GREAT SHIPS INITIATIVE
BENCH-SCALE TEST FINDINGS
Public Technical Report

Sodium Hydroxide Ballast Water Treatment

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ABSTRACT

This report describes findings from a bench-scale ballast water treatment (BWT) process dose effectiveness evaluation conducted by the Great Ships Initiative (GSI) beginning in July 2012. Researchers from the United States Geological Survey (USGS) are developing a BWT in which sodium hydroxide (NaOH) is added directly to a ship's ballast water to produce an alkaline environment in the ballast tanks. Treatment effects take place during the voyage and the treated water is neutralized with carbon dioxide (CO_2) prior to deballasting. To date, GSI has evaluated versions of the NaOH BWT at the bench, land-based and shipboard scales (TenEyck & Cangelosi, 2009; Cangelosi *et al*., 2011).

The tests reported here evaluated the dose effectiveness of 10 N NaOH in concentrations sufficient to increase the pH to 11.5 and 12.0. GSI conducted similar bench scale tests in 2008; however, the BWT developer expressed concerns that the CO_2 in the headspace in the exposure containers tended to neutralize the pH. They argued that the surface to volume ratio in a ballast tank is much different from that in an exposure container, so the results were not representative. To address this concern, GSI conducted the 2012 dose effectiveness tests reported here by creating pH 11.5 and 12.0 test solutions using 10 N NaOH and then sparging the headspace with ultra-pure compressed air (21 % oxygen and 79 % nitrogen) after the addition of test organisms. This sparging step created a headspace that was free of CO_2.

GSI measured the effects of the NaOH BWT at pH 11.5 and 12.0 with CO_2-free headspace on freshwater organisms known to be relatively resilient to stressors in filtered Duluth-Superior Harbor Water (the water was not filtered for microbial tests). Standard test organisms— the bacteria *Escherichia coli*, *Enterococcus faecium*, as well as total coliform and culturable heterotrophic bacteria; the zooplankton *Daphnia magna* (cladoceran) and *Eucyclops* spp. (copepod), and ephippia (resting eggs) of *D. magna,* and the green alga *Selenastrum capricornutum*—were exposed to the treated water under these revised experimental conditions and mortality and hatching rate was quantified.

The 2012 test results in which the head space was sparged tracked with GSI's 2008 test results for bacteria and zooplankton, indicating little to no effect of sparging. The NaOH BWT reduced the density of *E. coli, E. faecium,* and total coliform bacteria to less than the limit of detection within 48 hours. However, the NaOH BWT did not totally eliminate total heterotrophic bacteria during the 48 hour test period, though it did reduce their density from greater than 10,000 MPN/mL to 13 MPN/mL in the pH 12.0 treatment group.

In terms of zooplankton, the NaOH BWT caused 100 % mortality of the zooplankton *D. magna* and *Eucyclops* spp. at pH 11.5 and pH 12.0 within two hours. In an adverse effect, the NaOH BWT increased the hatching rate of *D. magna* ephippia by greater than 20 % at pH 11.5 and pH 12.0; likely the result of a weakening of the protective outside layer of the resting egg due to elevated pH.

With respect to the green alga *S. capricornutum*, the 2012 results differed from the 2008 results. In 2012 the NaOH BWT reduced survival of *S. capricornutum* cells from 100 % to 39 % at pH 11.5 and caused complete mortality at pH 12.0 within 48 hours. In 2008 tests, only a pH level of

12.5 resulted in 100 % mortality of this species following 48 hours of exposure (TenEyck & Cangelosi, 2009).

Overall, these 2012 results corroborate past GSI bench-scale and land-based findings that the NaOH treatment is most effective on *E. coli, E. faecium* and total coliform bacteria, and the zooplankton species tested, and least effective, particularly at the lower pH levels, on the green algae species tested, and total heterotrophic bacteria. Evacuation of CO_2 in exposure container head space could have increased treatment effects on the algal organism tested relative to 2008 tests in which CO_2 was not evacuated from exposure container head space.

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1. INTRODUCTION

This report describes findings from a Great Ships Initiative (GSI) bench-scale dose effectiveness evaluation of a ballast water treatment (BWT) process conducted beginning in July 2012. Researchers from the United States Geological Survey (USGS) are developing a BWT in which sodium hydroxide (NaOH) is added directly to a ship's ballast water to produce an alkaline environment in the ballast tanks. Treatment effects take place during the voyage and the treated water is neutralized with carbon dioxide (CO_2) prior to deballasting. To date, GSI has evaluated versions of the NaOH BWT at the bench, land-based and shipboard scales.

In 2008, GSI conducted bench-scale evaluations of the NaOH BWT using a wide range of cultured organisms in exposure beakers that were open to the environment (TenEyck & Cangelosi, 2009). Findings from these tests showed pH levels of 11.5, 12.0, and 12.5 were biologically effective against a broad range of aquatic organisms tested, though only the pH 12.5 treatment was effective at reducing the survival of adult rotifers, adult cladocerans and copepods, and did so within four hours (TenEyck & Cangelosi, 2009). The NaOH-treatment resulting in pH levels of 12.0 and 12.5 reduced the bacteria *Escherichia coli* and *Enterococcus faecium* to less than 1 Most Probable Number (MPN) within two hours (TenEyck & Cangelosi, 2009). While heterotrophic bacteria and the green alga *Selenastrum capricornutum,* were more resistant to treatment; bacteria were reduced by three logs, and there was complete *S. capricornutum* mortality at 48 hours only in the pH level of 12.5 (TenEyck & Cangelosi, 2009). There was no substantial change in elevated pH levels in NaOH-treated water (Duluth-Superior Harbor water or laboratory water) over 96 hours except where sediments were present (TenEyck & Cangelosi, 2009). Sediments led to a slight lowering of pH. These results indicate that treated water will remain toxic until it is actively neutralized or diluted. However, there was no acute residual toxicity observed at either dilution of 1:100 or 1:1,000 (TenEyck & Cangelosi, 2009).

In the summer of 2010, GSI conducted land-based research and development testing on a larger-scale version of the BWT that increased the alkalinity of ballast water to pH 12 (Cangelosi *et al*., 2011). During retention periods of two or three days in closed, 200 m^3 tanks with limited head space, the pH of the treated water remained relatively stable. Just prior to discharge, CO_2 injected into a recirculation stream between a Speece Cone-type carbonator and the retention tank neutralized alkalinity to pH levels between 6.5 and 8.5. Results of this land-based testing showed a significant ($p<0.05$) BWT effect of reducing live organism densities in treated discharge relative to control discharge in all size classes of organisms, with the most sensitive size class appearing to be zooplankton (Cangelosi *et al*., 2011).

Finally, in the summer of 2011, researchers installed a temporary version of the NaOH BWT onboard the motor vessel (M/V) *Indiana Harbor*. GSI conducted a single biological efficacy trial of the BWT with a target pH of 12.0, using two ballast tanks as control tanks and two ballast tanks as treatment tanks. In this shipboard trial, the NaOH BWT also showed a potential to reduce live densities of organisms relative to control discharge levels, with the greatest effectiveness associated with zooplankton and phytoplankton; bacteria results were inconclusive (GSI, 2013).

The BWT developer noted that the results from the 2008 bench-scale test may not have been as representative as they could be of the shipboard environment, since exposure containers were open to air and the surface-to-volume ratio was high relative to a ship's ballast tank, leading to ongoing partial neutralization by atmospheric CO_2. Minor reductions in the target pH for this BWT will translate into major operational cost savings when scaled up to a ship. Therefore, GSI conducted the bench-tests reported here on the NaOH BWT to answer the following specific dose effectiveness question: What is the effectiveness of water treated to pH 11.5 and pH 12.0 on select organisms if the headspace of the bench-scale experimental vessel is free of CO_2?

GSI created pH 11.5 and pH 12.0 test solutions using 10 N NaOH, and determined the effect of the increased pH, after sparging the headspace with compressed gas (21 % oxygen and 79 % nitrogen) to achieve a CO_2-free environment. Standard test organisms for these tests were the same as those used in the 2008 bench-scale tests, specifically: the bacteria *E. coli*, *E. faecium*, and total coliform and culturable heterotrophic bacteria; the zooplankton *Daphnia magna* (cladoceran) and *Eucyclops* spp. (copepod), and ephippia (resting eggs) of *D. magna;* and the green alga *S. capricornutum.* GSI exposed the organisms to the treated water under the revised experimental conditions and quantified mortality and hatching rate. The tests were performed using filtered Duluth-Superior Harbor water (FHW; bacteria tests were conducted using unfiltered Duluth-Superior Harbor water, HW), as well as, performance control water (PCW). GSI compared results from these 2012 tests to those from the 2008 tests with the original exposure vessel design.

2. BACKGROUND

2.1. Great Ships Initiative (GSI)

GSI is a regional effort managed by the Northeast-Midwest Institute (NEMWI) devoted to ending the problem of ship-mediated invasive species in the Great Lakes-St. Lawrence Seaway System and globally. Since its establishment in 2006, GSI has provided independent performance/verification testing services to developers of BWTSs at the bench, land-based and shipboard scales. GSI performs informal "status testing" for systems that are in the research and development stage, and formal certification/verification tests appropriate to market-ready BWMSs.

2.2. Organization

GSI is a project of NEMWI—a Washington, D.C-based private, non-profit, and non-partisan research organization dedicated to the economic vitality, environmental quality, and regional equity of Northeast and Midwest states. The project is carried out collaboratively with contracting entities including the University of Wisconsin-Superior (UWS), AMI Consulting Engineers, Broadreach Services, and the University of Minnesota-Duluth (UMD).

Ms. Allegra Cangelosi of NEMWI is GSI's Principal Investigator and Director (GSI PI). A GSI Advisory Committee comprising top-level officials of key stakeholder groups provides direct input to Ms. Cangelosi, advising on GSI award decisions, program direction, finances and fund-

raising. The American Great Lakes Ports Association advises the project, assuring that the GSI is meeting the needs of the maritime industry; and coordinating maritime industry and supply chain outreach. Researchers from UWS's Lake Superior Research Institute (LSRI) and the UMD's Natural Resources Research Institute (NRRI), among others, provide critical scientific and technical expertise and implementation services to the GSI PI. Dr. Mary Balcer of LSRI is the project's lead zooplankton ecologist. She is also the team leader for LSRI staff engaged in GSI research activities. Dr. Euan Reavie of UMD's Natural Resources Research Institute (NRRI) leads all phytoplankton analysis and NRRI staff. Mr. Matthew TenEyck of LSRI leads all bench-testing and Whole Effluent Toxicity (WET) tests. Ms. Deanna Regan of LSRI is responsible for GSI chemical analysis. Ms. Heidi Saillard of LSRI is responsible for GSI microbial analysis. Ms. Nicole Mays of NEMWI is GSI's Senior Quality Systems Officer and Ms. Kelsey Prihoda of LSRI is GSI's Senior Quality Assurance/Quality Control (QAQC) Officer.

3. GSI BENCH-SCALE TESTS

GSI bench-scale tests involve "status testing" to provide BWT system (BWTS) developers insight into the performance of BWTS processes and configurations at early stages of development relative to specific challenge conditions and scenarios. Findings are strictly the performance outcomes of the tests. That is, to maintain its independence as a testing facility, GSI does not engage in discussions with the BWTS developer on, or produce recommendations for, ways to improve the BWTS process subject to testing. Developers apply directly to GSI for these services, and GSI awards "Status Testing" services at no cost except for shipping the subject BWTS to and from the GSI testing facility, and costs involved in the installation and removal of the system from the GSI testing facility.

GSI bench-scale status tests take place year-round at the LSRI. The LSRI is amply equipped with staff expertise and resources to conduct the tests, and has a long history of successfully undertaking similar tests. The overarching goal of GSI bench-scale status testing is to explore dose effectiveness, chemical degradation, residual toxicity, and/or sensitivity to challenge conditions of a proposed BWTS or component thereof.

3.1. GSI Dose Effectiveness Tests

GSI bench-scale dose effectiveness tests help determine the range of concentrations of an active substance that is toxic to a variety of robust freshwater zooplankton, algae and bacteria known to be relatively resilient to stressors. Dose effectiveness test results for zooplankton and algae are typically expressed as percent survival and/or percent mortality, and in the case of resting eggs results are expressed as percent hatch. Where applicable, results may also be expressed in terms of a series of absolute quantifications: LC_{99}, i.e., the experimentally derived concentration of an active substance estimated to kill 99 % of the test population following 24 or 48 hours of continuous exposure; No Observed Effect Concentration (NOEC), i.e., the highest concentration of an active substance shown to have no significantly adverse effect on the test population compared to controls; and/or Lowest Observed Effect Concentration (LOEC), i.e., the lowest concentration of an active substance known to have a significantly adverse effect on the test population compared to controls.

3.2. GSI Active Substance Degradation Tests

GSI bench-scale active substance degradation tests determine the effect that various water quality or environmental parameters may have on the rate of active substance degradation and/or the rate of formation of disinfection by-products of a BWTS involving active substances. No organisms are used in association with these analytical assays; instead test solutions are analyzed for their concentration of active substance (or active component of the substance). Test results are typically expressed as the percent change in active ingredient concentration.

If a BWTS does not utilize an active substance, this stage of testing does not apply. Instead, preliminary water chemistry testing may be conducted as needed and include measurement of basic water quality parameters, such as, temperature, pH, conductivity, and dissolved oxygen.

3.3. GSI Residual Toxicity Tests

GSI bench-scale residual toxicity tests help estimate the effect that treated water (following neutralization of the active substance, a degradation period, and/or a dilution step) may have on non-target organisms in the receiving system. These test results are expressed in a manner similar to those for dose effectiveness assays. The principal difference between these tests and dose effectiveness tests is that the concentration of the active substance is adjusted to be consistent with potential discharge levels. The tests are also performed on sensitive organisms rather than robust species.

4. METHODS

4.1. Ballast Water Treatment System

Developers of the NaOH BWT propose it for use as a routine BWT. The BWT entails adding a solution of NaOH directly to ballast water, and retaining the water at an elevated pH during a voyage. The BWT then neutralizes the water prior to discharge. The bench-scale version of the BWT for these tests differed from the proposed shipboard BWT in that 10 N NaOH was used to increase the pH to 11.5 and 12.0, and no neutralization was carried out.

4.2. Test Objectives and General Test Methods

4.2.1. Test Objectives and Schedule

The objective of the dose effectiveness tests was to determine the survival relative to controls in acute, static toxicity tests of cultured zooplankton, phytoplankton species and microbes after treatment with 10 N NaOH to achieve pH 11.5 and pH 12.0, given a CO_2-free environment in the headspace of the test vessel.

GSI originally planned bench-scale testing of the NaOH BWT beginning in June 2012 at LSRI. However, massive flooding in the Duluth-Superior area delayed the effort until the beginning of

July for the microbial dose effectiveness test, and until the end of July for the zooplankton and green algae dose effectiveness tests due to population losses of cultured test organisms.

In total, the planned bench-scale testing of NaOH BWT comprised approximately six months of planning, test activities, and report writing. Table 1 summarizes the schedule of activities over the entire project period.

Table 1. NaOH Ballast Water Treatment Proposed Bench-Scale Test Schedule.

Test Milestones and Significant Events	Start Date	End Date
TQAP Development and Preliminary Methods Development	12 June 2012	28 June 2012
Microbial Dose Effectiveness Testing	03 July 2012	08 July 2012
Zooplankton and Green Algae Dose Effectiveness Testing	25 June 2012	27 June 2012
Data Entry, Data Reduction, and Discussion of Preliminary Results with Treatment System Developers	02 August 2012	19 November 2012
Final Report Writing	29 January 2013	20 March 2013

4.2.2. General Test Methods

4.2.2.1. Preparation of Test Water

Three types of test water were used during the NaOH BWT bench-scale testing: HW (for the microbial tests), FHW, and PCW. On 3 July 2012, approximately 12 L of HW in a clean 5 gallon container was collected from the GSI Land-Based Research, Development, Testing, and Evaluation Facility (RDTE; Superior, WI) using a submersible pump located at a depth of approximately three meters below the surface. The water was transported to LSRI for use in the microbial dose effectiveness test (i.e., this water was not filtered in order to retain the natural, ambient microbial population) and 4 L was warmed to approximately 25 °C prior to starting the test. On 24 July 2012, the FHW was prepared by collecting approximately 10 L of HW from the same location at the GSI Land-Based RDTE Facility using a submersible pump located at a depth of approximately three meters below the surface. The water was transported to LSRI, and filtered through a Whatman™ 934-AH™ glass microfiber filter (1.5 μm particle retention) to provide the water for acclimating *D. magna* and *Eucyclops spp.* and for dose effectiveness testing with *D. magna*, *Eucyclops spp*., ephippia of *D. magna* and *S. capricornutum*. The FHW was stored in a refrigerator at 4 °C and warmed to 25 °C just prior to use. The use of the PCW was a quality control measure. It was the optimal culture water for the species being tested; therefore, it varied for each dose effectiveness test conducted. The purpose of the PCW group was to provide information on the health of the test organisms. The PCW for each organism tested was:

- Dechlorinated Laboratory Water (LW): *Eucyclops spp.* and *S. capricornutum*

- Hard Reconstituted Water (HRW): *D. magna* and *D. magna* ephippia
- Non-Selective Broth Media (Tryptic Soy Broth, TSB): *E. coli* and *E. faecium*

4.2.2.2. Water Quality Measurements

Several water quality parameters were measured during the dose effectiveness experiments. These parameters were measured using calibrated meters, and included: temperature, dissolved oxygen (DO), pH, and specific conductivity.

The temperature of the test water was measured in the stock solutions at Hour 0 and in one "sacrificial" exposure vessel per day using a Fisher digital thermometer that was calibrated every seven days using an alcohol thermometer (that is calibrated against a certified National Bureau of Standards mercury thermometer).

The DO concentration of the test water was measured in the stock solutions at Hour 0 and in one "sacrificial" exposure vessel per day using an YSI DO Meter with Membrane Electrode Probe (YSI Incorporated; Yellow Springs, Ohio; USA). The DO meter was calibrated at least every seven days.

The pH of the test water was measured in the stock solutions at Hour 0 and daily in one "sacrificial" exposure vessel using a pH meter (i.e., Orion 3 Star) with a pH combination electrode and automatic-temperature-compensation (ATC) probe. The method outlined in *GSI/SOP/BS/RA/C/9, v. 3 – Procedure for pH Meter Calibration and pH Measurement* was used except that standard pH buffers 7.00, 10.00, and 12.45 were used for the three-point calibration and the meter was verified for accuracy using a pH 12.00 check buffer.

The specific conductivity was measured in the stock solutions at Hour 0 and daily in one "sacrificial" exposure vessel using the Oakton Model CON 110 Conductivity/TDS/Temperature Meter (Oakton Instruments; Vernon Hills, IL USA), hereafter referred to as the CON 110 Meter. The CON 110 Meter was calibrated on a monthly basis, and was verified for accuracy each day prior to sample analysis using the High Daily Check Standard (acceptance range of 1341 – 1483 μS/cm).

4.3. Experimental Methods

GSI conducted methods development experiments to determine the length of time needed to sparge the headspace above the test water and achieve a CO_2-free environment, and whether the closed environment would affect DO concentrations of the exposure solutions. GSI determined the required sparge time ranged from 1-3 minutes and depended upon the test vessel used. For example, a 50 mL test tube required one minute sparging while a 250 mL bottle required a three minute sparge. With respect to DO effects, GSI detected no depletion in the exposure solutions and no mortality due to the closed test chamber in 48 hour experiments using *S. capricornutum* and *Eucyclops spp*. (Nonetheless, GSI ran a FHW control to confirm these results during the definitive dose effectiveness tests.)

4.3.1. Dose Effectiveness Experiments

Test organisms in the bench experiments reported here were: *D. magna* (cladoceran zooplankton), *Eucyclops spp.* (copepod), ephippia (resting eggs) of *D. magna*, *S. capricornutum* (green algae), and raw HW spiked with *E. coli* (fecal indicator bacteria) and *E. faecium* (fecal indicator bacteria), which are both facultative anaerobic heterotrophic bacteria. The dose effectiveness tests were conducted using two water types (FHW and PCW; microbial testing was conducted in HW) at a temperature of 25 °C ± 3 °C. There were three to ten replicates per treatment group. The test water types were prepared just prior to testing according to the methods outlined in section 4.2.2.1. "Preparation of Test Water" of this report. The test water was mixed well and placed in a water bath until a temperature of 23 °C to 27 °C was established and maintained. Once the test water reached the test temperature, the following water quality measurements were made on the stock solutions: temperature, DO, pH, and specific conductivity.

4.3.1.1.Microbial Dose Effectiveness Methods

Dose effectiveness tests using *E. coli* and *E. faecium* were conducted using HW collected on the day that the test was started (i.e., 03 July 2012) according to the procedure detailed in the "Preparation of Test Water" section. Initial temperature, pH, DO, and conductivity were measured in the HW. In addition triplicate samples were collected from the HW to determine ambient microbial densities. A portion of the HW was filter-sterilized using 0.2 μm Stericups to be used as the procedural blank during the dose effectiveness test. Approximately 4 L of the HW was poured into a sterile polypropylene container and warmed to approximately 25 °C in a water bath, and then was spiked with 40 μL of *E. coli* (American Type Culture Collection #25922; Manassas, VA) and 80 μL *E. faecium* (American Type Culture Collection # 35667; Manassas, VA) inoculum suspensions (i.e., both organisms are culturable, heterotrophic bacteria) to prepare test water that contained approximately 10,000 cfu/mL. The PCW was prepared by spiking 1 L of TSB with *E. coli* and *E. faecium* inoculum suspensions to achieve approximately 10,000 cfu/mL. The spiked test water was aseptically transferred into three, 1 L sterile polypropylene bottles for each treatment group (HW Control, HW pH 11.5, HW pH 12.0). The pH 11.5 stock solution was adjusted from an initial pH of 7.54 to 11.53 using 0.5 mL of 10 N NaOH. Then, the pH of the 1 L HW pH 12.0 stock solution was adjusted from an initial pH of 7.58 to 12.01 using 1.3 mL of 10 N NaOH. Approximately 250 mL of each treated water type and the HW control were transferred using a glass pipette into three, 250 mL sterile, polypropylene screw cap bottles that had been sparged with compressed ultra-pure air (21 % oxygen and 79 % nitrogen) immediately prior for three minutes. An aliquot from each HW control replicate was immediately collected for analysis of the initial (0 HR) concentration of *E. coli*, *E. faecium*, total coliform bacteria and total heterotrophic bacteria. The headspace in each control and treatment group replicate was sparged for 30 seconds with ultra-pure air to achieve a CO_2-free headspace. The caps were tightened on the replicates and they were placed in a dark incubator set at 25 °C for 48 hours.

After the 48 hour exposure time, each sample was analyzed for *E. coli*, *E. faecium*, total coliform bacteria, and total heterotrophic bacteria. Once samples had been collected for analysis, the appropriate media capsule (Enterolert™ or Colilert®) was added to each 100 mL sample and

mixed. The sample was then poured into a Quanti-Tray/2000, and sealed using the Quanti-Tray sealer. All trays were incubated well-side down for 24 hours, Enterolert™ trays at 41ºC and Colilert® trays and SimPlates at 35ºC. After incubation time, total coliforms were calculated by recording the number of large and small, yellow wells in the Colilert trays. All trays were then examined under a handheld UV light (Spectroline E-series 6 watt, 110 volt), recording the number of small and large fluorescing (positive) wells. The Quanti-tray/2000 MPN table was referred to in order to find the MPN for each tray (sample). Total heterotrophic plate counts (HPC) were conducted using Simplate for HPC, which were run by pipetting 1mL or 0.1 mL of sample onto the center of a Simplate, and then pipetting 9 mL or 9.9 mL, respectively, of sterile rehydrated Simplate media directly onto the sample. In samples where counts were expected to be above the range of the Simplate (i.e., HW replicates, positive controls, PCW replicates), samples were diluted 1:10 using sterile harbor water (STH), and 0.1 mL and 1 mL of the diluted sample were also plated as described above. The plates were then gently swirled to allow sample to fill each well in the plate. Excess sample was poured into the absorbent pad within the plate. Plates were then incubated, upside down at 36 ºC for 48 hours. All plates were examined under a handheld UV light (Spectroline E-series 6 watt, 110 volt), recording the number of fluorescing (positive) wells. The Multi-dose IDEXX SimPlate for HPC MPN table was referred to in order to find the MPN. Note that no neutralizer was used prior to analysis, but a smaller volume was analyzed (i.e., samples were diluted to 100 mL with sterile deionized (DI) water for Colilert® and Enterolert™ tests, and dilutions with STH for HPC tests).

Each analysis day, a procedural blank (sterile HW for HPC, sterile DI +1 mL sterile HW for Colilert® and Enterolert™) and a positive and negative control sample were run for each Colilert®, Enterolert ™, and SimPlate for HPC test. An IDEXX quality control (QC) sample was analyzed as a quantitative positive control at time zero. The stock cultures used for preparing streak plates for test initiation were used at 48 hours (presence /absence) for QC samples. The MPNs for duplicate analysis were averaged and relative percent difference (RPD) calculated.

After the 48 hour bacterial analysis was complete, pH and temperature were recorded for each replicate and DO and conductivity was recorded for replicate two of each of the control and treatment groups.

4.3.1.2. Zooplankton and Green Algae Dose Effectiveness Methods

The dose effectiveness tests using zooplankton (*D. magna, Eucyclops spp.* and *D. magna* ephippia) and green algae (*S. capricornutum*) were all conducted on 25 July 2012 using FHW collected on 24 July 2012 and prepared according to the procedure detailed in section 4.2.2.1 “Preparation of Test Water” of this report. In preparation for these tests, 0-24 hour old *D. magna* neonates were harvested from the LSRI Hatchery cultures on 24 July 2012 and acclimated overnight in a 1:1 mixture of HRW (i.e., *D. magna* culture water) and FHW that was collected and prepared according the method previously described and referenced. The acclimation was conducted in a dark incubator set at 25 °C. Mixed-age male and female *Eucyclops spp.* from *Lumbriculus variegatus* Tank #5 in the LSRI Hatchery (i.e., *Eucyclops* are not cultured separately; they are naturally present in relatively high numbers in the *L. variegatus* cultures) were also harvested. The *Eucyclops spp.* were acclimated overnight in a 1:1 mixture of LW (i.e., *Eucyclops* culture water) and FHW in a dark incubator set at 25 °C. On 25 July 2012 (test start

day), *S. capricornutum* Batch F8 from the LSRI Algae Culture Laboratory were harvested and centrifuged at 4750 rpm to remove the growth media. A final rinse was conducted with sterile LW and the rinsed algae were centrifuged again to achieve a final concentrated volume of 300 mL *S. capricornutum*. A final volume of 521 mL of 1.0 x 10^8 cells/mL *S. capricornutum* inoculum was prepared.

The FHW was warmed in a water bath to approximately 25 °C, and then 2 L of water was poured into each of three, 1 gallon jugs to be used for the FHW control, FHW pH 11.5, and FHW pH 12.0 treatment groups. In addition, 1 L of LW was poured into a clean 1 gallon jug to be used for the *Eucyclops* and *S. capricornutum* PCW, and 1 L of HRW was poured into a clean 1 gallon jug to be used for the *D. magna* and *D. magna* ephippia PCW. Prior to adjusting the pH of the FHW pH 11.5 and FHW pH 12.0 treatment groups, the pH and temperature of the test water was measured. The pH of the FHW pH 11.5 treatment group was adjusted from a pH of 7.55 to 11.50 using 1.1 mL of 10 N NaOH (i.e., one increment of 1 mL and two increments of 0.05 mL) while stirring with a stir bar and stir plate. The pH of the FHW pH 12.0 treatment group was adjusted from a pH of 7.63 to 12.00 using 2.9 mL of 10 N NaOH (i.e., one increment of 1 mL, two increments of 0.2 mL, eight increments of 0.1 mL, and 14 increments of 0.05 mL) while stirring with a stir bar and stir plate. The temperature, pH, DO, and conductivity were measured in the control, PCW, and treated water stock solutions. The prepared test water was added into replicate test vessels using a pipette to avoid adding CO_2. The zooplankton dose effectiveness tests were conducted using 50 mL of test water in a 6 mL, screw-top tube, and the green algae (*S. capricornutum*) dose effectiveness test was conducted using 100 mL of test water in a 125 mL stoppered Erlenmeyer flask. The test containers were sparged with ultra-pure air for 1 or 2 minutes, respectively to achieve a CO_2-free head space. Then, the test organisms were added to each test container. The headspace was again sparged for 30 seconds, and the test containers were securely sealed and placed in an incubator set at 25 °C ± 3 °C in the dark. All of the organisms were exposed for a period of 48 hours, in the dark, with no renewal of test water. If all of the organisms in a treatment group died prior to 48 hours, then that treatment group was taken down prior to 48 hours. During exposure, the temperature and pH was measured at 24 and 48 hours in "sacrificial exposure vessels". At the end of the test period, temperature, pH, DO, and conductivity measurements were made (either on individual replicates or a composite) and the number of surviving test organisms was quantified. The *D. magna* ephippia were exposed for a period of 48 hours in the dark then transferred to PCW for a period of 72 hours in continuous light (6000 lux) to assess viability of the resting eggs. During exposure, the temperature and pH was measured at 24 and 48 hours. At the end of the 72 hour hatching period, the numbers of test organisms that have hatched were quantified.

4.3.2. Quality Control Samples and Quality Assurance Measures

The QC samples and quality assurance (QA) measures for NaOH BWT dose effectiveness testing were as follows:

- **QA Counts:**
  - All tests: At least 10 % of the replicates in each treatment group were counted a second time (QA count) by a qualified GSI staff member. The results of the first and second counts were recorded on a datasheet.

- **Method Blanks:**
  - Microbial tests only: Method blanks were prepared daily.
- **Positive/Negative Controls:**
  - Microbial tests only: A positive and negative control was analyzed once per test.
- **Reference Standards**:
  - Microbial tests only: Quanti-cult®/Quanti-cult PLUS® samples (IDEXX Laboratories, Inc.) were analyzed as a quantitative positive control at least once per test.
- **Duplicate Analysis:**
  - Microbial tests only: At least 10 % of the samples were analyzed duplicate (i.e., sample split and analyzed twice). The average RPD of all duplicates analyzed during the cycles was calculated separately for *E. coli* and *E. faecium*.
- **Reference Toxicant Tests**
  - *Eucyclops spp.* and *D. magna* tests only: In order to determine the health of the test organism population, reference toxicant tests were performed monthly using *Eucyclops spp.* and *D. magna* prior to the start of the definitive dose effectiveness tests. Results were compared to historical data using quality control charts.

4.3.3. Statistical Analysis

All basic descriptive statistics were conducted using Microsoft Excel. The mean pH value was calculated for all time points using the hydrogen ion concentration. The mean (standard deviation) microbial density at 0 HR and 48 HR was determined. In addition, the mean (standard deviation) survival of the *D. magna* and *Eucyclops spp.* at test takedown was calculated. The mean (standard deviation) live and dead *S. capricornutum* cell density was determined. Finally, the mean (standard deviation) number of hatched *D. magna* ephippia at 72 hours was calculated.

A One-Way Analysis of Variance (ANOVA) was conducted using SigmaStat for Windows, version 3.5 (Systat Software, Inc.; Chicago, IL). This analysis was only conducted on data that was normally distributed, had equal variance, and did not have complete mortality in both treatment groups (i.e., ‘pH 11.5’ and ‘pH 12.0’ treatments). In cases where these three requirements were met, all pairwise comparisons between experimental groups were made using the Holm-Sidak Method. The significance level for this analysis was set at $a = 0.05$.

5. FINDINGS

5.1. Microbial Dose Effectiveness

5.1.1. Water Chemistry Results

The HW collected on the day of the test had a temperature of 25.8 °C, pH of 7.50, DO of 7.6 mg/L, and conductivity of 115.9 μS/cm (Table 2); all measured values were within expected, historical ranges for this water type. The BWT raised pH of the 'HW pH 11.5' treatment group from 7.54 to 11.53 at the start of the test (Table 2). Likewise, the BWT raised pH of the 'HW pH 12.0' treatment group from 7.58 to 12.01 at Hour 0 of the test. Conductivity increased nearly 10-fold in the 'HW pH 11.5' treatment group; from 115.9 μS/cm in the untreated HW to 1068 μS/cm at the start of the test (Table 2). There was a greater than 25-times increase in conductivity in the 'HW pH 12.0' treatment group with a conductivity of 2960 μS/cm at the start of the test (Table 2). In contrast to 2008 test conditions, pH values held steady throughout the 48 hour dose effectiveness test for both pH-adjusted treatment groups. The 48 hour pH of the 'HW pH 11.5' treatment group was 11.49, while the 'HW pH 12.0' treatment group had a pH of 12.07 at the end of the test (Table 2).

Table 2. Water Chemistry Measurements during the NaOH BWT Microbial Dose Effectiveness Test. NM = Not Measured.

Treatment Group	Time Point (HR)	Temp. (°C)	pH	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)
Procedural Blank	Prior to Start of Test	25.8	7.50	7.6	115.9
Performance Control (TSB)	0	NM	7.50	NM	NM
	48	NM	5.94	NM	NM
HW Control	Prior to Start of Test	25.8	7.50	7.6	115.9
	48	NM	7.28	NM	NM
HW pH 11.5	Prior to pH Adjustment	26.2	7.54	NM	NM
	0	26.2	11.53	7.6	1068
	48	NM	11.49	NM	NM
HW pH 12.0	Prior to pH Adjustment	26.2	7.58	NM	NM
	0	26.5	12.01	7.5	2960
	48	NM	12.07	NM	NM

5.1.2. Biology Results

The fecal indicator bacteria (i.e., *E. coli*, *E. faecium*, and total coliform bacteria) all decreased in the treated HW groups to below the limit of detection (i.e., < 100 MPN/100 mL; Tables 3-5). The mean total coliform bacteria density, all of which were *E. coli*, at the start of the test was approximately 3.8 x 10^6 MPN/100 mL, and by 48 hours this density had decreased to less than

100 MPN/100 mL in the 'HW pH 11.5' and 'HW pH 12.0' treatment groups (Tables 3 and 4). In addition, the mean *E. faecium* density was 3.6 x 10^4 MPN/100 mL at the start of the test, and at 48 hours this density had decreased to less than 100 MPN/100 mL in the 'HW pH 11.5' and 'HW pH 12.0' treatment groups (Table 5). The procedural blank, sterile-filtered HW, remained sterile during the 48 hour dose effectiveness test with less than 1 MPN/100 mL total coliform bacteria/*E. coli* and *E. faecium* (Tables 3-5) .

The total heterotrophic bacteria also decreased in the treated HW groups. The mean total heterotrophic density at the start of the test was approximately 9.0 x 10^4 MPN/mL (Table 6). At 48 hours, the initial density had decreased to 130 MPN/mL in the 'HW pH 11.5' treatment group, and to 13 MPN/mL in the 'HW pH 12.0' treatment group (Table 6). However, there was contamination of the procedural blank in this test. At 48 hours, the procedural blank (i.e., sterile-filtered HW) had a density of greater than 738 MPN/mL, which was the upper limit of detection for the dilution level used for analysis (Table 6).

Table 3. Average (*n*=3) Density (± Std. Dev.) of *Escherichia coli* Exposed to the NaOH BWT at pH 11.5 and 12.0 with CO_2-Free Headspace in Duluth-Superior Harbor Water (HW). NM = Not Measured.

Treatment ID	0-Hour Density (MPN/100 mL)	48-Hour Density (MPN/100 mL)
Procedural Blank (Sterile HW)	NM	<1 (0)
Performance Control Water (TSB)	5.1 x 10^6 (1.3 x 10^6)	1.2 x 10^7 (0.0)
HW Control	3.8 x 10^6 (3.3 x 10^5)	5.1 x 10^4 (1.2 x 10^4)
HW pH 11.5	NM	<100 (0.0)
HW pH 12.0	NM	<100 (0.0)

Table 4. Average (*n*=3) Density (± Std. Dev.) of Total Coliform Bacteria Exposed to the NaOH BWT at pH 11.5 and 12.0 with CO_2-Free Headspace in Duluth-Superior Harbor Water (HW). NM = Not Measured.

Treatment ID	0-Hour Density (MPN/100 mL)	48-Hour Density (MPN/100 mL)
Procedural Blank (Sterile HW)	NM	<1 (0)
Performance Control Water (TSB)	5.1 x 10^6 (1.3 x 10^6)	1.2 x 10^7 (0.0)
HW Control	3.8 x 10^6 (3.3 x 10^5)	5.1 x 10^4 (1.2 x 10^4)
HW pH 11.5	NM	<100 (0)
HW pH 12.0	NM	<100 (0)

Table 5. Average (*n*=3) Density (± Std. Dev.) of *Enterococcus faecium* Exposed to the NaOH BWT at pH 11.5 and 12.0 with CO_2-Free Headspace in Duluth-Superior Harbor Water (HW). NM = Not Measured.

Treatment ID	0-Hour Density (MPN/100 mL)	48-Hour Density (MPN/100 mL)
Procedural Blank (Sterile HW)	NM	<1 (0)
Performance Control Water (TSB)	8.7×10^5 (9.6×10^4)	1.2×10^7 (0.0)
HW Control	3.6×10^4 (1.7×10^4)	1.4×10^4 (6.1 x 103)
HW pH 11.5	NM	67 (29)*
HW pH 12.0	NM	<100 (0)

*Two out of three replicates were below the limit of detection, and the median value was used in order to estimate average density for that treatment group.

Table 6. Average (*n*=3) Density (± Std. Dev.) of Total Heterotrophic Bacteria Exposed to the NaOH BWT at pH 11.5 and 12.0 with CO_2-Free Headspace in Duluth-Superior Harbor Water (HW). NM = Not Measured.

Treatment ID	0-Hour Density (MPN/mL)	48-Hour Density (MPN/mL)
Procedural Blank (Sterile HW)	NM	>738 (0)
Performance Control Water (TSB)	2.8×10^5 (1.3×10^5)	1.5×10^9 (1.4×10^8)
HW Control	9.0×10^4 (3.6×10^4)	1.2×10^5 (8.0×10^3)
HW pH 11.5	NM	130 (36)
HW pH 12.0	NM	13 (6)*

*Two out of three replicates were below the limit of detection, and the median value was used in order to estimate average density for that treatment group.

5.2. Zooplankton Dose Effectiveness

5.2.1. Eucyclops spp. and D. magna

5.2.1.1. WaterChemistry Results

For the *Eucyclops* spp. and *D. magna* tests, the CO_2-free headspace was successful at maintaining the elevated pH levels in the two treatment groups for the duration of the tests (i.e., tests were terminated at two hours due to 100 % mortality in the treatment groups). In the *Eucyclops* test at two hours, the pH in the 'FHW pH 11.5' treatment was 11.48 and the 'FHW pH 12.0' treatment was 12.04 (Table 7). In the *D. magna* test, the two hour pH in the 'FHW pH

11.5' treatment was 11.47 and the two hour pH in the 'FHW pH 12.0' treatment was 12.03 (Table 8).

At Hour 0, the 'FHW Control' stock solution for the *Eucyclops* and *D. magna* dose effectiveness tests had a temperature of 24.9 °C, pH of 7.74, DO of 7.0, and conductivity of 193.5 μS/cm (Tables 7 and 8); all measured values were within expected ranges for this water type. In both tests, the pH of the 'FHW pH 11.5' treatment group stock solution was successfully increased at Hour 0 from 7.75 to 11.47, thereby increasing the conductivity six-fold to 1161 μS/cm (Tables 7 and 8). The pH of the 'FHW pH 12.0' treatment group stock solution was also successfully increased at Hour 0 from 7.63 to 12.00, and the conductivity increased 17 times above that of the control to 3330 μS/cm (Tables 7 and 8).

5.2.1.2. Biology Results

The *Eucyclops* and *D. magna* dose effectiveness tests were terminated after two hours of exposure due to 100 % mortality in the 'FHW pH 11.5' and 'FHW pH 12.0' treatment groups (Tables 9 and 10). In both tests, the 'Performance Control' and 'FHW control' groups had 100 % survival at two hours (Tables 9 and 10). No statistical analysis was needed on these data due to complete mortality in the treatment groups.

Table 7. Water Chemistry Measurements during the NaOH BWT *Eucyclops spp.* Dose Effectiveness Test.

Treatment Group	Time Point (HR)	Temp. (°C)	pH	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)
Performance Control (LW)	0	25.4	7.50	5.8	154.3
	2	24.9 (0.1)	7.59	6.8 (0.1)	153.3 (1.6)
FHW Control	0	24.9	7.74	7.0	193.5
	2	24.9 (0.1)	7.91	7.2 (0.1)	168.0 (2.3)
FHW pH 11.5	Prior to pH Adjustment	24.6	7.75	NM	NM
	0	24.9	11.47	7.4	1161
	2	24.8 (0.1)	11.48	7.5 (0.1)	1087 (6.2)
FHW pH 12.0	Prior to pH Adjustment	25.4	7.63	NM	NM
	0	24.8	12.00	6.7	3330
	2	24.7 (0.1)	12.04	7.2 (0.1)	3180 (20)

Table 8. Water Chemistry Measurements during the NaOH BWT *Daphnia magna* Dose Effectiveness Test.

Treatment Group	Time Point (HR)	Temp. (°C)	pH	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)
Performance Control (HRW)	0	22.6	8.39	7.7	575
	2	25.6 (0.3)	8.35	7.3 (0.2)	575 (3.5)
FHW Control	0	24.9	7.74	7.0	193.5
	2	25.7 (0.3)	7.76	7.1 (0.1)	176.9 (1.0)
FHW pH 11.5	Prior to pH Adjustment	24.6	7.75	NM	NM
	0	24.9	11.47	7.4	1161
	2	25.8 (0.2)	11.47	7.1 (0.1)	1114 (21)
FHW pH 12.0	Prior to pH Adjustment	25.4	7.63	NM	NM
	0	24.8	12.00	6.7	3330
	2	25.5 (0.3)	12.03	6.9 (0.1)	3233 (50)

Table 9. Average (*n*=3) Percent Survival (± Std. Dev.) of *Eucyclops spp.* Exposed to the NaOH BWT at pH 11.5 and 12.0 with CO_2-Free Headspace in Filtered Duluth-Superior Harbor Water (FHW).

Treatment Group	Two-Hour Survival (%)
Performance Control (LW)	100 (0.0)
FHW Control	100 (0.0)
FHW pH 11.5	0 (0.0)
FHW pH 12.0	0 (0.0)

Table 10. Average (*n*=3) Percent Survival (± Std. Dev.) of *Daphnia magna* Exposed to the NaOH BWT at pH 11.5 and 12.0 with CO_2-Free Headspace in Filtered Duluth-Superior Harbor Water (FHW).

Treatment Group	Two-Hour Survival (%)
Performance Control (LW)	100 (0.0)
FHW Control	100 (0.0)
FHW pH 11.5	0 (0.0)
FHW pH 12.0	0 (0.0)

5.2.2. Resting Eggs (D. magna Ephippia)

5.2.2.1. Water Chemistry Results

At Hour 0, the 'FHW Control' stock solution for the *D. magna* ephippia dose effectiveness test had a temperature of 24.9 °C, pH of 7.74, DO of 7.0, and conductivity of 193.5 μS/cm (Table 11). The pH of the 'FHW pH 11.5' treatment group stock solution was successfully increased at Hour 0 from 7.75 to 11.47, thereby increasing the conductivity six-fold to 1161 μS/cm (Table 11). The pH of the 'FHW pH 12.0' treatment group stock solution was also successfully increased at Hour 0 from 7.63 to 12.00, and the conductivity increased 17 times above that of the control to 3330 μS/cm (Table 11).

The CO_2-free headspace was successful at maintaining the elevated pH levels in the two treatment groups for the duration of the 48 hour test. At 48 hours, the pH in the 'FHW pH 11.5' treatment was 11.51 and the 'FHW pH 12.0' treatment was 12.07 (Table 11).

5.2.2.2. Biology Results

The 'Performance Control' and 'FHW Control' groups had similar 72 hour hatching rates, at 34 % hatch and 29 % hatch, respectively (Table 12). Results from a one-way ANOVA indicate that these two values are not significantly (p<0.05) different from one another. In comparison, the hatch rates of the two treatment groups were higher than the 'FHW Control'. The 'FHW pH 11.5' treatment group had a 72 hour hatch rate of 56 %, which was a significantly (p<0.05) higher hatch percentage when compared to the 'FHW Control' hatch rate (Table 12). The 'FHW pH 12.0' treatment was slightly less than the 'FHW pH 11.5' treatment with a 72 hour hatch rate of 51 % (Table 12). Although this was not significantly (p<0.05) different from the 'FHW Control', it represents an increased hatch rate of 22 % when compared to the control (Table 12).

Table 11. Water Chemistry Measurements during the NaOH BWT *Daphnia magna* Ephippia Dose Effectiveness Test.

Treatment Group	Time Point (HR)	Temp. (°C)	pH	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)
Performance Control (HRW)	0	22.6	8.39	7.7	575
	24	24.7	8.35	NM	NM
	48	25.3 (0.1)	8.44	7.4	583 (7)
FHW Control	0	24.9	7.74	7.0	193.5
	24	24.6	7.97	NM	NM
	48	24.7 (0.3)	8.01	7.3	184.4 (5.0)
FHW pH 11.5	Prior to pH Adjustment	24.6	7.75	NM	NM
	0	24.9	11.47	7.4	1161
	24	24.4	11.48	NM	NM
	48	24.3 (0.4)	11.51	7.4	1086 (24)
FHW pH 12.0	Prior to pH Adjustment	25.4	7.63	NM	NM
	0	24.8	12.00	6.7	3330
	24	24.3	12.05	NM	NM
	48	23.1 (0.4)	12.07	7.3	3192 (51)

Table 12. Average (*n*=10) Percent Hatch (± Std. Dev.) of *Daphnia magna* Ephippia Exposed to the NaOH BWT at pH 11.5 and 12.0 with CO_2-Free Headspace in Filtered Duluth-Superior Harbor Water (FHW). In the "72-Hour Hatch (%)" column, treatment groups with different letters are significantly (*p*<0.05) different.

Treatment Group	72-Hour Hatch (%)
Performance Control (HRW)	34 (17)[a, b]
FHW Control	29 (21)[a]
FHW pH 11.5	56 (27)[b]
FHW pH 12.0	51 (22)[a, b]

5.3. Green Algae Dose Effectiveness

5.3.1. Water Chemistry Results

At Hour 0, the 'FHW Control' stock solution for the *S. capricornutum* dose effectiveness test had a temperature of 24.9 °C, pH of 7.74, DO of 7.0, and conductivity of 193.5 μS/cm (Table 13). The pH of the 'FHW pH 11.5' treatment group stock solution was successfully increased at Hour 0 from 7.75 to 11.47, thereby increasing the conductivity six-fold to 1161 μS/cm (Table 13). The pH of the 'FHW pH 12.0' treatment group stock solution was also successfully

increased at Hour 0 from 7.63 to 12.00, and the conductivity increased 17 times above that of the control to 3330 µS/cm (Table 13).

The CO_2-free headspace was successful at maintaining the elevated pH levels in the two treatment groups for the duration of the 48 hour test. At 48 hours, the pH in the 'FHW pH 11.5' treatment was 11.50 and the 'FHW pH 12.0' treatment was 12.05 (Table 13).

Table 13. Water Chemistry Measurements during the NaOH BWT *Selenastrum capricornutum* Dose Effectiveness Test.

Treatment Group	Time Point (HR)	Temp. (°C)	pH	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)
Performance Control (LW)	0	25.4	7.50	5.8	154.3
	24	25.0	8.46	NM	NM
	48	24.8 (0.3)	8.61	7.8 (0.1)	573 (8)
FHW Control	0	24.9	7.74	7.0	193.5
	24	24.8	8.04	NM	NM
	48	24.8 (0.2)	8.32	7.5 (0.1)	172.0 (1.3)
FHW pH 11.5	Prior to pH Adjustment	24.6	7.75	NM	NM
	0	24.6	11.47	7.4	11.61
	24	24.7	11.50	NM	NM
	48	24.3 (0.4)	11.50	7.4 (0.1)	1105 (26)
FHW pH 12.0	Prior to pH Adjustment	25.4	7.63	NM	NM
	0	24.8	12.00	6.7	3330
	24	23.9	12.05	NM	NM
	48	24.3 (0.2)	12.05	7.6 (0.0)	3297 (45)

5.3.2. Biology Results

The *S. capricornutum* dose effectiveness test began with 99.7 – 100 % live cells in all of the 0 Hour samples (Table 14). In the 'Performance Control' and 'FHW Control' groups, 100 % of the cells counted at 48 hours were live (Table 14). There was a significant ($p<0.05$) decrease in the number of live cells in both of the treatment groups when compared to the 'FHW Control' group. The 'FHW pH 11.5' treatment group had 38.7 % live cells at 48 hours, while there were no live cells counted in any of the 'FHW pH 12.0' treatment group replicates (Table 14).

Table 14. Average (*n*=3) Live and Dead Density (±Std. Dev.) of *Selenastrum capricornutum* Exposed to the NaOH BWT at pH 11.5 and 12.0 with CO_2-Free Headspace in Filtered Duluth-Superior Harbor Water (FHW). In the "Live Density" columns, treatment groups with different letters are significantly (*p*<0.05) different.

Treatment Group	0 Hour Average Density (cells/mL)		0 Hour Percent Live	48 Hour Average Density (cells/mL)		48 Hour Percent Live
	Live	Dead		Live	Dead	
Performance Control (LW)	171,429[a] (24,762)	0 (0)	100	179,048[a] (16,930)	0 (0)	100
FHW Control	187,302[a] (28,845)	0 (0)	100	192,381[a] (44,630)	0 (0)	100
FHW pH 11.5	166,349[a] (40,151)	0 (0)	100	62,857[b] (8,729)	99,683 (7,698)	38.7
FHW pH 12.0	200,635[a] (37,147)	635 (1,100)	99.7	0 (0)[c]	180,317 (4,794)	0

5.4. Comparison of Findings from 2008 and 2012 Bench-Scale Tests

5.4.1. Microbial Dose Effectiveness

It is difficult to make direct comparisons between the 2008 (CO_2 present in headspace) and 2012 (CO_2-free headspace) microbial dose effectiveness experiments because the 2012 experiment had higher control organism densities at test termination, was terminated at 48 hours, and had a higher limit of detection for *E. coli*, total coliform bacteria, and *E. faecium* (Table 15). Overall, the results appear to be quite similar with a general trend of decreasing organism density with increasing pH (Table 15). There is no obvious difference between the findings from the 2008 and 2012 test, which indicates that the presence or absence of CO_2 in the headspace of the test vessels was not a factor in the microbial effectiveness of the NaOH BWT (Table 15).

Table 15. Comparison of Findings from 2008 and 2012 Microbial Dose Effectiveness Tests using the NaOH BWT. The 2008 test did not have CO_2-free headspace while the 2012 test did.

Test Organism	Treatment Group	Avg. 24-48 Hour Density +CO_2 Headspace (2008)	Avg. 48-Hour Density CO_2-Free Headspace (2012)
Escherichia coli	**HW Control**	**24 HR:** 1550 MPN/100 mL	5.1 x 10^4 MPN/100 mL
	HW pH 11.5	**24 HR:** <1 MPN/100 mL	<100 MPN/100 mL
	HW pH 12.0	**24 HR:** <1 MPN/100 mL	<100 MPN/100 mL
Total Coliform Bacteria	**HW Control**	**24 HR:** 1730 MPN/100 mL	5.1 x 10^4 MPN/100 mL
	HW pH 11.5	**24 HR:** <1 MPN/100 mL	<100 MPN/100 mL
	HW pH 12.0	**24 HR:** <1 MPN/100 mL	<100 MPN/100 mL
Enterococcus faecium	**HW Control**	**48 HR:** 66 MPN/100 mL	1.4 x 10^4 MPN/100 mL
	HW pH 11.5	**48 HR:** 3 MPN/100 mL	67 MPN/100 mL
	HW pH 12.0	**48 HR:** 1 MPN/100 mL	<100 MPN/100 mL
Total Heterotrophic Bacteria	**HW Control**	**48 HR:** 4.2 x 10^4 MPN/mL	1.2 x 10^5 MPN/mL
	HW pH 11.5	**48 HR:** 18 MPN/mL	130 MPN/mL
	HW pH 12.0	**48 HR:** 5 MPN/mL	13 MPN/mL

5.4.2. Zooplankton Dose Effectiveness

The *Eucyclops spp.* and *D. magna* dose effectiveness tests were terminated at four hours in 2008 and at two hours in 2012, however, the results of both sets of tests are the same (Table 16). There was 0 % survival in the 'FHW pH 11.5' and 'FHW pH 12.0' treatment groups, indicating that regardless of the presence of CO_2 in the headspace of the test vessels, the NaOH BWT is completely effective against all species of adult zooplankton tested at pH 11.5 and pH 12.0 (Table 16).

The findings from dose effectiveness tests on resting eggs were not included as the 2008 tests used the cysts of the rotifer *Brachionus calyciflorus* and the 2012 tests used the ephippia of cladoceran *D. magna*. Therefore, direct comparisons between these two tests cannot be made.

Table 16. Comparison of Findings from 2008 and 2012 Zooplankton Dose Effectiveness Tests using the NaOH BWT. The 2008 test did not have CO2-free headspace while the 2012 test did.

Test Organism	Treatment Group	Avg. 4-Hour Survival (%) +CO_2 Headspace (2008)	Avg. 2-Hour Survival (%) CO_2-Free Headspace (2012)
Eucyclops spp.	**FHW Control**	100	100
	FHW pH 11.5	0	0
	FHW pH 12.0	0	0
D. magna	**FHW Control**	100	100
	FHW pH 11.5	0	0
	FHW pH 12.0	0	0

5.4.3. Green Algae Dose Effectiveness

Of all organisms tested, the impact of CO_2 in the headspace of the test vessels is most obvious with the green algae *S. capricornutum*. There was a decrease in survival in the 2012 test, conducted with CO_2-free headspace, when compared to the 2008 test (Table 17). Nearly all cells in the 'FHW pH 11.5' treatment survived during the 2008 test, while only 39 % of cells survived during the 2012 test (Table 17). At pH 12.0, there was complete mortality in the 2012 test while there was 9 % survival after 48 hours of exposure to pH 12.0 in the 2008 test (Table 17).

Table 17. Comparison of Findings from 2008 and 2012 *S. capricornutum* Dose Effectiveness Tests using the NaOH BWT. The 2008 test did not have CO2-free headspace while the 2012 test did.

Treatment Group	Avg. 48-Hour Survival (%) +CO_2 Headspace (2008)	Avg. 48-Hour Survival (%) CO_2-Free Headspace (2012)
FHW Control	100	100
FHW pH 11.5	95	38.7
FHW pH 12.0	9	0

6. GSI QUALITY MANAGEMENT

6.1. Standard Operating Procedures

Several GSI standard operating procedures (SOPs) were used to implement the NaOH BWT bench-scale test, in addition to the Test/Quality Assurance Plan (TQAP): *GSI/BS/QAQC/TQAP/3 – Test/Quality Assurance Plan for Sodium Hydroxide Ballast Water Treatment Bench-Scale Testing* (GSI, 2012). The use of the TQAP in combination with already existing GSI bench-scale SOPs facilitated consistent conformance to technical and quality system requirements, and

the bench-scale SOPs increase comparability if multiple bench-scale trials are conducted on the NaOH BWT.

The SOPs include both programmatic and technical processes and procedures such as organism culturing; sample collection, labeling, analysis and custody; and safety. GSI SOPs follow a common format and include specific QAQC procedures and metrics. They are grounded in published standard methods. They are also consistent with international and domestic guidelines where they exist. All GSI SOPs are subject to periodic review and revision to assure that the most up to date approaches are employed. Table 18 outlines the GSI SOPs utilized for the NaOH BWT bench-scale test.

Table 18. GSI Bench-Scale Standard Operating Procedures (SOPs) Utilized during the NaOH BWT Bench-Scale Test.

SOP Category	Subcategory	SOP Title	SOP Code
General	Administration	Procedure for Record Keeping	GSI/SOP/G/A/RK/1, v.1
		Procedure for Data Entry, Data Quality Control and Database Management	GSI/SOP/G/RA/DM/1, v.2
Research Activities	Sample Custody	Procedure for Labeling GSI Bench-Scale Samples (DRAFT)	GSI/SOP/G/RA/SC/4
	General Laboratory	Procedure for Verification of Laboratory Balances	GSI/SOP/BS/RA/GL/1
	Dose Effectiveness	Bench-Scale Procedure for Assessing Dose-Effectiveness of a Ballast Water Treatment System using a Copepod (DRAFT)	GSI/SOPBS/RA/EF/1
		Procedure for Assessing Dose Effectiveness of a Ballast Treatment System using the Cladoceran *Daphnia magna* (DRAFT)	GSI/SOP/BS/RA/EF/2
		Procedure for Assessing Dose Effectiveness of a Ballast Treatment System Using *Selenastrum capricornutum* (DRAFT)	GSI/SOP/BS/RA/EF/5, v.1
		Procedure for Assessing Dose Effectiveness of a Ballast Water Treatment using Daphnia magna Ephippia (DRAFT)	GSI/SOP/BS/RA/EF/8
	Microbial Preparation	General Microbiology Preparation Procedures	GSI/SOP/BS/RA/MP/1
	Microbial Analysis	Procedure for Conducting Heterotrophic Plate Counts (HPCs) using IDEXX's SimPlate® for HPC Method	GSI/SOP/BS/RA/MA/1, v.1
		Procedure for Assessing Antimicrobial Activity Using Time-Kill Method (DRAFT)	GSI/SOP/BS/RA/MA/2
		Procedure for the Detection and Enumeration of *Enterococcus* using Enterolert™	GSI/SOP/BS/RA/MA/3, v.1
		Procedure for the Detection and Enumeration of Total Coliforms and *E. coli* Using IDEXX's Colilert®	GSI/SOP/BS/RA/MA/4, v.1

6.2. Quality Assurance/Quality Control

6.2.1. Meter Calibration

Meter calibrations were recorded in a uniquely-identified laboratory notebook. The digital thermometer was calibrated eight days prior to the start of the zooplankton and green algae dose effectiveness experiments, which was one day greater than the seven day calibration frequency requirement (Table 19). The pH meters were successfully calibrated at the start of the microbial dose effectiveness experiment and daily during the zooplankton/green algae dose effectiveness experiments (Table 19). The DO meter was successfully calibrated with the appropriate frequency throughout the NaOH BWT bench-scale test (Table 19). The conductivity meter was verified to be accurate daily during the NaOH BWT bench-scale test (Table 19).

Table 19. Dates of Meter Calibrations for the NaOH BWT Bench-Scale Dose Effectiveness Test.

Meter Type	Meter ID	Date of Calibration	Calibrated By	Comments
Digital Thermometer	Fisher #6	17 July 12	Aduratomi Agbi	Successfully calibrated thermometer. No correction factor needed.
		26 July 12	Aduratomi Agbi	Successfully calibrated thermometer. Correction factor = -0.1°C.
pH	Ballast Microbes Orion 3 Star	03 July 12	Christine Polkinghorne and Kimberly Beesley	Successfully calibrated pH meter using buffers 7, 10, and 12.45. Used pH check buffer 12.00; after calibration check buffer read 12.00.
	Orion 3 Star #1	25 July 12	Matthew TenEyck	Successfully calibrated pH meter using buffers 7, 10, and 12.45. Used pH check buffer 12.00; after calibration check buffer read 12.05.
		26 July 12	Christine Polkinghorne	Successfully calibrated pH meter using buffers 7, 10, and 12.45. Used pH check buffer 12.00; after calibration check buffer read 12.00.
		27 July 12	Kimberly Beesley	Successfully calibrated pH meter using buffers 7, 10, and 12.45. Used pH check buffer 12.00; after calibration check buffer read 12.04.
Dissolved Oxygen	DO Meter #1	03 July 12	Cole Holstrom	Meter was successfully calibrated.
		24 July 12	Cole Holstrom	Meter was successfully calibrated.
Conductivity	Oakton #3	03 July 12	Christine Polkinghorne	Verified accurate using 'High Daily Check Standard'. Meter read 1352 µS/cm; acceptance range = 1341 – 1483 µS/cm.
		25 July 12	Matthew TenEyck	Verified accurate using 'Low Daily Check Standard'. Meter read 152.1 µS/cm; acceptance range = 139.7 – 154.4 µS/cm.
		26 July 12	Matthew TenEyck	Verified accurate using 'High Daily Check Standard'. Meter read 1409 µS/cm; acceptance range = 1341 – 1483 µS/cm.
		27 July 12	Christine Polkinghorne	Verified accurate using 'High Daily Check Standard'. Meter read 1429 µS/cm; acceptance range = 1341 – 1483 µS/cm.

6.2.2. Quality Control Samples and Quality Assurance Measures for Dose Effectiveness Tests

6.2.2.1. QA Counts

Overall, at least 10 % of the replicates in the zooplankton effectiveness experiments were counted by a second individual (Table 20). The *S. capricornutum* dose effectiveness experiment was slightly below the 10 % requirement with 8.5 % of replicates overall having a second count (Table 20). All QA counts met the data quality objective of equal to or greater than 90 % agreement between the primary and the QA count (Table 20).

Table 20. Results of Quality Assurance Counts of Test Organisms during the NaOH BWT Bench-Scale Test.

Test Type	Percentage of Replicates with QA Count	Primary and QA Count Agreement	Data Quality Objective	Data Quality Objective Met?
Eucyclops spp.	Hour 0 = 100% Hour 2 = 0%	100 %	≥ 90 % Agreement	YES
Daphnia magna	Hour 0 = 100% Hour 22 = 0%	100 %		YES
Selenastrum capricornutum	Hour 0 = 0% Hour 48 = 17%	99.6 %		YES
Ephippia of *Daphnia magna*	Hour 0 = 100% Hour 48 = 100% Hatch 72-Hour = 100%	100 % during test 92.5 % during 72 hour hatch		YES

6.2.2.2. Reference Toxicant Tests

Reference toxicant tests were required for *Eucyclops ssp.* and *D. magna* dose effectiveness experiments only. These tests are performed on a monthly basis and prior to the start of the dose effectiveness test to assess the health of the test organism population. A *Eucyclops spp.* reference toxicant test was performed on 10 July 12. In addition, a reference toxicant test was performed on *D. magna* on 24 July 12. A QC chart could not be produced for either of these two tests because there were insufficient data points as the chart was started over due to the flood that affected Duluth-Superior area (i.e., at least five tests are needed to produce a quality control chart). However, there was 100 % control organism survival of *Eucyclops spp.* and 93 % *D. magna* control organism survival during these two 48 hour tests, which was within the acceptance limits.

6.3. Data Audits, Management, and Archiving

Dose effectiveness, microbial preparation, and microbial analysis data were recorded on data collection forms and stored in uniquely-labeled, three-ring binders (12-06-18_GSI-BS_NaOH and 12-06-28_BLST-BS-NaOH_MA). The initial test water quality measurements, pH-adjustment data, and Hour 0 water quality measurements were recorded in a uniquely-labeled laboratory notebook (08-6-6_BLST_Lye). The GSI Senior QAQC Officer reviewed the dose

effectiveness datasheets and summarized water quality and survival data on November 19 – 20, 2012. All hard- and electronic-copies of data and records will be maintained by LSRI and archived for a period of seven years.

7. CONCLUSION

In this series of bench-scale dose effectiveness experiments, the pH was successfully adjusted in the test water to pH 11.5 and 12.0 using 10 N NaOH. The addition of 10 N NaOH elevated the conductivity in the treated water by at least 10 times in the pH 11.5 treatment group and at least 17 times in the pH 12.0 treatment group. The pH remained stable during the 48 hour test period by sparging the headspace with ultra-pure compressed air to eliminate the presence of CO_2. In addition, the temperature, DO, and conductivity remained stable in the test solutions during the 48 hour test period.

The NaOH BWT was effective against fecal indicator bacteria, reducing the density of *E. coli*/total coliform bacteria and *E. faecium* to less than the limit of detection (i.e., < 100 MPN/100 mL) within 48 hours. Although the NaOH BWT did not totally eliminate total heterotrophic bacteria during the 48 hour test period, the density was reduced from greater than 10,000 MPN/mL to 130 MPN/mL in the pH 11.5 treatment and to 13 MPN/mL in the pH 12.0 treatment.

The NaOH BWT was most effective on the zooplankton *D. magna* and *Eucyclops*, causing 100 % mortality at pH 11.5 and pH 12.0 within two hours. The NaOH BWT increased the hatching rate of *D. magna* ephippia by greater than 20 % at pH 11.5 and pH 12.0, which is likely the result of a weakening of the protective, outside layer of the resting egg due to elevated pH. Finally, the NaOH BWT reduced survival of *S. capricornutum* cells from 100 % to 39 % at pH 11.5 and caused completed mortality at pH 12.0.

When comparing the results from the 2008 dose effectiveness test (conducted in the presence of atmospheric CO_2) and the CO_2-free headspace results from 2012, it does not appear that the presence of CO_2 has any impact on the effectiveness of the NaOH BWT for microbes or zooplankton. However, the CO_2-free environment did appear to increase NaOH BWT effectiveness relative to *S. capricornutum* mortality at both pH 11.5 and pH 12.0 in comparison to the 2008 tests.

REFERENCES

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