INV11 Final Report

Project:

Innovative Technologies: Nano Bubble Ozone Technology (NBOT) Treatment Project in Jones Creek, Jupiter, FL.

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Final Submission:
April 12th, 2022

Reviewed by Florida Department of Environmental Protection (FDEP):
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Executive Summary

Nanobubble Ozone Technology (NBOT) has been proven as an effective bacteria and algaecide. Ozone nanobubble treatments have demonstrated water quality improvements in small scale experiments, and in larger-scale trials in freshwater lakes and canals. Applying this emerging technology to tidal water mitigation (i.e., Jones Creek trial) was a logical next step.

During the Jones Creek trial, NBOT was applied in recirculating tanks filled with Jones Creek water where NBOT effectively reduced enterococci bacteria and visibly improved water clarity under controlled conditions (study 1). The efficacy of NBOT in this tank study (study 1), as well as observations from previous NBOT trials, suggests that NBOT can effectively improve water quality. However, when NBOT was applied *in-situ* in Jones Creek we were unable to measure significant declines in enterococci bacteria concentrations and did not observe significant water quality improvements when compared to the reference creek or historical conditions (study 2). The lack of measurable treatment effects in Jones Creek (study 2) suggests: (1) we underestimated the number and/or scale of NBOT technology needed to address a significant source of bacteria, (2) the high organic load in Jones Creek required a much larger oxidative capacity of the ozone dose, (3) flushing of the open, tidal system diluted the effect of the NBOT, or (4) a combination of the three. Potential sources of bacteria include sewage, stormwater, and extensive muck/organic sediment accumulations. Previous molecular and tracer studies in Jones Creek revealed a small but persistent presence of human genetic markers, indicative of human waste (Arrington et al. 2021). This, alongside the extensive sampling of each stormwater outfall, catch basin, and other water source inputs throughout Jones Creek over the past 10 years has not identified a point or non-point source of high bacteria concentrations. Based on this, we hypothesize that the high enterococci bacteria concentrations in the water are due to accumulations of excessive organic matter and 'muck' being continuously resuspended in this shallow-water tidal system.

Our data shows a need to conduct additional, appropriately scaled NBOT studies to evaluate the capacity for treatment under various sediment and organic matter scenarios, with additional *in-situ* field studies to account for the effects of NBOT in a set area (e.g., known hydrodynamics, sediment resuspension, and organic content). For example, further field trials evaluating the effects of NBOT in different systems across environmental gradients (e.g., organic content, algal content, temperatures, salinities, etc.) would allow for a better understanding of how to effectively apply NBOT treatment under different environmental conditions.

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Introduction

Harmful Algal Blooms (HAB), Bacteria, Nutrients

Harmful Algal Blooms (HAB) describe the excessive growth of noxious algae which can include phytoplankton, cyanobacteria, or macroalgae. In natural environments bacteria co-exist with algae in a microbial matrix, comprised of planktonic algae, bacteria, and their carbohydrate exudates (see Ramanan et al. 2016). Bacterial-alga relationships are variable and can be directed by environmental conditions (e.g., Mayaki 2018). These micro-scale interactions can act as biological agents controlling HAB events (Sun et al 2018, Pal et al. 2020) and HAB species prevalence (e.g., Shin et al. 2018). Reductions in nitrogen and phosphorus have been suggested to minimize HAB conditions, with limiting nutrient inputs suggested as the first step to effectively mitigate HAB events (Anderson et al. 2002, Paerl et al. 2018). Similar to HABs, positive relationships between some microalga-bacteria and nutrients suggests that enhanced bacteria growth may accompany excess nutrients and algal growth in shallow estuarine waters (e.g., Kelly et al. 2020). The negative effects of HABs range from human health risks to fish die offs (see Heil and Muni-Morgan 2021) resulting in environmental, social, and economic concerns (Paerl et al. 2018). Based on these concerns, algal-bacteria relationships, interactions, and their drivers are a priority to coastal and estuarine management, in particular, for management of HAB conditions.

Estuarine Water Quality Monitoring

Long-term water quality and HAB monitoring programs have been useful in establishing location specific nutrient criteria and restoration targets in coastal and estuarine systems (e.g., Harding et al. 2019). Today many organizations monitor the larger estuarine systems across the United States to track long-term changes in estuarine water quality and attempt to tease out negative anthropogenic impacts. The historical information collected from these programs can then be used for larger programs such as Water Quality Assessment Reports which assess, identify, and address watershed 'impairments' of rivers, lakes, estuaries, and coastal waters under Sections 305(b) and 303(d) of the Clean Water Act. The Loxahatchee River District (LRD)'s long-term water quality monitoring program has been monitoring estuarine water quality in the Loxahatchee River estuary since the 1970's (https://loxahatcheeriver.org/river/river-keeper/). LRD's long-term water quality monitoring program has been through many changes over the years, adapting to new management concerns. For example, concern of high bacteria concentrations in recreational waterways has prompted additional work monitoring bacteria concentrations in the Loxahatchee River and estuary. In 2012 LRD, like many of the nation's other water quality monitoring programs, began to include enterococci bacterial testing into the existing estuarine water quality monitoring.

Impaired Waters in the Loxahatchee River Estuary

Directed by FDEP, regional entities have established a plan for addressing water quality impairments outlined in the Loxahatchee River Pollutant Reduction Plan (2020) as an adaptive estuarine management strategy aimed at minimizing and mitigating excess nutrients, chlorophyll, and bacteria in the Loxahatchee River estuary. Florida's Impaired Waters Rule provides a means of investigating water quality parameters that fall outside of an established criterion and/or local historical values of a given area (Chapter 62-303, Florida Administrative Code (F.A.C.)). Based on historical annual geometric means, in the mesohaline (5–18 psu) and oligohaline (0.5–5 psu) regions of the Loxahatchee River estuary, the numeric nutrient criteria standards approved by FDEP as annual geometric mean (AGM) are: 1.26 mg/L total nitrogen, 0.075 mg/L total phosphorus, 5.5 mg/L chlorophyll-a (subparagraph 62-302-532(1)(q)(4), F.A.C.; see NNC map) to not be exceeded more than once in a three year period. The objective being to maintain nutrient values below these levels to avoid HAB conditions.

There are different criteria used to evaluate enterococci bacteria concentrations. These criteria differ by agency, water type/designation (i.e., marine *verses* fresh), as well as by waterbody use or classification (i.e., recreational swimming *verses* harvestable shellfish). Enterococci bacteria are associated with the gut microbiome, abundant in feces of humans, mammals, and birds (Byappanahalli et al. 2012). Enterococci bacteria are used as an indicator of potential human pathogens in estuarine systems since these bacteria can survive in saline conditions (Byappanahalli et al. 2012). The criteria for enterococci bacteria concentrations were developed from epidemiological studies investigating the prevalence of gastrointestinal illness in swimmers by the US Environmental Protection Agency (EPA) in the 1970–80's (EPA Recreational Water Quality Review 2017). In Florida, the Department of Health (DOH) utilizes the Federal Beach Action Value (BAV), established by the EPA to advise recreational use/swimming along Florida's coastlines, where enterococci bacteria concentrations 71 or greater MPN/100mL based on the estimated illness rate of recreators (EPA National Beach Guidance and Required Performance Criteria for Grants, 2014) are considered 'poor', and a swimming advisory is issued (Florida Healthy Beaches Program). Outside of dedicated swimming beaches and the former Beachwatch Program, FDEP's water quality criterion for class III marine waters advises that MPN or MF counts shall not exceed a monthly geometric mean of 35 nor exceed the Ten Percent Threshold Value (TPTV) of 130 in 10% or more of the samples during any 30-day period (paragraph 62-302.530(6)(c), F.A.C.), and exceedances warrant further investigation. Recently, the recent World Health Organization (WHO) elaborated on the initial guidelines and assigned ranges of bacteria concentrations and the associated percent of gastrointestinal illness: 41–200 MPN/100mL as 1–5% gastrointestinal illness risk, 200–500 MPN/100mL as 5–10% gastrointestinal illness risk, and >500 MPN/100mL as >10% gastrointestinal illness risk (WHO Guidelines on Recreational Water Quality, 2021). These different criteria, or threshold values, help guide recreational use of waterways and help managers focus on areas of concern. Jones and Sims Creeks, located in the Southwest Fork of the Loxahatchee River estuary are included in FDEP watershed body identification (WIBID) 3226C, one of the areas of concern, listed as impaired for both chlorophyll (303(d) to be listed) and bacteria (Loxahatchee River Pollutant Reduction Plan, 2020).

Jones and Sims Creeks

Jones and Sims Creeks are both shallow water tidal creeks in the southwest fork of the Loxahatchee River estuary in Palm Beach County, southeast Florida (**Fig. 2**, top left). Both creeks wind through residential neighborhoods into the Loxahatchee River and experience mixed semidiurnal tidal inputs from Jupiter inlet, located approximately 5 km west/downstream of the creek mouths (**Fig. 2**, top right). Jones Creek is approximately 4 km in length winding with four segments branching off the main creek. Sims Creek is a smaller tidal creek (1 km length) approximately 200 meters directly west of the mouth of Jones Creek (**Fig. 2**, top right). Jones Creek would still be considered a small tidal creek with the upstream study area (south of Indiantown Road) at high tide totaling an area of approximately 46,670 m^2 and a total volume of 37,362 m^3. The estimated tidal prism, based on 2004/2005 survey, is 22,477 m^3, with approximately 60% of the creek volume exchanged during each tide cycle (see **Appendix L**). Based on these tidal volumes, the average volumetric flow rate in the upper portion of the creek over a given 6-hour tide cycle was calculated to be approximately 1.25 m^3 /sec (44 cfs). The 2004/2005 a dredging event in the upper portion of Jones Creek removed just under 10,000 m^3 of sediments and debris, which is roughly one quarter the calculated volume of water in Jones Creek during mean high tide (**Appendix L**). This material removed from the creek is equivalent to approximately 722 large dump trucks full of material. Jones Creek Residents report (pers. comm.) that the creek has returned to pre-dredge conditions because of shoreline erosion and the accumulation of organic sediments.

Urban, shallow tidal creeks can be more prone to water quality issues due to the increased anthropogenic pressure associated with heavy urbanization trends in these areas (Freeman et al. 2019). Jones and Sims Creeks in Jupiter FL are prime examples of two shallow water, urban tidal creeks with a history of water quality issues including elevated nutrients (both nitrogen and phosphorus), chlorophyll-a concentrations, and high bacteria concentrations (see https://loxahatcheeriver.org/jonescreek). These creeks are utilized by the residential community for a wide range of recreational activities such as boating, kayaking, paddle boarding, and fishing which is why the WIBID 3226C chlorophyll and bacteria impairments are such a concern. The residential community surrounding Jones Creek was converted from on-site sewage treatment and disposal systems (i.e., septic systems) to LRD's centralized sewer system in the 1980's–90's following a pollution transport study in 1995 (Lapointe and Krupa 1995). Today, all of the homes and businesses in the Jones Creek watershed are served by LRD's centralized sewer (i.e., there are no existing septic systems). Nonetheless, since 2011 when LRD began collecting enterococci bacteria samples 92% of enterococci bacteria samples collected from surface waters in Jones Creek have exceeded FL's Beach Action Value of 71 MPN/100mL. Between July 14, 2011 and July 13, 2021 LRD has collected 452 enterococci bacteria samples from Jones Creek with a mean value of 1,810 MPN/100mL and 85% of these samples exceeding FDEP's Ten Percent Threshold Value (TPTV) of 130 MPN/100mL (https://loxahatcheeriver.org/jonescreek).

To understand these high bacteria concentrations, LRD has been working with multiple agencies, including the FDEP and the Town of Jupiter (TOJ), to understand potential sources of poor water quality within the basin in an effort to guide water quality improvement projects. A recent Microbial Source Tracking (MST) study concluded that the lack of chemical tracers, combined with low levels of human genetic markers, indicative of human waste material, and high enterococci concentrations, indicated low volumes of human waste entering into the system; possibly from a small population, such as a single home, recreational vehicle, or homeless camp (Arrington et al. 2021).

Water Quality Improvement Technology

Ozone is a powerful oxidant and has been used successfully for decades in drinking water treatment. Ozone and its decay products that include hydroxy radicals and peroxides (both organic peroxides and hydrogen peroxide) represent the most powerful oxidants known, second only to Fluorine. These chemical agents are powerful at destroying activated organics and oxidize metals as well as acting as a most powerful micro-biocide against algae, bacteria, fungi and viruses. Ozone and its complements are indiscriminate oxidants. All activated chemicals are affected by ozone if they interact. At the same time, ozone represents a "green" biocide/chemical degradant, since there are no legacy residues left behind and though significant concentrations of disinfection byproducts may be produced (see Richardson et al. 2007). Micro (<100 µm size) and nano (<1 µm size) bubble treatment is a newer innovation that provides much higher surface area for gas exchange and much longer bubble lifetime. The combined ozone-nanobubble approach allows for ozone to persist for longer periods of time in the water column, diffuse more slowly, increase the production of hydroxyl radicals, and provide increased reaction times with potential contaminants without the use of legacy chemicals. Simply put, larger bubbles rise to surface and burst, but nanobubbles have been reported to take weeks to completely disappear in the water column. They collapse within the water column due to water pressure at negligible buoyancy coupled to chemical/physical factors such as pH, ionizing agents, temperature, sunlight and suspended particulate matter. The increased residence time of nanobubbles is thought to contribute to the bactericide efficacy of ozone nanobubbles (Seridou and Kalogerakis. 2021). To date ozone micro and nano bubbles have been successfully used as a disinfectant for drinking water, aquaculture, agriculture, and wastewater treatment (Khan et al. 2020, Seridou and Kalogerakis. 2021). When combined with ultrasound (to suspend particles), ozone nanobubbles have also been used to remediate organic pollutants in river sediments, where ozone oxidized contaminants with 92% treatment efficacy (Hewage et al. 2020). Each of these recent works concluded that large-scale, *in-situ* experimentation was needed to understand the efficacy and longevity of ozone nanobubble treatment. Specifically, Nanobubble Ozone Technology (NBOT) has been applied in large scale trials in freshwater aquatic systems at Port Mayaca Lock in Florida, and lakes in Ohio demonstrating substantial reductions in microcystins and nutrients with dramatic visible improvements in water quality (https://greenwatersolutions.org/team/, https://nbotsystems.com/studies-results/).

Project Monitoring Objectives

FDEP's Innovative Technology grant program provides local governmental entities financial support to evaluate and implement innovative technologies and short-term solutions to combat HABs and nutrient enrichment, restore and preserve Florida waterbodies, and implement water quality treatment technologies. The objective of this project, *INV11*, was to evaluate and implement NBOT as a solution to reduce bacteria concentrations, combat algal blooms, and reduce nutrient enrichment (further described in the grant documents provided in **Appendix A and B**). The project plan was to evaluate NBOT driven water quality improvements in Jones Creek, FL during a 60-day consecutive treatment using ozone nanobubbles. LRD used an adaptive monitoring plan that began with direct treatment comparisons to monitor water quality parameters before, during and after NBOT treatment. The initial proposed monitoring plan (further described in the grant's quality assurance project plan under **Appendix C and D**) was expanded to include supplementary studies of NBOT using a recirculation tank and additional sampling of NBOT diffuser heads. Collectively, this work resulted in three studies comparing: (1) NBOT treatment in recirculating tanks, (2) NBOT treatment compared to reference and historical data, and (3) differences between sample monitoring locations and NBOT diffusers.

Deviations from Original Project Monitoring Objectives

The additional studies (recirculating tank study 1 and diffuser locations in study 3) were not included in the original *INV11* grant agreement (**Appendix C**). Study 1 and study 3 were conducted by LRD staff, with assistance from Greenwater Solutions Inc., with advisement by Dr. Peter Moeller of NOAA. These studies were conducted while study 2 was in progress (as described in the funded FDEP *INV11* grant agreement, **Appendix C and D**). These additional studies were conducted to verify the efficacy of NBOT treatment in a controlled system using Jones Creek water (study 1) and confirm that the distance between established monitoring locations and NBOT diffusers did not dictate bacteria concentrations (i.e., if distance to diffuser was imperative, monitoring locations would always be lower than the corresponding diffuser).

We did not detect the expected strong treatment effect (defined in the grant agreement as <500 MPN/mL enterococci bacteria concentrations over consecutive weeks; **Appendix C**) in Jones Creek (study 2) by week 11 (see **Fig. 6**), therefore the initial grant agreement (**Appendix C**) was amended (**Appendix D**). In the initial grant agreement (**Appendix C**) extensive post NBOT monitoring (including metals analysis of surface waters and all sediment samples) was originally to be collected only 3 months after NBOT had effectively treated the watershed (original sampling timeline, **Table 3**, **Appendix C**). This timeline was proposed based on the expectation of measuring a significant treatment effect *in-situ* in Jones Creek, with a 3-month post NBOT treatment timeline set to capture any legacy treatment effects of NBOT. However, because we did not measure a significant treatment effect in Jones Creek, the three-month monitoring of metals and sediments was moved up to day 60 (as described in **Appendix D**). This amendment was made to ensure that all parameters measured the first week of NBOT treatment, were also measured again after 60 days of treatment for comparisons. In the original grant agreement plan both sediment and water samples were collected from established/regularly monitored LRD water quality locations (**Appendix C**). However, midway through the 60-days of NBOT treatment we decided to compare data collected from monitoring locations to data collected directly under diffusers (study 3). Consequently, when amendments (as described in **Appendix D**) were made to the initial grant agreement (**Appendix C**) we also collected all water quality and sediment parameter data from the NBOT diffusers on day 60.

Partners and Roles

Loxahatchee River District (LRD). Bud Howard (Bud.Howard@lrecd.org), Director of Information Services; Rachel Harris, Ph.D. (Rachel.Harris@lrecd.org) Senior Scientist; Susan Noel (Susan.Noel@lrecd.org), Laboratory Manager. LRD was designated as the local sponsor, contract administrator, and lead of environmental permitting and monitoring, including all surface water and sediment sample collection, and the analysis and reporting/manuscript development. As a TNI-NELAP certified laboratory, LRD collected and analyzed surface water grab samples for Fecal Indicator Bacteria (FIB; enterococci) in water and sediments, chlorophyll, color, and turbidity in water, as well as collected near-continuous, *in-situ* surface water measures using datasonde instrumentation. Additional surface water and sediment samples were also shipped to LRD's contract laboratory (Pace Analytical, Inc.) for analysis of nutrients, Total Organic Carbon (TOC), metals, and bromate.

Green Water Solutions, LLC. (https://greenwatersolutions.org/) Chas Antinone (cantinonejr@gmail.com), President, Green Water Solutions, LLC. Green Water was responsible for all aspects of the NBOT treatment including 6 NBOT machines, labor, generators, fuel, lodging, transportation, rentals, vehicles, site prep, cleanup, coordination with and permission from residents, development and implementation of the safety plan, sampling and testing of water and soil samples not performed by LRD, and any other associated project costs. Green Water obtained any necessary regulatory authorization, including the anticipated authorization required from the Florida Department of Environmental Protection (FDEP) and US Army Corps of Engineers (USACE).

Harmful Algal Bloom Monitoring and Reference Branch, Stressor Detection and Impacts Division, National Ocean Service/NOAA, Hollings Marine Laboratory. Peter Moeller, Ph.D. (peter.moeller@NOAA.gov). For the last seven years Dr. Moeller has been working on the development of nano bubble ozone technology (NBOT) as an efficient, green mitigation/remediation technology for treating harmful algal blooms and associated microbial consortia (algae, fungi, bacteria, viruses) as well the toxins associated with them. After evaluating multiple NBOT platforms, Dr. Moeller has focused his applied research on technology from Green Water Solutions, LLC. Dr. Moeller has been providing technical guidance in support of this project and will co-author the reporting of the findings.

Town of Jupiter (TOJ). Rebecca Wilder (RebeccaW@jupiter.fl.us) PE, Utilities Facilities Manager; TOJ is the local municipality that provides water and stormwater services to the study areas and is a local participant assisting with field sampling and stormwater infrastructure inspection.

Residents. Many of the waterfront residents along Jones Creek are actively engaged in water quality improvement efforts. The Jones Creek Restoration Group facilitated neighborhood coordination and assisted Green Water Solutions in providing NBOT deployment locations.

Methods

NBOTs. In order to treat water with NBOT, water was pumped using a 7.5HP pump through a flexible 5 cm diameter intake hose, pumping at a volumetric flow rate of approximately 14 m^3 hr^1 or 230 L/min (as described https://nbotsystems.com/about-nbot/; **Appendix N**). This flow rate was based on pump size and specifications (not based on target treatment goals). Pumps created a venturi that caused ozone to be mixed into pumped water from Jones Creek and returned to near shore waters of Jones Creek (**Fig. 1**) through diffuser heads. On July 21, 2021, we turned NBOT systems (Greenwater Solutions Inc. US Patent No. US11,247,923 B2) on and they ran continuously dosing Jones Creek with ozone nanobubbles until September 21, 2021. However, it should be noted that ozone is highly reactive, subject to dissolved and undissolved water borne contaminants. The reactive nature of ozone is ideal for immediate water treatment, but this makes it difficult to predict ozone remaining in the water since ozone actively reacts in the presence of both organic and inorganic compounds (i.e., if ozone oxidation is occurring, and all ozone oxidized, we would not expect to detect ozone).

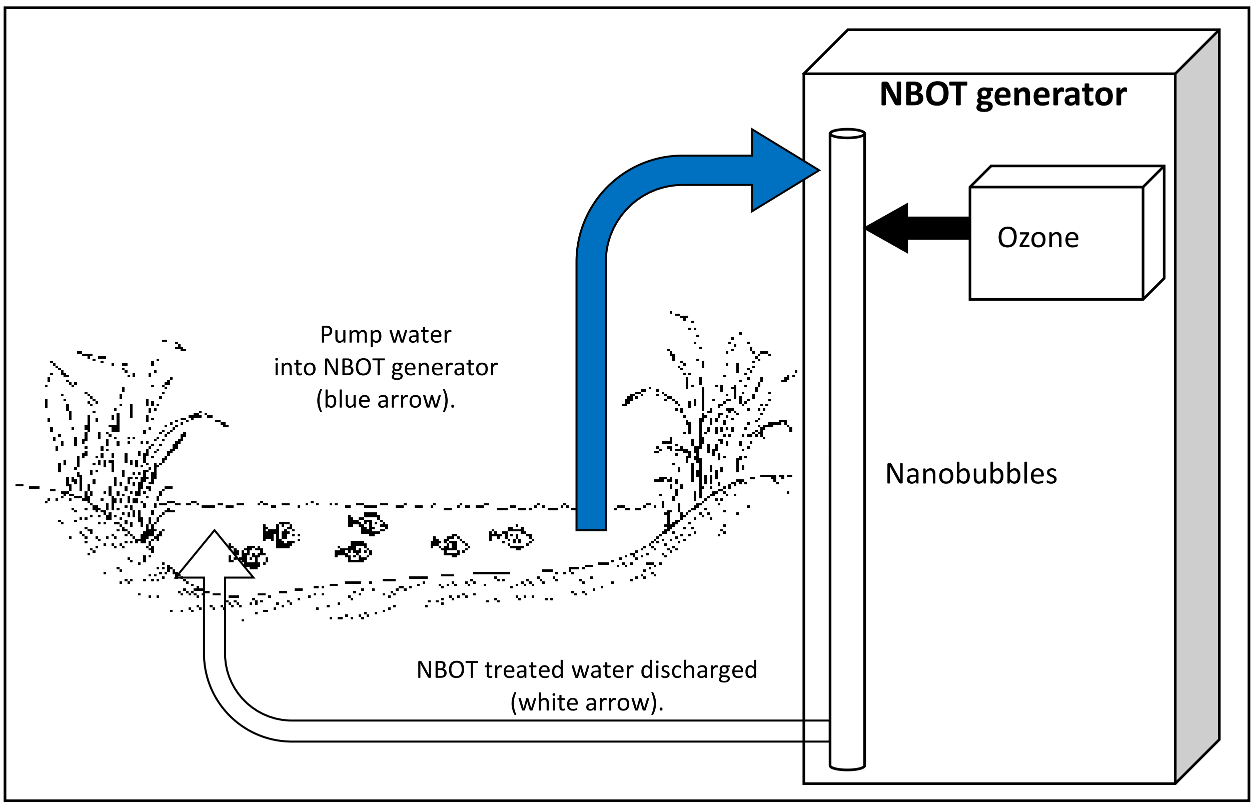


Figure 1. Schematic diagram showing NBOT treatment

Monitoring Locations

The monitoring locations in Jones and Sims Creeks in Jupiter Florida (**Fig. 2**) are part of LRD's ongoing water quality monitoring program and include extensive historical observations (https://loxahatcheeriver.org/river/river-keeper/). LRD's monitoring locations in Jones Creek were used to monitor changes in water quality and sediments associated with NBOT treatment. LRD's monitoring locations in nearby Sims Creek were used as reference sites to evaluate changes in water quality and sediments regardless of NBOT treatment.

A total of 6 NBOT generators were operated simultaneously in Jones Creek. All generators were engaged on July 20, 2021 and turned off on September 20, 2021 providing 60 days of cumulative NBOT treatment. There were brief periods when generators were turned off for maintenance (e.g., checking intake valves for leaf material and debris). Maintenance was conducted on one generator at a time, and 'off' periods generally lasted under 15 minutes where hoses were checked for debris. Two NBOT generators were deployed at 3 sites located within 500 meters of an established LRD surface water monitoring location (LRD Water Quality Stations 75, TPJ, CALC) as follows and illustrated in **Fig. 2**:

- NBOT 1 was originally deployed at 602 Caloosahatchee Ave (approximately 60 meters directly west/upstream of LRD's CALC monitoring location). At the request of the property owner, the NBOT 1 station was relocated on Week 4 (August 26, 2021) to the east to 1114 Sioux St (approximately 210 meters east/downstream of LRD's CALC monitoring location).
- NBOT 2 was deployed at 1007 Sioux St. (approximately 500 meters south/upstream of LRD's 75 monitoring location).
- NBOT 3 was deployed at 990 Mohican Blvd. (approximately 60 meters southwest/upstream of LRD's TPJ monitoring location).

The NBOT 1 location was shallow and became mostly exposed during extreme low tides and so NBOT 1 generators were turned off an hour before and after low tide for during the first 2-3 days. This required additional maintenance (e.g., checking intake valves for clogging by leaf material and sediments) and additional generator shut off times (i.e., generators could not be left running unsupervised during low tide to avoid risk of releasing ozone into the air) during the first 2-3 days. There were less than 3 isolated instances where one NBOT was turned off 4-8 hours due to tides and/or equipment checks. During those days input nozzles were buried in a basket of rocks to keep nozzles below the water level and still draw water in during low tide. A wooden platform was then built, and the input basket was set on top to access input nozzles more easily. This system was eventually relocated, at the request of the landowner, to an alternative address approximately 200 meters downstream (see **Fig. 2**). During this relocation NBOTs were turned off for approximately 24 hours.

LRD Water Quality Stations: 73, 735, and 74DW in nearby Sims Creek received no NBOT treatment and were monitored as a 'reference' (**Fig. 2**), to account for any regional differences in response measures. LRD Water Quality Station 71, 'downstream' near the mouth of Jones Creek was also monitored regularly. This downstream Jones Creek location is heavily influenced by marine tidal exchange and was expected to be least impacted (if at all) by NBOT ozone applications (**Fig. 2**).

Sample Collection Methods

To reduce variability within and between sites due to tidal flushing, surface water, and sediment samples were consistently collected on ebb tide from downstream to upstream within 3 hours of low tide (maximum of -3 hours from high tide, based on NOAA's predicted tides for the southwest fork of the Loxahatchee River; **Table 1**). All surface water observations were collected from < 0.3 meters depth in flowing water. Push cores were used to collect sediments from an area of approximately 10 meters along the intertidal banks of each sampling location during low tide. Sediment cores were collected as twenty 1.2 cm diameter cores pushed to 5 cm depth which were combined in the field and homogenized at the laboratory. Surface water and sediment samples were collected using LRD WildPine Laboratory's Field Sampling Quality Manual(FSQM) which conforms to FDEP field sample collections but has been modified to fit LRD staff operating procedures (see LRD-FSQM). All data collected conforms to the Final Quality Assurance Project Plan (QAPP) for INV11 established by LRD and DEP (**Appendix C** and **D**).

Field parameter measures were collected at each location during each sampling event using a handheld Hydrolab and/or YSI data sonde instruments pre- and post-calibrated (see FSQM link above and **Appendix G**). Additional data sonde instruments were deployed in Sims and Jones Creeks and continuously recorded temperatures and salinities in the middle of each creek. A third data sonde measuring temperature, salinity, turbidity, and chlorophyll-a was also deployed in Jones Creek: https://loxahatcheeriver.org/jonescreek.

A Model EZ-1X EcoZone Monitor calibrated by Gas Sensing (April 2021) was used to detect ozone in the air (photo in **Appendix J**). If ozone was detected in the air, NBOT generators were immediately shut off. This only happened during the recirculating tank studies (study 1). Ozone Sensafe Disposable Ozone Test Strips (481234) were used at all NBOT diffuser outflow locations with no ozone detected in the surface waters at NBOT diffusers.

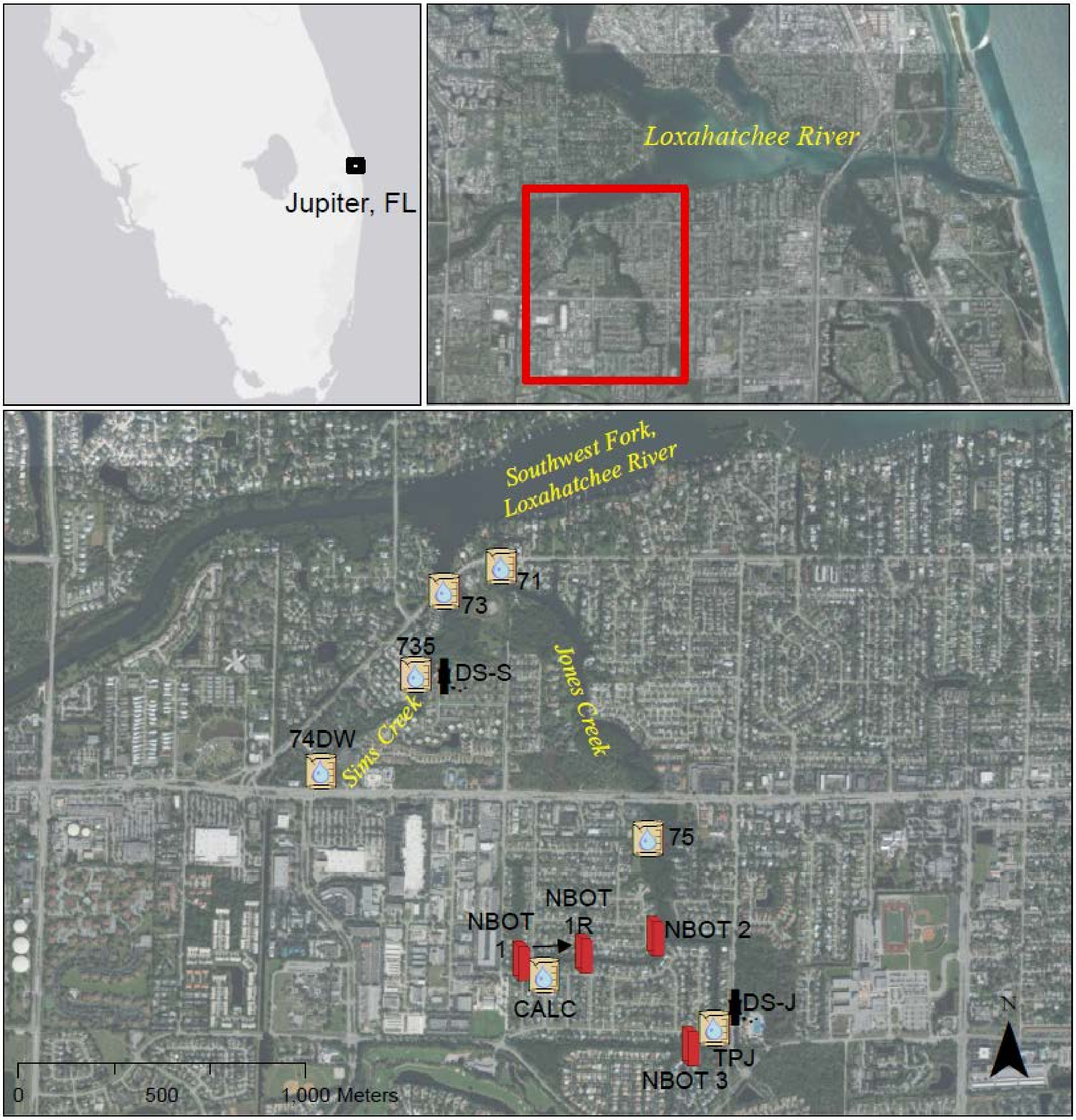


Figure 2. Top left map showing Jupiter, Florida. Top right map with red box showing the study area containing Jones and Sims Creeks. Bottom map showing Sims Creek reference monitoring locations (74DW, 735 and 73) and Jones Creek NBOT monitoring locations (CALC, TPJ, 75, and downstream Jones Creek 71) as beakers with water. Red boxes depict NBOT generator locations. Sample locations with data sondes continuously recording temperature, salinity and dissolved oxygen every 15 minutes are shown as using a black datasonde symbol (DS-J and DS-S).

Table 1. Environmental data shown by study.

	Scale (Locations)	**Data**	**Matrix** (Instrument)	**Frequency (Dates Collected)**	**Data Access**
Study 1 – NBOT Treatment in recirculating tanks	Tanks (NBOT 1, NBOT 2, NBOT 3)	Enterococci bacteria	Surface water (Lab analysis)	Sampling at time zero then every 15 min until ozone detected in air (less than 1 hr); NBOT 1 (9/16/21), NBOT 2 (8/24/21, 8,25/21, 9/2/21), NBOT 3 (9/8/21)	
Study 2 – NBOT treatment compared to reference and historical data	Monitoring Locations (Sims= reference= 73, 735, 74DW; Jones=downstream=71; Jones=NBOT= 75, TPJ, CALC)	Enterococci bacteria, Sal., temp., DO, pH.	Surface water (Lab analysis)	Weekly; Historical (7/1/16–10/1/20), Pre (7/20/21), Day 1(7/22/21), Day 2(7/23/21), Day 5(7/26/21), Week 1 (8/2/21), Week 2 (8/12/21), Week 3 (8/18/21), Week 4 (8/26/21), Week 5 (9/2/21), Week 6 (9/8/21), Week 7 (9/16/21), Week 8 (9/20/21), Week 9 (9/30/21), Week 10 (10/5/21), Week 11 (10/11/21), Week 12 (10/21/21)	Included data file and through data visualization tools at https://loxahatcheeriver.org/jonescreek
		TP, TN, chl-a, color, turb., TSS, TOC	Surface water (Lab analysis, Datasonde(s))	Quarterly; Historical (7/1/16–10/1/20)	
				Pre (7/20/21), Day 1 (7/22/21), Day 2 (7/23/21), Day 5 (7/26/21), Day 60 (9/20/21),	
		Bromate & metals	Surface water (Lab analysis)	Pre (7/20/21), Day 1 (7/22/21), Day 5 (7/26/21), Day 60 (9/20/21)	Included data file and FDEP's WIN/STORET https://floridadep.gov/dear/watershed-services-program/content/winstoret
		Enterococci bacteria, TP, TN, TOC, metals	Sediments (Lab analysis)	Pre (7/20/21), Day 1 (7/22/21), Day 5 (7/26/21), Day 60 (9/20/21)	Included data file and FDEP's WIN/STORET https://floridadep.gov/dear/watershed-services-program/content/winstoret
Study 2, Study 3 – differences between monitoring locations and NBOT systems	NBOTs (NBOT 1= 602 Caloosahatchee &1114 Sioux, NBOT 2=1007 Sioux, NBOT 3=990 Mohican)	Enterococci bacteria	Surface water (Lab analysis)	Week 1 (8/2/21), Week 2 (8/12/21), Week 4 (8/26/21), Week 5 (9/2/21), Week 6 (9/8/21), Week 7 (9/16/21), Week 8 (9/20/21)	
		Salinity	Surface water (Datasonde(s))	Week 2 (8/12/21), Week 8 (9/20/21)	
Supporting Data	Creeks (Sims= reference, Jones=NBOT)	Sal., temp., DO, pH, turbidity, chl-a	Surface water (Datasonde(s))	15 min (7/8/21–10/21/21)	Included data file and https://loxahatcheeriver.org/jonescreek
	Waterbasin (ID# 8722494)	Tidal Period	Surface water (NOAA)	Hourly	Included data file and https://tidesandcurrents.noaa.gov/noaatidepredictions
	Waterbasin (HydroID#10071063)	Rainfall	Rain (NEXRAD)	Daily (reported as monthly sum) (7/1/16–10/1/21)	Included data file and https://loxahatcheeriver.org/river/rainfall

Laboratory
Laboratory analysis of water and sediment samples was conducted at National Environmental Laboratory Accreditation Program (NELAP) laboratories. Samples were analyzed for bacteria, nutrients, and metal concentrations according to standard field and laboratory procedures (NELAC, FDEP, EPA) where applicable (see **Appendix E** for the full list of analytes and analytical methods). All enterococci bacteria, chlorophyll, turbidity, and color analysis were conducted at LRD's WildPine Laboratory (all TNI/NELAC certified except color and sediment enterococci). All TNI/NELAC certified analyses in the historical dataset were conducted at WildPine Ecological Laboratory except for Total Organic Carbon (TOC), which was analyzed by Pace Analytical Laboratory (Pace Analytical Inc.). Pace also analyzed all remaining samples for nutrients and metals pre-NBOT, Day 1, Day 2, and Day 5 (see **Appendix F** for Pace Laboratory Reports). For the Day 60 sampling event, LRD WildPine Laboratory analyzed all surface water nutrient parameters and Pace analyzed metals (in both surface water and sediments). All laboratory results, including QA/QC samples and laboratory data qualifiers can be accessed directly through FDEP's Watershed Information Network (WIN)/ Florida STORET (STOrage and RETrieval) WIN/STORET website and are provided as supplemental electronic datafiles (**Supplemental 1** and **Supplemental 2**). LRD field data collection sheets are provided in **Appendix G**. The official Planning Review Technical Audit Log (Exhibit D (5.b)) describing all field and laboratory issues related to individual laboratory analyses is provided as **Appendix H**.

Minimum Detection Limits (MDLs) and Data Analysis
Laboratory analyses generally include a Minimum Detection Limit (MDL). This provides a minimum value to which a laboratory can confidently report a result based on the laboratory analytical procedure. This MDL value may be consistent over time or may change with any given sample based on the laboratory practices used to analyze a particular sample. In data analysis the laboratory reported MDL is used instead of zero as a conservative approach to data analytics. Because the current study was designed to monitor changes in water quality and sediments over time, including multiple monitoring locations, we selected the highest recorded MDL per analyte (i.e., the maximum MDL per analyte was used rather than zero or a unique single sample MDL). All MDLs used for data analysis are reported in **Appendix E**. Some analytes were detected at, or just above MDLs; bromate (below MDL in water; not tested in sediments), beryllium, cobalt (below MDL in water), copper (below MDL in water), lead (below MDL in water), nickel (below MDL in water), potassium, selenium, silicon (not tested in sediments), silver, sodium, thallium, tin, vanadium (water), and zinc (water) (see **Appendix E**), so that although these parameters were analyzed in the laboratory, they were excluded from subsequent analysis.

<u>Study 1:</u> NBOT Treatment in Recirculating Tanks
Recirculating tank tests were conducted as a controlled assessment of NBOT generator performance. A total of 5 independent tank tests were conducted; one on August 24, 2021, August 25, 2021, and September 2, 2021 (tested using equipment at NBOT 3); September 8, 2021 (tested using equipment at NBOT 2); and September 16, 2021 (tested using equipment at NBOT 1) (**Table 1**). This purpose of this exploratory work was to verify the efficacy of NBOT treatment in reducing bacterial concentrations in Jones Creek water. We did not include a control tank in which no NBOT treatment occurred, which may have been an oversight. Recirculating tank tests

were conducted by continuously treating approximately 1,000 liters of Jones Creek water in a closed, recirculating system (see **Fig. 3**, left). To do this we filled a clean, 1,041-liter high density polyethylene (HDPE) tank container with water from Jones Creek (see **Fig. 3**, right, blue arrow). The water was pumped into the tank using the NBOT generator pump without ozone running (see **Fig. 3**, right, blue arrow, followed by white arrow). After the tank was filled with Jones Creek water, the ozone was turned on, and the NBOT generator effluent line deposited NBOT treated water into the HDPE tank (see **Fig. 3**, white arrow). The HDPE tank effluent line recirculated water from the HDPE tank back through the NBOT generator with the ozone nanobubble function of the NBOT generator turned on (see **Fig. 3**, dashed arrow). Water samples were collected to test for enterococci bacteria concentrations at time zero and every 15 minutes until the tank measured ozone saturation (i.e., the system was shut down once ozone was detected in the air immediately above the tank). Ozone saturation generally occurred in less than one hour of treatment. The Model EZ-1X EcoZone Monitor was used to detect ozone in the air just above the recirculating tank.

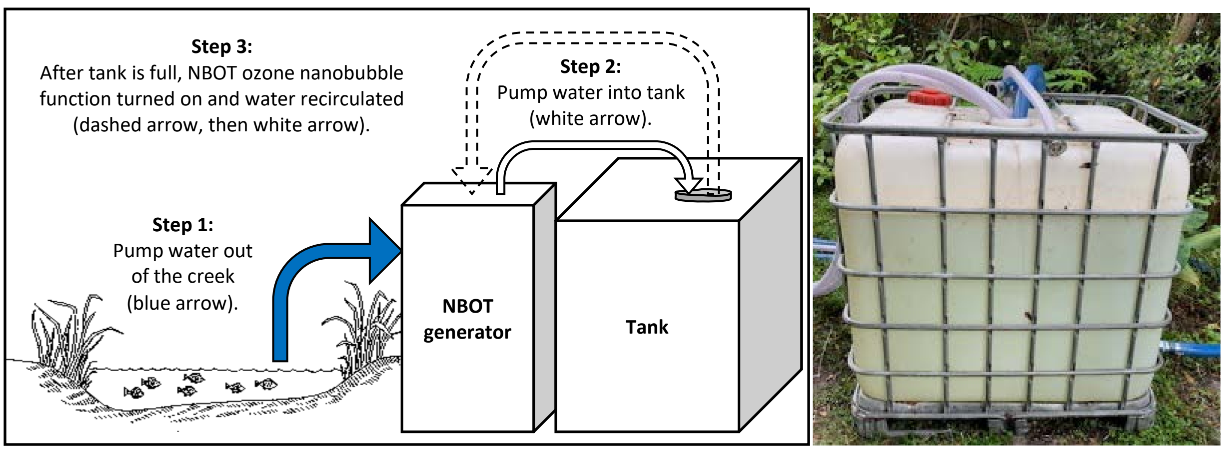


Figure 3. Simple schematic (line drawing) showing direction of water flow during a recirculating tank test (left) and photo of actual recirculating tank with NBOT hoses (right).

<u>Study 2:</u> In Situ NBOT Treatment of Jones Creek

This work was designed to evaluate the effectiveness of NBOT treatment on water quality improvements in a dynamic shallow water tidal system (i.e., Jones Creek) using weekly bacteria concentrations to monitor the effects of NBOT relative to results from reference site (Sims Creek) and historical data (previous LRD data collected in Jones Creek). Additional water quality and sediment parameters were monitored at set time intervals (pre-NBOT, Day 1, Day 2, Day 5, and Day 60 of NBOT treatment; see **Table 1**). This study was designed based on the expectation that we would detect significant improvements in water and/or sediment quality (measured as decreased bacteria, phytoplankton and/or metals) within the first 5 days of NBOT treatment and that we would cycle the NBOT systems as needed during the 60-day treatment period to maintain desired water quality results. These expected results were based on the results of prior NBOT success in lakes, canals, and ballast water systems (e.g., https://greenwatersolutions.org/team/, https://nbotsystems.com/about-nbot/), the general size of Jones Creek, water quality in Jones

Creek, and the capacity of treatment provided by the six NBOT generators deployed at 3 locations within Jones Creek.

Surface water enterococci bacteria samples were collected weekly for 12 weeks to monitor any changes in enterococci concentrations before, during, and after NBOT treatment (**Table 1**). Historical samples collected from the same study locations in Jones Creek dating back to 2016 (n=86) were used to contextualize data collected in Study 2. Surface water samples were collected from 7 locations in Jones and Sims Creek (**Fig. 2**); plus 1 field duplicate, and 1 field blank per sampling event (described in **Appendix C**). These locations were each sampled on 5 sampling events: one day prior to beginning NBOT treatment ('Pre' samples collected on July 20, 2021) and on Day 1 (July 22, 2021), Day 2 (July 23, 2021), Day 5 (July 22, 2021), and Day 60 (September 20, 2021) of continuous NBOT treatment (**Table 1**). Surface waters at each NBOT generator were also sampled on Day 60. Parameters measured in surface water samples included: dissolved oxygen (DO), pH, enterococci, salinity, total phosphorus (TP), total nitrogen (TN), chlorophyll-a (corrected), chlorophyll-a + non-photosynthetic phaeopigment (uncorrected), total organic carbon (TOC), color, turbidity, total suspended solids (TSS). Additional surface water and sediment samples were collected and analyzed for a suite of metals at Pre, Day 1, 5, and 60 of NBOT treatment (not at Day 2). These samples were collected from all 7 locations (plus 1 field duplicate, 1 field blank and 1 equipment blank per sampling event). The surface waters and sediments at each NBOT generator were also sampled on Day 60. The suite of Metals analyzed in surface water included: aluminum (Al), antimony (Sb), barium (Ba), boron (B), chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), silicon (Si), strontium (Sr), and titanium (Ti). Measures analyzed in sediments included: enterococci, total phosphorus (TP), total nitrogen (TN), total organic carbon (TOC), aluminum (AL), antimony (Sb), barium (Ba), boron (B), chromium (Cr), Copper (Cu), lead (Pb), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), Nickel (Ni), strontium (Sr), titanium (Ti), Vanadium (V), and Zinc (Zn) (see **Appendix E**).

Historical surface water data collected from Jones Creek was included in the analysis for comparisons. Laboratory analysis of historical data was conducted at LRD Wildpine laboratory (see Stoner and Arrington 2017). Only historical data collected from Jones Creek (75, TPJ, and CALC) for the months of July, August, September, or October, and during ebb to low tide, was included. This subset of data resulted in 86 records of historical surface water data collected from 2016 through 2020 and was plotted against the current NBOT data set for comparison and context.

Study 3: Diffusers

Because our environmental monitoring locations were located 35 to 500 meters away from each NBOT generator/diffuser, we collected a series of samples to determine if the distance between the NBOT diffuser was driving differences in the enterococci bacteria concentrations. We collected additional surface water samples within the NBOT effluent (i.e., water flowing out from the diffusers mixing with creek water). This was done six times; August 2, 2021, August 12, 2021, August 26, 2021, September 7, 2021, September 8, 2021, September 16, 2021, and September 20, 2021 (see **Table 1**).

Results

<u>Study 1:</u> NBOT Treatment in Recirculating Tanks

Based on the manufacturer's flow rate for the pump used in the NBOTs (227 L/min), we estimate that water in the tanks had recirculated once by 15 minutes and in each test, water clarity had visibly improved in most tank studies after 15 minutes and continued to show improvement until reaching ozone saturation (for a visual example see **Photo 1**, where ozone saturation in air was reached at 45 minutes). See **Table 2** for enterococci results.

Photo 1. Photo of water samples collected in recirculating tank test from NBOT 2 on September 2, 2021 showing increased water clarity over time from time zero to 45 minutes.

Table 2 shows that initial bacteria concentrations (657–6,867 enterococci/100mL) dramatically declined to <10 enterococci/100 mL within 45 minutes in all studies except the final tank study conducted on September 16, 2021. Leaf litter and/or some form of organic debris was observed on the top and sides of the tank on September 16, 2021 (see **Photo 2**). Some surface foam was visible during all tank studies and although we tried to avoid sampling the foam and/or debris it is possible that either the surface foam and/or some particulate matter was present in the samples from the final tank study conducted on September 16, 2021, which materialized the higher bacteria concentrations despite ozone air saturation (**Table 2**; **Fig. 4**). Regardless, enterococci bacteria concentrations declined from time zero in all recirculating tank studies (**Table 2**). For enterococci concentrations in the creek during the last 3 tank studies see **Table 3** (study 3).

Table 2. All observations collected from NBOT tank studies. Each study was concluded when ozone was detected in air above the tank, indicating ozone saturation was reached in the tank.

Date	NBOT	Time (Minutes)	Enterococci (MPN/100 ml)
8/24/2021	NBOT 3	0	1,616
		15	41
		30	31
		45	10
		60	10
		90	10
8/25/2021	NBOT 3	0	683
		15	272
		30	41
		45	10
		60	10
9/2/2021	NBOT 3	0	6,867
		15	213
		30	10
		45	20
9/8/2021	NBOT 2	0	657
		15	10
		30	10
9/16/2021	NBOT 1	0	763
		15	132
		30	189
		45	134

Photo 2. The tank from September 16, 2021 with leaf litter/organic matter observed on the sides and towards the surface of tank before and after the tank study.

Across the recirculating tank tests ozone saturation, measured by the presence of ozone in air above the water in the tank, was reached between 30 and 90 minutes, where % of samples reaching ozone saturation (on secondary y-axis) was 80% after 1 hour (blue line, secondary y-axis; **Fig. 4**). The test on September 16, 2021 reached ozone saturation in air by 45 minutes (**Fig. 4**), suggesting that the persistent bacteria detected in that sample (**Table 2**; **Fig. 4**) may be due to the inadvertent collection of organic matter (see **Photo 2**). It is likely that more complete NBOT treatment on this tank would have oxidized both the available organic matter and remaining bacteria.

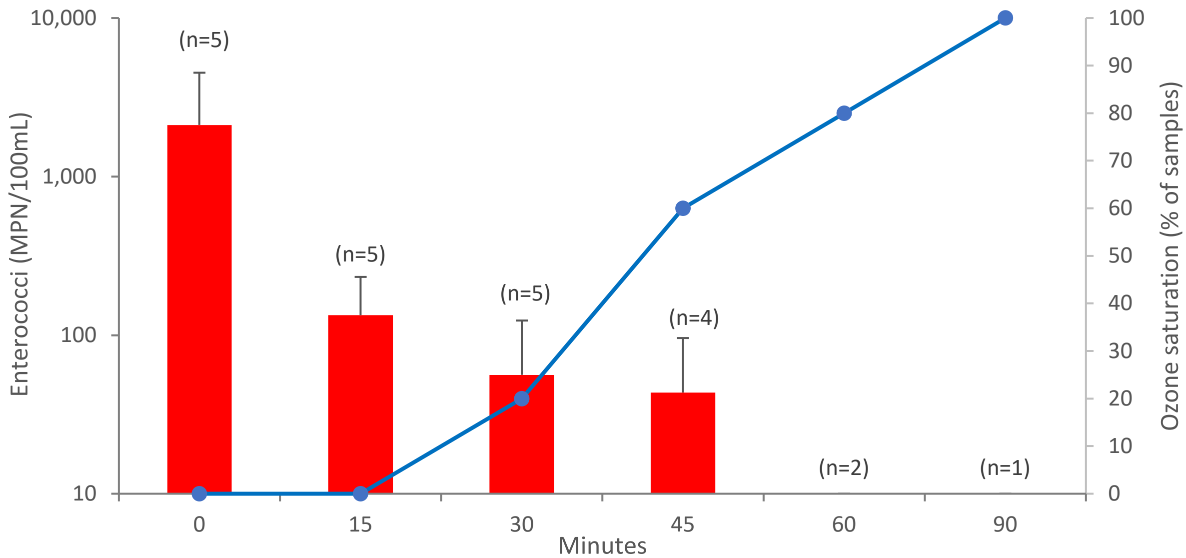


Figure 4. Average (+/- standard error) enterococci concentrations collected from recirculating tanks every 15 minutes (red bars). Ozone saturation (measured by ozone detection in air), shown as a percent of tests achieving ozone saturation per time step is shown on the secondary y-axis (blue line).

Study 2: NBOT Treatment in Jones Creek

This study was conducted during Florida's wet season (July–October). Because rainfall and associated stormwater flows can have profound effects on water quality, we compared the study period rainfall to historical rainfall. Localized, radar-based rainfall data (SFWMD NEXRAD) showed monthly rainfall totals fell within historical values (**Fig. 5**, top). Water quality data collected every 15 minutes from instruments deployed in Jones (75) and Sims (735) Creeks confirmed similar daily fluctuations and ranges in temperature, salinity, and dissolved oxygen in both the NBOT (Jones) and reference (Sims) tidal creeks (https://loxahatcheeriver.org/jonescreek). We also considered median values of daily average rainfall 3 days prior to sampling, where an increase in the median indicates greater average daily rainfall 3 days up to sample collection. During the 60 days of NBOT treatment we began measuring increases in 3-day rainfall at Week 2 of NBOT treatment (**Fig. 5**, bottom), and then again, a steady increase in 3-day rainfall during the final week of NBOT treatment through our post-NBOT treatment monitoring (**Fig. 5**, bottom).

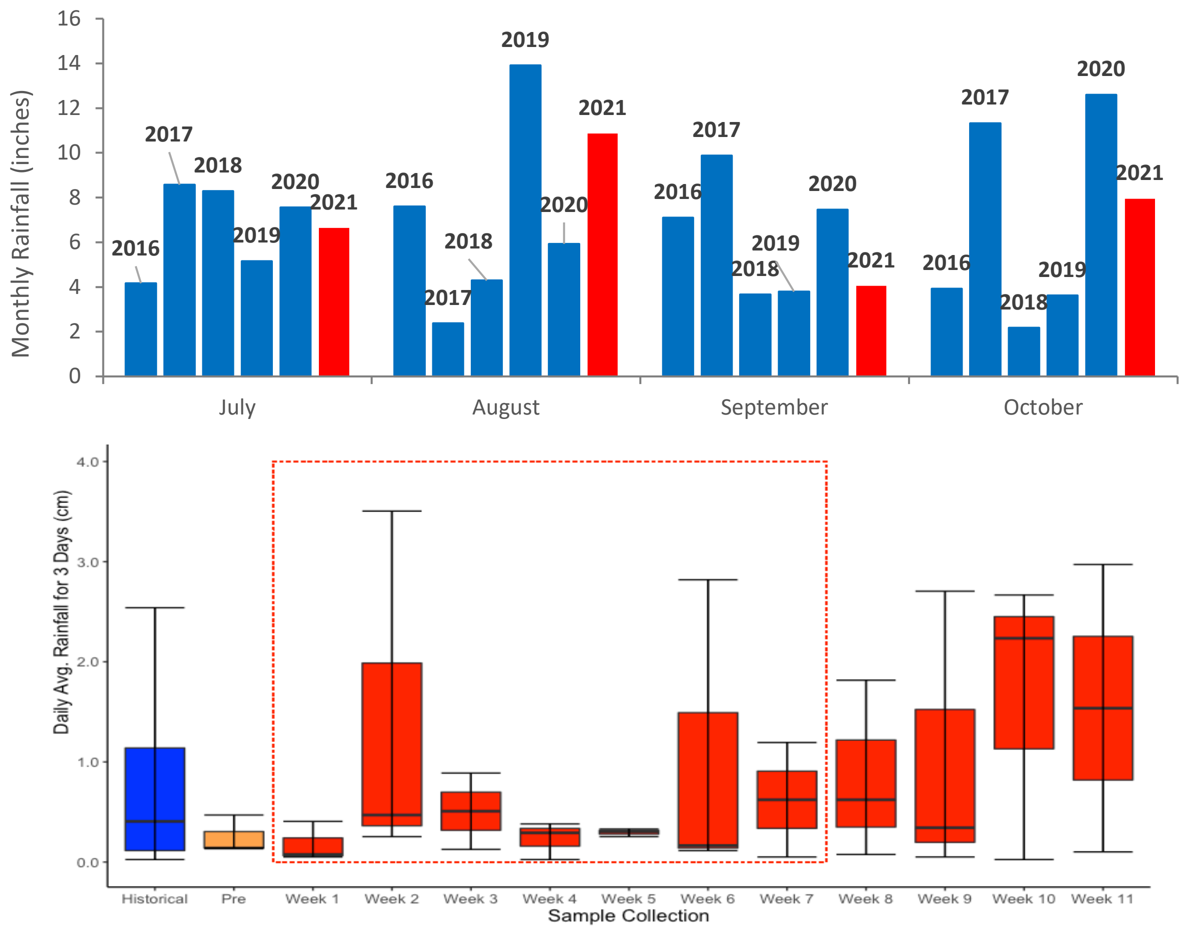


Figure 5. Top showing monthly rainfall from July –October for historical data (blue) and the NBOT study (red) in a historical context (by year in blue). Bottom figure showing the Daily Average Rainfall for 3 days prior to each sample event, the 60-days of NBOT treatment (Week 1–Week 7) are shown in the red dashed rectangle. All data from https://loxahatcheeriver.org/river/rainfall/.

In Jones Creek (NBOT locations 75, TPJ, CALC) the handheld field water quality meter indicated high temperatures (28–31°C), variable salinities (3–23 psu), pH ranging from 6.83–7.31, and dissolved oxygen was relatively low ranging from 0.39–1.96 mg/L (**Fig. 6**). Median values from Jones Creek during NBOT (median line in red interquartile range box; **Fig. 6**) fell within the more extreme historical ranges (upper and lower interquartile range whiskers in blue box; **Fig. 6**). The handheld field water quality meter readings revealed higher median (and some interquartile ranges) salinity, temperature, pH, and DOs in the reference creek (median line in gray interquartile range box; **Fig. 6**) compared to Jones Creek (NBOT median line in red interquartile range box; **Fig. 6**). These all point to a greater tidal input in the reference creek (Sims), compared to Jones Creek (NBOT), likely due to the greater area upstream in Jones than Sims Creek (see **Fig. 2**).

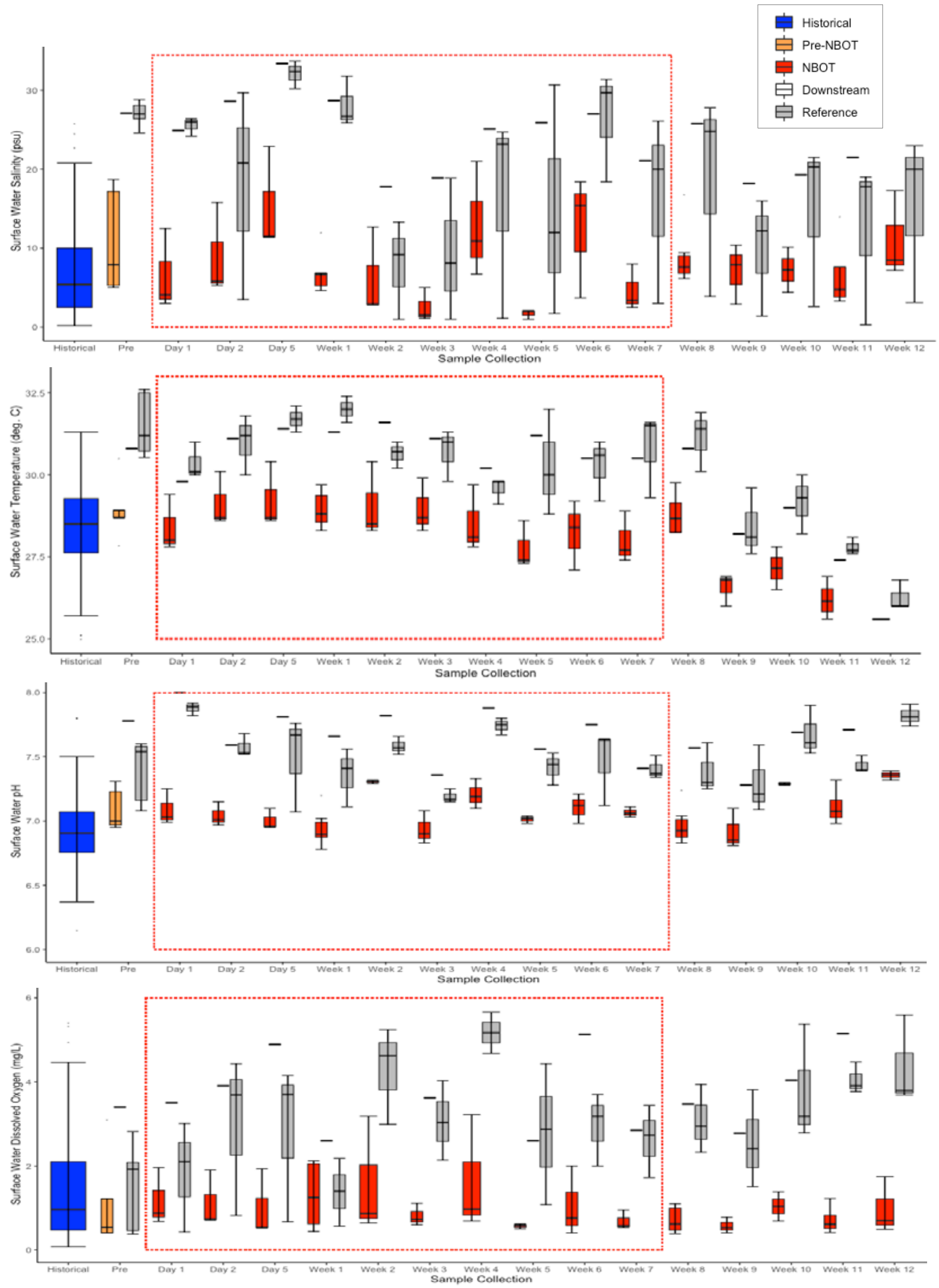


Figure 6. Box and whisker plots of surface water quality parameters collected weekly. Lines in boxes showing the median (or middle) value, boxes showing lower 75th percentile and 25th percentile interquartile range, and whiskers showing upper and lower range (1.5* interquartile range). Historical data in Jones Creek (data collected Jul., Aug., Sept, Oct, 2016–2020 on ebb tide from 75, TPJ, CALC; Jones Creek in blue), pre- NBOT treatment in Jones Creek (75, TPJ, CALC; Jones Creek in orange), NBOT treated in Jones Creek (75, TPJ, CALC; Jones Creek in red), reference locations (73, 735, 74DW; Sims Creek in gray), and downstream locations (71, Jones Creek shown as dashed lines) before NBOT treatment (Historical, Pre), during treatment (Days 1, 5 and Weeks 1, 2, 3, 4, 5, 6, 7), and after treatment (Weeks 8, 9, 10, 11, 12).

Weekly surface water enterococci bacteria counts were typically higher in Jones Creek (both median lines and red interquartile range boxes; **Fig. 7**) than in the reference creek (both median lines and gray interquartile range boxes; **Fig. 7**) and downstream locations (dashed lines showing the single measures collected downstream**; Fig. 7**). These findings are consistent with the historical data (upper and lower interquartile range shown as whiskers from blue box; **Fig. 7**), with historically higher median enterococci concentrations in Jones Creek than the reference locations (median line in gray interquartile range box; **Fig. 7**), and downstream in Jones Creek (black dashed line; **Fig. 7**). However, median enterococci bacteria concentrations in Jones Creek during NBOT (median line in red interquartile range box; **Fig. 7**) was greater than pre-NBOT (median line in orange interquartile range box; **Fig. 7**), and/or historical concentrations (median line in blue interquartile range box; **Fig. 7**). We did not measure immediate or systematic declines in enterococci bacteria concentrations over the 60 days of NBOT treatment (shown as dashed red NBOT box; **Fig. 7**). There was a marginal decline in median and interquartile range enterococci bacteria measured week 2 of NBOT treatment in both Jones Creek (median line and red interquartile range box) and the reference creek (median line and gray interquartile range box), which may be related to the increase in median average 3-day rainfall observed week 2 (**Fig. 5**).

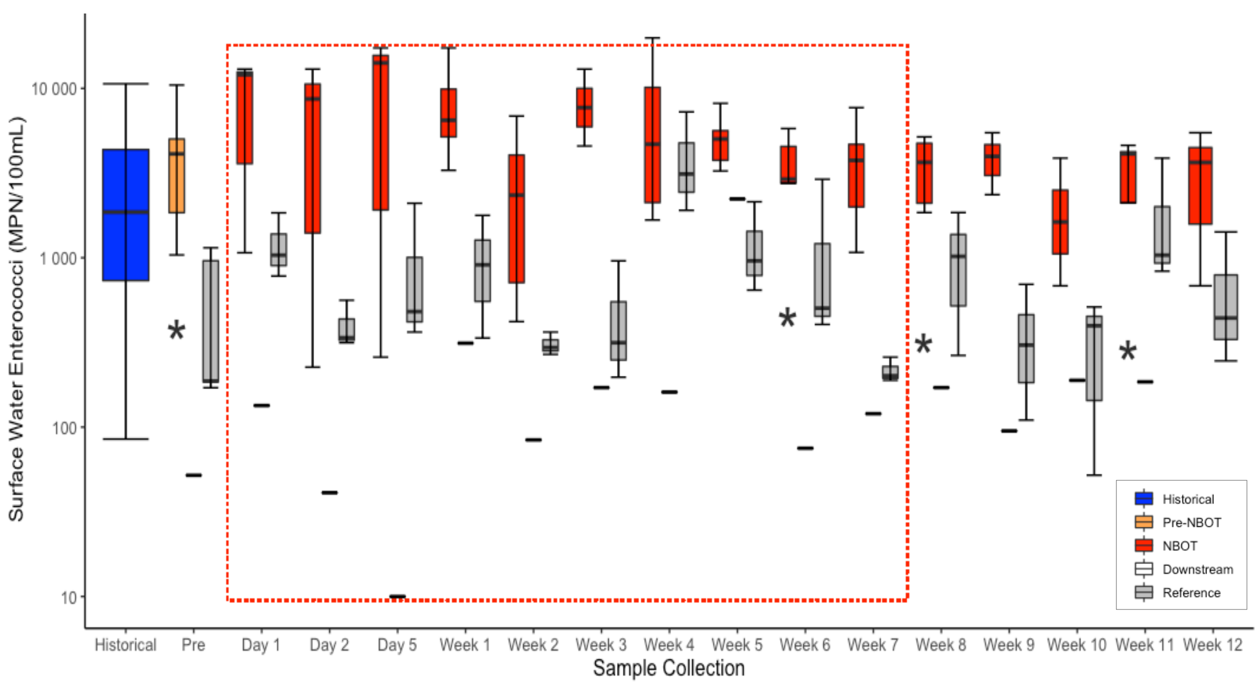


Figure 7. Box and whisker plots of surface water enterococci bacteria collected weekly in Jones Creek. Lines in boxes showing the median(middle) value, boxes showing lower 75th percentile and 25th percentile interquartile range, and whiskers showing upper and lower range (1.5* interquartile range); outliers shown as *. NBOT treatment period differentiated in red box. Historical data in Jones Creek (data collected Jul., Aug., Sept, Oct, 2016–2020 on ebb tide from 75, TPJ, CALC; Jones Creek in blue), pre- NBOT treatment in Jones Creek (75, TPJ, CALC; Jones Creek in orange), NBOT treated in Jones Creek (75, TPJ, CALC; Jones Creek in red), reference locations (73, 735, 74DW; Sims Creek in gray), and downstream locations (71, Jones Creek shown as dashed lines) before (Historical, Pre), during (Days 1, 5 and Weeks 1, 2, 3, 4, 5, 6, 7), and after (Weeks 8, 9, 10, 11, 12)

In general, median total nitrogen (TN), total phosphorus (TP), total organic carbon (TOC), and chlorophyll-a were lower at the downstream location (71) at the mouth of Jones creek (dashed lines showing the single measures collected downstream; **Fig. 8**). This corresponds with most observations in brackish water systems where bacteria, nutrients, and phytoplankton are typically not marine derived (Anderson et al. 2002). The main distinction between the two creeks was the higher median and interquartile ranges in TOC measured in surface waters of Jones Creek (both median lines and red interquartile range boxes; **Fig. 8**) compared to the reference Sims Creek (both median lines and gray interquartile range boxes; **Fig. 8**) throughout the study period. The strongest signal detected was a rather large increase in surface water TP and TOC after 60 days of NBOT treatment (both median lines and red interquartile range boxes on Day 60; **Fig. 8**). However, the values measured at Day 60 were comparable to historical medians (median line in red box on Day 60 falls within blue interquartile range box; **Fig. 8**). Median chlorophyll-a concentrations Pre-NBOT (median line and orange interquartile range box; **Fig. 8**) were higher than historical concentrations (median line in blue interquartile range box; **Fig. 8**), and further increased Day 1 and Day 2 (Day 1 and 2 median line and red interquartile range boxes; **Fig. 8**) compared to Pre-NBOT (median line and orange interquartile range box; **Fig. 8**) and Historical concentrations (median line and blue interquartile range box; **Fig. 8**). Median chlorophyll-a concentrations decreased at NBOT treatment locations from Day 1 (50 ug/L) to Day 60 (18 ug/L), but even after 60 days of treatment chlorophyll-a concentrations remained elevated, exceeding the 5.5 ug/L Numeric Nutrient Criteria ((NNC), Chapter 62-303, F.A.C.), and concentrations comparable to the historical median (**Fig. 8**).

The surface water metal results were inconsistent, suggesting background variability in surface waters of Jones (NBOT) and Sims (reference) creeks or potentially highlighting the variability in the oxidizing potential of some metals over others in solution. Surface water aluminum appeared to decrease after 1 day of NBOT treatment (median lines and orange and red interquartile range boxes greater Pre and Day 1 compared to red interquartile range boxes on Day 5 and Day 60; **Fig. 9**). Barium, manganese, and titanium appeared to increase in surface waters during NBOT treatment median lines and red interquartile range boxes higher on Day 60 than Day 1; **Fig. 9**), but these concentrations all fell within Pre-NBOT interquartile ranges (orange interquartile range box; **Fig. 9)**. Manganese was the only parameter that increased in surface waters after NBOT treatment, in concentrations (median lines and red interquartile range boxes on Days 5 and 60; **Fig. 9**) much higher than the reference monitoring location (median lines and gray interquartile range boxes; **Fig. 9**). Similarly surface water aluminum was the only metal that decreased (median lines and red interquartile range boxes on Days 5 and 60; **Fig. 9**) far below the reference at NBOT monitoring locations (median lines and gray interquartile range boxes; **Fig. 9**). As each metal maintains a unique oxidizing potential, it is likely these results reflect this physical parameter. However, one cannot discount the nano bubble collapse energy and its involvement in these processes as well.

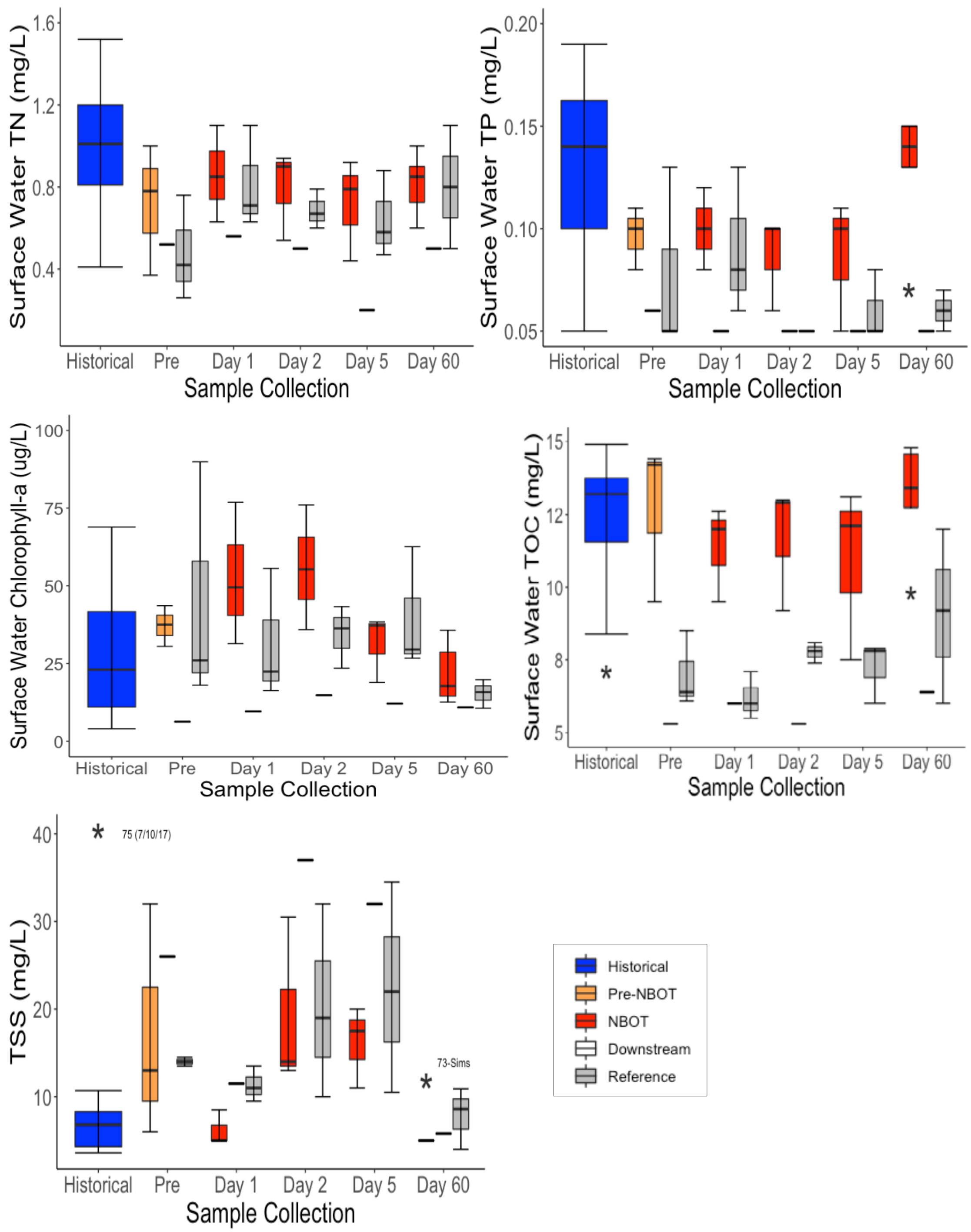


Figure 8. Box and whisker plots of surface water quality parameters. Lines in boxes showing the median (middle) value, boxes showing lower 75th percentile and 25th percentile interquartile range, and whiskers showing upper and lower range (1.5* interquartile range); outliers shown as *. Historical data in Jones Creek (data collected Jul., Aug., Sept, Oct, 2016–2020 on ebb tide from 75, TPJ, CALC; Jones Creek in blue), pre- NBOT treatment in Jones Creek (75, TPJ, CALC; Jones Creek in orange), NBOT treated in Jones Creek (75, TPJ, CALC; Jones Creek in red), reference locations (73, 735, 74DW; Sims Creek in gray), and downstream locations (71, Jones Creek shown as dashed lines) before (Historical, Pre), during (Days 1, 5 and Weeks 1, 2, 3, 4, 5, 6, 7), and after (Weeks 8, 9, 10, 11, 12) NBOT treatment.

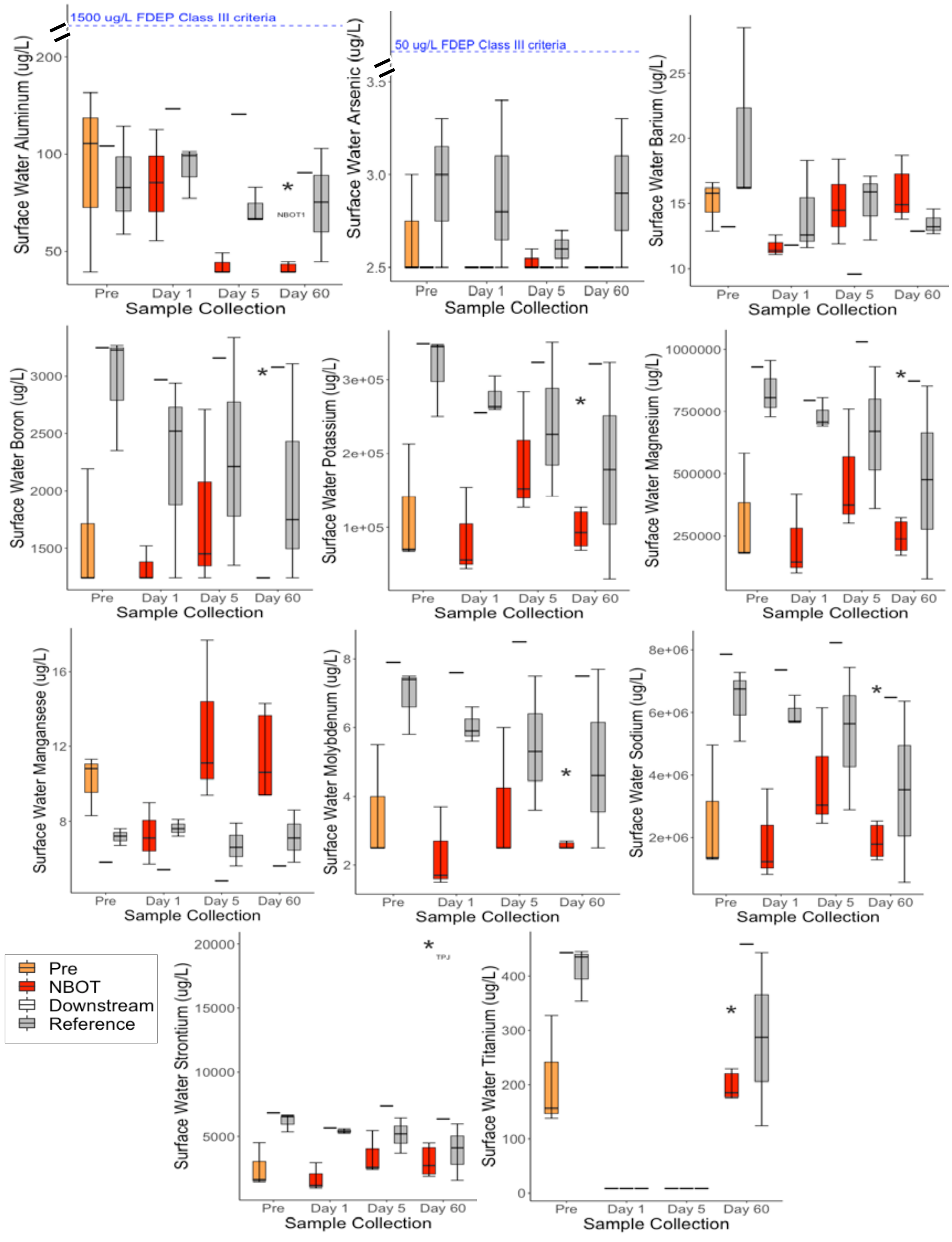


Figure 9. Box and whisker plots of metals in surface waters. Lines in boxes showing the median (middle) value, boxes showing lower 75th percentile and 25th percentile interquartile range, and whiskers showing upper and lower range (1.5* interquartile range); outliers shown as *. Pre- NBOT treatment in Jones Creek (75, TPJ, CALC; Jones Creek in orange), NBOT treated in Jones Creek (75, TPJ, CALC; Jones Creek in red), reference locations (73, 735, 74DW; Sims Creek in gray), and downstream locations (71, Jones Creek shown as dashed lines) before (Pre), during (Days 1, 5 and Weeks 1, 2, 3, 4, 5, 6, 7), and after (Weeks 8, 9, 10, 11, 12) NBOT treatment. FDEP criteria for class III surface waters: Recreation, Propagation, and Maintenance of a Healthy, Well-Balanced Population of Fish and Wildlife) are shown as blue dashed lines for reference (Chapter 62-302.530, F.A.C.).

There was a wide range in bacteria and nutrients measured in sediments (interquartile range boxes and upper and lower interquartile whiskers; **Fig. 10**). Median enterococci bacteria decreased in the reference creek over time (median line in gray interquartile range boxes; **Fig. 10**), while of the variability in enterococci bacteria in Jones Creek sediments increased over time (greater red interquartile range boxes and upper and lower interquartile whiskers on Day 60 than Day 5 or Day 1; **Fig. 10**). Compared to Pre-NBOT conditions (median line in orange interquartile range boxes; **Fig. 10**) both TOC and TN decreased in sediments Day 1 and 5 of NBOT treatment (median line in red interquartile range boxes; **Fig. 10**), but by Day 60 TOC were measured higher than both pre-NBOT and reference concentrations (median line in red interquartile range boxes higher than median line in orange interquartile range boxes; **Fig. 10**). Median TN in sediments declined over time from Pre-NBOT to Day 60, but by Day 60 the variability in TN of NBOT sediments was great (median line and red interquartile range box on day 60; **Fig. 10**). It is worth noting the very high bacteria concentrations measured in the sediments (**Fig. 10**); up to 10x higher than those measured in surface waters (**Fig. 8**).

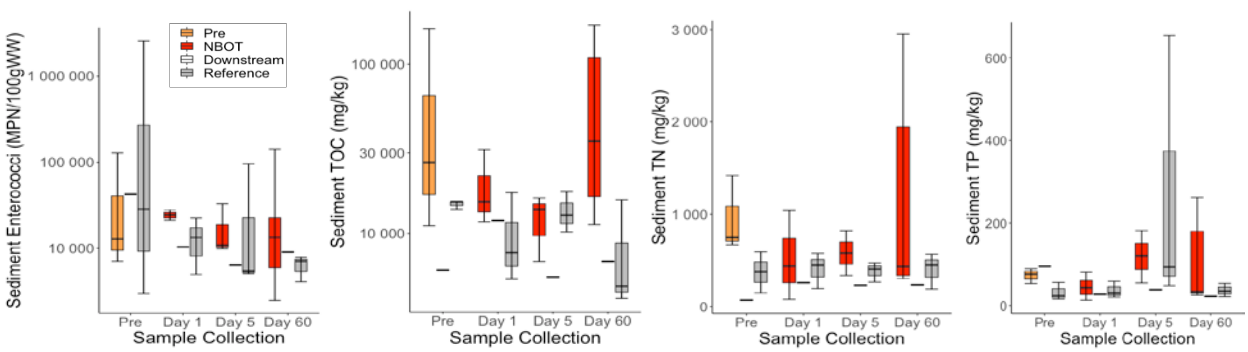


Figure 10. Box and whisker plots of bacteria and nutrients measured in sediments. Lines in boxes showing the median (middle) value, boxes showing lower 75th percentile and 25th percentile interquartile range, and whiskers showing upper and lower range (1.5* interquartile range). Pre- NBOT treatment in Jones Creek (75, TPJ, CALC; Jones Creek in orange), NBOT treated in Jones Creek (75, TPJ, CALC; Jones Creek in red), reference locations (73, 735, 74DW; Sims Creek in gray), and downstream locations (71, Jones Creek shown as dashed lines) before (Historical, Pre), during (Days 1, 5 and Weeks 1, 2, 3, 4, 5, 6, 7), and after (Weeks 8, 9, 10, 11, 12) NBOT treatment.

Metal concentrations in both Jones Creek (NBOT) and Sims Creek (reference) were variable over time with no clear pattern specific to NBOT treatment (**Fig. 11**); data that might be explained by continual water movement. By Day 60 we measured an increase in some median metal concentrations in NBOT sediments (median line in red interquartile range boxes higher on Day 60 than median line in orange interquartile range boxes; **Fig. 11**), which may support the theory that those metals are oxidized to an insoluble state fall out of solution. Although we observed general increases in median metal concentrations in NBOT sediments on Day 60, variance was also greater/higher (red interquartile range boxes higher on Day 60; **Fig. 11**). When available, sediment metal Toxic Effect Levels (TEL) established for Florida MacDonald et al. 1996) are shown (blue dashed line; **Fig. 11**). Apart from copper median values fell below the TELs, with 25% of the values at day 1 and day 60 of arsenic above the TEL (blue dashed line; **Fig. 11**).

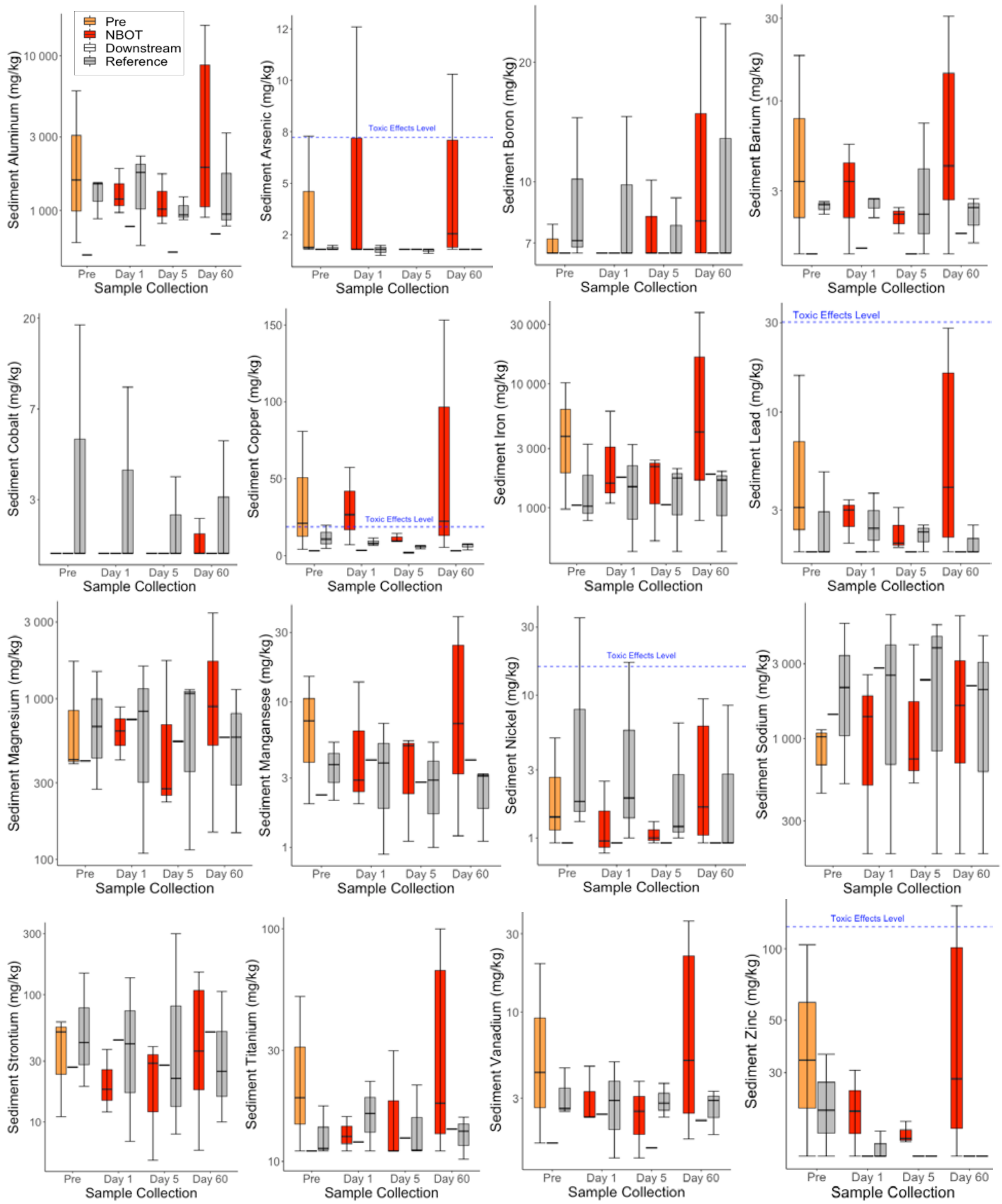


Figure 11. Box and whisker plots of metals measured in sediment. Lines in boxes showing the median (middle) value, boxes showing lower 75th percentile and 25th percentile interquartile range, and whiskers showing upper and lower range (1.5* interquartile range); outliers shown as Pre-NBOT treatment in Jones Creek (75, TPJ, CALC; Jones Creek in orange), NBOT treated in Jones Creek (75, TPJ, CALC; Jones Creek in red), reference locations (73, 735, 74DW; Sims Creek in gray), and downstream locations (71, Jones Creek shown as dashed lines) before (Historical, Pre), during (Days 1, 5 and Weeks 1, 2, 3, 4, 5, 6, 7), and after (Weeks 8, 9, 10, 11, 12) NBOT treatment. FDEP established Toxic Effects Levels are shown for reference (MacDonald et al. 1996).

Relationships between enterococci bacteria and salinity were examined to identify whether general patterns existed over time and across sample locations. We observed a strong negative relationship between surface water enterococci bacteria concentrations (log) and salinity (Pearson's r =-0.95) throughout Jones Creek over the course of the NBOT study (**Fig. 12**). Yet the high degree of co-variation among variables (e.g., enterococci concentrations, nutrients, chlorophyll biomass, salinity) along such salinity gradients makes it difficult to tease out the drivers since most variables either increase or decrease, either as a direct or indirect salinity response (correlation matrix **Appendix I**). We did not measure significant correlations between sediment and surface water enterococci bacteria (**Fig. 13**), suggesting that high enterococci bacteria concentrations measured in the surface waters are not the direct result of sediment enterococci concentrations (i.e., relationships likely rely on wind-driven resuspension, sediment type/organic content, and variations in hydrodynamics specific to monitoring locations and or select sampling times that would significantly impact these relationships).

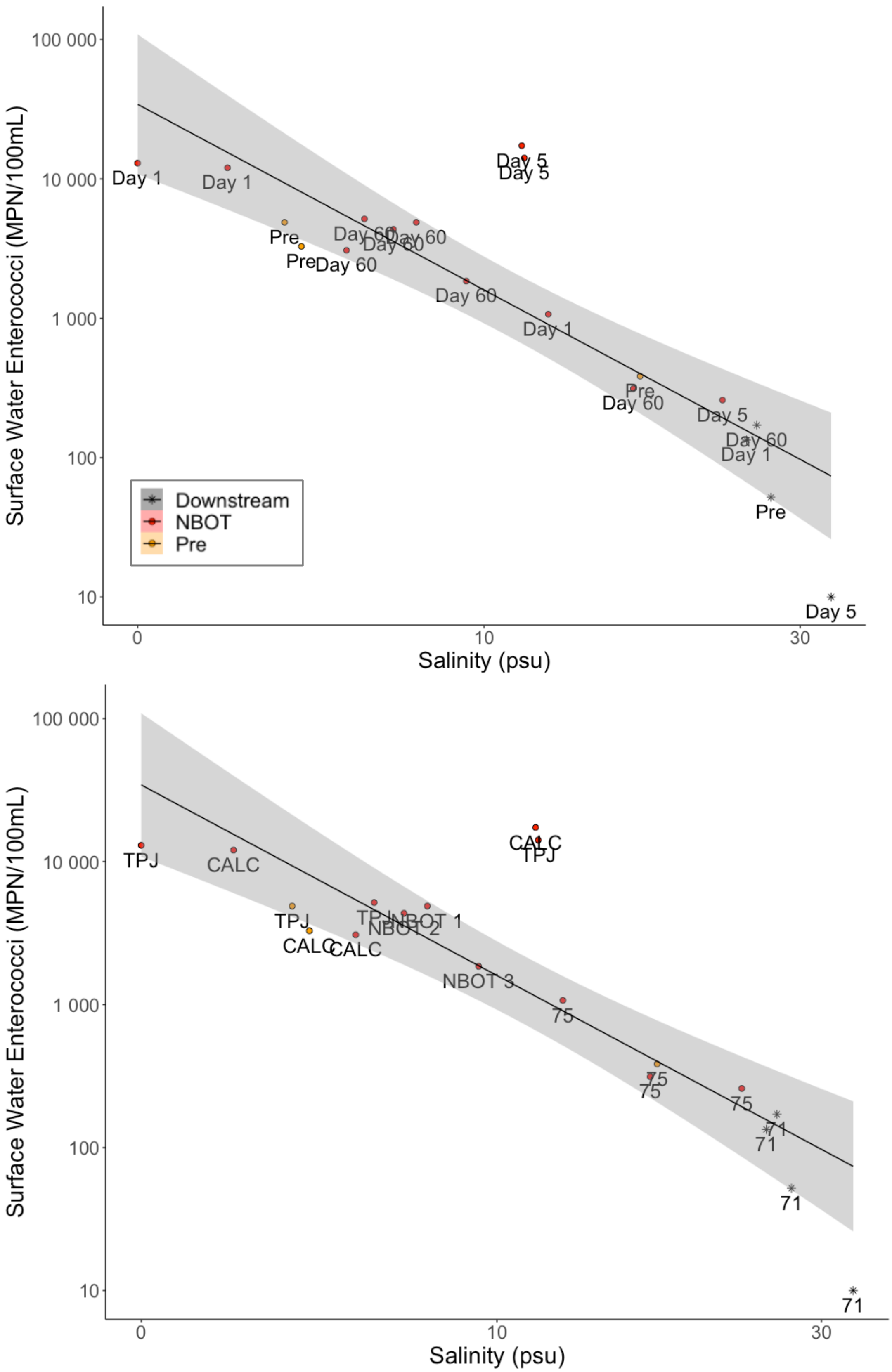
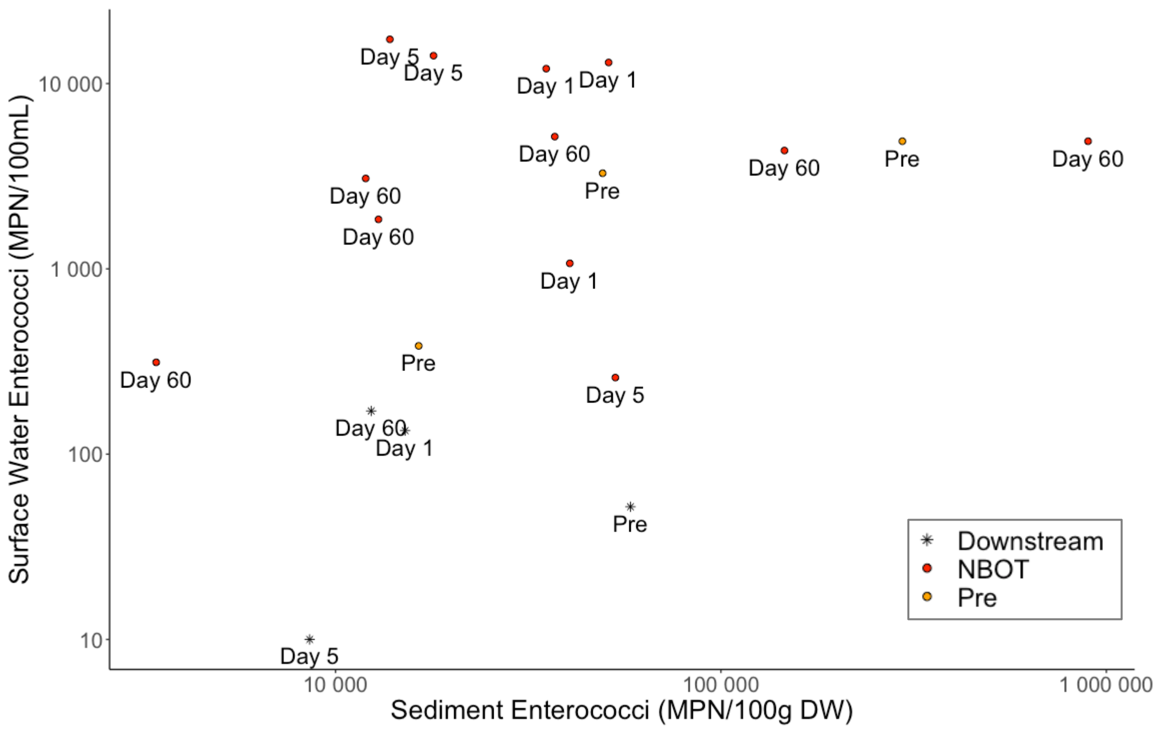


Figure 12. Relationship between surface water bacteria concentrations (y-axis) and salinity (x-axis), shown by time (top) and site (bottom). Significant surface water enterococci bacteria concentrations and salinity relationship (R^2=0.85, p≤0.0001). Visualized using: Wickham H (2016).



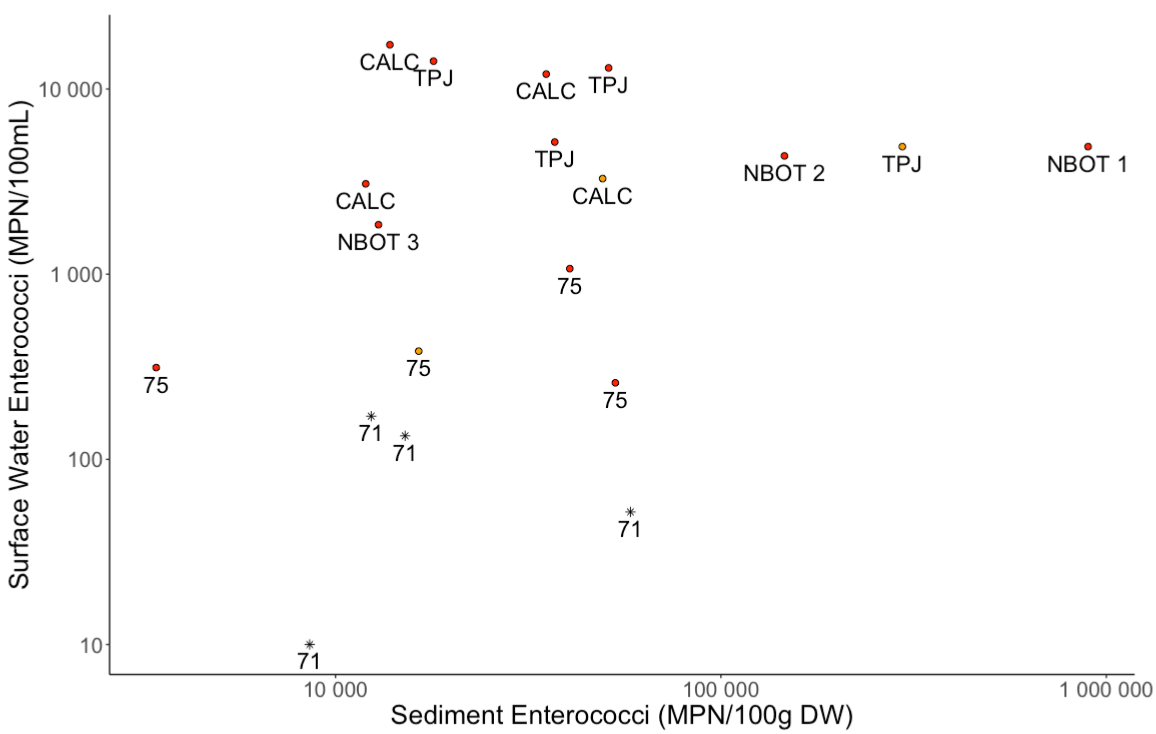


Figure 13. Relationship between surface water bacteria concentrations (y-axis) and sediment enterococci bacteria per 100 grams of Dry Weight (DW) (x-axis), shown by time (top) and site (bottom). Non-significant surface water and sediment enterococci bacteria relationships (R^2=0.12, p=0.14). Visualized using: Wickham H (2016).

<u>Study 3:</u> Diffusers

We evaluated the difference in salinities and enterococci bacteria concentrations directly from NBOT diffusers (NBOT 1, NBOT 2, and NBOT 3), compared to the respective LRD monitoring location (CALC, TPJ, and 75) (**Table 3**). This was done to document any discrepancies due to distance of the standard monitoring location to the NBOT diffuser and to assess a treatment effect at the NBOT discharge location. Average salinities were lower at NBOT 2 (8.1 psu) compared to the associated LRD monitoring location 75 (14.4 psu), which is not surprising because the monitoring sites were downstream of the NBOT units. On average pH was slightly lower at the NBOT units, but there were not large differences in pH (**Table 3**). Despite the difference in salinities, on average, enterococci bacteria concentrations differed slightly less between 75 and NBOT 2 (difference of 1,031 MPN/100mL), than TPJ and NBOT 2 (difference of 1,892 MPN/100mL), or CALC and NBOT 1 (difference of 2,589 MPN/100mL), but the bacteria concentrations are also generally lower at the downstream station 75 (**Table 3**). It should be noted that in some instances sometimes enterococci levels were lower at the monitoring location than at the diffuser (9/8/21 and 9/20/21 monitoring location 75 compared to NBOT 2; **Table 3**). This may be attributed to the disturbance and mobilization of bacteria-laden sediments at the diffuser site.

Table 3. Salinity (top), pH (middle), and enterococci bacteria (bottom) observations from NBOTs and associated LRD monitoring locations. Data shown for NBOT locations are derived from samples collected from within NBOT diffuser discharge (i.e., the area directly impacted by discharged nanobubble ozone treatment).

Salinity (ppt)

Date	CALC	NBOT 1	75	NBOT 2	TPJ	NBOT 3
8/2/21	6.7	6.6	12.0	6.8	4.8	4.6
9/20/21	6.2	7.9	16.8	9.4	6.6	7.3
Average	6.8	7.3	14.4	8.1	5.7	6.0

pH

Date	CALC	NBOT 1	75	NBOT 2	TPJ	NBOT 3
8/2/21	6.89	6.87	7.20	7.02	6.90	6.78
9/20/21	7.24	6.86	6.93	7.04	6.83	6.92
Average	7.07	6.87	7.07	7.03	6.87	6.85

Enterococci Bacteria (MPN/100mL)

Date	CALC	NBOT 1	75	NBOT 2	TPJ	NBOT 3
8/2/21	17,329	11,199	3,282	4,884	6,131	6,867
8/12/21	6,867	2,314	420	399	2,723	3,659
8/26/21	10,462	2,382	1,670	2,035	19,863	9,208
9/2/21	5,794	8,164	4,884	5,172	3,255	3,448
9/8/21	3,076	2,755	452	2,755	5,172	5,794
9/16/21	4,884	1,664	1,076	4,106	7,701	3,441
9/20/21	3,076	4,884	313	1,850	5,172	4,352
Average	7,355	4,766	1,728	3,029	7,145	5,253

Discussion

NBOT treatment effectively reduced enterococci bacteria concentrations in recirculating tanks (study 1). Based on the previous success of NBOT treatments in other mesocosms (e.g., Khan et al. 2020, Seridou and Kalogerakis. 2021) and freshwater systems (project examples include Ohio/port Mayaka, Lake Sylvan, Lake Newport in Youngstown Ohio, canals in Cape Coral; see https://greenwatersolutions.org/team/ and https://nbotsystems.com/studies-results/), the relatively small size of Jones Creek, and the capacity of treatment provided by the six NBOT generators, we anticipated measurable water quality improvements, particularly decreasing enterococci bacteria concentrations, in Jones Creek after 5 days of NBOT treatment. However, after 60 consecutive days of NBOT treatment we did not observe notable or lasting declines in enterococci bacteria in Jones Creek (study 2). In Jones Creek, after an initial increase in chlorophyll-a concentrations from Pre-NBOT to day 1 and 2, chlorophyll-a decreased over time and by Day 60 median concentrations of chlorophyll-a were comparable to the reference creek and historical values (study 2). A comparison between LRD monitoring locations and NBOT diffusers (study 3), confirmed that on average enterococci concentrations did not differ between NBOTs and monitoring locations. In other words, even when we sampled directly in the water immediately surrounding the NBOT discharge, we did not discern a notable decline in enterococci concentrations *in-situ* (study 3). Water quality parameters measured throughout the studies fell within seasonal patterns, were typical of brackish waters in South Florida, and did not reveal a noticeable improvement in the water quality in Jones Creek. These results highlight the need for further experimental work in dynamic tidal systems, and a need to further understand factors driving abundance and persistence of enterococci bacteria in tidally influenced estuarine systems and the capacity of treatment necessary to reduce bacteria concentrations. Also, we believe this study highlights the need to properly size NBOT to suit a given problem. In Jones Creek, it appears the NBOT systems were overwhelmed by the very large amount of organic matter (e.g., decaying vegetation, organic matter, leaf material) and associated bacterial concentrations.

In our recirculating tank tests of waters collected from Jones Creek, we observed rapidly decreased enterococci bacteria concentrations and visually observed improved water clarity (study 1). The tests in the recirculating tank studies showed that poor water quality conditions were effectively treated with NBOT under controlled conditions after approximately 15 minutes of treatment (study 1). NBOT application in the Jones Creek system applied the same technology *in-situ* in an open (tidally driven) shallow creek system (study 2). Even though we measured a marked decline in enterococci bacteria concentrations in the controlled recirculating tank tests (study 1), we did not observe a decline in Jones Creek enterococci bacteria concentrations during *in-situ* NBOT treatment (study 2). Recent work comparing water from a tilapia-cultured tank to de-chlorinated tap water spiked with specific bacteria indicated that the presence of tilapia feces (i.e., organic matter) greatly reduces the effectiveness of ozone nanobubble treatment on target bacteria (Jhunkeaw et al. 2021).. Furthermore, ozone reacts with dissolved organic matter and extracellular polymeric substances (e.g., biofilms, mucus, and polymers) before reacting with bacteria cells (Wu et al. 2021). Although enterococci bacteria are used as an indicator of potential human pathogens, these bacteria have also been documented in high concentrations in secondary environmental habitats, such as in sediments or organic debris (Byappanahalli et al. 2012). For example, in warmer marine waters enterococci bacteria are able to persist in organic rich environments abundant in plankton and plankton associated particles, serving as key detritivores (Mote et al. 2012). This is consistent with previous work on sediments, organic material, and enterococci in the Loxahatchee River (Harris et al. 2018). We suggest that the lack of detectable enterococci bacteria reductions with NBOT treatment in Jones Creek may be the result of excess organic matter (e.g., decaying leaf litter, dissolved organic matter, particulate organic matter, and substantial accumulations of muck sediments), where the organic matter may be reacting with (or consuming) available ozone before it is able to reduce bacteria concentrations.

The heavy bacteria load, combined with losses of diffused ozone with each tide cycle, appears to have constrained the treatment effectiveness of the six NBOT systems within Jones Creek. Post hoc analysis of the 2004/2005 survey data (Appendix L) was conducted to provide estimates of water volume exchange in the upper portions of Jones Creek. Based on available survey data we estimate approximately 37,362 m^3 of water in Jones Creek at high tide, and a tidal exchange/prism of 22,477 m^3. Using all 6 NBOT units, each pumping water at volumetric flow rates of 230 L/min, indicates that approximately 497,000 liters of water flowed through the NBOT treatment systems during each 6-hour tide cycle. Based on these estimates, without consideration of losses during tidal flux, and assuming effective treatment of water in direct contact with ozone, we estimate approximately 1.3% of the Jones Creek water volume was directly treated with ozone during each 6-hour tide cycle. It therefore appears the volume of treated water, as a ratio of total water in Jones Creek, was insufficient, given the persistently high bacteria concentrations, tidal exchange, and other flushing occurring in Jones Creek.

In Florida, enterococci can survive and thrive in biofilms in beach sands (Piggot et al. 2012), in aquatic sediments (Kelly et al. 2020), and bacteria concentrations can persist in sediments suspended in the water column (Desmarais et al. 2002). Enterococci bacteria concentrations in Jones Creek were up to 10 orders of magnitude greater in the sediments than enterococci measured in surface waters (**Fig. 13**). A dredging project conducted in 2004/2005 by the Town of Jupiter removed approximately 10,000 cubic meters of organic rich sediments from Jones Creek; equivalent to 722 large dump trucks full of material. Observations from Jones Creek residents report that present conditions are at, or even worse than, pre-dredge conditions, suggesting that the significant volumes organic sediments in Jones Creek, and these organic sediments contain high bacteria concentrations. The bathymetric survey data from the dredging project in 2004/2005 showed that, on average, the volume of sediment/muck is more than half of the volume of water at low tide (**Appendix L**). Further, the tidal exchange volume of water is less than double the sediment volume (**Appendix L**). This illustrates the magnitude of sediment accumulation and buildup of organic material within Jones Creek. Based on the literature, we suspect that the high bacteria concentrations measured in Jones Creek surface waters are the result of a buildup of sediments, and excessive detritus, combined with the high rates of resuspension. If organic material present as particulate matter is a significant source of enterococci bacteria, then disturbance of sediments (and any organic matter deposited along with the sediments), may have temporarily increased bacteria concentrations in the water column. Thus, it is possible that resuspension of particulates due to disturbance may have contributed to the slightly higher median concentrations and relatively high variability of enterococci concentrations during the first week of NBOT treatment (black lines and red boxes; **Fig. 7**). We suggest additional work investigating the persistence of enteric bacteria in sediments and organic materials to quantify (1) the general persistence of bacteria with sediments and organic matter under various conditions (e.g., temperatures, sunlight, resuspension, flushing, etc.) and (2) the ozone nanobubble treatment (amount, frequency, and duration) needed to reduce enteric bacteria concentrations under these conditions. Such fundamental studies would help us to understand how to effectively treat complex tidally driven systems.

The chlorophyll-a concentrations measured in this Jones Creek trial were much lower (< 115 ug/L, with no visible algal bloom) than other NBOT trials conducted during algal bloom events (based on visual observations, unpublished data). Although we did not observe the anticipated rapid decline in bacteria during or after NBOT treatment, we did measure a decline in median surface water chlorophyll concentrations from Day 2 to Day 60 at NBOT locations (study 2). The decrease in median chlorophyll concentrations by Day 60 occurred after an initial increase in chlorophyll-a concentrations from Pre-NBOT, to Day 1 and Day 2 of NBOT treatment (**Fig. 8**). By Day 60, median chlorophyll-a concentrations were comparable to historical values, and all median chlorophyll-a concentrations measured throughout this study fell within the historical range of variability (**Fig. 8**). Chlorophyll-a concentrations in Jones Creek are relatively high, with historical values commonly exceeding the Numeric Nutrient Criteria established for this section of the Loxahatchee River (NNC 5.5 ug/L; Chapter 62-303, F.A.C.). In Jones Creek discernable algal blooms were not visible during this study of Jones Creek. Under natural conditions, late summer–early fall declines in chlorophyll-a concentrations are to be expected due to seasonal decreased photosynthetic productivity and associated decreased biomass; a pattern we may have observed here in Jones Creek. Supplemental data using a sensor continuously deployed in Jones Creek also noted continued declines in chlorophyll-a concentrations after NBOT treatment (see https://loxahatcheeriver.org/jonescreek/). This, alongside the general decline in chlorophyll-a concentrations over time in the reference creek suggests that the measured reductions in chlorophyll-a were seasonal. However, without high frequency chlorophyll measures from our reference creek we cannot say for certain whether the reductions measured in chlorophyll were truly a seasonal trend, or of the result of NBOT treatment.

We documented metal concentrations in surface water and sediments before, during, and after NBOT treatment to note direct or indirect effects of NBOT due to changes in surface water chemistry. We did not observe any significant changes in metal concentrations of sediments or water that raised concern or could be solely attributed to NBOT. Metal concentrations in both creeks varied over time in water and sediments. Sediment metals (chromium, cobalt, zinc, molybdenum, cadmium, lead, and arsenic) measured in this study fell below maximum concentrations reported for similar urban–anthropogenically stressed mangrove systems in southern coastal China (Shi et al. 2019). Prior to NBOT treatment mean copper concentrations in Jones Creek sediments (35 mg/kg) exceeded Florida's Toxic Effects Levels (18.7 mg/kg; FDEP Florida Sediment Quality Assessment, 1994), yet were within maximum values reported in the

St. Lucie estuary (277 mg/Kg; He et al. 2006). We measured a decline in median surface water aluminum and increase in median surface water manganese both Day 5 and Day 60 of NBOT treatment compared to pre-treatment conditions, downstream, and reference locations (**Fig. 9**). The decrease in median aluminum concentrations measured in the water suggests that aluminum oxidation is occurring via ozonation. Manganese, common to soils and sediments, is generally associated with iron in mineral oxides. The combination of decreased aluminum and increased manganese suggests that ozone is reacting to generate mineral oxides or complexed metal – organics**.** By Day 60 there was an increase in median metal concentrations in NBOT sediments (aluminum, barium, copper, iron, lead, manganese, titanium, vanadium, and zinc; **Fig 10**). The form of these metal complexes was outside the scope of the grant agreement and has yet to be determined. The general increase in metal concentrations of NBOT sediments after 60 days suggests that ozonation was occurring and these metals may have precipitated out of the water column into the sediments, but the high degree of variation in metals over the course of the study makes it difficult to confidently pinpoint metal precipitates due to NBOT treatment.

Previous work has related available nutrients, tidal conditions, and short-term rainfall, to changes in chlorophyll and enterococci concentrations in the Loxahatchee (Kelly et al. 2020) and elsewhere (Jennings et al. 2018). Here, the strong significant negative correlation between enterococci bacteria concentrations and salinity indicates that tidal flux and/or an unidentified source (e.g., illicit single home waste discharge or simply organic loading) in the upstream reaches of Jones Creek are driving some fraction of the high enterococci bacteria concentrations (**Fig. 12**). Initially Stoner and Arrington (2017) suggested stormwater runoff driving poor water quality in Jones Creek. This was supported by Kelly et al. (2020) who used rainfall patterns to improve predictions of enterococci bacteria concentrations in a sub basin of the Loxahatchee River estuary. While stormwater runoff may be high in nutrients, in Jones Creek, increased precipitation does not appear to immediately result in higher enteric bacteria concentrations. Here, the observed increase in median 3-day average rainfall (**Fig. 5**) and decreased enterococci concentrations during week 2 of NBOT treatment (**Fig. 7**) suggests that freshwater inputs due to storm/precipitation events may be flushing out or diluting the high enteric bacteria concentrations in surface waters. This is supported by previous work in Jones Creek which found lower enterococci bacteria concentrations and lack of human genetic markers in the stormwater outflows (Arrington et al. 2021). LRD continues its efforts to identify and remedy the low-level human waste source identified in Arrington et al. (2021), though it is unlikely a major contributor to the high bacteria concentration observed throughout the Jones Creek system.

Conclusions

NBOT effectively reduced enterococci bacteria concentrations from Jones Creek waters in controlled recirculating tanks (study 1), indicating that NBOT can improve water quality, and specifically Jones Creek water, under controlled conditions with persistent treatment. However, the same effect was not measured on a larger scale, when treating water within Jones Creek (study 2). The discrepancy between the proven efficacy of NBOT (study 1), and our ability to apply this technology to effectively improve water quality in Jones Creek (study 2) suggests:

(1) a substantial (either point or non-point) source of the bacteria may have overwhelmed the NBOT system (i.e., bacteria concentrations, through external inputs or internal regeneration, exceeded the amount of water treated by NBOT),
(2) considerable organic load in Jones Creek overwhelmed the oxidative capacity of the ozone dose (i.e., ozone quickly reacted to the organic material present on the sediments in Jones Creek before coming into contact with/reacting to bacteria),
(3) flushing of the open, tidal system diluted the effect of the NBOT (i.e., insufficient ozone contact time), or
(4) likely some combination of the three theories.

These findings highlight a knowledge gap between a proven technology (ozone) and our ability to effectively apply this technology in a specific area with persistent high bacteria concentrations. Jones Creek is an urban/residential, shallow, brackish water, tidal ecosystem characterized by chronic high enteric bacteria concentrations, significant accumulations of organic debris (i.e., decaying vegetation) and organic sediments (i.e., muck). A better understanding of ozone dose, ozone contact time, organic load, bacterial load, and water column flushing rates are needed to determine why we did not measure the predicted effects of NBOT in Jones Creek.

Collectively, investigations of bacteria in Jones Creek over the past 10 years have found no evidence of point source pollution impairing Jones Creek. Molecular marker and chemical tracer studies conducted in coordination with FDEP fecal indicator bacteria experts revealed no significant source of human waste (Arrington et al. 2021). While these efforts identified low levels of human genetic material indicative of a single home, recreational vehicle, or homeless camp impacting Jones Creek (Arrington et al. 2021), it is doubtful that such a low-level source could be driving the extent of high bacteria at monitoring sites over 1 km apart and in separate legs of the creek. Like sewage, a stormwater point source would show spatial patterns pointing to a ‘hot spot’ for the source of bacteria concentrations and sampling of each of the stormwater outfalls, catch basins, and other inputs have not exposed a source of high bacteria. Enteric bacteria can persist in the sediments of aquatic systems and particle-bound bacteria can be resuspended with sediments into the water where the resuspended bacteria and viruses degrades water quality (Hassard et al. 2016). Given the spatial scale, separation among samples, and the high enterococci bacteria concentrations in sediments, we hypothesize that accumulations of excessive organic matter and ‘muck’ likely contribute to the chronic, high bacteria concentrations in Jones Creek.

Previous works have advocated the efficacy of ozone nanobubble treatment, recommending field applications as a logical next step (e.g., Khan et al. 2020, Seridou and Kalogerakis. 2021). We suggest additional *in-situ* experimental field studies of NBOT treatment in shallow water tidal environments under varying degrees of environmental conditions (e.g., organic matter, sediment types, salinities, flushing, etc.) or on a smaller scale with known/quantified conditions (e.g., blocking off a small segment/section of a creek where tidal flushing, freshwater inputs and organic matter has been quantified). This, as well as laboratory experiments quantifying the effects of NBOT on a set area (i.e., water volume, sediment volume, flushing, and organic load), would improve our ability to effectively apply NBOT treatment under various conditions (e.g., organic matter content, flow rates, etc.).

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Additional Website Links:

Loxahatchee River District (LRD) Riverkeeper data: https://loxahatcheeriver.org/river/river-keeper/
Loxahatchee River District (LRD) Field Sampling Quality Manuel (FQSM)
https://loxahatcheeriver.org/wp-content/uploads/2022/01/Wildpine-Lab-Field-Sampling-QM-v.1.0.pdf

FDEP's Watershed Information Network (WIN)/ Florida STORET (STOrage and RETrieval) website https://floridadep.gov/dear/watershed-services-program/content/winstoret