Final Shipboard Testing Report for the Severn Trent De Nora BalPure® BP-500 Ballast Water Treatment System, T/S *Golden Bear*

Golden Bear Facility
Vallejo, California

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CAL MARITIME
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Abbreviation	Description	Abbreviation	Description
AIS	Aquatic Invasive Species	m^3	Cubic Meters
ATP	Adenosine Triphosphate	MARAD	United States Maritime Administration
BWTS	Ballast Water Treatment System	mA	Milliamp
BSH	Bundesamt für Seeschifffahrt und Hydrographie	mg	Milligram
chl	Chlorophyll	MLML	Moss Landing Marine Laboratory
CFU	Colony Forming Units	MPN	Most Probable Number
CMA	California Maritime Academy	n.a.	Not Analyzed
CSU	California State University	ng	Nanogram
°C	Degree Centigrade	OIT	Operator Interface Terminal
DC	Direct Current	ORP	Oxidation Reduction Potential
DPD	Diethyl-p-phenylenediamine	PAM	Pulse Amplitude Modulated
ETV	Environmental Technology Verification Program	PLC	Programmable Logic Controller
FDA	Fluorescein Diacetate	POC	Particulate Organic Carbon
ft^2	Square Feet	PON	Particulate Organic Nitrogen
FSW	Filtered Sea Water	RDTE	Research, Development, Testing, and Evaluation
F_v/F_m	Variable Fluorescence / Maximum Fluorescence	STDN	Severn Trent De Nora
G8	IMO Guidelines for Approval of Ballast Water Management Systems	TMTC	Too Many To Count
HPC	Heterotrophic Plate Count	TSS	Total Suspended Solids
hr	Hour	µL	Microliters
IMO	International Maritime Organization	µm	Micron
L	Liter	UV	Ultraviolet
m	Meter		

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Section 1 Executive Summary

The BalPure® BP-500, a combination filter and electro-chlorination ballast water treatment system (BWTS) developed by Severn Trent De Nora (STDN), underwent third-party independent shipboard testing onboard the United States Training Ship *Golden Bear (Golden Bear*) between May 2010 and December 2010. Testing was performed in accordance with protocols based on the International Maritime Organization (IMO) *Guidelines for Approval of Ballast Water Management Systems (G8)* to demonstrate compliance with the D-2 standard. For each of the four biological efficacy test cycles conducted, the BalPure® BP-500 met or exceeded each of the D-2 standards specified in the *International Convention for the Control and Management of Ships' Ballast Water and Sediments* (2004). This document summarizes the test logistics, experimental design, and test results.

The *Golden Bear*, at 152.1 meters (m) overall length and 6,974 tonnes deadweight, has a ballast water capacity of 7,141 cubic meters (m^3) and typical flow rates ranging from 90 to 400 cubic meters per hour (m^3/hr). The ship underwent modifications in 2009 and 2010 to meet IMO guidelines for shipboard and land based testing of BWTSs. Testing on the *Golden Bear* is possible through collaboration between the U.S. Maritime Administration (MARAD) as the vessel owner and the California Maritime Academy (CMA) as part of the California State University (CSU) system, who jointly provide the testing facility known as the Golden Bear Facility. Also in collaboration are the science test team from Moss Landing Marine Laboratories (MLML), also a part of the CSU system, and The Glosten Associates who provide engineering and consulting services. To assist in developing the logistics and scientific protocols for testing the BalPure® BP-500, Dr. Stephan Gollasch provided invaluable guidance by conducting an independent review of the scientific testing protocols.

In addition to the required biological efficacy test cycles, the BalPure® BP-500 BWTS was operated during routine ballast water activities on the *Golden Bear* an average of once per month. The overall trial period took place during ten port calls between East Asia and the U.S. West Coast, including four biological efficacy test cycles and sixteen routine ballasting operations, for a total system treatment volume (uptake plus discharge) of approximately 17,000 m^3.

The four biological efficacy test cycles were conducted in four independent ports (Busan, S. Korea; Kobe, Japan; Guam, USA; and San Francisco Bay, USA). These ports represented variable challenge water conditions with a salinity range of 18-34 ‰, temperature range of 11-30°C, particulate organic carbon range of 0.2-1.0 mg C/L and total suspended solids range of 2-68 mg/L. Biological treatment efficacy was found to pass regulatory levels for all organism classes stipulated by IMO D-2 standards, specifically, organisms ≥50 µm, organisms <50 µm but ≥10 µm, and indicator microbe species, *Escherichia coli*, *Enterococci* and *Vibrio cholerae* (serotypes 01 and 0139). The BalPure® BP-500 BWTS showed particularly impressive inactivation/removal efficiencies for the larger zooplankton size class (≥50 µm) that are considered the more difficult organisms to inactivate. A total of only two (2) living organisms were observed in over 80 treated zooplankton samples analyzed collectively from all four ballast treatment test experiments during the course of this study. Corroborative viability measurements not specified in IMO guidelines, such as adenosine triphosphate (ATP), cultivable bacteria plate counts, and Pulse Amplitude Modulated (PAM) chlorophyll fluorescence also showed consistently large treatment reductions relative to challenge water conditions. Thus, it is our finding that the BalPure® BP-500 BWTS met, and in several cases, greatly exceeded the regulatory standards for living organism standards outlined by IMO D-2. The BWTS also operated without failure during the seven-month observation period under routine shipboard operating conditions.

Table 1 below provides a cumulative summary of results for all four biological efficacy test cycles.

Table 1 – Shipboard Data Summary for All Test Cycles

Parameter	Uptake			Treatment Discharge			IMO D-2 Standard	Control Discharge		
	Ave	Min	Max	Ave	Min	Max		Ave	Min	Max
Salinity (PSU)	29.1	18.7	34.8	29.1	18.7	34.8	-	29.1	18.7	34.8
TSS (mg/L)	21.1	1.9	67.5	15.0	3.2	33.0	-	15.0	2.3	40.0
POC (µg/L)	687	233	992	441	208	564	-	458	227	581
Plankton Count ≥50 µm org/m^3)	85212	37535	141700	0.6	0	2.1	<10 org/m^3	55914	21486	84952
Plankton Count <50 µm but ≥10 µm (org/1mL)	137.4	11.7	210.0	2.2	0.2	3.4	<10 org/mL	56.9	19.7	105.7
Escherichia coli (CFU/100mL)	11.2	1.5	28.1	<1	<1	<1	<250 CFU/100mL	18.6	4.8	41.5
Enterococci (CFU/100mL)	108.8	82.6	134.6	0.5	<1	1.0	<100 CFU/100mL	107.6	38.9	179.3
Vibrio cholera (CFU/100mL)	<1	<1	<1	<1	<1	<1	<1 CFU/100mL	<1	<1	<1

Section 2 Introduction to Test Facility – *Golden Bear*

The United States Training Ship *Golden Bear* is a 500 foot ship owned by the U.S. MARAD, operated by the CMA as a cadet training vessel. CMA serves a teaching/research campus within the CSU system. The ship is the primary asset of the organizational structure that is called the Golden Bear Facility (Facility). For the purposes of this report "*Golden Bear*" refers to the ship, and "Facility" refers to program activities.

The *Golden Bear* was built in 1989 by Bethlehem Steel Corporation at Sparrows Point, Maryland, and originally named the USNS *Maury* (T-AGS 39). Designed as an oceanographic survey ship for the U.S. Navy, its original mission was to conduct ocean surveys and provide essential geophysical bathymetric, gravity, and geomagnetic data. At the time of construction, the Maury was the largest and fastest oceanographic ship ever built, capable of maintaining speeds up to 20 knots.

The USNS *Maury* was transferred to the CMA in September of 1994 and renamed the *Golden Bear*. Upon transfer the vessel underwent modifications to adapt it to function in a training environment. As the vessel owner, the U.S. Maritime Administration provides maintenance and operational assistance to support CMA activities. Now based in Vallejo, California, the *Golden Bear* provides a comprehensive training platform to those interested in working in the marine industry.

Length Overall ..... 152.1 m

Molded Breadth ..... 21.9 m

Molded Depth ..... 12.8 m

Total Installed Power, Continuous.....10,740 kW

Design Speed, Calm Water.....20 knots

Design Deadweight .....6974 tonnes

Ballast Flow Rate ..... 90 to 400 m^3/hr

Ballast Capacity ..... 7141 m^3

Ballast Tanks 28

Training Ship *Golden Bear* —Vallejo, California

The Facility program was developed in spring 2010 to conduct research, development, testing, and evaluation (RDTE) of technologies and operational practices that serve to limit the impact of marine vessel operations on the environment. The *Golden Bear* has been outfitted to integrate containerized BWTSs with routine ship ballasting operations. In addition, two ballast tanks, each with capacities of approximately 435 m^3, and associated piping are outfitted to conduct controlled biological efficacy testing. The ship's pumping system can be varied between 90 m^3/hr and more than 400 m^3/hr and permits treatment on uptake and/or discharge, with treatment and control ballast tanks filled simultaneously. In this manner, the BWTS performs routine ballast water treatment under the stresses of normal ship operations during trans-oceanic voyages and biological efficacy testing cycles are performed under controlled conditions. The *Golden Bear* meets IMO G8 requirements for shipboard testing. The *Golden Bear* has also been designed to meet IMO, U.S. Coast Guard, and U.S. Environmental Protection Agency Environmental Technology Verification (ETV) requirements for land-based testing.

Specifically, the *Golden Bear* consists of:

- Dedicated onboard laboratory to enable rapid biological and chemical analysis to support Facility activities;
- Access to all ship's equipment; including the ballast water system, hull and apertures, bilge water and de-oiling equipment, sanitation system, and diesel engine exhaust systems; and
- Specialized equipment for Facility purposes. To support BWTS evaluation, the Facility installed a specialized pump and piping system, means of installing and integrating the BWTS, an automation system, and a ballast water sampling system.

2.1 Ballast Tank Sample and Data Collection

During testing of the BalPure® BP-500 BWTS, researchers had access to two existing ship ballast tanks (one treatment and one control) each with a capacity of approximately 435 m^3 (approximately 114,210 gallons). The seawater ballast tanks selected for use in this project were tanks 3-154-1 and 3-154-2. Both tanks are similar in construction and mirrored about the ship's centerline. During the four test cycles, the *Golden Bear* ballast water pumping system operated at an average total flow rate of 412 m^3/hr (treatment plus control) for ballast water uptake. Treatment and control tanks were filled simultaneously.

A slip stream sampling port installed in the ships ballast piping, and accessed on the main deck of the ship, allowed sampling of ballast water on uptake before treatment, control tank discharge, and ballast discharge after treatment. For testing of the BalPure® BP-500 BWTS, two sets of three replicate ballast water samples were collected in tubs located near the main deck sampling port. Each of the six sample tubs had a "flow through" design that allowed continuous ballast water sampling throughout the uptake and discharge of each test cycle.

2.2 Laboratory Facilities

The *Golden Bear* marine laboratory is located onboard the ship and provided researchers with the ability to assess the biological efficacy of the BWTS immediately after sample collection. The laboratory space, dedicated to ballast water testing measurements, is located on the same deck as the ballast water sampling points, approximately 25 m away. This close proximity enabled efficient processing and transport of all biological/chemical samples.

The *Golden Bear* laboratory (130 ft^2) served as the primary space for the majority of the sample processing/analysis. The laboratory provides bench space, a fume hood, refrigerator/freezer storage, high grade distilled water and a wet sink area. Ballast-related instruments installed in the ship's lab include: BD FACScan flow cytometer, Accuri C6 flow cytometer, Spex Fluoromax-2 high-

sensitivity spectrofluorometer, Turner Designs 20/20 ATP luminometer, Beckman pH meter, drying oven, microbiological culture incubator, microcentrifuge, filtration racks (2), Zeiss epifluorescence microscopes (2), Olympus dissecting stereomicroscopes (2), IDEXX bacterial MPN tray sealer, YSI portable temperature/salinity meter, and all pipets/labware required for sample processing.

Section 3 Overview of the BalPure® BP-500 Technology

The BalPure® BP-500 BWTS provides an electrolytic process for the on-site generation of a biocide solution from sea water to disinfect aquatic invasive species (AIS) in ballast water. Ballast water treatment is accomplished using a three-step process of filtration, injection of a biocide solution, and residual oxidant neutralization. The first phase is filtration using a 40 micron stainless steel mesh filter (BallastSafe, BSFc-V-16) to remove organisms, large particles, and sediment. The second phase of the treatment process is electrochemical generation of the biocide solution. This involves passing a small supply (1/100 of total ballast flow) of sea water, either from the incoming ballast water line or sea cooling water, through electrolytic cells. The resulting disinfectant solution (0.1 wt% active ingredient) is injected directly into the incoming ballast water line where it will oxidize potential AIS. The third and final treatment process phase is residual oxidant neutralization to ensure environmental acceptability. When the treated ballast water is ready to be discharged, sodium bisulfite is injected directly into the ballast water discharge line. The sodium bisulfite (oxidant neutralization) addition is controlled with Oxidation-Reduction Potential (ORP) and metering pump technology.

The BalPure® BP-500 is part of a product family developed to service ships with ballast water flow requirements from 100 – 10,000 m^3/hr. The BalPure® BP-500 BWTS can treat up to 500 m^3/hr of ballast water, with the treatment capacity and hypochlorite production rate designed to match to the actual measured ballast flow rate. That is, the BWTS has a 100% turn down capability. Ballast water treatment is done during ballasting operations allowing the biocide solution to be generated in a suitable machinery space on the ship and then injected into the ballast main as needed, even if located in a different machinery space. This would apply to ships with one or multiple ballast systems or ships with multiple ballast pumps mounted within separate ballast tanks. The system can be delivered as a complete system on one skid or divided into seven separate smaller skids based on specific unit operation (pumping, hypochlorite generation, hydrogen-hypochlorite separation, bisulfite storage, bisulfite metered addition, instrument and controls, and power rectification). This allows multiple skid locations for installation where space is available on the vessel. STDN considers the multiple skid design as most suitable for retrofit applications and the one skid system as most suitable for new builds.

Operational control of the BalPure® BP-500 BWTS is accomplished by the main control panel attached to the hypochlorite generation unit, or by a remote control panel that can be placed where most appropriate for a specific vessel. Together, the control panel and the transformer/rectifier unit comprise the main Programmable Logic Controller (PLC) control and rectification components. The control panel provides a visual display of the status of the rectifier DC voltage, current, and can alert the operator of system problems via the operator interface terminal (OIT) and audible alarms. The main control panel also has a local emergency stop push button. All major equipment such as booster pumps, bisulfite metering pumps, and hydrogen blowers can be controlled from the main control panel. All monitoring equipment such as flow meters and ORP probes send the output in the form of 4-20 mA signals. All system control screens are password protected to limit operator modification to existing control logic.

Other system monitoring and control devices include a flow transmitter to monitor the flow of seawater at the electrolyzer inlet, transformer temperature switches that monitor the internal temperature of the rectifier, and airflow switches that monitor the hydrogen gas dilution blowers.

Section 4 Shipboard Testing Execution and Logistics

This section summarizes the shipboard testing execution and logistics, which were conducted in accordance with the "*BalPure® BP-500 T/S Golden Bear Shipboard Testing Approach and Logistics Plan*" (Logistics Plan). The Logistics Plan was developed to meet the intentions of IMO G8 and was submitted to, and accepted by, Bundesamt für Seeschifffahrt und Hydrographie (BSH) prior to execution of the shipboard tests. The Logistics Plan is attached to this report as Appendix A. The executed Logistics Plan successfully demonstrated the consistent performance of the BalPure® BP-500 BWTS:

- During a test period from 23 May 2010 through 4 December 2010, exceeding the G8 minimum six month duration;
- In Pacific Ocean locations ranging from the Far East to Hawaii to the U.S. West Coast;
- Through controlled system testing that exceeded minimum system certification flow rates;
- While installed in the weather and on the 01 Deck of the *Golden Bear*, exposed to significant ship motions, vibrations, salt air and spray, humidity, and temperature fluctuations; and
- While used for all ballasting activities incidental to normal ship operations by ship officers and crew (See Appendix B, Ballasting Operations Log).

4.1 System Commissioning and Tank Flushing

The BalPure® BP-500 BWTS was commissioned by the *Golden Bear* Chief Engineer and the STDN technician while the *Golden Bear* traveled from Vallejo, California to Busan, S. Korea. During the commissioning, the ship's officers and crew were trained in the system operation and maintenance.

During this time, all active ballast tanks were flushed and treated in accordance with ship ballasting procedures accomplishing the following:

- Tanks in ballast were emptied and refilled with treated ballast water; and
- Tanks which were empty underwent a "rinse and spit." Tanks were filled to as close as practical to 25% volume with treated ballast water, held for approximately 24 hours, then emptied.

This was conducted for all active ballast tanks. The treatment and control tanks were opened and inspected to be free of sediment before testing began. It should be noted that ballast tanks which were kept normally full with fresh water as semi-permanent ballast were not flushed.

The single control tank was cleaned and found by inspection to be dry and free of sediment prior to departure from Vallejo. During the ocean transit, the control tank was filled with mid-ocean water without the use of the treatment system.

4.2 Test Cycles

Test cycles were defined as the ballast water uptake and discharge operations that specifically utilized the designated control and treatment tanks, and included biological efficacy testing to compare uptake and discharge organism counts for both control and treatment tanks. Test cycles were in addition to routine ballasting operations, which used the BWTS, and were conducted at flow rates, volumes, and durations similar to those routine ballasting operations. All test cycles were conducted with a holding time between 24 hours and 48 hours.

Following BWTS commissioning and tank flushing, Test Cycles 1, 2, and 3 were conducted. These tests demonstrated the three consecutive test cycles in accordance with IMO G8. These tests took place during voyages in the Far East. Test Cycle #1 was conducted in Busan, S. Korea; Test Cycle #2 was conducted in Kobe, Japan; Test Cycle #3 was conducted in Guam, USA.

A final and fourth test cycle was conducted not less than six months after Test Cycle #1 to meet the IMO G8 test duration requirements. During Test Cycle #4 the ship performed ballast water uptake/treatment in Vallejo, CA and discharge near Oakland, CA.

4.3 General Test Cycle Operations

The ballast water pumping rate for each test cycle was approximately 200-250 m^3/hr per tank (treatment and control) and each tank was filled to 95% capacity, or 410 m^3. This allowed the treatment system to operate for a period of about two hours. These ballast flow rates, ballast volumes, and ballasting times are typical of the *Golden Bear's* ballast operations. All vessel operational parameters were recorded using hand logs; an example Test Cycle Data Log providing operational data is attached to this report as Appendix C.

To characterize the incoming ballast water, the uptake water was sampled after the ballast pump. The incoming ballast water was then split into two equal and parallel streams, with one stream directed to the control (untreated) tank, and one stream directed to the BWTS / treatment tank. According to normal BWTS operation, the BalPure® BP-500 BWTS filtered the incoming ballast water, used a slip-stream of the ballast water to generate hypochlorite, and dosed the incoming ballast water with hypochlorite solution. This water was sampled as described in Section 2.1 above and analyzed in accordance with the protocol outlined in the Revised Science Test Plan (Appendix D).

Following a minimum holding time of 24 hours, the control tank was discharged and sampled at the main deck sample port. All control tank samples were analyzed in accordance with the protocol outlined in the Revised Science Test Plan (Appendix D).

Following a minimum holding time of 24 hours, and within two hours of the control tank discharge, the treatment tank was discharged. The treated ballast water was sampled at the main deck sample port and analyzed in accordance with the protocol outlined in the Revised Science Test Plan (Appendix D). During discharge, the BWTS monitored and data-logged any presence of hypochlorite in the treatment ballast tank discharge using ORP technology, which controlled the addition of sodium bisulfite to neutralize any residual hypochlorite. Also using ORP technology, the BWTS confirmed that no hypochlorite was present in the treated ballast tank discharge. This measurement was also verified with a hand-held unit, the Hach Colorimeter II, utilizing DPD chemistry. The level of hypochlorite on discharge was <0.1 mg/L as measured by the Hach colorimeter.

Section 5 Summary of IMO Success Criteria

IMO criteria for successful shipboard testing are given in Section 2.2.2 of IMO G8. Those guidelines identify: 1) sampling regime, 2) sample volumes, 3) total time period over which consecutive testing cycles are to be executed, 4) minimum concentrations of natural organisms in source water needed for successful testing, and 5) quantitative requirements for characterization of source water used for each test cycle. Characterization of tested source water is required by measurement of salinity, temperature, particulate organic carbon (POC) and total suspended solids (TSS).

The key biological criteria for successful ballast water treatment, as given in IMO Regulation D-2, are summarized in Table 2. The organisms of interest fall into three categories: 1) ≥50 µm, 2) <50 µm but ≥10 µm, and 3) indicator microbes. The indicator microbes are pathogens of human health concern. Additionally, organism concentrations in the uptake source water should meet minimum levels for proper shipboard evaluation of treatment efficacy. Specifically, for each shipboard ballast test cycle, the uptake ballast water should contain at least 10 times the concentration of living organisms ≥50 µm and <50 µm but ≥10 µm, as listed in Table 2. On discharge, the control ballast tank must have at least the concentration of living organisms listed in Table 2 for the two larger size classes. The methods used in evaluating treatment efficacy and organism concentrations are detailed in the Revised Science Test Plan (Appendix D).

Table 2 – IMO Regulation D-2 Ballast Water Discharge Standards, Maximum Organism Concentrations

Organisms ≥50 µm	Organisms <50 µm & ≥10 µm	Indicator Microbes
<10 viable organisms per m^3 for those organisms ≥50 µm in minimum dimension	<10 viable organisms per mL for those organisms ≥10 µm in minimum dimension, but <50 µm in minimum dimension	<1 CFU per 100 mL of *Vibrio cholorae* <100 CFU per 100 mL of intestinal *Enterococci* <250 CFU per 100 mL of *Escherichia coli*

Adequate access to sample control and treatment ballast water was available for successful completion of the above testing requirements. The sample volumes were digitally metered to 1% accuracy to ensure collection of the large quantitative volumes (i.e., at least 1 m^3) required for analysis of larger organisms such as zooplankton (see Table 2).

Section 6 Overview of Science Test Plan Modifications

Details of the scientific methodology were submitted prior to the shipboard trials for approval and acceptance by BSH. Those plans, including test procedures and quality control protocols, were followed closely during evaluation of the BalPure® BP-500 BWTS. However, during the course of the trial period, modifications to the test protocols were made in an effort to improve sampling and analytical capability, when possible. In some cases, methodological changes were introduced due to logistical constraints of at-sea shipboard testing. The "*Revised Science Test Plan for Type Approval of the BalPure® BP-500 Ballast Water Treatment System (Severn Trent De Nora, LLC) as per International Maritime Organization G-8 Guidelines, Regulation D-2, MEPC.174(58)*" is attached to this report as Appendix D. The revised Science Test Plan reflects the protocols and methods used during evaluation of the BalPure® BP-500. Below is a list of the primary Science Test Plan modifications that were made during the course of the trial period, accompanied by a brief explanation.

- Biological Sampling Protocol. The original Science Test Plan called for zooplankton sampling volumes and replication to match IMO minimum requirements. Those included specifically, a 1 m^3 zooplankton sample for each uptake and control discharge operation taken continuously throughout the ballasting operation, and *three* 1 m^3 zooplankton samples collected continuously throughout the treatment discharge operation. However, we were able to incorporate continuous 'triplicate' sampling during all ballast phases, yielding *three* 1 m^3 zooplankton samples for each of the uptake, control discharge and treatment discharge sampling events.

  The continuous sampling method was also utilized for microbe samples. That is, *three* 8 L microbe samples (whole, unfiltered water) were collected during uptake, control discharge and treatment discharge.

  Thus, both zooplankton and microbe sampling proceeded on a *continuous* basis during the entire two hour ballasting operation. This sampling approach integrates natural variability due to plankton patchiness in the ocean (during uptake operations), as well as within the ballast tanks (during control and treatment discharge operations).

- Sample Water Temperature Control. Space limitations prevented the installation of a temperature controlled illuminated incubator for sample water storage and chlorophyll-based Most Probable Number (MPN) growth experiments. Fortunately, ambient water temperatures (17-30°C) through the western Pacific ports closely matched laboratory and deck temperatures such that sample temperature could be maintained at ambient ±4°C. Likewise, December sampling in San Francisco Bay provided cooler water temperatures (ca. 11°C) which were matched by utilizing insulated sample storage tubs on the outside deck.

- Epifluorescence microscopy. The 10-50 µm live organisms were to be enumerated using two complementary methods, chlorophyll-based MPN grow-outs and fluorescein diacetate (FDA) vital probe analyzed primarily by flow cytometry. Corroborative FDA enumeration by epifluorescence microscopy was to be incorporated when possible to compare cytometry and microscopy. Logistics at sea during the first three tests allowed only one trial to be made by epifluorescence microscopy, which verified FDA visual marking of live cells but time constraints prevented routine incorporation of tedious microscopy in the science time schedule. However, extended dockside sample processing on the final test in San Francisco Bay provided the opportunity to make side-by-side comparisons of FDA cytometry to FDA epifluorescence microscopy. Refer to Sections 7.0 and 8.0 for results and discussion.

- Analysis of *Vibrio cholerae.* Standard assays for quantitative analysis of live *V. cholerae* are tedious and time consuming, and impose a requirement for multiple sterile plate transfers. This makes the general colony forming unit (CFU) methodology problematic while at sea. Prior to commencement of the extended sea trial, consultation with *V. cholerae* test kit manufacturer, New Horizons, Inc., (Dr. Larry Loomis, President) suggested that adequate sensitivity for CFU levels stipulated by the IMO standards (<1 CFU/100 mL) could be met with extended warm temperature incubations, e.g., >24 hr, 37°C, using their Cholera Smart™ and Bengal Smart™ test kits for *V. cholerae* 01 and 0139. A routine 48 hr, 37°C incubation protocol for all four test cycles was used. Verification samples from Kobe and Guam were air-shipped to New Horizons, Inc. to corroborate the onboard findings. The attached memo (see Appendix E) provides quantitative verification that the Cholera Smart™ and Bengal Smart™ test kits, as used onboard the *Golden Bear*, provided adequate sensitivity to check compliance levels against IMO Regulation D-2 cholera standards.
- Chain of custody. All samples were collected onboard the *Golden Bear* and analyzed onboard ship and/or at Moss Landing Marine Laboratories, in Dr. Welschmeyer's laboratory. Corroborative *V. cholera* samples shipped to New Horizons, Inc. were duplicates of samples already analyzed onboard ship during the Western N. Pacific sea-trial. Thus, sample tracking through a chain of custody form was not necessary.

Section 7 Biological Efficacy and Water Quality Results

The principal objective of the test cycles was to evaluate the biological efficacy of the BalPure® BP-500 BWTS relative to the IMO D-2 standards and in accordance with IMO G8. In addition, biological efficacy data was generated using corroborative, non-IMO methods that perhaps support the IMO-based conclusions. All data are presented in this section in figures and tables (Tables 3-16, Figures 1-13). The data will be discussed in sections conforming to: 1) IMO-based tests, 2) corroborative biological efficacy tests and 3) challenge water tests. Tables 3-7 and Figures 1-5 show IMO biological efficacy data for a) zooplankton ≥50 µm, b) organisms in the size range <50 µm but ≥10 µm and c) microbe indicator species.

A consistent routine of sample collection and sample processing was utilized for all four ballast test cycles. As noted in Section 2.1, the same ballast water sampling device, with three replicate sample collection tubs, was used during all four test cycles. This allowed for a consistent sample naming procedure for the scientific samples collected. The same sample identification procedure, resulting in a 3-character descriptor, is utilized to present and discuss test results in this report. Specifically, the four experimental locations (ports) were labeled 1, 2, 3 and 4 (in experimental chronology); uptake, control discharge, and treatment discharge samples were labeled U, C and T, respectively; the three replicate sample tubs for net collections and whole-water microbe collections were labeled A, B and C.

As an example, the complete sample suite for the second experiment (Kobe, Japan) included the labels 2UA, 2UB, 2UC (Test Cycle #2, uptake, 3 replicates), 2CA, 2CB, 2CC (Test Cycle #2, control discharge, 3 replicates), and 2TA, 2TB, 2TC (Test Cycle #2, treatment discharge, 3 replicates). This pattern is maintained in data tables and figures presented herein.

7.1 Organisms ≥50 µm

The concentration of living zooplankton ≥50 µm in uptake water (Table 3, Figure 1) exceeded 30,000 live organisms/m^3 in all four test experiments, thus meeting the minimum challenge water concentrations for IMO shipboard efficacy testing (i.e., >10x the standard of 10 live organisms/m^3). The treatment samples (held for a nominal 48 hr period in the ballast tank after treatment with the BalPure® BP-500 BWTS) yielded no observable living zooplankton in 10 of 12 total net samples (Table 3, Figure 1). A total of 81 zooplankton samples, analyzed microscopically in 10 mL Bogorov counting chambers, were processed throughout all *treatment* experiments (Table 3). In all, only two (2) live individual zooplankters (copepods) were observed through the course of this project in treatment samples, thus identifying an impressive treatment efficacy for the BalPure® BP-500 BWTS (Figure 1). Interestingly, the reduction in living zooplankton seemed dependent mostly on the action of biocidal chlorination rather than mechanical filtration since reasonably high concentrations of dead zooplankton (≥50 µm) persisted after treatment and were observed in all treated samples (Table 3).

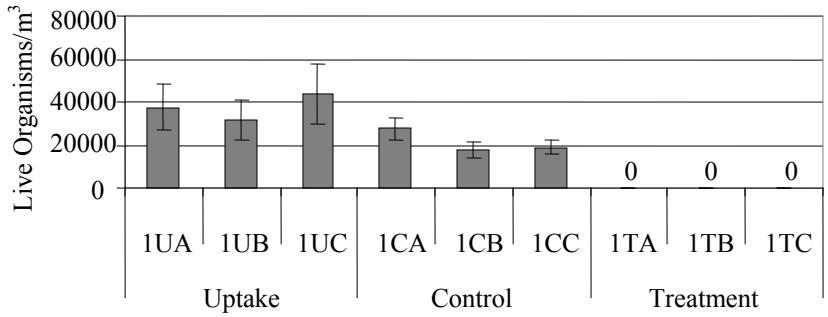
We noted a surprisingly low percentage of live zooplankton compared to dead zooplankton in uptake water during the Kobe experiment (15-30% of total counts, Table 3) which consisted largely of rotifers and tintinid loricas. However, this did not affect the concentration of living zooplankton in challenge water in Kobe relative to IMO minimum requirements. In fact the highest living zooplankton concentrations were measured in Kobe uptake water.

Table 3 – Results Summary for Organisms ≥50 µm, Test Cycles 1, 2, 3 and 4

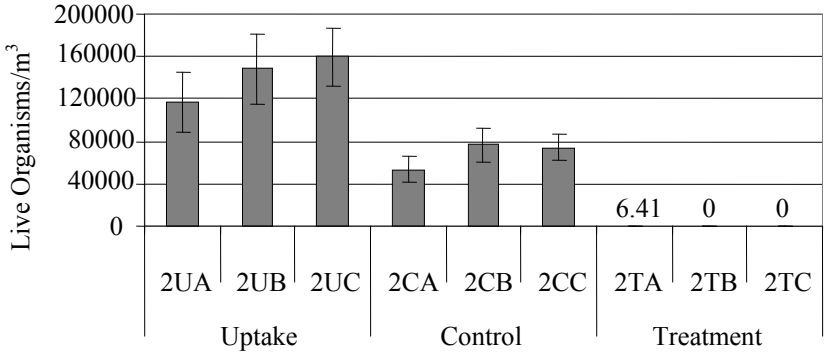
≥50 µm Organisms									
				Live Zoop. (#/m^3)		Dead Zoop. (#/m^3)			
Test Cycle	Location	Sample	Net Vol. (m^3)	Mean	S.D.	Mean	S.D.	% Live	n
1	Busan (Uptake)	1UA	1.32	37567	10635	12725	12055	74.7	6
		1UB	1.3	31636	8913	9414	9526	77.1	6
		1UC	1.32	43401	14042	9589	9416	81.9	6
1	Busan (Control)	1CA	0.74	27546	5375	14715	4141	65.2	6
		1CB	0.8	17939	3698	13016	1866	58	6
		1CC	0.77	18973	3525	10792	1590	63.7	6
1	Busan (Treatment)	1TA	1.04	0	0	4817	936	0	6
		1TB	1.07	0	0	3440	836	0	6
		1TC	1.1	0	0	5212	1906	0	6
2	Kobe (Uptake	2UA	1.02	116306	28278	616896	66856	15.9	6
		2UB	1.01	148794	32950	594912	45102	20	6
		2UC	1.01	160000	27033	388119	155870	29.2	6
2	Kobe (Control)	2CA	1.04	53436	12085	592935	162258	8.3	6
		2CB	1.04	76775	16308	669482	56452	10.3	6
		2CC	1.06	74424	12163	658467	79995	10.2	6
2	Kobe (Treatment)	2TA	1.02	6.41	n.a.	656026	85580	0	15
		2TB	1.02	0	0	654998	82632	0	6
		2TC	1.02	0	0	650772	82637	0	6
3	Guam (Uptake)	3UA	1.01	71764	9499	130751	34406	35.4	6
		3UB	1.01	76614	10269	177602	46788	30.1	6
		3UC	1.06	70633	8910	190368	43807	27.1	6
3	Guam (Control)	3CA	1.04	46095	9551	98425	39926	31.9	6
		3CB	1.05	51077	14532	108175	8739	32.1	6
		3CC	1.09	49847	15089	89134	17519	35.9	6
3	Guam (Treatment)	3TA	1.01	0	0	34257	16016	0	6
		3TB	1.02	0	0	31150	14076	0	6
		3TC	1.04	0	0	35094	14677	0	6
4	SF Bay (Uptake)	4UA	1.09	81074	12775	19988	7326	80.2	6
		4UB	1.04	100068	16224	20690	5252	82.9	6
		4UC	1.05	84683	5849	23796	4233	78.1	6
4	SF Bay (Control)	4CA	1.01	88770	13714	26854	4493	76.8	6
		4CB	1.03	92358	5784	23834	4470	79.5	6
		4CC	1.03	73727	7158	20833	2497	78	6
4	SF Bay (Treatment)	4TA	1.18	0	0	12072	5003	0	6
		4TB	1.19	0.84	n.a.	10159	1850	0	6
		4TC	1.2	0	0	12377	2163	0	6

n.a. = Not Analyzed

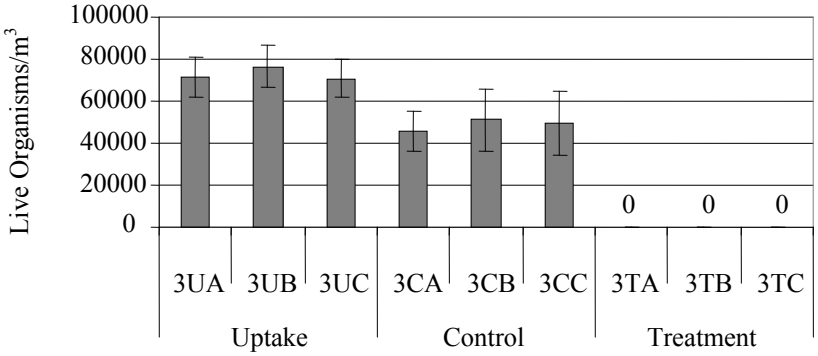
Test Cycle 1 (Busan, S. Korea) ≥50 µm organisms, Live Organisms/m^3



Test Cycle 2 (Kobe, Japan) ≥50 µm organisms, Live Organisms/m^3



Test Cycle 3 (Guam, USA) ≥50 µm organisms, Live Organisms/m^3



Test Cycle 4 (SF Bay, USA) ≥50 µm organisms, Live Organisms/m^3

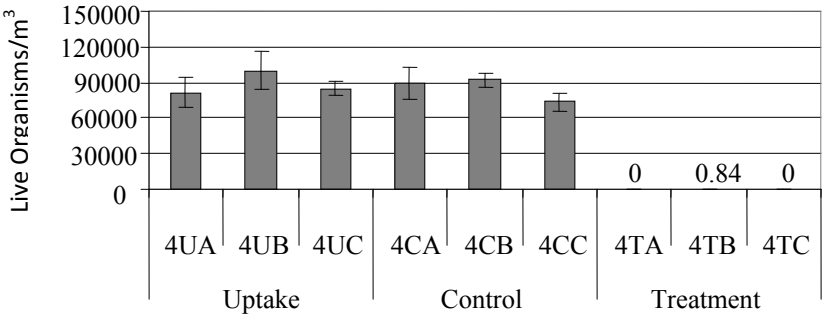


Figure 1 – Results for Organisms ≥50 µm, Test Cycles 1, 2, 3 and 4

Counts of live zooplankton ≥50 µm. Nominal 1 m^3 volumes were passed continuously through 35 µm plankton nets (50 µm on the diagonal of the square pore); actual volumes sampled are listed in Table 3. Samples were concentrated to a final volume of 400 mL and counted immediately in 10 mL Bogorov counting trays (6 replicates for each net sample). Quantitative dilutions were made with 0.2 µm filtered seawater when organism density was too high and when background particulate matter obscured the counting view. In the last trial in SF Bay, the net samples from uptake and control nets were concentrated to a final volume of 200 mL, six 5 mL aliquots were counted. Treated samples were further concentrated to 60 mL and counted in six, 10 mL replicates without dilution, thus effectively counting the entire 1 m^3 net volume.

7.2 Organisms <50 µm but ≥10 µm

Two independent experimental methods were used to enumerate live organisms in the <50 µm but ≥10 µm size class: a) FDA tagging of live cells with flow cytometric detection of fluorescein-marked cells (Table 4, Figure 2), and b) chlorophyll-based MPN grow-out experiments evaluated by whole cell chlorophyll (chl) fluorescence (Table 5, Figure 3). Uptake water, measured by cytometry, showed initial concentrations of live <50 µm but ≥10µm organisms that met the IMO G8 challenge criteria in three of four experiments (i.e., >10x the regulatory value of 10 live cells/mL). The oligotrophic waters near Guam yielded low counts of living organisms in the 10-50 µm size class. Encouragingly, the cytometry and MPN technique provided comparable results, including agreement on the relatively low number of protists observed in Guam (Tables 4 & 5, Figures 2 & 3). However, MPN results were generally lower than those from cytometry as might be expected, since the MPN technique is limited to detection of only those photoautotrophs that can successfully grow in the media and conditions provided (F/8 seawater media, 24 hr illumination 50 µEin m^{-2} s^{-1}, room temperature).

Most importantly, both experimental methods (FDA probe and chl-based MPN) provided estimates of live organism concentrations that passed the IMO D-2 discharge standard of <10 live organisms/mL for the <50 µm but ≥10 µm size class for all replicate samples measured over the course of all four ballast experiments (Tables 4 & 5, Figures 2 & 3).

7.2.1 Cytometry Results, Organisms <50 µm but ≥10 µm

Table 4 – Cytometry Results Summary for Organisms <50 µm but ≥10 µm Test Cycles 1, 2, 3 and 4

<50 µm but ≥10 µm Organisms (Cytometry)				
Test Cycle	**Location**	**Sample**	**Live Organisms/mL**	**n**
1	Busan (Uptake)	1UA	210	1
		1UB	n.a.	-
		1UC	n.a.	-
1	Busan (Control)	1CA	50.1	1
		1CB	54.4	1
		1CC	67.5	1
1	Busan (Treatment)	1TA	2.35	1
		1TB	4.02	1
		1TC	0.74	1
2	Kobe (Uptake)	2UA	166	1
		2UB	253	1
		2UC	34.2	1
2	Kobe (Control)	2CA	174	1
		2CB	105	1
		2CC	38.0	1
2	Kobe (Treatment)	2TA	0.54	1
		2TB	6.13	1
		2TC	1.76	1
3	Guam (Uptake)	3UA	16.8	1
		3UB	4.44	1
		3UC	14.0	1
3	Guam (Control)	3CA	24.2	1
		3CB	25.5	1
		3CC	9.35	1
3	Guam (Treatment)	3TA	0.22	1
		3TB	0.11	1
		3TC	0.13	1
4	SF Bay (Uptake)	4UA	197	1
		4UB	161	1
		4UC	172	1
4	SF Bay (Control)	4CA	51.2	1
		4CB	47.1	1
		4CC	36.1	1
4	SF Bay (Treatment)	4TA	5.40	1
		4TB	2.79	1
		4TC	1.94	1

n.a. = Not Analyzed

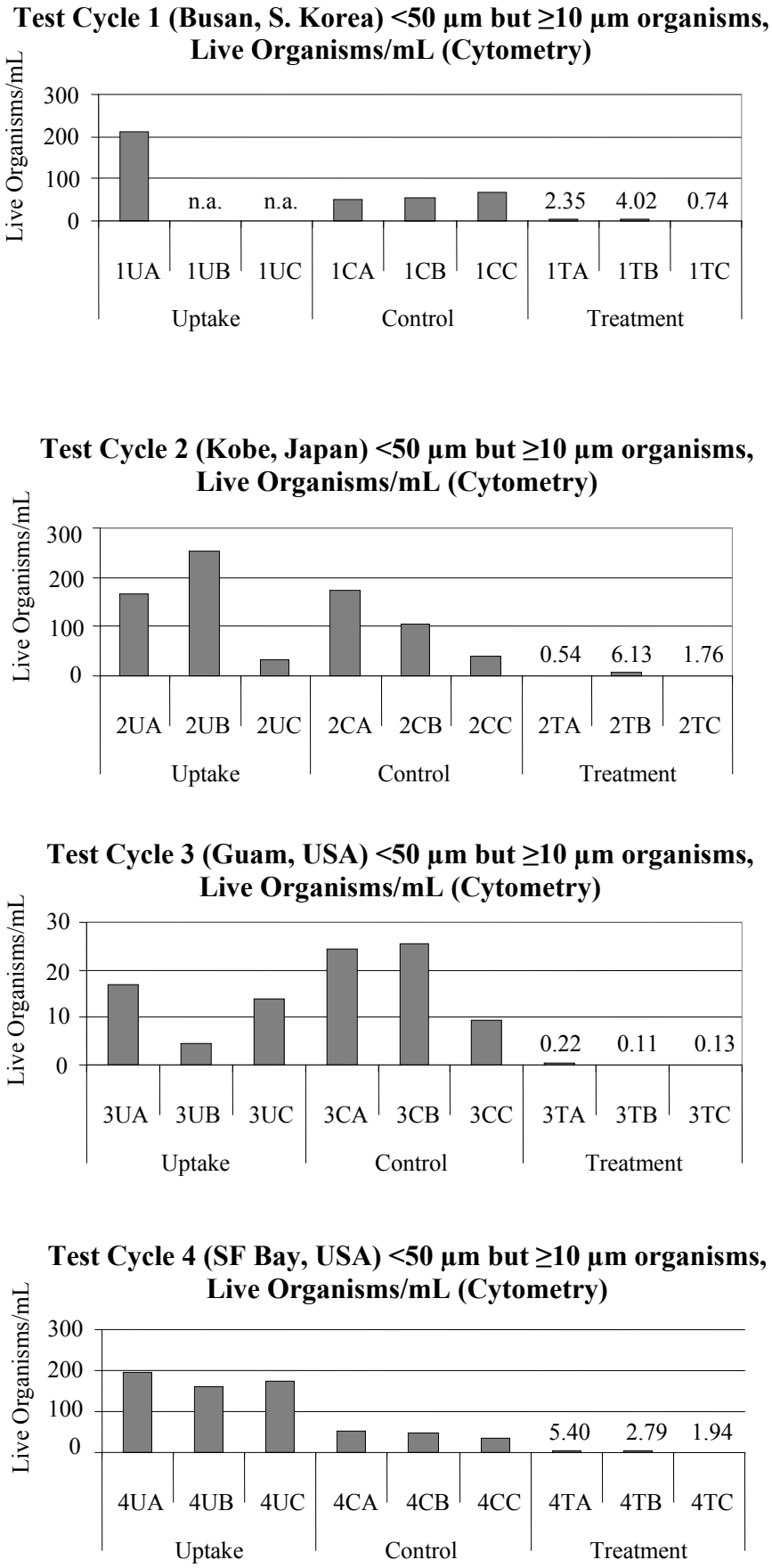


Figure 2 – Cytometry Results for Organisms <50 µm but ≥10 µm, Test Cycles 1, 2, 3 and 4

Flow cytometric determination of live organisms <50 µm but ≥10 µm. Analyses in Busan, Kobe and Guam were made on a BD FACScan cytometer, using 2 minute counts at 60 µL/min. An Accuri C6 cytometer was used onboard ship in San Francisco Bay at the same flow characteristics. (n.a. = Not Analyzed)

7.2.2 Chlorophyll-Based Results, Organisms <50 µm but ≥10 µm

Table 5 – Chlorophyll-based Results Summary, Organisms <50 µm but ≥10 µm, Test Cycles 1, 2, 3 and 4

<50 µm but ≥10 µm Organisms (chl-based MPN)				
Test Cycle	**Location**	**Sample**	**MPN/mL**	**n**
1	Busan (Uptake)	1UA	210	1
		1UB	290	1
		1UC	260	1
1	Busan (Control)	1CA	39	1
		1CB	130	1
		1CC	71	1
1	Busan (Treatment)	1TA	<0.220	1
		1TB	<0.220	1
		1TC	<0.220	1
2	Kobe (Uptake)	2UA	100	1
		2UB	120	1
		2UC	84	1
2	Kobe (Control)	2CA	61	1
		2CB	100	1
		2CC	71	1
2	Kobe (Treatment)	2TA	<0.220	1
		2TB	<0.220	1
		2TC	<0.220	1
3	Guam (Uptake)	3UA	0.3	1
		3UB	1.6	1
		3UC	1.6	1
3	Guam (Control)	3CA	0.8	1
		3CB	2.3	1
		3CC	0.6	1
3	Guam (Treatment)	3TA	0.008	1
		3TB	0.018	1
		3TC	0.008	1
4	SF Bay (Uptake)	4UA	49	1
		4UB	13	1
		4UC	3.1	1
4	SF Bay (Control)	4CA	0.2	1
		4CB	9.5	1
		4CC	3.4	1
4	SF Bay (Treatment)	4TA	0.003	1
		4TB	0.006	1
		4TC	0.014	1

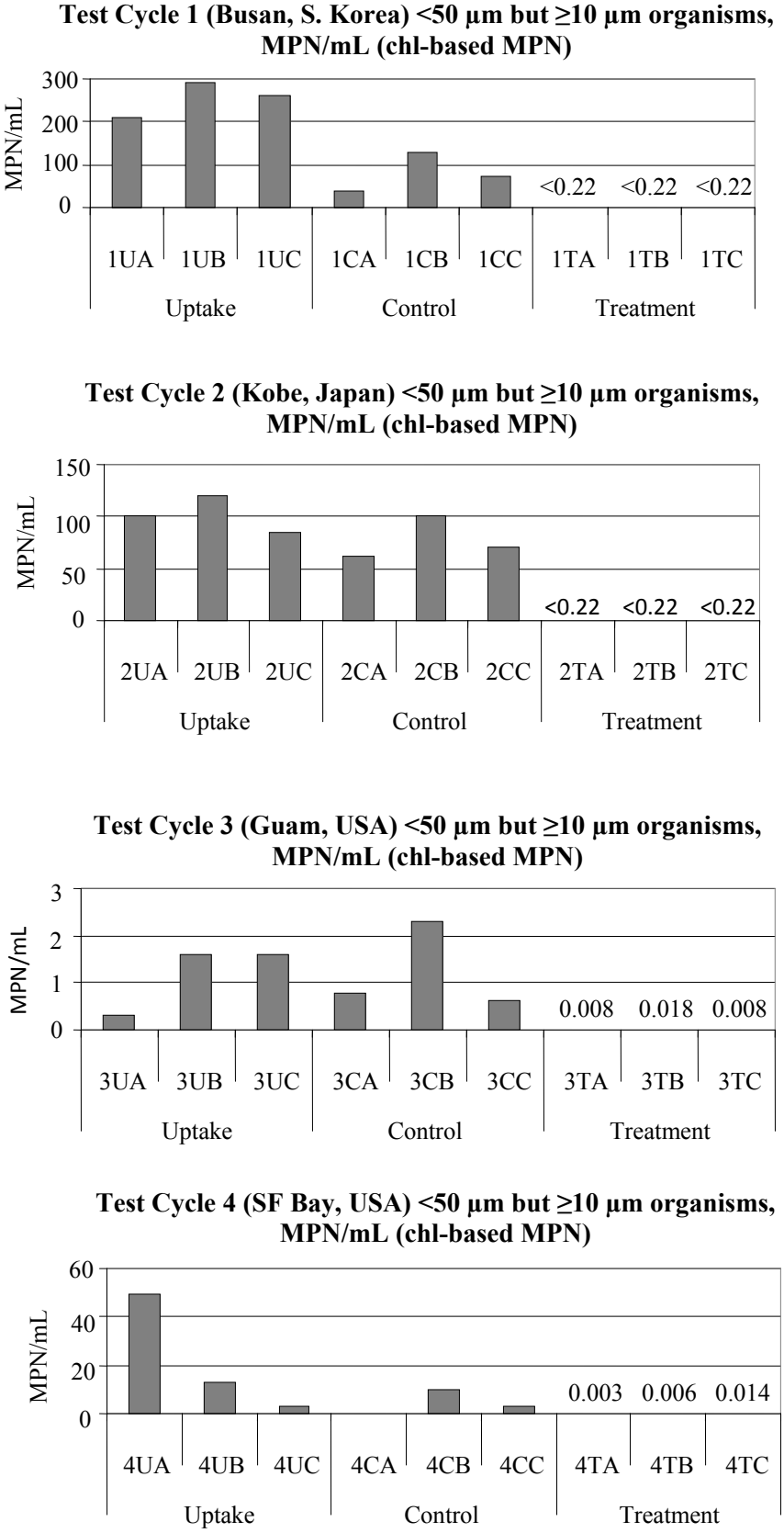


Figure 3 – Chl-based Results for Organisms <50 µm but ≥10 µm, Test Cycles 1, 2, 3 and 4

Chl-based most probable number (MPN) determination of living organisms <50 µm but ≥10 µm in size. Samples were passed through a Nitex 50 µm sieve to remove larger organisms prior to incubation. Uptake, control and treatment samples in Busan and Kobe were prepared from unconcentrated seawater samples; the measured treatment concentration of <0.22/mL represents the lower limit of detection for the observation of no observable growth in any MPN tube (6x6; replicates x dilutions, five-fold serial dilutions for each series). Treatment samples in Guam and San Francisco were concentrated 100-fold, permitting lower detection levels.

7.3 Indicator Microbes

Idexx MPN test kits, Colilert™ and Enterolert™, were used to enumerate the IMO-specified indicator microbe species, *Escherichia coli* and *Enterococci.* The maximum allowable concentration of *E. coli* and *Enterococci* specified in IMO D-2 discharge standards is <250 CFU/100mL and <100 CFU/100mL, respectively. All treatment samples easily met these regulation limits for all ballast experiments (Tables 6 & 7, Figures 4 & 5). In fact, the observed 'natural' challenge concentration in uptake water for *E. coli* met the IMO D-2 standard *without* treatment at all harbor locations, suggesting that the IMO ballast standard for *E. coli* may be set rather high. The MPN technique is known to produce numerical estimates with wide confidence limits (Woomer et al. 1990), thus we chose to make triplicate measurements for each of the triplicate sampling tubs (Tables 6 & 7). Not surprisingly, the resulting coefficients of variation often exceeded 100%, which is typical of MPN results. Even so, a strong 'treatment' effect was clearly evident in the results after treatment by the BalPure® BP-500 BWTS. Treatment samples for *E. coli* produced no detectable living organisms at all locations (Table 6) and treatment samples for *Enterococci* yielded at least a 2-log reduction from ambient uptake water (Table 7). Both tests easily passed the IMO D-2 standards.

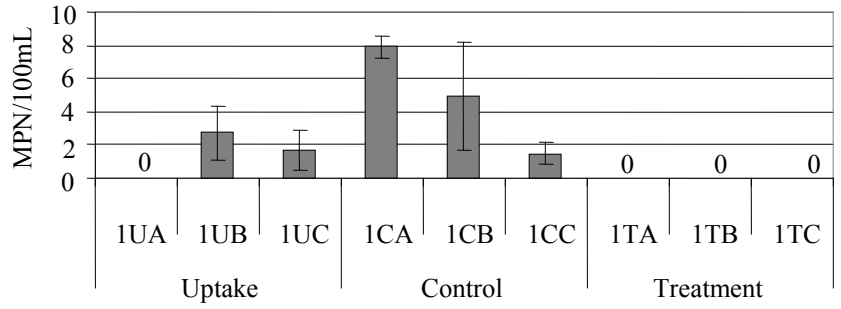
All tests for live *Vibrio cholerae* serotypes 01 and 0139 were negative at the level of IMO D-2 standards (<1 CFU/100mL). In all, 38 tests for *V. cholerae* 01 and 0139 were analyzed; each yielded negative results for uptake, control discharge and treatment discharge samples. Thus, data tables and figures are not presented for *V. cholerae*; we only need report that IMO D-2 standards for live *V. cholerae* 01 and 0139 were met in all samples analyzed, for all experiments. A figure showing an example of the BalPure® BP-500 BWTS results for *V. cholerae* is given in Appendix E. Therefore, although the test for *V. cholerae* passed the specified IMO regulation, the observed negative results for all ballast operations (uptake, control discharge and treatment discharge) provide little useful information regarding 'treatment' performance per se.

Attempts to utilize a direct count staining technique to detect total (live + dead) *V. cholerae* (Mourino-Perez et al. 2003) yielded very low, but positive responses in all samples analyzed at a level >1 cell/100 mL. This was observed in all samples measured, including uptake, control and treatment samples. The direct count method utilizes the same monoclonal antibody reagent (Hasan et al. 1995) used in the culture-based Cholera Smart™ and Bengal Smart™ test kits, but the direct count technique provides no distinction between live and dead cells. The analytical protocol was tedious and time-consuming (>1 hr labor per sample), since the entire 2.5 cm polycarbonate filter had to be visually scanned at high oil-immersion power (1000x) to find tagged cells in 100 mL seawater filtrations (generally, 1-3 total cells were observed per slide). The positive stained cells could not be differentiated as live vs. dead (Mourino-Perez et al. 2003), and there was no apparent difference between uptake and treatment. For these reasons the direct count method was not deemed appropriate for IMO D-2 testing. Therefore, data are not presented in this report and the method cannot be recommended in future ballast treatment studies.

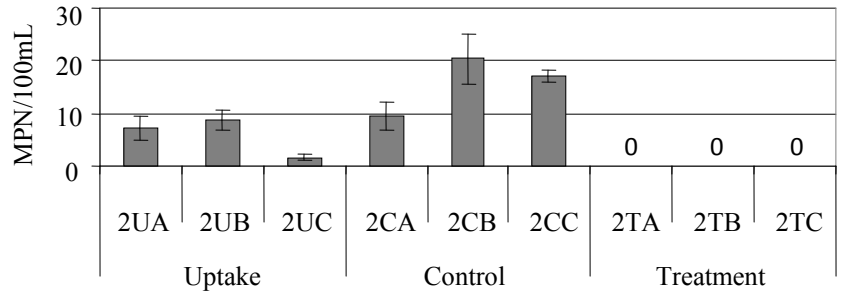
Table 6 – Results Summary for *E. coli*, Test Cycles 1, 2, 3 and 4

Indicator Microbes (*E. coli*)						
Test Cycle	**Location**	**Sample**	**MPN/100 mL**	**S.D.**	**C.V. (%)**	**n**
1	Busan (Uptake)	1UA	0	0	0	3
		1UB	2.77	1.63	58.8	3
		1UC	1.70	1.21	71.3	3
1	Busan (Control)	1CA	7.90	0.69	8.8	3
		1CB	4.97	3.23	65.1	3
		1CC	1.50	0.71	47.1	2
1	Busan (Treatment)	1TA	0	0	0	3
		1TB	0	0	0	3
		1TC	0	0	0	3
2	Kobe (Uptake)	2UA	7.20	2.40	33.4	3
		2UB	8.73	2.02	23.1	3
		2UC	1.67	0.58	34.6	3
2	Kobe (Control)	2CA	9.53	2.55	26.8	3
		2CB	20.33	4.61	22.7	3
		2CC	17.10	0.99	5.8	3
2	Kobe (Treatment)	2TA	0	0	0	3
		2TB	0	0	0	3
		2TC	0	0	0	3
3	Guam (Uptake)	3UA	27.07	9.24	34.1	3
		3UB	30.07	4.04	13.4	3
		3UC	27.30	5.99	22.0	3
3	Guam (Control)	3CA	66.87	28.12	42.0	3
		3CB	0	0	0	3
		3CC	57.55	2.19	3.8	3
3	Guam (Treatment)	3TA	0	0	0	3
		3TB	0	0	0	3
		3TC	0	0	0	3
4	SF Bay (Uptake)	4UA	6.40	1.91	29.8	3
		4UB	13.43	5.14	38.3	3
		4UC	8	8.56	107	3
4	SF Bay (Control)	4CA	16.03	4.33	27.0	3
		4CB	10.83	4.31	39.7	3
		4CC	10.10	5.15	51.0	3
4	SF Bay (Treatment)	4TA	0	0	0	3
		4TB	0	0	0	3
		4TC	0	0	0	3

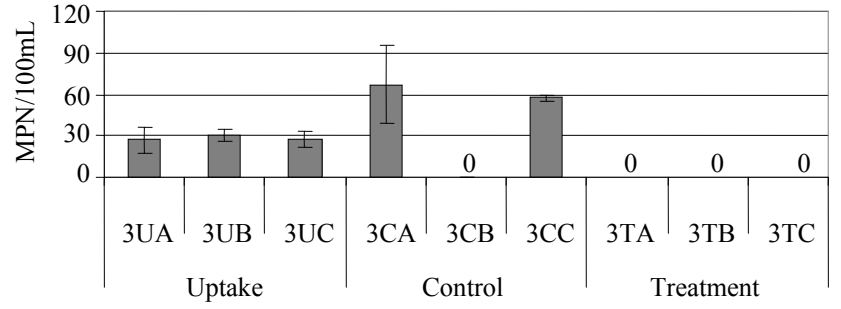
Test Cycle 1 (Busan, S. Korea) Indicator Microbes: *E. coli*, MPN/100 mL



Test Cycle 2 (Kobe, Japan) Indicator Microbes: *E. coli*, MPN/100 mL



Test Cycle 3 (Guam, USA) Indicator Microbes: *E. coli*, MPN/100 mL



Test Cycle 4 (SF Bay, USA) Indicator Microbes: *E. coli*, MPN/100 mL

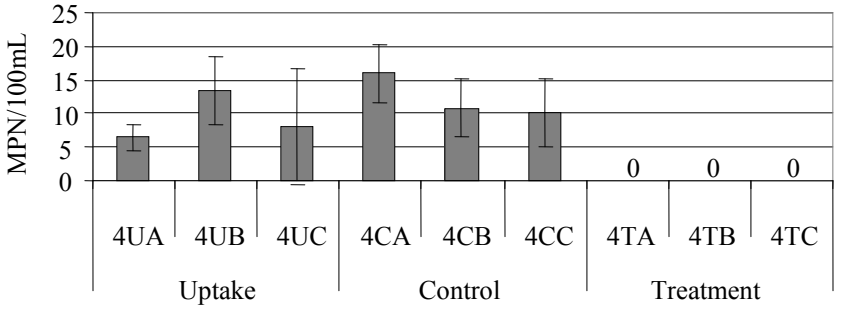


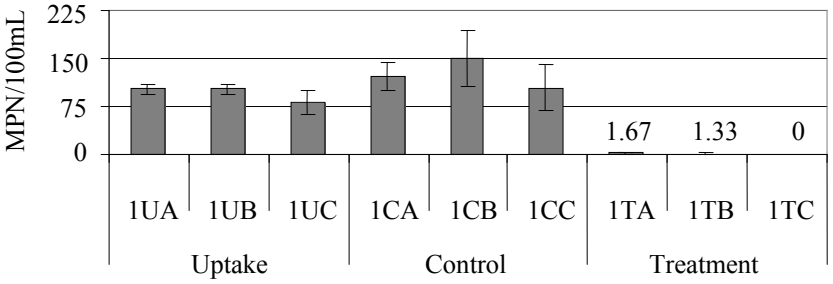
Figure 4 – Summary of *E. coli* Results, Test Cycles 1, 2, 3 and 4

Most probable number (MPN) determination of live *E. coli* concentrations. Idexx, Inc. Colilert® MPN kits were used with 366 nm UV detection after 24 hr dark incubations at 37°C in controlled incubator. No detectable growth was found in any of the treatment samples. The lowest limit of certainty for the 51 chamber Colilert® MPN tray is <1 MPN/100 mL when no growth is observed. Here, samples with no growth were scored as 0 MPN/100 mL to allow mean and standard deviation calculations for replicates. All samples were measured in triplicate.

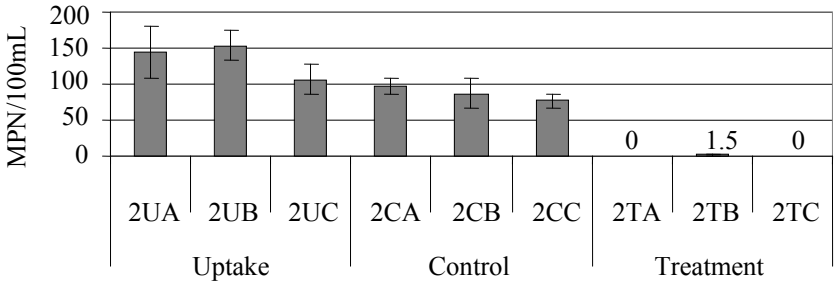
Table 7 – Results Summary for *Enterococci*, Test Cycles 1, 2, 3 and 4

Indicator Microbes (*Enterococci*)						
Test Cycle	**Location**	**Sample**	**MPN/100 mL**	**S.D.**	**C.V. (%)**	**n**
1	Busan (Uptake)	1UA	101.6	7.31	7.2	3
		1UB	101.6	7.31	7.2	3
		1UC	80.57	18.80	23.3	3
1	Busan (Control)	1CA	121.4	21.76	17.9	3
		1CB	149.6	44.48	29.7	3
		1CC	104.0	36.49	35.1	3
1	Busan (Treatment)	1TA	1.67	0.58	34.6	3
		1TB	1.33	0.58	43.3	3
		1TC	0	0	0	3
2	Kobe (Uptake)	2UA	143.9	36.89	25.6	3
		2UB	153.4	20.44	13.3	3
		2UC	106.6	21.12	19.8	3
2	Kobe (Control)	2CA	97.37	10.59	10.9	3
		2CB	87.30	20.03	22.9	3
		2CC	76.40	9.48	12.4	3
2	Kobe (Treatment)	2TA	0	0	0	3
		2TB	1.50	0.71	47.1	3
		2TC	0	0	0	3
3	Guam (Uptake)	3UA	81.47	2.83	3.5	3
		3UB	181.8	32.33	17.8	3
		3UC	107.4	81.70	76.1	3
3	Guam (Control)	3CA	200	0	0	3
		3CB	200	0	0	3
		3CC	137.8	55.60	40.3	3
3	Guam (Treatment)	3TA	0	0	0	3
		3TB	1.50	0.71	47.1	3
		3TC	0	0	0	3
4	SF Bay (Uptake)	4UA	83.60	8.49	10.2	3
		4UB	86.7	3.12	3.6	3
		4UC	77.53	11.31	14.6	3
4	SF Bay (Control)	4CA	37.13	4.24	11.4	3
		4CB	43.53	9.73	22.3	3
		4CC	36.1	8.02	22.2	3
4	SF Bay (Treatment)	4TA	0	0	0	3
		4TB	0	0	0	3
		4TC	0	0	0	3

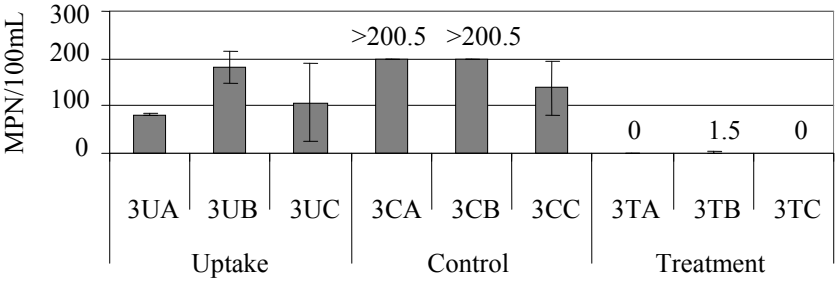
Test Cycle 1 (Busan, S. Korea) Indicator Microbes: *Enterococci*, MPN/100 mL



Test Cycle 2 (Kobe, Japan) Indicator Microbes: *Enterococci*, MPN/100 mL



Test Cycle 3 (Guam, USA) Indicator Microbes: *Enterococci*, MPN/100 mL



Test Cycle 4 (SF Bay, USA) Indicator Microbes: *Enterococci*, MPN/100 mL

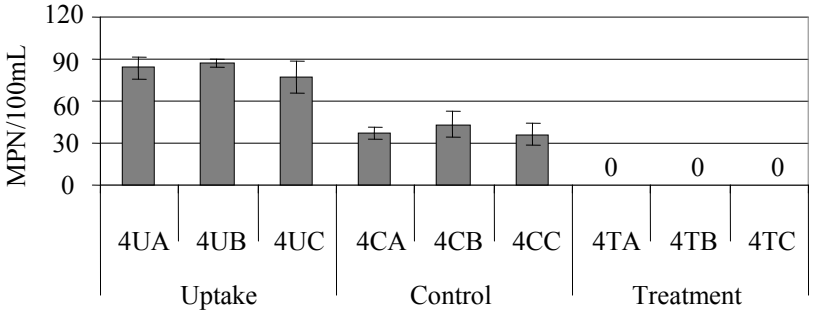


Figure 5 – *Enterococci* Results, Test Cycles 1, 2, 3 and 4

Most probable number (MPN) determination of live *Enterococci* concentrations. Idexx, Inc. Enterolert® MPN kits were used with 366 nm UV detection after 24 hr dark incubations at 37°C in controlled incubator. The lowest limit of certainty when no positive growth was detected for the 51 chamber Enterolert® MPN tray is <1 MPN/100 mL. Here, samples with no growth were scored as 0 MPN/100mL to allow mean and standard deviation calculations for replicates. All samples were measured in triplicate.

7.4 Corroborative Biological Efficacy Results

A small suite of methods for viability determination complementary to, but not required by IMO D-2 regulations, were executed to provide corroborative data to support the findings of IMO regulatory tests reported above. Tables 8-12 and Figures 6-9 show results for: cultivable total heterotrophic plate counts (CFU/mL) in Section 7.4.1; dark-adapted PAM fluorescence measurements of maximal phytoplankton photosystem II quantum efficiency, F_v/F_m (Maxwell and Johnson 2000) in Section 7.4.2; total ATP (ng/L) and ATP-derived estimates of living carbon in Section 7.4.3; and comparison of cytometric and epifluorescent microscopy methods for the <50 um but ≥10 um size class in Section 7.4.4.

7.4.1 Heterotrophic Plate Counts (HPC)

A 2- to 3-log reduction in cultivable bacteria was seen in treatment samples (relative to uptake samples) in 3 of the 4 test cycles, as determined from 24 hr, room temperature incubations on Difco 2216 sterile marine agar plates (Table 8, Figure 6). Treatment samples from Kobe showed relatively higher CFU concentrations, resulting in an apparent 1- to 2-log reduction in cultivable bacteria. The results were encouraging because a sterile workspace was not available onboard ship to provide confident, sterile streaking procedures, yet clear treatment effects were evident. In the three experiments with highest apparent biological treatment efficacy (Busan, Guam and San Francisco), treatment results yielded counts of only one or zero observed CFU per plate, indicating efficient inactivation of broadly cultivable marine bacteria. Generally, the bacterial HPC results complement the findings for the single species indicator microbes (*E. coli* and *Enterococci*) described above.

Table 8 – Results Summary for Heterotrophic Plate Counts, Test Cycles 1, 2, 3 and 4

Heterotrophic Plate Counts						
Test Cycle	**Location**	**Sample**	**CFU/mL**	**S.D.**	**C.V. (%)**	**n**
1	Busan (Uptake)	1UA	10900	n.a.	n.a.	1
		1UB	11380	n.a.	n.a.	1
		1UC	TMTC	n.a.	n.a.	1
1	Busan (Control)	1CA	3940	n.a.	n.a.	1
		1CB	10980	n.a.	n.a.	1
		1CC	12880	n.a.	n.a.	1
1	Busan (Treatment)	1TA	0	n.a.	n.a.	1
		1TB	0	n.a.	n.a.	1
		1TC	0	n.a.	n.a.	1
2	Kobe (Uptake)	2UA	28180	n.a.	n.a.	1
		2UB	11660	n.a.	n.a.	1
		2UC	8520	n.a.	n.a.	1
2	Kobe (Control)	2CA	4900	n.a.	n.a.	1
		2CB	4920	n.a.	n.a.	1
		2CC	5280	n.a.	n.a.	1
2	Kobe (Treatment)	2TA	540	n.a.	n.a.	1
		2TB	340	n.a.	n.a.	1
		2TC	80.0	n.a.	n.a.	1
3	Guam (Uptake)	3UA	TMTC	n.a.	n.a.	3
		3UB	TMTC	n.a.	n.a.	3
		3UC	TMTC	n.a.	n.a.	3
3	Guam (Control)	3CA	7060	3689	52.3	3
		3CB	10233	680	6.6	3
		3CC	TMTC	n.a.	n.a.	3
3	Guam (Treatment)	3TA	33.3	23.1	115	3
		3TB	0	0	0	3
		3TC	0	0	0	3
4	SF Bay (Uptake)	4UA	4163	439	10.6	3
		4UB	1330	164	12.3	3
		4UC	1603	90.7	5.7	3
4	SF Bay (Control)	4CA	147	112	76.6	3
		4CB	253	92.9	36.7	3
		4CC	190	145	76.5	3
4	SF Bay (Treatment)	4TA	10.0	10.0	100	3
		4TB	13.3	11.5	86.6	3
		4TC	0	0	0	3

n.a. = Not Analyzed; TMTC = Too many to count.

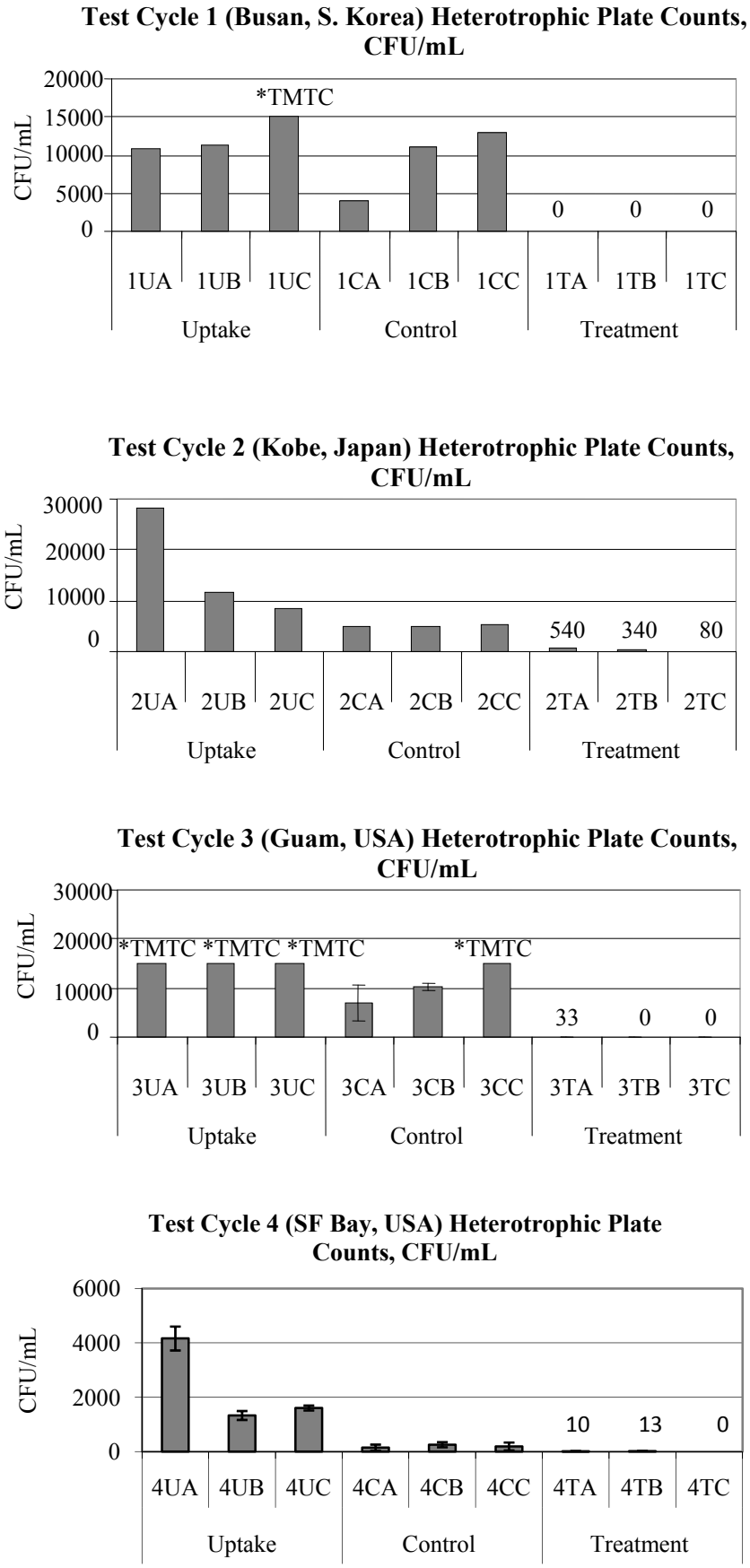


Figure 6 – Heterotrophic Plate Count Results, Test Cycles 1, 2, 3 and 4

Heterotrophic plate counts (HPC) made on 100 mm dia. sterile petri plates with Difco, Inc. 2216 marine agar. Plates were loaded quantitatively with 50 µL or 100 µL volumes (as necessary), spread with alcohol flamed glass rods on a rotating plate-spinner and incubated 24 hr room temperature (dark). (*TMTC = too many to count).

7.4.2 Pulse Amplified Modulated (PAM) Fluorescence

Table 9 and Figure 7 below show data for PAM fluorescence measurements of photosystem II quantum efficiency defined by the variable fluorescence ratio, F_v/F_m. Values of dark adapted F_v/F_m are expected to fall in the range of 0.5 - 0.7 for physiologically active algal cells (Maxwell and Johnson 2000). F_v/F_m for uptake and control discharge samples from Busan, Kobe and San Francisco Bay were all quite similar (ca. 0.65). This indicated normal physiological condition for ambient phytoplankton even after 48 hr dark storage in the control ballast tank. Guam showed slightly reduced values of F_v/F_m (ca. 0.5) possibly indicative of the nutrient-poor oligotrophic waters surrounding the Mariana Islands. Treatment discharge samples indicated F_v/F_m values near zero in many cases. Reported values of treatment F_v/F_m in Table 9 were calculated directly by automated PAM software. However, visual inspections of raw data signals showed treatment fluorescence responses that were within the noise of the background fluorescence. We conclude that the treatment measurements of F_v/F_m were near the level of detection, thus indicating severely compromised metabolic conditions for photoautotrophs present in the treated ballast water.

Table 9 – Results Summary for PAM Fluorescence, Test Cycles 1, 2, 3 and 4

PAM Fluorescence				
Test Cycle	**Location**	**Sample**	**Dark F_v/F_m**	**n**
1	Busan (Uptake)	1UA	0.701	1
		1UB	0.689	1
		1UC	0.690	1
1	Busan (Control)	1CA	0.698	1
		1CB	0.655	1
		1CC	0.672	1
1	Busan (Treatment)	1TA	0	1
		1TB	0.182	1
		1TC	0.004	1
2	Kobe (Uptake)	2UA	0.673	1
		2UB	0.671	1
		2UC	0.685	1
2	Kobe (Control)	2CA	0.655	1
		2CB	0.693	1
		2CC	0.674	1
2	Kobe (Treatment)	2TA	0	1
		2TB	0	1
		2TC	0	1
3	Guam (Uptake)	3UA	0.509	1
		3UB	0.480	1
		3UC	0.514	1
3	Guam (Control)	3CA	0.442	1
		3CB	0.511	1
		3CC	0.402	1
3	Guam (Treatment)	3TA	0	1
		3TB	0.210	1
		3TC	0.109	1
4	SF Bay (Uptake)	4UA	0.629	1
		4UB	0.597	1
		4UC	0.614	1
4	SF Bay (Control)	4CA	0.624	1
		4CB	0.642	1
		4CC	0.646	1
4	SF Bay (Treatment)	4TA	0.016	1
		4TB	0.073	1
		4TC	0	1

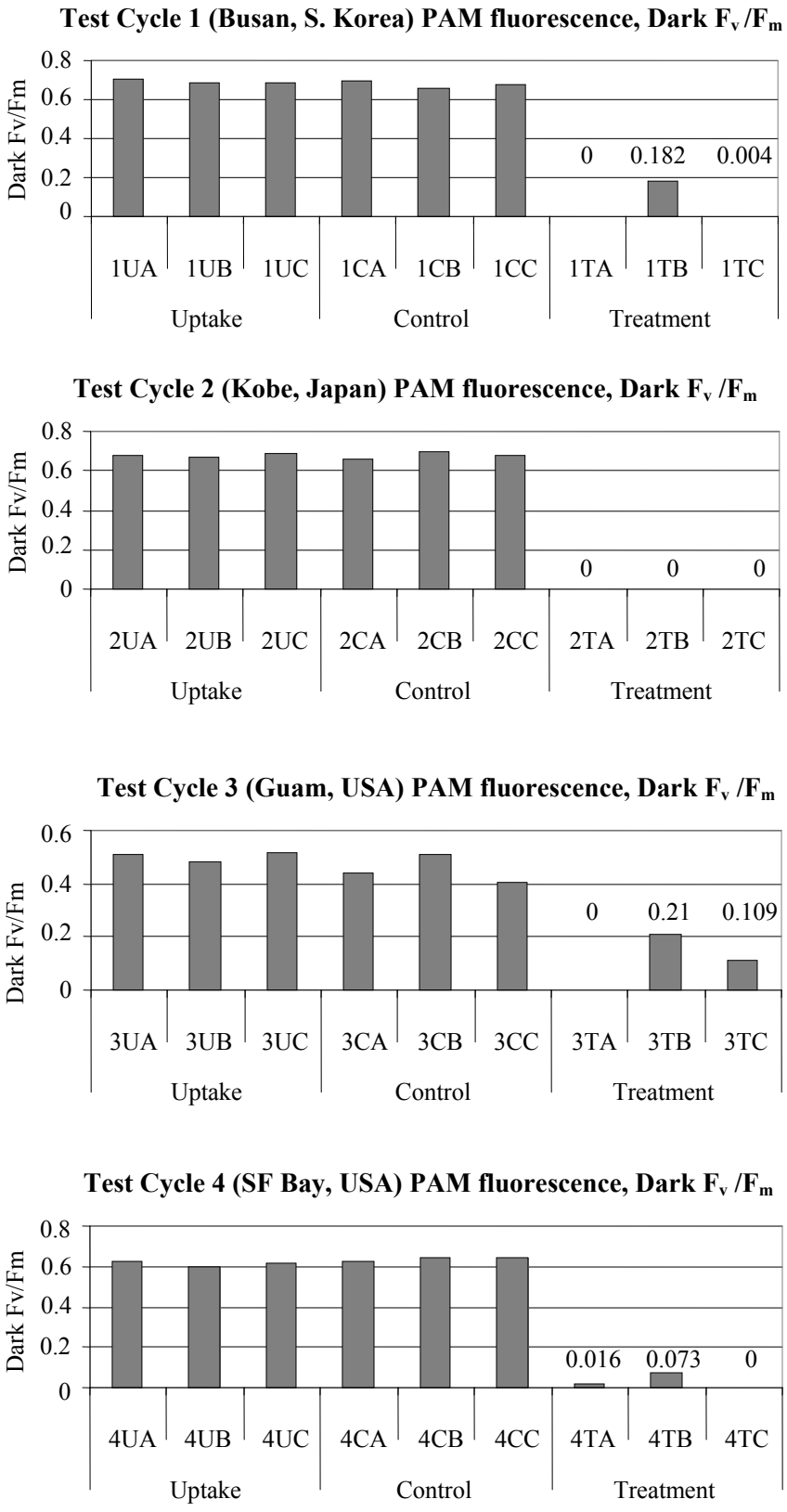


Figure 7 – PAM Fluorescence Results, Test Cycles 1, 2, 3 and 4

Dark adapted PAM fluorescence measurements of F_v/F_m, the maximum quantum yield of photosystem II. Samples were dark adapted at least 1 hr prior to measurement on a cuvet-based Water-PAM (Walz, Inc.) using a vendor-supplied stirrer to maintain cell suspension.

7.4.3 Adenosine Triphosphate (ATP)

ATP is known to serve as a quantitative indicator of total living microbial biomass and is also known to decay quickly from dead organisms (Karl 1993). During evaluation of the BalPure® BP-500 BWTS ATP was used as a bulk quantitative proxy for living biomass. As shown in Table 10 and Figure 8, total ATP concentrations (>0.7 µm, Whatman GF/F filter) in treatment samples were very low relative to uptake and control samples in all four ballast experiments. The results showed a 2- to 3-log reduction in ATP relative to challenge water ATP concentrations, similar to that noted in HPC counts (Table 8). It is likely that the 'total' ATP value measured here was largely representative of the microbial size classes (bacteria and protists) since the maximum volume filtered was 1 L which would have been too small to quantitatively include representative zooplankton contributions. The results highlight an impressive ballast treatment reduction in 'living biomass' as inferred from ATP analysis, thus corroborating measurements of numeric live counts specific to IMO standards presented earlier.

Table 10 – Results Summary for ATP, Test Cycles 1, 2, 3 and 4

Total ATP						
Test Cycle	**Location**	**Sample**	**ng/L**	**S.D.**	**C.V. (%)**	**n**
1	Busan	Uptake	85.26	11.25	13.2	3
		Control	27	n.a.	n.a.	1
		Treatment	0.33	n.a.	n.a.	1
2	Kobe	Uptake	110.5	27.55	24.9	2
		Control	90.08	3.58	4.0	2
		Treatment	0.13	0.27	208	2
3	Guam	Uptake	20.41	1.43	7.0	2
		Control	7.55	2.19	29.1	2
		Treatment	0.28	0.35	123	2
4	SF Bay	Uptake	n.a.	n.a.	n.a.	-
		Control	32.71	25.74	78.7	3
		Treatment	0.22	0.16	73.3	3

n.a. = Not Analyzed

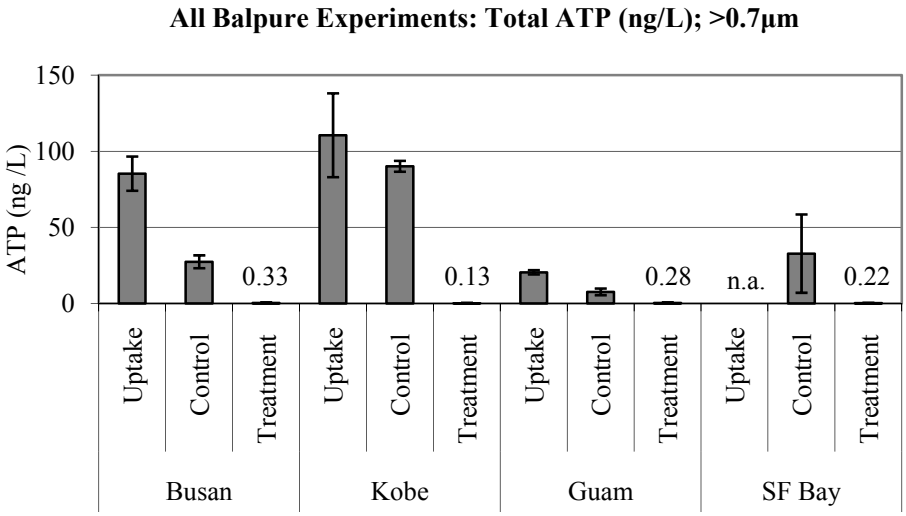


Figure 8 – ATP Results, Test Cycles 1, 2, 3 and 4
Adenosine triphosphate (ATP) determined on GF/F filters (0.7 µm nominal pore size). Extractions were made in boiling 0.25 mM Tris buffer (5 min); extracts were kept frozen (-20°C) until analysis on a Turner Designs 20/20 Luminometer using Promega Inc., Enlighten™ luciferin-luciferase enzyme preparations and ATP standards. (n.a. = Not Analyzed)

Several studies have documented a relatively constant cellular carbon/ATP ratio in unicellular organisms, suggesting that estimates of 'living' POC can be derived from ATP measurements (Holm-Hansen 1972; Karl 1993). Karl (1993) notes that the expected living POC/ATP ratio is ca. 250 (g/g) for most bacteria, phytoplankton and colorless protists. Below we present combined POC and ATP measurements that were used to calculate the fraction of total microbial 'living carbon' observed in the uptake, control and treatment samples. Figure 9A shows estimates of the percent living carbon calculated from total POC measurements (live + dead; CHN combustion analysis) and ATP-derived living POC (assumed cellular quota = 250 g C/g ATP) for test experiments made in Busan, Kobe, Guam and San Francisco Bay. The data from all uptake and control samples highlight the well known fact the majority of marine POC is detrital; e.g., only 1-4% of the POC was estimated to be alive in the challenge water. However, the living percent was reduced as low as 0.01% in most treatment samples, roughly a 2-log reduction in living carbon. The result suggests that a standardized measurement of percent living carbon might be useful in evaluating ballast treatment efficacy when numeric live counts cannot be made.

A further analysis of ATP is given here in an attempt to estimate upper and lower numeric live cell concentrations based on ATP measurements within the 10-50 um size class, for the purpose of corroboration with live numeric counts of the same size class presented earlier. In this case we use well known empirical relationships between protist cell size (biovolume) and carbon content (Strathmann 1967; Menden-Deuer and Lessard 2000).

In the context of the ballast-related <50 µm but ≥10 µm size class, the equations of Strathmann (1967) yield estimates of living carbon for 10 and 50 µm spherical cells corresponding to 90.3 pg C/cell and 8447 pg C/cell, respectively. Estimates of living carbon (from ATP) combined with sized-based estimates of cellular carbon content can be combined to yield estimates of numeric cell concentration for any protist size. We made such an attempt on all four BalPure® BP-500 treatment experiments using simple size fractionation technique to measure the ATP concentration within the <50 µm but ≥10 µm fraction for treatment discharge samples. Seawater (1 L) was passed through a Nitex 50 µm pore filter, collected onto a 10 µm Nitex pore filter and analyzed for ATP per methods described in the Revised Science Test Plan (Appendix D). The living POC/ATP ratio of 250 (g/g) combined with Strathmann equations for living carbon content as a function of biovolume for 10 µm and 50 µm equivalent spherical diameter cells yields an expected inverse cellular ATP quota of 2768 cells/ng ATP and 29.6 cells/ng ATP, respectively for the two size extremes. Those values were used to estimate the numeric cell densities of living organisms at the size extremes of the <50 µm but ≥10 µm range, based on the empirical, integrated ATP measured within the <50 µm but ≥10 µm range. The results given in Figure 9B and Figure 9C below show that the generous (Figure 9B) and conservative (Figure 9C) ATP-based estimates of cell concentration both pass the IMO D-2 discharge standard in the treatment samples.

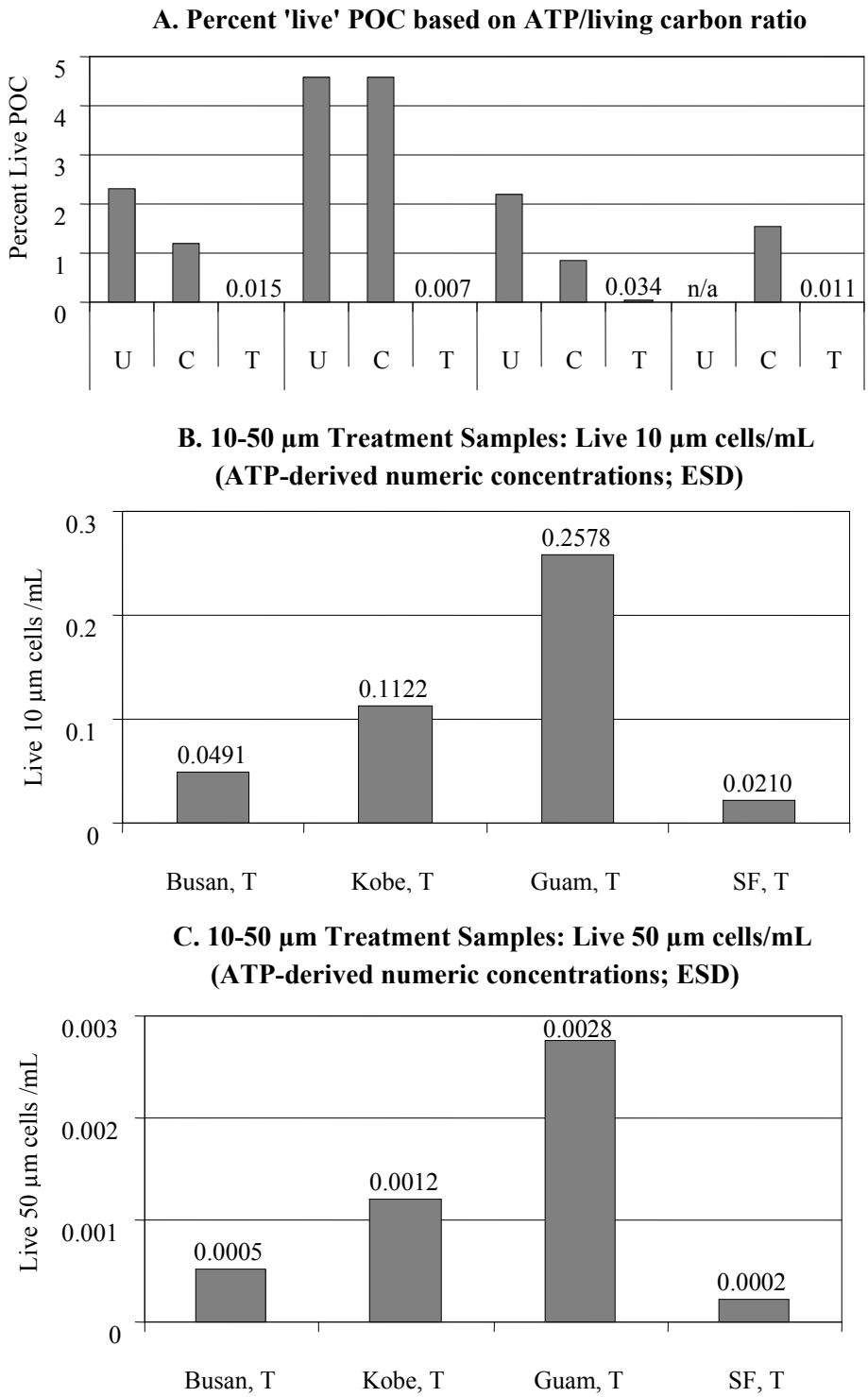


Figure 9 – ATP-derived Estimates of Living Organisms

A. Living carbon as a percentage of total particulate organic carbon (POC). Living carbon was estimated from empirical determination of total ATP and an expected ratio of living POC/ATP of 250 (g/g).

B. Numeric estimate of live 10 µm cells/mL in treatment samples based on empirical measurement of ATP in the size fraction <50 µm but ≥10 µm.

C. Numeric estimate of live 50 µm cells/mL in treatment samples based on empirical measurement of ATP in the size fraction <50 µm but ≥10 µm. Estimates of 10 µm (B) vs. 50 µm (C) numeric concentrations represent generous and conservative living cell densities, respectively, within the <50 µm but ≥10 µm size class (see text above).
(n.a. = Not Analyzed)

7.4.4 Cytometry and Epifluorescence Comparison, Organisms <50 µm but ≥10 µm

A comparison of cytometry and epifluorescence of organisms in the <50 µm but ≥10 µm size class was planned for all test cycles. Due to logistical reasons, we were unable to corroborate cytometric vs. microscopic identification of FDA-positive cells during at-sea trials on the Western N. Pacific cruise legs (Test Cycles 1, 2, and 3). However, comparisons were made during the final experiment in San Francisco Bay (Test Cycle #4) where time constraints were not as rigid. The results are shown in Table 11 and Table 12. Both detection methods yielded treatment concentrations that passed IMO D-2 discharge standards; however, epifluorescence detection yielded results two orders of magnitude lower than cytometry in the treatment samples. The epifluorescence method utilized a 100x concentration of cells onto 10 µm Nitex filters, resuspension in 1 mL FSW and visual microscopic examination of the entire three-dimensional 1 mL volume of the gridded Sedgewick-Rafter cell. The epifluorescence method is onerous, but results for Test Cycle #4 clearly yielded a strong treatment effect, suggesting a 4-log reduction in ambient numeric concentrations of <50 µm but ≥10 µm cells.

Table 11 – Cytometry Results for <50 µm but ≥10 µm Organisms, Method Comparison

<50 µm but ≥10 µm Organisms (Cytometry)				
Test Cycle	**Location**	**Sample**	**Live Organisms/mL**	**n**
4	SF Bay (Uptake)	4UA	196.50	1
		4UB	161.15	1
		4UC	172.38	1
4	SF Bay (Control)	4CA	51.19	1
		4CB	47.12	1
		4CC	36.08	1
4	SF Bay (Treatment)	4TA	5.40	1
		4TB	2.79	1
		4TC	1.94	1

Table 12 – Epifluorescence Results for <50 µm but ≥10 µm Organisms, Method Comparison

<50 µm but ≥10 µm Organisms (Epifluorescence)						
Test Cycle	**Location**	**Sample**	**Live Organisms/mL**	**S.D.**	**C.V. (%)**	**n**
4	SF Bay (Uptake)	4UA	497	23.09	4.65	3
		4UB	536	10.18	1.90	3
		4UC	498	119	23.82	3
4	SF Bay (Control)	4CA	590	20.00	3.39	3
		4CB	510	62.45	12.25	3
		4CC	487	83.86	17.23	3
4	SF Bay (Treatment)	4TA	0.008	0.010	133	4
		4TB	0.010	0.010	100	3
		4TC	0.007	0.006	86.60	3

7.5 Challenge Water Characteristics

Criteria for successful IMO D-2 shipboard testing require measurement of source water salinity, temperature, particulate organic carbon (POC) and total suspended solids (TSS). In contrast to land-based testing, shipboard testing does not require specific minimum challenge water characteristics other than the ambient organism concentrations be >10x the maximum allowable regulatory standard for the ≥50 µm and <50 but ≥10 µm size classes. Average challenge water characteristics were given in the Executive Summary (Table 1). In an effort to understand the fate of particulate material after ballast water treatment, measurements of TSS, POC and chlorophyll *a* (chl) were made in all uptake, control discharge and treatment discharge samples. POC analysis included measurements of particulate organic nitrogen (PON) which, when referenced against POC as C/N (g/g), can provide indications of qualitative transformations of the particulate material as a result of ballast treatment.

As shown in Tables 13 & 14 and Figures 10 & 11, POC and PON remained relatively constant over all ballast cycles (uptake, control and treatment). This is in contrast to the marked changes that were noted in numeric organism counts and biological viability metrics (ATP, PAM fluorescence) described earlier. Examination of C/N ratios in Table 13 shows that in three test cycles (Busan, Kobe and Guam) the treatment samples yielded an increase in C/N of about 50% relative to uptake challenge water. This indicates either a selective mechanical removal of particulate material characterized by low C/N and/or selective decomposition (solubilization) of PON relative to POC. The data corroborate the fact that the bulk of suspended organic material in marine waters is detrital (non-living) since all metrics for living organism concentrations (numeric or otherwise) decreased by orders of magnitude after treatment with the BalPure® BP-500 BWTS, while total POC decreased only modestly (30-50% reduction). This indicates that a substantial, possibly measureable, reduction in the ratio of living POC to total POC must have occurred due to treatment (see Section 8, Discussion of Results).

Changes in TSS after treatment as shown in Table 15 and Figure 12 were also relatively modest (less than two-fold changes). However, it was clear that a general increase in TSS was evident in the treatment samples for each of the first three experiments (Busan, Kobe and Guam). Ballast tanks were not cleaned between at-sea trials which may provide a partial explanation of the results in Kobe and Guam, e.g., accumulation of sediment in the ballast tank may have been re-suspended during the next fill cycle. This reasoning does not explain the contrasting results between the first test cycle (Busan) and the last test cycle (San Francisco Bay), which were both done with cleaned control and treatment tanks. Moreover, the possible explanation of sediment accumulation and re-suspension would suggest comparable effects in control and treatment tanks; however, the treatment clearly showed a larger TSS increase in the first three test cycles (Figure 12).

Chlorophyll *a* (chl) is commonly used to evaluate relative concentrations of phytoplankton biomass, but it is uncertain as to whether chl alone can serve as an indicator of live vs. dead algal biomass. As shown in Table 16 and Figure 13 chl decreased in all treatment samples by one or two orders of magnitude relative to uptake water. It is unlikely that BalPure® BP-500 BWTS *filtration* was responsible for removal of chl since the vast majority of algal biomass is smaller than the nominal 50 µm treatment filter and the results showed relatively little change to small particulates measured as POC (Table 13, Figure 10). Thus, the action of chlorination by the BalPure® BP-500 BWTS is the likely cause of chl loss suggesting that simple measurement of solvent-extracted chl can serve as a gross indicator of treatment efficacy.

Table 13 – Results Summary for POC, Test Cycles 1, 2, 3 and 4

Particulate Organic Carbon (POC)							
Test Cycle	**Location**	**Sample**	**µg/L**	**S.D.**	**C.V. (%)**	**C/N (g/g)**	**n**
1	Busan (Uptake)	1UA	652	n.a.	n.a.	7.63	1
		1UB	1415	n.a.	n.a.	8.43	1
		1UC	687	n.a.	n.a.	7.45	1
1	Busan (Control)	1CA	546	n.a.	n.a.	7.27	1
		1CB	595	n.a.	n.a.	7.60	1
		1CC	602	n.a.	n.a.	7.39	1
1	Busan (Treatment)	1TA	516	n.a.	n.a.	9.89	1
		1TB	574	n.a.	n.a.	11.30	1
		1TC	602	n.a.	n.a.	11.44	1
2	Kobe (Uptake)	2UA	651	n.a.	n.a.	6.01	1
		2UB	542	n.a.	n.a.	6.31	1
		2UC	624	n.a.	n.a.	6.51	1
2	Kobe (Control)	2CA	492	n.a.	n.a.	6.51	1
		2CB	509	n.a.	n.a.	6.69	1
		2CC	480	n.a.	n.a.	6.35	1
2	Kobe (Treatment)	2TA	415	n.a.	n.a.	8.62	1
		2TB	443	n.a.	n.a.	9.11	1
		2TC	522	n.a.	n.a.	8.60	1
3	Guam (Uptake)	3UA	233	n.a.	n.a.	5.50	1
		3UB	n.a.	n.a.	n.a.	n.a.	-
		3UC	n.a.	n.a.	n.a.	n.a.	-
3	Guam (Control)	3CA	227	n.a.	n.a.	6.68	1
		3CB	n.a.	n.a.	n.a.	n.a.	-
		3CC	n.a.	n.a.	n.a.	n.a.	-
3	Guam (Treatment)	3TA	208	n.a.	n.a.	9.82	1
		3TB	n.a.	n.a.	n.a.	n.a.	-
		3TC	n.a.	n.a.	n.a.	n.a.	-
4	SF Bay (Uptake)	4UA	994	193	19.4	8.50	3
		4UB	983	67.6	6.9	7.65	3
		4UC	1000	132	13.2	8.28	3
4	SF Bay (Control)	4CA	517	70.1	13.6	5.67	3
		4CB	546	131	23.9	5.79	3
		4CC	533	172	32.3	6.75	3
4	SF Bay (Treatment)	4TA	472	136	28.7	8.55	3
		4TB	515	270	52.5	8.11	3
		4TC	603	354	58.7	8.05	3

n.a. = Not Analyzed

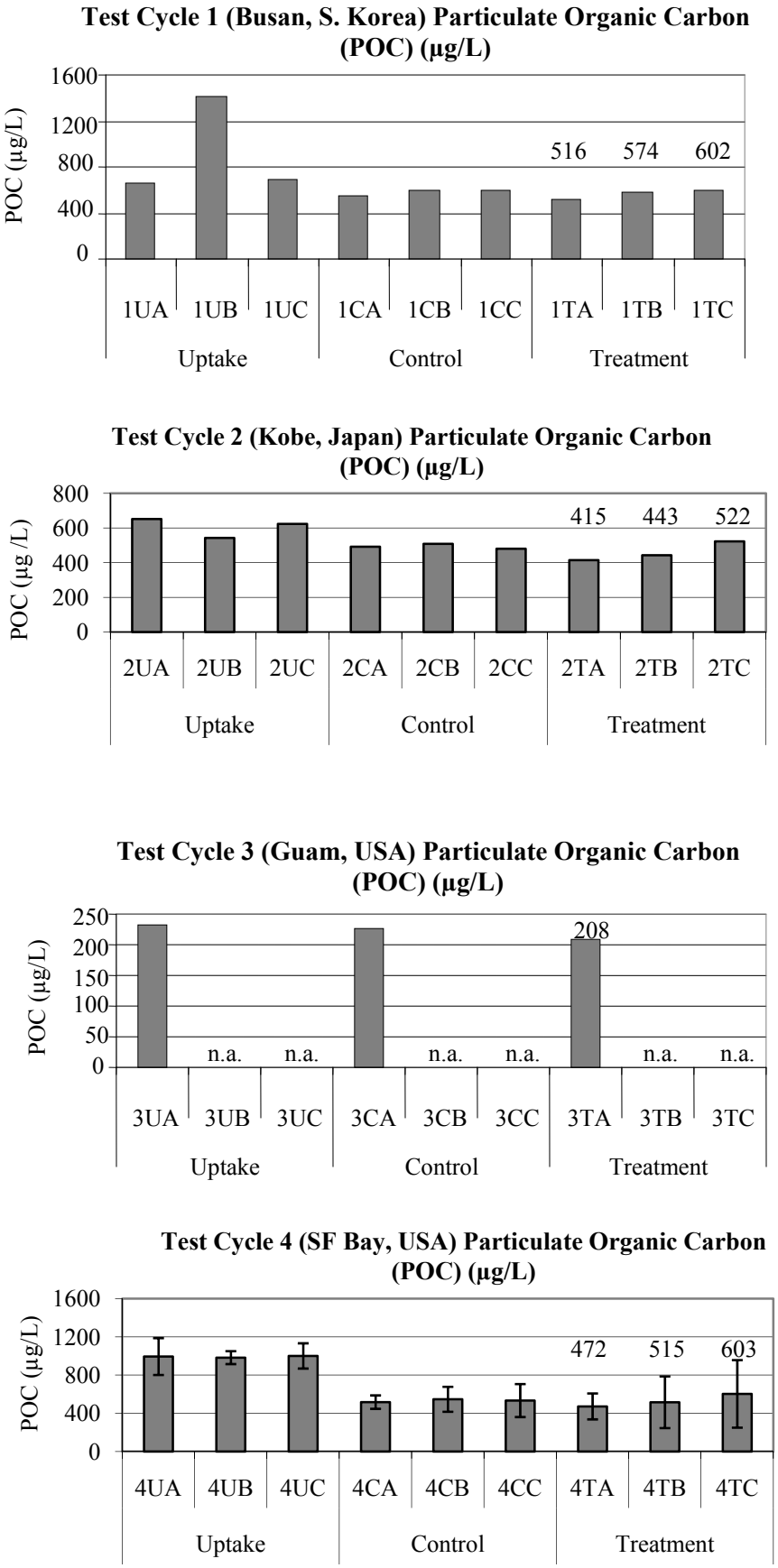


Figure 10 – POC Results, Test Cycles 1, 2, 3 and 4

Particulate organic carbon (POC) determined on GF/F filters (0.7 µm nominal pore size). Samples were dried at 65°C and stored under vacuum desiccation (room temperature) until analysis. Samples were run on a CEC 440 CHN analyzer, using gravimetrically determined L-cystine as standard. (n.a. = Not Analyzed)

Table 14 – Results Summary for PON, Test Cycles 1, 2, 3 and 4

Particulate Organic Nitrogen (PON)						
Test Cycle	**Location**	**Sample**	**µg/L**	**S.D.**	**C.V. (%)**	**n**
1	Busan (Uptake)	1UA	85.5	n.a.	n.a.	1
		1UB	168	n.a.	n.a.	1
		1UC	92.2	n.a.	n.a.	1
1	Busan (Control)	1CA	75.1	n.a.	n.a.	1
		1CB	78.3	n.a.	n.a.	1
		1CC	81.4	n.a.	n.a.	1
1	Busan (Treatment)	1TA	52.2	n.a.	n.a.	1
		1TB	50.8	n.a.	n.a.	1
		1TC	52.6	n.a.	n.a.	1
2	Kobe (Uptake)	2UA	108	n.a.	n.a.	1
		2UB	85.9	n.a.	n.a.	1
		2UC	95.8	n.a.	n.a.	1
2	Kobe (Control)	2CA	75.6	n.a.	n.a.	1
		2CB	76.0	n.a.	n.a.	1
		2CC	75.6	n.a.	n.a.	1
2	Kobe (Treatment)	2TA	48.1	n.a.	n.a.	1
		2TB	48.6	n.a.	n.a.	1
		2TC	60.7	n.a.	n.a.	1
3	Guam (Uptake)	3UA	42.4	n.a.	n.a.	1
		3UB	n.a.	n.a.	n.a.	-
		3UC	n.a.	n.a.	n.a.	-
3	Guam (Control)	3CA	34.0	n.a.	n.a.	1
		3CB	n.a.	n.a.	n.a.	-
		3CC	n.a.	n.a.	n.a.	-
3	Guam (Treatment)	3TA	21.2	n.a.	n.a.	1
		3TB	n.a.	n.a.	n.a.	-
		3TC	n.a.	n.a.	n.a.	-
4	SF Bay (Uptake)	4UA	117	8.6	7.3	3
		4UB	128	7.8	6.0	3
		4UC	121	1.9	1.6	3
4	SF Bay (Control)	4CA	91.2	10.7	11.7	3
		4CB	94.3	3.1	3.3	3
		4CC	79.0	3.2	4.1	3
4	SF Bay (Treatment)	4TA	55.2	2.6	4.7	3
		4TB	63.5	5.5	8.6	3
		4TC	74.8	5.8	7.8	3

n.a. = Not Analyzed

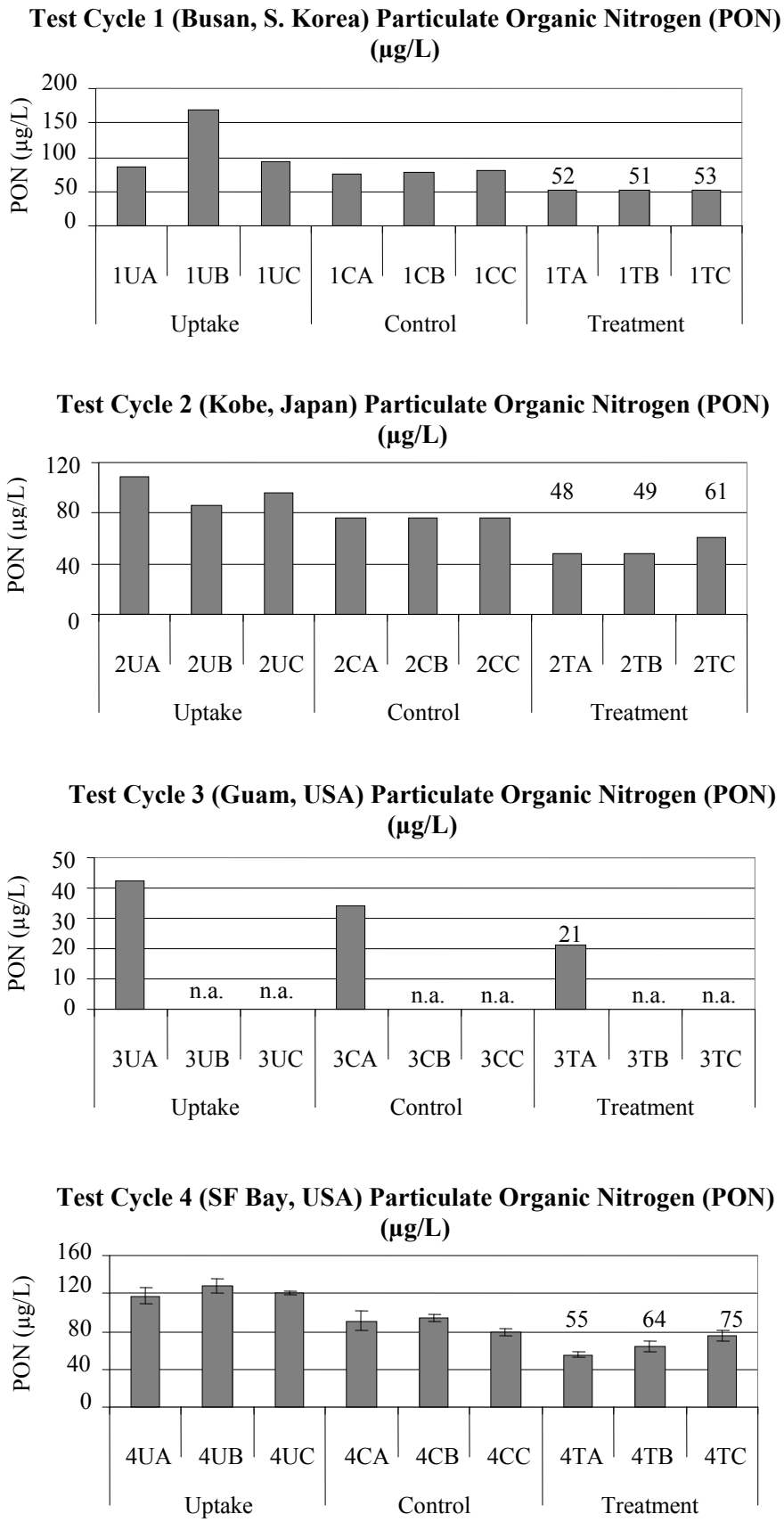


Figure 11 – PON Results, Test Cycles 1, 2, 3 and 4

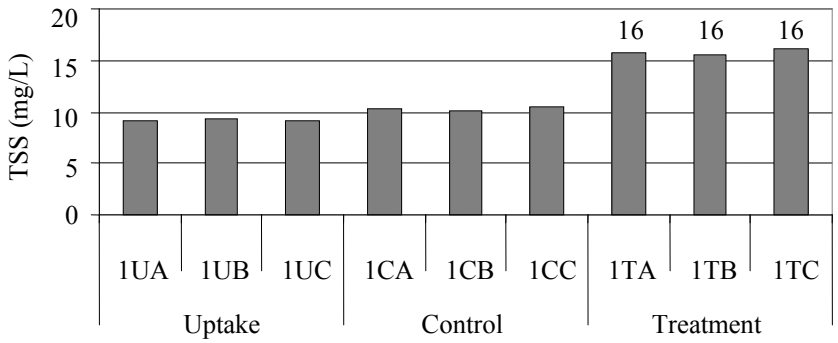
Particulate organic nitrogen (PON) determined on GF/F filters (0.7 µm nominal pore size). Samples were dried at 65°C and stored under vacuum desiccation (room temperature) until analysis. Samples were run on a CEC 440 CHN analyzer, using gravimetrically determined L-cystine as standard. (n.a. = Not Analyzed)

Table 15 – Results Summary for TSS, Test Cycles 1, 2, 3 and 4

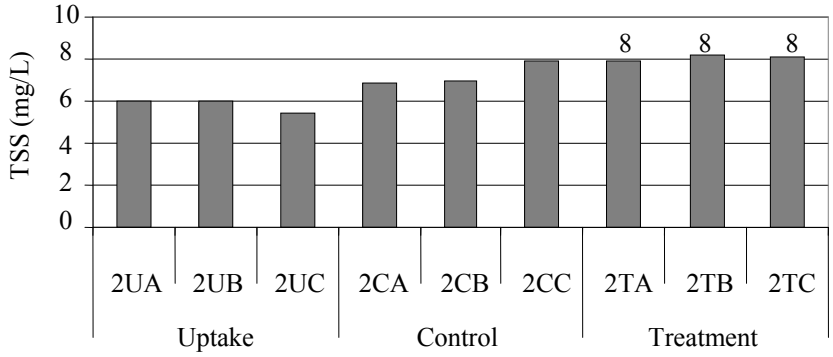
Total Suspended Solids, TSS						
Test Cycle	**Location**	**Sample**	**mg/L**	**S.D.**	**C.V. (%)**	**n**
1	Busan (Uptake)	1UA	9.06	n.a.	n.a.	1
		1UB	9.31	n.a.	n.a.	1
		1UC	9.19	n.a.	n.a.	1
1	Busan (Control)	1CA	10.21	n.a.	n.a.	1
		1CB	10.03	n.a.	n.a.	1
		1CC	10.56	n.a.	n.a.	1
1	Busan (Treatment)	1TA	15.68	n.a.	n.a.	1
		1TB	15.55	n.a.	n.a.	1
		1TC	16.13	n.a.	n.a.	1
2	Kobe (Uptake)	2UA	5.97	n.a.	n.a.	1
		2UB	6.04	n.a.	n.a.	1
		2UC	5.42	n.a.	n.a.	1
2	Kobe (Control)	2CA	6.87	n.a.	n.a.	1
		2CB	6.94	n.a.	n.a.	1
		2CC	7.93	n.a.	n.a.	1
2	Kobe (Treatment)	2TA	7.88	n.a.	n.a.	1
		2TB	8.17	n.a.	n.a.	1
		2TC	8.10	n.a.	n.a.	1
3	Guam (Uptake)	3UA	1.75	n.a.	n.a.	1
		3UB	2.00	n.a.	n.a.	1
		3UC	2.04	n.a.	n.a.	1
3	Guam (Control)	3CA	2.33	n.a.	n.a.	1
		3CB	n.a.	n.a.	n.a.	-
		3CC	n.a.	n.a.	n.a.	-
3	Guam (Treatment)	3TA	3.16	n.a.	n.a.	1
		3TB	n.a.	n.a.	n.a.	-
		3TC	n.a.	n.a.	n.a.	-
4	SF Bay (Uptake)	4UA	68.34	2.52	3.7	3
		4UB	68.62	2.11	3.1	3
		4UC	65.44	2.20	3.4	3
4	SF Bay (Control)	4CA	43.99	8.82	20.1	3
		4CB	37.09	2.13	5.7	3
		4CC	38.95	0.94	2.4	3
4	SF Bay (Treatment)	4TA	34.70	0.37	1.1	3
		4TB	31.09	1.82	5.9	3
		4TC	33.11	2.58	7.8	3

n.a. = Not Analyzed

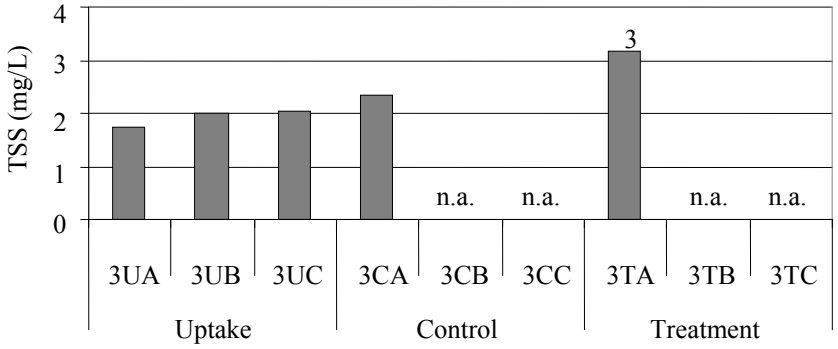
Test Cycle 1 (Busan, S. Korea) Total Suspended Solids (TSS) (mg/L)



Test Cycle 2 (Kobe, Japan) Total Suspended Solids (TSS) (mg/L)



Test Cycle 3 (Guam, USA) Total Suspended Solids (TSS) (mg/L)



Test Cycle 4 (SF Bay, USA) Total Suspended Solids (TSS) (mg/L)

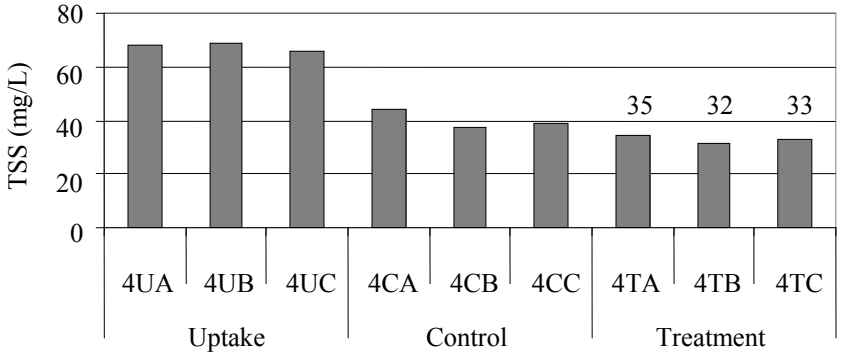


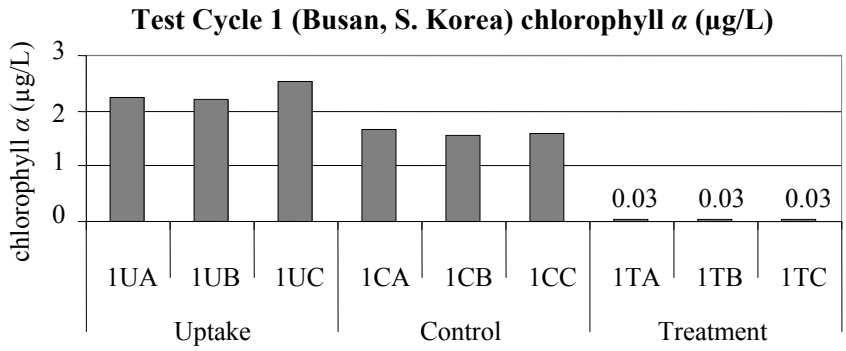
Figure 12 – TSS Results, Test Cycles 1, 2, 3 and 4

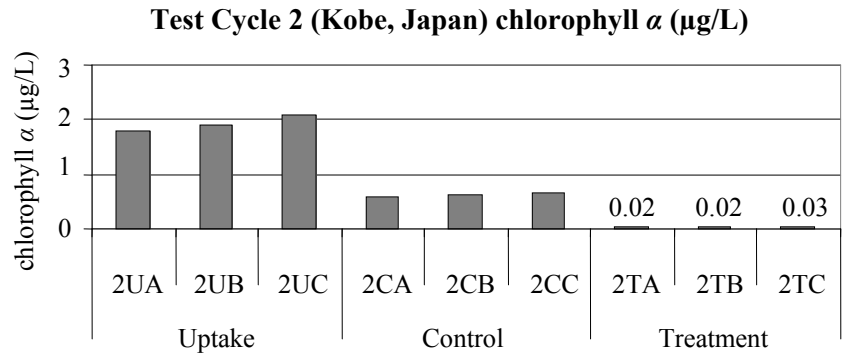
Total suspended solids (TSS). Samples were collected on pre-weighed, 47 mm diameter glass fiber filters (1.5 µm pore size, ProWeigh™, Environmental Express, Inc.), dried at 65°C and stored under vacuum desiccation until analysis on a calibrated balance (0.00001 g resolution). (n.a. = Not Analyzed)

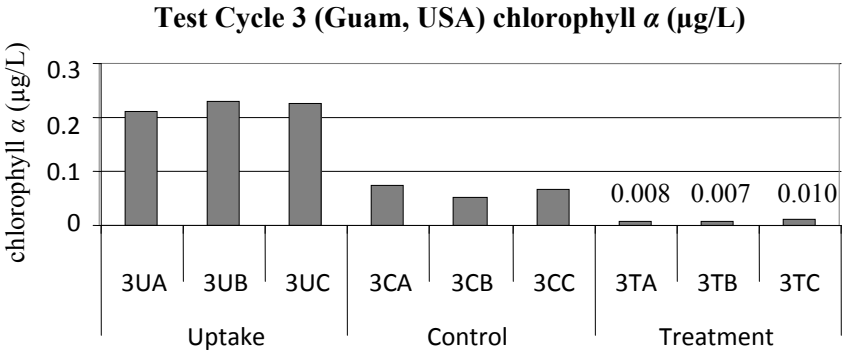
Table 16 – Results Summary for Chlorophyll *a*, Test Cycles 1, 2, 3 and 4

Chlorophyll *a*						
Test Cycle	**Location**	**Sample**	**µg/L**	**S.D.**	**C.V. (%)**	**n**
1	Busan (Uptake)	1UA	2.26	n.a.	n.a.	1
		1UB	2.21	n.a.	n.a.	1
		1UC	2.54	n.a.	n.a.	1
1	Busan (Control)	1CA	1.65	n.a.	n.a.	1
		1CB	1.55	n.a.	n.a.	1
		1CC	1.58	n.a.	n.a.	1
1	Busan (Treatment)	1TA	0.03	n.a.	n.a.	1
		1TB	0.03	n.a.	n.a.	1
		1TC	0.03	n.a.	n.a.	1
2	Kobe (Uptake)	2UA	1.81	n.a.	n.a.	1
		2UB	1.92	n.a.	n.a.	1
		2UC	2.07	n.a.	n.a.	1
2	Kobe (Control)	2CA	0.57	n.a.	n.a.	1
		2CB	0.61	n.a.	n.a.	1
		2CC	0.65	n.a.	n.a.	1
2	Kobe (Treatment)	2TA	0.02	n.a.	n.a.	1
		2TB	0.02	n.a.	n.a.	1
		2TC	0.03	n.a.	n.a.	1
3	Guam (Uptake)	3UA	0.21	n.a.	n.a.	1
		3UB	0.23	n.a.	n.a.	1
		3UC	0.23	n.a.	n.a.	1
3	Guam (Control)	3CA	0.07	n.a.	n.a.	1
		3CB	0.05	n.a.	n.a.	1
		3CC	0.07	n.a.	n.a.	1
3	Guam (Treatment)	3TA	0.01	n.a.	n.a.	1
		3TB	0.01	n.a.	n.a.	1
		3TC	0.01	n.a.	n.a.	1
4	SF Bay (Uptake)	4UA	2.31	0.237	10.3	3
		4UB	2.21	0.079	3.6	3
		4UC	2.32	0.175	7.5	3
4	SF Bay (Control)	4CA	1.39	0.063	4.5	3
		4CB	1.36	0.155	11.4	3
		4CC	1.25	0.036	2.8	3
4	SF Bay (Treatment)	4TA	0.06	0.011	18.5	3
		4TB	0.05	0.004	7.4	3
		4TC	0.05	0.007	14.2	3

n.a. = Not Analyzed







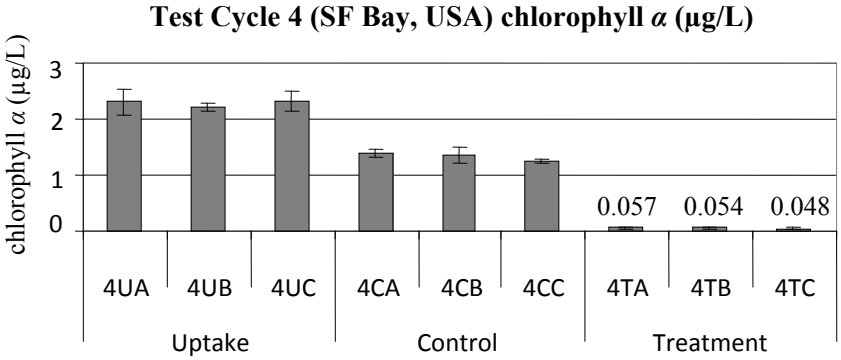


Figure 13 – Chlorophyll *a* Results, Test Cycles 1, 2, 3 and 4

Chlorophyll *a* determined fluorometrically from 90% acetone extracts. Samples were collected on GF/F filters (0.7 µm), extracted for a minimum of 24 hr (-20°C) and analyzed using single-step fluorometric protocol on a Spex Fluorolog 2 fluorometer. (n.a. = Not Analyzed)

Section 8 Discussion of Results

Criteria for successful shipboard ballast treatment efficacy include determinations of numeric live counts for organism groups including zooplankton ≥50 µm, protists in the <50 µm but ≥10 µm range and specific indicator microbe species (Table 2). The BalPure® BP-500 BWTS met, and in most cases, greatly exceeded IMO D-2 discharge standards for all test parameters measured in this study. With great regularity, e.g., in 79 of 81 microscope samples for zooplankton (Table 3), no living zooplankton were observed. The analysis was performed simultaneously, but independently, by two microscopists for all zooplankton samples for the sake of quality control. The data clearly indicate excellent performance in the inactivation of planktonic organisms ≥50 µm. The observation of living zooplankton in treatment samples was rare enough to question whether their appearance was due to random contamination from ship pipes and scientific labware, or whether it was due to incomplete sterilization from the BWTS. Regardless of cause, the data for those experiments where one (1) sole organism was found (Kobe and San Francisco Bay, Table 3) yielded a net 4-log and 5-log reduction, respectively, of living zooplankton relative to the source water. This is an impressive accomplishment.

As stated in the Science Test Plan, and as acknowledged by others (Dobroski et al. 2009), the numeric analysis of living organisms in the <50 µm but ≥10 µm size range is problematic because: 1) the organisms are small and difficult to identify, and 2) they do not present a reliable, visual indication of the live vs. dead state (in contrast to metazoans in the zooplankton size class). In an effort to account for these challenges, two independent methods were used to enumerate live <50 µm but ≥10 µm organisms. The FDA technique tags live cells for flow cytometric analysis on the basis of vital, intracellular esterase conversion of FDA to fluorescein (a green fluorescent marker). The chl-based MPN technique confirms viability on the basis of observable cell growth (population increase inferred from in situ chl fluorescence). Both methods yielded numeric treatment concentrations that were <10 live organisms/mL, thus satisfying IMO D-2 performance standards for that organism size class.

Though the two methods used for numeric counts of <50 µm but ≥10 µm organisms in treatment samples were below the allowable regulatory limit, the two techniques produced quite different absolute results. These assays are independent of each other and each follows a widely different method of execution. The FDA technique marks cells with a fluorescent tag that is not a stain per se; that is, fluorescein will freely diffuse out of the cell thereby requiring optical analysis (visual or cytometric) within 30 minutes after administering FDA; it is a nearly instantaneous marker for live cells. On the other hand, the chl-based MPN procedure requires an incubation time of at least 5-6 days to accumulate measurable evidence of phytoplankton growth, especially for the most dilute MPN tubes. Corroboration of multiple methods provides a reasonable foundation on which to build confidence in results representing the problematic <50 µm but ≥10 µm size class. Unfortunately, there is no single method that can irrefutably provide the true numeric concentration of the diverse taxa of organisms <50 µm but ≥10 µm (Dobroski et al. 2009). We therefore attempted to provide additional corroboration wherever possible.

A corroborative, indirect measure of living biomass, utilizing ATP, provided reasonable agreement with the cell-specific numeric counts of live organisms. It was shown that BalPure® BP-500 treatment resulted in a large decrease (ca. 2-log) in total ATP collected on GF/F filters (>0.7 µm) (Figure 8). Further, by utilizing filter size fractionation analysis of ATP, combined with well-established carbon/biovolume relations, we made both 'generous' and 'conservative' estimates of numeric cell densities in treatment discharge samples that fell below IMO limits in all cases for organisms <50 µm but ≥10 µm (Figure 9B and Figure 9C).

Thus, four corroborative procedures 1) FDA cytometry, 2) FDA microscopy, 3) chl-based MPN and 4) ATP-based size fractionation all yielded live cell densities in the <50 µm but ≥10 µm size category that pass the IMO D-2 discharge standard for treated samples, e.g., <10 living organisms/mL. We have taken measures to elaborate this discussion since, as stated, the methods for viable cell enumeration in the <50 µm but ≥10 µm size class are problematic (Dobroski et al. 2009). We conclude, on the basis of replicated, independent approaches, that treatment by the BalPure® BP-500 BWTS was successful in the inactivation/removal of <50 µm but ≥10 µm protists at the level required by IMO D-2 standards.

The discussion above, combined with the successful IMO biological efficacy results noted earlier for indicator microbes (Tables 6 & 7, Figures 4 & 5), leads to the conclusion that the BalPure® BP-500 BWTS reliably passed all IMO D-2 ballast discharge standards on each of four successful test cycles performed under real-time shipboard operations in the Eastern and Western Pacific.

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