Efficacy of **S**pray-**D**ried *Pseudomonas fluorescens*, **S**train CL145A (Zequanox®), for **C**ontrolling **Z**ebra **M**ussels (*Dreissena polymorpha*) within Lake Minnetonka, MN **E**nclosures

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August, 2016

Cooperator Project Completion Report

Funded by and Submitted to:

Legislative-Citizen Commission on Minnesota Resources
100 Rev. Dr. Martin Luther King Jr. Blvd.
Room 65 State Office Building
St. Paul, MN 55155

Acknowledgments

Any use of tradename, product, or firm names in this report are for descriptive purposes only and does not imply endorsement by the U.S. Government. Financial support for this study was provided by the State of Minnesota's Legislative-Citizen Commission on Minnesota Resources, Environment and Natural Resources Trust Fund and U.S. Geological Survey appropriated funds. The authors thank personnel from the Upper Midwest Environmental Science Center, including, Matthew Barbour, Mark Roth, Diane Waller, Kerry Weber, and Jeremy Wise for assisting with data collection. We extend sincere thanks to Gabe Jabbour and staff at the Tonka Bay Marina for allowing us to utilize their facilities and equipment which were essential for the completion of this project. We thank Eric Fieldseth and the staff at the Minnehaha Watershed District for their assistance in project coordination and execution as well as the use of their laboratory facilities as well as Megan Weber from Marrone Bio Innovations for technical assistance. Finally, we thank Dr. Barbara Bennie, Director of the University of Wisconsin at La Crosse Statistical Consulting Center, for her help with statistical analysis and data interpretation.

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Abstract

The efficacy of whole water column and subsurface applications of the biopesticide Zequanox®, a commercially prepared spray-dried powder formulation of *Pseudomonas fluorescens* (strain CL145A), were evaluated for controlling zebra mussels (*Dreissena polymorpha*) within 27-m^2 enclosures in Lake Minnetonka (Deephaven, Minnesota). Five treatments consisting of (1) two whole water column Zequanox applications, (2) two subsurface Zequanox applications, and (3) an untreated control were completed on each of three independent treatment days during September 2014. The two types of samplers used in the study were (1) type 1 samplers, which were custom built multi-plate samplers (wood, perforated aluminum, and tile substrates) that were placed into Robinson's Bay in June of 2013 to allow for natural colonization by zebra mussels, and (2) type 2 samplers, which consisted of zebra mussels adhering to perforated aluminum trays that were placed into mesh containment bags. One day prior to treatment, three individual samplers of each type were distributed to test enclosures and exposed to a randomly assigned treatment. Sampling to determine the zebra mussel biomass adhering to type 1 samplers and the survival assessments for zebra mussels contained in type 2 samplers were completed ~40 days after exposure. The zebra mussel biomass adhering to type 1 samplers and the survival of zebra mussels contained in type 2 samplers were significantly less in groups treated with the highest Zequanox concentrations and in groups that received whole water column applications than comparable groups treated with lower Zequanox concentrations and subsurface applications. However, standardization of biomass and survival results to the amount of Zequanox applied showed that the lower concentrations and subsurface applications were more cost efficient, with respect to product used, at reducing zebra mussel biomass and for inducing zebra mussel mortality. Although the subsurface application methods and lower treatment concentrations were more cost efficient, biological significance and management goals should be evaluated prior to selecting the application method. Development and refinement of additional application techniques may improve the utility of the subsurface Zequanox applications.

Introduction

Zebra mussels (*Dreissenia polymorpha*) are native to the Black, Caspian, and Aral Seas of eastern Europe (Gollasch and Leppäkoski, 1999) and were likely introduced into Lake Erie as veliger larvae in the summer or fall of 1985 (Mackie and Claudi, 2009). Their high reproductive capacity and planktonic larval stage enable zebra mussels to rapidly disperse (Birnbaum, 2011). Less than 10 years after introduction, zebra mussels were established in all of the Great Lakes and in the Arkansas,

Cumberland, Hudson, Illinois, Mississippi, Ohio, and Tennessee Rivers. Additionally, zebra mussels have been reported within the borders or in adjacent waterways of twenty-eight states (Benson et al., 2016). Many pathways have been implicated as overland dispersal mechanisms for transporting zebra mussels to inland lakes, including many references to small, trailered watercraft (Gollash and Leppäkoski, 1999). Once established in a waterbody, the likelihood of spreading to nearby waterbodies is greatly enhanced by the "dispersion kernel" phenomenon, which is a function of infestation probability and distance from a source population (Havel et al., 2015). As of April 2016, the Minnesota Department of Natural Resources listed 243 waterbodies as infested due to either confirmed zebra mussel presence or interconnection to a waterbody with a confirmed presence (http://www.dnr.state.mn.us/invasives/ais/infested.html, accessed May 16, 2016).

The myriad of ecosystem level impacts that zebra mussels can inflict on naïve systems have included significant increases in benthic algae and macrophyte biomass and increases of up to 2,000% in total zoobenthic biomass (Higgins and Vander Zanden, 2010). Zebra mussel attachment and pseudofeces deposition have been shown to result in interstitial space occlusion and subsequent habitat rejection by spawning fish, rendering once productive spawning shoals severely degraded (Einhouse et al., 2013, Marsden and Chotkowski, 2001). Due to their sedentary life style and their evolution in dreissenid-free waters, native Unionid mussels are particularly vulnerable to zebra mussel infestations and extirpation of Unionid mussels has been documented in some waterways following zebra mussel infestation (Napela, 1994; Ricciardi et al., 1996; Ricciardi et al., 1998). Of equal concern is the influence that zebra mussels have on harmful algal blooms (HABS) through selective rejection of *Microcystis* and other graze-resistant cyanobacteria species (Vanderploeg et al., 2001). HABS pose potential human health hazards in addition to causing domestic and wild animal mortalities (Wynne et al., 2015).

Substantial economic burdens are associated with biofouling control and damages related to zebra mussel infestations in water intake pipes, water filtration systems, and electrical generating facilities. Pimentel et al. (2005) cites costs of $1 billion/year related to damages and the control of biofouling dreissenid mussels (zebra and quagga mussel, *D. rostriformis bugensis*).

Upon introduction to a new environment, invasive species typically exhibit a population growth lag phase prior to a period of rapid growth (Crooks and Soulé, 1999). After this initial lag phase and establishment of the invasive species, eradication is nearly impossible (Bax, 2001; Crooks and Soulé, 1999). Until recently, no commercially available products were available to natural resource managers for use in controlling dreissenid mussel populations in open-water environments. The recently developed biopesticide, Zequanox®, may have potential use in integrated pest management programs designed to mitigate the detrimental effects of dreissenids. Zequanox is a commercially prepared spray-dried powder formulated product produced by Marrone Bio Innovations (Davis, CA) and it contains a specific strain (CL145A) of the common soil bacterium *Pseudomonas fluorescens* as the active ingredient (Luoma et al., 2015; Whitledge et al., 2015). Zequanox was registered in 2014 by the U.S. Environmental Protection Agency (registration number 84059-15) for controlling dreissenid mussels in open-water systems. Zequanox is readily ingested by zebra mussels as a food source and upon ingestion components associated with the active ingredient bacterial cells lyse the epithelial cells in the zebra mussel's digestive gland, resulting in death (Molloy et al., 2013). Zequanox applied at a concentration of 50 mg active ingredient (A.I.)/L in 350-L tanks containing lake water caused significant zebra mussel mortality within 6 hours of exposure (Luoma et al., 2015).

The objectives of this study were to evaluate the efficacy and application of Zequanox for controlling zebra mussels in open-water environments. To achieve these objectives, zebra mussel mortality and the reduction in zebra mussel biomass were assessed after whole water column and

subsurface applications of Zequanox were completed in replicated 27-m² enclosures positioned in Lake Minnetonka, Minnesota.

Materials and Methods

Overview

The study was conducted in Robinson's Bay, Lake Minnetonka, MN, (Fig. 1) where Zequanox treatments were applied to five 27-m² enclosures on three separate treatment days. On each of these days, the five treatments randomly applied to enclosures for 8 hours consisted of (1) an untreated control treatment, (2) a 50 mg Zequanox A.I./L whole water column treatment, (3) a 50 mg Zequanox A.I./L subsurface application treatment, (4) a 100 mg Zequanox A.I./L whole water column treatment, and (5) a 100 mg Zequanox A.I./L subsurface application treatment. The enclosures were the experimental units for the study (n = 15). Multi-plate samplers with adhering zebra mussels (type 1) and samplers with zebra mussels adhering to perforated aluminum trays contained within semi-rigid plastic mesh bags (type 2) were placed within each treatment replicate and used to assess the treatment-related impacts to zebra mussel biomass and zebra mussel mortality, respectively. Zequanox A.I. concentrations and water chemistry parameters were measured throughout the exposure period; sample collections for determining biomass and survival assessments were conducted approximately 40 days after exposure.

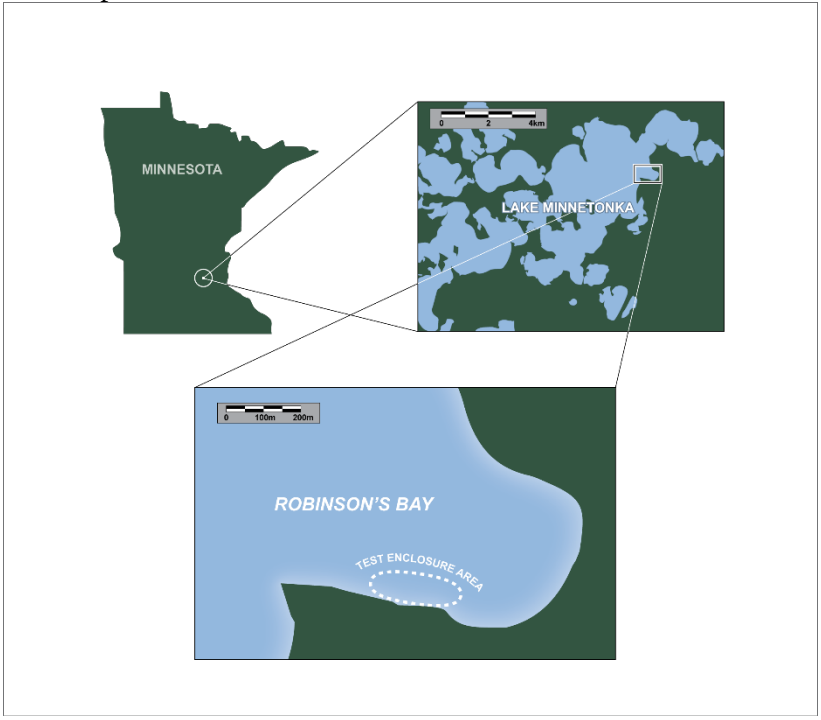

Figure 1. Location of test enclosures, Robinson's Bay, Lake Minnetonka, MN

Test Article

The test article, Zequanox, was produced by Marrone Bio Innovations, Inc. (Davis, CA) and was a spray-dried powder formulated product containing 50% (w/w) active ingredient (*Pseudomonas fluorescens*, strain CL145A). The test article was delivered directly to the test site and stored in a mobile refrigeration trailer at ~4°C during the course of the study. A sample of the test article was returned to Marrone Bio Innovations after each application day (n = 3) and retention of biological activity was confirmed in each sample using a Marrone Bio Innovations standard bioassay. Treatments and concentrations are reported as milligrams of active ingredient/L.

Test Animals and Test Samplers

Zebra mussels were the test animals evaluated in this study and they were either adhering to samplers (type 1 samplers; Figs. 2a, 2b) or placed in containment samplers (type 2 samplers; Figs. 3a, 3b). Type 1 samplers were custom built multi-plate samplers that consisted of a concrete base with

three attached metal rods. Attached to each metal rod were four square (~15.2 x 15.2 cm) substrates of the same type (wood, perforated aluminum, or stone tile). The substrates were separated from the concrete base using a 20 cm long poly vinyl chloride (PVC) pipe spacer and from each other using a 2.5 cm long PVC pipe spacer. The type 1 samplers extended ~46 cm from the lake bed. Type 2 samplers consisted of zebra mussels adhering to 15.2 x 15.2 cm perforated aluminum trays that were placed into semi-rigid plastic mesh containment bags (~20.3 x 25.4 x 5.1 cm, W x H x D; 0.31 x 0.31 cm openings). The type 2 samplers were suspended vertically within ~5 cm of the water/lake bed interface using a welded steel frame.

Figure 2. Example of multiple-plate (type 1) samplers before (A, Left) and after deployment for two growing seasons in Robinson's Bay, Lake Minnetonka, MN (B, right).

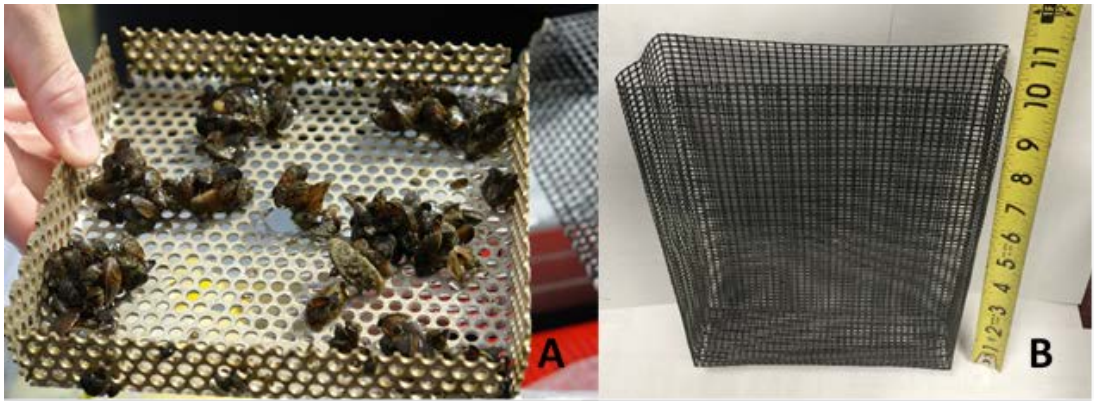

Figure 3. Example of type 2 sampler components consisting of a perforated aluminum tray with adhering zebra mussels (A, left) and a semi-rigid plastic mesh containment bag (B, right).

Type 1 samplers

In June of 2013, type 1 samplers were placed in Robinson's Bay of Lake Minnetonka in three independent groups (i.e. stockpiles) that were spatially separated ~20 meters, in water ~4.5 m deep. The type 1 samplers were then naturally colonized by zebra mussels over two growing seasons before use in this study. Three days prior to initiating the exposures, the type 1 samplers were collected from the stockpile locations using a boat mounted crane and placed into ~4.5 m of water near the southern shore of Robinson's Bay. Samplers from each stockpile were indiscriminately selected and uniquely identified with color and number coded tags. Three samplers, one from each stockpile, were assigned to each treatment replicate according to a randomization scheme (n = 3/enclosure, 45 total). The mean length of

zebra mussels adhering to type 1 samplers was determined by measuring 100 animals from each of the three material types on three untreated samplers (one from each stockpile; n = 900). The mean zebra mussel size for type 1 samplers was 8.62 ± 3.49 mm.

Type 2 samplers

Approximately 40 days prior to exposure, zebra mussel encrusted rocks were collected from Robinson's Bay and zebra mussels were removed by severing their byssus with a scalpel. Due to difficulty in assessing survival of smaller zebra mussels, they were excluded by sieving the zebra mussels in a plastic-mesh screen box (0.63 x 0.63 cm openings). The zebra mussels retained in the screen box were then indiscriminately distributed in groups of ~200 onto perforated aluminum trays (15.2 x 15.2 cm, 4.8 mm perforations, 51% open). A total of 90 trays were prepared and secured in groups of 10 onto threaded rods fixed to the base of holding cages that were constructed according to the methods described by Brady et al. (2010). The trays were separated from one another using 3.2-cm long PVC pipe spacers (1.9 cm i.d.). The holding cages were placed in ~1.5 m of water along the southern shore of Robinson's Bay and marked with a submerged hazard buoy. Three days prior to initiating the exposures, type 2 samplers were completed by removing the perforated aluminum substrates from the holding cages and placing individual substrates, with adhering zebra mussels, into uniquely identified semi-rigid plastic mesh containment bags (~20.3 x 25.4 x 5.1 cm, W x L x D; 0.31 x 0.31 cm openings). The type 2 samplers were then distributed to treatment replicates according to a randomization scheme (n = 3/enclosure, 45 total). The mean size of the zebra mussels in type 2 samplers was determined by measuring up to 100 live and 100 dead animals from each test replicate after the survival assessments were completed (n = 2,647). The mean size of zebra mussels in type 2 samplers was 16.20 ± 3.30 mm.

Type 1 and 2 samplers were placed into enclosures ~18 h prior to treatment initiation and removed approximately 12 h after treatment termination. The treated samplers were placed in clusters in ~1.5 m of water along the southern shore of Robinson's Bay for ~40 days before being sampled for biomass (type 1) or assessed for zebra mussel survival (type 2).

Test Enclosures

Five rectangular 27-m^2 (3 x 9 m) test enclosures were placed in ~1.5 m deep water along the southern shore of Robinson's Bay, Lake Minnetonka (between N 44° 56'37.4" W 093° 31'24.2" and N 44° 56'37.8" and W 093° 31'28.8"; Fig. 4). The enclosures were assembled by connecting welded aluminum frame panels (3 x 1.8 m, L x H) that were covered with an impermeable 30-mil ethylene propylene diene monomer **(**EPDM) pond liner membrane. Each enclosure consisted of eight panels that were interconnected and sealed with hook and loop fasteners sewn to the EPDM membranes and attachment flaps. A 0.3 m wide EPDM skirt on each panel created a seal with the lake bed. Ballast chain (~0.95 cm diameter) and sand bags placed on the skirts aided in creating the bottom seal and rebar stakes were passed through 2.5 cm diameter aluminum pipes welded to the frames and then driven into the lake bed to secure the panels. The enclosures were positioned adjacent to each other equidistant from the shoreline with ~3 m of separation.

Figure 4. Enclosures positioned in Robinson's Bay (Lake Minnetonka, MN) that are open to allow for water exchange.

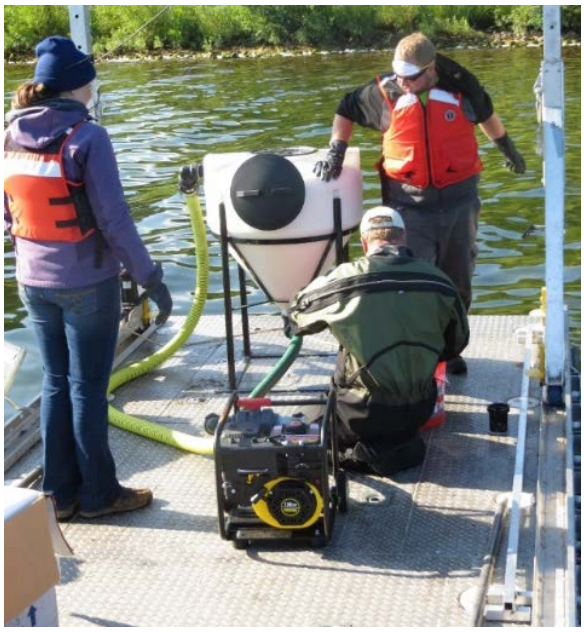

Figure 5. Zequanox suspension mixing system that was used to prepare the 5% (w/v) Zequanox suspensions for application to test enclosures in Robinson's Bay, Lake Minnetonka, MN.

Treatment Applications

Treatments were randomly assigned and applied to enclosures on three independent treatment days with a minimum of 48 h between applications. On each treatment day, the five 8 hour treatments applied were (1) an untreated control, (2) a 50 mg Zequanox A.I./L whole water column treatment, (3) a 50 mg Zequanox A.I./L subsurface treatment, (4) a 100 mg Zequanox A.I./L whole water column treatment, and (5) a 100 mg Zequanox A.I./L subsurface treatment. The amount of Zequanox applied to each enclosure ranged from ~1.9 to 7.7 kg in the 50 mg A.I./L subsurface applications and the 100 mg A.I./L whole water column application, respectively. Regardless of application method, Zequanox was applied as a 5% (w/v) suspension. The Zequanox was mixed into suspension for application by placing lake water into a 151-L conical mix tank (Ace Roto-Mold, Model IN0040-30, Den Hartog Industries, Inc. Hospers, IA) which was attached to a gas powered semi-trash pump (Champion Power Equipment, Model 66520, Champion Power Equipment, Santa Fe Springs, CA) using 5.1-cm i.d. suction hose with cam and groove connectors (Fig. 5). The pump recirculated the water in the mix tank which created a vortex into which the Zequanox was added. After ~10 minutes of mixing, the Zequanox suspensions were transferred into a 151-L application tank positioned on a 4.3 m flat-bottomed boat. The application tank was fitted with a commercially-available sprayer pump (Fimco Industries, Dakota Dunes, South Dakota; High-Flo Gold Series, 12-V, 14.4 LPM, duplex diaphragm) that was used to recirculate and deliver the Zequanox suspensions for all applications.

Whole water applications were applied to enclosures by connecting the application tank to a PVC pipe wand (~1.8 m L x 2.54 cm i.d.) which terminated with six horizontally placed 3-mm (i.d.) hose barb fittings. The applicator applied the Zequanox by walking on planks positioned on top of the enclosures and moving the wand throughout the water column for even application (Fig. 6). Care was taken during the applications to avoid the test samplers.

Figure 6. Zequanox being applied to whole water column enclosure in Robinson's Bay, Lake Minnetonka, MN.

Subsurface applications were applied ~91 cm from the lake bed using an application bar constructed from 2.54 cm i.d. PVC pipe with 3.97 mm holes drilled 30° below horizontal (Fig. 7). The bar was 2.5 m long and divided into two 1.25-m sections. Zequanox was pumped to each section through a length of 1.27-cm i.d. tubing and delivered through a total of 58 injection ports (14 paired holes + 1 end hole per section x 2 sections). Prior to application, air was removed by pumping untreated lake water through the application system. The appropriate amounts of the Zequanox suspensions were delivered to achieve the desired treatment concentrations (50 or 100 mg A.I./L) in the bottom 61 cm of the water column plus an additional 25% was applied to account for anticipated losses through drift. Twelve application positions for the application bar were pre-marked along the long sides of the enclosures 37.5 cm from one end and then every 75 cm thereafter. Zequanox was independently applied through each side of the application bar at each application position (i.e. 24 separate injections events were completed). A predetermined pump flow rate of 11.5 LPM was used to calculate the length of time the Zequanox was applied at each position (~9.5 seconds/section [50 mg A.I./L applications; 9.0 seconds/section [100 mg A.I./L applications]).To reduce potential mixing,the application bars were not removed from the enclosures until after the 8 hour exposure period.

Concentrations of active ingredient were verified during the exposure period by collecting water samples from the center of the enclosures at three equidistant locations along the length of each enclosure using a battery powered peristaltic pump and prepositioned peristaltic tubing. At each of these locations, samples were collected from three depths (15, 30, and 60 cm from the lake bed) at 2, 4, and 7.5 hours after Zequanox application. Concentrations of active ingredient were determined by comparing sample absorbance to a linear regression curve created from known concentrations of active ingredient (25, 50, 100, and 150 mg/L) using a Barnstead-Turner model SP-830 Plus spectrophotometer at 660 nm. At the termination of the 8-h exposure period, the end panels of the enclosures were opened to allow for Zequanox dissipation and water samples were collected one hour post-exposure to determine the dissipation rate.

Figure 7. An application bar being used to apply Zequanox to a subsurface application enclosure in Robinson's Bay, Lake Minnetonka, MN.

Water Chemistry

Dissolved oxygen, pH, and temperature were directly measured in each enclosure 2 and 7.5 hours after Zequanox application. Alkalinity, conductivity, and hardness were determined from pooled water samples that were collected 2 hours after application. Four hours after Zequanox application, 60-mL water samples were collected 15 cm from the lake bed from each enclosure and acidified with two drops of concentrated H_2SO_4. These samples were used for determining nitrogen and phosphorous content using the automated hydrazine reduction method and the automated ascorbic acid reduction method (Standard Method 4500-NO3-G and Method 4500-P-F in American Public Health Association et al., 2005). Seven and one-half hours after Zequanox application, ~15-mL water samples (n = 3) were collected 15 cm from the lake bed, pooled by enclosure, filtered through a 0.45-µm syringe filter, acidified to pH ≤ 2.5 with 10% sulfuric acid, and stored at ~4 ºC for later analysis of total ammonia nitrogen (TAN) content using the automated phenate method (Standard Method 4500G in American Public Health Association et al., 2012).

Type 1 Sampler Assessments

Approximately 40 days after exposure, type 1 multi-plate samplers were dismantled and all zebra mussels adhering to each individual substrate plate were removed and placed into a shallow fiberglass tray. Heavy colonization and the need to individually assess each zebra mussel for survival precluded hand sorting and enumeration of living and dead zebra mussels. Therefore, other invertebrates, algae, and debris were removed and the zebra mussels were placed into a uniquely labelled freezer bag and stored frozen until used for determining zebra mussel biomass. Due to constraints of time and expense, only the top plates of each substrate type (n = 3/sampler) on each sampler (n = 3/enclosure) were used to evaluate the zebra mussel biomass per square meter of substrate by treatment group.

Figure 8. Industrial mixer with custom stainless steel containers used to pulverize dried zebra mussels prior to burning in a muffle furnace for zebra mussel biomass determination.

The zebra mussel biomass was defined as the ash free dry weight (dry weight of sample – ash weight of sample). Zebra mussel samples from the top plates were dried at 60°C for 120 hours until a constant dry weight was observed, then the dried samples were pulverized in custom manufactured stainless steel containers (19 x 16.8 cm, H x dia.; 0.32 cm wall thickness) that contained twenty 1.27 cm diameter and twenty 0.635 cm diameter hardened (440C) stainless balls for homogenization media. Stainless steel covers were placed on the containers and secured with electrical tape, then the containers were placed in a Pacer dual-arm, bi-axial motion industrial mixer (Pacer model dual 15; Pacer Industrial Mixers, Inver Grove Heights, MN; Fig. 8) and shaken for 15 minutes. Four ~3 g replicate subsamples of the resultant homogeneous powder (Fig. 9) were

weighed to the nearest one hundredth of a milligram into clean, tared, 40-mL high-form crucibles (part number 60108; Coorstek, Golden, CO), and burned at 450°C for four hours in a Fisher Isotemp muffle furnace. After cooling to ambient temperature in a desiccator, each crucible was weighed again to determine ash weight. The mean percentage of zebra mussel biomass ([subsample dry weight – subsample ash weight] / subsample dry weight x 100) of the subsamples was then used to calculate the amount of zebra mussel biomass present in the entire sample. The zebra mussel biomass of each sample was then standardized by the mean surface area (m^2) of appropriate substrate type. Surface area was defined as the sum of the area for each side of the substrate (i.e. top, sides, and bottom). Mean substrate surface areas were calculated by measuring the area on a single plane, surface imperfections and perforations (aluminum substrates), except for the center mounting holes, were omitted from the calculations. The treatment groups were then compared using the biomass per square meter of substrate.

Figure 9. Dried and pulverized zebra mussel subsample in a crucible prior to burning in muffle furnace for determining zebra mussel biomass.

Type 2 Sampler Assessments

Approximately 40 days after exposure, all zebra mussels were removed from each type 2 sampler and placed into a shallow fiberglass tray. Each zebra mussel was individually assessed for survival by applying gentle pressure against the adductor muscle. Mussels that resisted opening when pressure was applied were considered to be alive. The number of dead and live zebra mussels in each sampler were then enumerated and compared by treatment group.

Data Analysis

Water chemistry (DO, pH, temperature, alkalinity, water hardness, conductivity, total ammonia nitrogen (TAN), un-ionized ammonia, phosphorus, and nitrogen) and exposure concentration data analyses were limited to simple descriptive statistics calculated using SAS software version 9.3 (SAS, 2010) and Microsoft Office Professional Plus 2013 Excel (Version 15.0.4833.1000 [64-bit]).

Statistical significance for all analyses was declared at $\alpha \leq 0.05$, and the treatment group replicates (test enclosures) were the experimental units in all analyses. A general linear mixed model was used with treatment type (i.e. subsurface injection or whole water column), target active ingredient concentration (including a treatment by concentration interaction), sampler source stockpile, and substrate material as fixed effects. The zebra mussel biomass per square meter of substrate was the response variable where the mean was computed across subsamples within each replicate before model fitting. Residuals followed a normal distribution and which was verified by inspecting a histogram and qqplot of the residuals. The relationship between mortality, treatment type, and target exposure concentration of zebra mussels in the type 2 samplers was analyzed with a binary logistic mixed model using SAS software version 9.3 (SAS, 2010).

Results and Discussion

The application of Zequanox to the test enclosures had minor impacts on water quality during the exposure period. The dissolved oxygen, pH, alkalinity, hardness, TAN, and un-ionized ammonia were all at acceptable levels for aquaculture (Timmons and Ebeling, 2013). The dissolved oxygen

ranged from 6.25 to 8.65 mg/L, the pH and temperature ranged from 7.87 to 8.45 and from 17.7 to 18.5°C, respectively (Table 1). Water hardness ranged from 154 to 157 mg/L as $CaCO_3$, alkalinity from 130 to 133 mg/L as $CaCO_3$, and conductivity from 324 to 340 µS/cm. The maximum observed TAN was 0.98 mg/L, and the un-ionized ammonia remained ≤ 0.02 mg/L in all treatment groups (Table 2). The total nitrogen and phosphorus were elevated in the treated groups with mean contributions from Zequanox treatments up to 7.79 mg/L and 3.39, respectively (Table 2). The significant contribution of nitrogen and phosphorus from the treatments may have implications for stimulating algal and other aquatic plant growth if multiple or large scale treatments are conducted.

Coefficients of determination (r^2) for the zero-intercept linear regressions used for determination of exposure concentrations exceeded 0.99 for all trials. With the exception of the 15-cm 100 mg A.I./L subsurface application 2 hour samples, the mean active ingredient concentrations in the 50 and 100 mg A.I./L whole water column treatment groups were consistently higher than the corresponding subsurface application treatment groups (Fig. 10).

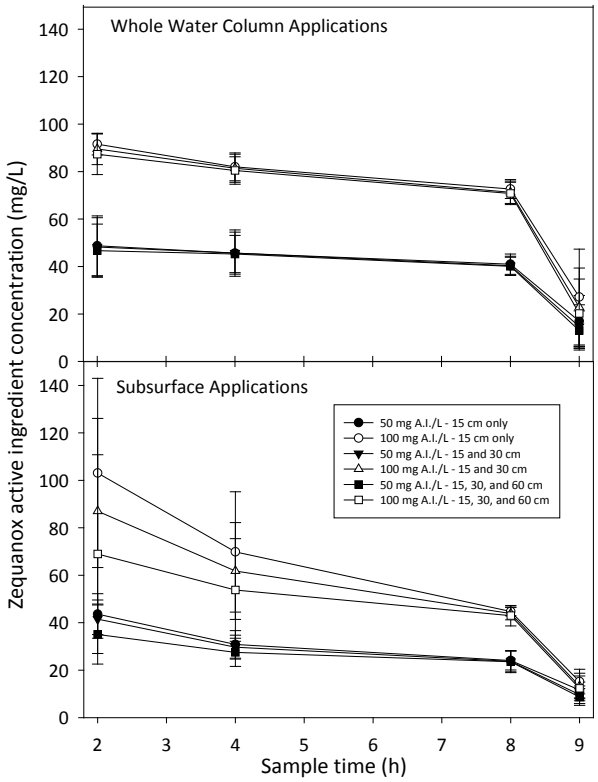

Figure 10. Mean Zequanox active ingredient concentrations (SD error bars; n = 3/sample depth/enclosure) measured in enclosures during the exposure period and 1-hour post exposure in the whole water column and subsurface applications conducted in Robinson's Bay, Lake Minnetonka, MN

In the 50 mg A.I./L whole water column treated group, the mean active ingredient concentration in all samples (15, 30, and 60 cm from the lake bed) was > 93% of target 2 hours after application and > 80% of target 8 hours after application. Likewise, in the 100 mg A.I./L whole water column treated group the mean active ingredient concentration in all samples was > 87% of target 2 hours after application and > 70% of target 8 hours after application. In both subsurface application groups, the mean active ingredient concentrations in the 15 and 30-cm samples were ≥ 83% of target 2 hours after application, however, the mean active ingredient concentrations for all samples were 70.1% and 68.9% of target for the 50 and 100 mg A.I./L subsurface application treated groups, respectively.

In the 50 mg A.I./L subsurface application group, the highest active ingredient concentrations 4 hours after application were observed at 15 cm and the 15-cm sample mean was 61.7% of target; the mean concentration for all sample depths was 55.0% of target. Similarly, in the 100 mg A.I./L subsurface application group the highest active ingredient concentrations 4 hours after application were observed at 15 cm and the mean 15-cm sample concentration was 69.8% of target; the mean active ingredient concentration for all sample depths was 53.8% of target. Eight hours after application, the highest active ingredient concentrations were observed at 15 cm and the mean 15-cm sample active ingredient concentrations were 48.1 and 44.7% of target for the 50 and 100 mg A.I./L subsurface application groups, respectively.

Table 1. Mean (SD) pH, dissolved oxygen, and temperature observed for each treatment group during the study period (n = 3/treatment group/sample time).

	Control			50 mg A.I./L Whole Water Column Application			50 mg A.I./L subsurface Application			100 mg A.I./L Whole Water Column Application			100 mg A.I./L subsurface Application		
Time	pH	DO (mg/L)	Temp. (°C)	pH	DO (mg/L)	Temp. (°C)	pH	DO (mg/L)	Temp. (°C)	pH	DO (mg/L)	Temp (°C)	pH	DO (mg/L)	Temp (°C)
Pre-Exposure	8.30 (0.13)	7.59 (0.61)	17.7 (0.4)	8.28 (0.11)	7.63 (0.36)	17.7 (0.5)	8.33 (0.10)	7.60 (0.48)	17.7 (0.5)	8.25 (0.08)	7.57 (0.23)	17.7 (0.5)	8.27 (0.05)	7.46 (0.29)	17.8 (0.5)
2	8.39 (0.21)	7.31 (0.47)	17.9 (0.5)	8.14 (0.12)	7.28 (0.07)	18.0 (0.6)	8.22 (0.11)	7.65 (0.86)	18.2 (0.5)	7.97 (0.10)	7.10 (0.19)	17.9 (0.5)	8.14 (0.07)	7.03 (0.22)	17.9 (0.6)
7.5	8.45 (0.20)	8.65 (0.39)	18.3 (1.1)	7.99 (0.13)	6.50 (1.30)	18.3 (1.2)	8.12 (0.15)	6.79 (0.24)	18.5 (1.3)	7.87 (0.12)	6.47 (0.47)	18.2 (1.4)	7.97 (0.05)	6.25 (0.41)	18.4 (1.3)

Table 2. Mean (SD) alkalinity, hardness, conductivity, total ammonia nitrogen (TAN), un-ionized ammonia (NH3-N), total phosphorus (P), and total nitrogen (N) observed during the study period (n = 3 treatment group).

Treatment group	Alkalinity (mg/L)[1,2]	Hardness (mg/L)[1,2]	Conductivity (µS/cm)[2,3]	TAN (mg NH_3-N/L)[4]	NH_3 (mg/L)[4]	Total P (mg/L)[4]	Total N (mg/L)[4]
Control	130 (0)	154 (0)	324 (10)	0.21 (0.07)	0.02 (<0.01)	0.03 (0.01)	0.69 (0.06)
50 mg A.I./L whole water column application	133 (2)	157 (3)	331 (9)	0.41 (0.10)	0.01 (<0.01)	1.89 (0.28)	6.01 (0.37)
50 mg A.I./L subsurface application	133 (1)	157 (3)	325 (8)	0.33 (0.07)	0.01 (<0.01)	1.34 (0.33)	4.51 (0.75)
100 mg A.I./L whole water column application	133 (1)	157 (2)	340 (5)	0.98 (0.33)	0.02 (<0.01)	3.42 (0.13)	8.44 (0.43)
100 mg A.I./L subsurface application	133 (0)	156 (2)	338 (7)	0.61 (0.24)	0.02 (<0.01)	3.29 (1.19)	8.48 (1.94)

[1] Reported as calcium carbonate ($CaCO_{3)}$.
[2] Measured 2 hours after application
[3] Temperature compensated to 25°C
[4] Measured 7.5 hours after application

Zequanox dissipated rapidly after the exposure period when the end panels of the enclosures were opened. The highest active ingredient concentrations measured 1 hour after exposure termination were at 15 cm and the mean 15-cm sample active ingredient concentrations in the whole water column and subsurface applications were 34.0 and 23.2% of target, respectively.

Wind direction and velocity on two of the three application days (N-NNW, 16-24 kph) made the enclosures on the southern shore of Robinson's Bay particularly vulnerable to wave action. Although the enclosures were constructed using rigid aluminum frame panels, the enclosure panels did not appear to damper wave action. Subsurface application of Zequanox on calm days without wave action would likely aid in maintaining a stratified Zequanox layer for a greater duration.

For type 1 samplers, an effect of stockpile location was observed on zebra mussel biomass ($p = 0.01$), however, the substrate material type with stockpile interaction was not statistically significant ($p = 0.13$). The differences observed in stockpiles was largely driven by the tile substrate, where stockpiles 1 and 2 were shown to have a significantly higher biomass than stockpile 3 ($p \leq 0.01$ and 0.02, respectively). Since the study design was balanced by randomly allocating one type 1 sampler from each stockpile to each treatment replicate, the biomass results for each enclosure were grouped by substrate type and then compared between treatments. The perforated aluminum substrates had considerably less biomass than both the wood and tile substrates, with a zebra mussel biomass in the control perforated aluminum substrate group of 77.42 g/m^2 compared to 169.00 and 188.30 g/m^2 for the tile and wood substrates, respectively (Table 3). The mean zebra mussel biomass adhering to the perforated aluminum substrates was approximately 50% of the tile and wood substrates, regardless of treatment group. The biomass of tile and wood substrates were similar in all treatment groups, with wood having slightly more biomass in all but one treatment group. When each substrate type was

compared to their respective substrate control group to determine the percent reduction in zebra mussel biomass/m^2, the perforated aluminum substrates were similar to the tile and wood substrates. On average, the zebra mussel biomass/m^2 was reduced 41.45 and 57.85% in the 50 and 100 mg A.I./L subsurface applications, respectively, and 61.88 and 78.87% in the 50 and 100 mg A.I./L whole water column applications, respectively.

Table 3. Type 1 sampler (n = 3/replicate, 9/treatment group, 45 total) mean (SD) zebra mussel biomass per square meter of substrate and the percent zebra mussel biomass reduction from the control groups by substrate type and treatment group.

	Mean Zebra Mussel Biomass per Square Meter by Substrate Type (g)			Mean Reduction of Zebra Mussel Biomass from Control Group by Substrate Type				
Treatment Group	Alum.	Tile	Wood	Alum. (%)	Tile (%)	Wood (%)	Mean Combined Reduction (%)	Standardized Mean Combined Reduction[1] (%/kg)
Control	77.42 (16.03)	169.00 (24.34)	188.30 (16.71)	N/A	N/A	N/A	N/A	N/A
50 mg A.I./L Whole Water Column Application	31.97 (12.38)	63.63 (25.45)	66.67 (26.45)	58.71	62.35	64.59	61.88	16.41
50 mg A.I./L Subsurface Application	50.88 (35.14)	90.45 (16.80)	106.2 (35.12)	34.28	46.48	43.60	41.45	21.73
100 mg A.I./L Whole Water Column Application	17.80 (3.24)	37.80 (16.61)	33.94 (6.89)	77.01	77.63	81.98	78.87	10.21
100 mg A.I./L Subsurface Application	34.97 (11.09)	70.35 (16.47)	74.64 (12.79)	54.83	58.37	60.36	57.85	13.95

[1] Standardized mean combined reductions were calculated by dividing the mean percent reduction by the mean amount of Zequanox applied (kg) in the treatment groups.

The amount of Zequanox applied in the subsurface applications was on average ~55% of the amount applied in the whole water column applications. The reductions in mean zebra mussel biomass per square meter of substrate were standardized to the amount of Zequanox applied by dividing the mean percent reduction by the average amount of Zequanox applied to each treatment group (Table 3). When standardized, the 50 mg A.I./L and the subsurface application treatments were more efficient, with respect to the amount of product used, for reducing the zebra mussel biomass. The zebra mussel biomass reductions were 21.73 and 13.95%/kg of Zequanox applied in the 50 and 100 mg A.I./L subsurface applications, respectively, versus 16.41 and 10.21%/kg of Zequanox applied in the 50 and 100 mg A.I./L whole water column applications, respectively. Although the 50 mg A.I./L treatments and the subsurface applications are slightly more efficient at reducing zebra mussel biomass when standardized to the amount of Zequanox applied, management goals, biological significance, and non-target impacts should be carefully considered before selecting treatment methods and application rates.

The mean survival of control group zebra mussels contained in type 2 samplers exceeded 98%. The mean mortality of zebra mussels contained in type 2 samplers that were in the treated groups ranged

from 27.83%, in the 50 mg A.I./L subsurface application group, to 73.25%, in the 100 mg A.I./L whole water column application group (Table 4). Similar to biomass reductions, when standardized to the amount of Zequanox applied, the 50 mg A.I./L and the subsurface application treatments were more efficient for inducing zebra mussel mortality (Table 4). Given the lower mortality observed in the subsurface application treatment groups (27.83 and 56.16% in the 50 and 100 mg A.I./L treatment groups, respectively), consideration should be given to restricting the use of subsurface Zequanox applications to quiescent waters when zebra mussels are actively feeding and for applying Zequanox as close to the target as feasible.

Table 4. Mean (SD) percent survival, percent mortality, and standardized percent mortality of zebra mussels contained in type 2 samplers (n = 3/replicate, 9/treatment group, 45 total).

Treatment Group	Mean Survival (%)	Mean mortality (%)	Standardized Mortality (%/kg)[1]
Control	98.02 (1.04)	1.98 (1.04)	N/A
50 mg A.I./L Whole Water Column Application	58.86 (18.29)	41.14 (18.29)	10.91
50 mg A.I./L Subsurface Application	72.17 (17.33)	27.83 (17.33)	14.59
100 mg A.I./L Whole Water Column Application	26.75 (5.22)	73.25 (5.22)	9.49
100 mg A.I./L Subsurface Application	43.84 (10.83)	56.16 (10.83)	13.55

[1]Standardized mortalities were calculated by dividing the mean mortality by the mean amount of Zequanox applied (kg) in the treatment groups.

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