Shipboard Trials of Hyde 'Guardian' system in Caribbean Sea and Western Pacific Ocean, April 5th - October 7th, 2008.

Final Report to Hyde Marine and Lamor Corp.

by

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Shipboard Trials of Hyde 'Guardian' system in Caribbean Sea and Western Pacific Ocean, April 5th - October 6th, 2008.

INTRODUCTION AND PROJECT DESCRIPTION

Three trials were conducted aboard the Princess Cruise Lines ship M/V *Coral Princess* in 2008 to test the efficacy of the Hyde Marine Inc. 'Guardian' Ballast Water Treatment system under normal working conditions. The system consists of a primary disc filter manufactured by Arkal Inc., Tel Aviv, Israel mounted in series with a medium pressure UV irradiation system rated by the manufacturer/vendor at 200 mJ cm^2 for treatment of ballast water at flow rates up to 250 m^3 h^{-1}. Trials took place during the vessel's regular spring schedule in the Caribbean Sea, the summer schedule in the N.W. Pacific Ocean between Whittier, Alaska and Vancouver, Canada, and during the repositioning cruise from the western Pacific to the vessel's winter base in Fort Lauderdale, Florida. Trials consisted of determination of water quality parameters and a comparison of biological endpoints in treated and untreated ballast water samples, with reference to both IMO G8 and the U.S. Coast Guard Shipboard Technology Evaluation Program (STEP). Sampling procedures and endpoint determinations followed IMO G8 guidelines for shipboard trials and the exercise was designed to supplement land-based trials being conducted concomitantly at NIOZ, Texel, Netherlands to determine the efficacy of the BWT system under varying water quality conditions. Shipboard trials were designed to document system performance under normal seagoing conditions and under different geographical and seasonal conditions, with the objective of determining the degree of compliance with IMO and STEP requirements. Whole Effluent Toxicity (WET) tests were also conducted as part of the third and last trial to determine whether any significant chemical changes in ballast water after exposure to UV irradiation, which resulted in subsequent residual toxicity

Background.

It is now widely accepted that ships' ballast water is the primary vector responsible for the introduction of non-indigenous aquatic species into coastal habitats (Carlton and Geller 1993, Cohen and Carlton 1998, Ruiz et al. 1997, 2000a, b). Pimental (2003) has estimated the total annual economic cost from invasive species to the U.S. is $123 billion, with $9 billion attributable to unwanted introductions of aquatic nuisance species (ANS) (Pimental et al. 2005). World-wide economic

costs associated with ANS are incomplete, but have been estimated at least in the tens of billions of dollars annually Raaymakers (2002).

The 2004 IMO *Convention for the Management of Ballast water and Sediment in Ships* is pending ratification by port states representing 30% of global shipping. Criteria for 'successful' management or treatment have been published as Regulation D-1, relating to Open Ocean Ballast Water Exchange and Regulation D-2, described as the Ballast Water Performance Standard pertinent to the efficacy of ballast water treatment**.** The criterion for successful exchange was defined as a better than 95% volumetric replacement of water either through an empty-refill procedure or a pass-through procedure involving 3x the volume of the tank (or less if the 95% exchange is satisfactorily met). 'Successful treatment' was defined as the discharge of less than 10 viable organisms/m^3 greater than or equal to 50 µm in minimum dimension and less than 10 viable organisms/ml less than 50 µm in minimum dimension and greater than or equal to 10 µm in minimum dimension. Specific bacteria are included as 'indicator microbes', namely serotypes O1 and O139 of *Vibrio cholerae* (standard: <1 cfu/100 ml or <1 cfu/gm wet weight zooplankton); *Escherichia coli* (standard: <250 cfu/100 ml); intestinal Enterococci (<100 cfu/100 ml).

The April 2008 U.S. Coast Guard authorization Act (HR 2830, Section 503, sub-section 1101; Ballast Water Management) contains standards that are up to 100x stricter than the IMO standards, and even more stringent standards were adopted in January 2008 by the state of California, essentially representing the complete elimination of plankton in the >50 µm (minimum dimension) size class and a live density of 0.01organisms/ml. in the >10 - <50 µm (minimum dimension) size class. California ballast water legislation also includes standards for total live bacteria post treatment, (less than 1,000 bacteria per 100 ml.) and viruses (less than 10,000 viruses per 100 ml.) as well as more rigorous standards relating to indicator bacteria, i.e. concentrations of microbes that are less than 126 colony forming units/100 ml. of *Escherichia coli*; 33 colony forming units per 100 ml. of intestinal enterococci and 1 colony forming unit per 100 ml. or 1 colony forming unit per gram of wet weight of zoological samples of toxicogenic *Vibrio cholerae* (serotypes 01 and 0139).

In Washington State the interim ballast water discharge treatment standard is 95% zooplankton and 99% phytoplankton/bacteria elimination, with the stipulation that "Vessels that have not adequately exchanged their ballast water must treat their ballast to meet or exceed the

Washington State interim ballast water discharge standard prior to discharging in Washington waters". Washington State legislation further states that only approved technologies may be used *on specified vessels* to discharge treated ballast in Washington waters. For approval, technologies must meet one of the following criteria:

- ✓ Previously approved by Washington Department of Fisheries and Wildlife for use in WA waters
- ✓ Approved by U.S. Coast Guard for use in national waters
- ✓ Enrolled in U.S. Coast Guard Shipboard Technology Evaluation Program (STEP).
- ✓ Approved by the State of California for use in California waters
- ✓ Approved by the International Maritime Organization (IMO) and authorized by U. S. State Department and U.S. Coast Guard for use in national waters.
- ✓ Vessel is enrolled in IMO approval process and is authorized by the U.S. State Department and U.S. Coast Guard for use in national waters.

Table 1 provides a summary of current legislation, both national and international, relating to ballast water treatment standards.

Table 1. 2008 Ballast Water Treatment Standards.

	IMO Regulation D-2 and Transport Canada	**2008 Ballast Water Management Act Section 1101 (f)i**	**2008 California Standard**	**Washington Administrative Code 222-170**
Management approach	Exchange moving towards treatment only	Exchange moving towards treatment only	Exchange moving towards treatment only	Exchange or treatment
Standard:	**Proposed**	**Proposed**	**Recommended Interim**	**Adopted Interim:**
1) Organisms greater than 50 microns in minimum dimension:	<10 viable organisms per cubic meter	< 0.1 living organisms per cubic meter	No detectable living organisms	Technology to inactivate or remove 95% zooplankton
2) Organisms 10-50 microns in minimum dimension:	<10 viable organisms per ml	< 0.1 living organisms per ml	$<10^{-2}$ living organisms per ml	
3) Organisms less than 10 microns in minimum	No standards	No standard	$< 10^{3}$ cfu bacteria/100 ml	99% bacteria & phytoplankton

dimension:			
4) *Escherichia coli*	< 250 cfu/100 ml	<126 cfu/100 ml	<126 cfu/100 ml
5) Intestinal Enterococci	<100 cfu/100 ml	< 33 cfu/100 ml	<33 cfu/100 ml
6) Toxicogenic *Vibrio cholerae* (O1& O139)	<1 cfu/100 ml <1 cfu/gram of wet zooplankton samples	<1 cfu/100 ml <1 cfu/gram of wet weight of zoological samples;	<1 cfu/100 ml < 1 cfu/gram of wet zoological samples <10^4 viruses/100 ml **Final standards** – no discharge of living organisms

MATERIALS AND METHODS.

<u>Disposition of treated and untreated ballast water.</u>

A matched pair of ballast tanks (5P, 5S) was identified for each of these trials. One tank (5S) was used for treated ballast water and the other for untreated water. Tank 5S was initially filled with treated water, followed by the filling of tank 5P with untreated water, as per normal ballasting procedure. The ‘treated first’ protocol was designed to eliminate any possible false ‘positives’ through carry-over of untreated organisms in the ballasting system downstream from the BWT unit. For untreated samples, water followed the same path as the treated samples, except that the filter was by-passed and the UV unit was deactivated during the ballasting of the untreated tank.

<u>Sample collection.</u>

The sampling regime adopted for the Hyde Guardian BWT system aboard the M/V *Coral Princess* essentially followed 3 ‘in tank’ replicate x 3 time period (during the de-ballasting operation) x 2 treatment (i.e. treated/untreated) x 3 trial matrix design. It should be noted that, for the Guardian system, a ‘treatment’ consists of [filtration + UV irradiation] during the ballasting cycle PLUS [UV irradiation] during the de-ballasting cycle. Therefore, a treatment is not deemed complete until after the water passes through the UV system during de-ballasting. Therefore the full sampling matrix (below) only applies to treated and untreated water during the de-ballasting

cycle, collected downstream from the BWT system following a residence time (4 days in Trial 1, 5 days in Trial 2 and 10 days in Trial 3) in the ballast tanks. A sampling port, downstream from the Guardian system, established in the machinery space of the vessel for this purpose, and was used to process replicate samples of water for biological examination.

Examination of 'challenge water' immediately following ballasting/treatment:

In order to obtain information on the effect of tank residence time on biota, a series of samples (5 in Trial 1, 9 in Trials 2 and 3) were obtained from treated and untreated (control) tanks immediately following the initial ballasting/treatment cycle (T=0). The condition/numbers of biota in these samples was compared with treated and untreated samples collected later following a period of residence in the ballast tanks.

Examination of treated and untreated water following residence time in the tanks.

Following the residence time in the tank, samples of treated and untreated water were collected according to the following sequence:

1. Treated tank; replicate T1a filtered/sampled at the **start** of the de-ballasting cycle.
2. Treated tank; replicate T1b filtered/sampled at the **start** of the de-ballasting cycle.
3. Treated tank; replicate T1c filtered/sampled at the **start** of the de-ballasting cycle.
4. Treated tank; replicate T2a filtered/sampled in the **middle** of the de-ballasting cycle.
5. Treated tank; replicate T2b filtered/sampled in the **middle** of the de-ballasting cycle.
6. Treated tank; replicate T2c filtered/sampled in the **middle** of the de-ballasting cycle.
7. Treated tank; replicate T3a filtered/sampled at the **end** of the de-ballasting cycle.
8. Treated tank; replicate T3b filtered/sampled at the **end** of the de-ballasting cycle.
9. Treated tank; replicate T3c filtered/sampled at the **end** of the de-ballasting cycle.

10. Untreated (Control) tank; replicate C1a filtered/sampled at the **start** of the de-ballasting cycle.
11. Untreated (Control) tank; replicate C1b filtered/sampled at the **start** of the de-ballasting cycle.
12. Untreated (Control) tank; replicate C1c filtered/sampled at the **start** of the de-ballasting cycle.
13. Untreated (Control) tank; replicate C2a filtered/sampled in the **middle** of the de-ballasting cycle.
14. Untreated (Control) tank; replicate C2b filtered/sampled in the **middle** of the de-ballasting cycle.
15. Untreated (Control) tank; replicate C2c filtered/sampled in the **middle** of the de-ballasting cycle.
16. Untreated (Control) tank; replicate C3a filtered/sampled at the **end** of the de-ballasting cycle.
17. Untreated (Control) tank; replicate C3b filtered/sampled at the **end** of the de-ballasting cycle.
18. Untreated (Control) tank; replicate C3c filtered/sampled at the **end** of the de-ballasting cycle.

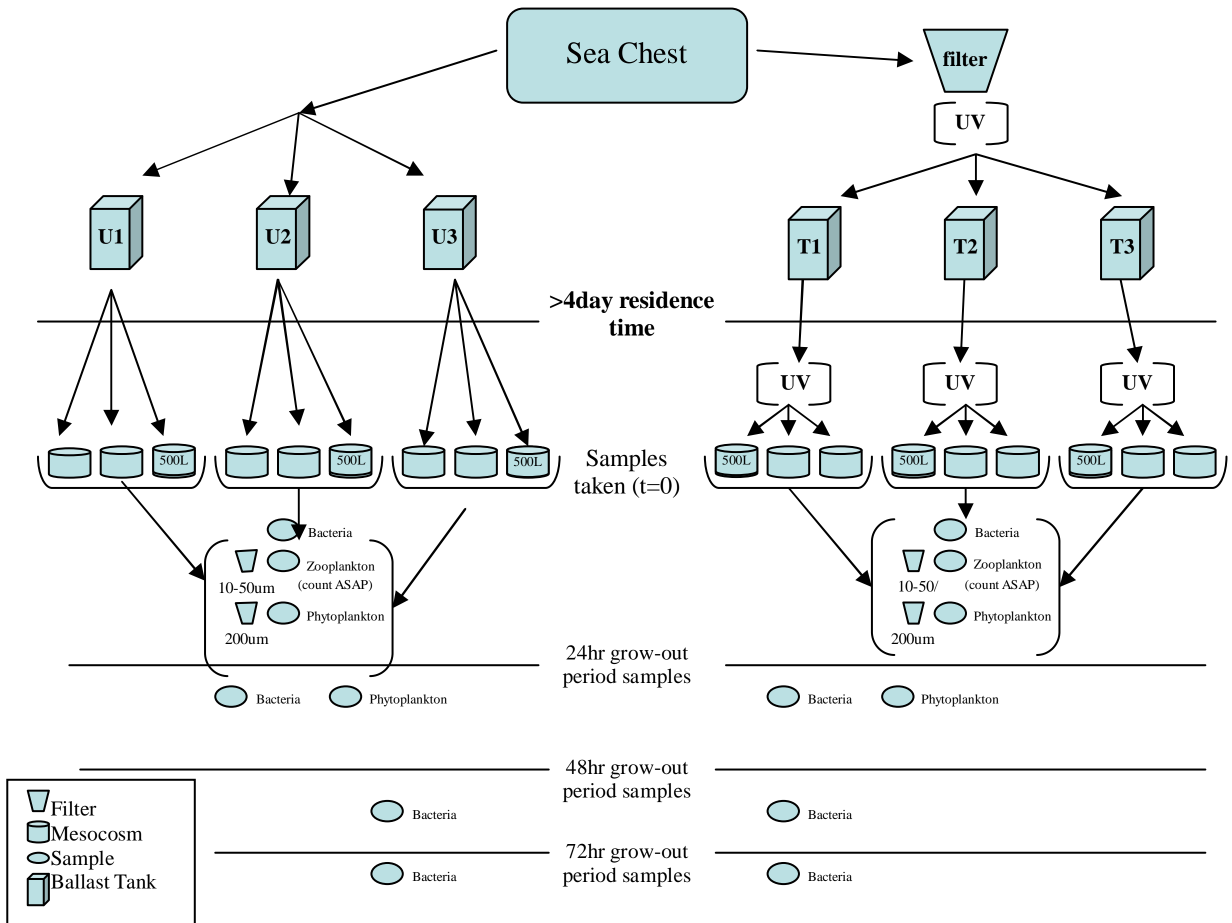
All samples were prepared for biological examination within 1 h of collection. This involved some pre-filtration of samples within the ship's machinery space, using 30 cm, 20 µm nets for zooplankton. Samples for phytoplankton and bacterial counts were unfiltered, although phytoplankton samples were further concentrated by filtration through 10 µm filters before grow-out and examination. These procedures are described below and a summary of the sampling scheme is shown in Figure 1.

Sample Preparation and Counting.

Zooplankton.

Samples for zooplankton counting were filtered through 30cm. nylon plankton nets consisting of 20µm mesh (nominal 50µm mesh nets can allow the passage of some >50µm organisms). During filtration, nets were submerged under the surface of a 125L plastic tub to soften the impact of the filtration procedure on the planktonic organisms. The 1L plastic bottle that forms the `cod-end' of the net also had 20µm mesh `windows' to facilitate the filtration process. Separate nets were used to sample from treated and untreated samples, and nets were rinsed with hot tap-water between sampling cycles. They were also examined for tears, leaks and imperfections and any repairs made. In the machinery space the contents of each mesocosm were filtered and concentrated to a volume suitable for manual transport up to the cabin space where samples were turned over to microscopists for examination. On receiving samples from the collection team, microscopists further concentrated samples through 10µm mesh filters to produce volumes suitable for microscopy, usually 10-20ml. Concentrated samples were transferred via Stempel pipets to counting wheels mounted on compound microscope stages for microscopical examination. For each sample, the appropriate multiplier was applied to each count for zooplankton densities to be expressed in terms of numbers of organisms per metric ton (m3). Records were kept of both alive and dead organisms in each sample, which were identified to the extent possible to the lowest taxonomic group (see Appendix A for taxonomic breakdown of zooplankton from each cruise), and records kept of the dimensions of each group of organisms, including maximum and minimum dimensions (µm). Dimensions of organisms were measures using reticule eye-pieces calibrated against National Institute of Standards and Technology certifies beads from 10 - 200µm in diameter. Sizing was also facilitated by seeding certified beads of known diameter into counting wheels. Live-dead status was assessed as movement of the organism, either as motility, heartbeat or movement of flagellum, velum or gut, following stimulation if necessary. This examination was supplemented by the use of the vital stain Neutral Red.

Figure 1. Schematic of Shipboard Trial sampling scheme design for Coral Princess Trials, 2008



Phytoplankton.

Live-dead status of phytoplankton for the determination of ballast water treatment efficacy remains problematic to the extent that, while some taxonomic groups, such as dinoflagellates, are clearly motile, many have vegetative stages that are immobile. The growth potential of non-motile forms can only be assessed by a variety of methods, including microscopic examination of chloroplast integrity, use of vital stain(s) and cell counts (of at least dominant groups) following a grow-out period under optimal growth conditions. The grow-out period for phytoplankton usually consists of a 24h (or 48h) period of irradiation under fluorescent lighting and non-limiting nutrient conditions through the addition of f/2 growth medium. Phytoplankton growth may be conveniently assessed by measuring *in vivo* chlorophyll a concentration before and after the grow-out period. While this represents a useful integrative determination of the status of the phytoplankton community as a whole, it does not provide information on individual taxonomic groups of phytoplankton that might have quite different characteristics in terms of size, shape, doubling time (growth rate) etc. Also, chlorophyll a data cannot be interpreted in terms of published standards.

Unfiltered 1L samples for phytoplankton analyses were taken from the same sample stream as that used for zooplankton sampling. Each sample was concentrated to a volume <10ml. for examination/grow-out, using a 10µm Nitex screen. Microscopic examination of cell/chloroplast integrity was supplemented by the use of Neutral Red as a vital stain. Unstained samples examined soon after collection were compared with 'splits' of the same sample that were stained with Neutral Red. Samples following a grow-out period were similarly examined, with and without Neutral Red. For these trials study *in vivo* chlorophyll a analyses was supplemented by individual cell counts of dominant phytoplankton taxa before and after a 24h grow-out period under fluorescent lights in ambient seawater supplemented with f/2 growth medium. Following initial examination of phytoplankton (before and after grow-out) to determine their general appearance, observations of chloroplast integrity and the activity of motile forms, samples were preserved in Lugol's Solution for more intensive taxonomy and determination of cell sizes. Typically counts were made of >200 squares of a 1000 square counting grid,. Determination of living phytoplankton was be made on the basis of (a) chlorophyll a analysis, (b) vital staining techniques and (c) cell counts before and after grow-out.

Bacteria.

Samples for bacterial analyses were be taken directly from the unfiltered discharge from the sampling port, and stored at temperatures just above freezing (1-4°C) prior to and during transport to the University of Maryland Center for Environmental Science, Chesapeake Biological Laboratory, although in Trial 3 some culturing/counting was carried out in cabin space aboard the vessel. Bacterial culture formed the basis of bacterial endpoints that were used to test the bactericidal effectiveness of the BWT system aboard this vessel. The following bactericidal endpoints were employed:

- Plate counts of cultural heterotrophic bacteria. It is emphasized that only approximately 1% of marine bacteria will culture successfully, and that this figure may vary according to geographical area. This, therefore, represents only an approximate measure of the efficacy of BWT technology, and is somewhat variable according to geographical location.
- Primary focus was on taxonomic groups specified in recently published IMO standards: colony-forming units of *E.Coli, Enterococcus* and *Vibrio Cholera* (with specific emphasis on virulent serotypes). Fluorescence-based techniques (IDEXX Laboatories.) were employed to quantify coliforms, *E. Coli* and *Enterococcus* in treated and untreated ballast water.

Samples were diluted an order of magnitude with sterile deionized water for the IDEXX protocols. The established detection range for this technique is 10 – 24,190 cfu / 100 mL of sea water sample.

Fluorescence and photometric counting of Coliform/E.coli was determined using the IDEXX Laboratories Inc. (ME) Colisure Quantitray counts following 18h incubation at 35°C.

Fluorescence counting of Enterococcus was determined by IDEXX Laboratories Inc. (ME) Enterolert Quantitray 2000 counts following 24h incubation at 41°C.

Further details of microbiological protocols are found in Appendix B.

Quantification of viable *Vibrio* cells was facilitated by the use of Polymerase Chain Reaction amplification techniques on refrigerated samples transported to the University of

Maryland Center for Environmental Science, Chesapeake Biological Laboratory, Molecular Biology Laboratory, directed by Dr. Carys Mitchelmore.

Water Quality Measurement.

The following water quality parameters were analyzed: temperature, salinity, pH, dissolved oxygen, total suspended solids, total particulate carbon, dissolved organic carbon phosphate, nitrate, nitrite (Trial 3) and UV transmittance.

Data Handling and Analysis.

Data were entered into a SAS (software) file and a header file created. This included a factor for the calculation of volume of filtered water, enabling raw counts to be appropriately multiplied to compute numbers of organisms per ton of water. This file is included in this report as Appendix A. Results from biological analyses from these trials were compared with published standards. Additionally, zooplankton mortalities in treated samples will expressed in terms of percentage removal relative to untreated controls. An assessment of the effect of ballast tank residence time was also made.

Whole Effluent Toxicity (WET) Testing.

In order to comply with IMO G-8 requirements for 'environmental acceptability of water treated by this technology, WET tests were conducted on standard test organisms exposed to treated and untreated water from Trial 3 to provide empirical information on possible residual toxicity on ballast discharge, resulting from chemical changes induced by UV irradiation during treatment. Details of these tests are provided on page 59.

TRIAL 1, April 5th-18th, 2008

Sampling Timetable

Ballasting took place close to the port of Aruba on April 8th, 2008, day 4 of a ten day cruise. Collection of samples 1-18 (see Materials and Methods) commenced immediately after the ballast pump began retrieving water from each of the respective tanks, following a residence time in the tanks of 96h. Ballast water was be taken on/treated after the vessel left Aruba on April 9th, 2008) and was discharged/sampled/analyzed four days later, after the ship departed Ocho Rios, Jamaica on April 13th. Samples for bacterial examination were kept on ice until their return to the Chesapeake Biological Laboratory on April 15th incubation and grow-out of these samples continued until April 18th, 2008.

Results and Discussion.

Zooplankton.

Results of zooplankton counts are summarized in table 2. These indicated a 98.6% mortality of zooplankton (>50µm minimum dimension) immediately following treatment on ballasting relative to untreated samples collected during the same sampling event, and a 100% mortality of zooplankton (>50µm minimum dimension) after a period of 4 days in the ballast tank followed by UV irradiation on de-ballasting. In contrast, untreated control samples demonstrated good survival following the 96h residence time in the tank. Overall there was no statistical decline in control numbers relative to those recorded from the intake water, although largevariations in organism numbers were apparent throughout the de-ballasting cycle, reflecting probable differences in plankton densities throughout the water column in the tanks.

Table 2. Summary of zooplankton results (>50µm minimum dimension) for Trial 1 of Hyde 'Guardian' BWT system aboard M/V *Coral Princess*, April 5th-18th, 2008.

Zooplankton	Alive (>50µm). Density/m³	Dead (>50µm) Density/m³
Control (untreated) N=5	**453±269**	**66±66**
Treated (UV + filter during ballasting) N=5	**6.4±6.7**	**1.6±3.6**
Control (untreated) Start de-ballast (N=3)	432±345	232±236
Control (untreated) Mid de-ballast (N=3)	296±343	536±385
Control (untreated) End de-ballast (N=3)	755±164	1,195±352
Mean Control No. at de-ballast	**494±284**	**654±324**
Treated (UV + filter during ballasting) → (UV at de-ballasting) Start de-ballast (N=3)	0	125±21
Treated (UV + filter during ballasting) → (UV at de-ballasting) Start de-ballast (N=3)	0	131±99
Treated (UV + filter during ballasting) → (UV at de-ballasting) Start de-ballast (N=3)	0	75±72
Mean treated No. at de-ballast		**110±64**

Phytoplankton.

Phytoplankton counts from treated and untreated (control) samples from T=0 and T=96h are shown in tables 3-5. Based on microscopic examination, cells were scored as 'live' based on morphological characteristics such as chloroplast integrity and the ability to concentrate the

Table 3. Summary of phytoplankton results (>10<50µm minimum dimension) for Trial 1 of Hyde 'Guardian' BWT system aboard M/V *Coral Princess*, April 5th-18th, 2008. T=0 data including grow-out cell concentrations.

Phytoplankton (>10µm-<50µm)	'Live' Dinoflagellates. Density/mL	'Live' Diatoms. Density/ mL	Total 'Live' phytoplankton. Density/ mL	Dead Dinoflagellates. Density/ mL	Dead Diatoms. Density/ mL	Total Dead phytoplankton. Density/ mL
Untreated Controls, T=0						
Rep. C1, T=0	0.01	0.17	0.18	0	0	0
Rep. C1, T=0 + 48h growout	0.016	0.032	0.048	0.0006	0.0006	0.001
Rep. C2 T=0	0.004	0.062	0.07	0	0	0
Rep. C2 T=0 + 48h growout	0.005	0.015	0.02	0	0.0008	0.0008
Rep. C3 T=0	0.024	0.13	0.15	0	0	0
Rep. C3 T=0 + 48h growout	0.003	0.063	0.066	0.0003	0.0006	0.0008
Mean 'Live' Phytoplankton. Control samples, T=0 (after grow-out)			**0.133±0.061 (0.45±0.023)**			
Treated, T=0						
Rep. T1, T=0	0.01	0.072	0.081	0	0	0
Rep. T1, T=0 + 48h growout	0.004	0.005	0.009	0.0008	0.0003	0.001
Rep. T2 T=0	0.008	0.076	0.084	0.002	0.0004	0.0025
Rep. T2 T=0 + 48h growout	0.003	0.006	0.009	0	0	0
Rep. T3 T=0	0.003	0.070	0.072	0.0004	0	0.0004
Rep. T3 T=0 + 48h growout	0.003	0.005	0.008	0	0	0
Mean 'Live' Phytoplankton. Treated samples, T=0 (after grow-out)			**0.080±0.006 (0.009±0.0006)**			

Table 4. Summary of phytoplankton results (>10<50µm minimum dimension) for Trial 1 of Hyde 'Guardian' BWT system aboard M/V *Coral Princess*, April 5th-18th, 2008. T=96h untreated (control) data.

Phytoplankton. (>10µm-<50µm)	**'Live' Dinoflagellates. Density/mL**	**'Live' Diatoms. Density/ mL**	**Total 'Live' phytoplankton. Density/ mL**	**Dead Dinoflagellates. Density/ mL**	**Dead Diatoms. Density/ mL**	**Total Dead phytoplankton. Density/ mL**
Untreated Controls, T=96h						
Start De-ballast. Rep. C1a, T=96	0.0003	0.005	0.006	0	0	0
Rep. C1b T=96	0.0003	0.005	0.005	0	0.0006	0.0006
Rep. C1c T=96	0	0.003	0.003	0	0	0
Mean C1, T = 96h samples			0.004±0.002			
Mid De-ballast. Rep. C2a, T=96	0.0003	0.019	0.019	0	0.003	0.003
Rep. C2b T=96	0.0008	0.014	0.015	0	0.0025	0.0025
Rep. C2c T=0	0.001	0.009	0.01	0	0.004	0.004
Mean C2, T = 96h samples			0.015±0.005			
End De-ballast. Rep. C3a, T=96	0.001	0.015	0.016	0.0006	0.006	0.006
Rep. C3b T=96	0.0008	0.016	0.017	0.0003	0.004	0.004
Rep. C3c T=96	0.0006	0.021	0.021	0	0.004	0.004
Mean C3, T = 96h samples			0.018±0.003			
Mean Live Phytoplankton			**0.012±0.007 (n=9)**			

Table 5 Summary of phytoplankton results (>10<50µm minimum dimension) for Trial 1 of Hyde 'Guardian' BWT system aboard M/V *Coral Princess*, April 5th-18th, 2008. T=96h data from treated samples, including 45-47h grow-out cell concentrations.

Phytoplankton. (>10µm-<50µm)	'Live' Dinoflagellates. Density/mL	'Live' Diatoms. Density/ mL	Total 'Live' phytoplankton. Density/ mL	Dead Dinoflagellates. Density/ mL	Dead Diatoms. Density/ mL	Total Dead phytoplankton . Density/ mL
Treated, T=96h						
Start De-ballast. Rep. T1a, T=96	0.0006	0.004	0.004	0.0003	0	0.0003
After 48h grow-out	0	0.002	0.002	0	0.0006	0.0006
Rep. T1b T=96	0.0003	0.007	0.007	0	0.0006	0.0006
After 48h grow-out	0	0.0008	0.0008	0	0.0003	0.0003
Rep. T1c T=96	0	0.006	0.006	0.0006	0	0.0006
After 48h grow-out	0	0.001	0.001	0	0	0
Mean T1, T=96h samples (after grow-out)			0.006±0.002 (0.001±0.0004)			
Mid De-ballast. Rep. T2a, T=96	0.0003	0.007	0.008	0.0003	0.0003	0.0006
After 48h grow-out	0	0.001	0.001	0	0	0
Rep. T2b T=96	0	0.006	0.006	0	0	0
After 48h grow-out	0	0.004	0.004	0	0.0003	0.0003
Rep. T2c T=96	0.0003	0.005	0.006	0.0003	0	0.0003
After 48h grow-out	0.0006	0.0006	0.001	0	0.003	0.003
Mean T2, T = 96h samples (after grow-out)			0.006±0.001 (0.002±0.002)			

End De-ballast. Rep. T3a, T=96	0.0006	0.004	0.004	0	0.003	0.003
After 48h grow-out	0	0.002	0.002	0	0.002	0.002
Rep. T3b T=96	0.0003	0.007	0.007	0	0.001	0.001
After 48h grow-out	0.0003	0.005	0.005	0	0.0006	0.0006
Rep. T3c T=96	0.0006	0.008	0.009	0	0	0
After 48h grow-out	0.0006	0.001	0.002	0	0.0003	0.0003
Mean T3, T = 96h samples (after grow-out)			0.007±0.002 (0.003±0.002)			
Mean Live Phytoplankton (after grow-out)			**0.006±0.001 (n=9) (0.002±0.001) (n=9)**			

vital stain Neutral Red. Based solely on these criteria, initial treatment (filter + UV during ballasting) resulted in an immediate 41% reduction in live cell numbers relative to untreated samples at T=0. However, following a 96h residence time in the tank, untreated 'live' cell numbers had fallen to 7% of the initial, untreated T=0 density, and treated 'live' cell numbers had fallen to 4.7% of that initial concentration (i.e. 95.3% removal). Under such circumstances a comparison between treated and control 'live' densities at 96h probably has little meaning as it is clear that the ballast tank provides an inhospitable environment for treated and untreated cells alike. If it is assumed that viability is best described by growth potential this assumption is further reinforced by cell counts following grow-out. Concentrations of treated cells following grow-out, shown in red in table 5, clearly indicate a failure to grow, based on the fact that they represent a mean *reduction* in cell numbers of 65% relative to the corresponding samples before grow-out. Based on growth potential, treated phytoplankton at 96h could reasonably be described as non-viable. (Grow-out data were not available for untreated (control) samples after 96h in the tank). This assessment is reinforced by measurement of chlorophyll *a* concentrations before and

after grow-out (table 6). While some positive growth was seen in untreated control samples at T=0, 2/3 control samples exhibited a small amount of growth after 96h, while no growth relative to intake water was recorded from treated samples at either T=0 or T=96h.

If 'live' cell counts based on morphological examination are used as the basis for regulatory compliance, the 'live' phytoplankton count of 6,327 recorded for treated samples at 96h (table 5) would comply with both the current IMO standard of 10^7 live cells per m^3 (10 live cells/mL) the U.S. Coast Guard Standard standard of 10^5 live cells per m^3 (0.1 live cells/mL), and the much more rigorous standard of 10^4 live cells per m^3 (0.01 live cells/mL) currently employed by the state of California. We conclude that cell numbers based on counts made after grow-out are even lower and probably best represent the criterion defining the term "live", i.e. having potential for growth.

Overall phytoplankton cell numbers were exceptionally low in challenge water samples, and we observe that, in this trial, even untreated samples would comply with IMO and U.S. Coast Guard standards at discharge, but not the more rigorous California standard.

Table 6. Chlorophyll *a* concentrations from measurement of in vivo fluorescence. (all values are means of 3 determinations)

T=0		**T=0, Pre grow-out chlorophyll *a* conc. (µg L^{-1})**	**chlorophyll *a* conc. (µg L^{-1}) after 24h grow-out (T=0 + 24)**	**chlorophyll *a* conc. (µg L^{-1}) after 48h grow-out (T=0 + 48)**
Control 1	start ballasting	2.75	4.33	4.3
Control 2	mid ballasting	1.81	1.95	1.98
Control 3	end ballasting	7.78	8.58	8.76
Treated 1	start ballasting	1.31	0.92	0.88
Treated 2	mid ballasting	1	0.98	0.82
Treated 3	end ballasting	1.13	1	0.94
T=96h		**T=96h, Pre grow-out Chlorophyll *a* conc. (µg L^{-1})**	**Chlorophyll *a* conc. (µg L^{-1}) after 24h grow-out (T=96 + 24)**	**Chlorophyll *a* conc. (µg L^{-1}) after 48h grow-out (T=96 + 48)**
Control 1	start de-ballasting	1.2	1.29	1.4

Control 2	mid de-ballasting	1.43	1.86	1.77
Control 3	end de-ballasting	1.26	1.45	1.22
Treated 1	start de-ballasting	1.34	1.26	1.22
Treated 2	mid de-ballasting	1.31	1.23	1.15
Treated 3	end de-ballasting	1.22	1.22	1.07

Bacteria.

Based on raw counts of intestinal *Enterococci,* and coliforms (table 7), no viable cfus of these indicator microbes were detected in treated ballast water samples from this trial and only one each cfu of coliforms and Enterococci were recorded from control samples. No *Vibrio cholerae* cells, live or dead, were found in any of the samples examined.

Low densities of cultural bacteria were detected in both control and treated samples (12.2 ± 12.9cfu per 100ml. and 19.9 ± 10.5 per 100ml. respectively). By 96h mean cfu per 100ml. had fallen to 5 and 1 respectively (table 8).

Table 7. Trial 1. *Enterococci, Vibrio cholerae* and colifom counts from treated and untreated (control) samples at T=0 and T=96h.

			Coliforms		***E. Coli***		**Enterrococci**		***Vibrio Cholerae***
Treatment	**Time**	**Replicate**	**Large**	**Small**	**Large**	**Small**	**Large**	**Small**	
Control	T=0	C1	**1**	0	0	0	0	0	0
Control	T=0	C2	0	0	0	0	0	0	0
Control	T=0	C3	0	0	0	0	0	0	0
Control	T=0	C4	0	0	0	0	0	0	0
Control	T=0	C5	0	0	0	0	0	**1**	0
Treated	T=0	T1	0	0	0	0	0	0	0
Treated	T=0	T2	0	0	0	0	0	0	0
Treated	T=0	T3	0	0	0	0	0	0	0
Treated	T=0	T4	0	0	0	0	0	0	0
Treated	T=0	T5	0	0	0	0	0	0	0
Control	T=96h	C1a	0	0	0	0	0	0	0
Control	T=96h	C1b	0	0	0	0	0	0	0
Control	T=96h	C1c	0	0	0	0	0	0	0
Control	T=96h	C2a	0	0	0	0	0	0	0
Control	T=96h	C2b	0	0	0	0	0	0	0

Control	T=96h	C2c	0	0	0	0	0	0	0
Control	T=96h	C3a	0	0	0	0	0	0	0
Control	T=96h	C3b	0	0	0	0	0	0	0
Control	T=96h	C3c	0	0	0	0	0	0	0
Treated	T=96h	T1a	0	0	0	0	0	0	0
Treated	T=96h	T1b	0	0	0	0	0	0	0
Treated	T=96h	T1c	0	0	0	0	0	0	0
Treated	T=96h	T2a	0	0	0	0	0	0	0
Treated	T=96h	T2b	0	0	0	0	0	0	0
Treated	T=96h	T2c	0	0	0	0	0	0	0
Treated	T=96h	T3a	0	0	0	0	0	0	0
Treated	T=96h	T3b	0	0	0	0	0	0	0
Treated	T=96h	T3c	0	0	0	0	0	0	0

Table 8. Trial 1. Cultural bacteria in treated and untreated (control) samples at T=0 and T=96h. (10ml. samples were filtered through 0.45µm. Plates were read after 72h incubation at 25°

			Colony forming units	Colony forming units	Colony forming units
			100%	**10%**	**1%**
Treatment	**Time**	**Replicate**			
Control	T=0	C1	8	2	0
Control	T=0	C2	34	2	2
Control	T=0	C3	4	0	0
Control	T=0	C4	2	0	0
Control	T=0	C5	13	1	0
Treated	T=0	T1	33	1	0
Treated	T=0	T2	27	3	0
Treated	T=0	T3	18	0	0
Treated	T=0	T4	11	1	0
Treated	T=0	T5	8	0	0
Control	T=96h	C1a	2	0	0
Control	T=96h	C1b	3	3	1
Control	T=96h	C1c	8	0	0
Control	T=96h	C2a	19	2	0
Control	T=96h	C2b	3	3	0
Control	T=96h	C2c	3	0	0
Control	T=96h	C3a	0	0	0
Control	T=96h	C3b	3	1	0
Control	T=96h	C3c	4	0	0
Treated	T=96h	T1a	2	0	0
Treated	T=96h	T1b	0	0	0
Treated	T=96h	T1c	0	0	0
Treated	T=96h	T2a	0	0	0
Treated	T=96h	T2b	5	0	0
Treated	T=96h	T2c	0	0	0
Treated	T=96h	T3a	0	0	0
Treated	T=96h	T3b	0	0	0
Treated	T=96h	T3c	2	0	0

Water Chemistry.

Ballast water during trial 1 was taken from near Aruba Harbor on April 8th, 2008. The salinity of challenge water (untreated water at T=0) varied between 36.4 – 37.1 PSU (mean 36.8), water temperatures ranged from 25.2 – 29.0^o C (mean 26.8) and pH from 7.3-7.4. Dissolved oxygen (DO) concentrations ranged from 5.4mg L^{-1} to 6.6mg L^{-1} (mean 6.0mg L^{-1}). DO and pH measured in ballast water immediately after treatment (T=0) showed no noticeable change from untreated water (table 9). UV transmittance was determined to be 95% in untreated water at T=0 Nutrient levels in challenge water extremely low, ranging between 0.6 – 1.2 µg L^{-1} for nitrate and 2.2 – 4.0 µg L^{-1} for phosphate. A higher nitrate level (9.72µg L^{-1}) was recorded from treated samples at T=0.

Table 9. Trial 1. Water Chemistry in untreated challenge water and treated ballast water at time of ballasting (T=0) near Aruba, April 8th, 2008. (DO = Dissolved O2; TSS = Total Suspended Solids; POC – Particulate Organic Carbon; DOC = Dissolved Organic Carbon; NO_3 = Nitrate; PO_4 = Phosphate)

	pH	DO (mg L^{-1})	Salinity (PSU)	Temp(^{O}C)	TSS (mg L^{-1})	POC (mg L^{-1})	DOC (mg L^{-1})	NO_3 (µgN L^{-1})	PO_4 (µgP L^{-1})
C1, T=0 (begin ballast)	7.3	6.47	36.8	26.8	4.12±0.09	3.24±0.38	1.37±0.11	1.1±0.4	2.8±0.4
C2, T=0 (mid ballast)					4.51±0.20	2.58±0.62	0.85±0.05	0.6±0.2	4.0±0.5
C3, T=0 (end ballast)					4.46±0.38	2.56±1.16	0.92±0.07	1.2±0.8	2.2±0.4
T1, T=0 (begin ballast)	7.4	5.41			4.50±0.23	3.00±0.90	1.75±0.36	9.7±0.37	4.4±0.7

GENERAL CONCLUSIONS (Trial 1).

Plankton densities in challenge water reflect oligotrophic conditions typified by low zooplankton and phytoplankton densities. However, zooplankton densities in untreated challenge water did exceed the value of 10 x D-2 required by IMO G8 guidelines. Based on results obtained from the first trial, the Hyde 'Guardian' system would comply with those portions of IMO Regulation D-2 and current California regulations relating to plankton. Viable bacteria, measured as colony forming units (cfu) of named indicator bacteria were within the prescribed limits published in current IMO Regulation D-2, U.S.C.G. and California regulations. Only 1 cfu

each of coliforms intestinal Enterococci were detected in untreated samples and none were seen in any treated samples. Culturable heterotrophic bacterial numbers were low in both treated samples, and actually decreased in both treated and untreated samples between T=0 and de-ballasting at T=96h. Cfus in treated samples declined more rapidly than in untreated samples over this time. At the time of de-ballasting treated and untreated samples contained 10 cfu/100 ml. and 50 cfu/100 ml. respectively. No determination was made of performance of the BWT system against viruses, as per California regulations.

TRIAL 2. June 30th – July 13th, 2008

Sampling Timetable.

In Trial 2 the same matched pair of ballast tanks (5P, 5S) was employed as in Trial 1, i.e. tank (5S) was used for treated ballast water and the other (5P) for untreated water. Ballasting took place southeast of the port of Whittier, Alaska on July 1st, 2008, day 2 of an eight day cruise. As with Trial 1, tanks 5S and 5P were filled with treated and untreated water respectively, as per normal ballasting procedure, and for untreated samples, water followed the same path as the treated samples, except that the filter was by-passed and the UV unit was deactivated during the ballasting of the untreated tank.

Sample collection.

The same sampling regime was adopted as for Trial 1, i.e. 3 'in tank' replicates x 3 time period (during the de-ballasting operation) x 2 treatment (i.e. treated/untreated), except that nine untreated samples and seven treated samples were obtained from the respective tanks immediately following the initial ballasting/treatment cycle. In the second trial a residence time of 114h elapsed before samples were retrieved from the tanks according to the sequence previously described. Ballast water was taken up/treated the day after the vessel left Whittier on July 1st, 2008, and was discharged/sampled/analyzed 4.5days later, after the ship departed Ketchekan on July 6th. Samples for bacteriological analysis were kept on ice following collection and accompanied the scientific team to Dr. Mitchelmore's laboratory at the University of Maryland Center for Environmental Science, Chesapeake Biological Laboratory, Solomons, Maryland. Culture and analysis of these samples continued until July 13th 2008.

Preparation of samples for biological examination followed procedures described for Trial 1 (see Figure 1.) Methods for sample examination and analysis were as previously described.

Results and Discussion

Zooplankton.

Results of zooplankton counts are summarized in table 10. Results indicated a 99.99% mortality/removal of zooplankton (>50µm minimum dimension) immediately following treatment on ballasting relative to untreated samples collected during the same sampling event. Samples were characterized by a dramatic difference in biomass between the treated and untreated samples. In treated samples a mean of 1.14 live organisms >50µm in minimum dimension per ton were found at T=0. No dead organisms were found in this size range in treated samples, indicating that the large majority were removed by the filter. In control samples at T=0, 15,373±6118 live organisms >50µm and 141±117 dead organisms >50µm per ton were found. It is of interest to note that the live density of organisms >50µm in the challenge water showed a 35-fold increase relative to the much more oligotrophic conditions encountered in Caribbean waters during the first, April 2008, trial.

Unlike trial 1, there was a dramatic (98.4%) decline in overall numbers of live organisms in the untreated (control) tank after the 114h residence period. Both treated and untreated samples retrieved from the tanks after 114h were quite different in character from the samples examined at T=0. In treated samples, after 114h in the tank, and following UV irradiation on de-ballasting, numerous zooplankters in the >50µm minimum dimension size range were found in treated samples, although these all appeared to be dead, as judged by motility and many had begun to disintegrate. Many of these organisms were not seen in the T=0 untreated samples, and we conclude that many were living in the tanks before the start of the trial.

Table 10. Summary of zooplankton results (>50µm minimum dimension) for Trial 2 of Hyde 'Guardian' BWT system aboard M/V *Coral Princess*, June 30th – July 8th, 2008.

Zooplankton	**Alive (>50µm). Density/m^3**	**Dead (>50µm) Density/m^3**
Control (untreated) N=9	**15,373±6118**	**141±117**
Treated (UV + filter during ballasting) N=7	**1.14±2.8**	**0**
Control (untreated) Start de-ballast (N=3)	504±356	0
Control (untreated) Mid de-ballast (N=3)	464±670	1,123±572
Control (untreated) End de-ballast (N=3)	43±74	971±654
Mean control (untreated) No. at de-ballast	**337±441**	**698±683**
Treated (UV + filter during ballasting) → (UV at de-ballasting) Start de-ballast (N=3)	0	75±20
Treated (UV + filter during ballasting) → (UV at de-ballasting) Mid de-ballast (N=3)	0	256±374
Treated (UV + filter during ballasting) → (UV at de-ballasting) End de-ballast (N=3)	0	533±9
Mean treated No. at de-ballasting	**0**	**288±274**

Counts indicated live residuals of smaller zooplankton, the great majority of which were marine nematodes, in both treated and untreated samples taken at 114h, although these did not fall into the >50µm size category. Live/dead nematode numbers are shown in table 11. Nematode numbers were almost completely absent from the T=0 samples, and were seen to increase in T=114h samples from the beginning to the end of the de-ballasting cycle, reflecting a probable difference in plankton densities throughout the water column in the tanks. The most likely explanation for this is that, as the tank approaches empty, increasing numbers of organisms living in or on the surface of the residual sediment are stirred up and appear in the later samples.

In trial 1 it was noted that overall zooplankton densities at the end of the de-ballasting cycle were nearly double those at the beginning of the de-ballasting cycle, perhaps reflecting a similar phenomenon, although it was noteworthy that no nematodes were found in trial 1 samples.

Table 11. Densities of living nematode worms in treated and untreated samples.
Trial 2. June 30th-July 11th 2008.

	Nematode densities (concentrations per m^3)	
	Live	**Dead**
T=0h		
Control	0	**0**
Treated	0	**0**
T=114h		
Control (untreated). Start de-ballast (N=3)	0	**0**
Control (untreated) Mid de-ballast (N=3)	0	**0**
Control (untreated) End de-ballast (N=3)	0	**11±15**
Treated (UV + filter during ballasting) → (UV at de-ballasting). Start de-ballast (N=3)	0	**0**
Treated (UV + filter during ballasting) → (UV at de-ballasting) Mid de-ballast (N=3)	27±27	**69±98**
Treated (UV + filter during ballasting) → (UV at de-ballasting) End de-ballast (N=3)	**80±39**	**160±48**

Phytoplankton.

Phytoplankton counts from treated and untreated (control) samples from T=0 and T=114h are shown in tables 12-15. Based on microscopic examination, cells were scored as 'live' based on morphological characteristics such as chloroplast integrity and the ability to concentrate the vital stain Neutral Red. Unlike trial 1, initial treatment (filter + UV during ballasting) resulted in a dramatic (97%) reduction in live cell numbers based solely on morphological characteristics. Following a 114h residence time in the tank, untreated 'live' cell numbers had fallen to 30% of

the initial, untreated T=0 density (compared with 7% in trial 1). This, despite the presence of exceptionally high numbers of apparently viable diatoms in the first sample of this series (table 14), perhaps reflecting the release of a 'pulse' of 'live' cells at the beginning of that sampling sequence. After 114h treated 'live' cell numbers had fallen to 1.9% of the initial (T=0) concentration (i.e. 98.1% removal). As with trial 1, it is clear that the ballast tank provides a poor environment for treated and untreated cells alike. Concentrations of treated cells following grow-out, shown in red in tables 12 and 13, indicate some growth capacity in 3/9 T=0 controls and 5/9 T=0 treated samples, although taken overall cell numbers after grow-out show reductions in control cell densities in control and treated samples of 70% and 58% respectively. This is reinforced by measurement of chlorophyll a concentrations before and after grow-out (table 16). While some positive growth was seen in untreated control samples at T=0, treated samples at T=0, no growth relative to intake water was recorded from treated samples at either T=0 or T=114h.

If 'live' cell counts based on morphological examination are used as the basis for regulatory compliance, the 'live' phytoplankton count of 189,500 per m^3 (0.189 live cells/mL) recorded for treated samples at 114h (table 15) would comply with current IMO standard of 10^7 live cells per m^3, (10 live cells/mL) but not the 2008 U.S. Coast Guard regulations, 10^5 live cells per m^3 (0.1 live cells/mL) or the much more rigorous standard of 10^4 live cells per m^3 (0.01 live cells/mL)currently employed by the state of California. However, if cell counts after grow-out are taken into account, this number falls to 75,667 per m^3 (0.076 live cells/mL; table 15 - total phytoplankton cells after grow-out), which does comply with U.S. Coast Guard requirements of 0.1 live cells/mL). Again, California standards would not be met at this level, based on morphological characteristics alone. However, cell counts after grow-out indicated a 60% decrease in treated cell numbers compared with concentrations before grow-out, indicating that the treated phytoplankton population could not sustain growth. In contrast, untreated samples showed a small increase in numbers following grow-out (table 14), which correlated closely with chlorophyll *a* determinations made on the same samples (table 16).

Table 12. Summary of phytoplankton results (>10<50µm minimum dimension) for Trial 2 of Hyde 'Guardian' BWT system aboard M/V *Coral Princess*, June 30th-July 8th, 2008. Untreated (control) T=0 data, including live grow-out cell concentrations (in red). Due to time constraints, dead cell numbers were not recorded from C2 orC3 samples following grow-out.

Phytoplankton. (>10µm-<50µm)	'Live' Dinoflagellates. Density/mL	'Live' Diatoms. Density/ mL	Total 'Live' phytoplankton. Density/ mL	Dead Dinoflagellates. Density/ mL	Dead Diatoms. Density/ mL	Total Dead phytoplankton. Density/ mL
Rep. C1a, T=0	0.26	1.2	12.3	0	0	0
After42h growout	0.77	6.5	7.3	0	0.30	0.30
Rep. C1b, T=0	0.45	19.9	20.3	0	0	0
After42h growout	0.48	11.8	12.3	0.03	0.03	0.06
Rep. C1c, T=0	0.19	17.4	17.6	0	0	0
After42h growout	0.36	12.7	13.1	0.1	0.13	0.23
Mean C1 samples (after grow-out)			16.8±4.1 (10.9±3.2)			
Rep. C2a T=0	0.39	19.8	20.2	0	0	0
After42h growout	0.45	29.2	30.0	0	0.03	0.03
Rep. C2b, T=0	0.64	3.5	3.6	0	0.06	0.06
After42h growout	0.29	6.8	7.1	0	0.13	0.13
Rep. C2c, T=0	0.74	4.8	5.6	0	0	0
After42h growout	0.13	3.7	3.8	0.1	0.13	0.23
Mean C2 samples (after grow-out)			9.8±9.0 13.5±14.1			
Rep. C3a, T=0	0.97	2.5	2.6	0	0	0
After42h growout	0.22	7.4	7.6	0	0.40	0.40
Rep. C3b, T=0	0.12	2.3	2.4	0	0	0
After42h growout	0.21	0.88	1.1	0	0.06	0.06
Rep. C3c, T=0	0.14	2.7	2.8	0	0.02	0.02
After42h growout	0.17	2.4	2.6	0.07	0.1	0.16
Mean C3 samples (after grow-out)			2.6±0.22 3.8±3.4			

Mean 'Live' Phytoplankton **(after grow-out)**			**9.7±7.9** **2.9±6.6**			

Table 13. Summary of phytoplankton results (>10<50µm minimum dimension) for Trial 2 of Hyde 'Guardian' BWT system aboard M/V *Coral Princess*, June 30th-July 8th, 2008. Treated (control) T=0 samples, including live grow-out cell concentrations (in red).

Phytoplankton (>10µm-<50µm)	**'Live' Dinoflagellates. Density/mL**	**'Live' Diatoms. Density/ mL**	**Total 'Live' phytoplankton. Density/ mL**	**Dead Dinoflagellates. Density/ mL**	**Dead Diatoms. Density/ mL**	**Total Dead phytoplankton . Density/ mL**
Rep. T1a, T=0	0.13	0.06	0.19	0	0	0
After42h growout	0.03	0.10	0.13	0.06	64,500.060	0.13
Rep. T1b, T=0	0.02	0.05	0.07	0	0	0
After42h growout	0.10	0.03	0.13	0.03	0	0.03
Rep. T1c, T=0	0.15	0	0.15	0	0	0
After42h growout	0.06	0.46	0.51	0	0.06	0.06
Mean treated No. at T=0 **(after grow-out)**			0.14 ± 0.06 0.26±0.22			
Rep. T2a, T=0	0.13	0.22	0.35	0	0	0
After42h growout	0.29	0.23	0.52	0.14	0	0.14
Rep. T2b, T=0	0.08	0.22	0.30	0.03	0.08	0.11
After42h growout	0.32	0.45	0.77	0.03	0.10	0.13
Rep. T2c, T=0	0.13	0.26	0.39	0	0	0
After42h growout	0.09	0.13	0.21	0	0	0
Mean treated No. at T=0 **(after grow-out)**			0.35±0.04 0.50±0.28			
Rep. T3a, T=0	0.19	0.06	0.26	0	0	0
After42h growout	0.16	0.21	0.36	0.13	0.03	0.16
Rep. T3b, T=0	0.32	0.32	0.64	0	0.05	0.05
After42h growout	0.19	0.14	0.33	0.14	0.09	0.23
Rep. T3c, T=0	0.07	0.61	0.61	0.02	0.10	0.12
After42h growout	0.10	0.03	0.13	0.03	0.03	0.06

Mean treated No. at T=0			0.53±0.23			
(after grow-out)			0.27±0.13			
Mean 'Live' Phytoplankton			**0.29±0.23**			
(after grow-out)			**0.13±0.14**			

Table 14. Summary of phytoplankton results (>10<50µm minimum dimension) for Trial 2 of Hyde 'Guardian' BWT system aboard M/V *Coral Princess*, June 30th-July 8th, 2008. T=114h untreated (control) samples. Due to time constraints, dead cell numbers were not recorded from C3 samples following grow-out.

Phytoplankton. (>10µm-<50µm). Untreated, T=114h	'Live' Dinoflagellates. Density/mL	'Live' Diatoms. Density/ mL	Total 'Live' phytoplankton. Density/ mL	Dead Dinoflagellates. Density/ mL	Dead Diatoms. Density/ mL	Total Dead phytoplankton. Density/ mL
Start De-ballast. Rep. C1a, T=114h	1.2	18.1	19.3	0.82	0.38	0.46
After 42h grow-out	1.0	17.8	18.8	0.10	0.30	0.40
Rep. C1b T=114h	0.025	0.65	0.68	0	0.025	0.025
After 42h grow-out	0	0.35	0.35	0.05	0.05	0.10
Rep. C1c T=114h	0.08	0.06	0.67	0	0.03	0.03
After 42h grow-out	0.04	0.60	0.64	0	0.04	0.04
Mean C1, T= 114h samples			**6.9±10.8**			
After grow-out			**6.6±8.6**			
Mid De-ballast. Rep. C2a, T=114h	0.05	0.90	0.90	0	0.08	0.08
After 42h grow-out	0.05	1.24	1.29	0	0.04	0.04
Rep. C2b T=114h	0	0.62	0.62	0	.0.02	0.02
After 42h grow-	0	0.79	0.79	0	0.04	0.04

out						
Rep. C2c T=114h	0	0.65	0.65	0	0.08	0.08
After 42h grow-out	0	1.05	1.05	0	0.05	0.05
Mean C2, T= 114h samples			**0.74±0.18**			
After grow-out			**1.04±0.21**			
End De-ballast. Rep. C3a, T=114h	0.13	0.80	0.93	0	0.13	0.13
After 42h grow-out	0.88	1.24	1.33			
Rep. C3b T=114h	0.17	1.10	1.30	0	0.02	0.23
After 42h grow-out	0.14	1.26	1.40			
Rep. C3c T=114h	0.09	0.72	0.81	0	0.12	0.12
After 42h grow-out	0.10	0.90	1.0			
Mean C3, T= 114h samples			**1.0±0.23**			
After grow-out			**1.2±0.17**			
Mean Live Phytoplankton			**2.9±6.2**			
After grow-out			**3.0±5.6**			

Table 15 Summary of phytoplankton results (>10<50µm minimum dimension) for Trial 2 of Hyde 'Guardian' BWT system aboard M/V *Coral Princess*, June 30th-July 8th, 2008. T=114h data from treated samples, including grow-out cell concentrations.

Phytoplankton. (>10µm-<50µm) Treated, T=114h	'Live' Dinoflagellates. Density/mL	'Live' Diatoms. Density/ mL	Total 'Live' phytoplankton. Density/ mL	Dead Dinoflagellates. Density/ mL	Dead Diatoms. Density/ mL	Total Dead phytoplankton. Density/ mL
Start De-ballast. Rep. T1a, T=114h	0.05	0.98	0.15	0	0.05	0.05
After42h growout	0.02	0.04	0.06	0	0.06	0.06
Rep. T1b, T=114h	0.03	0.21	0.23	0	0.05	0.05
After42h growout	0.02	0.03	0.05	0.02	0.07	0.09
Rep. T1c, T=114h	0.05	0.20	0.25	0	0	0
After42h growout	0	0.04	0.04	0	0.02	0.02
Mean T1, T= 114h samples			**0.21±0.06**			
After grow-out			**0.05±0.007**			
Mid De-ballast. Rep. T2a, T=114h	0.02	0.24	0.26	0	0.02	0.02
After42h growout	0	0.02	0.02	0	0.02	0.02
Rep. T2b, T=114h	0	0.16	0.16	0	0	0
After42h growout	0	0.09	0.09	0	0	0
Rep. T2c T=114h	0	0.09	0.09	0	0.05	0.05
After42h growout	0	0.06	0.06	0	0.06	0.06
Mean T1, T= 114h samples			**0.17±0.08**			
After grow-out			**0.06±0.03**			

End De-ballast. Rep. T3a, T=114h	0.03	0.07	1.0	0	0	0
After42h growout	0.03	0.04	0.07	0	0	0
Rep. T3b, T=114h	0	0.18	0.18	0	0	0
After42h growout	0	0.12	0.12	0	0	0
Rep. T3c, T=114h	0	0.28	0.28	0	0	0
After42h growout	0	0.16	0.16	0	0	0
Mean T3, T= 114h samples			**0.19±0.09**			
(After grow-out)			**(0.12±0.04)**			
Mean Live Phytoplankton (After grow-out)			**0.19±0.07** **(0.08±0.04)**			

Table 16. Trial 2. Chlorophyll *a* concentrations from measurement of in vivo fluorescence. (all values are means of 3 determinations)

T=0	T=0, Pre grow-out chlorophyll *a* conc. (µg L^{-1})	chlorophyll *a* conc. (µg L^{-1}) after 24h grow-out (T=0 + 24)	chlorophyll *a* conc. (µg L^{-1}) after 42h grow-out (T=0 + 42)
Control 1 start ballasting	1.32	1.36	1.38
Control 2 mid ballasting	2.2	2.54	2.9
Control 3 end ballasting	1.43	1.65	1.84
Treated 1 start ballasting	0.38	0.24	0.1
Treated 2 mid ballasting	0.47	0.32	0.14
Treated 3 end ballasting	0.42	0.27	0.09
T=114h	**Pre grow-out chlorophyll *a* conc. (µg L^{-1})**	**chlorophyll *a* conc. (µg L^{-1}) after 24h grow-out (T=114 + 24)**	**chlorophyll *a* conc. (µg L^{-1}) after 42h grow-out (T=114 + 42)**
Control 1 start de-ballasting	0.46	0.48	0.49
Control 2 mid de-ballasting	0.56	0.65	0.71
Control 3 end de-ballasting	0.57	0.63	0.72
Treated 1 start de-ballasting	0.021	ND	ND
Treated 2 mid de-ballasting	0.017	ND	ND
Treated 3 end de-ballasting	0.016	ND	ND

Bacteria.

No coliform bacteria, including *E. Coli*, were detected in any treated or untreated samples in this trial. Treated and untreated samples collected immediately following ballasting/treatment were all negative for intestinal enterococci, although both large and small cfus of Enterococci were detected in both untreated and treated samples retrieved from ballast tanks following a residence time of 114h (table 17). No *Vibrio cholerae* cells were detected in any of the samples examined.

No determination was made of performance of the BWT system against viruses, as per California regulations.

Table 17. Trial 2. *Enterococci,* colifoms and *Vibrio cholerae* from treated and untreated (control) samples at T=0 and T=114h.

			Coliforms		***E. Coli***		**Enterrococci**		***Vibrio cholerae***
Treatment	**Time**	**Replicate**	**Large**	**Small**	**Large**	**Small**	**Large**	**Small**	
Control	T=0	C1a	0	0	0	0	0	0	0
Control	T=0	C1b	0	0	0	0	0	0	0
Control	T=0	C1c	0	0	0	0	0	0	0
Control	T=0	C2a	0	0	0	0	0	0	0
Control	T=0	C2b	0	0	0	0	0	0	0
Control	T=0	C2c	0	0	0	0	0	0	0
Control	T=0	C3a	0	0	0	0	0	0	0
Control	T=0	C3b	0	0	0	0	0	0	0
Control	T=0	C3c	0	0	0	0	0	0	0
Treated	T=0	T1a	0	0	0	0	0	0	0
Treated	T=0	T1b	0	0	0	0	0	0	0
Treated	T=0	T1c	0	0	0	0	0	0	0
Treated	T=0	T2a	0	0	0	0	0	0	0
Treated	T=0	T2b	0	0	0	0	0	0	0
Treated	T=0	T2c	0	0	0	0	0	0	0
Treated	T=0	T3a	0	0	0	0	0	0	0
Treated	T=0	T3b	0	0	0	0	0	0	0
Treated	T=0	T3c	0	0	0	0	0	0	0
Control	T=114h	C1a	0	0	0	0	2	0	0
Control	T=114h	C1b	0	0	0	0	1	3	0
Control	T=114h	C1c	0	0	0	0	1	0	0
Control	T=114h	C2a	0	0	0	0	0	3	0
Control	T=114h	C2b	0	0	0	0	2	0	0
Control	T=114h	C2c	0	0	0	0	0	2	0
Control	T=114h	C3a	0	0	0	0	0	0	0
Control	T=114h	C3b	0	0	0	0	0	0	0
Control	T=114h	C3c	0	0	0	0	0	0	0

Treated	T=114h	T1a	0	0	0	0	2	0	0
Treated	T=114h	T1b	0	0	0	0	1	0	0
Treated	T=114h	T1c	0	0	0	0	1	0	0
Treated	T=114h	T2a	0	0	0	0	6	1	0
Treated	T=114h	T2b	0	0	0	0	0	0	0
Treated	T=114h	T2c	0	0	0	0	5	2	0
Treated	T=114h	T3a	0	0	0	0	3	1	0
Treated	T=114h	T3b	0	0	0	0	2	2	0
Treated	T=114h	T3c	0	0	0	0	4	1	0

Culturable heterotrophic bacteria showed markedly different characteristics in the second trial, compared with Trial 1. Numbers of cfus varied from 2->200 in T=0 controls, which did not differ significantly from treated samples examined at T=0. After a 144h residence time in the ballast tanks, both treated and untreated samples showed elevated counts, although this was particularly evident in treated samples where, in all but one (the last) sample investigators had to estimate the cfus as either >100 or >200 (table 18).

Table 18. Trial 2. Culturable bacteria counts from treated and untreated (control) samples at T=0 and T=114h.

				Colony-forming Units	**Colony-forming Units**	**Colony-forming Units**
				100%	**10%**	**1%**
Trial 2	**Treatment**	**Time**	**Replicate**			
	Control	T=0	C1a	3	0	0
	Control	T=0	C1b	5	7	1
	Control	T=0	C1c	18	4	0
	Control	T=0	C2a	>200	23	2
	Control	T=0	C2b	8	3	2
	Control	T=0	C2c	2	1	0
	Control	T=0	C3a	45	4	0
	Control	T=0	C3b	74	5	0
	Control	T=0	C3c	4	0	0
	Treated	T=0	T1a	23	2	0
	Treated	T=0	T1b	4	3	0
	Treated	T=0	T1c	4	0	0
	Treated	T=0	T2a	84	8	0
	Treated	T=0	T2b	9	0	0
	Treated	T=0	T2c	1	0	0
	Treated	T=0	T3a	8	3	0
	Treated	T=0	T3b	56	3	1
	Treated	T=0	T3c	5	0	0

Control	T=114h	C1a	>200	28	6
Control	T=114h	C1b	64	6	0
Control	T=114h	C1c	81	0	0
Control	T=114h	C2a	>200	34	3
Control	T=114h	C2b	86	13	1
Control	T=114h	C2c	94	9	1
Control	T=114h	C3a	81	11	1
Control	T=114h	C3b	8	0	0
Control	T=114h	C3c	15	3	0
Treated	T=114h	T1a	>200	25	2
Treated	T=114h	T1b	>200	24	3
Treated	T=114h	T1c	>100	10	2
Treated	T=114h	T2a	>200	17	5
Treated	T=114h	T2b	>200	23	1
Treated	T=114h	T2c	>100	17	5
Treated	T=114h	T3a	>200	38	4
Treated	T=114h	T3b	>100	11	0
Treated	T=114h	T3c	35	13	1

Water Chemistry.

Ballast water during trial 2 was taken south of Whittier, Alaska on July 1st, 2008. The salinity of challenge water was 30.8 – 31.9 PSU, water temperatures ranged from 12.1 = 14.0^{o} C and pH from 8.25 – 8.36. Dissolved oxygen (DO) concentrations ranged from 6.85 mg L^{-1} to 10.46 mg L^{-1} (table 19). Total Suspended Solids (TSS) ranged from 5.37 – 9.95 in challenge water, with a tendency to the higher value early in the ballasting cycle. A similar pattern was seen in Particulate Organic Carbon (POC) levels, which ranged from 3.62 – 7.28 mg L^{-1}. UV Transmittance was high (94%).

Nutrient levels in challenge water were significantly higher in the first trial, reflecting the overall greater productivity at this ballasting location.

Table 19. Trial 2. Water Chemistry in untreated challenge water and treated ballast water at time of ballasting (T=0) near Whittier AK, July 1st 2008. (DO = Dissolved O2; TSS = Total Suspended Solids; POC – Particulate Organic Carbon; DOC = Dissolved Organic Carbon; NO_3 = Nitrate; PO_4 = Phosphate)

	pH	DO (mg L^{-1})	Salinity (PSU)	Temp (^{O}C)	TSS (mg L^{-1})	POC (mg L^{-1})	DOC (mg L^{-1})	NO_3 (µgN L^{-1})	PO_4 (µgP L^{-1})
C1a, T=0 (begin ballast)	8.36	6.85	30.8	14.0	9.95±0.05	7.28±0.34	1.34±0.03	21.3±6.7	22.4±8.7
C2a, T=0 (mid ballast)	8.29				5.51±0.20	3.62±0.29	1.33±0.07	24.7±5.5	12.5±0.8
C3a, T=0 (end ballast	8.25				5.37±0.18	4.0±0.15	1.72±0.73	11.0±3.6	13.2±4.2
T1a, T=0 (begin ballast)	8.25	10.46	31.9	12.1					
T2a, T=0 (mid ballast)	8.32								
T3a, T=0 (end ballast	8.35								

GENERAL CONCLUSIONS (Trial 2)

Plankton concentrations in challenge water reflected higher densities of organisms compared with trial 1. However, zooplankton densities in untreated challenge water did exceed the value of 10 x D-2 required by IMO G8 guidelines. Based these results, the Hyde Guardian system would comply with those portions of IMO Regulation D-2 and current California regulations relating to plankton.

The appearance of intestinal Enterococci in samples collected at de-ballasting although none were detected in any T=0 samples poses a problem of interpretation. Clearly, no live enterococci were introduced into the tanks, i.e. none were detected in T=0 samples. We therefore interpret the appearance of these bacteria at de-ballasting as the result of their association with planktonic organisms present in the tanks before the trial took place. As with the culturable heterotrophic bacteria, treatment was actually associated with an *increase* in bacterial flora relative to controls. Mean numbers of Enterococci were 10 cfus per 100ml. and 36 cfus per 100ml. for untreated and treated samples respectively. These data are consistent with the unusual nature of the samples collected during de-ballasting. Samples were characterized by a large amount of flocculated material, particularly in the treated tank, including several specimens of decaying zooplankton, some >>1000µm in the smallest dimension.

TRIAL 3. September 17th-October 6th, 2008

Sampling Timetable.

In Trial 3 the same matched pair of ballast tanks (5P, 5S) was employed as in Trials 1 and 2, i.e. tank (5S) was used for treated ballast water and the other (5P) for untreated water. The scientific team joined the vessel in Long Beach, CA on September 17th. Ballasting took place on September 19th, 2008, day 3 of a seventeen day cruise. As with Trials 1 and 2, tanks 5S and 5P were filled with treated and untreated water respectively, as per normal ballasting procedure, and for untreated samples, water followed the same path as the treated samples, except that the filter was by-passed and the UV unit was deactivated during the ballasting of the untreated tank.

Sample collection.

The same sampling regime was adopted as for Trial 2, i.e. 3 'in tank' replicates x 3 time period (during the de-ballasting operation) x 2 treatment (i.e. treated/untreated), except that nine samples were obtained from both untreated and treated tanks immediately following the initial ballasting/treatment cycle. In the third trial a residence time of 10 days elapsed before samples were retrieved from the tanks in Aruba Harbor on September 29th, according to the sequence previously described. For trial 3, Dr. Carys Mitchelmore and graduate student Jon Bearr joined the scientific team and performed all coliform and Enterococcus assays, and culturable heterotrophic bacterial assays for T=0 and T=10 Day samples in an air-conditioned passenger cabin. Other samples were carried over ice to the University of Maryland Center for Environmental Science, Chesapeake Biological Laboratory, Solomons, Maryland for further analysis. Culture and analysis of these samples continued until October 6th, 2008.

Otherwise, preparation of samples for biological examination followed procedures described for previous trials (see Figure 1.) Methods for sample examination and analysis were as previously described.

Results and Discussion

Zooplankton.

Results of zooplankton counts are summarized in table 20. These indicated 100% mortality of zooplankton (>50µm minimum dimension) immediately following treatment on ballasting relative to untreated samples collected during the same sampling event (T=0). Unlike Trial 1 and similar to Trial 2 a massive die-off (99.7%) of zooplankton in the >50µm size category was recorded in the untreated tank at the time of de-ballasting (T=10 days) and no survivors in this size class appeared in the treated tank. While some smaller zooplankton, largely nematodes, survived, these fell into the >10 - <50µm size category. These organisms appeared only in the last sample taken and may have reflected some re-suspension from sediment present in the bottom of the tank. Such a conclusion is supported by the fact that nematodes appeared only at the end of the de-ballasting process, when the ballast tank was nearly empty (Table 21).

Table 20. Summary of zooplankton results (>50µm minimum dimension) for Trial 3 of Hyde 'Guardian' BWT system aboard M/V *Coral Princess*, September 17th-October 6th, 2008.

Zooplankton	**Alive (>50µm). Density/m^3**	**Dead (>50µm) Density/m^3**
Control (untreated) during ballasting N=9	1,391±918	192±233
Treated (UV + filter during ballasting) N=7	0	11±4
Control (untreated) Start de-ballast (N=3)	24±0	32±16
Control (untreated) Mid de-ballast (N=3)	8±7	13±19
Control (untreated) End de-ballast (N=3)	16±17	13±10
Mean Control No. at de-ballasting	**16±13**	**20±18**
Treated (UV + filter during ballasting) → (UV at de-ballasting)	0	0

Start de-ballast (N=3)		
Treated (UV + filter during ballasting) → (UV at de-ballasting) Mid de-ballast (N=3)	0	3±4
Treated (UV + filter during ballasting) → (UV at de-ballasting) End de-ballast (N=3)	0	3±4
Mean Treated Nos. at de-ballasting	**0**	**1.8±3.5**

Table 21. Densities of living nematode worms in treated and untreated samples. Trial 3. September 17th-October 6th, 2008.

	Nematode densities (concentrations per m^3)	
	Live	**Dead**
T=0h		
Control	6±5	0
Treated	0	0
T= 10 Days		
Control (untreated). Start de-ballast (N=3)	0	0
Control (untreated) Mid de-ballast (N=3)	0	0
Control (untreated) End de-ballast (N=3)	0	0
Treated (UV + filter during ballasting) → (UV at de-ballasting). Start de-ballast (N=3)	0	0
Treated (UV + filter during ballasting) → (UV at de-ballasting) Mid de-ballast (N=3)	0	0
Treated (UV + filter during ballasting) → (UV at de-ballasting) End de-ballast (N=3)	3±4	13±14

Phytoplankton.

Phytoplankton counts from trial 3 are shown in tables 22 – 25. Due to time constraints a full suite of grow-out data was only available for untreated samples collected at ballasting (table 22). Only three treated samples at T=0 were subjected to grow-out (table 23). At de-ballasting (T=10 Days), grow-out information was obtained from four untreated and four untreated samples each (tables 24 and 25).

Based on morphological characteristics treated samples at T=0 treated samples show a 77% reduction in live cell counts relative to untreated samples. 'Live' cell numbers following grow-out had fallen to 2,469,400/m^3 (2.4 cells/mL) and 1,181,737/m^3 (1.2 cells/mL) in control and treated samples respectively. Following a 10 day residence period in the tank both treated and untreated samples showed a >50% drop in live cell numbers based on morphological characteristics, relative to corresponding T=0 samples. Following grow-out, further reductions of 55% and 83% in 'live' cell numbers were reported from untreated and treated samples respectively. Based on grow-out data the number of viable cells in T= 10 Day treated samples after grow-out exceeded by 3% the 2008 standard published by the U.G. Coast Guard and the state of California, 103,000 live cells/m^3 (0.103 cells/mL) vs. 100,000 live cells/m^3 (0.100 cells/mL). However, this number represents an 83% decrease in cell numbers relative to pre-grow-out concentrations, indicating a predominantly non-viable phytoplankton population in treated samples.

Chlorophyll *a* concentrations (table 26) indicate a small degree of growth potential in untreated samples at T=0, although treated samples appear non-viable as demonstrated by a 75% decrease in chlorophyll a following a 48h grow-out period. After 10 days residence in the ballast tank, untreated samples demonstrated no growth potential (90% decrease in chlorophyll *a* following a 48h grow-out period), while treated samples indicated negligible chlorophyll *a* after 10 days in the tank.

Table 22. Summary of phytoplankton results (>10<50µm minimum dimension) for Trial 3 of Hyde 'Guardian' BWT system aboard M/V *Coral Princess*, September 15th-October 6th, 2008. Untreated (control) T=0 data.

Phytoplankton. (>10µm-<50µm). Untreated, T=0	'Live' Dinoflagellates. Density/mL	'Live' Diatoms. Density/ mL	Total 'Live' phytoplankton. Density/ mL	Dead Dinoflagellates. Density/ mL	Dead Diatoms. Density/ mL	Total Dead phytoplankton. Density/ mL
Start Ballast. Rep. C1a, T=0	0.55	5.32	5.87	0.10	0.55	0.65
After 48h grow-out	0.25	0.12	0.37	0.19	0.04	0.63
Rep. C1b T=0	0.49	7.83	8.32	0	0.82	0.82
After 48h grow-out	0.18	0.85	1.03	0	0.09	0.09
Rep. C1c T=0	0.49	3.42	3.91	0.24	0.24	0.49
After 48h grow-out	0.38	0.34	0.72	0	0.04	0.04
Mean C1, T=0 samples	0.51	4.50	**6.03± 2.21**	0.12	0.54	0.65
(After grow-out)			**(0.71± 0.33)**			
Mid De-ballast. Rep. C2a, T=0	0.04	9.0	9.4	0	0.4	0.4
After 48h grow-out	0.3	2.0	2.3	0	0.2	0.2
Rep. C2b T=0	2.8	10.3	13.1	0.4	0.5	0.9
After 48h grow-out	1.2	2.3	3.5	0.1	0.5	0.6
Rep. C2c T=0	0.6	3.8	4.4	0.21	0.42	0.63
After 48h grow-out	0.72	4.44	5.16	0.11	0.45	0.56
Mean C2, T=0 samples	1.27	7.70	**8.96±4.33**	193,037	460,741	653,778
(After grow-out)			**(3,65±1.45)**			
End De-ballast. Rep. C3a, T=0	1.0	0.5	1,5	1.0	0.4	1.4
After 48h grow-out	1.02	0.70	1.72	0.06	0.3	0.36
Rep. C3b T=0	1.34	3.25	4.59	0	0.38	0.38
After 48h grow-	0.99	0.96	1.95	0	0.19	0.19

out						
Rep. C3c T=0 **After 48h grow-out**	0.79 0.48	6.69 5.0	7.46 5.48	0.19 0.20	0.39 0.45	0.60 0.65
Mean C3, T=0 samples **(After grow-out)**	1.0	3.5	**4.5±2.98** **(3.05±2.11)**	0.98	0.39	0.48
Mean Live Phytoplankton **(After grow-out)**	0.94	4.91	**6.5±3.46** **(2.47±1.90(n=9))**	0.14	0.46	0.60

Table 23. Summary of phytoplankton results (>10<50µm minimum dimension) for Trial 3 of Hyde 'Guardian' BWT system aboard M/V *Coral Princess*, September 15th-October 6th, 2008. Treated T=0 data. Grow-outs were recorded for one sample each from T1, T2 and T3.

Phytoplankton (10µm-50µm) Treated, T=0	'Live' Dinoflagellates. Density/mL	'Live' Diatoms. Density/ mL	Total 'Live' phytoplankton. Density/ mL	Dead Dinoflagellates. Density/ mL	Dead Diatoms. Density/ mL	Total Dead phytoplankton. Density/ mL
Rep. T1a, T=0	1.80	0.12	1.92	0	0.08	0.08
After 48h grow-out			ND			
Rep. T1b, T=0	1.32	0.23	1.56	0	0	0
After 48h grow-out	2.48	0	2.48	0	0.16	0.16
Rep. T1c, T=0	2.48	0	2.48	0	0.16	0.16
After 48h grow-out			ND			
Mean T1 T=0 samples			**1.98±0.46**			
Rep. T2a, T=0	0.81	0.26	1.06	0	0	0
After 48h grow-out			ND			
Rep. T2b, T=0	1.61	0.18	1.79	0	0	0
After 48h grow-out	0	0.33	0.33	0	0	0
Rep. T2c, T=0	1.73	0.23	1.97			
After 48h grow-out			ND			
			1.61±0.48			
Rep. T3a, T=0	1.44	0	1.44	0	0	0
After 48h grow-out			ND			
Rep. T3b, T=0	2.15	0	2.15	0	0	0
After 48h grow-out	0.73	0	0.73	0	0	0
Rep. T3c, T=0	0.85	0	0.85			
After 48h grow-out			ND			
			1.48±0.65			
Mean 'Live' Phytoplankton	1.37	0.12	**1.51±0.72 (n=9)**	0	0.03	0.03
(After grow-out)			**(1.18±1.14 (n=3))**			

Table 24. Summary of phytoplankton results (>10<50µm minimum dimension) for Trial 3 of Hyde 'Guardian' BWT system aboard M/V *Coral Princess*, September 15th-October 6th, 2008. Untreated (control) T=10 Days data, including live grow-out cell concentrations (in red). Due to time constraints, no record was made of dead cell numbers following grow-out.

Phytoplankton (10µm-50µm) Untreated Controls, T=0	'Live' Dinoflagellates. Density/mL	'Live' Diatoms. Density/ mL	Total 'Live' phytoplankton. Density/ mL	Dead Dinoflagellates. Density/ mL	Dead Diatoms. Density/ mL	Total Dead phytoplankton. Density/ mL
Rep. C1a, T=10 Days	0.17	1.54	1.71	0	0.68	0.68
After 48h grow-out	0.39	2.16	2.55			
Rep. C1b, T=10 Days	0.73	1.47	2.20	0.20	0	0.20
After 48h grow-out	0.66	0.50	1.16			
Rep. C1c, T= 10 Days	0.54	4.90	5.44	0.15	0	0.15
After 48h grow-out			ND			
Mean C1, T=10 Days			**3.12±2.03**			
Rep. C2a T= 10 Days	1.66	2.50	3.16	0	0.16	0.16
After 48h grow-out			ND			
Rep. C2b, T= 10 Days	0.44	1.29	1.73	0	0	0
After 48h grow-out	0.83	0	0.83			
Rep. C2c, T= 10 Days	0.83	3.88	4.71	0	0.16	0.16
After 48h grow-out			ND			
Mean C2, T=10 Days			**3.20±1.49**			
Rep. C3a, T= 10 Days	0.4	2.18	2.58	0	0.21	0.21
After 48h grow-out			ND			
Rep. C3b, T= 10 Days	0	0.66	0.66	0	0	0

After 48h grow-out	0	0.33	0.33			
Rep. C3c, T= 10 Days	0	3.15	3.15	0	0.33	0.33
After 48h grow-out			ND			
Mean C3, T=10 Days	0.67	1.98	**2.22±1.36**			
Mean 'Live' Phytoplankton **(After grow-out)**	0.17	2.40	**2.82±1.50 (n=9)** **(1.27±0.95 (n=4))**	0.04	0.02	0.02

Table 25. Summary of phytoplankton results (>10<50µm minimum dimension) for Trial 3 of Hyde 'Guardian' BWT system aboard M/V *Coral Princess*, September 15th-October 6th, 2008. Treated T=10 Days data, including live grow-out cell concentrations (in red).

Phytoplankton. (>10µm-<50µm) Treated, T=10 Days	'Live' Dinoflagellates. Density/mL	'Live' Diatoms. Density/ mL	Total 'Live' phytoplankton. Density/ mL	Dead Dinoflagellates. Density/ mL	Dead Diatoms. Density/ mL	Total Dead phytoplankton. Density/ mL
Start De-ballast. Rep. T1a, T=10 Days	0.43	0.65	1.01	0.22	0.22	0.44
After 48h grow-out	0.10	0.06	0.16	0.20	0.17	0.37
Rep. T1b, T=10 Days	0	0	0	2.16	0.54	2.7
After 48h grow-out	0	0	0	1.70	0.22	1.92
Rep. T1c, T=10 Days	0.65	0.43	1.09	0	0.65	0.65
After 48h grow-out	0.14	0.10	0.25	0	0.50	0.50
Mean T1, T=10 Day samples	**0.36**	**0.36**	**0.72±0.62**	**0.79**	**0.50**	**1.26**
(After grow-out)			**(0.14±0.12)**			
Mid De-ballast. Rep. T2a, T=10 Days	0.17	0	0.17	0.17	0.87	1.04
After 48h grow-out	0.06	0	0.06	0.16	0.43	0.59
Rep. T2b, T=10 Days	0.89	1.09	1.98	0.19	0	0.19
After 48h grow-out	0.16	0.11	0.26	0.12	0	0.12
Rep. T2c T=10 Days	0	0	0	0	0.42	0.42
After 48h grow-out	0	0	0	0	0.33	0.33
Mean T2, T=10 Day samples	**0.36**	**0.36**	**0.72±1.1**	**0.12**	**0.43**	**0.55**
(After grow-out)						

			(0.11±0.14)			
End De-ballast. Rep. T3a, T=10 Days	0	0.84	0.84	0.17	0	0.17
After 48h grow-out	0	0.10	0.10			
Rep. T3b, T=10 Days	0	0.24	0.24	0.15	0	0.15
After 48h grow-out	0	0.10	0.10			
Rep. T3c, T=10 Days	0	0.14	0.14	0.14	0	0.14
After 48h grow-out		0	0			
Mean T3, T=10 Day samples	0	0.49	**0.41±0.38**	0.15	0	0.15
(After grow-out)			**(0.07±0.06)**			
Mean Live Phytoplankton	0.24	0.38	**0.61±0.68**	0.36	0.30	0.66
(After grow-out)			**(0.10±0.10)**			

Table 26. Trial 3. Chlorophyll *a* concentrations from measurement of *in vivo* fluorescence. (all values are means of 3 determinations)

T=0	T=0, Pre grow-out chlorophyll *a* conc. (µg L^{-1})	chlorophyll *a* conc. (µg L^{-1}) after 24h grow-out (T=0 + 24)	chlorophyll *a* conc. (µg L^{-1}) after 42h grow-out (T=0 + 42)
Control 1 start ballasting	0.257	0.260	0.2
Control 2 mid ballasting	0.180	0.221	0.220
Control 3 end ballasting	0.171	0.231	0.24
Treated 1 start ballasting	0.141	0.051	0.044
Treated 2 mid ballasting	0.142	0.049	0.034
Treated 3 end ballasting	0.106	0.069	0.022
T=10 Days	**Pre grow-out chlorophyll *a* conc. (µg L^{-1})**	**chlorophyll *a* conc. (µg L^{-1}) after 24h grow-out (T=10D + 24h)**	**chlorophyll *a* conc. (µg L^{-1}) after 42h grow-out (T=10D + 42h)**
Control 1 start de-ballasting	0.284	0.065	0.028
Control 2 mid de-ballasting	0.282	0.087	0.044
Control 3 end de-ballasting	0.287	0.039	0.02
Treated 1 start de-ballasting	0.147	0.05	0.02
Treated 2 mid de-ballasting	0.167	0.043	ND
Treated 3 end de-ballasting	0.125	0.029	ND

Bacteria.

No coliform bacteria or, *E. Coli* were found in either treated or untreated samples immediately following ballasting/treatment in Trial 3, although a small number of cfus were detected in untreated samples following de-ballasting 10 days later (Table 27). No coliforms or *E. Coli* were found in any treated samples at de-ballasting, and no intestinal enterococci appeared in any samples examined during this trial. No *Vibrio cholerae* cells were found in any samples, treated or untreated.

Cfus of aerobic cultural bacteria were found in approximately equal numbers in both treated and untreated samples at T=0 (Table 28). However, both showed marked declines after 10 days in the tanks. Of the treated samples, only one showed a positive response in terms of cultural bacteria. All indicator bacteria were within national and international standards. No determination was made of performance of the BWT system against viruses, as per California regulations.

Table 27 . Trial 3. *Enterococci,* colifoms and *Vibrio cholerae* from treated and untreated (control) samples at T=0 and T=10 Days

			Coliforms		***E. Coli***		**Enterrococci**		***Vibrio cholerae***
Treatment	**Time**	**Replicate**	**Large**	**Small**	**Large**	**Small**	**Large**	**Small**	
Control	T=0	C1a	0	0	0	0	0	0	0
Control	T =0	C1b	0	0	0	0	0	0	0
Control	T =0	C1c	0	0	0	0	0	0	0
Control	T =0	C2a	0	0	0	0	0	0	0
Control	T =0	C2b	0	0	0	0	0	0	0
Control	T =0	C2c	0	0	0	0	0	0	0
Control	T =0	C3a	0	0	0	0	0	0	0
Control	T =0	C3b	0	0	0	0	0	0	0
Control	T =0	C3c	0	0	0	0	0	0	0
Treated	T =0	T1a	0	0	0	0	0	0	0
Treated	T =0	T1b	0	0	0	0	0	0	0
Treated	T =0	T1c	0	0	0	0	0	0	0
Treated	T =0	T2a	0	0	0	0	0	0	0
Treated	T =0	T2b	0	0	0	0	0	0	0
Treated	T =0	T2c	0	0	0	0	0	0	0
Treated	T =0	T3a	0	0	0	0	0	0	0

Treated	T =0	T3b	0	0	0	0	0	0	0
Treated	T =0	T3c	0	0	0	0	0	0	0
Control	T=10 Days	C1a	0	1	0	1	0	0	0
Control	T=10 Days	C1b	0	1	0	1	0	0	0
Control	T=10 Days	C1c	0	1	0	0	0	0	0
Control	T=10 Days	C2a	0	0	0	0	0	0	0
Control	T=10 Days	C2b	0	1	0	0	0	0	0
Control	T=10 Days	C2c	0	0	0	0	0	0	0
Control	T=10 Days	C3a	0	0	0	0	0	0	0
Control	T=10 Days	C3b	0	0	0	0	0	0	0
Control	T=10 Days	C3c	0	1	0	1	0	0	0
Treated	T=10 Days	T1a	0	0	0	0	0	0	0
Treated	T=10 Days	T1b	0	0	0	0	0	0	0
Treated	T=10 Days	T1c	0	0	0	0	0	0	0
Treated	T=10 Days	T2a	0	0	0	0	0	0	0
Treated	T=10 Days	T2b	0	0	0	0	0	0	0
Treated	T=10 Days	T2c	0	0	0	0	0	0	0
Treated	T=10 Days	T3a	0	0	0	0	0	0	0
Treated	T=10 Days	T3b	0	0	0	0	0	0	0
Treated	T=10 Days	T3c	0	0	0	0	0	0	0

Table 28. Trial 3. Culturable heterotrophic bacteria counts from treated and untreated (control) samples at T=0 and T=10 Days.

			Colony-forming Units	**Colony-forming Units**	**Colony-forming Units**
			25mL	**10mL**	**1mL**
Treatment	**Time**	**Replicate**			
Control	t=0	C1a	51	28	5
Control	t=0	C1b	>100	17	4
Control	t=0	C1c	30	17	3
Control	t=0	C2a	>100	22	1
Control	t=0	C2b	9	27	7
Control	t=0	C2c	>100	88	15
Control	t=0	C3a	2	2	0
Control	t=0	C3b	0	97	24
Control	t=0	C3c	6	9	1
Treated	t=0	T1a	>100	10	2
Treated	t=0	T1b	9	5	1
Treated	t=0	T1c	>100	9	6
Treated	t=0	T2a	4	2	1

Treated	t=0	T2b	>100	22	4
Treated	t=0	T2c	3	4	0
Treated	t=0	T3a	3	0	0
Treated	t=0	T3b	23	10	1
Treated	t=0	T3c	>100	2	0
Control	T=10 Days	C1a	8	0	0
Control	T=10 Days	C1b	2	0	0
Control	T=10 Days	C1c	4	0	0
Control	T=10 Days	C2a	0	0	0
Control	T=10 Days	C2b	8	0	0
Control	T=10 Days	C2c	23	4	0
Control	T=10 Days	C3a	12	4	0
Control	T=10 Days	C3b	4	1	1
Control	T=10 Days	C3c	0	0	0
Treated	T=10 Days	T1a	0	0	0
Treated	T=10 Days	T1b	0	0	0
Treated	T=10 Days	T1c	0	0	0
Treated	T=10 Days	T2a	0	0	0
Treated	T=10 Days	T2b	0	0	0
Treated	T=10 Days	T2c	0	0	0
Treated	T=10 Days	T3a	18	0	0
Treated	T=10 Days	T3b	0	0	0
Treated	T=10 Days	T3c	0	0	0

Water Chemistry.

Ballast water during trial 3 was taken south of Long Beach, S. California on September 19th, 2008. The salinity of challenge water was 33.3 PSU, water temperatures ranged from 23.7 – 24.3^{o} C and pH from 7.5 - 7.82. Dissolved oxygen (DO) concentrations ranged from 7.5 mg L^{-1} to 8.83 mg L^{-1} (table 28). Total Suspended Solids (TSS) ranged from 3.59 – 7.3 in challenge water, will a tendency to the higher value early in the ballasting cycle. A similar pattern was seen in Particulate Organic Carbon (POC) levels, which ranged from 2.04 – 4.37 mg L^{-1}. As in earlier trials percentage UV Transmittance was high (95%).

Nitrate levels in challenge water declined sharply throughout the initial ballasting sequence, from an initial concentration of 71.4µg L^{-1} to 5.2 ± 3.7 µg L^{-1}. In treated samples at T=0 nitrate levels ranged from 2.1 – 6.4 µg L^{-1}. At de-ballasting, ten days later, nitrate concentrations in untreated, control samples varied between 1.2 and 18.7 µg L^{-1}. However, treated samples at T=10 days contained much higher nitrate levels, possibly due to an increase in nutrient release from dying/dead organisms in these samples (table 29). Nitrite levels, although

much lower, also showed a 3-4 fold increase in treated samples after 10 days (table 29). Phosphate concentrations in treated samples at T= 10 days also indicated higher concentrations than untreated (control) samples. DOC levels were slightly higher in both treated and untreated samples at T=0 than at T= 10 Days and showed no apparent relationship to treatment (table 29).

Table 29. Trial 3. Water Chemistry in untreated challenge water and treated ballast water at time of ballasting (T=0) near Long Beach CA, September 19th, 2008. (DO = Dissolved O2; TSS = Total Suspended Solids; POC – Particulate Organic Carbon; DOC = Dissolved Organic Carbon; NO_3 = Nitrate; NO_2 = Nitrite; PO_4 = Phosphate)

	pH	DO (mg L^{-1})	Salinity (PSU)	Temp (^{O}C)	TSS (mg L^{-1})	POC (mg L^{-1})	DOC (mg L^{-1})	NO_3 (µgN L^{-1})	NO_2 (µgN L^{-1})	PO_4 (µgP L^{-1})
C1, T=0 (begin ballast)	7.5	8.83	33.3	23.7	7.3±0.176	4.37±0.11	4.08±1.43	71.4±17.2	0.8±0.2	11.6±2.3
C2, T=0 (mid ballast)	7.78				3.75±0.073	2.04±0.026	3.16±1.21	25.1±18.5	0.63±0.06	10.8±2.1
C3, T=0 (end ballast	7.73				3.59±0.066	2.11±0.06	1.87±0.88	5.2±3.7	0.6±0.0	10.8±1.0
T1, T=0 (begin ballast)	7.82	7.5	33.3	24.3			2.45±0.89	2.4±0.5	0.6±0.0	12.1±0.3
T2, T=0 (mid ballast)	7.78						3.01±1.27	2.1±0.8	0.6±0.0	13.4±0.06
T3, T=0 (end ballast	7.73						2.05±0.42	6.4±6.8	0.63±0.06	9.5±0.9
C1, T= 10 Days (begin de-ballast)							1.5±0.4	1.2±0.8	0.6±0	1.76±0.4
C2, T= 10 Days (mid de-ballast)							1.5±0.8	8.1±1.1	0.7±0.1	4.2±0.5
C3, T= 10 Days (end de-ballast)							1.38±0.4	18.7±3.2	0.77±0.15	5.2±0.9
T1, T=10 Days (begin de-ballast)							1.3±0.2	41.6±13.8	1.75±1.62	11.15±5.6
T2, T=10 Days (mid de-ballast)							1.3±0.2	51.8±10.3	2.33±1.36	14.7±2.9
T3, T=10 Days (end de-ballast)							1.5±0.3	52.6±11.8	2.73±1.34	15.65±1.6

GENERAL CONCLUSIONS (Trial 3)

Zooplankton densities >50 µm in size range in trial 3 were initially only about 15% of concentrations seen in trial 2, but still exceed G-8 D2 regulations for challenge water. As in trial 2, zooplankton mortalities (>50 µm) were 100% in treated samples at T=0 in trial 3, and numbers of untreated organisms in this size category declined precipitously over the period of residence in the ballast tanks.

Phytoplankton cell numbers in trial 3 challenge water, 6.5 x 10^6 cells m^3 (6.5 cells/mL) were approximately 2/3 of those seen in trial 2, 9.7 x 10^6 cells m^3 (9.7 cells/mL). However, somewhat surprisingly, they fell less precipitously than in the earlier trial despite a residence time in the tank approximately twice as long. Based on morphological characteristics, live cell numbers at trial 3 de-ballasting, 2.81 x 10^6 cells m^3 (2.8 cells/mL) were comparable to the numbers seen in trial 2 after 114h residence time 2.88 x 10^6 cells m^3 (2.9 cells/mL). Based on morphological characteristics, cell numbers in treated vs. untreated samples at de-ballasting had further decreased by respectively 93.4% and 78.2% in trials 2 and 3. If grow-out is considered as a criterion for phytoplankton cell viability, the viable cell count in treated samples is further reduced to 103,000 cells per m^3 (0.103 cells/mL), just exceeding the U.S. Coast Guard standard of 0.100 cells/mL) for cells in the >10 - <50µm size category. The IMO D-2 standard is easily met for this phytoplankton population.

All indicator bacteria were within national and international standards following treatment.

Test for Environmental Acceptence.

Whole Effluent Toxicity Testing.

Rationale.

Whole effluent toxicity tests were conducted on treated and untreated water samples collected during the shipboard ballasting and de-ballasting procedures described in Trial 3. The objective of these bioassays was to identify any residual chemical toxicity that could have resulted from UV irradiation of ballast water. A new (October 10, 2008) G8 resolution adopted by the Marine Environmental Protection Committee (MEPC 58) states:

> *" -- if it can reasonably be concluded that the treatment process could result in changes to the chemical composition of the treated water such that adverse impacts to receiving waters might occur upon discharge, the documentation should include results of toxicity tests of treated water. The toxicity tests should include assessments of the effects of hold time following treatment, and dilution, on the toxicity. Toxicity tests of the treated water should be conducted in accordance with paragraphs 5.2.3 to 5.2.7 of the Procedure for approval of ballast water management systems that make use of Active Substances (G9), as revised, (resolution MEPC.169(57))"*

While, to date, no potentially toxic chemical changes resulting from UV irradiation have been identified, this resolution adopts an approach requiring tests for residual toxicity that essentially follow IMO G-9 guidelines, even for systems not involving the addition of active substances. The resolution was adopted too late for such testing to be incorporated into land-based testing of the system, which ended in July, 2008. Hence, three tests, one chronic (growth-based) and two acute toxicity bioassays, were incorporated into the third trial in order to provide empirical toxicological evidence on this point.

Using the convention adopted for sample collection and examination in this trial. Four 'treatments' were tested:

Untreated, T=0

Treated, T=0

Untreated, T=10 Days

Treated, T=10 Days

As the prescribed treatment by the Hyde 'Guardian' system consists of two passes through the UV irradiation unit (on both ballasting and de-ballasting) the *definitive* comparison is regarded as that between treated and untreated water on final discharge (T=10 Days). Nevertheless, from an experimental standpoint, T=0 samples, untreated vs. treated, also represent a valid comparison, and provide a useful means of controlling for any possible toxic agents that might be introduced into the water as a result of prolonged storage in the ballast tanks.

Sample collection and storage.

For WET tests 20L of each water sample to be tested was collected in the middle of each ballasting/deballasting operation. Thus, treated and untreated T=0 samples were collected during sequence T2a-T2c for treated samples and between sequence C2a-C2c for untreated samples. The logistics of being at sea during ballasting and de-ballasting demanded a deviation from standard U.S. EPA protocols for dealing with storage/shipment of whole effluent test samples. Standard practice stipulates that samples should be shipped over ice, with the temperature not to exceed $6^{O}C$. In order to preserve the integrity of the samples to the greatest degree possible it was therefore decided to freeze the samples as soon as possible after collection, pending toxicity bioassays. On landing at the destination port of Fort Lauderdale on October 2nd, 2008 frozen water samples in plastic 'Cubitainers' were transported overnight to the respective bioassay laboratories, where samples were thawed and testing commenced (October 3rd,2008 for larval fish and mysid shrimp assays; October 4th, 2008 for phytoplankton assay – Appendix C). Tests were accompanied by standard water quality measurements as well as nitrate, nitrite and phosphate analyses.

The following three bioassays were performed according to standardized U.S.EPA procedures for Acute (EPA 821-R-02-012) and Chronic (EPA 821-R-02-014) bioassays:

Invertebrate.

48-hour Acute Static Renewal Definitive Test using larval mysid shrimp, *Americamysis bahia* (Method 2007.0)

Vertebrate.

96-hour Acute Static Renewal Definitive Test using larval topsmelt, *Atherinops affinis* (Method 2006.0)

Phytoplankton.

96-hour Chronic Static Non-renewal Definitive Test using the marine brown alga (diatom) *Isochrysis galbana*, strain T. Iso. (adapted from Method 1003.0)

The invertebrate and vertebrate tests were carried out at the University of Maryland Center for Environmental Science, Chesapeake Biological Laboratory, and the phytoplankton assay was conducted at the Maryland Department of Natural Resources, Harmful Algal Bloom (HAB) Laboratory, Annapolis, MD.

Raw treatment water was subjected to a series of dilution steps with clean, reconstituted seawater matched to the salinity of the treatment water, in order to create a dilution series representing 100 %, 50 %, 25 %, 12.5 %, 6.25 % of the original raw treatment water including a negative control (reconstituted seawater) Static renewal 48 h and 96 h LC_{50} assays were conducted on 4-day-old mysid shrimp (*Americamysis bahia*) and 14-day-old topsmelt (*Antherinops affinis*) larvae, respectively. These organisms were obtained from Aquatic Research Organisms (ARO) Ltd., Maine. A culture of marine acclimated (30PSU) *Isochrysis galbana* (Tahitian strain T. Iso) was obtained from the Provasoli-Guillard National Center for the Culture of Marine Phytoplankton (CCMP), Bigelow Laboratory, New Hampshire. Prior to the test the culture was grown to log phase in the test laboratory at 33PSU and $21^{O}C$. Tests were conducted at $21^{O}C$ on a 14:10 light/dark cycle

Results of Whole Effluent Toxicity Tests.

Summary data from invertebrate *Americamysis bahia* and vertebrate *Atherinops affinis* assays are given below. Neither treated nor untreated samples resulted in any significant toxicity to either species. > 97% mysid shrimp survival was recorded from all samples, both treated and untreated, retrieved from the tanks at the time of de-ballasting. Topsmelt survival in undiluted treated and untreated water at time of discharge was 100% and 95.2% respectively with no noticeable toxicity associated with other treatments in the dilution series.

WET Test Summary Data.

Zooplankton.

Invertebrate Assay

(1) TEST SAMPLE ID: 10.03.08/CO/S

48 hour mysid test for C=0 control water

Initial Water Quality tests and characterization

Criteria	Test Water: CO water	Dilution water
Final pH (SU)	8.00	8.15
Salinity (ppt)	33	33
Total ammonia (mg / L)	0.003	<0.003
Temperature:	19.6	21.6
Dissolved oxygen (DO mg /L):	5.71	7.27

SU = standard units

ppt = parts per thousand

Ranges of water quality tests for all treatments

Criteria	Treatment Waters
pH (SU)	7.93 - 8.18
Salinity (ppt)	31 - 33
Total ammonia (mg / L)	0.003-0.006
Temperature:	20.0 - 22.3
Dissolved oxygen (DO mg /L):	6.40 – 7.62

Survival of *Americamysis bahia* at test termination (48 hours)

% test water	% survival 48-h	Statistics
0	97.5	LC50: N/A
6.25	100	LOEL:N/A

12.5	95	NOEL:N/A
25	100	
50	97.5	
100	97.5	

1: LC50 = median-lethal concentration (95% confidence interval) as measured by the trimmed spearman- Karber method.
2: LOEL = lowest observable effect level as measured by steel many-one rank method
3: NOEL = no observable effect level as measured by steel many-one rank method

Protocol Deviations / problems: None.

1) TEST SAMPLE ID: 10.03.08/TO/S

48 hour mysid test for T=0 treated water

Initial Water Quality tests and characterization

Criteria	Test Water: CO water	Dilution water
Final pH (SU)	8.09	8.15
Salinity (ppt)	33	33
Total ammonia (mg / L)	<0.003	<0.003
Temperature:	21.0	21.6
Dissolved oxygen (DO mg /L):	5.85	7.27

SU = standard units
ppt = parts per thousand

Ranges of water quality tests for all treatments

Criteria	Treatment Waters
pH (SU)	7.93 – 8.27
Salinity (ppt)	32 - 33
Total ammonia (mg / L)	<0.003
Temperature:	21.1 - 22.3
Dissolved oxygen (DO mg /L):	6.05 – 7.62

Survival of *Americamysis bahia* at test termination (48 hours)

% test water	% survival 48-h	Statistics
0	100	LC50:N/A
6.25	97.5	LOEL:N/A
12.5	100	NOEL:N/A
25	100	
50	97.5	
100	100	

1: LC50 = median-lethal concentration (95% confidence interval) as measured by the trimmed spearman- Karber method.

2: LOEL = lowest observable effect level as measured by steel many-one rank method
3: NOEL = no observable effect level as measured by steel many-one rank method

Protocol Deviations / problems: None.
(1) TEST SAMPLE ID: 10.03.08/C12/S

48 hour mysid test for C=10 Day control water

Initial Water Quality tests and characterization

Criteria	Test Water: CO water	Dilution water
Final pH (SU)	8.11	8.15
Salinity (ppt)	30	33
Total ammonia (mg / L)	0.004	<0.003
Temperature:	20.6	21.6
Dissolved oxygen (DO mg /L):	6.08	7.27

SU = standard units
ppt = parts per thousand

Ranges of water quality tests for all treatments

Criteria	Treatment Waters
pH (SU)	7.93 - 8.25
Salinity (ppt)	29 - 33
Total ammonia (mg / L)	0.003-0.014
Temperature:	21.1 - 22.3
Dissolved oxygen (DO mg /L):	6.30 – 7.62

Survival of *Americamysis bahia* at test termination (48 hours)

% test water	% survival 48-h	Statistics
0	100	LC50:N/A
6.25	97.5	LOEL:N/A
12.5	100	NOEL:N/A
25	100	
50	97.5	
100	100	

1: LC50 = median-lethal concentration (95% confidence interval) as measured by the trimmed spearman- Karber method.
2: LOEL = lowest observable effect level as measured by steel many-one rank method
3: NOEL = no observable effect level as measured by steel many-one rank method

Protocol Deviations / problems: None.
(1) TEST SAMPLE ID: 10.03.08/T12/S

48 hour mysid test for T=10 Day treated water

Initial Water Quality tests and characterization

Criteria	Test Water: CO water	Dilution water
Final pH (SU)	7.92	8.15
Salinity (ppt)	33	33
Total ammonia (mg / L)	<0.003	<0.003
Temperature:	21.0	21.6
Dissolved oxygen (DO mg /L):	6.41	7.27

SU = standard units
ppt = parts per thousand

Ranges of water quality tests for all treatments

Criteria	Treatment Waters
pH (SU)	7.93 - 8.22
Salinity (ppt)	31 - 34
Total ammonia (mg / L)	<0.003
Temperature:	21.4 - 22.3
Dissolved oxygen (DO mg /L):	6.11 – 7.62

Survival of *Americamysis bahia* at test termination (48 hours)

% test water	% survival 48-h	Statistics
0	100	LC50:N/A
6.25	97.5	LOEL:N/A
12.5	100	NOEL:N/A
25	100	
50	97.5	
100	100	

1: LC50 = median-lethal concentration (95% confidence interval) as measured by the trimmed spearman- Karber method.
2: LOEL = lowest observable effect level as measured by steel many-one rank method
3: NOEL = no observable effect level as measured by steel many-one rank method

Protocol Deviations / problems: None.

Vertebrate Assay.

(1) TEST SAMPLE ID: 10.03.08/CO/F

96 hour larval fish test for T=0 control water

Initial Water Quality tests and characterization

Criteria	Test Water: CO water	Dilution water
Final pH (SU)	8.00	8.15
Salinity (ppt)	33	33
Total ammonia (mg / L)	0.003	<0.003
Temperature:	19.6	21.6
Dissolved oxygen (DO mg /L):	5.71	7.27

SU = standard units
ppt = parts per thousand

Ranges of water quality tests for all treatments

Criteria	Treatment Waters
pH (SU)	7.93 - 8.18
Salinity (ppt)	31 - 33
Total ammonia (mg / L)	0.003-0.006
Temperature:	20.0 - 22.3
Dissolved oxygen (DO mg /L):	6.40 – 7.62

Survival of *Atherinops affinis* at test termination (96 hours)

% test water	% survival 48-h	Statistics
0	90	LC50:N/A
6.25	95	LOEL:N/A
12.5	90	NOEL:N/A
25	100	
50	95	
100	90	

1: LC50 = median-lethal concentration (95% confidence interval) as measured by the trimmed spearman- Karber method.
2: LOEL = lowest observable effect level as measured by steel many-one rank method
3: NOEL = no observable effect level as measured by steel many-one rank method

Protocol Deviations / problems: None.

(1) TEST SAMPLE ID: 10.03.08/TO/F

96 hour larval fish test for T=0 treated water

Initial Water Quality tests and characterization

Criteria	Test Water: CO water	Dilution water
Final pH (SU)	8.00	8.15
Salinity (ppt)	33	33
Total ammonia (mg / L)	<0.003	<0.003
Temperature:	19.6	21.6
Dissolved oxygen (DO mg /L):	5.71	7.27

SU = standard units
ppt = parts per thousand

Ranges of water quality tests for all treatments

Criteria	Treatment Waters
pH (SU)	7.93 - 8.18
Salinity (ppt)	31 - 33
Total ammonia (mg / L)	<0.003
Temperature:	20.0 - 22.3
Dissolved oxygen (DO mg /L):	6.40 – 7.62

Survival of *Atherinops affinis* at test termination (96 hours)

% test water	% survival 48-h	Statistics
0	95	LC50:N/A
6.25	100	LOEL:N/A
12.5	90	NOEL:N/A
25	80	
50	75	
100	100	

1: LC50 = median-lethal concentration (95% confidence interval) as measured by the trimmed spearman- Karber method.
2: LOEL = lowest observable effect level as measured by steel many-one rank method
3: NOEL = no observable effect level as measured by steel many-one rank method

Protocol Deviations / problems: None.

(1) TEST SAMPLE ID: 10.03.08/C12/F

96 hour larval fish test for T=10 Day control water

Initial Water Quality tests and characterization

Criteria	Test Water: CO water	Dilution water
Final pH (SU)	8.11	8.15
Salinity (ppt)	30	33
Total ammonia (mg / L)	0.004	<0.003

Temperature:	20.6	21.6
Dissolved oxygen (DO mg /L):	6.08	7.27

SU = standard units
ppt = parts per thousand

Ranges of water quality tests for all treatments

Criteria	**Treatment Waters**
pH (SU)	7.93 - 8.25
Salinity (ppt)	29 - 33
Total ammonia (mg / L)	0.003-0.014
Temperature:	21.1 - 22.3
Dissolved oxygen (DO mg /L):	6.30 – 7.62

Survival of *Atherinops affinis* at test termination (96 hours)

% test water	**% survival 48-h**	**Statistics**
0	100	LC50:N/A
6.25	100	LOEL:N/A
12.5	100	NOEL:N/A
25	95.2	
50	85.7	
100	95.2	

1: LC50 = median-lethal concentration (95% confidence interval) as measured by the trimmed spearman- Karber method.
2: LOEL = lowest observable effect level as measured by steel many-one rank method
3: NOEL = no observable effect level as measured by steel many-one rank method

Protocol Deviations / problems: None.

(1) TEST SAMPLE ID: 10.03.08/T12/F

96 hour larval fish test for T=10 Day treated water

Initial Water Quality tests and characterization

Criteria	**Test Water: CO water**	**Dilution water**
Final pH (SU)	7.92	8.15
Salinity (ppt)	33	33
Total ammonia (mg / L)	<0.003	<0.003
Temperature:	21.0	21.6
Dissolved oxygen (DO mg /L):	6.41	7.27

SU = standard units
ppt = parts per thousand

Ranges of water quality tests for all treatments

Criteria	Treatment Waters
pH (SU)	7.93 - 8.22
Salinity (ppt)	31 - 34
Total ammonia (mg / L)	<0.003
Temperature:	21.4 - 22.3
Dissolved oxygen (DO mg /L):	6.11 – 7.62

Survival of *Atherinops affinis* at test termination (96 hours)

% test water	% survival 48-h	Statistics
0	100	LC50:
6.25	95	LOEL:
12.5	100	NOEL:
25	90	
50	95	
100	100	

1: LC50 = median-lethal concentration (95% confidence interval) as measured by the trimmed spearman- Karber method.
2: LOEL = lowest observable effect level as measured by steel many-one rank method
3: NOEL = no observable effect level as measured by steel many-one rank method

Protocol Deviations / problems: +None.

Phytoplankton Assay.

Results from the phytoplankton whole effluent test are summarized in table 30. Results indicate variable growth in T=0 control (untreated) samples, with no clear relationship to the dilution series, and a slight positive relationship between degree of dilution and growth rate in treated T=0 samples. Definitive samples taken during de-ballasting indicate some growth inhibition, although this appears not to be related to UV irradiation.

Table 30. Summary data from Phytoplankton WET bioassay. T=0 samples collected September 19th, 2008. T=10 Days samples collected September 29th, 2008. Test begum October 4th, 2008. Growth was determined as percentage change in cell count before and after grow-out

Untreated, T=0h	**Phytoplankton Cell Counts**				
	Before Grow-out	**72h**	**After 96h Grow-out Mean ± S.D. 5 reps**	**% T. Iso @ 96h/0h**	**% growth relative to controls @ 96h**
6.25%	251,697	1066547	1,227,844±315,040	488	72.21
12.50%	249,546	1195623	1,597,287±366,670	640	93.94
25%	255,761	1369875	1,511,954±379,578	591	88.92
50%	249,546	1647627	2,207,671±349,221	885	129.84
100%	270,820	1015395	1,486,712±243,092	549	87.44
Treated, T=0h					
6.25%	266,517	1698904	2,112,251±253,418	793	124.22
12.50%	268,908	1726984	2,171,482±267,665	808	127.71
25%	259,107	1497038	1,631,086±230,473	630	95.93
50%	248,112	953725	1,173,489±235,826	473	69.01
100%	245,722	1026390	1,127,643±176,926	459	66.32
Untreated, T=10 Days					
6.25%	244,527	1111246	2,057,322±324,840	841	120.99
12.50%	256,956	1164310	1,961,950±144,565	764	115.38
25%	253,371	1318245	1,908,216±301,344	753	112.22
50%	248,351	1135627	1,429,680±470,361	576	84.08
100%	266,995	924086	1,028,255±84,855	385	60.47
Treated, T=10 Days					
6.25%	261,498	1377285	1,861,175±337,341	712	109.46
12.50%	248,829	1357684	1,973,662±128,668	793	116.07
25%	268,430	1109333	1,596,570±290,540	595	93.9
50%	263,171	1105270	1,553,019±223,125	590	91.33
100%	247,634	998663	1,363,134±55,263	550	80.17
Lab Controls	256,275	1214745	1,700,356	675	

SUMMARY CONCLUSIONS.

In conducting these trials investigators were aware of several different requirements and interests that guided the testing timetable and other procedures. For example, under IMO G-8 guidelines there is a requirement to perform three tests, the first and last of which should be separated by a period of not less than six months, in order to provide a seasonal dimension to the testing regime. Under U.S. Coast Guard Shipboard Technology Evaluation Program (STEP) requirements, two tests should be conducted in two different water bodies in which the vessel normally operates. These requirements were met by conducting these three trials between April 5th and October 6th, and by testing water picked up respectively in the Caribbean Sea, the North West Pacific off Alaska, and the Western Pacific off Long Beach. The distributors of the system, Hyde Marine Inc. expressed an interesting in varying the retention time of ballast water in the tanks in order to investigate any possible latent effects of UV irradiation. This was accommodated by varying the retention times by as much as 6 days: 96h in Trial 1, 114h in Trial 2 and 10 days in Trial 3. Actual ballasting times were at the discretion of the officers and crew of the vessel, according to their schedule and other logistics.

In all three trials the numbers of live zooplankton >50µm (narrowest dimension) complied with all published ballast water treatment regulations, in that no live zooplankton in this size class were seen at the time of ballast water discharge. While some smaller taxa survived treatment, e.g. marine nematodes, these were very much narrower than 50µm. In making live/dead assessment investigators relied on movement of some part of the organism to confirm viability, although microscopic examination of this group was aided by the vital stain neutral red, which greatly facilitated the location of potentially live organisms in the microscope field of view. Trial 2 was characterized by a large amount of detritus appearing in samples collected during the de-ballasting operation close to the end of the cruise. Several large organisms were present in samples from both treated and untreated tanks, although these were dead in treated samples. These are discussed later in the context of bacterial counts.

Planktonic organisms in the >10µm - <50µm represent, in several respects, the most problematic of all the endpoints measured. This size range primarily, but not exclusively, consists of phytoplankton, and is subject to standards that differ by as much as three orders of magnitude among jurisdictions (table 1). Natural populations of phytoplankton may have an even greater

concentration range among different water bodies, and cell densities encountered during these trials demonstrated 100-fold differences between Caribbean waters and Alaskan waters. Alaskan water (trial 2) showed the highest densities encountered throughout these trials (ca. 10^8 cells/m^3), although very much higher cell concentrations would not be unusual in Pacific waters. We, therefore, categorize, phytoplankton densities throughout this sequence of trials as moderately low (trial 2) to extremely low (trial 1), with trial 3 intermediate. In view of the fact that most of the groups comprising this size class are non-motile, the primary problem confronting scientific investigation is that of viability. Several discussions of this issue exist (e.g. Wright 2007, Veldhuis et al. 2001, Veldhuis and Brussaard 2006). Further problems concern the fact that some phytoplankton taxa are actually larger than 50µm (Wright 2007) and in many cases a significant proportion of phytoplankton fall into the <10 µm size category as was the case in the current trials.

In all of these trials phytoplankton numbers following treatment (at de-ballasting) were well below the standard of 10^7 live cells/m^3 set by IMO under G-8 guidelines. When compared to the U.S. Coast Guard standard of 10^5 live cells/m^3 (=100,000 live cells/m^3), the grand mean from all three trials (2,222, 75,667, 103,300/3 = 60,398 live cells/m^3) complies with the standard, although the individual value from trial 3 (103,300 live cells/m^3) is marginally higher than the 100,000 live cells/m^3 U.S. Coast Guard standard. Another way of looking at these data takes note of the fact that cell densities following grow-out represent reductions in cell densities of 65%, 60% and 83% for trials 1-3, compared with cell numbers before grow-out. A case can, therefore, be made for assuming that *all* these natural populations of phytoplankton are incapable of growth, and therefore non-viable. A similar result was seen in 2004 trials of the Hyde BWT system aboard *Coral Princess* (Wright et al. 2007), where a comprehensive analysis was made of individual genera/species of phytoplankton in treated and untreated ballast water. In untreated samples 27 out of 43 genera for which data were available showed positive growth rate (average increase after grow-out 1.25), whereas no treated samples exhibited positive growth of any phytoplankton genus (average decline of 75% in cell numbers after grow-out). Several analytical techniques are currently available to differentiate between dead and live, but non-motile cells (Bruussard et al 2001, Veldhuis et al. 2001, Veldhuis and Bruussard 2006), although quantification is difficult within the context of current published standards

Bacterial counts illustrate some of the difficulties involved with conducting trials aboard

working ships where, unlike land-based trials, there is limited ability to control some test parameters. A summary of bacterial counts from all three trials is shown in table 31.

As judged by indicator bacteria, water examined from trials 1 and 3 indicate relatively pristine conditions. Trial 1 showed very low densities of coliforms and Enterococci in control (untreated) samples at T=0, but there was no evidence of these groups in any other samples from this trial. Counts were made of heterotrophic cultural bacteria in all samples. Although they are not regulated by IMO or the U.S. Coast Guard, January 2008 California regulations set a standard of 1000cfu/100ml for this group (Table 1). Cfus for cultural heterotrophic bacteria nominally comply with this standard in all three trials, thereby illustrating the sparse bacterial flora in areas from which ballast water was obtained. Noticeably higher concentrations of culturable bacteria were recorded from treated samples at 114h in trial 2, although the mean figure of 148.3 must be regarded as approximate in view of the '>100cfu' and '>200cfu' designations given the most densely populated plates (Table 18).

Table 31 . Summary of Bacterial endpoints for Trial 1 (April), Trial 2 (July) and Trial 3 (September). Numbers are reported as cfu/100ml.

			Total culturable heterotrophic	**Coliforms**	***E. Coli***	**Enterococci**	***Vibrio cholerae***
Treatment	**Time**	**Trial**					
Control	t=0	1	12.2±12.9	0.2±0	ND	0.2±0	0
Treated	t=0	1	19.4±10.5	ND	ND	ND	0
Control	t=96h	1	5.0±5.7	ND	ND	ND	0
Treated	t=96h	1	1.0±1.7	ND	ND	ND	0
Control	t=0	2	39.6±65.0	ND	ND	ND	0
Treated	t=0	2	21.6±29	ND	ND	ND	0
Control	t=114h	2	94.1±68.3	ND	ND	1.6±1.4	0
Treated	t=114h	2	148.3±64.4	ND	ND	3.4±2.6	0
Control	t=0	3	44.2±44.75	ND	ND	ND	0
Treated	t=0	3	49.1±48.1	ND	ND	ND	0
Control	t=10 days	3	6.8±7.3	0.6±0.53	0.3±0.5	ND	0
Treated	t=10 days	3	2.0±6.0	ND	ND	ND	0

Samples from trial 3 were similar to trial 1. No indicator bacteria were reported in either

treated or untreated samples at T=0, although low numbers of coliforms, including *E. Coli*, appeared in untreated samples at the time of discharge. No indicator bacteria were reported from any treated samples from trial 3. Cultural bacteria were present in both treated and untreated samples at T=0 in trial 3 (Table 31) although, as in trial 1, numbers at the time of discharge were lower than the California standard (table 30), particularly in treated samples.

Bacterial counts from trial 2 differed from the other two trials, and were characterized by higher counts of cultural bacteria at the time of discharge (de-ballasting) and the appearance of intestinal enterococci in both treated and untreated samples at time of de-ballasting, despite the fact that no cfus from this taxa were recorded in the respective T=0 samples. No coliforms were recorded from any samples in this trial. With respect to Enterococci, numbers of cfus in treated samples at de-ballasting, 3.4±2.6 cfu/100ml (table 31), were higher than in untreated samples (1.6±1.4cfu/100 ml.). The figure of 3.4±2.6 cfu/100ml is below the standard of 100cfu/100ml set for this group by IMO and also complies with the corresponding standard of 33 cfu/100ml proposed by the U.S. Coast Guard and adopted by the state of California (table 1). Appearance of intestinal enterococci in both treated and untreated samples at time of de-ballasting, despite no observation of cfus from this taxa in the respective T=0 samples should be viewed within the context of the zooplankton samples, which had high detritus loads and large zooplankton specimens that were not seen in T=0 samples. These included several harpacticoid copepod adults >1000µm (some >2000µm), and indicated a quite different population that that sampled at T=0. Despite the presence of these organisms at the time of de-ballasting, all specimens in the treated samples were dead; some recently, others in varying states of decay. It must be borne in mind that the filtered was turned off at de-ballasting as per normal de-ballasting protocol, and would not have filtered out large organisms during the discharge cycle.

It seems reasonable to conclude that the appearance of Enterococci in samples withdrawn from the tank at discharge would result from the decay/disintegration of these planktonic organisms with the concomitant release of endogenous bacteria that had been shielded from the effect of UV irradiation. In contrast, the corresponding T=0 samples were free from Enterococci and were remarkably "clean", indicating that the filter appeared to be working correctly. Two explanations could explain the presence of large planktonic organisms identified at discharge but not previously seen:

1. An earlier ballasting operation (before trial 2) may not have properly employed the filter, or

2. Small eggs and/or juvenile stages (<50µm) may have passed through a correctly functioning filter rated for a 50µm cut-off, then subsequently grew and formed a live population within the tank.

A similar situation has been observed by this author in previous shipboard trials (Wright et al. 2007 and unpublished). A drawback of conducting shipboard tests, therefore, relates to the fact that it is impossible to know if residual flora/fauna inhabit the tank prior to the onset of a trial. If such a situation exists, there may be significant, extraneous, qualitative and quantitative differences between the discharged water and that characterized by T=0 sampling. Such differences are avoided in land-based testing, where the collection/storage tanks can be vigorously rinsed between trials. In shipboard trials a similar problem relates to the flushing of the, often lengthy, piping and associated dead-space that constitutes the ballasting system.

Current IMO shipboard testing protocols simply require a comparison between treated and untreated water in shipboard trials following a certain defined residence time in the tanks, although there is an additional requirement to characterize the challenge (untreated) water at ballasting (T=0). For a system such as the one tested here, where treatment is not deemed complete until the second pass through the UV system at de-ballasting, it is clear that, from a regulatory standpoint, that the definitive samples should be those collected at discharge. Nevertheless, problems such as those described above illustrate the importance of inline sampling and analysis of a representative number of <u>treated</u> samples as well as untreated samples at T=0, particularly where a filter is involved.

Results of Whole Effluent Toxicity (WET) bioassays conducted during trial 3 indicated no significant differences between the toxicity of treated vs. untreated water samples at the time of discharge from the vessel. In invertebrate and vertebrate larval assays undiluted water from both treated and untreated tanks appeared not to show any significant toxicity relative to laboratory controls. In the case of phytoplankton there appeared to be a small degree of toxicity associated with water retrieved from ballast waters following the (10 day) residence time, although the toxicity did not differ significantly between treated and untreated water. It might be concluded from those data that any toxic element present in discharged water did not result from UV irradiation. Such results have been supported by an extensive literature search that has

revealed no evidence of residual chemical toxicity in water resulting from prior UV irradiation of that water. While some changes in nutrient levels appeared to be associated with water treatment it was not possible to definitively conclude that these changes directly resulted from UV irradiation. For example, both phosphate and nitrate levels at de-ballasting were higher in treated samples relative to untreated samples at the time of discharge from the ship. It is possible that part of this difference could be attributed to an increase in the degradation of planktonic organisms resulting from the treatment process, rather than UV irradiation directly. Nitrite levels were also higher in treated samples 10 days after treatment. While UV photolysis of nitrate to nitrite has been demonstrated in UV-irradiated drinking water (e.g. Sharpless and Linden 2001) the levels involved are very small relative to the U.S. drinking water standard of 1000µg L^{-1}, or the European standard of 100µg L^{-1}. Nitrate levels reported in this trial were negligible relative to these standards and pose no toxicological threat associated with discharge.

Table 32 provides a summary of compliance/non-compliance of the Hyde 'Guardian' BWT system with current published national and international standards. Under the conditions encountered during these trials, the system is seen to comply with all IMO G-8 standards relating to the elimination of biota and with respect the issue of residual toxicity of treated water related to chemicals generated during treatment. A bacterial (Enterococcus) excursion seen in Trial 2 (see table 32 footnote) and minimally exceeding U.S. Coast Guard standards, was interpreted as an artifact related to the presence of a population of large zooplankton living in the tank prior to the test. Several of these organisms were probably alive until UV irradiation on de-ballasting. In view of the fact that no Enterococci were detected in T=0 samples, endogenous bacteria were probably released from dead plankton at de-ballasting.

Problems such as this illustrate one of the drawbacks of shipboard trials, i.e. the problem of not being able to rigorously control all conditions related to the test. Another concerns the relative lack of flexibility in certain cases in determining the exact timing of a ballasting/de-ballasting event. Biological productivity in such cases may be less than optimal. Within these constraints, shipboard trials nevertheless represent a useful and informative exercise providing critical information regarding the performance of a ballast water treatment system under 'real world conditions'. In this case, under the ambient conditions and for the flow rates encountered throughout these trials, the Hyde Marine 'Guardian' system appears to be a highly effective means of treating ballast water to remove potentially invasive species from the ballast stream.

Table 32. A summary of compliance (√) / non-compliance (X) with current published national and international ballast water management standards. NT = Not Tested

	IMO Regulation D-2 and Transport Canada	Ballast Water Management Act Section 1101 (f) i	2008 California Standard	IMO G-9 Discharge Standard for residual Toxicity
Trial 1 Zooplankton	√	√	√	
Trial 1 Phytoplankton	√	√	√	NT
Trial 1 Bacteria	√	√	√	
Trial 2 Zooplankton	√	√	√	
Trial 2 Phytoplankton	√	√	X[2]	NT
Trial 2 Bacteria	√	√	√	
Trial 3 Zooplankton	√	√	√	
Trial 3 Phytoplankton	√	X[1]	X[2]	√
Trial 3 Bacteria	√	√	√	

[1] – 'Live' phytoplankton present in treated samples after 10 days in tank + UV irradiation on de-ballasting. Residuals exceed U.S, C.G. standard by 3% following 83% reduction in cell concentration after grow-out. Such a reduction in cell count following a grow-out period could be interpreted as non-viable (Wright *et al*. 2007; Perrins *et al*. 2006).

[2] – While phytoplankton cells can be scored as 'live' based on morphological characteristics their lack of growth potential determined through cell counts before and after grow-out can be interpreted as 'non-viable', in which case California regulations would be met.

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Appendix A. Raw zooplankton counts. *Coral Princess*.

Trial 1

CORAL PRINCESS BALLAST WATER TRIAL #1 APR 5-15 2008 (updated 10/08/2008)
CONSOLIDATED SPECIES LIST

GROUP	SPECNAME	LIFESTAGE
ACTINULA	ACTINULA	
BIVALVE	BIVALVE	D-HINGE
CHAETOGNATH	CHAETOGNATH	
CLADOCERAN	CLADOCERAN	
COPEPOD	ACARTIA HELGOLANDICA	ADULT
COPEPOD	CALANOID	ADULT
COPEPOD	CALANOID	COPEPODITE
COPEPOD	CANDACEA	ADULT
COPEPOD	CENTROPAGES HAMATUS	ADULT
COPEPOD	CLAUSOCALANUS	ADULT
COPEPOD	COPEPOD	ADULT
COPEPOD	COPEPOD	COPEPODITE
COPEPOD	CORYCAEUS	
COPEPOD	CORYCAEUS	ADULT
COPEPOD	CORYCAEUS	COPEPODITE
COPEPOD	CYCLOPOID	COPEPODITE
COPEPOD	FARANULA CARINATA	ADULT
COPEPOD	HALICYCLOPS	ADULT
COPEPOD	HALICYCLOPS	COPEPODITE
COPEPOD	HARPACTICOID	
COPEPOD	HARPACTICOID	ADULT
COPEPOD	HARPACTICOID	UNDETERMINED
COPEPOD	LABIDOCERA	ADULT
COPEPOD	MACROSETELLA	
COPEPOD	MACROSETELLA	ADULT
COPEPOD	MICROSETELLA	ADULT
COPEPOD	OITHONA	ADULT
COPEPOD	OITHONA	COPEPODITE
COPEPOD	ONCAEA	
COPEPOD	ONCAEA	ADULT
COPEPOD	ONCAEA-POPEYE	ADULT
COPEPOD	PARACALANUS	ADULT
COPEPOD	SAPHIRELLA	COPEPODITE
COPEPOD	TEMORA	UNDETERMINED
COPEPOD NAUPLII	COPEPOD	NAUPLIUS
EGGS	EGGS	
FORAMINIFERA	GLOBIGERINA	
GASTROPOD	GASTROPOD	
NEMATODE	NEMATODE	
NOZOO	NOZOOPLANKTON	
POLYCHAETE	POLYCHAETE	
POLYCHAETE	POLYCHAETE	LARVAE
PROTOZOAN	PROTOZOAN	
TINTINNID	TINTINNID	

CORAL PRINCESS BALLAST WATER TRIAL #1 (CARIBBEAN SEA) APR 5-15 2008 (updated 10/08/2008)

Event	Treatment	Rep	Group	Num. Alive	Num. Dead	Total	Total alive	Total dead	Grand total
T0									
	CONTROL	1	COPEPOD	328	136	464			
		1	COPEPOD NAUP	72	0	72			
		1	EGGS	312	0	312			
		1	NEMATODE	8	0	8			
		1	POLYCHAETE	48	0	48	768	136	904
		2	CLADOCERAN	16	0	16			
		2	COPEPOD	128	24	152			
		2	COPEPOD NAUP	168	8	176			
		2	EGGS	224	104	328			
		2	POLYCHAETE	80	0	80	616	136	752
		3	BIVALVE	16	0	16			
		3	COPEPOD	104	40	144			
		3	COPEPOD NAUP	24	0	24			
		3	TINTINNID	96	0	96	240	40	280
		4	COPEPOD	32	0	32			
		4	COPEPOD NAUP	64	0	64			
		4	EGGS	88	0	88			
		4	POLYCHAETE	16	0	16			
		4	TINTINNID	112	0	112	312	0	312
		5	BIVALVE	32	8	40			
		5	COPEPOD	40	0	40			
		5	COPEPOD NAUP	80	8	88			
		5	EGGS	368	0	368			
		5	NEMATODE	8	0	8			
		5	POLYCHAETE	24	0	24	552	16	568
	TREATED	1	COPEPOD	16	0	16			
		1	TINTINNID	0	8	8	16	8	24
		2	COPEPOD	8	0	8			
		2	POLYCHAETE	0	8	8	8	8	16
		3	NOZOO	0	0	0	0	0	0
		4	NOZOO	0	0	0	0	0	0
		5	COPEPOD	16	0	16	16	0	16
T96									
	CONTROL	1	BIVALVE	8	0	8			
		1	COPEPOD	40	104	144			
		1	COPEPOD NAUP	24	8	32			
		1	EGGS	384	0	384			
		1	POLYCHAETE	8	0	8	464	112	576
		2	BIVALVE	8	8	16			
		2	COPEPOD	48	72	120			
		2	EGGS	16	0	16	72	80	152
		3	BIVALVE	16	8	24			

CORAL PRINCESS BALLAST WATER TRIAL #1 (CARIBBEAN SEA) APR 5-15 2008 (updated 10/08/2008)

Event	Treatment	Rep	Group	Num. Alive	Num. Dead	Total	Total alive	Total dead	Grand total
T96		3	COPEPOD	24	272	296			
		3	COPEPOD NAUP	24	0	24			
		3	EGGS	624	240	864			
		3	POLYCHAETE	64	0	64			
		3	PROTOZOAN	8	0	8			
		3	TINTINNID	16	0	16	776	520	1296
		4	BIVALVE	8	8	16			
		4	COPEPOD	8	88	96			
		4	EGGS	32	0	32	48	96	144
		5	BIVALVE	16	8	24			
		5	COPEPOD	128	80	208			
		5	COPEPOD NAUP	0	8	8			
		5	POLYCHAETE	8	0	8			
		5	TINTINNID	8	0	8	160	96	256
		6	CHAETOGNATH	0	8	8			
		6	COPEPOD	0	328	328			
		6	COPEPOD NAUP	680	344	1024			
		6	FORAMINIFERA	8	0	8			
		6	POLYCHAETE	24	0	24			
		6	TINTINNID	0	24	24	712	704	1416
		7	CHAETOGNATH	16	8	24			
		7	COPEPOD	0	344	344			
		7	COPEPOD NAUP	584	440	1024			
		7	EGGS	0	8	8			
		7	NEMATODE	24	0	24			
		7	POLYCHAETE	16	0	16			
		7	TINTINNID	0	8	8	640	808	1448
		8	BIVALVE	8	8	16			
		8	CHAETOGNATH	24	8	32			
		8	COPEPOD	80	648	728			
		8	COPEPOD NAUP	552	752	1304			
		8	EGGS	0	40	40			
		8	FORAMINIFERA	16	0	16			
		8	POLYCHAETE	32	8	40			
		8	TINTINNID	0	32	32	712	1496	2208
		9	CHAETOGNATH	8	0	8			
		9	COPEPOD	8	672	680			
		9	COPEPOD NAUP	856	568	1424			
		9	EGGS	0	8	8			
		9	FORAMINIFERA	16	0	16			
		9	POLYCHAETE	48	16	64			
		9	TINTINNID	0	16	16	936	1280	2216

CORAL PRINCESS BALLAST WATER TRIAL #1 (CARIBBEAN SEA) APR 5-15 2008 (updated 10/08/2008)

Event	Treatment	Rep	Group	Num. Alive	Num. Dead	Total	Total alive	Total dead	Grand total
T96	TREATED	1	ACTINULA	0	16	16			
		1	BIVALVE	0	8	8			
		1	COPEPOD	0	104	104			
		1	GASTROPOD	0	8	8			
		1	NEMATODE	0	16	16	0	152	152
		2	ACTINULA	0	16	16			
		2	COPEPOD	0	80	80	0	96	96
		3	COPEPOD	0	128	128			
		3	COPEPOD NAUP	0	8	8			
		3	TINTINNID	0	8	8	0	144	144
		4	COPEPOD	0	176	176			
		4	COPEPOD NAUP	0	8	8			
		4	FORAMINIFERA	0	8	8	0	192	192
		5	COPEPOD	0	184	184	0	184	184
		6	COPEPOD	0	16	16	0	16	16
		7	COPEPOD	0	64	64			
		7	COPEPOD NAUP	0	16	16	0	80	80
		8	COPEPOD	0	144	144	0	144	144
		9	NOZOO	0	0	0	0	0	0

Trial 2

CORAL PRINCESS BALLAST WATER TRIAL #2 JUN 30-JUL 7 2008 (updated 10/08/2008)
CONSOLIDATED SPECIES LIST

GROUP	SPECNAME	LIFESTAGE
AMPHIPOD	HYPERIA SP	ADULT
B?	B?	
BIVALVE	BIVALVE	LARVA
BIVALVE	BIVALVE	UMBO
COPEPOD	CALANOID	
COPEPOD	CALANOID	ADULT
COPEPOD	CALANOID	COPEPODITE
COPEPOD	CALANOID2	ADULT
COPEPOD	CALANOID3	ADULT
COPEPOD	COPEPOD	EGG
COPEPOD	OITHONA	ADULT
COPEPOD	OITHONA	COPEPODITE
COPEPOD	ONCAEA	ADULT
COPEPOD NAUPLIUS	COPEPOD	NAUPLIUS
DECAPOD	SHRIMP	POSTLARVA
EGGS	COPEPOD	EGG
EGGS	EGG	DARK
EGGS	EGG	GREEN
EGGS	EGG	MASS
EGGS	FISH EGGS	
FORAMINIFERA	GLOBEGERINA	
GASTROPOD	LAMACINA	
GASTROPOD	SNAIL	
NEMATODE	NEMATODE	
NOZOO	NOZOOPLANKTON	
POLYCHAETE	POLYCHAETE	LARVA
PROTOZOAN	PROTOZOAN	
ROTIFER	ROTIFER	
TINTINNID	TINNINNID	V-SHAPE

CORAL PRINCESS BALLAST WATER TRIAL #2 ALASKAN COAST JUN 30-JUL 7 2008 (as of 10/08/2008)

Event	Treatment	Rep	Group	Total*	Num* Alive*	Num Dead*	Sample grand total*	Sample total alive*	Sample total dead*
T0	CONTROL	1	B?	16	16	0			
			COPE NAUPLII	2992	2944	48			
			COPEPOD	4136	3992	144			
			EGGS	48	48	0			
			POLYCHAETE	8	8	0			
							7200	7008	192
		2	BIVALVE	40	40	0			
			COPE NAUPLII	7100	6880	220			
			COPEPOD	13220	12840	380			
			DECAPOD	40	40	0			
							20400	19800	600
		3	B?	48	48	0			
			COPE NAUPLII	2064	2064	0			
			COPEPOD	2928	2928	0			
			EGGS	208	208	0			
			PROTOZOA	32	32	0			
							5280	5280	0
		4	COPE NAUPLII	11504	11472	32			
			COPEPOD	7808	7760	48			
							19312	19232	80
		5	COPE NAUPLII	13600	13536	64			
			COPEPOD	9600	9536	64			
			DECAPOD	32	32	0			
			EGGS	320	320	0			
							23552	23424	128
		6	AMPHIPOD	64	64	0			
			BIVALVE	32	32	0			
			COPE NAUPLII	10976	10976	0			
			COPEPOD	6912	6720	192			
			DECAPOD	32	32	0			
			EGGS	576	576	0			
			GASTROPOD	32	32	0			
							18624	18432	192
		7	BIVALVE	32	32	0			
			COPE NAUPLII	8144	8144	0			
			COPEPOD	11712	11712	0			
			EGGS	176	176	0			
							20064	20064	0
		8	COPE NAUPLII	11584	11584	0			
			COPEPOD	3952	3872	80			
			EGGS	128	128	0			
			GASTROPOD	16	16	0			
							15680	15600	80
		9	COPE NAUPLII	4464	4464	0			
			COPEPOD	4656	4656	0			
			EGGS	368	368	0			
			PROTOZOA	32	32	0			
							9520	9520	0
T0	TREATED	1					0	0	0
		2					0	0	0
		3					0	0	0

CORAL PRINCESS BALLAST WATER TRIAL #2 ALASKAN COAST JUN 30-JUL 7 2008 (as of 10/08/2008)

Event	Treatment	Rep	Group	Total*	Num* Alive*	Num Dead*	Sample grand total*	Sample total alive*	Sample total dead*
		4					0	0	0
		5					0	0	0
		6	COPEPOD	8	8	0			
							8	8	0
		7					0	0	0
T114	CONTROL	1	COPE NAUPLII	8	8	0			
			COPEPOD	192	192	0			
							200	200	0
		2	COPE NAUPLII	160	160	0			
			COPEPOD	240	240	0			
			EGGS	16	16	0			
							416	416	0
		3	COPE NAUPLII	256	256	0			
			COPEPOD	624	624	0			
			EGGS	16	16	0			
							896	896	0
		4	COPE NAUPLII	504	336	168			
			COPEPOD	1224	808	416			
			EGGS	40	40	0			
			POLYCHAETE	16	8	8			
			PROTOZOA	48	24	24			
			ROTIFERA	32	16	16			
							1864	1232	632
		5	COPE NAUPLII	704	32	672			
			COPEPOD	1120	96	1024			
			GASTROPOD	32	32	0			
							1856	160	1696
		6	COPE NAUPLII	544	0	544			
			COPEPOD	544	0	544			
			EGGS	32	0	32			
							1120	0	1120
		7	COPE NAUPLII	416	0	416			
			COPEPOD	896	0	896			
			EGGS	128	0	128			
			NEMATODE	32	0	32			
							1472	0	1472
		8	COPE NAUPLII	512	0	512			
			COPEPOD	704	64	640			
			EGGS	64	0	64			
			GASTROPOD	96	64	32			
							1376	128	1248
		9	COPE NAUPLII	32	0	32			
			COPEPOD	192	0	192			
							224	0	224

CORAL PRINCESS BALLAST WATER TRIAL #2 ALASKAN COAST JUN 30-JUL 7 2008 (as of 10/08/2008)

Event	Treatment	Rep	Group	Total*	Num* Alive*	Num Dead*	Sample grand total*	Sample total alive*	Sample total dead*
T114	TREATED	1	COPEPOD	56	0	56			
							56	0	56
		2	COPEPOD	96	0	96			
							96	0	96
		3	COPE NAUPLII	8	0	8			
			COPEPOD	64	0	64			
							72	0	72
		4	COPE NAUPLII	16	0	16			
			COPEPOD	24	0	24			
							40	0	40
		5	COPE NAUPLII	8	0	8			
			COPEPOD	32	0	32			
			NEMATODE	16	16	0			
							56	16	40
		6	BIVALVE	48	0	48			
			COPE NAUPLII	80	0	80			
			COPEPOD	240	0	240			
			EGGS	64	0	64			
			FORAMINIFERA	16	0	16			
			NEMATODE	272	64	208			
			PROTOZOA	16	16	0			
			TINTINNID	32	0	32			
							768	80	688
		7	COPE NAUPLII	80	0	80			
			COPEPOD	320	0	320			
			FORAMINIFERA	16	0	16			
			NEMATODE	192	80	112			
							608	80	528
		8	COPE NAUPLII	32	0	32			
			COPEPOD	224	0	224			
			GASTROPOD	80	0	80			
			NEMATODE	336	128	208			
							672	128	544
		9	COPEPOD	144	0	144			
			EGGS	32	0	32			
			NEMATODE	384	32	352			
							560	32	528

*Density expressed as numbers per ton of water.

CORAL PRINCESS BALLAST WATER TRIAL #2 ALASKAN COAST JUN 30-JUL 7 2008 (as of (as of 10/08/2008)

ANALYST=MARCIA. NUM DEAD=DEAD PINK + DEAD CLEAR

Event	Treatment	Rep	Group	Num Alive*	Num Dead*	Dead pink	Dead clear
T0	CONTROL	4	COPE NAUPLII	11472	32	.	.
			COPEPOD	7760	48	.	.
		5	COPE NAUPLII	13536	64	.	.
			COPEPOD	9536	64	.	.
			DECAPOD	32	0	.	.
			EGGS	320	0	.	.
		6	AMPHIPOD	64	0	.	.
			BIVALVE	32	0	.	.
			COPE NAUPLII	10976	0	.	.
			COPEPOD	6720	192	.	.
			DECAPOD	32	0	.	.
			EGGS	576	0	.	.
			GASTROPOD	32	0	.	.
		8	COPE NAUPLII	11584	0	.	.
			COPEPOD	3872	80	.	.
			EGGS	128	0	.	.
			GASTROPOD	16	0	.	.
T0	TREATED	3					
		4					
		5					
		6	COPEPOD	8	0	.	.
T114	CONTROL	5	COPE NAUPLII	32	672	672	0
			COPEPOD	96	1024	928	96
			GASTROPOD	32	0	0	0
		6	COPE NAUPLII	0	544	544	0
			COPEPOD	0	544	480	64
			EGGS	0	32	0	32
		7	COPE NAUPLII	0	416	416	0
			COPEPOD	0	896	896	0
			EGGS	0	128	0	128
			NEMATODE	0	32	0	32
		8	COPE NAUPLII	0	512	512	0
			COPEPOD	64	640	640	0
			EGGS	0	64	0	64
			GASTROPOD	64	32	32	0
		9	COPE NAUPLII	0	32	32	0
			COPEPOD	0	192	192	0
T114	TREATED	6	BIVALVE	0	48	0	48
			COPE NAUPLII	0	80	80	0
			COPEPOD	0	240	240	0
			EGGS	0	64	0	64
			FORAMINIFERA	0	16	0	16
			NEMATODE	64	208	64	144
			PROTOZOA	16	0	0	0
			TINTINNID	0	32	0	32
		7	COPE NAUPLII	0	80	80	0
			COPEPOD	0	320	48	272
			FORAMINIFERA	0	16	0	16
			NEMATODE	80	112	32	80

CORAL PRINCESS BALLAST WATER TRIAL #2 ALASKAN COAST JUN 30-JUL 7 2008 (as of (as of 10/08/2008)

ANALYST=MARCIA. NUM DEAD=DEAD PINK + DEAD CLEAR

Event	Treatment	Rep	Group	Num Alive*	Num Dead*	Dead pink	Dead clear
		8	COPE NAUPLII	0	32	0	32
			COPEPOD	0	224	96	176
			GASTROPOD	0	80	0	80
			NEMATODE	128	208	32	176
		9	COPEPOD	0	144	0	144
			EGGS	0	32	0	32
			NEMATODE	32	352	288	64

*Density expressed as numbers per ton of water.

Trial 3

CORAL PRINCESS BALLAST WATER TRIAL #3 SEP 19-29 2008 (updated 10/08/2008)

CONSOLIDATED SPECIES LIST

GROUP	SPECNAME	LIFESTAGE
BARNACLE	BARNACLE	CYPRIS STAGE
BARNACLE	BARNACLE	NAUPLIUS
BIVALVE	BIVALVE	D-HINGE
COPEPOD	CALANOID	COPEPODITE
COPEPOD	CALANOID4	ADULT
COPEPOD	CALANOID5	ADULT
COPEPOD	CALANOID5	COPEPODITE
COPEPOD	COPEPOD	ADULT
COPEPOD	COPEPOD	COPEPODITE
COPEPOD	CORYCEUS	ADULT
COPEPOD	CYCLOPOID	ADULT
COPEPOD	CYCLOPOID1	ADULT
COPEPOD	CYCLOPOID1	COPEPODITE
COPEPOD	CYCLOPOID2	COPEPODITE
COPEPOD	HARPACTICOID	ADULT
COPEPOD	HARPACTICOID	COPEPODITE
COPEPOD	SAPHIRELLA	COPEPODITE
COPEPOD NAUPLIUS	COPEPOD	NAUPLIUS
DECAPOD	SHRIMP	POST LARVA
ECHINODERMATA	STARFISH	LARVA
NEMATODE	NEMATODE	
NOZOO	NOZOOPLANKTON	
POLYCHAETE	POLYCHAETE	
POLYCHAETE	POLYCHAETE	ADULT
POLYCHAETE	POLYCHAETE	LARVA
POLYCHAETE	POLYCHAETE	TROCHOPHORE
ROTIFER	ROTIFER	

CORAL PRINCESS BALLAST WATER TRIAL #3 PACIFIC COAST SEP 19-29 2008 (as of 10/08/2008)

Event	Treatment	Rep	Group	Total*	Num* Alive*	Num Dead*	Sample grand total*	Sample total alive*	Sample total dead*
T0	CONTROL	1	BIVALVE	16	16	0			
			COPEPOD	200	160	40			
			COPEPOD NAUPLII	3696	3216	480			
			NEMATODE	8	8	0			
			POLYCHAETE	24	24	0			
							3944	3424	520
		2	COPEPOD	32	32	0			
			COPEPOD NAUPLII	1832	1776	56			
			NEMATODE	8	8	0			
							1872	1816	56
		3	COPEPOD	32	32	0			
			COPEPOD NAUPLII	1608	1600	8			
			NEMATODE	8	8	0			
							1648	1640	8
		4	COPEPOD	72	64	8			
			COPEPOD NAUPLII	1096	1024	72			
			POLYCHAETE	24	24	0			
							1192	1112	80
		5	COPEPOD	16	16	0			
			COPEPOD NAUPLII	736	736	0			
			ROTIFERA	16	16	0			
							768	768	0
		6	COPEPOD	184	184	0			
			COPEPOD NAUPLII	1064	1016	48			
			NEMATODE	8	8	0			
			POLYCHAETE	8	8	0			
							1264	1216	48
		7	COPEPOD	232	232	0			
			COPEPOD NAUPLII	1448	1440	8			
			NEMATODE	8	8	0			
							1688	1680	8
		8	BIVALVE	8	8	0			
			COPEPOD	72	72	0			
			COPEPOD NAUPLII	224	184	40			
			POLYCHAETE	8	8	0			
							312	272	40
		9	COPEPOD	138	138	0			
			COPEPOD NAUPLII	492	492	0			
			ECHINODERMATA	15	15	0			
			NEMATODE	15	15	0			
							662	662	0
T0	TREATED	1	COPEPOD	8	0	8			
			COPEPOD NAUPLII	8	0	8			
							16	0	16
		2					0	0	0
		3					0	0	0
		4	COPEPOD NAUPLII	16	0	16			
							16	0	16
		5	BIVALVE	8	0	8			

CORAL PRINCESS BALLAST WATER TRIAL #3 PACIFIC COAST SEP 19-29 2008 (as of 10/08/2008)

Event	Treatment	Rep	Group	Total*	Num* Alive*	Num Dead*	Sample grand total*	Sample total alive*	Sample total dead*
			COPEPOD NAUPLII	40	0	40			
							48	0	48
		6					0	0	0
		7					0	0	0
		8					0	0	0
		9					0	0	0
T240	CONTROL	1	COPEPOD	32	16	16			
			POLYCHAETE	8	8	0			
							40	24	16
		2	COPEPOD	48	16	32			
			POLYCHAETE	8	8	0			
							56	24	32
		3	COPEPOD	72	24	48			
							72	24	48
		4	BARNACLE	8	8	0			
			DECAPOD	8	8	0			
							16	16	0
		5	COPEPOD	48	8	40			
							48	8	40
		6					0	0	0
		7	BIVALVE	8	0	8			
			COPEPOD	8	0	8			
			COPEPOD NAUPLII	8	8	0			
							24	8	16
		8	BARNACLE	8	8	0			
			COPEPOD	24	16	8			
			COPEPOD NAUPLII	16	16	0			
			ECHINODERMATA	8	0	8			
			POLYCHAETE	8	0	8			
							64	40	24
		9					0	0	0
T240	TREATED	1					0	0	0
		2					0	0	0
		3					0	0	0
		4	BIVALVE	8	0	8			
							8	0	8
		5					0	0	0
		6					0	0	0
		7	BARNACLE	8	0	8			

CORAL PRINCESS BALLAST WATER TRIAL #3 CARIBBEAN SEP 19-29 2008 (as of 10/08/2008)

Event	Treatment	Rep	Group	Total*	Num* Alive*	Num Dead*	Sample grand total*	Sample total alive*	Sample total dead*
			NEMATODE	32	0	32			
							40	0	40
		8	NEMATODE	8	8	0			
							8	8	0
		9	NEMATODE	8	0	8			
							8	0	8

*Density expressed as numbers per ton of water.

CORAL PRINCESS BALLAST WATER TRIAL #3 PACIFIC COAST SEP 19-29 2008 (as of 10/08/2008)
ANALYST=MARCIA. NUM DEAD=DEAD PINK + DEAD CLEAR

Event	Treatment	Rep	Group	Num Alive*	Num Dead*	Dead pink	Dead clear
T0	CONTROL	1	BIVALVE	16	0	.	.
			COPEPOD	160	40	24	16
			COPEPOD NAUPLII	3216	480	280	200
			NEMATODE	8	0	.	.
			POLYCHAETE	24	0	.	.
		2	COPEPOD	32	0	.	.
			COPEPOD NAUPLII	1776	56	0	56
			NEMATODE	8	0	.	.
		3	COPEPOD	32	0	.	.
			COPEPOD NAUPLII	1600	8	0	8
			NEMATODE	8	0	.	.
T0	TREATED	1	COPEPOD	0	8	0	8
			COPEPOD NAUPLII	0	8	0	8
		2					
		3					
		4	COPEPOD NAUPLII	0	16	0	16
		5	BIVALVE	0	8	.	.
			COPEPOD NAUPLII	0	40	0	40
		6					
T240	CONTROL	7	BIVALVE	0	8	0	8
			COPEPOD	0	8	0	8
			COPEPOD NAUPLII	8	0	.	.
		8	BARNACLE	8	0	.	.
			COPEPOD	16	8	8	0
			COPEPOD NAUPLII	16	0	.	.
			ECHINODERMATA	0	8	0	8
			POLYCHAETE	0	8	8	0
		9					
T240	TREATED	3					
		4	BIVALVE	0	8	.	8
		5					
		6					
		7	BARNACLE	0	8	.	8
			NEMATODE	0	32	24	8
		8	NEMATODE	8	0	.	.
		9	NEMATODE	0	8	8	0

*Density expressed as numbers per ton of water.

Appendix B.

Bacterial Sampling Protocol for Ballast Water testing (Trial 3) showing mold counts not included in culturable bacteria counts.

(1) General sample details:

Name : Carys Mitchelmore
Date of test : 19th September 2008
Test ID : Princess Cruise/Hyde Maine – trial 3, T=0
Type of sample : Ballasting of treated and control tanks; 3 samples each tank; early, mid and end samples (n=3 each time point; n=9 total each tank).
Time and details of sample collection :
Three sets (A-C) of 55ml sterile PP tubes filled from water outlet for collection, stored in cold cooler once collected.

(1) Treated tank; : Start of collection: 11.40am (in cold cooler)
: Number of samples: 9
: Id of samples: T1, T2, T3….through T9 (start-end time in order)
: End of collection: 12.00pm

(2) Control tank: : Start of collection: 12.05pm (in cold cooler)
: Number of samples: 9
: Id of samples: C1, C2, C3….through C9 (start-end time in order)
: End of collection: 12.25pm

Sample processing/storage:

Samples processed immediately after sampling for Idexx : Coliform/*E.coli*/Enterococci (Tube set A), tube set A placed in fridge after processing. Other samples (Tubes set B and C) stored in fridge until processing <24hr (see specific tests).

(2) Idexx Tests:

(1) Coliform / E.coli (colilert 18)

Sample ID: Trial 3, T=0
Date: 19th September 2008 samples collected
Storage: N/A; samples processed immediately
Time: 15.40-18.10; processed Treated first (9 samples T1-T9), then control samples (9 samples C1-C9). Distilled water used as control.
Protocol: As per standard Idexx method.

Specific details: 90ml of DI water placed in sterile Idexx bottles. 10ml of test water (at RT) added (one bottle per sample. Vial opened and added to water, mixed till dissolved. Samples placed in trays and tray sealer used. Specific times made noted. Treated samples processed first then control samples. Samples read at 18 hours and later (up to 22 hours), scored for coliform (yellow wells) and E.coli (blue fluorescence). Positive wells scored as (large; L or small; S). After 22 hours tests are invalid.

Incubation times: Treated samples placed in incubator at 17.15 at 35°C (19th Sept 2008)
Control samples placed in incubator at 18.10pm at 35°C (19th Sept 2008)
Read times: Treated samples read at 11.15 (20th Sept 2008) – 18 hours
Control samples read at 12.00 (20th Sept 2008) ~ 18 hours

Sample ID	Coliform counts		MPN Coliform	E.coli counts		MPN E.coli
	Large	Small		Large	Small	
T1	0	0	0	0	0	0
T2	0	0	0	0	0	0
T3	0	0	0	0	0	0
T4	0	0	0	0	0	0
T5	0	0	0	0	0	0
T6	0	0	0	0	0	0
T7	0	0	0	0	0	0
T8	0	0	0	0	0	0
T9	0	0	0	0	0	0
C1	0	0	0	0	0	0
C2	0	0	0	0	0	0
C3	0	0	0	0	0	0
C4	0	0	0	0	0	0
C5	0	0	0	0	0	0
C6	0	0	0	0	0	0
C7	0	0	0	0	0	0
C8	0	0	0	0	0	0
C9	0	0	0	0	0	0
DI water	0	0	0	0	0	0

Additional notes:
Samples also scored at 23.20; C3 positive coliform (11B, 14S) (but this was at 28 hours!)

(2) Idexx Tests:

(2) Enterobacteriacae (Enterolert)

Sample ID: Trial 3, T=0
Date: 19th September 2008; sample collection
Storage: N/A; processed immediately
Time: 17.40-18.50; processed Treated first (9 samples T1-T9), then control samples (9 samples C1-C9). Distilled water used as control.
Protocol: As per standard Idexx method.

Specific details: 90ml of DI water placed in sterile Idexx bottles. 10ml of test water (at RT) added (one bottle per sample). Vial opened and added to water, mixed till dissolved. Samples placed in trays and tray sealer used. Specific times made noted. Treated samples processed first then control samples. Samples read at 24 hours and later (up to 28 hours), scored for Enterobacteriacea (blue fluorescence). Positive wells scored as (large; L or small; S). After 28 hours tests are invalid.

Incubation times: Treated samples placed in incubator at 18.30pm at 41C (19th Sept 2008)
Control samples placed in incubator at 18.55pm at 41C (19th Sept 2008)
Read times: Treated samples read at 20.10pm (20th Sept 2008) ~ 25 hours
Control samples read at 20.30pm (20th Sept 2008) ~ 25 hours

Sample ID	Entero. counts		MPN Entero.
	Large	Small	
T1	0	0	0
T2	0	0	0
T3	0	0	0
T4	0	0	0
T5	0	0	0
T6	0	0	0
T7	0	0	0
T8	0	0	0
T9	0	0	0
C1	0	0	0
C2	0	0	0
C3	0	0	0
C4	0	0	0
C5	0	0	0
C6	0	0	0
C7	0	0	0
C8	0	0	0
C9	0	0	0
DI water	0	0	0

Additional notes:
Samples also scored at 23.30pm; no positives (but this was 29 hours).

(4) *Vibrio cholera* sample tests:

Sample ID: Trial 3, T=0
Date: 19th September 2008 (sample collection)
Storage: Samples stored at 4C until processing (on September 20th 2008).
Time: 16.00-17.40; processed Treated first (9 samples T1-T9), then control samples (9 samples C1-C9). Distilled water used as control.
Protocol: As per standard methods.

Specific details: 100ml of each sample filtered onto 0.2um filters. Filters folded onto themselves in half, then half again. Placed in foil and frozen. Stored at -20°C until processing. Filters at 4°C until freezing on September 21st 2008.

(4) Aerobic (culturable bacteria) sample tests:

Sample ID: Trial 3, T=0
Date: 19th September 2008 (sample collection)
Storage: Samples stored at 4°C until processing (on September 20th 2008).
Time: 11.40-14.40; processed Treated first (9 samples T1-T9), then control samples (9 samples C1-C9). Distilled water used as control.

Protocol: As per standard plate counting methods. Four plates used per sample. Four dilutions / volumes used; 25ml (A), 10ml (B), 1ml (C) and 0.1ml (D) of sample per plate. Dilutions prepared as follows ; for 1ml volume (C), 1.1ml of sample and 9.9ml of DI. For 0.1ml sample (D) use 1ml of sample (C) and 9ml of DI (1:10 dilution). Blackfield 0.45um filters placed on vacuum manifold and 25ml or 10ml samples slowly filtered onto filters. Filters placed in 47mm sterile plates, with pads containing (soaked in) 2ml of sterile marine broth. Plates placed upside down in humid incubator at 35C. Plates read at 48 hours (or longer times also). Scored for colonies; I note the colored versus clear colonies, also make note of fungi. Score mid-large colonies very tiny ones (which do not grow at longer incubations) do not score, but make note.
Incubation times: Treated samples placed in incubator at 13.00 at 35°C (20th Sept 2008)
Control samples placed in incubator at 14.40 at 35°C (20th Sept 2008)
Read times: Treated samples read at 14.05 (22nd Sept 2008) ~ 49 hours
Control samples read at 14.50pm (22nd Sept 2008) ~ 49 hours

Sample Id	**counts**		**counts**		**Notes**
	25ml	**10ml**	**1ml**	**0.1ml**	
T1	? (m)	10 (m)	2 (m)	0	mold
T2	9 (m)	5 (m)	1	1	mold
T3	? (m)	9 (m)	6	0	mold
T4	4 (m)	2 (m)	1	0	mold
T5	?	22 (m)	4	0	mold
T6	3 (m)	4 (m)	0	0	mold
T7	3 (m)	0 (m)	0	0	mold
T8	23 (m)	10	1	0	mold
T9	?	2	0	0	
		7.11±6.31			
C1	51	28	5	0	
C2	? (m)	17	4	0	
C3	30	17 (m)	3	0	mold
C4	? (m)	22	1	1	
C5	9	27	7	0	V.small-25ml
C6	tntc	88	15	1	
C7	? 2	2 ?	0	0	
C8		97	24	2	

C9	6 (m)	9	1	0	mold
		34.1±32.2			
DI water	0	0	0	0	

2nd Read times: Treated samples read at 23.00 (22nd Sept 2008) ~ 58 hours
Control samples read at 23.30pm (22nd Sept 2008) ~ 58 hours

Sample ID	**counts**		**counts**		**Notes**
	25ml	**10ml**	**1ml**	**0.1ml**	
T1	15 (m)	11 (m)	2 (m)	0	mold
T2	11 (m)	6 (m)	1	1	mold
T3	5 (m)	7	5	0	mold
T4	6 (m)	3 (m)	1	0	mold
T5	30 (m)	37 (m)	3	0	mold
T6	5 (m)	4 (m)	0	0	mold
T7	3 (m)	2 (m)	0	0	mold
T8	44 (m)	12	1	0	mold
T9	?	3 (m)	0	0 (m)	
Mean		8.74±10.6			
C1	86	23	4	0	
C2	61	39 (m)	6	0	
C3	28 (m)	32	3	0	mold
C4	26 ?	32	1	1	v.small-25/10ml
C5	39 ?	38	10	0	v.small- 25/ml
C6	tntc	87	17	1	
C7	? 14	54	0	0	v.small-25ml
C8	164	98	22	2	
C9	15 ? (m)	14	5	0	mold
		46.3±26.9			
DI water	0	0	0	0	

3rd **Read times:** Treated samples read at 09.30 (23rd Sept 2008) ~ 67 hours
Control samples read at 09.30 (23rd Sept 2008) ~ 67 hours

Sample ID	**counts**		**counts**		**Notes**
	25ml	**10ml**	**1ml**	**0.1ml**	
T1	15 (12m)	11 (5m)	2 (1m)	0	mold
T2	14 (8m)	4 (6m)	1	1	mold
T3	? (m)	9 (m)	6	0	mold
T4	4 (m)	2 (m)	1	0	mold
T5	28 (1m)	46 (3m)	4	(2m)	mold
T6	4 (1m)	3 (4m)	0	0	mold
T7	3 (12m)	0 (5m)	0	0	mold
T8	34 (3m)	12 (2m)	1	0	mold
T9	?	2 (2m)	0	0 (1m)	
C1	46?	28	4 (1m)	0	
C2	49 (4m)	18	4	0	
C3	31 (2m)	17 (1m)	3	0	mold
C4	23 (4m)	17 (1m)	2	1	
C5	33	28	12	0	V.small- 25ml
C6	tntc	80 (1m)	19	2	
C7	? 43	46 ?	0	0	
C8	152 (1m)	99	25	2	
C9	13 (1m)	9	9	1	mold
DI water	0	0	0	0	

4th **Read times:** Treated samples read at 17.20 (23rd Sept 2008) ~ 75 hours
Control samples read at 17.50 (23rd Sept 2008) ~ 75 hours
(NB not score prior negative plates)

Sample ID	**counts**		**counts**		**Notes**
	25ml	**10ml**	**1ml**	**0.1ml**	
T1	16 (9m)	12 (5m)	2 (1m)	0	mold
T2	9 (5m)	5 (5m)	1	1	mold
T3	4 (3m)	7	4		mold
T4	6 (3m)	2 (2m)	1	0	mold
T5	24 (1m)	25 (3m)	4		mold
T6	5 (1m)	3 (4m)	0	0	mold
T7	4 (13m)	0 (6m)	0		mold
T8	36 (3m)	12 (2m)	2		mold
T9	?	2 (2m)	0	0 (1m)	
C1	44?	19	4	0	
C2	? (4m)	24	4	0	
C3	33 (2m)	19 (1m)	3	0	mold
C4	20 (4m)	20 (1m)	2	3	
C5	33	28	12	0	V.small- 25ml
C6	tntc	81 (1m)	14 (5m)	2	
C7	? 27	12 ?	0	0	
C8	152 (1m)	99	25	2	
C9	9	8 (1m)	11	1	mold
DI water	0	0	0	0	

DEPARTMENT OF NATURAL RESOURCES

Harmful Algal Blooms Investigation Unit
Maryland Department of Natural Resources
Tawes State Office Building
580 Taylor Avenue
Annapolis
MD 21401

Date : 9 November 2008

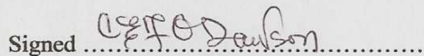
Chain of Custody Letter

Whole Effluent Toxicity Testing of Treated Ballast Water

Four seawater samples were received by the laboratory in a frozen state, October 2nd, 2008. Experiments began October 4th. After thawing to room temperature,(20 degrees centigrade), the average salinity was recorded as 33.1 ppt, the dissolved oxygen levels ranged from 8.3 – 8.9 mg/l and the average pH was 7.8.

Prior to receipt of the samples the test species of phytoplankton (Tahitian Isochrysis) had been maintained in growth phase culture at equivalent salinity, temperature, pH and oxygen levels with adequate nutrients.

Phytoplankton numbers and growth was monitored on the basis of in vivo fluorescence (measured with the hand-held, Turner Designs, Aquafluor fluorescence detector) and microscopy for the following four days and the data analyzed and reported.


Signed ....

Celia E.F. Orano-Dawson
Harmful Algal Blooms
Project Manager

Appendix D.

QA/QC Protocols Related to Shipboard Trials of the Hyde Marine 'Guardian' BWTS, April – October 2008.

The testing and quality assurance procedures described in this document are based on Standard Operating Procedures (SOP) of the University of Maryland Center for Environmental Science, Guidelines of the U.S. Environmental Protection Agency (USEPA) and the American Society for Testing and Materials (ASTM).

Shipboard testing of the Hyde Marine Guardian Ballast Water Treatment System (BWTS) was conducted by a team led by Dr. David Wright, Professor of Environmental Toxicology at the University of Maryland Center for Environmental Science (UMCES), Chesapeake Biological Laboratory, Solomons Maryland 20688, U.S.A. Dr. Wright has more than 20 years research experience with Aquatic Nuisance Species and has conducted more than 15 shipboard trials of a variety of BWTS. The test team and associates for IMO G-8 trials of the Hyde Marine 'Guardian' BWTS is summarized in table 1, below:

Table 1. Shipboard testing team and associates for G-8 shipboard trial of the Hyde Marine 'Guardian' BWTS, April October 2008.

Personnel/Title	Role
David.A. Wright, Professor UMCES.	Project Director,
Dr. Rodger. Dawson, UMCES (ret.). Environmental Research Services.	Consultant Marine Chemist, water quality, nutrient analysis, logistics
Dr. Carys Mitchelmore. Associate Professor, UMCES	Microbiologist, Toxicologist responsible for WET testing (zooplankton)
Jonathon Bearr, PhD student, UMCES	Assistant for microbiology and WET testing (phytoplankton
Marcia Olson MS. Morgan State University, Environmental Research Laboratory, Lusby, MD.	Plankton microscopy, data entry, QA/QC
Celia Orano-Dawson MS. MD Department of Natural Resources, Harmful Algal Bloom Laboratory	Plankton microscopy, data entry. WET testing (phytoplankton)
UMCES Analytical Services Division. Dr. Walter R. Boynton, Professor and Principal Investigator; Carl F. Zimmerman, Advanced Faculty Research Assistant; Carolyn Keefe, Advanced Faculty Research Assistant; Kathy Wood, Advanced Faculty Research Assistant; Nancy Kaumeyer, Advanced Faculty Research Assistant; Adriene Capers, Senior Faculty Research Assistant; Maggie Weir, Advanced Faculty Research Assistant	Nutrient analyses, QA/QC

Objectives and Background.

The objective of the shipboard trials was to measure the biological effectiveness of shipboard filtration/separation and ultraviolet radiation equipment as a ballast water treatment method. The sampling protocol was designed to satisfy IMO G8 requirements, with the objective of comparing biological endpoints to those published under regulation D-2 of the IMO Ballast Water Convention (IMO 2004). Investigators were also aware of other standards set by U.S. federal and state regulatory bodies described below (table 2).

In order to comply with IMO G-8 guidelines for shipboard testing, three trials were conducted aboard the Princess Cruise Lines ship M/V *Coral Princess* between April and October 2008. Samples of treated and untreated ballast water were taken from a customized sampling port mounted downstream of the BWTS in the machinery space of the vessel. These samples were compared in order to test the efficacy of the system to IMO and other standards (table 2) standards under normal working conditions. The BWTS system consists of a primary disc filter manufactured by Arkal Inc., Tel Aviv, Israel mounted in series with a medium pressure UV irradiation system rated by the manufacturer/vendor at 200 mJ cm^2 for treatment of ballast water at flow rates up to 250 $m^3 h^{-1}$. Trials took place during the vessel's regular spring schedule in the Caribbean Sea, the summer schedule in the N.W. Pacific Ocean between Whittier, Alaska and Vancouver, Canada, and during the repositioning cruise from the western Pacific to the vessel's winter base in Fort Lauderdale, Florida. Trials consisted of determination of water quality parameters and a comparison of biological endpoints in treated and untreated ballast water samples, with reference to both IMO G8 and the U.S. Coast Guard Shipboard Technology Evaluation Program (STEP). Sampling procedures and endpoint determinations followed IMO G8 guidelines for shipboard trials and the exercise was designed to supplement land-based trials being conducted concomitantly at NIOZ, Texel, Netherlands to determine the efficacy of the BWT system under varying water quality conditions. Shipboard trials were designed to document system performance under normal seagoing conditions and under different geographical and seasonal conditions, with the objective of determining the degree of compliance with IMO and STEP requirements. Whole Effluent Toxicity (WET) tests were also conducted as part of the third and last trial to determine whether any significant chemical changes in ballast water after exposure to UV irradiation, which resulted in subsequent residual toxicity.

Table 2. . 2008 IMO and U.S. Ballast Water Treatment Standards.

	IMO Regulation D-2 and Transport Canada	**2008 Ballast Water Management Act Section 1101 (f)i**	**2008 California Standard**	**Washington Administrative Code 222-170**
Management approach	Exchange moving towards treatment only	Exchange moving towards treatment only	Exchange moving towards treatment only	Exchange or treatment
Standard:	**Proposed**	**Proposed**	**Recommended Interim**	**Adopted Interim:**
1) Organisms greater than 50	<10 viable organisms per	< 0.1 living organisms per cubic	No detectable living organisms	Technology to inactivate or remove

microns in minimum dimension:	cubic meter	meter		95% zooplankton
2) Organisms 10-50 microns in minimum dimension:	<10 viable organisms per ml	< 0.1 living organisms per ml	$<10^{-2}$ living organisms per ml	
3) Organisms less than 10 microns in minimum dimension:	No standards	No standard	$< 10^{3}$ cfu bacteria/100 ml	99% bacteria & phytoplankton
4) *Escherichia coli*	< 250 cfu/100 ml	<126 cfu/100 ml	<126 cfu/100 ml	
5) Intestinal Enterococci	<100 cfu/100 ml	< 33 cfu/100 ml	<33 cfu/100 ml	
6) Toxicogenic *Vibrio cholerae* (O1& O139)	<1 cfu/100 ml <1 cfu/gram of wet zooplankton samples	<1 cfu/100 ml <1 cfu/gram of wet weight of zoological samples;	<1 cfu/100 ml < 1 cfu/gram of wet zoological samples $<10^{4}$ viruses/100 ml **Final standards** – no discharge of living organisms	

Endpoints Measured.

The primary measurements in 2008 shipboard trials were:

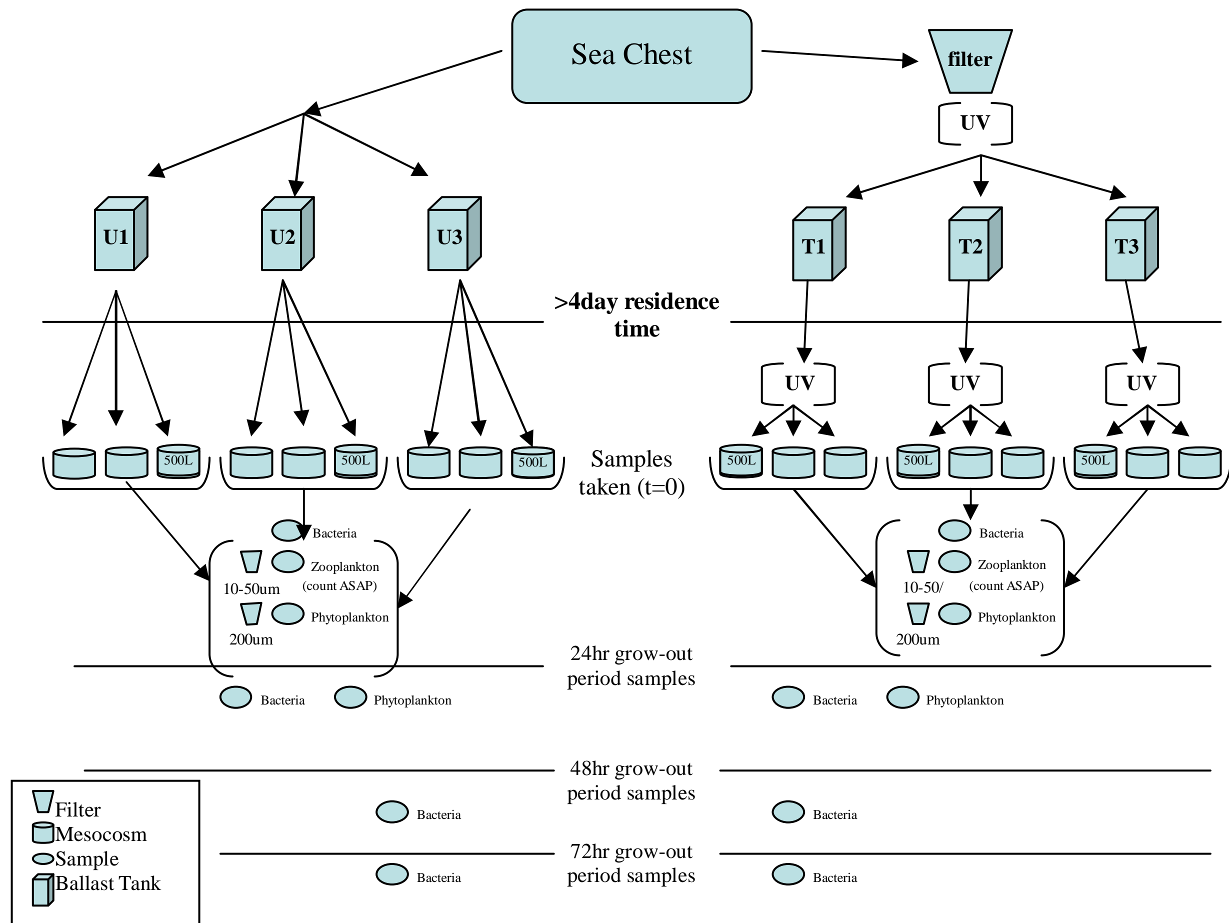
- Plankton Viability/Growth Potential (Plankton Abundance)
- Microbial activity as determined by plate counts and MPN
- Water Quality Measurements, e.g. Salinity, Temperature, DO, TSS, POC, Nitrate

To assess biological effectiveness, the following protocols were employed in order to comply with IMO G-8 guidelines and produce results that were compatible with IMO D-2 regulations (IMO 2004). Procedures were modified in certain cases in order to account for problems encountered aboard a working vessel. For example, logistics dictated that it was not always possible to ballast the vessel in port, and that the challenge water was not ‘optimal’ in terms of organism densities, bacterial flora and TSS. The following procedures describe both sampling strategies and analytical techniques employed during shipboard trials. Most biological endpoint analyses began aboard the vessel, an essential requirement where rapid assessment of live plankton was required. However, in two out of three trials, samples for bacterial analysis

were preserved pending analysis using internationally recognized standards/chain of custody procedures. While standard water quality parameters were measured aboard ship, nutrient analyses were conducted by the University of Maryland Analytical Services Division, which has been an independent, nationally and internationally recognized analytical resource for over thirty years.

Biological Endpoints

Figure 1 depicts the basic sampling strategy employed in the 2008 shipboard trials. As a measure of accuracy a 3 x 3 sampling matrix was employed, with U1, U2 and U3 representing untreated ballast water sampled in triplicate at the beginning, middle and end of a de-ballasting cycle, and T1, T2 and T3 representing treated samples taken at the beginning, middle and end of a de-ballasting cycle.



Sampling Strategy.

Triplicate samples were taken at the beginning, middle and end of a collection cycle. It must be emphasized that treatment by the Hyde 'Guardian' system is only complete following a second 'pass' through the UV system at the time on discharge from the ship. Therefore, definitively treated samples can only be collected and examined during de-ballasting. Related to this is the fact that shipboard testing cannot be subject to the same degree of control as land-based testing, e.g. the test team cannot start with clean 'rinsed' tanks. It is, therefore of particular importance to define the nature of the intake (challenge) water as well as discharged water, in order to identify important differences between the biology and chemistry of water at the time of uptake, and that discharged from the vessel.

Biological Endpoints.

Zooplankton.

Samples for zooplankton counting were taken from a sampling port located downstream from the Ballast Water Treatment System (BWTS) in the machinery space of the vessel. At that location samples were filtered through 30cm. nylon plankton nets consisting of 20µm mesh (nominal 50µm mesh nets can allow the passage of >50µm organisms). During filtration, each net was submerged under the surface of a 125L plastic tub to soften the impact of the filtration procedure on the planktonic organisms. The 1L plastic bottle that forms the `cod-end' of the net also had 20µm mesh `windows' to facilitate the filtration process. Separate nets were used to sample from treated and untreated samples, and nets were rinsed with hot tap-water between sampling cycles. They were also examined for tears, leaks and imperfections and any repairs made. In the machinery space the contents of each mesocosm were filtered and concentrated to a volume suitable for manual transport up to the cabin space where samples were turned over to microscopists for examination. On receiving samples from the collection team, microscopists further concentrated samples through 20µm mesh filters to produce volumes suitable for microscopy, usually 10-20ml. Concentrated samples were transferred via Stempel pipets to counting wheels mounted on compound microscope stages for microscopical examination. Using these procedures, it was possible to begin microscopic examination for plankton density/activity within one hour of collection. As a measure of precision, a majority of plankton samples are split and processed as duplicates to indicate the amount of variability sample processing may add to the results. Counts are made of at least 100 individuals from at least five taxonomic groups.

Sample-sharing between microscopists was practiced throughout these trials. Organism sizes were recorded as the minimum dimension of the cell/organism, using calibrated reticule eyepieces mounted in the eyepiece lens of each microscope. However, a particularly useful means of cross-calibration involved the 'seeding' of counting slides with glass beads of specific sizes (10 µm, 20 µm, 50 µm, 100 µm, 200 µm). These glass beads are certified by the U.S. National Institute for Standards as calibration standards for particle sizing technology. Their use as a supplement to microscopy provides an excellent cross-calibration tool among microscopists and has been referenced in recent shipboard studies (Wright et al. 2009). In order to address precision associated with counting a majority of plankton samples are split and processed as duplicates to indicate the amount of variability sample processing may add to the results.

Precision of nutrient analyses is addressed through regular running of standards between samples batches to ensure consistent equipment operation and calibration.

QA/QC considerations for shipboard sampling include rigorous washing of the sampling nets with freshwater and examination of nets and other sampling devices containers for flaws/leaks between sampling runs. Sampling aboard a working vessel involves pumping water through numerous pipes/tank segments, which may involve dead spaces. Unlike land-based operations, extensive rinsing of tanks/pipes is not possible between sampling episodes. Therefore, to the extent possible under the constraints of the sampling regime, sufficient time should be given to flushing water through the ballasting system of the shipping before sampling commences. An important consideration in this respect is that the collection of treated samples always precedes the collection of untreated samples. This minimizes the potential of carry-over of live individuals (i.e. Type 1 error) that might have been trapped in dead-spaces. Conversely, carry-over of dead organisms (Type 2 error) through a 'treated first' protocol is likely to insignificant in the context of current published standards. Given the rigor of current standards, type 1 errors should be avoided at all costs. These and other aspects of shipboard sampling and testing are discussed by Wright (2007).

Phytoplankton.

Samples for phytoplankton counting/assessment consisted of 1L of unfiltered water collected from the sampling port. These samples were passed through a 200µm mesh net to remove any large predatory zooplankton. Experience with shipboard trials has indicated that grazing can be a factor, even within the context of ballast tank residence time and sample holding time prior to examination. Each sample was concentrated to a volume <10ml. for examination/grow-out, using a 10µm Nitex screen. This enabled unusually low densities of phytoplankton to be counted with greater precision, as it resulted in greater cell densities on the counting slide. Typically counts were made of >200 squares of a 1000 square counting grid,. Determination of living phytoplankton was be made on the basis of (a) chlorophyll a analysis, (b) vital staining techniques and (c) cell counts before and after grow-out.

Use of grow-out technique.

Live-dead status of phytoplankton for the determination of ballast water treatment efficacy remains problematic to the extent that, while some taxonomic groups, such as dinoflagellates, are clearly motile, many have vegetative stages that are immobile. The growth potential of non-motile forms can only be assessed by a variety of methods, including microscopic examination of chloroplast integrity, use of vital stain(s) and cell counts (of at least dominant groups) following a grow-out period under optimal growth conditions. Other, more sophisticated techniques, such as flow cytometry are also available, but do not present a realistic option for shipboard trials. For shipboard trials, it was determined that growth potential represents a robust indicator of phytoplankton viability within the constraints of shipboard analyses, although this endpoint remains difficult to quantify in terms of regulatory endpoints. The grow-out period for phytoplankton usually consisted of a 24h (or 48h) period of irradiation under fluorescent lighting and non-limiting nutrient conditions through the addition of f/2 growth medium. This medium had the following constituents: $FeCl_3.6H_2O$, 1.45 mg L^{-1}; KNO_3 200 mg L^{-1} ; $NaH_2PO_4.2H_20$, 34.8 mg L^{-1}; H_3BO_3, 34.2 mg L^{-1}; EDTA.$2H_20$, 30 mg L^{-1}; $MnCl_2.4H_20$, 4.3 mg L^{-1}; $CoCl_2.6H_20$, 0.13 mg L^{-1}; $ZnSO_4{\cdot}7H_20$, 0.364 mg L^{-1}; H_2SeO_3, 0.00173 mg L^{-1};

Thiamine HCl, 0.1 mg L^{-1}; Vit B12, 0.001 mg L^{-1}; Biotin, 0.002 mg L^{-1}.

An effort was made to identify and count as many taxonomic groups as possible, with particular focus on dominant taxa, although individual cell counts before and after grow-out are still subject to different interpretation. While 'live' counts depended on such parameters as chloroplast integrity, motility and neutral red staining (useful for several but not all taxonomic groups), numbers of viable cells determined in this way often declined rather than increased following 'grow-out'. Under such circumstances it may be concluded that the population as a whole was non-viable, i.e. incapable of growth.

<u>Other determinants of phytoplankton viability.</u>

Microscopic examination of cell/chloroplast integrity was supplemented by the use of Neutral Red as a vital stain, which proved a useful stain for many, but not all taxonomic groups.. Unstained samples examined soon after collection were compared with 'splits' of the same sample that were stained with Neutral Red. Samples following a grow-out period were similarly examined, with and without Neutral Red. Following initial examination of phytoplankton (before and after grow-out) to determine their general appearance, observations of chloroplast integrity and the activity of motile forms, samples were preserved in Lugol's Solution for more intensive taxonomy and determination of cell sizes.

<u>Chlorophyll *a*</u>

Phytoplankton growth may be conveniently assessed by measuring *in vivo* chlorophyll *a* concentration before and after the grow-out period. While this represents a useful integrative determination of the status of the phytoplankton community as a whole, it does not provide information on individual taxonomic groups of phytoplankton that might have quite different characteristics in terms of size, shape, doubling time (growth rate) etc. Also, chlorophyll a data cannot be interpreted in terms of published standards, which are based on cell numbers. While good quantitative relations can be drawn between chlorophyll a and cell numbers in monocultures (Wright et al. 2006), natural phytoplankton communities may include taxa which differ in volume by more than two orders of magnitude. Under such circumstances chlorophyll a levels would be biased towards larger cells. We, nevertheless find that useful information can be obtained from chlorophyll a analyses, particularly with respect to growth capacity. While two analytical methodologies are described in UMCES standard operating procedures (see Appendix E), the method described by Welschmeyer was used for these trials. For this study, chlorophyll *a* analyses were made aboard the vessel, using a Turner Designs Aquafluor fluorimeter, Model # 8000-001 calibrated aboard the vessel using a solid standard, which in turn was calibrated against a chlorophyll *a* standard at the UMCES Analytical Services Division.

<u>Bacteria</u>.

250ml. samples for bacterial analyses were be taken directly from the unfiltered discharge from the sampling port, and stored at temperatures just above freezing (1-4°C) prior to and during transport to the University of Maryland Center for Environmental Science, Chesapeake Biological Laboratory, although in Trial 3 some culturing/counting was carried out in cabin space aboard the vessel. Bacterial culture formed the basis of bacterial endpoints that were used to test the bactericidal effectiveness of the BWT system aboard this vessel. The following bactericidal endpoints were employed:

- <u>Cultural bacteria.</u> For total culturable heterotrophic bacteria, samples were

serially diluted three to four orders of magnitude. 20 ml. full strength and diluted samples were filtered through 0.45 um black field filters to isolate the bacteria on the filter. The filter was then transferred to a Petri dish that contained a sterile absorbent pad soaked in 2ml of autoclaved marine broth (Difco™ Marine Broth 2216). The filter paper was placed so as to expose the side inoculated with bacteria. The Petri dishes were then closed and placed upside down in a 25 ±0.5 ^{o}C incubator. After 72 hours, the dishes were removed and colony forming units were enumerated using a colony counter.

It is emphasized that only approximately 1% of marine bacteria will culture successfully, and that this figure may vary according to geographical area. This, therefore, represents only an approximate measure of the efficacy of BWT technology, and is somewhat variable according to geographical location.

Primary focus was on taxonomic groups specified in published IMO (2004) D-2 regulations: colony-forming units of *E.Coli, Enterococcus* and *Vibrio Cholera* (with specific emphasis on virulent serotypes). Fluorescence-based techniques (IDEXX Laboatories.) were employed to quantify coliforms, *E. Coli* and *Enterococcus* in treated and untreated ballast water.

Samples were diluted an order of magnitude with sterile deionized water for the IDEXX protocols. The established detection range for this technique is 10 – 24,190 cfu / 100 mL of sea water sample.

- Total coliform and *E.coli* levels were enumerated using IDEXX Colilert-18 Quantitray/2000 test kits (IDEXX Laboratories Inc., ME), which are US EPA approved standard methods for *E.coli* analysis. Samples were diluted an order of magnitude with sterile deionized water , added with the detection media into the Quantitray and sealed and incubated at 37 ^{O}C for 18 hours. The analytical method is a multi-well Most Probable Number (MPN) method involving exposure to a mixture of *o*-nitrophenol-*β-D*-galactopyranoside (ONPG) and 4-methyl-umbelliferyl-*β-D*-gluronide (MUG). Coliform bacteria produce a yellow color resulting from the formation of *β*-galactosidase. In addition to the yellow color, *E.coli* also produce fluorescence (under a 6 watt, 365nm UV light) as a result of the action of *β*-glucuronidase. Yellow and fluorescent squares were counted and converted to cfu/100ml.
- Counting of a variety of Enterococci sps. including *faecalis, faecium, avium* and *gallinarum* is achieved using a MUG-based nutrient-indicator and a fluorescent end-product. Like the coliform method, the IDEXX Laboratories Inc. Enterolert Quantitray 2000 (ASTM method # D6503-99) relies on a MPN endpoint, following 24h incubation at 41°C. A blue fluorescence signifies a positive result for Enterococci.

Quantification of viable *Vibrio* cells was facilitated by the use of Polymerase Chain Reaction amplification techniques on refrigerated samples transported to the University of Maryland Center for Environmental Science, Chesapeake Biological Laboratory, Molecular Biology Laboratory, directed by Dr. Carys Mitchelmore.

Water Quality and Nutrient Analyses.

Only chlorophyll a and standard water quality criteria were determined aboard the vessel. As previously reported chlorophyll a analyses were cross-calibrated via chlorophyll a standard measurements conducted both aboard ship and at the UMCES Analytical Services Division, Solomons MD. pH, dissolved oxygen (DO), salinity, temperature were all measured using a Yellow Springs Instruments 'Multimeter', which was calibrated against laboratory-based instrumentation before and after each trial, with pH/salinity re-calibrations against known standards between sampling batches. Spare membranes for the DO meter were available 'in the field'.

Apart from chlorophyll a other nutrient analyses were performed by the UMCES Analytical Services Division, Chesapeake Biological Laboratory, Solomons, MD, U.S.A. Standard Operating Procedures from this Laboratory have been published as comprehensive document, available online at http://cbl.umces.edu.nasl/Methods (Appendix E), and only those analyses relevant to the 2008 shipboards trials are summarized below

Total Suspended Solids (TSS).

Total Suspended Solids (TSS) and Particulate Organic Carbon (POC) were measured using GF/C filters that were initially dried to (102-105°C), pre-weighed (gm.) to 4 decimal places and brought aboard the vessel in individual sealed Petri dishes. Filter volumes varied between 500ml. -1-5 L. Filters were rinsed with freshwater to remove salt and brought back to the laboratory over ice. Each filter was then dried at 60°C for 24h and re-weighed (APHA 1975, USEPA 1979a). Total Suspended Solids (TSS) were calculated from the difference in between this weight and that of the pre-weighed filter.
For POC measurement filters were then combusted in a 500°C oven overnight, stored in a desiccators and then re-weighed. POC was calculated as the loss in weight relative to the TSS weight.
Both TSS and POC are expressed as mg L^{-1}.

Dissolved Organic Carbon (DOC).
The Shimadzu TOC-5000 uses a high temperature combustion method to analyze aqueous samples for TIC, TOC and non-purgeable organic carbon. Samples for DOC analysis are treated with hydrochloric acid and sparged with ultra pure carrier grade air to drive off inorganic carbon. High temperature combustion (680 °C) on a catalyst bed of platinum-coated alumina balls breaks down organic carbon into carbon dioxide (CO_2). The CO_2 is carried by ultra pure air to a non-dispersive infrared detector (NDIR) where CO_2 is detected (Sugimura and Suzuki 1988).

Nitrite + Nitrate
Filtered samples are passed through a granulated copper-cadmium column to reduce nitrate to nitrite. The nitrite (originally present plus reduced nitrate) then is determined by diazotizing with sulfanilamide and coupling with N-1- naphthylethylenediamine dihydrochloride to from a colored azo dye. Nitrate concentration is obtained by subtracting the corresponding nitrite value from the nitrite + nitrate concentration. (Technicon 1977, USEPA 1979b).

Orthophosphate
Ammonium molybdate and antimony potassium tartrate react in an acid medium with dilute solutions of phosphorus to form an antimony-phospho-molybdate complex which is reduced to an intensely blue-colored complex by ascorbic acid. Color is proportional to phosphorus concentration. (Technicon 1973, USEPA 1979c).

Ammonium (analyzed) in conjunction with Whole Effluent Testing (WET) only (Trial 3).

Determination of ammonium is by the Berthelot Reaction in which a blue-colored compound similar to indophenol forms when a solution of ammonium salt is added to sodium phenoxide, followed by the addition of sodium hypochlorite. The addition of a potassium sodium tartrate and sodium citrate solution prevents precipitation of hydroxides of calcium and magnesium (Technicon 1986, Kerouel and Aminot 1987).

University of Maryland Analytical Services Division - Statement of Quality Assurance/Quality Control Procedures.

The analytical services division of the University of Maryland Center for Environmental Science (UMCES) is a nationally and internationally recognized resource for aquatic chemical analyses, which for over thirty years has provided this service to numerous State and Federal agencies and other clients. Standard Operating Procedures (SOP) used by this group are available online at http://cbl.umces.edu.nasl/Methods and are reproduced here as Appendix E. A representative summary of QA/QC components practices by this laboratory is given below.

A constant consideration of the UMCES Analytical Services laboratory is assuring the quality of data generated by the procedures presented in this manual. Further, indication of data quality is accomplished by analyzing duplicates, spikes, standards-as-samples, standard reference materials and participating in cross-calibration exercises.

Laboratory Duplicates

Approximately 5% of the total number of samples analyzed consist of laboratory duplicates. For dissolved analytes, after a sample is analyzed, the same sample container is placed farther along in the automatic sampler and re-analyzed. The mean of the two values is reported as the concentration for that sample. If a difference of >10% is observed between replicates, then all of the replicates for that particular analytical run are carefully reviewed. If only one of the duplicate pairs is in question, then only that sample is re-analyzed. If all show a similar trend, then instrumentation/reagent problems are suspected and the analytical run is halted until such time as the problem is resolved. This procedure is practiced for all dissolved analytes that are not consumed completely in the analytical procedure. For those that are completely consumed and for particulate analytes, duplicate samples constitute actual duplicate samples collected in the field and analyzed in the same analytical run.

Values for each duplicate analyzed are recorded in a separate QA/QC data file along with the sample number, sample collection date and analysis date. The mean concentration and standard deviation of the replicates are calculated in this data file. In the case of particulate carbon and nitrogen, total suspended solids and chlorophyll a, 10 percent of the total number of

samples are analyzed as duplicates. This generates sufficient quality assurance data to compensate for the omission of laboratory spikes for these non-aqueous samples.

Laboratory duplicates serve as an indicator of instrument stability, consistency in laboratory sample preparation and analysis, as well as an estimate of field proficiency.

Cross Calibration Exercises

UMCES Analytical Services Division has participated in many cross calibration exercises. Participation in such programs is an excellent means of determining accuracy of results. Examples of such cross calibration exercises include the Chesapeake Bay Quarterly Split Sample Program, US EPA Method Validation Studies and International Council for the Exploration of the Sea Intercomparison Exercise for Nutrients in Sea Water.

Sample Custody

Upon arrival at the laboratory, samples are counted, observed for potential problems (melting, broken containers, etc.) and placed in a freezer until analysis. Sample information and date of arrival are recorded on a log sheet.

Instrument Maintenance

Analytical instruments are maintained on a regular basis and records are kept of hours of operation, scheduled maintenance, pump tube changes, etc. A critical spare parts inventory is maintained for each instrument. Instrument down-time is minimized by troubleshooting instrument problems telephonically with manufacturers and service representatives. Spare parts can be received within 24 hours via next-day air service.

Statement on Instrument Comparability

The Nutrient Analytical Services Laboratory develops a data quality maintenance program for each analyte whenever new instrumentation is acquired. It is the policy of the Nutrient Analytical Services Laboratory to report any data from new instrumentation only after thorough and satisfactory side-by-side comparisons with existing instrumentation are preformed. No predetermined number of data pairs are used to make the assessment on data comparability between new and existing methodology. Even in the case of instrumentation with similar methods of detection (i.e., automated colorimetric), no specific number of data pairs is used. Comparability at low and high concentrations, salinity and other possible matrix interferences, sensitivity and precision are all factors in determining the number of pairs that must be addressed before bringing an instrument on-line and in determining instrument comparability.

The analyst who performs these comparisons should be experienced, open-minded and impartial. This person can give an evaluation of ease of instrument operation and a very important general statement of comparability. This statement on comparability must then be substantiated via statistical analysis of the data. As previously mentioned, these data must encompass the entire concentration range, matrix interferences, percent recovery, results of standard reference material analyses, etc. The data interpretation must support comparability. The analyst and laboratory QA/QC officer must concur and finally, some sort of presentation

(written or verbal) must be given to the contractor explaining what procedure was followed and the results that were obtained to bring this instrument on-line.

Temperature Logs.

Temperature logs of freezers, refrigerators and drying ovens are kept on a monthly basis. Thermometers used in this equipment are calibrated against a certified NBS thermometer.

Laboratory Spikes

Approximately 5% of the total number of samples analyzed consist of laboratory spikes. A spike is prepared by adding a known volume of standard to a known volume of pre-analyzed sample. We routinely add enough concentrated standard to provide a significant response on our instruments that is distinguishable from the original concentration of the sample. This concentrated standard is used to minimize any possible change in sample matrix by the addition of spike. The spiked sample is analyzed and its expected concentration calculated as the sum of the original concentration and the spike concentration, normalized for the constituent volumes. A comparison is made between the actual value and the expected value. These concentrations (original, expected and actual) are recorded in a separate QA/QC data file along with sample number, sample collection date, analysis date and the amount of spike added. In the case of particulate phosphorus, the volume filtered is not used in the calculation to determine percentage recovery. If a value of >115% or <85% is observed for percentage recovery of the spike, then all of the spikes for that particular analytical run are carefully reviewed. If only one of the spikes is in question, then only that sample is re-analyzed. If all show poor recovery, then instrumentation/reagent problems are suspected and the analytical run is halted until such time that the problem is resolved. This procedure is adhered to for all dissolved analytes and for particulate phosphorus and biogenic silica.

Documentation of Slopes

A running record of the slopes of the standard curves (the so-called "F," "S" and "K" factors) is maintained for each analysis. Random up and down movement within a predetermined range as a function of time indicates the analysis is under control. Consistent upward or downward movement of these factors indicates the analysis is out of control and requires immediate attention.

Standard as Sample

Standards are analyzed as samples throughout the analytical run. This is an excellent means of evaluating instrument performance during the course of an analytical run. Standards are analyzed every 12 - 20 samples, depending on the instrument and analyte.

Limits of Detection.

Limits of detection, the lowest concentration of an analyte that the analytical procedure can reliably detect, have been established for all parameters routinely measured by Nutrient Analytical Services. The limit of detection is 3 times the standard deviation of a minimum of 7 replicates of a single low concentration sample.

Standard Reference Materials

Particulate Carbon, Nitrogen and Phosphorus: BCSS-1 is a marine sediment reference material prepared by the National Research Council of Canada. It is certified by the Council for carbon content, gives a non-certified range of results for phosphorus, but no information for nitrogen. We have analyzed this sediment for many years and maintain a substantial database for nitrogen and phosphorus, as well as carbon values. We analyze this sediment quarterly and compare these results to the certified value, non-certified range of values and our historical values. Dissolved Analytes and Hardness: Standard reference materials for ammonium, nitrite + nitrate, nitrite, orthophosphate, dissolved nitrogen, dissolved phosphorus, dissolved organic carbon, sulfate and chloride and hardness are supplied by SPEX, a US EPA certified company. The samples arrive in ampules and we prepare final concentrations to approximate typical estuarine concentrations. The samples are then placed in pre-cleaned poly bottles, frozen and analyzed on a quarterly basis.The analysis of these frozen standard reference materials as a function of time also provides data on the effect of our preservation technique (freezing) on the integrity of the concentration of samples. The US EPA recommends a holding time of 28 days for many of the parameters we routinely analyze.

Organic compounds are included with each dissolved nitrogen and dissolved phosphorus digestion to determine the completeness of the digestion procedure. Glutamic acid and glycerophosphate are used as the N and P sources, respectively.

Data Management.

In general, Standard Methods (APHA, 1998), section 9020B.10-11, guided the procedures for documentation, recordkeeping and data handling. Data were analyzed using desktop and software such as Microsoft Excel, QuattroPro and system software such as Statistics and Analysis System (SAS).

The project director was present throughout every aspect of shipboard trials, a total of thirty-one days at sea over three trials, and was an integral member of the microscopy team. The responsibility for maintenance of quality for a project lies with every member of the project. All project personnel aid in identifying perceived problems that may affect quality and report such problems to the project director. A team approach is adopted in order to correct observed deviations for which there is an obvious solution during the routine implementation of the sampling procedures. During data validation and assessment, deviations in the project data are corrected as appropriate. If data are determined to be invalid, they are denoted as such and not included in the data set. The project director is responsible for assigning/conducting QA assessments and is responsible for correcting any deviations observed when accuracy, precision, completeness, and comparability are not to specifications, and for inspection of the final reports to confirm that the methods, procedures, and observations are accurately and completely described, and that the reported results accurately and completely reflect the raw data of the studies.

Responsible persons for zooplankton, phytoplankton and microbial assessments provide preliminary reports on data derived from the first day of testing to the project director to allow the test team to correct any readily apparent problems. They also prepare a report to the project manager within one month of the period of ship trials. The report includes but is not limited to the following:

1. An introduction stating an overview of the procedures performed;

2. Methods/SOPs followed for the analysis;

3. Brief analytical assessment of the results highlighting pertinent information about the data (i.e. outliers, deviations, unexpected results etc);

4. Compliance with SOPs and chain-of-custody procedures (see following sample form)

UMCES, Chesapeake Biological Laboratory

FIELD CHAIN-OF-CUSTODY - Sample Custodian

Project Title: Coral Princess BW Testing Trial, _________2008 #:________ Time_________
Sample Type:

1 = Preserved Zooplankton
2 = Live Phytoplankton
3 = Preserved Phytoplankton
4 = Microbial (Macrophage)
5 = Microbial (TPC)

1. Collected by:____________________________

Date:_____________ Time:_______________

Relinquished by:________________________

Date:_____________ Time:_______________

2. Received by:__________________________

Date:_____________ Time:_____________

Relinquished by:______________________

Date:_____________ Time:_______________

3. Received by:__________________________

Date:_____________ Time:_______________

Relinquished by:_______________________

Date:_____________ Time:_______________

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Appendix E.

Standard Operating Procedures for UMCES Analytical Services Division.

Filename: PCL Final Reportcorrected April 2009.doc
Directory: C:\Documents and Settings\David Wright\My Documents\Princess trials 2008
Template: C:\Documents and Settings\David Wright\Application Data\Microsoft\Templates\Normal.dotm
Title: Protocols To Determine Efficacy of The Hyne Marine Inc
Subject:
Author: David Wright
Keywords:
Comments:
Creation Date: 5/4/2009 10:28:00 AM
Change Number: 6
Last Saved On: 7/6/2009 3:44:00 PM
Last Saved By: David Wright
Total Editing Time: 28 Minutes
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As of Last Complete Printing
Number of Pages: 122
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