BENCH-SCALE TECHNICAL REPORT

TESTS OF THE NANO BUBBLE OZONE TECHNOLOGY (2.5 HP UNIT)

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ABSTRACT

This technical report presents the bench-scale evaluation of the Nano Bubble Ozone Technology 2.5-horsepower unit (NBOT 2.5-HP) developed by NanoClear Group Inc. of Rockville, Maryland. This evaluation was the first to assess NBOT 2.5-HP as a potential in-tank, recirculating ballast water treatment method for the Laurentian Great Lakes.

The evaluation began in September 2019 and ended in March 2020. All analyses occurred at the Lake Superior Research Institute (LSRI) at the University of Wisconsin-Superior (UWS) in Superior, Wisconsin, USA. The NBOT 2.5-HP uses cavitation to create ultrafine microbubbles (nanobubbles) containing ozone (O_3) generated by the system. According to the developer, the resulting ozone and hydroxyl radical biproducts destroy all chemicals containing activated functional groups (aldehydes, ketones, amines, nitrates, etc.), RNA, DNA, peptides, steroids, as well as activated organic compounds (herbicides and pesticides), and microbial toxins.

The ability of NBOT 2.5-HP to increase dissolved ozone in a 1,000-L treatment tank was tested at two water temperatures (~15°C and ~25°C) using both dechlorinated laboratory water (LW) and the more challenging amended dechlorinated laboratory water (LW-TMH). In LW, NBOT 2.5-HP increased ozone (<15 minutes) upon treatment and reached equilibrium after approximately 2 hours of treatment under both temperature conditions. In LW-TMH, no increase in ozone was observed initially upon treatment. Instead, ozone increased after approximately 2 hours and reached equilibrium after 5 to 7 hours under both temperature conditions.

Degradation rates of dissolved ozone in LW and LW-TMH were examined at two water temperatures (~15°C and ~25°C). In general, ozone degradation rates were lower at 15°C than at 25°C while degradation occurred more rapidly in LW-TMH than in LW.

Biological effectiveness tests examined the ability of NBOT 2.5-HP to induce mortality in biological organisms over time in both LW and LW-TMH. Three classes of organisms were tested: bacteria (*Escherichia coli* and *Enterococcus faecium*), green algae (*Selenastrum capricornutum*), and zooplankton (*D. magna* neonates, *D. magna* ephippia, and *Eucyclops spp.*). In LW, the algae and bacteria experienced 100% mortality, or no live organisms (a count of <1 MPN/100 mL) after 30 minutes of treatment. In LW, *D. magna* neonates and *Eucyclops spp*. experienced 100% mortality after 30 – 60 minutes of treatment. In LW-TMH, the algae and *E. coli* experienced 100% mortality, or no live organisms (a count of <1 MPN/100 mL) after 240 minutes of treatment. In LW-TMH, only one sample replicate had an *E. faecium* count of 3 MPN/100 mL at 240 minutes and no live organisms were detected after 390 minutes of treatment. In LW-TMH, *D. magna* neonates and *Eucyclops spp*. experienced 100% mortality after 240 – 390 minutes of treatment. In both water types, the *D. magna* ephippia had a hatch rate of 22.5 - 36% following treatment. These results demonstrate that NBOT 2.5-HP is effective at inducing mortality in a wide range of organisms within size classes regulated in ballast water discharge in two different water qualities.

Chronic Residual Toxicity (CRT) testing examined the potential for water treated with NBOT 2.5-HP to cause toxicity to organisms in receiving water upon discharge. This testing was conducted using LW

treated with the NBOT 2.5-HP system. Three classes of organisms were tested: green algae (*Selenastrum capricornutum*), zooplankton (*Ceriodaphnia dubia*), and vertebrate (*Pimephales promelas*). No statistically significant effects on growth, survival or reproduction were seen.

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ACKNOWLEDGEMENTS

LSRI-GWRC would like to thank the American Marine University Research Institute Inc. for their application to our laboratory-based testing program and for providing the Nano Bubble Ozone Technology 2.5 horsepower (NBOT 2.5-HP) ballast water treatment (BWT) testing unit. Brian Domrese of NanoPure Tech provided installation and operational training and technical support prior to the start of and during testing. Dr. Peter Moeller of the National Oceanic and Atmospheric Administration's (NOAA) National Ocean Service (NOS) in Charleston, South Carolina provided technical advice and assistance during implementation of bench scale testing.

This work was supported by the United States Maritime Administration (United States Department of Transportation; Washington, D. C.) and the United States Environmental Protection Agency's Great Lakes Restoration Initiative.

1 INTRODUCTION

The Lake Superior Research Institute's (LSRI) Great Waters Research Collaborative (GWRC) aims to provide unbiased and independent data to accelerate the development of technologies with potential to prevent the introduction and/or control the spread of non-indigenous organisms within the Laurentian Great Lakes. This document describes the bench-scale evaluation of the Nano Bubble Ozone Technology 2.5 horsepower unit (NBOT 2.5-HP) as developed by NanoClear Group Inc. (Rockville, Maryland, USA) and provided by American Marine University Research Institute, Inc. (AMURI).

The NBOT 2.5-HP ballast water treatment (BWT) technology produces nanobubbles impregnated with ozone (O_3) that can react with water to generate hydroxyl (OH) radicals. Ozone and hydroxyl radicals are known to have antiseptic properties and can destroy algae, fungi, bacteria, and viruses. The NBOT 2.5-HP BWT is a patented technology that has been tested in a laboratory setting by Dr. Peter Moeller of the National Oceanic and Atmospheric Administration's (NOAA) National Ocean Service (NOS) in Charleston, South Carolina. The system has also been applied to commercial field trial treatments of ponds, lakes, and contaminated canals in Florida, Ohio, South Carolina, and Washington D.C. From September 2019 to March 2020, the NBOT 2.5-HP BWT was evaluated for its applicability to treat Laurentian Great Lakes ballast water as part of GWRC's technology testing program. The NBOT 2.5-HP BWT is a proposed in-tank treatment technology, which would treat ballast water on a Great Lakes vessel during the voyage from one port to another. Laboratory, or bench-scale, tests took place at the LSRI of UWS in Superior, Wisconsin, USA. Test objectives included:

1. Determination of the NBOT 2.5-HP dissolved oxygen and ozone concentrations in simulated ballast water over time.
2. Determination of the degradation rate of dissolved oxygen and ozone following treatment.
3. Determination of the biological effectiveness of the NBOT 2.5-HP system in freshwater at Great Lakes relative challenge conditions.
4. Determination of the chronic residual toxicity of NBOT 2.5-HP treated water to non-target organisms in receiving water.

2 TEST METHODS

2.1 TEST PLAN AND SOPS

A test plan (Schaefer et al., 2019) and standard operating procedures (SOPs) were used to implement all test activities. These procedures facilitate consistent conformance to technical and quality system requirements and increase data quality in addition to providing unbiased, independent data. The test plan details sample and data collection, sample analysis, sample handling and preservation, and quality assurance/quality control (QAQC) requirements. The test plan was approved by both LSRI-GWRC and AMURI on September 17, 2019, prior to the start of bench-scale test activities. Revisions were made to the test plan on January 14, 2020, March 9, 2020, and March 23, 2020 with both parties agreeing to the

revisions prior to continuation of testing. The procedures followed throughout testing are described in the *Test Methods* section and listed in the *References* section of this report.

2.2 BALLAST WATER TREATMENT TECHNOLOGY AND EXPERIMENTAL APPARATUS

Functioning as an in-tank treatment system, NBOT 2.5-HP intakes test water from the treatment tank into the system via the liquid pump and the nano-bubble generator creates nano-sized bubbles of the oxygenated ozone rich water that are then released back into the treatment tank. During normal operation of the NBOT 2.5-HP system, water was recirculated from the system to the treatment and back at rate of 1.6 m^3/hr (as measured by LSRI staff).

The NBOT 2.5-HP system (Figure 1) has both static and dynamic parameters associated with its operation which, according to the developer, are dependent on the application of the system. These parameters can be controlled from the control panel (Figure 2). The nano-bubble generator motor speed is not adjustable (Figure 3). According to the developer, the ozone production rate can be adjusted from 0-15 grams/hr during operation. The pump speed is not adjustable. Additional system settings during normal operation described in the *User's Manual* (NanoPure, 2019) are:

- ***Gas Flowmeter (Bubble Generator Flowmeter):*** set to 1.5-2.5 L/min flow
- ***Ozone Consistency Control (Ozone Adjustment):*** set to 80% function for this evaluation
- ***Ozone Generator Flow Meter:*** set to completely open
- ***Emergency Stop (Figure 3):*** disengaged position

Note: some of the system component names listed in the User's Manual do not exactly match those labeled on the cabinets. Names in parentheses are those from the User's Manual.

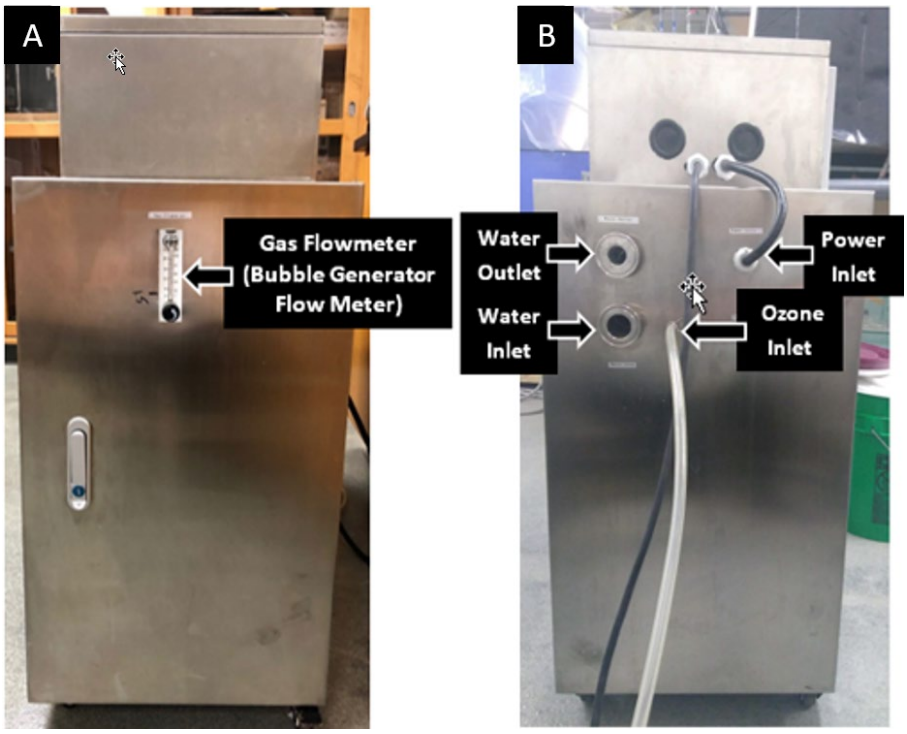

Figure 1. A.) Front View of Pump Cabinet of the NBOT 2.5-HP. B.) Back View of the Pump Cabinet of the NBOT 2.5-HP.

Figure 2. Control panel on the oxygen/ozone generator cabinet of the NBOT 2.5-HP.

Figure 3. Top view of the pump cabinet of the NBOT 2.5-HP.

2.3 EXPERIMENTAL WATER PREPARATION

During testing, three experimental water types were prepared. The first type, performance control water (PCW), was used to represent optimal growth conditions for a test organism and to demonstrate organism viability as a quality control measure. The other two water types were used to test the system at two challenge levels. Laboratory water (LW) provides a low water quality challenge to treatment technologies, generally, and amended lab water (LW-TMH) provides a higher challenge. These solutions are defined by a range of chemical parameters including organic carbon, suspended solids, and UV-transmittance. Table 1 outlines the target ranges for each parameter within samples collected prior to the start of each test trial. Treatment processes may alter water quality, therefore, the targets described in Table 1 apply only to water sampled at test initiation. These water types were prepared as described below:

Performance Control Water (PCW): The use of PCW is a quality control measure. It is the optimal culture water for the species being tested; therefore, it will vary for each biological effectiveness test conducted. The purpose of the PCW group is to provide information on the health of the test organisms. The PCW for each test organism was:

- LW: *S. capricornutum, Eucyclops spp.*

- Tryptic Soy Broth (TSB): *E. coli*
  - Prepared following manufacturer instructions and the LSRI *General Microbiology Laboratory Procedures Handbook* (LSRI, 2019a).
- Brain Heart Infusion Broth (BHB): *E. faecium*
  - Prepared following manufacturer instructions and the LSRI *General Microbiology Laboratory Procedures Handbook* (LSRI, 2019a).
- Moderately Hard Reconstituted Water (MHRW): *D. magna* neonates and ephippia
  - Prepared following LSRI/SOP/AC/37 - *Preparation of Moderately-Hard Reconstituted Water for Use in Amphipod and Cladoceran Culturing* (LSRI, 1995a).

The PCW for all chronic residual test organisms is *C. dubia* Moderately Hard Reconstituted Water (CMHRW) prepared following LSRI SOP AC/37.

Laboratory Water (LW): Prior to each test, the 1,000-L control and treatment tanks were filled with LW at the approximate test temperature. The LW is municipal water from the City of Superior, Wisconsin, that is passed through an activated carbon column to remove the majority of the chlorine. The remaining residual chlorine is removed through injection of sodium sulfite, resulting in a total residual chlorine concentration of < 5.4 µg/L Cl_2 (LSRI 2019 detection limit for chlorine analysis). Typically, LW has a very low concentration of organic carbon, solids, and a very high UV transmittance.

Amended Laboratory Water (LW-TMH), Water-Only Tests: Prior to each water-only test, the 1,000-L control and treatment tanks were filled with approximately 200 L of LW at the approximate test temperature. While the tank was filling, LW-TMH was prepared by amending the LW in the tanks with 12 mg/L pre-sterilized ISO 12103-1, A2 Fine Test Dust (Powder Technology, Inc.; Arden Hills, MN, USA), 12 mg/L pre-sterilized Micromate™ (micronized humate for liquid suspension; Mesa Verde Humates; Bernalillo, NM, USA), and 20 mg/L humic acid (ACROS organics, New Jersey, USA). The amended water was mixed thoroughly in the control and treatment tanks until few visible clumps of Fine Test Dust or Micromate™ remained and a homogenous solution was achieved. Then, both tanks were filled to the 1,000-L mark. LW-TMH is used to achieve challenge conditions like those stipulated in the U.S. Environmental Protection Agency (USEPA) Environmental Technology Verification (ETV) Program's *Generic Protocol for the Verification of Ballast Water Treatment Technology*, version 5.1 (USEPA, 2010).

Amended Laboratory Water (LW-TMH), Biological Effectiveness Tests: The method used to prepare the LW-TMH was changed during the biological effectiveness tests in order to increase dissolution and homogenization of the Test Dust, Micromate, and humic acid in the LW and achieve water chemistry values within the target ranges listed below (Table 1). Prior to each biological effectiveness test, the 1,000-L control and treatment tanks were filled with approximately 200 L of LW at the approximate test temperature. LW-TMH was prepared in a 1-L bottle for each tank following LSRI/SOP/AT/46- *Preparing Amended Lab Water using Test Dust, Micromate™, and Humic Acid Sodium Salt for use in Bench-Scale Testing* (LSRI, 2020) and was then added to the LW in each of the tanks. Both tanks were filled to the 1,000-L mark, allowing the Test Dust, Micromate, and humic acid to thoroughly mix in the tanks while filling. LW-TMH is used to achieve challenge conditions like those stipulated in the U.S. Environmental Protection Agency (USEPA) Environmental Technology Verification (ETV) Program's *Generic Protocol for the Verification of Ballast Water Treatment Technology*, version 5.1 (USEPA, 2010).

Table 1. Target Ranges for LW and LW-TMH Water Chemistry and Water Quality Parameters.

Parameter	Units	Water Type	Acceptable Range for Initiating Bench-Scale Testing
Temperature	°C	LW	22 – 28, 10-15*
		LW-TMH	
pH	NA	LW	6.5 - 9.0
		LW-TMH	
Specific Conductivity	µS/cm	LW	120-170
		LW-TMH	
Salinity	ppt	LW	< 1
		LW-TMH	
Dissolved Oxygen (DO)	mg/L	LW	4 - 12
		LW-TMH	
Total Suspended Solids (TSS)	mg/L	LW	Less than reporting limit
		LW-TMH	11.9 - 30.3
Particulate Organic Matter (POM)	mg/L	LW	Less than reporting limit
		LW-TMH	4.1-12.1
Dissolved Organic Carbon (DOC)	mg/L	LW	Less than detection - 2
		LW-TMH	4.4-6.9
Non-Purgeable Organic Carbon (NPOC)	mg/L	LW	Less than detection - 2
		LW-TMH	5.1-13.1
Percent UV Transmittance at 254 nm (%T)	%	LW	93.0-100 (filtered and unfiltered)
		LW-TMH	25.5-35.5 (filtered and unfiltered)

*Tests occurred at two temperatures, ranges are for 25 °C and 10 °C tests, respectively. 10°C tests were done with the coldest water available to the lab. The lower temperature tests are referred to as 15°C tests throughout this report.

2.4 BWT INSTALLATION AND COMMISSIONING

Prior to conducting commissioning of the NBOT 2.5-HP, the system was installed in LSRI's Aquatic Toxicology Testing Laboratory by LSRI staff members. In lieu of formal training on the system, the developer sent GWRC a training video describing the system components and the operation of the unit. The developer also provided LSRI with the *User's Manual* for NBOT 2.5-HP. During commissioning, LSRI staff ran the system as directed by the developer and observed system connections and output levels to ensure the system was operating as expected. The NBOT 2.5-HP BWT system was operating at an acceptable level upon completion of the installation and details were recorded on GWRC/FORM/22 – *Bench-Scale Ballast Water Management System (BWMS) Installation Acceptance Form* on September 17, 2019.

2.5 BWT TECHNOLOGY TEST DESIGN

2.5.1 WATER-ONLY EXPERIMENTS

LSRI-GWRC tested NBOT 2.5-HP's effect on ozone, ORP, and dissolved oxygen (DO) concentrations in a 1,000-L tank through water-only testing (no biological organisms present). Testing occurred in LW at 15°C (LW-15), LW at 25°C (LW-25), LW-TMH at 15°C (LW-TMH-15), and twice in LW-TMH at 25°C (LW-TMH-25 (1) and LW-TMH-25 (2)). Outflow from NBOT 2.5-HP was recirculated into the treatment tank throughout the system operation time. During treatment, ozone, ORP, DO, temperature, pH, and specific conductivity were measured prior to the start of system operation and every 15 minutes thereafter until ozone concentrations stopped increasing. In LW-TMH-25 (1) NBOT 2.5-HP was not operated for a long enough duration to see ozone increases. Due to this, LW-TMH-25 (2) was performed, but for the first 120 minutes of LW-TMH-25(2) test, samples were collected every 30 minutes instead of 15. After 120 minutes, samples were collected every 15 minutes in LW-TMH-25 (2).

For all water-only experiments, an additional 1,000-L tank served as the control and was sampled for ozone and ORP prior to initiation of the treatment system, 15 minutes after initiation, at an approximate mid-point of the run time, and immediately prior to treatment system shut down. DO, temperature, pH, and specific conductivity were measured in the control tank at the same time points as the treatment tank.

Samples to be analyzed for total suspended solids (TSS), particulate organic matter (POM), total non-purgeable organic carbon (NPOC), dissolved organic carbon (DOC), percent transmittance at 254 nm (%T), hardness, and alkalinity were collected from both the control and treatment tanks prior to initiation of the treatment system, 15 minutes after initiation, at an approximate mid-point of the run time, and immediately prior to treatment system shut down. In all tests, except LW-TMH-25 (1), once ozone concentrations plateaued, the system was shut off. In LW-TMH-25 (1), no ozone was observed after 135 minutes so the test was terminated.

Once ozone concentrations plateaued, the system was shut off and three replicate, one-gallon (3.785 L), bottles of water were collected from both the treatment and control tanks immediately prior to system shut down. The water was held in a dark incubator at the test temperature for a period of 48 hours. The DO, temperature, pH, specific conductivity, ozone, and ORP were measured in each bottle at multiple time points. For LW-25, LW-15, and LW-TMH-25 (1), measurements were collected at 30 minutes, 1 hour, 2 hours, 4 hours, 24 hours, and 48 hours post-treatment. For LW-TMH-15, and LW-TMH-25 (2), measurements were collected at 30 minutes, 1 hour, 1.75 hours, 2.5 hours, 24 hours, and 48 hours post-treatment. Once ozone measurements in treated water were below the detection limit, no further ozone measurements were collected.

2.5.2 BIOLOGICAL EFFECTIVENESS EXPERIMENTS

Biological effectiveness tests measured treatment effects of NBOT 2.5-HP on organisms traditionally used for laboratory toxicity testing. All organisms used during the biological effectiveness tests were

from in-house cultures except for *D. magna* ephippia (purchased from EBPI, Environmental Bio-detection Products, Inc., Mississauga, Ontario, Canada). In-house cultures were raised using LSRI organism specific SOPs and the General Microbiological Procedures Handbook (LSRI, 2019a). The overall experimental design and target organism concentration is outlined in Table 2.

Prior to test initiation, stock solutions of test water were prepared as described in section 2.3. Once the control and treatment tanks were filled to the 1,000 L mark with 25°C ± 3°C LW or LW-TMH, samples for TSS, POM, MM, NPOC, DOC, %T, temperature, conductivity, DO, pH, ozone, ORP (SC-LW and M-LW only), hardness, and alkalinity analysis were collected from the control tank, treatment tank, and the PCW stock solutions prior to the addition of the organisms to verify water quality parameters were acceptable in LW and LW-TMH for test initiation (Table 1). TSS, POM, MM, NPOC, DOC, and % T were not collected on the PCW for bacteria tests due to limited volumes available and lack of established acceptance parameters.

During all biological effectiveness tests, target organisms were added to both the control and treatment tank for exposure. It should be noted that all organism types were tested individually except bacteria, *E. coli* and *E. faecium,* which were tested simultaneously. Additionally, for each organism, PCW samples (optimal culture water) were spiked with the organism and held in an incubator through the duration of each test to demonstrate the health of the test organisms.

Table 2. Type and Numbers of Organisms Analyzed in Biological Effectiveness Experiments using the NBOT 2.5-HP.

Major Taxonomic Group	Type	Species	Sample location	Number of Organisms per Exposure/Control	Number of Replicates per Exposure/Control	Sample Volume (mL)	Test Abbreviation
Algae	Green alga	*S. capricornutum*	1) Untreated LW and LW-TMH 2) LW and LW-TMH treated with NBOT 2.5-HP 3) Untreated PCW	200,000 cells/mL	3	500 mL	SC-LW and SC-LW-TMH
Bacteria	Gram-negative	*E. coli*		≥ 10,000 MPN/mL	5 per exposure/2 per control	1,000	M-LW and M-LW-TMH
	Gram-positive	*E. faecium*				1,000	
Zooplankton	Adult copepods	*Eucyclops spp.*		10	3 plus one sacrificial chemistry sample	1,000	EU-LW and EU-LW-TMH
	Water flea	*D. magna neonate*		10	3 plus one sacrificial chemistry sample	1,000	DM-LW and DM-LW-TMH
	Water flea	*D. magna* ephippia		10	10	0. Transferred from teabags to well plates.	EDM-LW and EDM-LW-TMH

For bacteria biological effectiveness tests, a 1-L whole water sample was collected from the control and treatment tanks prior to spiking to verify the absence of *E. coli* and *E. faecium* in LW and LW-TMH. The control and treatment tanks were then spiked with *E. coli* and *E. faecium* to bring the density of each organism to its target concentration in both tanks (LSRI/SOP/GWRC/14-*Assessing Antimicrobial Effectiveness* (LSRI, 2017a)). Two replicate samples were collected from the control tank and five replicate samples were collected from the treatment tank prior to the start of NBOT 2.5-HP operation to document the initial density of each tank. The control and treatment tank replicate samples were disposed of following the initial density determination.

For the algae (*S. capricornutum*) concentrated algae stock was added to the control and treatment tanks (LSRI/SOP/GWRC/17-*Exposing Test Organisms to Potential Ballast Water Treatment Processes using a Bench-Scale Flow-Through System* (LSRI, 2017b)) to obtain the target concentration. Following addition of algae or bacteria, the control and treatment tanks were manually mixed for two minutes.

For zooplankton tests, rather than adding organisms to the entire 1,000-L tank, zooplankton were exposed in flow-through chambers, suspended in the tanks, designed to allow continuous exposure during treatment. *Daphnia magna* neonates and *Eucyclops spp.* were exposed in one-liter bottles with two 10 cm x 10 cm sections removed from the sides and replaced with 153 µm mesh adhered to the inside of the bottle using silicone (Red Devil, Pryor, OK) (Figure 4.A.). *Daphnia magna* ephippia were exposed using unbleached teabags (Figure 4.B.). The chambers were suspended in the tank using the apparatus pictured in Figure 4.C. The following LSRI SOPs were utilized for zooplankton testing: GWRC/09 – *Assessing Bench-Scale Dose-Effectiveness of Potential Ballast Water Treatment Processes on* Eucyclops spp. (LSRI, 2018a), GWRC/10 – *Assessing Bench-Scale Dose-Effectiveness of Potential Ballast Water Treatment Processes on* Daphnia magna (LSRI, 2019b), GWRC/15 – *Assessing Bench-Scale Dose-Effectiveness of Potential Ballast Water Treatment Processes on* Daphnia magna *Ephippia* (LSRI, 2019c).

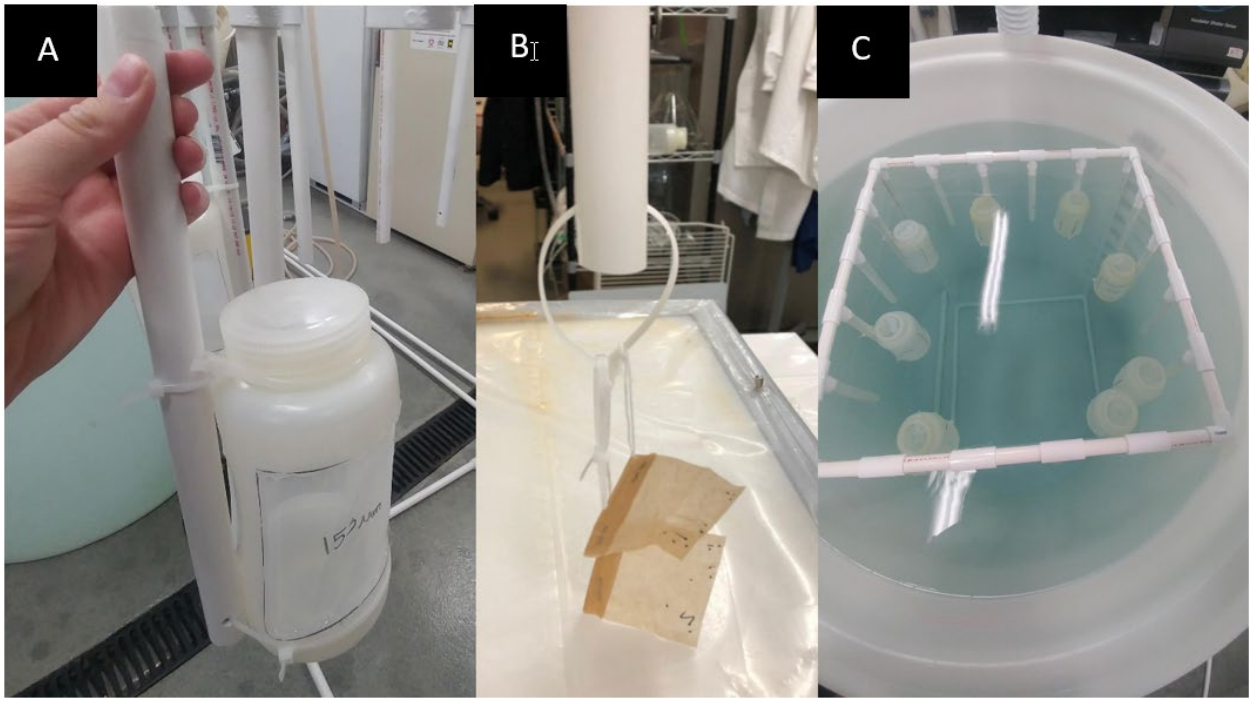

Figure 4. A. Mesh-sided bottle used for *Daphnia magna* neonate and *Eucyclops spp.* exposures. B. Two unbleached teabags used for *Daphnia magna* ephippia exposure. C. The suspension apparatus used to suspend the bottles or teabags in the tanks.

After organisms were added, the treatment tank was treated by NBOT 2.5-HP and the control tank was held for the same period of time with the lid on the control tank to prevent the treatment from affecting the control water. Based on the results from the water-only experiments, the developer determined that for the biological effectiveness testing, the NBOT 2.5-HP should run until the ozone concentration plateaued or 100% mortality occurred. For all LW tests, the NBOT 2.5-HP system was operated for a

maximum of 120 minutes or until 100% mortality of the target organism was observed. For LW-TMH tests, the system was operated for a maximum of 390 minutes or until 100% mortality of the target organism was observed.

To initiate testing, the NBOT 2.5-HP was turned on and run according to the specifications described in section 2.2. Water quality (temperature, pH, DO, specific conductivity, ozone, and ORP) parameters were measured and recorded at each exposure time point (30, 60, and 120 minutes for LW and 60, 120, 240, and 390 minutes for LW-TMH). For algae, bacteria, and *D. magna* ephippia tests, a designated beaker was submerged in the appropriate tank to collect water for measurements. For *D. magna* neonate and *Eucyclops spp*. tests these measurements were made in the water from the exposure containers, which were designated at each time point and removed from both the control and treatment tank during sampling (Figure 4.). Treatment via the NBOT 2.5-HP occurred as water was recirculated from the treatment tank, through the NBOT 2.5-HP system and back into the treatment tank. Prior to test initiation/addition of sampling apparatus, the control tank was manually mixed using a canoe paddle for approximately two minutes for all tests in LW-TMH and those involving algae and bacteria. Throughout treatment operation, for algae and bacteria tests only, the water in the control tank was manually mixed using a canoe paddle at three-minute intervals. The control tank was not mixed during the zooplankton tests due to the sampling apparatus obstructing the ability to manually mix the tank. The treatment tank was not manually mixed during the treatment operation because it was greatly agitated by the system and there is a health-risk associated with inhaling ozone produced by the NBOT 2.5-HP system.

During all algae and bacteria testing, samples for enumeration were collected by submerging bottles or beakers into the control and treatment tank at the designated time points. Staff collected either three algae replicates from both the control and treatment tanks or two control and five treatment replicate bacteria samples for the analysis of live organisms at each time point. Staff also collected one chemistry replicate from each tank in 1-L Teflon beakers for water quality measurements.

During *D. magna* neonate and *Eucyclops spp.* tests, one bottle was removed from each side of the exposure apparatus, for a total of 4 bottles collected at each time point (Figure 4.C.). The bottles were numbered, color coded, and arranged in the same order in both the control and treatment tanks so that bottles in the same position could be pulled from each tank at each time point. For *D. magna* ephippia testing, teabags were attached in groups of two and three to the sample posts on the exposure apparatus (Figure 4.B.). One post (containing either two or three bags) was removed from each side of the exposure apparatus in a manner that allowed 10 teabags to be collected at each time point.

Organisms were counted in each bottle or teabag upon removal from the control or treatment tank. For ephippia testing, the ephippia were added to well plates containing PCW water and placed in an incubator for 72 hours to facilitate hatching of the young following LSRI/SOP/GWRC/15 - *Assessing Bench-Scale Dose-Effectiveness of Potential Ballast Water Treatment Processes on* Daphnia magna *Ephippia (*LSRI, 2019c*).*

Organism survival was determined in the PCW before treatment and after the completion of the test. For algae and zooplankton tests, after testing, the PCW was also analyzed for ozone, hardness, and alkalinity. The PCW was not analyzed after treatment in the bacteria tests due to the color of the PCW interfering with the color endpoints for ozone, hardness and alkalinity.

Table 3. Dates, Water Type, Organisms Tested, and Exposure Time for NBOT 2.5-HP Bench-Scale Tests at 25°C ± 3°C.

Date	Water Type	Organisms Tested	Treatment Exposure Time	Analysis Times
17 January 2020	LW	*S. capricornutum*	30 minutes	Initial and 30-minute treatment
23 January 2020	LW	*E. coli*, *E. faecium*	120 minutes	Initial, 30, 60, and 120-minute treatment
30 January 2020	LW-TMH	*E. coli*, *E. faecium*	390 minutes	Initial, 60, 120, 240, and 390-minute treatment
6 February 2020	LW-TMH	*S. capricornutum*	240 minutes	Initial, 60, 120, and 240-minute treatment
19 February 2020	LW	*Eucyclops spp.*	120 minutes	Initial, 30, 60, and 120-minute treatment
20 February 2020	LW	*D. magna*	120 minutes	Initial, 30, 60, and 120-minute treatment
25 February 2020	LW-TMH	*D. magna*	390 minutes	Initial, 60, 120, 240 and 390-minute treatment
26 February 2020	LW-TMH	*Eucyclops spp.*	390 minutes	Initial, 60, 120, 240 and 390-minute treatment
27 February 2020	LW-TMH	*D. magna* ephippia	390 minutes	Initial, 60, 120, 240 and 390-minute treatment
28 February 2020	LW	*D. magna* ephippia	120 minutes	Initial, 30, 60, and 120-minute treatment

2.5.3 CHRONIC RESIDUAL TOXICITY TESTING

GWRC conducted testing to determine if water treated by NBOT 2.5-HP results in non-target effects on three different size classes of organisms. Testing was done in LW at 25 ± 3°C. Organisms used were the green alga *Selenastrum capricornutum*, the zooplankton *Ceriodaphnia dubia*, and the fish *Pimephales promelas*. The *S. capricornutum* and *C. dubia* used were from in-house cultures while the *P. promelas* were from a local supplier (Environmental Consulting and Testing, Inc. Superior, WI). All organisms were tested with water treated in one batch. Water was treated for 120 minutes in the 1,000 L treatment tank before being left to off-gas for ~18 hours prior to initiating the chronic residual toxicity test. The off-gassing step was used to mimic hold time on board a vessel, to answer the question of toxicity of ozone trapped in nanobubbles, and to protect the staff doing transfers from over-exposure to high ozone concentrations. In the same manner as previous tests, the 1,000-L control tank was also filled and held through the off-gassing time. After the off-gassing period, water (i.e., mock ballast discharge water) was collected from both the treatment and control tanks for use in the chronic residual toxicity (CRT) tests. Water was retained in 50-L carboys in a fridge at less than 4°C but greater than 0°C, until needed daily for testing at which point sufficient water was warmed to 25°C ± 3°C to prepare exposure solutions for one day. The system was operated on March 12, 2020 and CRT testing for all organisms began on March 13, 2020.

All CRT testing procedures followed LSRI SOPS AT/43 – *Conducting a Chronic Whole Effluent Toxicity Test with* Pimephales promelas (LSRI, 2017c), AT/44 – *Conducting a Chronic Whole Effluent Toxicity Test with* Ceriodaphnia dubia (LSRI, 2017d), and AT/45 – *Conducting a Chronic Whole Effluent Toxicity Test with* Selenastrum capricornutum (LSRI, 2017e).

2.6 ANALYTICAL METHODS

Water chemistry parameters that may have an impact on BWT performance or may by impacted by the treatment process were measured during this evaluation. These parameters included TSS, percent transmittance (%T), POM, NPOC, DOC, total alkalinity, total hardness, DO, temperature, specific conductivity, and pH.

2.6.1 TOTAL SUSPENDED SOLIDS, PARTICULATE ORGANIC MATTER, AND MINERAL MATTER

TSS analysis was conducted according to LSRI/SOP/SA/66 – *Analyzing Total Suspended Solids (TSS), Particulate Organic Matter (POM), and Mineral Matter (MM)* (LSRI, 2017f). Accurately measured sample volumes (±1%) were vacuum filtered through pre-washed, dried, and pre-weighed glass fiber filters (Whatman 934-AH, 1.5 µm pore diameter). After each sample was filtered, it was dried in an oven and brought to constant weight. TSS values were determined based on the weight of particulates collected on the filter and the volume of water filtered. To determine POM, the residue from the TSS analysis was ignited to a constant weight at 550°C in a muffle furnace. The concentration of POM was determined by the difference of the dry weight of the particulates on the filter before and after ignition (the mass lost to combustion). Mineral matter was defined, and calculated, as the difference between TSS and POM.

2.6.2 PERCENT TRANSMITTANCE AT 254 NM

Percent transmittance analysis was conducted according to LSRI/SOP/SA/69 – *Laboratory Determination of Percent Transmittance (%T) of Light in Water at 254 nm* (LSRI, 2018b). The %T was measured on both unfiltered and filtered aliquots of each sample collected using a Perkin Elmer Lambda 35 UV-Vis spectrophotometer. For analysis of the filtered aliquot, an appropriate volume of sample was filtered through a glass fiber filter (Whatman 934-AH, 1.5 µm pore diameter). Deionized water was used as a reference to adjust the spectrophotometer to 100%T, and then each aliquot was measured in a pre-rinsed sample cuvette with a 1-cm path length.

2.6.3 ORGANIC CARBON ANALYSIS

NPOC/DOC analysis was conducted according to LSRI/SOP/SA/47 – *Measuring Organic Carbon in Aqueous Samples* (LSRI, 2006) and using a Shimadzu Model TOC-L Total Organic Carbon Analyzer. After collection, DOC samples were filtered through a glass fiber filter (Whatman GF/F, 0.7 µm effective pore size). Before analysis, the samples were acidified to a pH <2 with concentrated hydrochloric acid (HCl; ~ 0.2% v/v). Samples were then purged with high-purity air to remove the inorganic carbon and purgeable organic carbon and injected into the analyzer. Amended samples (LW-TMH) were sonicated for a minimum of 30 minutes and were stirred continuously, using a stir bar and stir plate, while being manually injected into the instrument. A 1,000 mg/L total organic carbon stock solution was used to prepare a working standard of 50 mg/L carbon, which was also acidified to a pH <2 with concentrated HCl. The standard was used to generate a calibration curve from which the organic carbon concentration of the samples was determined.

2.6.4 HARDNESS AND ALKALINITY

Total hardness was analyzed using the ethylenediaminetetraacetic acid (EDTA) titrimetric method through manual titration according to the method described in LSRI/SOP/GLM/02 – *Procedure for Measuring Total Hardness* (LSRI, 1991a). Total hardness is reported as mg/L $CaCO_3$. Analysis of total alkalinity was conducted using the sulfuric acid titrimetric method through manual titration and according to the method described in LSRI/SOP/GLM/01 – *Procedure for Measuring Alkalinity* (LSRI, 1991b). Total alkalinity is reported as mg/L $CaCO_3$.

2.6.5 DISSOLVED OXYGEN ANALYSIS

Analysis of DO was conducted using a YSI ProSolo handheld meter and optical dissolved oxygen/temperature probe (YSI ProSolo) or a Hach® HQ30D meter and luminescent dissolved oxygen LDO101 probe. The YSI ProSolo was calibrated daily following LSRI/SOP/GLM/34 – *Calibrating, Maintaining and Using the YSI ProSolo Handheld Meter and Optical Dissolved Oxygen/Temperature Probe* (LSRI, 2019d). The Hach HQ30d was calibrated daily following LSRI/SOP/GLM/30 – *Calibrating Maintaining and Using the HQ30d and HQ40d Meter and LDO101 Optical Electrode to Measure Dissolved Oxygen in Water Samples* (LSRI, 2017g).

2.6.6 TEMPERATURE, CONDUCTIVITY, AND pH

Temperature was measured using a Fisher digital thermometer that was verified quarterly following LSRI/SOP/GLM/17– *Procedure for Thermometer Verification and Calibration* (LSRI, 1995b). Specific conductivity was measured using an Oakton Model CON 110 Conductivity/TDS/Temperature Meter that was calibrated on a monthly basis following LSRI/SOP/GLM/26- *Procedures for Calibrating and Using the Oakton CON 110 Conductivity/TDS/Temperature Meter* (LSRI, 2011a). Its accuracy was verified daily prior to sample analysis using a potassium chloride check standard. pH analysis was conducted using an Orion 3 Star meter and Orion 8157BNUMD pH probe. The pH meter was calibrated daily following LSRI/SOP/GLM/05– *Procedure for Calibration and Operation of pH Meters Utilizing Automatic Temperature Compensation (ATC)* (LSRI, 1992). A check buffer of 8.00 was measured after calibration to verify the accuracy of the calibration.

2.6.7 OXIDATION REDUCTION POTENTIAL

The ORP was measured following LSRI/SOP/SA/54 - *Determination of Oxidation-Reduction Potential (ORP)* (LSRI, 2011b). ORP was measured using a Thermo Scientific Orion Epoxy Refillable ORP/ATC Triode, with a platinum indicator electrode and a silver/silver chloride reference electrode. Calibration was performed daily with an ORP standard (Thermo Scientific, Orion #967901). Accuracy was verified daily using an externally sourced reference standard (600 or 200 mV vs Ag/AgCl; RICCA Chemical Company).

2.6.8 OZONE

Ozone concentration was measured according to the method in LSRI/SOP/SA/73 - *Analyzing Ozone Concentrations in Water* (LSRI, 2019e). Test water was reacted immediately with an Indigo Reagent. Ozone reacts quickly with the reagent so a decrease in absorbance at 600 nm can be related to ozone concentration. The detection range of this method is 0.05-0.5 mg/L. To measure ozone at higher concentrations, samples were diluted so ozone concentrations were within the measurable range. It should be noted that according to Baird et al. (2017) this method measures residual ozone in aqueous solutions. It is unknown if this method is able to measure ozone confined within nanobubbles. Due to this, it is possible ozone concentrations were underestimated by this method.

2.7 ANALYTICAL METHODS FOR BIOLOGICAL EFFECTIVENESS TESTING

2.7.1 BACTERIAL ENUMERATION

From each whole water sample, subsamples were collected at designated analysis periods (Table 3) and placed in sterile 120-mL sample vessels. *E. coli* and *Enterococcus* were enumerated according to LSRI/SOP/SA/56 – *Detection and Enumeration Total Coliforms and E. coli Using IDEXX Colilert®* (LSRI, 2019f) and LSRI/SOP/SA/62 *Detection and Enumeration of Enterococcus using IDEXX Enterolert®* (LSRI, 2018c). Results are given as Most Probable Number (MPN), a common method of obtaining quantitative data on concentrations of discrete items from positive/negative (incidence) data, and in this case correlates well with colony forming units (CFU). The Colilert and Enterolert assays have a detection limit of 1 MPN/100 mL *E. coli or Enterococci*, respectively. Both tests use Defined Substrate Technology®

(DST) in which the bacteria metabolize the enzymes in the specific media causing the sample to fluoresce. Microbial densities (as MPN/100 mL) over the test period were calculated and reported for each species.

2.7.2 ALGAE ENUMERATION

Whole water algae samples were analyzed by staining a subsample of *S. capricornutum* cells from each sample with the vital stain SYTOX® Green. LSRI/SOP/GWRC/11 - *Assessing Bench-Scale Dose-Effectiveness of Potential Ballast Water Treatment Processes on* Selenastrum capricornutum (LSRI, 2017h) was followed for staining and counting. Counting was conducted by enumerating the number of live and dead cells within a known area using a compound microscope equipped with epifluorescence able to excite samples at 450-490 nm under 400x magnification. The epifluorescence was turned on or off depending on the type of count being done and at the discretion of the trained algae counter. It was noticed during counts on treated samples that very few cells were visible with the epifluorescence. On treated samples, counts were made with the epifluorescence to establish if the cells were dead/alive and then counts were done with the epifluorescence off to ensure cells were present in the sample at the same concentration as at test initiation. Many cells were visible without the epifluorescence in the treated samples. These cells were reasoned to be dead as they did not react with the stain at all.

2.8 DEVIATIONS

During the course of NBOT 2.5-HP testing, several deviations from the test plan and SOPs occurred. These deviations are listed in Table 4 along with corrective actions that were taken as a response to the deviations and the perceived impact of the deviations on the test results.

Table 4. Deviations Encountered During NBOT 2.5-HP Bench-Scale Testing, Potential Impact, and Corrective Actions.

Test Date(s) Project ID	Description and Root Cause of Deviation or Quality Control Failure	Description of Corrective Action(s)	Describe the Impact on the Project/Test	Do the Data Need to be Qualified?
LW-TMH-15	TSS, DOC, and POM in the Control Stock and DOC in the Treatment Stock were above the target ranges for initiating bench-scale testing that were listed in the test plan.	Add TMH to the tanks when they have 200 liters of lab water in them so the TMH is allowed to mix as the tank is filling the rest of the way. Be especially sure samples are collected well beneath the surface of the water in the Control and Treatment tanks and that they are collected from the same spot within the tank each time.	Minimal, all other water chemistry parameters were within the acceptable range for initiating bench-scale testing.	No
LW-TMH-25 (2)	DOC in the Treatment Stock was above the target range listed in the test plan for initiation of bench-scale testing.	Try a new method of mixing the tanks to help the TMH dissolve better. Conduct a test to determine if this mixing does in fact provide more consistently acceptable DOC, NPOC,	Minimal, all other water chemistry parameters were within the acceptable ranges	No

Test Date(s) Project ID	Description and Root Cause of Deviation or Quality Control Failure	Description of Corrective Action(s)	Describe the Impact on the Project/Test	Do the Data Need to be Qualified?
		and TSS values for the LW-TMH stock solutions.	for initiating bench-scale testing.	
LW-15 and LW-TMH-15	Water temperatures were not within the acceptable range for test initiation (10-15°C). During test LW-15 the initiation temperature of the control tank was 16.7°C and the treatment tank was 16.5°C. During test LW-TMH-15 the initiation temperature of the control and treatment tanks was 15.2°C. Root Cause: This was a deviation was caused by the chilled laboratory water not being cold enough to reach the desired temperature.	Test code was updated on all test materials to reflect the appropriate temperature range.	Minimal, testing temperatures were warmer than the target temperatures.	Yes
M-LW-TMH	%T unfiltered values in the PCW, Control and Treatment Stocks and %T filtered values in the Control Stock were below the target range listed in the test plan for initiation of bench-scale testing. Root cause: The target ranges were developed based on historical data so this may have occurred in part due to the new LW-TMH preparation method. The new method may lead to more complete suspension of solids and dissolutions of solutes causing lower %T than was historically observed.	No change needed. If %T continues to be lower than historical data in future tests, the target ranges may need to be reviewed to reflect the new LW-TMH preparation method.	Minimal, all other water chemistry parameters were within the target range for initiating bench-scale testing.	Yes
SC-LW-TMH	%T unfiltered values in the Control and Treatment Stocks was below the target range. DOC in the Control Stock was above the target range listed in the test plan for initiation of bench-scale testing. Root Cause: The target ranges were developed based on historical data so this may have occurred in part due to the new LW-TMH preparation method. The new method may lead to more	No change needed. If %T and DOC continue to be different than historical data in future tests, the target ranges may need to be reviewed to reflect the new LW-TMH preparation method.	Minimal, all other water chemistry parameters were within the target range for initiating bench-scale testing.	Yes

Test Date(s) Project ID	Description and Root Cause of Deviation or Quality Control Failure	Description of Corrective Action(s)	Describe the Impact on the Project/Test	Do the Data Need to be Qualified?
	complete suspension of solids and dissolutions of solutes causing lower %T than was historically observed.			
DM-LW-TMH	%T unfiltered and filtered values in the Treatment Stock and %T unfiltered values in the Control and Control duplicate stocks were below the target range listed in the test plan for initiation of bench-scale testing. Root cause: The target ranges were developed based on historical data so this may have occurred in part due to the new LW-TMH preparation method. The new method may lead to more complete suspension of solids and dissolution of solutes causing lower %T than was historically observed.	No change needed. If %T continues to be lower than historical data in future tests, the target ranges may need to be reviewed to reflect the new LW-TMH preparation method.	Minimal, all other water chemistry parameters were within the target range for initiating bench-scale testing.	Yes
EU-LW-TMH	%T unfiltered values in the Control and Treatment Stocks were below the target range listed in the test plan for initiation of bench-scale testing. Root cause: The target ranges were developed based on historical data so this may have occurred in part due to the new LW-TMH preparation method. The new method may lead to more complete suspension of solids and dissolution of solutes causing lower %T than was historically observed.	No change needed. If %T continues to be lower than historical data in future tests, the target ranges may need to be reviewed to reflect the new LW-TMH preparation method.	Minimal, all other water chemistry parameters were within the target range for initiating bench-scale testing.	Yes
EDM-LW-TMH	%T unfiltered values in the Control and Treatment Stocks were below the target range listed in the test plan for initiation of bench-scale testing. Root cause: The target ranges were developed based on historical data so this may have occurred in part due to the new LW-TMH preparation method. The	No change needed. If %T continues to be lower than historical data in future tests, the target ranges may need to be reviewed to reflect the new LW-TMH preparation method.	Minimal, all other water chemistry parameters were within the target range for initiating bench-scale testing.	Yes

Test Date(s) Project ID	Description and Root Cause of Deviation or Quality Control Failure	Description of Corrective Action(s)	Describe the Impact on the Project/Test	Do the Data Need to be Qualified?
	new method may lead to more complete suspension of solids and dissolution of solutes causing lower %T than was historically observed.			
SC-CRT-LW	Coefficient of Variation (CV) for growth in the *S. capricornutum* LW and CMHRW controls (PCW) was above the ≤20% quality assurance parameter. Root cause: Not able to be determined.	Growth was acceptable in all LW and CMHRW controls (PCW). CV was acceptable in all concentrations other than the LW and CMHRW controls (PCW).	Minimal, the chronic reference toxicant test passed all QA parameters.	No
CD-CRT-LW	Neither the LW nor CMHRW controls (PCW) in the *C. dubia* portion met all of the quality assurance parameters. Both met some, neither met all. Root cause: Not able to be determined.	The test would be repeated. Due to COVID-19 the test was not able to be repeated.	Minimal, the chronic reference toxicant test passed all QA parameters.	Yes
RTT – *P. promelas*	Survivorship of control organisms was below the quality assurance range. Root cause: Not able to be determined.	The reference toxicant test needs to be repeated or data obtained from the facility culturing the organisms used for testing.	None, data was received from the culturing facility proving the health of the organisms.	No

3 TEST RESULTS

3.1 NBOT 2.5-HP BWT OPERATIONAL PERFORMANCE

On September 23, 2019 (after LW-15) strong ozone odors were observed in the laboratory during the operation of NBOT 2.5-HP. Following this, LSRI staff conducted leak tests on NBOT 2.5-HP and a leak was discovered in the line connecting the NBOT 2.5-HP ozone generator to the pump. This leak was repaired by LSRI staff who bypassed the affected tubed with existing tubing. On September 25, 2019 during LW-TMH-25(1), after around 120 minutes of treatment an increase in ozone odor was observed. An inspection of NBOT 2.5-HP on September 26, 2019 revealed a broken connector within the pump cabinet of NBOT 2.5-HP. The connector was replaced by LSRI staff. After replacement of the connector, the UWS Environmental Health and Safety Director measured ozone levels in the laboratory air while the NBOT 2.5-HP system was running. The operating environment was determined to be safe. No other operational issues were encountered by LSRI staff during the remaining tests.

3.2 WATER-ONLY TESTING

3.2.1 WATER QUALITY

Water quality parameters during water-only testing were within target ranges for all tests except LW-TMH-15 and LW-TMH-25 (2) tests (Table 5). In LW-TMH-15, initial POM, TSS, and DOC were all greater than the target ranges in the control stock and initial DOC was greater than the target range in the treatment stock. In LW-TMH-25 (2), initial DOC was greater than the target range in the treatment stock. The effects of these deviations are discussed in the *Deviations* section but are not believed to significantly affect the conclusions of this report.

During the LW tests, TSS, POM, MM, NPOC, DOC, and %T were similar between the treatment and control tanks (Table 6). During LW-TMH tests, changes were observed in all parameters. Relative to the control tank, TSS, POM, MM, DOC, and NPOC in the treatment tank had decreased, and both filtered and unfiltered %T increased, by the conclusion of all LW-TMH tests (Table 6). These observations suggest complex bulk chemical changes in the water chemistry of solutions treated by NBOT 2.5-HP, however, isolating the causes of decreases in POM, TSS, MM, and the increase in %T during the LW-TMH tests, was beyond the scope of this work.

Table 5. Water Quality Parameters Measured in Treatment and Control Tanks during NBOT 2.5-HP Water-Only Tests.

Trial	Duration (min.)	TSS (mg/L)		Percent Transmittance Filtered/Unfiltered (%)		NPOC (mg/L)		DOC (mg/L)		POM (mg/L)		MM (mg/L)	
		Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control
LW-25	0	<1.25	<1.25	98.3/98.0	98.3/98.3	0.77[J]	0.76[J]	0.97[J]	0.86[J]	<1.25	<1.25	<1.25	<1.25
	15	<1.25	<1.25	98.8/99.4	98.2/98.4	0.75[J]	0.83[J]	0.72[J]	0.74[J]	<1.25	<1.25	<1.25	<1.25
	75	<1.25	<1.25	99.2/99.2	98.5/98.8	<0.70	<0.70	<0.70	<0.70	<1.25	<1.25	<1.25	<1.25
	135	<1.25	<1.25	99.0/99.2	98.4/98.4	<0.70	<0.70	<0.70	<0.70	<1.25	<1.25	<1.25	<1.25
LW-15	0	<1.25	<1.25	97.2/97.7	99.6/99.5	0.96[J]	<0.70	0.74[J]	<0.70	<1.25	<1.25	<1.25	<1.25
	15	<1.25	<1.25	98.8/100.1	97.9/100.5	<0.70	<0.70	<0.70	0.79[J]	<1.25	<1.25	<1.25	<1.25
	75	<1.25	<1.25	99.1/96.9	97.2/98.0	0.74[J]	<0.70	0.80[J]	0.71[J]	<1.25	<1.25	<1.25	<1.25
	135	<1.25	<1.25	97.7/97.4	99.3/99.2	<0.70	0.73[J]	<0.70	0.75[J]	<1.25	<1.25	<1.25	<1.25
LW-TMH- 25 (1)	0	18.4	20.0	29.8/26.1	28.5/25.8	9.0	9.1	4.5	7.5	7.7	8.2	10.7	11.8
	15	16.7	18.6	33.4/29.7	28.2/25.5	8.8	9.1	4.8	7.7	6.6	7.6	10.0	11.0
	75	12.9	17.7	48.3/44.5	28.2/25.7	7.8	9.2	5.8	7.6	5.2	7.6	7.7	10.2
	135	10.9	18.5	60.4/55.9	28.3/25.6	7.5	8.9	5.4	8.1	4.3	7.6	6.6	10.9
LW-TMH- 15	0	18.8	36.8	30.1/26.8	30.0/26.2	9.4	9.4	9.1	8.7	7.5	21.5	11.3	15.3
	15	17.0	18.6	34.4/30.9	29.4/26.3	9.0	9.6	6.7	6.6	6.8	7.7	10.2	10.8
	75	14.5	16.6	56.1/51.0	29.4/26.4	8.1	9.4	5.9	7.0	5.7	7.0	8.8	9.5
	360	5.1	17.0	68.8/49.9	28.9/26.3	5.4	9.6	4.9	6.9	0.9	7.2	4.3	9.8
LW-TMH- 25 (2)	0	19.9	18.5	29.5/27.0	29.4/26.8	9.1	8.9	6.9	6.8	8.9	7.8	11.0	10.7
	15	14.0	18.9	38.1/35.2	29.4/26.8	8.6	9.1	6	6.2	6.1	8.4	7.9	10.4
	135	10.3	19.3	72.9/68.2	29.4/26.7	7.7	9.0	5.2	6.0	4.1	8.1	6.2	11.2
	405	4.2	13.4	88.3/84.7	29.3/26.5	4.6	9.2	3.7	6.6	<1.25	6.3	3.5	7.1

[J] Indicates values above the detection limit but below the limit of quantitation of the analysis method.

3.2.2 MEASUREMENTS DURING TREATMENT (WATER-ONLY TESTING)

During treatment, ozone, ORP, pH, DO, conductivity, and temperature were measured (Table 6-Table 8 and Figure 5 and Figure 6). Ozone was measured during treatment to test the ability of NBOT 2.5-HP to produce ozone and introduce it into the treated solution (Table 6). ORP was measured to estimate the ability of NBOT 2.5-HP to change the oxidation potential of the treated solution; the presence of ozone or hydroxyl radicals should lead to an increase in ORP (Table 6).

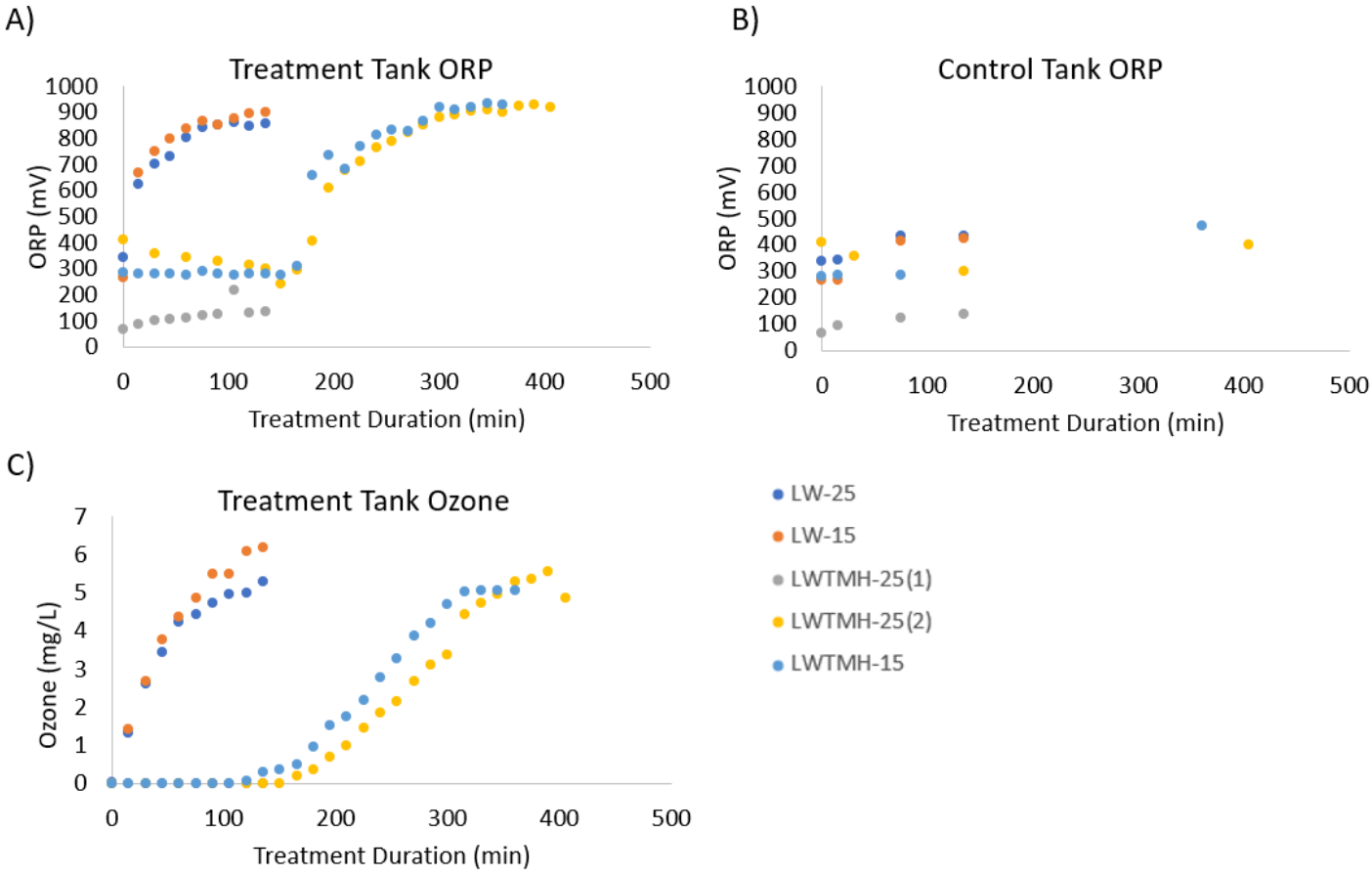

Figure 5. Time Series of A) ORP in the Treatment Tank, B) ORP in the Control Tank, C) Ozone in the Treatment Tank. Control Tank Ozone is not Shown because all values were Less than the Detection Limit (<DL).

In the control tanks for both LW-15 and LW-25 tests, no appreciable change in ORP, DO, or ozone was observed. In the treatment tanks, ozone increased with treatment time to peak values of 5.29 mg/L in LW-25 and 6.19 mg/L in LW-15 (Table 6, Figure 5). The greater concentration of ozone in colder water is explained by the solubility of ozone in water, which is inversely related to temperature (Roth and Sullivan, 1981). In both LW tests, ORP also increased with treatment time to peak values of 860.8 mV in LW-25 and 897.9 mg/L in LW-15 (Table 6, Figure 5). Dissolved oxygen also increased quickly during treatment, with stable values reached at ~75 minutes in both experiments (Table 7, Figure 6). In the LW

tests, the LW-25 had a lower maximum DO (38.6 mg/L) than the LW-15 test (41.3 mg/L), again explained by the inverse relationship between gas solubility and temperature (Table 7, Figure 6).

In the control tanks of all LW-TMH tests, no large change in ORP, DO, or ozone was observed (Figure 5 and Figure 6, Table 6 and Table 7). In the LW-TMH-25 (1) treatment tank, no increase in ozone or ORP was observed. Therefore, this test was repeated using a longer treatment duration. In LW-TMH-25 (2) and LW-TMH-15 treatment tanks, ozone increased to peak values of 5.54 mg/L and 5.07 mg/L and ORP increased to peak values of 928.3 mV and 933.7 mV, respectively (Table 6, Figure 5). Dissolved oxygen also increased during treatment in all LW-TMH tests, with stable values reached at ~75 minutes in all experiments (Table 7, Figure 6). Maximum DO concentrations were slightly lower within the LW-TMH tests than the LW tests; LW-TMH-25 (2) had the lowest maximum DO (35.3 mg/L), followed by LW-TMH-25 (1)(35.6 mg/L), then the LWMTH-15 (40.9 mg/L) (Table 7, Figure 6). The lower solubility of oxygen in higher temperature water may explain the slightly lower DO concentrations in the 25°C tests.

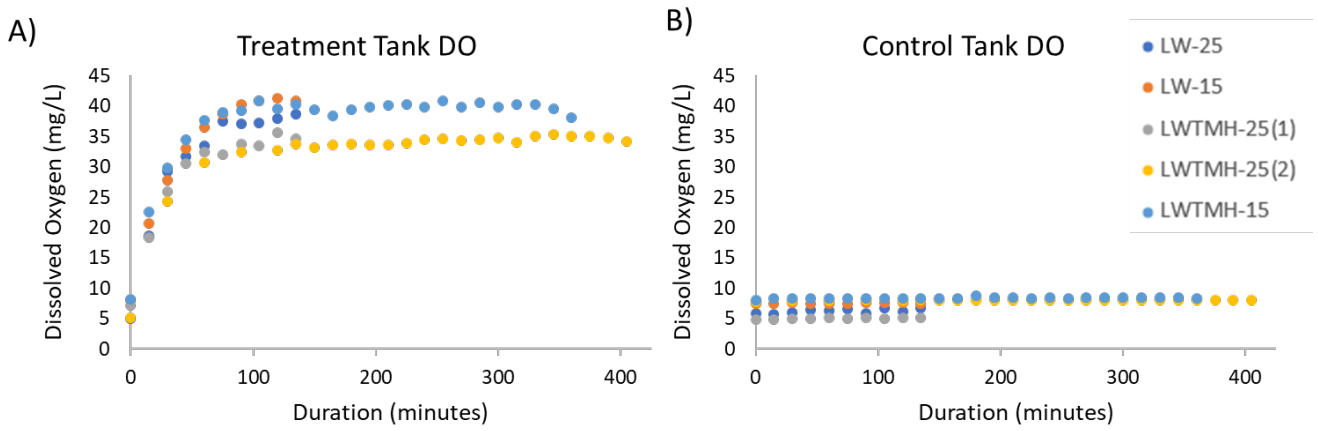

Figure 6. Time Series of A) DO in the Treatment Tank and B) DO in the Control Tank during all NBOT 2.5-HP Tests.

Other parameters measured during treatment include pH, temperature, conductivity, total hardness, and total alkalinity. For conductivity, alkalinity, and hardness, no large differences between water type, temperatures, or treatment and control tanks were observed (Table 8). For pH, decreases were observed in the treatment tank in all LW-TMH tests but no large change occurred in LW tests or within control tanks of any test (Table 7). The causes of pH changes are unclear but could be a result of ozone-driven chemistry in the treatment tanks. For example, if NBOT 2.5-HP was completely oxidizing organic molecules to CO_2, the pH may decrease (Caldeira et al., 2003). The specific mechanisms of this pH change are beyond the scope of this work.

Small changes in temperature were observed during treatment. These changes are likely driven by the treatment process, as heat from the microbubble generation process and pumps could be transferred to the solution.

Table 6. Ozone and ORP Measurements Collected during Treatment of 1,000-L Tanks with NBOT 2.5-HP BWT Water-Only Tests.

Treatment Duration (min.)	LW-25				LW-15				LW-TMH-25 (1)				LW-TMH-25 (2)				LW-TMH-15			
	Treatment		Control		Treatment		Control		Treatment		Control		Treatment		Control		Treatment		Control	
	ORP (mV)	Ozone* (mg/L)	ORP (mV)	Ozone* (mg/L)	ORP (mV)	Ozone* (mg/L)	ORP (mV)	Ozone* (mg/L)	ORP (mV)	Ozone* (mg/L)	ORP (mV)	Ozone* (mg/L)	ORP (mV)	Ozone* (mg/L)	ORP (mV)	Ozone* (mg/L)	ORP (mV)	Ozone* (mg/L)	ORP (mV)	Ozone* (mg/L)
0	342.4	0.05	339.7	<0.9	267.2	<0.9	267.6	<0.9	69.6	<0.05	67.8	<0.05	412.6	<0.05	408.3	<0.05	283.7	<0.05	280.5	<0.05
15	622.2	1.33	344.7	<0.9	669.2	1.43	265.7	<0.9	85.1	<0.05	95.4	<0.05	NM	NM	NM	NM	282.3	<0.05	286.2	<0.05
30	699.4	2.62	NM	NM	750.1	2.67	NM	NM	103.2	<0.05	NM	NM	359.0	<0.05	355.6	<0.05	280.4	<0.05	NM	NM
45	728.2	3.43	NM	NM	796.8	3.76	NM	NM	106.7	<0.05	NM	NM	NM	NM	NM	NM	280.6	<0.05	NM	NM
60	800.9	4.24	NM	NM	835.8	4.38	NM	NM	113.1	<0.05	NM	NM	341.3	<0.05	NM	NM	276.4	<0.05	NM	NM
75	839.2	4.43	431.5	<0.9	863.5	4.86	413.7	<0.9	118.6	<0.05	124.2	<0.05	NM	NM	NM	NM	289.8	<0.05	286.3	<0.05
90	850.3	4.71	NM	NM	853.0	5.48	NM	NM	124.1	<0.05	NM	NM	328.7	<0.05	NM	NM	280.1	<0.05	NM	NM
105	860.8	4.95	NM	NM	874.1	5.48	NM	NM	218.3	<0.05	NM	NM	NM	NM	NM	NM	276.0	<0.05	NM	NM
120	845.3	5.00	NM	NM	893.4	6.10	NM	NM	130.1	<0.05	NM	NM	312.0	<0.05	NM	NM	279.2	0.08	NM	NM
135	858.1	5.29	435.7	<0.9	897.9	6.19	426.1	<0.9	135.4	<0.05	138.6	<0.05	300.8	<0.05	300.5	<0.05	279.7	0.29	NM	NM
150	Test concluded at 135 min				Test concluded at 135 min				Test concluded at 135 min				242.1	<0.05	NM	NM	277.3	0.37	NM	NM
165													295.3	0.19	NM	NM	310.7	0.49	NM	NM
180													406.2	0.35	NM	NM	655.9	0.94	NM	NM
195													611.1	0.70	NM	NM	736.8	1.53	NM	NM
210													678.0	1.01	NM	NM	683.2	1.74	NM	NM
225													712.5	1.46	NM	NM	768.3	2.19	NM	NM
240													762.9	1.85	NM	NM	810.4	2.79	NM	NM
255													789.5	2.15	NM	NM	829.2	3.26	NM	NM
270													821.7	2.69	NM	NM	828.2	3.88	NM	NM
285													850.7	3.10	NM	NM	866.2	4.21	NM	NM
300													878.8	3.36	NM	NM	916.9	4.69	NM	NM
315													891.9	4.44	NM	NM	907.5	5.02	NM	NM
330													902.6	4.73	NM	NM	917.2	5.07	NM	NM
345													909.9	4.96	NM	NM	933.7	5.07	NM	NM
360													898.7	5.29	NM	NM	927.2	5.07	470.6	<0.9
375													924.6	5.35	NM	NM	Test concluded at 375 min			
390													928.3	5.54	NM	NM				
405													918.7	4.87	399.8	<0.05				

NM= Not Measured

* Ozone reporting limits for below detection limit values vary due to sample dilution. The method detection limit is 0.05 mg/L for an undiluted sample.

Table 7. Dissolved Oxygen, pH, and Temperature (Temp.) Measurements during Treatment of 1,000-L Tanks with NBOT 2.5-HP BWT Water-Only Tests.

Treatment Duration (min.)	LW-25						LW-15						LW-TMH-25 (1)					
	Treatment			Control			Treatment			Control			Treatment			Control		
	DO (mg/L)	pH	Temp. (°C)	DO (mg/L)	pH	Temp. (°C)	DO (mg/L)	pH	Temp. (°C)	DO (mg/L)	pH	Temp. (°C)	DO (mg/L)	pH	Temp. (°C)	DO (mg/L)	pH	Temp. (°C)
0	5.00	7.17	25.8	5.8	7.27	25.7	8.1	7.39	16.7	7.5	7.32	17.0	7.2	7.29	25.2	4.8	7.07	25.8
15	18.7	7.18	24.9	5.7	7.27	25.5	20.7	7.40	17.3	7.4	7.35	17.4	18.3	7.23	25.3	4.9	7.09	25.8
30	29.2	7.17	25.2	6.0	7.27	25.4	27.8	7.39	17.4	7.6	7.38	17.5	25.9	7.18	25.6	5.0	7.10	25.9
45	31.7	7.19	25.6	6.5	7.26	25.4	33.0	7.40	17.7	7.5	7.36	17.5	30.5	7.08	25.7	5.0	7.10	25.9
60	33.5	7.20	25.2	6.3	7.27	25.4	36.5	7.42	18.0	7.5	7.34	17.6	32.4	7.04	25.9	5.1	7.09	25.8
75	37.5	7.20	25.6	6.6	7.27	25.3	38.7	7.42	18.3	7.5	7.35	17.8	32.0	7.02	26.0	5.0	7.10	25.7
90	37.1	7.20	26.0	5.9	7.28	25.2	40.2	7.41	18.4	7.6	7.35	17.6	33.8	6.95	26.3	5.1	7.09	25.8
105	37.2	7.20	26.2	6.7	7.26	25.4	40.9	7.41	18.7	7.6	7.36	17.8	33.5	6.92	26.4	5.0	7.09	25.7
120	38.0	7.21	26.3	6.1	7.29	25.3	41.3	7.42	19.0	7.6	7.35	17.9	35.6	6.88	26.5	5.1	7.07	25.7
135	38.6	7.20	26.1	6.8	7.26	25.0	40.8	7.41	19.3	7.5	7.35	17.9	34.6	6.88	26.8	5.1	7.07	25.6

Treatment Duration (min.)	LW-TMH-25 (2)						LW-TMH-15					
	Treatment			Control			Treatment			Control		
	DO (mg/L)	pH	Temp. (°C)	DO (mg/L)	pH	Temp. (°C)	DO (mg/L)	pH	Temp. (°C)	DO (mg/L)	pH	Temp. (°C)
0	5.1	7.11	23.7	7.7	7.37	24.3	8.2	7.73	15.8	8.1	7.54	15.5
15	NM	NM	NM	NM	NM	NM	22.6	7.55	15.9	8.3	7.72	15.6
30	24.3	6.96	24.0	7.9	7.35	24.3	29.8	7.41	16.0	8.3	7.7	15.7
45	NM	NM	NM	NM	NM	NM	34.5	7.32	16.3	8.4	7.73	15.6
60	30.7	6.89	24.7	7.9	7.35	24.2	37.7	7.22	16.5	8.3	7.72	15.6
75	NM	NM	NM	NM	NM	NM	38.9	7.11	16.9	8.4	7.70	15.8
90	32.5	6.82	25.1	8.0	6.95	24	39.3	7.05	17.1	8.4	7.69	15.7
105	NM	NM	NM	NM	NM	NM	40.9	7.00	17.2	8.4	7.71	15.7
120	32.7	6.74	24.7	7.9	7.32	23.6	39.6	6.96	17.6	8.4	7.73	15.8

Treatment Duration (min.)	LW-TMH-25 (2)						LW-TMH-15					
	Treatment			Control			Treatment			Control		
	DO (mg/L)	pH	Temp. (°C)	DO (mg/L)	pH	Temp. (°C)	DO (mg/L)	pH	Temp. (°C)	DO (mg/L)	pH	Temp. (°C)
135	33.7	6.72	24.9	8.0	7.36	23.5	40.3	6.89	17.8	8.4	7.76	16.0
150	33.1	6.71	25.3	8.0	7.36	23.8	39.4	6.86	18.0	8.4	7.74	16.0
165	33.6	6.66	25.5	8.0	7.37	23.8	38.4	6.85	18.2	8.4	7.74	16.2
180	33.7	6.64	25.5	8.0	7.37	23.4	39.4	6.82	18.6	8.8	7.69	16.2
195	33.6	6.60	25.5	8.0	7.36	23.4	39.9	6.74	18.7	8.5	7.71	16.5
210	33.6	6.61	25.6	8.0	7.37	23.6	40.1	6.79	19.0	8.5	7.74	16.5
225	33.9	6.60	25.6	8.0	7.34	23.6	40.3	6.78	19.1	8.4	7.68	16.6
240	34.4	6.63	25.9	8.0	7.36	23.1	39.8	6.76	19.3	8.5	7.73	16.6
255	34.6	6.55	26.0	8.0	7.34	23.5	40.8	6.76	19.6	8.4	7.71	16.5
270	34.3	6.58	26.0	8.0	7.37	23.3	39.8	6.75	19.8	8.5	7.74	16.7
285	34.4	6.58	26.3	8.0	7.36	23.5	40.6	6.76	19.9	8.5	7.74	16.8
300	34.7	6.57	26.2	8.0	7.35	23.5	39.8	6.75	20.2	8.5	7.69	16.7
315	34.0	6.58	26.5	8.0	7.34	23.3	40.3	6.78	20.4	8.5	7.70	16.9
330	35.0	6.60	26.2	8.0	7.38	23.4	40.3	6.77	20.5	8.5	7.70	16.8
345	35.3	6.59	26.4	8.0	7.36	23.4	39.6	6.79	20.8	8.5	7.74	16.8
360	35.1	6.61	26.5	8.0	7.36	23.3	38.1	6.77	20.7	8.4	7.72	16.5
375	35.0	6.61	26.9	8.0	7.35	23.5						
390	34.8	6.61	27.1	8.0	7.36	23.4	LW-TMH test concluded at 360 minutes					
405	34.2	6.63	26.9	8.0	7.37	23.2						

NM= Not Measured

Table 8. Conductivity, Alkalinity, and Hardness Average (Min, Max) Summary Statistics during Treatment of 1,000-L Tanks with NBOT 2.5-HP BWT Water-Only Tests

Test	Conductivity (µS/cm)	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)
Control LW-25	135.6 (133.2, 136.2)	45.9 (44.6, 47.6)	48.2 (46.1, 49.1)
Treatment LW-25	133.5 (131.2, 142.9)	45.7 (44.4, 47.8)	47.0 (45.9, 48.3)
Control LW-15	136.8 (133.3, 140.3)	48.6 (46.4, 51.2)	48.6 (47.7, 49.3)
Treatment LW-15	135.7 (135.3, 136.4)	47.9 (46.8, 49.4)	48.9 (46.7, 50.4)
Control LW-TMH-25 (1)	126.4 (122.6, 127.7)	44.9 (41.2, 47.6)	45.5 (43.5, 46.9)
Treatment LW-TMH-25 (1)	128.4 (127.8, 128.8)	46.2 (45.2, 48.2)	45.3 (42.3, 47.9)
Control LW-TMH-25 (2)	132.2 (130.1, 136.2)	49.0 (46.8, 52.4)	53.2 (51.6, 55.0)
Treatment LW-TMH-25 (2)	133.7 (132.8, 134.7)	49.4 (48.6, 50.4)	49.8 (40.5, 54.6)
Control LW-TMH-15	127.0 (124.8, 128.5)	46.1 (45.4, 46.4)	49.1 (44.9, 51.0)
Treatment LW-TMH-15	129.6 (128.3, 130.7)	47.8 (46.8, 48.2)	45.9 (42.3, 50.2)

3.2.3 POST-TREATMENT AQUATIC DEGREDATION

After treatment, the degradation of ORP, ozone, and DO were monitored (Table 9). Ozone concentration decreases were used to calculate half-lives ($t_{1/2}$) of ozone in this system from an exponential decay fit of the degradation data. The final measurement of each test and post treatment measurements until 4 hours post treatment were used in the fit and to calculate $t_{1/2}$. The post-treatment measurements collected after 4 hours were not used in the calculation because these measurements were below the detection limit of the method.

Degradation of ozone in the LW experiments after treatment was relatively rapid; 240-minute post treatment the ozone concentrations had decreased from 5.29 to 0.27 mg/L in LW-25 and from 6.19 to 1.06 mg/L in LW-15 (Table 6 and Table 9). The $t_{1/2}$ of ozone in LW-25 was shorter, $t_{1/2}$=60 minutes, than LW-15, $t_{1/2}$ = 100 minutes, suggesting that colder temperatures reduce the rate of ozone removal from treated solutions.

Degradation of ozone in the LW-TMH experiments after treatment was more rapid than in LW. At 150 minutes post treatment the ozone concentrations had decreased from 4.87 to 0.19 mg/L in LW-TMH-25 (2) and from 5.07 to 0.67 mg/L in LW-TMH-15 (Table 6 and Table 9). The $t_{1/2}$ of ozone in LW-TMH-25 (2) was shorter, $t_{1/2}$=33 minutes, than in the LW-TMH-15, $t_{1/2}$=50 minutes. Notably, $t_{1/2}$ of ozone in the LW-TMH trials were nearly half of those observed in the LW trials, suggesting ozone degrades faster in waters with high carbon and/or solids concentrations.

In LW, ORP change was relatively slow, at 240 minutes post treatment ORP only decreased from 858.1 mV to 712.1 mV in LW-25 and from 897.9 mV to 788.0 mV in LW-15. In LW-TMH tests ORP change was similar; at 150 minutes hours post treatment ORP decreased from 918.7 mV to 657.8 mV in LW-TMH-25 (2) and from 927.2 to 841.0 in LW-15 (1) (Table 6 and Table 9). The slow degradation of ORP relative to

ozone suggests that water treated by NBOT 2.5-HP may remain highly oxidizing even after ozone has dissipated. DO decreased slowly with all tests having higher DO in treatment samples than in control 48 hours post treatment (Table 10).

Table 9. Water-Only Degradation Average (Standard Deviation) Ozone Concentration and ORP Measured in Samples after NBOT 2.5-HP Treatment.

Post Treatment Duration (min.)	LW-25				LW-15				LW-TMH-25 (1)				LW-TMH-25 (2)				LW-TMH-15			
	Treatment		Control		Treatment		Control		Treatment		Control		Treatment		Control		Treatment		Control	
	ORP (mV)	Ozone* (mg/L)	ORP (mV)	Ozone* (mg/L)	ORP (mV)	Ozone* (mg/L)	ORP (mV)	Ozone* (mg/L)	ORP (mV)	Ozone* (mg/L)	ORP (mV)	Ozone* (mg/L)	ORP (mV)	Ozone* (mg/L)	ORP (mV)	Ozone* (mg/L)	ORP (mV)	Ozone* (mg/L)	ORP (mV)	Ozone* (mg/L)
30	848.4 (16.7)	2.46 (0.40)	NM	NM	872.8 (9.8)	3.94 (0.19)	NM	NM	137.1 (5.2)	NM	140.9 (4.2)	NM	859.8 (27.6)	2.02 (0.20)	401.6 (8.6)	NM	839.0 (18.7)	3.27 (0.03)	454.6 (3.8)	<0.05
60	829.6 (10.1)	1.98 (0.05)	NM	NM	869.5 (5.1)	2.92 (0.05)	NM	NM	138.0 (2.1)	NM	141.6 (0.2)	NM	856.3 (8.3)	1.25 (0.05)	393.8 (5.9)	NM	837.6 (19.2)	2.00 (0.08)	413.8 (2.9)	NM
120	786.5 (25.8)	1.06 (0.03)	NM	NM	835.5 (17.0)	2.03 (0.12)	NM	NM	135.4 (1.3)	NM	138.0 (0.5)	NM	692.3 (34.6)	0.53 (0.03)	381.3 (5.8)	NM	840.6 (32.0)	1.01 (0.24)	493.5 (179.9)	NM
240	712.1 (32.2)	0.27 (0.05)	NM	NM	788.0 (30.3)	1.06 (0.10)	NM	NM	122.8 (2.0)	NM	121.8 (0.2)	NM	657.8 (8.4)	0.19 (0.02)	401.6 (6.1)	NM	841.0 (7.3)	0.67 (0.08)	396.1 (1.4)	NM
1440	423.9 (17.0)	<0.05	395.7 (11.2)	NM	313.1 (11.7)	<0.05	297.9 (3.4)	NM	316.0 (7.1)	NM	308.8 (0.9)	NM	157.4 (14.4)	<0.05	158.4 (1.0)	NM	189.6 (8.8)	<0.05	195.3 (0.2)	NM
2880	290.1 (2.0)	NM	288.0 (2.4)	NM	289.6 (3.7)	NM	297.5 (3.4)	NM	258.7 (3.2)	NM	265.1 (1.9)	NM	150.0 (1.0)	NM	140.0 (8.4)	NM	393.9 (1.7)	NM	391.9 (0.5)	NM

NM= Not Measured because values were below detection at prior time point

* Ozone reporting limits for below detection limit values vary due to sample dilution. The method detection limit is 0.05 mg/L for an undiluted sample

Table 10. Temperature (Temp.), pH, DO, and Conductivity (Cond.) Average (min, max) Summary Statistics from Water-Only Degradation Experiments with NBOT 2.5-HP.

Exposure	Time (hours)	LW-25				LW-15			
		Temp (˚C)	pH	DO (mg/L)	Cond. (µS/cm)	Temp (˚C)	pH	DO (mg/L)	Cond. (µS/cm)
Control	0-4	24.8 (24.6, 25.1)	7.30 (7.26, 7.32)	6.3 (6.1, 6.6)	136.2 (135.8, 137.8)	17.1 (16.8, 17.3)	7.22 (7.11, 7.32)	7.6 (7.5, 7.7)	136.2 (135.7, 137.1)
	24	25.1 (25.0, 25.1)	7.32 (7.31, 7.34)	7.1 (6.9, 7.3)	136.4 (136.2, 136.6)	16.7 (16.7, 16.7)	7.35 (7.35, 7.36)	7.9 (7.9, 7.9)	128.5 (127.6, 129.1)
	48	25.1 (25.0, 25.1)	7.32 (7.31, 7.33)	6.9 (6.9, 7.0)	127.8 (127.7, 128.0)	16.5 (16.5, 16.5)	7.42 (7.39, 7.44)	8.1 (8.0, 8.2)	127.8 (127.2, 128.6)
Treatment	0-4	25.2 (24.8, 25.7)	7.23 (7.22, 7.25)	33.1 (29.5, 36.7)	132.6 (132.4, 133.2)	18.0 (17.1, 18.5)	7.4 (7.37, 7.42)	37.7 (34.6, 40.3)	136.0 (135.5, 136.4)
	24	25 .0 (24.9, 25.1)	7.31 (7.28, 7.33)	23.4 (22.8, 24.6)	133.0 (132.7, 133.3)	16.8 (16.8, 16.9)	7.41 (7.40, 7.41)	27.9 (27.3, 28.8)	127.9 (127.7, 128.1)
	48	25.3 (25.2, 25.3)	7.37 (7.32, 7.41)	15.6 (14.1, 17.9)	124.5 (124.3, 124.8)	16.7 (16.7, 16.7)	7.48 (7.47, 7.49)	20.3 (19.6, 21.4)	127.6 (127.1, 128.1)

Exposure	Time (hours)	LW-TMH-25 (1)				LW-TMH-15				LW-TMH-25 (2)			
		Temp (˚C)	pH	DO (mg/L)	Cond. (µS/cm)	Temp (˚C)	pH	DO (mg/L)	Cond. (µS/cm)	Temp (˚C)	pH	DO (mg/L)	Cond. (µS/cm)
Control	0-4	25.2 (24.9, 25.4)	7.12 (7.09, 7.15)	5.6 (5.3, 5.9)	126.4 (124.8, 127.1)	15.7 (15.5, 15.9)	7.69 (7.65, 7.72)	8.6 (8.4, 8.7)	133.6 (133.1, 134.0)	23.9 (23.5, 24.3)	7.40 (7.37, 7.42)	8.1 (8.0, 8.3)	127.5 (126.8, 128.0)
	24	25.1 (25.1, 25.1)	7.20 (7.17, 7.22)	6.3 (6.2, 6.4)	126.6 (126.2, 126.8)	15.4 (15.3, 15.5)	7.69 (7.68, 7.70)	8.9 (8.9, 8.9)	133.9 (133.3, 134.9)	24.8 (24.5, 25.0)	7.44 (7.44, 7.45)	8.0 (8.0, 8.1)	127.8 (127.4, 128.4)
	48	24.9 (24.8, 24.9)	7.15 (7.09, 7.23)	7.1 (7.1, 7.1)	126.9 (126.2, 127.7)	15.2 (15.2, 15.2)	7.79 (7.78, 7.81)	9.2 (9.2, 9.2)	134.1 (133.4, 134.7)	25.0 (24.9, 25.2)	7.45 (7.33, 7.54)	8.0 (8.0, 8.0)	127.1 (126.5, 127.5)
Treatment	0-4	25.6 (25.0, 26.3)	6.91 (6.80, 6.95)	28.5 (24.3, 32.2)	128.7 (127.8, 129.0)	18.1 (17.0, 19.4)	6.74 (6.62, 6.81)	37.3 (35.2, 39.2)	134.5 (133.2, 135.2)	25.7 (25.1, 26.6)	6.66 (6.64, 6.70)	30.8 (28.2, 33.7)	130.6 (129.2, 131.2)
	24	25.0 (25.0, 25.0)	7.00 (6.98, 7.02)	20.4 (18.1, 22.8)	128.8 (128.6, 128.9)	15.3 (15.2, 15.3)	6.86 (6.85, 6.88)	27.7 (27.4, 28.1)	135.6 (135.3, 136.1)	24.9 (24.8, 25.1)	6.74 (6.71, 6.76)	20.6 (19.5, 22.2)	130.9 (130.6, 131.2)
	48	24.8 (24.8, 24.8)	7.10 (7.07, 7.13)	13.9 (12.6, 16.0)	129.9 (129.4, 130.6)	15.2 (15.2, 15.3)	6.92 (6.91, 6.93)	22.0 (21.4, 22.5)	135.7 (135.3, 136.2)	24.7 (24.5, 24.8)	6.84 (6.79, 6.88)	14.5 (13.2, 16.0)	131.1 (130.7, 131.5)

3.3 BIOLOGICAL EFFECTIVENESS TEST RESULTS

3.3.1 WATER CHEMISTRY

Water chemistry parameters during biological effectiveness testing were within target ranges for all LW tests, however, during all LW-TMH tests percent transmittance and/or DOC values fell outside of the acceptable target ranges (Table 11). In the SC-LW-TMH, filtered %T in both the treatment and control tank were lower than the target range and in the control tank DOC was higher than the target range (Table 1 and Table 11). In M-LW-TMH, DM-LW-TMH, EU-LW-TMH, and EDM-LW-TMH filtered %T and/or unfiltered %T in the control tank and/or treatment tanks were below the target range. The effects of these deviations are discussed in the *Deviations* section but are not believed to significantly affect the conclusions of this report.

Table 11. Water Chemistry Parameters at the Initiation of Biological Effectiveness Tests.

Test	TSS (mg/L)			Percent Transmittance Filtered/Unfiltered (%)			NPOC (mg/L)			DOC (mg/L)			POM (mg/L)			MM (mg/L)		
	Treat.	Cont.	PCW	Treat.	Cont.	PCW	Treat.	Cont.	PCW	Treat.	Cont.	PCW	Treat.	Cont.	PCW	Treat.	Cont.	PCW
SC-LW	<2.5	<2.5	<2.5	97.3/ 97.5	97.0/ 97.3	97.1/ 98.0	1.2[J]	1.0[J]	0.7[J]	1.1[J]	1.1[J]	0.8[J]	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5
M-LW	<1.25	<1.25	NM	97.4/ 97.5	97.8/ 97.8	NM	1.2[J]	1.5[J]	NM	1.1[J]	1.5[J]	NM	<1.25	<1.25	NM	<1.25	<1.25	NM
SC-LW-TMH	19.7	21.7	<1.25	26.2/23.6	25.8/22.2	97.8/97.8	9.9	10.2	1.0[J]	6.6	7.0	0.8[J]	8.1	10.1	<1.25	11.6	11.6	<1.25
M-LW-TMH	18.8	20.2	NM	26.7/24.3	24.5/22.5	NM	9.1	9.9	NM	5.8	6.7	NM	7.6	7.6	NM	11.2	12.6	NM
EU-LW	<1.25	<1.25	<1.25	96.7/96.9	96.9/96.9	97.8/97.1	1.5[J]	1.6[J]	0.8	1.4[J]	1.4[J]	0.8[J]	<1.25	<1.25	<1.25	<1.25	<1.25	<1.25
EU-LW-TMH	18.7	20.3	<1.25	27.0/24.5	26.1/23.7	97.0/97.0	7.6	8.0	0.9[J]	6.3	5.6	1.0[J]	8.0	8.1	<1.25	10.7	12.2	<1.25
DM-LW	<1.25	<1.25	<1.25	96.6/96.5	96.6/96.6	99.5/99.4	1.2[J]	1.2[J]	<0.48	1.0[J]	1.1[J]	<0.48	<1.25	<1.25	<1.25	<1.25	<1.25	<1.25
DM-LW-TMH	16.6	21.3	<1.25	24.8/22.5	26.4/24.2	99.4/99.2	8.8	8.7	0.6[J]	6.4	6.5	<0.48	6.8	8.5	<1.25	9.8	12.8	<1.25
EDM-LW	<1.25	<1.25	<1.25	97.3/97.3	97.4/97.4	99.8/99.6	0.8[J]	1.7[J]	<0.48	0.9[J]	1.4[J]	<0.48	<1.25	<1.25	<1.25	<1.25	<1.25	<1.25
EDM-LW-TMH	19.2	19.2	<1.25	26.7/23.8	25.8/23.1	99.5/99.2	8.1	7.9	<0.48	6.1	6.4	<0.48	8.1	8.0	<1.25	11.1	11.2	<1.25

[J] Indicates sample is above the limit of detection but below the limit of quantitation

NM= Not Measured

3.3.2 GREEN ALGAE (*SELENASTRUM CAPRICORNUTUM*)

The ability of NBOT 2.5-HP to cause mortality in *S. capricornutum* was examined in tests SC-LW and SC-LW-TMH as detailed in the *Test Methods* section. DO, pH, temperature, hardness, alkalinity, and conductivity parameters measured at 0 minutes were acceptable for test initiation based on the Test Plan (Schaefer et al., 2019). After test initiation, these parameters displayed the same general trends observed and discussed in the *Measurements During Treatment (Water-Only Testing)* section.

In LW-SC, *S. capricornutum* experienced 100% mortality after 30 minutes of treatment with NBOT 2.5-HP (Table 12). *S. capricornutum* concentrations in controls and PCW decreased negligibly (<1% mortality) over the same time period. The mortality observed in the treatment, but not the control or PCW, demonstrates the potential effectiveness of NBOT 2.5-HP to induce mortality in *S. capricornutum* in water quality with low challenge conditions.

In SC-LW-TMH, *S. capricornutum* experienced 0.8% mortality after 60 minutes of treatment and 100% mortality after 240 minutes (Table 12). Total numbers of algae in the 240-minute treated samples were lower than total numbers at the lower treatment durations. It was difficult at the final time point to see the organisms through the microscope, possibly due to ozone decolorizing organisms after their death. *S. capricornutum* mortality in the control at 240 minutes was 0% and in the PCW mortality at 240 minutes was 0.69 %, indicating the organisms were healthy. The mortality observed during treatment suggests that under challenging water quality conditions, the NBOT 2.5-HP requires a longer operating time to overcome these challenges and induce mortality in *S. capricornutum*. Notably, detectable ozone concentrations (Table 13) were only observed at the 240-minute measurement when complete mortality was also observed. The measurable presence of ozone may indicate the system's effectiveness and could reveal when NBOT 2.5-HP has overcome water quality challenges.

Table 12. Concentrations of *S. capricornutum* in LW and LW-TMH during Treatment in 1,000-L Tanks with NBOT 2.5-HP BWT.

SC-LW									
Treatment Duration (min)	Treatment			Control			PCW		
	Alive	Dead	% Mortality	Alive	Dead	% Mortality	Alive	Dead	% Mortality
0	172,857 (17,321)	952 (825)	0.58 (0.50)	198,095 (38,791)	476 (825)	0.23 (0.40)	182,381 (7,047)	0 (0)	0 (0)
30	0 (0)	315,000 (36,000)	100 (0)	188,095 (3,595)	952 (825)	0.50 (0.43)	200,952 (14,662)	0 (0)	0 (0)
SC-LW-TMH									
Treatment Duration (min)	Treatment			Control			PCW		
	Alive	Dead	% Mortality	Alive	Dead	% Mortality	Alive	Dead	% Mortality
0	310,000 (87,283)	0 (0)	0 (0)	259,524 (4,364)	476 (825)	0.19 (0.32)	251,905 (20,718)	952 (825)	0.36 (0.31)

60	370,476 (69,066)	2857 (1429)	0.80 (0.42)	251,429 (65,341)	0 (0)	0 (0)	NM	NM	NM
120	236,667 (70,455)	107,619 141,928	26.6 (31.4)	240,000 (42,594)	0 (0)	0 (0)	NM	NM	NM
240	0 (0)	39,524 (4,592)	100 (0)	296,190 (136,944)	0 (0)	0 (0)	274,762 (34,533)	1905 (1650)	0.69 (0.61)

NM= Not Measured

As detailed in the *Analytical Methods* section, water chemistry and water quality were measured on stock solutions of the water prior to initiation of testing (Table 11, Table 13). Water quality was additionally measured during the exposure period. The results of the water quality measurements taken during the tests with *S. capricornutum* are shown in Table 13. Water quality parameters measured at 0 minutes were acceptable for test initiation based on the Test Plan (Schaefer et al., 2019). After test initiation, water quality parameters displayed the same general trends observed and discussed in the *Measurements During Treatment (Water-Only)* section. Ozone concentrations in the LW treatment tank increased from <0.05 mg/L to 2.8 mg/L in the 30-minute treatment period. As observed in water only testing, ozone concentrations increased more slowly in the LW-TMH treatment tank, with samples measuring near or below the ozone detection limit until the 240-minute sample time when the ozone concentration was 1.49 mg/L. Ozone was below the detection level at all time points in the control and PCW samples. The dissolved oxygen concentrations in the treated LW increased to a maximum of 27.6 mg/L and in the LW-TMH increased to a maximum of 34.4 mg/L. During treatment, slight increases in temperature and slight decreases in pH are also observed. Conductivity, hardness, and alkalinity were not affected by the treatment process.

Table 13. Water Quality Results of Stock and Exposure Solutions Measured during Biological Effectiveness Tests with NBOT 2.5-HP Involving *S. capricornutum* in LW and LW-TMH at 25°C ± 3°C.

SC-LW								
Exposure	Treatment Duration (min.)	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)	Ozone (mg/L)	Hardness (mg/L $CaCO_3$)	Alkalinity (mg/L $CaCO_3$)
PCW (LW)	0	22.5	7.64	7.2	170.5	<0.05	56.7	60.5
	30	24.6	8.13	8.4	169.8	<0.05	59.9	59.9
Control	0	22.4	7.42	8.0	143.2	<0.05	54.1	53.1
	30	24.5	7.39	7.9	143.1	<0.05	52.3	53.9
Treatment	0	22.2	7.36	7.9	139.8	<0.05	49.1	50.2
	30	23.5	7.30	27.6	138.1	2.8	51.1	50.9
SC-LW-TMH								
Exposure	Treatment Duration (min.)	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)	Ozone (mg/L)	Hardness (mg/L $CaCO_3$)	Alkalinity (mg/L $CaCO_3$)
PCW (LW)	0	24.2	7.23	5.4	180.9	<0.05	55.3	54.9
	240	NM	NM	NM	NM	<0.05	52.7	50.9
Control	0	25.6	7.18	8.4	137.6	<0.05	47.7	48.6
	60	24.5	7.20	9.2	137.7	<0.05	NM	NM
	120	24.4	7.22	9.2	137.4	<0.05	NM	NM
	240	24.2	7.20	9.2	137.4	<0.05	47.9	51.1
Treatment	0	24.1	7.04	6.1	145.3	0.06*	50.9	52.3
	60	23.7	6.86	32.4	145.3	<0.05	NM	NM
	120	24.4	6.64	34.4	145.6	<0.05	NM	NM
	240	25.4	6.55	33.3	149.6	1.49	51.9	44.4

NM= Not Measured

*Likely a false detection. The background in LW-TMH is often variable, adding additional uncertainty to low concentration measurements.

3.3.3 BACTERIA (*ESCHERICHIA COLI* AND *ENTEROCOCCUS FAECIUM*)

The ability of NBOT 2.5-HP to induce mortality of *E. coli* and *E. faecium* was tested in trials M-LW and M-LW-TMH as detailed in the *Analytical Methods for Biological Effectiveness Testing* and *Biological Effectiveness Experiments* sections, results are shown below (Table 14). DO, pH, temperature, hardness, alkalinity, and conductivity parameters measured at 0 minutes were acceptable for test initiation based on the Test Plan (Schaefer et al., 2019). After test initiation, these parameters displayed the same general trends observed in water-only tests and discussed in section 3.2.2.

In the M-LW test, *E. coli and E. faecium* experienced complete mortality (<1 MPN/100 mL) after 30 minutes of treatment with NBOT 2.5-HP (Table 14). As expected, there was no significant increase or decrease in *E. coli and E. faecium* densities in M-LW control samples over the 120-minute testing period and live *E. coli* and *E. faecium* concentrations increased in the performance controls indicating test organisms were healthy. The mortality observed in the treatment, but not the control or PCW, demonstrates the effectiveness of NBOT 2.5-HP to induce mortality in *E. coli and E. faecium* in water quality with low challenge conditions.

In M-LW-TMH, *E. coli* and *E. faecium* both experienced approximately 91% mortality after 60 minutes of treatment and 91.95% and 99.98% mortality, respectively, after 120 minutes. Complete mortality (<1 MPN/100 mL) of *E. coli* and near complete mortality (99.99995%) of *E. faecium* occurred by 240 minutes. There were no live *E. coli* or *E. faecium* detected after 390 minutes of treatment (Table 14). *E. coli* densities increased over the 390-minute treatment period in M-LW-TMH as is often seen in the first 24 hours of bench scale tests using amended water. As expected, *E. faecium* densities experienced no significant increase or decrease in M-LW or M-LW-TMH control samples and live *E. coli* and *E. faecium* concentrations increased significantly in the performance controls indicating test organisms were healthy. In contrast to the *S. capricornutum* tests, some *E. coli and E. faecium* mortality was observed at 60 minutes while ozone was still below detection (Table 15). This suggests that unlike with *S. capricornutum*, NBOT 2.5-HP induces *E. coli* and *E. faecium* mortality even when ozone is not measurable in the solution.

Table 14. Average *E. coli and E. faecium* Densities (± Standard Error of the Mean (SEM) in LW and LW-TMH during Treatment of 1,000-L Tanks with NBOT 2.5-HP BWT.

M-LW						
Treatment Duration (min)	Treatment (n=5)		Control (n=2)		Performance Control (n=5)	
	E. coli MPN/100 mL	*E.* faecium MPN/100 mL	*E. coli* MPN/100 mL	*E.* faecium MPN/100 mL	*E. coli* MPN/100 mL	*E. faecium* MPN/100 mL
0	4.7E+06 (8.1E+05)	3.7E+06 (3.6E+05)	6.3E+06 (1.4E+06)	3.0E+06 (2.5E+05)	5.9E+06 (6.1E+05)	3.1E+06 (2.6E+05)
30	<1	<1	6.7E+06 (1.9E+05)	3.0E+06 (2.5E+05)	NM	NM
60	<1	<1	7.5E+06 (6.5E+05)	3.9E+06 (4.5E+05)	NM	NM

120	<1	<1	4.5E+06 (1.3E+05)	4.0E+06 (1.1E+06)	1.4E+07 (1.5E+06)	1.1E+07 (1.6E+06)
M-LW-TMH						
Treatment Duration (min)	Treatment (n=5)		Control (n=2)		Performance Control (n=5)	
	E. coli MPN/100 mL	*E. faecium* MPN/100 mL	*E. coli* MPN/100 mL	*E. faecium* MPN/100 mL	*E. coli* MPN/100 mL	*E.* faecium MPN/100 mL
0	6.2E+06 (7.0E+05)	2.3E+06 (6.6E+04)	4.7E+06 (1.4E+05)	2.3E+06 (3.1E+05)	7.9E+06 (4.3E+05)	3.5E+06 (4.2E+05)
60	5.6E+05 (4.8E+04)	2.1E+05 (3.8E+04)	5.8E+06 (0)	1.9E+06 (3.1E+04)	NM	NM
120	3.4E+03 (4.9E+02)	3.8E+02 (1.3E+02)	6.5E+06 (3.7E+05)	2.4E+06 (9.0E+03)	NM	NM
240	<1	1.1* (0.5)	9.2E+06 (0.0E+00)	3.1E+06 (5.9E+05)	NM	NM
390	<1	<1	1.4E+07 (1.7E+06)	2.9E+06 (3.9E+05)	6.6E+08 (9.2E+07)	8.0E+07 (1.1E+07)

*One or more values were below the limit of detection (LOD) so half of LOD (0.5) used to calculate the average and SEM.
NM=Not Measured

Water chemistry and water quality were measured on stock solutions of the water prior to initiation of testing with bacteria (Table 11, Table 15). Water quality was also measured during the exposure period. The results of water quality measurements made during the tests with *E. coli* and *E. faecium* are presented in Table 15. Water quality measurements in the stock solutions met the criteria defined in the test plan. Ozone concentrations increased rapidly in the LW treatment tank, with the initial sample being <0.05 mg/L and increasing to 2.43 mg/L by the 30-minute sample time. The ozone continued to increase throughout the treatment time, from 4.71 mg/L at 60 minutes to 5.72 mg/L at 120 minutes. Ozone levels in the LW-TMH treatment tank were below the detection limit from test initiation until 240 minutes of NBOT 2.5-HP operation when the ozone concentration was 1.60 mg/L. The ozone concentration in the LW-TMH treatment tank was 4.63 mg/L at 390 minutes. Ozone was below the detection level at all time points in the control and PCW samples. Dissolved oxygen concentrations increased in the treated LW to a maximum of 32.5 mg/L and in the treated LW-TMH to a maximum of 34.0 mg/L. Temperature increased slightly and pH decreased slightly during treatment in both the LW and LW-TMH tests. Conductivity, hardness, and alkalinity were not affected by the treatment process.

Table 15. Water Quality Results of Stock and Exposure Solutions Measured during Biological Effectiveness Tests with NBOT 2.5-HP in 1,000-L Tanks Involving *E. coli* and *E. faecium* in LW and LW-TMH at 25°C ± 3°C.

M-LW								
Exposure	Treatment Duration (min.)	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)	Ozone (mg/L)	Hardness (mg/L $CaCO_3$)	Alkalinity (mg/L $CaCO_3$)
PCW (TSB)	0	23.0	7.13	9.1	13220	NM	NM	NM
	120	25.0	7.11	7.2	13140	NM	NM	NM
PCW (BHB)	0	22.5	7.20	9.4	12650	NM	NM	NM
	120	25.1	7.16	5.8	12680	NM	NM	NM
Control	0	23.8	7.24	7.5	139.6	<0.05	53.9	48.6
	30	24.9	7.20	7.5	140.4	<0.05	NM	NM
	60	24.5	7.24	7.4	139.8	<0.05*	NM	NM
	120	24.5	7.25	7.7	140.1	<0.05	54.3	48.8
Treatment	0	23.3	7.23	7.0	136.0	<0.05	54.5	50.0
	30	23.9	7.13	28.4	137.4	2.43	NM	NM
	60	23.8	7.13	32.5	137.5	4.71*	NM	NM
	120	24.8	7.17	31.3	136.8	5.72	54.3	45.4
M-LW-TMH								
Exposure	Treatment Duration (min.)	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)	Ozone (mg/L)	Hardness (mg/L $CaCO_3$)	Alkalinity (mg/L $CaCO_3$)
PCW (TSB)	0	18.3	7.16	10.1	14120	NM	NM	NM
	390	25.2	7.09	4.3	13130	NM	NM	NM
PCW (BHB)	0	18.4	7.24	10.6	13730	NM	NM	NM
	390	25.2	7.16	8.0	12470	NM	NM	NM
Control	0	24.6	7.30	9.8	142.3	<0.05	50.7	50.2
	60	24.3	7.32	9.6	142.3	<0.05	NM	NM
	120	24.0	7.31	9.7	143.7	<0.05	NM	NM
	240	23.4	7.29	9.6	142.9	<0.05	NM	NM
	390	23.1	7.33	8.7	150.4	<0.05	52.7	46.8
Treatment	0	23.2	7.20	6.3	151.6	<0.05	59.3	52.9
	60	23.8	6.96	31.5	NM	<0.90	NM	NM
	120	24.0	6.79	34.0	152.6	<0.45	NM	NM
	240	24.6	6.66	33.5	152.7	1.60	NM	NM
	390	25.8	6.77	32.6	155.0	4.63	57.9	46.2

*Samples collected at 70 minutes due to a sample handing error with the initial samples.
NM= Not Measured

3.3.4 BIOLOGICAL EFFECTIVENESS: ZOOPLANKTON (*EUCYCLOPS SPP.*)

Table 16 reports the *Eucyclops spp*. average percent live, dead, and recovered (*n*=3, ten organisms per replicate) with standard deviations during LW and LW-TMH tests. For the zooplankton testing, although ten organisms were exposed in each replicate, ten organisms were not recovered in all cases. This was especially true in LW-TMH samples as the particles used to amend the water obscured the very small organisms. Average percent recovery is determined by dividing the number of organisms observed in the exposure vessel following the treatment time by the number of organisms exposed. Percent live and percent dead calculations were based on the number of organisms recovered from the exposure vessels.

Low percent mortality (Average % Dead) in PCW indicates the organisms used for testing were healthy. Low percent mortality in the control and high percent mortality in the treatment in both LW and LW-TMH indicate that NBOT 2.5-HP effectively induced mortality. Percent recovery in the control samples during the LW test ranged from an average of 93-97% and in the LW-TMH test ranged from an average of 80-83%. Percent recovery in the treatment samples was lower than in the control samples (LW recovery 67-83% and LW-TMH recovery 67-87%), however by the 60-minute exposure time points in the LW test and the 390-minute exposure time point in the LW-TMH test all the organisms that were recovered were dead. As indicated in Table 17, these exposure time points (i.e., 60 and 390 minutes for LW and LW-TMH, respectively) mark when ozone concentrations near 4.0 mg/L were measured in the treatment bottles.

Table 16. Average Percent Live, Dead, and Recovered (Standard Deviation) of *Eucyclops spp*. in LW and LW-TMH during Treatment in 1,000-L Tanks with NBOT 2.5-HP BWT.

EU-LW									
Treatment Duration (min)	Treatment			Control			PCW		
	Average % Live	Average % Dead	Average % Recovered	Average % Live	Average % Dead	Average % Recovered	Average % Live	Average % Dead	Average % Recovered
30	4 (7)	96 (7)	83 (6)	100 (0)	0 (0)	93 (6)			
60	0 (0)	100 (0)	67 (6)	100 (0)	0 (0)	97 (6)			
120	0 (0)	100 (0)	67 (6)	100 (0)	0 (0)	93 (6)	100 (0)	0 (0)	100 (0)
EU-LW-TMH									
Treatment Duration (min)	Treatment			Control			PCW		
	Average % Live	Average % Dead	Average % Recovered	Average % Live	Average % Dead	Average % Recovered	Average % Live	Average % Dead	Average % Recovered
60	100 (0)	0 (0)	87 (6)	100 (0)	0 (0)	80 (0)			
120	90 (10)	10 (10)	67 (21)	100 (0)	0 (0)	83 (6)			

240	54 (22)	46 (22)	73 (15)	100 (0)	0 (0)	80 (10)			
390	0 (0)	100 (0)	80 (20)	100 (0)	0 (0)	80 (10)	100 (0)	0 (0)	100 (0)

Water chemistry and water quality were measured on stock solutions of the water prior to initiation of testing with zooplankton (Table 11, Table 17). Water quality was also measured during the exposure period. The results of water quality measurements made during the test with *Eucyclops spp.* are presented in Table 17. Ozone concentration in the LW treatment tank increased throughout the treatment period from 3.24 mg/L at 30 minutes to a maximum of 5.62 mg/L ozone at 120 minutes. In the LW-TMH treatment tank ozone was first measurable at 240 minutes (2.12 mg/L) and peaked at 4.96 mg/L after 390 minutes of treatment. Throughout the treatment periods in both LW and LW-TMH, ozone was below the detection limit in control and PCW samples. Dissolved oxygen concentrations increased in the treated LW to a maximum of 32.1 mg/L and in the treated LW-TMH to a maximum of 33.0 mg/L. Temperature increased slightly and pH decreased slightly during treatment in both the LW and LW-TMH tests. Conductivity, hardness, and alkalinity were not affected by the treatment process.

Table 17. Water Quality Results of Stock and Exposure Solutions Measured during Biological Effectiveness Tests Involving *Eucyclops spp.* during Treatment in 1,000-L Tanks with NBOT 2.5-HP BWT in LW and LW-TMH at 25°C ± 3°C.

EU-LW								
Exposure	Treatment Duration (min.)	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)	Ozone (mg/L)	Hardness (mg/L $CaCO_3$)	Alkalinity (mg/L $CaCO_3$)
PCW (LW)	0	23.6	7.58	7.9	177.4	<0.05	52.1	54.3
	120	24.6	7.52	8.1	167.2	<0.05	52.5	52.9
Control	0	24.4	7.20	8.0	139.1	<0.05	48.9	51.3
	30	23.9	7.25	7.8	138.8	<0.05	NM	NM
	60	24.6	7.25	8.0	137.2	<0.05	NM	NM
	120	24.8	7.23	7.7	137.4	<0.05	49.7	50.0
Treatment	0	24.1	7.14	7.2	135.1	<0.05	48.9	49.6
	30	23.2	7.15	24.2	136.6	3.24	NM	NM
	60	24.6	7.10	31.3	134.7	4.67	NM	NM
	120	24.6	7.13	32.1	135.1	5.62	49.1	48.6
EU-LW-TMH								
Exposure	Treatment Duration (min.)	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)	Ozone (mg/L)	Hardness (mg/L $CaCO_3$)	Alkalinity (mg/L $CaCO_3$)
PCW (LW)	0	24.2	7.72	8.4	138.8	<0.05	49.5	52.9
	390	24.1	7.78	8.4	142.0	<0.05	46.7	50.9
Control	0	25.9	7.37	8.4	130.2	<0.05	49.5	52.5
	60	25.6	7.34	9.1	138.4	<0.05	NM	NM
	120	25.6	7.40	8.2	137.1	<0.05	NM	NM

	240	25.4	7.38	8.3	137.3	<0.05	NM	NM
	390	24.6	7.78	8.6	136.1	<0.05	50.3	54.5
	0	24.8	7.32	8.9	133.4	<0.05	44.7	52.5
Treatment	60	25.2	7.04	29.4	134.0	<0.05	NM	NM
	120	25.5	6.90	29.0	132.5	<0.05	NM	NM
	240	26.6	6.67	33.0	134.1	2.12	NM	NM
	390	26.5	6.76	29.8	133.7	4.96	50.3	44.0

NM= Not Measured

3.3.5 BIOLOGICAL EFFECTIVENESS: ZOOPLANKTON (*DAPHNIA MAGNA* NEONATE)

Table 18 displays the treatment effect of the NBOT 2.5-HP on the *Daphnia magna* neonates. Low percent mortality (Average % Dead) in PCW indicates the organisms used for testing were healthy. Low percent mortality in the control and high percent mortality in the treatment in both LW and LW-TMH indicate that NBOT 2.5-HP effectively induced mortality. Percent recovery in the control samples during the LW test ranged from an average of 80-83% and in the LW-TMH test averaged 80%. Percent recovery in the treatment samples was the same as in the control samples for the LW test (80-83%) but lower than in the control samples for the LW-TMH test (63-87%), however by the 60-minute exposure time point in the LW test and the 390-minute exposure time point in the LW-TMH test all the organisms that were recovered were dead. As indicated in Table 19, these exposure time points (i.e., 60 and 390 minutes in LW and LW-TMH, respectively) mark when ozone concentrations near 4.0 mg/L were measured in the treatment bottles.

Table 18. Average Percent Live, Dead, and Recovered (Standard Deviation) of *Daphnia magna* Neonates in LW and LW-TMH during Treatment in 1,000-L Tanks with NBOT 2.5-HP BWT.

DM-LW									
Treatment Duration (min)	Treatment			Control			PCW		
	Average % Live	Average % Dead	Average % Recovered	Average % Live	Average % Dead	Average % Recovered	Average % Live	Average % Dead	Average % Recovered
30	26 (27)	74 (27)	83 (12)	100 (0)	0 (0)	80 (10)			
60	0 (0)	100 (0)	83 (12)	100 (0)	0 (0)	83 (12)			
120	0 (0)	100 (0)	80 (0)	100 (0)	0 (0)	80 (0)	100 (0)	0 (0)	100 (0)
DM-LW-TMH									
Treatment Duration (min)	Treatment			Control			PCW		
	Average % Live	Average % Dead	Average % Recovered	Average % Live	Average % Dead	Average % Recovered	Average % Live	Average % Dead	Average % Recovered
60	100 (0)	0 (0)	87 (12)	100 (0)	0 (0)	80 (17)			

120	100 (0)	0 (0)	80 (17)	100 (0)	0 (0)	80 (10)			
240	19 (3)	81 (3)	70 (17)	100 (0)	0 (0)	80 (10)			
390	0 (0)	100 (0)	63 (15)	100 (0)	0 (0)	80 (10)	100 (0)	0 (0)	100 (0)

Water chemistry results measured prior to the initiation of the testing with *D. magna* neonates are shown in Table 11. The results of water quality measurements made during the test with *Daphnia magna* neonates are presented in Table 19. Ozone levels in LW during treatment of *D. magna* neonates increased from 2.38 mg/L at 30 minutes to 4.67 mg/L at 120 minutes. As in previous tests, ozone levels in the LW-TMH treatment tank increased more slowly than in the LW treatment tank. Ozone was first measurable in the LW-TMH treatment tank at 120 minutes (0.15 mg/L) and increased to a maximum of 4.02 mg/L after 390 minutes of treatment. Ozone was not detected in control or PCW samples in either LW or LW-TMH. Dissolved oxygen concentrations increased in the treated LW to a maximum of 30.9 mg/L and in the treated LW-TMH to a maximum of 28.9 mg/L. Temperature increased slightly during treatment in the LW-TMH tests. pH decreased slightly in both the LW and LW-TMH tests. Conductivity, hardness, and alkalinity were not affected by the treatment process.

Table 19. Water Quality Results of Stock and Exposure Solutions Measured during Biological Effectiveness Tests with NBOT 2.5-HP Involving *Daphnia magna* Neonates in LW and LW-TMH during Treatment in 1,000-L Tanks with NBOT 2.5-HP BWT at 25°C ± 3°C.

DM-LW								
Exposure	Treatment Duration (min.)	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)	Ozone (mg/L)	Hardness (mg/L $CaCO_3$)	Alkalinity (mg/L $CaCO_3$)
PCW (MHRW)	0	24.5	8.10	8.4	400	<0.05	119.6	51.9
	120	24.4	7.98	8.8	392	<0.05	123.6	52.7
Control	0	25.7	7.48	8.8	134.5	<0.05	51.5	52.7
	30	25.3	7.51	8.8	139.4	<0.05	NM	NM
	60	25.2	7.49	9.0	136.9	<0.05	NM	NM
	120	24.9	7.53	8.9	137.1	<0.05	50.1	52.5
Treatment	0	26.1	7.46	8.5	135.7	<0.05	48.9	50.9
	30	25.5	7.41	21.2	136.6	2.38	NM	NM
	60	25.6	7.37	28.6	136.0	3.81	NM	NM
	120	25.9	7.31	30.9	137.4	4.67	50.9	50.9
DM-LW-TMH								
Exposure	Treatment Duration (min.)	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)	Ozone (mg/L)	Hardness (mg/L $CaCO_3$)	Alkalinity (mg/L $CaCO_3$)
PCW (MHRW)	0	24.3	8.07	8.2	394	<0.05	120.2	52.5
	390	24.5	7.93	8.3	395	<0.05	121.8	52.1
Control	0	24.5	7.08	8.5	127.3	<0.05	43.9	48.8
	60	24.3	7.30	8.6	128.9	<0.05	NM	NM
	120	24.3	7.01	8.3	127.0	<0.05	NM	NM
	240	24.0	6.97	8.3	127.1	<0.05	NM	NM
	390	23.6	7.11	8.8	128.1	<0.05	45.5	48.0
Treatment	0	24.2	6.86	5.0	130.7	<0.05	43.5	46.4
	60	24.4	6.85	23.3	131.9	<0.05	NM	NM

	120	25.3	6.69	28.9	129.9	0.15	NM	NM
	240	25.8	6.52	28.3	127.8	0.81	NM	NM
	390	26.3	6.57	28.2	131.2	4.02	46.3	41.6

NM= Not Measured

3.3.6 BIOLOGICAL EFFECTIVENESS: ZOOPLANKTON (DAPHNIA MAGNA EPHIPPIA)

Hatch rate of *D. magna* ephippia treated by the NBOT 2.5-HP and then transferred to optimal hatching conditions for 72 hours are presented in Table 20. Hatch rates of the ephippia in PCW ranged from 27-36% across water types. Control hatch rates ranged from 23-32% in LW and from 28-31% in LW-TMH. The average hatch rate of ephippia exposed to the NBOT 2.5-HP system was very similar to the PCW and control average hatch rate and ranged from 27-30% in LW and 27-33% in LW-TMH. T-tests were run in Microsoft Excel and no significant differences (p<0.05) were found between any of the control and treatment hatch rates at each designated sampling time point. No significant differences (p<0.05) were found when comparing the hatch rate of the PCW samples at zero minutes and 120 minutes as well.

Table 20. Average Percent Recovered and Total Percent Hatch (Standard Deviation) of *Daphnia magna* Ephippia in LW and LW-TMH following Treatment in 1,000-L Tanks with NBOT 2.5-HP BWT.

EDM-LW						
Treatment Duration (min)	Treatment		Control		PCW	
	Average % Recovered	Total % Hatch	Average % Recovered	Total % Hatch	Average % Recovered	Total % Hatch
0					100 (0)	33 (11)
30	99 (3)	27 (9)	100 (0)	23 (10)		
60	99 (3)	30 (9)	100 (0)	30 (13)		
120	100 (0)	30 (15)	100 (0)	32 (9)	100 (0)	27 (12)
EDM-LW-TMH						
Treatment Duration (min)	Treatment		Control		PCW	
	Average. % Recovered	Total % Hatch	Average % Recovered	Total % Hatch	Average % Recovered	Total % Hatch
0					100 (0)	36 (15)
60	100 (0)	27 (9)	100 (0)	31 (12)		
120	100 (0)	31 (10)	100 (0)	28 (6)		
240	100 (0)	29 (10)	99 (3)	31 (13)		

390	97 (9)	33 (14)	100 (0)	31 (14)	100 (0)	35 (10)

Water chemistry results measured on stock solutions of the water prior to initiation of testing with ephippia are shown in Table 11. Water quality was also measured during the exposure period. The results of water quality measurements made during the test with *Daphnia magna* Ephippia are presented in Table 21. Ozone levels in the LW treatment tank increased from 3.58 mg/L at 30 minutes to a maximum concentration of 4.95 mg/L at 60 minutes. At the 120-minute treatment point in LW, the ozone level was 4.72 mg/L. In LW-TMH, ozone levels were 2.26 mg/L at 240 minutes and 4.87 mg/L at 390 minutes. Ozone was below the detection limit in all control and PCW samples in both LW and LW-TMH. Dissolved oxygen concentrations increased in the treated LW to a maximum of 34.6 mg/L and in the treated LW-TMH to a maximum of 34.0 mg/L. Temperature increased slightly and pH decreased slightly during treatment in both the LW and LW-TMH tests. Conductivity, hardness, and alkalinity were not affected by the treatment process.

Table 21. Water Quality Results of Stock and Exposure Solutions Measured during Biological Effectiveness Tests with NBOT 2.5-HP Treatment in 1,000-L Tanks Involving *Daphnia magna* Ephippia in LW and LW-TMH at 25°C ± 3°C.

EDM-LW								
Exposure	Treatment Duration (min.)	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)	Ozone (mg/L)	Hardness (mg/L $CaCO_3$)	Alkalinity (mg/L $CaCO_3$)
PCW (MHRW)	0	23.4	7.34	8.4	390	<0.05	120.6	53.3
	120	21.3	8.10	9.3	409	<0.05	128.2	49.2
Control	0	23.5	8.14	7.4	133.7	<0.05	49.9	52.5
	30	23.1	7.32	7.5	133.2	<0.05	NM	NM
	60	23.2	7.31	7.6	133.5	<0.05	NM	NM
	120	23.1	7.28	7.6	132.8	<0.05	51.1	51.7
Treatment	0	24.2	7.48	9.4	131.1	<0.05	47.1	50.0
	30	24.1	7.42	28.3	131.1	3.58	NM	NM
	60	24.4	7.42	33.2	130.6	4.95	NM	NM
	120	24.7	7.42	34.6	129.2	4.72	47.5	49.6
EDM-LW-TMH								
Exposure	Treatment Duration (min.)	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)	Ozone (mg/L)	Hardness (mg/L $CaCO_3$)	Alkalinity (mg/L $CaCO_3$)
PCW (MHRW)	0	23.4	8.15	8.5	394	<0.05	119.8	52.9
	390	24.7	8.04	8.6	402	<0.05	122.6	50.9
Control	0	22.8	7.47	8.7	135.8	<0.05	48.3	55.3
	60	23.5	7.46	8.7	136.7	<0.05	NM	NM
	120	23.4	7.44	8.6	133.9	<0.05	NM	NM
	240	22.9	7.47	8.7	136.0	<0.05	NM	NM
	390	23.1	7.45	9.6	137.2	<0.05	49.9	54.1

Treatment	0	24.4	7.35	7.3	137.3	<0.05	49.5	52.1
	60	24.5	7.05	31.5	130.8	<0.05	NM	NM
	120	24.9	6.85	32.0	135.2	<0.05	NM	NM
	240	25.7	6.71	32.6	133.6	2.26	NM	NM
	390	26.0	6.77	34.0	130.1	4.87	51.5	48.8

NM= Not Measured

3.4 CHRONIC RESIDUAL TOXICITY TEST

In order to determine whether water treated with NBOT 2.5-HP could cause toxicity to organisms in receiving water upon ballast water discharge, chronic residual toxicity testing was conducted. Water chemistry measurements taken in stock waters prior to initiating treatment of water for use in chronic residual toxicity testing is shown in Table 22. All parameters were within acceptable ranges shown in Table 1.

Table 22. Water Chemistry Parameters at the Initiation of the Chronic Residual Toxicity Test.

CRT-LW						
Exposure	TSS (mg/L)	Percent Transmittance Filtered/Unfiltered (%)	NPOC (mg/L)	DOC (mg/L)	POM (mg/L)	MM (mg/L)
PCW (CMHRW)	<1.25	99.2/99.2	<0.48	0.9	<1.25	<1.25
Control	<1.25	97.1/97.2	1.2	1.0	<1.25	<1.25
Treatment	<1.25	96.9/97.3	1.1	1.2	<1.25	<1.25

Water quality measurements taken during the 120-minute treatment period of LW for use in chronic residual toxicity tests are shown in Table 23. These results agree with measurements made during water only tests with LW.

Table 23. Water Quality Results during NBOT 2.5-HP system running for the Chronic Residual Toxicity Test.

CRT-LW								
Exposure	Treatment Duration (min.)	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)	Ozone (mg/L)	Hardness (mg/L $CaCO_3$)	Alkalinity (mg/L $CaCO_3$)
PCW (CMHRW)	0	NM	NM	NM	NM	<0.05	85.1	61.0
	120	NM	NM	NM	NM	<0.05	84.3	57.4
Control	0	25.3	7.36	10.6	135.5	<0.05	47.5	49.5
	30	25.6	7.41	10.2	129.5	NM	NM	NM
	60	25.7	7.38	10.5	129.1	<0.05	NM	NM
	120	25.1	7.38	10.3	129.3	<0.05	45.9	51.8
Treatment	0	24.7	7.19	8.4	128.6	<0.05	45.9	47.9

CRT-LW								
Exposure	Treatment Duration (min.)	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)	Ozone (mg/L)	Hardness (mg/L $CaCO_3$)	Alkalinity (mg/L $CaCO_3$)
	30	25.0	7.19	27.7	128.6	NM	NM	NM
	60	25.2	7.18	32.8	128.9	4.26	NM	NM
	120	25.1	7.22	33.2	128.9	5.29	43.9	50.2

NM= Not Measured

3.4.1 *S. CAPRICORNUTUM* CHRONIC RESIDUAL TOXICITY RESULTS

The *S. capricornutum* were held in incubator that had an average temperature of 25.5°C with a minimum of 25.3°C and a maximum of 25.9°C (n=7). Light intensity was measured in multiple locations in the incubator daily and had an overall average of 383-foot candles (265 ft. cd. minimum, 455 ft. cd. maximum). The water quality measurements taken on stock solutions on Day 0 of the *S. capricornutum* test are shown in Table 24.

Table 24. Water Quality Results from Day 0 Stock Solution Exposures used for the *S. capricornutum* Chronic Residual Toxicity Test.

Exposure	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)
PCW	24.0	8.17	8.6	396
LW Control	24.4	7.75	8.9	225
12.5 % Treated LW	24.3	7.82	8.7	226
25% Treated LW	24.3	7.85	8.9	222
50% Treated LW	24.3	7.88	9.2	217
75% Treated LW	24.2	7.92	9.6	214
100% Treated LW	24.0	7.95	10.3	232

Growth in all *S. capricornutum* exposures exceeded the minimum required growth of 1,000,000 cells/mL. The Coefficient of Variation (CV) in both controls were above the quality assurance parameter of ≤20%. No statistically significant differences were noted between the LW control and any of the treated LW concentrations for *S. capricornutum* growth (Table 25). The reference toxicant test done by LSRI on March 3, 2020 met both quality assurance parameters with a mean growth in the control of 3,404,000 cells/mL and a control CV of 10%.

Table 25. Average Concentrations of *S. capricornutum* upon Completion of the Chronic Residual Toxicity Test.

Exposure	*S. capricornutum* Concentration	
	Average Cells/mL	CV (%)
PCW	3,110,417	35
LW Control	3,921,250	33
12.5 % Treated LW	4,395,000	14
25% Treated LW	4,435,000	16
50% Treated LW	4,471,250	14
75% Treated LW	5,372,500	24
100% Treated LW	5,266,250	23

3.4.2 *C. DUBIA* AND *P. PROMELAS* CHRONIC RESIDUAL TOXICITY RESULTS

The incubator that *C. dubia* and *P. promelas* were held in during chronic residual tests had an average temperature of 24.9°C with a minimum of 24.2°C and a maximum of 25.6°C (n=16). Water quality measurements for stock solutions prepared daily are reported in Table 26.

Table 26. Average (min, max) Water Quality Results from Stock Solution Exposures used for the *C. dubia* and *P. promelas* Chronic Residual Toxicity Tests.

Exposure	Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)
PCW	24.3 (23.3, 24.8)	8.03 (7.71, 8.27)	8.5 (8.3, 8.8)	311 (307, 317)
LW Control	24.9 (24.0, 25.5)	7.67 (7.47, 7.81)	9.0 (8.6, 9.4)	131.5 (124.8, 139.4)
12.5 % Treated LW	24.7 (23.5, 25.4)	7.72 (7.63, 7.83)	9.0 (8.6, 9.3)	130.0 (127.9, 133.9)
25% Treated LW	24.7 (23.7, 25.4)	7.74 (7.62, 7.85)	8.9 (8.6, 9.3)	129.5 (128.2, 132.4)
50% Treated LW	24.6 (23.7, 25.3)	7.78 (7.72, 7.83)	9.0 (8.5, 9.5)	129.6 (128.5, 131.9)
75% Treated LW	24.6 (23.8, 25.4)	7.81 (7.71, 7.88)	9.2 (8.6, 9.8)	129.1 (128.6, 129.9)
100% Treated LW	24.8 (23.7, 25.4)	7.83 (7.79, 7.90)	9.5 (8.7, 10.7)	129.3 (128.4, 129.7)

Temperatures in each of the four corner exposure cups and one cup in the middle of the exposure trays were measured immediately after removing *C. dubia* and *P. promelas* exposure trays from the incubator daily. The average daily temperatures are reported in Table 27. The average daily temperatures were within the acceptable range of 25.0 ± 1.0°C for all but one day for both organisms. The average temperature over all the days for *C. dubia* was 24.4°C (23.2°C minimum, 25.1°C maximum). The average temperature over all the days for *P. promelas* was 24.2°C (23.2°C minimum, 24.5°C maximum). One

corner of both trays was often colder than the others upon removal from the incubator. The *P. promelas* test runs for seven days, so data is only reported for seven days.

Table 27. Average (min, max) Daily Tray Temperatures for *C. dubia* and *P. promelas* Trays in the Chronic Residual Toxicity Test.

Day	*C. dubia*	*P. promelas*
1	23.9 (23.2, 24.3)	24.2 (23.5, 24.7)
2	24.3 (24.2, 24.5)	23.8 (23.2, 24.2)
3	24.5 (24.3, 24.7)	24.2 (23.2, 24.6)
4	24.6 (24.3, 24.8)	24.3 (23.7, 24.6)
5	24.5 (24.2, 24.7)	24.2 (23.8, 24.6)
6	24.9 (24.6, 25.1)	24.4 (23.6, 24.8)
7	24.4 (24.2, 24.6)	24.3 (24.0, 24.7)
8	24.1 (23.4, 24.6)	NA

Post exposure solutions were measured within 15 minutes of completing transfers and averaged according to exposure type across all days (Table 28). In all cases, pH and DO values for *P. promelas* solutions were lower than comparable *C. dubia* solutions. This is likely due to *P. promelas* using up larger amounts of oxygen and respiring carbon dioxide, decreasing pH levels.

Table 28. Average (min, max) Water Quality Results from Post-Exposure Solutions used in the *C. dubia* and *P. promelas* Chronic Residual Toxicity Test.

Exposure	*C. dubia* (n=8)		*P. promelas* (n=7)	
	pH	DO (mg/L)	pH	DO (mg/L)
PCW	8.06 (7.83, 8.30)	8.7 (8.3, 9.2)	7.79 (7.60, 8.00)	8.1 (7.5, 8.5)
Control LW	8.14 (8.01, 8.49)	8.7 (8.3, 9.2)	7.69 (7.34, 7.90)	7.9 (7.4, 8.4)
12.5 % Treated LW	8.19 (8.05, 8.40)	8.7 (8.3, 9.1)	7.70 (7.37, 7.91)	7.9 (7.3, 8.3)
25% Treated LW	8.17 (8.06, 8.40)	8.6 (8.3, 9.0)	7.69 (7.36, 7.89)	7.8 (7.3, 8.4)
50% Treated LW	8.17 (8.05, 8.38)	8.6 (8.3, 9.0)	7.71 (7.39, 7.88)	7.9 (7.4, 8.4)

75% Treated LW	8.19 (8.08, 8.41)	8.6 (8.3, 9.0)	7.70 (7.37, 7.89)	7.8 (7.2, 8.3)
100% Treated LW	8.16 (8.04, 8.42)	8.6 (8.3, 9.0)	7.69 (7.36, 7.91)	7.7 (7.1, 8.2)

Survival of *C. dubia* adults and average number of young produced at each exposure concentration during chronic residual testing is reported in Table 29. No statistically significant differences (p<0.05) were noted between the LW control and any of the treated LW concentrations for adult *C. dubia* survival or number of young produced per adult, although, average number of young in the 100% treated LW was the lowest of any of the exposure concentrations. At the completion of the test, those adults that had not produced young were examined and no males were observed. The *C. dubia* portion of the CRT test did not pass LSRI's quality control parameters listed in AT/44 – *Conducting a Chronic Whole Effluent Toxicity Test with* Ceriodaphnia dubia (LSRI, 2017d). Specifically, it did not pass due to one control failing to meet the necessary criteria: ≥ 80% adult survival, ≥ 15 young per adult, ≥ 80% of adults with a third brood, and ≤ 40% CV for reproduction. Neither the LW Control nor the PCW control met the reproduction CV requirement. The LW control met the requirement for survivorship and number of broods, but did not produce enough young per adult. The PCW control did not meet the survivorship requirement, but had ≥ 15 young per adult. The Reference Toxicant Test conducted by LSRI on March 3, 2020 passed all quality control requirements. The 0% control had 100% adult survival, 16 young produced per adult on average, 80% had three broods, and the CV for young production was 33.9%.

Table 29. Average Percent Survival and Number of Young per Adult *C. dubia* upon Completion of the Chronic Residual Toxicity Test.

Exposure	*C. dubia* Adult Survival		Number of Young per Adult	
	Average %	CV	Average	CV
PCW	70	69	16.3	57.6
LW Control	100	0	13.4	70.3
12.5 % Treated LW	90	35.1	12.1	80.2
25% Treated LW	100	0	14.7	62.5
50% Treated LW	100	0	11.3	71.3
75% Treated LW	100	0	14.2	63.1
100% Treated LW	90	35.1	9.8	88.1

Table 30 displays survival and growth of *P. promelas* exposed for seven days to a dilution series created using water treated with the NBOT-2.5HP system. No statistically significant differences (p<0.05) were noted between the LW control and any of the treated LW concentrations for both *P. promelas* survival or growth. The LW Control met all LSRI quality assurance parameters listed in the SOP: one control must have ≥ 80% survival, ≤ 40% CV for growth, and ≥ 0.250 mg average biomass. The PCW control growth was slightly below the required average biomass. The Reference Toxicant Test conducted by LSRI staff on March 3, 2020 did not meet the survival requirements for the controls. Due to time limits, LSRI staff requested reference toxicant test data from the laboratory the *P. promelas* were acquired from to ensure the *P. promelas* used for testing were healthy. The reference toxicant test data received from

Environmental Counseling and Testing, from a test conducted the week of March 13, 2020, passed LSRI's quality assurance parameters.

Table 30. Average Percent Survival and Growth by Weight of *P. promelas* upon Completion of the Chronic Residual Toxicity Test.

Exposure	*P. promelas* Survival		Growth by Weight	
	Average %	CV	Average per fish (mg)	CV
PCW	100	0	0.24	5.42
LW Control	100	0	0.26	0.14
12.5 % Treated LW	100	0	0.25	0.14
25% Treated LW	100	0	0.25	0.04
50% Treated LW	70	59	0.23	0.56
75% Treated LW	90	15	0.27	0.14
100% Treated LW	95	12	0.26	0.14

4 QUALITY ASSURANCE/QUALITY CONTROL – DATA QUALITY OBJECTIVES

4.1 WATER CHEMISTRY AND WATER QUALITY

The data quality objectives (DQO) for water quality and chemistry analyses conducted during the evaluation of the NBOT 2.5-HP are summarized in Table 31. Quality objectives are established by the TQAP (Schaefer et al., 2019), analyte specific SOPs, and LSRI's Quality Management Plan (LSRI, 2018d). Quality control requirements are specified in each SOP outlined in section 2.6 and those requirements are used to determine whether the DQOs for the overall evaluation were met. Duplicate samples were analyzed on 23.9% of the samples analyzed for TSS, POM, NPOC, DOC, and %T. Average Relative Percent Difference of duplicates for those parameters was less than 20%, therefore meeting the DQO for TSS, POM, NPOC, DOC, and %T. Ozone and ORP were measured in duplicate on 15.5% and 17.3% of the samples, respectively. Hardness and alkalinity were measured in duplicate on 15.7% and 16.7% of the samples, respectively. The DQOs were met on ozone, ORP, hardness and alkalinity measurements. The DQOs were also met on method blanks analyzed for TSS/POM, NPOC, DOC, and %T. To measure accuracy, NPOC/DOC spikes were prepared on the stock solutions for 22.5% of the samples and reference standards were analyzed for NPOC, DOC, TSS, POM, ORP, hardness, and alkalinity. The spike recovery for NPOC/DOC and the percent difference between the nominal and measured concentrations met the DQO for accuracy. One hundred percent completeness was achieved for analysis of TSS, %T, NPOC/DOC, hardness, and alkalinity.

Table 31. Data Quality Objectives (DQOs), Criteria, and Performance Measurement Results from Water Chemistry and Water Quality Analyses Conducted during the NBOT 2.5-HP Testing.

Data Quality Indicator	Evaluation Process/Performance Measurement	Data Quality Objective	Performance Measurement Result	
Precision	Samples (10%) were collected and analyzed in duplicate with performance measured by average relative percent difference (RPD).	< 20% average RPD	Percentage of Samples Collected and Analyzed in Duplicate:	Duplicate Average Relative Percent Difference
			%TF: 23.9%	%TF: 0.3 ± 0.3%
			%TU: 23.9%	%TU: 0.4 ± 0.4%
			NPOC: 23.9%	%NPOC: 10.9 ± 9.1%
			DOC: 23.9%	%DOC: 9.4 ± 7.8%
			POM: 23.9%	POM: 2.6 ± 2.0%
			TSS: 23.9%	TSS: 2.7 ± 1.7%
			Hardness: 15.7%	Hardness: 3.0 ± 3.3%
			Alkalinity: 16.7%	Alkalinity: 2.9 ± 2.6%
			Ozone: 15.5%	Ozone: 5.7 ± 5.6%
			ORP: 17.3%	ORP: 2.2 ± 2.1%

Data Quality Indicator	Evaluation Process/Performance Measurement	Data Quality Objective	Performance Measurement Result	
Bias, Method Blanks	%T method blanks were prepared by filtering deionized water samples from sample bottles (one per analysis date).	> 98% average %T	Number of %T Method Blanks Analyzed: 16	Method blanks (%T): 99.7 ± 0.3%
	TSS/POM method blanks were prepared by filtering deionized water samples from sample bottles (one per analysis date) and then drying, weighing, ashing and weighing the filter again.	< 0.63 mg/L average TSS/POM	Number of TSS Method Blanks Analyzed: 16	Method Blanks (TSS): <0.63 ± 0
			Number of POM Method Blanks Analyzed: 16	Method Blanks (POM): <0.63 ± 0
	NPOC blanks were prepared by acidifying a volume of deionized water to 0.2% with concentrated hydrochloric acid.	< 0.70 mg/L average NPOC	Number of NPOC Blanks Analyzed: 58	Blanks (NPOC): <0.70 ± 0
	DOC method blanks were prepared by filtering deionized water samples from sample bottles (one per analysis date).	< 0.70 mg/L average DOC	Number of DOC Method Blanks Analyzed: 16	Method Blanks (DOC): <0.70 ± 0
Accuracy	Samples (10%) were spiked with a total organic carbon spiking solution with performance measured by average spike-recovery (SPR).	75% - 125% average SPR	Percentage of NPOC/DOC Samples Spiked: 22.5%	NPOC/DOC Spike Recovery: 101.1 ± 5.6
	Performance was measured by average percent difference (%D) between all measured and nominal reference standard values.	< 20% average D	Percentage of Analysis Days Containing a Reference Standard:	Reference Standard Percent Difference
			TSS: 100%	TSS: 1.7 ± 1.3%
			POM: 100%	POM: 3.2 ± 4.7%
			NPOC: 106%	NPOC: 6.2 ± 1.8%
			ORP: 100%*	ORP: 13.7 ± 4.8%
	A hardness/alkalinity reference standard was analyzed once per bench-scale test type per analyst. Performance was measured by ensuring the	Within acceptance range (lot dependent)	Percentage of Analysis Days Containing a Reference Standard: 94%	Hardness: DQO met 100% of the time

Data Quality Indicator	Evaluation Process/Performance Measurement	Data Quality Objective	Performance Measurement Result	
	titrated value was within the acceptance range for the standard.		Test types: 3	Alkalinity: DQO met 100% of the time
Represent-ativeness	All samples were collected, handled, and analyzed in the same manner.	Not Applicable – Qualitative.	All water chemistry/quality samples were collected, handled, transported and analyzed in the same manner using the appropriate SOPs.	
Comparability	Routine procedures were conducted according to appropriate SOPs to ensure consistency between tests.	Not Applicable – Qualitative.	The SOPs listed in the methods and references section were used for all water chemistry and water quality analyses.	
Completeness	Percentage of valid (i.e., collected, handled, analyzed correctly and meeting DQOs) water chemistry samples measured out of the total number of water chemistry samples collected. Performance is measured by percent completeness (%C).	> 90% C	TSS: 100%	
			%T, Filtered: 100%	
			%T, Unfiltered: 100%	
			NPOC: 100%	
			DOC: 100%	
			Hardness: 100%	
			Alkalinity: 100%	
Sensitivity	The limit of detection (LOD) and limit of quantitation (LOQ) for each analyte and analytical method utilized was determined annually unless a reporting limit was used based on the amount filtered as was the case with TSS/POM.	Not Applicable	TSS/POM RL: 1.25 mg/L based on filtering 800 mL of sample	
			NPOC/DOC LOD: 0.70 mg/L	
			NPOC/DOC LOQ: 2.3 mg/L	
			Determined 7 February 2019	

*ORP analysis was only conducted during the water-only and LW biological effectiveness tests. After that, the probe was not working properly, and the developer authorized the elimination of ORP measurements beginning with the M-LW-TMH test.

4.2 ALGAE TESTING

During *S. capricornutum* testing, data quality was measured by analyzing a minimum of 10% of samples in duplicate and by having a second individual conduct quality assurance counts on a minimum of 10% of samples. The precision of the individual counting the *S. capricornutum* cells was checked via the duplicate counts. The operator bias of the analyst was checked via the quality assurance counts done by a second analyst. For all testing with S. capricornutum, the minimum number of duplicate and quality assurance samples were met or exceeded with the exception of the duplicate agreement of SC-LW-TMH which was 20.1% agreement, slightly above the acceptable limit (Table 32).

Table 32. Average Relative Percent Difference (RPD) ± Std. Dev. for *S. capricornutum* Counts Conducted during the NBOT 2.5-HP Bench-Scale Testing.

Test	Duplicate or Quality Assurance Count	Percent of Samples with QA counts	DQO	Relative Percent Difference (%)	
				Live	Dead
SC-LW	Duplicate	11%	RPD ≤ 20%, when greater than 10 cells of live/dead are counted	17.0 ± 11.4	0.0 ± 0.0
	Quality Assurance	11%		3.7 ± 3.1	0.0 ± 0.0
SC-LW-TMH	Duplicate	13%		20.1 ± 19.9	116.7 ± 100.0*
	Quality Assurance	10%		2.8 ± 1.9	22.2 ± 38.5*
CRT-LW	Duplicate	11%		13.2 ± 5.1	NA
	Quality Assurance	11%		1.2 ± 0.5	NA

* The RPD values for dead cells are >20% because only few dead cells were counted, the DQO acceptance limit does not apply.

4.3 BACTERIA TESTING

Data quality objectives for precision, bias, accuracy, and completeness were within acceptable limits for bacteria testing (Table 33). As a measure of precision, a minimum of 10% of reported *E. coli* and *E. faecium* samples were analyzed in duplicate with and all duplicate analyses had a range of logarithms (R_{log}) well within the acceptable calculated precision criteria (PC) with an average R_{log} of 0.0939 for *E. coli* (PC= 0.4043) and 0.1222 for *E. faecium (*PC = 0.5597). All media blanks and method blanks run coincident with LW and LW-TMH bacteria tests were negative and 15% of the *E. coli* and *E. faecium* samples analyzed were counted by a second qualified analyst and all QA counts for NOBT 2.5-HP bacteria tests matched the initial analysts counts. Quality control kits purchased from IDEXX and analyzed with each bacteria test were within the acceptable values provided by the manufacturer indicating that selective media was working properly and that accurate *E. coli* and *E. faecium* values were achieved during testing.

Table 33. Data Quality Objective Summary for the NBOT 2.5-HP Bench-Scale Tests using *E. coli* and *E. faecium*.

Data Quality Indicator	Evaluation Process/ Performance Measurement	Data Quality Objective	Performance Measurement Result
Precision	Samples (10%) are analyzed in duplicate – with performance measured R_{log} not greater than precision criterion (PC)	R_{log} not greater than 0.4043 for *E. coli and* not greater than 0.5597 for *E. faecium*	***E. coli:*** 9 of 84 of (11%) reported samples were analyzed in duplicate; Average R_{log} = 0.0939
			E. faecium: 8 of 84 of (10%) reported samples were analyzed in duplicate; Average R_{log} = 0.1222
Bias, Operator	Samples (10%) are counted by two separate analysts with performance measured by	<20% average RPD	***E. coli***: 18 of 118 (15%) samples analyzed counted by 2nd analyst; Average RPD=0%

Data Quality Indicator	Evaluation Process/ Performance Measurement	Data Quality Objective	Performance Measurement Result
	average relative percent difference (RPD) of all second counts.		***E. faecium:*** 18 of 117 (15%) analyzed samples counted by 2nd analyst; Average RPD=0%
Bias, Positive Control	Qualitative positive control samples (American Type Culture Collection) are analyzed on each analysis date or IDEXX-QC samples are analyzed as a quantitative positive control at least once per ballast water treatment system test.	Results must be greater than the limit of detection.	***E. coli:*** Qualitative Positive controls >1 MPN/100 mL n=3
			E. faecium: Qualitative Positive controls >1 MPN/100 mL n=2
Bias, Negative Control	Qualitative negative control samples (American Type Culture Collection) are analyzed on each analysis date or IDEXX-QC samples are analyzed as a negative control at least once per ballast water treatment system test.	Results must be less than the limit of detection.	***E. coli:*** Qualitative Negative controls <1 MPN/100 mL, n=2
			E. faecium: Qualitative Negative controls <1 MPN/100 mL, n=3
Bias, Method	Sterilized water (similar matrix sample) analyzed using same method as samples on each analysis date.	Results must be less than the limit of detection.	***E. coli:*** All method blanks <1 MPN/100 mL, n=12
			E. faecium: All method blanks <1 MPN/100 mL, n=12
Bias, Diluent Blank	One per analysis day, diluent (e.g., sterile deionized water) blank run analyzed using same media as samples	Results must be less than the limit of detection.	***E. coli:*** Blank <1 MPN/100 mL, n=2
			E. faecium: Blank <1 MPN/100 mL, n=2
Accuracy	IDEXX-QC samples are analyzed as a quantitative positive control at least once per ballast water treatment system test.	***E. coli:*** 19-461 MPN/100 mL	***E. coli:*** All quantitative analyses within IDEXX acceptance ranges (n=3)
			23 Jan. 2020; 201.4 MPN/100 mL 30 Jan. 2020; 156.5 MPN/100 mL
		E. coli: 319-1141 MPN/100 mL	30 Jan. 2020; 410.6 MPN/100 mL
		E. faecalis: 53-179 MPN/100 mL	***E. faecalis:*** All quantitative analyses within IDEXX acceptance ranges (n=2)
			23 Jan. 2020; 88.2 MPN/100 mL 30 Jan. 2020; 90.6 MPN/100 mL

Data Quality Indicator	Evaluation Process/ Performance Measurement	Data Quality Objective	Performance Measurement Result
Representativeness	All samples are collected, handled, and analyzed in the same manner.	Not Applicable – Qualitative.	All bacterial samples were collected, handled, and analyzed in the same manner (using the appropriate LSRI/GWRC SOPs).
Comparability	Routine procedures are conducted according to appropriate SOPs to ensure consistency between tests.	Not Applicable – Qualitative.	The LSRI/GWRC SOPs listed in section 2.7.1were used for all bacterial analyses conducted.
Completeness	Percentage of valid (i.e., collected, handled, analyzed correctly and meet DQOs) bacterial samples measured out of the total number of bacterial samples collected. Performance is measured by percent completeness (%C).	>90% C.	***E. coli:*** 84 of 84 samples = 100% Completeness ***E. faecium: 84 of 84*** samples = 100% Completeness
Sensitivity	The limit of detection (LOD) for the analytical method used is reported.	Dependent upon the analytical technique used. Adjusted for volume used.	***E. coli:*** **LOD:** <1 MPN/100 mL ***E. faecium:*** **LOD:** <1 MPN/100 mL

4.4 ZOOPLANKTON AND FATHEAD MINNOW TESTING

During all tests involving zooplankton (Biological Effectiveness or Chronic Residual Toxicity), data quality was measured by having a second individual conduct quality assurance counts on a minimum of 10% of samples. For all zooplankton testing, the minimum number of duplicate and quality assurance samples were met or exceeded (Table 34-Table 36).

Table 34. Data Quality Objective Summary for the NBOT 2.5-HP Bench-Scale Tests with *D. magna* neonates and *Eucyclops spp.*

Test	Percent of Samples with QA counts	DQO	Relative Percent Difference (%)	
			Live	Dead
EU-LW	75%	<20% RPD	0.0 ± 0.0	0.0 ± 0.0
DM-LW	70%		0.0 ± 0.0	0.0 ± 0.0
DM-LW-TMH	65%		0.0 ± 0.0	0.0 ± 0.0
EU-LW-TMH	65%		0.0 ± 0.0	0.0 ± 0.0

Table 35. Data Quality Objective Summary for the NBOT 2.5-HP Bench-Scale Tests with *D. magna* Ephippia.

Test	Ephippia Present or Young Hatched QA Count	Percent of Samples with QA counts	DQO	Relative Percent Difference (%)
EDM-LW-TMH	Ephippia Present	56%	<20% RPD	0.0 ± 0.0
	Young Hatched	10%		0.0 ± 0.0
EDM-LW	Ephippia Present	56%		0.0 ± 0.0
	Young Hatched	10%		0.0 ± 0.0

Table 36. Data Quality Objective Summary for the NBOT 2.5-HP Chronic Residual Toxicity Test with *C. dubia* and *P. promelas*.

Chronic Residual Toxicity Test Organism	Adult Survival or Young Produced	Percent of Samples with QA counts	DQO	Relative Percent Difference (%)
C. dubia	Adult Survival	28%	<20% RPD	0.0 ± 0.0
	Young Produced	19%		0.0 ± 0.0
P. promelas	NA	31%		0.0 ± 0.0

5 CONCLUSIONS

The LSRI-GWRC evaluation of the in-tank NBOT 2.5-HP met the stated objectives, as outlined in the Test Plan (Schaefer et al., 2019). The reported deviations and quality control failures do not impact LSRI-GWRC's ability to draw conclusions on the performance of NBOT 2.5-HP BWT during testing. The system was fully operational during all reported tests and was operated in accordance with the developer's instructions.

Objective 1: Determination of the dissolved oxygen and ozone concentrations in simulated Great Lakes ballast water over time.

The data generated during this evaluation supports the NBOT 2.5-HP BWT's mechanism of action as stated by the developer. In LW and LW-TMH, the NBOT 2.5-HP BWT increased dissolved oxygen and ozone concentrations, reaching an equilibrium state for dissolved ozone in LW after 135 minutes of treatment at both ~25°C and ~15°C. The maximum ozone concentration achieved was 5.29 mg/L in LW-25 and 6.19 in LW-15. In LW-TMH, no increase in ozone was observed in the first 135 minutes. However, ozone concentrations in LW-TMH increased after longer treatment times revealing NBOT 2.5-HP is capable of overcoming challenge conditions with appropriate treatment length. The maximum ozone concentration achieved was 5.54 mg/L in LW-TMH-25 (2) and 5.07 in LW-TMH-15, slightly lower, but similar to those observed in LW.

DO was observed to increase in all trials, even when ozone was not detected, which indicates that the technology was effectively introducing oxygenated nanobubbles. The absence of an increase in ozone while DO was increasing, in the first two hours of LW-TMH tests, suggests that the presence of ozone-reactive species (i.e., dissolved and particulate organic compounds) was a large enough challenge to overcome all ozone introduced by NBOT 2.5-HP.

A potential drawback of the elevated oxygen levels in ballast water treatment tanks is that it may increase the rate of corrosion within the ballast tanks. Generally, lower dissolved oxygen levels will decrease the rate of corrosion while elevated dissolved oxygen levels will increase corrosion rates (Lysogorski, D., et al., 2011). Great Lakes vessels are made with steel that lacks the coatings applied to the ballast water tanks of seagoing vessels to inhibit corrosion (Malewitz, J., 2019). The bare metal within the ballast tanks would be exposed to a high-oxygen environment, which could increase the rate of corrosion within the tanks.

Objective 2: Determination of the degradation rate of dissolved oxygen and ozone following treatment.

The speed at which the degradation of the dissolved oxygen concentration, after NBOT 2.5-HP treatment, occurred was determined in LW and LW-TMH. In LW and LW-TMH (2) at 25°C, DO average concentrations at 48 hours post-treatment remained at 15.6 mg/L and 14.5 mg/L, respectively. In LW and LW-TMH at 15°C, DO concentrations at 48 hours post-treatment degraded at a slower rate, remaining at average concentrations of 20.3 mg/L and 22.0 mg/L, respectively. These results show that a complete degradation of the dissolved oxygen concentration (proxy for superoxide) following treatment would take more than 48 hours.

The degradation rate of ozone concentrations, after NBOT 2.5-HP treatment, was determined in LW and LW-TMH. In LW and LW-TMH (2) at 25°C ozone concentrations remained at 0.27 mg/L in LW and 0.19 mg/L in LW-TMH after 240 minutes post-treatment. In LW and LW-TMH at 15°C ozone concentrations were higher after 240 minutes post-treatment due to greater concentrations achieved during treatment. Ozone concentrations in LW and LW-TMH at 15°C after 240 minutes post-treatment remained at 1.06 mg/L in LW and 0.67 mg/L in LW-TMH. At 24 hours post-treatment ozone concentrations, in both water types at both temperatures, were below the detection limit, indicating that complete degradation of ozone following treatment would occur within 24 hours.

Objective 3: Determination of the biological effectiveness of the NBOT 2.5-HP system in freshwater at Great Lakes relative challenge conditions.

The effectiveness tests with green algae, *S. capricornutum*, in LW and LW-TMH at 25°C showed that the NBOT 2.5-HP is effective when operated for 30 minutes in LW and when operated for 240 minutes in LW-TMH, with complete mortality in the treatment tank (0% survival) immediately following treatment.

The results of the antibacterial effectiveness of the NBOT 2.5-HP on two species of pathogen indicator organisms showed that in LW treatment for 30 minutes was successful in killing *E. coli* and *E. faecium,* and in LW-TMH, treatment for 240 minutes was successful in killing *E. coli* and *E. faecium.* The complete mortality induced during these tests, indicate that this technology has the ability to reduce the number

of pathogen indicator bacteria from as many as 6.2E+06 *E. coli and* 3.7E+06 *E. faecium* per 100 mL to densities well below the Ballast Water Discharge Standard of <250 *E. coli* (CFU/100 mL) and <100 *E. faecium* (CFU/100 mL) when treating LW or LW-TMH for 30 or 240 minutes, respectively.

The biological effectiveness tests with the zooplankton, *Eucyclops spp*., in LW and LW-TMH indicated that in LW treatment for 60 minutes was successful in producing 100% mortality in the 67% of organisms that were recovered. In LW-TMH treatment for 390 minutes was successful in producing 100% mortality in the 80% of organisms recovered.

The biological effectiveness tests with zooplankton, *Daphnia magna* neonates, in LW and LW-TMH indicated that in LW treatment for 60 minutes was successful in producing 100% mortality in the 83% of organisms that were recovered. In LW-TMH treatment for 390 minutes was successful in producing 100% mortality in the 63% of organisms recovered.

The biological effectiveness tests with zooplankton, *Daphnia magna* ephippia, in LW and LW-TMH indicated that treatment with NBOT 2.5-HP and then transferring to optimal hatching conditions had no effect on the hatch rate as the hatch rates among PCW, control, and treatment samples were very similar and had no statistically significant differences.

The overall conclusion from the biological effectiveness testing with NBOT-2.5HP is that the system is highly effective at controlling concentrations of algae, bacteria and motile zooplankton in both low and high water quality challenge conditions in time frames less than Great Lakes trade voyages. These promising results in varying challenge conditions at the bench scale, provide support for further research into determining the effectiveness of this technology as an in-tank treatment system at larger scales (e.g., land-based) or for the potential treatment of Great Lakes ballast water on board Great Lakes vessels. While the NBOT 2.5-HP showed no significant effectiveness at treating *D. magna* ephippia, treatment of resting stages of zooplankton has been a historic challenge for BWMS (Raikow et al., 2007a and 2007b; Branstrator et al., 2013), including treatment technologies tested as part of GWRC's bench-scale technology testing program.

Objective 4: Determination of the chronic residual toxicity of NBOT 2.5-HP treated water to non-target organisms in receiving water.

The results from the CRT test using off-gassed treated water showed that in LW there was no statistically significant effects on any of the three organism types tested.

When relating the results from this CRT to the potential application of Nano Bubble Ozone Technology as a ballast water treatment for U.S. and Canadian Laker vessels, the hold time on the voyage would have to exceed the time for treatment of the ballast water and off-gassing to occur to the point where ozone levels are below the detection limit before the treated water could be discharged into the receiving water body. This time requirement will depend on water chemistry/quality as treatment takes a longer period in higher challenge water conditions but degradation of ozone also happens more quickly in higher water challenge conditions. If proper off-gassing occurred, the results from this

laboratory-based study suggest that discharged water would contain no residual toxicity from the treatment process.

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