WET-tests on UV-treated ballast water

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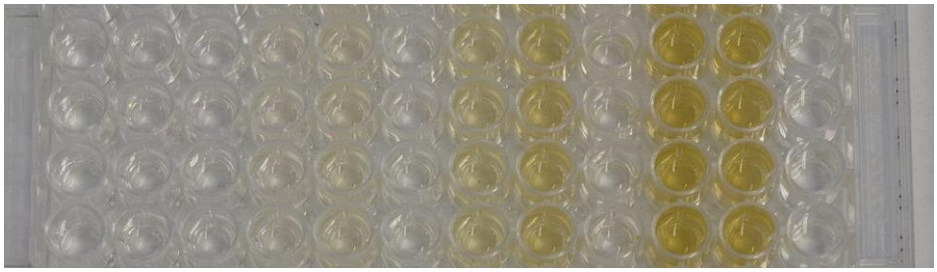

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Summary

Damen Shipyards has developed a barge-based ballast water management system (BWMS) that enables direct treatment of ballast water during discharge in a receiving harbour. The treatment is based upon filtration and a once-through UV-treatment. As part of the Type Approval process, the Dutch Authorities (IL&T) required an Environmental Acceptability document, based upon Whole Effluent Toxicity (WET) testing on freshwater and marine water.

A suit of standardised bioassays that have regularly been used for the assessment of treated ballast water, were used for these WET-tests. Samples of control and treated ballast water were taken on-board of the MEA-Innovator during test runs and were transported to the IMARES laboratories for the WET-tests.

No negative effects were found in the tests exposing control water. Most of the tests showed no sign of negative effects due to exposure to the treated ballast water. Slight, but significant effects were observed in the highest test concentrations of the marine bacteria test and freshwater algae test. The freshwater algae test result is reported as chronic value. The test results are summarised in Table 1 below.

Table 1 Summary of test results of treated ballast water, form marine and freshwater type approval tests, including the maximum exposure of the test organisms.

Acute effects	**Species**	**Marine tests - max exposure**	**Freshwater tests - max exposure**
Bacteria	*Vibrio fischeri*	13% effect at 50% sample- 30 min	No effects - 30 min
Algae	*Phaedactylum tricornutum*	No effects - 72 h	
	Pseudokirchneriella subcapitata		12% effect at 96% sample- 72 h
Rotifera	*Brachionus plicatilis*	No effects - 48 h	
	Brachionus calyciflorus		No effects - 24 h
Crustacea	*Artemia franciscana*	No effects - 48 h	
	Daphnia magna		No effects - 48 h

Chronic effects	**Species**	**Marine tests - max exposure**	**Freshwater tests - max exposure**
Algae	*Phaedactylum tricornutum*	No effects - 72 h	
	Pseudokirchneriella subcapitata		NOEC 70% - 72h
Rotifera	Brachionus plicatilis	No effects - 48 h	

1 Introduction

Damen Shipyards has developed a barge-based ballast water management system (BWMS) that enables direct treatment of ballast water during discharge in a receiving harbour. The treatment is based upon filtration and a once-through UV-treatment. As part of the Type Approval process, the Dutch Authorities (IL&T) required an Environmental Acceptability document, based upon Whole Effluent Toxicity (WET) testing on freshwater and marine water, in order to ensure that no harmful levels of dis-infection by-products (DBP) are formed by the UV-treatment.

IMARES has conducted the WET-tests, using a suit of standardised bioassays that have regularly been used for the assessment of treated ballast water derived from land-based and shipboard tests of several BWMSs. Water samples for the WET-tests were taken during land-based tests for Type Approval that were conducted by MEA, using the MEA Innovator test barge. As the BWMS developed by Damen is developed to treat ballast water during discharge in a once-through treatment, the water for the WET-tests was sampled during uptake, directly after the treatment. Control water was sampled before treatment during the same uptake event. The WET-tests were conducted the next day at the IMARES laboratories in Den Helder.

2 Materials and Methods

2.1 Sampling

Samples were taken on board of the MEA Innovator by the MEA personnel during a freshwater and a marine uptake cycle. The samples were collected in pre-washed 30 L PE jerrycans (90% filled) and stored in a sheltered place. A relatively large sample volume was chosen in order to provide some buffer during storage.

At the end of the working day the MEA Innovator returned to the harbour in Den Oever, the samples were handed over to IMARES and transported to the IMARES laboratories in Den Helder. Here they were stored in a climate controlled room at 15±2°C in the dark until further processing the next day.

2.2 Environmental parameters

Environmental parameters were measured in the water samples to ensure the boundary conditions for the bioassays were met. Dissolved oxygen (% saturation) and salinity (‰) were measured using a Hach HQ40d multimeter. The oxygen electrode was used to measure the temperature. A Mettler Toledo SevenGo handheld meter with pH glass electrode was used to measure the acidity in the samples.

2.3 WET-tests

The WET-tests consisted of bio-assays with four organism groups: bacteria, algae, rotifera and crustacea. Treated water, as well as control water was tested in dilution series, using artificial water as diluent.

The bacteria *Vibrio fisheri* was used, following the Microtox® Basic test procedure. This test measures loss of luminescence due to exposure to toxicants. Since *Vibrio* is a marine species, the salinity of the freshwater samples is adjusted with concentrated dilution water containing 20% NaCl.

Algae are tested using the growth-inhibition test, comparing growth in a treated sample with that in a control. For freshwater samples, the green algae *Pseudokirchneriella subcapitata* was used, while the diatom *Phaeodactylum tricornutum*, cultured at a salinity of 30 ‰ was used for the marine tests.

For tests with rotifers, the standard test kits provided by MicroBioTests were used, in which survival of newly hatched rotifer nauplii is assessed. For the freshwater tests Rotoxkit F, based upon the freshwater species *Brachionus calyciflorus*, was used. Rotoxkit M was used for the marine tests. This is based upon the saltwater species *Brachionus plicatilis*.

Two species of crustacean were selected. For the freshwater tests, juvenile *Daphnia magna* hatched from ephippia (winter-eggs), were used. After 24h and 48h, changes in mobility of the individuals was assessed. The intention was to use the calanoid copepod *Acartia tonsa* as test species for the marine tests. The quality of the animals delivered by the supplier was, however, not sufficient. Therefore, we used instar II-III larvae of the brine shrimp *Artemia franciscana*, freshly hatched at a salinity of 35 ‰. After 24h and 48h, the survival of the instar II-III larvae was assessed.

2.3.1 Bacteria luminescence inhibition test with Vibrio fischeri (Microtox®)

Description
The bacteria test with *Vibrio fisheri* (Microtox® Basic test) exposes luminescent bacteria in reagent to aqueous samples, and measures the increase or decrease in light output by the test organisms, which is an indicator of the organism's condition. The test procedures are based upon ISO 11348-3 (2007) and AZUR Environmental Basic Test (1998). Reagent contains living luminescent bacteria that have been grown under optimal conditions, harvested, and then lyophilized (freeze-dried). The lyophilized bacteria are rehydrated with Reconstitution Solution to provide a ready-to-use suspension of organisms. The test system measures the light output of the luminescent bacteria after they have been exposed to a sample and compares it to the light output of a control (reagent blank) that contains no sample. A difference in light output (between the sample and the control) is attributed to the effect of the sample on the organisms.

Inhibition effects may be masked when saltwater samples are tested. This is probably caused by alkali and alkali-earth metal ions as described in Annex D of ISO 11348-3 (2007), resulting in stimulation effects on the luminescence of the bacteria. Therefore, adapted reagents for the saltwater samples as described in the standard procedure have been used. The highest test concentration of environmental samples used is 45-50% sample due to dilution with the reconstitution solution.

Quality assurance
For each test series, the viability of the bacteria is tested using phenol. The results are compared with the specifications of the bacteria batch used and previous reference tests.

Characteristics

Test organism	*Vibrio fischeri* (lyophilized)
Salinity range	0–35‰
Test duration	30 minutes
Test volume	1 ml
Number of replicates	1
Temperature	15±2°C
Standard test water	Freshwater test (x<5‰): Diluent (2%-NaCl) Brackish test (5‰<x<20‰): ABW* Seawater test (20‰<x<35‰): ASW*
Test protocol(s)	AZUR Environmental Basic Test (1998) and ISO 11348-3 (2007)
Reference toxicant	Phenol
Toxicological observations	Inhibition of luminescence
Toxicological parameters	EC_{50} (5, 15, 30 min)

** ABW: Artificial Brackish Water; ASW: Artificial Sea Water*

Statistics
The EC_{50} is calculated using the Microtox Omni software provided with the Microtox® basic test.

2.3.2 Freshwater algal growth inhibition test with Pseudokirchneriella subcapitata

Description
Algal growth inhibition by exposure to a sample is determined by the so-called algal growth inhibition test according to ISO 8692 (2004) procedures using the freshwater green algae *Pseudokirchneriella subcapitata*. After 24, 48 and 72 hours, the growth of algae is analyzed with means of fluorescence readings. The standard test procedure has been adapted by reducing the test volume to 96-well plate levels based on Blaise & Vasseur (2005), Peterson *et al*. (2005) and SCA (2008). Each concentration is replicated eight times and every well contains a total of 0.25 ml in volume. Each test concentration is inoculated with algae from a log-phase growing culture. The start concentration is approx. 10,000 cells/ml, determined microscopically. The 96-well plate is incubated under continuous light at 23±2°C and shaken. For each test concentration, a number of wells is not inoculated with algae, in order to allow for correction for context values and water colour. A difference in growth (between the sample and the control) is attributed to the effect of the sample on the organisms.

Quality assurance
The quality of algae is stipulated by exposing the algae to a substance of which the toxicity is known (potassium dichromate; $K_2Cr_2O_7$). The results are compared with the specifications of the algae used on bases of previous reference tests. Moreover the growth of the algae in the control group is checked and compared to the standard conditions (Factor 67; μ_c>1.4 r^{-1}).

Characteristics

Test organism	*Pseudokirchneriella subcapitata* (exponential growing culture)
Salinity range	0-5‰
Test duration	72 hours
Test volume	0.25 ml
Number of replicates	8
Test chamber	96 polystyrene multiwell plate
Temperature	23±2°C
Standard test water	Algal medium ISO
Method	Fluorescence readings
Photoperiod	Culture and test: Permanent illumination
Test protocol(s)	ISO 8692 and OECD 201 (adapted for small volume)
Reference toxicant	Potassium dichromate
Control growth	Factor 67 μ_c>1.4 r^{-1}
Toxicological observations	Inhibition of growth
Toxicological parameters	E_rC_{50} (72h), NOE_rC

Statistics
Individual growth rates of the algae are determined using the least squares method by Weisstein (2008a). Secondly, percentages of inhibition are calculated using the following formula:

$$I_{\mu i} = \frac{\mu_c - \mu_i}{\mu_c} \times 100\% \quad (1)$$

Where: $I_{\mu i}$ = percentage inhibition for each test concentration i (%)
μ_i = growth rate of each test concentration i (r-1)
μ_c = average growth rate of the control (r-1)

The E_rC_{50} and the confidence intervals are calculated by using a 'sigmoidal dose response curve' with variable slope (ISO, 2006). The calculated effect concentrations are corrected for dilution (ca. 4%) by added nutrient stock and algal inoculum. Percentages of inhibition are cut-off at 100%, to correct for negative growth (mortality). To calculate the E_rC_{50} the following formula is used:

$$y = \frac{100}{1 + 10^{(\log EC_{50} - x) * Hillslope}} \quad (2)$$

Where: E_rC_{50} = Concentration in which 50% effect is found (v/v%)
x = Concentration of the dilution (v/v%)
y = Specific percentage inhibition μ (%)
Hillslope = slope of the logistic curve (-)

The E_rC_{50} and the Hillslope is determined using Gaus-Newton algorithm for non-linear regression. The unknown values are estimated iterative using the Jacobian matrix as described by Weisstein (2008b).

NOE_rC (No observed effect concentration): The highest concentration at which no significant effect is observed. The NOE_rC is derived from the data noting that the effect at the NOE_rC should not exceed 10% (ECB, 2003).

2.3.3 Marine algal growth inhibition test with Phaeodactylum tricornutum

Description
Algal growth inhibition by exposure to a sample is determined by the so-called algal growth inhibition test according to ISO 10253 (2006) procedures using the marine diatom *Phaeodactylum tricornutum*. After 24, 48 and 72 hours, the growth of algae is analyzed with means of fluorescence readings.

The standard test procedure has been adapted by reducing the test volume to 96-well plate levels based on Blaise & Vasseur (2005), Peterson *et al*. (2005) and SCA (2009). Each concentration is replicated eight times and every well contained a total of 0.25 ml in volume. Each test concentration is inoculated with algae from a log-phase growing culture. The start concentration is approx. 10,000 cells/ml, determined microscopically. The 96-well plate is incubated under continuous light at 20±2°C and shaken. For each test concentration, a number of wells is not inoculated with algae, in order to allow for correction for context values and water colour. A difference in growth (between the sample and the control) is attributed to the effect of the sample on the organisms.

Quality assurance
The quality of algae is stipulated by exposing the algae to a substance of which the toxicity is known (potassium dichromate; $K_2Cr_2O_7$). The results are compared with the specifications of the algae used on bases of previous reference tests. Moreover the growth of the algae in the control group is checked and compared to the standard conditions (Factor 16; μ_c>0.9 r^{-1}).

Characteristics

Test organism	*Phaeodactylum tricornutum* (exponential growing culture)
Salinity range	25-35‰
Test duration	72 hours
Test volume	0.25 ml
Number of replicates	8
Test chamber	96 polystyrene multiwell plate
Temperature	20±2°C
Standard test water	Algal medium F2 in Artificial Sea Water
Method	Fluorescence readings
Photoperiod	Culture and test: Permanent illumination
Test protocol(s)	ISO 10253 (adapted for small volume)
Reference toxicant	Potassium dichromate
Control growth	Factor 16 μ_c>0,9 r^{-1}
Toxicological observations	Inhibition of growth
Toxicological parameters	E_rC_{50} (72h), NOE_rC

Statistics
Individual growth rates of the algae are determined using the least squares method by Weisstein (2008a). Secondly, percentages of inhibition are calculated using the following formula:

$$I_{\mu i} = \frac{\mu_c - \mu_i}{\mu_c} \times 100\% \qquad (3)$$

Where: $I_{\mu i}$ = percentage inhibition for each test concentration i (%)
μ_i = growth rate of each test concentration i (r-1)
μ_c = average growth rate of the control (r-1)

The E_rC_{50} and the confidence intervals are calculated by using a 'sigmoidal dose response curve' with variable slope (ISO-TS 20281, 2006). The calculated effect concentrations are corrected for dilution (ca. 4%) by added nutrient stock and algal inoculum. Percentages of inhibition are cut-of at 100%, to correct for negative growth (mortality). To calculate the E_rC_{50} the following formula is used:

$$y = \frac{100}{1 + 10^{(\log EC_{50} - x) * Hillslope}} \qquad (4)$$

Where: E_rC_{50} = Concentration in which 50% effect is found (v/v%)
x = Concentration of the dilution (v/v%)
y = Specific percentage inhibition μ (%)
Hillslope = slope of the logistic curve (-)

The E_rC_{50} and the Hillslope is determined using Gaus-Newton algorithm for non-linear regression. The unknown values are estimated iterative using the Jacobian matrix as described by Weisstein (2008b).

NOE_rC (No observed effect concentration): The highest concentration at which no significant effect is observed. The NOE_rC is derived from the data noting that the effect at the NOE_rC should not exceed 10% (ECB, 2003).

2.3.4 Freshwater acute mortality test with the rotifer Brachionus calyciflorus

Description
The test is performed in a multiwell testplate using freshly hatched larvae of the rotifer *Brachionus calyciflorus*, obtained as dried cysts from Microlan B.V., The Netherlands, conform the Standard Operational Procedure for ROTOXKIT F™ (MicroBioTests Inc.). Cysts are incubated in artificial freshwater 28-30h prior to the start of the tests, at 25°C with continuous illumination. After 28-30 hours, most cysts are hatched. Approx. 50 larvae are transferred to a rinsing well containing test medium. From the rinsing well, 5 larvae are transferred to the six wells per concentration/dilution for the test. The well plate is incubated at 25°C in the dark. After 24 hours living and dead larvae in the test wells are counted. Rotifers are considered dead if they do not exhibit any internal or external movement in 5 seconds of observation.

Quality assurance
For each test series, the quality of the test organisms is determined by exposing the larvae to a substance whose toxicity is known (potassium dichromate; $K_2Cr_2O_7$). The results are compared with specifications of the test organism batch and results from previous reference tests with the species. Additionally, the results of the control exposure are assessed for acceptability (<10% effect).

Characteristics

Test organism	*Brachionus calyciflorus* (<4 hours old)
Salinity range	0-5‰
Test duration	24 hours
Test volume	0.3 ml
Number of organisms/replicate	5
Number of replicates	6
Temperature	25±2°C
Standard test water	AFW (Artificial Fresh Water)
Test protocol(s)	MicroBioTests Inc.
Reference toxicant	Potassium dichromate
Toxicological observations	Mortality
Toxicological parameters	LC_{50} (24h)

Statistics
The LC_{50} and its confidence intervals were calculated by using a 'sigmodal dose response curve' with variable slope (ISO TS 20281, 2006) built into the software program GraphPad Prism (Version 5.04, Nov 2010). This is based on a maximum effect of 100% and a minimum effect between 0 and 10% (blank condition). GraphPad Prism uses the following formula:

$$y = Bottom + \frac{100 - Bottom}{1 + 10^{(\log LC_{50} - x) * Hillslope}} \quad (5)$$

Where: LC_{50} = Concentration in which 50% lethality is found (mg/l)
x = Concentration of the dilution (mg/l)
y = Specific percentage effect μ (%)
Bottom = Minimum percentage effect (%)
Hillslope = slope of the logistic curve (-)

2.3.5 Marine acute mortality test with the rotifer Brachionus plicatilis

Description
The test is performed in a multiwell testplate using freshly hatched larvae of the rotifer Brachionus plicatilis, obtained as dried cysts from Microlan B.V., The Netherlands, conform the Standard Operational Procedure for ROTOXKIT M™ (MicroBioTests Inc. V071090). Cysts are incubated in artificial seawater (salinity 20 ‰) 28-30h prior to the start of the tests at 25°C with continuous illumination. After 28-30 hours, most cysts are hatched.

Approx. 50 larvae are transferred to a rinsing well containing test medium. From the rinsing well, 5 larvae are transferred to the six wells per concentration/dilution for the test. The well plate is incubated at 25°C in the dark. After 24 hours living and dead larvae in the test wells are counted. Rotifers are considered dead if they do not exhibit any internal or external movement in 5 seconds of observation.

Quality assurance
For each test series, quality of the rotifers is determined by exposing the larvae to a substance whose toxicity is known (potassium dichromate; $K_2Cr_2O_7$). Moreover, control mortality is checked on the basis of acceptability in aquatic toxicity tests (<10%).

Characteristics

Test organism	*Brachionus plicatilis* (28-30 hours after hatching starts)
Salinity range	3-40 ‰
Test duration	24-48 hours
Test volume	0.3 ml
Number of organisms/replicate	5
Number of replicates	6
Temperature	25±2°C
Standard test water	ASW (Artificial Sea Water)
Photoperiod	Hatching: permanent light Test: permanent dark
Test protocol(s)	MicroBioTests Inc. V071090
Reference toxicant	Potassium dichromate
Toxicological observations	Mortality
Toxicological parameters	LC_{50} (24h, 48h), NOEC

Statistics
The LC_{50} and its confidence intervals were calculated by using a 'sigmodal dose response curve' with variable slope (ISO TS 20281, 2006) built into the software program GraphPad Prism (Version 5.04, Nov 2010). This is based on a maximum effect of 100% and a minimum effect between 0 and 10% (blank condition). GraphPad Prism uses the following formula:

$$y = Bottom + \frac{100 - Bottom}{1 + 10^{(\log LC_{50} - x) * Hillslope}} \quad (6)$$

Where: LC_{50} = Concentration in which 50% lethality is found (mg/l)
x = Concentration of the dilution (mg/l)
y = Specific percentage effect μ (%)
Bottom = Minimum percentage effect (%)
Hillslope = slope of the logistic curve (-)

NOEC (No observed effect concentration): The highest concentration at which no significant effect is observed. The NOEC is derived from the data noting that the effect at the NOEC should not exceed 10% (ECB, 2003).

2.3.6 Freshwater acute immobility test with the crustacean Daphnia magna

Description
The acute Daphnia-test is based upon the guideline ISO-NEN 6341 (1998), adapted for small volumes. Ephippia (winter resting-eggs) are hatched 3 days prior to testing in artificial freshwater. Each concentration is tested using four 4 ml replicates with 5 test organisms each. Juvenile Daphnids (<24h old) are exposed to the test concentrations for 48h. After 24h and 48h the (im)mobility of the organisms is assessed, as an indication for the viability of the test organisms. Daphnids are considered immobile if they are not able to swim after gentle agitation of the liquid in 15 seconds of observation.

Quality assurance
For each test series, the quality of the test organisms is determined by exposing the organisms to a substance with a known toxicity (potassium dichromate; $K_2Cr_2O_7$). The results are compared with specifications of the test organism batch and results from previous reference tests with the species. Additionally, the results of the control exposure are assessed for acceptability (<10% effect).

Characteristics

Test organism	*Daphnia magna* (<24 hours oud)
Salinity range	0-3‰
Test duration	48 hours
Test volume	4 ml
Number of organisms/replicate	5
Number of replicates	4
Temperature	20±2°C
Standard test water	DSW (Dutch Standard Water)
Test protocol(s)	ISO 6341 (adapted for small volume)
Reference toxicant	Potassium dichromate
Toxicological observations	Immobility
Toxicological parameters	EC_{50} (24h, 48h)

Statistics
The EC_{50} and its confidence intervals were calculated by using a "sigmodal dose response curve with variable slope (ISO, 2006) built into the software program GraphPad Prism (Version 5.04, Nov 2010). This is based on a maximum effect of 100% and a minimum effect between 0 and 10% (blank condition). GraphPad Prism uses the following formula:

$$y = Bottom + \frac{100 - Bottom}{1 + 10^{(\log EC_{50} - x) * Hillslope}} \quad (7)$$

Where: EC_{50} = Concentration in which 50% effect is found (mg/l)
x = Concentration of the dilution (mg/l)
y = Specific percentage effect μ (%)
Bottom = Minimum percentage effect (%)
Hillslope = slope of the logistic curve (-)

2.3.7 Marine acute mortality test with the crustacean Artemia franciscana

Description
For the acute Artemia test, dried cysts are incubated in artificial seawater of the desired salinity. After 30 hours, stage II-III larvae are available for testing. Each concentration is tested using four 4 ml replicates with 5 test organisms each. After 24±2 hours the number of surviving and dead organisms is counted. An Artemia is considered dead if they do not exhibit any internal or external movement in 5 seconds of observation.

Quality assurance
For each test series, the quality of the test organisms is determined by exposing the organisms to a substance with a known toxicity (potassium dichromate; $K_2Cr_2O_7$). The results are compared with specifications of the test organism batch and results from previous reference tests with the species. Additionally, the results of the control exposure are assessed for acceptability (<10% effect).

Characteristics

Test organism	*Artemia franciscana* (stage II-III larvae)
Salinity range	5-35‰
Test duration	48 hours
Test volume	1 ml
Number of organisms/replicate	10
Number of replicates	3
Temperature	25±2°C
Standard test water	ASW (Artificial Sea Water)
Photoperiod	Hatching: Permanent illumination Test: Dark
Test protocol(s)	Standard Operating Procedure MicroBioTests
Reference toxicant	Potassium dichromate
Toxicological observations	Mortality
Toxicological parameters	LC_{50} (24h, 48h)

Statistics
The LC_{50} and its confidence intervals were calculated by using a 'sigmodal dose response curve' with variable slope (ISO, 2006) built into the software program GraphPad Prism (Version 5.04, Nov 2010). This is based on a maximum effect of 100% and a minimum effect between 0 and 10% (blank condition). GraphPad Prism uses the following formula:

$$y = Bottom + \frac{100 - Bottom}{1 + 10^{(\log LC_{50} - x) * Hillslope}} \quad (8)$$

Where: LC_{50} = Concentration in which 50% lethality is found (mg/l)
x = Concentration of the dilution (mg/l)
y = Specific percentage effect μ (%)
Bottom = Minimum percentage effect (%)
Hillslope = slope of the logistic curve (-)

3 Results

3.1 Samples SET 1

The first set of samples was collected at Den Oever on 4 June 2015 and was stored overnight at 15°C. The environmental parameters were within the ranges suitable for the bioassays, although the salinity was lower than expected (Table 2). According to the specifications of the IMO, the test water is classified as "Brackish".

Table 2 Environmental parameters of the marine water samples prior to test initiation on 5 June 2015

Parameter	Control	Treated
Temperature	14.2 °C	14.5 °C
Dissolved Oxygen	101.7 %	94.8 %
pH	8.4	8.4
Salinity	28.0 ‰	28.6 ‰

The tests were initiated on June 5th, 2015. All QA criteria for the tests were met. In the bio-assays, no effects were observed for the control water and for the treated water sample. The various concentration steps with increasing level of sample showed the same response as the blanks, except for the bacteria test where significant inhibition was observed at the highest concentration tested (50% treated water; t-test, $p<0.05$). The results are summarized in Table 3.

*Table 3 Summary of test results (as % of blank) for marine WET-tests initiated 5 June 2015. * significant ($p<0.05$)*

Bacteria, Microtox; inhibition of luminescence

[C]	Control			Treated		
	5 min	15 min	30 min	5 min	15 min	30 min
6.25 %	-0.1	-2.7	-0.3	3.9	4.4	3.8
12.5 %	1.6	0.5	1.0	3.6	3.2	2.6
25 %	-0.7	-0.2	0.6	1.8	2.6	2.8
50 %	0.9	2.0	4.4	7.6	11.9*	13.0*
EC_{50}	>50	>50	>50	>50	>50	>50

Algae, *Phaeodactylum tricornutum; inhibition of growth*

[C]	Control		Treated	
	48h	72h	48h	72h
30.2 %	-17.2	-12.2	-44.0	-29.4
40.4 %	-16.8	-12.5	-21.0	-14.4
53.7 %	-21.4	-15.7	-19.9	-13.3
71.7 %	-18.4	-13.5	-21.8	-14.6
95.6 %	-12.5	- 9.6	-14.1	- 9.3
E_rC_{50}	>95.6	>95.6	>95.6	>95.6
NOE_rC	≥95.6	≥95.6	≥95.6	≥95.6

Crustacea, *Artemia franciscana; mortality*

[C]	Control		Treated	
	24 h	48 h	24 h	48 h
0.0 %	0.0	0.0	5.7	3.3
31.6 %	3.3	3.3	0.0	3.3
42.2 %	0.0	0.0	0.0	0.0
56.2 %	3.3	6.7	0.0	0.0
75 %	0.0	0.0	0.0	0.0
100 %	0.0	0.0	0.0	0.0
LC_{50}	>100	>100	>100	>100

Rotifera, *Brachionus plicatilis; mortality*

[C]	Control		Treated	
	24 h	48 h	24 h	48 h
0.0 %	3.4	3.4	0.0	0.0
31.6 %	6.7	6.7	0.0	0.0
42.2 %	3.6	3.6	0.0	0.0
56.2 %	0.0	0.0	0.0	0.0
75 %	0.0	0.0	0.0	0.0
100 %	0.0	0.0	0.0	3.4
LC_{50}	>100	>100	>100	>100
NOEC	-	≥100	-	≥100

3.2 Samples SET 2

A second set of samples were collected at Den Oever on 11 June 2015 and were stored overnight at 15°C. The environmental parameters were within the ranges suitable for the bioassays (Table 4). According to the specifications of the IMO, the test water is classified as "Freshwater".

Table 4 Environmental parameters of the freshwater samples prior to test initiation on 5 June 2015

Parameter	Control	Treated
Temperature	18.1 °C	-
Dissolved Oxygen	100.8 %	106.6 %
pH	8.7	8.8
Salinity	0.40 ‰	0.55 ‰

The tests were initiated on June 12th, 2015, except for the bacteria, which had to be conducted June 13th. All QA criteria for the bacteria, algae and rotifer tests were met. The batch of *Daphnia magna* ephippia used, did not hatch sufficiently, but QA criteria were met. Therefore, only a limited concentration series was used for the control exposure. No effects of the treatment were observed, although relatively high random mortality was seen after 48h.

In the bacteria test and the rotifer test, no effects were observed for the control water nor for the treated water sample. All blanks performed as required and the various concentration steps with increasing level of sample showed the same response as the blanks.

The test with the freshwater algae *Pseudokirchneriella subcapitata* showed a significant reduction of the growth rate at the highest concentration tested (t-test, $p<0.05$). After 48h exposure, the effect was twice as strong compared to the 72h exposure.

The results are summarized in Table 5.

*Table 5 Summary of test results (as % of blank) for freshwater WET-tests initiated at 12 June 2015 (Bacteria 13 June). * significant (p<0.05)*

Bacteria, Microtox, *Vibrio fischeri*; inhibition of luminescence

[C]	Control			Treated		
	5 min	15 min	30 min	5 min	15 min	30 min
5.625	-0.3	-0.8	2.3	-11.9	-12.6	-6.2
11.25	-3.3	-6.6	-2.9	-12.8	-13.3	-5.1
22.5	-6.3	-11.2	-9.7	-23.8	-25.2	-20.2
45	-16.1	-25.6	-25.2	-12.7	-17.7	-9.5
EC_{50}	>45	>45	>45	>45	>45	>45

Algae, *Pseudokirchneriella subcapitata; inhibition of growth*

[C]	Control		Treated	
	48h	72h	48h	72h
29.6 %	- 3.0	- 5.2	-12.5	- 8.9
39.4 %	-13.5	-12.7	-13.3	- 9.5
52.6 %	- 2.7	- 3.5	- 8.9	- 7.5
70.2 %	-18.1	-16.3	- 0.5	- 2.3
93.6 %	-14.8	-14.6	24.0*	12.0*
E_rC_{50}	>93.6	>93.6	>93.6	>93.6
NOE_rC	≥93.6	≥93.6	70.2	70.2

Crustacea, *Daphnia magna; immobility*

[C]	Control		Treated	
	24 h	48 h	24 h	48 h
0.0 %	-	-	0.0	0.0
31.6 %	-	-	0.0	15.0
42.2 %	-	-	0.0	5.9
56.2 %	-	-	5.0	15.0
75 %	0.0	0.0	0.0	5.0
100 %	0.0	10.0	4.8	4.8
EC_{50}	>100	>100	>100	>100

Rotifera, *Brachionus calyciflorus* 24h mortality

[C] (%)	Control	Treated
0.0 %	0.0	0.0
31.6 %	0.0	0.0
42.2 %	0.0	0.0
56.2 %	0.0	0.0
75 %	6.7	0.0
100 %	0.0	0.0
LC_{50}	>100	>100

4 Discussion

In general, no negative effects were seen in the bio-assays. For the bacterial luminescence test and the algae growth inhibition tests some stimulation was observed (negative inhibition). This occurred both in control as in treated water and is probably caused by additional availability of specific nutrients.

In two tests a significant negative effect was found in treated ballast water. As the effect was not present in the control water, it is treatment-related. Bacterial luminescence was significantly inhibited after 15 and 30 min exposure to the maximum concentration of brackish water tested and clear, but not significant after 5 min exposure. The maximum inhibition is only 13%, but it should be noted that the maximum concentration of treated water that could be tested was 50% due to dilution with the bacteria suspension. It was not possible to derive an acute value (EC_{50}), based upon these data.

The second test showing negative effects in response to exposure to treated ballast water, was the algae growth inhibition test with the freshwater algae *P. subcapitata*. In this test too, significant effects were only seen at the highest concentration tested, which was 94% after correction for added medium and algae. After 72 h exposure, the effect was much reduced compared to 48 h exposure. Suggesting the algae were not fatally affected and started catching up. No acute value (EC_{50}) could be established, but the algae test may be used as chronic value and a NOEC of 70.2% treated ballast water was reported. Freshwater contains only a fraction of the halogen concentration in sea water, but higher levels of dissolved and particulate organic carbon (as is reflected by the different requirements for challenge waters in G8). These are known to be precursors for DBP (Neil *et al.*, 1990; Kleiser & Frimmel, 2000; IMO, 2008).

5 Quality Assurance/ Quality Control

IMARES utilises an ISO 9001:2008 certified quality management system (certificate number: 124296-2012-AQ-NLD-RvA). This certificate is valid until 15 December 2015. The organisation has been certified since 27 February 2001. The certification was issued by DNV Certification B.V. Furthermore, the chemical laboratory of the Fish Division has NEN-EN-ISO/IEC 17025:2005 accreditation for test laboratories with number L097. This accreditation is valid until 1th of April 2017 and was first issued on 27 March 1997. Accreditation was granted by the Council for Accreditation.

As of April 29th, 2015, the IMARES ballast water test facilities have been accepted as subcontractor of the CUC Independent Laboratory by the US Coast Guard.

The quality of the test organisms is checked using reference tests and control performance as described in the test procedures. Reference tests are performed in conjunction with the WET-tests and are registered in control charts.

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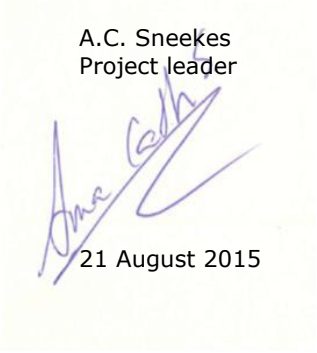
Justification

Report: C118/15
Project Number: 430.51125.01

The scientific quality of this report has been peer reviewed by the a colleague scientist and the head of the department of IMARES.

Approved: A.C. Sneekes
Project leader

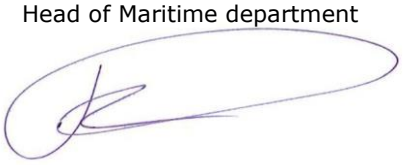
Signature:



Date: 21 August 2015

Approved: Drs. F.C. Groenendijk
Head of Maritime department

Signature:



Date: 21 August 2015