

# Testing Toxicity of Ballast Water treated by the Greenship Ballast Water Treatment System

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# Summary

Greenship B.V. has developed a ballast water management system consisting of a sedimentor (multi hydrocyclone system) and an electrolytic cell, producing hypochlorite for killing organisms. The residual effects of sea water treated with the electrolytic cell was investigated with bioassays, using three marine organisms: an algae (*Skeletonema costatum*) a crustacean (*Artemia franciscana*) and a fish (*Solea solea*).

The tests were performed at the laboratory of IMARES in Den Helder, using treated ballast water aged for 24 h, 48 h, 72 h and 96 h. Each test day dilutions of 10%, 18%, 33%, 56% and 100% treated ballast water were made, using untreated ballast water (only hydrocyclone treated) as dilution medium and blank. The salinity of the test water was adjusted to approx. 30‰ by adding NaCl.

Treated ballast water showed no negative effects in the tests with the crustacean and the fish.

In the algal growth inhibition test, significant negative effects of treated ballast water were found. The algae did not survive exposure to 100% treated ballast water in any of the test runs. Exposure to a dilution of 56% treated ballast water resulted in complete mortality in the test run with 24 h aged ballast water, but recovery was observed in older ballast water. In 96 h aged ballast water, full recovery was observed during the course of the test (3 days).



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# 1 Introduction

Ballast water is used by ships to compensate for differences in cargo load. With the ballast water non-indigenous organisms are transported world-wide from ships transporting cargo between regions, which may result in disturbances of regional ecosystems. IMO has set out a mandatory framework for treatment of ballast water on board of ships, in order to minimize the risk of ballast water discharges. Bioassays have to be conducted to assess the effects of treated ballast water on the receiving ecosystems.

Greenship B.V. has developed a ballast water management system consisting of a sedimentor (multi hydrocyclone system) and an electrolytic cell, producing hypochlorite for killing organisms. The residual effects of sea water treated with the electrolytic cell was investigated with bioassays, using three marine organisms: an algae (*Skeletonema costatum*) a crustacean (*Artemia franciscana*) and a fish (*Solea solea*), one to four days after the treatment.





## 2 Materials and Methods

### 2.1 General

On Tuesday, August 21<sup>st</sup>, 2007, a treatment run was performed in Harlingen Harbour, The Netherlands. From IMARES, A.C. Sneekes and J. Cuperus were present to collect the water samples. The Treated Ballast Water (TBW) was sea water from Harlingen Harbour, treated with the hydrocyclone and the electrolytic cell. It was collected in four 25 L PE jerrycans, coded TBW1 to TBW4. A 5<sup>th</sup> jerrycan was taken as back-up. The Untreated Ballast Water (UBW), was taken at the same location in Harlingen Harbour, but was only treated with the hydrocyclone. The UBW was collected in four 25 L PE jerrycans, coded UBW1 to UBW4 and a 5<sup>th</sup> as back-up.

After filling the last jerrycan, the water samples were directly transported to the laboratory of IMARES in Den Helder and stored in the dark at approx. 16°C.

### 2.2 Bioassays

Bioassays were initiated approximately 24h (August 22<sup>nd</sup>), 48h (August 23<sup>rd</sup>), 72h (August 24<sup>th</sup>) and 96h (August 25<sup>th</sup>) after the water was collected and treated in Harlingen Harbour. As the oxygen content of the water was too low for the tests, before test initiation, the water used for the tests was aerated overnight. Before use, water quality parameters were measured in the jerrycans. As the salinity of the water in Harlingen Harbour was too low for the test organisms (approx. 19‰), 10 L was tapped into a large bottle and the salinity was adjusted with NaCl (p.a.-grade) to approx. 29‰. After mixing for at least 30 min. water quality parameters were measured again (see Appendix Table 6).

Each test day, dilution series were made by mixing TBW with UBW in the following proportions (Table 1):

Table 1 Dilutions of treated ballast water (TBW) with untreated ballast water (UBW)

Code	TBW	UBW
BW0	0%	100%
BW1	10%	90%
BW2	17.8%	82.2%
BW3	32.6%	67.4%
BW4	56.2%	43.8%
BW5	100%	0%

The dilution series were made directly from stored water (appr. 16°C) and was then set away at the conditions of different bioassays for acclimatisation. Water parameters were measured in the dilution series for *Skeletonema costatum* and *Solea solea* at start and termination of the test. The test water for *Artemia franciscana* was taken from the dilution series of the fish test.

With each testrun, reference tests were executed. The following concentrations were used (Table 2):

Table 2 Concentrations of reference toxicants

Code	Skeletonema costatum	Artemia franciscana	Solea solea
	K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub>	K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub>	H <sub>2</sub> O <sub>2</sub>
PC0	0.0 mg/L	0.0 mg/L	0 mg/L
PC1	1.0 mg/L	10 mg/L	18 mg/L
PC2	1.8 mg/L	18 mg/L	36 mg/L
PC3	3.2 mg/L	32 mg/L	72 mg/L
PC4	5.6 mg/L	56 mg/L	143 mg/L
PC5	10.0 mg/L	100 mg/L	285 mg/L

### 2.2.1 *Skeletonema costatum*

The test protocol is based upon OECD 201 (1984) and ISO10253 (2006). The test species used was the marine diatom *Skeletonema costatum*. An active growing culture was obtained from TNO Industry (J. Klijnstra). With this inoculum, cultures were started in F2-medium, incubated under the conditions of the test. In the temperature-controlled shaking-cabinet normally used for algal-growth tests, only one test series (incl. reference test) could be placed. Therefore, part of the tests was performed in a climate chamber. In this location, the test flasks had to be shaken by hand several times a day to facilitate gas exchange. The control growth rate on this location was actually too slow (less than 16x multiplication in 72h), due to a slightly lower temperature (18°C vs 20°C), a different light source (cool white vs fluoro) and the lack of continuous shaking. Therefore, test series I (24h after treatment) and IV (96h after treatment) were performed in the shaking cabinet, while only test series II (24 h) and III (72 h) were performed in the climate chamber.

The tests were performed in 250 mL Erlenmeyer flask, filled with appr. 150 mL test medium. For the algal growth test, the TBW was diluted with UBW. The UBW was 0.2µm filtered in order to remove the natural phytoplankton. Each concentration was tested in 3-fold. To each concentration, the same amount of F2-growth medium was added.

Each test concentration was inoculated with algae from a log-phase growing culture. The start concentration was appr. 20 µg/L chlorophyll-a, as measured with a fluorometer before filling the replicate flasks. The flasks were incubated in continuous light at 18°C or 20°C.

The development of the algal biomass, measured as chlorophyll-a concentration, was measured each 24 h.

With each test series, a reference test was conducted, using potassiumdichromate (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>) as toxicant.

Summary of test conditions for the algal growth inhibition test with the marine diatom *Skeletonema costatum*

Test organism	<i>Skeletonema costatum</i>
Test organism source	TNO Industry, continuous culture
Test organism life stage	Log-phase growth, ca. 4 day old culture
Test duration	72 h
Test chamber	250 mL Erlenmeyer
Test solution volume	200 mL per replicate
Initial concentration	Approx. 20 µg/L chlorophyll-a
Replicates	3
Method	Fluorescence measurements
Endpoint	Growth (as concentration chlorophyll-a)
Test temperature	20°C (series I and IV) 18°C (series II and III)
Dilution water	UBW, 0.2 µm filtered/autoclaved
Media stock solutions	F2-medium
Photoperiod	Permanent light
Type of light	Fluoro (series I and IV) Cool white (series II and III)
Shaking	130 rpm (series I and IV) by hand, several times a day (series II and III)
Test protocol	OECD 201 (1984) and ISO10253 (2006)
Control acceptability criteria	Minimum chlorophyll-a increase by factor 16 after 72 h
Reference test	K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub>

### Statistics

Growth rates were calculated by fitting an exponential growth curve through the individual samples (replicates). The curve was fitted with the statistical program GraphPad Prism (v 4.03, Jan. 2005), using the following formula:

$$\mu = \frac{\ln(N_L) - \ln(N_0)}{t_L - t_0}$$

Wherein:

µ = specific growth rate (day<sup>-1</sup>)

N<sub>L</sub> = chlorophyll-a concentration at t<sub>L</sub> (at 24, 48, or 72 h).

N<sub>0</sub> = chlorophyll-a concentration at t<sub>0</sub> (initial concentration)

t<sub>L</sub> = time of measurements (days)

t<sub>0</sub> = start of measurements (0 days).

EC<sub>50</sub>s were calculated as being the concentration/dilution that causes 50% growth inhibition, using the formula for a dose-response curve with variable slope built-in in GraphPad Prism. GraphPad Prism uses the following formula:

$$y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log EC_{50} - x) * \text{Hillslope}}}$$

Wherein: x = Concentration or dilution

y = Specific growth rate µ

Top = Maximum growth rate

Bottom = Minimum growth rate (0)

Bottom was set at a fixed value of 0, to correct for negative growth (mortality).

The NOEC was determined as the highest test concentration in which the growth rate was not significantly reduced compared to the control (UBW). This was assessed by means of a one-way ANOVA followed by Dunnett's multiple comparison post-test.

### 2.2.2 *Artemia franciscana*

The test was performed in a multiwell testplate using instar II-III larvae of the brine shrimp *Artemia franciscana*, obtained as dried cysts from Microlan B.V., The Netherlands, conform the Standard Operational Procedure for ARTOXKIT M™ (MicroBioTests Inc. V100603).

The cysts were incubated in artificial sea water (salinity 35‰) 30 h prior to the start of the tests at 25°C with continuous illumination. After 30h, most cysts were hatched and the larvae developed into instar II-III stages. Appr. 50 larvae were transferred to a rinsing well containing test medium. From the rinsing well, 10 larvae were transferred to the three test wells per concentration/dilution for the test. The well plate was incubated at 25°C in the dark. After 24h living and dead larvae in the test wells were counted.

With each test series, a reference test was conducted, using potassiumdichromate (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>) as toxicant.

Summary of test conditions for the ARTOXKIT M wit the brine shrimp *Artemia franciscana*

Test organism	<i>Artemia franciscana</i>
Test organism source	ARTOXKIT M, MicroBioTests Inc.
Test organism life stage	Larvae <24h old
Test duration	24 h
Test chamber	24-well plate
Test solution volume	1 mL per replicate
Number of organisms/chamber	10
Replicates	3
Endpoints	Survival
Test temperature	25±2°C
Dilution water	UBW
Photoperiod	Permanent dark
Test protocol	Standard Operating Procedure MicroBioTests
Control acceptability criteria	<10% mortality
Reference test	K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub>

### Statistics

EC<sub>50</sub>s were calculated as being the concentration/dilution that causes 50% mortality, using the formula for a dose-response curve with variable slope built-in in GraphPad Prism. GraphPad Prism uses the following formula:

$$y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log EC_{50} - x) * \text{Hillslope}}}$$

Wherein: x = Concentration or dilution  
y = Mortality (as %)  
Top = Maximum mortality (100)  
Bottom = Minimum mortality (0)

The NOEC was determined as the highest test concentration in which the growth rate was not significantly reduced compared to the control (UBW). This was assessed by means of a one-way ANOVA followed by Dunnett's multiple comparison post-test.

### 2.2.3 *Solea solea*

The protocol is based upon OECD 203 (1992). Freshly fertilized eggs of the sole (*Solea solea*) were obtained from SOLEA/IMARES, IJmuiden, from a batch produced August 19<sup>th</sup>, 2007. The eggs were stored at 12-13°C and slight aeration until use in the tests.

The test was performed in 100 mL glass beakers, containing approx. 80 mL of the test concentration. Each concentration was replicated 4-fold. To prevent dilution of the test concentrations, first 40 fertilised eggs, or yolk-sac larvae (depending on availability) were transferred to a rinsing beaker containing the test concentration. From there, 10 organisms were transferred to each replicate.

The test beakers were covered with perforated microwave foil to prevent evaporation, and put away at 12-13°C with a 16h light – 8h dark regime.

After 4 days exposure, surviving organisms were counted in each beaker.

With each test series, a reference test was conducted, using hydrogenperoxide (H<sub>2</sub>O<sub>2</sub>) as toxicant.

Summary of test conditions for the acute fish test with eggs/larvae of the sole *Solea solea*

Test organism	<i>Solea solea</i>
Test organism source	Commercial hatchery SOLEA/IMARES, Netherlands
Test organism life stage	Egg – yolk-sac larvae
Test duration	96 h
Test chamber	100 mL glass beakers
Test solution volume	80 mL
Number of organisms/chamber	10
Replicates	4
Endpoint	Survival
Test temperature	12-15 °C
Dilution water	UBW
Photoperiod	16 hours light/8 hours dark
Aeration	None
Test protocol	OECD 203 (1992)
Control acceptability criteria	<10% mortality
Reference test	H <sub>2</sub> O <sub>2</sub>

### Statistics

EC<sub>50</sub>s were calculated as being the concentration/dilution that causes 50% mortality, using the formula for a dose-response curve with variable slope built-in in GraphPad Prism. GraphPad Prism uses the following formula:

$$y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log EC_{50} - x) * \text{Hillslope}}}$$

Wherein: x = Concentration or dilution  
y = Mortality (as %)  
Top = Maximum mortality (100)  
Bottom = Minimum mortality (0)

The NOEC was determined as the highest test concentration in which the growth rate was not significantly reduced compared to the control (UBW). This was assessed by means of a one-way ANOVA followed by Dunnett's multiple comparison post-test.

## 2.3 Quality Assurance

As a control on the viability of the test organisms, reference tests were performed simultaneously with the ballast water tests. For *Skeletonema costatum* and *Artemia franciscana* potassiumdichromate ( $K_2Cr_2O_7$ ) was used as reference toxicant. The results were compared with standard values: ISO for *S. costatum* and manufacturer specifications of the batches for *A. franciscana*.

For the sole early life stage test, no standards were available. Therefore,  $H_2O_2$  was used as reference toxicant and the results were compared with previous tests with sole larvae conducted at the IMARES laboratory.

## 3 Results

### 3.1 Reference tests

The results of the reference tests are summarised in Table 3. *Skeletonema costatum* appears to be rather insensitive, with 72h EC<sub>50</sub> values two to four times higher than is indicated in the ISO guidelines. K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> is a yellow solution causing some disturbance in the fluorometric measurements. This probably resulted in chlorophyll-a measurements that were too high for the treatments with 3.2 and 5.6 mg/L, as measurements of cell activity did not support the relatively high levels of chlorophyll-a in these treatments (Appendix Table 9). It was the intention to verify the fluorometric measurements by performing cellcounts after the final measurements. However, the cells of *S. costatum* tended to form aggregates during the test which made cell countings impossible.

Table 3: Results of reference tests as EC50 in mg/L

Test initiation date	<b>S. costatum</b> EC <sub>50</sub> K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub>	<b>A. franciscana</b> EC <sub>50</sub> K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub>	<b>S. solea</b> EC <sub>50</sub> H <sub>2</sub> O <sub>2</sub>
Expected EC <sub>50</sub>	2.5±1.1	26.7-35.6	84-150
22-08-2007	5.6	28.4	87.4
23-08-2007	9.2	54.0	87.6
24-08-2007	8.7	37.8	145.6
25-08-2007	5.6	46.9	-

The EC<sub>50</sub> values obtained in the reference tests with *Artemia franciscana* were close to or within the indicated range specified for the batch of cysts used at 22-08 and 24-08, but were higher for the other two tests. The latter were initiated by a less experienced analyst, who probably introduced too much hatching medium into the rinsing wells, causing dilution of the test solutions. The results of the reference test are summarised in Table 11.

The EC<sub>50</sub> values for H<sub>2</sub>O<sub>2</sub> in the reference tests with sole eggs and larvae, were within the range established in previous tests with this species (Sneekes *et al.*, 2007). In the test initiated on August 25<sup>th</sup>, the concentrations were accidentally only 10% of those intended. Therefore, the highest concentration was well below no-effect level and effects were not observed. The results of the reference test are summarised in Table 15.

### 3.2 Ballast water tests

#### 3.2.1 *Skeletonema costatum*

*S. costatum* responded directly to exposure to treated ballast water. Each test day, a clear dose response relationship was obvious at the initial measurements that were performed within half an hour after the algae were introduced into the test medium (see Table 4).

Table 4 Measurements of chlorophyll-a (chl-a) and cell activity (% act) at initiation of the tests.

Start day	24h		48h		72h		96h	
% TBW	chl-a	% act	chl-a	% act	chl-a	% act	chl-a	% act
0	20.83	31.53	23.22	30.89	21.50	24.24	29.04	27.75
10	19.69	32.11	20.80	38.16	18.80	26.02	29.79	28.74
18	19.51	32.16	21.69	34.74	14.82	23.88	28.91	28.89
33	19.97	30.79	21.23	33.43	13.74	19.58	28.53	30.13
56	16.38	13.56	21.77	35.43	12.16	10.43	25.98	24.09
100	14.31	12.03	20.87	26.00	11.55	10.37	18.86	11.57

In the tests with 24h and 48h old ballast water the algae seemed affected in all ballast water dilutions, but recovered during the test in the most diluted treatments (Figure 1). Algae in 33% TBW grew a little less than in 10% and 18% TBW, but the growth rate was only significantly different from UBW in the test with 48h old ballast water.

In 56% TBW the same pattern was observed, but more severe. In the test with 24h old ballast water, the algae died and chlorophyll-a concentrations decreased to levels below the detection limit. In the other tests, however, the algae recovered after an initial decrease in density, resulting in a complete recovery after 3 days in the test with 96h old ballast water.

In 100% TBW, the algae died within 24h exposure and no recovery was observed in any of the four tests. The results of the ballast water tests are summarised in Table 8.

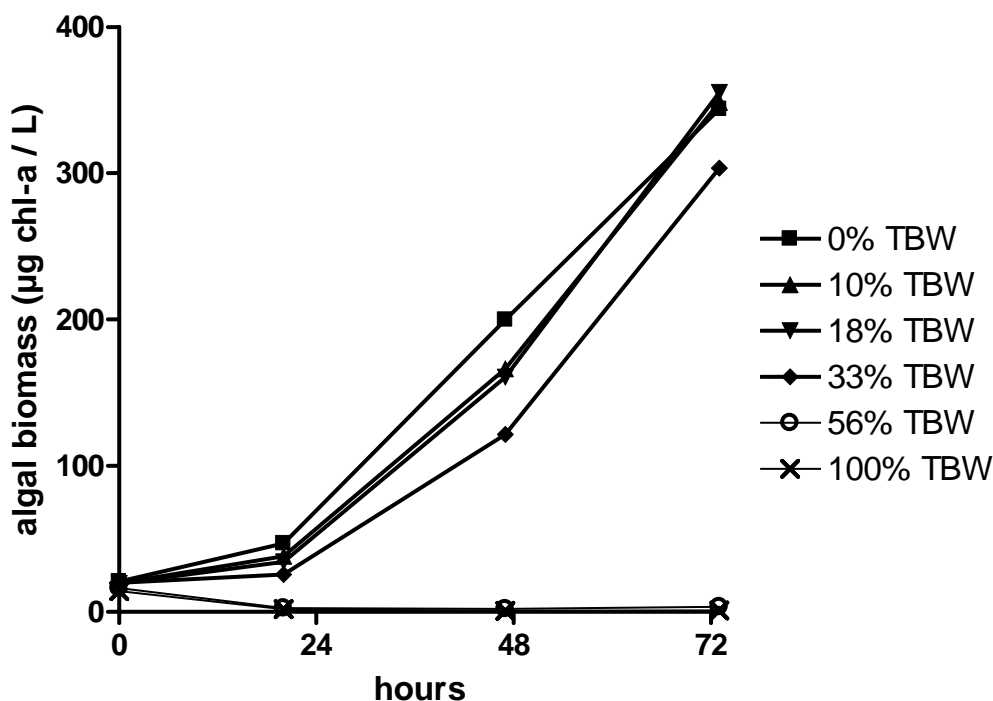


Figure 1 Growth of *S. costatum* introduced into ballast water dilutions 24 h after treatment

From the obtained growth rates, the following EC<sub>50</sub> and NOEC values (as % TBW) were calculated (Table 5):



Table 5 *EC<sub>50</sub> values based upon algal growth rate*

Time after BW treatment	72 h EC <sub>50</sub>	72 h NOEC
24 h	34.8 %	32.6 %
48 h	59.4 %	17.8 %
72 h	56.7 %	32.6 %
96 h	67.6 %	56.2 %

The first and last test (24h and 96h old ballast water) were incubated in a temperature-controlled shaking cabinet with fluoro-type lights at 20°C. The second and third test were incubated in a temporary location with cool-white lights at ca. 18°C and had to be shaken manually. Under these circumstances, the algae grew slower and did not reach the minimum growth rate of 0.9/d.

However, the response to TBW (and the reference toxicant) was similar in all tests, indicating that the lower growth was not due to a lower viability of the algae, but to less than optimal culture conditions.

### 3.2.2 *Artemia franciscana*

The brine shrimp *A. franciscana* was not affected by treated ballast water. Survival was at least 90% in all treatments, with two exceptions: UBW in the 48 h test (87%) and 10% TBW in the 72 h test (77%). The results of the ballast water tests are summarised in Table 10.

### 3.2.3 *Solea solea*

Survival of sole larvae was good (87.5% to 100%) throughout all tests. No effects of ballast water treatment were observed. The results of the ballast water tests are summarised in Table 14.



## 4 Conclusions

At 24 h after treatment of the ballast water with the electrolytic cell, no effects were observed of the treatment on larvae of the brine shrimp (*Artemia franciscana*) and eggs and yolk-sac larvae of the sole (*Solea solea*). The diatom *Skeletonema costatum* was severely affected by the treated ballast water, resulting in complete mortality of the algae in undiluted treated ballast water even in the tests initiated 96 h after treatment. In time, however, the effects were gradually alleviated, resulting in complete recovery of the algae in 56% TBW during the 3 day exposure in the test initiated with ballast water 96 h after treatment.



## 5 References

ISO (2006) Water quality – Marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricorutum*. NEN-ENISO 10253.

MicroBioTest Inc. (s.a.) Artemia toxicity screening test for estuarine and marine waters. Standard Operational Procedure ARTOXKIT M™ V100603.

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## 6 Referees and Authors

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This report has been professionally prepared by Wageningen IMARES. The scientific validity of this report has been internally tested and verified by another researcher and evaluated by the Scientific Team at Wageningen IMARES.

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Date: 6 November, 2007

Approved: Drs. J.H.M. Schobben  
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Date: 6 November 2007

## Appendix A Data Tables

Table 6 Water conditions of stored ballast water (BW) before and after adjusting of salinity for the four test series (I=BW 24h after treatment; II=BW 48h; III=BW 72h; IV=BW 96h)

	Temp (°C)	O2 (mg/L)	O2 (%)	Conductivity (mS/cm)	Salinity (‰)	pH
Before adjustment						
<b>I-TBW</b>	17.1	10.04	104.5	31.2	19.2	7.90
<b>I-UBW</b>	16.6	9.45	98.6	31.3	19.2	8.15
After adjustment						
<b>I-TBW</b>	17.0	9.97	104.6	46.1	29.5	7.89
<b>I-UBW</b>	17.0	9.89	102.0	45.9	29.4	8.10
Before adjustment						
<b>II-TBW</b>	14.1	10.22	104.4	31.5	19.2	8.07
<b>II-UBW</b>	14.2	10.06	99.7	31.4	19.2	7.98
After adjustment						
<b>II-TBW</b>	15.5	10.76	113.8	-	29.3	7.93
<b>II-UBW</b>	15.8	10.40	116.7	-	29.1	7.89
Before adjustment						
<b>III-TBW</b>	14.3	10.40	102.5	31.6	19.3	8.10
<b>III-UBW</b>	14.8	10.20	102.0	31.4	19.2	7.96
After adjustment						
<b>III-TBW</b>	14.8	10.17	101.0	46.4	29.5	8.03
<b>III-UBW</b>	15.3	10.15	102.4	46.2	29.4	7.97
Before adjustment						
<b>IV-TBW</b>	15.8	9.47	94.4	31.9	19.6	7.66
<b>IV-UBW</b>	15.6	9.35	92.0	31.4	19.2	7.66
After adjustment						
<b>IV-TBW</b>	16.8	9.90	100.7	46.7	29.8	7.64
<b>IV-UBW</b>	17.3	9.65	99.1	46.2	29.5	7.64



Table 7 Water conditions algal growth tests. BW: Ballast water tests, PC: reference tests.

<b>22-8-2007</b>	<b>Temp (°C)</b>	<b>pH (start)</b>	<b>pH (end)</b>		<b>Temp (°C)</b>	<b>pH (start)</b>	<b>pH (end)</b>
BW0	17.4	7.62	9.20	PC0	17.2	7.94	9.08
BW1	17.7	7.74	9.22	PC1	17.9	7.72	9.09
BW2	17.9	7.67	9.09	PC2	18.3	7.65	9.09
BW3	18.2	7.56	8.96	PC3	18.5	7.65	8.48
BW4	18.3	7.49	8.17	PC4	18.9	7.78	8.11
BW5	18.5	7.45	8.08	PC5	19.2	7.70	8.03
<b>23-8-2007</b>	<b>Temp (°C)</b>	<b>pH (start)</b>	<b>pH (end)</b>		<b>Temp (°C)</b>	<b>pH (start)</b>	<b>pH (end)</b>
BW0	20.5	7.40	9.20	PC0	17.5	7.57	9.02
BW1	19.6	7.38	9.19	PC1	17.8	7.57	9.06
BW2	19.6	7.37	9.19	PC2	18.0	7.54	9.08
BW3	20.0	7.43	9.20	PC3	18.5	7.56	9.10
BW4	20.0	7.43	9.06	PC4	18.9	7.53	8.79
BW5	20.2	7.52	8.02	PC5	19.3	7.49	8.08
<b>24-8-2007</b>	<b>Temp (°C)</b>	<b>pH (start)</b>	<b>pH (end)</b>		<b>Temp (°C)</b>	<b>pH (start)</b>	<b>pH (end)</b>
BW0	20.5	7.45	9.18	PC0	19.2	7.59	8.98
BW1	20.5	7.45	9.24	PC1	18.9	7.57	9.01
BW2	20.6	7.48	9.23	PC2	19.1	7.61	9.02
BW3	20.4	7.45	9.22	PC3	19.0	7.60	9.00
BW4	20.3	7.42	8.77	PC4	18.8	7.55	8.42
BW5	20.4	7.43	8.03	PC5	19.3	7.55	8.04
<b>25-8-2007</b>	<b>Temp (°C)</b>	<b>pH (start)</b>	<b>pH (end)</b>		<b>Temp (°C)</b>	<b>pH (start)</b>	<b>pH (end)</b>
BW0	18.5	6.98	9.06	PC0	15.6	7.18	8.87
BW1	18.6	7.03	9.28	PC1	15.6	7.19	8.58
BW2	18.0	7.03	8.78	PC2	15.6	7.19	8.71
BW3	17.9	6.99	9.29	PC3	15.7	7.18	9.11
BW4	17.7	6.96	9.03	PC4	15.8	7.15	8.10
BW5	17.3	6.88	8.10	PC5	15.9	7.13	8.06

Table 8 Growth (chlorophyll-a in µg/L) and activity (%) of algae in ballast water treatments

<b>Test 24 h</b>	<b>Day 0 chl-a</b>	<b>22-8-2007 %activity</b>	<b>Day 1 chl-a</b>	<b>23-8-2007 %activity</b>	<b>Day 2 chl-a</b>	<b>24-8-2007 %activity</b>	<b>Day 3 chl-a</b>	<b>25-8-2007 %activity</b>
BW0	20.83	31.53	46.76	37.70	199.8	40.27	343.8	23.58
BW1	19.69	32.11	38.23	36.16	166.3	39.90	348.5	23.16
BW2	19.51	32.16	34.39	38.83	160.6	39.28	355.2	25.11
BW3	19.97	30.79	25.57	36.25	121.3	39.32	303.4	26.05
BW4	16.38	13.56	2.62	22.65	2.16	32.85	3.59	34.88
BW5	14.31	12.03	1.89	34.03	0.85	-	1.09	-
<b>Test 48 h</b>	<b>Day 0 chl-a</b>	<b>23-8-2007 %activity</b>	<b>Day 1 chl-a</b>	<b>24-8-2007 %activity</b>	<b>Day 2 chl-a</b>	<b>25-8-2007 %activity</b>	<b>Day 3 chl-a</b>	<b>26-8-2007 %activity</b>
BW0A	23.22	30.89	61.09	32.30	225.9	28.06	285.5	21.81
BW1A	20.80	38.16	55.80	33.80	171.8	35.33	233.9	23.92
BW2A	21.69	34.74	56.39	34.92	159.7	34.04	252.4	24.28
BW3A	21.23	33.43	45.93	33.94	143.7	33.79	226.4	21.07
BW4A	21.77	35.43	29.29	33.97	108.0	33.21	174.3	23.78
BW5A	20.87	26	0.62	-	0.18	-	0.20	-
<b>Test 72 h</b>	<b>Day 0 chl-a</b>	<b>24-8-2007 %activity</b>	<b>Day 1 chl-a</b>	<b>25-8-2007 %activity</b>	<b>Day 2 chl-a</b>	<b>26-8-2007 %activity</b>	<b>Day 3 chl-a</b>	<b>27-8-2007 %activity</b>
BW0A	21.50	24.24	52.06	31.97	147.3	32.61	207.3	25.70
BW1A	18.80	26.02	54.20	31.56	152.0	33.08	209.8	24.03
BW2A	14.82	23.88	50.16	32.73	141.7	33.88	205.3	22.06
BW3A	13.74	19.58	39.43	33.23	133.4	33.13	205.6	20.77
BW4A	12.16	10.43	2.56	29.64	12.45	29.43	54.71	29.24
BW5A	11.55	10.37	0.35	-	0.11	-	0.27	-
<b>Test 96 h</b>	<b>Day 0 chl-a</b>	<b>25-8-2007 %activity</b>	<b>Day 1 chl-a</b>	<b>26-8-2007 %activity</b>	<b>Day 2 chl-a</b>	<b>27-8-2007 %activity</b>	<b>Day 3 chl-a</b>	<b>28-8-2007 %activity</b>
BW0A	29.04	27.75	72.14	30.86	285.9	34.74	350.5	35.97
BW1A	29.79	28.74	69.35	30.18	277.3	34.20	342.3	33.93
BW2A	28.91	28.89	61.79	29.51	284.0	32.09	396.7	33.84
BW3A	28.53	30.13	55.11	29.29	269.1	27.87	370.4	34.62
BW4A	25.98	24.09	38.24	29.76	151.6	36.97	381.9	34.88
BW5A	18.86	11.57	0.48	-	0.45	-	1.21	-

Table 9 Growth (chlorophyll-a in µg/L) and activity (%) of algae in reference tests

<b>Test 24 h</b>	<b>Day 0 chl-a</b>	<b>22-8-2007 %activity</b>	<b>Day 1 chl-a</b>	<b>23-8-2007 %activity</b>	<b>Day 2 chl-a</b>	<b>24-8-2007 %activity</b>	<b>Day 3 chl-a</b>	<b>25-8-2007 %activity</b>
PC0	19.62	28.97	32.91	37.41	136.5	41.15	367.2	31.45
PC1	21.24	31.73	33.33	38.88	146.5	41.37	334.7	31.27
PC2	20.65	33.38	35.67	38.01	163.7	38.40	381.3	28.94
PC3	19.96	34.86	35.56	33.49	200.0	37.22	659.6	21.51
PC4	19.17	34.58	33.53	31.29	99.38	14.54	81.28	6.60
PC5	18.95	33.75	21.90	30.15	30.36	10.07	12.21	5.48
<b>Test 48 h</b>	<b>Day 0 chl-a</b>	<b>23-8-2007 %activity</b>	<b>Day 1 chl-a</b>	<b>24-8-2007 %activity</b>	<b>Day 2 chl-a</b>	<b>25-8-2007 %activity</b>	<b>Day 3 chl-a</b>	<b>26-8-2007 %activity</b>
PC0A	19.95	39.51	57.20	32.62	129.5	29.40	161.2	23.88
PC1A	20.26	33.76	58.50	31.91	130.3	29.62	154.7	21.67
PC2A	20.01	35.25	58.06	32.01	132.5	28.62	159.7	25.48
PC3A	19.81	35.73	57.29	32.69	152.1	29.50	183.4	26.21
PC4A	18.77	36.05	53.68	28.32	223.4	12.73	174.9	7.39
PC5A	19.00	31.70	33.18	24.50	35.69	3.52	5.53	10.73
<b>Test 72 h</b>	<b>Day 0 chl-a</b>	<b>24-8-2007 %activity</b>	<b>Day 1 chl-a</b>	<b>25-8-2007 %activity</b>	<b>Day 2 chl-a</b>	<b>26-8-2007 %activity</b>	<b>Day 3 chl-a</b>	<b>27-8-2007 %activity</b>
PC0A	25.08	27.60	62.60	31.25	103.3	31.38	127.7	29.76
PC1A	24.48	30.88	59.30	31.22	102.3	29.19	128.2	29.55
PC2A	23.25	32.98	56.27	31.33	106.2	29.42	131.8	29.08
PC3A	21.82	34.10	54.85	29.66	130.4	27.84	156.5	27.76
PC4A	21.01	34.59	46.53	26.91	161.9	13.31	133.3	6.52
PC5A	19.86	34.51	23.22	20.06	15.97	9.68	4.44	13.67
<b>Test 96 h</b>	<b>Day 0 chl-a</b>	<b>25-8-2007 %activity</b>	<b>Day 1 chl-a</b>	<b>26-8-2007 %activity</b>	<b>Day 2 chl-a</b>	<b>27-8-2007 %activity</b>	<b>Day 3 chl-a</b>	<b>28-8-2007 %activity</b>
PC0A	30.80	37.41	53.47	30.42	293.8	37.73	358.9	39.61
PC1A	30.65	36.19	47.33	30.11	257.0	38.08	341.7	41.14
PC2A	29.83	36.51	43.82	29.02	239.9	36.49	372.4	40.25
PC3A	29.87	35.75	36.65	27.62	207.8	30.87	417.9	35.53
PC4A	29.26	36.12	33.05	24.45	94.68	22.45	112.7	8.67
PC5A	27.76	33.99	28.10	14.30	24.15	9.46	12.27	7.13

Table 10 Survival of *Artemia franciscana* in ballast water treatments

Test 24h	22-8-2007		Alive 23-8-2007			Batch Survival	AF030 % Survival
	In	A	B	C			
BW0	30	10	10	10	10	30	100.0%
BW1	30	10	10	10	10	30	100.0%
BW2	30	10	10	10	10	30	100.0%
BW3	30	10	10	10	10	30	100.0%
BW4	30	10	10	10	9	29	96.7%
BW5	30	10	10	10	10	30	100.0%
Test 48h	23-8-2007		Alive 24-8-2007			Batch Survival	AF030 % Survival
	In	A	B	C			
BW0	30	10	7	9	9	26	86.7%
BW1	30	10	9	9	9	28	93.3%
BW2	30	9	10	10	10	29	96.7%
BW3	30	10	10	9	9	29	96.7%
BW4	30	10	10	10	10	30	100.0%
BW5	30	10	10	10	10	30	100.0%
Test 72h	24-8-2007		Alive 25-8-2007			Batch Survival	AF030 % Survival
	In	A	B	C			
BW0	30	9	9	9	9	27	90.0%
BW1	30	7	8	8	8	23	76.7%
BW2	30	10	10	9	9	29	96.7%
BW3	30	8	10	9	9	27	90.0%
BW4	30	10	10	10	10	30	100.0%
BW5	30	10	10	10	10	30	100.0%
Test 96h	25-8-2007		Alive 26-8-2007			Batch Survival	AF/F 2006 % Survival
	In	A	B	C			
BW0	30	10	10	9	9	29	96.7%
BW1	31	10	9	10	10	29	93.5%
BW2	30	8	10	10	10	28	93.3%
BW3	30	10	10	7	7	27	90.0%
BW4	30	10	8	10	10	28	93.3%
BW5	30	10	10	10	10	30	100.0%

Table 11 Survival of *Artemia franciscana* in reference tests

<b>Test</b>	<b>22-8-2007</b>	<b>Alive 23-8-2007</b>			<b>Batch AF030</b>	
<b>24h</b>	<b>In</b>	<b>A</b>	<b>B</b>	<b>C</b>	<b>Survival</b>	<b>% Survival</b>
PC0	30	10	9	10	29	96.7%
PC1	30	10	10	9	29	96.7%
PC2	30	7	8	9	24	80.0%
PC3	30	5	3	3	11	36.7%
PC4	30	0	0	2	2	6.7%
PC5	30	1	3	2	6	20.0%
<b>Test</b>	<b>23-8-2007</b>	<b>Alive 24-8-2007</b>			<b>Batch AF030</b>	
<b>48h</b>	<b>In</b>	<b>A</b>	<b>B</b>	<b>C</b>	<b>Survival</b>	<b>% Survival</b>
PC0	30	10	10	10	30	100.0%
PC1	30	10	10	10	30	100.0%
PC2	30	10	10	10	30	100.0%
PC3	30	4	4	6	14	46.7%
PC4	30	2	4	5	11	36.7%
PC5	30	5	2	7	14	46.7%
<b>Test</b>	<b>24-8-2007</b>	<b>Alive 25-8-2007</b>			<b>Batch AF030</b>	
<b>72h</b>	<b>In</b>	<b>A</b>	<b>B</b>	<b>C</b>	<b>Survival</b>	<b>% Survival</b>
PC0	30	9	10	10	29	96.7%
PC1	30	10	10	10	30	100.0%
PC2	30	5	7	10	22	73.3%
PC3	30	5	6	5	16	53.3%
PC4	30	5	3	2	10	33.3%
PC5	29	0	1	4	5	17.2%
<b>Test</b>	<b>25-8-2007</b>	<b>Alive 26-8-2007</b>			<b>Batch AF/F 2006</b>	
<b>96h</b>	<b>In</b>	<b>A</b>	<b>B</b>	<b>C</b>	<b>Survival</b>	<b>% Survival</b>
PC0	30	10	10	10	30	100.0%
PC1	30	10	10	10	30	100.0%
PC2	30	8	10	9	27	90.0%
PC3	31	5	5	5	15	48.4%
PC4	30	5	3	4	12	40.0%
PC5	30	3	5	2	10	33.3%

Table 12 Water conditions fish test with ballast water

Test 24h	22-8-2007				26-8-2007			
	Temp (°C)	O2 (%)	Salinity (‰)	pH	Temp (°C)	O2 (%)	Salinity (‰)	pH
BW0	13.5	98.4	29.4	8.19	13.4	95.5	29.9	8.02
BW1	13.4	99.8	29.5	8.18	13.3	95.5	29.7	8.01
BW2	13.4	99.7	29.5	8.17	13.0	95.8	30.0	8.01
BW3	13.4	99.9	29.5	8.13	12.7	96.3	29.9	8.02
BW4	13.4	99.7	29.5	8.08	13.1	98.3	30.0	8.02
BW5	13.4	100.1	29.5	7.98	13.0	97.2	29.8	8.02
Test 24h	23-8-2007				27-8-2007			
	Temp (°C)	O2 (%)	Salinity (‰)	pH	Temp (°C)	O2 (%)	Salinity (‰)	pH
BW0	15.2	97.4	29.3	7.91	12.8	86.4	29.7	7.95
BW1	15.1	98.3	29.4	7.91	12.8	86.7	29.7	7.97
BW2	15.1	98.5	29.5	7.91	12.8	89.6	29.7	7.98
BW3	15.1	98.5	29.5	7.92	12.7	30.1	29.7	7.99
BW4	15.1	99.5	29.5	7.94	12.7	89.7	29.7	8.00
BW5	15.0	99.8	29.5	7.96	12.7	90.2	29.7	8.00
Test 24h	24-8-2007				28-8-2007			
	Temp (°C)	O2 (%)	Salinity (‰)	pH	Temp (°C)	O2 (%)	Salinity (‰)	pH
BW0	15.6	101.1	29.5	8.00	12.9	97.9	29.6	7.96
BW1	15.5	101.1	29.4	8.01	12.9	96.6	29.7	7.97
BW2	15.5	102.0	29.5	8.02	12.8	96.3	29.7	7.99
BW3	15.5	102.1	29.5	8.03	12.7	96.2	29.8	7.99
BW4	15.5	102.3	29.6	8.04	12.7	96.1	29.8	7.99
BW5	15.5	102.5	29.6	8.05	12.7	95.4	29.8	7.99
Test 24h	25-8-2007				29-8-2007			
	Temp (°C)	O2 (%)	Salinity (‰)	pH	Temp (°C)	O2 (%)	Salinity (‰)	pH
BW0	13.3	98.9	29.5	7.77	12.8	98.0	29.8	7.98
BW1	13.3	97.9	29.5	7.78	12.8	97.9	29.8	7.99
BW2	13.4	98.2	29.6	7.78	12.7	97.9	29.8	8.00
BW3	13.6	98.6	29.6	7.78	12.8	97.7	29.8	8.00
BW4	13.6	98.8	29.7	7.77	12.8	97.9	29.9	8.02
BW5	13.6	99.1	29.9	7.77	12.8	97.0	30.0	8.01

Table 13 Water condition fish reference test

Test 24h	22-8-2007				26-8-2007			
	Temp (°C)	O2 (%)	Salinity (‰)	pH	Temp (°C)	O2 (%)	Salinity (‰)	pH
PC0	12.8	99.6	32.5	9.16	13.0	97.1	32.9	8.03
PC1	12.7	98.4	32.4	8.15	13.0	98.5	32.7	8.03
PC2	12.8	98.6	32.3	8.15	13.0	99.0	32.6	8.03
PC3	12.9	99.0	32.0	8.15	13.0	99.2	32.2	8.03
PC4	13.0	99.2	31.3	8.15	12.9	109.0	31.6	8.05
PC5	13.2	99.4	30.0	8.15	12.7	109.7	30.3	8.06
Test 48h	23-8-2007				27-8-2007			
	Temp (°C)	O2 (%)	Salinity (‰)	pH	Temp (°C)	O2 (%)	Salinity (‰)	pH
PC0	13.7	98.6	32.3	7.99	12.8	89.8	32.8	8.00
PC1	13.5	98.1	32.5	7.98	12.8	91.3	32.8	8.01
PC2	13.4	98.3	32.5	7.98	12.8	91.5	32.7	8.02
PC3	13.4	98.3	32.5	7.98	12.8	92.9	32.7	8.03
PC4	13.5	99.0	32.5	7.97	12.7	99.3	32.6	8.04
PC5	13.6	99.1	32.3	7.96	12.6	102.9	32.6	8.05
Test 72h	24-8-2007				28-8-2007			
	Temp (°C)	O2 (%)	Salinity (‰)	pH	Temp (°C)	O2 (%)	Salinity (‰)	pH
PC0	14.1	100.4	32.5	8.10	12.7	94.4	32.8	7.99
PC1	14.0	100.4	32.5	8.10	12.9	113.2	32.8	7.99
PC2	14.1	101.2	32.6	8.11	12.9	96.7	32.8	8.00
PC3	14.1	101.5	32.5	8.10	12.8	99.0	32.7	8.02
PC4	14.2	102.1	32.5	8.10	12.8	101.7	32.7	8.02
PC5	14.2	102.7	32.3	8.09	-	-	-	-
Test 96h	25-8-2007				29-8-2007			
	Temp (°C)	O2 (%)	Salinity (‰)	pH	Temp (°C)	O2 (%)	Salinity (‰)	pH
PC0	12.2	100.9	32.7	8.14	13.0	94.9	32.9	8.01
PC1	12.1	100.7	32.6	8.14	12.7	95.4	32.8	8.03
PC2	12.1	100.7	32.7	8.15	12.7	95.4	32.9	8.03
PC3	12.2	101.4	32.6	8.15	12.7	97.9	32.8	8.03
PC4	12.2	101.3	32.6	8.16	12.8	102.2	32.8	8.04
PC5	12.2	101.9	32.4	8.16	12.8	97.4	32.6	8.03

Table 14 Survival of fish larvae in ballast water tests. Test 24h, BW4C and D, were lost. Test 48h: organisms introduced as fertilized eggs.

Test 24h	22-8-2007 introduced				26-8-2007 survival				% Survival
	A	B	C	D	A	B	C	D	
BW0	10	10	10	10	10	9	9	10	95.0%
BW1	9	10	9	10	9	9	8	9	92.1%
BW2	10	10	10	10	9	9	10	10	95.0%
BW3	10	10	10	10	10	10	10	9	97.5%
BW4	10	10	-	-	9	10	-	-	95.0%
BW5	10	10	9	10	10	9	9	9	94.9%
Test 48h	23-8-2007 introduced				27-8-2007 survival				% Survival
	A	B	C	D	A	B	C	D	
BW0	10	10	10	10	10	10	6	9	87.5%
BW1	10	10	10	10	10	10	9	9	95.0%
BW2	10	10	10	10	10	9	10	10	97.5%
BW3	10	10	10	10	10	10	10	10	100.0%
BW4	10	10	11	10	10	10	11	9	97.6%
BW5	10	10	10	10	10	10	10	10	100.0%
Test 72h	24-8-2007 introduced				28-8-2007 survival				% Survival
	A	B	C	D	A	B	C	D	
BW0	10	10	10	10	10	9	10	9	95.0%
BW1	10	10	10	10	10	10	10	8	95.0%
BW2	10	10	11	10	10	10	11	10	100.0%
BW3	10	10	10	10	10	10	10	10	100.0%
BW4	10	10	10	10	10	10	10	10	100.0%
BW5	10	11	10	10	10	10	10	10	97.6%
Test 96h	25-8-2007 introduced				29-8-2007 survival				% Survival
	A	B	C	D	A	B	C	D	
BW0	10	10	10	10	10	10	10	10	100.0%
BW1	10	10	10	10	9	10	10	10	97.5%
BW2	10	10	10	10	10	9	10	9	95.0%
BW3	11	10	10	10	10	10	9	10	95.1%
BW4	10	10	10	11	10	10	10	11	100.0%
BW5	10	11	10	10	10	11	10	10	100.0%



Table 15 Survival of fish larvae in reference tests. Test 48h: organisms introduced as fertilized eggs.

<b>Test 24h</b>	<b>22-8-2007 introduced</b>				<b>26-8-2007 survival</b>				<b>% Survival</b>
	<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>	<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>	
PC0	9	9	9	10	9	8	7	5	78.4%
PC1	10	10	10	9	9	8	7	6	76.9%
PC2	10	10	10	10	9	9	7	8	82.5%
PC3	10	10	10	10	10	8	9	9	90.0%
PC4	10	9	10	10	0	0	0	0	0.0%
PC5	10	10	10	10	0	0	0	0	0.0%
<b>Test 48h</b>	<b>23-8-2007 introduced</b>				<b>27-8-2007 survival</b>				<b>% Survival</b>
	<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>	<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>	
PC0	10	10	10	10	10	10	10	10	100.0%
PC1	10	10	10	10	10	10	9	10	97.5%
PC2	10	10	10	10	10	9	10	10	97.5%
PC3	10	10	10	10	10	9	10	10	97.5%
PC4	10	10	10	10	0	0	0	0	0.0%
PC5	10	10	10	10	0	0	0	0	0.0%
<b>Test 72h</b>	<b>24-8-2007 introduced</b>				<b>28-8-2007 survival</b>				<b>% Survival</b>
	<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>	<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>	
PC0	10	10	10	10	9	9	10	9	92.5%
PC1	10	10	10	10	9	10	9	9	92.5%
PC2	10	10	10	10	10	10	9	8	92.5%
PC3	10	10	10	10	10	10	8	9	92.5%
PC4	10	10	10	10	6	4	4	7	52.5%
PC5	10	10	10	10	0	0	0	0	0.0%
<b>Test 96h</b>	<b>25-8-2007 introduced</b>				<b>29-8-2007 survival</b>				<b>% Survival</b>
	<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>	<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>	
PC0	10	10	10	10	8	10	9	9	90.0%
PC1	10	10	10	10	10	9	10	9	95.0%
PC2	10	10	10	10	9	9	10	10	95.0%
PC3	10	10	10	10	10	10	10	10	100.0%
PC4	10	10	10	10	10	10	10	10	100.0%
PC5	10	10	10	10	10	10	9	10	97.5%