APPENDIX H4

Ecotoxicology

APPENDIX H4.1

Toxicity testing on larval kingfish

The Provision of Reverse Osmosis Brine Toxicity Testing on Larval Kingfish (*Seriola lalandi*)

Prepared for

ARUP

Report ECX08-1809 and ECX08-0910

Marine Toxicity Tests

24th October 2008

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DISCLAIMER

This report presents a study initiated by Arup Partners on behalf of BHP Billiton to determine the toxicity of reverse osmosis brine to be discharged into Spencer Gulf from a proposed desalination plant located at Point Lowly.

Geotech has endeavored to achieve high accuracy results using certified techniques and equipment. However, Geotech shall not be held responsible or liable for the results of any actions taken on the basis of the information contained in this document. Moreover, this report should not be the sole reference when considering issues that may have commercial implications.

All data and information will remain proprietary to Arup Partners and is regarded as strictly confidential by all Geotech personnel. Any queries related to this report may be directed to David Strom at Geotech.

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Executive Summary

This report presents a study initiated by Arup on behalf of BHP Billiton to determine the toxicity of reverse osmosis brine (RO brine) to be discharged into Spencer Gulf from a proposed desalination plant located at Point Lowly. The potential for adverse biological effects resulting from exposure to the RO brine and the chemical antiscalant 'Nalco Permatreat PC-1020T' were assessed concurrently using a locally relevant fish species *Seriola lalandi* (Yellowtail Kingfish).

Growth inhibition bioassays with early life stage Kingfish larvae were performed to quantify any sub-lethal toxicity resulting from exposure to the RO brine and antiscalant. The larvae were continuously exposed to combinations of the RO brine and antiscalant over the 7-day duration. Toxicity tests were also performed using pulsed exposures of 2 days that aimed to replicate *in situ* wind and tidal conditions found in the Upper Spencer Gulf.

The RO brine with antiscalant did not appear to be more toxic than the RO brine without antiscalant for both the continuous and pulsed exposure regimes. Based on the more representative pulsed exposure results for the RO brine with antiscalant, a 99% level of protection for the Kingfish larvae may be achieved by diluting the brine discharge with local seawater by a factor of 8.3. This dilution factor of 8.3 is well within the previously recommended diffuser configuration of 43.5 and would be expected to provide adequate environmental protection for *S. lalandi* larvae at the mixing zone boundary.

1 Introduction

Geotechnical Services were requested by ARUP Pty Ltd to study the ecotoxicological impacts of the reverse osmosis (RO) return water produced by the proposed BHP Billiton desalination plant to be located at Point Lowly in Upper Spencer Gulf on several marine species. The ambient salinity at Point Lowly (40-43 g/L) is greater than the majority of Australian marine waters (34-37 g/L), thus the return water salinity approximates 78 g/L.

One of the species initially tested was the Yellowtail Kingfish *Seriola lalandi*. However, excess mortality in the control treatments (>80%) using eggs sourced from South Australia indicated these tests had failed. It was unclear whether the tests failed due to the egg quality, acclimation issues associated with transferring larvae hatched in oceanic water or the elevated salinity of the control water sourced from Point Lowly.

BHP Billiton therefore requested a further test, whereby the eggs were transferred from a South Australian hatchery to Fremantle, WA in water of a similar salinity to the control water used in the testing to approximate the maximum ambient salinity expected from Point Lowly.

2 Methods

2.1 Experimental Design

Growth inhibition bioassays with early life stage Yellowtail Kingfish were used to assess any sub-lethal toxicity resulting from exposure to the RO brine and Nalco Permatreat PC-1020T (referred to herein as 'Nalco'). Yellowtail Kingfish eggs were sent to Geotech's Fremantle Laboratory in Western Australia from Upper Spencer Gulf on the 18th September 2008 and the 9th October 2008. Toxicity from the RO brine and Nalco were examined individually and in combination to include (i) RO brine without Nalco, (ii) the RO brine with Nalco and (iii) Nalco without RO brine treatments (Table 1). Kingfish larvae were continuously exposed to the toxicants for 7 days for all bioassays performed in September. However, the experimental design was not considered to be representative of an *in situ* exposure given the larvae are more likely to pass through the RO brine plume borne by currents and tides. Therefore, tests performed in October used a pulsed exposure of 2 days, after which the larvae were transferred to clean seawater (without RO brine or Nalco). The pulsed exposure aimed to replicate conditions found in the Upper Spencer Gulf when wind and tidal movement is minimal and exposure may be extended. The Nalco treatment in the absence of the RO brine was not repeated in the 2-day pulsed experiments. Selected dilutions for the concentration series are shown in Table 2.

Table 1 Combinations and exposure durations of RO Brine and Nalco tested

Date	Treatments	Duration
19/09/08	RO Brine with Nalco	Continuous / 7-days
19/09/08	RO Brine without Nalco	Continuous / 7-days
19/09/08	Nalco without RO Brine	Continuous / 7-days
10/10/08	RO Brine with Nalco	Pulsed / 2 days
10/10/08	RO Brine without Nalco	Pulsed / 2 days

Table 2 Summary of concentration-response series used in bioassays

Concentration Series	Dilution						
	1	2	3	4	5	6	7
Continuous (%)	1.6	3.1	6.3	12.5	25.0	50.0	100.0
Pulsed (%)	0.8	1.6	3.1	6.3	12.5	25.0	50.0
Nalco (mg/L)	0.1	0.2	0.4	0.9	1.8	3.5	7.0

2.2 Seawater Diluent, RO Brine and Nalco Treatments

Seawater diluent and RO brine were sourced on site from Point Lowly and transported in a refrigerated truck at 4°C from South Australia to to Geotech's Welshpool Laboratory in Perth, Western Australia on 23rd June 2008. A sample of Nalco Permatreat PC-1020T was sourced from the Perth Desalination Plant (Water Corporation of WA) and used to spike subsamples of the RO brine and Point Lowly diluent to final concentrations of 7.0 mg/L (15th September 2008). The seawater diluent, RO brine and Nalco treatments were stored at 4°C prior to testing.

2.3 Physico-chemical Measurements

The salinity and pH was measured on delivery to the laboratory (Table 3). The diluent seawater was filtered to 0.45 µm and transported to Geotech's Fremantle Ecotoxicology Laboratory in 25 L HDPE containers for use in the bioassays. The RO brine sample was tested as received.

Nominal salinity measurements were made using a refractometer with an accuracy of ±1 ppt, followed by more accurate measurements (post-testing) using an Autolab Salinometer, considered by BHP Billiton to have an accuracy of ±0.02 ppt.

Table 3 Sample information sheet for diluent and RO brine

Contact Company	ARUP
Contact Person	James Brook
Number of Samples	1 x Diluent + 1 x RO brine
Sample Type	RO brine and Point Lowly seawater
Date Sampled	15/09/08 and 16/06/08
Seawater Collected	Point Lowly, South Australia
Sampled by	Paul Fildes and James Brook
Sample pH	RO Brine: 7.80 Diluent: 7.97
Nominal Sample Salinity	RO Brine: 78 ppt Diluent: 41.9 ppt
Transport Conditions	Transported at 4ºC
Date of Arrival at Geotech	23/06/08
Time of Arrival at Geotech	11 am
Sample Temp on Arrival	4ºC
Sample Received by	Max Offer
Tests Requested	Kingfish Larval Growth

2.4 Kingfish Larval Growth Inhibition Bioassays

The effect of RO brine and Nalco on the growth of fish larvae was determined using the marine species *Seriola lalandi* (Yellowtail Kingfish). The continuous and pulsed exposure endpoints were based on USEPA Method 1004.0 (USEPA, 2003) which are summarised in Table 4.

Fish stock culture

Yellowtail Kingfish eggs were transported from Upper Spencer Gulf on the 18th September and the 9th October, 2008. The eggs were transported in two batches, one batch in seawater of nominal salinity 43 ppt (to be used in the bioassays) and one batch transported in hatchery seawater of nominal salinity 37 ppt (to be used as additional controls). The eggs arrived in Perth at 11 am via air freight and were immediately rinsed and transferred into clean seawater at the appropriate nominal salinities.

Fish larvae bioassays

A 400 mL aliquot of each control, concentration and reference toxicant solution was dispensed into acid washed 500 mL borosilicate glass beakers. To each of the four replicates per treatment, 20 newly hatched fish larvae of equal size were added. Larvae were fed a concentrated rotifer stock (0.5 mL) daily after Day 2. The test was incubated at 21°C on a 12-h light:12-h dark photoperiod. On Day 7, the remaining fish larvae were removed and the length measured (mm) using a microscope mounted digital camera and Digimizer® imaging software package. Inhibition of larval growth was reported as a percentage of the control.

Quality assurance

A spiked chromium reference bioassay was performed concurrently to ensure *S. lalandi* was responding as expected to a known toxicant. The spiked chromium concentration series consisted of a Control (0), 0.6, 1.2, 2.5, 5, 10 mg/L treatments. Test acceptability was achieved when (i) survival in the control treatments was >80% and (ii) larval growth effects from the reference toxicant were within the expected response range.

Table 4 Summary of the test protocol for the *Seriola lalandi* continuous and pulsed growth inhibition bioassays

Test Parameter	Sub-lethal Specification
Organism	*Seriola lalandi*
Test Type	Static
Test Duration	7-day
Temperature	21 ± 1°C
Light Quality	Ambient illumination
Photoperiod	12 hour light : 12 hour dark
Test Chamber Size	500 mL
Test Solution Volume	400 mL
Renewal of Test Solutions	None
Age of Test Organisms	Larvae < 24-h
Organisms per Replicate	20
No. of Replicates	4
Feeding	Daily
Dilution Water	0.45 µm filtered seawater (43 ‰)
Serial Dilution Factor	2
Endpoint	Percentage Growth Inhibition (length)
Test Acceptability	>80% Control Survival / Acceptable Chromium Reference

3 Results

This following is a summary of results for all Yellowtail Kingfish bioassays performed.. Data from the bioassays and statistical analyses are shown in Appendices 1 and 2, respectively.

3.1 Physico-chemical Measurements

The nominal salinity of the diluent seawater was 41.9 ppt (later determined to be 41.3 ppt). Seawater at this salinity was used to transport the Kingfish eggs. The control and diluent water used in test solutions was evaporated to a nominal salinity of 43 ppt (the maximum salinity likely to be encountered near Point Lowly), and later measured to be 44.3 ppt. The nominal salinity of the RO brine was 78 ppt (later measured to be 77.6 ppt)

The physicochemical data for the RO brine tests are shown in Table 5. The nominal salinity values have been adopted for the remainder of this report, reflecting the understanding at the time of the toxicity tests.

Table 5 Physico-chemical measurements for RO Brine concentration series

RO Brine (%)	DO (ppm)	Nominal Salinity (ppt)	pH
0	5.9 -7.0	43.0	7.95 – 8.10
0.8	5.9 - 7.0	43.0	7.95 – 8.10
1.6	5.8 - 7.0	43.5	7.95 – 8.10
3.1	5.8 - 7.0	44.0	7.95 – 8.11
6.3	5.9 - 7.0	45.0	7.94 – 8.11
12.5	5.7 - 7.0	48.0	7.90 – 8.12
25.0	5.8 - 7.0	53.5	7.80 – 8.12
50.0	5.8 - 7.0	63.5	7.80 – 7.98
100.0	5.9 - 7.0	78.0	7.67 – 7.97

3.2 Kingfish Larval Growth Inhibition Bioassays

The percentage growth results for Kingfish larvae exposed to RO brine treatments (with and without Nalco) for continuous and pulsed durations are shown in Tables 6 and 7, respectively. The percentage growth results for Kingfish larvae exposed to the Nalco treatment (without RO brine) continuously are shown in Table 8.

Acceptable growth in all controls indicated that the Kingfish eggs were of good quality. Slightly more growth was observed in the 37 ppt continuous and pulsed controls (10% and 3%, respectively) in comparison the 43 ppt diluent controls. For this reason and in keeping with salinity of the Point Lowly

discharge environment, the 43 ppt diluent control was used for all statistical analyses.

The RO brine without Nalco significantly inhibited larval growth in both the continuous and pulsed exposures with EC50 values (i.e. the effect concentration at which larval growth is reduced by 50% compared to the control) of 8.1% and 16.0%, respectively. However, the larval growth inhibition EC50 was not significantly different to the RO brine treatments with Nalco, with continuous and pulsed exposure EC50 values of 6.9% and 15.8%, respectively (Table 9). This implied that additive effects from the antiscalant were negligible at the concentrations tested for both continuous and pulsed exposures and toxicity was likely due to the RO brine. Further to this, larval growth inhibition appeared to be greater in the continuous exposure compared to the pulsed exposure.

For the continuous Nalco treatment without RO brine, the EC50 was greater than the highest concentration tested (>7.0 mg/L). However, significant inhibition of larval growth (approx 22±7%) was observed at concentrations >0.4 mg/L (Table 8).

Table 6 Percentage growth results for Kingfish larvae continuously exposed to RO Brine (with or without Nalco) for 7-days

Concentration (% Sample)	RO Brine with Nalco (% Growth)	RO Brine without Nalco (% Growth)
Control (43 ppt)	100 ± 1	100 ± 8
Control (37 ppt)	110 ± 3	110 ± 3
1.6	86 ± 4	95 ± 8
3.1	65 ± 5	87 ± 7
6.3	64 ± 1	70 ± 5
12.5	48 ± 2	19 ± 7
25.0	0 ± 0	0 ± 0
50.0	0 ± 0	0 ± 0
100.0	0 ± 0	0 ± 0

Table 7 Percentage growth results for Kingfish larvae pulsed with RO Brine (with or without Nalco) for 2-days

Concentration (% Sample)	RO Brine with Nalco (% Growth)	RO Brine without Nalco (% Growth)
Control (43 ppt)	100 ± 2	100 ± 6
Control (37 ppt)	103 ± 4	103 ± 4
0.8	84 ± 2	73 ± 3
1.6	79 ± 1	79 ± 3
3.1	75 ± 2	99 ± 1
6.3	79 ± 1	86 ± 5
12.5	73 ± 3	81 ± 5
25.0	0 ± 0	0 ± 0
50.0	0 ± 0	0 ± 0

Table 8 Percentage growth results for Kingfish larvae continuously exposed to Nalco without RO Brine for 7-days

Concentration (mg/L)	Nalco without RO Brine (% Growth)
Control (43 ppt)	100 ± 6
Control (37 ppt)	110 ± 3
0.1	110 ± 6
0.2	91 ± 3
0.4	83 ± 1
0.9	66 ± 4
1.8	83 ± 1
3.5	76 ± 3
7.0	82 ± 1

Table 9 Summary of statistical effects data for larval Kingfish growth inhibition bioassays

Sample	EC50	EC10	LOEC	NOEC
Continuous / 7-days RO Brine with Nalco	6.9%	1.5%	1.6%	<1.6%
Continuous / 7-days RO Brine without Nalco	8.1%	4.4%	6.3%	3.1%
Continuous / 7-days Nalco without RO Brine	>7.0 mg/L	0.3 mg/L	0.2 mg/L	0.1 mg/L
Pulsed / 2 days RO Brine with Nalco	15.8%	12.1%	0. 8 %	<0.8%
Pulsed / 2 days RO Brine without Nalco	16.0%	12.5%	6.3%	3.1%

4 Discussion

4.1 Continuous 7-day Exposures to RO Brine and Nalco

The eggs delivered in September were used in the continuous 7-day exposure bioassays with RO brine (with and without Nalco) and Nalco (without RO Brine). This is the same methodology that was used in the 2007 larval fish growth bioassays (Geotechnical Services 2008), with the exception that the nominal salinity of the diluent was 43 ppt for all tests and the toxicity of Nalco without RO brine was also assessed. Three replicates of larvae were maintained in seawater of nominal salinity 37 ppt to compare growth over the 7-day period with the 43 ppt diluent. Thus there were two controls, nominally of 6 ppt difference (later measured to be 8 ppt difference).

Results indicated that additive effects from the Nalco were negligible at the concentrations tested and toxicity was likely due to the RO brine. In the absence of RO brine (i.e. salinity of 43 ppt) larval growth was significantly inhibited by 22±7% for Nalco concentrations exceeding 0.4 mg/L. However,

the additional 22±7% growth inhibition observed for Nalco concentrations >0.4 mg/L did not translate into additive effects when in combination with the RO brine. For example, the RO brine with Nalco EC50 concentration of 6.9% approximated 0.5 mg-Nalco/L in solution. At this concentration of Nalco, an additional 22±7% inhibition of growth would be expected if additive effects were occurring. This was not observed when the percentage growth inhibition in treatments bracketing the EC50 concentrations for RO brine with and without Nalco were compared (Table 6). At the 6.3% dilution there was no significant difference between RO brine with or without Nalco and the 12.5% RO brine treatment with Nalco was significantly less toxic. It was unclear if the antiscalant was less bioavailable in the hypersaline brine or if biodegradation of Nalco had occurred in the unfiltered RO brine.

The 2008 results were also compared to the tests undertaken in 2007. Due to the poor quality of Kingfish eggs received from South Australia, the 2007 bioassay used larvae sourced from the Aquaculture Development Unit of Challenger TAFE in Fremantle, WA. In addition, this test was performed using 35 ppt seawater diluent collected from Rottnest Island. The EC50 for the 2007 continuous exposure to RO brine with Nalco was calculated to be 16.4%. The 2007 sample appeared to be slightly less toxic compared to the 2008 sample, where an EC50 of 6.9% was calculated. Larvae maintained in the 37 ppt seawater control for the current testing also showed a 10% increase in growth compared to the larvae in 43 ppt seawater control. Assuming the RO brine sample preparation and physico-chemistry was similar between testing years; the higher salinity diluent used in 2008 may have promoted additional osmotic stress on the larvae which is known to increase the sensitivity of test organisms. However, given the larvae were also sourced from different hatcheries, the variability in broodstock should not be discounted. Despite the differences between the 2007 and 2008, the testing may be considered within the range of acceptable variability for biological testing.

4.2 Pulsed 2-day Exposures to RO Brine and Nalco

The eggs delivered in October were used in the pulsed 2-day exposure bioassays for comparing toxicity of the RO brine with and without the Nalco antiscalant. The pulsed bioassays also used the 43 ppt nominal salinity diluent.

The larvae in these bioassays were exposed to the respective treatments for 2 days post hatch. The larvae were then gently transferred into clean seawater with a nominal salinity of 43 ppt. Three replicates of larvae were maintained in seawater of nominal salinity 37 ppt to compare growth over the 7-day period with the 43 ppt diluent. The larvae did not respond well to handing and high mortality in the control treatments was apparent by Day 4. The bioassays were therefore terminated after 5 days.

No significant differences in the EC50 concentrations were observed between the RO brine treatments with Nalco (15.8%) or RO brine without Nalco (16.0%), indicating that additive effects were negligible at the concentrations tested and toxicity was likely due to the RO brine. Conclusions regarding the

additive effects from RO brine and Nalco were in good agreement with the continuous 7-day exposure results. Larval growth in the 37 ppt control was again slightly greater than the 43 ppt nominal salinity controls, though not statistically significant for the pulsed exposures.

While the pulsed experiment was considered to be more representative of an *in situ* discharge, it can not be concluded that more toxicity was observed in the 7-day continuous exposure. The reduced pulsed exposure duration (5 days in total) was not comparable to the 7-day continuous exposure duration. For example, it is not possible to delineate between mortality caused through handling or toxicity considering the experimental designs were not identical. Regardless of such comparisons, the pulsed bioassay may still be considered a more representative exposure scenario and the RO brine with Nalco does not appear to be any more toxic than the RO brine without Nalco..

5 References

Geotechnical Services (2008). The Provision of Reverse Osmosis Brine Toxicity Testing, Report ECX07-1805 prepared for Arup, inserted as Appendix O10.4 of BHP Billiton (2009). Olympic Dam Expansion Draft Environmental Impact Statement.

USEPA (2003) Short-term methods for assessing the chronic toxicity of effluents and receiving water to marine and estuarine organisms. US Environmental Protection Authority, Washington DC, USA.

Appendix 1

S. lalandi Larval Growth Inhibition Bioassay Data

GEOTECHNICAL SERVICES

Ecotoxicology Laboratory Test Report

Report Date: 24th October 2008

Sample Details

Lab ID No. ECX08- 1809	**Sample:** RO Brine
Client: ARUP	**Date Sampled:** 15th September 2008
Attn: James Brook	**Date Received:** 15th September 2008
Level 2	**Sampled By:** P. Fildes
431 – 439 King William St	**pH:** 7.8
Adelaide SA 5000	**Nominal Salinity:** 43 – 78 ppt
Phone No.	**Test Started:** 19th September 2008
Mobile: 0417 822 705	**Test Finished:** 25th September 2008
Order No.: Contract	**Test Temperature:** 17.0 ± 1.0°C

Test Performed	7-day Continuous Fish Larval Growth
Test Protocol	WIENV-64
Reference	USEPA 1004.0 Larval Fish Growth Test
Test Species	Yellowtail Kingfish
Deviations from Protocol	Nil

7-day Continuous Larval Fish Test Results (RO brine with and without Nalco)

Concentration Tested %	RO Brine + Nalco Av. Length mm	RO Brine + Nalco % Growth n = 30	RO Brine Av. Length mm	RO Brine % Growth n = 30
Initial	4.26 ± 0.07			
Control (43 ppt)	5.15 ± 0.01	100.0 ± 0.9	5.06 ± 0.06	99.9 ± 7.9
Control (37 ppt)	5.24 ± 0.03	110 ± 3.3	5.24 ± 0.03	110 ± 3.3
1.56	5.00 ± 0.01	86.0 ± 3.7	5.02 ± 0.07	95.0 ± 8.1
3.13	4.84 ± 0.04	65.4 ± 5.3	4.95 ± 0.06	86.8 ± 7.3
6.25	4.83 ± 0.01	63.9 ± 1.1	4.81 ± 0.05	69.6 ± 4.6
12.5	4.68 ± 0.02	48.2 ± 1.6	4.01 ± 0.13	18.6 ± 6.8
25	0.0	0.0	0.0	0.0
50	0.0	0.0	0.0	0.0
100	0.0	0.0	0.0	0.0

Sample	EC50 %	EC10 %	LOEC %	NOEC %
RO Brine + Nalco	6.92	1.48	1.6	<1.6
RO Brine	8.14	4.40	6.25	3.13

Results apply to the sample in the condition as received by Geotech

Quality Assurance Limits for the Larval Fish Toxicity Test.

	EC50	Cusum Chart Limits	Coefficient of Variation
Chromium	3.20 ppm	2.29 – 3.77 ppm	12.1

GEOTECHNICAL SERVICES

Ecotoxicology Laboratory Test Report

Report Date: 24th October 2008

Sample Details

Lab ID No. ECX08- 1809	**Sample:** Nalco
Client: ARUP	**Date Sampled:** 8th November 2007
Attn: James Brook	**Date Received:** 8th November 2007
Level 2	**Sampled By:** S. Christie
431 – 439 King William St	**pH:** NA
Adelaide SA 5000	**Nominal Salinity:** 43 ppt
Phone No.	**Test Started:** 19th September 2008
Mobile: 0417 822 705	**Test Finished:** 26th September 2008
Order No.: Contract	**Test Temperature:** 17.0 ± 1.0°C

Test Performed	7-day Continuous Fish Larval Growth
Test Protocol	WIENV-64
Reference	USEPA 1004.0 Larval Fish Growth Test
Test Species	Yellowtail Kingfish
Deviations from Protocol	Nil

7-day Continuous Larval Fish Test Results (Nalco)

Concentration Tested ppm	Nalco Av. Length mm	Nalco % Growth n = 30
Initial	4.26 ± 0.07	
Control (43 ppt)	5.14 ± 0.06	99.9 ± 6.2
Control (37 ppt)	5.23 ± 0.09	109.5 ± 2.5
0.11	5.24 ± 0.05	110.3 ± 5.9
0.22	5.06 ± 0.03	90.9 ± 3.3
0.44	4.99 ± 0.01	83.0 ± 1.4
0.88	4.84 ± 0.04	65.8 ± 3.7
1.75	4.99 ± 0.01	83.4 ± 0.8
3.5	4.93 ± 0.03	76.0 ± 3.1
7.0	4.98 ± 0.01	81.7 ± 0.6

Sample	EC50 ppm	EC10 ppm	LOEC ppm	NOEC ppm
Nalco	>7	0.26	0.22	0.11

Results apply to the sample in the condition as received by Geotech

Quality Assurance Limits for the Larval Fish Toxicity Test.

	EC50	Cusum Chart Limits	Coefficient of Variation
Chromium	3.20 ppm	2.29 – 3.77 ppm	12.1

GEOTECHNICAL SERVICES

Ecotoxicology Laboratory Test Report

Report Date: 24th October 2008

Sample Details

Lab ID No. ECX08- 1809	**Sample:** RO Brine
Client: ARUP	**Date Sampled:** 15th September 2008
Attn: James Brook	**Date Received:** 15th September 2008
Level 2	**Sampled By:** P. Fildes
431 – 439 King William St	**pH:** 7.8
Adelaide SA 5000	**Nominal Salinity:** 43 – 78 ppt
Phone No.	**Test Started:** 10th October 2008
Mobile: 0417 822 705	**Test Finished:**15th October 2008
Order No.: Contract	**Test Temperature:** 17.0 ± 1.0°C

Test Performed	2-day Pulsed Fish Larval Growth
Test Protocol	WIENV-64
Reference	USEPA 1004.0 Larval Fish Growth Test
Test Species	Yellowtail Kingfish
Deviations from Protocol	2 Day Pulse Exposure

2 Day Pulse Larval Fish Test Results (RO brine with and without Nalco)

Concentration Tested %	RO Brine + Nalco Av. Length mm	RO Brine + Nalco % Growth n = 30	RO Brine Av. Length mm	RO Brine % Growth n = 30
Initial	4.11 ± 0.03			
Control (43 ppt)	4.74 ± 0.01	99.8 ± 1.7	4.75 ± 0.04	99.9 ± 6.4
Control (37 ppt)	4.71 ± 0.02	102.9 ± 3.7	4.71 ± 0.02	102.9 ± 3.7
0.78	4.64 ± 0.01	84.0 ± 1.8	4.58 ± 0.02	73.0 ± 2.5
1.56	4.61 ± 0.01	78.9 ± 1.4	4.62 ± 0.02	79.0 ± 3.1
3.13	4.59 ± 0.02	75.1 ± 2.4	4.74 ± 0.01	98.7 ± 0.5
6.25	4.61 ± 0.01	79.1 ± 1.4	4.66 ± 0.03	85.9 ± 5.2
12.5	4.57 ± 0.02	72.8 ± 2.6	4.63 ± 0.03	80.8 ± 4.9
25	0.0	0.0	0.0	0.0
50	0.0	0.0	0.0	0.0

Sample	EC50 %	EC10 %	LOEC %	NOEC %
RO Brine + Nalco	15.76	12.13	0.78	<0.78
RO Brine	15.99	12.45	6.25	3.13

Results apply to the sample in the condition as received by Geotech

Quality Assurance Limits for the Larval Fish Toxicity Test.

	EC50	Cusum Chart Limits	Coefficient of Variation
Chromium	3.43 ppm	2.29 – 3.77 ppm	12.1

Appendix 2

Statistical Analyses

Larval Growth

Start Date:	19/09/2008	Test ID:	ECX08-1809	Sample ID:	Brine + Nalco
End Date:	26/09/2008	Lab ID:	Freo	Sample Type:	Mixture
Sample Date:	19/09/2008	Protocol:	Geotech WI ENV-64	Test Species:	Yellowtail kingfish
Comments:					

Conc-%	1	2	3
Control	0.9690	1.0000	1.0000
1.6	0.8180	0.8890	0.8720
3.13	0.7100	0.6050	0.6450
6.25	0.6360	0.6300	0.6520
12.5	0.4950	0.4640	0.4870
25	0.0000	0.0000	0.0000

			Transform: Arcsin Square Root						1-Tailed		Number
Conc-%	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp
Control	0.9963	1.0000	1.5024	1.4657	1.5208	2.116	3				1
*1.6	0.8597	0.8628	1.1687	1.1301	1.2311	4.413	3	10.052	2.470	0.0771	42
*3.13	0.6533	0.6557	0.9419	0.8912	1.0021	5.952	3	17.956	2.470	0.0771	104
*6.25	0.6393	0.6417	0.9266	0.9169	0.9398	1.280	3	18.449	2.470	0.0771	108
*12.5	0.4820	0.4838	0.7674	0.7494	0.7804	2.099	3	23.551	2.470	0.0771	155
25	0.0000	0.0000	0.0500	0.0500	0.0500	0.000	3				300

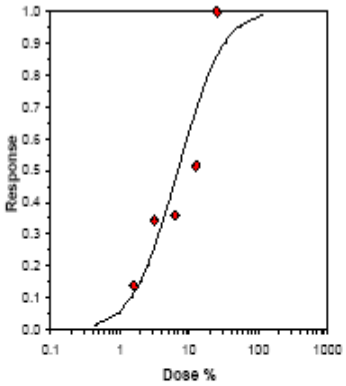
Auxiliary Tests					Statistic		Critical		Skew
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)					0.965759		0.835		-0.18075
Bartlett's Test indicates equal variances (p = 0.28)					5.064292		13.2767		
Hypothesis Test (1-tail, 0.05)	**NOEC**	**LOEC**	**ChV**	**TU**	**MSDu**	**MSDp**	**MSB**	**MSE**	**F-Prob**
Dunnett's Test	<1.6	1.6			0.016343	0.016419	0.247129	0.001461	3.9E-09

Maximum Likelihood-Probit

Parameter	Value	SE	95% Fiducial Limits		Control	Chi-Sq	Critical	P-value	Mu	Sigma
Slope	1.916191	0.603321	-0.00385	3.836231	0.003333	120.0495	7.814725	7.5E-26	0.84063	0.521869
Intercept	3.389193	0.546682	1.649404	5.128982						
TSCR	0.004394	0.023931	-0.07176	0.080552						

Point	Probits	%	95% Fiducial Limits
EC01	2.674	0.423235	
EC05	3.355	0.959914	
EC10	3.718	1.485348	
EC15	3.964	1.994103	
EC20	4.158	2.520078	
EC25	4.326	3.080595	
EC40	4.747	5.109939	
EC50	5.000	6.928347	
EC60	5.253	9.393848	
EC75	5.674	15.56205	
EC80	5.842	19.04782	
EC85	6.036	24.07197	
EC90	6.282	32.317	
EC95	6.645	50.00655	
EC99	7.326	113.4168	

Significant heterogeneity detected (p = 7.53E-26)



Larval Growth

Start Date:	19/09/2008	Test ID:	ECX08-1809	Sample ID:	Brine - Nalco
End Date:	25/09/2008	Lab ID:	Freo	Sample Type:	RO Brine
Sample Date:	19/09/2008	Protocol:	Geotech WI ENV-64	Test Species:	Yellowtail kingfish
Comments:					

Conc-%	1	2	3
Control	0.9590	0.9470	1.0000
1.6	0.9370	1.0000	0.8770
3.13	0.8730	0.9390	0.7930
6.25	0.7180	0.6300	0.7390
12.5	0.1620	0.2620	0.1330
25	0.0000	0.0000	0.0000

			Transform: Arcsin Square Root						1-Tailed		Number
Conc-%	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp
Control	0.9687	1.0000	1.4087	1.3385	1.5208	6.962	3				9
1.6	0.9380	0.9683	1.3501	1.2125	1.5208	11.613	3	0.668	2.470	0.2168	18
3.13	0.8683	0.8964	1.2087	1.0985	1.3212	9.217	3	2.279	2.470	0.2168	40
*6.25	0.6957	0.7182	0.9875	0.9169	1.0346	6.304	3	4.799	2.470	0.2168	91
*12.5	0.1857	0.1917	0.4416	0.3733	0.5373	19.334	3	11.018	2.470	0.2168	245
25	0.0000	0.0000	0.0500	0.0500	0.0500	0.000	3				300

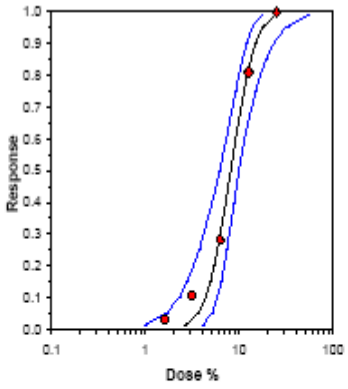
Auxiliary Tests					Statistic		Critical		Skew
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)					0.953042		0.835		0.406975
Bartlett's Test indicates equal variances (p = 0.62)					1.51711		13.2767		
Hypothesis Test (1-tail, 0.05)	**NOEC**	**LOEC**	**ChV**	**TU**	**MSDu**	**MSDp**	**MSB**	**MSE**	**F-Prob**
Dunnett's Test	3.13	6.25	4.422952	31.94888	0.110763	0.113724	0.460242	0.011556	4.1E-06

Maximum Likelihood-Probit

Parameter	Value	SE	95% Fiducial Limits		Control	Chi-Sq	Critical	P-value	Mu	Sigma
Slope	4.7923	0.657387	2.700198	6.884402	0.03	14.42499	7.814725	2.4E-03	0.910682	0.208668
Intercept	0.63574	0.642335	-1.40846	2.679938						
TSCR	0.060656	0.019687	-0.002	0.123307						

Point	Probits	%	95% Fiducial Limits	
EC01	2.674	2.66224	0.962631	4.05489
EC05	3.355	3.693629	1.701513	5.152068
EC10	3.718	4.396077	2.29728	5.873826
EC15	3.964	4.947789	2.806483	6.432139
EC20	4.158	5.433282	3.283967	6.9273
EC25	4.326	5.887586	3.750532	7.39678
EC40	4.747	7.208025	5.17585	8.836793
EC50	5.000	8.141075	6.195257	9.973386
EC60	5.253	9.194904	7.298401	11.43668
EC75	5.674	11.25709	9.198463	14.96074
EC80	5.842	12.19835	9.958039	16.85262
EC85	6.036	13.3953	10.85262	19.4873
EC90	6.282	15.06956	12.00744	23.56234
EC95	6.645	17.94362	13.8195	31.51336
EC99	7.326	24.89524	17.71187	55.22054

Significant heterogeneity detected (p = 2.38E-03)



Larval Growth

Start Date:	19/09/2008	Test ID:	ECX08-1809	Sample ID:	SW + Nalco
End Date:	26/09/2008	Lab ID:	Freo	Sample Type:	Nalco
Sample Date:	19/09/2008	Protocol:	Geotech WIENV-64	Test Species:	Yellowtail kingfish
Comments:					

Conc-mg/L	1	2	3
Control	0.9330	1.0000	1.0000
0.11	1.0000	1.0000	1.0000
0.219	0.9050	0.8780	0.9430
0.44	0.8330	0.8410	0.8150
0.875	0.6240	0.6960	0.6510
1.75	0.8400	0.8380	0.8250
3.5	0.7650	0.7700	0.7260
7	0.8240	0.8140	0.8130

			Transform: Arcsin Square Root						1-Tailed		Number
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp
Control	0.9777	1.0000	1.4502	1.3090	1.5208	8.432	3				7
0.11	1.0000	1.0228	1.5208	1.5208	1.5208	0.000	3	-1.660	2.560	0.1089	0
*0.219	0.9087	0.9294	1.2671	1.2140	1.3297	4.614	3	4.305	2.560	0.1089	28
*0.44	0.8297	0.8486	1.1455	1.1262	1.1606	1.539	3	7.163	2.560	0.1089	52
*0.875	0.6577	0.6727	0.9462	0.9107	0.9890	4.191	3	11.851	2.560	0.1089	103
*1.75	0.8343	0.8534	1.1517	1.1392	1.1593	0.946	3	7.018	2.560	0.1089	50
*3.5	0.7603	0.7777	1.0597	1.0199	1.0887	3.364	3	9.181	2.560	0.1089	72
*7	0.8170	0.8357	1.1288	1.1236	1.1379	0.699	3	7.557	2.560	0.1089	56

Auxiliary Tests	Statistic	Critical	Skew
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.01)	0.876966	0.884	-1.17103
Equality of variance cannot be confirmed			

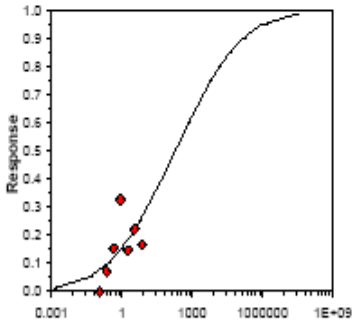
Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob
Dunnett's Test	0.11	0.219	0.15521		0.037273	0.03782	0.113081	0.002713	4.2E-09

Maximum Likelihood-Probit

Parameter	Value	SE	95% Fiducial Limits		Control	Chi-Sq	Critical	P-value	Mu	Sigma
Slope	0.447061	0.314066	-0.36027	1.254393	0.023333	118.5887	11.07048	6.3E-24	2.281412	2.236832
Intercept	3.98007	0.228102	3.393716	4.566424						
TSCR	0.01886	0.038441	-0.07996	0.117676						

Point	Probits	mg/L	95% Fiducial Limits
EC01	2.674	0.001196	
EC05	3.355	0.040008	
EC10	3.718	0.259894	
EC15	3.964	0.918512	
EC20	4.158	2.505226	
EC25	4.326	5.925049	
EC40	4.747	51.84626	
EC50	5.000	191.1667	
EC60	5.253	704.8674	
EC75	5.674	6167.834	
EC80	5.842	14587.4	
EC85	6.036	39786.9	
EC90	6.282	140613.7	
EC95	6.645	913426.4	
EC99	7.326	30553634	

Significant heterogeneity detected (p = 6.25E-24)



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Larval Fish Growth

Start Date:	10/10/2008	Test ID:	ECX08-0910	Sample ID:	Brine + Nalco
End Date:	15/10/2008	Lab ID:	Freo	Sample Type:	2 Day Pulse
Sample Date:	10/10/2008	Protocol:	GEOTECH WIENV-64	Test Species:	Yellowtail Kingfish
Comments:					

Conc-%	1	2	3
Control	1.0000	0.9790	1.0000
0.78	0.8560	0.8210	0.8440
1.56	0.7870	0.7770	0.8050
3.13	0.7740	0.7530	0.7260
6.25	0.7910	0.7760	0.8040
12.5	0.7620	0.7120	0.7100
25	0.0100	0.0200	0.0000
50	0.0000	0.0000	0.0000

			Transform: Arcsin Square Root						1-Tailed		Number
Conc-%	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp
Control	0.9930	1.0000	1.4890	1.4254	1.5208	3.699	3				2
*0.78	0.8403	0.8463	1.1601	1.1340	1.1816	2.082	3	11.749	2.530	0.0708	48
*1.56	0.7897	0.7952	1.0945	1.0790	1.1134	1.597	3	14.092	2.530	0.0708	62
*3.13	0.7510	0.7563	1.0487	1.0199	1.0754	2.651	3	15.730	2.530	0.0708	75
*6.25	0.7903	0.7959	1.0953	1.0776	1.1122	1.571	3	14.063	2.530	0.0708	63
*12.5	0.7280	0.7331	1.0225	1.0021	1.0612	3.273	3	16.663	2.530	0.0708	82
*25	0.0100	0.0101	0.0974	0.0500	0.1419	47.249	3	49.713	2.530	0.0708	297
50	0.0000	0.0000	0.0500	0.0500	0.0500	0.000	3				300

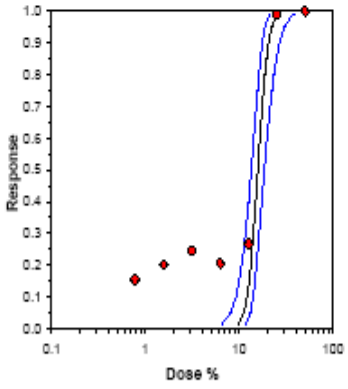
Auxiliary Tests					Statistic		Critical		Skew
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)					0.969141		0.873		-0.41618
Bartlett's Test indicates equal variances (p = 0.68)					3.962718		16.81187		
Hypothesis Test (1-tail, 0.05)	**NOEC**	**LOEC**	**ChV**	**TU**	**MSDu**	**MSDp**	**MSB**	**MSE**	**F-Prob**
Dunnett's Test	<0.78	0.78			0.01644	0.016551	0.550181	0.001175	2.6E-15

Maximum Likelihood-Probit

Parameter	Value	SE	95% Fiducial Limits		Control	Chi-Sq	Critical	P-value	Mu	Sigma
Slope	11.27103	1.900397	6.38591	16.15615	0.006667	22.60878	11.07048	4.0E-04	1.197685	0.088723
Intercept	-8.49915	2.277436	-14.3535	-2.64482						
TSCR	0.166665	0.020462	0.114066	0.219265						

Point	Probits	%	95% Fiducial Limits	
EC01	2.674	9.801321	6.52908	11.82853
EC05	3.355	11.26546	8.226455	13.22726
EC10	3.718	12.13339	9.269111	14.09363
EC15	3.964	12.75645	10.02297	14.74417
EC20	4.158	13.27438	10.64707	15.30917
EC25	4.326	13.73544	11.19675	15.83457
EC40	4.747	14.96951	12.61633	17.36925
EC50	5.000	15.76469	13.47452	18.47374
EC60	5.253	16.6021	14.32219	19.74297
EC75	5.674	18.09373	15.69574	22.2667
EC80	5.842	18.72218	16.22959	23.42323
EC85	6.036	19.48233	16.84654	24.88905
EC90	6.282	20.48276	17.61889	26.92133
EC95	6.645	22.06082	18.76679	30.34333
EC99	7.326	25.35631	20.97834	38.24567

Significant heterogeneity detected (p = 4.01E-04)



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Larval Fish Growth

Start Date:	10/10/2008	Test ID:	ECX08-0910	Sample ID:	Brine - Nalco
End Date:	15/10/2008	Lab ID:	Freo	Sample Type:	2 Day Pulse
Sample Date:	10/10/2008	Protocol:	GEOTECH WIENV-64	Test Species:	Yellowtail Kingfish
Comments:					

Conc-%	1	2	3
Control	0.9927	0.9385	1.0000
1.56	0.8244	0.7667	0.7786
3.13	0.9821	0.9911	0.9883
6.25	0.8965	0.8803	0.7993
12.5	0.7605	0.8054	0.8583
25	0.0000	0.0100	0.0200
50	0.0000	0.0000	0.0000

			Transform: Arcsin Square Root						1-Tailed		Number
Conc-%	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp
Control	0.9771	1.0000	1.4421	1.3202	1.5208	7.422	3				7
*1.56	0.7899	0.8084	1.0953	1.0667	1.1384	3.466	3	6.622	2.500	0.1309	63
3.13	0.9872	1.0103	1.4584	1.4366	1.4763	1.382	3	-0.313	2.500	0.1309	4
*6.25	0.8587	0.8789	1.1890	1.1063	1.2433	6.123	3	4.833	2.500	0.1309	42
*12.5	0.8081	0.8270	1.1194	1.0594	1.1849	5.619	3	6.162	2.500	0.1309	57
*25	0.0100	0.0102	0.0974	0.0500	0.1419	47.249	3	25.680	2.500	0.1309	297
50	0.0000	0.0000	0.0500	0.0500	0.0500	0.000	3				300

Auxiliary Tests	Statistic	Critical	Skew
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)	0.962346	0.858	-0.64765
Bartlett's Test indicates equal variances (p = 0.46)	4.669957	15.08632	

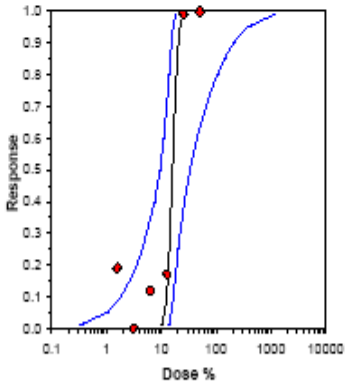
Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob
Dunnett's Test	3.13	6.25	4.422952	31.94888	0.04943	0.050258	0.751526	0.004113	6.8E-11

Maximum Likelihood-Probit

Parameter	Value	SE	95% Fiducial Limits		Control	Chi-Sq	Critical	P-value	Mu	Sigma
Slope	11.79537	3.765414	1.340881	22.24986	0.023333	74.43747	9.487728	2.6E-15	1.203972	0.084779
Intercept	-9.20129	4.490077	-21.6678	3.265189						
TSCR	0.096666	0.036799	-0.00551	0.198836						

Point	Probits	%	95% Fiducial Limits	
EC01	2.674	10.15655	0.336091	13.77701
EC05	3.355	11.60172	1.036757	15.44573
EC10	3.718	12.4544	1.857562	16.70372
EC15	3.964	13.06483	2.716384	17.84785
EC20	4.158	13.57125	3.62834	19.05072
EC25	4.326	14.02132	4.59073	20.41231
EC40	4.747	15.22275	7.618668	26.47903
EC50	5.000	15.99453	9.520126	33.60978
EC60	5.253	16.80545	11.17757	45.40336
EC75	5.674	18.24543	13.28129	62.26257
EC80	5.842	18.85052	13.94644	106.2023
EC85	6.036	19.5812	14.64149	144.2296
EC90	6.282	20.54094	15.42864	213.8614
EC95	6.645	22.05061	16.48098	387.9253
EC99	7.326	25.1882	18.27445	1209.93

Significant heterogeneity detected (p = 2.62E-15)



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APPENDIX H4.2

Toxicity testing on the sponge *Aplysine* sp.

The Provision of Reverse Osmosis Brine Toxicity Testing on the Sponge *Aplysina* sp.

Prepared for

ARUP

Report ECX08-1909

Marine Toxicity Tests

12th October 2008

Prepared by

Dr Jill Woodworth

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DISCLAIMER

This report presents a component of a study initiated by Arup on behalf of BHP Billiton to determine the toxicity of reverse osmosis brine (RO brine) to be discharged into Spencer Gulf from a proposed desalination plant located at Point Lowly.

Geotech has endeavored to achieve high accuracy results using certified techniques and equipment. However, Geotech shall not be held responsible or liable for the results of any actions taken on the basis of the information contained in this document. Moreover, this report should not be the sole reference when considering issues that may have commercial implications.

All data and information will remain proprietary to Arup Partners and is regarded as strictly confidential by all Geotech personnel. Any queries related to this report may be directed to David Strom at Geotech.

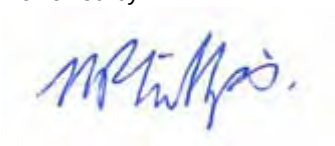
Report finalised by:	Reviewed by:
	
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Table of Contents

Executive Summary

This report presents a component of a study initiated by Arup on behalf of BHP Billiton to determine the toxicity of reverse osmosis brine (RO brine) to be discharged into Spencer Gulf from a proposed desalination plant located at Point Lowly. The potential for adverse biological effects resulting from exposure to the RO brine and the chemical antiscalant 'Nalco Permatreat® PC-1020T' were assessed concurrently using the locally relevant sponge *Aplysina sp*.

A sub-lethal endpoint assessing the ability of sponge cells to re-aggregate when exposed to a chemical stressor was developed for *Aplysina sp.* The test involved exposing small cubes of sponge to the RO brine treatments with and without the antiscalant.

While the RO brine significantly inhibited the ability of *Aplysina sp.* to re-attach, the RO brine with antiscalant did not appear to be more toxic than the RO brine without antiscalant. However, it was difficult to draw robust conclusions regarding the significance of biological effects given the lack of quality assurance data for the newly developed sponge bioassay.

1 Introduction

Geotechnical Services were requested by ARUP Pty Ltd to study the environmental impacts of the reverse osmosis (RO) return water produced by the proposed BHP Billiton desalination plant to be located at Point Lowly in Upper Spencer Gulf. The ambient salinity at Point Lowly (40-43 g/L) is greater than the majority of Australian marine waters (34-37 g/L), thus the return water salinity approximates 78 g/L.

A component of this project was to develop a test to determine the toxicity of the return water to a representative sponge.

2 Biology of Sponges (from Brusca and Brusca 1990)

Sponges occur at all depths. Most littoral sponges are encrusting, forming thick or thin layers on hard surfaces. Benthic sponges that live on soft substrata are often upright and tall, thus avoiding burial by shifting sediments.

2.1 Taxonomy of Sponges

There are 3 classes of Porifera. Class Calcarea or the calcareous sponges have spicules of mineral skeleton composed entirely of calcium carbonate laid down as calcite with skeletal elements often not differentiated into megascleres and microscleres. Body form can be asconoid, synconoid or leuconoid. Calcareous sponges are largely limited to shallow waters (less than 100m) as the secretion of calcareous skeletons becomes more difficult at greater depths due to the increased solubility of calcium carbonate needed for a firm substratum for attachment.

Class Hexactinellida, or the glass sponges, have spicules that are siliceous and basically six-rayed with both megascleres and microscleres always present. Their body wall is cavernous with trabecular network. They are exclusively marine and primarily found in deep water.

Class Demospongiae contain siliceous spicules and their spicule skeleton may be supplemented or replaced by an organic collagenous network called spongin. They inhabit marine, brackish or freshwater and occur at all depths.

Poriferans are sessile, suspension-feeding metazoans that utilize flagellated cells called choanocytes to circulate water through a unique system of water canals. Most of the body cells of a sponge retain a high degree of mobility and are capable of changing form and function. Most individual sponge cells are capable of radically altering their form and function and grow by continually adding new cells that differentiate rapidly.

The outer squamous surface cells of a sponge make up the pinacoderm and are called pinacocytes. The inner surface called the choanoderm and is composed of flagellated cells called choanocytes. Both of these epithelial

layers are a single cell thick and between these two cellular sheets is the bulk of the sponge body called the mesohyl. The pinacoderm is perforated by small holes called dermal pores (opening surrounded by several cells) or ostia (surrounded by a single cell). The choanocytes pump large volumes of water through the sponge body at very low pressures, establishing a water current (aquiferous) system. The one-cell-thick choanoderm may remain simple and continuous (the asconoid condition), folded (the synconoid condition) or greatly subdivided into separate flagellated chambers (the leuconoid condition).

H.V. Wilson (1891) first demonstrated the remarkable ability of sponge cells to re-aggregate after being mechanically dissociated. Almost any sponge dissociated and maintained under proper conditions will form aggregates, and many will eventually reconstitute their aquiferous system. Furthermore, if cell suspensions of two different species are mixed, the cells resort themselves to reconstitute individuals of each separate species.

Sponges are size-selective particle feeders and the arrangement of the aquiferous system creates a series of sieves with decreasing mesh size. Excretion and gas exchange are by simple diffusion.

Sponges are capable of responding to a variety of environmental stimuli by closure of the ostia, canal constriction, backflow and reconstruction of flagellated chambers. During major growth phases such a canal or chamber reorganisation, activity levels typically fall and pumping rates can cease completely within a few minutes. The response aims to reduce or stop the flow of water through the aquiferous system.

2.2 Reproduction of Sponges

Asexual reproduction processes enable sponges to regenerate viable adults from fragments. Some branching species 'pinch' off branch ends by a process of cellular reorganisation after which dislocated pieces regenerate into new individuals. This regenerative ability is used by commercial sponge farmers. Additional asexual processes of poriferans include formation of gemmules (small spherical structures that are resistant to freezing and drying), budding and possibly the formation of asexual larvae.

Most sponges are hermaphroditic but they produce eggs and sperm at different times. Sexual reproduction (sequential hermaphroditism) may take the form of protogyny or protandry and sex change may occur once or repeatedly. In some species, individuals appear to be permanently male or female. Release of the larvae is through either the excurrent plumbing of the aquiferous system or a rupture in the body wall. Larvae may then settle directly, swim for several hours or simply reside in the substratum until attachment conditions are favourable.

2.3 Growth of Sponges

Growth rates vary widely among species. Some species are annuals (especially Calcarea of colder waters) and grow from larvae or gemmules to reproductive adulthood in a matter of months. Others are perennials and grow so slowly that almost no change can be seen from one year to the next. This growth pattern is especially true of tropical and polar demosponges.

The suggested primary defence mechanism of sponges is biochemical. Previous studies have shown that sponges manufacture a surprisingly broad spectrum of biotoxins and antimicrobial agents which they use to reduce predation, prevent infection and compete for space with other sessile invertebrates.

2.4 Sponge Aquaculture

Environmental conditions to be considered in optimising sponge growth and survival in laboratory based closed systems include water movement, light intensity, temperature and nutrient availability. Another important factor is the type of sponge and how it reacts to damage. The type and size of sponge explants have a large influence on survival and growth of sponges in aquaculture and may differ between species and sponge types.

When a sponge is damaged, healing of the surface pinacoderm must occur rapidly. For an explant to survive it requires consolidation of collagen below the surface layer. Some species additionally need to incorporate foreign matter into surface tissue (Bergquist, 1980) and require melanin cells to develop below the healed pinacoderm. Such reinforcement processes can be directly monitored to provide information on the health of explants (Duckworth *et al.,* 1997).

Seawater temperature may also influence the healing time of the explant surface pinacoderm. In laboratory experiments with *Psammocinia hawere* (a massive cup-shape sponge found below 10m in exposed areas along the north eastern coast of New Zealand), healing of each cut side of explants was complete after 110 days when farmed at 14°C, compared to 80% healing for explants farmed at 19°C (Duckworth *et al.* 1997).

The same study also showed that the number of cut sides on an explant affected the healing time of the pinacoderm. Cut surfaces are more susceptible to disease, algal and fungal fouling and damage from UV radiation. Healing of explants with <4 cut sides were indistinguishable, while explants with 5 cut sides achieved approximately one-third the level of healing. None of the explants with 6 cut sides survived. As explant size increased, the ratio of cut surface area to volume decreased. Proportionate decreases in tissue stress were observed for lower surface area to volume ratio explants which resulted in greater survival. These larger explants appeared to have a greater tissue reserve and capacity to divert energy into healing than the smaller high surface area to volume ratio explants. High

light intensities have also been demonstrated as unfavourable for explant survival.

2.5 Sponge Suitability for Laboratory Use

Sponges are generally very slow growing organisms. The morphological plasticity of a species in response to changing environmental conditions may be an indicator of suitability for use in aquaculture. A study by Duckworth *et al.* (1997) suggested that encrusting and amorphous sponges have the greatest potential for success in aquaculture or experimental conditions. The study used three morphologically distinct sponge species including *Psammocinia hawere* (a massive cup shaped fibrous sponge), *Raspailia topsenti* (a branching digitate siliceous sponge) and *Raspailia agminata* (a thickly encrusting siliceous sponge). Explants of *R. agminata* exhibited the highest growth with some explants more than doubling their weight in 262 days. Encrusting sponges often respond to damage by growing many times faster than their undisturbed growth rate (Ayling 1978). However increased growth rates after damage is less common, for large or digitate sponges not amenable to cutting (Verdenal and Vacelet, 1990).

2.6 Measurements of Sponge Growth

The simplest way to measure growth of sponge explants is by wet weight. Studies by Duckworth *et al.* (1997) showed that dry weights of sponge explants correlated well with wet weights and can be wet-weighed with an acceptable degree of accuracy. Wet weighing is therefore a consistently reliable and non-destructive measure of sponge growth.

Another suitable method of determining sponge growth involves measuring explant size as a two-dimensional projection of body area determined from photographs (Osinga *et al.* 1999, Ayling 1993). The method is advantageous in that sponges are not removed from the water. Exposure to air can cause serious damage to sponge tissue which may affect the rate of growth.

In contrast to these studies, Hausmann *et al.* (2006) showed that sponge fragments or explants that initially attached to an experimental substrate gradually developed an increasing contact area. However, the increase in contact area was not linked to an increase in volume. The applicability of projected body area measurements may be unreliable especially given the morphological variability of some sponges is very high.

As for many other invertebrates, sponge populations relying on larval settlement stages may be more sensitive to contaminants than the larger and more established individuals. The sub-lethal effect of contaminants on larval stages may have drastic repercussions at an ecological level. For example, an acute exposure to a toxicant may result in mortality for half of the population with little ongoing ecological significance. While consistent exposure to low levels of a toxicant may not cause immediate acute effects, ongoing inhibition of early life stage development may adversely affect the

biological processes responsible for successive populations (Moriarty, 1983).

Therefore larval settlement has been demonstrated to be a relevant and useful method of measuring the ecological impacts from pollutants. However, several methods should be considered given the many different environmental variables known to influence the growth of sponges.

3 Methods

3.1 Sponge Samples

Sponge samples were received by Geotech's Fremantle Ecotoxicology Laboratory on the 23rd July 2008 and 19th September 2008 for use in method development and the RO brine with and without antiscalant bioassays (Tables 1 and 2, respectively). The antiscalant is referred to as Nalco herein. Samples were transported in bags of seawater.

The first sample received contained 4 large fragments of a sponge identified as the *Aplysina* species (Table 1). The family Aplysinidae comprises massive, tubular or ramose sponges with a skeleton of pithed, amber coloured fibres forming a regular reticulum of polygonal meshes without specialised arrangement near the sponge surface. Most Aplysinids are yellow to green, brown or purple in colour. However, when preserved or exposed to air, Aplysinids may undergo a conspicuous oxidative colour change to brown, dark purple or black.

The *Aplysina sp.* received was a branching form with tapering cylindrical ends. The external pinacoderm was dark brown in colour with the internal mesohyl being bright yellow. The specimen appeared to be aerophobic and changed colour from bright yellow to black when exposed to air.

The second sample received contained encrusting and finger sponges (Table 2). No further testing could be undertaken with these sponges given the samples died within 3 days of receipt.

Table 1 Sample information sheet for *Aplysina sp*.

Contact Details	ARUP
Contact Person	James Brook
Number of Samples	2
Sample Type	Sponges (*Aplysina* sp.)
Date Collected	22/07/08
Location Collected	Point Lowly
Sample Collected by	James Brook
pH	Bag 1: pH 7.60 Bag 2: pH 6.60 (larger piece of sponge)
Nominal Salinity	Bag 1: 41.9 ppt Bag 2: 41.9 ppt
Temperature	Bag 1: 11.3°C Bag 2: 12.3°C
Transport Conditions	Esky - Air Freight
Date of Arrival at Geotech	23/07/08 4 pm Received by JW

Table 2 Sample information sheet for encrusting and finger sponges

Company	ARUP
Contact Person	James Brook
Number of Samples	2 x sponges
Sample Type	1 x Encrusting 1x Finger
Date Collected	18/09/08
Location Collected	Point Lowly
Sample Collected by	James Brook
pH	Encrusting: pH 7.62 Finger: pH 6.82
Nominal Salinity	Encrusting: 40 ppt Finger: 40 ppt
Temperature	Encrusting: 19.9°C Finger: 19.9°C
Transport Conditions	Air Freight
Date of Arrival at Geotech	19/09/08
Time of Arrival at Geotech	5:30 pm
Sample Received by	JW
Tests Required	RO brine toxicity

3.2 Seawater Diluent, RO Brine and Nalco Treatments

Seawater diluent and RO brine were sourced on site from Point Lowly and transported in a refrigerated truck at 4°C from South Australia to to Geotech's Welshpool Laboratory in Perth, Western Australia on 23rd June 2008. A sample of Nalco Permatreat® PC-1020T was sourced from the Perth Desalination Plant (Water Corporation of WA) and used to spike sub-samples of the RO brine and Point Lowly diluent to final concentrations of 7.0 mg/L (15th September 2008). The seawater diluent, RO brine and Nalco treatments were stored at 4°C prior to testing.

3.3 Physico-chemical Measurements

The salinity and pH was measured on delivery to the laboratory. The diluent seawater was filtered to 0.45 µm and transported to Geotech's Fremantle Ecotoxicology Laboratory in 25 L HDPE containers for use in the bioassays. The RO brine sample was tested as received.

Nominal salinity measurements were made using a refractometer with an accuracy of ±1 ppt, followed by more accurate measurements (post-testing) using an Autolab Salinometer, considered by BHP Billiton to have an accuracy of ±0.02 ppt.

3.4 Maintenance of Sponge Samples

The specimens were maintained at 15°C in 100 L culturing containers filled with 0.45 µm filtered seawater diluent sourced from South Australia. The cultures were aerated and seawater was renewed daily. The sponge cultures were fed using a mixture of two marine microalgae species (cultured in-house) which included *Isochrysis sp.* and *Nannochloropsis sp.* Each sponge fragment was transferred to separate 5L glass containers and similarly maintained.

3.5 Sponge Bioassay Method Development

Explants were cut using a sterile surgical scalpel blade and transferred to separate smaller culture vessels. Unless otherwise specified, small cubes (approximately 5 mm^3) were cut from parent material. Explants were placed into each well of a 6 well microplate containing 15 mL of seawater diluent or test solution. The explants were photographed, measured then incubated using a 12-h light:12-hr dark photoperiod at 15°C.

Explant survival was determined qualitatively, with bacterial and fungal fouling indicative of explant mortality. Explant growth was measured using percentage increases in wet weight and re-attachment was identified by gentle agitation of the tissue under a dissection microscope.

3.5.1 Suitability of sponge bioassay endpoints

The literature outlined sponge survival, growth and re-attachment as potential endpoints that could be measured in a bioassay. Observational trials were therefore conducted to determine if explant growth, survival or re-attachment endpoints were suitable for use in the sponge bioassay. The preliminary experiments aimed to determine if morphological changes of the explant responding to a mechanical disturbance could be used as an endpoint for further method development of the sponge bioassay.

Explant cubes (approx 3 mm^3) were monitored over 5 days to identify which tissue types were most capable of surviving, growing and re-attaching. Explant samples containing both brown coloured pinacoderm and yellow mesohyl were compared to explants of yellow mesohyl tissue only (independent of the external pinacoderm cells).

The survival, growth and re-attachment experiments were then co-varied with (i) surface area to volume (SA:V) ratio and (ii) exposed surface cuts. Six explant treatments were cut from the parent sponge to include pinacoderm and mesohyl tissue. For each of the three differing SA:V ratio treatments (3 and 5 mm^3 cubes and 2 mm thick slices), explants were prepared to include one cut surface (whole tips of the sponge finger) and two cut surfaces (below the initial incisions). The explants were transferred to 1 L glass beakers containing 800 mL seawater and monitored until complete mortality was observed.

3.5.2 Explant response to chromium reference toxicant

The response of the re-attachment endpoint was further examined by exposing explants to Control (seawater) and 10 mg/L chromium reference treatments. For each treatment, five sponge explants were placed in each well of a six well microplate (Figure 1, Appendix 2). The number of sponge pieces that re-attached was determined after 19 hours. To reduce the exposure duration, the experiment was repeated by increasing the chromium reference concentration to 20 mg/L. Re-attachment of the sponge pieces was assessed after 1 and 2 hours.

To ensure that the type of sponge tissue did not influence the ability of explants to re-attach after a reduced exposure time, a small scale experiment was performed using internal and external sponge tissue. Six pieces of internal and external sponge tissue were placed in microplate wells containing seawater (in triplicate). The number of sponge pieces that re-attached was determined after 2 hours.

3.5.3 Toxicity of RO brine and Nalco to the sponge Aplysina sp.

The toxicity of the RO brine both (i) with Nalco antiscalant and (ii) without Nalco antiscalant to the sponge *Aplysina sp.* was assessed. Each sample was serially diluted to a concentration series of 100, 50, 25, 12.5, 6.3, 3.1 and 1.5%. Diluent seawater controls were also included. Each concentration contained 6 replicates, to which 6 pieces of *Aplysina sp.* sponge tissue were added. The number of sponge pieces that re-attached was determined after 2 hours.

4 Results

4.1 Suitability of Sponge Bioassay Endpoints

Observations over 5 days revealed that the explants underwent visible morphological changes, with sharp edges rapidly becoming rounded and smooth overnight. Separate pieces placed in close proximity were also observed to physically join and remain fixed. The ability to regroup after mechanical separation was a well known attribute of sponges and was in good agreement with the literature.

However, both tissue types tested did not survive beyond 5 days with visible decomposition occurring rapidly. Complete mortality was observed after 2 days for explants independent of the pinacoderm with high levels of bacterial and fungal growth evident. Therefore, the following experiments used explants containing pinacoderm and mesohyl tissue.

Percentage growth, survival and re-attachment of the explants were observed with SA:V ratio co-varying with one cut and two cuts (Tables 3 and 4, respectively). The 5 mm^3 explant with one cut surface was observed to have the greatest percentage growth (21±4%), survival duration (21 days) and ability to re-attach. Poor growth or survival was observed in other explant treatments. Of the possible endpoints, re-attachment was observed more rapidly after <24 hours. Explant re-attachment was therefore selected as the preferred endpoint for further development of the sponge bioassay given it could be easily determined and rapidly observed.

Table 3 Percentage growth, survival duration and re-attachment of explants (1 cut surface)

Explant Size	Percent Growth (%)	Survival Duration (Days)	Re-attachment
3 mm Cube	0	19	NO
2 mm Slice	0	10	NO
5 mm Cube	21 ± 4	21	YES

Table 4 Percentage growth, survival duration and re-attachment of explants (2 cut surfaces)

Explant Size	Percent Growth (%)	Survival Duration (Days)	Re-attachment
3 mm Cube	0	1	NO
2 mm Slice	0	4	NO
5 mm Cube	0	8	NO

4.2 Explant Response to Chromium Reference Toxicant

Inhibition of explant re-attachment was further examined by exposure to the reference toxicant chromium. There was no significant difference between the Control and 10 mg/L chromium treatment after 19 hours of exposure (Table 5). Similar results were observed between the Control and 20 mg/L chromium reference after exposure for 1 and 2 hours (Table 6), indicating that the endpoint was not sensitive to the concentrations of chromium tested.

Less replicate variability was evident in controls exposed for 2 hours. In addition, there were no significant differences in the number of attachments between the internal and external explant tissues after 2 hours (Table 7). Therefore, the 2 hour exposure duration was selected to enable optimal re-attachment in control treatments.

Table 5 Number of explants re-attached after a 19 hour exposure to 10 mg/L chromium

Replicate	Control (No. attachments $_{n=4}$)	10 mg/L Chromium (No. attachments $_{n=4}$)
1	4	3
2	4	4
3	2	3
4	4	4
5	3	3
6	4	2
Mean ± S.D.	3.5 ± 0.8	3.2 ± 0.8

Table 6 Number of explants re-attached after 1 and 2 hour exposures to 20 mg/L chromium

Replicate	Control		20 mg/L Chromium	
	1 hour $_{n=4}$	2 hour $_{n=4}$	1 hour $_{n=4}$	2 hour $_{n=4}$
1	2	2	2	2
2	4	4	4	2
3	3	4	2	2
4	2	3	3	4
5	0	3	0	1
6	3	4	0	1
Mean ± S.D.	2.3 ± 1.4	3.3 ± 0.8	1.8 ± 1.6	2.0 ± 1.1

Table 7 Number of internal and external tissue re-attachments after 2 hour exposure

Replicate	Internal Tissue $_{n=5}$	External Tissue $_{n=5}$
1	4	5
2	5	4
3	4	4
Mean ± S.D.	4.3 ± 0.6	4.3 ± 0.6

4.3 Toxicity of RO Brine and Nalco to *Aplysina sp.*

The nominal salinity of the diluent seawater was 41.9 ppt (later measured to be 41.3 ppt). The control and diluent water used in test solutions was evaporated to a nominal salinity of 43 ppt (the maximum salinity likely to be encountered near Point Lowly) and later measured to be 44.3 ppt. The nominal salinity of the RO brine was 78 ppt (later measured to be 77.6 ppt).

The physicochemical data for the RO brine tests are shown in Table 8. The nominal salinity values have been adopted in this table, reflecting the understanding at the time of the toxicity tests. The sponges were delivered in water of nominal salinity 40 ppt (later measured to be 41.0 ppt).

Table 8 Physico-chemical data for RO brine concentration series

RO Brine Concentration (%)	pH	Nominal Salinity (‰)
Control	8.04	43.0
1.6	8.04	43.5
3.1	8.04	44.1
6.3	8.03	45.2
12.5	8.02	47.4
25.0	8.00	51.8
50.0	7.95	60.5
100.0	7.84	78.0

The number of explant re-attachments in each replicate of the RO brine concentration series (with and without Nalco) is shown in Tables 9 and 10, respectively. While control re-attachment after 2 hours was acceptable for both bioassays (>80%), there was high inter-replicate variability (>20%) observed for most concentrations (Table 11).

Although the RO brine appeared to inhibit sponge explant re-attachment, no significant differences in the EC50 concentration (i.e. the effect concentration at which explant re-attachment is reduced by 50% compared to the control) was observed between RO brine with Nalco (EC50 of 48.6%) or RO brine without Nalco (EC50 of 37.7%). This result indicated that additive effects from Nalco in the RO brine were negligible to sponge re-attachment (Table 12).

Table 9 Number of *Aplysina sp.* explants re-attached for RO brine with Nalco

RO Brine Concentration (%)	Replicate					
	1 n=5	2 n=5	3 n=5	4 n=5	5 n=5	6 n=5
Control	4	4	5	4	3	4
1.6	3	4	3	4	1	4
3.1	4	4	3	3	4	3
6.3	2	3	5	5	3	2
12.5	3	2	1	3	4	3
25.0	1	2	4	3	4	2
50.0	1	1	4	3	2	2
100.0	0	1	0	1	1	0

Table 10 Number of *Aplysina sp.* explants re-attached for RO brine without Nalco

RO Brine Concentration (%)	Replicate					
	1 n=5	2 n=5	3 n=5	4 n=5	5 n=5	6 n=5
Control	2	5	2	4	4	2
1.6	1	2	1	1	2	3
3.1	1	3	2	4	3	4
6.3	3	3	4	5	2	4
12.5	1	3	2	1	3	3
25.0	2	1	2	1	0	3
50.0	0	1	1	1	1	1
100.0	1	1	1	0	2	0

Table 11 Percentage of *Aplysina sp.* explants re-attached for RO brine without Nalco

RO Brine Concentration (%)	Percent Re-attachment (%)	
	RO Brine with Nalco	RO Brine without Nalco
Control	80 ± 13	63 ± 27
1.6	63 ± 23	33 ± 16
3.1	70 ± 11	57 ± 23
6.3	67 ± 27	70 ± 21
12.5	53 ± 21	43 ± 20
25.0	53 ± 24	30 ± 21
50.0	43 ± 23	17 ± 8
100.0	10 ± 11	17 ± 15

Table 12 Statistical effects data for *Aplysina sp.* exposed to RO brine with and without Nalco

RO Brine Concentration (%)	RO Brine with Nalco	RO Brine without Nalco
EC50	48.6	37.7
EC10	14.6	7.9
LOEC	50.0	25.0
NOEC	25.0	12.5

5 Discussion

Caution is advised when using the results presented in this study. The newly developed endpoint was not considered to be sensitive to the chromium reference toxicant with no significant inhibition of explant re-attachment being evident at concentrations as high as 20 mg/L. In comparison, EC50 concentrations <5 mg/L are generally observed for other marine organism endpoints when exposed to chromium (e.g. kingfish larval growth, mussel larvae development or copepod reproduction). If this limited sensitivity to chromium extends to the RO brine, the threshold of significant biological effects observed in this study may be underprotective when implemented in-situ. In addition, the results could not be confirmed or further refined given the lack of viable sponge tissue remaining after the initial developmental phase. Despite this, the RO brine with Nalco did not appear to be any more toxic than the RO brine without Nalco.

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APPENDIX 1

Statistical Analyses

Explants Attached

Start Date:	22/09/2008	Test ID:	ecx08-1809	Sample ID:	RO Brine with antiscalant
End Date:	22/09/2008	Lab ID:	Freo	Sample Type:	RO Brine
Sample Date:	15/09/2008	Protocol:	GEOTECH	Test Species:	APLYSINA Sp
Comments:					

Conc-%	1	2	3	4	5	6
Control	0.8000	0.8000	1.0000	0.6000	0.6000	0.8000
1.5	0.6000	0.8000	0.6000	0.8000	0.2000	0.8000
3.125	0.8000	0.8000	0.6000	0.6000	0.8000	0.6000
6.25	0.4000	0.6000	1.0000	1.0000	0.6000	0.4000
12.5	0.6000	0.4000	0.2000	0.6000	0.8000	0.6000
25	0.2000	0.4000	0.8000	0.6000	0.8000	0.4000
50	0.2000	0.2000	0.8000	0.6000	0.4000	0.4000
100	0.0000	0.2000	0.0000	0.2000	0.2000	0.0000

			Transform: Arcsin Square Root						1-Tailed		Number
Conc-%	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp
Control	0.8000	1.0000	1.1392	0.8861	1.5208	18.150	6				120
1.5	0.6333	0.7917	0.9262	0.4636	1.1071	27.117	6	1.474	2.420	0.3497	220
3.125	0.7000	0.8750	0.9966	0.8861	1.1071	12.150	6	0.987	2.420	0.3497	180
6.25	0.6667	0.8333	1.0305	0.6847	1.5208	37.872	6	0.752	2.420	0.3497	200
12.5	0.5333	0.6667	0.8190	0.4636	1.1071	26.799	6	2.217	2.420	0.3497	260
25	0.5333	0.6667	0.8222	0.4636	1.1071	31.378	6	2.194	2.420	0.3497	260
*50	0.4333	0.5417	0.7150	0.4636	1.1071	34.895	6	2.936	2.420	0.3497	340
*100	0.1000	0.1250	0.2568	0.0500	0.4636	88.210	6	6.107	2.420	0.3497	540

Auxiliary Tests	Statistic	Critical	Skew
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)	0.971764	0.929	0.200301
Bartlett's Test indicates equal variances (p = 0.51)	6.260219	18.47532	

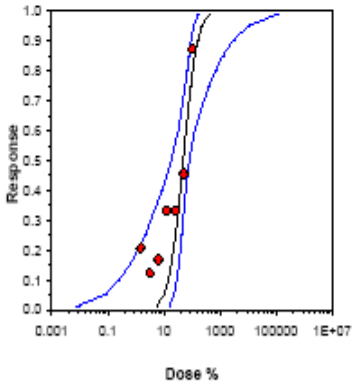
Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob
Dunnett's Test	25	50	35.35534	4	0.320884	0.388933	0.440782	0.06264	1.8E-05

Maximum Likelihood-Probit

Parameter	Value	SE	95% Fiducial Limits		Control	Chi-Sq	Critical	P-value	Mu	Sigma
Slope	2.457645	0.703201	0.650013	4.265276	0.2	75.91285	11.07048	6.0E-15	1.687152	0.406894
Intercept	0.853581	1.222538	-2.28905	3.99621						
TSCR	0.311545	0.038496	0.212589	0.410501						

Point	Probits	%	95% Fiducial Limits	
EC01	2.674	5.502695	0.007007	15.96858
EC05	3.355	10.42008	0.076715	23.55803
EC10	3.718	14.64524	0.272724	29.20054
EC15	3.964	18.4261	0.638126	33.94478
EC20	4.158	22.11573	1.247256	38.46832
EC25	4.326	25.86462	2.20304	43.08646
EC40	4.747	38.37659	8.767182	60.41431
EC50	5.000	48.65772	18.42035	80.86286
EC60	5.253	61.69317	33.03996	126.844
EC75	5.674	91.53714	58.14869	402.1665
EC80	5.842	107.0538	67.35756	686.8534
EC85	6.036	128.4902	78.14541	1311.373
EC90	6.282	161.6616	92.40938	3016.331
EC95	6.645	227.2125	116.0995	10579.3
EC99	7.326	430.2571	173.1578	114565.9

Significant heterogeneity detected (p = 6.00E-15)



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Explants Attached

Start Date:	22/09/2008	Test ID:	ecx08-1809	Sample ID:	RO Brine without antiscalant
End Date:	22/09/2008	Lab ID:	Freo	Sample Type:	RO Brine
Sample Date:	15/09/2008	Protocol:	GEOTECH	Test Species:	APLYSINA Sp
Comments:					

Conc-%	1	2	3	4	5	6
Control	0.4000	1.0000	0.4000	0.6000	0.8000	0.4000
1.5	0.2000	0.4000	0.2000	0.2000	0.4000	0.6000
3.125	0.2000	0.6000	0.4000	0.6000	0.6000	0.8000
6.25	0.6000	0.6000	0.8000	1.0000	0.4000	0.8000
12.5	0.2000	0.6000	0.4000	0.2000	0.6000	0.6000
25	0.4000	0.2000	0.4000	0.2000	0.0000	0.6000
50	0.0000	0.2000	0.2000	0.2000	0.2000	0.2000
100	0.2000	0.2000	0.2000	0.0000	0.4000	0.0000

			Transform: Arcsin Square Root						1-Tailed		Number
Conc-%	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp
Control	0.6333	1.0000	0.9649	0.6847	1.5208	35.450	6				220
*1.5	0.3333	0.5263	0.6077	0.4636	0.8861	28.652	6	2.444	2.420	0.3536	400
3.125	0.5667	0.8947	0.8558	0.4636	1.1071	29.153	6	0.746	2.420	0.3536	260
6.25	0.7000	1.1053	1.0320	0.6847	1.5208	27.862	6	-0.459	2.420	0.3536	180
12.5	0.4333	0.6842	0.7117	0.4636	0.8861	29.137	6	1.733	2.420	0.3536	340
*25	0.3000	0.4737	0.5388	0.0500	0.8861	53.366	6	2.916	2.420	0.3536	420
*50	0.1667	0.2632	0.3947	0.0500	0.4636	42.781	6	3.902	2.420	0.3536	500
*100	0.1667	0.2632	0.3626	0.0500	0.6847	70.826	6	4.122	2.420	0.3536	500

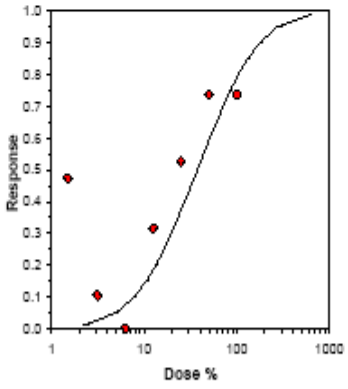
Auxiliary Tests					Statistic		Critical		Skew
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)					0.96615		0.929		0.022758
Bartlett's Test indicates equal variances (p = 0.79)					3.914017		18.47532		
Hypothesis Test (1-tail, 0.05)	**NOEC**	**LOEC**	**ChV**	**TU**	**MSDu**	**MSDp**	**MSB**	**MSE**	**F-Prob**
Dunnett's Test	12.5	25	17.67767	8	0.346273	0.512507	0.380693	0.06405	8.8E-05

Maximum Likelihood-Probit

Parameter	Value	SE	95% Fiducial Limits		Control	Chi-Sq	Critical	P-value	Mu	Sigma
Slope	1.896843	1.107674	-0.95052	4.744205	0.366667	232.709	11.07048	2.8E-48	1.576259	0.527192
Intercept	2.010085	1.845255	-2.73328	6.753456						
TSCR	0.446947	0.086084	0.225662	0.668232						

Point	Probits	%	95% Fiducial Limits
EC01	2.674	2.237829	
EC05	3.355	5.118058	
EC10	3.718	7.954907	
EC15	3.964	10.71172	
EC20	4.158	13.56947	
EC25	4.326	16.62161	
EC40	4.747	27.7138	
EC50	5.000	37.69282	
EC60	5.253	51.26502	
EC75	5.674	85.47598	
EC80	5.842	104.7019	
EC85	6.036	132.6349	
EC90	6.282	178.6002	
EC95	6.645	277.5952	
EC99	7.326	634.8781	

Significant heterogeneity detected (p = 2.81E-48)



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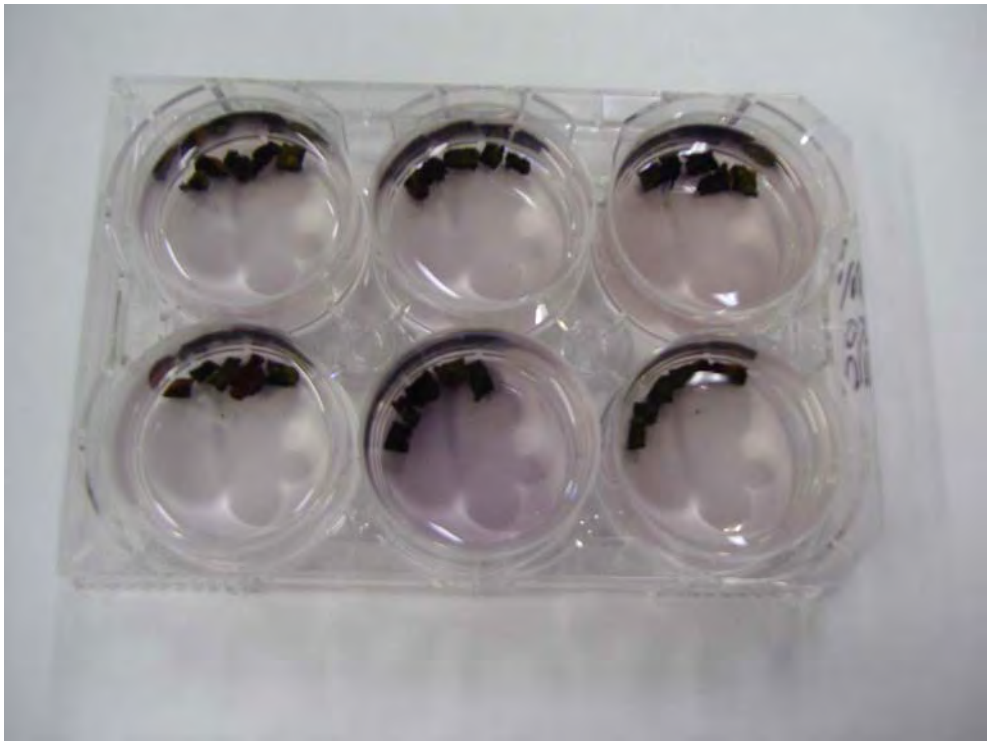
APPENDIX 2

Photograph of Explant Exposure

Figure 1 Replicate of Explant Exposure



APPENDIX H4.3

Refined assessment of safe dilutions

A refined assessment of the selection of species and other factors that affect dilution factors for the proposed desalination plant at Point Lowly, South Australia.

Michael Warne
CLW Report 07/10
October 2010

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Important Disclaimer

Cover Photograph:

From CSIRO's ScienceImage: www.scienceimage.csiro.au
File: AS2842.jpg
Description: Port survey sampling..
Photographer: CSIRO Marine and Atmospheric Research

CONTENTS

EXECUTIVE SUMMARY

Sixteen organisms were tested and evaluated as part of the Environmental Impact Statement for the proposed desalination plant at Point Lowly for their appropriateness to calculate dilution factors for the return water. This report provides an assessment of all the direct toxicity assessment (DTA) results, and the species protection values presented here use the most appropriate dataset available and thus supercede all previous values.

Seven of the tested sixteen species comprise the best dataset; being the unicellular alga *Isochrysis galbana*, the macroalga *Ecklonia radiata*, the Western King Prawn *Melicertus latisulcatus*, the Pacific Oyster *Crassostrea gigas*, the Pink Snapper *Chrysophrys*[1] *auratus*, the Mulloway *Argyrosomus japonicus* and the Giant Australian Cuttlefish *Sepia apama*.

A second dataset which retained the previous species but added the macroalga *Hormosira banksii*, the copepod *Gladioferens imparipes* and the Yellowtail Kingfish *Seriola lalandi* was also evaluated as this maximized the number of test species but contained toxicity data from a mixture of exposure durations from acute to chronic tests and included data derived using diluent water with different salinities.

Both the best and second best datasets contain more species belonging to more taxonomic groups than the minimum required by the Australian and New Zealand water quality guidelines and used in the evaluation of the Victorian and Western Australia desalination plants. Therefore there will be greater confidence in the dilution factors being derived for the proposed desalination plant at Point Lowly than for the others.

Use of the best dataset (i.e. that comprising the first seven species listed above) resulted in a concentration that should protect 99% of species (PC99) of 2.35 % return water and a dilution factor of 45 at 41.2 ppt diluent salinity. The corresponding values for the second best dataset are 2.48 % return water and a dilution factor of 40 respectively. The best dataset is recommended for use, even though it contained fewer species than the second best dataset, because all the toxicity data it contains are based upon sub-chronic or chronic exposure, all tests were conducted at one salinity (i.e. 41.2 ppt) and it results in a more conservative (larger) dilution factor.

A dilution factor of 45 would theoretically protect 99 % of marine species typical of Upper Spencer Gulf from experiencing a sub-chronic or chronic toxic effect of greater than 10 % in

[1] Reported previously (Warne et al., 2008) as *Pagrus auratus.*

receiving water with a salinity of 41.2 ppt. However, this salinity is just below the median of the range of salinities reported at Point Lowly (i.e. 40 – 43 ppt) and it may therefore underestimate the dilution factor required when the receiving water has a salinity of 41.2 to 43 ppt. Therefore the toxicity data in the best dataset were corrected to estimate the toxicity when the receiving water has a salinity of 43 ppt. This resulted in a PC99 of 1.56 % return water and a dilution factor of 65 for receiving waters with a salinity of 43 ppt. The PC99 value of 1.56 % return water was higher than the lowest toxicity value of 1.48 % return water generated by the study (which corresponds to a dilution factor of 68) and therefore the PC99 would not prevent this toxic effect occurring. However, this toxicity value is most probably an overestimate as it was conducted at a salinity greater than the maximum salinity at Point Lowly. Nonetheless, in order to be conservative and ensure protection of this species the recommended safe dilution factor was increased to 70.

If a dilution of 70 is achieved it would:

- theoretically protect more than 99 % of marine species typical of Upper Spencer Gulf from experiencing sub-chronic or chronic toxic effects of greater than 10% in sea water with a salinity of 43 ppt; and
- cause less than a 0.3 % reduction in post-hatch survival of the Giant Australian Cuttlefish in seawater with a salinity of 43 ppt and an even lower effect at 41 ppt (the measured salinity during breeding at Point Lowly).

Given the range of salinities (40 to 43 ppt) that occur at Point Lowly the appropriate degree of dilution (that will protect at least 99% of marine species and will provide a high level of protection to the Giant Australian Cuttlefish) is 45 (when the salinity is 41.2 ppt) to 70 (when the salinity is 43 ppt). In contrast, the safe dilution factor based on the most ecologically appropriate toxicity value for the Giant Australian Cuttlefish is 16 and thus the recommended safe dilution factors should provide a high degree of protection to this species.

The natural salinity variation at Point Lowly complicates the calculation of a single safe dilution factor. The best solution given the available toxicity data was to derive a range of dilution factors that accurately reflect the fluctuating salinity at Point Lowly. The adopted approach and assumptions made should theoretically overestimate the toxicity of the return water.

BACKGROUND

Dr Warne (CSIRO) was approached in 2008 by consultants acting on behalf of BHP Billiton to review two years of studies undertaken as part of the Draft Environmental Impact Statement (EIS) for the proposed desalination plant at Point Lowly, South Australia and to provide his expert opinion on a number of issues related to the toxicity tests. Specifically, it was requested that the following issues be addressed:

- which species should be used to derive dilution factors;
- how do the species tested for this project compare with those undertaken for other Australian desalination plants;
- what role if any could a lack of test species acclimation have on the toxicity results;
- what effect if any could the use of diluent water with different salinities have on the toxicity results;
- what effect does exposure duration have on toxicity data;
- whether it is possible to combine EC10 and NOEC type toxicity data to derive dilution factors; and
- to derive a set of dilution factors to protect 99% of species and provide information on how these were derived.

A report (Warne, 2008a) that addressed each of these issues was included as Appendix O10.5 of the Draft EIS for the proposed expansion of the Olympic Dam mine (BHP Billiton, 2009). That report determined species protection trigger values (SPTVs) and safe dilution factors for the marine ecosystem of Upper Spencer Gulf when the receiving water had a salinity of 41.2 parts per thousand (ppt), which is slightly lower than the median of the range of salinities experienced at Point Lowly. It was therefore possible that the resulting SPTVs and safe dilution factors may have underestimated the toxicity of the return water from the proposed desalination plant when released into receiving water at the maximum salinity that occurs at Point Lowly (i.e. 43 ppt). Subsequent to the earlier report by Warne (2008a), Dr Warne was asked by consultants acting on behalf of BHP Billiton to undertake further analysis of the toxicity data, which included additional data for a new species, the sponge *Aplysina* sp. (Woodworth, 2008a) and the Yellowtail Kingfish (Woodworth, 2008b). The current report provides a refined assessment and corrects the toxicity data to estimate the SPTV and safe dilution needed in receiving waters with a salinity of 43 ppt. Discussion by Warne (2008a) on a number of issues relating to the selection of species used to derive the dilution factor has been updated in the present report to consider the additional data (see Appendix 1).

TYPES OF DIRECT TOXICITY ASSESSMENT TESTING

There are two different approaches that can be used to conduct direct toxicity assessment (DTA) which is also called whole effluent toxicity testing (WET).

- to use generic species that occur in that environmental media. For example, a DTA test at Point Lowly would use species that occur within Australian marine waters. This is also called the Standard DTA approach (Van Dam and Chapman, 2001).
- to use endemic organisms that actually occur in the ecosystem that is being assessed. For example, a DTA test at Point Lowly would use species that are found in the marine waters around Point Lowly or closely related organisms. This is also called the Site-specific DTA approach (Van Dam and Chapman, 2001).

There are strengths and limitations to both approaches. The key limitations of the generic species approach are that:

- the resulting toxicity data may not be relevant to the particular ecosystem being considered – as the species tested may not be present or closely related species may not be present; and
- usually the dilution water is not from the particular ecosystem and therefore site-specific characteristics of the water can not be taken into account.

The strength of this approach is that toxicity data for many generic species are often available and therefore there is greater confidence in the outcomes as more species can be tested.

The limitations of the endemic species approach are that:

- toxicity tests may not already be developed for endemic species and developing tests takes considerable time and money; and
- generally, toxicity data is generated for the minimum acceptable number of species for the desired purpose.

The effect of the above limitations decreases as the number of species used in DTA increases (e.g. Van Dam and Chapman, 2001). An excellent review of the status of DTA within Australia and New Zealand is the work by Van Dam and Chapman (2001).

The strength of the endemic species approach is that the toxicity data are directly relevant to the particular ecosystem being studied.

It is generally accepted within ecotoxicology that the endemic species approach is the preferred approach providing toxicity data are available for a similar number of species and taxonomic groups of organisms. Van Dam and Chapman (2001) state that:

> "For the purposes of Australian water managers, who generally oversee specific geographical regions and are concerned with local water quality, site-specific DTA is likely to be the most appropriate approach."

This is certainly the approach recommended for conducting DTA by the Australian and New Zealand guidelines for marine and fresh water quality (ANZECC and ARMCANZ, 2000).

Overall, the initial toxicity testing undertaken to assess the toxicity of the return water for Point Lowly followed the generic species approach with the exception of the Giant Australian Cuttlefish *Sepia apama*. The species used were (Geotechnical Services, 2006a) *S. apama* - cephalopod, *Penaeus monodon* – crustacean; *Seriola lalandi* – fish; *Nitzschia closterium* – diatom; *Hormosira banksii* – brown macroalga; *Heliocidaris tuberculata* – echinoid; and *Saccostrea commercialis* – bivalve (Hydrobiology, 2006). The use of the above generic organisms caused some problems mainly as they were acclimated to normal salinity marine water (i.e. 35 – 36 ppt), while the salinity of the Point Lowly region varies between 40 and 43 ppt. At the salinities that naturally occur at Point Lowly, two of the tested species (i.e. the oyster and the sea urchin) died – thus highlighting their unsuitability as test organisms. Also, neither of these species was endemic to the Point Lowly region. Given the above, I recommended that it would be desirable to (1) conduct further toxicity tests, preferably using species found in Upper Spencer Gulf, (2) increase the number of species for which there are toxicity data and (3) increase the relevance of the resulting dilution factors.

As a result of my previous recommendation subsequent toxicity testing was undertaken to follow the endemic species approach (see Appendix O10.4 of the Draft EIS, BHP Billiton, 2009). A list of all the species that have been used to determine the toxicity of return water and whether they are endemic to Upper Spencer Gulf (where Point Lowly is located) is presented in Table 1. The information on the distribution of species was provided by consultants acting on behalf of BHP Billiton.

Table 1. Information on the test organisms used in the direct toxicity assessment of return water for the proposed Point Lowly desalination plant.

Species	Present in USG[a]	Notes	Phase[b]
Microalga - *Nitzschia closterium*	Yes	Widely distributed in Australian waters	1
Microalga - *Isochrysis galbana*	Genus yes, species unknown		2
Microalga - *Ecklonia radiata*	No	Widely distributed throughout SA waters, but not recorded to occur north of Arno Bay (which is to the south of Point Lowly)	2
Macroalga - *Hormosira banksii*	Yes	Widely distributed throughout SA waters	1
Copepod - *Gladioferens imparipes*	Unknown		2
Tiger Prawn - *Penaeus monodon*	No		1
Western King Prawn - *Melicertus latisulcatus*	Yes		2
Blue Swimmer Crab - *Portunus armatus*[c]	Yes		2
Pacific Oyster - *Crassostrea gigas*	Yes	In aquaculture	2
Sydney Rock Oyster - *Saccostrea commercialis*	No		1
Sea urchin - *Heliocidaris tuberculata*	No	Distributed on rocky reefs from Southern Queensland to central New South Wales	1
Yellowtail Kingfish - S*eriola lalandi*	Yes	Also an important aquaculture species	1, 2 & 3
Snapper - *Chrysophrys auratus*	Yes		2
Mulloway - *Argyrosomus japonicus*	Yes		2
Giant Australian Cuttlefish - *Sepia apama*	Yes	Important breeding habitat at Point Lowly	1 & 2
Sponge - *Aplysina* sp.	Yes	This is a newly developed toxicity test.	3

[a]USG = Upper Spencer Gulf

[b]Phases 1, 2 and 3 refer to testing conducted in 2006, 2007 and 2008 respectively.

[c]Formerly *P. pelagicus* (Lai et al., 2010).

RECOMMENDED SPECIES FOR THE CALCULATION OF DILUTION FACTORS AND THE RATIONALE

There are a number of limitations associated with some of the DTA data that have been discussed in Appendix 1. These revolve around the fact that some of the DTA tests were conducted using diluent water with salinity outside the range found at Point Lowly, that some of the DTA tests only use acute exposure and QAQC issues. It is the author's opinion that the most internally consistent dataset which permits the largest number of species should be used to derive the dilution factors. By internally consistent it is meant that:

- toxicity data for only one type of exposure (i.e. chronic or acute or pulse) and
- data determined using diluent water with a single salinity within the range of Point Lowly (i.e. 40 – 43 ppt).

Based on these criteria, the best dataset was that using chronic toxicity data measured in diluent water with a salinity of 41.2 ppt (Table 2). An *a priori* decision was made to use, whenever possible, the concentration that causes a 10 % effect (EC10) rather than no observed effect concentration (NOEC) data to derive the PC99 and safe dilution factors. A justification for this decision is provided in Appendix 2 of this report. For the Giant Australian Cuttlefish there were limitations associated with the toxicity data for both phases I and II (see Appendix 1). Given the selection criteria (Appendix 1) the toxicity data from phase II were used to calculate the safe dilution factors. However, it is acknowledged that the EC10 values from phase I are lower than those of phase II. Therefore, even though the lowest EC10 value from phase I was conducted at a salinity exceeding the maximum found at Point Lowly, it was considered when ground-truthing the safe dilution factors derived in the current project (see the section "Ground Truthing The Safe Dilution Factor") to ensure that the safe dilution factor calculated will protect this species.

The second best dataset was considered to be that which permitted the most species to be used to derive the dilution factors even if some acute, chronic, and values measured in different salinity diluent water were combined (Table 2). In addition to the chronic toxicity values measured at 41.2 ppt the best toxicity values for *H. banksii, G. imparipes* and *S. lalandi* were included in the second best dataset. In the case of *S. lalandi* the toxicity values from phases I, II and III not ideal (see previous explanation). The lowest EC10 value was 1.48 % return water however, this was determined in diluent water with a salinity of 44.3 ppt which is higher than the highest reliably measured salinity at Point Lowly (i.e. 43 ppt). The EC10 and NOEC values from phases I (conducted using diluent water with a salinity of 41.2 ppt) and II (where the diluent water had a salinity of 35 ppt) were 12.5 (10.6% when

recalculated by the author, see Table 2) and 11.1 % return water, respectively. The close agreement of the EC10 values from phase I and II tends to indicate that the phase III result was atypical and therefore as the recalculated phase II EC10 of 10.6% return water was the lower of the two value it was adopted. However, this atypical value was the lowest EC10 for this species, in fact it was the lowest of any of the test species. Therefore, the phase III EC10 value was used to ground-truth the safe dilution factors derived in the current project (see the section “Ground Truthing The Safe Dilution Factor”) to ensure that the safe dilution factor calculated by the current project will protect this species. *H. banksii* was included as it has regional relevance and the toxicity data from salinity controls shows that there was no difference in the toxicity measured within the range 37 to 45 ppt. Therefore the toxicity of the return water measured in diluent water with a salinity of 37 ppt could be used to estimate the toxicity when tested in diluent water with a salinity of 43 ppt. The acute EC10 value for *G. imparipes* was included due to regional relevance. The organisms and toxicity values presented in Table 2 are those recommended for the derivation of concentrations that should protect 99% of species (PC99) and safe dilution factors.

The best dataset contains toxicity data for seven species that belong to six taxonomic groups of organisms. The second best dataset contains toxicity data for ten species that belong to six taxonomic groups of organisms. Thus both datasets exceed the minimum data requirements of the BurrliOZ method (Campbell et al., 2000) and the Australian and New Zealand water quality guidelines (ANZECC and ARMCANZ, 2000) to derive site-specific trigger values (i.e. at least five species that belong to at least four taxonomic groups of organisms).

Table 2. The species and the toxicity values for the two preferred datasets used to derive the dilution factors.

Test species	Taxonomic group	EC10 and NOEC values (% return water)	
		Best dataset	2nd best dataset
H. banksii	Macroalga		16[a]
I. galbana	Diatom	84.4	84.4
E. radiata	Macroalga	27.6	27.6
C. gigas	Bivalve	3.3	3.3
G. imparipes	Crustacean		10.9[b]
C. auratus	Fish	22.2	22.2
S. lalandi	Fish		10.6[c]
A. japonicus	Fish	11.0[d]	11.0 [d]
M. latisulcatus	Crustacean	7.5[e]	7.5[e]
S. apama	Cephalopod	6.3	6.3

[a] the NOEC for *H. banksii* was measured in diluent water with a salinity of 37 ppt. [b] the EC10 for *G. imparipes* is an acute toxicity value. [c] the EC10 value for *S. lalandi* was measured in diluent water with a salinity of 35 ppt and calculated by the author using data generated by Geotechnical Services (2008) (Appendix O10.4 of the Draft EIS, BHP Billiton, 2009). The method used fits a log-logistic distribution to the data (Barnes et al., 2003).[d] in Warne (2008a) the reported value was 11.6 % return water. The reason for the change is discussed in the text on this species that following this table. [e] the EC10 value for *M. latisulcatus* was calculated by the author using data generated by Geotechnical Services (2008) (Appendix O10.4 of the Draft EIS, BHP Billiton, 2009). The method of Barnes et al (2003) was used.

SCIENTIFIC APPROPRIATENESS OF THE TOXICITY DATA

The toxicity data related to the best or second best datasets were reviewed to verify whether they met the assumptions of the statistical tests used to derive these values. Specific findings of this review are:

- there are no problems with the data used in the preferred dataset for the unicellular alga *I. galbana*, the macroalga *E. radiata*, the Pacific Oyster *C. gigas* and the Giant Australian Cuttlefish *S. apama*;
- in the cases of the Western King Prawn *M. latisulcatus*, the Pink Snapper *C. auratus* and the Mulloway *A. japonicus*, the only issue that could be interpreted as invalidating the assumptions of the statistical methods is the statement that the data have 'significant heteroscedasity'. The statistical method used to derive the EC10 values in these cases was Probit which does not assume homogeneous variance (Newman, 1995; Bromaghin and Engemann, 1989; Environment Canada 2005). These authors

state that the only assumption of the method is normally distributed data. If this is the criticism of the data, it does not appear to be appropriate;

- in the case of the Pink Snapper (*C. auratus*) the probit line models the data well, particularly in the region of concern (i.e. the region below the 50% effect level). Therefore there is no problem with the use of this data point;
- for the Western King Prawn (*M. latisulcatus*) the probit line did not fit the data particularly well. Therefore it might be more appropriate to use the NOEC value which is 12.7. Using the NOEC of 12.7 rather than 11.8 would have little or no effect on the calculation of the safe dilution factor and if anything would lead to a decrease in the dilution factor required. Therefore the original EC10 value was retained;
- for Mulloway (*A. japonicus*) the probit line does not fit the data particularly well. Therefore the EC10 value was recalculated using the method of Barnes et al. (2003) which fits a logistic distribution to the data. This method is used extensively by CSIRO in calculating toxicity values. The key assumption in this method is that the residuals have a random distribution. Using this method the EC10 value was 11.04 % return water with 95% confidence intervals of 8.73 to 13.95 % return water. This is very similar to the value derived by Geotechnical Services Pty Ltd of 11.6 % return water. But the fit of the logistic distribution to the toxicity data was still not particularly good. However, in order to be conservative (protective of the environment) the value of 11.0 % return water was used in all subsequent calculations; and
- in the case of the sponge *Aplysina* sp. the method used to determine the EC10 value did not fit the toxicity data well and this is reflected in the fact that no confidence limits were generated. For this reason and the limitations of the sponge toxicity data discussed in Appendix 1, these data were not included in either the best or second best datasets.

DERIVATION OF PROTECTIVE CONCENTRATION VALUES AND SAFE DILUTION FACTORS

It is appropriate given the close proximity of the breeding ground of the Giant Australian Cuttlefish (*S. apama*) to the proposed discharge point that 99% of species should be protected at Point Lowly. This level of protection is that applied to aquatic ecosystems with high conservation value by the Australian and New Zealand Guidelines for Fresh and Marine Water Quality (ANZECC and ARMCANZ, 2000). The concentrations that should theoretically protect 99% of marine species (PC99) and the corresponding safe dilution factors (the extent the return water must be diluted in order to meet the PC99) were calculated using the

BurrliOZ species sensitivity distribution (SSD) method (Campbell et al., 2000) that was used to derive the Australian and New Zealand water quality guidelines (ANZECC and ARMCANZ, 2000). Only the PC99 and PC95 values and the corresponding safe dilution factors are presented in the following text, rather than other possible levels of protection used for aquatic ecosystems (e.g., PC90 and PC80 see ANZECC and ARMCANZ, 2000). The PC99 and safe dilution factor for the best dataset were 2.35 % return water and 45 (rounded up from 42.6) respectively. The PC95 and safe dilution factor for the best dataset were 3.37 % return water and 30 (rounded up from 29.6) respectively. The PC99 value and dilution factor for the second best dataset were 2.51 % return water and 40 (rounded up from 39.8) respectively. The PC95 value and dilution factor for the second best dataset were 3.91 % return water and 26 (rounded up from 25.6) respectively. The SSD plots used to generate these PC values and safe dilution factors for the best and second best datasets are presented in Figures 1 and 2 respectively.

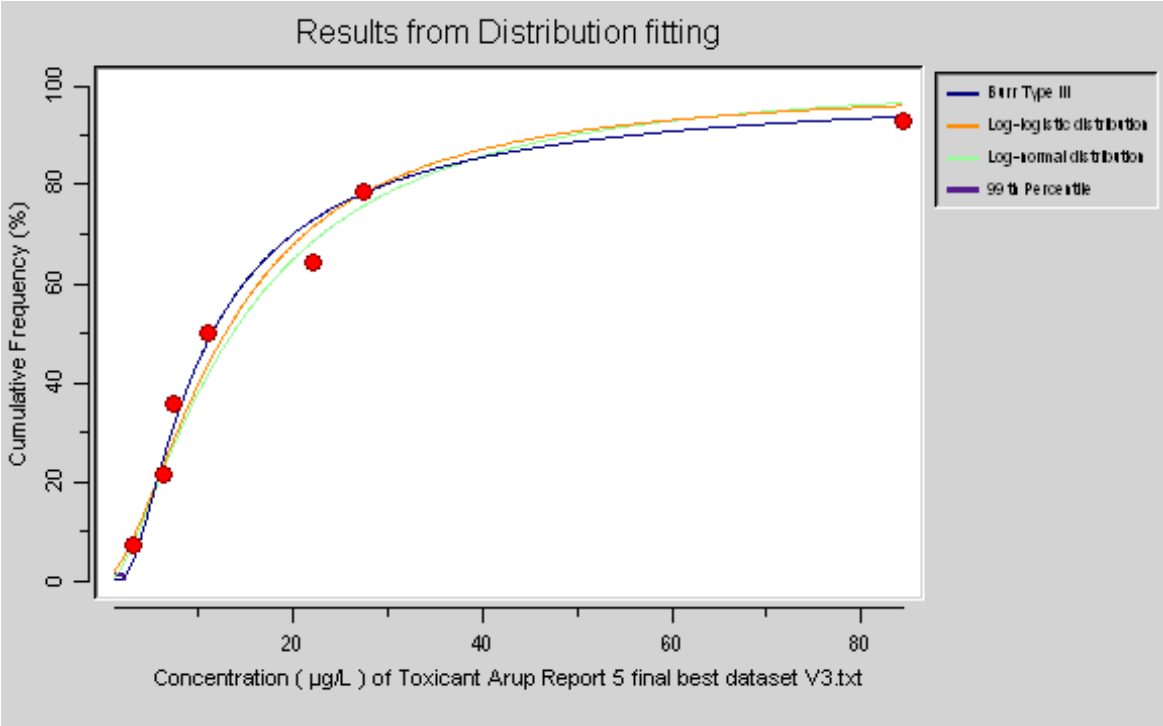

Figure 1. The species sensitivity distribution plot of the concentrations of return water that cause a 10% effect (EC10) for the best dataset.

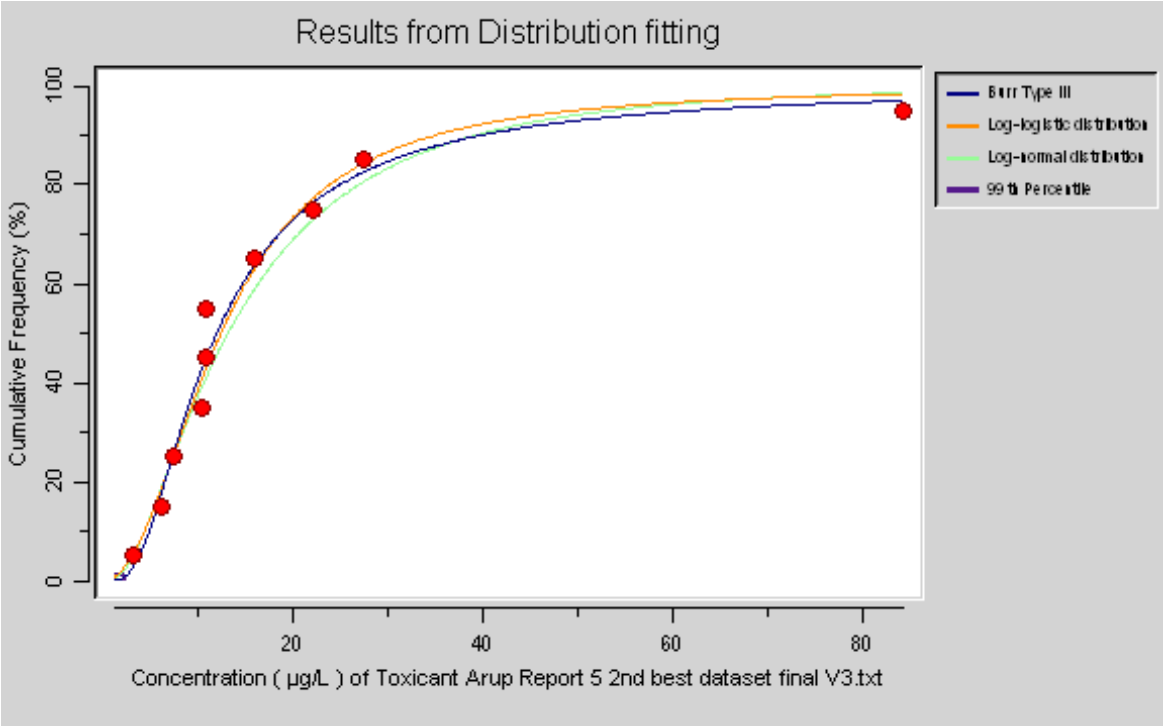

Figure 2. The species sensitivity distribution plot of the concentrations of return water that cause a 10% effect (EC10) for the second best dataset.

It is worth noting that the PC99 and safe dilution factors derived using the best dataset (even though they are based on toxicity data for fewer species) are more conservative (i.e. requiring a greater dilution of the return water) than those derived using the second best dataset. Therefore, in order to be conservative the PC99 and safe dilution factor of the best dataset are preferred. The close agreement of the PC99 and safe dilution factors generated by the two datasets increases the confidence associated with using the values from the best dataset.

If the PC99 and dilution factor for the best dataset are achieved then theoretically 99% of marine organisms typical of Upper Spencer Gulf should be protected from experiencing sub-chronic or chronic toxic effects of greater than 10% magnitude caused by the discharge of return water into water with a salinity of 41.2 ppt. It is important to note however, that the salinity of the diluent water used in the preceding calculations (41.2 ppt) is slightly below the mean of the range of salinities experienced at Point Lowly. Therefore, it is likely that the PC99 and safe dilution factor underestimate those that would be derived using toxicity data generated using diluent water with a salinity of 43 ppt (the maximum salinity reached at Point Lowly).

CORRECTING THE TOXICITY DATA, PROTECTIVE CONCENTRATION VALUES AND SAFE DILUTION FACTORS TO THE MAXIMUM SALINITY RECORDED AT POINT LOWLY

The ratio of the salinity of the return water and any other additives present in the return water is fixed until dilution occurs. Therefore, it is generally possible to estimate the EC10 of the return water when it is discharged into water with different salinities (Appendix 2 states the conditions when this does not apply). The toxicity data in the best dataset were corrected as if the toxicity tests were conducted using diluent water with a salinity of 43 ppt and thus representing the situation where return water is discharged in seawater with the maximum reliably measured salinity at Point Lowly (i.e., 43 ppt). This was done in a two-step process. First, the salinity at the EC10 value, when the diluent water had a salinity of 41.2 ppt, was calculated. This was done using the formula:

Salinity at EC10 (41.2 ppt) = {[salinity of return water x EC10 (% return water)] + [100 - EC10 (% return water) x diluent water salinity]} ÷ 100

= {[78 x EC10 (% return water)] + [100 - EC10 (% return water) x 41.2]} ÷ 100

Second, the salinity of the EC10 was corrected to that which would occur if the diluent water had a salinity of 43 ppt using the following formula:

Salinity of EC10 (43 ppt) = 100 x (salinity of EC10 – 43 ppt) ÷ (return water salinity – 43 ppt)

= 100 x (salinity of EC10 – 43 ppt) ÷ (78 – 43)

The EC10 values for *C. gigas*, *E. radiata* and *S. apama* were not corrected but those the other four species (*I. galbana, C. auratus, A. japonicus* and *M. latisculatus*) were corrected. The EC10 value for *C. gigas* was not corrected as it breeds during summer (Mark Gluis, South Australian Research & Development Institute (SARDI) Aquatic Sciences, *pers. comm*.; Wiltshire et al., 2008) when maximum salinities do not exceed 41.2 ppt (Nunes, 1985). The EC10 value for *E. radiata* was not corrected as its presence has not recorded north of Arno Bay (Carolyn Ricci, SA Herbarium, *pers. comm.*; Draft EIS; CHAH, 2010) and therefore it would not experience salinities exceeding 41.2 ppt (Nunes, 1985). The EC10 value for *S. apama* was not corrected as the lifestage that corresponds to the most sensitive endpoint

occurs when the salinity is approximately 41 ppt (Draft EIS, BHP Billiton, 2009). The original and corrected EC10 values for the best dataset are presented in Table 3.

Table 3. The percentages of return water (78 ppt) that cause a 10% effect when diluted in diluent water with a salinity of 41.2 (original) and 43 ppt (corrected) and the final EC10 values of the best dataset that were used to calculate the most environmentally relevant safe dilution factor.

Species	Original EC10 (% return water)	Salinity at original EC10 (ppt)	Corrected EC10 (% return water)	Final EC10 (% return water)
I. galbana	84.4	72.3	83.6	83.6
E. radiata	27.6		nc	27.6
C. auratus	22.2	49.4	18.2	18.2
A. japonicus	11.0	45.2	6.4	6.4
M. latisculatus	7.5	44.0	2.7	2.7
C. gigas	3.3		nc	3.3
S. apama	6.3		nc	6.3

nc = not corrected

The SSD plot of this data is presented in Figure 3. The resulting PC99 and safe dilution factor derived using the final dataset (Table 3) are 1.56 % return water and 65 (rounded up from 64.1), respectively. Thus correcting for salinity, in this case, led to an increase in the dilution factor of approximately 40 %. The PC95 and corresponding dilution factor are 2.30 return water and 44 (rounded up from 43.5).

It should also be noted that the correction of the toxicity data to a salinity of 43 ppt is likely to have overestimated the toxicity. This is because the toxicity of the controls was not corrected. It is highly likely that the percent effect in the controls at a salinity of 43 ppt would be greater than at 41.2 ppt. This in turn would decrease the percent effect (as it is expressed as a percentage of the control) at a given concentration of return water and mean that a higher percentage of return water would be required to cause a 10% [or 50%] effect.

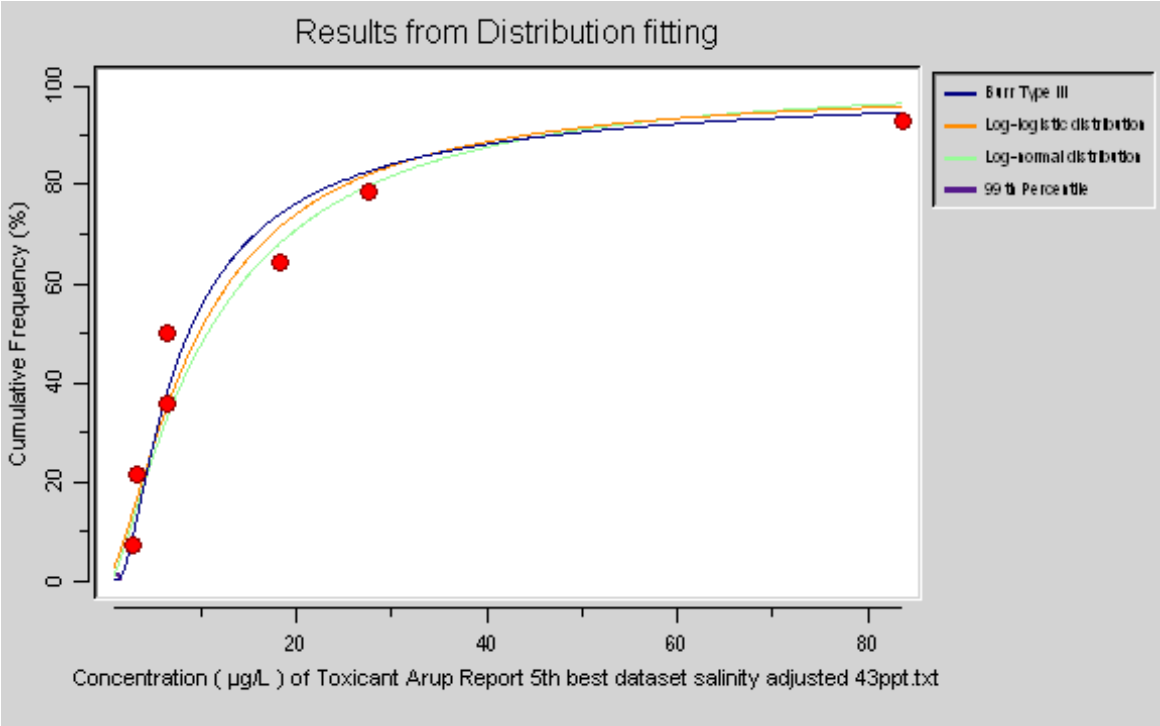

Figure 3. The species sensitivity distribution plot of the concentrations of return water that cause a 10% effect (EC10) for the salinity corrected (43 ppt) best dataset.

WHAT IMPACT MIGHT THE GIANT AUSTRALIAN CUTTLEFISH EXPERIENCE AT DILUTIONS EXCEEDING THE PC99 VALUES?

Due to the close proximity of the Giant Australian Cuttlefish's breeding ground to the proposed discharge site, it was decided to ascertain what level of protection the PC99 values, calculated in this report (i.e., 40, 45, and 65), would provide based on the most sensitive set of toxicity data for that species (i.e., post-hatch survival). This dataset was generated using diluent water with a salinity of 45 ppt (Geotechnical Services, 2006b) which is higher than the highest salinity reliably measured at Point Lowly (Appendix 09.2 of the Draft EIS, BHP Billiton, 2009). Therefore determining the effect on the Giant Australian Cuttlefish using this data set would most likely be an overestimate. To overcome this problem, the toxicity dataset could be corrected down to the salinity experienced by the Giant Australian Cuttlefish during its breeding season (40 – 41 ppt). However, it has been shown that this method may underestimate the toxicity when the corrected salinity is below 43.5 ppt (see Appendix 3). Given this limitation, the level of protection provided by the three PC99 values was determined using diluent water of two salinities: 43.5 and 45 ppt.

This was done by plotting the concentrations of return water against the percent reduction in post-hatch survival values (Appendix 010.3 of the Draft EIS, BHP Billiton, 2009) at the two

salinities and regressing. The plot for the 45 ppt salinity data is presented in Figure 4. The resulting regression equations could predict approximately 97 % of the variation in toxicity (i.e. $R^2 = 0.97$) and therefore accurately fitted the data (Table 4).

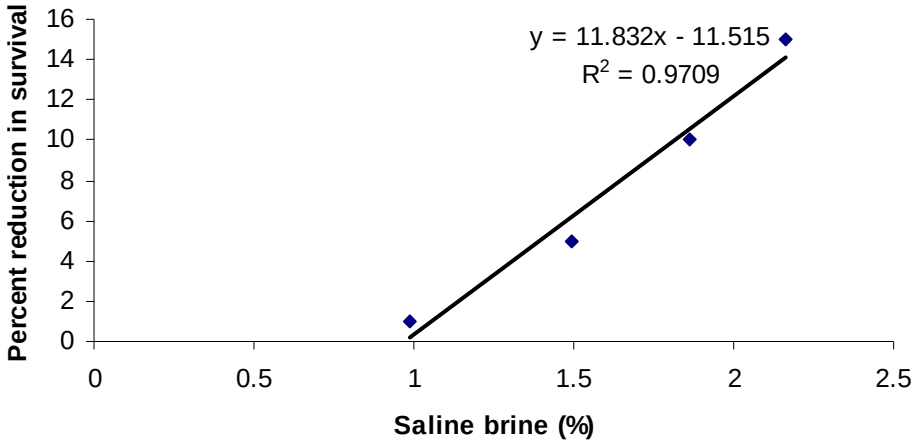

Figure 4. Plot of percent return water in diluent water at 45 ppt against the percent reduction in post-hatch survival of the Giant Australian Cuttlefish (*S. apama*) and the regression line and equation for this data.

Table 4. The regression equations between percent reduction in post-hatch survival of the Giant Australian Cuttlefish (y parameter) and the percentage of return water (x parameter) from toxicity tests conducted using diluent water with different salinities (i.e. 43.5 and 45 ppt). The coefficient of determination (r^2) for both equations was 0.97.

Diluent water salinity (ppt)	Regression equation (y =)
45	11.8 x – 11.5
43.5	12.4 x – 65.3

However, the relationship below the 1 % reduction in post-hatch survival may not conform to the relationship observed above this level (e.g. Figure 4). A conservative approach would be to assume there is a linear relationship between the control and the 1 % effect level. A plot of this for the toxicity dataset using diluent water with a salinity of 45 ppt is presented in Figure 5.

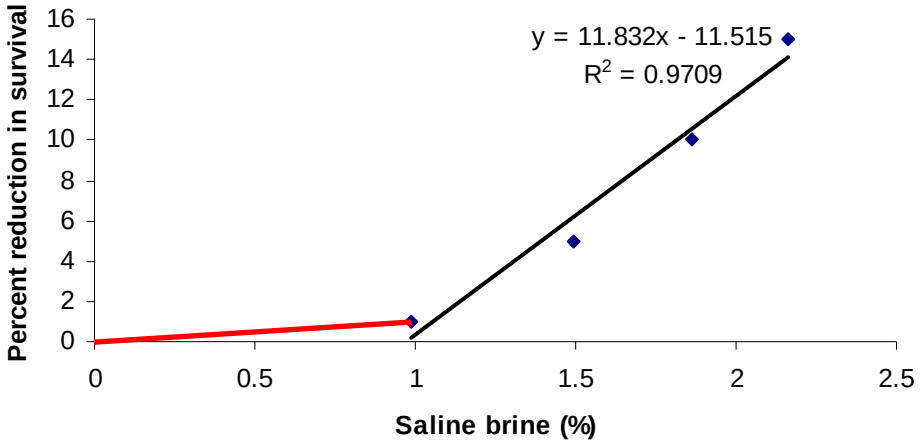

Figure 5. Plot of percent return water in seawater at 45 ppt against the percent reduction in post-hatch survival of the Giant Australian Cuttlefish (*Sepia apama*) and the regression lines for this for % saline values between 0 and 1 % return water (in red) and greater than 1 % return water (in black).

By making this assumption and regressing the data at the different salinities two equations were obtained and are presented in Table 5.

Table 5. The regression equations between percent reduction in post-hatch survival of the Australian Giant Australian Cuttlefish (y parameter) that is less than 1% and the percentage of return water (x parameter) from toxicity tests conducted using diluent water with different salinities (i.e. 43.5 and 45 ppt).

Diluent water salinity (ppt)	Regression equation (y =)
45	1.01 x
43.5	0.19 x

By substituting the PC99 values into each regression equation (Tables 4 and 5) the toxic effect that the Giant Australian Cuttlefish would experience was calculated (Table 6). The predicted percentage reduction values in post-hatch survival of the Giant Australian Cuttlefish ranged from approximately 18 to 7 % when the diluent water had a salinity of 45 ppt. The predicted percentage reductions in post-hatch survival were considerably smaller when the salinity of the diluent water was 43.5 ppt with values always being less than 0.5 %.

Table 6. The predicted percentage reduction in post-hatch survival of the Giant Australian Cuttlefish using different toxicity datasets and Giant Australian Cuttlefish toxicity data conducted using diluent water with different salinities.

PC value	Safe dilution factors	% reduction in post-hatch survival of *S. apama*	
		Diluent water salinity @ 43.5 ppt	Diluent water salinity @ 45 ppt
PC99 (best dataset at 41.2 ppt)	45	0.42	14.8
PC99 (2^{nd} best dataset at 41.2 ppt)	40	0.47	18.1
PC99 (best dataset at 43 ppt)	65	0.29	6.7

The predicted percentage reduction in the post-hatch survival of the Giant Australian Cuttlefish based on the Giant Cuttlefish toxicity data conducted using diluent water with a salinity of 45 ppt will overestimate the actual effect, as the salinity is higher than the highest reliable measured salinity at Point Lowly. The predicted percentage reduction in the post-hatch survival of the Giant Australian Cuttlefish when toxicity tests were conducted using diluent water with a salinity of 43.5 ppt provides the best estimate of the effect at the maximum reliably measured salinity at Point Lowly and will overestimate the effect at lower salinities. This set of data were therefore viewed as the most appropriate estimate of the likely percentage reduction in post-hatch survival of the Giant Australian Cuttlefish that would occur at Point Lowly.

Therefore if a dilution factor of 65 is achieved then:

- theoretically 99 % of marine species typical of Upper Spencer Gulf would be protected from experiencing sub-chronic or chronic toxic effects of greater than 10 % in sea water with a salinity of 43 ppt; and

- there would be less than a 0.5 % reduction in post-hatch survival of the Giant Australian Cuttlefish in seawater with a salinity of 43.5 ppt (and therefore even less with a salinity of 41 ppt).

GROUND TRUTHING THE SAFE DILUTION FACTOR

The last step in the derivation of the Australian and New Zealand water quality guidelines was to ground-truth the trigger values (Warne, 2001) and if necessary to adjust the trigger values by manipulating the calculations or the data in various ways (e.g. by increasing the level of protection from 95 % to 99 % or using a larger assessment factor). This was done by comparing the trigger values to all the raw toxicity data paying particular attention to field-based, mesocosm or microcosm toxicity data (Warne, 2001). A similar ground-truthing was conducted in the current study.

The PC99 value for the best dataset that had been corrected to a salinity of 43 ppt was compared to all the toxicity data that had been generated by the DTA testing (i.e. Appendices O10.2 to O10.4 of the Draft EIS, BHP Billiton, 2009; Woodworth 2008a, b). The lowest ecologically relevant toxicity value derived by the DTA testing was an EC10 value of 1.48 % return water for *S. lalandi* which corresponds to a dilution factor of 68 (rounded up from 67.6) which is slightly larger than the safe dilution factor of 65 derived in this report. However, this toxicity value was determined using diluent water with a salinity of 44.3 ppt, which is greater than the salinity range at Point Lowly and therefore the value most probably overestimates the toxicity that would occur at Point Lowly. Nonetheless, in order to be conservative and ensure protection of this species the recommended safe dilution factor was increased to 70. This will provide adequate protection given the available toxicity data.

COMPARISON WITH THE SAFE DILUTION FACTOR RECOMMENDED IN THE DRAFT EIS

In a previous report (Warne, 2008a) and the Draft EIS (BHP Billiton, 2009) the toxicity data were not corrected to seawater with a salinity of 43 ppt (the maximum salinity measured at Point Lowly). There was, therefore, a concern that the resulting PC99 and safe dilution factors may not protect 99 % of species if return water was discharged into seawater with a salinity of 43 ppt. As a means of overcoming this potential underestimation, the percentage of the return water that would protect all species (i.e. 100 % of marine species typical of Upper Spencer Gulf, the PC100) was determined. This resulted in a recommended safe dilution factor of 85 (Warne 2008a).

The current report opted to correct the toxicity data to a salinity of 43 ppt (the maximum salinity measured at Point Lowly) and recommends a maximum safe dilution factor of 70 that should:

- theoretically protect more than 99 % of marine species typical of Upper Spencer Gulf from experiencing sub-chronic or chronic toxic effects of greater than 10 % in sea water with a salinity of 43 ppt; and

- permit less than a 0.5 % reduction in post-hatch survival of the Giant Australian Cuttlefish in seawater with a salinity of 43.5 ppt (and therefore even less with a salinity of 41 ppt).

This provides a very high level of protection of the Upper Spencer Gulf ecosystems that is greater than that required by the Australian and New Zealand water quality guidelines (ANZECC and ARMCANZ, 2000) for high conservation waterbodies. Further, it indicates that the previously recommended safe dilution factor of 85 was overly conservative.

COMPARISON WITH THE SPECIES USED FOR THE VICTORIAN AND WA DESALINATION PLANT

The species that were used to assess the return water from the Perth Seawater Desalination Plant were: the marine bacteria *Vibrio fischeri*; the macroalga *E. radiata*; the Blue Mussel *Mytilis edulis*; the unicellular algae *N. closterium* and *Isochrysis* sp; the copepod *G. imparipes*; and the Pink Snapper *C. auratus* (Geotechnical Services, 2006a, 2007a, 2007b). The *V. fischeri* was only used to determine the range of concentrations to be used for the other species and was not used in the calculations of the dilution factors (Geotechnical Services, 2006a; Warne, 2008b). Thus only five species that belonged to five different taxonomic groups were used to derive the dilution factors. This meets the minimum data requirements to use the BurrliOZ species sensitivity distribution method (Campbell et al., 2000) and to derive a trigger value in accordance with the Australian and New Zealand water quality guidelines (ANZECC and ARMCANZ, 2000).

The two rounds of DTA testing conducted for the Victorian Desalination Plant each used six species consisting of the amphipod *Allorchestes compressa*, the Doughboy Scallop *Mimachlamys asperrima*, the macro-alga *H. banksii*, the micro-alga *N. closterium*, the sea-urchin *Heliocidaris tuberculata* and the Yellowtail Kingfish *S. lalandi* or the Sand Whiiting *Sillago ciliate* or the Australian Bass *Macquaria ambigua* (Warne, 2010).

In comparison, the best and second best datasets used in the current study to calculate the PC99 and safe dilution factors consisted of data for 7 species that belonged to 6 taxonomic groups and 10 species that belonged to 6 taxonomic groups, respectively. These also meet the minimum data requirements of the Australian and New Zealand water quality guidelines (ANZECC and ARMCANZ, 2000). However, there should be greater confidence in the dilution factors calculated for Point Lowly than for the Victorian and WA desalination plants as toxicity data for more species and more taxonomic groups is being used. The inclusion of toxicity data for *S. apama* in the derivation is very important and appropriate as there is a breeding ground located close to the proposed desalination plant site.

SENSITIVITY OF THE CALCULATIONS

There are a number of factors that can affect the protective concentration values and safe dilution factors that are derived in studies such as the present study. These include: variability in the results of the toxicity tests; variability in the measurement of salinity and variability in the calculation of the protective concentration values and safe dilution factors themselves. The toxicity data are reported to one decimal place to reflect this variability. Statistical methods such as the BurrliOZ SSD method (Campbell et al., 2000) that was used to derive the PC99 values and safe dilution factors generally provide more reliable and accurate results as the number of data used increase. This is one reason that there are recommended minimum data requirements to use BurrliOZ (ANZECC and ARMCANZ, 2000). The safe dilution values presented in this report have been rounded up to at least the nearest whole unit but in most cases they have been rounded up to the nearest multiple of 5.

Given the above variability it is pertinent to briefly examine the sensitivity of the safe dilution factors to variations in the toxicity data. The safe dilution factors generated by SSD methods are particularly sensitive to changes in the toxicity of the most sensitive species. Therefore four scenarios were tested to see what effect they would have on the resulting safe dilution factors based on the PC99 values derived using the best dataset (that had not been salinity corrected). The scenarios were to decrease and increase:

- the lowest EC10 value by 10%;
- the lowest EC10 value by 50%;
- the lowest two EC10 values by 10%; and
- the lowest two EC10 values by 50%.

The results of this sensitivity analysis are shown in Table 7.

Table 7. The variation in safe dilution factors that should protect 99 percent of aquatic species as a result of a sensitivity analysis and the current safe dilution factors.

Scenario	Safe dilution factors based on variation of		Current safe dilution factor	% variation
	- 10%	+10%		
Lowest value PC99	46	40	43	± 7.5
Two lowest values PC99	47	39	43	± 9
Scenario	Safe dilution factors based on		Current safe dilution factor	% variation
	-50%	+50%		
Lowest values PC99	182	31	43	- 325, + 28
Two lowest values PC99	182	28	43	-325, + 35

Small variations (10%) in the lowest toxicity value and the two lowest values led to commensurately small variations in the resulting safe dilution factors. However, larger variations (50%) led to considerably larger variations in the safe dilution factors.

CONCLUSIONS

A series of direct toxicity assessment (DTA) tests have been conducted using sixteen species. Different subsets of these species have been combined in various reports to produce a range of species protection values and safe dilution factors (refer to Warne, 2008a and Appendices O10.2 to O10.4 of the Draft EIS, BHP Billiton, 2009). The current report provides a refined assessment of all the DTA results, and the species protection values presented here use the most appropriate dataset available and thus supercede all previous values.

The suite of organisms tested as part of the Draft Environmental Impact Statement for the proposed desalination plant at Point Lowly were evaluated for their appropriateness to calculate safe dilution factors for the return water. Some additional toxicity data were generated following the previous report (Warne, 2008a) and this data has been incorporated, where appropriate, in the current assessment. The best possible dataset is based solely on sub-chronic and chronic toxicity data measured in diluent water with a salinity of 41.2 ppt. Based on this the recommended species are *I. galbana, E. radiata, M. latisulcatus, C. gigas, C. auratus, A. japonicus* and *S. apama*. However, a second dataset which retained the previous species but added *H. banksii, G. imparipes* and *S. lalandi* was also evaluated as this maximized the number of test species. Both datasets contain more species belonging to more taxonomic groups than that used in the evaluation of the Victorian and Western Australia desalination plants and that exceed the minimum data requirements of the Australian and New Zealand water quality guidelines. Therefore there will be greater

confidence in the safe dilution factors being derived for the proposed Olympic Dam Mine desalination plant being examined in this report than for the Victorian and WA plants.

Use of the best dataset determined in receiving water with a salinity of 41.2 ppt yielded a concentration that should protect 99 % of species (PC99) of 2.35 % return water and a dilution factor of 45. The corresponding values for the second best dataset are 2.48 % and 40 respectively. The best dataset yielded larger dilution factors then the second dataset, and it is therefore recommended for deriving dilution factors. If the PC99 and dilution factor for the best dataset are achieved then theoretically 99 % of marine organisms typical of Upper Spencer Gulf will be protected from experiencing sub-chronic or chronic toxic effects of greater than 10 % caused by the discharge of return water into water with a salinity of 41.2 ppt.

The salinity of seawater at Point Lowly ranges from 40 to 43 ppt. Therefore the dilution factor derived for seawater with a salinity of 41.2 ppt may underestimate that required for the range 41.2 to 43 ppt. Therefore the toxicity data for appropriate species in the best dataset were corrected to estimate the toxicity if the receiving water had a salinity of 43 ppt. This resulted in a PC99 of 1.56 % return water and a dilution factor of 65. This PC99 was larger than the lowest toxicity value generated in the current project (1.48 % return water determined in receiving water with a salinity of 44.3 ppt) therefore if it is desired to prevent that toxic event occurring a higher dilution factor may be advisable. The value of 1.48 % return water corresponds to a dilution factor of 68, therefore the recommended safe dilution factor was increased to 70. If a dilution of 70 was achieved it would:

- theoretically protect more than 99 % of marine species typical of Upper Spencer Gulf from experiencing sub-chronic or chronic toxic effects of greater than 10% in sea water with a salinity of 43 ppt; and

- cause less than a 0.3 % reduction in post-hatch survival of the Giant Australian Cuttlefish in seawater with a salinity of 43.5 ppt and a lower effect at the ecologically relevant salinity for the Giant Australian Cuttlefish of 41 ppt (the lowest salinity that occurs at Point Lowly).

The most appropriate safe dilution factors for Point Lowly that will protect at least 99% of species and provide a high degree of protection to the Giant Australian Cuttlefish range between 45 (when the receiving sea water has a salinity of 41.2 ppt) to 70 when the receiving sea water has a salinity of 43 ppt). In contrast, the safe dilution factor based on the most ecologically appropriate toxicity value for the Giant Australian Cuttlefish is 16 and thus

the recommended safe dilution factors based on protecting the marine ecosystems of Upper Spencer Gulf should provide a high degree of protection to this species.

The natural salinity variation at Point Lowly complicates the calculation of a single safe dilution factor. The best solution given the available toxicity data was to derive a range of dilution factors that accurately reflect the fluctuating salinity at Point Lowly. The adopted approach and assumptions made should theoretically overestimate the toxicity of the return water.

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APPENDIX 1 – FACTORS TO CONSIDER IN THE SELECTION OF SPECIES USED TO DERIVE THE DILUTION FACTOR

A series of criteria were used to determine the most appropriate species to be used in deriving dilution factors. These were:

- did the test species have regional relevance?
- were the toxicity tests conducted in water similar to that at Point Lowly?
- were the exposure scenarios relevant and appropriate?
- were the endpoints appropriate?
- did the tests meet appropriate quality assurance and quality control criteria?
- were the test species exposed to the toxicant for the same duration?

Another consideration is that the species sensitivity distribution (SSD) method used to derive the dilution factors becomes more reliable and more representative as the number of species for which there are toxicity data increases. The Australian and New Zealand water quality guidelines (ANZECC and ARMCANZ, 2000) recommend using chronic tests for a minimum of five species representing at least four taxonomic groups in order to derive a high reliability trigger value.

Finally, it is important to adopt a pragmatic approach to DTA testing (Chapman et al., 2001; van Dam and Chapman, 2001). For example, it will rarely be possible to generate regionally relevant toxicity data for more than five species due to time and cost considerations. However, the limited number of species is offset by the greater environmental relevance of the toxicity data to the site being considered.

Did the test species have regional relevance?

Based on the occurrence of the test organisms within Upper Spencer Gulf toxicity data for the following ten species could be used: *N. closterium*; *H. banksii*; *M. latisulcatus; P. armatus; C. gigas; S. lalandi; C. auratus; A. japonicus; S. apama;* and *Aplysina* sp.

The Pacific Oyster (*C. gigas*) does not occur naturally in Upper Spencer Gulf, however it is cultured there and is therefore a commercially important species to this region. The available evidence suggests that the range of salinities experienced at Point Lowly is at the upper tolerance threshold for this species (Helm and Millican, 1977; Nell and Holiday, 1988; PIRSA, 2003; Wiltshire et al., 2008) and this combined with the generally low algal densities in these waters leads to very poor survival of larvae and spat and is the reason that there are very

limited natural colonies of this species in Upper Spencer Gulf. Other oysters do occur naturally in Upper Spencer Gulf (PIRSA, 2003), but there are no ecotoxicity tests available for these species. *Crassostrea gigas* was used as it is a commercially important species and it is representative of other oysters and bivalves that occur naturally in Upper Spencer Gulf. The inclusion of this species is likely to overestimate the toxicity of the return water compared to other oyster and bivalve species as it is very close to its salinity tolerance threshold. This species represents species that are very close to their maximum salinity tolerance which is relevant given the Upper Spencer Gulf acts as a reverse estuary with salinity increasing as you move north. The inclusion of *C. gigas* is likely to increase the safe dilution factor required.

Based on unidentified members of the same genus of algae being present in Upper Spencer Gulf *I. galbana* could also be used. *E. radiata,* as far as it is known, does not occur in Upper Spencer Gulf, but it is widely distributed throughout South Australian waters so it could also be used.

In terms of the regional relevance, the copepod *G. imparipes* could also be considered for use in determining dilution factors. While it is not clear that this particular species is present in Upper Spencer Gulf it still has regional relevance. The reasons for this are that:

- it is an herbivorous calanoid copepod (Rippingale and Hodgkin, 1974) found in south-western Australian marine waters and copepods in general play important roles in coastal marine ecosystems (e.g. Willis, 1999) as they take in energy through the consumption of phytoplankton and algae, transfer energy to higher trophic levels by being consumed by birds, fishes and mammals; and
- copepods are planktonic crustaceans. Thus while they are motile they generally move with the surrounding water. All crustacea spend at least the early part of their life as plankton and move with the water – however for most macrocrustaceans (e.g. barnacles, crabs, lobsters) only the early lifestages (which are generally the more sensitive lifestages) are planktonic. Therefore, it is argued that copepods are appropriate indicators of the early life stages of crustaceans. There definitely are crustaceans present in Upper Spencer Gulf.

Therefore 13 species were suitable for use as endemic organisms.

Were the toxicity tests conducted in water similar to that of Point Lowly?

According to information provided by consultants acting on behalf of BHP Billiton the salinity of the water at Point Lowly ranges from 40 to 43 ppt. The salinity of the diluent water used for the recommended test species from the previous section are presented in Table A1.

As salinity can act as a toxicant it is likely that the toxicity data for at least some of the recommended test species will underestimate and some overestimate the toxicity measured using 43 ppt diluent water.

Table A1. The salinity of the diluent water used in the toxicity tests for the species that have been recommended for use in deriving the dilution factors for the return water.

Recommended test species	Salinity of diluent water (ppt)
Isochrysis galbana	41.2[a]
Ecklonia radiata	41.2
Hormosira banksii	37
Gladioferens imparipes	41.2
Melicertus latisulcatus	41.2
Crassostrea gigas	41.2
Seriola lalandi	40[b], 35[c,] 44.3[d]
Chrysophrys auratus	41.2
Argyrosomus japonicus	41.2
Sepia apama	45[b] and 41.2[c]
Aplysina sp.	41[d]

[a] all salinities of 41.2 ppt in this table were previously reported in Warne (2008a) to be either 39.9 or 40 ppt. Subsequent work to determine the most accurate method of measuring the salinity of the Point Lowly water showed that the conductivity-based field instruments had proved unreliable in the elevated salinities of Upper Spencer Gulf and that the most reliable methods were the salinometer and density measurements (Appendix 09 of the Draft EIS, BHP Billiton, 2009). Samples of the diluent water used for the *I. galbana, E. radiata, G. imparipes, M. latisulcatus, C. gigas, C. auratus, A. japonicus* and *S. apama* toxicity tests were re-analysed using the salinometer and the reported salinity values of 39.9 and 40 ppt were revised to 41.2 ppt. [b] conducted in phase I. [c] conducted in phase II. [d] conducted in phase III.

The toxicity tests conducted by Geotechnical Services (Appendix O10.3 of the Draft EIS, BHP Billiton, 2009) showed that the salinity of the diluent water affected the toxicity to *S. apama*. They recalculated the toxicity of the return water at 42 ppt and found that it was 2

- 3.2 fold lower (i.e. the EC50 values were 2-3.2 times smaller) at 45 ppt (i.e. the salinity of the diluent water) than at 42 ppt (i.e. the then perceived upper end of the range of salinities found at Point Lowly). However, as in indicated in Appendix 3, later data showed that correcting the toxicity data to salinities below 43.5 ppt may underestimate the toxicity. The *S. apama* toxicity tests conducted in phase II were reported as being conducted using diluent water with a salinity of 39.9 ppt (Geotechnical Services; see Appendix O10.4 of the Draft EIS, BHP Billiton, 2009) but have been re-assessed as 41.2 ppt (see Table A1 foot-note). This salinity is close to the middle of the range of salinities measured at Point Lowly (i.e. 40 to 43 ppt). While those from phase I (at 45 ppt) are above the highest reliable measured salinity values at Point Lowly (Appendix 09.2 of the Draft EIS, BHP Billiton, 2009). Therefore the toxicity results for *S. apama* from phase II are the more appropriate for deriving dilution factors when assessed in terms of the water being similar to that at Point Lowly.

Hydrobiology (see Appendix O10.2 of the Draft EIS, BHP Billiton, 2009) conducted DTA testing but did not adjust their toxicity values to salinities other than 36 ppt (as Geotechnical Services did). However, for five of the six species salinity controls were conducted (the exception was *S. lalandi).* The effect of increasing salinity was not consistent for all species. For some species (i.e. *H. tuberculata, N. closterium, P. monodon*, and *S. commercialis*), increased salinity increased toxicity, while for others (i.e. *H. banksii* and *S. lalandi*), increased salinity had no statistically significant effect ($p \leq 0.05$) within the range of salinities reported as occurring in the Spencer Gulf (Geotechnical Services; see Appendix O10.3 of the Draft EIS, BHP Billiton, 2009), but above this range toxicity increased with increased salinity. There is, therefore, the potential that the toxicity values for *H. tuberculata, N. closterium, P. monodon* and *S. commercialis* from phase I underestimate the toxicity of the return water at Point Lowly. Therefore, all four of these species should not be included in the derivation of the dilution factors as they underestimate the toxicity of the return water at 40 – 43 ppt.

For the Yellowtail Kingfish (*S. lalandi*) toxicity results were generated in phases I, II and III. From phase I the no observed effect concentration (NOEC) was 12.5 % return water. The phase II test yielded a concentration that causes a 10% effect (EC10) of 11.1 % return water. However neither of these tests was ideal. The phase I test was conducted at 40 ppt but the exposure was acute (96 hour exposure of larvae) while for phase II the exposure was sub-chronic but it was conducted at 35 ppt (see Appendices O10.2 and O10.4 of the Draft EIS, BHP Billiton, 2009). In phase III additional chronic (7-day) and pulse (2-day) tests were conducted at 44.3 ppt which is higher than the highest reliably measured salinity at Point Lowly.

The toxicity tests for the sponge *Aplysina* sp. were conducted using diluent water with a salinity of 44.3 ppt which is higher than the range of salinities reported at Point Lowly.

Were the exposure scenarios relevant and appropriate?

Continuous (both acute and chronic) exposures were used in all the DTA tests conducted bar one set of tests for *S. lalandi* and *G. imparipes* that used a pulse (2-day) exposure regime. Which exposure scenario is the best depends on how the toxicant(s) in question exerts its toxicity, how the organisms deal with the chemical and the likely exposure of organisms. If the toxicant is metabolised and/or excreted rapidly (shorter duration than the pulses) then the pulsed experiments will provide a lower estimate of the toxicity than the continual exposure experiments. If the toxicant is not excreted rapidly then pulsed exposures will again give a lower estimate of toxicity.

It is possible that with prolonged exposure some organisms can develop tolerance either physiological or genetic, but this generally occurs over generations. However, the development of tolerance is unlikely to be without adverse effects as explained by the metabolic cost hypothesis of Calow and Sibly (1990). For example with elevated salinity higher energy demands are likely to placed on the organism and therefore some other aspect of the organism will have less energy to expend (e.g. reproduction, growth, ability to detoxify toxicants). There are many examples of exactly this occurring.

It is well established in ecotoxicology that the magnitude of any adverse effect on organisms, be they osmoregulatory or toxic, is a function of both the length of exposure and concentration of the waste stream or toxicant. The nature of this relationship is that the shorter the duration of the exposure the higher the aqueous concentration needs to be to cause adverse effects and conversely the longer the exposure duration the lower the aqueous concentration needs to be to cause the same adverse effect (e.g. Connell, 1984; Newman, 1998). Finally, at this point the exact exposure regime of organisms in the vicinity of the discharge is not known. Given the above, using continual exposures was deemed the most appropriate and conservative.

A related exposure scenario issue is that of acclimatising the test organisms. Acclimation is routinely conducted when organisms are collected from the wild and subsequently used in toxicity tests or were there are marked changes in experimental conditions. This is done to ensure that the change in experimental conditions do not contribute or minimally contribute to the measured toxic effects and that only healthy test organisms are used in the toxicity tests.

The test organisms used in the DTA testing were either not acclimated to the test conditions or were not acclimatised for the usual duration (i.e. 2 to 7 days). The test organisms that were included in the best dataset to calculate the dilution factors were all conducted in water with a salinity of 41.2 ppt. These organisms, with the exception of the cuttlefish and the kingfish, were all transferred from normal marine water with a salinity of approximately 35-36 ppt to water with a salinity of 41.2 ppt. Not acclimatising the test organisms would correspond to organisms moving instantaneously from regions where the background salinity occurs into the desalination plant discharge zone and then remaining there for the duration of the toxicity test. While this exposure scenario may occur it is not the most likely to occur and the resulting toxicity data would tend to overestimate the actual toxicity. As such not acclimatising the organisms is a conservative approach (i.e. protective of the environment). It is not possible to estimate the extent of this overestimation of the toxicity given the data currently available.

Were the measured endpoints appropriate?

The Australian and New Zealand Water Quality Guidelines (ANZECC and ARMCANZ, 2000) recommend that only toxicity data based on ecologically relevant endpoints be used to derive national and site-specific trigger values. Ecologically relevant endpoints were “lethality, immobilisation, growth, population growth, and reproduction or the equivalent” (Warne, 2001). Of the 13 species endemic to Upper Spencer Gulf only the toxicity data for the sponge, which measured the attachment of extants (small cubes of sponge cut from live, intact sponges) to a substrate, does not meet the ANZECC and ARMCANZ (2000) requirement.

Did the tests meet appropriate quality assurance and quality control criteria?

All the tests conducted met the quality assurance and quality control criteria (QAQC) except for three species: *P. armatus*, *S. apama* and *Aplysina* sp. The issues associated with each of these species are presented below.

Portunus armatus (Blue Swimmer Crab) can not be used as the test failed due to excessive mortality in the controls.

The percentage hatch of *S. apama* in the phase II toxicity tests was not optimal (i.e. % hatch for the control was 61.8 % while values for the 0.4 to 6.3 % return water treatments ranged from 56.3 to 67.2 %) (Appendix O10.4 of the Draft EIS, BHP Billiton, 2009) and was much less than that reported for the phase I toxicity tests (i.e. 100 % hatch in the control and 75.5

to 89.1 % for the 0.4 to 6.3 % return water treatments). Generally, standardised toxicity tests have a set of validation criteria on which it is determined whether the test is of suitable quality or not and should therefore be accepted or rejected. A key validation criterion is always a stated level of toxic effect for the control - generally permitting a maximum effect of 10 to 20 %. The permitted variation in the percent effect in the controls reflects the innate variability of the test species. The toxicity test for *S. apama* is not standardised and therefore I am not aware that it has such validation criteria, however, it is unlikely that any validation criterion would permit a 40 % effect. We therefore have the situation where the *S. apama* results from phase I with their greater percent hatch in the control are more reliable, but they were measured at 45 ppt and thus may overestimate the toxicity at 40 – 43 ppt. The results from phase II are less reliable but were measured in diluent water with a salinity of 41.2 ppt and thus are within the range of measured salinities at Point Lowly.

The toxicity tests for *Aplysina* sp. were developed for this project by Geotechnical Services Pty. Ltd. and there are no reference toxicants data (which are usually deemed to be essential QAQC procedures). Therefore we have no idea about the relative sensitivity of this batch of sponges compared to others. In addition, no acceptability criteria have been developed for this species and therefore it is not known if the levels of extant attachment observed in the controls is adequate and indicative of healthy sponges.

Were the test species exposed to the toxicant for the same duration?

Acute and chronic toxicity data were not combined to derive the Australian and New Zealand water quality guidelines as they would have different statistical distributions (ANZECC and ARMCANZ, 2000; Warne, 2001). The toxicity tests for the species that have so far been recommended to derive dilution factors (Table A1) are all classed as either chronic (i.e. *N. closterium*) or sub-chronic toxicity tests with the exception of the *G. imparipes, Aplysina* sp. and phase I *S. lalandi* tests which are acute and the phase III tests which included some pulse-exposure tests. Sub-chronic tests are not strictly chronic tests, which require a prolonged exposure of the test organisms to the toxicant. Generally, sub-chronic tests are markedly more sensitive (i.e. they can detect toxicity at considerably lower concentrations) than acute toxicity tests because they expose sensitive early life-stages of the test organisms to a toxicant. For the purposes of deriving water quality guidelines and dilution factors, sub-chronic data can be treated as chronic estimates of toxicity (USEPA, 2002; Stauber, 2003; Warne, 2008b) and this has been done in the assessments of toxicity of the return water from all Australian desalination plants.

If the *G. imparipes, Aplysina* sp. and/or *S. lalandi* acute or pulse toxicity data were used to derive dilution factors, then it would mean that toxicity data that used different exposure periods were being combined. This is not appropriate as stated in the Australian and New Zealand water quality guidelines (ANZECC and ARMCANZ, 2000). It might be possible to use a default assessment factor to convert the acute values to chronic values but the magnitude of these is arbitrary and there is little scientific basis for this (Warne, 1998). It is the author's opinion that it would be preferable to only use sub-chronic and chronic toxicity data rather than use estimates of chronic toxicity.

APPENDIX 2 – USE OF EC10 AND/OR NOEC TOXICITY DATA

The current Australian and New Zealand Water Quality Guidelines use no observed effect concentration (NOEC) data to derive high reliability Trigger Values (TVs) but EC/LC50 toxicity data to derive moderate and both classes of low reliability TVs (ANZECC & ARMCANZ, 2000; Warne, 2001). The relative merits of NOEC and lowest observed effect concentration (LOEC) toxicity data (which are collectively called hypothesis-based toxicity values) have been discussed in the literature. Critics of NOEC data such as Hoekstra and Van Ewijk (1993), Noppert et al. (1994) and Chapman et al. (1996) feel that such data should not be used for regulatory purposes. They prefer point estimates of toxicity such as the concentration that is lethal to 5% of a population (i.e. LC5) or the concentration that causes a 10% effect (i.e. EC10). The problems with the use of NOEC and LOEC data are that:

- only tested concentrations can be NOEC or LOEC values (therefore such values are somewhat predetermined by the concentrations used in the toxicity test);
- the term NOEC is misleading. A NOEC is the highest concentration used in a toxicity test that causes an effect not significantly different to the control(s). It therefore does not correspond to 'no effect'. Typically, the NOEC corresponds to a 10 to 30% effect (Moore and Caux, 1997; USEPA, 1991 and Hoekstra and Van Ewijk, 1993);
- this measure of toxicity can easily be manipulated and does not encourage high quality work. For instance, less rigorous procedures would increase the variability between replicates. This in turn, would increase the size of the difference needed between the treatment and control means in order for a statistically significant difference to be found (i.e. the NOEC value is likely to increase).
- a problem related to the third dot point is that TVs derived using this data do not have as clear a definition as those derived using EC10 data. The TVs based on NOECs would theoretically protect X% of species from experiencing statistically significant inhibitory impacts. The TVs based on EC10 data would theoretically protect X% of species from experiencing inhibitory impacts greater than 10%.

An example of the problems that can arise with using hypothesis-based toxicity data compared to point estimates is provided by the toxicity data for return water to the Mulloway. For that species the NOEC is < 1.6 % return water while the EC10 is 11.56 % return water. The hypothesis based method compared the values for each treatment to the control and found that the first treatment (i.e. 1.6 % return water) was significantly different to the control – hence the NOEC became < 1.6 % return water. However, the concentration response curve is unusual – in that there is a marked difference between the control and the lowest

treatment but then with subsequent increases in the return water content there was very little increase in toxic effect until above 12.7 % return water at which point all growth essentially stopped. This tends to indicate that there was possibly another toxicant present in the diluent water which caused this initial low level effect. So the point estimates of toxicity were calculated using the growth rate of the first treatment as the starting point from which the toxicity values were determined.

Despite the above problems NOEC data were recommended in preference to toxicity data such as EC10 values in the Australian and New Zealand guidelines (ANZECC and ARMCANZ, 2000) for the following reasons:

- there was a general lack of EC10 type data in the scientific literature; and
- there are large amounts of NOEC data available in the literature.

However, the Australian and New Zealand WQGs (ANZECC & ARMCANZ, 2000) point out that the methods used to derive the trigger values are not data specific. Thus, TVs could be derived using EC10 values if there was sufficient data. In fact, these same documents suggested that the use of NOEC data "be phased out" as EC10 type data become available (Warne, 1998; ANZECC & ARMCANZ, 2000).

Recently the NOEC and LOEC type data and the hypothesis-based statistical methods used to derive them have come under further attack. Newman (2008) has written a scathing article which reveals that the methods used to derive the NOEC and LOEC are statistically flawed and that these methods should be replaced 'whenever possible' by confidence interval-based methods. Warne and Van Dam (2008) and Fox (2008) also argue strongly that NOEC and LOEC data should not be generated from now on and that any that is generated should be rejected by regulators and journals.

APPENDIX 3 – DISCUSSION OF THE SALINITY CORRECTION FOR TOXICITY DATA

Ecotoxicology studies of saline return water are unusual because a major toxicant (in this case salt) is already present in the receiving water but at a level that is not toxic to local organisms (although some may be near their limit of tolerance). Each aquatic species has its own unique tolerance for salinity (the maximum salinity before toxic effects commence). In the toxicity tests conducted in this study both the salinity in the return water and in the receiving water contribute to reaching the tolerance for salinity. Therefore, the lower the salinity of the receiving water the more salinity that can be present in the return water before the tolerance limit is reached. Conversely, the higher the salinity of the receiving water the less salinity that can be present in the return water before the tolerance limit is reached. This means that the toxicity values generated by the toxicity tests are affected by both the salinity of the receiving water and that of the return water. The toxicity data generated using receiving water with a salinity lower than the salinities that occur at Point Lowly may be underestimates. Equally the toxicity data generated using receiving waters with a higher salinity than occurs at Point Lowly may be over-estimates. When conducting environmental impact statements toxicity tests should be conducted using receiving waters with salinities that reflect the highest found at Point Lowly (i.e., 43 ppt). However, for various reasons this did not happen and the best way to overcome this is to correct the toxicity data to a salinity of 43 ppt. If there are other toxicants present in the return water (e.g. anti-scalant), then the corrected EC10 values may potentially over- or under-estimate the effect of those toxicants. For the purpose of impact assessment, correcting toxicity data for the salinity of the receiving water should be acceptable providing this does not lead to underestimation of the toxicity of any toxicant present.

Ecotoxicology studies, including a suite of tests on Giant Australian Cuttlefish *Sepia apama*, were undertaken in 2007 using diluent of salinity 41.2 ppt (see Appendix O10.4 of the Draft EIS, BHP Billiton, 2009). Correcting the toxicity data from a lower receiving water salinity to a higher salinity will not underestimate the toxicity of any toxicant. Therefore salinity corrections can safely be made for species that are likely to experience salinities up to 43 ppt (refer to the section “Correcting the toxicity data, protective concentration values and safe dilution factors to the maximum salinity recorded at Point Lowly”). Correcting the toxicity data for the Giant Australian Cuttlefish to a salinity of 43 ppt was not considered necessary as the lifestage that corresponds to the most sensitive endpoint occurs when the salinity is approximately 41 ppt (Draft EIS, BHP Billiton, 2009).

However, the most sensitive estimate of the toxicity of return water to the Giant Australian Cuttlefish (an EC10 of EC10 value of 1.48 %) was measured at a salinity of 45 ppt, which is higher than the maximum measured salinity at Point Lowly. Therefore, it was necessary to determine if the salinity correction could be used to adjust toxicity data to a salinity of 41 ppt. To do this the two sets of toxicity data for the Giant Australian Cuttlefish, (2006 and 2007) that were measured in receiving waters with salinities of 41.2 and 45 ppt were compared. If the causes of the toxicity were the same in both cases then you would expect that either the salinities or the EC10 values (and hence anti-scalant concentrations) would be the same or similar. Yet the salinities at the EC10 vary by more than 2 ppt and the EC10 values differ by approximately three-fold. Potential causes for these differences are temporal variation in toxicity determinations, but also it could be due to differences in the cause of the toxicity.

Table A2. The percentage of return water that causes a 10% reduction in post-hatch survival of the Giant Australian Cuttlefish determined in 2006 and 2007 and the corresponding salinities and anti-scalant concentrations.

Year determined	EC10 (% return water)	Salinity at EC10 (ppt)	Anti-scalant concentration at EC10 (mg/L)[a]
2006	1.9	45.6	0.13
2007	6.3	43.5	0.44

[a] The concentration of anti-scalant in all the undiluted return waters tested was 7 mg/L.

Based on the data presented in Table A2:

- if it is assumed that a salinity of 43.5 ppt causes the toxicity in the 2007 data then the salinity at the 2006 EC10 should be the same and hence the EC10 value should be lower than current value of 1.9% return water.
- if it is assumed that the salinity causes the toxicity in the 2006 sample then the salinity at the 2007 EC10 should be higher than the current value of 43.5 ppt.
- the results of testing the above two assumptions argues that the anti-scalant in the 2007 determination is contributing to the toxicity and may be the major contributor. From this it would also be argued that the anti-scalant in the 2006 determination, present at a concentration markedly lower than that in the 2007 determination, would not be a major contributor to the toxicity of the 2006 determination.

The above has been interpreted as indicating that the salinity correction of toxicity data for the *S. apama* test conducted using diluent of 45 ppt would be appropriate, but only down to a

salinity corresponding to the highest EC10 concentration of 6.3%, namely 43.5 ppt. Correcting to the toxicity of diluent water with a salinity of less than 43.5 ppt, however, potentially does not fully account for the contribution to the toxicity of the anti-scalant and could underestimate the toxicity.

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