Good Harbor Bay Dreissenid Mussel Control Demonstration Project Final Project Report

LimnoTech

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Good Harbor Bay
Dreissenid Mussel Control
Demonstration Project
Final Project Report

Prepared for:
Great Lakes Commission
and
Invasive Mussel Collaborative Partners, including

In association with:
Great Lakes Commission
U.S. Geological Survey
National Oceanic and Atmospheric Administration
Great Lakes Fishery Commission
Underwater Construction Corporation
University of Wisconsin-Milwaukee School of Freshwater Sciences
National Park Service
University of Michigan

Prepared by:
LimnoTech

University of Wisconsin-Milwaukee School of Freshwater Sciences
University of Michigan, Department of Ecology and Evolutionary Biology

In association with:
Underwater Construction Corporation
Great Lakes Environmental Center

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Executive Summary

Zebra and quagga mussels (*Dreissena polymorpha* and *D. rostriformis bugensis*, respectively) are two aquatic invasive species (AIS) that have resulted in significant ecological and economic impacts to Great Lakes systems. The Invasive Mussel Collaborative (IMC), a binational partnership formed by federal, state, provincial, and tribal agencies and other entities, released its *Strategy to Advance Management of Invasive Zebra and Quagga Mussels* in 2018. This Dreissenid Mussel Control Demonstration Project (Project) represents a first step towards implementation of the strategy, with funding provided by the Great Lakes Restoration Initiative through the U.S. Geological Survey and Great Lakes Commission (GLC).

The Project location is a rocky reef in Good Harbor Bay in Lake Michigan near the Sleeping Bear Dunes National Lakeshore (SBDNL). The primary objective was to test the feasibility and effectiveness of a unique application of a targeted treatment method for causing site-specific mortality of invasive mussels. The Project deployed a benthic containment barrier injected with an AIS-specific molluscicide, Zequanox®. Zequanox® is a registered biopesticide (EPA registration number 84059-15) derived from a particular strain of the naturally occurring soil bacterium, *Pseudomonas fluorescens*. The effects of Zequanox® are highly selective (i.e., targeted) to invasive dreissenid mussels, and the product is low in toxicity to freshwater fish, native mollusks, insects, plants, crustaceans and other aquatic organisms (MBI, 2017).

Treatment was conducted on a 300 m² area of the reef over the course of several days in August 2019, in waters 8-10 meters (26 – 33 feet) deep. Substrate in the study area is dominated by rocky outcrops surrounded by sands, with extensive biofouling across Good Harbor Bay. The rocky reef area is dominated by dense distributions of quagga mussels, as well as nuisance levels of *Cladophora* algae. SBDNL has seen an increase in avian botulism outbreaks, so the effect that the treatment may have on the bacterial biological community and the *Clostridium botulinum* type E toxin gene was also of particular interest to the Project.

Monitoring of physical, chemical, and biological conditions before, during and after the Zequanox® treatment was conducted, to evaluate the effectiveness of the treatment, assess water quality impacts, and meet regulatory requirements. Treatment resulted in a ~95% decrease in mussel density. There were also declines in other benthic invertebrates and algae. Limited changes in water chemistry (temperature, pH, dissolved oxygen, turbidity, and conductivity) were observed during treatment but did not persist. Quantitative polymerase chain reaction (qPCR) showed no presence of the *Clostridium botulinum* type E toxin gene in microbial communities sampled from the water column and benthic biota. Results suggest that treatment with Zequanox® within an underwater containment structure may be an environmentally compatible method to reduce dreissenid abundance in targeted areas within the Great Lakes.

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1 Introduction

This report describes 2019 activities for conducting a Dreissenid Mussel Control Demonstration Project located in Good Harbor Bay in Lake Michigan near Sleeping Bear Dunes National Lakeshore. The Project included testing of a targeted molluscicide treatment method on an offshore rocky reef with invasive dreissenid mussels and nuisance levels of *Cladophora* algae.

1.1 Background

Zebra and quagga mussels (*Dreissena polymorpha* and *D. rostriformis bugensis*, respectively) are two aquatic invasive species (AIS) that have resulted in significant ecological and economic impacts to Great Lakes systems. In response to the negative impacts of these invasive mussels in Lake Michigan, a 2016 partnership effort led by the National Park Service (NPS) and University of Wisconsin-Milwaukee School of Freshwater Sciences (UWM) was implemented in Good Harbor Bay, MI, near the Sleeping Bear Dunes National Lakeshore (SBDNL), to physically remove mussels from selected test areas for research and outreach activities. This partnership effort is being expanded with the expert input and support of additional resources and capacity provided through the Invasive Mussel Collaborative (IMC), a partnership established and coordinated by the Great Lakes Commission (GLC), U.S. Geological Survey (USGS), National Oceanic and Atmospheric Administration (NOAA), and Great Lakes Fishery Commission (GLFC).

This Dreissenid Mussel Control Demonstration Project (Project), is also located in Good Harbor Bay near SBDNL, and is aligned with the IMC's recently released Strategy to Advance Management of Invasive Zebra and Quagga Mussels (IMC 2018). The Strategy identifies a set of goals and objectives to advance science and management to control invasive mussel populations and restore critical habitats in Great Lakes coastal areas. This Project, with funding provided by the Great Lakes Restoration Initiative through the USGS and GLC, aligns with and is in collaboration with IMC members.

1.2 Goals and Objectives

The Project's primary objective was to test the feasibility and effectiveness of a unique application of a targeted treatment method for causing site-specific mortality of invasive mussels. The Project deployed a benthic containment barrier injected with an AIS-specific biopesticide, Zequanox®. Treatment was conducted on Good Harbor Bay reef, where the bottom is predominantly rocky substrate and invasive mussel and *Cladophora* densities are high.

While the primary objective of this project was to test the effectiveness and feasibility of removing mussels in the open waters of Lake Michigan, a second objective was to understand how the application might affect the composition and function of the biological community, including benthic algae, benthic invertebrates, and fish. SBDNL has seen an increase in avian botulism outbreaks, so the effect that the treatment had on the bacterial biological community and the *Clostridium botulinum* type E toxin gene was also of particular interest to the Project.

1.3 Permits and Regulatory Compliance

Implementation of the Project required acquisition of National Pollutant Discharge Elimination System (NPDES), Great Lakes Submerged Lands, and Department of the Army (Corps of Engineers) permits, as well as consultation with state and federal agencies, as described below. The necessary permits were obtained prior to the treatment, and procedures specified in the permits were followed.

1.3.1 National Pollutant Discharge Elimination System (NPDES) Permit

Chemical control of nuisance aquatic species in waters of the state of Michigan is a regulated activity and requires a permit from the Michigan Department of Environment, Great Lakes, and Energy (EGLE) Aquatic Nuisance Control Program. While some aquatic nuisance control activities may be covered under a general NPDES permit, the location of this project in Lake Michigan near SBDNL meant that an individual NPDES permit was required.

An NPDES application was prepared, along with a detailed Work Plan describing the Project (LimnoTech, 2019a). EGLE developed a draft NPDES permit, which was placed on public notice for 30 days. No comments were received during the public notice period.

The final NPDES permit, issued on August 6, 2019, included maximum and minimum pH limits, and minimum dissolved oxygen limits, as well as monitoring requirements for temperature, carbonaceous biological oxygen demand ($CBOD_5$), total suspended solids, total phosphorus, specific conductance, turbidity, ammonia nitrogen, visual observation, and Zequanox® levels, as determined from turbidity measurements. The permit also required that Zequanox® be administered by a certified aquatic pesticide applicator. The permit also required development and implementation of a Pesticide Discharge Management Plan and preparation of a Discharge Summary Report (LimnoTech, 2019b).

1.3.2 Great Lakes Submerged Lands (Part 325) Permit

Part 325 of Michigan's Natural Resources and Environmental Protection Act requires a permit from the state for any alteration of the bottomlands of the Great Lakes, including dredging, deposition of fill materials, or construction of structures. Because this project included both installation of temporary structures on the bottom of Lake Michigan and placement of sand fill, a Part 325 Great Lakes Submerged Lands permit was required.

Permit No. WRP017379 was obtained from the EGLE Water Resources Division. This permit, issued on July 23, 2019 and effective through July 23, 2024, authorized conducting invasive species treatment testing by installing temporary enclosures, sealed with sandbags, then removing the enclosures and discharging a total of approximately 4.7 cubic yards of sandbag sand on the bottomlands of Lake Michigan.

1.3.3 Department of the Army Permit

Section 10 of the Federal Rivers and Harbors Act of 1899 prohibits the obstruction or alteration of navigable waters of the Unites States without a permit from the U.S. Army Corps of Engineers (USACE). Section 404 of the Clean Water Act prohibits the discharge of dredged or fill materials into waters of the United States without a permit; USACE retains Section 404 jurisdiction over navigable waters of the U.S. Therefore, this project required a permit from the Department of the Army, Corps of Engineers, Detroit District. Permit No. LRE-2019-00365-56-A19 was issued by the U.S. Army Engineer District on July 30, 2019. This permit authorized the temporary installation of containment structures to allow application of molluscicide, placement of sandbags around the perimeter to seal the enclosures, installation of monitoring sondes and subsurface buoys, and discharge of approximately 4.7 cubic yards of sand from the sandbags onto the lake bed near the study plots.

1.3.4 National Historic Preservation Act

Section 106 of the National Historic Preservation Act of 1966 (NHPA) requires federal agencies to take into account the effects of federal undertakings on historic properties. Because this project was undertaken using federal funds, a NHPA evaluation was needed. NHPA is implemented at the state level,

with the State Historic Preservation Office (SHPO) reviewing the undertaking and determining whether historic properties are affected.

The GLC submitted a request to the SHPO research office to identify any historic properties in the vicinity of the Project; no properties were identified within 2 miles of the Project site. On behalf of the USGS, GLC prepared and submitted an Application for Section 106 Review to the SHPO (June 27, 2019), determining that no historic properties were affected. The SHPO concurred with the determination of the USGS that no historic properties are affected within the area of potential effects of the undertaking (SHPO, 2019).

1.3.5 National Environmental Policy Act

The National Environmental Policy Act (NEPA) requires federal agencies to assess the environmental effects of their proposed actions prior to making decisions. Federal agencies must prepare statements assessing the environmental impact of and alternatives to major federal actions significantly affecting the environment. Federal actions may be categorically excluded from detailed environmental analyses if the action does not have a significant effect on the environment.

The USGS determined that the Project qualified for a categorical exclusion under the USGS NEPA policy, because USGS has concurrence or co-approval with another Department of the Interior bureau and the action is a categorical exclusion for that bureau (USGS Categorical Exclusion CE 516 DM 9.N). In this case, the National Park Service Sleeping Bear Dunes National Lakeshore Research Permit Programmatic Categorical Exclusion (CatEx 3.3.E.(5)) applied. The Categorical Exclusion was approved on July 23, 2019.

1.3.6 FWS Section 7 Consultation

The Endangered Species Act (ESA) directs all federal agencies to work to conserve endangered and threatened species. Under Section 7 of the Act, federal agencies must consult with the U.S. Fish and Wildlife Service (USFWS) when an action the agency carries out, funds, or authorizes may affect a listed endangered or threatened species. GLC requested informal consultation and carefully reviewed the U.S. Fish and Wildlife technical assistance and Information for Planning and Consultation websites on April 11, 2019, for federally listed threatened and endangered species. The consultation concluded that the Project would have no effect on listed species, their habitats, or proposed or designated critical habitat.

1.4 Project Team

The Project points of contact include the GLC and USGS as project funders and coordinators, individual IMC members as project advisors, and LimnoTech and Underwater Construction Corporation (UCC) as the primary implementation team, with additional support from the University of Wisconsin-Milwaukee School of Freshwater Sciences (UWM) and the National Park Service (NPS). LimnoTech managed design and implementation of the Project, working closely with UCC and Marrone Bio Innovations (MBI) on control site location selection, implementation, monitoring during implementation, and removal of the treatment method after project completion. The pre- and post-treatment effectiveness assessments were conducted by partners, including the UWM, the Michigan Department of Natural Resources (MDNR), and the University of Michigan (UM), with logistic support from the NPS. Key agencies and personnel are summarized in Figure 1-1.
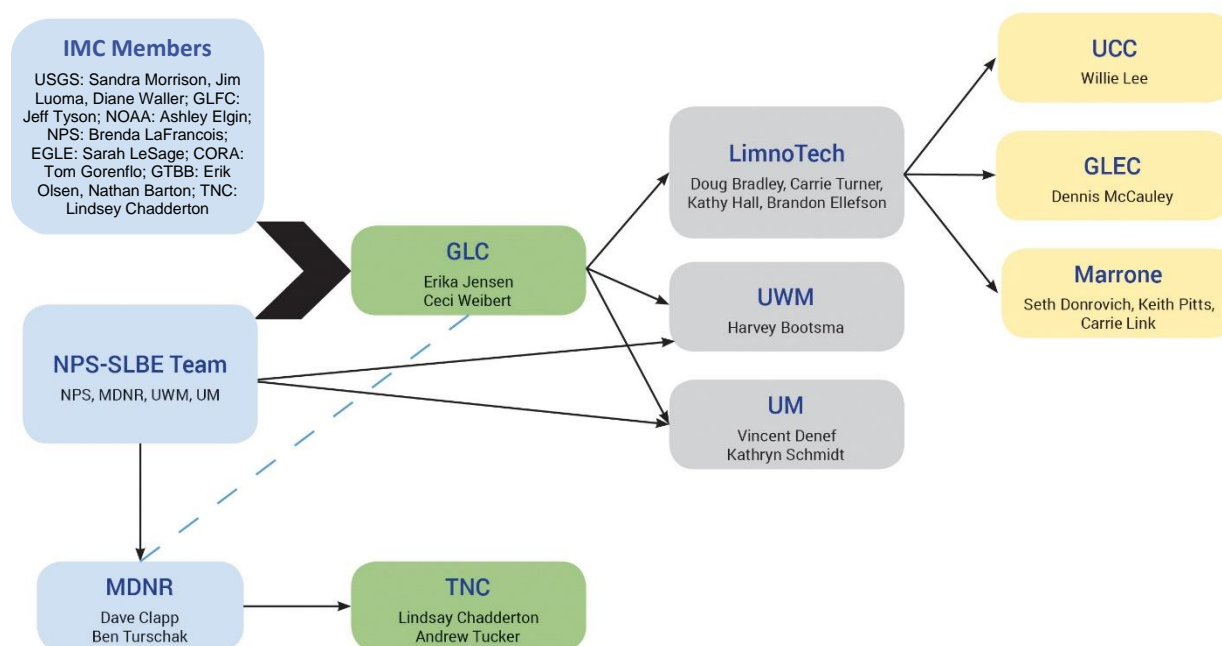
Figure 1-1. Project Team and Partners.

2 Site Description

The Project site is located at a reef in Good Harbor Bay (aka, Good Harbor Reef), offshore of Sleeping Bear Dunes National Lakeshore, MI (Figure 2-1). The reef site is located approximately two kilometers from shore (44°59'07.0"N 85°49'30.0"W) and approximately four miles southwest of Leland, MI.

Good Harbor Reef was identified as the Project site by the IMC because of its easy access from Leland's harbor, its size for accommodating the study plots (three 10 meter x 10 meter treatment areas, as well as a control area), ideal conditions as a rocky reef covered with invasive mussels, and the familiarity of Project partners with the site conditions.
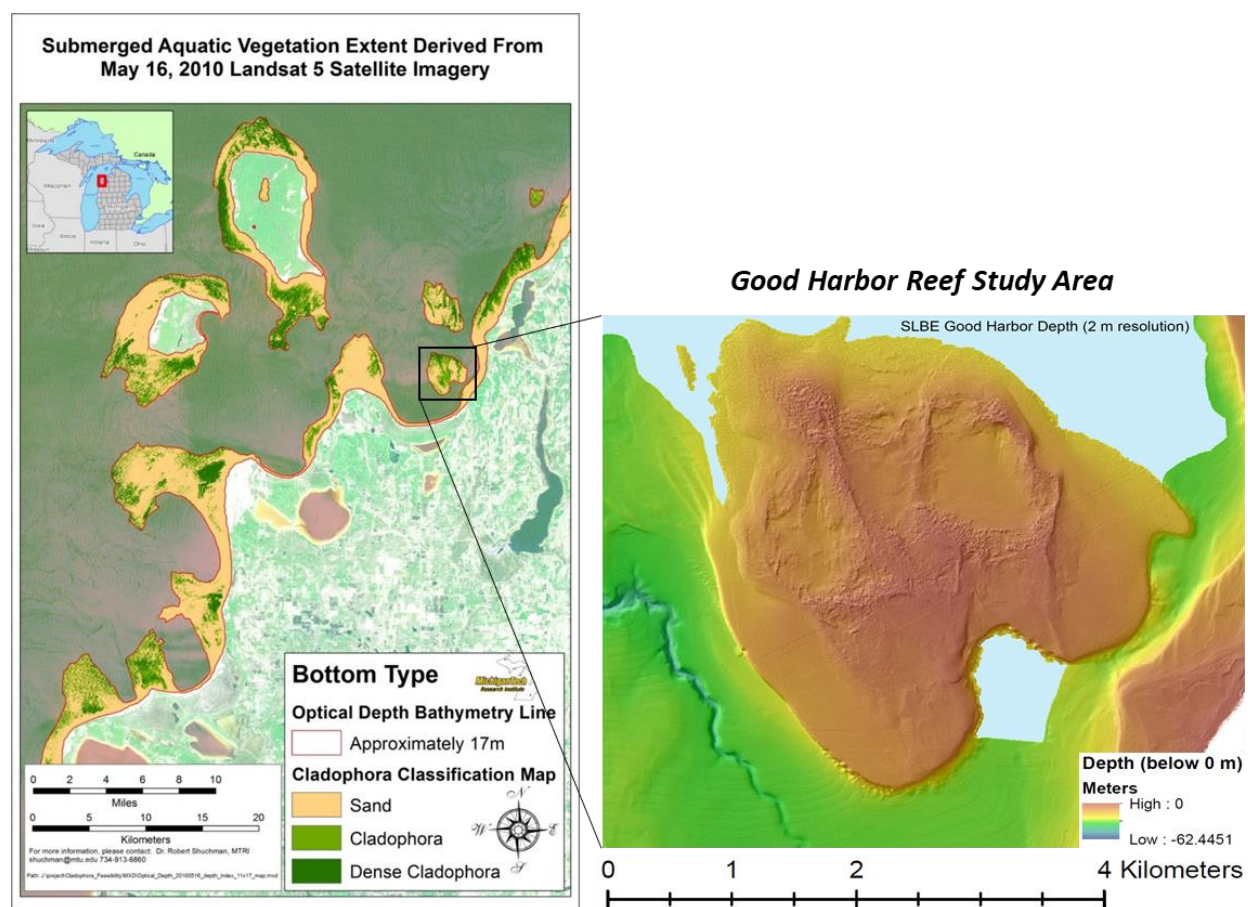
Figure 2-1. Good Harbor Reef Study Area.

2.1 Treatment Sites

The implementation site is located in Good Harbor Bay, in waters 8-10 meters (26 – 33 feet) deep. Substrate is dominated by rocky outcrops surrounded by sands, with extensive biofouling across the bay. The rocky reef area is dominated by dense distributions of quagga mussels, as well as nuisance levels of *Cladophora* (Figure 2-2).

Figure 2-2. Substrate in Treatment Area Prior to Treatment.

The center coordinates of the 10 m x 10 m treatment site locations are as follows:

- Treatment plot 1: N 44°59.697', W085°49.781', 33.4 ft deep

- Treatment plot 2: N 44°59.684' , W085°49.770', 34.3 ft deep

- Treatment plot 3: N 44°59.672' , W085°49.783', 30.0 ft deep

The depths were recorded at the time of a site reconnaissance conducted prior to treatment implementation. These locations are shown in Figure 2-3.
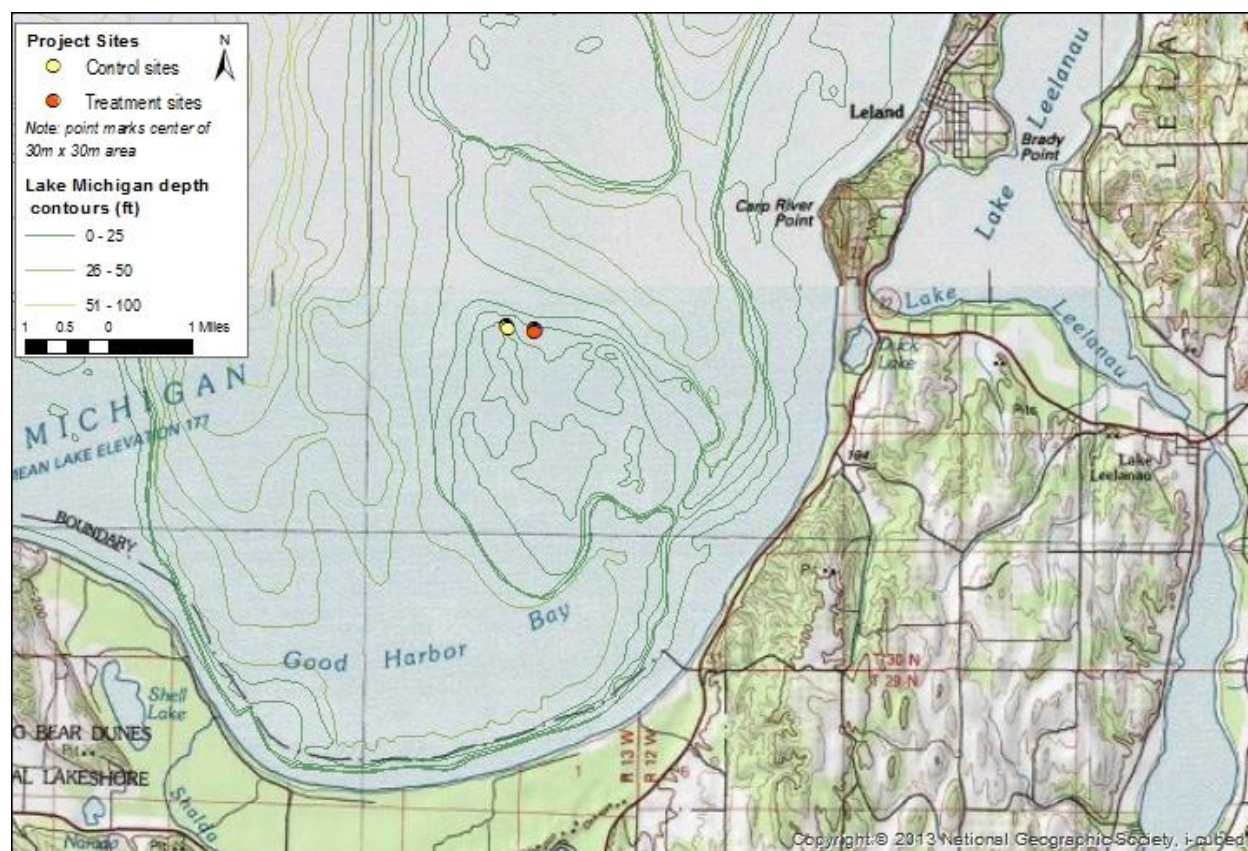
Figure 2-3. Treatment and Control Site Locations.

2.2 Control Site

The control site was locally identified, unaffected by treatment method plots, and used to provide reference information pre- and post-treatment. The site is located in close proximity to the treatment sites for ease of access and monitoring, but far enough from the treatment sites that it was unlikely to be affected during treatment activities or treatment site demobilization. The control site selected was located to the west of the treatment area, in an area that is regularly monitored by UWM, NPS, and the MDNR, and serves as a control for other ongoing, long term monitoring studies in Good Harbor Bay.

The center of the control site was located at N 44°59.770', W085°50.005'; this location is shown in Figure 2-3.

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3 Treatment Implementation

The primary objective of the Project was to test the feasibility and effectiveness of a unique application of a targeted treatment method for causing site-specific mortality of invasive mussels. The Project deployed a benthic containment barrier injected with an AIS-specific biopesticide, Zequanox®. Project activities were conducted in accordance with a Work Plan (LimnoTech, 2019a) approved by EGLE.

3.1 Treatment Material Information

3.1.1 Zequanox® Material Background

Zequanox® molluscicide is a biopesticide developed and registered by Marrone Bio Innovations (MBI) for control of invasive mussels. The product is derived from a particular strain of the naturally occurring soil bacterium, *Pseudomonas fluorescens*. Zequanox® is a commercially-prepared, spray-dried powder formulation containing dead *P. fluorescens* cells. The product is ingested by the invasive mussels as a food source; upon ingestion, the bacterial cells destroy the dreissenid mussels' digestive systems, leading to death. Zequanox® was registered in 2014 by the U.S. Environmental Protection Agency (registration number 84059-15) for controlling dreissenid mussels in open-water systems.

Studies conducted by MBI show the effects of Zequanox® are highly selective (i.e., targeted) to invasive dreissenid mussels, and the product is low in toxicity to freshwater fish, native mollusks, insects, plants, crustaceans and other aquatic organisms (MBI, 2017).

Zequanox® is stable as a dry powder. Because it is composed of dead bacterial cells, breakdown occurs quickly within the environment, and when mixed with water, it begins to lose activity within hours (MBI, 2017). According to the manufacturer, waterbodies treated with Zequanox® can continue to be used immediately after treatment for recreation purposes (i.e., swimming) as well as for irrigation of crops and turf (MBI, 2018). Zequanox® is also noncorrosive, reducing adverse impacts to boat surfaces and equipment. Finally, *P. fluorescens* is already prevalent in food and in surface water, so eating fish from Zequanox® treated waters should pose no risk to humans (MBI, 2018). The American Type Culture Collection and the American Biological Safety Association define *P. fluorescens* as "having no known potential to cause disease in humans or animals." U.S. and international health and safety regulators consider *P. fluorescens* species to be of the lowest possible risk to human health and the environment (MBI, 2018).

The effects of Zequanox® treatment on dreissenid mussels are visible within a few days of treatment, with full effect occurring over 3-21 days following application (MBI, undated). The rate of mortality varies with mussel metabolic (or biological) activity and water temperature. This mode of action prevents a quick mass kill, reducing the risk of causing anoxic conditions in the treated water body (MBI, 2018).

Zequanox® has been demonstrated to control invasive zebra and quagga mussels, and has been used by others within the Great Lakes region, including applications in Illinois, Minnesota, and Michigan. Open water applications in Michigan have included La Plaisance Bay, Lake Erie (Weber, 2015) and Round Lake in Emmett County (Tip of the Mitt Watershed Council, 2017).

Note that, while applications of Zequanox® elsewhere have required the use of an anti-foaming agent, no foaming issues were anticipated for this project, because of the application design and associated methodology. The primary need for anti-foaming agents in other projects was to address foaming responses of the Zequanox® during application, causing a quick dispersal upwards in the water column.

Because this study was designed for treatments under submerged barriers, such dispersal was not anticipated, nor observed during the treatment application.

3.1.2 Good Harbor Reef Project Zequanox® Material Handling

Prior to conducting the Zequanox® treatment, LimnoTech's project manager completed the required coursework and successfully passed the Michigan Department of Agriculture and Rural Development (MDARD) Commercial Core Applicator and Aquatic Pesticide Applicator (Category 5) exams. The relevant pesticide applicator license information is:

Name: Doug Bradley (LimnoTech)
Contact information: (734) 332-1200 (LimnoTech Ann Arbor office)
MI Certified Applicator #: 3190776

MBI prepared 80 kilograms of dry Zequanox® product, divided into 6-kg bags, and then transferred the dry product to the Good Harbor Reef location. Product mixing was conducted on-site and applied as described below. A representative from MBI, experienced and familiar with Zequanox® applications and product safety, remained on-site and accompanied the boat crew during treatment applications. The MBI representative also functioned as an additional resource advisor during the treatments.

All mixed (i.e., liquid) product was delivered during treatment to the treatment plots. All dry, empty Zequanox® containers were returned to shore and placed in trash cans for landfill disposal. Unused, dry Zequanox® product was transported back to LimnoTech offices for disposal, following manufacturer recommendations.

3.2 Treatment Distribution and Containment System Design

To ensure treatment effectiveness and minimize exposure of non-target organisms, Zequanox® treatment was conducted within contained enclosures (containment areas) placed on the reef. A critical Project component was designing a system to prepare and distribute the Zequanox® solution within and throughout the containment system while minimizing losses and leaks outside of the treatment area. The primary elements of the delivery system included (1) treatment strategy, (2) delivery system design, and (3) containment area design. Other project design elements included the monitoring strategy and deployment of caged mussels for quantitative mortality assessment.

3.2.1 Treatment Strategy

The Project was scoped to support an assessment of three 10 m x 10 m areas treated with Zequanox®. The strategy devised in the Work Plan was to treat one area per day (Table 3-1). The containment structure for the first treatment replicate was set up on the first day in the field, with treatment to be conducted on the second day. While the treatment was occurring at the first replicate, the containment structure for the second replicate was to be set up, with treatment ideally occurring the following day. Similarly, the third structure was to be set up during the treatment of the second treatment plot.

Table 3-1. Planned Treatment Schedule.

		Planned Field Implementation Schedule			
Treatment	Replicate	Set up Treatment and Under Barrier Monitoring Equipment	Conduct Treatment & Under Barrier Monitoring	Disassembly of Treatment Equipment and Under Barrier Monitoring Equipment	Notes
Barrier + Zequanox® (Triplicate)	A	Day 1	Day 2	Day 3	Treatment with Zequanox® will be conducted for 6-8 hours, and the barrier will be re-opened immediately following the 6-8 hour contact time.
	B	Day 2	Day 3	Day 4	
	C	Day 3	Day 4	Day 5	
Control (Triplicate)		Day 1-3	Day 2-4	n/a	"Treatment" = marking out a 10m x 10m grid and securing markers

At the Project kick-off meeting, interest was expressed in setting up the treatment in adjoining areas to create a larger continuous treated area. The Project implementation team determined that breaking each 10 m x 10 m area into quadrants, each 5 m x 5 m, offered several advantages over treating a single larger 10 m x 10 m area, specifically:

1. Higher probability of uniform application of Zequanox®;

2. Monitoring more likely to be representative of the enclosure conditions;

3. More manageable materials (e.g. containment framing and tarp) and handling (mixing volume of Zequanox®); and,

4. Less likelihood of containment disruption (e.g. leakage, tears, etc.) and other potential contamination (spills, wind-blown dispersal of Zequanox® dry product).

For operating purposes, the three treatment plots were identified as "A", "B", and "C". Each 5 m x 5 m quadrant was labeled 1 through 4, as shown in Figure 3-1. Since the containment system around each quadrant was secured with sandbags, it was not possible to treat all four quadrants within a given treatment area on the same day without leaving the sandbag area untreated. Therefore, the treatment strategy was devised to minimize overlap of contained quadrant areas, as shown in Figure 3-1.
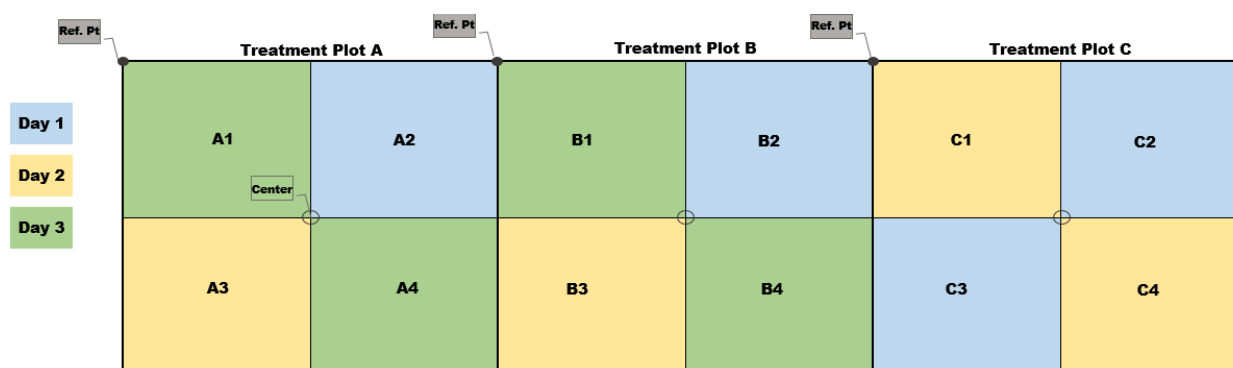
Figure 3-1. Zequanox® Treatment Area Quadrants and Corresponding Treatment Day.

This planned treatment strategy was consistent with the strategy articulated in the Work Plan and shown in Table 3-1 in that each day, a total of 100 m² was treated with Zequanox® and the treatment was

conducted over three days. Once this strategy was endorsed by the Project partners, the treatment distribution and containment systems were designed.

3.2.2 Zequanox® Delivery System

The delivery system has two major components: 1) mixing and pump delivery system; and 2) distribution system. The objectives of the delivery system were to

1. Generate a well-mixed, homogenous Zequanox® solution for application using lake water pumped on site;

2. Minimize losses from the delivery system during mixing and treatment;

3. Have sufficient power to deliver each Zequanox® solution to the corresponding treatment area in less than 30 minutes; and

4. Provide users at each end of the system with the ability to control the flow of the Zequanox® solution into the treatment area.

A schematic of the delivery system design is shown in Figure 3-2. Photos of the system during the design and testing phase are shown in Figure 3-3 and Figure 3-4. A photo of the pump mixing and delivery system in the field during one of the treatment days is shown in Figure 3-5. The procedures used and resulting performance of the delivery system are discussed in more detail in Section 3.3.

The spray distribution system consisted of a single polyvinyl chloride (PVC) wand approximately 8 feet (~2.5 m) long that could be inserted through the containment tarp on two sides of the target treatment area (see Figure 3-4). Multiple spray ports were drilled into the wand to ensure a broad and uniform distribution of the Zequanox® within the contained treatment plot (see Figure 3-17 in Section 3.3). The delivery system (pumps and wand spray ports) was designed to deliver and distribute the product quickly within the contained plots. Typically the Zequanox® was delivered to the divers and into the containment plots within 15 minutes.
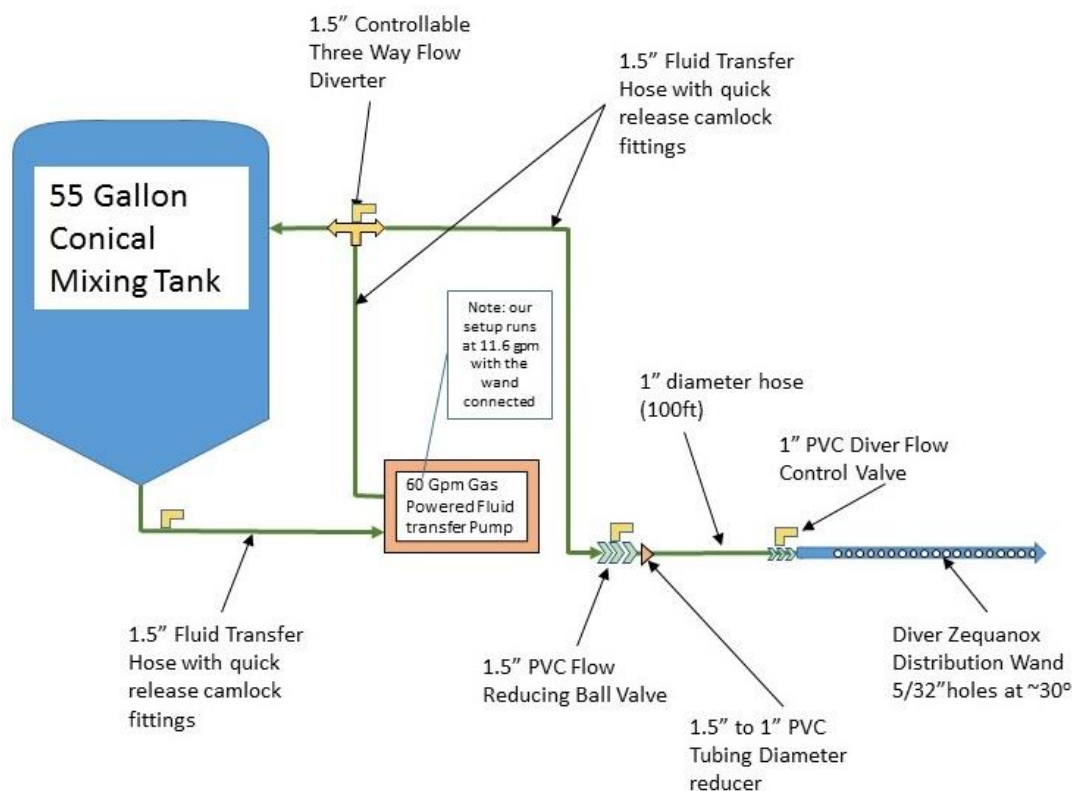
Figure 3-2. Schematic Diagram of Zequanox® Delivery System.

Figure 3-3. Pump Mixing and Delivery System during Design.

Figure 3-4. Zequanox® Distribution Wand during Design.

Figure 3-5. Pump Mixing and Delivery System in the Field.

3.2.3 Zequanox® Treatment Containment Area Design

Figure 3-6 provides a conceptual diagram of the containment and distribution system from the Project Work Plan. As described in Section 3.2.1, the design was modified so that each 10 m x 10 m treatment site was split into quadrants for containment and treatment. The frame for the enclosure was constructed as a PVC frame sized for each 5 m by 5 m quadrant within the 10 m by 10 m treatment area. Each PVC frame was constructed in sections with overall dimension of 5 meters long by 6 meters wide by 1 meter high (Figure 3-7). The slightly longer width (6 m) was used to ensure that the enclosure could be appropriately secured with sandbags and entire 10 m x 10 m area would still be exposed to the Zequanox® treatment. The height of the frame was set to 1 m (~3 feet) to accommodate the variable morphology of the reef, to minimize the overall volume treated and the associated Zequanox® product, and for ease of treatment and distribution using the wand system. The frame was constructed from 1.75-inch PVC in pieces linked with shock cords (Figure 3-8). This structure allowed the frame to be easily transported to the site and then assembled onsite on the boat and placed by the UCC dive team.
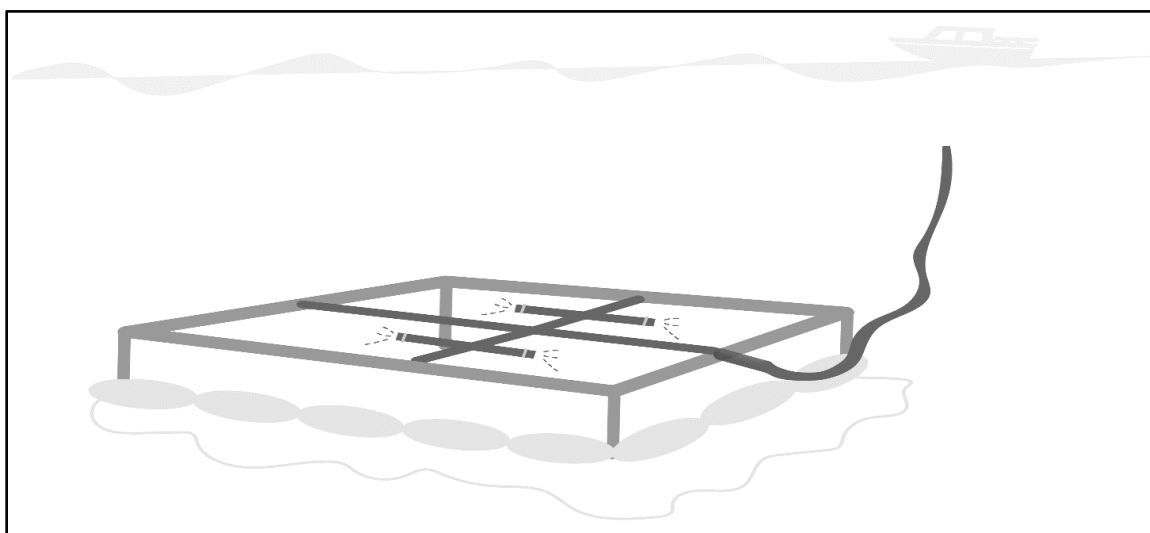
Figure 3-6. Conceptual Diagram of Zequanox® Injection and Containment System (LimnoTech, 2019)

Figure 3-7. Treatment Containment Frame during Design and Testing.

Figure 3-8. Treatment Containment Area Frame Kits.

The frame was covered by a heavy-duty (7 ounces/yard density), semi-transparent vinyl tarp (Figure 3-9) to contain the product and maintain target concentrations for the treatment duration. Two access ports (i.e., ~2-inch slots cut into each tarp that self-sealed when not in use) on opposite sides of the quadrant served as wand insertion points for Zequanox® injection, and access for water sample collections.

Figure 3-9. Tarp Material for Treatment Area Containment System.

The tarp perimeter was sealed and secured using sandbags to minimize substrate disturbance and ensure a seal given the variable, rocky substrate. The sand bags were filled with certified clean, fine aggregate sand. The sealed edges contained the Zequanox® within the enclosure for the target exposure periods. In the Work Plan, a continuous perimeter of sandbags was planned, including approximately 35 sandbags,

each weighing 30 pounds, for each treatment plot quadrant. A total of 140 sandbags was estimated to be needed to completely enclose one 10 m x 10 m treatment area or four (4) treatment plot quadrants. In practice, the tarp was successfully sealed with a smaller number of sandbags (Figure 3-10) so only 30-32 sandbags were used per treatment plot quadrant (approximately 120 sandbags spanning four treatment plot quadrants). This decreased the volume of sand disposed on site by approximately 15% from the original amount estimated in the permit application. Visual observation and turbidity measurements confirmed a successful seal of the enclosures.

Figure 3-10. Treatment Area with Containment System in Place.

3.2.4 Other Design Considerations

Caged mussels

Caged mussels were implemented as part of the Project to provide a quantitative measure of mussel mortality and to help assess Zequanox® effectiveness. Project partners collected 150 quagga mussels from a nearby location (in-situ) and placed them in small cages. Each cage was flagged and numbered, and cable-tied to a brick, which served as an anchor for the cage (Figure 3-11). The cage lids were secured with zip ties and bolt snap hardware. Five cages were placed in one of the treatment quadrants, and five were placed in the control area. The cages were left in place through the duration of the treatment and follow-up monitoring, and used as one metric of treatment effectiveness. The cage design was intended to make it easy for the divers to assess mortality over the duration of their deployment.
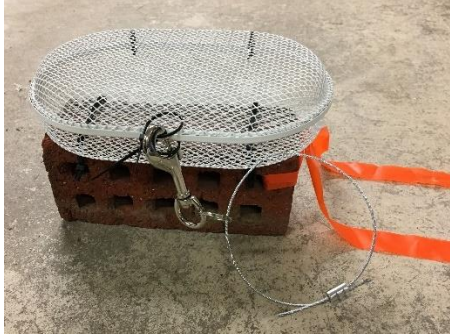
Figure 3-11. Photo of Mussel Cage and Brick Anchor.

<u>Monitoring during treatment</u>

Four water quality sondes were used within the treatment areas, with one located in each treated plot during the treatment periods. The sondes provided continuous monitoring, as described in the Monitoring section (Section 4 of this report). One water quality sonde was also deployed in the control area. The sondes were used to collect the following parameters: dissolved oxygen (DO), turbidity, conductivity, pH, and temperature.

3.3 Treatment Implementation

The Project team and boats initially staged equipment and materials and launched from Leland Harbor, MI. For implementation, four boats owned and operated by Project partners were used for the study:

1. One boat (UCC) was dedicated to supporting the divers and monitoring water quality during the treatment and exposure period;

2. One boat (UCC) was dedicated to making runs back and forth from Leland Harbor to the Project site for materials and personnel;

3. One boat (GLEC) was dedicated to transporting the dry Zequanox® to the site, mixing the product, and delivering the Zequanox® to the treatment sites;

4. One boat (UWM) was dedicated to conducting monitoring activities before and after treatments, and at the control area.

3.3.1 Treatment Implementation Schedule

The treatment period was planned for August 2019, and was dependent upon receipt of all permits and calm weather conditions. August is historically a month with relatively calm lake conditions, offering a better environment for staging, setup, and implementation of the study. Additionally, the Zequanox® product is most effective at temperatures greater than 10°C, when the invasive mussels are actively feeding; these temperatures are more likely to occur in late summer. This target treatment period also avoided potentially interfering with native fish (e.g., lake trout) spawning activities that usually start in mid-September.

A pre-mobilization meeting was held on August 5, 2019, before conducting any treatment-related activities at the site. Roles, responsibilities, site activity sequencing, product handling, pesticide spill procedures, and the health and safety plan were reviewed with the field team at this meeting.

Throughout the study, spill prevention and health and safety protocols were followed to ensure boat, worker, diver, and environment protections. Further, divers made a conscious effort to minimize substrate disturbance during all stages of the study. The frame and tarp were placed and secured one day

before the planned Zequanox® injection to allow for ambient water quality conditions to stabilize. Table 3-2 summarizes the actual treatment schedule. As noted in the table, unsafe boating weather forced a several day delay between the first and second days of treatment.

Table 3-2. Zequanox® Treatment Implementation Dates.

Treatment Area ID	Scheduled Treatment Day	Treatment Area Set-up Date	Treatment Area Start Date-Time
A1	3	8/12/19	8/13/19 11:13
A2	1	8/6/19	8/7/19 9:15
A3	2	8/7/19[a]	8/12/19 13:20
A4	3	8/12/19	8/13/19 11:44
B1	3	8/12/19	8/13/19 12:01
B2	1	8/6/19	8/7/19 9:54
B3	2	8/7/19 [a]	8/12/19 13:38
B4	3	8/12/19	8/13/19 12:18
C1	2	8/7/19 [a]	8/12/19 14:00
C2	1	8/6/19	8/7/19 10:26
C3	1	8/6/19	8/7/19 10:53
C4	2	8/7/19 [a]	8/12/19 14:17

[a] Planned treatment on 8/8/19 could not be conducted due to bad weather.

The containment frame orientation for the treatment areas is shown in Figure 3-12 for the first treatment day, Figure 3-13 for the second treatment day, and Figure 3-14 for the third treatment day. The frames have a long edge of 18 feet (6 m) and a short edge of 15 feet (5 m) so that, when rotated between quadrants, the entire 10 m x 10 m treatment site area was exposed to Zequanox®.
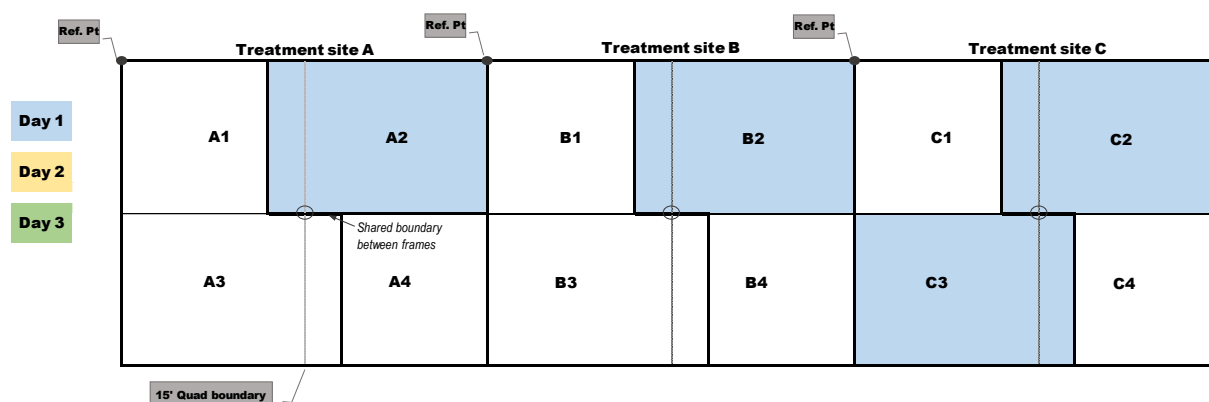
Figure 3-12. Treatment Area Setup for First Day of Treatment.
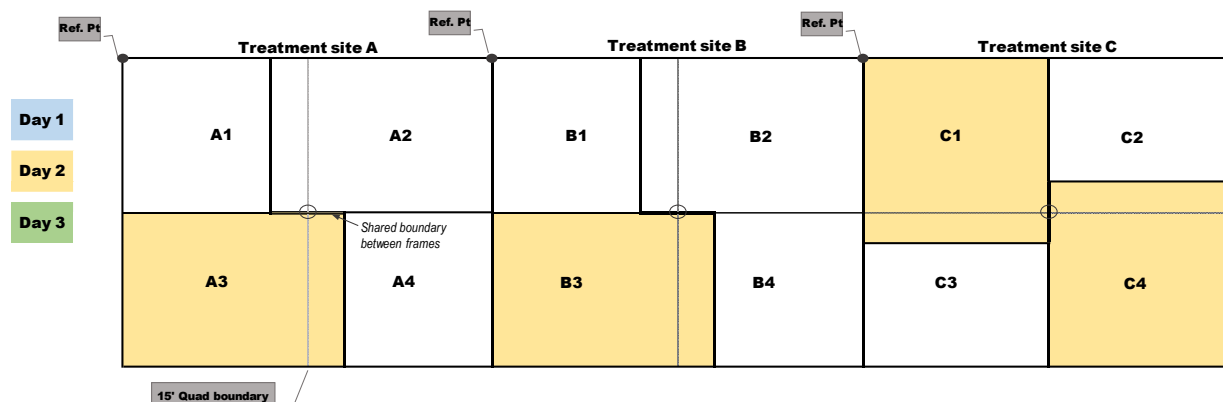
Figure 3-13. Treatment Area Setup for Second Day of Treatment.
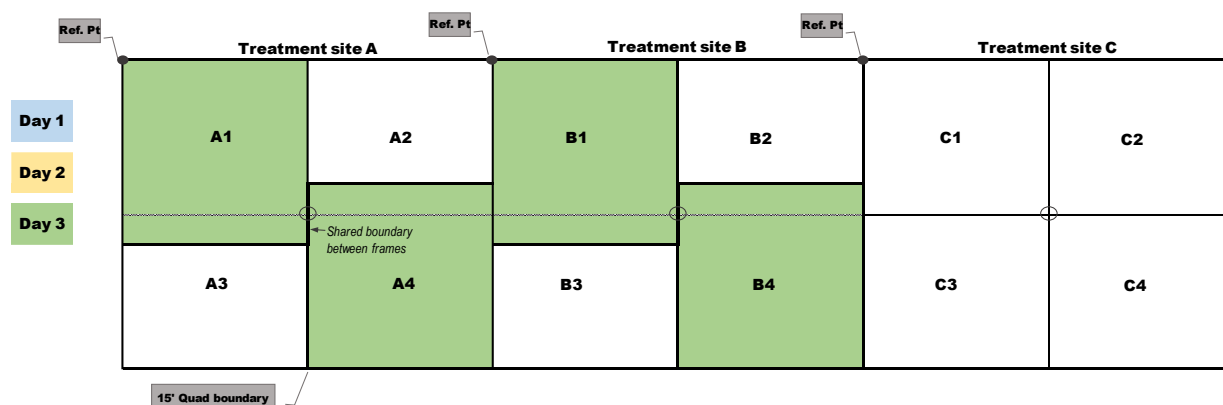
Figure 3-14. Treatment Area Setup for Third Day of Treatment.

3.3.2 Turbidity-Zequanox® Relationship

Zequanox® concentrations are strongly correlated with turbidity measurements, and turbidity can be used as a surrogate to monitor Zequanox® levels throughout the treatment period (MBI, undated). A site-specific relationship was developed on site, in accordance with manufacturer instructions, using ambient water from Lake Michigan. The turbidity-Zequanox® relationship was developed prior to beginning the treatment to allow for real-time assessment of Zequanox® concentrations.

Upon arriving at the site, the Project team collected ambient water to develop the turbidity-Zequanox® relationship. The process included the preparation of four Zequanox® solutions, each 500 mL in volume, with the following concentrations:

1. 200 mg/L active ingredient
2. 100 mg/L active ingredient
3. 50 mg/L active ingredient
4. 10 mg/L active ingredient

The turbidity of each solution was measured using a YSI EXO3 sonde similar to the sondes deployed in the field for real-time logging within the barriers (Table 3-3).

Table 3-3. Turbidity-Zequanox® Relationship Data.

Product concentration (mg/L)	Active Ingredient concentration (mg/L)	Turbidity (NTU)
400	200	107.34
200	100	53.40
100	50	27.46
20	10	5.81
0	0	0

The turbidity measurement and corresponding Zequanox® concentration were plotted and fit to a linear regression (Figure 3-15).
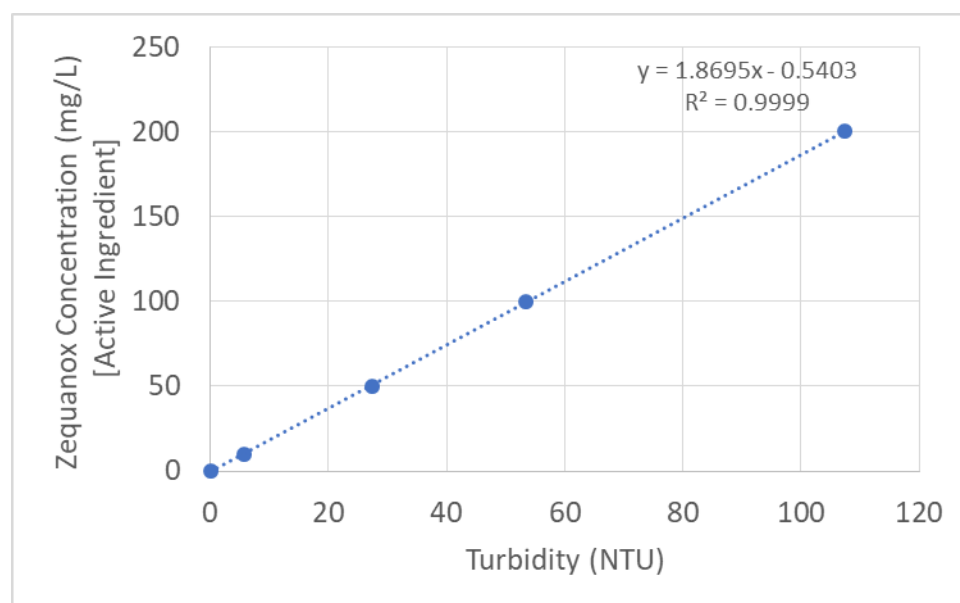
Figure 3-15. On-site Turbidity-Zequanox® Relationship.

Turbidity and Zequanox® concentration were strongly correlated, with a coefficient of determination (R^2) of 0.9999. The linear relationship defined by the fitted line was

$$y = 1.8695x - 0.5403 \qquad \text{(Equation 1)}$$

where

y = Zequanox® concentration, mg/L

x = Turbidity, NTU

Turbidity was measured at a 5 to 15-second frequency in each contained treatment area quadrant just prior to and during the treatment period. The turbidity-Zequanox® relationship defined in Equation 1 was used to estimate the Zequanox® concentration from the turbidity data.

3.3.3 Zequanox® Application

A total of 72 kilograms of the dry Zequanox® product was used for the study. Batches of treatment solution containing 6 kilograms of dry Zequanox® product mixed with 100 liters (~26 gallons) of ambient lake water were prepared for each treatment quadrant (Figure 3-16). The resulting solution was a 10% weight to volume ratio of Zequanox® dry product. The active ingredient concentration was 5% weight to volume because the dry product contains 50% active ingredient; a 5% active ingredient concentration was recommended by the manufacturer.

Figure 3-16. Preparation of a Batch of Zequanox® Treatment Solution.

Prior to treatment, the delivery system was set up in the boat and tested for proper operation before adding the Zequanox® product. A spill containment tub was set up underneath the delivery system to catch any potential leaks or spills that could have occurred during the delivery process. The large tank was secured to the side wall of the boat using ratchet straps and bungee cords. All tubing was attached and seals were checked during a leak test to prevent spills. Once the system was checked and in proper working order, the dosing procedure began.

The mixing tank was filled with 26 gallons of water, and the pump was used to circulate water through the tank. Wearing appropriate personal protective equipment, the operator slowly added 6 kg of Zequanox® product into the tank. The solution was vigorously mixed to minimize product clumping. Appendix A provides additional detail on mixing and delivery procedures.

Once the Zequanox® solution was fully mixed and the divers were positioned with the treatment wand in the enclosure, the solution was pumped into the containment area at a rate of approximately 5-10 gallons per minute (Figure 3-17). When half (50 liters/13 gallons) of the solution had been pumped, the delivery system was shut off at the pump and at the wand while the divers moved to the opposite side of the

enclosure. Once the divers again had the treatment wand inserted into the enclosure, the pumping commenced until all of the solution was pumped out of the tank. A second pump was used to pump lake water into the mixing tank to rinse the tank and ensure all of the product was delivered to the treatment area. All product safety, storage, and delivery protocols were strictly followed during the treatment application. Approximately 26 gallons (100 liters) of mixed product solution at 5% active ingredient weight to volume were pumped into each contained quadrant area to achieve a target concentration within the containment area of 100 mg/L active ingredient. These procedures were used to prepare and apply the Zequanox® mixture to each quadrant (repeated 12 times for each of the 12 quadrant areas).
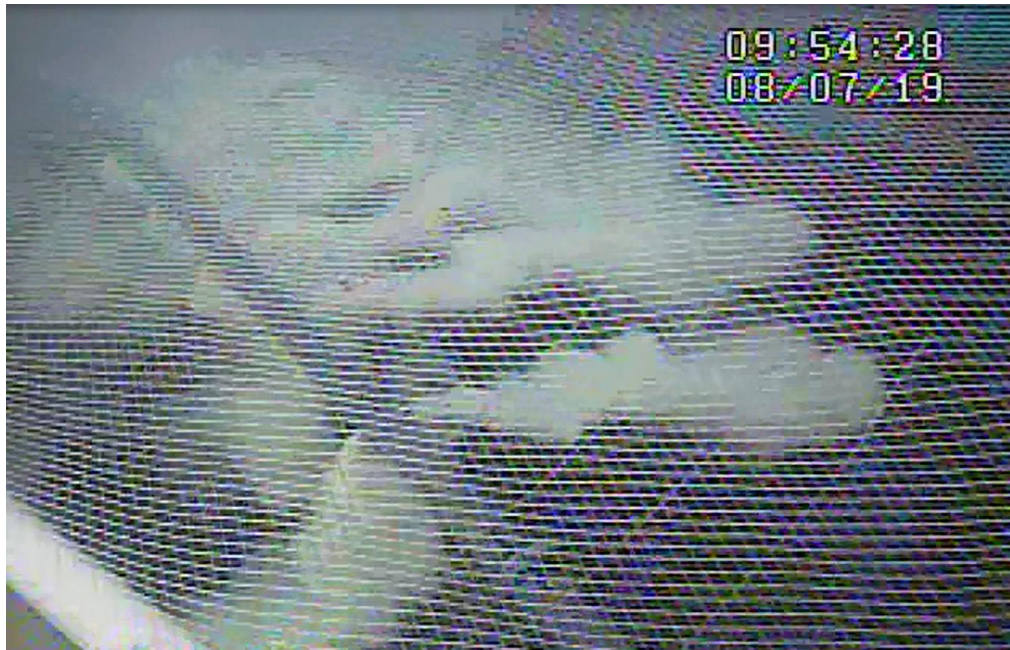
Figure 3-17. Zequanox® Solution Being Distributed within Containment Area.

After the Zequanox® was injected into the enclosure, the ports were sealed with the sandbags, and the enclosure was left sealed and in place with a target exposure duration of 8 hours, consistent with MBI and EPA guidelines. As noted previously, adverse weather conditions and high waves prevented treatment on concurrent days. The first treatment was conducted on August 7, 2019. Adverse conditions on August 8 resulted in postponing the second and third treatment days until the following week.

On the second day of treatment (8/12/19), the treatment time had to be shortened due to foul weather moving into the area. The Project team consulted with MBI regarding the effect of limiting the exposure time to less than 8 hours. Based on prior experience with the product, the MBI representative indicated that the resulting treatment time for each quadrant, which ranged between 5 and 6 hours, was likely sufficient for mussel exposure and expected to induce mussel mortality.

On the third day of treatment (8/13/19), the treatment was completed, but the crews could not return at the end of the planned exposure duration period because of the unexpected onset of hazardous conditions. In this situation, the Project team, which included MBI, decided that the best course of action was to leave the quadrants contained until the following morning. The resulting treatment time for each quadrant was approximately 20 hours. Turbidity was monitored during the exposure period in each treatment area quadrant. During the extended exposure that occurred on the third day of treatment, turbidity values tended to approach ambient values after approximately 6-9 hours.

After treatment exposure, the sandbags were removed from the edges of the enclosure and the tarps were lifted, allowing mixing with the surrounding ambient waters. Any remaining Zequanox® was rapidly

diluted through dispersion and dilution by ambient lake water. Because Zequanox® begins to lose activity within hours after being mixed with water, and the product rapidly and completely biodegrades, minimizing the risk of impacts to non-target organisms.

The tarps and PVC structures were removed from the site after the final day of treatment. Sandbags were moved by the divers to a suitable location outside the perimeter of the study area, and cut open. The sand was distributed onto nearby sandy areas of the reef and the bags were removed from the water for disposal. Several sand bags were kept intact to anchor the buoys marking the center of each treatment site for post-treatment monitoring dive crews. As indicated in the Work Plan, the distribution of the sand outside the perimeter of the study area resulted in a small area with a deposited sand layer less than two inches deep.

3.3.4 Zequanox® Delivery Results

During the exposure period, turbidity within the enclosure was monitored at 15-second intervals to ensure that the desired Zequanox® concentration was maintained. Some decrease in turbidity was expected as the product settled and the mussels began to ingest the material. No significant drops in turbidity indicative of a leak in the enclosure were observed. It is worth noting that the quadrants (quadrants A1, A4, B1, and B4) treated on the third day (8/13/19) were enclosed for an extended duration (~20 hours) due to foul weather, and the turbidity levels tended to return to ambient levels before the tarps were lifted.

Table 3-4 presents a summary of the turbidity and corresponding Zequanox® concentrations observed at each treatment area quadrant. The turbidity sensor used in quadrant A2 was initially deployed with the turbidity cap inadvertently left on, so only a partial data set was obtained in this treatment area after the cap was removed. The sensor data from quadrant C3 for all parameters was anomalous and deemed unusable by the Project team. For example, the dissolved oxygen data indicated very low dissolved oxygen prior to the treatment, which is inconsistent with other observations. The data suggest that the sonde was isolated in some fashion from the rest of the containment area volume, such as being partially buried.

The average and maximum Zequanox® concentration for each quadrant were calculated during the treatment period and are presented in Table 3-4. The target concentration of 100 mg/L (active ingredient) was reached in all ten of the treatment quadrants for which complete data are available. Four of the ten quadrants had an average Zequanox® concentration above 100 mg/L. The time series of calculated Zequanox® concentrations in each treatment quadrant are shown in Figure 3-18 for treatment site A, Figure 3-19 for treatment site B, and Figure 3-20 for treatment site C.

It should be noted that the data density of the turbidity measurements was between 5 and 15 seconds, which may not have allowed sufficient time for the turbidity reading to stabilize before recording a measurement. As a result, there is some scatter evident in the calculated Zequanox® concentrations. Also, note that the results reflect measures from a single sonde within each treatment area quadrant, so the turbidity data are reflective of only conditions in the immediate vicinity of the sonde.

Table 3-4. Zequanox® Concentration Statistics for Each Treatment Area Quadrant.

Plot	Sonde No.	Treatment Start Date-Time	Treatment End Date-Time (Tarps Opened)	Treatment Duration (hrs)	During Treatment Period			
					Maximum Turbidity (NTU)	Average Turbidity (NTU)	Maximum Zequanox® Concentration (mg/L)	Average Zequanox® Concentration (mg/L)
A1	1	8/13/19 11:13	8/14/19 8:10	20.95	304	25	567	47
A2*	1	8/7/19 9:15	8/7/19 17:00	7.75	85*	23*	159*	43*
A3	1	8/12/2019 13:20	8/12/19 19:20	6.00	177	37	330	68
A4	3	8/13/19 11:44	8/14/19 8:10	20.43	253	34	472	62
B1	2	8/13/19 12:01	8/14/19 8:10	20.15	2530	29	4728	53
B2	4	8/7/19 9:54	8/7/19 17:00	7.10	207	71	386	133
B3	4	8/12/19 13:38	8/12/19 19:20	5.70	156	80	292	148
B4	4	8/13/19 12:18	8/14/19 8:10	19.87	262	37	489	70
C1	2	8/12/19 14:00	8/12/19 19:20	5.33	256	75	478	140
C2	3	8/7/19 10:26	8/7/19 17:00	6.57	287	90	536	168
C3	2	8/7/19 10:53	8/7/19 17:00	6.12	No data-unrepresentative	N/A	N/A	N/A
C4	3	8/12/19 14:17	8/12/19 19:20	5.05	137	46	255	85

* Partial data available
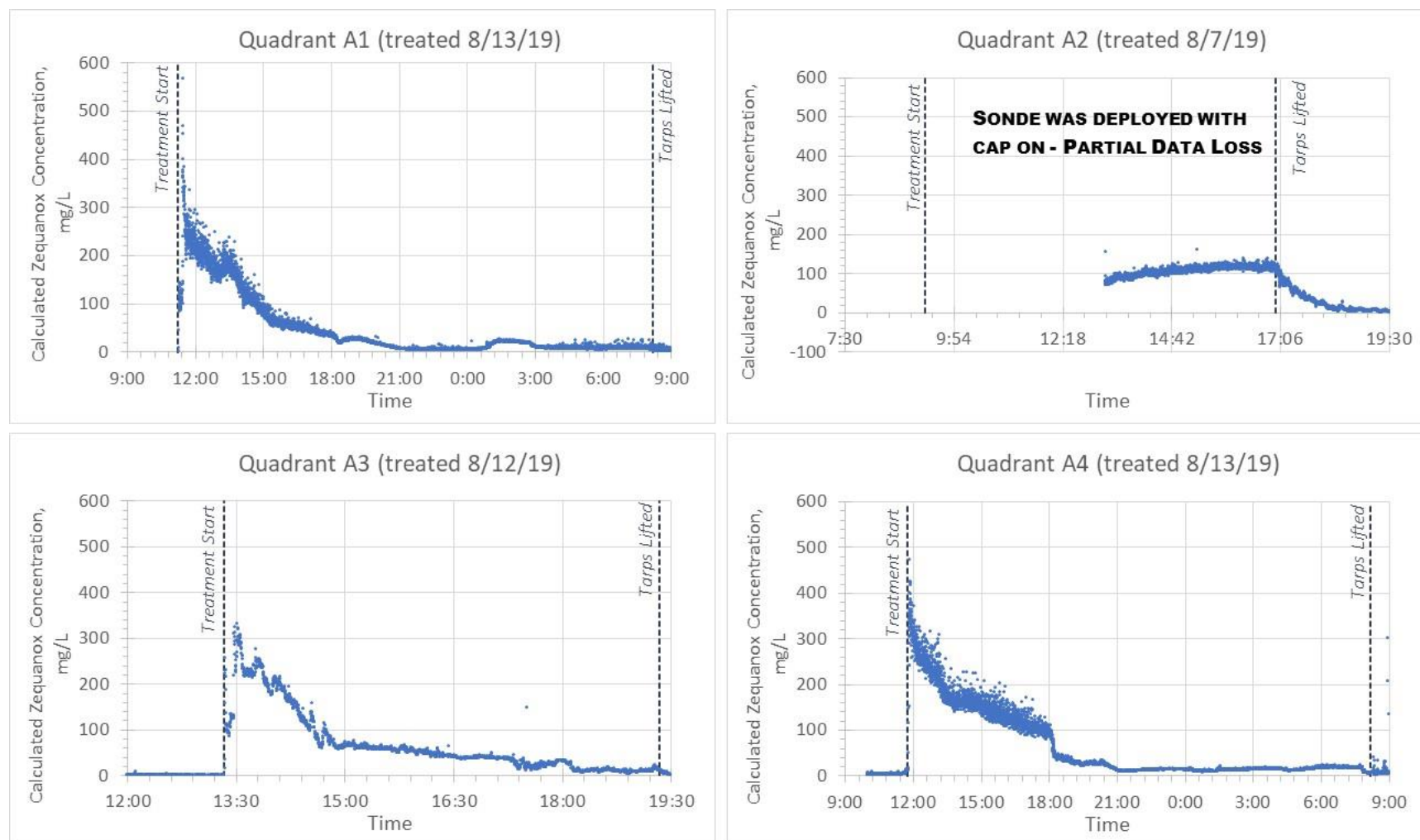
Figure 3-18. Estimated Zequanox® Concentrations in Treatment Site A Quadrants.
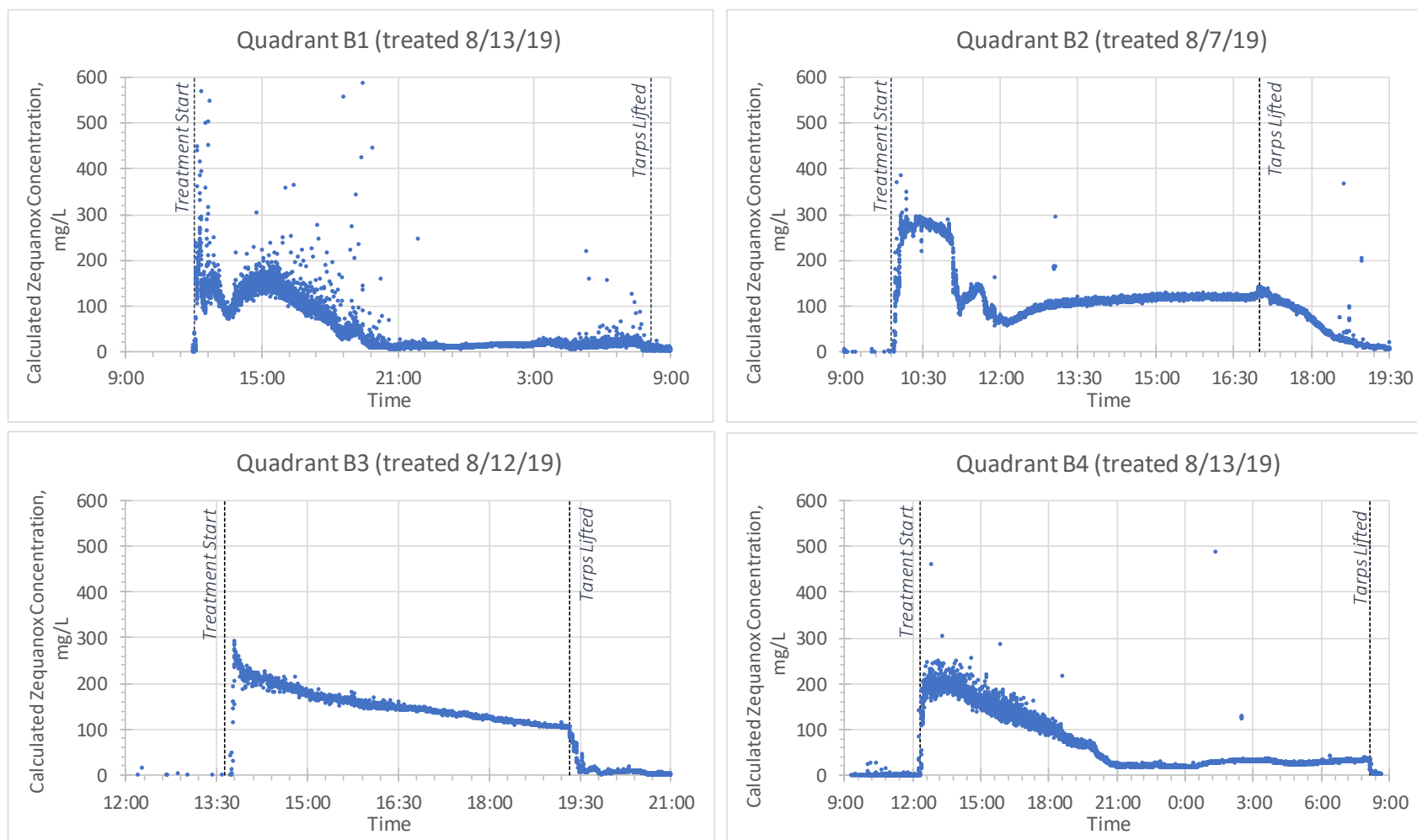
Figure 3-19. Estimated Zequanox® Concentrations in Treatment Site B Quadrants.
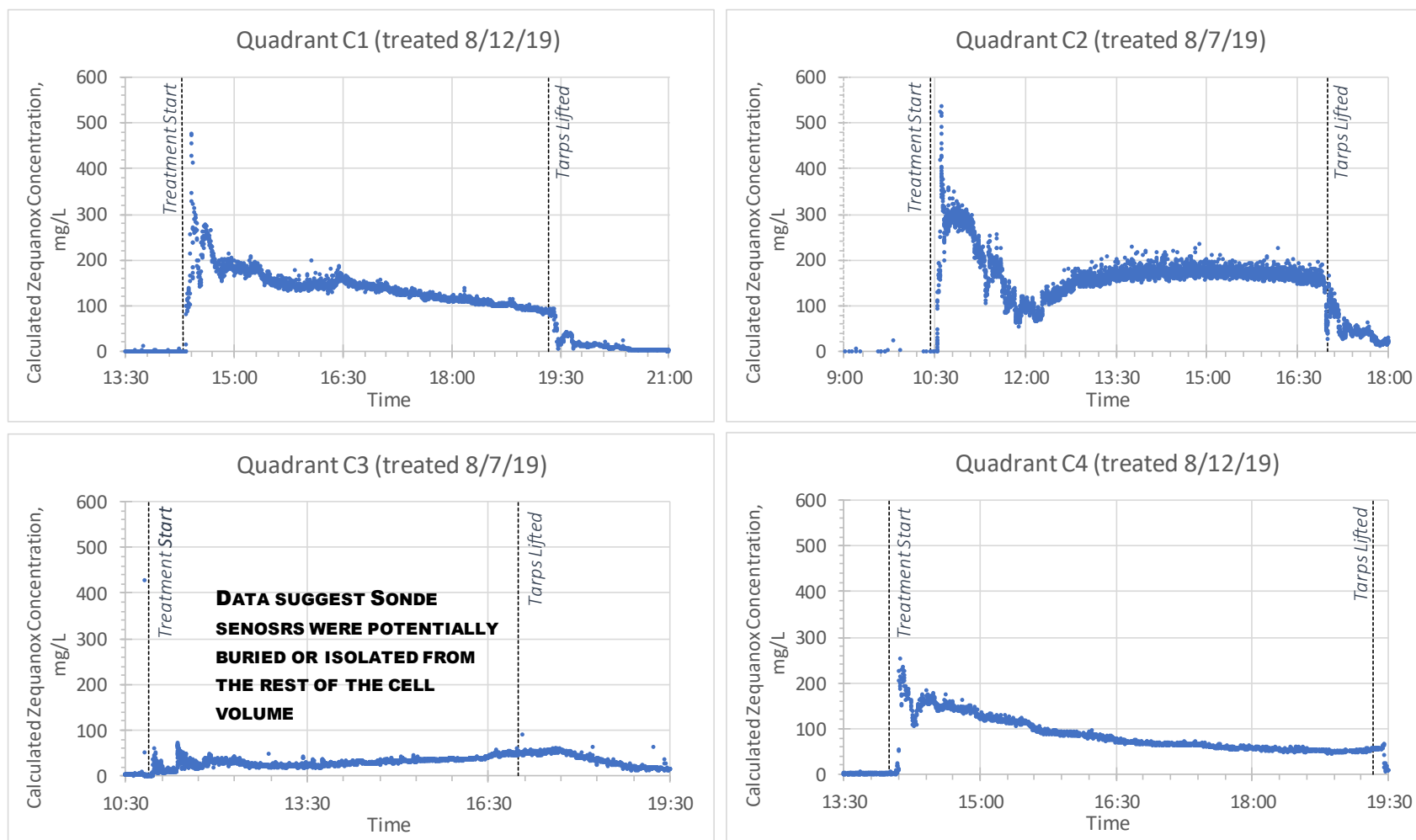
Figure 3-20. Estimated Zequanox® Concentrations in Treatment Site C Quadrants.

4 Monitoring

Monitoring of physical, chemical and biological conditions was conducted before, during and after the Zequanox® treatment to evaluate the effectiveness of the treatment, assess water quality impacts, understand how the treatment may affect the composition and function of the biological community, including benthic algae, benthic invertebrates, and fish for up to six weeks post-treatment, and meet NPDES permit requirements.

4.1 Overview

Monitoring of the Project was conducted primarily by Project partners, including the University of Wisconsin-Milwaukee (UWM), the Michigan Department of Natural Resources (MDNR), and the University of Michigan (UM), with logistic support from the National Park Service (NPS). Table 4-1 summarizes the constituents monitored within the treatment plots.

Table 4-1. Monitoring of Treatment Plots.

Constituent	Pre-treatment	During treatment	One day post-treatment	One week post-treatment	Two weeks post-treatment	One month post-treatment
Dissolved oxygen	●	●	●	●	●	●
Ammonia-Nitrogen (as N)	●	●				
Temperature	●	●[2]	●	●	●	●
pH	●	●[2]	●	●	●	●
Conductivity	●	●[2]	●	●	●	●
Turbidity[1]	●	●[2]	●	●	●	●
Total Suspended Solids	●		●	●		
CBOD$_5$	●		●	●		
Total Phosphorus	●		●	●		
Dissolved Phosphorus (SRP, TDP)	●		●	●	●	●
Mussel density & size distribution	●			●		●
Benthic invertebrate density & taxonomic composition	●			●		●
Benthic DO	●	●[3]	●	●	●	●
Bacteria	●		●	●	●	●
Video documentation	●		●	●	●	●
Caged mussel assessment		●				

[1] Turbidity was used as a surrogate to monitor Zequanox® levels throughout the treatment period

[2] During treatment, these parameters were monitored by LimnoTech/UCC under the barrier; additional water column data were collected by UWM-deployed sonde.

[3] LimnoTech/UCC monitored DO under the barrier during treatment, as described in Section 4.3.1. UWM conducted additional DO monitoring before and after the treatments.

Analytical methods, holding times, and detection limits for the chemical parameters listed in Table 4-1 are summarized in Table 4-2.

Table 4-2. Analytical Methods and Detection Limits

Parameter	Description	Type of Measure-ment	Sample Container	Preser-vative	Sample Volume	Sample Holding Time	Reference Method[1]	Minimum Detection Limit[2]
TSS	Total suspended solids	Grab	Plastic	None	1000 mL	7 days	EPA 160.2	1 mg/L
$CBOD_5$	5-day Carbonaceous oxygen demand	Grab	Plastic	None	1000 mL	48 hours	SM 5210 B-2011	2 mg/L
TP	Total phosphorus	Grab	Plastic	H_2SO_4	500 ml	48 hours	EPA 365.1	0.01 mg/L-P
SRP	Soluble reactive phosphorus	Grab	Plastic	H_2SO_4	500 ml	48 hours	EPA 365.1	0.01 mg/L-P
TDP	Total dissolved phosphorus						EPA 365.1	0.01 mg/L-P
PP	Particulate phosphorus						EPA 365.1	0.05 mg/L-P
$NH_3 - N$	Total ammonia as nitrogen	Grab	Plastic	H_2SO_4	500 ml	28 days	EPA 350.1	0.05 mg/L-N
Temp	Water temperature	*In-situ*	n/a	n/a	n/a	n/a	sonde	0.1 °C
pH	pH	*In-situ*	n/a	n/a	n/a	n/a	sonde	0.1 S.U.
Turbidity	Turbidity	*In-situ*	n/a	n/a	n/a	n/a	sonde	0.1 NTU
Cond	Conductivity	*In-situ*	n/a	n/a	n/a	n/a	sonde	1 uS/cm
DO	Dissolved oxygen	*In-situ*	n/a	n/a	n/a	n/a	sonde	0.1 mg/L

[1] 40 CFR Part 136 method or equivalent

[2] Sensitivity value listed for field parameters – instrument must be capable of reporting to the nearest incremental sensitivity value listed

4.2 Mussels

To help assess Zequanox® effectiveness, UWM divers placed five small mussel cages (Figure 4-1) in one of the treatment enclosures and five cages at the control site, as described in Section 3.2.4. Each cage contained 15 quagga mussels that had been collected near the treatment site. These caged mussels remained in place throughout the treatment and were used as controlled effectiveness checks for the Zequanox® treatment. The divers checked the status of the mussels in these cages before treatment (August 2), one day after treatment (August 8), approximately one week after treatment (August 12), one month after treatment (September 5), and two months after treatment (October 8). Figure 4-2 compares mortality in the cages within the treatment plot and at the control site, showing substantially higher mortality in the treatment site. At the treatment site, on average, approximately 41% of the caged mussels had died five days after treatment. Mortality increased to ~63% after one month, and remained at that level between one and two months post-treatment.
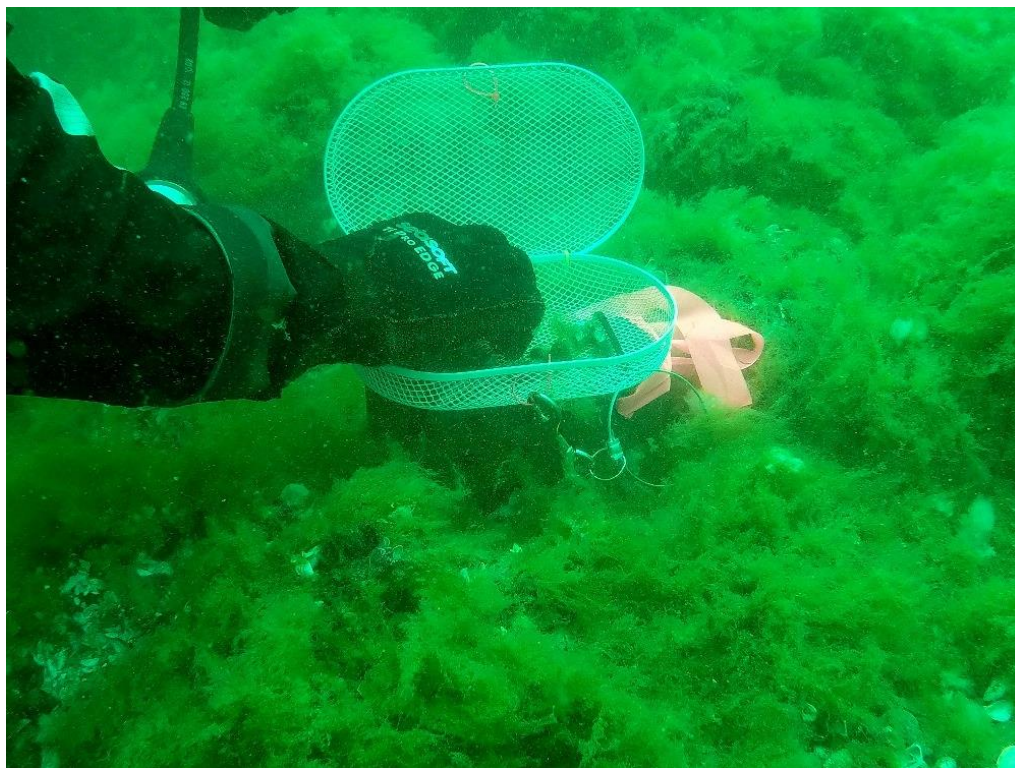
Figure 4-1. Caged Mussels used to Evaluate Treatment Effectiveness.
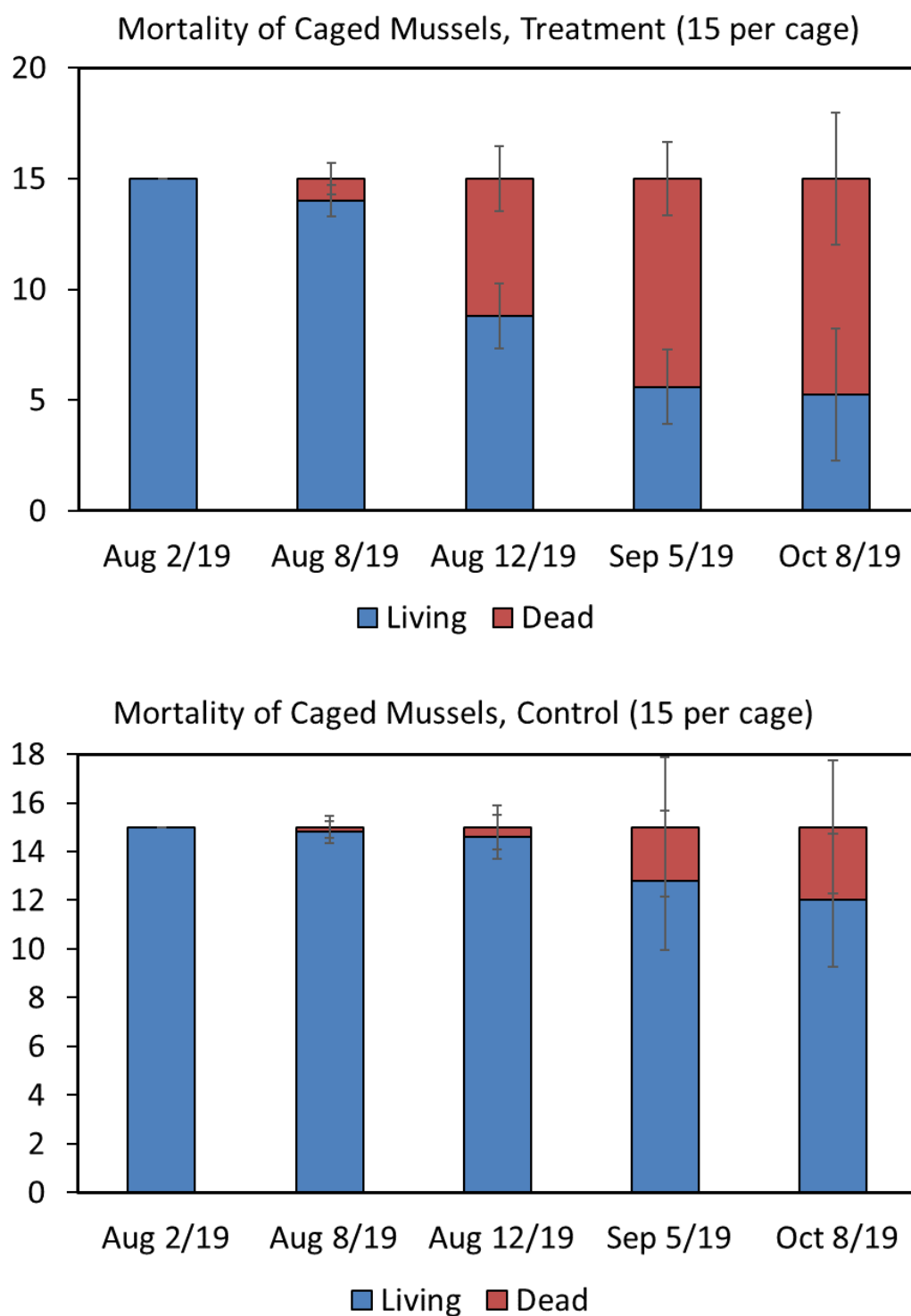
Figure 4-2. Caged Mussel Mortality at Treatment (top) and Control (bottom) Sites.

In addition to the caged mussels, mussels attached to natural substrate were sampled by hand scraping. This method has been used at a nearby experimental invasive mussel removal site for the past several years, and involves placing 20 x 20 cm quadrats on the tops of rocks with an upper horizontal surface area of at least 40 cm x 40 cm. All biota within each quadrat were hand-scraped by a scuba diver and placed in a Whirl-Pak bag. Upon return to the laboratory, the mussels were separated out, counted, and sized.

Figure 4-3 compares mussel density on the natural substrate in the control and treatment plots over time. Similar to the caged mussel data, mortality was much higher in the treatment area. Mortality on the natural substrate appears to have been substantially higher than mortality of the caged mussels. On the natural substrate, mortality was 97%, as compared to 63% average mortality of caged mussels over the same time period. Possible explanations for this difference include:

1. The cages were elevated above the substrate, and as the Zequanox® settled during the treatment period, the concentration in the cages may have been lower than at the natural substrate.

2. Turbulence within the cages was likely less than in the ambient water, which may also have reduced the effective concentration of Zequanox® to which the mussels were exposed.

3. Conversely, after the tarps were removed, the slightly elevated position of the cages may have allowed them to be flushed more quickly than the benthic boundary layer immediately above the natural substrate.

Mussel mortality in both the mussel cages and on the natural substrate within the treatment area indicates that the treatment was effective.
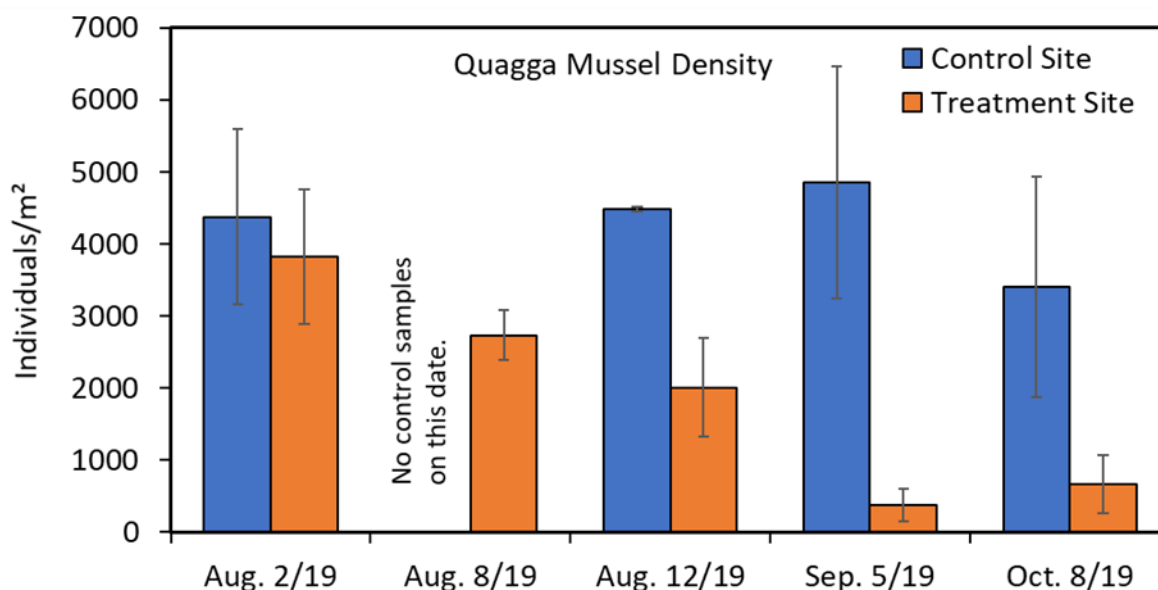
Figure 4-3. Comparison of Mussel Density in Control and Treatment Plots.

The quagga mussel length-weight relationship can serve as an index of mussel nutritional status (Nalepa et al. 2010). The UWM team determined the length-weight relationship of mussels collected at both the control and treatment sites on five (5) dates, once before Zequanox® treatment and four (4) times post-treatment. For each date / site combination a minimum of 50 mussels, ranging from 5 millimeters (mm) to 30 mm in length, were selected and measured for length (to the nearest mm) and shell-free tissue dry weight (to the nearest milligram [mg]). Temporal differences in the length-weight relationship were greatest between the initial and final dates, and so only data for those dates are presented here. Mussels smaller than 15 mm varied little in weight between dates and control vs. treatment (Figure 4-4).

However, mussels larger than 15 mm tended to be heavier at the control site than at the treatment site, both before and after the treatment date of August 7. The cause of this apparent spatial difference is uncertain, but may reflect differences in physical conditions (temperature, currents) and food supply at the two sites, as the two sites are separated by a slightly shallower spit.

At both sites there was an apparent decrease in mussel weight per unit length over time for mussels larger than 15 mm. While few studies of dreissenid spawning behavior have been conducted in the Great Lakes, Nalepa et al. (2010) found that, within the epilimnion, quagga mussels spawn primarily between August and September, and spawning results in a significant decline in mussel condition. Our conclusion is that, over the period of this study, mortality of mussels on the treatment site was high, but there was little effect of the treatment on the condition of the mussels that survived, and the weight loss of mussels >15 mm between August and October was likely the result of spawning.
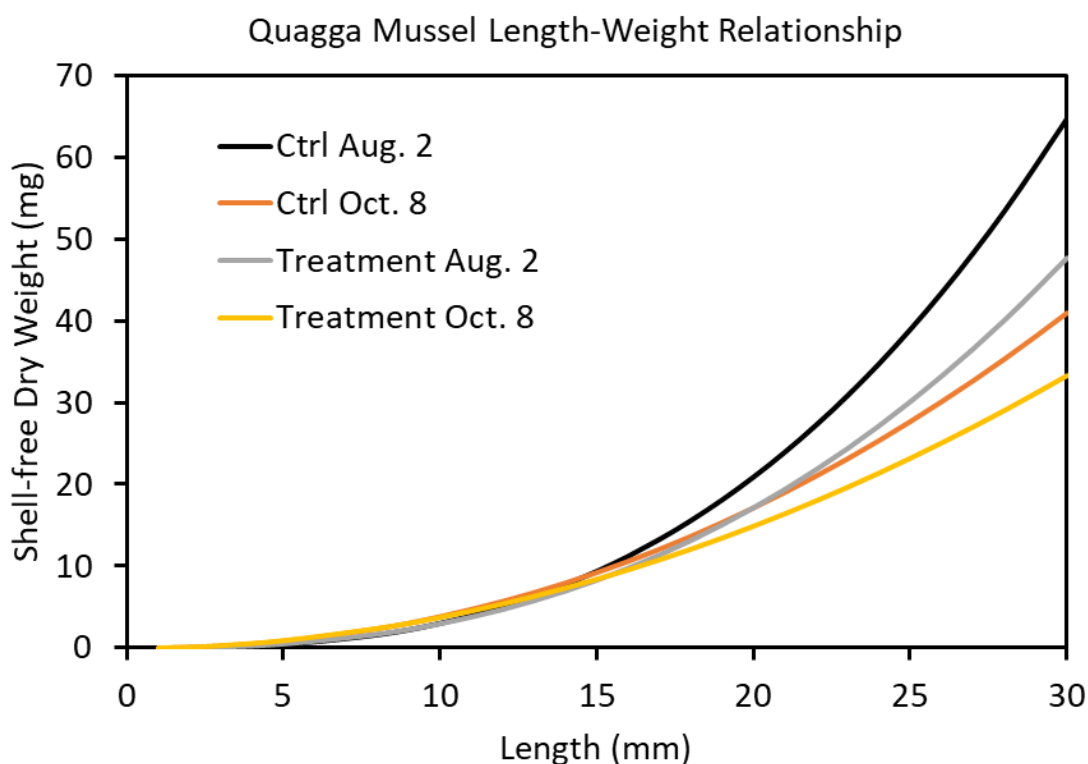
Figure 4-4. Length-Weight Relationship of Quagga Mussels on the Control Site and the Treatment Site Before and After Zequanox® Treatment.

The quagga mussel length distribution at both the control and treatment sites was also monitored over time to determine whether the mortality response to treatment varied among size classes. Figure 4-5 presents those data, which indicate that the few mussels that survived the treatment were the larger ones; virtually all of the smaller mussels died.
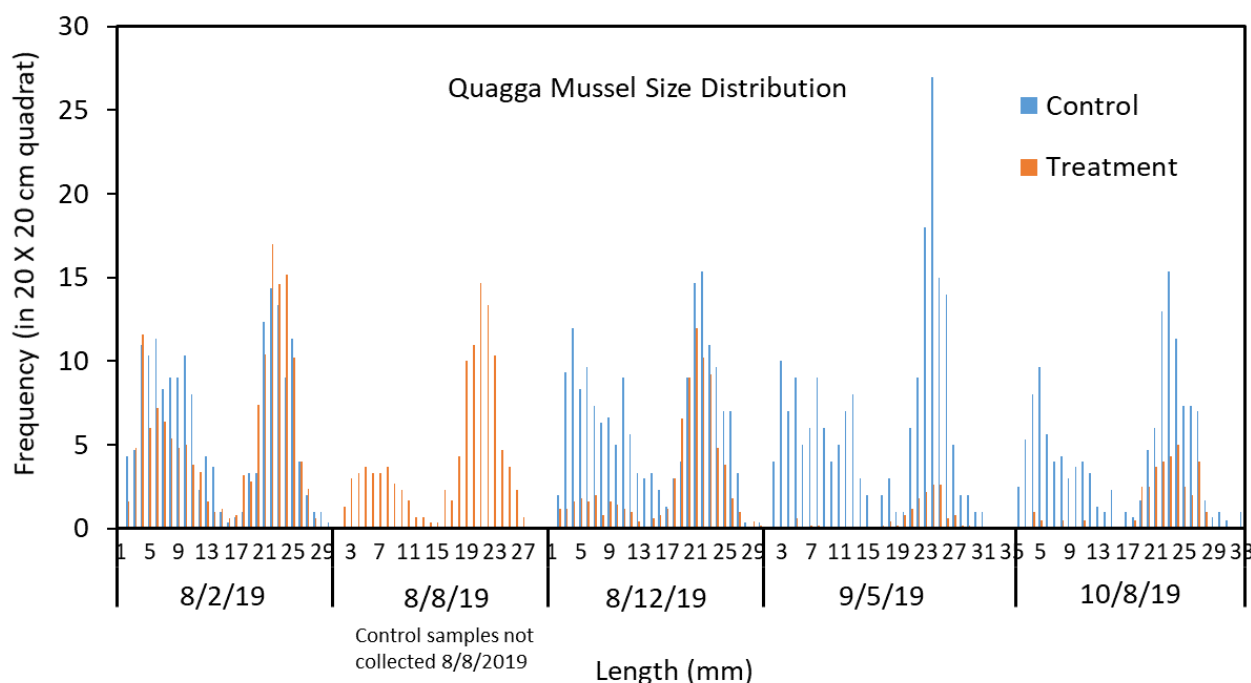
Figure 4-5. Size Distribution of Quagga Mussels in the Control and Treatment Areas.

4.3 Water Quality

4.3.1 Dissolved Oxygen

Each treatment plot contained a water quality sonde that recorded dissolved oxygen (DO). In general, the data show that DO typically began to drop a few hours after the Zequanox® was applied, and increased once the enclosures were opened (Figure 4-6, Figure 4-7, and Figure 4-8). The NPDES permit for the Project included a minimum DO limitation of 7.0 mg/L. Over the course of the treatment, there were short-term, localized excursions below the minimum DO limit. These were reported to EGLE in accordance with permit requirements.

Table 4-3 summarizes the DO results for the 12 treatment quadrants (labeled A1, A2, A3, A4, B1, B2, etc.). The table shows, for each treatment plot, the date and time that the treatment started, the time the tarp covering the enclosure was removed, the treatment duration (difference between treatment start and tarp removal), the time that the DO excursion started, the time the DO excursion ended, and the total duration of the non-compliance. In some cases, the DO excursion was intermittent; in these cases, the duration of non-compliance was determined by summing only the periods in which DO fell below 7.0 mg/L. (Note that the table does not include excursions for plot C3; data for this plot appear to be anomalous. Within plot C3, DO was well below 7.0 before treatment began, and fluctuated widely through the treatment period. It is surmised that the sensor may have been inadvertently covered by sediment during deployment.)
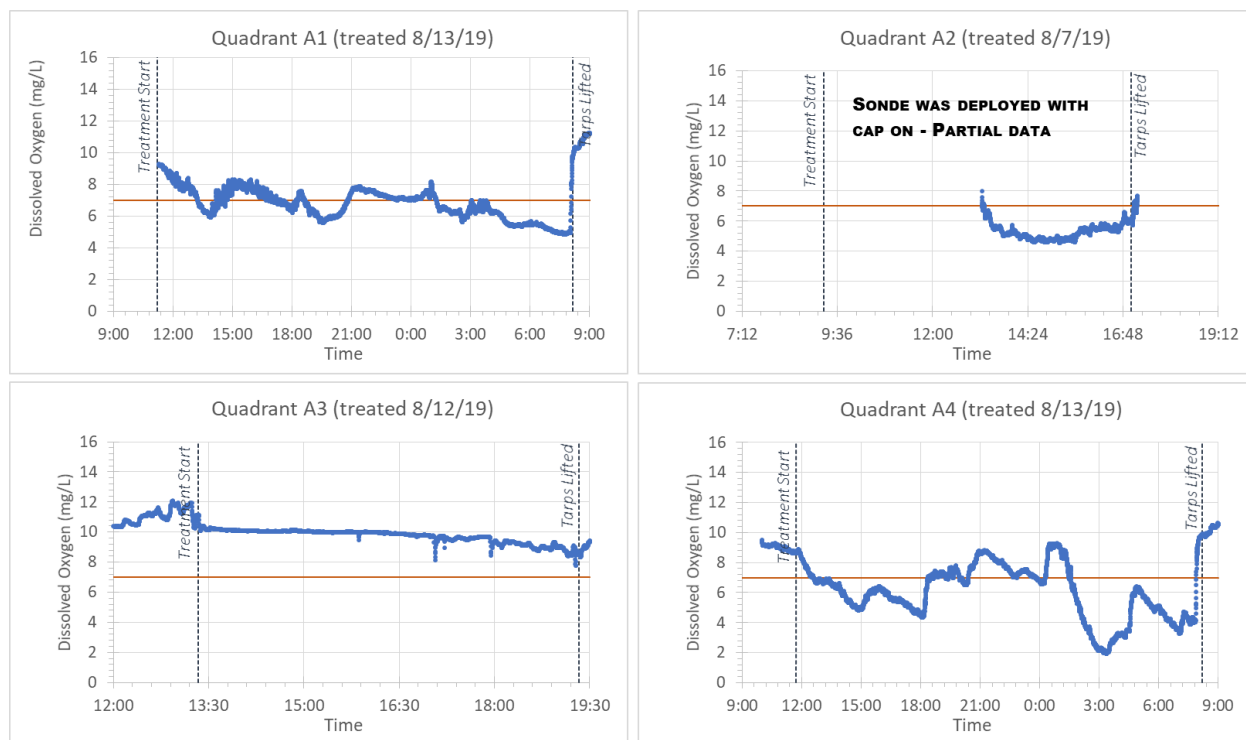
Figure 4-6. Dissolved Oxygen Concentrations in Treatment Site A.
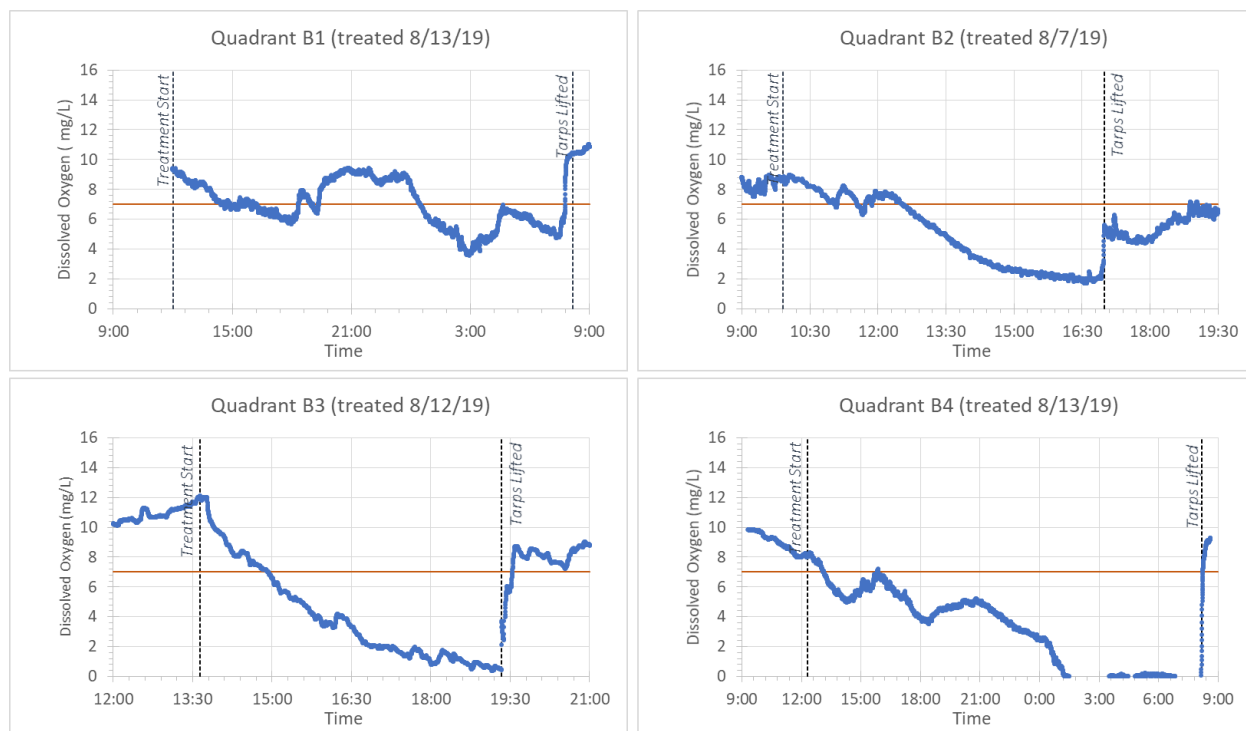
Figure 4-7. Dissolved Oxygen Concentrations in Treatment Site B.
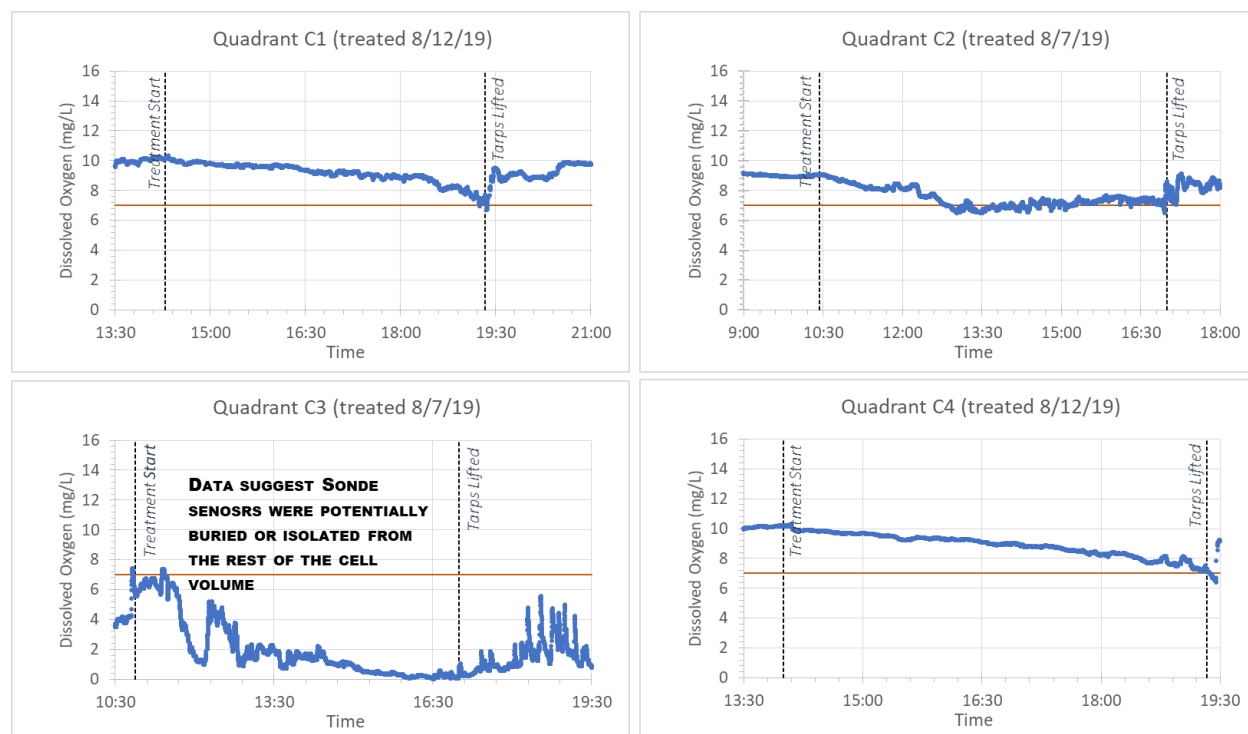
Figure 4-8. Dissolved Oxygen Concentrations in Treatment Site C.

Target treatment durations were up to eight hours for each treatment site; however, dangerous wave conditions on Lake Michigan prevented removal of the tarps after eight hours for the treatments conducted on August 13, 2019. The enclosures were left in place until the following morning, resulting in a longer duration of DO excursions. In all cases, dissolved oxygen increased immediately upon removal of the tarps. No aquatic species other than quagga mussels and round goby were observed during the staging, setup, treatment, or conclusion of the study, and there is expected to be little effect to non-target organisms as a result of the short-term DO excursions within the enclosures. Dissolved oxygen concentrations increased and met the minimum permit limit of 7.0 mg/L upon conclusion of the treatment.

Table 4-3. Summary of Dissolved Oxygen Excursions.

Plot	Treatment Start Date-Time	Treatment End Date-Time (Tarps Opened)	Treatment Duration (hrs)	DO Excursion	Excursion Start	Excursion End	Excursion Duration (hrs)
A1	8/13/19 11:13	8/14/19 8:10	20.95[1]	Yes	13:18	7:45[1]	11.2[1,2]
A2	8/7/19 9:15	8/7/19 17:00	7.75	Yes	13:18	17:05	3.8
A3	8/12/2019 13:20	8/12/19 19:20	6.00	No	-	-	0.0
A4	8/13/19 11:44	8/14/19 8:10	20.43[1]	Yes	12:36	7:45[1]	13.2[1,2]
B1	8/13/19 12:01	8/14/19 8:10	20.15[1]	Yes	14:36	7:45[1]	10.9[1,2]
B2	8/7/19 9:54	8/7/19 17:00	7.10	Yes	12:32	20:08	7.6
B3	8/12/19 13:38	8/12/19 19:20	5.70	Yes	14:56	19:32	4.6
B4	8/13/19 12:18	8/14/19 8:10	19.87[1]	Yes	13:11	8:14[1]	19.1[1]

Plot	Treatment Start Date-Time	Treatment End Date-Time (Tarps Opened)	Treatment Duration (hrs)	DO Excursion	Excursion Start	Excursion End	Excursion Duration (hrs)
C1	8/12/19 14:00	8/12/19 19:20	5.33	Yes	19:21	19:22	0.02
C2	8/7/19 10:26	8/7/19 17:00	6.57	Yes	12:50	14:25	1.6
C3	8/7/19 10:53	8/7/19 17:00	6.12	Uncertain due to apparent sensor malfunction			
C4	8/12/19 14:17	8/12/19 19:20	5.05	Yes	19:23	19:27	0.1

[1] Weather and wave conditions precluded removing the tarps until the morning following the treatment, extending the duration of noncompliance

[2] DO excursions were intermittent over the treatment period; "excursion start" and "excursion end" indicate the first and last excursion time, while the duration indicates only the times when DO was below 7.0 mg/L.

Dissolved oxygen concentration within the benthic substrate was measured using a fiber optic probe connected to a NewFox Sport spectrometer, which was contained in an underwater housing (Figure 4-9A). The probe was used to measure dissolved oxygen concentrations in benthic microhabitats - the algal mats (Figure 4-9B), the mussel matrix on rocks (Figure 4-9C), the interface between rocks and the sediment (Figure 4-9D), accumulations of dead mussel shells (not shown), and the water column. A minimum of five (5) measurements were made within each microhabitat type on both control and treatment sites (plot C2), before and after treatment.
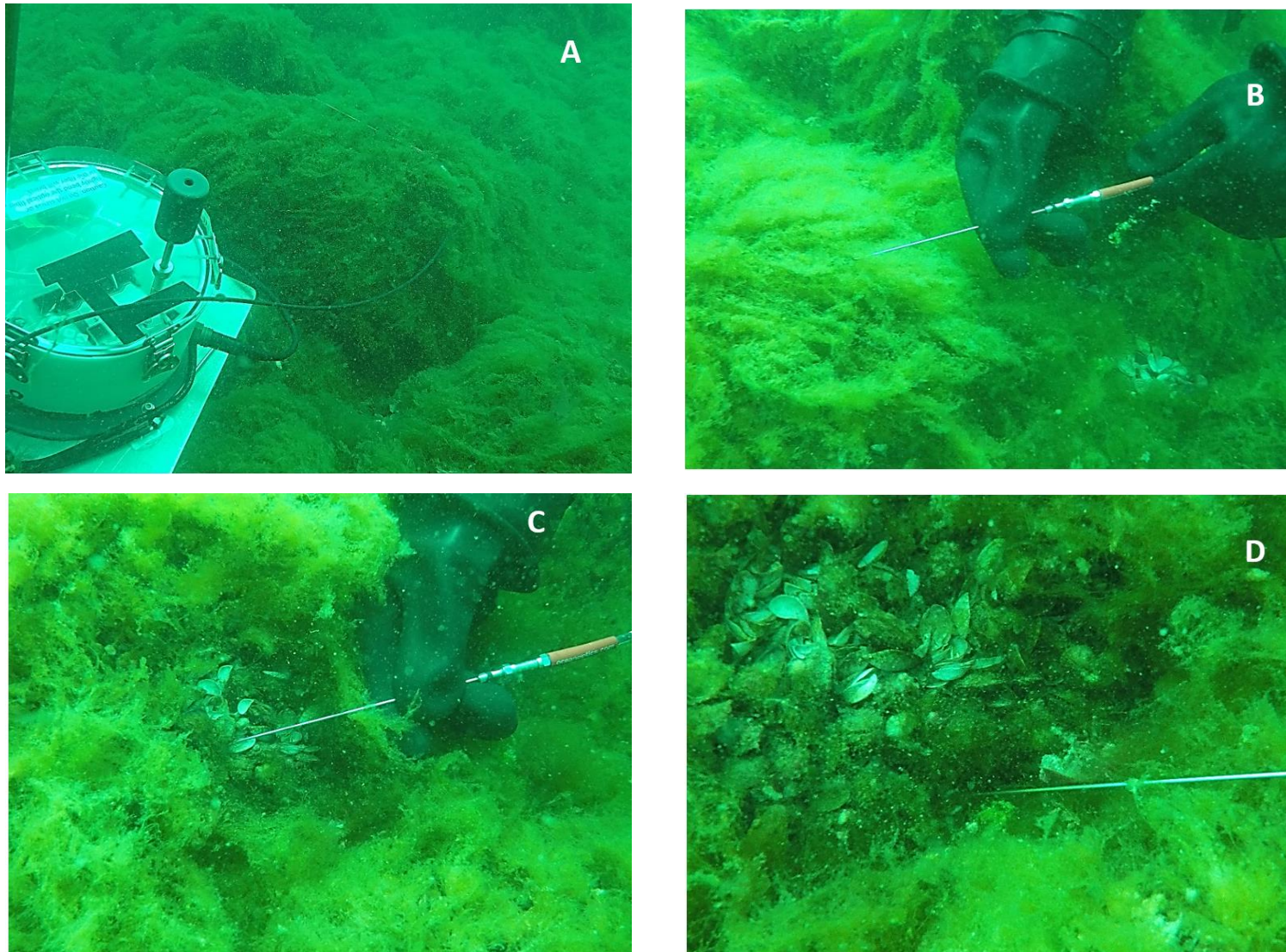
Figure 4-9. Dissolved Oxygen Measurements in the Benthos.

A = Spectrometer with fiber optic cable. B = Fiber optic probe inserted into algal mat. C = Probe inserted into mussel bed. D = Probe inserted beneath rock.

As shown in Figure 4-10, benthic dissolved oxygen (DO) concentrations varied by microhabitat types and sample date. For example, on August 8 (one day post-treatment), DO concentrations under rocks on the treatment site were significantly lower than in other treatment microhabitats, while DO concentration within the mussel bed was only slightly lower than the ambient DO concentration in the water column. On August 12, the DO concentration beneath rocks at the control site was low relative to other microhabitats on the control site, and also low relative to the same microhabitat on the treatment site. While there is an apparent general decrease in the benthic DO concentration on the treatment site between August 5 (before treatment) and August 12 (after treatment), the DO concentrations within the mussel matrix and beneath rocks on the treatment site are similar to those in the same microhabitats on the control site, making post-treatment DO patterns challenging to decipher.
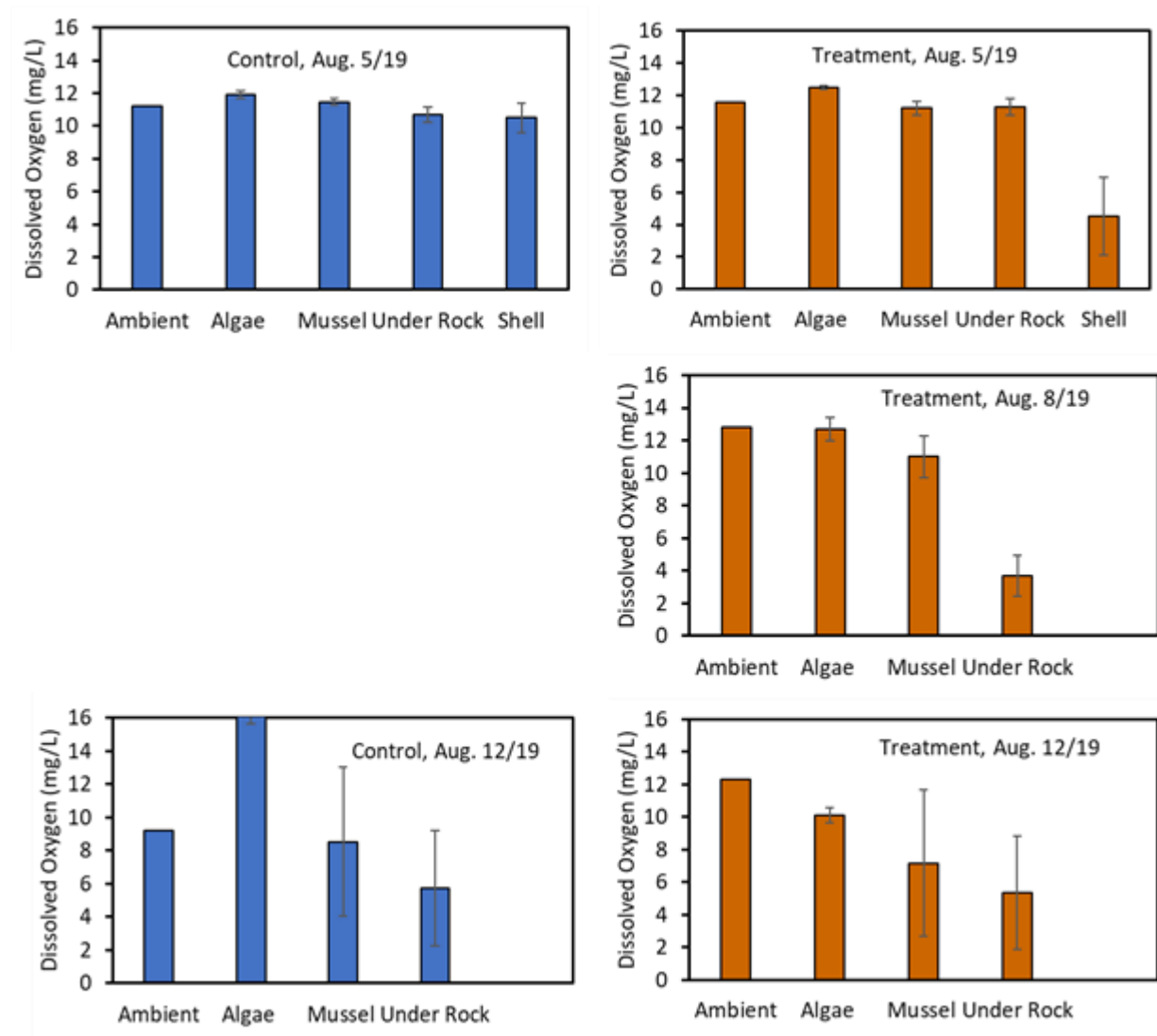
Figure 4-10. Dissolved Oxygen Concentrations in Various Benthic Microhabitats Before and After Zequanox® Treatment.

Treatment occurred on 8/7/19; the top figures present data before treatment, while the center and bottom figures show data after treatment

Several factors may confound the DO concentration comparisons. For example, within each microhabitat there is a large amount of spatial variability with regard to reef structure. As a result, the variability of replicate measurements is high at times. Within the mussel, under-rock, and shell microhabitats it was noted that the DO measurement varied significantly depending on how deep the fiber optic probe was inserted into the matrix, and it was difficult to standardize the sampling method to avoid this variability. With the data available, it appears that mortality of mussels, and possibly other benthic biota such as algae, may have resulted in declines in the DO concentration in some benthic microhabitats, but the high spatial and temporal variability did not allow for statistically robust conclusions.

4.3.2 Ammonia

Ammonia concentrations in the treatment area were measured prior to and during the treatment, at intervals of one hour, four hours, and eight hours after application of the Zequanox®. These data are shown in Table 4-4. In all three treatment plots, ammonia concentrations increased after the Zequanox® was applied, and remained elevated through the treatment period, with concentrations appearing to decrease toward the end of the treatment. No sample was collected on the day following the treatment, due to a communication error. However, measurements of other water quality constituents clearly show rapid recovery of the treatment area after removal of the tarps, suggesting that ammonia concentrations also returned to ambient levels after completion of the treatments.

Table 4-4. Ammonia Concentrations (mg/L) within the Treatment Plots.

Monitoring Period	Plot A	Plot B	Plot C
pre-treatment	<0.05	<0.05	<0.05
1 hour post-treatment	2.63	2.47	1.05
4 hours post-treatment	1.46	2.11	2.42
8 hours post-treatment	1.00	1.85	1.95

4.3.3 Total Suspended Solids

Total suspended solids (TSS) concentrations in the treatment area were measured prior to treatment and at intervals of one day and one week after application of the Zequanox®. These data are summarized in Table 4-5. In all three treatment plots, TSS was similar pre- and post-treatment. Compared to the control area, TSS concentrations in the treatment area appeared slightly elevated one day after the treatment, perhaps due to disturbance of the sediments in the area during the treatment.

Table 4-5. Total Suspended Solids Concentrations (mg/L) within the Treatment Plots.

Monitoring Period	Control	Plot A	Plot B	Plot C
pre-treatment	6	4	4	4
one day post-treatment	1	5	5	5
one week post-treatment	2	2	3	5

4.3.4 Phosphorus

Total phosphorus concentrations in the treatment area were measured prior to treatment and at intervals of one day and one week after application of the Zequanox®. These data are summarized in Table 4-6. Total phosphorus was not detected in any of the control or treatment plot samples.

Table 4-6. Total Phosphorus Concentrations (µg/L) within the Treatment Plots.

Monitoring Period	Control	Plot A	Plot B	Plot C
pre-treatment	<30	<30	<30	<30
one day post-treatment	<30	<30	<30	<30
one week post-treatment	<30	<30	<30	<30

Additional phosphorus monitoring was conducted within the treatment and control areas two days prior to the treatment, approximately one week after the treatment, and approximately one month after the treatment. This monitoring was conducted by UWM and included measurements of soluble reactive phosphorus (SRP), total dissolved phosphorus (TDP), and particulate phosphorus (PP), as well as chlorophyll–a. Total phosphorus (TP) was determined by summing the TDP and PP results. These data are presented in Table 4-7 and generally indicate comparable control and treatment area concentrations.

Table 4-7. UWM Phosphorus and Chlorophyll-a Data.

Date	Depth	Control Site SRP (µg/L)	Treatment Site SRP (µg/L)	Control Site TDP (µg/L)	Treatment Site TDP (µg/L)	Control Site PP (µg/L)	Treatment Site PP (µg/L)	Control Site TP (µg/L)	Treatment Site TP (µg/L)	Control Site Chl-a (µg/L)	Treatment Site Chl-a (µg/L)
8/5/19	Surface	0.415	0.303	0.590	1.100	1.974	1.295	2.564	2.395	0.863	0.959
	Bottom	<0.3	<0.3	0.638	0.670	1.487	1.919	2.125	2.589	0.911	1.295
8/12/19	Surface	<0.3		0.845		1.763		2.608		1.151	
	Bottom	0.686	0.367	0.845	0.590	2.543	4.692	3.389	5.282	1.103	1.343
	Bottom		0.718		0.463		3.654		4.117		1.007
	Bottom		0.861		0.494		2.736		3.231		1.007
9/5/19	Surface	<0.3		<0.3		1.850		1.864		0.6953	
	Bottom	<0.3	<0.3	<0.3	<0.3	1.991	1.830	2.006	1.844	0.6474	0.6953

4.3.5 Carbonaceous Biochemical Oxygen Demand

Carbonaceous biochemical oxygen demand (CBOD$_5$) in the treatment area was measured prior to treatment and at intervals of one day and one week after application of the Zequanox®. These data are summarized in Table 4-8. CBOD$_5$ was below detection in all of the control and treatment plot samples.

Table 4-8. Carbonaceous Biochemical Oxygen Demand (mg/L) in the Treatment Area.

Monitoring Period	Control	Plot A	Plot B	Plot C
pre-treatment	<3	<3	<3	<3
one day post-treatment	<3	<3	<3	<3
one week post-treatment	<3	<3	<3	<3

4.3.6 pH

The NPDES permit requires pH in the range of 6.5 to 9.0 SU. These limits were maintained throughout the study. Table 4-9 shows the minimum and maximum pH in each monitoring plot. The sonde data showed similar patterns within the quadrants during the treatment periods, with a decrease in pH within the enclosures during treatment, and a return to pre-treatment levels after removal of the enclosure. Figure 4-11 presents pH data for treatment quadrant B3, illustrating this pattern.

Table 4-9. Summary of pH Measurements within the Treatment Plots

Location	Minimum pH (SU)	Maximum pH (SU)
A1	7.77	8.73
A2	7.63	8.59
A3	8.04	8.90
A4	7.46	8.91
B1	7.69	8.62
B2	7.61	8.53
B3	7.65	8.90
B4	7.54	8.59
C1	7.47	8.69
C2	7.67	8.62
C3	No data-unrepresentative	
C4	8.07	8.72
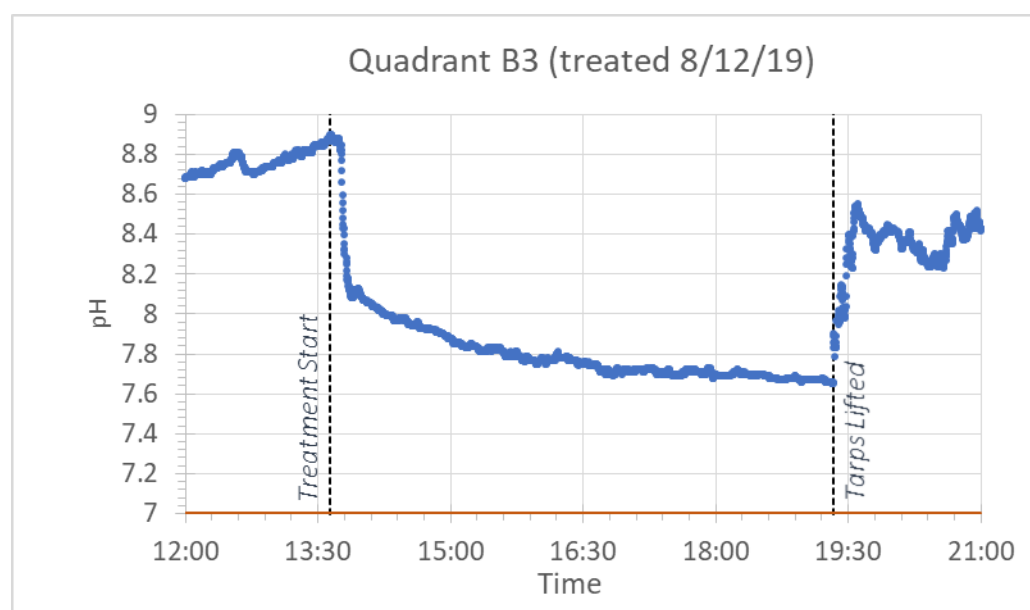
Figure 4-11. pH at Treatment Quadrant B3.

4.3.7 Temperature, Turbidity, and Chlorophyll *a*

4.3.7.a Treatment Implementation Monitoring

Table 4-10 summarizes temperatures in the treatment quadrants during the treatment period. Temperatures were within expected ranges for August temperatures in Lake Michigan and indicated compliance with Michigan Rule 70 standards (23.9°C). Temperatures showed relatively little variation (2-3°C over the course of the treatment period) during the first two days of treatment. On the third day (treatment quadrants A1, A4, B1, and B4), there was greater variation, with a substantial temperature drop during the evening hours (Figure 4-12), presumably due to the stormy conditions. Nonetheless, all temperatures were greater than the desired minimum of 10°C to ensure Zequanox® effectiveness.

Table 4-10. Summary of Temperature Data within the Treatment Quadrants.

Location	Minimum Temperature (°C)	Maximum Temperature (°C)
A1	12.1	19.6
A2	19.7	21.8
A3	19.3	21.9
A4	11.8	21.0
B1	11.4	20.9
B2	19.0	21.5
B3	19.1	21.3
B4	13.3	19.8
C1	18.6	21.6
C2	18.9	21.8
C3	No data-unrepresentative	
C4	18.7	21.6
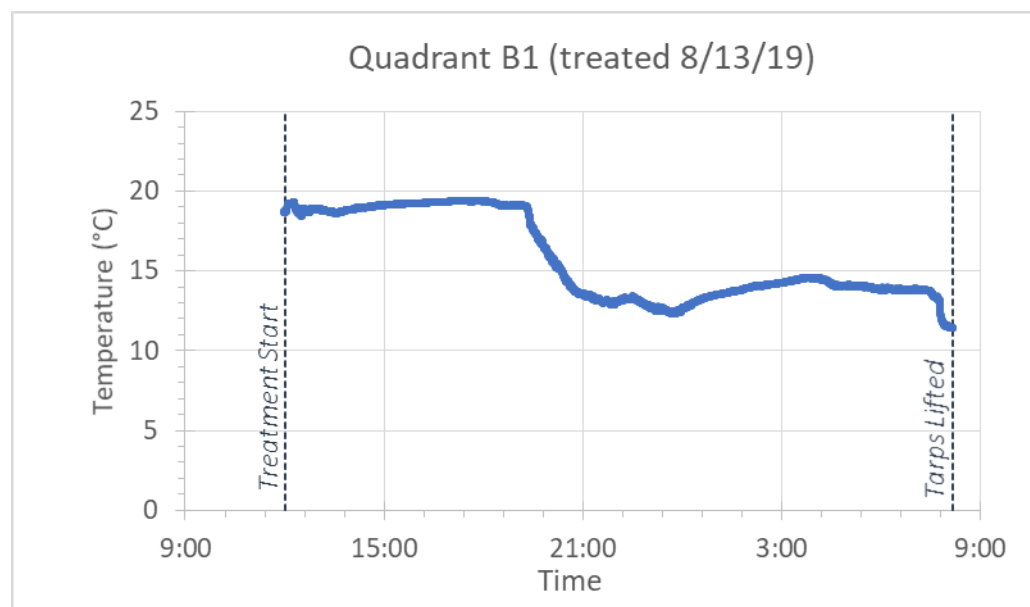
Figure 4-12. Temperature at Treatment Quadrant B1.

Turbidity monitoring during treatment was primarily conducted to assess Zequanox® concentrations (see Section 3.3.4). Table 4-11 provides a summary of the turbidity data collected within the enclosures during the treatments. Turbidity within the enclosures decreased over the course of the treatment, and returned rapidly to ambient levels upon removal of the enclosures, as shown by the turbidity-based Zequanox® concentration estimates presented in Figure 3-18, Figure 3-19, and Figure 3-20.

Table 4-11. Summary of Turbidity Measurements During Treatment.

Plot	Sonde No.	Treatment Start Date-Time	Treatment End Date-Time (Tarps Opened)	Treatment Duration (hrs)	During Treatment Period	
					Maximum Turbidity (NTU)	Average Turbidity (NTU)
A1	1	8/13/19 11:13	8/14/19 8:10	20.95	304	25
A2	1	8/7/19 9:15	8/7/19 17:00	7.75	85*	23*
A3	1	8/12/2019 13:20	8/12/19 19:20	6.00	177	37
A4	3	8/13/19 11:44	8/14/19 8:10	20.43	253	34
B1	2	8/13/19 12:01	8/14/19 8:10	20.15	2530	29
B2	4	8/7/19 9:54	8/7/19 17:00	7.10	207	71
B3	4	8/12/19 13:38	8/12/19 19:20	5.70	156	80
B4	4	8/13/19 12:18	8/14/19 8:10	19.87	262	37
C1	2	8/12/19 14:00	8/12/19 19:20	5.33	256	75
C2	3	8/7/19 10:26	8/7/19 17:00	6.57	287	90
C3	2	8/7/19 10:53	8/7/19 17:00	6.12	No data-unrepresentative	
C4	3	8/12/19 14:17	8/12/19 19:20	5.05	137	46

* Based on partial data during treatment period

4.3.7.b *Pre- and Post-Treatment Monitoring*

Water quality monitoring instruments were deployed at both the control and treatment site for the month of August. A YSI 6600 sonde equipped with temperature, turbidity and chlorophyll a sensors was deployed at the control site and a Sea-Bird ECO fluorometer / turbidity sensor was deployed at the treatment site. All sensors were set up to record measurements every half hour. Not surprisingly, bottom temperatures at both sites were similar over time. During the treatment period, temperatures remained around 20°C (Figure 4-13), which is likely optimal with regard to efficacy of treatment, as Zequanox® needs to be ingested by mussels to cause mortality, and mussel filter feeding rate increases with temperature (Tyner et al. 2015). During the month of August, there were cold water incursions during August 14-17 and August 24-27. There was significant mussel mortality between August 12 and September 5 (see section 4.2), so these temporary drops in temperature do not appear to have negatively affected treatment efficacy.
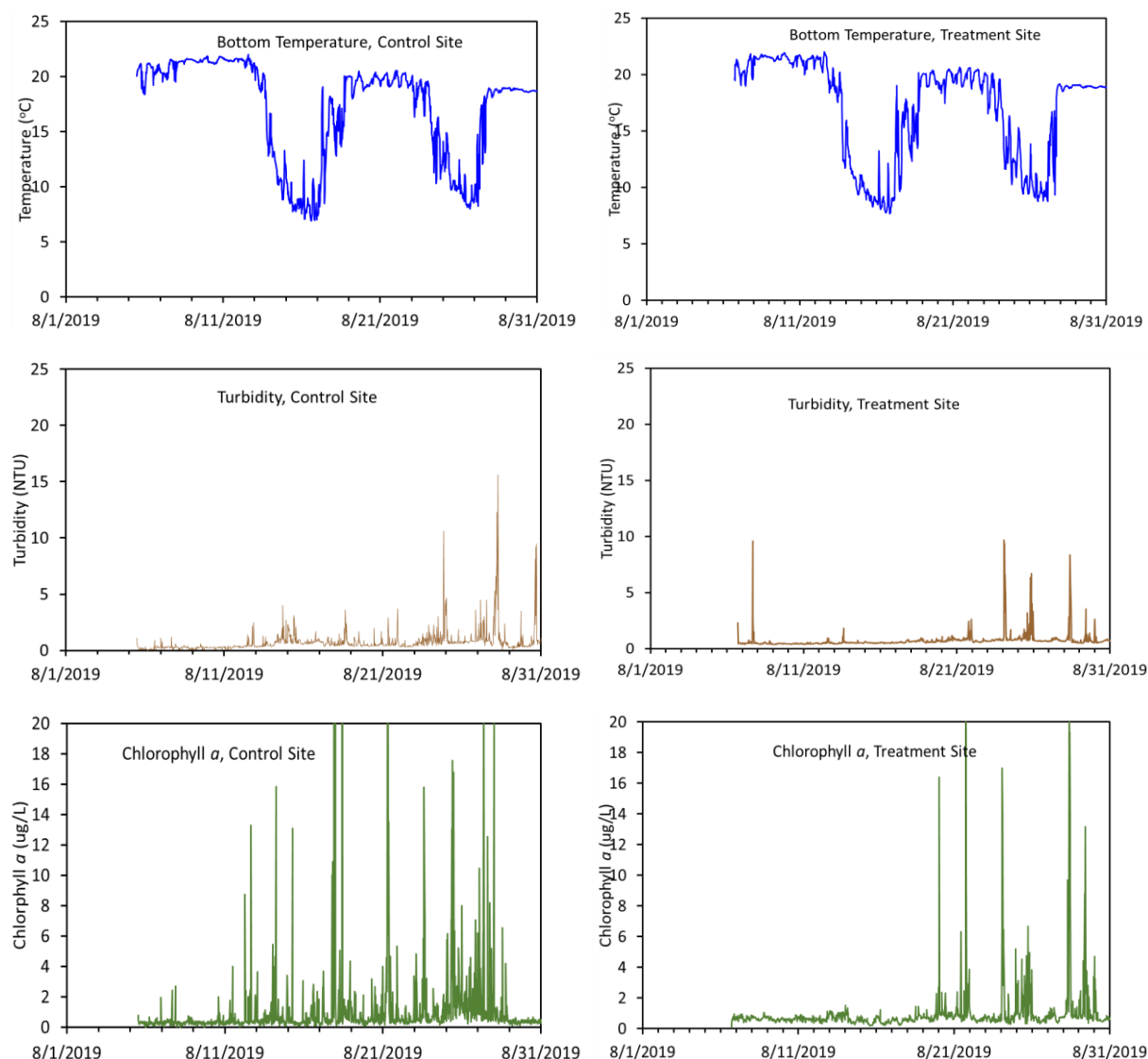
Figure 4-13. Continuous Measurement of Bottom Temperature, Turbidity and Chlorophyll *a* Fluorescence at the Control and Treatment Sites.

Turbidity was low at both the control and treatment sites through most of August, although there were brief spikes that were likely due to either short-lived resuspension events or advection of turbid water resulting from shoreline erosion, which was visibly obvious during field work (Figure 4-13). Although high-turbidity events were not always simultaneous at both sites, there is no evidence to suggest that Zequanox® treatment altered the turbidity at the treatment site following removal of the enclosures.

Chlorophyll *a* fluorescence was also similar at the two locations, being lower in the first half of the month and increasing in the second half (Figure 4-13). Variability was greater at the control site. While this may suggest a real difference between the sites, it more likely is due to the deployment of different fluorometers at the two sites. The sonde sensor deployed at the control site is not as sensitive or as accurate as the Sea-Bird ECO fluorometer deployed at the treatment site, and has a less efficient anti-biofouling system. Despite the greater variability observed at the two sites, mean fluorescence was similar at the two sites, and the fluorescence-derived estimates of chlorophyll *a* concentration are within the normal range for Lake Michigan nearshore waters.

4.3.8 Conductivity

Conductivity data for the treatment plots is summarized in Table 4-12. Conductivity data within the enclosures generally follow a similar pattern to turbidity, with an increase in conductivity when the Zequanox® was applied, and a decrease over the course of the treatment. Figure 4-14 shows this relationship for Treatment Quadrant A4.

Table 4-12. Summary of Conductivity Data within the Treatment Quadrants.

Location	Minimum Conductivity (µS/cm)	Maximum Conductivity (µS/cm)
A1	288	332
A2	261	288
A3	259	301
A4	290	345
B1	208	277
B2	282	322
B3	278	323
B4	228	281
C1	247	290
C2	295	356
C3	No data-unrepresentative	
C4	259	295

Figure 4-14. Turbidity (blue) and Conductivity (yellow) within Treatment Plot A4.

4.4 Biota

4.4.1 Benthic Macroinvertebrates

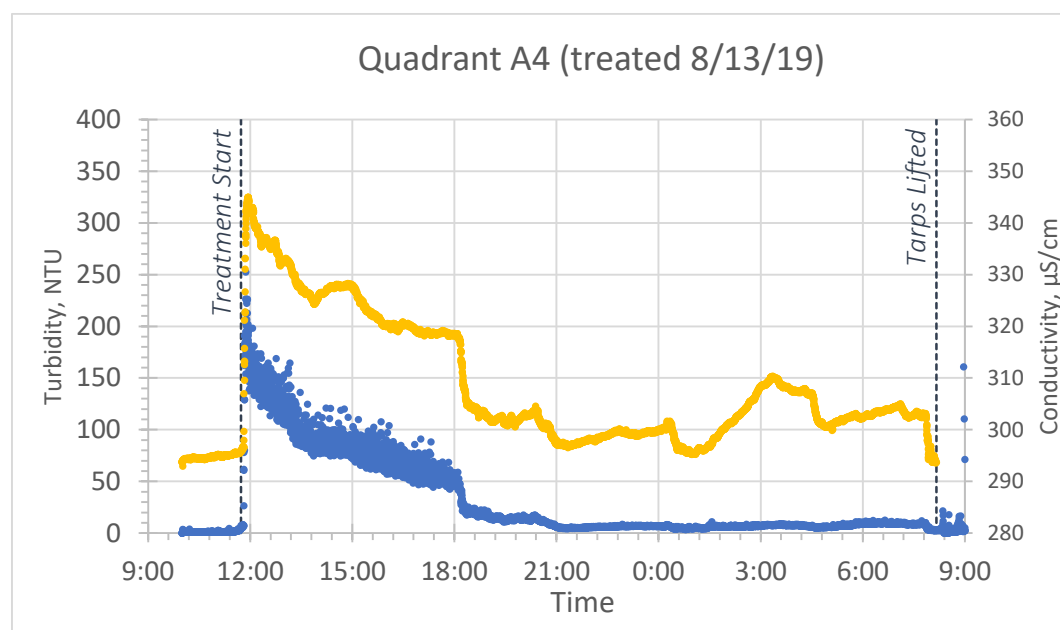Samples for quantitative measurements of benthic algae biomass, dreissenid mussels, and other benthic macroinvertebrates were collected by the UWM research team from the control and treatment areas. Samples were collected from 20 x 20 cm quadrats from rock surfaces (Figure 4-15). Additional sampling details are provided in Section 4.2 (Mussels). Samples from each location were collected in triplicate from rocks within two to four meters of each other. The initial intent was to collect one sample from each of the treatment plots. However, because foul weather delayed the treatment of plots A and B, the three triplicate treatment samples were all collected from plot C. Within six hours of collection, samples were placed in ceramic sorting pans with water, which allowed for the suspension of algae, and forceps were used to pick out benthic macroinvertebrates. Macroinvertebrates were sorted into the following taxonomic groups: 1) Amphipods; 2) Isopods; 3) Chironomids; 4) Oligochaetes; 5) Planarians; 6) Leeches (Hirudinae); 7) Snails (Gastropoda); 8) Insect nymphs (family specific). Water mites (Hydrachnidia) and ostracods were also observed but were not counted as they represented a negligible fraction of total non-dreissenid macroinvertebrate biomass.

Figure 4-15. Diver Collecting Benthic Samples from Rock Surfaces, Using a 20 x 20 cm Frame to Delineate Sampling Quadrats.

Prior to application of Zequanox®, the composition and abundance of benthic macroinvertebrates was similar at the treatment and control sites (Figure 4-16 and Figure 4-17). Amphipods were the most abundant taxa, and made up the largest portion of biomass, followed by isopods. On the first day after treatment (which was applied to plot C on August 7, 2019), macroinvertebrate abundance and composition on the treatment site was similar to that before treatment. Five days after treatment, there was a noticeable decline in the abundance of amphipods and isopods. These declines were not statistically significant, due to the high variability among replicates resulting from high natural spatial variability. However, the declines were likely real, as the mean abundance and biomass values five days post-treatment are in line with the declining trajectories observed between day 1 and day 29. By day 29

(September 5), benthic macroinvertebrates had virtually disappeared from the treatment site (Figure 4-17), and this persisted until the final sample collection on October 8 (62 days post-treatment).

Over the 62 days post-treatment, there was also a gradual decline in the abundance and biomass of benthic macroinvertebrates at the control site (Figure 4-16, Figure 4-17, Figure 4-18). It is possible that some of the decline at the treatment site was due to natural causes related to the balance between predation and production. Benthic macroinvertebrates may benefit from the protection provided by benthic algae and the microhabitats created within the dreissenid mussel matrix (Kuhns and Berg 1999). These two cover types were also reduced to a large extent on the treatment site (see Sections 4.2 and 4.4.3). It is possible that some of the decline in benthic macroinvertebrates on the treatment site was due to loss of predation shelter, which exposed the organisms to predation by round gobies and other scavenging fish. There may be other factors, such as particulate matter, which also contributed to the decline, as these have been suggested to contribute to toxicity in some organisms (Molloy et al., 2013). With the data collected in this study, it is not possible to determine the degree to which non-dreissenid benthic invertebrate loss was due to mussel mortality, increased predation, or other factors related to Zequanox® application.
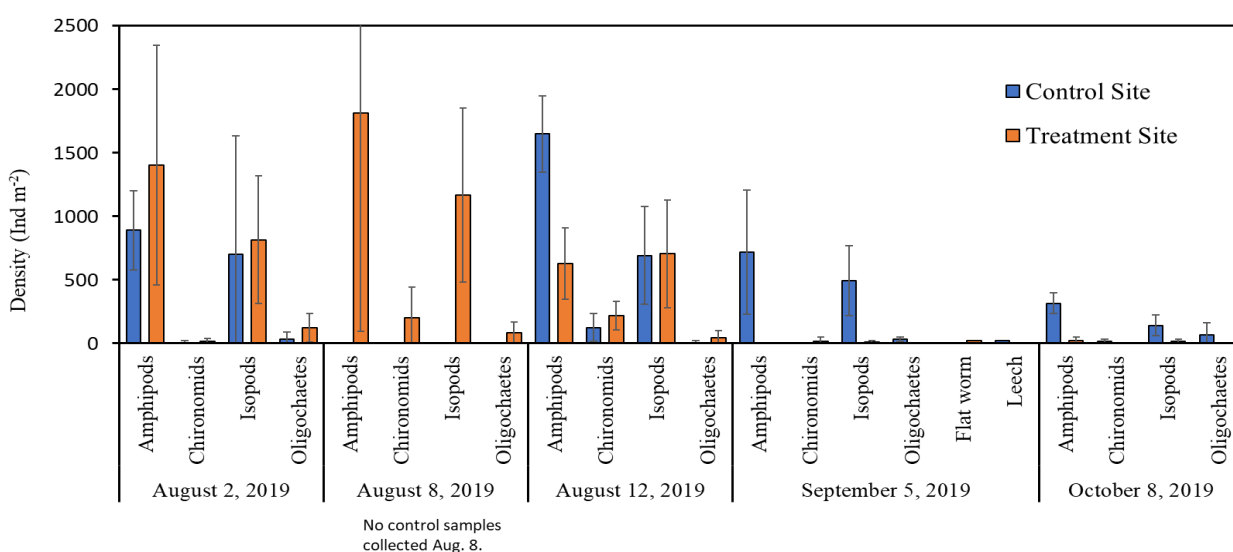
Figure 4-16. Abundance of Various Benthic Macroinvertebrate Taxa on Rock Surfaces over Time.

Note: Zequanox® treatment was applied on August 7.
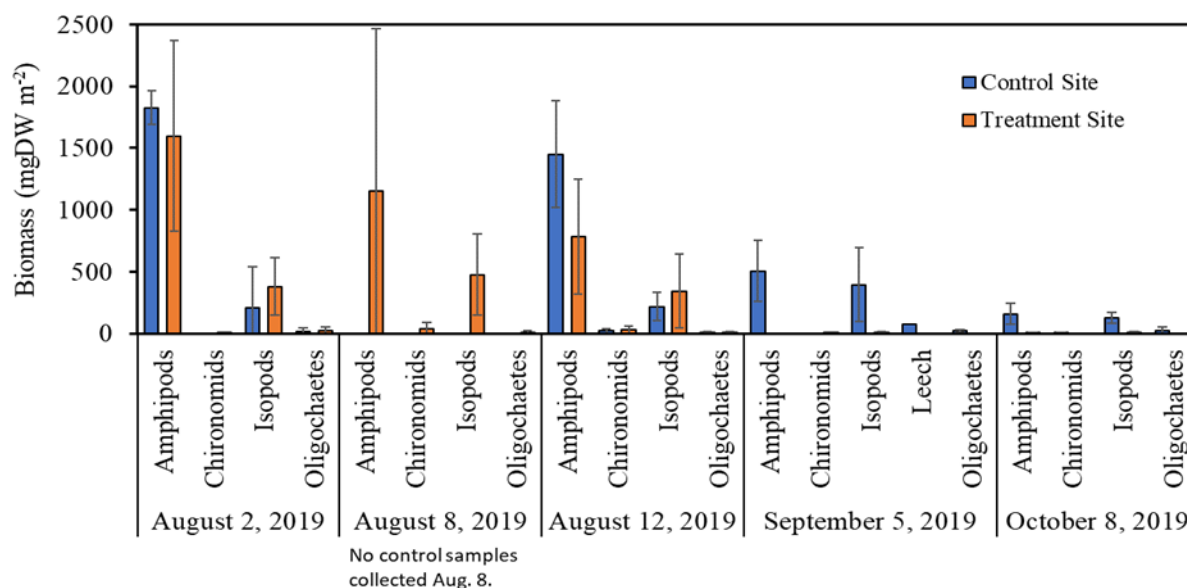
Figure 4-17. Biomass of Various Benthic Macroinvertebrate Taxa on Rock Surfaces Over Time.
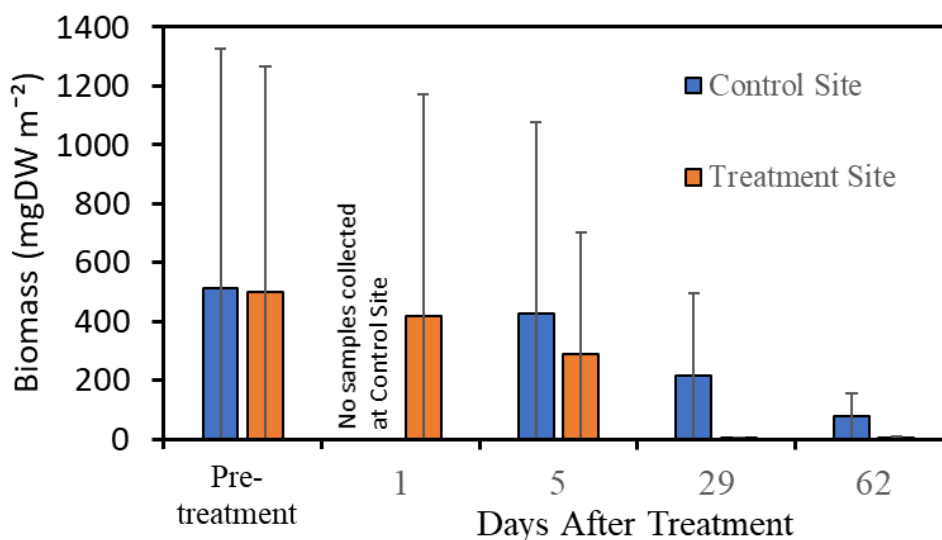
Figure 4-18. Total Biomass of Benthic Macroinvertebrate on Rock Surfaces Over Time (Excluding Dreissenids).

4.4.2 Bacteria

Bacterial community monitoring was conducted by researchers from the University of Michigan (UM). Bacteria communities are a valuable response metric because they can rapidly respond to changes in chemical and physical environmental conditions. These responses are observed via changes in (a) bacterial physiology, which can be assessed by analyzing changes in gene expression, and (b) growth and death rates, which can be assessed by evaluating bacterial community composition shifts. Gene expression and composition shifts for bacteria are measured by genetic sequencing of universal marker genes, and

typically using the small subunit of the ribosomal RNA gene (16S rRNA). Because of the known context of the problems with avian botulism outbreaks in the Sleeping Bear Dunes region (Moraska-Lafrancois, 2011), genetic analyses targeted changes in abundance of the causative agent, *Clostridium botulinum*, the gene encoding the botulism type E toxin (*bont*E), and its expression for processed sample detection. To assess impacts of the Zequanox® treatment, various biotic substrates were sampled from both control and treatment plots before, during, and after Zequanox® treatment. Bacterial community composition was analyzed using 16S rRNA gene sequencing and *Clostridium botulinum* type E toxin (*bont*E) gene for both presence and expression using quantitative polymerase chain reaction (qPCR), as well as reverse transcription-quantitative PCR (RT-qPCR).

Samples for microbial analysis were taken from triplicate benthic scrapes of benthic algae, macroinvertebrates (i.e., Chironomids) and dreissenid mussel tissue. These were collected at three time-points: pre, mid-day during, and one-day post Zequanox® application. During the Zequanox® applications, samples of the water column were collected at Hour 0 (0h), Hour 4 (4h), and Hour 8 (8h). Samples included one replicate from each matrix (algae, macroinvertebrates, quagga mussels, water) in each experimental plot as well as the control area before, during, and after treatment. All analyses were performed using molecular methods without cultivation. Microbial analyses of DNA and RNA extracted from collected samples using qPCR and RT-qPCR quantified the bontE gene and its expression, and characterized the overall bacterial community composition to understand shifts in toxin gene abundance and expression in a whole community context. The details of the methods for bacterial analyses are described in Appendix 2.

The analysis found no *C. botulinum* nor bontE gene expressions in any samples taken during monitoring (Figure 4-19). Further, no inhibition of the qPCR or RT-qPCR assays were detected by chemicals that may be co-extracted with DNA and RNA from the benthic sample matrix (Figure 4-20). These findings support the observations that there were no sample processing or environmental inhibiting factors that would impact the detection of *bontE* toxin gene's abundance or expression observed in Figure 4-19. In addition, the bacterial community composition analysis showed no significant difference between the treatment and control site over the course of the experiment associated with benthic algae matrices, macroinvertebrates, or dreissenid mussel tissues (Figure 4-21 and Figure 4-22). Finally, water column analysis during the treatment also revealed no major shifts in community composition at the control site, and dominance by the Zequanox® bacterium, (i.e., *Pseudomonas fluorescens*) at the treatment site (Figure 4-23). These results indicate there was no dispersal of the Zequanox® treatment to the control plots.
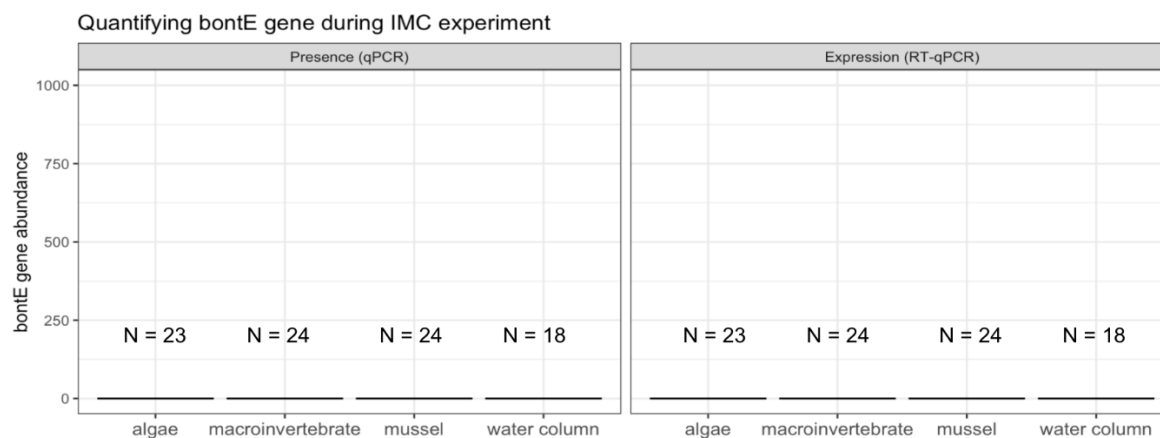
Figure 4-19. Quantification of the *bont*E Gene Presence and Expression across Sample Matrix, Determined Using qPCR and RT-qPCR, respectively.

Note: The bar plot shows the absence and lack of expression of the *bont*E toxin gene in any matrix examined. Numbers above each matrix represent the number of samples analyzed. Potential matrix inhibition was ruled out based on matrix inhibition tests.
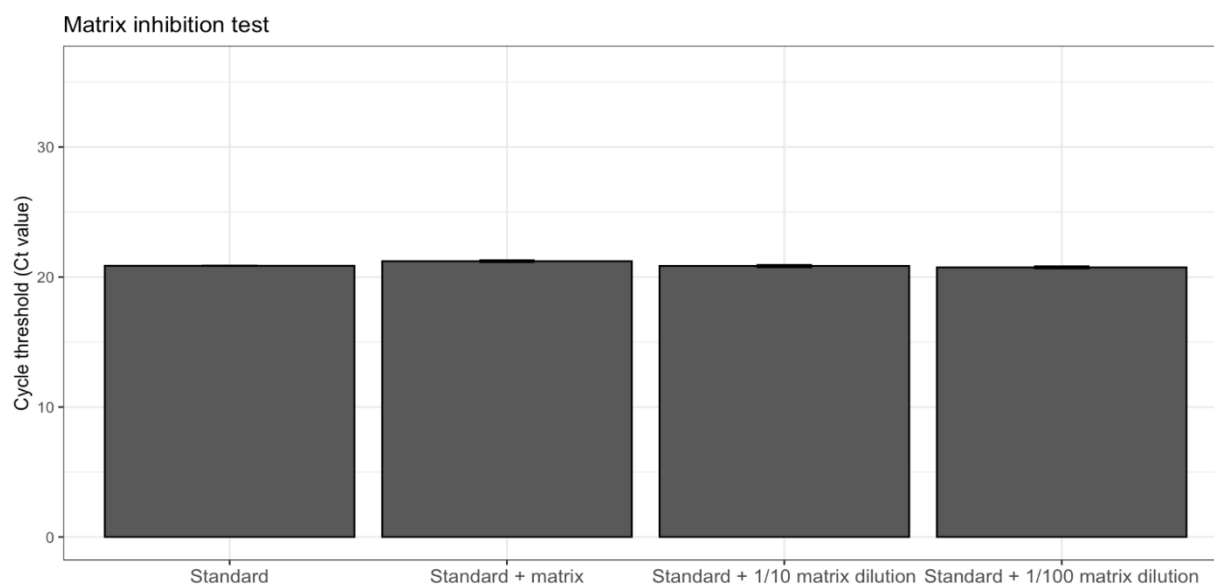
Figure 4-20. Bar Plot Showing the Results of the Matrix Inhibition Test Performed for the RT-qPCR Assay.

Note: The cycle threshold (Ct) value of the standard template with a known copy number of *bont*E ($1.3x10^6$) was compared to the Ct value of the standard spiked into the sample matrix, 1/10 dilution of the sample matrix, and 1/100 dilution of the sample matrix. The bars represent the mean Ct value of all the matrices (algae, mussel, macroinvertebrate, and water) examined for each reaction mixture, and the standard error of the mean. No significant difference was seen between control (template only) and treatments, indicating chemicals co-extracted with DNA/RNA from our sample matrices did not inhibit the qPCR reaction.
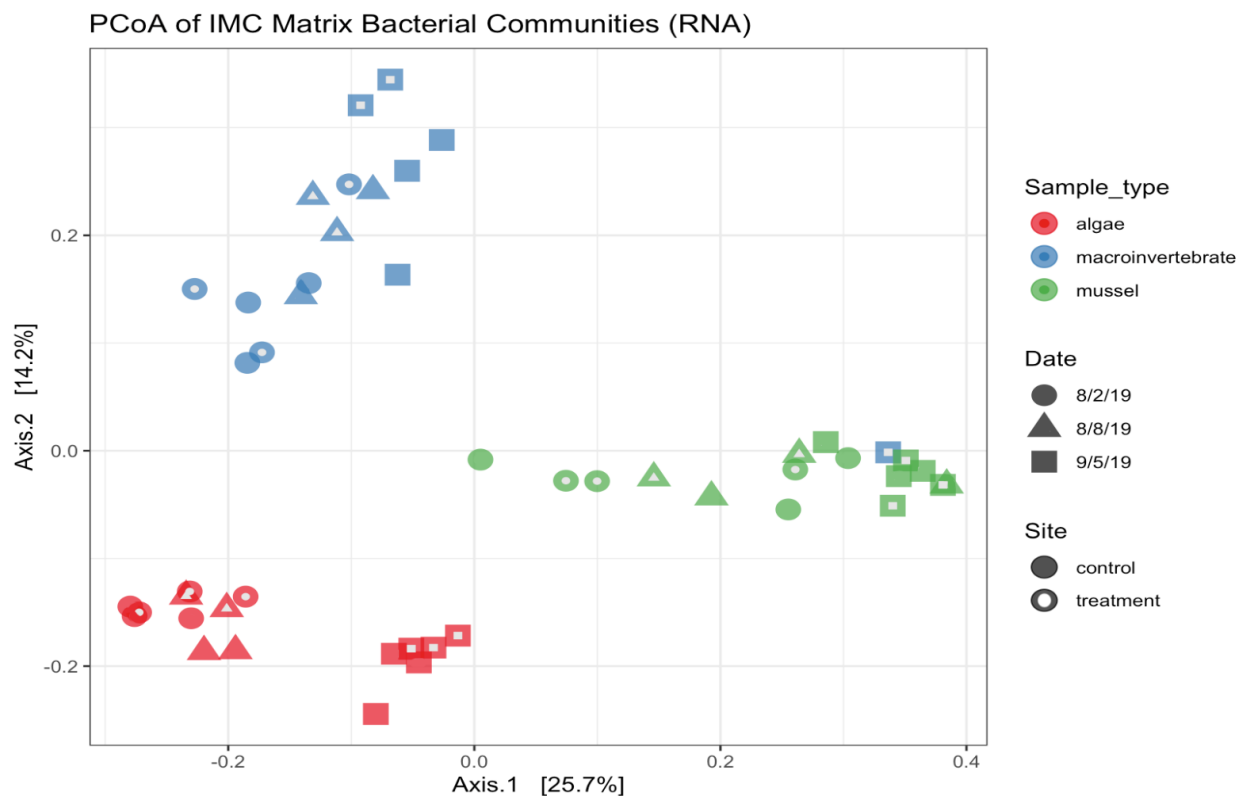
Figure 4-21. Principal Coordinates Analysis Displaying Bacterial Community Similarity Between Samples Based on Bray-Curtis Dissimilarities Between Samples from Three Matrices: Algae, Macroinvertebrates, and Mussels Sampled During Three Timepoints.

Note: Colors represent matrix type, shape represents the date sampled, and open/closed circle represent the sampling location. Samples were taken from benthic scrapes at both the Good Harbor Reef IMC treatment and control sites. Permanova by Site: Adonis P= 0.489; betadisper: P=0.519, which indicates that there was no difference in community composition between treatment and control plots.
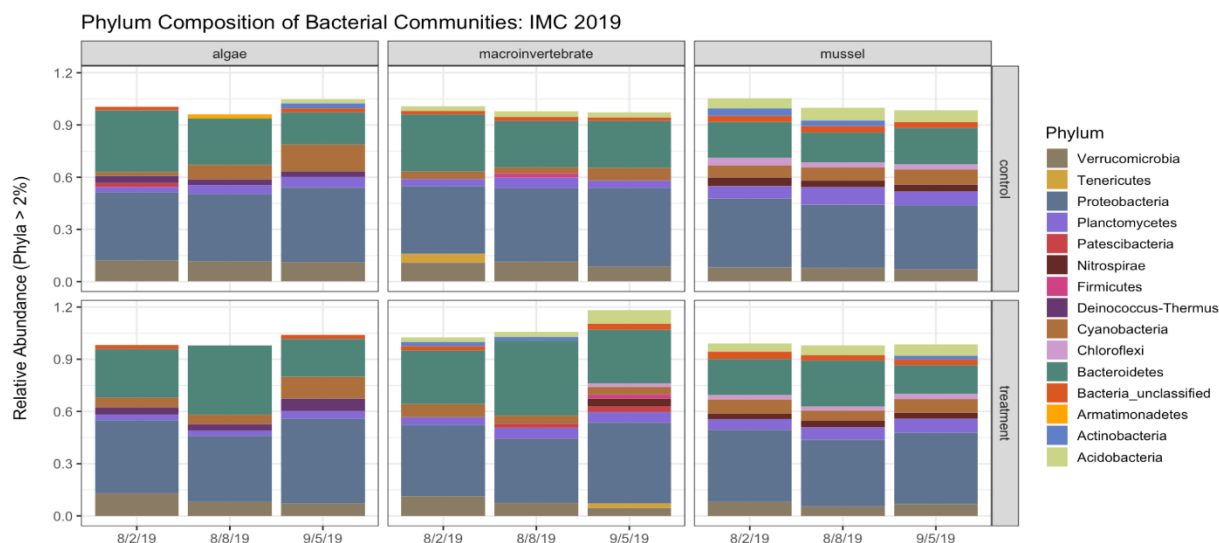
Figure 4-22. Bar Plot Comparing the Relative Abundance of Bacterial Phyla Based On 16S rRNA Sequencing Data Across Sample Matrix in the Control and Treatment Sites Before Treatment (8/2/19), the Day After (8/8/19), and One Month After Zequanox® Application (9/5/19).

Note: No significant differences in community composition were observed.
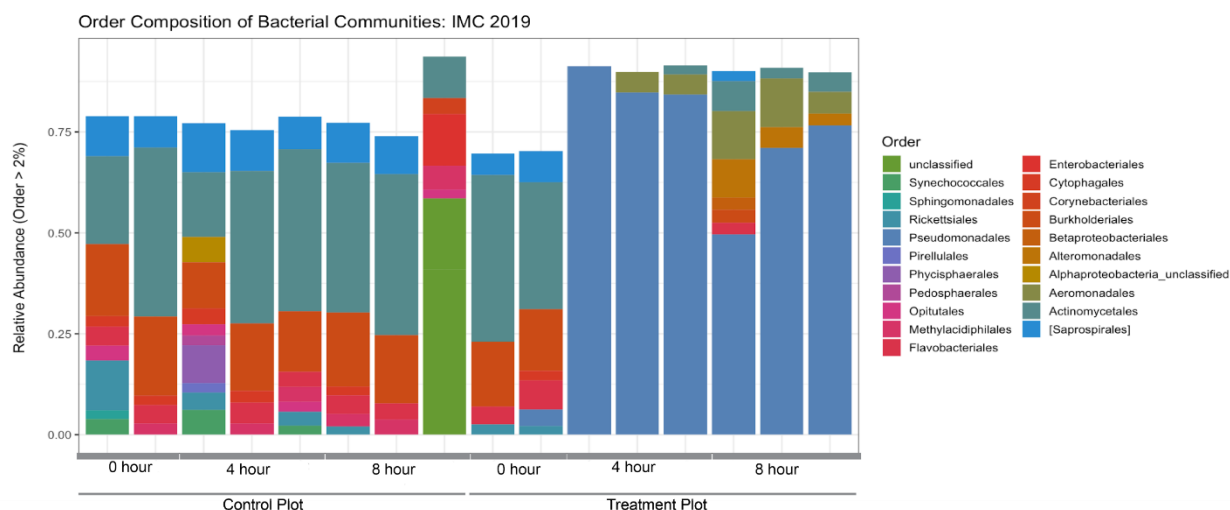
Figure 4-23. Bar Plot Showing the Relative Abundance of Bacterial Orders Based on 16S rRNA Gene Sequencing Data in the Water Column over the Course of the Zequanox® Treatment (Hours 0, 4, 8).

Note: The two time series (0-8 hours) on the x-axis represents the change in bacterial phyla in the control and treatment plot respectively. Multiple bars within the same hour represent biological replicates taken from the water column. As expected, water samples during treatment are dominated by Pseudomonadales, however, there is no enrichment in Pseudomonadales in the control plot, indicating containment of the Zequanox® treatment.

4.4.3 Benthic Algae (*Cladophora*)

Benthic algal samples were collected from both the treatment and control sites as described above (Section 4.4.1). Following separation from dreissenids, other invertebrates, detritus, and inorganic material, algae were briefly rinsed with distilled water, freeze dried, and then weighed to determine dry

mass. Following drying, each sample was ground to homogenize, after which a subsample was taken for the measurement of tissue phosphorus (P) content. To determine P content, samples were combusted in Pyrex tubes at 550°C for one hour, followed by digestion in a dilute HCl solution (2 ml of 1 N HCl + 10 ml distilled water) at 105°C for 2 hours. Samples were then analyzed for soluble reactive phosphorus using the molybdate method (Stainton et al. 1977).

Prior to application of Zequanox®, *Cladophora* biomass was high at the treatment site (Figure 4-24) and equal to the biomass measured at the control site (Figure 4-25). These concentrations are similar to what has been measured near the control site in early August of previous years, and well above the concentration of 50 g dry weight/m² that is nominally considered the nuisance threshold for *Cladophora* in Lake Michigan (Bootsma et al. 2015). Following Zequanox® application, there was a rapid decline of *Cladophora* on the treatment site (Figure 4-25 and Figure 4-26). Biomass on the treatment site one day post-treatment was less than 20% of that before treatment and remained at that level for the next two months. By contrast, *Cladophora* biomass on the control site remained relatively high into September (Figure 4-25 and Figure 4-26), and then declined in October, similar to the temporal pattern observed at this site in previous years.

Figure 4-24. Benthic Substrate on the Treatment Site (plot C2) One Day Before Zequanox® Application, Showing Mussel-Covered, Algae-Covered Rock, Round Gobies, and Mussel Shells Between Rocks.

The cause of the rapid decrease in biomass following treatment is not obvious. Zequanox® is not known to negatively affect algae, and in some cases, the application of Zequanox® has actually resulted in small increases in the abundance of both phytoplankton and periphytic algae (Nicholson 2018). *Cladophora* is a periphytic alga that is attached to the substrate. In areas of high mussel density, a significant fraction of *Cladophora* filaments can be attached to the shells of living mussels. Hence, much of the *Cladophora* loss was likely due to the mortality and detachment of mussels from rocks.

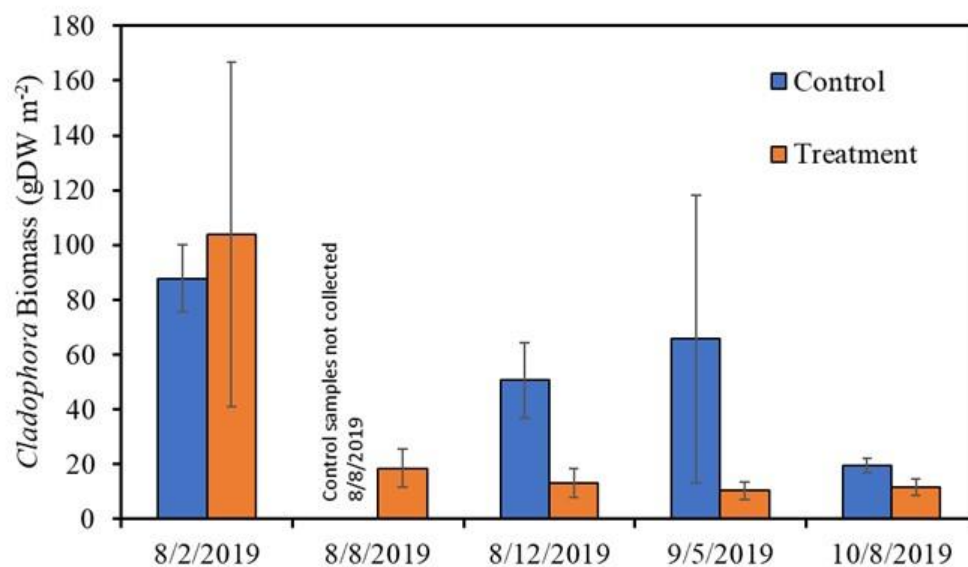**Figure 4-25. Benthic Algal Biomass Variation Between August 2 and October 8, 2019.**

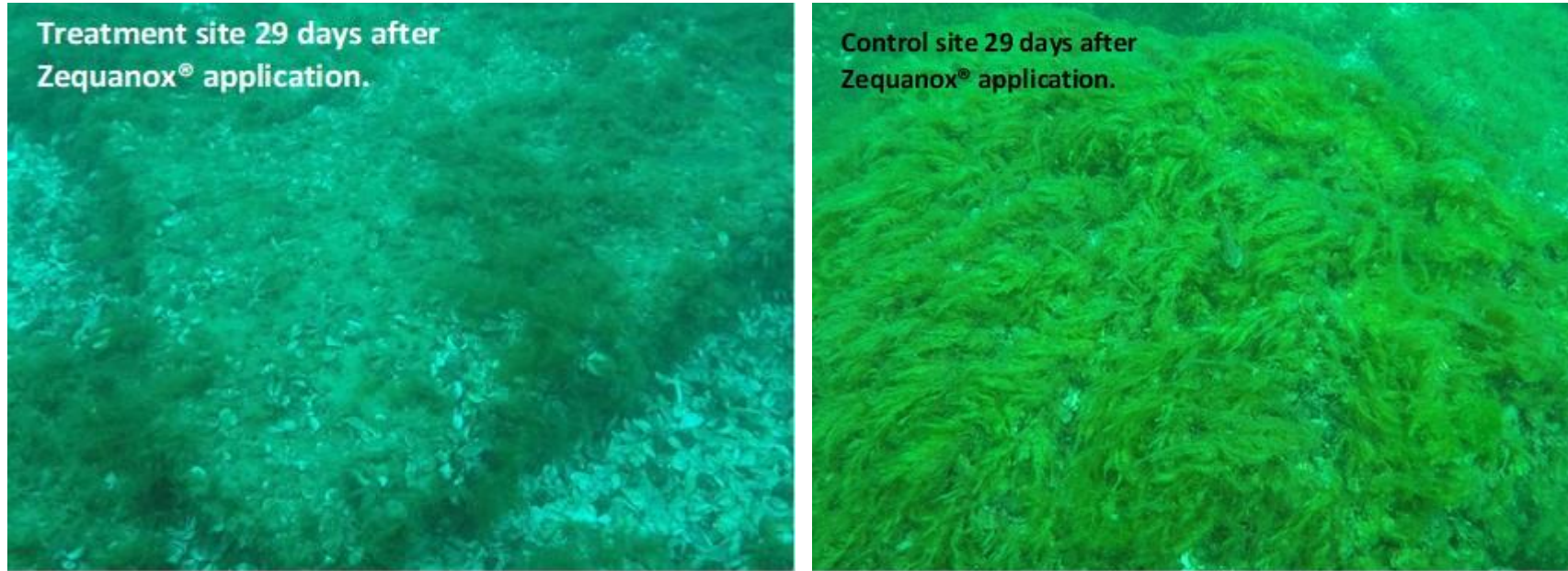Note: Zequanox® was applied to the treatment site on August 7.

Figure 4-26. Benthic Substrate on the Treatment Site (plot C2) [left] and on the Control Site [right] on September 5 (29 Days Post-Treatment).

Note sparseness of algae and mussel on the rock upper surface, relative to the substrate at the control site.

Mean *Cladophora* phosphorus content was slightly greater at the treatment site than the control site before treatment (Figure 4-27), but the difference was not statistically significant (α=0.05). Within one day of Zequanox® application, the P content of algae on the treatment site increased dramatically and remained high for at least the next four days (Figure 4-27). By one month post-treatment, the P content of *Cladophora* on the treatment site returned to values similar to those measured on the control site.
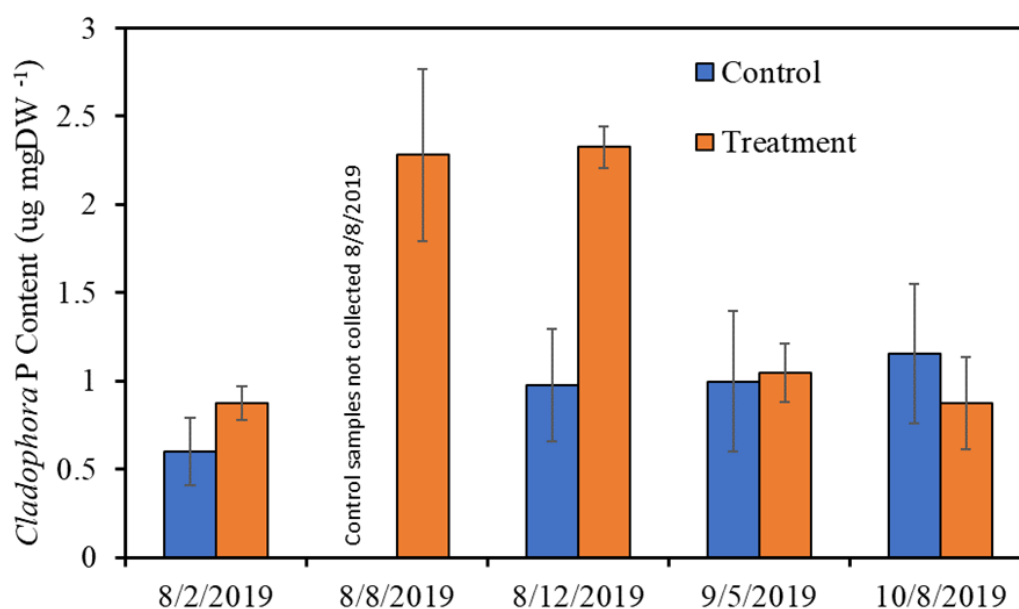
Figure 4-27. *Cladophora* **Phosphorus Content on the Treatment and Control Sites Between August 2 and October 8, 2019.**

The minimum P content required by *Cladophora* to grow is ~0.4 µg/mgDW (Tomlinson et al. 2010) and maximum growth rate is achieved when algal P content is >2 µg/mgDW (Auer and Canale 1982). The growth rate response to phosphorus is strongly modulated by light, which is likely the reason for an increase in the *Cladophora* P content on the control site between early August and October. As daily irradiance decreases, growth becomes more light-limited and less phosphorus-limited, so that the algae accumulate some P rather than immediately allocating it to increased biomass.

The rapid increase in algal P content after Zequanox® application likely reflects an increased availability of dissolved P that may have resulted from mussel stress and/or mortality and decomposition. While dissolved P concentrations were not monitored under the enclosures during the period of treatment, monitoring of dissolved ammonium (NH_4+) revealed a spike in concentrations from below detection limit (0.05 mg/L) before treatment to as high as 2.63 mg/L during treatment (see Section 4.3.2). This spike in ammonium was likely accompanied by a spike in dissolved P concentration. While dissolved P concentrations in the water column were low following treatment (see Section 4.3.4), the P content of *Cladophora* at the treatment site remained elevated for at least 5 days following treatment (Figure 4-27). Although the *Cladophora* P content in the five days following treatment was at the level required for maximum growth rate, this was not reflected in algal biomass, which remained low (Figure 4-26). This may have been due in part to the sparsity of algae following the biomass loss during treatment (i.e. there was a small seed population to take advantage of the increased phosphorus availability), but the remaining algal filaments were still short 5 days after treatment, suggesting growth rate was low. The lack of growth may have also been due to low light conditions, or sloughing of any biomass that was produced.

One month following treatment, the P content of benthic algae at the treatment site was once again similar to that at the control site (Figure 4-27). In a parallel experiment, UWM researchers have been

monitoring the biomass and P content of benthic algae in a plot cleared of mussels in 2016, located close to the control site. In that experiment, the P content of algae growing on rocks without mussels has been consistently lower than that of algae growing on rocks with mussels, which is attributed to the fact that mussel grazing and phosphorus excretion is a significant source of dissolved P that supports benthic algal growth. In the two months following the Zequanox® treatment, this effect was not observed (Figure 4-27). Again, this may be due to low light conditions in September and October. If further measurements can be made at the treatment site in the summer of 2020, they will indicate whether the decrease in mussel abundance resulting from Zequanox® treatment has a negative effect on P supply to benthic algae.

4.4.4 Round Gobies

Round goby densities were measured at the treatment and control sites before and after Zequanox® application (Figure 4-28). At the control site, gobies were counted by a scuba diver along a 20 meter transect. Transect lines were laid down with the starting point at least 10 m away from the area where benthic scrape samples were collected, to avoid fish attraction or deterrent that might result from disturbance during collection of benthic scrape samples. Following the deployment of the 20-meter long transect line, the diver waited several minutes to minimize any disturbance that may have occurred during positioning of the line. The diver then swam two to three meters above the line and counted gobies within one meter on each side of the line, resulting in a total area counted of 40 m². This was repeated in triplicate. This has been shown to be the most reliable method for determining round goby densities (Johnson et al. 2005). However, because small gobies can remain hidden under rocks and algae, the densities determined with this method are likely conservative, as some of the smaller fish would not have been counted. The same procedure was followed at the treatment site, except the transect distance was limited to 10 m at the treatment site, corresponding to the length of each of the three treatment plots. At the treatment site, gobies were counted before any benthic sampling was done, to minimize disturbance of the goby community. One 10-meter transect was counted on each of plots A, B, and C.
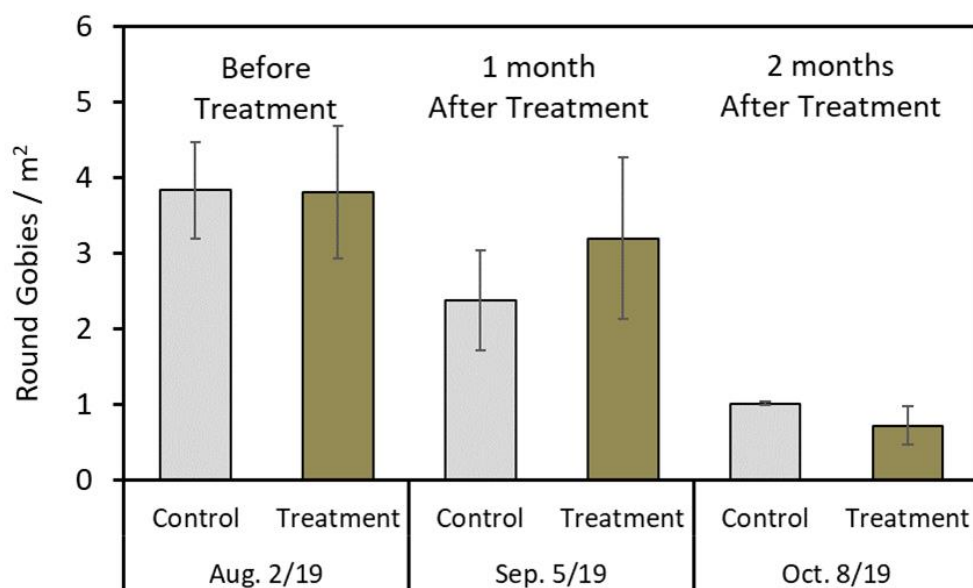

Figure 4-28. Round Goby Population Density on the Treatment and Control Sites.

Note: Zequanox® treatment was applied on August 7.

Prior to treatment, round goby population densities were equal at the control and treatment sites. In the two months following treatment, round goby numbers slowly declined on the treatment site, but they declined at a similar rate on the control site (Figure 4-28), so there was no significant difference between goby numbers on the treatment and control sites across the study. The declining trend in numbers at both locations over time was likely due to goby migration to deeper waters in the fall as nearshore temperatures began to cool (Figure 4-28; Walsh et al. 2007). The persistence in similarities of goby numbers on the treatment and control sites across time are somewhat surprising, considering the large decline in quagga mussels and other benthic invertebrates at the treatment sites shortly following Zequanox® treatment (see sections 4.2 and 4.4.1). Quagga mussels and benthic macroinvertebrates are the main food sources for round gobies, and so a decline of these fish on the treated sites would be expected.

There are two possible explanations for the persistence of round gobies, post-treatment, at the treated sites:

1. Benthic invertebrates may have remained at relatively high concentrations in protected microhabitats, such as crevices and underneath rocks. Sampling was confined primarily to the upper surfaces of rocks, but visual inspection indicates that quagga mussels on the sides and bottoms of rocks may not have been decimated to the extent that mussels on the tops of rocks were.

2. The treated area may not have been sufficiently large to affect local round goby numbers. Round gobies move frequently while foraging for food. Within the treatment area, the furthest distance from untreated substrate with ambient concentrations of benthic invertebrates was 5 m, and so goby numbers within this area may simply reflect the average regional concentration, even if the fish are finding little food in the treated area.

A full understanding of how the loss of quagga mussels will affect round goby feeding and abundance will likely require mussel removal from a larger area, although mechanistic models can also be used to explore this question if there is sufficient knowledge of goby bioenergetics.

4.5 Visual Observations

Visual assessments were used to ensure that there were no impacts on non-target organisms as a result of the treatment, and to make qualitative observations regarding the effectiveness of the treatment. The following observations were made by the UCC and UWM divers:

1. Prior to the treatment, the divers inspected the treatment area and found no evidence of native fish or fish spawning activity.

2. Visual observations during the survey confirmed that the containment areas were sealed, and Zequanox® was not released during the treatment period.

3. During post-treatment monitoring activities, the UWM divers observed the effects of the treatment, (Figure 4-26). The bottom substrate at the control site and the substrate within the treatment area approximately one month after the Zequanox® treatment clearly shows both an abundance of dead quagga mussels and a much lower level of *Cladophora*.

5 Conclusions & Recommendations

The Dreissenid Mussel Control Demonstration Project was successfully implemented in Good Harbor Bay near Sleeping Bear Dunes National Lakeshore. The demonstration Project included testing of a targeted treatment method, Zequanox® molluscicide, on an offshore rocky reef with invasive dreissenid mussels and nuisance levels of *Cladophora* algae.

The two objectives of the Project were achieved: (1) to test the feasibility and effectiveness of deploying a benthic containment barrier at 30 feet deep, on an offshore Great Lakes reef, injected with Zequanox®; and (2) to understand how the treatment may affect the composition and function of the biological community, including fish, benthic algae, benthic invertebrates, bacteria and the *Clostridium botulinum* type E toxin gene. The Project demonstrated that such a treatment is feasible, and resulted in a ~95% decrease in mussel density in the weeks following treatment. It also brought to light some coordination and implementation challenges associated with working offshore on northern Lake Michigan, and questions for further investigation around declines in non-target, benthic invertebrates and algae following treatment. Changes in water chemistry (temperature, pH, dissolved oxygen, turbidity, and conductivity) were observed during treatment but were minor and of limited duration.

Project results further suggest that treatment with Zequanox®, under the conditions applied in this study, does not carry risk of increased botulism toxin production. Quantitative polymerase chain reaction (qPCR) showed no presence of the *Clostridium botulinum* type E toxin gene or its expression in microbial communities sampled from the water column or benthic biota in control and treatment plots. Additionally, microbial community composition analysis using marker gene sequencing indicated limited shifts in bacterial community composition in the weeks following treatment, and successful containment of the Zequanox® treatment within the treatment plots. The latter indicates promise in the use of this experimental design in other open water applications.

Overall results suggest that application of Zequanox® within an underwater containment structure can produce effective, targeted treatment at a small scale (relative to Lake Michigan), and may serve as a viable, environmentally compatible option to reduce dreissenid abundance on offshore reefs within the Great Lakes. Additional monitoring is needed to assess the long-term effectiveness of the Project and whether repeated or additional treatments are needed to maintain mussel suppression at the site.

The innovative nature of this project resulted in successes, challenges, and ideas on next steps and approaches. Drawing on the breadth of experience provided by the Project partners throughout the planning, implementation, and monitoring phases, the team was able to successfully implement the Project, gaining valuable insights that can be used in future invasive mussel control efforts. This section provides observations, lessons learned, and recommendations for future activities.

5.1 Planning and Preparation

The Project team conducted extensive and detailed planning prior to mobilizing for the on-site Zequanox® treatment. This degree of planning accomplished several benefits: 1) ensured that all materials were ready for the field application; 2) defined the roles and responsibilities of each Project partner and facilitated communication among all Project participants; and 3) allowed the underwater activities to be conducted with a high degree of efficiency, which protected diver safety and limited time in the water. Some key takeaways from the planning and preparation component of the Project are:

1. Coordinating with research partners experienced with the site was critical for initial design planning. Details of the site conditions such as size and extent of reef area, topographic relief,

substrate type and size, locations of control areas, and potential treatment locations all aided efficient treatment site selections.

2. Conducting an initial site visit early in the planning process was extremely valuable for subsequently informing boat numbers (i.e., dive boat, mixing and pumping boat, materials and personnel shuttle boat), boat type requirements and positioning, and anchoring logistics. The early visit also provided critical planning with the marina managers for timing and staging of the field activities, parking, signage, etc. at the popular and small marina.

3. Conducting a reconnaissance dive prior to mobilizing for the Zequanox® application provided both above and below water site condition assessments to evaluate working depth, wind and wave exposure, and subsurface conditions for safety and field application planning. The Project team also used this reconnaissance dive to select and mark the site boundaries as well as understand and adapt project-specific approaches that are best developed on-site.

Like other complex field studies, several challenges were encountered during the Project that could be improved in future efforts, including:

1. Typically, August is a very calm month in Lake Michigan, so implementation was planned for early to mid-August. Changing regional climate conditions make planning of offshore field studies more difficult to predict, so future offshore projects may need to build in a broader implementation window.

2. The Project partners would have benefited from having more consensus and details regarding the definition of bad (i.e., less safe) weather and the associated protocols prior to starting the field work. With four boats spanning three different owner organizations, it became clear that each had a somewhat different comfort level for declining weather conditions and ambient light levels. Further, each boat had a different role for the Project, so details such as boat stability and anchoring needs differed, depending on the vessel's task. Having a clearer agreement on what constituted declining and foul weather might have improved decision-making for the challenging and quickly changing conditions.

3. Because the Project site was offshore of a national park and the treatment was done in August, which is during the peak tourist season, finding lodging was a challenge. If any future work is done in the SBDNL during peak season (June – August), lodging reservations should be made as early as possible in the planning process.

5.2 Permitting and Regulatory Considerations

This project benefited from a high degree of interest by Michigan's EGLE and the U.S. Army Corps of Engineers. The Project team had exceptional cooperation from these permitting authorities, who made an extra effort to expedite the review process so that permits did not delay the start of field work. The Project also benefited from having several federal agency partners involved, which helped to facilitate the turnaround of several permit activities, as described in Section 1.3.

The time between initiating and completing the permit process was approximately three months. It may not be prudent to assume a similar schedule is feasible for future mussel-related experiments. This project's permitting effort included a voluntary public meeting, which was planned and coordinated by the Great Lakes Commission, and may have benefited the public's understanding of the Project. Pro-active public engagement of this type is recommended for future projects. In addition, the Project team recommends allowing four to six months to obtain the necessary permits. The NPDES permit, for example, required a 30-day public notice period before the final permit could be issued. Starting the

permit process as early as possible and engaging with the permit authorities throughout the preparation and review periods is important for timely acquisition of permits.

Additionally, an aquatic pesticide applicator certification was required and included a two to three month span between ordering materials needed for the exam, applying for the state-required license, scheduling and taking the exam, and having the license issued. Future projects that include the use of Zequanox®, or other pesticide-based treatments, will also require a licensed aquatic pesticide applicator. The Project team recommends that at least one pesticide applicator be identified early in the planning process and that this person begin the certification procedures as soon as possible.

5.3 Implementation and Logistics

Thanks to the high degree of preparation and planning, the implementation of the Zequanox® treatment in the field went very well, even with the challenges posed by the inclement weather. Key to the success of the implementation was having individual boats dedicated to specific project tasks. One boat was dedicated to maintaining diver equipment and activities, while a separate boat handled Zequanox® mixing and delivery. A third boat was responsible for monitoring activities, and an occasional fourth boat served as a shuttle for materials or additional personnel, when needed. However, this also presented challenges for communication between the boats, since the Zequanox® material to be delivered to the treatment plots was on one boat and the divers actually applying the Zequanox® to the treatment plots were being directed by a boat captain from a different boat. Similarly, communication between the boats working in the treatment area and the boats conducting the monitoring could have been improved to ensure smooth collection of samples at the appropriate intervals.

Including a MBI representative on site was extremely helpful in making decisions on product mixing and delivery. Minimizing monitoring activities while the divers were in the water was also a good practice to maintain their safety, reduce occurrences of their lines getting tangled up or constricted, and minimize substrate disturbances during Zequanox® application.

As the data and subsequent discussion in Section 4.2 indicate, future treatments using caged mussels would benefit from identifying a better method to deploy the caged mussels so they have similar positioning to provide comparable exposure to that of the uncaged mussels. This might include coarser mesh cages and positioning the cages within the matrix of the reef, rather than on a brick. Additionally, a greater number of cages, randomly located across the plot, with more individuals within each cage would also increase the statistical rigor during data evaluation.

5.4 Monitoring and Assessment

The monitoring conducted for the Project was extensive, as discussed in Section 4. It would have been helpful to have more water quality sensors deployed in each enclosed area during treatment. Additional turbidity sensors would allow for a more representative assessment of Zequanox® concentrations across the site to capture potential variability in micro-level exposure during the containment period. One of the key concerns during the treatment was the length of time and extent of anoxic conditions, so additional dissolved oxygen sensors would also be beneficial. A low cost option could be using multiple PME MiniDOT sondes in each enclosure, rather than the larger, more costly multiparameter probes.

One lesson learned during the Project was that the distance between treatment area and control site made it difficult for the same crew to collect water chemistry samples from both areas, as originally planned. It would have been more efficient to have one boat and crew deployed at the control site to collect samples and another boat and crew deployed at the treatment site, but this also comes with an added expense.

Another unforeseen complication was trying to work with an analytical laboratory's schedule for conducting $CBOD_5$ analysis, which has specific timing requirements to complete the analysis. The location of the laboratory, relative to the field site, provided some logistical challenges for transport. The field activity schedule was planned in part to accommodate the laboratory's regular $CBOD_5$ analysis schedule to the extent possible. In hindsight, it would have been better to provide additional flexibility in scheduling for the field crews, and pay extra fees to the laboratory if needed, to have staff conduct the $CBOD_5$ analysis using overtime.

The caged mussel component of the monitoring was more challenging than anticipated and required more diver time to set up than desired. MBI would have preferred more mussels per cage and more cages to boost the statistical power of the caged mussel mortality data. There was also some discussion regarding the value of the caged mussel data in light of the extensive post-treatment monitoring activities. This would be a good topic for the IMC to discuss and develop a consensus decision regarding the use of caged mussels in future treatment experiments.

5.5 Recommended Future Activities

The results and lessons learned from this project provide a foundation for a next set of questions for further discussion and investigation by the IMC and its partners:

1. How does the Zequanox® treatment method compare in effectiveness, non-target impacts, cost, and feasibility to other methods or approaches for Great Lakes coastal reefs?

2. What is the effect of leaving the dead mussels and their shells on site on mussel recolonization, goby predation, and fish spawning; and what effect does dead, decomposing tissue have on water quality and nutrient levels? In previous mussel removal projects in Good Harbor Bay, mussels were manually scraped off the rocks, harvested, and physically removed from the site. In this project, the dead mussels in the treatment area were left on site, except for the areas that were monitored for mortality effectiveness as described in Section 4.2.

3. What are the long-term effects of treatment, including understanding if, when, and how mussels may recolonize the treated site, and what other changes are seen at the site (e.g., round goby activity, *Cladophora* growth, habitat use by desirable species)?

4. Are there other monitoring methods that provide information on the biological community but are less expensive than the current approach of using divers to manually collect and analyze biological community samples?

The first question might be addressed by comparing this approach for Zequanox® treatment to the hand scraping project, or through other experiments using different treatment technologies or approaches. Suggestions discussed by the IMC include deploying a benthic barrier alone for an extended period of time, a low-dose copper treatment, and hot water pressure washing. Modifications to the Zequanox® approach, given the depth of the site, may include a short (1-2 meter), walled containment area, without a top, that might be simpler to set up and treat, under the assumption that the observed density of the Zequanox® would stay within the containment areas and allow the recommended exposure. Further, open site applications (no containment) might be tested to understand the level of treatment under short exposure periods.

A second recommendation related to future mussel removal projects is to use the results of the Project partners' detailed bottom bathymetry and habitat classification mapping to identify potential target areas for future mussel removal experiments. In this study, the IMC and partners like NPS, MDNR, and NOAA collaborated within Good Harbor Bay, and this type of collaborative approach should be used in the future to identify other target areas for future mussel removal experiments.

The second and third questions could be addressed with additional monitoring at the manual removal and Zequanox® treated sites. Additional monitoring activities could include assessing stomach contents of round gobies between the two sites to identify potential changes in predation before and after treatment. In addition, future monitoring activities would provide information on potential mussel recolonization of the treated site. If mussels do return, depending on the extent, it could indicate the need for additional treatments or modifications in the approach to sustain mussel suppression over a longer period of time. Due to the long-term investment of research and interest in restoration activities within Good Harbor Bay, the Project team is optimistic that there will be opportunities to return to the site and collect additional data on mussel populations, round goby activity, and fish spawning.

Several ideas for additional monitoring have been suggested with respect to the fourth question, including monitoring coregonid use of treated sites, expanding deployment of egg mats or use of genetic tools. This might specifically assess egg predation (qPCR) to see if target species eggs or their relative abundance change before and after treatment. Another example might include hatching success of known sites using eRNA methods to assess differences in spawning success before and after treatment. Discussions are ongoing for potential future collaborative projects incorporating these ideas.

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6 References

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Appendix A
Zequanox® Mixing and Delivery Details

Prior to treatment, the delivery system was setup in the boat and tested for proper operation before adding the Zequanox® product. A spill containment tub was setup underneath the delivery system to catch any potential leaks or spills that might occur during the delivery process. The large tank was secured to the side wall of the boat using ratchet straps and bungee cords. All tubing was attached and seals were checked during a leak test to prevent spills. The engine was filled with gas and run for a brief period to ensure proper operation. Once the system was checked and in proper working order, the dosing procedure could begin.

The following steps were followed for mixing and delivery of the Zequanox®:

1. Using a 1/3 horsepower submersible pump, fill the conical mixing tank with 26 gallons of lake water.

2. Before turning on the water pump, set the conical mixing tank T-Valve to recycle the water (point the valve handle towards the tank).

3. Turn on the water pump and increase the throttle to approximately half speed. Let the pump run for a brief period and look in the tank to ensure water is being recycled back into the tank.

4. Before handling Zequanox®, put on all necessary PPE (long sleeved shirt, long pants, face mask, and nitrile gloves). Take one 6 kg bag of Zequanox® (prepared by MBI prior to arrival at the site) and holding the bag over the opening in the tank, cut off the corner of the bag, making an approximately two inch hole.

5. Slowly empty the Zequanox® product into the tank while vigorous mixing is occurring to minimize product clumping.

6. Once the contents of the bag have been emptied, use the stirring paddle to provide additional mixing and break up remaining clumps. If after a few minutes of mixing, there still appear to be large clumps of product, increase the pumping rate to further homogenize the contents. (In some cases, gloved hands were used to manually break up larger persistent chunks of Zequanox®.)

7. Once the product is fully mixed and all large clumps have been broken up, reduce the engine throttle to the minimum speed setting for product delivery.

8. At this point, the divers need to be signaled to get into position for product delivery. Once they are ready, the tank T-Valve is rotated 180 degrees to point toward the delivery line. On the deck a few feet down the delivery line is a butterfly flow reduction valve. This valve needs to be opened to approximately 1/2 flow for delivery.

9. At this point, product is being delivered to the divers. The diver will have the distribution wand inside the containment area and have control of the flow rate with a butterfly valve at the entrance to the distribution wand. The product needs to be distributed as evenly as possible by moving the wand back and forth across the treatment area. During this process, the tank needs to be watched carefully and shut off once half of the volume has been delivered.

10. Once half of the product has been delivered, the tank T-Valve needs to be rotated back to point towards the tank and the ground butterfly valve will be shut off. The divers will be signaled that flow has stopped, and they will now need to move to the opposite side of the containment area.

11. The diver closes the valve on the distribution wand and moves to the port on the opposite side of the enclosure.

12. Once the diver has inserted the wand into the opposite side of the containment area, the process can be repeated. Turn the T-Valve 180 degrees to deliver flow to the diver. Turn the deck butterfly valve to 1/2 open for delivery.

13. Once the tank is mostly drained, use the submersible pump spray nozzle to spray down the inside of the tank with lake water. This will clean the tank and remove any clinging solids from the tank wall. Once the tank is clean and empty, shut off the deck butterfly valve, turn the T-Valve back towards the tank, and shut off the engine. This concludes the Zequanox® delivery process for a single containment area. This process will be repeated until all treatment areas have been dosed appropriately.

Appendix B
Analysis Methods

DNA/RNA Extractions

Extractions were performed using the Qiagen RNeasy PowerSoil Total RNA Kit with DNA co-extraction on samples of benthic algae, macroinvertebrates, and dreissenid mussel tissue according to manufacturer's instructions. Samples were stored in the -80°C until extracted. One-half gram of wet biomass was added to each reaction tube. DNA/RNA extractions from water filters were performed using the Qiagen Allprep DNA/RNA/MiRNA Kit according to manufacturer's instructions.

Quantitative PCR Assay Template Preparation

The DNA standard template was synthesized from a synthetic oligonucleotide containing the T7 promoter region, *bont*E target region, spanning the region between and including the previously developed qPCR primer (*bont*E 1467F and 1605R; Wijesinghe et al. 2015). The dsDNA *bont*E template was purified using the Qiagen MinElute Reaction Cleanup Kit. Then, the RNA *bont*E standard template was synthesized using the TranscriptAid T7 Kit and purified with Zymo RNA Clean and Concentrator kit following manufacturer's instructions. Copies of the *bont*E gene were estimated based on DNA *bont*E PCR product or in vitro generated RNA transcript concentrations and product length.

Reaction Conditions for qPCR/RT-qPCR Assays

An Applied Biosystems QuantStudio 3 was used to amplify the *bont*E target sequence in both the qPCR and RT-qPCR assays. All samples were run with a triplicate six-point standard curve ranging from 106 to 10 gene copies and triplicate negative template controls. Each 20 µL qPCR assay contained the following reagents: 5 µL DNA template, 10 µL 2X qPCR Fast Master Mix, 1 µL of 18uM forward and reverse primers *bont*E_1467F (5'-AATATTGTTTCTGTAAAAGGC ATAAGGAA-3') and 1605R (5'-AAGTTACTGTATCGTCAATTTCTTT AGGAG-3'), 1 µL of 4 µM *bont*E taqman probe (5'-56FAM-AATGGTGAG-ZEN-TTATTTTTTGTGGCTTCCGAGAAT- 3IABkFQ-3'), and 4 µg bovine serum albumin. The thermocycling conditions were as follows: 2 min at 50 °C and 2 min at 95 °C for denaturing, followed by 50 cycles at 95 °C for 1 s for denaturation, 60 °C for 20 s for annealing and extension, and a final hold step at 4 °C. Each RT-qPCR assay contained the following reagents: 5 µL RNA template, 10 µL 2X RT-qPCR Master Mix, 0.5 µL 40X Enzyme Mix, 1 µL of 18 µM forward and reverse primers bontE_1467F and 1605R, 1 µL 4 µM bontE taqman probe, 4 µg bovine serum albumin, and nuclease-free water. The thermocycling conditions were as follows: 15 min at 48 °C and 10 min at 95 °C for denaturing, followed by 40 cycles at 95 °C for 15 s for denaturation, 60 °C for 1 min for annealing and extension, and a final hold step at 4 °C.

Quality Control and Limits of Detection/Quantification for qPCR/RT-qPCR

The sample matrix inhibition of the qPCR/RT-qPCR assays was tested by spiking in a known concentration of *bont*E standard template into a dilution gradient of each matrix. Deviations in the *bont*E standard template concentrations based on matrix would indicate inhibition in the qPCR assay. All matrix and template combinations were run in triplicate to determine potential inhibition (Figure 4-23). The limit of detection (LOD) for both assays was 1 copy *bont*E. The LOD was calculated as the lowest concentration of known target that meets the criteria that (i) the standard deviation of Ct values (cycle thresholds are the number of cycles at which the fluorescence signal exceeds the background level) is less than 1, (ii) the number of replicates with detections is greater than 95%. The limit of quantification (LOQ)

is 10 copies and 20 copies for the qPCR and RT-qPCR assays respectively. The LOQ was established by calculating the average Ct value of the LOD and subtracting two-times the standard deviation of those Ct values, represented by the following equation: $CtLoQ = CtLoD - 2(\sigma(CtLoD))$.

16S rRNA Gene Sequencing and Analysis

To determine bacterial community composition, amplicon sequencing targeting the V4 region of the 16S rRNA gene (515F/806R) was performed at the University of Michigan Medical School. Pooled libraries were sequenced on an Illumina MiSeq sequencer, using v2 chemistry 2x250 (500 cycles) paired-end reads. RTA v1.17.28 and MCS v2.2.0 software were used to generate data. Analyses were performed with mothur v.1.34.3 using the MiSeq standard operating protocol for the generation of the operational taxonomic unit (OTU, 97% sequence similarity) table. Only bacterial sequences were retained. For classification, we used a hybrid protocol using a freshwater-specific taxonomy (https://github.com/mcmahon-uw/FWMFG) and the SILVA release 119 taxonomy. Further analyses were carried out in R version 3.2.1 using phyloseq and vegan. All figures were generated using the ggplot2 R package. All beta-diversity analyses were performed using Bray-Curtis dissimilarities based on OTU read count data. Differences in assemblage composition based on treatment were performed using a permutational multivariate analysis of variance (PERMANOVA) using the adonis function (vegan).