001772	0.455318

Log-Logit Interpolation (200 Resamples)

Point	mg/L	SD	95% CL(Exp)	Skew
IC05	>50			
IC10	>50			
IC15	>50			
IC20	>50			
IC25	>50			
IC40	>50			
IC50	>50			



Ceriodaphnia Survival and Reproduction Test-48 Hr Survival

Start Date:	3/07/2008 18:00	Test ID:	PR391/1	Sample ID:	Phoslock
End Date:	5/07/2008 18:00	Lab ID:	2763	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	101-ESA SOP101	Test Species:	CD-Ceriodaphnia dubia
Comments:					

		Auxiliary Data Summary					
Conc-mg/L	**Parameter**	**Mean**	**Min**	**Max**	**SD**	**CV%**	**N**
DMW-Control	% survival	100.00	100.00	100.00	0.00	0.00	4
Diln-Control		100.00	100.00	100.00	0.00	0.00	4
0.25		100.00	100.00	100.00	0.00	0.00	4
0.5		100.00	100.00	100.00	0.00	0.00	4
0.75		100.00	100.00	100.00	0.00	0.00	4
1		100.00	100.00	100.00	0.00	0.00	4
2		100.00	100.00	100.00	0.00	0.00	4
20		100.00	100.00	100.00	0.00	0.00	4
50		95.00	80.00	100.00	10.00	3.33	4
DMW-Control	Temp C	25.50	25.50	25.50	0.00	0.00	1
Diln-Control		25.50	25.50	25.50	0.00	0.00	1
0.25		25.50	25.50	25.50	0.00	0.00	1
0.5		25.50	25.50	25.50	0.00	0.00	1
0.75		25.50	25.50	25.50	0.00	0.00	1
1		25.50	25.50	25.50	0.00	0.00	1
2		25.50	25.50	25.50	0.00	0.00	1
20		25.50	25.50	25.50	0.00	0.00	1
50		25.50	25.50	25.50	0.00	0.00	1
DMW-Control	pH	7.90	7.90	7.90	0.00	0.00	1
Diln-Control		7.70	7.70	7.70	0.00	0.00	1
0.25		7.70	7.70	7.70	0.00	0.00	1
0.5		7.70	7.70	7.70	0.00	0.00	1
0.75		7.60	7.60	7.60	0.00	0.00	1
1		7.60	7.60	7.60	0.00	0.00	1
2		7.60	7.60	7.60	0.00	0.00	1
20		7.50	7.50	7.50	0.00	0.00	1
50		7.50	7.50	7.50	0.00	0.00	1
DMW-Control	Cond uS/cm	211.00	211.00	211.00	0.00	0.00	1
Diln-Control		100.80	100.80	100.80	0.00	0.00	1
0.25		100.20	100.20	100.20	0.00	0.00	1
0.5		100.60	100.60	100.60	0.00	0.00	1
0.75		103.10	103.10	103.10	0.00	0.00	1
1		101.40	101.40	101.40	0.00	0.00	1
2		101.60	101.60	101.60	0.00	0.00	1
20		101.90	101.90	101.90	0.00	0.00	1
50		102.40	102.40	102.40	0.00	0.00	1
DMW-Control	DO %sat	104.00	104.00	104.00	0.00	0.00	1
Diln-Control		102.20	102.20	102.20	0.00	0.00	1
0.25		102.50	102.50	102.50	0.00	0.00	1
0.5		103.40	103.40	103.40	0.00	0.00	1
0.75		103.20	103.20	103.20	0.00	0.00	1
1		102.60	102.60	102.60	0.00	0.00	1
2		107.70	107.70	107.70	0.00	0.00	1
20		107.90	107.90	107.90	0.00	0.00	1
50		107.10	107.10	107.10	0.00	0.00	1

Appendix B: Statistical analyses for the 7-d *Ceriodaphnia dubia* reproductive impairment test

Ceriodaphnia Survival and Reproduction Test-7 Day Survival

Start Date:	3/07/2008 19:00	Test ID:	PR391/1	Sample ID:	Phoslock
End Date:	10/07/2008 19:00	Lab ID:	2763	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	102-ESA SOP102	Test Species:	CD-Ceriodaphnia dubia
Comments:					

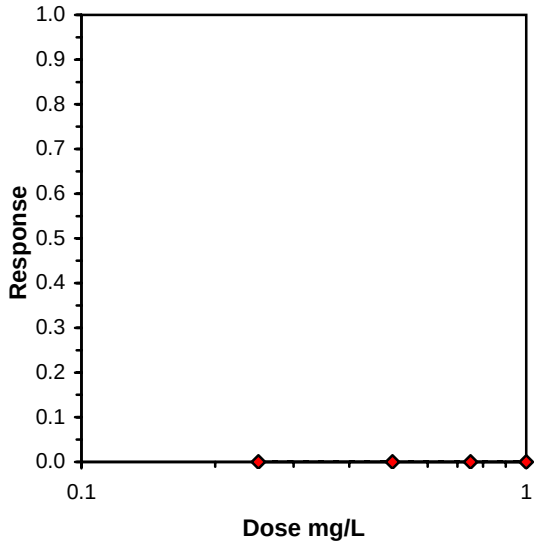
Conc-mg/L	1	2	3	4	5	6	7	8	9	10
DMW-Control	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Diln-Control	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
0.25	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
0.5	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
0.75	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
1	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000

Conc-mg/L	Mean	N-Mean	Resp	Not Resp	Total	N	Fisher's Exact P	1-Tailed Critical	Isot Mean
DMW-Control	1.0000	1.0000	0	10	10	10	0.6238		
Diln-Control	1.0000	1.0000	0	10	10	10			1.0000
0.25	1.0000	1.0000	0	10	10	10	1.0000	0.0500	1.0000
0.5	1.0000	1.0000	0	10	10	10	1.0000	0.0500	1.0000
0.75	1.0000	1.0000	0	10	10	10	1.0000	0.0500	1.0000
1	1.0000	1.0000	0	10	10	10	1.0000	0.0500	1.0000

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU
Fisher's Exact Test	1	>1		

Log-Logit Interpolation (200 Resamples)

Point	mg/L	SD	95% CL	Skew
IC05	>1			
IC10	>1			
IC15	>1			
IC20	>1			
IC25	>1			
IC40	>1			
IC50	>1			



Ceriodaphnia Survival and Reproduction Test-7 Day Survival

Start Date:	3/07/2008 19:00	Test ID:	PR391/1	Sample ID:	Phoslock
End Date:	10/07/2008 19:00	Lab ID:	2763	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	102-ESA SOP102	Test Species:	CD-Ceriodaphnia dubia
Comments:					

Auxiliary Data Summary

Conc-mg/L	Parameter	Mean	Min	Max	SD	CV%	N
DMW-Control	% survival	100.00	100.00	100.00	0.00	0.00	10
Diln-Control		100.00	100.00	100.00	0.00	0.00	10
0.25		100.00	100.00	100.00	0.00	0.00	10
0.5		100.00	100.00	100.00	0.00	0.00	10
0.75		100.00	100.00	100.00	0.00	0.00	10
1		100.00	100.00	100.00	0.00	0.00	10
DMW-Control	Temp C	25.50	25.50	25.50	0.00	0.00	1
Diln-Control		25.50	25.50	25.50	0.00	0.00	1
0.25		25.50	25.50	25.50	0.00	0.00	1
0.5		25.50	25.50	25.50	0.00	0.00	1
0.75		25.50	25.50	25.50	0.00	0.00	1
1		25.50	25.50	25.50	0.00	0.00	1
DMW-Control	pH	7.90	7.90	7.90	0.00	0.00	1
Diln-Control		7.70	7.70	7.70	0.00	0.00	1
0.25		7.70	7.70	7.70	0.00	0.00	1
0.5		7.70	7.70	7.70	0.00	0.00	1
0.75		7.60	7.60	7.60	0.00	0.00	1
1		7.60	7.60	7.60	0.00	0.00	1
DMW-Control	Cond uS/cm	211.00	211.00	211.00	0.00	0.00	1
Diln-Control		100.80	100.80	100.80	0.00	0.00	1
0.25		100.20	100.20	100.20	0.00	0.00	1
0.5		100.60	100.60	100.60	0.00	0.00	1
0.75		103.10	103.10	103.10	0.00	0.00	1
1		101.40	101.40	101.40	0.00	0.00	1
DMW-Control	DO %sat	104.00	104.00	104.00	0.00	0.00	1
Diln-Control		102.20	102.20	102.20	0.00	0.00	1
0.25		102.50	102.50	102.50	0.00	0.00	1
0.5		103.40	103.40	103.40	0.00	0.00	1
0.75		103.20	103.20	103.20	0.00	0.00	1
1		102.60	102.60	102.60	0.00	0.00	1

Ceriodaphnia Survival and Reproduction Test-Reproduction

Start Date:	3/07/2008 19:00	Test ID:	PR391/1	Sample ID:	Phoslock
End Date:	10/07/2008 19:00	Lab ID:	2763	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	102-ESA SOP102	Test Species:	CD-Ceriodaphnia dubia
Comments:					

Conc-mg/L	1	2	3	4	5	6	7	8	9	10
DMW-Control	15.000	16.000	10.000	16.000	14.000	18.000	16.000	16.000	16.000	16.000
Diln-Control	12.000	16.000	16.000	14.000	20.000	8.000	22.000	20.000	10.000	20.000
0.25	18.000	22.000	10.000	19.000	20.000	20.000	8.000	18.000	19.000	19.000
0.5	20.000	13.000	19.000	11.000	8.000	10.000	17.000	12.000	19.000	12.000
0.75	16.000	18.000	19.000	16.000	10.000	11.000	22.000	19.000	15.000	8.000
1	17.000	17.000	16.000	17.000	16.000	16.000	16.000	19.000	18.000	8.000

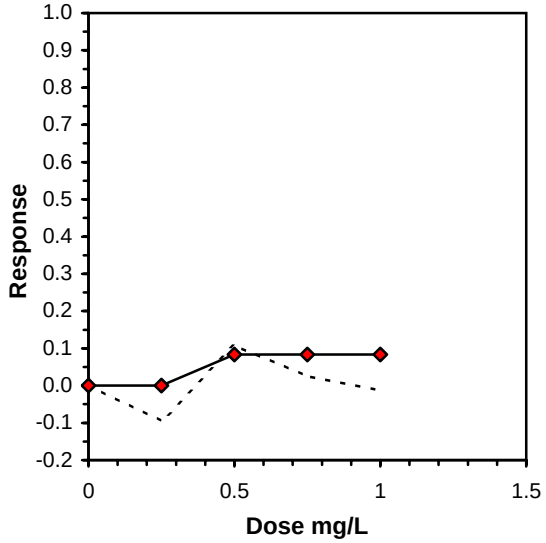
			Transform: Untransformed					1-Tailed			Isot
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Mean
DMW-Control	15.300	0.9684	15.300	10.000	18.000	13.796	10				
Diln-Control	15.800	1.0000	15.800	8.000	22.000	30.103	10				16.550
0.25	17.300	1.0949	17.300	8.000	22.000	26.285	10	-0.788	2.223	4.231	16.550
0.5	14.100	0.8924	14.100	8.000	20.000	30.358	10	0.893	2.223	4.231	15.167
0.75	15.400	0.9747	15.400	8.000	22.000	29.072	10	0.210	2.223	4.231	15.167
1	16.000	1.0127	16.000	8.000	19.000	18.634	10	-0.105	2.223	4.231	15.167

Auxiliary Tests	Statistic	Critical	Skew
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)	0.937956	0.93	-0.59799
Bartlett's Test indicates equal variances (p = 0.72)	2.100546	13.2767	
The control means are not significantly different (p = 0.76)	0.303853	2.100922	

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob
Dunnett's Test	1	>1			4.231478	0.267815	13.27	18.11111	0.574461

Linear Interpolation (200 Resamples)

Point	mg/L	SD	95% CL	Skew
IC05	0.3995			
IC10	>1			
IC15	>1			
IC20	>1			
IC25	>1			
IC40	>1			
IC50	>1			



Ceriodaphnia Survival and Reproduction Test-Reproduction

Start Date:	3/07/2008 19:00	Test ID:	PR391/1	Sample ID:	Phoslock
End Date:	10/07/2008 19:00	Lab ID:	2763	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	102-ESA SOP102	Test Species:	CD-Ceriodaphnia dubia
Comments:					

Auxiliary Data Summary

Conc-mg/L	Parameter	Mean	Min	Max	SD	CV%	N
DMW-Control	# young	15.30	10.00	18.00	2.11	9.50	10
Diln-Control		15.80	8.00	22.00	4.76	13.80	10
0.25		17.30	8.00	22.00	4.55	12.33	10
0.5		14.10	8.00	20.00	4.28	14.67	10
0.75		15.40	8.00	22.00	4.48	13.74	10
1		16.00	8.00	19.00	2.98	10.79	10
DMW-Control	Temp C	25.50	25.50	25.50	0.00	0.00	1
Diln-Control		25.50	25.50	25.50	0.00	0.00	1
0.25		25.50	25.50	25.50	0.00	0.00	1
0.5		25.50	25.50	25.50	0.00	0.00	1
0.75		25.50	25.50	25.50	0.00	0.00	1
1		25.50	25.50	25.50	0.00	0.00	1
DMW-Control	pH	7.90	7.90	7.90	0.00	0.00	1
Diln-Control		7.70	7.70	7.70	0.00	0.00	1
0.25		7.70	7.70	7.70	0.00	0.00	1
0.5		7.70	7.70	7.70	0.00	0.00	1
0.75		7.60	7.60	7.60	0.00	0.00	1
1		7.60	7.60	7.60	0.00	0.00	1
DMW-Control	Cond uS/cm	211.00	211.00	211.00	0.00	0.00	1
Diln-Control		100.80	100.80	100.80	0.00	0.00	1
0.25		100.20	100.20	100.20	0.00	0.00	1
0.5		100.60	100.60	100.60	0.00	0.00	1
0.75		103.10	103.10	103.10	0.00	0.00	1
1		101.40	101.40	101.40	0.00	0.00	1
DMW-Control	DO %sat	104.00	104.00	104.00	0.00	0.00	1
Diln-Control		102.20	102.20	102.20	0.00	0.00	1
0.25		102.50	102.50	102.50	0.00	0.00	1
0.5		103.40	103.40	103.40	0.00	0.00	1
0.75		103.20	103.20	103.20	0.00	0.00	1
1		102.60	102.60	102.60	0.00	0.00	1

Appendix C: Test guidance document prepared by Phoslock Water Solutions Ltd.

Phoslock toxicity tests on the water flea (*Ceriodaphnia dubia*) using Fitzroy Falls Reservoir water

Objectives

- To investigate the effects of a Phoslock application on *Ceriodaphnia dubia* using Fitzroy Falls Reservoir water

Proposed Phoslock dose to Fitzroy Falls

- 0.72 ppm

Toxicity tests

1. **Acute tests**
    a. 48 h
2. **Chronic tests**
    a. ~7 d

Conducted by

- Ecotox Services Australia
    - A NATA endorsed laboratory
    - Rigorous QA/QC components are incorporated into routine test procedures

Test organism

- Water flea (*Ceriodaphnia dubia*)

Water sample collection

- 20 L of water will be collected from Fitzroy Falls Reservoir on Tuesday 1st July 2008 and delivered to Ecotox Services Australia labs during that afternoon
- During collection of the FF water, the sample containers will be tripled rinsed with FF water before completely filling each container
- During transportation from FF Reservoir to the Ecotox Services Australia labs the sample containers will be placed in an esky containing ice to keep the water chilled
- Ecotox Australia will use this water for all ecotox work (including the preparation of the Phoslock stock solutions and test dilution series)

Phoslock sample

- A subsample of the batch of Phoslock that will be applied to FF will also be dropped off at the Ecotox Services Australia labs with the FF Reservoir water on the afternoon of Tuesday 1st July, 2008
- A copy of the current MSDS, Certificate of Analysis and the batch number of Phoslock sample to be used for the test will be supplied to Ecotox Services Australia

Sample Preparation

Phoslock Concentrations (ppm) to be used in the Ecotox test for the DECC application: 0, 0.25, 0.5, 0.75, 1, 2, 20, 50

Step 1: Preparation of slurry

- Add 100 g Phoslock granules to a volumetric flask and add Fitzroy Falls water to make up to one litre (i.e. 100 g/L concentration)
- Mix for 10 minutes using a magnetic stirrer on a slow stir (to make sure all Phoslock granules are dissolved)
- Phoslock concentration: 100 g/L or 1 g/10 ml or 0.5 g/5 ml

Step 2: Preparation of stock solution

- Add 5 ml (i.e. 0.5 g Phoslock) slurry to 995 ml Fitzroy Falls water
- Mix for 5 minutes using a magnetic stirrer on a slow stir
- Phoslock concentration: 500 mg/L or 1 mg/2 ml or 0.5 mg/1 ml

Step 3:

- **Preparation of 0.25 ppm solution**
  - Add 0.5 ml stock solution to 999.5 ml Fitzroy Falls water
  - Homogenise sample by inversion
  - Transfer 20 ml to each experimental vial

- **Preparation of 0.50 ppm solution**
  - Add 1 ml stock solution to 999 ml Fitzroy Falls water
  - Homogenise sample by inversion
  - Transfer 20 ml to each experimental vial

- **Preparation of 0.75 ppm solution**
  - Add 1.5 ml stock solution to 998.5 ml Fitzroy Falls water
  - Homogenise sample by inversion
  - Transfer 20 ml to each experimental vial

- **Preparation of 1 ppm solution**
  - Add 2 ml stock solution to 998 ml Fitzroy Falls water
  - Homogenise sample by inversion
  - Transfer 20 ml to each experimental vial

- **Preparation of 2 ppm solution**
  - Add 4 ml stock solution to 996 ml Fitzroy Falls water
  - Homogenise sample by inversion
  - Transfer 20 ml to each experimental vial

- **Preparation of 20 ppm solution**
  - Add 40 ml stock solution to 960 ml Fitzroy Falls water
  - Homogenise sample by inversion
  - Transfer 20 ml to each experimental vials

- **Preparation of 50 ppm solution**
  - Add 100 ml stock solution to 900 ml Fitzroy Falls water
  - Homogenise sample by inversion
  - Transfer 20 ml to each experimental vials

Methods

Acute test

- Phoslock concentrations (ppm)- 0, 0.25, 0.5, 0.75, 1, 2, 20, 50
- Juvenile *Ceriodaphnia dubia* (<24-h neonates)
- 20 mL test chambers
- Four replicates for each treatment
- Static, non-renewal system used
- 48-h exposure period
- No feeding during the test
- Monitor pH, temperature and dissolved oxygen for each test chambers for every 24 h
- After 48 h, the number of surviving *Ceriodaphnia* are counted
- Statistical analysis are then applied to the test data to determine for example, the concentration of the test material causing 50% mortalities in the test population
- Ecotox Services Australia will take sub-samples at 48-h for the analysis of dissolved La, Hardness and filterable reactive phosphorus (and a record of temp, DO, pH should be included).
- Analyses of dissolved La, hardness and FRP will be carried out by Envirolab Services, who will supply sample bottles and relevant

instructions. As test replicates are 20-mL volumes, all 4 replicates will be pooled at the end of the test to yield 80mL of solution for analyses.

- Samples should not be filtered at any stage. Unfiltered samples should be sent to Envirolab in acid washed bottles (supplied by Envirolab) with a Chain of Custody form (complete with clearly labelled bottles and sample numbers).

Chronic test

- Phoslock concentrations (ppm)- 0, 0.25, 0.5, 0.75, 1
- Static, 24 h renewal system used
- Juvenile *Ceriodaphnia dubia*
- 20 mL test chamber
- Ten replicates for each treatment
- Test run until controls have 3-broods, and an average of 15 young (about 7 or 8 days).
- Test organisms fed daily with renewals of solutions
- Monitor pH, temperature and dissolved oxygen for each test chambers for every 24 h
- Test duration – about 7 d at constant temperature
- Test will continue until 80% of control organisms produce 3rd brood of young (about 7 d)
- After each 24 h, the number of surviving *Ceriodaphnia* and offspring are counted
- Statistical analysis are then applied to the test data to determine for example, the concentration of the test material causing 50% mortalities in the test population
- Ecotox Services Australia will collect sub-samples for **dissolved La, hardness and FRP from each of the renewal waters (before renewal) every day until the end of the experiment**.
- Ecotox Services Australia will pool replicate water samples after each renewal period on days 2,5 and 7, and sun-sample for **dissolved La, hardness and filterable reactive phosphorus**.
- Samples should not be filtered at any stage. Unfiltered samples should be sent to Envirolab in acid washed bottles (supplied by Envirolab) with a Chain of Custody form (complete with clearly labelled bottles and sample numbers).

Appendix D: Certificate of Analysis

PHOSLOCK®

Phoslock Water Solutions Ltd.
ABN 88 099 555 290

Certificate of Analysis for Phoslock®

Date of Issue: 13th September, 2007

Quantity Shipped: 294 metric tonnes

Client Name: SCA

Sampling Method: A composite sample from each batch taken during production.

Test Methods: Testing of Filterable Reactive Phosphorus (FRP) uptake and Moisture Content conducted as per PWS Standard PWS/FH-FRP05 and PWS/MC05.

Batch Number	FRP Uptake	Moisture Content	Total Batch Size (tonnes)
180407	92.3	3.0	7.35
190407	93.0	5.8	7.35
200406	94.0	6.0	8.4
210407	96.0	6.0	12.6
220407	91.0	6.9	15.75
230407	94.0	7.0	13.65
240407	93.0	6.4	13.65
250407	96.0	5.2	14.7
260407	89.0	6.5	15.75
270407	95.0	5.8	14.7
280407	93.0	6.2	14.7
290407	92.0	6.3	13.65
300407	94.0	8.2	12.6
10507	94.0	6.5	11.55
20507	90.0	6.8	9.45
30507	91.0	4.9	10.5
50507	93.0	5.5	13.3
60507	96.0	5.7	15.75
70507	91.0	5.1	12.6
80507	92.0	5.5	10.5
90507	91.0	7.0	10.5
100507	94.0	4.0	12.6
110507	93.0	4.5	14.7
120507	95.0	6.0	5.25
120507A	93.0	7.0	2.1
130507A	93.0	4.0	11.55
140507A	93.0	5.0	6.3

Laboratory Analyst: Mr Li

Signature: Li Shao

Authorised By: Terry Vulles

Signature: T Vulles

Position: China Operations Manager