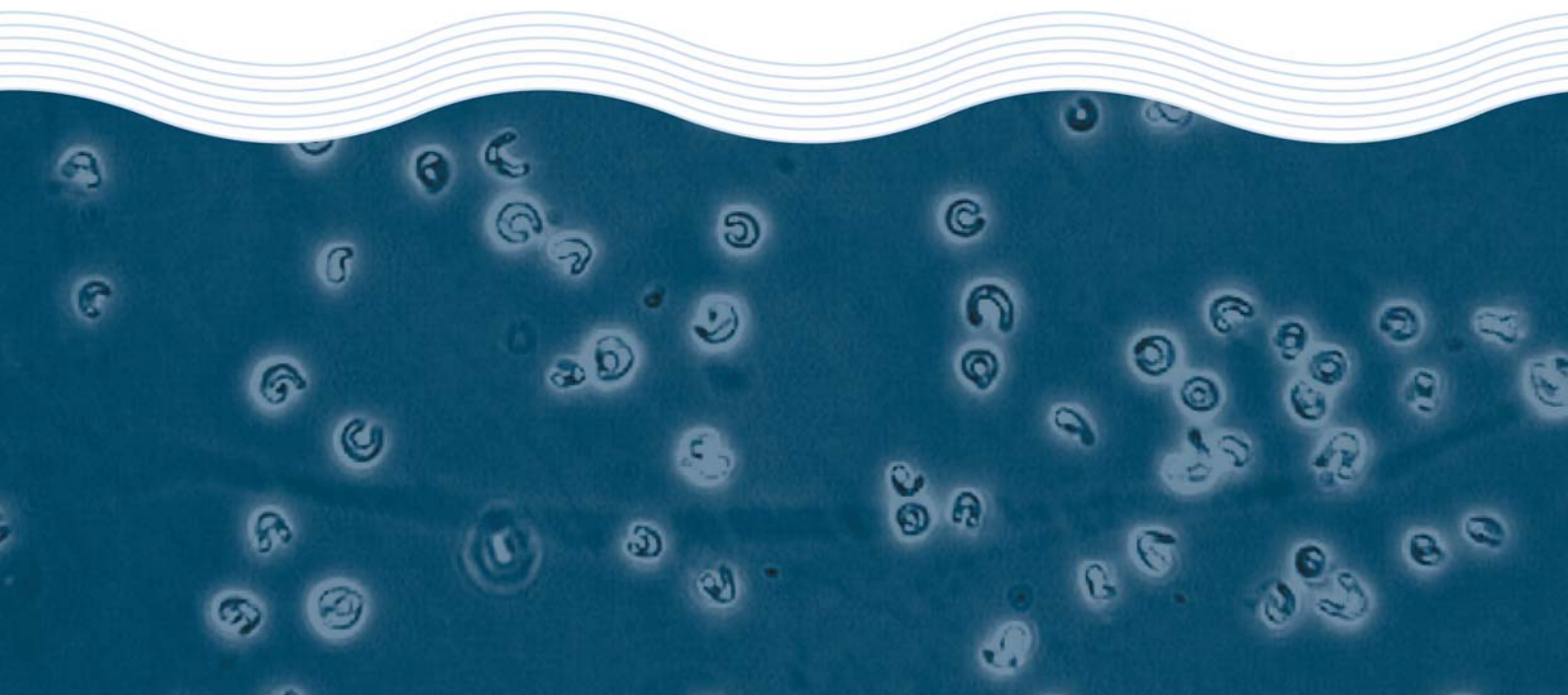


**Toxicity Assessment of Pulp Mill  
Effluent for the Proposed  
Tasmanian Pulp Mill- Brazil Pulp  
Mill Sample**

**Gunns Limited**

**Test Report**

**December 2009**



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# 1. Executive Summary

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## 1.1 Executive Summary

A Direct Toxicity Assessment (DTA) programme was implemented by Gunns Limited to assess the ecotoxicity of an effluent generated by a Brazilian pulp mill, which is similar to the proposed Bell Bay Pulp Mill under development by Gunns Limited. This round of sampling and testing was undertaken to address Condition 34 of the Commonwealth of Australia Decision to Approve the Taking of an Action-Gunns Pulp Mill (EPBC2007/3385). In summary, Condition 34 addresses temporal variability in effluent toxicity with parallel determination of effluent chemical characteristics. The sample was collected on the 17 November 2009, organised by Gunns Limited, and received by ESA on 19 November 2009. The toxicity tests undertaken were initiated on the day of arrival at ESA. Repeat testing for the micro-algal growth inhibition test and Microtox<sup>®</sup> test was necessary and were initiated within 33 h of arrival. The sample was tested for toxicity using the following bioassays:

- ❑ 1-hr sea urchin fertilisation success using *Heliocidaris tuberculata*
- ❑ 72-hr sea urchin larval development using *Heliocidaris tuberculata*
- ❑ 72-hr macroalgal germination assay using *Hormosira banksii*
- ❑ 48-hr scallop larval development using the *Mimachlamys asperrima*
- ❑ 96-hr acute amphipod toxicity tests using *Allorchestes compressa*
- ❑ 96-hr fish imbalance test using the pink snapper *Pagrus auratus*
- ❑ 72-hr microalgal growth inhibition test using *Nitzschia closterium*
- ❑ 72-hr macro-algal germination assay using *Ecklonia radiata*
- ❑ 48-hr Acute *Ceriodaphnia dubia* immobilisation test
- ❑ Acute Microtox test using the marine bacterium *Vibrio fischeri*

Test data are summarised in **Table 1.1**. The concentration of ammonia in the Brazil pulp mill effluent sample was less than 2.0mg/L. Therefore, no assessments of the relative contribution of ammonia to observed toxicity have been made. The bioassays were performed at the ESA laboratory in Lane Cove. This report describes the results of each of the toxicity tests performed. Statistical printouts for each test are given in **Appendices A to J**. Chain-of-custody documentation is given in **Appendix K**.

Test results indicated the following:

- ❑ **Sea Urchin Fertilisation Success:** The Brazil pulp mill effluent sample did not exhibit toxicity to the sea urchin fertilisation success test end-point. There was no significant difference in fertilisation success in the sample, compared with the artificial seawater control. The NOEC and LOEC for the pulp mill effluent sample was therefore, 100% and >100%, respectively. The 1-hr EC10 and EC50 for the sample were >100%.
- ❑ **Sea Urchin Larval Development:** There was a significant decrease in normal development of larvae to the pluteus stage of urchin larvae exposed to the Brazil pulp mill effluent sample, compared with the artificial seawater control treatment. Decreased normal larval development was only apparent in the 100% (un-diluted) sample (resulting in 43.0 ± 7.8% normal larvae). The NOEC

and LOEC was therefore, 50% and 100%, respectively. The 1-hr EC10 and EC50 values for the sample were 62.3 (53.2-68.7)% and 96.6 (92.3-101.1)%, respectively.

- ***Hormosira Macro-algal Germination Success:*** There was no significant difference in germination success of *Hormosira banksii* exposed to the Brazil pulp mill effluent sample, compared with the artificial seawater control treatment. The NOEC and LOEC for the pulp mill effluent sample was therefore, 100% and >100%, respectively. The 72-hr EC10 and EC50 values for the sample were 89.7% and >100%, respectively.
- ***Scallop Larval Development:*** Some toxicity was observed in the Brazil pulp mill effluent sample using the scallop larval development test end-point. There was a significant decrease in normal development of larvae to the D-veliger stage, compared with the artificial seawater control treatment. Decreased normal larval development was only apparent in the 100% (un-diluted) sample (resulting in 57.8 ± 10.1% normal larvae). The NOEC and LOEC for the pulp mill effluent sample was therefore, 50% and 100%, respectively. The 48-hr EC10 and EC50 values for the sample were 69.0 (32.2-81.7)% and >100%, respectively.
- ***Amphipod Acute Survival:*** The Brazil pulp mill effluent sample exhibited some reduced amphipod survival in the undiluted sample, however this was not statistically significant. The NOEC and LOEC for the pulp mill effluent sample was 100% and >100%, respectively. The 96-hr IC10 and EC50 values for the sample were 65.3% and >100%, respectively.
- ***Fish Imbalance:*** The Brazil pulp mill effluent sample did not exhibit toxicity using the larval fish imbalance end-point. The NOEC and LOEC for the pulp mill effluent sample was therefore, 100% and >100%, respectively. The 96-hr EC10 and EC50 values for the sample were >100%.
- ***Micro-algal Growth Inhibition:*** The un-diluted Brazil pulp mill effluent sample exhibited a similar cell yield of *N. closterium* compared with the filtered seawater control treatment, despite an effect observed in the artificial seawater control treatment. This indicated that the un-diluted Brazil pulp mill effluent sample did not exhibit toxicity. There was a statistically significant reduction in cell yield in the 1.6% and 3.1% concentrations, however, no such reduction was observed in the 6.3% concentration and above. Subsequently, NOEC and LOEC values were 100% and >100%, respectively. The 72-hr IC10 and IC50 values were >100%.
- ***Microtox:*** The Brazil pulp mill effluent sample exhibited some toxicity to the Microtox<sup>®</sup> acute toxicity test. A slight but significant reduction to the luminescence of the bacteria *V. fischeri* was measured in the 45% and 90% concentrations at both 5- and 15-minutes exposure. The EC50, NOEC and LOEC values at 5 and 15 minutes were >90%, 22.5% and 45%, respectively.
- ***Ecklonia Macro-algal Germination Success:*** No toxicity was observed in the Brazil pulp mill effluent sample using the macro-algal germination end-point. There was no significant difference in germination success in the sample, compared with the artificial seawater control treatment. The NOEC and LOEC for the pulp mill effluent sample was therefore, 100% and >100%, respectively. The 72-hr EC10 and EC50 values for the sample were >100%.
- ***Ceriodaphnia Acute Immobilisation:*** The pulp mill effluent sample did not exhibit toxicity to the 48-hr *Ceriodaphnia dubia* acute toxicity test endpoint. The 48-hr EC10, EC50, NOEC and LOEC estimates were >100%, >100%, 100% and >100%, respectively.

**Table 1.1. Summary of toxicity test data for the Brazil pulp mill effluent sample.**

[Note that the EC/ IC 10 and 50 values provide an estimate of the sample concentration where 10% and 50% of test organisms, respectively, are affected. If the sample exhibited little or no toxicity, the estimates will be >100%] Refer to Glossary of Terms in the Section for an explanation of terms used.

Toxicity Test	Endpoint	Value (%)
		Brazil
1-hr sea urchin fertilisation success test	EC10	>100
	EC10	>100
	NOEC	100
	LOEC	>100
72-hr sea urchin larval development test	EC10	62.3 (53.2-68.7)
	EC50	96.6 (92.3-101.1)
	NOEC	50
	LOEC	100
72-hr <i>Hormosira banksii</i> macro-algal germination success test	EC10	89.7*
	EC50	>100
	NOEC	100
	LOEC	>100
48-hr scallop larval development test	EC10	69.0 (32.2-81.7)
	EC50	>100
	NOEC	50
	LOEC	100
96-hr acute amphipod survival test	IC10	65.3*
	EC50	>100
	NOEC	100
	LOEC	>100
96-hr fish imbalance test	EC10	>100
	EC50	>100
	NOEC	100
	LOEC	>100
72-hr micro-algal growth inhibition test	IC10	>100
	IC50	>100
	NOEC	100
	LOEC	>100
Acute Microtox test **	EC50	>90
	NOEC	22.5
	LOEC	45
72-hr <i>Ecklonia radiata</i> macro-algal germination success test	EC10	>100
	EC50	>100

	NOEC	100
	LOEC	>100
-----		
48-hr Acute <i>Ceiodaphnia dubia</i> test	EC10	>100
	EC50	>100
	NOEC	100
	LOEC	>100
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\* 95% confidence limits are not available

\*\* 15-min Microtox® toxicity data is presented, and is the same for 5-minute Microtox® exposure data

## 1.2 Glossary of Terms

The following glossary is based on that provided by Environment Canada (1999)

**Acute toxicity** is an adverse effect (lethal or sub-lethal) induced in the test organisms within a short period of exposure to a test material, usually a few days.

**Bioassay** is a test (=assay) in which the strength or potency of a substance is measured by the response of living organisms or living system. **Toxicity test** is a more specific and preferred term for environmental work.

**Chronic** toxicity implies long-term effects that are related to changes in metabolism, growth, reproduction, or ability to survive

**Control** is a treatment in an investigation that duplicates all the factors that might affect results, except the specific condition being studied. In toxicity tests, the control must duplicate all the conditions in the exposure treatment(s) but must contain no test material. The control is used as a check for toxicity due to basic conditions such as quality of dilution water or health and handling of the test organisms. Control is synonymous with **negative control**. See also **positive control**.

**EC50** is the median effective concentration. That is the concentration of material in water (e.g. mg/L) that is estimated to cause some defined toxic effect to 50% of the test organisms. In most instances the EC50 and its 95% confidence limits are statistically derived by analysing the percentages of organisms affected at various test concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g. 48h).

**Endpoint** means the measurement(s) or value(s) that characterise the results of a test (LC50, EC50, IC50). It also means the reaction of the organism to show the effect which is intended to mark completion of the test (eg. death, number of shell abnormalities).

**ICp** is the inhibiting concentration for a specified percent effect (e.g. IC50). It represents a point estimate of a concentration of test material that causes a designated percent inhibition ( $p$ ) compared to the control, in a quantitative biological measurement such as microalgal cell yield attained at the end of a test.

**LC50** is the median effective concentration. That is the concentration of material in water (e.g. mg/L) that is estimated to be lethal to 50% of the test organisms. In most instances the EC50 and its 95% confidence limits are statistically derived by analysing the percentage mortality at various test concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g. 96-hr).

**LOEC** is the lowest-observed-effect concentration. This represents the lowest concentration of a test material for which a statistically significant effect on the test organisms was observed, relative to the control.

**NOEC** is the no-observed-effect concentration. This represents the highest test concentration of a test material for which no statistically significant effect on the test organisms was observed, relative to the control.

**Positive Control** is a toxicity test with a reference toxicant, used to assess the sensitivity of the organisms at the time of the test material is evaluated and the precision of the results obtained by the laboratory for that chemical.

**Reference toxicant** is a standard chemical used to measure the sensitivity of the test organisms to establish confidence in the toxicity data obtained for a test material. In most instances, a toxicity test with a reference toxicant is performed to assess the sensitivity of the organisms at the time the test material is evaluated and the precision of the results obtained by the laboratory for that chemical.

**Replicate** is a single test chamber containing a prescribed number of test organisms in either one concentration of test solution or in dilution water as a control. In a toxicity test comprising five test concentrations and a control, and using four replicates, 18 test chambers would be used. For each concentration or control, there would be 4 test chambers or replicates. A replicate must be an independent unit, and therefore, any transfer of test material or organisms from one replicate to another would invalidate a statistical analysis based on replication.

**Static** describes toxicity tests in which test solutions are not renewed during the test.

**Sub-lethal** means detrimental to the organism, but below the level that directly causes death within the test period.

**Toxic** means poisonous. A toxic material can cause adverse effects on living organisms, if present in sufficient amount at the right location.

**Toxicant** is a toxic material.

## 2. Introduction

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A Direct Toxicity Assessment (DTA) programme was implemented by Gunns Limited to assess the ecotoxicity of an effluent generated by a Brazil pulp mill, which is similar to the proposed Bell Bay Pulp Mill under development by Gunns Limited. This round of sampling and testing was undertaken to address Condition 34 of the Commonwealth of Australia Decision to Approve the Taking of an Action-Gunns Pulp Mill (EPBC2007/3385). In summary, Condition 34 addresses temporal variability in effluent toxicity with parallel determination of effluent chemical characteristics. The sample was collected on the 17 November 2009, organised by Gunns Limited, and received by ESA on 19 November 2009. The toxicity tests undertaken were initiated on the day of arrival at ESA. Repeat testing for the micro-algal growth inhibition test and Microtox<sup>®</sup> test were initiated within 33 h of arrival.

The sample was collected in five 2L glass schott bottles, prepared and provided by Gunns Limited. The bottles were received by ESA in an esky with ice bricks and a temperature logger at a temperature of 7.5°C. A completed Chain-of-Custody form accompanied the samples, and is provided in **Appendix K**. The sample collection details and characteristics of the sample on arrival at ESA are given in **Tables 2.1** and **2.2** below.

**Table 2.1 Sample collection details**

Sample ID	Date of collection	Collection notes	Date & time of arrival at ESA
Brazil	17/11/09	Grab sample	19/11/2009 at 0915h

**Table 2.2 Sample Characteristics on arrival at ESA\***

Sample ID	pH	Conductivity (µS/cm)	Dissolved oxygen (% saturation)	Total Ammonia (mg N/L)
Brazil	8.5	2950	98.3	<2.0

\* determined prior to the raising of sample salinity

Given that the salinity of the sample was not sufficient to sustain marine organisms (this excludes the test with the freshwater cladoceran *Ceriodaphnia dubia*), the salinity of the sample was raised to approximately 35‰ using GP-2 artificial sea salts (USEPA 1993) prior to the preparation of test dilutions for toxicity testing. The samples were tested for toxicity using the following bioassays:

- ❑ 1-hr sea urchin fertilisation success using *Heliocidaris tuberculata*
- ❑ 72-hr sea urchin larval development using *Heliocidaris tuberculata*
- ❑ 72-hr macroalgal germination assay using *Hormosira banksii*
- ❑ 48-hr scallop larval development using the *Mimachlamys asperrima*
- ❑ 96-hr acute amphipod toxicity test using *Allorchestes compressa*
- ❑ 96-hr fish imbalance test using the pink snapper *Pagrus auratus*

- 72-hr microalgal growth inhibition test using *Nitzschia closterium*
- 72-hr macro-algal germination assay using *Ecklonia radiata*
- 48-hr Acute *Ceriodaphnia dubia* immobilisation test
- Acute Microtox test using the marine bacterium *Vibrio fischeri*

The bioassays were performed at the ESA laboratory in Lane Cove, Sydney. This report describes the results of each of the toxicity tests performed. Statistical printouts for each test are given in **Appendices A to J**. All toxicity tests were initiated on the 19 November 2009, with repeat testing undertaken on the 20 November 2009 for the 72-hr micro-algal growth inhibition test and Microtox<sup>®</sup> acute test.

### 3. 1-hr Sea Urchin Fertilisation Success Test

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#### 3.1 Summary of Test Methodology

The sea urchin fertilisation success test using the gametes of *Heliocidaris tuberculata* was undertaken in accordance with ESA Standard Operating Procedure 104, which is based on methods described by USEPA (2002a) and adapted for use with *H. tuberculata* by Simon and Laginestra (1997) for the National Pulp Mills Research Program. The test was performed in a constant temperature chamber of  $20\pm 1^\circ\text{C}$ . Clean seawater was collected from the Sydney region and filtered to  $0.45\mu\text{m}$  on return to the laboratory. Sea urchins used for the test were obtained by field collection in Maroubra, NSW and spawned within 6 h of collection. The conditions of the test are summarised in **Table 3.1**.

The test was undertaken in 9mL borosilicate glass tissue culture vials. Seven concentrations of the Brazil pulp mill effluent sample were prepared and tested using 4 replicate vials. The test concentrations were 100, 50, 25, 12.5, and 6.3, 3.1 and 1.6%. A filtered seawater (FSW) control, consisting of seawater collected from the Sydney region and an artificial Seawater (ASW) control were tested concurrently with the sample.

The pH, salinity and dissolved oxygen concentration of a representative sample from each concentration was measured. Salinity was measured using a WTW LF330 salinity/conductivity meter with a WTW Tetracon 325 probe. The pH was 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.

Gametes were obtained by injecting the sea urchins with a KCl solution, stimulating the urchins to spawn almost immediately. Viable gametes were selected on the basis of fertilisation success trials and visual examination of gamete maturity. Spermatozoa were exposed to each of the test treatments for 1 h, following which 2000 eggs were introduced to each test vessel. Eggs were then left in the test vessels for a period of 20 minutes before the addition of buffered formalin. The test was performed in a constant temperature chamber set at  $20\pm 1^\circ\text{C}$  for the duration of the test exposure period.

One milliliter of test solution was drawn directly from the bottom of each test vessel and placed in a Sedgwick-Rafter counting chamber. The first 100 eggs were examined and the number of fertilised and un-fertilised eggs was recorded. The concentration resulting in the inhibition of fertilisation to 10% and 50% of the test population (1-hr EC10 and EC50) was determined by Non-linear Interpolation method using TOXCALC V5.0 software. The concentration of the effluent causing no significant toxicity (No Observed Effect Concentration, NOEC) and the lowest concentration of the effluent causing significant toxicity (Lowest Observed Effect Concentration, LOEC) were determined by performing Dunnett's test.

**Table 3.1. Summary of test conditions for the 1-hr sea urchin fertilisation success test**

Test species	Sea urchin <i>Heliocidaris tuberculata</i>
Test type	Static, non-renewal
Test duration	1- hour, plus 20 minutes fertilisation time
Test end-point	Fertilisation success
Test temperature	20 ± 1°C
Test salinity	35 ± 1‰
Test chamber size / volume	5mL in 9mL tissue culture tube
Source of test organisms	Field collection, Sydney coastal region (Maroubra)
Test concentrations	100, 50, 25, 12.5, 6.3, 3.1 and 1.6%, plus filtered seawater (FSW) control and artificial seawater (ASW) control
Test acceptability criteria	>70% fertilisation in controls, reference toxicant results within prescribed range

To test the relative sensitivity of the test organisms and the proficiency of the Laboratory Technician, a separate positive (toxic) control test was conducted using copper sulphate. The test was performed in the same manner as for the effluent sample. The results of this test were compared with the results from previous testing using a control chart.

### 3.2 Results

The results for the sea urchin fertilisation success test are summarised in **Tables 3.2** and **3.3** below. The statistical print-outs are given in **Appendix A**.

**Table 3.2. Summary of toxicity data for the Brazil pulp mill effluent sample using the 1-hr sea urchin fertilisation success test.**

Sample Concentration (%)	Percentage of Fertilised Eggs (Mean ± SD)
FSW Control	93.3 ± 1.7
ASW Control	93.0 ± 1.4
1.6	93.8 ± 2.2
3.1	93.8 ± 3.1
6.3	92.5 ± 2.7
12.5	93.5 ± 3.1
25	92.3 ± 1.5
50	94.5 ± 2.7
100	92.5 ± 2.7

The Brazil pulp mill effluent sample did not exhibit toxicity to sea urchin fertilisation success test end-point. There was no significant difference in fertilisation success in the sample, compared with the artificial seawater control. The NOEC and LOEC for the pulp mill effluent sample was therefore, 100% and >100%, respectively. The 1-hr EC10 and EC50 for the sample were >100% (**Table 3.3**).

**Table 3.3. The 1-hr EC10, EC50, NOEC and LOEC for the Brazil pulp mill effluent samples to the sea urchin fertilisation success test.**

Sample	1-hr EC10 (%)	1-hr EC50 (%)	NOEC (%)	LOEC (%)
Brazil	>100	>100	100	>100

### 3.3 Quality Assurance

The sea urchin fertilisation success test met all quality assurance criteria. The mean percentage of fertilised eggs in the laboratory filtered seawater controls was 93.3%, which exceeded the minimum control criteria of 70%. Water quality parameters were also within test acceptability ranges.

The 1-hr EC50 estimate for the copper sulphate reference toxicant test run concurrently with the effluent sample test fell within the reference toxicant cusum chart control limits (**Table 3.4**). This indicated that the toxicity test was within the expected range with respect to performance and sensitivity.

**Table 3.4. Quality Assurance criteria for the 1-hr sea urchin fertilisation success test.**

QA/QC Parameter	Criterion	This Test	Criterion Met?
Control % fertilised	≥70%	93.3%	Yes
Reference toxicant within Cusum limits	16.9-95.1µg Cu/L	43.2 µg Cu/L	Yes

## 4. 72-hr Sea Urchin Larval Development Test

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### 4.1 Summary of Test Methodology

The sea urchin larval development test using the fertilised eggs of *Heliocidaris tuberculata* was undertaken in accordance with ESA Standard Operating Procedure 105, which is based on methods described by ASTM (1995) and APHA (1998), and adapted for use with *H. tuberculata* by Simon and Laginestra (1997) for Sydney Water for use with the 72-hr larval development endpoint by Doyle *et al.* (2003). The test was performed in a constant temperature chamber of  $20\pm 1^{\circ}\text{C}$ . Clean seawater was collected from the Sydney region and filtered to  $0.45\mu\text{m}$  on return to the laboratory. Sea urchins used for the test were obtained by field collection in Maroubra, NSW and spawned within 6 h of collection. The conditions of the test are summarised in **Table 4.1**.

The test was undertaken in 9mL borosilicate glass tissue culture vials. Seven concentrations of the Brazil pulp mill effluent sample were prepared and tested using 4 replicate vials. The test concentrations were 100, 50, 25, 12.5, 6.3, 3.1 and 1.6%. A filtered seawater (FSW) control, consisting of seawater collected from the Sydney Region, and an Artificial Seawater (ASW) control were tested concurrently with the effluent sample.

The pH, salinity and dissolved oxygen concentration of a representative sample from each concentration was measured. Salinity was measured using a WTW LF330 salinity/conductivity meter with a WTW Tetracon 325 probe. The pH was 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.

The methodology for extracting gametes and fertilising eggs was the same as for the sea urchin fertilisation success test. Fertilised eggs were exposed to each of the test treatments for 72 h, followed by the addition of buffered formalin.

One milliliter of test solution was drawn directly from the bottom of each test vessel and placed in a Sedgwick-Rafter counting chamber. The first 100 larvae were examined and the number of normally developed larvae was recorded. The concentration resulting in the inhibition of normal larval development to 10% and 50% of the test population (72-hr EC10 and EC50) was determined by the Maximum Likelihood Probit method using TOXCALC V5.0 software. The concentration of effluent causing no significant toxicity (No Observed Effect Concentration - NOEC) and the lowest concentration causing significant toxicity (Lowest Observed Effect Concentration - LOEC) were determined by performing a Dunnett's test.

**Table 4.1. Summary of test conditions for the 72-hr sea urchin larval development test**

Test species	Sea urchin <i>Heliocidaris tuberculata</i>
Test type	Static, non-renewal
Test duration	72 hours
Test end-point	Larval development to pluteus stage
Test temperature	20 ± 1°C
Test salinity	35 ± 1‰
Test chamber size / volume	5mL in 9mL tissue culture tube
Source of test organisms	Field collection, Sydney coastal region (Maroubra)
Test concentrations	100, 50, 25, 12.5, 6.3, 3.1 and 1.6%, plus filtered seawater (FSW) control and artificial seawater (ASW) control
Test acceptability criteria	>70% larval development in controls, reference toxicant results within prescribed range

To test the relative sensitivity of the test organisms and the proficiency of the Laboratory Technician, a separate positive (toxic) control test was conducted using copper sulphate. The test was performed in the same manner as for the effluent sample. The results of this test were compared with the results from previous testing using a control chart.

## 4.2 Results

The results for the sea urchin larval development test is summarised in **Tables 4.2** and **4.3** below. The statistical print-outs are given in **Appendix B**.

**Table 4.2. Summary of toxicity data for the Brazil pulp mill effluent sample using the 72-hr sea urchin larval development test.**

Sample Concentration (%)	Percentage of Normal Larvae (Mean ± SD)
FSW Control	92.8 ± 1.7
ASW Control	93.0 ± 2.6
1.6	93.0 ± 2.9
3.1	94.5 ± 2.7
6.3	92.5 ± 2.1
12.5	93.5 ± 3.1
25	94.8 ± 2.8
50	91.0 ± 2.2
100	43.0 ± 7.8*

\* Significantly lower % normal larvae compared with the ASW control treatment (Dunnett's Test, 1 tailed, P=0.05).

The Brazil pulp mill effluent sample did exhibit some toxicity to the sea urchin larval development test end-point. There was a significant decrease in the normal development of urchin larvae to the pluteus stage, exposed to the sample, compared with the artificial seawater control treatment. Decreased normal larval development was only apparent in the 100% (un-diluted) sample (resulting in  $43.0 \pm 7.8\%$  normal larvae). The NOEC and LOEC for the pulp mill effluent sample was therefore, 50% and 100%, respectively. The 72-hr EC10 and EC50 values for the sample were 62.3 (53.2-68.7)% and 96.6 (92.3-101.1)%, respectively (Table 4.3).

**Table 4.3. The 72-hr EC10, EC50, NOEC and LOEC for the Brazil pulp mill effluent sample to the sea urchin larval development test.**

Sample	72-hr EC10 (%)	72-hr EC50 (%)	NOEC (%)	LOEC (%)
Brazil	62.3 (53.2-68.7)	96.6 (92.3-101.1)	50	100

### 4.3 Quality Assurance

The sea urchin larval development test met all quality assurance criteria. The mean percentage of normal larvae in the laboratory filtered seawater controls was 92.8%, which exceeded the minimum control criteria of 70%. Water quality parameters were also within test acceptability ranges.

The 72-hr EC50 estimate for the copper sulphate reference toxicant test run concurrently with the effluent sample test fell within the reference toxicant cusum chart control limits (Table 4.4). This indicated that the toxicity test was within the expected range with respect to performance and sensitivity.

**Table 4.4. Quality Assurance criteria for the 72-hr sea urchin larval development test.**

QA/QC Parameter	Criterion	This Test	Criterion Met?
Control % normal larvae	$\geq 70\%$	92.8%	Yes
Reference toxicant within Cusum limits	7.0-13.1 $\mu\text{g Cu/L}$	8.7 $\mu\text{g Cu/L}$	Yes

## 5. 72-hr *Hormosira banksii* Macro-algal Germination Test

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### 5.1 Summary of Test Methodology

The macro-algal germination test using the gametes of *Hormosira banksii* was undertaken in accordance with ESA Standard Operating Procedure 116, which is based on methods described by USEPA (1985) for *Champia sp.* and adapted for use with *H. banksii* by Kevekordes and Clayton (1996). The 72-hr germination test endpoint is generally performed in preference to the fertilisation assay described by Gunthorpe *et al* (1997) for the National Pulp Mills Research Program given the germination assay is generally more sensitive to pollutants and is less susceptible to seasonal variation. *H. banksii* broodstock was collected from the lower intertidal zone in Bilgola, Sydney, NSW and held at 4°C prior to spawning. Clean seawater for spawning the broodstock was collected from the Sydney region and filtered to 0.45µm on return to the laboratory. The conditions of the test are summarised in **Table 5.1**.

The macro-alga were induced to spawn by washing individual alga in filtered seawater that had been warmed to 30-40°C for 30 seconds and then drying the washed alga at room temperature. Gamete extrusion usually occurred within 10 minutes of washing. Viable gametes were selected based on visual examination of gamete maturity and fertilisation success trials. The eggs were fertilised by adding spermatozoa to the egg suspension such that the final egg to sperm ratio was 1:100. The density of the egg suspension was determined using a Sedgwick-Rafter counting chamber and adjusted to a density of 2, 500 fertilised eggs/mL prior to initiating the tests.

The toxicity test was undertaken in small glass Petri dishes containing 5 mL of test solution. Seven concentrations of the Brazil pulp mill effluent sample were prepared and tested using 4 replicate vials. The test concentrations were 100, 50, 25, 12.5, 6.3, 3.1 and 1.6%. A filtered seawater (FSW) control, consisting of seawater collected from the Sydney region, and an artificial seawater (ASW) control were tested concurrently with the effluent sample. The test was performed in a constant temperature chamber set at 18±1°C for the duration of the exposure period.

The pH, salinity and dissolved oxygen concentration of a representative sample from each concentration was measured. Salinity was measured using a WTW LF330 salinity/conductivity meter with a WTW Tetracon 325 probe. The pH was 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.

The test vessels were inoculated with 200 µL of the fertilised egg suspension, thereby adding 500 eggs per test vessel. The test vessels were then incubated in a constant temperature chamber of 18±1°C with a 16:8 hr light: dark photoperiod for the entire 72-hr exposure. After 72-hr exposure, the Petri dish was placed under a microscope. The embryos were examined live under the microscope and the number of germinated and un-germinated spores were recorded.

The concentration resulting in the inhibition of normal larval development to 10% and 50% of the test population (72-hr EC10 and EC50) was determined by the Maximum Likelihood Probit method using TOXCALC V5.0 software. The concentrations of effluent causing no significant toxicity (No Observed Effect Concentration, NOEC) and the lowest concentration of effluent causing significant toxicity (Lowest Observed Effect Concentration, LOEC) were determined by performing Dunnett's test.

**Table 5.1. Summary of test conditions for the 72-hr *Hormosira banksii* macro-algal germination test**

Test species	<i>Hormosira banksii</i>
Test type	Static, non-renewal
Test duration	72 hours
Test end-point	Germination
Test temperature	18 ± 1°C
Test salinity	35 ± 1 ‰
Test chamber size / volume	5mL in Petri dishes
Test Concentrations	100, 50, 25, 12.5, 6.3, 3.1 and 1.6%, plus filtered seawater (FSW) control and artificial seawater (ASW) control
Source of test organisms	Field collection, Bilgola, NSW
Test acceptability criteria	≥70% germination in controls, reference toxicant results within prescribed range

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 copper sulphate. The test was performed in the same manner as the effluent test. The results of this test were compared with results from previous testing from the same laboratory using a control chart.

## 5.2 Results

The results for the *H. banksii* macroalgal growth inhibition test are summarised in **Tables 5.2** and **5.3** below. Statistical print-outs for the toxicity test are provided in **Appendix C**.

**Table 5.2. Summary of toxicity data for the Brazil pulp mill effluent sample using the 72-hr *Hormosira banksii* macro-algal germination test.**

Sample Concentration (%)	Percent Germinated (Mean ± SD)
FSW Control	74.3 ± 7.3
ASW Control	74.3 ± 10.5
1.6	82.5 ± 7.1
3.1	77.5 ± 5.8
6.3	76.5 ± 7.9
12.5	77.8 ± 8.9
25	73.0 ± 7.4
50	75.8 ± 9.1
100	68.5 ± 3.7

There was no significant difference in germination success of *Hormosira banksii* exposed to the Brazil pulp mill effluent sample, compared with the artificial seawater control treatment. The NOEC and LOEC for the pulp mill effluent sample was therefore, 100% and >100%, respectively. The 72-hr EC10 and EC50 values for the sample were 89.7% and >100%, respectively (Table 5.3).

**Table 5.3. The 72-hr EC10, EC50, NOEC and LOEC for the Brazil pulp mill effluent sample with the *Hormosira banksii* macro-algal germination test.**

Sample	72-hr EC10 (%)	72-hr EC50 (%)	NOEC (%)	LOEC (%)
Brazil	89.7*	>100	100	>100

\* 95% confidence limits are not available

### 5.3 Quality Assurance

The *H. banksii* germination test undertaken concurrently with the effluent sample met all quality assurance criteria for the test. The mean germination in the filtered seawater control treatment was 74.3%, which exceeded the minimum control germination of 70%. Water quality parameters were also within test acceptability ranges.

The 72-hr EC50 estimate for the copper sulphate reference toxicant run concurrently with the test fell within the reference toxicant cusum chart control limits (Table 5.4). This indicated that the toxicity test was within the expected range with respect to performance and sensitivity.

**Table 5.4 The Quality Assurance criteria for the 72-hr *Hormosira banksii* macro-algal germination test.**

QA/QC Parameter	Criterion	This Test	Criterion Met?
Control germination minimum	≥70%	74.3%	Yes
Reference toxicant within Cusum limits	1.5-739.9µg Cu/L	84.1µg Cu/L	Yes

## 6. 48-hr Doughboy Scallop Larval Development Test

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### 6.1 Summary of Test Methodology

The 48-hr larval development toxicity test using the larvae of the doughboy scallop *Mimachlamys asperrima* was undertaken in accordance with ESA Standard Operating Procedure 106, which is based on methods described by USEPA (1996a) and APHA (1998), and adapted for use with *M. asperrima* by Krasso *et al.* (1996) for the National Pulp Mills Research Program. Doughboy scallops were obtained by a commercial scallop supplier from Mercury Passage, Tasmania. Clean seawater for spawning the broodstock was collected from the Sydney region and filtered to 0.45µm on return to the laboratory. The conditions of the test are summarised in **Table 6.1**.

The scallops were induced to spawn by a 0.1mL injection of a 400mg/L serotonin solution to the distal portion of sexually mature adult gonads. Viable gametes were selected on the basis of fertilisation success trials and visual examination of gamete maturity. The eggs were fertilised by adding spermatozoa to the egg suspension such that the final egg to sperm ratio was 1:100. The density of the egg suspension was determined using a Sedgwick-Rafter counting chamber to calculate the volume required to achieve a final density of 30±5 eggs/mL in the test vessels.

The test was undertaken in 9mL borosilicate glass tissue culture vials. Seven concentrations of the Brazil pulp mill effluent sample were prepared and tested using 4 replicate vials. The test concentrations were 100, 50, 25, 12.5, 6.3, 3.1 and 1.6%. A filtered seawater (FSW) control, consisting of seawater collected from the Sydney region, and an artificial seawater (ASW) control were tested concurrently with the effluent sample. The test was performed in a constant temperature chamber set at 18±1°C for the duration of the exposure period.

The temperature, pH, salinity and dissolved oxygen concentration of a representative sample from each concentration was measured. Salinity was measured using a WTW LF330 salinity/conductivity meter with a WTW Tetracon 325 probe. The pH was 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.

After the 48-hr exposure period, a formalin solution was added to each vessel. One milliliter of test solution was drawn directly from the bottom of each test vessel and placed in a Sedgwick-Rafter counting chamber. The first 100 scallop larvae were examined and the number of normal and abnormal D-veliger larvae were recorded. The concentration of effluent sample affecting 10% and 50% of the test population (48-hr EC10 and EC50) was determined by the Maximum Likelihood Probit method using TOXCALC V5.0 software. The concentration causing no significant toxicity (No Observed Effect Concentration - NOEC) and the lowest concentration causing significant toxicity (Lowest Observed Effect Concentration - LOEC) was determined by performing Dunnett's test.

**Table 6.1. Summary of test conditions for the 48-hr doughboy scallop larval development test.**

Test species	Doughboy scallop <i>Mimachlamys asperimma</i>
Test type	Static, non-renewal
Test duration	48 hours
Test end-point	Larval development to D-veliger stage
Test temperature	18 ± 1°C
Test salinity	35 ± 1 ‰
Test chamber size / volume	5mL in 9mL tissue culture tube
Source of test organisms	Scallop farm, Mercury Passage, Tasmania
Test Concentrations	100, 50, 25, 12.5, 6.3, 3.1 and 1.6%, plus filtered seawater (FSW) control and Artificial Seawater (ASW) control
Test acceptability criteria	≥70% normally developed larvae in controls, reference toxicant results within prescribed range

To test the relative sensitivity of the test organisms and the proficiency of the Laboratory Technician, a separate positive (toxic) control test was conducted, using copper sulphate. The test was performed in the same manner as for the effluent sample. The results of this test were compared with the results from previous testing using a control chart.

## 6.2 Results

The results from the doughboy scallop larval development test are summarised in **Tables 6.2** and **6.3** below. Statistical printouts are given in **Appendix D**.

**Table 6.2. Summary of toxicity data for the Brazil pulp mill effluent sample using the 48-hr doughboy scallop larval development test.**

Sample Concentration (%)	Percentage Normal Larvae (Mean ± SD)
FSW Control	79.8 ± 3.3
ASW Control	77.5 ± 4.5
1.6	77.8 ± 5.1
3.1	78.0 ± 1.8
6.3	77.0 ± 4.4
12.5	77.0 ± 3.2
25	76.8 ± 4.0
50	74.8 ± 1.5
100	57.8 ± 10.1*

\* Significantly lower % normally developed larvae compared with the ASW Control (Dunnett's Test, 1 tailed, P=0.05).

The Brazil pulp mill effluent sample exhibited some toxicity to the scallop larval development test end-point. There was a significant decrease in normal development of larvae to the D-veliger stage, which had been exposed to the sample, compared with the artificial seawater control treatment. Decreased normal larval development was only apparent in the 100% (un-diluted) sample (resulting in  $57.8 \pm 10.1\%$  normal larvae). The NOEC and LOEC for the pulp mill effluent sample was therefore, 50% and 100%, respectively. The 48-hr EC10 and EC50 values for the sample were 69.0 (32.2-81.7)% and >100%, respectively (**Table 6.3**).

**Table 6.3. The 48-hr EC10, EC50, NOEC and LOEC for the effluent sample to the doughboy scallop larval development test.**

Sample	48-hr EC10 (%)	48-hr EC50 (%)	NOEC (%)	LOEC (%)
Brazil	69.0 (32.2-81.7)	>100	50	100

### 6.3 Quality Assurance

The doughboy scallop larval development toxicity test met all quality assurance criteria. The mean percentage of normally developed D-veliger larvae in the filtered seawater controls was 79.8%, which exceeded the minimum control criteria of 70%. Water quality parameters for control samples were also within test acceptability ranges.

The 48-hr EC50 estimate for the copper sulphate reference toxicant test run concurrently with the effluent sample fell within the reference toxicant cusum chart control limits (**Table 6.4**). This indicated that the toxicity test was within the expected range with respect to performance and sensitivity.

**Table 6.4. Quality Assurance criteria for the 48-hr doughboy scallop larval development test.**

QA/QC Parameter	Criterion	This Test	Criterion Met?
Control % normal larvae	$\geq 70\%$	79.8%	Yes
Reference toxicant within Cusum limits	2.8-10.3 $\mu\text{g}$ Cu/L	4.5 $\mu\text{g}$ Cu/L	Yes

## 7. 96-hr Acute Amphipod Toxicity Test

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### 7.1 Summary of Test Methodology

The 96-hr toxicity test using the amphipod *Allorchestes compressa* was undertaken in accordance with ESA Standard Operating Procedure 108, which is based on methods described by USEPA (1993) and the Department of Transport and Communications (1990). The conditions of the test are summarised in **Table 7.1**.

Amphipods of approximately 1-5 mm in length, used in the toxicity test, were isolated from laboratory maintained cultures. The toxicity test was undertaken in 100 mL glass beakers containing 80 mL of test solution. Seven concentrations of the effluent samples were prepared and tested using 4 replicate vials. The test concentrations were 100, 50, 25, 12.5, 6.3, 3.1 and 1.6%. A filtered seawater (FSW) control, consisting of seawater collected from the Sydney region, and an artificial seawater (ASW) control were tested concurrently with the effluent sample.

The temperature, pH, salinity and dissolved oxygen concentration of a representative sample from each concentration was measured. Salinity was measured using a WTW LF330 salinity/conductivity meter with a WTW Tetracon 325 probe. The pH was 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.

Five amphipods were introduced into each of the test beakers. The beakers were covered with cling-wrap film to minimise evaporation and placed in a constant temperature chamber of  $20 \pm 1^\circ\text{C}$ . The condition of test organisms was assessed at 24-hr intervals, and the number of surviving amphipods was recorded. The test was terminated after 96 h, and the pH, salinity and dissolved oxygen concentration of a representative sample from each concentration was measured, as detailed above.

The concentration of the effluent sample affecting 10% and 50% of amphipod survival (the 96-hr IC10 and EC50) was determined by Non-linear Interpolation method using TOXCALC v5.0 (Tidepool Scientific Software). The concentration of the effluent causing no significant toxicity (No Observed Effect Concentration, NOEC) and the lowest concentration of the effluent causing significant toxicity (Lowest Observed Effect Concentration, LOEC) were determined by performing a Dunnett's test.

**Table 7.1 Summary of test conditions for the 96-hr acute amphipod toxicity test using *Allorchestes compressa*.**

Test species	<i>Allorchestes compressa</i>
Test type	Static, non-renewal
Test duration	96 hours
Test end-point	Survival
Test temperature	$20 \pm 1^\circ\text{C}$
Test salinity	$35 \pm 1 \text{‰}$
Test chamber size / volume	80mL in 100mL borosilicate glass beakers
Test concentrations	100, 50, 25, 12.5, 6.3, 3.1 and 1.6%, plus filtered seawater (FSW) control and artificial seawater (ASW) control

Source of test organisms	Laboratory cultured
Test acceptability criteria	≥90% survival in controls

To test the relative sensitivity of the test organisms and the proficiency of the Laboratory Technician, a separate positive (toxic) control test was conducted, using Sodium Dodecyl Sulphate (SDS). The test was performed in the same manner as for the effluent sample. The results of this test were compared with the results from previous testing using a control chart.

## 7.2 Results

The results for acute amphipod test are summarised in **Table 7.2** and **7.3** respectively. The statistical print-outs are given in **Appendix E**.

**Table 7.2. Summary of toxicity data for the Brazil pulp mill effluent sample using the 96-hr acute amphipod toxicity test with *Allorchestes compressa*.**

Sample Concentration (%)	Percent Survival (Mean ± SD)
FSW Control	95.0 ± 10.0
ASW Control	90.0 ± 11.6
1.6	95.0 ± 10.0
3.1	100 ± 0.0
6.3	90.0 ± 11.6
12.5	95.0 ± 10.0
25	95.0 ± 10.0
50	90.0 ± 11.6
100	75.0 ± 25.2

The pulp mill effluent sample exhibited some mortalities in the undiluted (100%) concentration using the amphipod survival end-point. However, no significant difference in survival rate was detected in the undiluted sample, compared with the artificial seawater control treatment. The NOEC and LOEC values for the pulp mill effluent sample were 100% and >100%, respectively. The 96-hr IC10 and EC50 values for the sample were 65.3% and >100%, respectively (**Table 7.3**).

**Table 7.3. The 96-hr EC10, EC50, NOEC and LOEC of the Brazil pulp mill effluent sample for the acute amphipod toxicity test with *Allorchestes compressa*.**

Sample	96-hr IC10 (%)	96-hr EC50 (%)	NOEC (%)	LOEC (%)
Brazil	65.3*	>100	100	>100

\* 95% confidence limits are not available

### 7.3 Quality Assurance

The amphipod acute survival test undertaken met all quality assurance criteria. The percentage survival in the laboratory controls was 95.0%, exceeding the minimum control survival criteria of  $\geq 90\%$ . Water quality parameters for control samples were also within test acceptability ranges (**Table 7.4**).

The 72-hr EC50 estimate for the sodium dodecyl sulphate reference toxicant run concurrently with the test fell within the reference toxicant cusum control limits (**Table 7.4**). This indicated that the toxicity test was within the expected range with respect to performance and sensitivity.

**Table 7.4. Quality Assurance criteria for the 96-hr acute amphipod toxicity test with *Allorchestes compressa*.**

QA/QC Parameter	Criterion	This Test	Criterion Met?
Control % survival	$\geq 90\%$	95.0%	Yes
Reference toxicant within Cusum limits	1.4-10.5mg SDS/L	6.2mg SDS/L	Yes

## 8. 96-hr Fish Imbalance Test

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### 8.1 Summary of Test Methodology

The 96-hr toxicity test using the larvae of the pink snapper *Pagrus auratus* was undertaken in accordance with ESA Standard Operating Procedure 117, which is based on the method described by USEPA (2002). Research with vertebrates in the state of New South Wales is subject to the Animal Research Act, and the toxicity test with larval fish was performed by ESA under the Animal Research Authority issued to ESA by the Director-General of NSW Department of Primary Industries (valid from 27 May 2008 to 27 May 2010) and Certificate of Approval from the Animal Care and Ethics Committee of the Director-General of the NSW Department of Primary Industries (valid from 16 May 2008 to 16 May 2010). The conditions of the test are summarised in **Table 8.1**.

Larvae less than 24 h old were obtained from a commercial hatchery in Western Australia. The eggs were shipped same-day express in a foam box containing an ice brick and fish were contained within an air inflated bag containing approximately 4 L of seawater. The fish larvae were transferred to a holding tank at 21°C on arrival, and provided gentle aeration using a Schego air pump. Clean seawater for holding the larval fish was collected from the Sydney region and filtered to 0.45µm on return to the laboratory. The seawater was raised to 21°C prior to use. The fish were used for the toxicity test within 8 h of arrival at the ESA laboratory.

The toxicity test was undertaken in 250 mL glass beakers containing 100 mL of test solution. Seven concentrations of the Brazil pulp mill effluent sample were prepared and tested using 4 replicate beakers. The test concentrations were 100, 50, 25, 12.5, 6.3, 3.1 and 1.6%. A filtered seawater (FSW) control, consisting of seawater collected from the Sydney Region, and an Artificial Seawater (ASW) control were tested concurrently with the effluent samples.

The pH, salinity and dissolved oxygen concentration of a representative sample from each concentration was measured. Salinity and conductivity were measured using a WTW LF330 salinity/conductivity meter with a WTW Tetracon 325 probe. The pH was 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.

Ten larval fish were selected and introduced into each of the test beakers. The beakers were covered with cling-wrap film to minimise evaporation and placed in a constant temperature chamber of 20±1°C. The test vessels were monitored three times per day to examine fish for signs of distress or imbalance. Larval fish demonstrating such signs were removed and euthanised in accordance with ESA SOP 117. The beakers were examined every 24 h and the number of surviving and apparently healthy larval fish recorded. The test was terminated after 96 h, and the pH, salinity and dissolved oxygen concentration of a representative sample from each concentration was measured, as detailed above. At the termination of the test, the larval fish were euthanised by the addition of clove oil.

The concentration of the effluent sample affecting 10% and 50% of the larval fish (the 96-hr EC10 and EC50) was determined by the Non-linear Interpolation method using TOXCALC v5.0 (Tidepool Scientific Software). The concentrations of the effluent causing no significant toxicity (No Observed Effect Concentration, NOEC) and the lowest concentration of the effluent causing significant toxicity (Lowest Observed Effect Concentration, LOEC) were determined by performing Dunnett's test.

**Table 8.1 Summary of test conditions for the 96-hr fish-Imbalance test using the pink snapper *Pagrus auratus*.**

Test species	Pink snapper <i>Pagrus auratus</i>
Test type	Static, non-renewal
Test duration	96 hours
Test end-point	Imbalance, including survival
Test temperature	20 ± 1°C
Test salinity	35 ± 1 ‰
Test chamber size / volume	100mL in 250mL glass beakers
Test concentrations	100, 50, 25, 12.5, 6.3, 3.1 and 1.6%, plus filtered seawater (FSW) control and artificial seawater (ASW) control
Source of test organisms	Hatchery reared, WA
Test acceptability criteria	≥90% survival in controls

## 8.2 Results

The results for fish imbalance test are summarised in **Table 8.2** and **8.3** respectively. The statistical print-outs are given in **Appendix F**.

**Table 8.2. Summary of toxicity data for the Brazil pulp mill effluent sample using the 96-hr fish imbalance test with pink snapper *Pagrus auratus*.**

Sample Concentration (%)	Percent Un-affected (Mean ± SD)
FSW Control	97.5 ± 5.0
ASW Control	89.2 ± 12.5
1.6	92.5 ± 9.6
3.1	80.0 ± 21.6
6.3	95.0 ± 5.8
12.5	95.0 ± 10.0
25	87.5 ± 5.0
50	95.0 ± 10.0
100	82.5 ± 20.6

The Brazil pulp mill effluent sample did not exhibit toxicity using the larval fish imbalance end-point. There was no significant difference in un-affected fish in the sample, compared with the artificial seawater control treatment. The NOEC and LOEC values for the sample were therefore, 100% and >100%, respectively. The 96-hr EC10 and EC50 values for the sample were >100% (**Table 8.3**).

**Table 8.3. The 96-hr EC10, EC50, NOEC and LOEC for the Brazil pulp mill effluent sample to the larval fish imbalance test with pink snapper *Pagrus auratus*.**

Sample	96-hr EC10 (%)	96-hr EC50 (%)	NOEC (%)	LOEC (%)
Brazil	>100	>100	100	>100

### 8.3 Quality Assurance

The larval fish imbalance test met all quality assurance criteria. The percentage survival in the laboratory controls was 97.5%, exceeding the minimum control survival criteria of  $\geq 90\%$  (Table 8.4). Water quality parameters for control samples were also within test acceptability ranges.

**Table 8.4. Quality Assurance criteria for the 96-hr fish imbalance test with pink snapper *Pagrus auratus*.**

QA/QC Parameter	Criterion	This Test	Criterion Met?
Control % survival	$\geq 90\%$	97.5%	Yes

## 9. 72-hr *Nitzschia closterium* Microalgal Growth Inhibition Test

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### 9.1 Summary of Test Methodology

The 72-hr micro-algal growth inhibition test with *Nitzschia closterium* was undertaken in accordance with ESA Standard Operating Procedure 110. The procedure described here is based on Stauber et al. (1994). The conditions for the test are summarised in **Table 9.1**.

*N. closterium* in log-growth phase were obtained from cultures maintained at the ESA laboratory. The inoculum was prepared prior to the commencement of the test, using cells harvested from a 4 to 7 day old stock culture. The algal cells were 'washed' of culture media by centrifugation at 3000 rpm for 5 minutes, the supernatant decanted and discarded, and the algal pellet re-suspended in filtered seawater (FSW). This process was repeated a second time to ensure that none of the culturing media remained in the inoculum. The density of the algal cells was diluted with FSW water such that each millilitre of inoculum contained approximately 1, 000,000 cells. One hundred microlitres of this inoculum was added to 10mL of test solution to provide an initial cell density of 10,000 cells/mL. The inoculum was prepared no more than 3 h prior to the beginning of the test.

The test was undertaken in 20mL borosilicate glass vials containing 10mL of test solution. Seven concentrations of the pulp mill effluent sample were prepared and tested using 4 replicate vials. The test concentrations were 100, 50, 25, 12.5, 6.3, 3.1 and 1.6%. A filtered seawater (FSW) control consisting of seawater collected from the Sydney region were tested concurrently with the effluent sample.

The pH and salinity of a representative sample from each concentration was measured. Salinity was measured using a WTW LF330 salinity/conductivity meter with a WTW Tetracon 325 probe. The pH was measured using a WTW pH330 meter, with a WTW SenTix 41 electrode.

The test vials were then incubated in a constant temperature chamber at  $21 \pm 1$  °C. The test vials were provided with continuous lighting of 4300 Lux. The test was terminated after 72 h, and the algal growth in each test vessel was determined by using absorbance at 750nm on a spectrophotometer as a surrogate to algae biomass.

The 72-hr IC10 and IC50 estimates were determined using the Linear Interpolation method using TOXCALC v5.0 (Tidepool Scientific Software). The concentration of effluent causing no significant toxicity (NOEC) and the lowest concentration of effluent causing significant toxicity (LOEC) were determined by performing Dunnett's test.

**Table 9.1 Summary of test conditions for the 72-hr *Nitzschia closterium* micro-algal growth inhibition (cell yield) test**

Test Type	Static, non-renewal
Test duration	72 hours
Temperature	21 ± 1°C
Light quality	cool-white fluorescent tube lighting
Light intensity	4300 Lux
Photoperiod	12 hr light : 12 hr dark
Test Chamber size	20mL
Test solution volume	10mL
Age of test organisms	Cells in log-growth phase
Initial cell density	10,000 cells /mL
No. of replicates per treatment	4
Aeration	none
Shaking rate	Twice daily by hand
Dilution water	Filtered seawater with sodium nitrate stock and potassium dihydrogen phosphate
Dilution series	100, 50, 25, 12.5, 6.3, 3.1 and 1.6% sample
Endpoint	Cell yield, as determined via cell counts or absorbance at 750nm
Test acceptability criterion	Mean cell density of at least 160,000 cells per mL in controls, and variability (CV%) in controls should not exceed 20%.

To test the relative sensitivity of the test organisms and the proficiency of the Laboratory Technician, a separate positive (toxic) control test was conducted using copper sulphate. The test was performed in the same manner as the toxicity test. The results of this test were compared with the results from previous testing using a control chart.

## 9.2 Results

The results for the *N. closterium* micro-algal growth inhibition test are summarised in **Tables 9.2 and 9.3** below. Statistical print-outs for the toxicity test are provided in **Appendix G**.

**Table 9.2. Summary of toxicity data for the Brazil pulp mill effluent sample using the 72-h *Nitzschia closterium* micro-algal growth inhibition test.**

Sample Concentration (%)	Cell Yield (x10 <sup>4</sup> cells/mL) (Mean ± SD)
FSW Control	39.1 ± 3.5
ASW Control	4.9 ± 0.3*
Colour Control	34.0 ± 2.6
1.6	31.8 ± 1.6 **
3.1	31.8 ± 2.7 **
6.3	36.0 ± 0.8
12.5	40.1 ± 5.7
25	46.3 ± 1.6
50	47.5 ± 4.0
100	45.8 ± 3.3

\* Significantly lower cell yield compared with the FSW Control (Steel's Many One Rank Test, 1 tailed, P=0.05).

\*\* Significantly lower cell yield compared with the FSW Control (Dunnett's Test, 1 tailed, P=0.05).

Significantly reduced cell yield was observed in the artificial seawater control treatment compared with the filtered seawater control treatment. This reduction in cell yield was not reflected in the 100% sample, which had artificial sea salts added directly to it. Sample concentrations 50, 25, 12.5, 6.3, 3.1 and 1.6% contained decreasing amounts of artificial sea salts as they were diluted with natural filtered seawater to achieve the test concentrations (i.e. higher concentrations contained a higher fractions of artificial sea salts). Hence, significantly reduced cell yield in the 3.1% and 1.6% concentrations is not suspected to be due to the addition of artificial sea salts, as significantly reduced cell yield was not observed in higher test concentrations containing greater fractions of artificial sea salts. Subsequently, the sample treatments were compared against the filtered seawater control treatment.

The Brazil pulp mill effluent sample exhibited a significant decrease in cell yield of *N. closterium* in the 3.1% and 1.6% concentrations, however, no toxicity was observed in the 6.3% concentration and above. Subsequently, NOEC and LOEC values were 100% and >100%, respectively (Table 9.3). The 72-hr IC10 and IC50 values were >100%.

**Table 9.3. The 72-hr IC10, IC50, NOEC and LOEC for the pulp mill effluent sample with the *Nitzschia closterium* micro-algal growth inhibition test.**

Sample	72-hr IC10 (%)	72-hr IC50 (%)	NOEC (%)	LOEC (%)
Brazil	>100	>100	100	>100

### 9.3 Quality Assurance

The *N. closterium* growth inhibition test undertaken concurrently with the effluent sample met all quality assurance criteria for the test, with the exception of mean cell density in the artificial seawater control (4.9x10<sup>4</sup>). Despite this, the toxicity observed in the artificial seawater control was not reflected in the sample. The mean cell density in the filtered seawater control treatment was 40.1x10<sup>4</sup>. This value exceeded the

minimum control criteria of  $>16 \times 10^4$  cells/mL. Water quality parameters for control samples were also within test acceptability ranges.

The 72-hr IC50 estimate for the copper sulphate reference toxicant run concurrently with the test fell within the reference toxicant cusum chart control limits (**Table 9.4**). This indicated that the toxicity test was within the expected range with respect to performance and sensitivity.

**Table 9.4 The Quality Assurance criteria for the marine micro-alga *Nitzschia closterium* growth inhibition (cell yield) test.**

QA/QC Parameter	Criterion	This Test	Criterion Met?
Control mean cell density	$>16 \times 10^4$ cells/mL	$40.1 \times 10^4$ cells/mL	Yes
Coefficient of variation	<20%	9.0%	Yes
Reference toxicant within Cusum limits	1.0-37.6 $\mu$ g Cu/L	6.8 $\mu$ g Cu/L	Yes

## 10. Microtox® Acute Test

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### 10.1 Summary of Test Methodology

The microtox® acute toxicity test with the luminescent bacteria *Vibrio fischeri* was undertaken in accordance with ESA Standard Operating Procedure 119. The Microtox® Acute Toxicity Test involves exposing freeze-dried bacteria (*V. fischeri*) to the test material for up to 30-minutes. Readings of the light output of the bacteria are taken using a Microbics Model 500 Analyser immediately prior to exposure to the test material, and then again following 5- and 15- minutes exposure. A significant decrease in luminescence relative to the controls is an indication of toxicity. The conditions for the test are summarised in **Table 10.1**.

The test was undertaken in 4mL cuvettes. Seven concentrations of the Brazil pulp mill effluent sample were prepared and tested using 2 replicate cuvettes. The test concentrations were 90, 45, 22.5, 11.3, 5.6, 2.8 and 1.4%. A filtered seawater (FSW) control and an artificial seawater (ASW) control were tested concurrently with the effluent sample.

Freeze dried bacteria was reconstituted with 1mL of reconstitution solution. A dilute reagent containing the bacteria was then prepared by addition of the concentrated bacteria reagent to reconstitution solution. One hundred microlitres of the diluted reagent was then added to each of the cuvettes containing sample. Following a 15 minute stabilisation period, the luminescence of the bacteria is measured at 5-, and 15-minutes exposure to the sample.

The pH and salinity of a representative sample from each concentration was measured. Salinity was measured using a WTW LF330 salinity/conductivity meter with a WTW Tetracon 325 probe. The pH was measured using a WTW pH330 meter, with a WTW SenTix 41 electrode.

The concentration resulting in the inhibition of luminescence to 50% of the test population (5- and 15-min EC50) was determined using MicrotoxOmni software. The concentration of the effluent causing no toxicity (No observed Effect Concentration, NOEC) and the lowest concentration of the effluent causing significant toxicity (Lowest Observed Effect Concentration, LOEC) were determined by performing Dunnett's test (TOXCALC v5.0 software).

**Table 10.1 Summary of test conditions for the Microtox<sup>®</sup> acute toxicity test.**

Test parameter	Test Condition
Test Type	Static non-renewal
Test duration	5-, 15- min
Temperature	15 ± 0.5°C
Salinity	35 ± 1‰
Test Chamber size	4mL
Test solution volume	1mL
Source of test organisms	Commercially supplied
No. of replicates per treatment	2
Dilution water	Natural sea water filtered to 0.45µm
Test concentrations	90, 45, 22.5, 11.3, 5.6, 2.8 and 1.4%, plus filtered seawater (FSW) and artificial seawater (ASW)
Endpoint	Luminescence
Test acceptability criterion	Reference toxicant within cusum chart limits

To test the relative sensitivity of the batch of bacteria and the proficiency of the Laboratory Technician, a separate positive (toxic) control test was conducted using zinc sulphate. The test was performed in the same manner as the toxicity test. The results of this test were compared with the results from previous testing using a control chart.

## 10.2 Results

The results for the Microtox<sup>®</sup> acute toxicity test are summarised in **Table 10.2** below. Statistical print-outs for the toxicity test are provided in **Appendix H**.

The Brazil pulp mill effluent sample exhibited some toxicity to the Microtox<sup>®</sup> acute toxicity test. A slight but significant reduction to the luminescence of the bacteria *V. fischeri* was measured in the 45% and 90% concentrations at both 5- and 15-minutes exposure. The EC50, NOEC and LOEC values at 5 and 15 minutes were >90%, 22.5% and 45%, respectively (**Table 10.3**).

**Table 10.2. The 5- and 15- minute EC50 for the Brazil pump mill effluent sample with the Microtox<sup>®</sup> acute toxicity test.**

Sample	5-min EC50 (%)			15-min EC50 (%)		
	EC50 (%)	NOEC (%)	LOEC (%)	EC50 (%)	NOEC (%)	LOEC (%)
Brazil	>90	22.5	45	>90	22.5	45

### 10.3 Quality Assurance

The Microtox<sup>®</sup> acute toxicity test undertaken concurrently with the effluent sample met all quality assurance criteria for the test. Water quality parameters for control samples were also within test acceptability ranges.

The EC50 estimate for the zinc sulphate reference toxicant run concurrently with the test fell within the reference toxicant cusum chart control limits (**Table 10.3**). This indicated that the toxicity test was within the expected range with respect to performance and sensitivity.

**Table 10.3 The Quality Assurance criteria for the Microtox<sup>®</sup> acute toxicity test.**

QA/QC Parameter	Criterion	This Test	Criterion Met?
Reference toxicant within Cusum limits	2.9-14.8mg Zn/L	11.1mg Zn/L	Yes

## 11. 72-hr *Ecklonia radiata* Macro-algal Germination Test

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### 11.1 Summary of Test Methodology

The macro-algal germination test using the zoospores of *Ecklonia radiata* was undertaken in accordance with ESA Standard Operating Procedure 116, which is based on methods described by Bidwell *et al.* (1998) and BurrIDGE *et al.* (1999). *E. radiata* broodstock was collected from an intertidal zone in Tasmania and transported to ESA Laboratories in polyethylene bags within a foam box. Clean seawater for spawning the broodstock was collected from the Sydney region and filtered to 0.45µm on return to the laboratory. The conditions for the test are summarized in **Table 11.1**.

The sori of the macro-algae were induced to spawn by washing the alga in filtered seawater (FSW) that had been chilled to approximately 10°C, and then drying the washed algae at room temperature for 30 minutes. Once dried, sori were placed back into chilled FSW for 10 minutes, hand dried and placed back into FSW at room temperature. The solution was then checked at 10 min intervals until moving spores were observed. Sori was then placed into a fresh beaker of filtered seawater and allowed to spawn until the desired density of spores was achieved. The density of the zoospore suspension was determined using a haemocytometer.

Toxicity test was undertaken in 60 mm diameter glass Petri dishes containing 5 mL of test solution. A cover slip was placed on the bottom surface of each Petri dish to provide a site of attachment and to permit easy recovery of the developing embryos. Seven concentrations of the effluent samples were prepared and tested using 4 replicate vials. The test concentrations were 100, 50, 25, 12.5, 6.3, 3.1 and 1.6%. A FSW control, consisting of seawater collected from the Sydney region, and an artificial seawater (ASW) control were tested concurrently with the effluent samples. The test was performed in a constant temperature chamber set at 18±1°C for the duration of the exposure period.

The pH, salinity and dissolved oxygen concentration of a representative sample from each concentration was measured. Salinity was measured using a WTW LF330 salinity/conductivity meter with a WTW Tetracon 325 probe. The pH was 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.

Five milliliters of spore suspension was then placed into petri dishes, and allowed to settle for a period of at least 1 h. Following this period, the solution was gently removed and 5mL of the test solution was placed into each of the test vessels immediately. The test vessels were then incubated in a constant temperature chamber of 18±1°C with a 16:8 hr light: dark photoperiod for the entire 72-hr exposure. After 72-hr exposure, the coverslip from each Petri dish was removed and placed under a microscope. The embryos were examined live under the microscope and the number of germinated and un-germinated spores was recorded.

The concentration of the effluent inhibiting germination in 10% and 50% of the test population (the 72-hr EC10 and EC50) were determined by the non-linear interpolation method using TOXCALC v5.0 (Tidepool Scientific Software). The concentration of effluent causing no significant toxicity (No Observed Effect Concentration, NOEC) and the lowest concentration of effluent causing significant toxicity (Lowest Observed Effect Concentration, LOEC) were determined by performing Dunnett's test.

**Table 11.1. Summary of test conditions for the 72-hr *Ecklonia radiata* macro-algal germination test.**

Test species	<i>Ecklonia radiata</i>
Test type	Static, non-renewal
Test duration	72 hours
Test end-point	Germination
Test temperature	18 ± 1°C
Test salinity	35 ± 1 ‰
Test chamber size / volume	5mL in Petri dishes
Test Concentrations	100, 50, 25, 12.5, 6.3, 3.1 and 1.6%, plus filtered seawater (FSW) control and artificial seawater (ASW) control
Source of test organisms	Field collection, Tasmania
Test acceptability criteria	≥70% germination in controls, reference toxicant results within prescribed range

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 copper sulphate. The test was performed in the same manner as the effluent test. The results of this test were compared with results from previous testing from the same laboratory using a control chart.

## 11.2 Results

The results for the *E. radiata* macro-algal growth inhibition test are summarised in **Tables 11.2** and **11.3** below. Statistical print-outs for the toxicity test are provided in **Appendix I**.

**Table 11.2. Summary of toxicity data for the Brazil pulp mill effluent sample using the 72-hr *Ecklonia radiata* macro-algal germination test.**

Sample Concentration (%)	Percentage of Fertilised Eggs (Mean ± SD)
FSW Control	93.5 ± 2.1
ASW Control	95.5 ± 2.7
1.6	95.3 ± 2.1
3.1	94.3 ± 2.2
6.3	95.0 ± 2.9
12.5	94.8 ± 1.7
25	92.8 ± 2.5
50	94.0 ± 3.4
100	94.0 ± 1.8

The Brazil pulp mill effluent sample did not exhibit toxicity using the macro-algal germination success end-point. There was no significant difference in germination success in the sample, compared with the artificial seawater control treatment. The NOEC and LOEC for the pulp mill effluent sample was therefore, 100% and >100%, respectively. The 72-hr EC10 and EC50 values for the sample were >100% (Table 11.3).

**Table 11.3. The 72-hr EC10, EC50, NOEC and LOEC for the Brazil pulp mill effluent sample with the *Ecklonia radiata* macro-algal germination test.**

Sample	72-hr EC10 (%)	72-hr EC50 (%)	NOEC (%)	LOEC (%)
Brazil	>100	>100	100	>100

### 11.3 Quality Assurance

The *E. radiata* germination test undertaken concurrently with the pulp mill effluent sample met all quality assurance criteria for the test. The mean germination in the filtered seawater control treatment was 93.5%. These values exceeded the minimum control germination of 70%. Water quality parameters for control samples were also within test acceptability ranges.

The 72-hr EC50 estimate for the copper sulphate reference toxicant run concurrently with the test fell within the reference toxicant cusum chart control limits (Table 11.4). This indicated that the toxicity test was within the expected range with respect to performance and sensitivity.

**Table 11.4 The Quality Assurance criteria for the 72-hr *Ecklonia radiata* macro-algal germination test.**

QA/QC Parameter	Criterion	This Test	Criterion Met?
Control germination minimum	≥70%	93.5%	Yes
Reference toxicant within Cusum limits	233.6-793.2µg Cu/L	459.6µg Cu/L	Yes

## 12. 48-hr *Ceriodaphnia dubia* Acute Test

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### 12.1 Summary of Test Methodology

An acute toxicity test with the freshwater cladoceran *Ceriodaphnia dubia* was undertaken in accordance with ESA Standard Operating Procedure 101. This procedure is based on methods described by the USEPA (2002b) and adapted for use with *C. dubia* (referred to as the Sydney Clone) by Bailey *et al.* (2000). The conditions for the test are summarised in **Table 12.1**.

Dilute Mineral Water (DMW) was used as the diluent for the toxicity test and as the culture medium for the culturing of the test organisms. DMW was prepared 24-48 h prior to use in accordance with the procedure described by the USEPA (1993). 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 2µg/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.

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 test. The neonates to be used in the toxicity test were isolated approximately 2 h prior to test initiation in a 250 mL beaker containing 200 mL of DMW.

Seven concentrations of the Brazil pulp mill effluent sample were prepared and tested using 4 replicate vials. The test concentrations were 100, 50, 25, 12.5, 6.3, 3.1 and 1.6%. A DMW control was tested concurrently with the effluent sample. The solutions were prepared in 250 mL beakers by diluting with DMW.

The pH, conductivity and dissolved oxygen concentration of a representative sample from each concentration was measured. Conductivity was measured using a WTW LF330 salinity/conductivity meter with a WTW Tetracon 325 probe. The pH was 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.

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°C. A 16:8 h light/dark cycle was provided. The condition of the test organisms was assessed at 24 h and again at 48 h, and the number of immobilised organisms was recorded. The test was terminated after 48 h of exposure.

The concentration of the effluent inhibiting germination in 10% and 50% of the test population (the 72-hr EC10 and EC50) were determined by the non-linear interpolation method using TOXCALC v5.0 (Tidepool Scientific Software). The concentration of effluent causing no significant toxicity (No Observed Effect Concentration, NOEC) and the lowest concentration of effluent causing significant toxicity (Lowest Observed Effect Concentration, LOEC) were determined by performing Shapiro Wilk's test.

**Table 12.1 Summary of test conditions for the *Ceriodaphnia dubia* acute toxicity test.**

Test Type	Static, non-renewal
Test duration	48 hours
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 <i>Selenastrum</i> and trout chow while holding prior to test. Newly-released neonates should have food available at least 2 hr prior to testing
Dilution water	Dilute Mineral Water (DMW), prepared by mixing 20% Perrier mineral water with deionised water, with vitamin B12 and selenium supplements
Dilution series	100, 50, 25, 12.5, 6.3, 3.1 and 1.6% plus control
Endpoint	Immobilisation
Test acceptability criterion	≥ 90% survival in controls. Reference toxicant EC50 within Cusum chart control limits

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 (KCl). This test was performed in the same manner and run concurrently with the effluent sample. The results of this reference toxicant test were compared with the results from previous testing using a control chart.

## 12.2 Results

The results for the 48-hr *C. dubia* acute toxicity test are summarised in **Tables 12.2 and 12.3** below. Statistical print-outs for the toxicity test are provided in **Appendix J**.

**Table 12.2. Summary of survival data for the pulp mill effluent sample using the 48-hr *Ceriodaphnia dubia* acute toxicity test.**

Sample Concentration (%)	Percent Survival (Mean ± SD)
Control	100 ± 0.0
1.6	100 ± 0.0
3.1	100 ± 0.0
6.3	100 ± 0.0
12.5	100 ± 0.0
25	100 ± 0.0
50	100 ± 0.0
100	100 ± 0.0

The Brazil pulp mill effluent sample did not exhibit toxicity to the 48-hr *C. dubia* acute immobilisation test endpoint (Table 12.3). The 48-hr EC10, EC50, NOEC and LOEC estimates were >100%, >100%, 100% and >100%, respectively.

**Table 12.3. The 48-h EC10, EC50, NOEC and LOEC for the effluent sample with the *Ceriodaphnia dubia* acute toxicity test.**

Sample	48-hr EC10 (%)	48-hr EC50 (%)	NOEC (%)	LOEC (%)
Brazil	>100	>100	100	>100

### 12.3 Quality Assurance

The toxicity test performed with the effluent 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.

The 48-hr EC50 estimate for the potassium chloride reference toxicant run concurrently with the test fell within the reference toxicant cusum chart control limits. This indicated that the toxicity test was within the expected range with respect to performance and sensitivity (Table 12.4).

**Table 12.4 The Quality Assurance criteria for the 48-hr *Ceriodaphnia dubia* acute toxicity test.**

QA/QC Parameter	Criterion	This Test	Criterion Met?
Control % survival	>90%	100%	Yes
Reference toxicant within Cusum limits	184.4-282.6mg KCl/L	204.9mg KCl/L	Yes

## 13. References

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## **Appendix A: Statistical Print-outs for the Sea Urchin Fertilisation Success Test**

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**Sea Urchin Fertilisation Test-Proportion Fertilized**

Start Date:	19/11/2009 18:45	Test ID:	PR0490/02	Sample ID:	Brazil
End Date:	19/11/2009 20:05	Lab ID:	3891Q	Sample Type:	AQ-Aqueous
Sample Date:		Protocol:	ESA 104	Test Species:	HT-Heliocidaris tuberculata
Comments:					

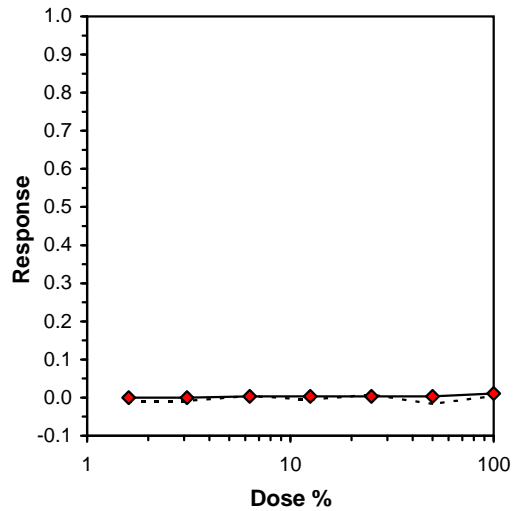
Conc-%	1	2	3	4
FSW Control	0.9300	0.9500	0.9100	0.9400
ASW Control	0.9500	0.9200	0.9300	0.9200
1.6	0.9300	0.9500	0.9100	0.9600
3.1	0.9200	0.9400	0.9100	0.9800
6.3	0.9300	0.9100	0.9000	0.9600
12.5	0.9200	0.9500	0.9000	0.9700
25	0.9100	0.9400	0.9300	0.9100
50	0.9800	0.9200	0.9500	0.9300
100	0.9300	0.9600	0.9000	0.9100

Conc-%	Mean	N-Mean	Transform: Arcsin Square Root					t-Stat	1-Tailed Critical	MSD	Isotonic	
			Mean	Min	Max	CV%	N				Mean	N-Mean
FSW Control	0.9325	1.0027	1.3094	1.2661	1.3453	2.570	4				0.9350	1.0000
ASW Control	0.9300	1.0000	1.3041	1.2840	1.3453	2.214	4	*			0.9350	1.0000
1.6	0.9375	1.0081	1.3210	1.2661	1.3694	3.461	4	-0.443	2.480	0.0944	0.9350	1.0000
3.1	0.9375	1.0081	1.3256	1.2661	1.4289	5.499	4	-0.565	2.480	0.0944	0.9350	1.0000
6.3	0.9250	0.9946	1.2969	1.2490	1.3694	4.113	4	0.189	2.480	0.0944	0.9319	0.9967
12.5	0.9350	1.0054	1.3188	1.2490	1.3967	4.962	4	-0.386	2.480	0.0944	0.9319	0.9967
25	0.9225	0.9919	1.2896	1.2661	1.3233	2.203	4	0.380	2.480	0.0944	0.9319	0.9967
50	0.9450	1.0161	1.3403	1.2840	1.4289	4.802	4	-0.952	2.480	0.0944	0.9319	0.9967
100	0.9250	0.9946	1.2969	1.2490	1.3694	4.113	4	0.189	2.480	0.0944	0.9250	0.9893

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.05)	0.936483	0.93	0.618722	-0.56113
Bartlett's Test indicates equal variances (p = 0.77)	4.066401	18.47531		
The control means are not significantly different (p = 0.82)	0.240779	2.446912		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	100	>100		1	0.055339	0.059469	0.001217	0.002895	0.880021	7, 24
Treatments vs ASW Control										

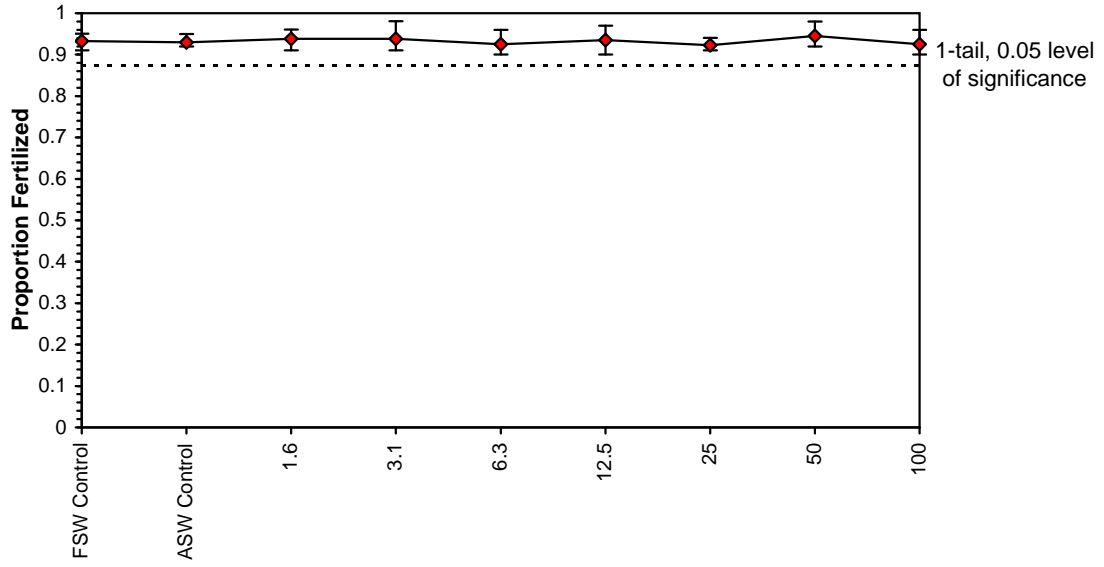
Log-Logit Interpolation (200 Resamples)				
Point	%	SD	95% CL(Exp)	Skew
IC05	>100			
IC10	>100			
IC15	>100			
IC20	>100			
IC25	>100			
IC40	>100			
IC50	>100			



**Sea Urchin Fertilisation Test-Proportion Fertilized**

Start Date: 19/11/2009 18:45    Test ID: PR0490/02    Sample ID: Brazil  
End Date: 19/11/2009 20:05    Lab ID: 3891Q    Sample Type: AQ-Aqueous  
Sample Date:    Protocol: ESA 104    Test Species: HT-Heliocidaris tuberculata  
Comments:

**Dose-Response Plot**



**Sea Urchin Fertilisation Test-Proportion Fertilized**

Start Date:	19/11/2009 18:45	Test ID:	PR0490/02	Sample ID:	Brazil
End Date:	19/11/2009 20:05	Lab ID:	3891Q	Sample Type:	AQ-Aqueous
Sample Date:		Protocol:	ESA 104	Test Species:	HT-Heliocidaris tuberculata
Comments:					

**Auxiliary Data Summary**

Conc-%	Parameter	Mean	Min	Max	SD	CV%	N
FSW Control	% Fertilised	93.25	91.00	95.00	1.71	1.40	4
ASW Control		93.00	92.00	95.00	1.41	1.28	4
1.6		93.75	91.00	96.00	2.22	1.59	4
3.1		93.75	91.00	98.00	3.10	1.88	4
6.3		92.50	90.00	96.00	2.65	1.76	4
12.5		93.50	90.00	97.00	3.11	1.89	4
25		92.25	91.00	94.00	1.50	1.33	4
50		94.50	92.00	98.00	2.65	1.72	4
100		92.50	90.00	96.00	2.65	1.76	4
FSW Control	pH	8.00	8.00	8.00	0.00	0.00	1
ASW Control		8.00	8.00	8.00	0.00	0.00	1
1.6		8.00	8.00	8.00	0.00	0.00	1
3.1		8.00	8.00	8.00	0.00	0.00	1
6.3		7.90	7.90	7.90	0.00	0.00	1
12.5		7.90	7.90	7.90	0.00	0.00	1
25		7.80	7.80	7.80	0.00	0.00	1
50		8.00	8.00	8.00	0.00	0.00	1
100		8.00	8.00	8.00	0.00	0.00	1
FSW Control	Salinity ppt	34.40	34.40	34.40	0.00	0.00	1
ASW Control		34.40	34.40	34.40	0.00	0.00	1
1.6		34.50	34.50	34.50	0.00	0.00	1
3.1		34.50	34.50	34.50	0.00	0.00	1
6.3		34.50	34.50	34.50	0.00	0.00	1
12.5		34.70	34.70	34.70	0.00	0.00	1
25		35.00	35.00	35.00	0.00	0.00	1
50		34.20	34.20	34.20	0.00	0.00	1
100		34.40	34.40	34.40	0.00	0.00	1
FSW Control	% DO	96.70	96.70	96.70	0.00	0.00	1
ASW Control		96.90	96.90	96.90	0.00	0.00	1
1.6		96.70	96.70	96.70	0.00	0.00	1
3.1		96.60	96.60	96.60	0.00	0.00	1
6.3		96.90	96.90	96.90	0.00	0.00	1
12.5		98.70	98.70	98.70	0.00	0.00	1
25		101.50	101.50	101.50	0.00	0.00	1
50		96.50	96.50	96.50	0.00	0.00	1
100		96.20	96.20	96.20	0.00	0.00	1

## **Appendix B: Statistical Print-outs for the Sea Urchin Larval Development Test**

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**Sea Urchin Larval Development Test-Proportion Normal**

Start Date: 19/11/2009 19:05    Test ID: PR0490/03    Sample ID: Brazil  
 End Date: 22/11/2009 19:05    Lab ID: 3891Q    Sample Type: AQ-Aqueous  
 Sample Date:    Protocol: ESA 105    Test Species: HT-Helicidaris tuberculata  
 Comments:

Conc-%	1	2	3	4
FSW Control	0.9500	0.9200	0.9100	0.9300
ASW Control	0.9600	0.9200	0.9400	0.9000
1.6	0.9500	0.9000	0.9100	0.9600
3.1	0.9200	0.9500	0.9300	0.9800
6.3	0.9200	0.9000	0.9300	0.9500
12.5	0.9000	0.9200	0.9700	0.9500
25	0.9600	0.9300	0.9800	0.9200
50	0.9400	0.9000	0.8900	0.9100
100	0.3500	0.5300	0.4500	0.3900

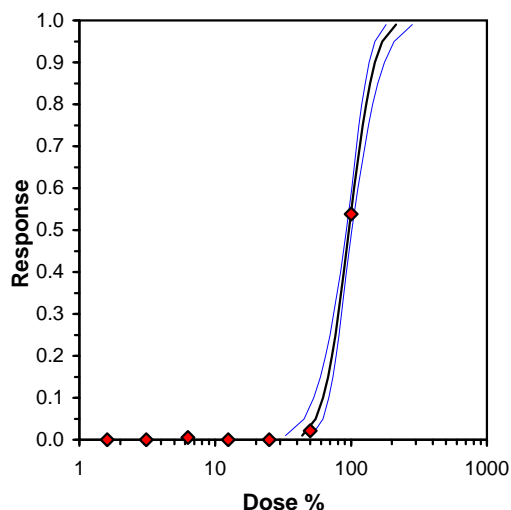
Conc-%	Mean	N-Mean	Transform: Arcsin Square Root					t-Stat	1-Tailed Critical	MSD	Number Resp	Total Number
			Mean	Min	Max	CV%	N					
FSW Control	0.9275	0.9973	1.2996	1.2661	1.3453	2.614	4					
ASW Control	0.9300	1.0000	1.3065	1.2490	1.3694	3.965	4	*			28	400
1.6	0.9300	1.0000	1.3075	1.2490	1.3694	4.502	4	-0.024	2.480	0.1045	28	400
3.1	0.9450	1.0161	1.3403	1.2840	1.4289	4.802	4	-0.804	2.480	0.1045	22	400
6.3	0.9250	0.9946	1.2954	1.2490	1.3453	3.096	4	0.264	2.480	0.1045	30	400
12.5	0.9350	1.0054	1.3188	1.2490	1.3967	4.962	4	-0.292	2.480	0.1045	26	400
25	0.9475	1.0188	1.3464	1.2840	1.4289	4.909	4	-0.947	2.480	0.1045	21	400
50	0.9100	0.9785	1.2678	1.2327	1.3233	3.111	4	0.918	2.480	0.1045	36	400
*100	0.4300	0.4624	0.7146	0.6331	0.8154	11.094	4	14.052	2.480	0.1045	228	400

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.05)	0.939977	0.93	0.35319	-1.073
Bartlett's Test indicates equal variances (p = 0.95)	2.126783	18.47531		
The control means are not significantly different (p = 0.83)	0.221103	2.446912		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test Treatments vs ASW Control	50	100	70.71068	2	0.061697	0.066217	0.180798	0.003549	7.1E-13	7, 24

Parameter	Value	SE	95% Fiducial Limits		Maximum Likelihood-Probit						
					Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter
Slope	6.733193	0.881257	5.00593	8.460456	0.07	2.493435	11.0705	0.78	1.98496	0.148518	5
Intercept	-8.36512	1.745397	-11.7861	-4.94414							
TSCR	0.064588	0.005022	0.054746	0.074431							

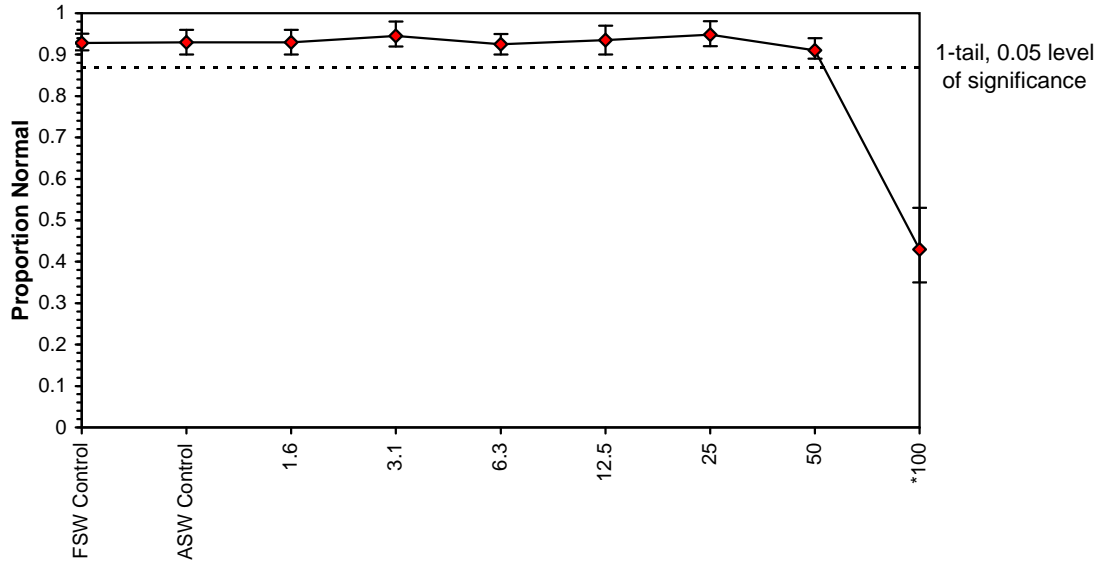
Point	Probits	%	95% Fiducial Limits	
EC01	2.674	43.5969	33.02736	51.47892
EC05	3.355	55.0389	45.10192	62.08637
EC10	3.718	62.31988	53.21121	68.65998
EC15	3.964	67.769	59.45387	73.52998
EC20	4.158	72.43762	64.89219	77.69527
EC25	4.326	76.69838	69.90024	81.51724
EC40	4.747	88.57954	83.72356	92.63754
EC50	5.000	96.5962	92.34827	101.1021
EC60	5.253	105.3384	100.6733	111.6423
EC75	5.674	121.6561	114.3107	133.8329
EC80	5.842	128.8119	119.9181	144.1803
EC85	6.036	137.6857	126.6993	157.3835
EC90	6.282	149.7247	135.6764	175.8599
EC95	6.645	169.5315	150.033	207.4912
EC99	7.326	214.025	180.9394	283.3619



**Sea Urchin Larval Development Test-Proportion Normal**

Start Date: 19/11/2009 19:05 Test ID: PR0490/03 Sample ID: Brazil  
End Date: 22/11/2009 19:05 Lab ID: 3891Q Sample Type: AQ-Aqueous  
Sample Date: Protocol: ESA 105 Test Species: HT-Heliocidaris tuberculata  
Comments:

**Dose-Response Plot**



**Sea Urchin Larval Development Test-Proportion Normal**

Start Date:	19/11/2009 19:05	Test ID:	PR0490/03	Sample ID:	Brazil
End Date:	22/11/2009 19:05	Lab ID:	3891Q	Sample Type:	AQ-Aqueous
Sample Date:		Protocol:	ESA 105	Test Species:	HT-Heliocidaris tuberculata
Comments:					

**Auxiliary Data Summary**

Conc-%	Parameter	Mean	Min	Max	SD	CV%	N
FSW Control	% Normal	92.75	91.00	95.00	1.71	1.41	4
ASW Control		93.00	90.00	96.00	2.58	1.73	4
1.6		93.00	90.00	96.00	2.94	1.84	4
3.1		94.50	92.00	98.00	2.65	1.72	4
6.3		92.50	90.00	95.00	2.08	1.56	4
12.5		93.50	90.00	97.00	3.11	1.89	4
25		94.75	92.00	98.00	2.75	1.75	4
50		91.00	89.00	94.00	2.16	1.62	4
100		43.00	35.00	53.00	7.83	6.51	4
FSW Control		pH	8.00	8.00	8.00	0.00	0.00
ASW Control	8.00		8.00	8.00	0.00	0.00	1
1.6	8.00		8.00	8.00	0.00	0.00	1
3.1	8.00		8.00	8.00	0.00	0.00	1
6.3	7.90		7.90	7.90	0.00	0.00	1
12.5	7.90		7.90	7.90	0.00	0.00	1
25	7.80		7.80	7.80	0.00	0.00	1
50	8.00		8.00	8.00	0.00	0.00	1
100	8.00		8.00	8.00	0.00	0.00	1
FSW Control	Salinity ppt		34.40	34.40	34.40	0.00	0.00
ASW Control		34.40	34.40	34.40	0.00	0.00	1
1.6		34.50	34.50	34.50	0.00	0.00	1
3.1		34.50	34.50	34.50	0.00	0.00	1
6.3		34.50	34.50	34.50	0.00	0.00	1
12.5		34.70	34.70	34.70	0.00	0.00	1
25		35.00	35.00	35.00	0.00	0.00	1
50		34.20	34.20	34.20	0.00	0.00	1
100		34.40	34.40	34.40	0.00	0.00	1
FSW Control		% DO	96.70	96.70	96.70	0.00	0.00
ASW Control	96.90		96.90	96.90	0.00	0.00	1
1.6	96.70		96.70	96.70	0.00	0.00	1
3.1	96.60		96.60	96.60	0.00	0.00	1
6.3	96.90		96.90	96.90	0.00	0.00	1
12.5	98.70		98.70	98.70	0.00	0.00	1
25	101.50		101.50	101.50	0.00	0.00	1
50	96.50		96.50	96.50	0.00	0.00	1
100	96.20		96.20	96.20	0.00	0.00	1

## **Appendix C: Statistical Print-outs for the *Hormosira banksii* Germination Success Test**

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**Macroalgal Germination Success Test-Proportion Germinated**

Start Date: 19/11/2009 22:00 Test ID: PR0490/04 Sample ID: Brazil  
 End Date: 22/11/2009 22:00 Lab ID: 3891Q Sample Type: AQ-Aqueous  
 Sample Date: Protocol: ESA 116 Test Species: HB-Hormosira banksii  
 Comments:

Conc-%	1	2	3	4
FSW Control	0.6500	0.7200	0.7900	0.8100
ASW Control	0.6200	0.8200	0.6900	0.8400
1.6	0.8400	0.9100	0.7400	0.8100
3.1	0.6900	0.8200	0.8000	0.7900
6.3	0.7200	0.8100	0.8500	0.6800
12.5	0.7300	0.7500	0.7200	0.9100
25	0.8400	0.7100	0.6800	0.6900
50	0.8900	0.6900	0.7400	0.7100
100	0.6900	0.7300	0.6800	0.6400

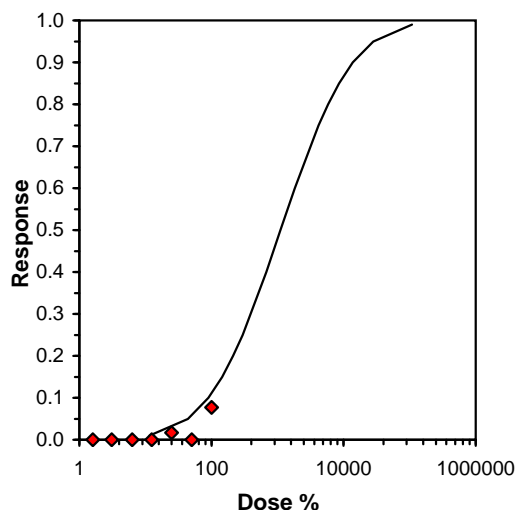
Conc-%	Mean	N-Mean	Transform: Arcsin Square Root					N	t-Stat	1-Tailed Critical	MSD	Number Resp	Total Number
			Mean	Min	Max	CV%							
FSW Control	0.7425	1.0000	1.0414	0.9377	1.1198	7.944	4						
ASW Control	0.7425	1.0000	1.0447	0.9066	1.1593	11.604	4	*			103	400	
1.6	0.8250	1.1111	1.1452	1.0357	1.2661	8.352	4	-1.474	2.480	0.1691	70	400	
3.1	0.7750	1.0438	1.0787	0.9803	1.1326	6.256	4	-0.499	2.480	0.1691	90	400	
6.3	0.7650	1.0303	1.0689	0.9695	1.1731	8.780	4	-0.355	2.480	0.1691	94	400	
12.5	0.7775	1.0471	1.0877	1.0132	1.2661	11.010	4	-0.631	2.480	0.1691	89	400	
25	0.7300	0.9832	1.0278	0.9695	1.1593	8.629	4	0.248	2.480	0.1691	108	400	
50	0.7575	1.0202	1.0627	0.9803	1.2327	10.879	4	-0.264	2.480	0.1691	97	400	
100	0.6850	0.9226	0.9754	0.9273	1.0244	4.089	4	1.017	2.480	0.1691	126	400	

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.05)	0.942139	0.93	0.567622	-0.59985
Bartlett's Test indicates equal variances (p = 0.79)	3.897174	18.47531		
The control means are not significantly different (p = 0.97)	0.045414	2.446912		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	100	>100		1	0.158101	0.211412	0.009648	0.009296	0.431593	7, 24
Treatments vs ASW Control										

Parameter	Value	SE	95% Fiducial Limits	Maximum Likelihood-Probit						
				Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter
Slope	1.171499	0.692043	-0.18491 2.527903	0.2575	8.490202	11.0705	0.13	3.046756	0.853607	4
Intercept	1.430728	1.293121	-1.10379 3.965245							
TSCR	0.222286	0.011923	0.198916 0.245655							

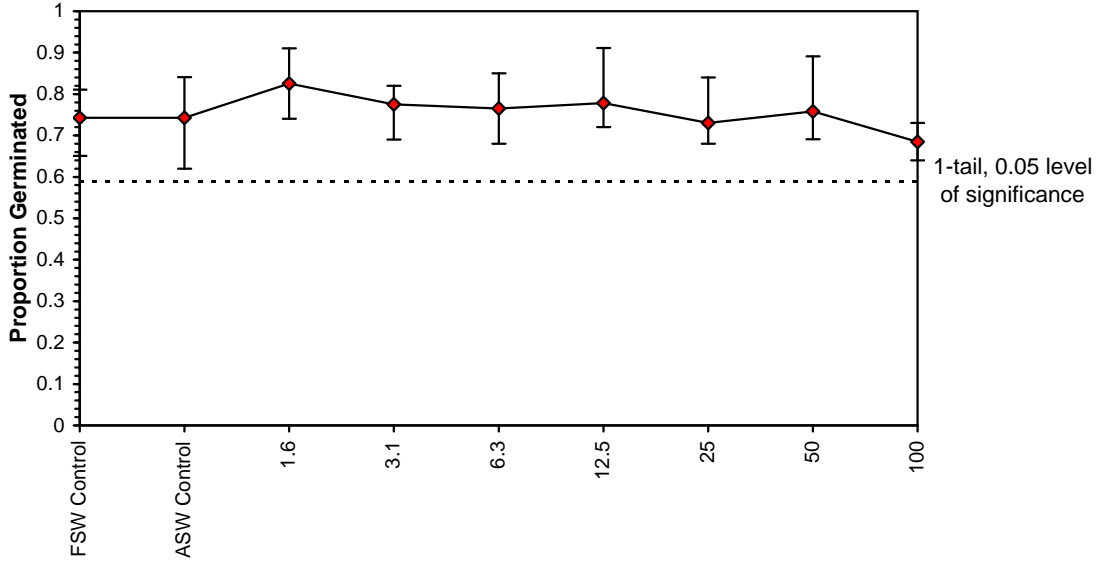
Point	Probits	%	95% Fiducial Limits
EC01	2.674	11.50719	
EC05	3.355	43.92356	
EC10	3.718	89.70462	
EC15	3.964	145.2277	
EC20	4.158	212.9818	
EC25	4.326	295.8061	
EC40	4.747	676.8578	
EC50	5.000	1113.669	
EC60	5.253	1832.379	
EC75	5.674	4192.813	
EC80	5.842	5823.313	
EC85	6.036	8540.106	
EC90	6.282	13826.04	
EC95	6.645	28236.76	
EC99	7.326	107781.4	



**Macroalgal Germination Success Test-Proportion Germinated**

Start Date: 19/11/2009 22:00 Test ID: PR0490/04 Sample ID: Brazil  
End Date: 22/11/2009 22:00 Lab ID: 3891Q Sample Type: AQ-Aqueous  
Sample Date: Protocol: ESA 116 Test Species: HB-Hormosira banksii  
Comments:

**Dose-Response Plot**



**Macroalgal Germination Success Test-Proportion Germinated**

Start Date: 19/11/2009 22:00    Test ID: PR0490/04    Sample ID: Brazil  
 End Date: 22/11/2009 22:00    Lab ID: 3891Q    Sample Type: AQ-Aqueous  
 Sample Date:    Protocol: ESA 116    Test Species: HB-Hormosira banksii  
 Comments:

**Auxiliary Data Summary**

Conc-%	Parameter	Mean	Min	Max	SD	CV%	N
FSW Control	% Germinated	74.25	65.00	81.00	7.27	3.63	4
ASW Control		74.25	62.00	84.00	10.53	4.37	4
1.6		82.50	74.00	91.00	7.05	3.22	4
3.1		77.50	69.00	82.00	5.80	3.11	4
6.3		76.50	68.00	85.00	7.85	3.66	4
12.5		77.75	72.00	91.00	8.92	3.84	4
25		73.00	68.00	84.00	7.44	3.74	4
50		75.75	69.00	89.00	9.07	3.98	4
100		68.50	64.00	73.00	3.70	2.81	4
FSW Control	pH	8.00	8.00	8.00	0.00	0.00	1
ASW Control		8.00	8.00	8.00	0.00	0.00	1
1.6		8.00	8.00	8.00	0.00	0.00	1
3.1		8.00	8.00	8.00	0.00	0.00	1
6.3		7.90	7.90	7.90	0.00	0.00	1
12.5		7.90	7.90	7.90	0.00	0.00	1
25		7.80	7.80	7.80	0.00	0.00	1
50		8.00	8.00	8.00	0.00	0.00	1
100		8.00	8.00	8.00	0.00	0.00	1
FSW Control	Salinity ppt	34.40	34.40	34.40	0.00	0.00	1
ASW Control		34.40	34.40	34.40	0.00	0.00	1
1.6		34.50	34.50	34.50	0.00	0.00	1
3.1		34.50	34.50	34.50	0.00	0.00	1
6.3		34.50	34.50	34.50	0.00	0.00	1
12.5		34.70	34.70	34.70	0.00	0.00	1
25		35.00	35.00	35.00	0.00	0.00	1
50		34.20	34.20	34.20	0.00	0.00	1
100		34.40	34.40	34.40	0.00	0.00	1
FSW Control	% DO	96.70	96.70	96.70	0.00	0.00	1
ASW Control		96.90	96.90	96.90	0.00	0.00	1
1.6		96.70	96.70	96.70	0.00	0.00	1
3.1		96.60	96.60	96.60	0.00	0.00	1
6.3		96.90	96.90	96.90	0.00	0.00	1
12.5		98.70	98.70	98.70	0.00	0.00	1
25		101.50	101.50	101.50	0.00	0.00	1
50		96.50	96.50	96.50	0.00	0.00	1
100		96.20	96.20	96.20	0.00	0.00	1

## **Appendix D: Statistical Print-outs for the Doughboy Scallop Larval Development Test**

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**Bivalve Larval Development Test-Proportion Normal**

Start Date:	19/11/2009 18:00	Test ID:	PR0490/01	Sample ID:	Brazil
End Date:	21/11/2009 18:00	Lab ID:	3891Q	Sample Type:	AQ-Aqueous
Sample Date:		Protocol:	ESA 106	Test Species:	MA-Mimachlamys asperrima
Comments:					

Conc-%	1	2	3	4
FSW Control	0.7900	0.8400	0.7600	0.8000
ASW Control	0.8300	0.7200	0.7700	0.7800
1.6	0.7900	0.7600	0.8400	0.7200
3.1	0.7700	0.7900	0.8000	0.7600
6.3	0.7200	0.7500	0.7900	0.8200
12.5	0.7600	0.7900	0.8000	0.7300
25	0.8100	0.7200	0.7500	0.7900
50	0.7400	0.7600	0.7300	0.7600
100	0.5300	0.4600	0.6400	0.6800

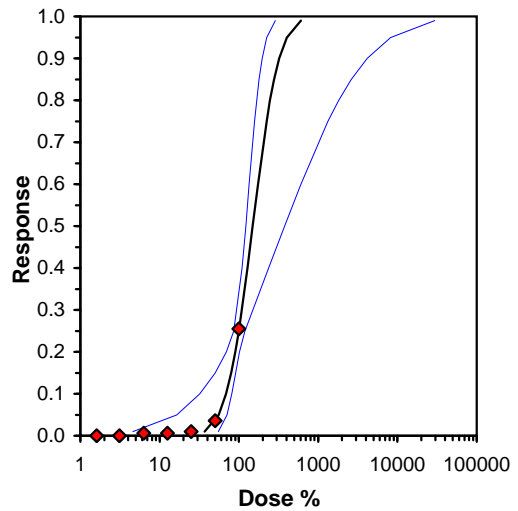
Conc-%	Mean	N-Mean	Transform: Arcsin Square Root					t-Stat	1-Tailed Critical	MSD	Number Resp	Total Number
			Mean	Min	Max	CV%	N					
FSW Control	0.7975	1.0290	1.1050	1.0588	1.1593	3.763	4					
ASW Control	0.7750	1.0000	1.0781	1.0132	1.1458	5.045	4	*			90	400
1.6	0.7775	1.0032	1.0815	1.0132	1.1593	5.701	4	-0.088	2.480	0.0970	89	400
3.1	0.7800	1.0065	1.0828	1.0588	1.1071	2.037	4	-0.122	2.480	0.0970	88	400
6.3	0.7700	0.9935	1.0720	1.0132	1.1326	4.898	4	0.156	2.480	0.0970	92	400
12.5	0.7700	0.9935	1.0713	1.0244	1.1071	3.489	4	0.173	2.480	0.0970	92	400
25	0.7675	0.9903	1.0687	1.0132	1.1198	4.465	4	0.238	2.480	0.0970	93	400
50	0.7475	0.9645	1.0444	1.0244	1.0588	1.650	4	0.859	2.480	0.0970	101	400
*100	0.5775	0.7452	0.8644	0.7454	0.9695	11.869	4	5.460	2.480	0.0970	169	400

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.05)	0.988523	0.93	-0.08206	0.118206
Bartlett's Test indicates equal variances (p = 0.16)	10.51495	18.47531		
The control means are not significantly different (p = 0.46)	0.78732	2.446912		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	50	100	70.71068	2	0.08557	0.110238	0.021973	0.003062	1.1E-04	7, 24
Treatments vs ASW Control										

Parameter	Value	SE	95% Fiducial Limits	Maximum Likelihood-Probit						
				Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter
Slope	3.828064	1.328309	1.224577 6.43155	0.225	0.244132	11.0705	1	2.17343	0.261229	6
Intercept	-3.32003	2.630815	-8.47643 1.836368							
TSCR	0.226374	0.008715	0.209292 0.243455							

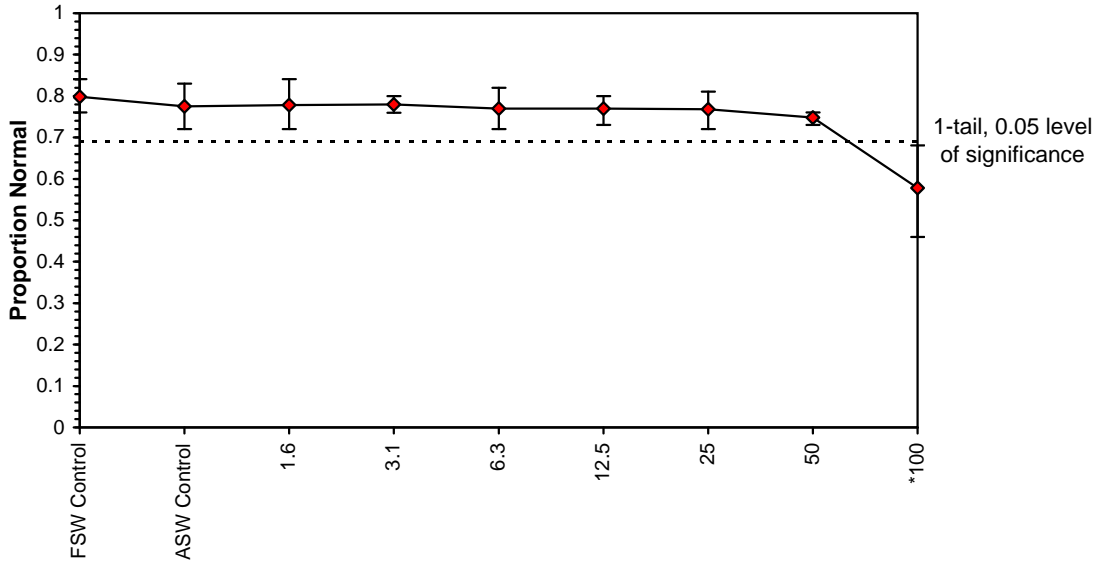
Point	Probits	%	95% Fiducial Limits
EC01	2.674	36.78928	4.607301 55.12167
EC05	3.355	55.43029	16.46651 70.89936
EC10	3.718	68.96871	32.24195 81.65553
EC15	3.964	79.92525	50.14302 90.88114
EC20	4.158	89.86168	69.30519 101.6936
EC25	4.326	99.36514	85.75424 119.4734
EC40	4.747	128.0114	110.4248 238.1493
EC50	5.000	149.0837	122.4104 378.7687
EC60	5.253	173.6248	134.864 606.1403
EC75	5.674	223.6795	157.6604 1330.871
EC80	5.842	247.3351	167.6073 1819.838
EC85	6.036	278.0842	179.9387 2621.66
EC90	6.282	322.2613	196.6839 4151.541
EC95	6.645	400.9711	224.3071 8209.148
EC99	7.326	604.142	286.7662 29518.11



**Bivalve Larval Development Test-Proportion Normal**

Start Date: 19/11/2009 18:00 Test ID: PR0490/01 Sample ID: Brazil  
End Date: 21/11/2009 18:00 Lab ID: 3891Q Sample Type: AQ-Aqueous  
Sample Date: Protocol: ESA 106 Test Species: MA-Mimachlamys asperrima  
Comments:

**Dose-Response Plot**



**Bivalve Larval Development Test-Proportion Normal**

Start Date:	19/11/2009 18:00	Test ID:	PR0490/01	Sample ID:	Brazil
End Date:	21/11/2009 18:00	Lab ID:	3891Q	Sample Type:	AQ-Aqueous
Sample Date:		Protocol:	ESA 106	Test Species:	MA-Mimachlamys asperrima
Comments:					

**Auxiliary Data Summary**

Conc-%	Parameter	Mean	Min	Max	SD	CV%	N
FSW Control	% Normal	79.75	76.00	84.00	3.30	2.28	4
ASW Control		77.50	72.00	83.00	4.51	2.74	4
1.6		77.75	72.00	84.00	5.06	2.89	4
3.1		78.00	76.00	80.00	1.83	1.73	4
6.3		77.00	72.00	82.00	4.40	2.72	4
12.5		77.00	73.00	80.00	3.16	2.31	4
25		76.75	72.00	81.00	4.03	2.62	4
50		74.75	73.00	76.00	1.50	1.64	4
100		57.75	46.00	68.00	10.08	5.50	4
FSW Control	pH	8.00	8.00	8.00	0.00	0.00	1
ASW Control		8.00	8.00	8.00	0.00	0.00	1
1.6		8.00	8.00	8.00	0.00	0.00	1
3.1		8.00	8.00	8.00	0.00	0.00	1
6.3		7.90	7.90	7.90	0.00	0.00	1
12.5		7.90	7.90	7.90	0.00	0.00	1
25		7.80	7.80	7.80	0.00	0.00	1
50		8.00	8.00	8.00	0.00	0.00	1
100		8.00	8.00	8.00	0.00	0.00	1
FSW Control	Salinity ppt	34.40	34.40	34.40	0.00	0.00	1
ASW Control		34.40	34.40	34.40	0.00	0.00	1
1.6		34.50	34.50	34.50	0.00	0.00	1
3.1		34.50	34.50	34.50	0.00	0.00	1
6.3		34.50	34.50	34.50	0.00	0.00	1
12.5		34.70	34.70	34.70	0.00	0.00	1
25		35.00	35.00	35.00	0.00	0.00	1
50		34.20	34.20	34.20	0.00	0.00	1
100		34.40	34.40	34.40	0.00	0.00	1
FSW Control	% DO	96.70	96.70	96.70	0.00	0.00	1
ASW Control		96.90	96.90	96.90	0.00	0.00	1
1.6		96.70	96.70	96.70	0.00	0.00	1
3.1		96.60	96.60	96.60	0.00	0.00	1
6.3		96.90	96.90	96.90	0.00	0.00	1
12.5		98.70	98.70	98.70	0.00	0.00	1
25		101.50	101.50	101.50	0.00	0.00	1
50		96.50	96.50	96.50	0.00	0.00	1
100		96.20	96.20	96.20	0.00	0.00	1

## **Appendix E: Statistical Print-outs for the Acute Amphipod Toxicity Test**

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**Amphipod Acute Toxicity Test-96-h survival**

Start Date: 19/11/2009 19:00	Test ID: PR0490/06	Sample ID: Brazil
End Date: 23/11/2009 19:00	Lab ID: 3891Q	Sample Type: AQ-Aqueous
Sample Date:	Protocol: ESA 108	Test Species: AC-Allorchestes compressa

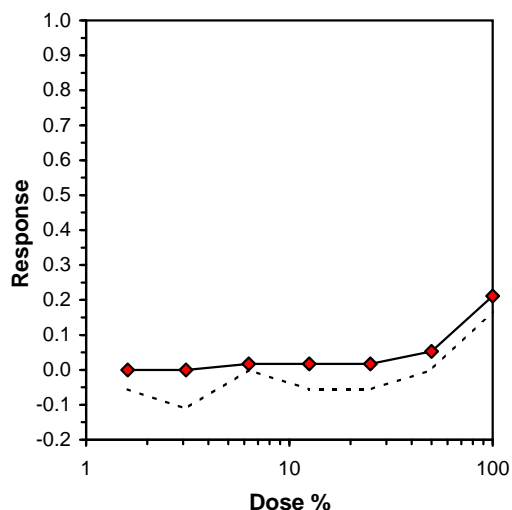
Conc-%	1	2	3	4
FSW Control	1.0000	0.8000	1.0000	1.0000
ASW Control	1.0000	0.8000	0.8000	1.0000
1.6	1.0000	1.0000	1.0000	0.8000
3.1	1.0000	1.0000	1.0000	1.0000
6.3	0.8000	0.8000	1.0000	1.0000
12.5	1.0000	0.8000	1.0000	1.0000
25	0.8000	1.0000	1.0000	1.0000
50	0.8000	0.8000	1.0000	1.0000
100	1.0000	0.8000	0.4000	0.8000

Conc-%	Mean	N-Mean	Transform: Arcsin Square Root					t-Stat	1-Tailed Critical	MSD	Isotonic	
			Mean	Min	Max	CV%	N				Mean	N-Mean
FSW Control	0.9500	1.0556	1.2857	1.1071	1.3453	9.261	4					
ASW Control	0.9000	1.0000	1.2262	1.1071	1.3453	11.212	4	*			0.9500	1.0000
1.6	0.9500	1.0556	1.2857	1.1071	1.3453	9.261	4	-0.570	2.480	0.2592	0.9500	1.0000
3.1	1.0000	1.1111	1.3453	1.3453	1.3453	0.000	4	-1.139	2.480	0.2592	0.9500	1.0000
6.3	0.9000	1.0000	1.2262	1.1071	1.3453	11.212	4	0.000	2.480	0.2592	0.9333	0.9825
12.5	0.9500	1.0556	1.2857	1.1071	1.3453	9.261	4	-0.570	2.480	0.2592	0.9333	0.9825
25	0.9500	1.0556	1.2857	1.1071	1.3453	9.261	4	-0.570	2.480	0.2592	0.9333	0.9825
50	0.9000	1.0000	1.2262	1.1071	1.3453	11.212	4	0.000	2.480	0.2592	0.9000	0.9474
100	0.7500	0.8333	1.0611	0.6847	1.3453	25.905	4	1.580	2.480	0.2592	0.7500	0.7895

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.05)	0.910258	0.93	-0.70925	1.088879
Equality of variance cannot be confirmed				
The control means are not significantly different (p = 0.54)	0.654654	2.446912		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	100	>100		1	0.20825	0.235075	0.028506	0.021849	0.290499	7, 24

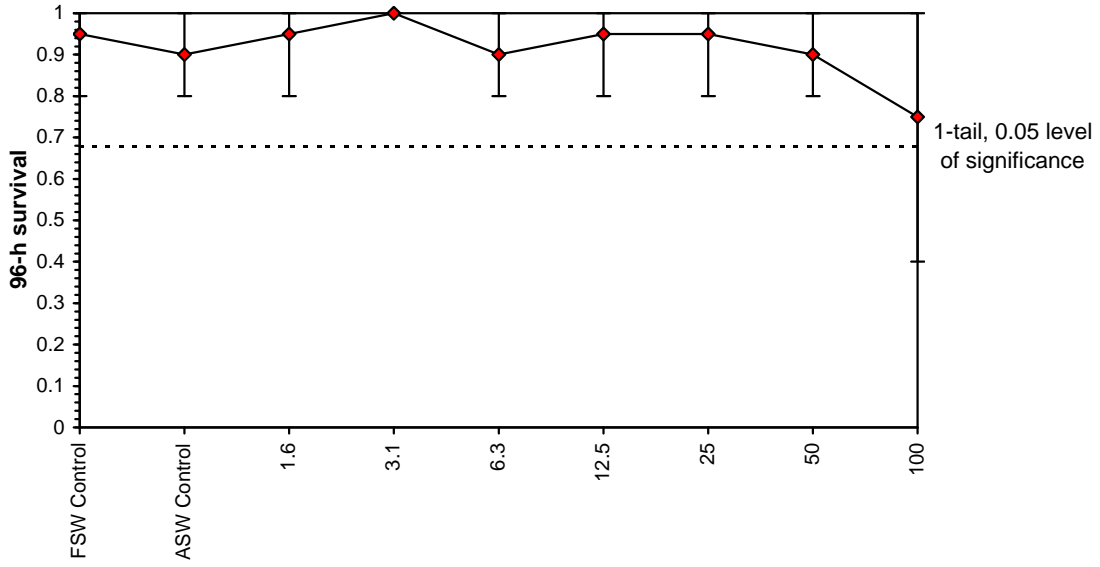
Log-Logit Interpolation (200 Resamples)				
Point	%	SD	95% CL(Exp)	Skew
IC05	47.862			
IC10	65.342			
IC15	80.992			
IC20	96.660			
IC25	>100			
IC40	>100			
IC50	>100			



**Amphipod Acute Toxicity Test-96-h survival**

Start Date: 19/11/2009 19:00 Test ID: PR0490/06 Sample ID: Brazil  
End Date: 23/11/2009 19:00 Lab ID: 3891Q Sample Type: AQ-Aqueous  
Sample Date: Protocol: ESA 108 Test Species: AC-Allorchestes compressa  
Comments:

**Dose-Response Plot**



**Amphipod Acute Toxicity Test-96-h survival**

Start Date:	19/11/2009 19:00	Test ID:	PR0490/06	Sample ID:	Brazil
End Date:	23/11/2009 19:00	Lab ID:	3891Q	Sample Type:	AQ-Aqueous
Sample Date:		Protocol:	ESA 108	Test Species:	AC-Allorchestes compressa
Comments:					

**Auxiliary Data Summary**

Conc-%	Parameter	Mean	Min	Max	SD	CV%	N
FSW Control	% Survival	95.00	80.00	100.00	10.00	3.33	4
ASW Control		90.00	80.00	100.00	11.55	3.78	4
1.6		95.00	80.00	100.00	10.00	3.33	4
3.1		100.00	100.00	100.00	0.00	0.00	4
6.3		90.00	80.00	100.00	11.55	3.78	4
12.5		95.00	80.00	100.00	10.00	3.33	4
25		95.00	80.00	100.00	10.00	3.33	4
50		90.00	80.00	100.00	11.55	3.78	4
100		75.00	40.00	100.00	25.17	6.69	4
FSW Control	pH	8.00	8.00	8.00	0.00	0.00	1
ASW Control		8.00	8.00	8.00	0.00	0.00	1
1.6		8.00	8.00	8.00	0.00	0.00	1
3.1		8.00	8.00	8.00	0.00	0.00	1
6.3		7.90	7.90	7.90	0.00	0.00	1
12.5		7.90	7.90	7.90	0.00	0.00	1
25		7.80	7.80	7.80	0.00	0.00	1
50		8.00	8.00	8.00	0.00	0.00	1
100		8.00	8.00	8.00	0.00	0.00	1
FSW Control	Salinity ppt	34.40	34.40	34.40	0.00	0.00	1
ASW Control		34.40	34.40	34.40	0.00	0.00	1
1.6		34.50	34.50	34.50	0.00	0.00	1
3.1		34.50	34.50	34.50	0.00	0.00	1
6.3		34.50	34.50	34.50	0.00	0.00	1
12.5		34.70	34.70	34.70	0.00	0.00	1
25		35.00	35.00	35.00	0.00	0.00	1
50		34.20	34.20	34.20	0.00	0.00	1
100		34.40	34.40	34.40	0.00	0.00	1
FSW Control	% DO	96.70	96.70	96.70	0.00	0.00	1
ASW Control		96.90	96.90	96.90	0.00	0.00	1
1.6		96.70	96.70	96.70	0.00	0.00	1
3.1		96.60	96.60	96.60	0.00	0.00	1
6.3		96.90	96.90	96.90	0.00	0.00	1
12.5		98.70	98.70	98.70	0.00	0.00	1
25		101.50	101.50	101.50	0.00	0.00	1
50		96.50	96.50	96.50	0.00	0.00	1
100		96.20	96.20	96.20	0.00	0.00	1

## **Appendix F: Statistical Print-outs for the Fish Imbalance Test**

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**Fish Imbalance Test-96-hr Imbalanced**

Start Date: 19/11/2009 19:00	Test ID: PR0490/07	Sample ID: Brazil
End Date: 23/11/2009 19:00	Lab ID: 3891Q	Sample Type: AQ-Aqueous
Sample Date:	Protocol: ESA 117	Test Species: PA-Pagrus auratus

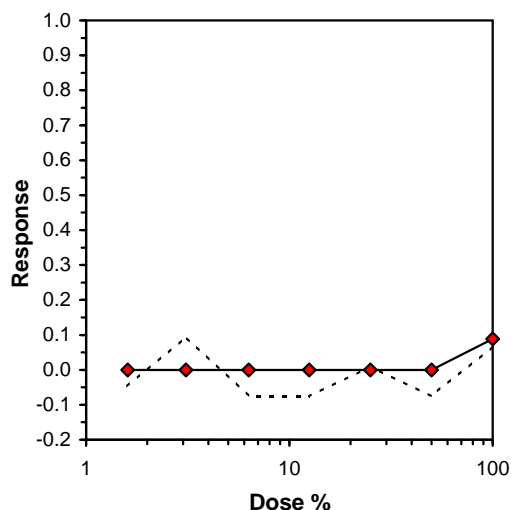
Conc-%	1	2	3	4
FSW Control	1.0000	1.0000	1.0000	0.9000
ASW Control	1.0000	0.8000	1.0000	0.7692
1.6	0.8000	1.0000	1.0000	0.9000
3.1	0.8000	0.5000	1.0000	0.9000
6.3	0.9000	1.0000	0.9000	1.0000
12.5	0.8000	1.0000	1.0000	1.0000
25	0.8000	0.9000	0.9000	0.9000
50	1.0000	1.0000	0.8000	1.0000
100	1.0000	0.7000	0.6000	1.0000

Conc-%	Mean	N-Mean	Transform: Arcsin Square Root					t-Stat	1-Tailed		Isotonic	
			Mean	Min	Max	CV%	N		Critical	MSD	Mean	N-Mean
FSW Control	0.9750	1.0927	1.3713	1.2490	1.4120	5.942	4					
ASW Control	0.8923	1.0000	1.2502	1.0697	1.4120	14.993	4	*			0.9048	1.0000
1.6	0.9250	1.0366	1.2951	1.1071	1.4120	11.347	4	-0.349	2.480	0.3189	0.9048	1.0000
3.1	0.8000	0.8966	1.1384	0.7854	1.4120	23.390	4	0.870	2.480	0.3189	0.9048	1.0000
6.3	0.9500	1.0647	1.3305	1.2490	1.4120	7.072	4	-0.625	2.480	0.3189	0.9048	1.0000
12.5	0.9500	1.0647	1.3358	1.1071	1.4120	11.411	4	-0.666	2.480	0.3189	0.9048	1.0000
25	0.8750	0.9806	1.2136	1.1071	1.2490	5.846	4	0.285	2.480	0.3189	0.9048	1.0000
50	0.9500	1.0647	1.3358	1.1071	1.4120	11.411	4	-0.666	2.480	0.3189	0.9048	1.0000
100	0.8250	0.9246	1.1753	0.8861	1.4120	23.539	4	0.583	2.480	0.3189	0.8250	0.9118

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.05)	0.947148	0.93	-0.43932	-0.55556
Bartlett's Test indicates equal variances (p = 0.42)	7.117376	18.47531		
The control means are not significantly different (p = 0.28)	1.184499	2.446912		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	100	>100		1	0.256827	0.285141	0.023943	0.033067	0.653102	7, 24

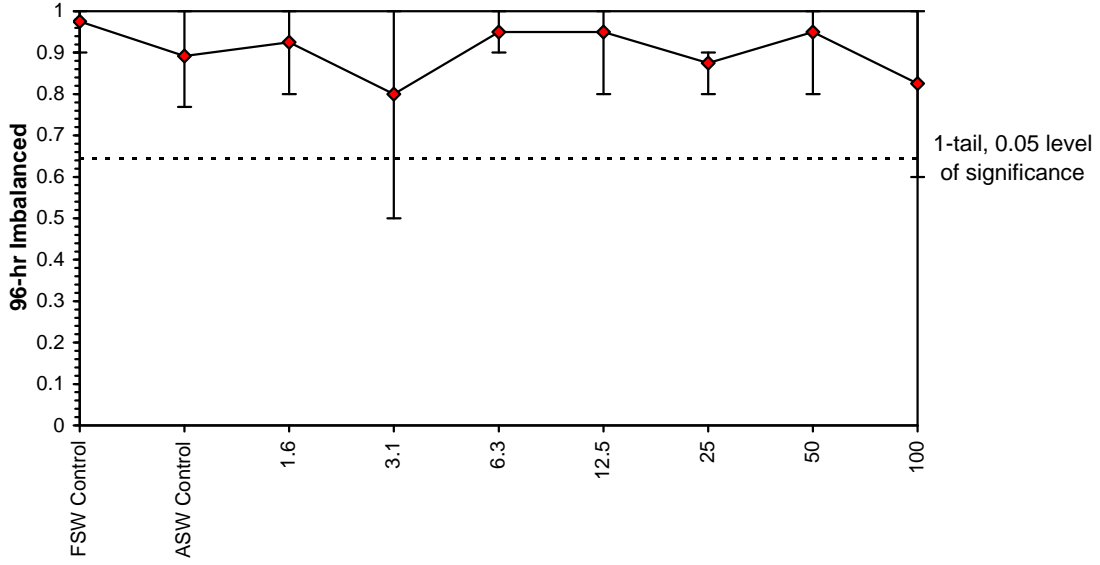
Log-Logit Interpolation (200 Resamples)				
Point	%	SD	95% CL(Exp)	Skew
IC05	77.309			
IC10	>100			
IC15	>100			
IC20	>100			
IC25	>100			
IC40	>100			
IC50	>100			



**Fish Imbalance Test-96-hr Imbalanced**

Start Date: 19/11/2009 19:00 Test ID: PR0490/07 Sample ID: Brazil  
End Date: 23/11/2009 19:00 Lab ID: 3891Q Sample Type: AQ-Aqueous  
Sample Date: Protocol: ESA 117 Test Species: PA-Pagrus auratus  
Comments:

**Dose-Response Plot**



**Fish Imbalance Test-96-hr Imbalanced**

Start Date:	19/11/2009 19:00	Test ID:	PR0490/07	Sample ID:	Brazil
End Date:	23/11/2009 19:00	Lab ID:	3891Q	Sample Type:	AQ-Aqueous
Sample Date:		Protocol:	ESA 117	Test Species:	PA-Pagrus auratus
Comments:					

**Auxiliary Data Summary**

Conc-%	Parameter	Mean	Min	Max	SD	CV%	N
FSW Control	% un-affected	97.50	90.00	100.00	5.00	2.29	4
ASW Control		89.23	76.92	100.00	12.50	3.96	4
1.6		92.50	80.00	100.00	9.57	3.35	4
3.1		80.00	50.00	100.00	21.60	5.81	4
6.3		95.00	90.00	100.00	5.77	2.53	4
12.5		95.00	80.00	100.00	10.00	3.33	4
25		87.50	80.00	90.00	5.00	2.56	4
50		95.00	80.00	100.00	10.00	3.33	4
100		82.50	60.00	100.00	20.62	5.50	4
FSW Control		pH	8.00	8.00	8.00	0.00	0.00
ASW Control	8.00		8.00	8.00	0.00	0.00	1
1.6	8.00		8.00	8.00	0.00	0.00	1
3.1	8.00		8.00	8.00	0.00	0.00	1
6.3	7.90		7.90	7.90	0.00	0.00	1
12.5	7.90		7.90	7.90	0.00	0.00	1
25	7.80		7.80	7.80	0.00	0.00	1
50	8.00		8.00	8.00	0.00	0.00	1
100	8.00		8.00	8.00	0.00	0.00	1
FSW Control	Salinity ppt		34.40	34.40	34.40	0.00	0.00
ASW Control		34.40	34.40	34.40	0.00	0.00	1
1.6		34.50	34.50	34.50	0.00	0.00	1
3.1		34.50	34.50	34.50	0.00	0.00	1
6.3		34.50	34.50	34.50	0.00	0.00	1
12.5		34.70	34.70	34.70	0.00	0.00	1
25		35.00	35.00	35.00	0.00	0.00	1
50		34.20	34.20	34.20	0.00	0.00	1
100		34.40	34.40	34.40	0.00	0.00	1
FSW Control		% DO	96.70	96.70	96.70	0.00	0.00
ASW Control	96.90		96.90	96.90	0.00	0.00	1
1.6	96.70		96.70	96.70	0.00	0.00	1
3.1	96.60		96.60	96.60	0.00	0.00	1
6.3	96.90		96.90	96.90	0.00	0.00	1
12.5	98.70		98.70	98.70	0.00	0.00	1
25	101.50		101.50	101.50	0.00	0.00	1
50	96.50		96.50	96.50	0.00	0.00	1
100	96.20		96.20	96.20	0.00	0.00	1

## **Appendix G: Statistical Print-outs for the *Nitzschia closterium* Growth Inhibition Test**

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**Microalgal Cell Yield-Growth-Cell Yield**

Start Date: 20/11/2009 17:30    Test ID: PR0490/16    Sample ID: BRAZIL  
 End Date: 23/11/2009 17:30    Lab ID: 3891Q    Sample Type: AQ-Aqueous  
 Sample Date:    Protocol: ESA 110    Test Species: NC-Nitzschia closterium  
 Comments:

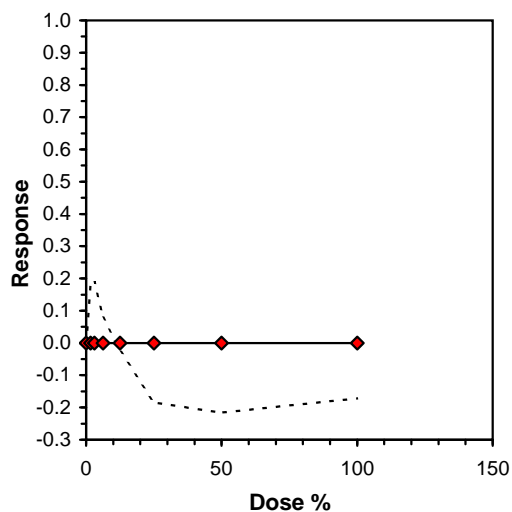
Conc-%	1	2	3	4
FSW Control	409600	409600	338500	406000
ASW Control	45300	49600	49600	51000
1.6	333400	309700	299700	329100
3.1	283100	311900	342800	333400
6.3	358600	357200	371500	354300
12.5	330600	382300	463500	426100
25	464200	448400	453400	485100
50	444800	443400	484300	528900
100	481500	462100	410300	477100

Conc-%	Transform: Untransformed							1-Tailed		Isotonic		
	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Mean	N-Mean
FSW Control	390925	7.9985	390925	338500	409600	8.951	4	*			397950	1.0000
ASW Control	48875	1.0000	48875	45300	51000	5.060	4					
*1.6	317975	6.5059	317975	299700	333400	5.019	4	3.167	2.480	57122.71	397950	1.0000
*3.1	317800	6.5023	317800	283100	342800	8.340	4	3.175	2.480	57122.71	397950	1.0000
6.3	360400	7.3739	360400	354300	371500	2.113	4	1.325	2.480	57122.71	397950	1.0000
12.5	400625	8.1969	400625	330600	463500	14.297	4	-0.421	2.480	57122.71	397950	1.0000
25	462775	9.4685	462775	448400	485100	3.518	4	-3.119	2.480	57122.71	397950	1.0000
50	475350	9.7258	475350	443400	528900	8.504	4	-3.665	2.480	57122.71	397950	1.0000
100	457750	9.3657	457750	410300	481500	7.145	4	-2.901	2.480	57122.71	397950	1.0000

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.05)	0.969154	0.93	-0.32264	0.523242
Bartlett's Test indicates equal variances (p = 0.11)	11.7927	18.47531		
The control means are significantly different (p = 1.18E-06)	19.502	2.446912		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test Treatments vs FSW Control	100	>100		1	57122.71	0.146122	1.6E+10	1.06E+09	2.1E-07	7, 24

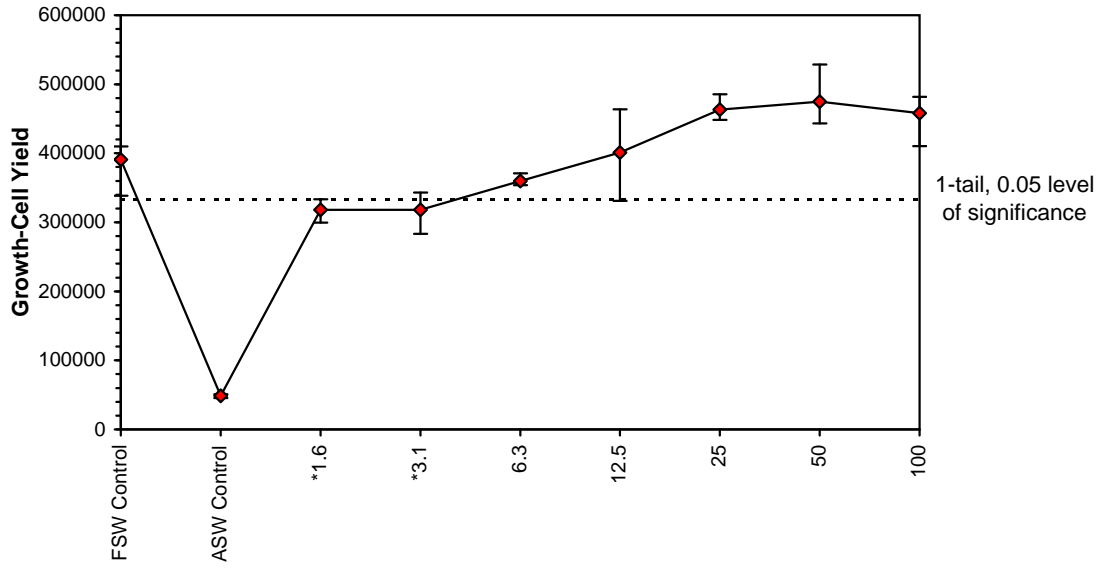
Linear Interpolation (200 Resamples)				
Point	%	SD	95% CL(Exp)	Skew
IC05	>100			
IC10	>100			
IC15	>100			
IC20	>100			
IC25	>100			
IC40	>100			
IC50	>100			



**Microalgal Cell Yield-Growth-Cell Yield**

Start Date: 20/11/2009 17:30 Test ID: PR0490/16 Sample ID: BRAZIL  
End Date: 23/11/2009 17:30 Lab ID: 3891Q Sample Type: AQ-Aqueous  
Sample Date: Protocol: ESA 110 Test Species: NC-Nitzschia closterium  
Comments:

**Dose-Response Plot**



**Microalgal Cell Yield-Growth-Cell Yield**

Start Date: 20/11/2009 17:30	Test ID: PR0490/16	Sample ID: BRAZIL
End Date: 23/11/2009 17:30	Lab ID: 3891Q	Sample Type: AQ-Aqueous
Sample Date:	Protocol: ESA 110	Test Species: NC-Nitzschia closterium

Comments:

**Auxiliary Data Summary**

Conc-%	Parameter	Mean	Min	Max	SD	CV%	N
FSW Control	Cell Yield	39.09	33.85	40.96	3.50	4.79	4
ASW Control		4.89	4.53	5.10	0.25	10.17	4
1.6		31.80	29.97	33.34	1.60	3.97	4
3.1		31.78	28.31	34.28	2.65	5.12	4
6.3		36.04	35.43	37.15	0.76	2.42	4
12.5		40.06	33.06	46.35	5.73	5.97	4
25		46.28	44.84	48.51	1.63	2.76	4
50		47.54	44.34	52.89	4.04	4.23	4
100		45.78	41.03	48.15	3.27	3.95	4
FSW Control	pH	8.00	8.00	8.00	0.00	0.00	1
ASW Control		8.00	8.00	8.00	0.00	0.00	1
1.6		8.00	8.00	8.00	0.00	0.00	1
3.1		8.00	8.00	8.00	0.00	0.00	1
6.3		8.00	8.00	8.00	0.00	0.00	1
12.5		8.00	8.00	8.00	0.00	0.00	1
25		8.00	8.00	8.00	0.00	0.00	1
50		7.90	7.90	7.90	0.00	0.00	1
100		7.90	7.90	7.90	0.00	0.00	1
FSW Control	Salinity ppt	34.30	34.30	34.30	0.00	0.00	1
ASW Control		34.50	34.50	34.50	0.00	0.00	1
1.6		34.50	34.50	34.50	0.00	0.00	1
3.1		34.50	34.50	34.50	0.00	0.00	1
6.3		34.40	34.40	34.40	0.00	0.00	1
12.5		34.60	34.60	34.60	0.00	0.00	1
25		34.50	34.50	34.50	0.00	0.00	1
50		34.50	34.50	34.50	0.00	0.00	1
100		34.50	34.50	34.50	0.00	0.00	1

## **Appendix H: Statistical Print-outs for the Microtox<sup>®</sup> Acute Toxicity Test**

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**Microtox Acute Toxicity Test-5 minutes**

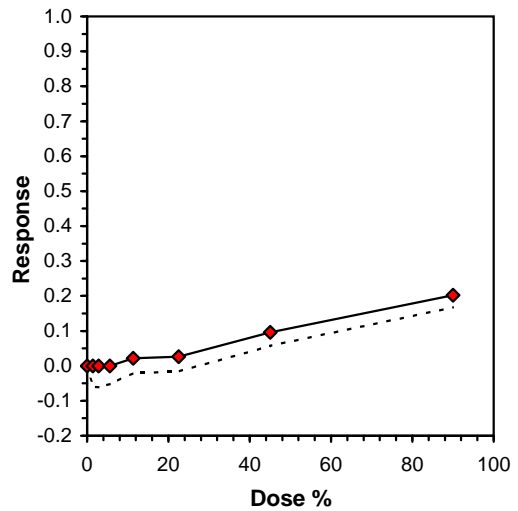
Start Date:	20/11/2009 14:50	Test ID:	PR0490/21	Sample ID:	Brazil
End Date:	20/11/2009 14:55	Lab ID:	3891Q	Sample Type:	AQ-Aqueous
Sample Date:		Protocol:	ESA 119	Test Species:	VF-Vibrio fischerii

Conc-%	1	2
FSW Control	1.1158	1.1348
ASW Control	1.0562	1.0899
1.4	1.1364	1.1364
2.8	1.1250	1.1512
5.6	1.1446	1.1124
11.3	1.1000	1.0899
22.5	1.0920	1.0879
45	1.0225	1.0000
90	0.8876	0.8977

Conc-%	Mean	N-Mean	Transform: Untransformed				N	t-Stat	1-Tailed		Isotonic	
			Mean	Min	Max	CV%			Critical	MSD	Mean	N-Mean
FSW Control	1.1253	1.0487	1.1253	1.1158	1.1348	1.197	2					
ASW Control	1.0730	1.0000	1.0730	1.0562	1.0899	2.221	2	*			1.1190	1.0000
1.4	1.1364	1.0590	1.1364	1.1364	1.1364	0.000	2	-4.231	2.810	0.0421	1.1190	1.0000
2.8	1.1381	1.0606	1.1381	1.1250	1.1512	1.626	2	-4.346	2.810	0.0421	1.1190	1.0000
5.6	1.1285	1.0517	1.1285	1.1124	1.1446	2.019	2	-3.704	2.810	0.0421	1.1190	1.0000
11.3	1.0949	1.0204	1.0949	1.0899	1.1000	0.653	2	-1.464	2.810	0.0421	1.0949	0.9785
22.5	1.0899	1.0157	1.0899	1.0879	1.0920	0.262	2	-1.129	2.810	0.0421	1.0899	0.9740
*45	1.0112	0.9424	1.0112	1.0000	1.0225	1.571	2	4.129	2.810	0.0421	1.0112	0.9037
*90	0.8927	0.8319	0.8927	0.8876	0.8977	0.799	2	12.050	2.810	0.0421	0.8927	0.7978

Auxiliary Tests	Statistic	Critical	Skew	Kurt						
Normality of the data set cannot be confirmed										
Equality of variance cannot be confirmed										
The control means are not significantly different (p = 0.11)	2.700625	4.302653								
<b>Hypothesis Test (1-tail, 0.05)</b>	<b>NOEC</b>	<b>LOEC</b>	<b>ChV</b>	<b>TU</b>	<b>MSDu</b>	<b>MSDp</b>	<b>MSB</b>	<b>MSE</b>	<b>F-Prob</b>	<b>df</b>
Dunnett's Test	22.5	45	31.81981	4.444444	0.042057	0.039194	0.013822	0.000224	2.5E-06	7, 8
Treatments vs ASW Control										

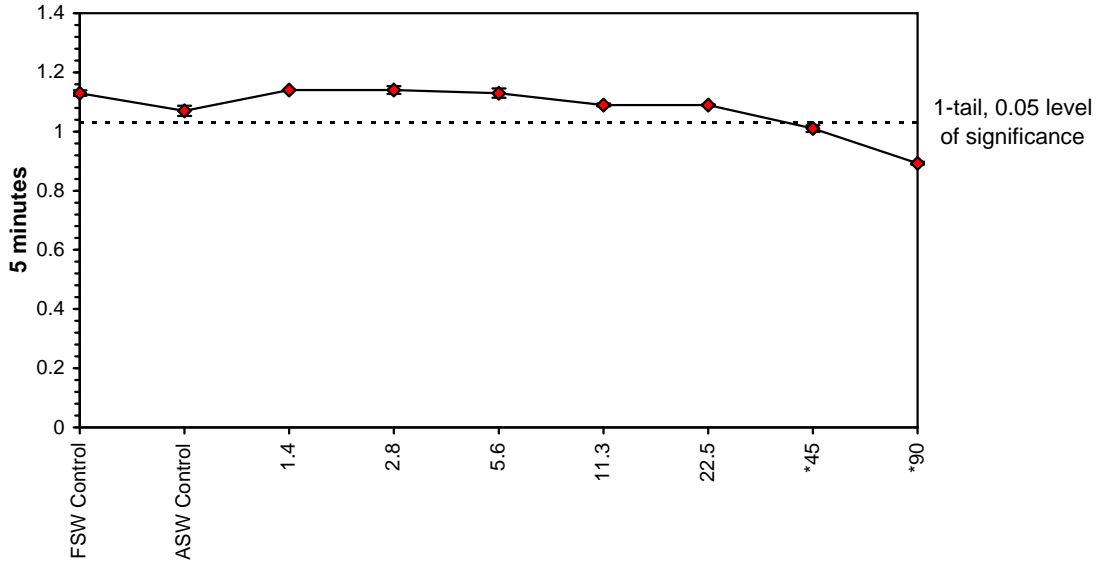
Linear Interpolation (200 Resamples)					
Point	%	SD	95% CL(Exp)	Skew	
IC05	30.190	1.659	17.682	44.567	0.1932
IC10	46.574	3.060	26.595	70.953	0.1614
IC15	67.812	2.404	48.108	84.317	-0.2382
IC20	89.049				
IC25	>90				
IC40	>90				
IC50	>90				



**Microtox Acute Toxicity Test-5 minutes**

Start Date: 20/11/2009 14:50 Test ID: PR0490/21 Sample ID: Brazil  
End Date: 20/11/2009 14:55 Lab ID: 3891Q Sample Type: AQ-Aqueous  
Sample Date: Protocol: ESA 119 Test Species: VF-Vibrio fischerii  
Comments:

**Dose-Response Plot**



**Microtox Acute Toxicity Test-5 minutes**

Start Date:	20/11/2009 14:50	Test ID:	PR0490/21	Sample ID:	Brazil
End Date:	20/11/2009 14:55	Lab ID:	3891Q	Sample Type:	AQ-Aqueous
Sample Date:		Protocol:	ESA 119	Test Species:	VF-Vibrio fischerii
Comments:					

**Auxiliary Data Summary**

Conc-%	Parameter	Mean	Min	Max	SD	CV%	N
FSW Control	5 min	103.50	101.00	106.00	3.54	1.82	2
ASW Control		95.50	94.00	97.00	2.12	1.53	2
1.4		100.00	100.00	100.00	0.00	0.00	2
2.8		99.00	99.00	99.00	0.00	0.00	2
5.6		97.00	95.00	99.00	2.83	1.73	2
11.3		98.00	97.00	99.00	1.41	1.21	2
22.5		97.00	95.00	99.00	2.83	1.73	2
45		90.50	90.00	91.00	0.71	0.93	2
90		79.00	79.00	79.00	0.00	0.00	2
FSW Control		pH	0.00	0.00	0.00	0.00	
ASW Control	0.00		0.00	0.00	0.00		0
1.4	0.00		0.00	0.00	0.00		0
2.8	0.00		0.00	0.00	0.00		0
5.6	0.00		0.00	0.00	0.00		0
11.3	0.00		0.00	0.00	0.00		0
22.5	0.00		0.00	0.00	0.00		0
45	0.00		0.00	0.00	0.00		0
90	0.00		0.00	0.00	0.00		0
FSW Control	Salinity ppt		0.00	0.00	0.00	0.00	
ASW Control		0.00	0.00	0.00	0.00		0
1.4		0.00	0.00	0.00	0.00		0
2.8		0.00	0.00	0.00	0.00		0
5.6		0.00	0.00	0.00	0.00		0
11.3		0.00	0.00	0.00	0.00		0
22.5		0.00	0.00	0.00	0.00		0
45		0.00	0.00	0.00	0.00		0
90		0.00	0.00	0.00	0.00		0

**Microtox Acute Toxicity Test-15 minutes**

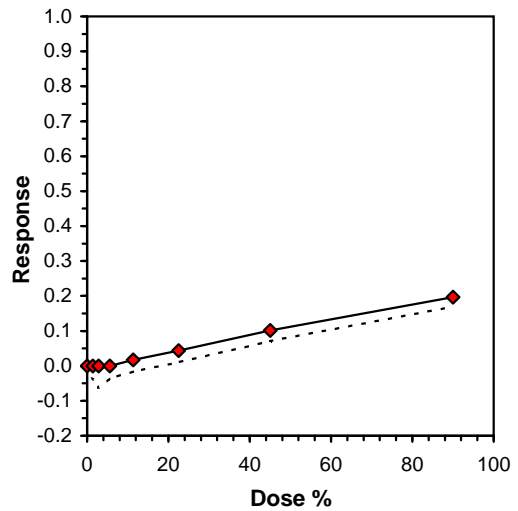
Start Date: 20/11/2009 14:50	Test ID: PR0490/21	Sample ID: Brazil
End Date: 20/11/2009 14:55	Lab ID: 3891Q	Sample Type: AQ-Aqueous
Sample Date:	Protocol: ESA 119	Test Species: VF-Vibrio fischerii

Conc-%	1	2
FSW Control	1.0316	1.0674
ASW Control	1.0225	1.0337
1.4	1.0682	1.0682
2.8	1.0795	1.1047
5.6	1.0602	1.0674
11.3	1.0444	1.0449
22.5	1.0115	1.0220
45	0.9551	0.9556
90	0.8539	0.8523

Conc-%	Mean	N-Mean	Transform: Untransformed				N	t-Stat	1-Tailed Critical	MSD	Isotonic	
			Mean	Min	Max	CV%					Mean	N-Mean
FSW Control	1.0495	1.0208	1.0495	1.0316	1.0674	2.415	2					
ASW Control	1.0281	1.0000	1.0281	1.0225	1.0337	0.773	2	*			1.0630	1.0000
1.4	1.0682	1.0390	1.0682	1.0682	1.0682	0.000	2	-5.284	2.810	0.0213	1.0630	1.0000
2.8	1.0921	1.0623	1.0921	1.0795	1.1047	1.626	2	-8.436	2.810	0.0213	1.0630	1.0000
5.6	1.0638	1.0348	1.0638	1.0602	1.0674	0.477	2	-4.710	2.810	0.0213	1.0630	1.0000
11.3	1.0447	1.0162	1.0447	1.0444	1.0449	0.034	2	-2.188	2.810	0.0213	1.0447	0.9827
22.5	1.0167	0.9890	1.0167	1.0115	1.0220	0.729	2	1.496	2.810	0.0213	1.0167	0.9564
*45	0.9553	0.9292	0.9553	0.9551	0.9556	0.037	2	9.592	2.810	0.0213	0.9553	0.8986
*90	0.8531	0.8298	0.8531	0.8523	0.8539	0.138	2	23.062	2.810	0.0213	0.8531	0.8025

Auxiliary Tests	Statistic	Critical	Skew	Kurt						
Normality of the data set cannot be confirmed										
Equality of variance cannot be confirmed										
The control means are not significantly different (p = 0.37)	1.140001	4.302653								
<b>Hypothesis Test (1-tail, 0.05)</b>	<b>NOEC</b>	<b>LOEC</b>	<b>ChV</b>	<b>TU</b>	<b>MSDu</b>	<b>MSDp</b>	<b>MSB</b>	<b>MSE</b>	<b>F-Prob</b>	<b>df</b>
Dunnett's Test	22.5	45	31.81981	4.444444	0.021322	0.020739	0.011996	5.76E-05	2.1E-08	7, 8
Treatments vs ASW Control										

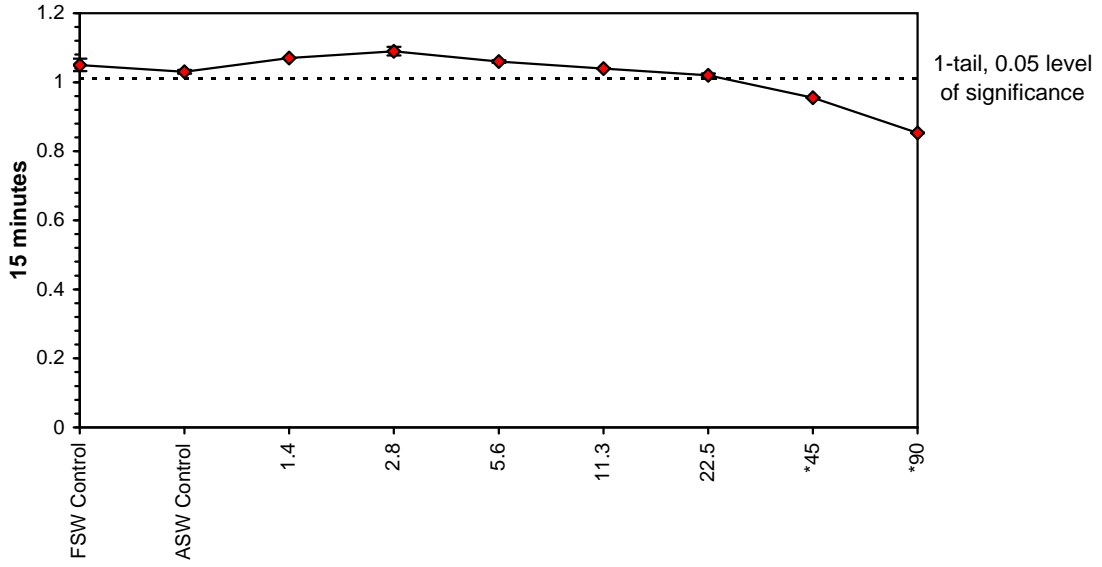
Linear Interpolation (200 Resamples)					
Point	%	SD	95% CL(Exp)		Skew
IC05	25.005	1.582	12.109	36.561	-0.2656
IC10	44.473	1.013	37.156	51.210	-0.0551
IC15	67.770	1.123	58.847	74.575	-0.1711
IC20	>90				
IC25	>90				
IC40	>90				
IC50	>90				



**Microtox Acute Toxicity Test-15 minutes**

Start Date: 20/11/2009 14:50 Test ID: PR0490/21 Sample ID: Brazil  
End Date: 20/11/2009 14:55 Lab ID: 3891Q Sample Type: AQ-Aqueous  
Sample Date: Protocol: ESA 119 Test Species: VF-Vibrio fischerii  
Comments:

**Dose-Response Plot**



**Microtox Acute Toxicity Test-15 minutes**

Start Date:	20/11/2009 14:50	Test ID:	PR0490/21	Sample ID:	Brazil
End Date:	20/11/2009 14:55	Lab ID:	3891Q	Sample Type:	AQ-Aqueous
Sample Date:		Protocol:	ESA 119	Test Species:	VF-Vibrio fischerii
Comments:					

**Auxiliary Data Summary**

Conc-%	Parameter	Mean	Min	Max	SD	CV%	N
FSW Control	15 min	96.50	95.00	98.00	2.12	1.51	2
ASW Control		91.50	91.00	92.00	0.71	0.92	2
1.4		94.00	94.00	94.00	0.00	0.00	2
2.8		95.00	95.00	95.00	0.00	0.00	2
5.6		91.50	88.00	95.00	4.95	2.43	2
11.3		93.50	93.00	94.00	0.71	0.90	2
22.5		90.50	88.00	93.00	3.54	2.08	2
45		85.50	85.00	86.00	0.71	0.98	2
90		75.50	75.00	76.00	0.71	1.11	2
FSW Control		pH	0.00	0.00	0.00	0.00	
ASW Control	0.00		0.00	0.00	0.00		0
1.4	0.00		0.00	0.00	0.00		0
2.8	0.00		0.00	0.00	0.00		0
5.6	0.00		0.00	0.00	0.00		0
11.3	0.00		0.00	0.00	0.00		0
22.5	0.00		0.00	0.00	0.00		0
45	0.00		0.00	0.00	0.00		0
90	0.00		0.00	0.00	0.00		0
FSW Control	Salinity ppt		0.00	0.00	0.00	0.00	
ASW Control		0.00	0.00	0.00	0.00		0
1.4		0.00	0.00	0.00	0.00		0
2.8		0.00	0.00	0.00	0.00		0
5.6		0.00	0.00	0.00	0.00		0
11.3		0.00	0.00	0.00	0.00		0
22.5		0.00	0.00	0.00	0.00		0
45		0.00	0.00	0.00	0.00		0
90		0.00	0.00	0.00	0.00		0

## **Appendix I: Statistical Print-outs for the *Ecklonia radiata* Macroalgal Germination Success Test**

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**Macroalgal Germination Success Test-Proportion Germinated**

Start Date: 19/11/2009 13:30    Test ID: PR0490/05    Sample ID: Brazil  
 End Date: 22/11/2009 13:30    Lab ID: 3891Q    Sample Type: AQ-Aqueous  
 Sample Date:    Protocol: ESA 116    Test Species: ER-Ecklonia radiata  
 Comments:

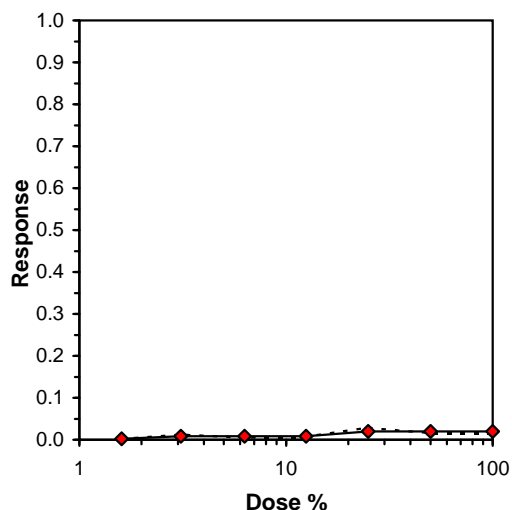
Conc-%	1	2	3	4
FSW Control	0.9400	0.9100	0.9600	0.9300
ASW Control	0.9500	0.9800	0.9200	0.9700
1.6	0.9500	0.9300	0.9800	0.9500
3.1	0.9200	0.9700	0.9500	0.9300
6.3	0.9600	0.9800	0.9500	0.9100
12.5	0.9400	0.9700	0.9300	0.9500
25	0.9000	0.9600	0.9200	0.9300
50	0.9800	0.9300	0.9500	0.9000
100	0.9500	0.9300	0.9600	0.9200

Conc-%	Mean	N-Mean	Transform: Arcsin Square Root					t-Stat	1-Tailed Critical	MSD	Isotonic	
			Mean	Min	Max	CV%	N				Mean	N-Mean
FSW Control	0.9350	0.9791	1.3155	1.2661	1.3694	3.274	4				0.9550	1.0000
ASW Control	0.9550	1.0000	1.3637	1.2840	1.4289	4.643	4	*				
1.6	0.9525	0.9974	1.3556	1.3030	1.4289	3.891	4	0.204	2.480	0.0986	0.9525	0.9974
3.1	0.9425	0.9869	1.3323	1.2840	1.3967	3.754	4	0.791	2.480	0.0986	0.9467	0.9913
6.3	0.9500	0.9948	1.3524	1.2661	1.4289	4.986	4	0.284	2.480	0.0986	0.9467	0.9913
12.5	0.9475	0.9921	1.3421	1.3030	1.3967	3.002	4	0.544	2.480	0.0986	0.9467	0.9913
25	0.9275	0.9712	1.3014	1.2490	1.3694	3.886	4	1.568	2.480	0.0986	0.9358	0.9799
50	0.9400	0.9843	1.3316	1.2490	1.4289	5.701	4	0.809	2.480	0.0986	0.9358	0.9799
100	0.9400	0.9843	1.3254	1.2840	1.3694	2.937	4	0.963	2.480	0.0986	0.9358	0.9799

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.05)	0.963982	0.93	0.17058	-0.74185
Bartlett's Test indicates equal variances (p = 0.96)	2.07053	18.47531		
The control means are not significantly different (p = 0.25)	1.260382	2.446912		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test Treatments vs ASW Control	100	>100		1	0.048298	0.05043	0.001583	0.003162	0.824786	7, 24

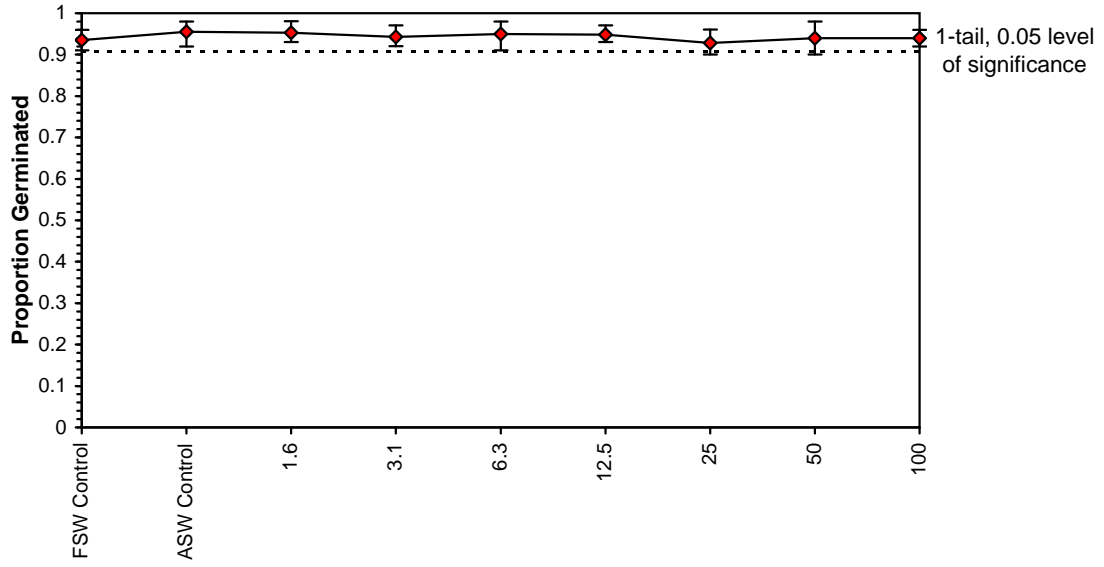
Log-Logit Interpolation (200 Resamples)				
Point	%	SD	95% CL(Exp)	Skew
IC05	>100			
IC10	>100			
IC15	>100			
IC20	>100			
IC25	>100			
IC40	>100			
IC50	>100			



**Macroalgal Germination Success Test-Proportion Germinated**

Start Date: 19/11/2009 13:30 Test ID: PR0490/05 Sample ID: Brazil  
End Date: 22/11/2009 13:30 Lab ID: 3891Q Sample Type: AQ-Aqueous  
Sample Date: Protocol: ESA 116 Test Species: ER-Ecklonia radiata  
Comments:

**Dose-Response Plot**



**Macroalgal Germination Success Test-Proportion Germinated**

Start Date: 19/11/2009 13:30    Test ID: PR0490/05    Sample ID: Brazil  
 End Date: 22/11/2009 13:30    Lab ID: 3891Q    Sample Type: AQ-Aqueous  
 Sample Date:    Protocol: ESA 116    Test Species: ER-Ecklonia radiata  
 Comments:

**Auxiliary Data Summary**

Conc-%	Parameter	Mean	Min	Max	SD	CV%	N
FSW Control	% Germinated	93.50	91.00	96.00	2.08	1.54	4
ASW Control		95.50	92.00	98.00	2.65	1.70	4
1.6		95.25	93.00	98.00	2.06	1.51	4
3.1		94.25	92.00	97.00	2.22	1.58	4
6.3		95.00	91.00	98.00	2.94	1.81	4
12.5		94.75	93.00	97.00	1.71	1.38	4
25		92.75	90.00	96.00	2.50	1.70	4
50		94.00	90.00	98.00	3.37	1.95	4
100		94.00	92.00	96.00	1.83	1.44	4
FSW Control	pH	8.00	8.00	8.00	0.00	0.00	1
ASW Control		8.00	8.00	8.00	0.00	0.00	1
1.6		8.00	8.00	8.00	0.00	0.00	1
3.1		8.00	8.00	8.00	0.00	0.00	1
6.3		7.90	7.90	7.90	0.00	0.00	1
12.5		7.90	7.90	7.90	0.00	0.00	1
25		7.80	7.80	7.80	0.00	0.00	1
50		8.00	8.00	8.00	0.00	0.00	1
100		8.00	8.00	8.00	0.00	0.00	1
FSW Control	Salinity ppt	34.40	34.40	34.40	0.00	0.00	1
ASW Control		34.40	34.40	34.40	0.00	0.00	1
1.6		34.50	34.50	34.50	0.00	0.00	1
3.1		34.50	34.50	34.50	0.00	0.00	1
6.3		34.50	34.50	34.50	0.00	0.00	1
12.5		34.70	34.70	34.70	0.00	0.00	1
25		35.00	35.00	35.00	0.00	0.00	1
50		34.20	34.20	34.20	0.00	0.00	1
100		34.40	34.40	34.40	0.00	0.00	1
FSW Control	% DO	96.70	96.70	96.70	0.00	0.00	1
ASW Control		96.90	96.90	96.90	0.00	0.00	1
1.6		96.70	96.70	96.70	0.00	0.00	1
3.1		96.60	96.60	96.60	0.00	0.00	1
6.3		96.90	96.90	96.90	0.00	0.00	1
12.5		98.70	98.70	98.70	0.00	0.00	1
25		101.50	101.50	101.50	0.00	0.00	1
50		96.50	96.50	96.50	0.00	0.00	1
100		96.20	96.20	96.20	0.00	0.00	1

## Appendix J: Statistical Print-outs for the *Ceriodaphnia dubia* Acute Test

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**Ceriodaphnia Acute Toxicity Test-48 Hr Survival**

Start Date: 19/11/2009 16:00	Test ID: PR0490/13	Sample ID: Brazil
End Date: 21/11/2009 16:00	Lab ID: 3891Q	Sample Type: AQ-Aqueous
Sample Date:	Protocol: ESA 101	Test Species: CD-Ceriodaphnia dubia

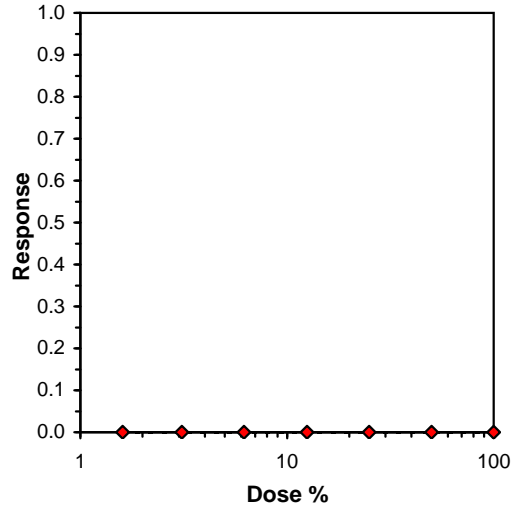
Conc-%	1	2	3	4
DMW Control	1.0000	1.0000	1.0000	1.0000
1.6	1.0000	1.0000	1.0000	1.0000
3.1	1.0000	1.0000	1.0000	1.0000
6.2	1.0000	1.0000	1.0000	1.0000
12.5	1.0000	1.0000	1.0000	1.0000
25	1.0000	1.0000	1.0000	1.0000
50	1.0000	1.0000	1.0000	1.0000
100	1.0000	1.0000	1.0000	1.0000

Conc-%	Transform: Arcsin Square Root							Isotonic	
	Mean	N-Mean	Mean	Min	Max	CV%	N	Mean	N-Mean
DMW Control	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	1.0000	1.0000
1.6	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	1.0000	1.0000
3.1	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	1.0000	1.0000
6.2	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	1.0000	1.0000
12.5	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	1.0000	1.0000
25	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	1.0000	1.0000
50	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	1.0000	1.0000
100	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	1.0000	1.0000

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.05)	1	0.93		
Equality of variance cannot be confirmed				

**Log-Logit Interpolation (200 Resamples)**

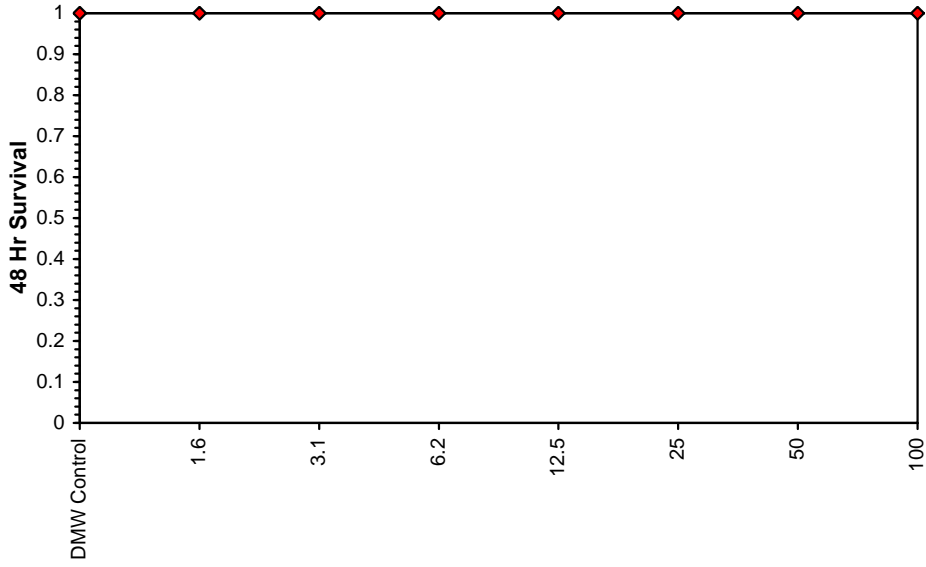
Point	%	SD	95% CL(Exp)	Skew
IC05	>100			
IC10	>100			
IC15	>100			
IC20	>100			
IC25	>100			
IC40	>100			
IC50	>100			



**Ceriodaphnia Acute Toxicity Test-48 Hr Survival**

Start Date: 19/11/2009 16:00 Test ID: PR0490/13 Sample ID: Brazil  
End Date: 21/11/2009 16:00 Lab ID: 3891Q Sample Type: AQ-Aqueous  
Sample Date: Protocol: ESA 101 Test Species: CD-Ceriodaphnia dubia  
Comments:

**Dose-Response Plot**



**Ceriodaphnia Acute Toxicity Test-48 Hr Survival**

Start Date:	19/11/2009 16:00	Test ID:	PR0490/13	Sample ID:	Brazil
End Date:	21/11/2009 16:00	Lab ID:	3891Q	Sample Type:	AQ-Aqueous
Sample Date:		Protocol:	ESA 101	Test Species:	CD-Ceriodaphnia dubia
Comments:					

**Auxiliary Data Summary**

Conc-%	Parameter	Mean	Min	Max	SD	CV%	N
DMW Control	% Survival	100.00	100.00	100.00	0.00	0.00	4
1.6		100.00	100.00	100.00	0.00	0.00	4
3.1		100.00	100.00	100.00	0.00	0.00	4
6.2		100.00	100.00	100.00	0.00	0.00	4
12.5		100.00	100.00	100.00	0.00	0.00	4
25		100.00	100.00	100.00	0.00	0.00	4
50		100.00	100.00	100.00	0.00	0.00	4
100		100.00	100.00	100.00	0.00	0.00	4
DMW Control	pH	7.90	7.90	7.90	0.00	0.00	1
1.6		8.00	8.00	8.00	0.00	0.00	1
3.1		8.00	8.00	8.00	0.00	0.00	1
6.2		8.10	8.10	8.10	0.00	0.00	1
12.5		8.10	8.10	8.10	0.00	0.00	1
25		8.20	8.20	8.20	0.00	0.00	1
50		8.30	8.30	8.30	0.00	0.00	1
100		8.50	8.50	8.50	0.00	0.00	1
DMW Control	DO %	99.60	99.60	99.60	0.00	0.00	1
1.6		98.70	98.70	98.70	0.00	0.00	1
3.1		98.30	98.30	98.30	0.00	0.00	1
6.2		98.50	98.50	98.50	0.00	0.00	1
12.5		98.60	98.60	98.60	0.00	0.00	1
25		97.90	97.90	97.90	0.00	0.00	1
50		97.10	97.10	97.10	0.00	0.00	1
100		98.30	98.30	98.30	0.00	0.00	1
DMW Control	Cond uS/cm	184.70	184.70	184.70	0.00	0.00	1
1.6		234.00	234.00	234.00	0.00	0.00	1
3.1		281.00	281.00	281.00	0.00	0.00	1
6.2		379.00	379.00	379.00	0.00	0.00	1
12.5		561.00	561.00	561.00	0.00	0.00	1
25		921.00	921.00	921.00	0.00	0.00	1
50		1596.00	1596.00	1596.00	0.00	0.00	1
100		2950.00	2950.00	2950.00	0.00	0.00	1

## **Appendix K: Chain-of-Custody Documentation**

# Sample Receipt Notification

**Attention** : Lawson Harding

**Client** : Gunns Limited  
PO Box 572  
Launceston TAS 7250

**Email** : Lawson.harding@gunns.com.au  
**Telephone** : (03) 6335 5446  
**Facsimile** : (03) 6335 5498

**Date** : 19/11/2009

**Re** : Receipt of Sample for Toxicity Testing

**Pages** : 2

**ESA Project** : PR0490

For Review

Additional Documentation Required - Please Respond

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## **Sample Delivery Details**

**Completed Chain of Custody accompanied samples:** YES  
**Samples received in apparent good condition and correctly bottled:** YES  
**Security seals on sample bottles and esky intact:** YES

**Date samples received** : 19/11/2009  
**Time samples received** : 9:15  
**No. of samples received** : 1  
**Sample matrix** : aqueous  
**Sample temperature** : 6-10°C

**Comments** : Includes 5 x 2L Brazil sample (ESA ID# 3891Q)

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## **Contact Details**

Customer Services Officer : Tina Micevska  
Telephone : 61 2 9420 9481  
Facsimile : 61 2 9420 9484  
Email : tmicevska@ecotox.com.au

Please contact customer services officer for all queries or issues regarding samples

**Note that the chain-of-custody provides definitive information on the tests to be performed**

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## **Ecotox Services Australia**

ABN 45 094 714 904  
Unit 27, 2 Chaplin Drive  
Lane Cove NSW 2066 Australia

Phone : 61 2 9420 9481  
Fax : 61 2 9420 9484  
Email : info@ecotox.com.au

# Chain-of-Custody / Service Request Form

Customer: **Guas Ltd.** Ship To: **ESA**  
 Contact Name: **Kauroa Harding** Attention: **Dr. Rick Kauroa**  
 Phone: **61 407 827 958** Email: **kauroa.harding@ecotox.com.au** (please provide an email address for sample receipt notification)  
 Sampled by: **Kauroa Harding**

Sample Date (day/month/year)	Sample Time	Sample Name (exactly as written on the sample vessel)	Sample Method (eg. Grab, composite etc.)	Number and Volume of Containers (eg 2 x 1L)	Tests Requested (See reverse for guidance)			Comments / Instructions
17/11/09		Brazil 1,2,3,4,5	Grab	5 x 2L				Note that testing will be delayed if an incomplete chain of custody is received <ul style="list-style-type: none"> <li>• Additional treatment of samples (i.e. spiking)</li> <li>• Sub-contracted services (i.e. chemical analyses)</li> <li>• Dilutions required (if different than 100% down to 6.25%)</li> <li>• Sample holding time restriction (if applicable)</li> <li>• Sample used for litigation (if applicable)</li> </ul> Note: An MSDS must be attached if Available ESA Project Number: PR Requested by L. Harding Ref Quoted by PL 0499101

1) Released By: <i>[Signature]</i> Date: 19/11/09 Time: 09:15	2) Received By: <i>[Signature]</i> Date: 19/11/09 Time: 0915
Of: <i>Guas Ltd.</i>	Of: <i>ESA</i>
3) Released By: _____ Date: _____ Time: _____	4) Received By: _____ Date: _____ Time: _____
Of: _____	Of: _____

Note that the chain-of-custody documentation will provide definitive information on the tests to be performed.