M.L. 2013 Project Abstract

For the Period Ending June 30, 2016

PROJECT TITLE: Zebra Mussel Control Research and Evaluation in Minnesota Waters
PROJECT MANAGER: Jeff Meinertz
AFFILIATION: U.S. Geological Survey
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FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: M.L. 2013, Chp. 52, Sec. 2, Subd. 06f
APPROPRIATION AMOUNT: $600,000

Overall Project Outcome and Results

Zebra mussels (*Dreissena polymorpha*) continue to rapidly expand their range within Minnesota's lakes and rivers disrupting aquatic food webs, threaten native species, and damage infrastructure. Zequanox®, which contains killed cells of *Pseudomonas fluorescens* as the active ingredient, is a potential tool for controlling dreissenid mussels (zebra and quagga mussels *D. rostriformis bugensis*. The project goals were to determine the safety and efficacy of Zequanox for controlling zebra mussels and to evaluate the use of molecular tools to inform control efforts. Project studies are summarized in supplemental attachments with the final report.

The Zequanox non-target animal impacts database was expanded by evaluating the exposure-related impacts on three life stages of fathead minnow (*Pimephales promelas*), and on the survival of adult scuds (*Gammarus lacustris*) and mayfly nymphs (*Hexagenia* sp.) after applications were conducted in outdoor 1,000-L mesocosm tanks. No significant treatment related impacts were observed in survival of invertebrates or fathead minnows or in hatchability and growth of fathead minnows.

Detailed maps were prepared for portions of Lake Le Homme Dieu and Maple Lake (Douglas County), which had different zebra mussel infestation levels. Maps of depth, substrate hardness, and submerged aquatic vegetation (SAV) depth and biovolume were generated using side-scanning sonar and parallel sonar data transects were collected and processed into component data categories. Processed sonar data and resulting maps are available on the vendor's cloud-based server network and could be combined with new or existing data to generate additional mapping products. Sonar data were used to generate a geospatial database of map characteristics in ArcGIS, and spatial analyses of the data were used to generate additional map products in ArcMap. Conversion to ArcGIS allowed for spatial analysis and sharing in GIS format. Zebra mussel populations were correlated with depth and substrate and submerged aquatic vegetation was found to be an important component of zebra mussel habitat in shallow areas in Lake Le Homme Dieu.

The use of environmental DNA to detect and identify application locations for Zequanox that might have the greatest impact on zebra mussel populations was also evaluated. The use of eDNA could assist management agencies to identify infestations, however, eDNA was found to not be effective for targeting control efforts.

Methods to apply Zequanox under the surface were first evaluated in controlled laboratory and pond-scaled mesocosm studies and further evaluated in 27-m^2 enclosures placed in Robinson's Bay (Lake Minnetonka, MN). Whole water column and subsurface applications were evaluated by comparing zebra mussel mortality and biomass reduction between treated and control groups. Approximately 73 and 56% of the zebra mussels in contained samples were killed in the highest whole water column and subsurface Zequanox applications, respectively, and the similarly the adhering zebra mussel biomass was reduced ~79 and 57%, respectively.

Overall, we found that Zequanox has the potential to be used as a management tool for zebra mussels in quiescent water environments, however, Zequanox is not likely to be effective for eradication of zebra mussels in an open water environment. Additionally, eDNA may have utility as a tool for the detection of zebra mussels in a waterbody but it is not an effective tool for determining the biomass of zebra mussels present or for prioritizing the location of zebra control efforts.

Project Results Use and Dissemination

Three oral presentations describing study methods and results were prepared and disseminated at professional scientific meetings including the Upper Midwest Invasive Species Conference and the Annual Conference of the International Association of Great Lake Research. One webinar entitled "The potential use of eDNA to guide site selection for zebra mussel control treatments" was presented during a USGS hosted Environmental DNA Webinar Series. One peer-reviewed manuscript entitled "Safety of the molluscicide Zequanox® to nontarget macroinvertebrates *Gammarus lacustris* (Amphipoda: Gammaridae) and *Hexagenia* spp. (Ephemeroptera: Ephemeridae)" was prepared and published online on June 23, 2016 in the Management of Biological Invasions and is included as a supplemental attachment to the project final report. Five peer-reviewed reports that summarize study methods and results were prepared and are supplemental attachments to the project final report.

A model was developed for selecting the proper concentration (w/v) of Zequanox to be used in stocks prepared for subsurface applications waters between 7 and 22°C. This prediction model is described in supplemental attachments with the final report .

Molecular markers for the detection of zebra mussels were found to be highly specific to zebra mussels. A water sampling protocol was also developed to improve the probability of detecting zebra mussels. The use of environmental DNA (eDNA) did correlate with zebra mussel biomass. Zebra mussel DNA did accumulate in depositional areas. This suggests that our zebra mussel eDNA assay could assist management agencies to identify infestations, but not inform control efforts. The molecular markers, sampling protocol and depositional areas are described in supplemental attachments with the final report.

Environment and Natural Resources Trust Fund (ENRTF)
M.L. 2013 Work Plan Final Report

Date of Status Update Report: August 12, 2016

Final Report

Date of Work Plan Approval: June 11, 2013

Project Completion Date: June 30, 2016

PROJECT TITLE: Zebra Mussel Control Research and Evaluation in Minnesota Waters

Project Manager: Jeff Meinertz

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Statewide

Total ENRTF Project Budget:		
	ENRTF Appropriation:	**$600,000**
	Amount Spent:	**$600,000**
	Balance:	**$0**

Legal Citation: M.L. 2013, Chp. 52, Sec. 2, Subd. 06f

Appropriation Language:
$600,000 the first year is from the trust fund to the commissioner of natural resources for an agreement with the United States Geological Survey, Upper Midwest Environmental Sciences Center, to assess the ecological impacts of a commercially available molluscicide formulation on the reproduction and development of native fish, as well as impacts on larval aquatic insect survival, and to evaluate the effectiveness of these treatment options for detection and control of zebra mussels. The United States Geologic Survey is not subject to the requirements in Minnesota Statutes, section 116P.10. This appropriation is available until June 30, 2016, by which time the project must be completed and final products delivered.

I. PROJECT TITLE: Zebra Mussel Control Research and Evaluation in Minnesota Waters

II. PROJECT STATEMENT: There is an immediate need for safe and effective control measures to reduce the impact of dreissenid mussels (zebra *Dreissena polymorpha* and quagga mussels *D. rostriformis bugensis*) whose attachment and feeding behavior disrupt aquatic food webs and foul spawning habitats, behaviors that threaten native aquatic species like mussels and fish. The range expansion of dreissenid mussels within Minnesota lakes and rivers continues (e.g. ~27 lakes were added to the list since 2009, bringing the total to about 90 waters with confirmed or interconnected dreissenid populations) while management agencies lack access to effective tools to control dreissenid mussel populations in open waters.

One potential tool for limited open-water control of dreissenid mussels is the commercially formulated product, Zequanox®, which contains the killed cells of a specific strain (*Pf*-CL145A) of the common soil bacterium *Pseudomonas fluorescens*. Zequanox® is produced by Marrone Bio Innovations (Davis, CA) and it is registered by the U.S. Environmental Protection Agency for control of dreissenid mussels in defined discharges (e.g. in cooling and service water systems for industrial facilities). Reference to any specific commercial products, process, or service by trade name, trademark, manufacturer, or otherwise, does not constitute or imply its endorsement, recommendation, or favoring by the United States government. Throughout the remainder of this document the formulated *Pf*-CL145A product (Zequanox®) will be referred to as sprayed dried powder (SDP). A 3-year, multiagency (U.S. Geological Survey, U.S. Fish and Wildlife Service, and New York State Museum) research project is in progress to further assess the potential impacts of SDP on native fish and mussel species during open water applications (see http://cida.usgs.gov/glri/projects/invasive_species/zm_control.html).

The overall goal of the proposed project is to determine the safety and efficacy of SDP for control of dreissenid mussels in limited, high-value Minnesota waters. The existing non-target animal impacts database of SDP will be expanded by evaluating the impacts of SDP on the reproductive success of native fish populations and on the survival of native aquatic invertebrates. Fathead minnows, a representative test species, and their eggs will be exposed to an environmentally relevant concentration and exposure duration of SDP and the impacts on spawning and development assessed. Separate but similar exposures will be completed to assess the potential acute effects of SDP to aquatic invertebrates.

Adult fathead minnows (*Pimephales promelas)* will be exposed to SDP in outdoor mesocosms. Treatment groups will be exposed to a single dose of SDP at the expected environmental treatment concentration (e.g. 0, 50, and 100 mg/L active ingredient [A.I.]) for 8 hours. Fish in each treatment group will be observed for up to 30 days after exposure to assess reproduction. Fish in each treatment group will have access to spawning tiles and the number of eggs deposited on each tile will be determined. Resulting eggs will be monitored to determine percent hatch. A subset of the resulting fry (F_1 generation) from each treatment concentration will be reared to adulthood to compare development and reproductive success to that of untreated controls.

Separately, eggs (at selected development stage[s]) from naïve fathead minnows will be exposed to a single static dose of SDP in an outdoor mesocosm setting at the expected environmental treatment concentration (e.g. 0, 50, and 100 mg/L A.I.) for 8 hours to assess the potential impact of SDP open water application on fish embryo development. A subset of the resulting fry from each treatment group will be reared to adulthood to compare development between exposed and control groups.

Laboratory and pond-scale studies will compare treatment application techniques (i.e., injection vs. whole water column treatment) to determine the minimum amount of SDP required during field applications. These studies will focus on determining techniques that maintain an effective concentration of SDP for the required exposure duration. Delivery techniques will be evaluated in the laboratory then evaluated under field conditions in 0.01-acre outdoor research ponds.

Detailed maps of two Minnesota lakes, or portions thereof, (1 high and 1 low level infestation, to be identified in 2013) will be prepared using a combination of bathymetric (using high-resolution sonar systems to characterize habitat), physical (to determine zebra mussel densities) and molecular surveys (using environmental DNA [eDNA]). The maps will be used to correlate zebra mussel populations within the lake to bathymetric and substrate data.

Habitats in Robinson's Bay, Lake Minnetonka (Deephaven, MN) will be selected to evaluate efficacy of SDP application techniques for controlling zebra mussels in limited, high-value, open water. Five replicated enclosures (~24 m^2) will be prepared on 3 independent treatment days at selected locations and assigned 1 of 5 treatments (control, 50 mg/L A.I. SDP injection, 100 mg/L A.I. SDP injection, 50 mg/L A.I. SDP complete water column, or 100 mg/L A.I. SDP complete water column) according to a randomized study design. Treatment efficacy will be assessed from pre- and post-application surveys with a focus on impacts on zebra mussel survival and colonization rates. In addition to evaluating treatment efficacy, the project will evaluate the capacity of molecular monitoring (using eDNA) to identify locations where the application of a control, such as SDP, might have the greatest impact on zebra mussel populations.

III. PROJECT STATUS UPDATES:

Periodic work plan status update reports will be submitted not later than December 31, 2013, June 30, 2014, December 31, 2014, June 30, 2015, December 31, 2015, and June 30, 2016. A final report and associated products will be submitted by June 30, 2016 or as requested by the LCCMR.

Project Status as of December 31, 2013:

USGS policy does not allow the obligation of funds until an executable agreement is established. The agreement, which authorized the USGS to proceed with LCCMR funds, was signed on September 13, 2013. Due to the timing of the project agreement, activities originally planned to commence in 2013 were delayed until 2014. Activities conducted to date have been funded through USGS cost share funds and have included the acquisition of equipment and supplies, construction and deployment of zebra mussel colonization substrates in Lake Minnetonka, and selection of test lakes (Lake Le Homme Dieu and Maple Lake) for bathymetric mapping and eDNA analysis and protocol development.

Amendment Request (12/18/2013)

A budget realignment is requested to shift the funds for each activity originally indicated for equipment and supplies and contracts to salaries. The total amount for each activity will remain the same. The USGS will provide the necessary equipment and supplies for the project from cost share funds. Additionally, the amended request includes the withdrawal of a project partner (Mayer [NYSM]) and the associated funds ($20,000) listed under Professional/Technical/Service Contracts. These funds will be shifted to salaries as previously described. The amended request also includes a USGS personnel change from Amberg to Rees as the USGS PCR expert. Although the personnel budget associated with each activity increases, the total activity cost remains unchanged. This shift in budget activity assignment of project funds significantly reduces USGS accounting expenses to document each expenditure activity as required by the agreement. The documentation requirements were unknown at the time of project proposal submission and were not accounted for in the original budget submission. The proposed change in activity assignment of project funds will not change the level of work planned for the project but will minimize study-related but unbudgeted costs to USGS.

Amendment approved January 3, 2014

Project Status as of June 30, 2014:
Mesocosm test systems have been constructed and studies initiated for activity 1, non-target animal impacts. Year 1 exposures for activity 1 are expected to be completed by July 15, 2014. The resulting fathead minnow fry will be reared and assessed for growth, development and reproductive success (2015). Sonar equipment for bathymetric mapping has been installed and evaluated; however, field activities for bathymetry and eDNA marker studies for activity 2 have experienced minor weather related delays. Field operations for bathymetry, eDNA sampling and physical sampling are expected to be completed by August 15, 2014. Several markers have been designed to detect for the presence of zebra mussel DNA. Currently, the specificity of the markers is being evaluated with DNA from native unionids. Specific markers will be chosen for analyzing eDNA from collected water samples. Injection technique development studies for activity 3 are scheduled for initiation in July 2014 and additional samplers for adult zebra mussels and native invertebrates will be placed in Lake Minnetonka during August, 2014. Field treatments for activity 3 are scheduled for September 8-22, 2014.

Amendment Request (9/26/2014)
An amendment is requested to properly align the budget with personnel changes and additions. Additionally, this amendment reflects the use of USGS cost share funds to cover travel expenses and salary expenses previously identified as being charged to ENRTF funds (bill was cancelled). The funds identified as travel expenses have been moved to salary costs, however, the total reimbursement request from the ENRTF and the project outcomes do not change.

Amendment Approved October 3, 2014

Project Status as of December 31, 2014:
Activity 1: Non-target invertebrates, fathead minnow (FHM) brood fish, and newly deposited FHM eggs SDP exposures were conducted. Resulting FHM from both exposure trials are being held for continued growth and development. Analyses and review of the data have not been completed. Problem encountered: The feed used for FHM fry was deficient in vitamin C, most likely the cause of some scoliosis in developing fry which will confound comparison of growth and development between treatment groups.

Activity 2: Bathymetry mapping, eDNA sampling, and physical surveys on Maple Lake and Lake Le Homme Dieu were completed. Water samples were collected and frozen at -20°C until centrifuged and analyzed for zebra mussel DNA in octet using quantitative real-time polymerase chain reaction. Zebra mussel density estimates have been completed and biomass determinations are pending.

Activity 3: Application technique development included construction of a SDP mixing system, an indoor test system, and mesocosm enclosures panels. Seven injection apparatuses were evaluated with various parameters. Two subsurface injection bar delivery systems were constructed for use during the field application. Zequanox field applications were conducted within 27-m^2 enclosures positioned in Robinson's bay (Lake Minnetonka. Five treatment groups were completed on each treatment day. Samplers were assessed for zebra mussel mortality (type 2 samplers) and sampled for zebra mussel biomass (type 1 samplers). Data analyses and review have not been completed. Problem encountered: Maintenance of benthic layers of SDP by sub-surface applications in dynamic, high energy environments are problematic. Additional work is planned to

evaluate the maximum concentration of SDP that can be applied during a subsurface application to increase the duration of acceptable SDP concentration.

Amendment Request (6/30/2015)
An amendment is requested to reflect (1) a project manager change from Mark Gaikowski to Jeff Meinertz and (2) to align the budget (Attachment A) with personnel departures, additions, and time allocation revisions.

- In September 2014 Mark Gaikowski became the Center Director for the Upper Midwest Environmental Sciences Center (UMESC). Project management duties were transferred to Jeffery Meinertz, the acting Branch Chief of UMESC Aquatic Ecosystems Health (AEH) Branch.

- Four personnel previously identified in Schedule A (Boma, Vang, Rees, and Weber) are no longer employed at UMESC and therefore their time has been reduced to actual hours worked. Personnel allocations within the project activities have been revised to reflect actual amounts for completed activities (Allen, Black, Boma, and Roth) and an additional staff member (Smerud) has been added for activity 3. Salary estimates for remaining personnel have been adjusted to current work assignments. The previous FTE calculations on Schedule A and in Section VI (project budget Summary) were incorrectly computed and have been revised.

Amendment Approved: July 08, 2015

Project Status as of June 30, 2015:

Activity 1: Non-target invertebrates, fathead minnow brood fish, and newly deposited fathead minnow eggs Zequanox exposures were conducted. Final review of the invertebrate data has been completed and the preparation of the final report is in progress. Review of the data collected in 2014 for the fathead minnow brood fish was completed; statistical analysis of adult mortality, egg deposition and egg hatchability data was completed. Final review of the data collected in 2014 for the newly deposited eggs was completed; summarization and statistical analysis of egg hatchability data is in progress. Resulting fish from both trials were placed into outdoor concrete ponds to assess spawning ability. An exposure to determine the impacts of Zequanox exposure on the survival, growth, and development of new hatched fathead minnow fry was conducted on June 24, 2015 and the fish will be held in outdoor mesocosms until October, 2015.

Activity 2: A suite of eDNA markers were tested *in silico* for specificity to zebra mussels from samples collected from Lake Minnetonka. Two primer sets (Dre2 and Dre5) with one hydrolysis probe (Dpo1) were selected as the most efficient and specific. Both primer sets with the probe were tested on environmental samples collected at seven sites from multiple depths. At each site, samples were collected from the surface, from mid-water column, and from six inches off the bottom. The near-bottom samples resulted in highest concentrations of zebra mussel DNA, so samples from Maple Lake and Lake Le Homme Dieu were collected from near the bottom. Additional environmental DNA samples were collected from Maple Lake and Lake Le Homme Dieu through the ice in March of 2015 at the same locations sampled during 2014. The samples were processed using the techniques used for the 2014 samples. Preliminary correlations between summer and winter samples have been completed. Bathymetry mapping data have been imported into ArcGIS to form the basis of an interactive ArcMap product for each lake. ArcMap products for each lake are under development and are expected to be completed by August 30, 2015.

Activity 3: Preliminary data summarization and analyses of water chemistry, Zequanox concentration, and zebra mussel survival during the field applications conducted within 27-m^2 enclosures positioned in Robinson's bay (Lake Minnetonka) have been completed. Methods to determine the living zebra mussel biomass adhering to

multi-plate samplers placed in each treated group replicate are under development and samples are expected to be processed by February, 2016. Additional work to assess the optimum concentration for sub-surface application of Zequanox in quiescent waters is planned for completion in 2015.

Project Status as of December 31, 2015:

Activity 1: Non-target invertebrates, fathead minnow brood fish, newly deposited fathead minnow eggs, and fathead minnow fry Zequanox exposures have been completed. A manuscript of the invertebrate data has been drafted and is in review. Statistical analysis of the data collected in 2014 for the fathead minnow brood fish (adult mortality, egg deposition, egg hatchability data, and F1 spawning) and for the newly deposited fathead minnow eggs (hatchability and spawning) has been completed and a peer-reviewed publication is in preparation. A Zequanox exposure to newly hatched fathead minnow fry was conducted on June 24, 2015 and data summarization and statistical analysis is in progress. The results will be included in a peer-reviewed publication describing the effects of Zequanox exposure on the survival, growth, and reproduction of fathead minnows.

Activity 2: A suite of eDNA markers were tested *in silico* for specificity to zebra mussels from samples collected from Lake Minnetonka. Two primer sets (Dre2 and Dre5) with one hydrolysis probe (Dpo1) were selected as the most efficient and specific. Both primer sets with the probe were tested on environmental samples collected at seven sites from multiple depths. At each site, samples were collected from the surface, from mid-water column, and from six inches off the bottom. The near-bottom samples resulted in highest concentrations of zebra mussel DNA, so samples from Maple Lake and Lake Le Homme Dieu were collected from near the bottom. Additional environmental DNA samples were collected from Maple Lake and Lake Le Homme Dieu through the ice in March of 2015 at the same locations sampled during 2014. The samples were processed using the techniques used for the 2014 samples. Preliminary correlations between summer and winter samples have been completed. Zebra mussels samples collected from Maple Lake and Lake Le Homme Dieu were cleaned and are being processed for ash-free dry weight analysis.

Bathymetry mapping data were imported into ArcGIS to form the basis of an interactive ArcMap product for each lake. Mapping data from Maple Lake and Lake Le Homme Dieu were combined with existing bathymetry in an ArcGIS ArcMap product for each lake. Data for depth, substrate type, and vegetation bio-volume were combined with other available geo-spatial data in an ArcMap final product. The maps of each lake will be included with the final project completion report.

Activity 3: Data summarization of water chemistry, Zequanox concentration, and zebra mussel survival during the field applications conducted in Robinson's bay (Lake Minnetonka) are in process. Methods to determine the living zebra mussel biomass adhering to multi-plate samplers in each treated group replicate have been developed and sample processing has been initiated. Laboratory studies to optimize subsurface applications of Zequanox at various water temperatures were completed and a temperature-dependent regression was prepared to determine the temperature-dependent Zequanox stock concentration to use for subsurface Zequanox applications in quiescent waters. Validation trials were conducted at three water temperatures (~9, 14, and 20 °C) to assess the use of the regression to select temperature-dependent Zequanox stock concentrations for subsurface applications in quiescent waters. The validation trials were conducted within replicated 3-m^2 enclosures placed in 0.01 acre concrete. Zequanox concentrations in the enclosures were

monitored at 3 heights (7.5, 30, and 60 cm from the bottom) for 8 hours. Data summarization and analysis is in progress.

Overall Project Outcomes and Results

Zebra mussels (*Dreissena polymorpha*) continue to rapidly expand their range within Minnesota's lakes and rivers disrupting aquatic food webs, threaten native species, and damage infrastructure. Zequanox®, which contains killed cells of *Pseudomonas fluorescens* as the active ingredient, is a potential tool for controlling dreissenid mussels (zebra and quagga mussels *D. rostriformis bugensis*. The project goals were to determine the safety and efficacy of Zequanox for controlling zebra mussels and to evaluate the use of molecular tools to inform control efforts. Project studies are summarized in supplemental attachments 1 through 6.

The Zequanox non-target animal impacts database was expanded by evaluating the exposure-related impacts on three life stages of fathead minnow (*Pimephales promelas*), and on the survival of adult scuds (*Gammarus lacustris*) and mayfly nymphs (*Hexagenia* sp.) after applications were conducted in outdoor 1,000-L mesocosm tanks. No significant treatment related impacts were observed in survival of invertebrates or fathead minnows or in hatchability and growth of fathead minnows.

Detailed maps were prepared for portions of Lake Le Homme Dieu and Maple Lake (Douglas County), which had different zebra mussel infestation levels. Maps of depth, substrate hardness, and submerged aquatic vegetation (SAV) depth and biovolume were generated using side-scanning sonar and parallel sonar data transects were collected and processed into component data categories. Processed sonar data and resulting maps are available on the vendor's cloud-based server network and could be combined with new or existing data to generate additional mapping products. Sonar data were used to generate a geospatial database of map characteristics in ArcGIS, and spatial analyses of the data were used to generate additional map products in ArcMap. Conversion to ArcGIS allowed for spatial analysis and sharing in GIS format. Zebra mussel populations were correlated with depth and substrate and submerged aquatic vegetation was found to be an important component of zebra mussel habitat in shallow areas in Lake Le Homme Dieu.

The use of environmental DNA to detect and identify application locations for Zequanox that might have the greatest impact on zebra mussel populations was also evaluated. The use of eDNA could assist management agencies to identify infestations, however, eDNA was found to not be effective for targeting control efforts.

Methods to apply Zequanox under the surface were first evaluated in controlled laboratory and pond-scaled mesocosm studies and further evaluated in 27-m^2 enclosures placed in Robinson's Bay (Lake Minnetonka, MN). Whole water column and subsurface applications were evaluated by comparing zebra mussel mortality and biomass reduction between treated and control groups. Approximately 73 and 56% of the zebra mussels in contained samples were killed in the highest whole water column and subsurface Zequanox applications, respectively, and the similarly the adhering zebra mussel biomass was reduced ~79 and 57%, respectively.

Overall, we found that Zequanox has the potential to be used as a management tool for zebra mussels in quiescent water environments, however, Zequanox is not likely to be effective for eradication of zebra mussels in an open water environment. Additionally, eDNA may have utility as a tool for the detection of zebra mussels in a waterbody but it is not an effective tool for determining the biomass of zebra mussels present or for prioritizing the location of zebra control efforts.

Project Results Use and Dissemination

Three oral presentations describing study methods and results were prepared and disseminated at professional scientific meetings including the Upper Midwest Invasive Species Conference and the Annual Conference of the International Association of Great Lake Research. One webinar entitled "The potential use of

eDNA to guide site selection for zebra mussel control treatments" was presented during a USGS hosted Environmental DNA Webinar Series. One peer-reviewed manuscript entitled "Safety of the molluscicide Zequanox® to nontarget macroinvertebrates *Gammarus lacustris* (Amphipoda: Gammaridae) and *Hexagenia* spp. (Ephemeroptera: Ephemeridae)" was prepared and published online on June 23, 2016 in the Management of Biological Invasions and is included as a supplemental attachment to the project final report. Five peer-reviewed reports that summarize study methods and results were prepared and are supplemental attachments to the project final report.

A model was developed for selecting the proper concentration (w/v) of Zequanox to be used in stocks prepared for subsurface applications waters between 7 and 22°C. This prediction model is described in supplemental attachment report number five.

Molecular markers for the detection of zebra mussels were found to be highly specific to zebra mussels. A water sampling protocol was also developed to improve the probability of detecting zebra mussels. The use of environmental DNA (eDNA) did correlate with zebra mussel biomass and zebra mussel DNA did accumulate in depositional areas. This suggests that our zebra mussel eDNA assay could assist management agencies to identify infestations, but not inform control efforts. The molecular markers, sampling protocol and depositional areas are described in supplemental report number 4.

IV. PROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1: Non-target animal impacts endeavors

Description: Three experimental trials will be conducted to evaluate the impacts of SDP exposure on non-target animals. The first trials will evaluate the potential acute toxicity of SDP to aquatic invertebrates. Mayfly larvae (*Order: Ephemerotera*) and adult amphipods *(Order: Amphipoda*) will be obtained from the upper Mississippi River basin and a known number of animals placed into outdoor mesocosms (~1,000 L) containing sediment and water from a UMESC research pond. The invertebrates will be exposed to a single static application of SDP. Replicated exposures will be applied at expected environmental concentrations (e.g. 0, 50 and 100 mg/L A.I.) and at the expected environmental exposure duration (8h) to assess the potential effect of open water application of SDP to control dreissenid mussels on the survival of aquatic invertebrates. The second set of trials will evaluate the potential reproductive impacts of SDP exposure to fathead minnows. Adult fathead minnows will be exposed to a single static application of SDP in outdoor mesocosms (~1,000 L) containing pond water from a UMESC research pond. The replicated exposures will be conducted at expected environmental concentrations (e.g. 0, 50, and 100 mg/L A.I.) and at the expected environmental exposure duration (8h). Fish will be observed for reproductive activity for up to 30 days after exposure and the number of eggs deposited and the portion that hatches will be determined. A subset of the resulting fry (F_1 generation) from each treatment group will be reared to adulthood to compare development and reproductive success to that of untreated controls. The third set of trials will be conducted to evaluate the potential effects of SDP exposure on fathead minnow larval development. Fathead minnow eggs spawned from naive fish will be exposed to a single static application of SDP in outdoor mesocosms (~1,000 L) containing water from a UMESC research pond. The replicated exposures will be conducted at expected environmental concentrations (e.g. 0, 50 and 100 mg/L A.I.) and at the expected environmental exposure duration (8h) during selected embryo developmental periods to determine the impact on embryo development. A subset of the resulting fry from each treatment group will be reared to adulthood to compare development between exposed and control groups.

Summary Budget Information for Activity 1:	**ENRTF Budget:**	**$ 163,500**
	Amount Spent:	**$ 163,500**
	Balance:	**$0**

Activity Completion Date:

Outcome	Completion Date	Budget
1. Determine the survival of aquatic invertebrates following exposure to SDP in outdoor mesocosms	October 2014	$35,270
2. Determine reproductive success (egg deposition and % egg hatch) of adult FHM (F_0 generation) following exposure to SDP pre-spawn in mesocosm	October 2015	$79,280
3. Determine reproductive success of fathead minnows embryo survival following egg exposure to SDP in mesocosm tanks	October 2015	$48,950
4. Publish results	February 2016	$ USGS

Activity Status as of December 31, 2013:
USGS policy does not allow the obligation of funds until an executable agreement is established. The agreement, which authorized the USGS to proceed with LCCMR funds, was signed on September 13, 2013. Due to the timing of the project agreement, activities originally planned to commence in 2013 were delayed until 2014. Activities conducted to date have been funded through USGS cost share funds. The USGS has identified and obtained equipment for water filtration and delivery to outdoor mesocosms.

Activity Status as of June 30, 2014:
A mesocosm system to provide filtered pond water to 1,000 L test tanks was designed and installed at UMESC. Invertebrate test specimens were obtained from sources (Lincoln Bait; Staples, MN and Hilger and Sons, Inc; Antigo, WI) and Zequanox exposures were conducted on May 23, 2014. Survival was assessed on groups of test animals at the conclusion of the 8-h exposure period and at 96-h post exposure. Data analysis and reviews have not been completed.
Zequanox exposures to fathead minnow eggs were initiated on June 14, 2014 in the mesocosm test tanks. Due to development of fungus on some of the fish eggs, a second exposure will be attempted and egg incubation methods will be altered (addition of aeration, formalin egg treatments to reduce fungus) in an attempt to reduce fungal infection. Exposures are expected to be completed by July 15, 2014. A subset of resulting fry will be maintained and reared in separate 1,000-L mesocosm test tanks.
Fathead minnow brood fish were distributed to mescosm test tanks on June 19, 2014. Spawning condition of the test animals was verified in each test tank by observing egg deposition in each test tank. Zequanox exposures to the fathead minnow brood fish in the mesocosm test tanks was completed on June 25, 2014. Fathead minnow spawning will be observed in the test tanks for up to 21 days after treatment. A subset of post-treatment F1 generation fry will be maintained and reared in separate 1,000-L mesocosm test tanks.

Activity Status as of December 31, 2014:
Gammarus lacustris adults and *Hexagenia* sp. nymphs were exposed to SDP in outdoor mesocosm tanks for 8 hours and then held for an additional 96 hours. Data analyses of 8-h, 16-h and 96-h survival following SDP exposure have been completed. Results indicate that 8-h exposure to 50 or 100 mg/L (A.I.) SDP did not cause significant mortality to the test species. Final review of the data has not been completed. SDP exposure to the fathead minnow brood fish in mesocosm test tanks was completed on June 25, 2014. Spawning activity of fish

was observed for 21 days after exposure to SDP (ending July 16, 2014). Spawning tiles were observed daily for newly deposited eggs. The first 10 spawns with >50 eggs observed on spawning tiles were removed from the brood fish tank, photographed for enumeration and then placed into a separate 1,000-L mesocosm rearing tank. The resulting F1 generation fry were maintained in the mesocosm rearing tanks until October 8, 2014. Samples of fry were taken from each rearing tank every 30 days to determine average length and weight of the fry. Fry were transferred to indoor rearing facilities on October 8, 2014. The fry from each of the 3 replicate rearing tanks were indiscriminately combined into two 320-L tanks. Fry will be maintained and reared to adulthood. Enumeration of egg deposition is complete. Analyses and review of the data have not been completed.
SDP exposures to fathead minnow eggs were initiated on July 7, 2014 in the mesocosm test tanks. Hatch of fry was complete within 1 week. A subset of resulting fry were maintained in separate 1,000-L mesocosm rearing tanks. The resulting F1 generation fry were maintained in the mesocosm rearing tanks until October 8, 2014. Samples of fry were taken from each replicate tank every 30 days to determine average length and weight of the fry. Fry were transferred to indoor rearing facilities on October 8, 2014. The fry from each of the three replicate rearing tanks were indiscriminately combined into two 320-L tanks. Fry will be maintained and reared to adulthood. Enumeration of deposited and developed eggs is in progress. Analyses and review of the data have not been completed. Problem encountered: The supplemental feed used during the early lifestages of the fathead minnow fry rearing was deficient in vitamin C content which was most likely the cause for varying degrees of scoliosis in the developing fry, including control tanks. The feed was replaced upon observing scoliosis in the test animals and it was independently verified to be lacking in Vitamin C content. The vitamin deficiency and resulting scoliosis of the fry will confound comparison of growth and development between treatment groups.

Activity Status as of June 30, 2015:

Outcome 1: Determine the survival of aquatic invertebrates following exposure to SDP in outdoor mesocosms

Gammarus lacustris adults and *Hexagenia* sp. macroinvertebrate acute exposure non-target animal trial: Final review of the data has been completed and the preparation of the final report is underway. Results show that 8-h exposure to 50 or 100 mg/L (A.I.) SDP did not cause significant mortality to the test species

Outcome 2: Determine reproductive success (egg deposition and % egg hatch) of adult FHM (F_0 generation) following exposure to SDP pre-spawn in mesocosm

Resulting fathead minnow F1 fry from eggs deposited from adult fathead minnows exposed to SDP in 2014 were transferred to outdoor 0.01 acre concrete ponds in May 2015 to monitor spawning. Egg deposition occurred in F1 fish from all three SDP treatment populations. Enumeration of egg deposition is underway. Review of the data collected in 2014 was completed; statistical analysis of adult mortality, egg deposition and egg hatchability data was completed. The analyses showed no significant effect of SDP treatment at 50 or 100 mg/L on adult mortality, average egg deposition and egg hatchability.

2015 Exposure to fathead minnow fry

Due to problems encounter regarding vitamin C deficiency induced scoliosis in developing F1 in 2014, an exposure to newly hatched fathead minnow fry was conducted on June 24, 2015. This test will use the same exposure concentrations used in previous test and it will 1) determine potential exposure-related impacts on the survival of newly hatched fathead minnow fry and 2) determine the potential exposure-related impacts on the growth and development of newly hatched fathead minnow fry.

Outcome 3: Determine reproductive success of fathead minnows embryo survival following egg exposure to SDP in mesocosm tanks

Fathead minnow fry resulting from eggs that were exposed to SDP in 2014 were transferred to outdoor ponds in May 2015 to monitor spawning. Egg deposition has occurred in the control treatment and 100 mg/L group and monitoring of egg deposition is continuing in all treatment groups. Review of the data collected in 2014 was completed; summarization and statistical analysis of egg hatchability data is in progress.

Outcome 4: Publish results

The results of the data will be included in the final project completion report and prepared for a peer-reviewed publication.

Problem encountered: Fathead minnow fry from both trials experienced significant mortality from an *Aeromonas* bacterial infection, particularly during January and February 2015. Fry were treated for the infection with an oxytetracycline bath and Aquaflor medicated feed. The disease will confound comparison of mortality, growth and development of fry among treatment groups.

Activity Status as of December 31, 2015:

Outcome 1: Determine the survival of aquatic invertebrates following exposure to SDP in outdoor mesocosms.

Histological samples of the digestive tracts of *Gammarus lacustris* adults and *Hexagenia spp*. from the acute Zequanox exposures (2014) were prepared and examined. There was no evidence of pathology associated with the Zequanox treatments. A manuscript has been prepared and is in review.

Outcome 2: Determine reproductive success (egg deposition and % egg hatch) of adult FHM (F0 generation) following exposure to SDP pre-spawn in mesocosm.

F1 fish resulting from adult fathead minnow exposure (2014) were transferred to outdoor 0.01 acre concrete ponds in May 2015 to monitor spawning. Egg deposition occurred from fish in all three treatment groups. Enumeration of egg deposition was completed.

Due to problems related to vitamin C deficiency induced scoliosis in developing F1 fish in 2014, an exposure with newly hatched fathead minnow fry was conducted on June 24, 2015. Fry from each treatment replicate were sampled at 45 days to assess growth, condition, and survival. After 90 days, remaining fry in each treatment replicate were enumerated and a total wet weight was recorded;subsamples of 20 fish from each treatment replicate were individually weighed and measured to assess body condition. Review of the data was completed; statistical analysis and summarization of the data are underway.

Outcome 3: Determine reproductive success of fathead minnows embryo survival following egg exposure to SDP in mesocosm tanks

Fathead minnows that hatched from eggs that were exposed to Zequanox (2014) were transferred to outdoor ponds in May 2015 to monitor spawning. Egg deposition occurred in fish from all treatment

groups. Enumeration of egg deposition was completed. Summarization of egg hatchability data (2014) is in progress.

Outcome 4: Publish results

The results of the data will be included in the final project completion report and prepared for a peer-reviewed publication. A manuscript regarding the exposure of invertebrates to Zequanox (Outcome 1) has been prepared and is in review.

Final Report Summary:

Overview

In order to determine the impacts of SDP (Zequanox®) exposure on non-target animals we evaluated the acute toxicity of SDP to two species of aquatic macroinvertebrates including burrow mayflies nymphs (*Hexagenia* species; *Ephemerotera:Ephemeridae*) and adult amphipods (*gammarus lacustris*; *Amphipoda: Gammaridae*). We also evaluated the effects of SDP exposure on the reproduction and early lifestage development of the fathead minnow (*Pimphales promelas*). The results of these studies are summarized in supplimental attachments 1 and 2 which are a peer-reviewed manuscript decribing the safety of SDP exposure to aquatic macroinvertebrates and a peer-reviewed report describing the effects of SDP exposure on the reproduction and early lifestage development of the fathead minnow, respectively.

Invertebrate Acute Toxicity Trials

Invertebrates were exposed to static applications of SDP (Zequanox®) which included six replicated concentrations of 0 (control), 50 and 100 mg active ingredient (A.I.)/L for 8 hours. Exposures were conducted in 1,000 L outdoor mesocosms tanks that were supplied filtered pond water from a 0.10 hectare UMESC pond. Test animals were obtained from either an independent bait supplier (mayflies) or a private aquaculture facility (amphipods). Concentrations of SDP were determined in each treatment replicate by spectroscopy. The invertebrates were assess for survival and histopathological changes in their digest tracts 96 hours post exposure. Unrecovered invertebrates were treated as a mortality in all analyses. The survival of *G. lacustris* exceeded 80% in all control and treated groups and no treatment-related mortality was detected. Survival of *Hexagenia* species mayfly nymphs ranged from 70-73% in all control and treated groups and no treatment-related mortality was detected. *G. lacustris* treated with SDP exhibited intact digestive epithelium tissues in the stomach, midgut, and hindcut with no treatment related impacts observed. Similarly, no treatment related impacts were observed in *Hexagenia* species mayflies as the epithelium and cell structures appeared intact and comparable to the control groups.

Conclusion

Epithelial tissue necrosis after ingestion is the causative agent in the toxicity SDP to zebra mussels. Molloy et al. (2013) observed histopathological changes in zebra mussels including hemocyte infiltration within 24 hours and degradation of digestive epithelium within 48 hours of Zequanox exposure. In our study, food was confirmed in the digestive tract of specimens examined for hispathology and therefore, ingestion of SDP likely occurred. The presence of food in the digestive tract and the lack of treatment-related histopathological changes or mortality provides strong evidence that exposure to SDP at concentrations and durations expected during open-water applications does not cause significant mortality to either *G. lacustris* or *Hexagenia* species nymphs.

Fathead Minnow Trials

Overview

Three separate SDP (Zequanox®) exposures were conducted with various lifestages of fathead minnows including newly deposited eggs, newly hatched fry, and reproductively active adults. Initial plans included a trial to evaluate the potential reproductive impacts of SDP exposure to fathead minnows *by exposing* adult fathead minnows to a single application of SDP and then determining their reproductive activity for up to 30 days after exposure. This trial was completed as planned and egg deposition and hatchability were successfully determined. Another set of trials initially planned included a trial to evaluate the potential effects of SDP exposure on fathead minnow larval development. In this trial, fathead minnow eggs (≤ 24-h old) were successfully spawned from naive fish, exposed to a single application of SDP, and evaluated for hatching success. A subset of the resulting fry (F1 generation) from each of these trials were reared to adulthood to compare development between exposed and control groups, however, rearing was confounded by several factors. Confounding factors included (1) scoliosis developed in fish from all control and treated groups from both trials and was likely a result of insufficient vitamin C content in the supplemental feed, and (2) upon over winter indoor rearing, a bacterial infection (*columnaris* sp.) was observed in fish, resulting in significant mortality and subsequent therapeutic chemical treatments. Although spawning was observed in the F1 generation from all treated and control groups in both trials, no reliable reproductive success data could be obtained and growth of the resulting F2 generation was not attempted. Due to these complications, an additional trial was completed to evaluate the effects of SDP exposure on fathead minnow larval growth and survival. In this trial, newly hatched fathead minnow fry (≤ 24-h old) were exposed to SDP and reared for 90 days to evaluate growth and survival between the treated and control groups.

All fathead minnow trials utilized static SDP applications which included replicated concentrations of 0 (control), 50 and 100 mg A.I./L for 8 hours. Exposures were conducted in 1,000 L outdoor mesocosms tanks that were supplied filtered water from a 0.10 hectare UMESC aquaculture pond. Test animals were obtained from internal stocks. In all exposures, concentrations of SDP were determined in each treatment replicate by spectroscopy.

2014 Adult Fathead Minnow Reproduction Trial

In 2014, a trial was initiated to determine the effects of SDP exposure on the reproduction of fathead minnows. Spawning condition fathead minnows were placed into 1,000 L tanks and pretreatment baseline spawning was observed for 5 days before SDP exposures were conducted. After the exposures, adult fish mortality and spawning were observed for 21 days. Each day spawning tiles with ≥ 50 deposited eggs were removed, photographed and transferred to a separate corresponding rearing tank. Substrates were photographed again after 48-72 h and used to enumerate the number of eyed eggs for determination of fertilization and hatchability. The resulting F1 generation fry were grown to adulthood and spawning was observed from all treatment groups in 2015. Due to previously mentioned complications, further evaluation of the F1 and F2 generations were not completed. Mean mortality of adult fish in the ranged from 3 to 6% and was not correlated with treatment. Twenty one days after exposure, the condition of the fish did not differ between treatment groups (P=0.9327). Spawning was observed on every day of the 21-day holding period in at least one tank. The number of spawns observed was not correlated to treatment and there was no correlation between treatment and the development of eggs to the eyed stage.

2014 Larval Fathead Minnow Development Trial

In 2014, a trial was initiated to determine the effects of SDP exposure on the larval development of fathead minnows. Ten substrates containing ≥ 50 newly deposited fathead minnows eggs (≤24-h old) were photographed and placed into 1,000 L tanks and SDP exposures were initiated within 4 hours of transfer. Substrates were photographed again after 48-72 h and used to enumerate the number of eyed eggs for determination of fertilization and hatchability. The resulting F1 generation fry were grown to adulthood and spawning was observed from all treatment groups in 2015. Due to previously mentioned complications, further evaluation of the F1 and F2 generations were not completed. The percentage of deposited eggs that developed to the eyed stage ranged from 92.6 to 94.8% and did not differ between the treated and control groups (*P*=0.82).

2015 Larval Fathead Minnow Development Trial

In 2015, a trial was initiate to further determine the effects of SDP exposure on the larval development of fathead minnows. In this trial, newly hatched fathead minnows fry (≤24-h old) were transferred into 100-L stainless steel tanks that were placed into the 1,000 L mesocosm tanks and allowed to acclimate overnight. SDP exposures were applied and after treatment, the fry were released into the 1,000 L mesocosm tank. Mortality was observed for 90 days after exposure and fish condition was determined on a subset (n =20) fish from each treatment replicate 45 and 90 days after exposure. Mean survival in all treatment groups was ≥ 81% and did not differ between treatment groups (*P*=0.54). Similarly, fish condition did not differ between the treated and control groups at 45 or 90 days (*P*=0.75 and 0.30, respectively).

Conclusion

The lack of treatment-related impacts on (1) fry and adult fathead minnow survival, (2) the development of eggs to the eyed stage, and (3) larval development, provides strong evidence that exposure to SDP at concentrations and durations expected during open-water applications does not harm fathead minnows.

ACTIVITY 2: Bathymetric mapping, environmental DNA and physical surveys

Description: Detailed bathymetric maps of two Minnesota lakes or portions thereof, (1 high and 1 low level infestation, to be identified in 2013) will be prepared using high-resolution side-scanning sonar systems to characterize bottom substrate and vegetated habitat. Physical (e.g. divers) and eDNA sampling will be conducted over various habitat types determined from bathymetric survey. Survey (physical and eDNA) and bathymetry data will be compared to identify potential SDP application locations and to determine the potential for eDNA as a treatment prioritization and evaluation tool.

Summary Budget Information for Activity 2:	**ENRTF Budget:**	**$ 160,580**
	Amount Spent:	**$ 160,580**
	Balance:	**$0**

Activity Completion Date:

Outcome	Completion Date	Budget
1. Identify study lakes and sampling locations	July 2014	$5,000
2. Optimize zebra mussel eDNA primers and sampling protocol	July 2014	$12,500

3. Complete bathymetric surveys and data processing	January 2015	$60,000
4. Complete physical surveys and eDNA surveys and data processing	October 2015	$83,080
5. Publish results	May 2016	$ USGS

Activity Status as of December 31, 2013:
USGS policy does not allow the obligation of funds until an executable agreement is established. The agreement, which authorized the USGS to proceed with LCCMR funds, was signed on September 13, 2013. Due to the timing of the project agreement, activities originally planned to commence in 2013 were delayed until 2014. Activities conducted to date have been funded through USGS cost share funds and include the selection of test lakes. Lake Le Homme Dieu will be used for the lake of high zebra mussel abundance and Maple Lake will be used as the lake of low zebra mussel abundance. Both lakes have adequate launch access, are within close proximity for ease of travel, have diverse substrate types, and fit all other criteria for study lake assignment.

Activity Status as of June 30, 2014:
Lake Le Homme Dieu and Maple Lake (Douglas County) have been identified as the test lakes for conducting bathymetric mapping, physical surveys and eDNA surveys. Bathymetry sonar equipment installation on the mapping boat has been completed and cloud computing software for the rapid upload and processing of sonar data has been installed and evaluated with the sonar equipment. Field bathymetry mapping activities have been hampered due to weather conditions and are currently scheduled to be completed by July 15, 2014.
Several markers have been designed to detect for the presence of zebra mussel DNA. Currently, the specificity of the markers is being evaluated with DNA from native unionids. Specific markers will be chosen for analyzing eDNA from collected water samples. Weather conditions and resulting high waters and untreated discharge have delayed eDNA sampling depth determination studies designed to determine the optimum sampling depth for eDNA sample collection. This work is currently scheduled to be completed by July 15, 2014. Full scale eDNA and physical sampling on Lake Le Homme Dieu and Maple Lake are currently scheduled for completion by August 15, 2014. Laboratory processing of water samples and results from eDNA analysis are expected to be completed by November 2014. Completion dates are subject to change due to inclement weather and adverse field conditions.

Activity Status as of December 31, 2014:
Bathymetry mapping data collected from Maple Lake between July and the end of September, 2014, produced maps of substrate hardness, vegetation bio-volume, and total depth for 210 acres of lake surface area. This area represents 25% of the total surface area of Maple Lake. Point estimates for all three parameters were collected at over 6,800 points in the lake (Table 1). Detailed information on the distribution of aquatic vegetation from two focus areas in Maple Lake illustrate variation in the percent of area covered by vegetation in depths less than five meters (Table 2). Mapping efforts in this lake focused on low-infestation areas with suitable substrates for zebra mussel. The northern end of Maple Lake was the farthest location from the initial point of zebra mussel infestation, providing a potential reference site for low density zebra mussel populations.
Table 1.

	Type	PAC	Avg BVp	SD BVp	Avg BVw	SD BVw	Depth Range	Avg Depth	Distance	No. Points
Full Survey	Point	39.80%	25.50%	±13.2%	10.10%	±15%	1.14-20.58 m	4.76 m	11.91 km	6,894
	Grid	42%	19.60%	±9.2%	8.20%	±11.4%	0.02-20.64 m	5.69 m	-	3,466

Table 2.

	Depth	Type	Count	PAC	Avg BVp	SD BVp	Avg BVw	SD BVw
Maple Lake Area 1	1-2m		210	9.50%	59.30%	±27.7%	5.60%	±19.4%
	2-3m		930	16.20%	31.30%	±26.1%	5.10%	±15.6%
	3-4m		568	93%	27.50%	±12.3%	25.60%	±13.8%
	4-5m		432	93.80%	24.30%	±11.3%	22.80%	±12.4%
	5-6m		234	0%	-	-	0%	±0%
	6-7m		256	0%	-	-	0%	±0%
	7-8m		163	0%	-	-	0%	±0%
	8-9m		64	0%	-	-	0%	±0%
	>9m		58	0%	-	-	0%	±0%
	Depth	Type	Count	PAC	Avg BVp	SD BVp	Avg BVw	SD BVw
Maple Lake Area 2	1-2m		289	9%	13.80%	±12.4%	1.20%	±5.4%
	2-3m		1591	50.10%	22.90%	±11.5%	11.50%	±14%
	3-4m		710	94.90%	25%	±10.1%	23.70%	±11.3%
	4-5m		150	93.30%	29.10%	±9.2%	27.20%	±11.5%
	5-6m		133	0%	-	-	0%	±0%
	6-7m		233	0%	-	-	0%	±0%
	7-8m		118	0%	-	-	0%	±0%
	8-9m		101	0%	-	-	0%	±0%
	>9m		10	0%	-	-	0%	±0%

Bathymetry mapping efforts for Lake Le Homme Dieu during the same period produced maps of substrate hardness, vegetation bio-volume, and total depth for over 334 acres of lake surface area. This area represents 19% of the total surface area of the lake. Point estimates for all three parameters were collected at over 12,400 points in the lake (Table 3). Composite data from these three focus areas illustrate that average plant cover and plant biovolume are highly variable over large areas.

Table 3. Summary data from three mapping areas in Lake Le Homme Dieu.

	Area	PAC	Avg BVp	SD BVp	Avg BVw	SD BVw	Depth Range	Avg Depth	Distance	No. Points
Full	1	71.60%	34.40%	±23.8%	24.60%	±25.4%	0.88-15.34 m	3.47 m	8.27 km	5,599
	2	85%	52.60%	±27.1%	44.50%	±31.3%	0.94-17.67 m	2.31 m	4.11 km	2,759
	3	58%	40.50%	±25.5%	23.30%	±27.8%	0.93-16.08 m	3.7 m	14.97 km	4,086

Table 4. Summary of sampling trips for zebra mussel habitat mapping in Maple Lake and Lake Le Homme Dieu, MN.

Lake Mapping 2014								
Date	Equipment	Transducers	Algorithm	Depth	Substrate	Plant Biovolume	Shore survey	Lake
7/10/2014	Lowrance HDS 10 w/ StructureScan	50/200 kHz; 455kHz	BioBase	X	X	X		ML
7/15/2014	Lowrance HDS 10 w/ StructureScan	50/200 kHz; 455kHz	BioBase	X	X	X		ML
7/18/2014	Lowrance HDS 10 w/ StructureScan	50/200 kHz; 455kHz	BioBase	X	X	X		ML; LHD
7/23/2014	Lowrance HDS 10 w/ StructureScan	50/200 kHz; 455kHz	BioBase	X	X	X		ML; LHD
7/29/2014	Lowrance HDS 10 w/ StructureScan	50/200 kHz; 455kHz	BioBase	X	X	X		LHD
7/31/2014	Lowrance HDS 10 w/ StructureScan	50/200 kHz; 455kHz	BioBase	X	X	X		LHD
8/5/2014	Lowrance HDS 10 w/ StructureScan	50/200 kHz; 455kHz	BioBase	X	X	X		LHD
8/7/2014	Lowrance HDS 10 w/ StructureScan	50/200 kHz; 455kHz	BioBase	X	X	X		LHD
8/12/2014	Lowrance HDS 10 w/ StructureScan	50/200 kHz; 455kHz	BioBase	X	X	X		LHD
9/9/2014	Lowrance HDS 10 w/ StructureScan	50/200 kHz; 455kHz	BioBase	X	X	X		LHD
9/16/2014	Lowrance HDS 10 w/ StructureScan	50/200 kHz; 455kHz	BioBase	X	X	X		LHD
9/17/2014	Lowrance HDS 10 w/ StructureScan	50/200 kHz; 455kHz	BioBase	X	X	X		LHD
9/23/2014	Lowrance HDS 10 w/ StructureScan	50/200 kHz; 455kHz	BioBase	X	X	X		LHD

Glossary

AOI

Area of Interest: Defines the individual transects or contiguous data samples as depicted by the color coding of each trip line. Separate areas of interest can be generated through merging of multiple trips, appending data to a single sonar log or lapses in time (greater than five minutes) within a sonar log.

BVp

Biovolume (Plant):: Refers to the percentage of the water column taken up by vegetation when vegetation exists. Areas that do not have any vegetation are not taken into consideration for this calculation.

BVw

Biovolume (All water): Refers to the average percentage of the water column taken up by vegetation regardless of whether vegetation exists. In areas where no vegetation exists, a zero value is entered into the calculation, thus reducing the overall biovolume of the entire area covered by the survey.

PAC

Percent Area Covered: Refers to the overall surface area that has vegetation growing.

Grid

Geostatistical Interpolated Grid: Interpolated and evenly spaced values representing kriged (smoothed) output of aggregated data points. The gridded data is most accurate summary of individual survey areas.

Point

Individual Coordinate Point: A single point represents a summary of sonar pings and the derived bottom and canopy depths. Individual point data create an irregularly spaced dataset that may have overlaps and/or gaps in the data resulting in an increased potential for error.

Qualitative surveys were also carried out during the bathymetry work to obtain an initial characterization of the zebra mussel population in each lake. This was accomplished by taking dredge samples and pulling vegetation at several sites on each study lake. The qualitative surveys were also intended to direct eDNA water sampling and dive efforts.

Water samples were collected from Lake Le Homme Dieu and Maple Lake (Douglas County) for eDNA analysis on September 22 and 23, 2014. A total of 60 water samples were collected from near the benthos over at least 3

different substrates at depths between 0.5 and 6.0 m at each lake. All water samples were frozen at -20°C within three hours of collection and stored at -20°C until further processed. All samples were centrifuged to concentrate cellular debris into a pellet and the supernatant was discarded. DNA was extracted from each pellet using a commercially available DNA isolation kit. Resulting DNA was then analyzed for zebra mussel DNA in octet using quantitative real-time polymerase chain reaction. Copy numbers of DNA were estimated using a standard curve generated by serially diluting known amounts of the target DNA transcript. All eDNA data are currently being analyzed and are expected to be summarized for submission in a peer-reviewed journal by April 1, 2015.

Physical sampling on Lake Le Homme Dieu and Maple Lake was completed on September 24 and 25, 2014. All zebra mussels were collected from three 0.25 m^2 quadrats at each of the locations that eDNA was collected. Zebra mussel specimens were collected using SCUBA. All zebra mussels were frozen and stored at -20°C. General density estimates have been completed and biomass determinations for samples are expected to be completed by March 1, 2015.

Activity Status as of June 30, 2015:

Outcome 1: Identify study lakes and sampling locations

Completed July, 2014.

Outcome 2: Optimize zebra mussel eDNA primers and sampling protocol

A suite of eDNA markers developed by Wendylee Stott (USGS-Great Lakes Science Center) were tested *in silico* for specificity to zebra mussels using primer-BLAST. Two primer sets (Dre2 and Dre5) with one hydrolysis probe (Dpo1) were selected as the most efficient and specific. Both primer sets with the probe were tested on environmental samples collected at seven sites from multiple depths. At each site in Lake Minnetonka, samples were collected from the surface, from mid-water column, and from six inches off the bottom. Both primer sets performed equally, so further analysis was carried out using the Dre2 primer set. Both the surface samples and near-bottom samples had 100% detection accuracy with the mid-column samples failing to detect at one known-positive site. The near-bottom samples resulted in higher concentrations of zebra mussel DNA than the surface samples, so samples collected at Maple Lake and Lake Le Homme Dieu were obtained from near the bottom at all sites.

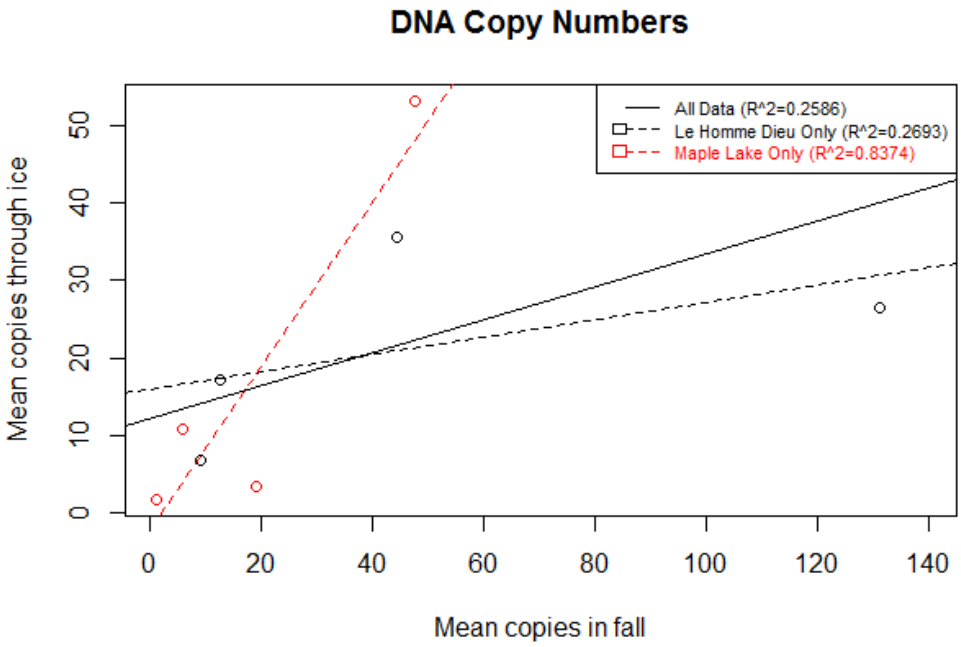
Outcome 3: Complete bathymetric surveys and data processing

Bathymetry mapping survey data were completed in Maple Lake and Lake Le Homme Dieu between July September 20, 2014. Data on substrate hardness, vegetation bio-volume, and total depth were collected for 210 (Maple Lake) and 334 acres (Lake Le Homme Dieu). The resulting Map data from Maple Lake and Lake Le Homme Dieu have been successfully imported into ArcGIS to form the basis of an interactive ArcMap product for each lake. ArcMap products for each lake are under development and are expected to be completed by August 30, 2015.

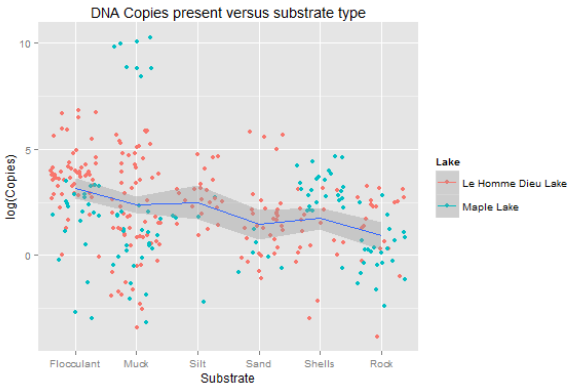
Outcome 4: Complete physical surveys and eDNA surveys and data processing

Qualitative zebra mussel surveys were also carried out during the bathymetry work conducted in 2014. Water samples were collected from Lake Le Homme Dieu and Maple Lake (Douglas County) for eDNA analysis on September 22 and 23, 2014. Water samples were analyzed by quantitative real-time polymerase chain reaction in quadruplicate (not octet as previously described). Additional water samples were collected through the ice in March 2015 using the GPS coordinates of the summer samples. Preliminary results suggest there is a very strong correlation between the DNA copy numbers

detected in the samples collected through the ice compared to samples collected on open water in the fall at the same sites for Maple Lake and a weaker, but still positive correlation on Le Homme Dieu Lake. Figure 1 shows the mean DNA copy number detected across samples for a given site plotting spring through-ice sample means against fall open-water sample means. Maple Lake is plotted in red and Le Homme Dieu Lake is plotted in black. The solid line is linear regression of all data points collectively, and the dashed lines are considering each lake separately.



Preliminary results also show a negative correlation between DNA copy numbers and sediment grain size. Figure 2 shows log-transformed copy numbers for each replicate reaction plotted against categorical substrate types over which the samples were collected. The substrate types are arranged from smallest grain size to largest as you move from left to right on the plot. The points are in red for Le Homme Dieu Lake and in blue for Maple Lake. The line represents a loess polynomial regression of the data with 95% credible interval shown in the shaded area.



Zebra mussels collected for biomass determination have been cleaned and documented and the methods to determine living biomass are under development with samples expected to be completed by February 28, 2016.

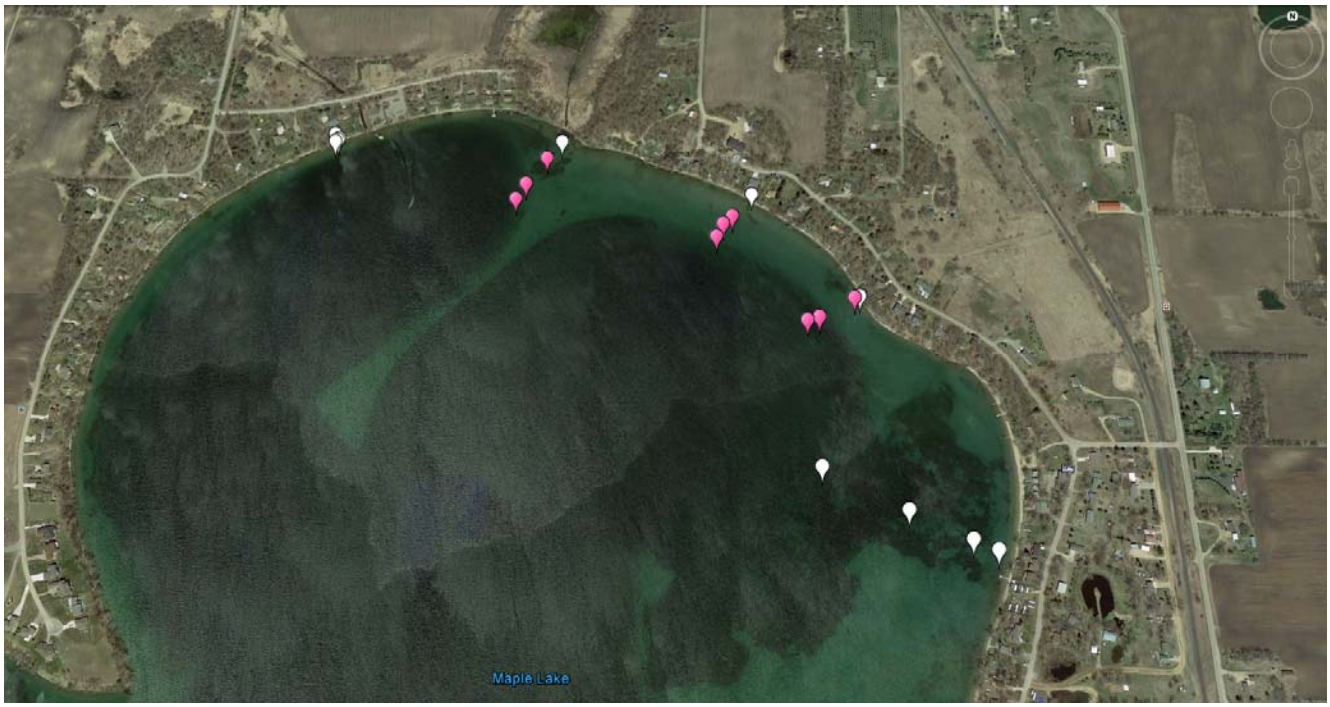
Outcome 5: Publish results

The results of the data will be included in the final project completion report and prepared for a peer-reviewed publication. A bathymetric map from outcome 3 of each lake will be included with the final projection completion report.

Activity Status as of December 31, 2015:

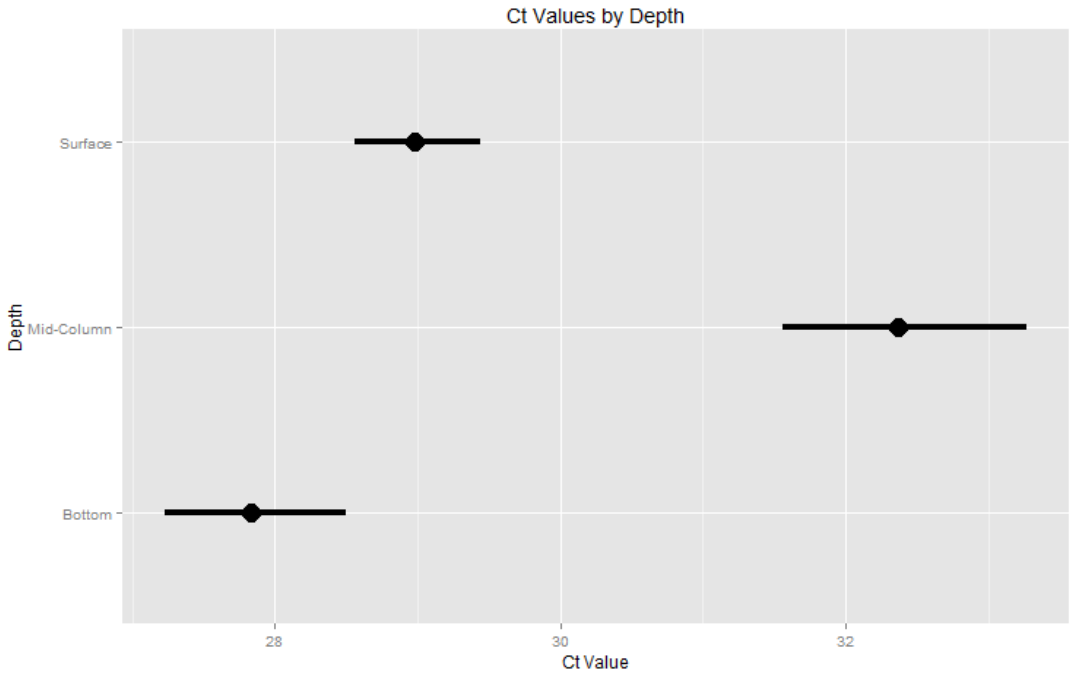
Outcome 1: Identify study lakes and sampling locations

Completed July, 2014. Sites were selected on Lake Le Homme Dieu and Maple Lake near Alexandria, MN and are shown on the maps below. Five transects were chosen on each lake attempting to cover a range of different substrate types and zebra mussel densities. Sampling points were selected on each transect as the water depth passed 3, 7, 12, or 18 feet. All points were sampled in the fall, 2014 sampling. The points shown in pink were selected for sampling through the ice in March, 2015.



Outcome 2: Optimize zebra mussel eDNA primers and sampling protocol

Completed August, 2014. Thirty water samples were collected at varying depths in Lake Minnetonka above zebra mussel beds. Ten samples each were collected at the surface, 6 inches above the bottom, or from the middle of the water column using a Van Dorn sampler. Each water sample was analyzed in 4 replicates with each of 2 sets of primers. Both primer sets performed equally well. Water samples collected near the bottom or at the surface had a 100% detection rate while mid-column samples had an 85% detection rate. However, samples collected near the bottom were found to contain higher concentrations of zebra mussel DNA as indicated by the plot below.



For a qPCR, lower Ct values indicate higher concentrations of target DNA. The different depths tested are shown on the y-axis with Ct values on the x-axis. The means are plotted with 95% credible intervals. The credible intervals are non-overlapping indicating a statistically significant difference with samples collected near the bottom having the most zebra mussel eDNA and mid-column samples having the least.

Outcome 3: Complete bathymetric surveys and data processing

Mapping data from Maple Lake and Lake Le Homme Dieu have been successfully combined with existing bathymetry and additional mapping data into an ArcGIS ArcMap product for each lake. Data for depth, substrate type, and vegetation bio-volume have been combined with other available geo-spatial data in an ArcMap final product. ArcMap and pdf file maps of each lake will be included with the final project completion report.

Outcome 4: Complete physical surveys and eDNA surveys and data processing

Environmental DNA analysis has been completed on all water samples from both sampling periods. Zebra mussels collected at each site have been cleaned, documented, and are currently being processed for ash-free dry weight analysis to obtain biomass measurements.

Outcome 5: Publish results

The results of the data will be included in the final project completion report and prepared for peer-reviewed publication(s). A bathymetric map from outcome 3 of each lake will be included with the final projection completion report.

Final Report Summary:

Overview

Detailed maps were prepared for portions of Lake Le Homme Dieu and Maple Lake (Douglas County), which had high and low zebra mussel infestation levels, respectively. Depth, substrate hardness, and submerged aquatic vegetation (SAV) depth and biovolume were compared to Zebra mussel populations and submerged aquatic vegetation was an important component of zebra mussel habitat in shallow habitats in Lake Le Homme Dieu. The results of this study are summarized in supplimental attachment 3 which is a peer-reviewed report.

In order to determine the utility of using molecular markers for the detection and/or correlation of zebra mussel biomass, we first evaluated the specificity of molecular primers and probes for detection of zebra mussel DNA and the optimal depth to collect water samples for DNA analysis. After determining the appropriate markers and sampling protocol, water samples were collected from the same locations in Maple Lake and Lake Le Homme Dieu during the summer of 2014 and in March of 2015 and the DNA content was correlated to the estimated zebra mussel biomass resulting from physical surveys conducted by SCUBA divers. The results of this study are summarized in supplimental attachment 4 which is a peer-reviewed report.

Mapping Activities

Data Collection Platform Configuration

Data were collected using a commercial sonar platform from Lowrance™ High Definition System (HDS®) consumer echo-sounder (Contour Innovations LLC 2013). Transect data were collected using an HDS® 10 unit equipped with a 200 kHz transducer operating with a 20 degree beam angle and a 455kHz side-scanning transducer for vegetation characterization. The HDS® unit was configured using the shallow water setting which provides a constant 75% ping speed or 15 pings per second. Ping speed is the rate at which the transducer transmits a sound pulse into the water column and receives the response echo, controlling the along-transect coverage. Pings are combined into an ensemble and values are averaged to produce a reported data value. The actual density of data collection is controlled by the velocity of the boat as it drives along the sampling transect. During data collection for this project, boat speed was maintained at five miles per hour providing a forward velocity of approximately seven feet per second. An estimate of the average sonar data density used for this project can be calculated by combining the forward velocity estimate with ping speed, resulting in an average sonar data density of approximately one sample every two feet of linear distance along a sampling transect. Pulse width (i.e., band width) is not user controlled with the Lowrance™ system but is dynamic and varies depending on depth. Software algorithms for ciBioBase are optimized at 3200 bytes per second with a range window set to Auto on the HDS®-10 unit. This configuration of the unit provided the optimal range resolution of the sonar return signal for the range of depths available to the system.

Data Acquisition

Data were collected along transects spaced 30 meters apart using an eighteen-foot aluminum-hulled boat with a modified V hull configuration. Sonar transducers were mounted on the transom of the boat approximately 20 cm below the water line. Tracks for data collection were laid-out in the navigation display and followed at five miles per hour during data acquisition.

Data ensembles were acquired at one second intervals. Feature data from pings that occurred between position reports were reported as an average value for each GPS geo-referenced data point. As a result, attribute data for a specific feature class including depth, bottom hardness, and plant height represents the average of 15 values from sonar returns during the sampling interval.

During acquisition, sonar data were simultaneously paired with global position (GPS) data using a built-in, time-referenced GPS unit on the HDS®-10. The GPS unit for the HDS®-10 was set to differentially correct the GPS satellite data using the Wide Area Augmentation System (WAAS) navigation system. Acoustic signal data and GPS position data were logged to SD data storage cards using the (.sl2) format for later upload to the Contour Innovations cloud server for post processing using the ciBioBase software tool.

Post Processing and Map Development

Unprocessed data files were uploaded to the Contour Innovations centralized servers using a client software program supplied as part of the ciBioBase GIS software system. Raw data files for GPS positional data and quality-assured sonar response data were processed using proprietary algorithms into estimates of bottom depth, plant height, plant bio-volume, and bottom hardness features. Within the ciBioBase software package, each data ensemble goes through a quality-assurance test to determine whether the feature data for the sampling period can be extracted and used for further analysis. If the data pass the internal data filters, values are sent on to the respective feature detection algorithms. Data failing to meet the quality assurance tests are removed from consideration for summarization

Processed data were used to generate geo-referenced, surface response maps for each feature class using kriging to generate a grid of equal-area cells referred to as a raster grid. Kriging is a geo-spatial analysis method that uses the actual statistical relationship of neighboring data points to make predictions in un-sampled locations. The following explanation from the ArcGIS online help manual helps explain the kriging process:

> "Kriging assumes that the distance or direction between sample points reflects a spatial correlation that can be used to explain variation in the surface. The Kriging tool fits a mathematical function to a specified number of points, or all points within a specified radius, to determine the output value for each location. Kriging is a multistep process; it includes exploratory statistical analysis of the data, variogram modeling, creating the surface, and (optionally) exploring a variance surface. Kriging is most appropriate when you know there is a spatially correlated distance or directional bias in the data." (http://desktop.arcgis.com/)

Once kriging had produced x,y,z grids of raster cells for each feature class, the data were used to project the data in three dimensions, producing a map of each attribute class for each survey. Maps were used to choose potential eDNA sampling sites prior to preliminary field data collection.

In order to be able to reproduce the statistical analysis used in the ciBioBase software, processed data files from the cloud server were incorporated into an ArcGIS project and subjected to kriging analysis following the reported analysis criteria from ciBioBase. This independent analysis of the feature class and GPS location data confirmed the proprietary mapping program included in the ciBioBase processing software. Once data were geo-referenced in ArcGIS, the results were combined with existing Minnesota DNR GIS base layers for lake bathymetry and satellite imagery to generate ArcMap geo-spatial databases and map products. The ArcMap data platform provides the end user with the option of changing the parameters used to generate the feature maps. For example, the buffer width surrounding the transect data can be changed during kriging analysis, providing for a more narrow or a wider map footprint.

Results

Bathymetry from the data collection effort provided one-foot contour resolution within the survey areas. This level of resolution was more than three times the available bathymetry resolution from MN DNR historical mapping efforts conducted prior to their use of the ciBioBase software. In addition to higher-resolution bathymetry, the data for substrate hardness and vegetation bio-volume in the water column provided important data on habitat quality for zebra mussels. Zebra mussels in Lake Le Homme Dieu and in the connected Lake Carlos have been observed to preferentially colonize hard substrates (Kiesling, unpublished video surveys). In addition to substrate hardness, observed zebra mussel density (> 10 individuals per plant) in vertical (>40cm) vegetation stands in Lake Le Homme Dieu suggest that vegetation biomass in the water column is a potential axis of zebra mussel distribution in the study lakes. The amount of plant biovolume at the survey sites is significant, accounting for more than half of the water column in many location in Lake Le Homme Dieu.

Maps generated by the ciBioBase software identify both Maple Lake and Lake Le Homme Dieu as having a wide range of available depths, substrate types, and vegetation height and cover. This range of feature-class values provided the eDNA sampling teams with a broad choice of habitat class combinations to guide their sampling plans. Sampling transects were located in or very near to sonar survey data collection areas. Maps from multiple areas in Lake Le Homme Dieu have also made it clear that feature characteristics such as vegetation bio-volume do not track depth or substrate hardness directly (e.g., east launch area maps) suggesting a need for follow-up analyses of the factors controlling the vegetation distribution.

Development of an ArcGIS geo-spatial data product provides a common platform for future spatial analysis of the mapping datasets from this project. In addition to the GIS database, the ability to generate the same maps in ArcMap as were produced by the commercial software product provides an important statistical data archive

for the results of the kriging analysis in ciBioBase. Data from the project are also available through the Contour Innovation cloud server and can be incorporated into future map enhancements for the lakes by requesting permission to use the data.

Molecular Detection Activities

Development of the eDNA assay and protocol

During 2014, we tested a suite of eDNA markers developed by Wendylee Stott (USGS-Great Lakes Science Center) for specificity to zebra mussels using primer-BLAST. Two primer sets (Dre2 and Dre5) with one hydrolysis probe (Dpo1) were selected as the most efficient and specific.

During 2014, we sampled Lake Minnetonka to develop a sampling protocol. Water was collected from the surface, mid-water and near the bottom directly above a known colony of Zebra Mussels. Ten 50 mL sterile conical tubes were placed just below the water surface to collect the surface film. Mid-water samples were collected using a 2.2 L horizontal Van Dorn water sampler. The water sampler was lowered to mid-depth and sealed. Ten 50 mL water samples were collected from the water sampler. The bottom samples were collected using a separate 2.2 L horizontal Van Dorn water sampler. This water sampler was lowered to approximately 9 cm above the bottom where the water was collected and brought to the surface. Again, ten 50 mL water samples were collected from the sampler. Once each sample was collected it was capped and stored on ice and transported to the U.S. Geological Survey Upper Midwest Environmental Sciences Center in La Crosse, Wisconsin (UMESC) for further processing. DNA was extracted from individual samples and DNA quantified using quantitative real-time polymerase chain and our Zebra Mussel eDNA assay.

Once at UMESC, we centrifuged the 50 mL water samples at 5,000 x g for 30 minutes and decanted the supernatant. We extracted DNA from the remaining pellet and residual water using the commercially available genomic DNA extraction kit. We also extracted 100 µL of deionized water as an extraction negative control with each extraction batch. All samples had a final elution volume of 100 µL. We analyzed each DNA extract in four replicate qPCRs with 1 µL of template in 20 µL reactions with four no template controls and two replicate standard curves. The standard curves contained synthetic DNA of the target sequence in a 5-fold dilution series from 31,250 copies down to 10 copies per reaction. We then used this standard curve to estimate the number of copies of Zebra Mussel DNA present in each sample.

Our Zebra Mussel-specific eDNA assay only detected DNA from Zebra Mussels and no detections were observed in any of the native mussels or fish species tested. We concluded that this marker was adequate for detecting the presence of Zebra Mussel DNA in Minnesota waters. We also determined that water samples collected near the bottom or at the surface were the best sampling depths for detecting Zebra Mussel DNA. However, water samples collected near the bottom had slightly higher amounts of DNA than those collected from the surface. This suggests that sampling near the bottom may provide improved detection and improve the probability of correlating DNA copies with biomass. Therefore, we used benthic sampling for our 2015 water collections from Lake La Homme Dieu and Maple Lake.

Physical Surveys and eDNA Surveys for Mapping Infested Lakes

Qualitative zebra mussel surveys were carried once bathymetry work was completed in 2014. Water samples were collected from Lake Le Homme Dieu and Maple Lake (Douglas County) for eDNA analysis autumn of 2014. At each lake, we collected water from near the bottom using a horizontal Van Dorn water sampler in triplicate. Water samples (50 mL) were collected at depths of approximately 1, 2, 4 and 6 m along four transects in each lake. Transects covered different substrate types from loose flocculent to cobble in each lake. Immediately following water sampling at each sample point, we placed a brick, tied to a buoy, and recorded GPS

coordinates so that we could use SCUBA divers to collect Zebra Mussels at each sampling location and confirm the substrate type. For eDNA, we sampled each lake twice; once in the autumn and again in late winter of 2015. Once water samples were collected and placed on ice. All water samples were frozen within 12 hours. All samples were transported to UMESC for DNA extraction and quantification using qPCR.

Once at UMESC, we centrifuged the 50 mL water samples at 5,000 x g for 30 minutes and decanted the supernatant. We extracted DNA from the remaining pellet and residual water using the commercially available genomic DNA extraction kit. We also extracted 100 µL of deionized water as an extraction negative control with each extraction batch. All samples had a final elution volume of 100 µL. We analyzed each DNA extract in four replicate qPCRs with 1 µL of template in 20 µL reactions with four no template controls and two replicate standard curves. The standard curves contained synthetic DNA of the target sequence in a 5-fold dilution series from 31,250 copies down to 10 copies per reaction. We then used this standard curve to estimate the number of copies of Zebra Mussel DNA present in each sample.

The day following 2014 autumn water sampling, we used SCUBA divers to collect all the Zebra Mussels in three 0.25 m^2 quadrants near each brick. Zebra Mussels from each quadrant were brought to the surface and placed into separate plastic storage containers and placed on wet ice. All Zebra Mussel samples were frozen (-20°C) within 4 h of collection. SCUBA divers also verified substrate at each sampling location. Mussel samples were then transported to UMESC so that biomass could be estimated for each sampling site.

During 2015, we processed and prepared each sample for estimating biomass. Biomass for each sample was estimated by ash-free dry weight (AFDW). Each Zebra Mussel sample was weighed to determine total wet-weight. The moisture content was determined according to AOAC Official Method 934.01. Subsequently, ash weight was determined according to AOAC Official Method 942.05 for each sample. AFDW was calculated subtracting AW from DW for the subsample and adjusting to the mass (wet-weight) of the whole sample.

In Lake La homme Dieu, AFDW decreased with increased depth. No correlation between AFDW and substrate type or between AFDW and the number of copies of Zebra Mussel DNA for both fall and winter were found. DNA copy numbers were not found to accurately predict the biomass of Zebra Mussels in this lake. However, the number of positive detections was a negatively correlated with substrate type, which suggests one has a higher probability of detecting Zebra Mussel DNA in areas that have softer substrates.

Like in Lake La Homme Dieu, AFDW decreased with increased depth in Maple Lake. No correlation between AFDW and substrate type or between AFDW and the number of Zebra Mussel DNA copies was found for Maple Lake. Again, this suggests that DNA cannot accurately predict the biomass of Zebra Mussels in a lake. Unlike Lake Le Homme Dieu, no correlation was found between the number of hits for a sample and substrate type in Maple Lake, which suggests one has an equal probability of detecting Zebra Mussel DNA in areas with soft substrates as those with harder substrates.

ACTIVITY 3: SDP application technique development and validation and field efficacy

Description: Laboratory, pond-scale and field studies will be completed to develop and validate the use of injection versus whole water column treatment application techniques to achieve SDP treatment concentrations and to potentially reduce the quantity of SDP applied during field application. Laboratory studies will compare injection technology and techniques for treatment administration to reduce the quantity of SDP applied relative to whole water column treatments. Refined injection techniques will be further evaluated pond-scale (0.01 acre) to compare injection application methods with whole water column SDP application. The pond-scale studies will refine the selected injection techniques to confirm that effective concentrations of SDP are maintained for the required exposure duration. The efficacy of dreissenid mussel control through SDP application will be validated under field conditions through in-lake testing in Lake Minnetonka (Deephaven, MN). The developed/refined injection application technique and whole water column SDP application will be evaluated within replicated

enclosures (~24m^2). Applications will be conducted in September 2014 and treatment success will be evaluated through the completion of pre-and post-treatment assessments.

Summary Budget Information for Activity 3:

ENRTF Budget:	**$ 275,920**
Amount Spent:	**$ 275,920**
Balance:	**$0**

Activity Completion Date:

Outcome	Completion Date	Budget
1. Identify enclosure areas and place colonization substrates	August 2014	$10,500
2. Complete laboratory and pond scale evaluations of SDP injection application techniques	October 2015	$100,932
3. Perform field treatments with SDP	September 2014	$82,244
4. Perform post-treatment assessments and compile data	February 2015	$82,244
5. Publish results	May 2016	$USGS

Activity Status as of December 31, 2013:
USGS policy does not allow the obligation of funds until an executable agreement is established. The agreement, which authorized the USGS to proceed with LCCMR funds, was signed on September 13, 2013. Due to the timing of the project agreement, activities originally planned to commence in 2013 were delayed until 2014. Activities conducted to date have been funded through USGS cost share funds and include 1) the construction and placement of colonization substrate samplers to be used for the treatment effectiveness determination, 2) the completion of a research protocol for the in-lake treatments and 3) outreach to local groups, organizations and the public to provide information regarding research objectives.

Activity Status as of June 30, 2014:
Activities conducted to date have included 1) the construction and placement of colonization substrate samplers to be used for the treatment effectiveness determination, 2) the completion of a research protocol for the in-lake treatments and 3) outreach to local groups, organizations and the public to provide information regarding research objectives. Additional outreach activities to adjacent landowners will be conducted in July 2014. Initiation of injection application studies is scheduled for July, 2014 and adult zebra mussel and native invertebrate samplers will be placed in August, 2014. Field treatments are scheduled to be conducted from September 8-22, 2014.

Activity Status as of December 31, 2014:
Laboratory and pond scale evaluations of SDP injection application techniques:
Application technique development included construction of a large scale (~150 L) system to mix SDP into the desired application concentration. An indoor test system consisting of 15 350-L test tanks was constructed and used to compare 5 different injection apparatuses at multiple Zequanox stock concentrations (3, 4 and 5 %). Aluminum framed, impermeable membrane-covered mesocosm enclosures panels were constructed and placed in 0.01 acre concrete ponds and used to evaluate 7 different injection apparatuses at two SDP stock concentrations (4 and 5 %), two injection rates (~ 2 and 4 GPM) and two application heights (~60 and 90 cm).

Two suspended injection bar delivery systems, which provided acceptable treatment layer during mesocosm evaluations, were constructed for delivering a 5% SDP solution at ~90 cm during the field application.

SDP field applications were conducted within 27-m^2 enclosures erected from aluminum framed, impermeable membrane-covered enclosure panels positioned in Robinson's bay (Lake Minnetonka) on September 12, 15 and 17, 2014. Five treatment groups (control, 50 and 100 mg SDP/L (A.I.) whole water and 50 and 100 mg SDP/L (A.I.) subsurface application were completed on each treatment day. Samplers placed in each treatment enclosure were held in the bay after SDP application and assessed for zebra mussel mortality (type 2 samplers) and sampled for zebra mussel biomass (type 1 samplers) from October 22-26, 2014. Data analyses and review have not been completed. Problem encountered: Preliminary data review and field observations indicate that sub-surface SDP applications in dynamic, high energy environments are problematic for obtaining a benthic layer of SDP for a suitable duration to achieve acceptable dreissenid mussel mortality. Additional work is planned to evaluate the maximum concentration of SDP that can be used during subsurface application. Use of a higher viscosity solution during subsurface applications may increase the duration of acceptable SDP concentration in the benthic treatment zone.

Activity Status as of June 30, 2015:

Outcome 1: Identify enclosure areas and place colonization substrates

Completed August, 2014.

Outcome 2: Complete laboratory and pond scale evaluations of SDP injection application techniques

Initial trials were completed in 2014. Additional work to determine the maximum mix ratio at various water temperatures will be completed by September 30, 2015. Additional pond trials to determine sub-surface application methods for quiescent waters will be completed by October 30, 2015.

Outcome 3: Perform field treatments with SDP

SDP field applications were completed in Robinson's bay (Lake Minnetonka) in September, 2015. Mortality data from zebra mussels placed in containment bags within each enclosure and treatment concentration data have been reviewed. Mean Zequanox concentration in the bottom 15 cm over the entire exposure period (8h) in the 50 mg/L sub-surface application treatment group was 33 mg/L compared to 45 mg/L for the whole water treatment group. Similarly, the mean Zequanox treatment concentration in the bottom 15 cm for the entire exposure period in the 100 mg/L sub-surface application treatment group was 73 mg/L compared to 82 mg/L for the whole water treatment group. Mean survival of zebra mussels in the 50 mg/L sub-surface application treatment group was 72% compared to 59% for the whole water treatment group. Similarly, the mean survival of zebra mussels in the 100 mg/L sub-surface application treatment group was 44% compared to 27% in the whole water treatment group. Methods and equipment to determine living biomass in zebra mussel samples are being determined and acquired. Once the procedures are optimized, the samples collected to determine the living zebra mussel biomass adhering to multi-plate samplers will be processed and compared by treatment group. Processing of these samples is expected to begin in October, 2015.

Outcome 4: Perform post-treatment assessments and compile data

Outcome 2 data computation is expected to be completed by December 31, 2015. Outcome 3 data computation for water chemistry, Zequanox concentrations, and mortality of zebra mussels is completed. Biomass data computation is expected to be completed by February 28, 2016.

Outcome 5: Publish results

The results of the data will be included in the final project completion report and prepared for a peer-reviewed publication.

Activity Status as of December 31, 2015:

Outcome 1: Identify enclosure areas and place colonization substrates

Completed August, 2014.

Outcome 2: Complete laboratory and pond scale evaluations of SDP injection application techniques

Initial trials were completed in 2014. Further tests to assess the optimum concentration for subsurface applications of Zequanox at a range of environmental temperatures were performed in temperature controlled environmental chambers at the Upper Midwest Environmental Sciences Center. Four Zequanox stock concentrations (5 to 25 % w/w) were evaluated at temperatures of 7, 12, 17, and 22 °C. Observations of Zequanox stock viscosity, Zequanox stock concentration sink rate, and of the characteristics of the Zequanox layer formed within the water column were made for four Zequanox stock concentrations at each temperature. Zequanox stock concentrations were prepared by mixing Zequanox into water with an immersion blender. After preparation, the viscosity of each concentration was measured using Zahn cup viscometers. Zequanox stocks were injected into graduated cylinders containing temperature acclimated water and the sink rate and qualitative observations were collected for 8 hours. The data collected from the environmental chamber studies were analyzed and used to create a temperature-concentration linear regression which was then used to predict the optimum concentration of Zequanox to be used in subsurface applications at specific water temperatures from 7-22 °C.

The utility of the linear-regression to predict the optimum concentration of Zequanox to be used in subsurface applications at specific water temperatures was evaluated in replicated studies conducted in outdoor 3-m^2 enclosures placed in 0.01 acre concrete ponds. The validation studies were conducted at three environmental temperatures (~9, 14, and 20 °C) and applications at each temperature included 3 Zequanox treated enclosures and a control enclosure. The treated enclosures utilized an application system (peristaltic delivery pump with delivery tubes attached to a welded aluminum frame consisting of 16 delivery points spaced equally throughout the enclosure) to apply the Zequanox stock at 90 cm above the bottom. All enclosures utilized a collection system (peristaltic pump with collection tubes attached to a welded aluminum frame) to collect water at three depths (7.5, 30, and 60 cm) to verify Zequanox concentrations for an 8 hour period. Preliminary results of the enclosure validation studies indicate that subsurface application of Zequanox to quiescent waters using stock concentrations obtained from the laboratory derived concentration/temperature curve maintained lethal Zequanox concentrations near the bottom of the enclosure. Mean Zequanox concentrations at 7.5 cm from the bottom of pond were near the target of 100 mg/L active ingredient as evident by a mean Zequanox concentration of 116.1 ± 15.4 mg/L. Little Zequanox was observed to migration out of the treatment zone as evident by mean Zequanox concentrations of 12.3 ± 12.3 mg/L at a depth of 60 cm from the pond bottom.

Outcome 3: Perform field treatments with SDP

SDP field applications were completed in Robinson's bay (Lake Minnetonka) in September, 2015. Summarization of mortality data from contained zebra mussels and Zequanox concentrations within each enclosure are being completed. Methods to determine living biomass in zebra mussel adhering to multi-plate samplers have been determined and will utilize a ball mill to homogenize the dried samples. After homogenization, quadruplicate subsamples will be oxidized using a muffle furnace and the resulting ash free dry weight (AFDW) of each subsample will be determined. AFDW of each sampler/material type will be statistically compared between treatment replicates and comparisons to mortality zebra mussel in contained samplers will be conducted. Furthermore, the reduction in living biomass per square meter will be conducted for each treatment group.

Outcome 4: Perform post-treatment assessments and compile data

Outcome 2 data computation is expected to be completed by December 31, 2015. Outcome 3 data computation for water chemistry, Zequanox concentrations, and mortality of zebra mussels is completed. Biomass data computation is expected to be completed by February 28, 2016.

Outcome 5: Publish results

The results of the data will be included in the final project completion report and prepared for peer-reviewed publication(s).

Final Report Summary:

Overview

After preliminary work was conducted in 2014, two additional studies were conducted to evaluate the application and efficacy of SDP (Zequanox®). The first study involved creating a laboratory-derived model to select the appropriate concentration of Zequanox to use in application suspensions at various water temperatures and then validating the model and associated application techniques by conducting subsurface Zequanox applications in experimental ponds at three different water temperatures. The second study evaluated the efficacy of Zequanox applications for open-water zebra mussel control within 27-m^2 experimental enclosures located in Robinson's Bay (Lake Minnetonka, MN) using both whole water column and subsurface application techniques. The results of these studies are summarized in peer-reviewed reports which are supplimental attachments 5 and 6, respectively.

2014 Subsurface Application Activities

Initial work in 2014 to develop subsurface Zequanox application techniques included developing (1) a Zequanox mixing system, (2) an indoor test system that was used to compare several different injection apparatuses at multiple Zequanox stock concentrations (3, 4 and 5 % w/v), and (3) an outdoor enclosure test system (9-m^2 enclosures placed in 0.004 hectare concrete ponds) which were used to evaluate injection apparatuses at two Zequanox stock concentrations (4 and 5 % w/v), two injection rates (~8 and 16 LPM), and two application heights (~60 and 90 cm from pond bottom). Results from this initial work provided direction for the construction of two subsurface Zequanox application systems which were used to deliver a 5% w/v Zequanox solution ~91 cm from the lake bottom during the 2014 field applications (described below).

2015 Subsurface Application Activities

After analyzing the results of the field applications, a study was initiated in 2015 to further refine subsurface application techniques of Zequanox by developing and validating a water-temperature dependent model for selecting the temperature-dependent Zequanox concentrations in suspensions prepared for subsurface applications. In this study, a range of Zequanox concentrations (5-25% w/v) were evaluated in a climate-controlled laboratory at temperatures of 7, 12, 17, and 22°C to determine the effects of temperature and Zequanox concentration on the viscosity, settling, stratification, and air entrainment of Zequanox suspension. Results from the climate-controlled laboratory study were used to develop a two-step linear regression model for selecting the temperature-specific Zequanox concentrations to be used in suspensions prepared for subsurface Zequanox applications. The first-step of this model plotted a linear regression of each test temperature's viscosity and Zequanox concentration data. The second-step plotted the temperature-specific Zequanox concentration values predicted from the first-step regressions that would yield 180 cSt viscosity Zequanox suspensions at each of the corresponding temperatures (180 cSt was selected as the optimal viscosity as a result of the climate-controlled laboratory study). This second-step regression was then used to predict the concentration of Zequanox required to achieve a suspension of 180 cSt at water temperatures ranging from 7 to 22°C. The utility of the model was evaluated in three separate outdoor pond trials that were conducted at three temperatures (~9, 14, and 20 °C). During these outdoor trials, concentrations of Zequanox predicted by the model were used in subsurface applications of Zequanox to 9-m^2 enclosures that were positioned in 0.004 hectare concrete ponds. Water samples were collected at various depths within the enclosures throughout the eight hour exposure period to determine the dispersion and concentration of Zequanox. In this study, air entrainment in Zequanox suspensions was found to cause buoyancy and the addition of a silicone-based aquaculture defoaming agent to the suspensions at 0.1% (v/v) reduced air entrainment and allowed for the use of the predicted concentrations of Zequanox. The pond applications demonstrated the ability to maintain desired Zequanox concentrations within 7.5 cm of the pond bottom eight hours after application and also within 30 cm of the pond bottom for a minimum of three hours after application.

Additionally, a revised two-step model that better fit the data was developed to more accurately predict 180 cSt Zequanox concentrations than the original two-step linear prediction model. This study demonstrated that viscosity of Zequanox suspensions are highly dependent upon water temperature and that mitigation of air entrainment in more viscous suspensions is required. Furthermore, the use of this methodology to select the concentration of Zequanox for subsurface applications in quiescent waters should allow for the retention of lethal Zequanox concentrations near the sediment-water interface.

2014 Field Application Trial

In the fall of 2014, a trial was initiated to evaluate the efficacy of whole water and subsurface Zequanox applications for zebra mussel control in open-water environments. In this study five Zequanox treatments were applied to 27-m^2 enclosures positioned in Robinson's Bay (Lake Minnetonka, MN) on three independent treatment days. The 8-hour, single application treatments consisted of (1) an untreated control treatment, (2) a 50 mg Zequanox active ingredient (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. All applications were conducted using a 5% (w/v) Zequanox suspension. Whole water column treatments were applied to enclosures by hand by moving an application wand throughout the water column for even distribution. Subsurface applications were applied ~90 cm from the lake bed using a rolling application bar constructed from PVC pipe with holes drilled 30° below horizontal. The bar was 2.5 m long and divided into two sections. Zequanox was pumped to each section through a length of tubing and delivered through a total of 58 injection ports (14 paired holes + 1 end hole per

section x 2 sections). 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 ~60 cm of the water column, plus an additional 25% was applied to account for anticipated losses through drift. Zequanox concentrations were verified by collecting water samples from the enclosures 2, 4, and 7.5 hours after Zequanox application and comparing sample absorbance to a standard curve created from known concentration Zequanox standards.

Two types of samplers, type 1 and 2, were used in the study to evaluate the efficacy of Zequanox treatments for reducing living zebra mussel biomass and inducing zebra mussel morality, respectively. 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 either wood, perforated aluminum, or stone tile. The substrates were separated from the concrete base using a 20 cm long PVC pipe spacer and from each other using 2.5 cm long PVC pipe spacers. 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 lake bed using a welded steel frame.

Type 1 Sampler Assessments

Approximately 40 days after exposure, type 1 samplers were dismantled and all zebra mussels adhering to individual substrate plates were collected and frozen for later determination of zebra mussel living biomass after all other invertebrates, algae, and debris were removed and discarded. The living zebra mussel biomass (ash free dry weight) of each top plate was determined and compared by treatment group. Samples were dried at 60°C and then pulverized in custom manufactured stainless steel containers that were placed in a Pacer dual-arm, bi-axial motion industrial mixer and shaken for 15 minutes. Subsamples of the resultant homogeneous powder were burned at 450°C for four hours in a muffle furnace. The mean percentage of living 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 living zebra mussel biomass present in the entire sample. The living zebra mussel biomass of each sample was then standardized by the mean surface area (m^2) of substrate. The treatment groups were then compared using the biomass per square meter of substrate.

Type 2 Sampler Assessments

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

Data Analysis and Results

Water chemistry and exposure concentration data analyses were limited to simple descriptive statistics. A general linear mixed model was used to compare the living zebra mussel biomass per square meter of substrate and the relationship between mortality, treatment type, and target exposure concentration. The survival of zebra mussels in the type 2 samplers was analyzed with a binary logistic mixed model. The applications of Zequanox to the test enclosures had minor impacts on water quality during the exposure period. The dissolved oxygen, pH, alkalinity, hardness, and un-ionized ammonia were all at acceptable levels for aquaculture. On average, the living 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. The amount of Zequanox applied in the subsurface applications was on average ~55% of the amount applied in the whole water column applications. When the reductions in mean

living zebra mussel biomass per square meter of substrate were standardized to the amount of Zequanox applied, the 50 mg A.I./L and the subsurface application treatments were more efficient with respect to the amount of Zequanox applied. The living 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 living zebra mussel biomass, 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% and the mean mortality of treated zebra mussels contained in type 2 samplers ranged from 27.83 to 73.25%. Similar to biomass reductions, standardization to the amount of Zequanox applied demonstrated that the 50 mg/L and the subsurface application treatments were more efficient at inducing zebra mussel mortality. However, 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), management goals, biological significance, and multiple applications should be considered when using this technique.

V. DISSEMINATION:

Description: Results will be communicated to local groups, state agencies and national peer groups through presentations at regional and national meetings including state resource management meetings. Details of results will be available as a final project report to the LCCMR, fact sheet summaries and scientific journal articles.

Status as of December 31, 2013:
No reportable activities have been completed.

Status as of June 30, 2014:
Results from studies completed to date have not been complied, analyzed and reviewed.

Status as of December 31, 2014:
An oral presentation entitled "*Pseudomonas fluorescens* (strain CL145A) exposure impacts on survival of non-target invertebrates" was presented by Diane Waller at Upper Midwest Invasive Species Conference, Duluth, MN on October 20th, 2014.

Status as of June 30, 2015:
An oral presentation entitled "Efficacy and Application Overview of Zequanox in USGS Field Trials" was presented by James Luoma at the 58th Annual Conference of the International Association of Great Lake Research. Burlington, VT. May 27, 2015.
An oral presentation entitled "Evaluation of the Impacts of Zequanox on Nontarget organisms" was presented by Diane Waller at the 58th Annual Conference of the International Association of Great Lake Research. Burlington, VT. May 27, 2015.

Status as of December 31, 2015:
A webinar entitled "The potential use of eDNA to guide site selection for zebra mussel control treatments" was presented by Christopher M. Merkes during the Environmental DNA Webinar Series, December 17, 2015.

Final Report Summary:
Dissemination of research results throughout the project period included:

Oral presentations:

"*Pseudomonas fluorescens* (strain CL145A) exposure impacts on survival of non-target invertebrates" was presented by Diane Waller at Upper Midwest Invasive Species Conference, Duluth, MN on October 20th, 2014.

"Efficacy and Application Overview of Zequanox in USGS Field Trials" was presented by James Luoma at the 58th Annual Conference of the International Association of Great Lake Research. Burlington, VT. May 27, 2015.

"Evaluation of the Impacts of Zequanox on Nontarget organisms" was presented by Diane Waller at the 58th Annual Conference of the International Association of Great Lake Research. Burlington, VT. May 27, 2015.

Webinars:

"The potential use of eDNA to guide site selection for zebra mussel control treatments" was presented by Christopher M. Merkes during the Environmental DNA Webinar Series, December 17, 2015

Peer-reviewed Journal Articles:

Waller, D.L., Luoma, J.A. and Erickson, R., 2016. Safety of the molluscicide Zequanox® to nontarget macroinvertebrates *Gammarus lacustris* (Amphipoda: Gammaridae) and *Hexagenia* spp. (Ephemeroptera: Ephemeridae), Management of Biological Invasions, Volume 7.

VI. PROJECT BUDGET SUMMARY:

The project includes a combination of ENRTF funds in addition to USGS overhead (48%), USGS in-kind for project management and Marrone Bio Innovations in-kind for project facilitation, implementation, equipment, and test product. Additionally, see the attached Marrone Bio Innovations letter of commitment.

A. ENRTF Budget:

Budget Category	$ Amount	Explanation
Personnel:	$520,000	≥5.1 FTE
Professional/Technical/Service Contracts:	$80,000	Bathymetric mapping, technical support
TOTAL ENRTF BUDGET:	$600,000	

Add or remove rows as needed

Explanation of Use of Classified Staff: N/A

Explanation of Capital Expenditures Greater Than $3,500:
The purchase of a single item >$3,500 is not anticipated

Number of Full-time Equivalent (FTE) funded with this ENRTF appropriation:
≥ 5.2 FTE

Number of Full-time Equivalent (FTE) estimated to be funded through contracts with this ENRTF appropriation:
0.5 FTE

B. Other Funds:

Source of Funds	$ Amount Proposed	$ Amount Spent	Use of Other Funds
Non-state			
Marrone Bio Innovations	$39,500	$39,500	Project support, test product, equipment
USGS overhead expenses (54%)	$324,000	$324,000	Project overhead costs
USGS in-kind	$102,000	$544,763	Project management, computer, equipment, supplies, methods development, travel
TOTAL OTHER FUNDS:	**$465,500**	**$842,079**	

Add or remove rows as needed

VII. PROJECT STRATEGY:

A. Project Partners: This project is a continuing partnership between the United States Geological Survey (USGS), MN DNR and Marrone Bio Innovations. Team members from the USGS include Mark Gaikowski (USGS-UMESC project manager), Dr. Richard Kiesling (USGS-MN WSC, bathymetric mapping manager; $60,000 ENRTF), Jim Luoma (USGS-UMESC project coordinator), Dr. Jon Amberg (USGS-UMESC, eDNA project manager), Mr. Chris Rees, (USGS-UMESC, eDNA project coordinator), Mr. Steve Redman (USGS-UMESC, fish culturist), and Ms. Irene Nissalke (USGS-UMESC, project budget analyst). Dr. Diane Waller (USGS-UMESC) will manage the fathead minnow life cycle and invertebrate toxicology. Gary Montz (MN DNR- Ecological and Water Resources, Aquatic Invertebrate Biologist) and Mark Ranweiler (MN DNR- Ecological and Water Resources, Invasive Species Specialist) will assist in test lake selection, permitting and field applications. Carolyn Link and Megan Weber (Marrone Bio Innovations -Zequanox product development manager and open water development scientist, respectively; $20,000 ENRTF) will provide test product, project support and field treatment equipment. Dr. Wendylee Stott (USGS-GLSC, geneticist) will provide assistance in the development of the molecular markers for the detection of zebra mussel DNA in water samples. Mr. Jeffrey Allen (USGS-GLSC, biologist/diver) and Mr. Glen Black (USGS-GLSC, biologist/diver) will provide support for mapping using SCUBA. All team members will participate in writing the final report and communicating results to state and national user groups.

B. Project Impact and Long-term Strategy:

1) The project determines the potential magnitude of non-target animal responses to acute SDP exposure by assessing the potential effects of intermittant SDP exposure on the reproductive success of fathead minnows (*Pimephales promelas*) and survival of mayfly larvae (*Order: Ephemerotera*) and adult amphipod *(Order: Amphipoda*), common to Minnesota's aquatic ecosystems.

Much of the previous non-target animal impact data is limited to acute exposures in laboratory settings. The proposed work will expand the non-target animal database by assessing the potential effects of intermittant SDP exposure on reproduction and success of fathead minnows and on the survival of mayfly larvae and adult amphipods. Exposures will be completed in mesocosms at environmentally relevant concentration and duration.

2) The project directly provides treatment protocols and optimization techniques by assessing multiple treatment application techniques, development of high resolution baythmetric and environmental DNA maps, and field application to various substrates.

The application of SDP using injection techniques will be evaluated in laboratory or mesocosm and pond-scale trials then validated through in-lake field trials using multiple zebra mussel attachment substrates. The use of injection techniques has the potential to deliver effective SDP applications while significantly reducing the

amount of applied product, lowering treatment costs and reducing potential impacts to non-target organisms. The use of eDNA to detect the presence of specific fish species is becoming widespread. The proposed work includes the use of eDNA to both determine the presence of zebra mussels and to potentially target treatment locations to optimize efficacy.

C. Spending History:

Funding Source	M.L. 2007 or FY08	M.L. 2008 or FY09	M.L. 2009 or FY10	M.L. 2010 or FY11	M.L. 2011 or FY12-13

(add or remove rows and columns as needed)

VIII. ACQUISITION/RESTORATION LIST: N/A

IX. MAP(S):

Maps of test lakes will be provided after creation for the test lakes upon completion of Activity 2 (Bathymetric mapping, environmental DNA and physical surveys).

X. RESEARCH ADDENDUM:

See Attachment B

XI. REPORT REQUIREMENTS:

Periodic work plan status update reports will be submitted not later than December 31, 2013, June 30, 2014, December 31, 2014, June 30, 2015, December 31, 2015, and June 30, 2016. A final report and associated products will be submitted by June 30, 2016 or as requested by the LCCMR.

Attachment A: Budget Detail for M.L. 2013 Environment and Natural Resources Trust Fund Projects

Project Title: Zebra Mussel Control Research and Evaluation in Minnesota Waters

Legal Citation: M.L. 2013, Chp. 52, Sec. 2, Subd. 06f

Project Manager: Jeff Meinertz

M.L. 2013 ENRTF Appropriation: $ 600,000

Project Length and Completion Date: 3 yr, June 30, 2016

Date of Update: Final Report

	Activity 1 Budget 6/30/2016	Amount Spent 6/30/2016	Balance 6/30/2016	Activity 2 Budget 6/30/2016	Amount Spent 6/30/2016	Balance 6/30/2016	Activity 3 Budget 6/30/2016	Amount Spent 6/30/2016	Balance 6/30/2016	Total Budget 6/30/2016	TOTAL BALANCE 6/30/2016
Budget Item 12/31/2015											
Personnel (Wages and Benefits)	163,500	163,500	0	100,580	100,580	0	255,920	255,920	0	520,000	0
Amberg(UMESC)/Research Fisheries Biologist (PCR expert) $16,995 (79% salary & 21% benefits) 11% FTE											
Luoma(UMESC)/Research Fisheries Biologist $112,354 (72% salary & 28% benefits) 60% FTE											
Waller(UMESC)/Research Biologist $85,004 (71% salary & 29% benefits) 50% FTE											
Weber(UMESC)/Biologist/project implementation $42,573 (74% salary & 26% benefits) 35% FTE											
Rees (UMESC)/Research Fisheries Biologist (PCR expert) $12,729 (76% salary & 24% benefits) 10% FTE											
Severson (UMESC)/Biologist $58,055 (75% salary & 25% benefits) 60% FTE											
Redman(UMESC)/Fish Culurist $12,669 (71% salary & 29% benefits) 10% FTE											
Merkes(UMESC)/Research Biologist $30,005 (74% salary & 26% benefits) 30% FTE											
Nissalke (UMESC)/Budget analyst $5,014 (93% salary & 7% benefits) 5% FTE											
Roth (UMESC)/Biologist project implementation $8,906 (76% salary & 24% benefits) 10% FTE											
Fisher (UMESC) research assistant/project implementation $25,916 (93% salary & 7% benefits) 100% FTE											
Wise (UMESC) Biologist $59,000 (75% salary & 25% benefits) 70% FTE											
Boma (UMESC) biologist $2,920 (72% salary & 28% benefits) 2% FTE											
McCalla (UMESC) Geneticist $ 20,294 (74% salary & 26% benefits) 20% FTE											
Vang (UMESC) research assistant/project implementation $8,275 (93% salary & 7% benefits) 20% FTE											
Black (GLSC) Biologist/SCUBA diver $2,198 (83% salary & 17% benefits) 2% FTE											
Allen (GLSC) Biologist/SCUBA diver $2,654 (83% salary & 17% benefits) 2% FTE											
Smerud (UMESC)/Biologist $14,440 (73% salary & 27% benefits) 20% FTE											
Professional/Technical/Service Contracts											
Minnesota Water Sciences Center (USGS) high resolution substrate mapping				60,000	60,000	0				60,000	0
Marrone Bio Innovations, permitting, project design and implementation							20,000	20,000	0	20,000	0
Equipment/Tools/Supplies											
Travel expenses in Minnesota											
	0	0									0
	$163,500	**$163,500**	**0**	**$160,580**	**$160,580**	**0**	**$275,920**	**$275,920**	**0**	**600,000**	**0**

Management of Biological Invasions (2016) Volume 7 in press

Open Access

Research Article

CORRECTED PROOF

Safety of the molluscicide Zequanox® to nontarget macroinvertebrates *Gammarus lacustris* (Amphipoda: Gammaridae) and *Hexagenia* spp. (Ephemeroptera: Ephemeridae)

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Abstract

Zequanox® is a commercial formulation of the killed bacterium, *Pseudomonas fluorescens* (strain CL145A), that was developed to control dreissenid mussels. In 2014, Zequanox became the second product registered by the United States Environmental Protection Agency (USEPA) for use in open water environments as a molluscicide. Previous nontarget studies demonstrated the safety and selectivity of *P. fluorescens* CL154A, but the database on the toxicity of the formulation (Zequanox) is limited for macroinvertebrate taxa and exposure conditions. We evaluated the safety of Zequanox to the amphipod *Gammarus lacustris lacustris*, and nymphs of the burrowing mayfly, *Hexagenia* spp. at the maximum approved concentration (100 mg/L active ingredient, A.I.) and exposure duration (8 h). Survival of animals was assessed after 8 h of exposure and again at 24 and 96 h post-exposure. Histopathology of the digestive tract of control and treated animals was compared at 96 h post-exposure. The results showed no significant effect of Zequanox on survival of either species. Survival of *G. lacustris* exceeded 85% in all concentrations at all three sampling time points. Survival of *Hexagenia* spp. ranged from 71% (control) to 91% at 8 h, 89–93% at 24 h post-exposure, and 70–73% at 96 h post-exposure across all treatments. We saw no evidence of pathology in the visceral organs of treated animals. Our results indicate that application of Zequanox at the maximum approved concentration and exposure duration did not cause significant mortality or treatment-related histopathological changes to *G. lacustris* and *Hexagenia* spp.

Key words: *Pseudomonas fluorescens*, molluscicide, nontarget, macroinvertebrate, amphipod, Ephemeroptera

Introduction

Zebra mussels, (*Dreissena polymorpha* Pallas, 1771) and quagga mussels, (*Dreissena rostriformis bugensis* Andrusov, 1898) were introduced to North American freshwaters through the release of ballast water from transoceanic vessels entering the Great Lakes (Carlton 2008). Since their arrival in the 1980s, dreissenid mussels have expanded from the east to west coast of the United States and into Canada (USGS 2015), significantly altering the dynamics of the freshwater systems where they have established (Higgins and Vander Zanden 2010; Mayer et al. 2014; Colvin et al. 2015). The ecological and economic impacts of dreissenids continue to mount in North America, as well as in invaded systems in Europe (Nalepa and Schloesser 2014).

A suite of treatment options is available to control dreissenid populations in closed systems of intake lines and cooling systems (Claudi and Mackie 1994; Mackie and Claudi 2009; Glomski 2015). However, there are currently only two molluscicides registered by the United States Environmental Protection Agency (USEPA) to control dreissenids in open water. Earthtec QZ® (Earth Science Laboratories, Inc., Bentonville, AR) is a copper-based product that has demonstrated effectiveness for killing dreissenid mussels; however, exposures of up to 96 h are required to kill adult mussels (Claudi et al. 2014) and the product can be toxic to fish and other aquatic invertebrates (USEPA 2008). Zequanox® (Marrone Bio Innovations (MBI), Davis, CA), the most recently approved molluscicide for dreissenid control, requires a shorter application period (e.g., 8 h), reportedly

has fewer nontarget impacts, and toxicity of the aquaeous product degrades within 24 h (Marrone Bio Innovations 2012a; Molloy et al. 2013a). The active ingredient of Zequanox is killed cells of a specific strain (CL154A) of the common soil bacterium, *Pseudomonas fluorescens.* Researchers at the NewYork State Museum found *P. fluorescens* CL154A to be toxic and relatively selective for dreissenid mussels (Molloy et al. 2013a, b, c). The toxic component of the bacterium has not been reported, but its mode of toxicity is lysis and degradation of the digestive gland and stomach epithelium of the mussels when ingested (Molloy et al. 2013b). Initial non-target toxicity trials were conducted using the unformulated cells (live and dead) of *P. fluorescens* with a variety of invertebrates that included seven species of unionid mussels, the ciliate *Colpidium colpoda (*Ehrenberg, 1838), the cladoceran *Daphnia magna,* (Straus, 1820), and the amphipod *Hyallela azteca* (Saussure, 1858) (Molloy et al. 2013c). Exposure durations of 24–72 h were tested at concentrations that were efficacious to dreissenids (100 or 200 mg/L active ingredient, A.I.). Mortality was insignificant in all species, except *H. azteca;* however, mortality (3–27%) in the amphipod was considered unrelated to *Pf*-CL145A toxicity. Following commercial production of *Pf*-CL145A by MBI as Zequanox, additional non-target trials were conducted to expand the database on selectivity of the product for dreissenids (Marrone Bio Innovations 2012a). Meehan et al. (2014) tested the duck mussel (*Anodonta*), a non-biting midge (*Chironomus plumosus* Linnaeus, 1758), and the white-clawed crayfish (*Austropotamobius pallipes* Lereboullet, 1858) in 72-h static tests and found that Zequanox was safe to these species at concentrations that equaled (100 mg/L A.I.) or exceeded the approved open water label (up to 750 mg/L A.I.). The most comprehensive non-target testing has been conducted on native unionid mussels including, trials on adults (Luoma et al. 2015a), newly transformed juveniles (Weber et al. 2015), and the glochidia (Luoma et al. 2015b). Adult and subadult mussels survived 24-h exposure at the maximum concentration of 100 mg/L A.I. (Luoma et al. 2015a). Juveniles and glochidia of several species were more sensitive (Luoma et al. 2015b; Weber et al. 2015), suggesting a need for further testing. Safety evaluation of Zequanox to other macroinvertebrate taxa requires an effort similar to that given to unionid mussels. An expanded database on non-target animal safety will assist resource managers in assessing risks of Zequanox exposure to the broader macroinvertebrate community in a dreissenid control program.

The goal of our study was to determine the safety of the commercial formulation of *Pf*-CL145A, Zequanox, at open-water application rates (100 mg/L A.I.) to high-value, non-target invertebrate species, the amphipod *Gammarus lacustris lacustris* (Sars, 1864) and burrowing mayflies, *Hexagenia bilineata* (Say, 1824) and *H. limbata* (Serville, 1829). *Gammarus lacustris* is distributed from the Great Lakes region into the western United States and north into most of Canada (Holsinger 1972). This is one the most abundant amphipods in the pothole region of the Upper Midwest (Kantrud et al. 1989) and is a significant dietary component for fish and waterfowl (Anteau and Afton 2006; Anteau and Afton 2008; Anteau et al. 2011). *Gammarus* feed on suspended coarse organic particulates, along with epibenthic algae, zooplankton and bacteria (Mathias and Papst 1981) and are at risk for ingestion of adsorbed Zequanox on these food items. Currently, Zequanox toxicity data for macroinvertebrate crustaceans is limited to the aforementioned laboratory tests with *A. pallipes* (Meehan et al. 2014), *D. magna* and *H. azteca* (Molloy et al. 2013c), and *Asellus aquaticus* Linnaeus, 1758 (Marrone Bio Innovations 2012a).

Hexagenia spp. are distributed throughout the United States (McCafferty 1975) and the nymphal stage represents a benthic-dwelling detritus feeder in the macroinvertebrate community of rivers and lakes. Nymphs dislodge deposited detrital material with their forelegs or beating movements of the gills and transfer particulates to the mouth parts for ingestion (Zimmerman and Wissing 1980). The pre-emergent nymph can reside in sediments for 1–2 years in northern lakes and rivers (Fremling 1960, Hilsenhoff 1981; Heise et al. 1987). Since applications of Zequanox would target the benthic zone where dreissenids densities are highest, *Hexagenia* nymphs have a high probability of exposure to and ingestion of the particles. Toxicity information on Zequanox for mayflies is limited to trials with *Pf*-CL145A on a single non-burrowing species, *Baetis* spp. (Marrone Bio Innovations 2012a).

We simulated an open-water application of Zequanox by conducting trials in outdoor mesocosms and testing maximum approved exposure concentrations (50 and 100 mg/L A.I.) and duration (8 h). In addition to comparing survival rates, we compared histological sections of the digestive tract among control and treated animals of both species in order to assess sublethal effects of Zequanox. Degradation of the digestive gland of dreissenids is evident within 24–48 h of ingesting *P. fluorescens*, though mortality may not occur for a week or more (Molloy et al. 2013b). Histological changes would be expected to occur within 96 h of exposure if the toxin in the product has a similar mode of action in macroinvertebrates.

Figure 1. Test system in outdoor concrete ponds. Two ponds held nine 1000-L tanks in a thermal water jacket. Water was supplied to test tanks from an adjacent earthen pond. Amphipod test chambers (arrow) were suspended in the test tank at mid-depth. Mayfly chambers (not visible) were placed on the tank bottom. Each pond contained three replicates of each treatment (0, 50, and 100 mg/L A.I. Zequanox).

Methods

Test animals and test system

Mayfly nymphs (mean length = 26.9 mm, standard deviation = 3.6 mm, range = 19–35 mm) were obtained from an independent bait supplier (Hilger and Sons, Inc., Antigo, WI) and identified as a mixture of *H. limbata* and *H. bilineata* (McCafferty 1975). Adult amphipods (mean length = 20.3 mm, standard deviation = 1.6 mm, range = 16.8–26.4) were obtained from a private aquaculture facility (Lincoln Bait Supply, Staples, MN) and identified as *G. lacustris lacustris* (Holsinger 1972). Before testing, mayflies and amphipods were held in separate raceways at the Upper Midwest Environmental Sciences Center (UMESC), La Crosse, WI, and supplied with a semi-recirculating chilled well water (12°C); water temperature in the raceways was increased gradually to 14°C over 24 h and maintained at this temperature during the holding period. Mayflies were contained in aluminum pans (36.8 × 27.0 × 7.6 cm; L × W × H) that were filled with 4–5 cm of sand/silt substrate. Dried alfalfa tablets (Hikari® algal wafers, Kyorin Co., Hayward, CA) and aged leaf litter were provided as a food source. Amphipods were contained in mesh cages (90 × 90 × 30 cm; L × W × H) and provided with leaf packs of aged birch leaves for cover and food. Feeding was supplemented once a day with Tetramin® flaked fish food (Tetra US, Blacksburg, VA). Animals were quarantined in the laboratory at UMESC for about 1 week and then transferred in their respective holding containers to outdoor raceways and acclimated to the test water and temperature for 1 week. The outdoor raceways were supplied with water from the same 0.10 ha earthen pond that supplied the test system.

The test system consisted of 18 1000-L circular high density polyethylene (HDPE) tanks (175 cm diameter × 64 cm height). Nine tanks were placed into each of two 0.004-ha concrete ponds (Figure 1). Test water was pumped from a nearby 0.1 ha earthen pond, filtered through a 200-µm filter to remove particulates and other invertebrates, and delivered to a head box above each concrete pond. Water was delivered from the head box to each tank at a rate of approximately 3.8 L/min. Daily and diurnal fluctuations in water temperature were minimized by filling the concrete ponds with well water to provide a thermal jacket for the test tanks and by covering the ponds with black shade cloth.

Amphipod test chambers were constructed of poly vinyl chloride (PVC) pipe (25.4 cm length, 10.2 cm inner diameter) with 1500-µm Nitex® screen covering each end. A peristaltic pump and tubing was connected to a threaded hose barb inserted at the midpoint of each chamber. To ensure that Zequanox

was mixed uniformly inside the chambers, water from the treatment tank was drawn through the peristaltic pump, into the top of the test chamber, and out each end of the chamber. A cylindrical roll (4 cm × 10 cm; diameter × length) of semi-rigid plastic mesh (3.0 mm diameter opening) was packed with aged birch leaves and placed inside each chamber to provide cover and substrate for the amphipods. Chambers were suspended in the test tank at approximately mid-depth (Figure 1). Mayfly test chambers consisted of plastic dishpans (28.5 cm × 12.1 cm; diameter × depth) filled with 2.5–3.0 cm of conditioned sand/silt substrate and dry leaf litter. The top of the chamber was covered with mesh screen (3.0 mm diameter opening) to prevent loss of mayflies during the test; the chamber was then placed on the bottom of the test tank.

Each pond contained three blocks of each treatment (0, 50, and 100 mg/L A.I. Zequanox) for a total of six replicates per treatment for each species. Treatments were randomly allocated to a tank according to a randomized block design. Amphipods and mayflies were tested simultaneously. Twenty-four hours before exposure, amphipods and mayflies were transferred from the outdoor raceways to the test tanks. Groups of 10 amphipods were removed from the raceway and distributed into 18 10-L buckets, in three separate rounds, according to a predetermined randomization schedule. Amphipods that were paired (male and female) were not selected. The 30 amphipods were then transferred into a test chamber in each test tank. Mayfly nymphs were distributed in the same manner, following a unique randomization schedule. In both species, only animals that were actively swimming were selected for testing. Ten additional animals of each species were randomly assigned to each test tank to assess mortality at 8 h and 24 h post-exposure without disturbance of the larger test group. These were placed into separate mesh bags, with aged leaf litter, and suspended mid-depth within each tank.

Exposure and assessments

The test material, Zequanox was a spray-dried powder formulation, made of 50% active ingredient (MBI-401 SDP, MBI, Davis, CA). A dosing stock was prepared for each individual tank by removing 10 L of water from the tank and adding the specified weight of dry Zequanox to the water. The stock was stirred for approximately 5 minutes using a paint mixer attachment on an electric drill. The solution was poured through a strainer and funnel into a second bucket. Undissolved test product was mechanically broken apart within the strainer using a pestle. The dosing stock was added to the test tank within 5 minutes of preparation. The stock was thoroughly mixed within each tank using a boat paddle. Control tanks (i.e., no test material added) were mixed using a boat paddle in a manner identical to the treatment tanks. Water flow to each tank was halted during the 8 h treatment and re-established for the remainder of the test period.

Water quality parameters (dissolved oxygen, pH, and temperature) were measured immediately before exposure, at 1, 4, and 8 h during the exposure, and once daily thereafter. Dissolved oxygen was measured with a YSI® 550A dissolved oxygen meter (YSI, Inc., Yellow Springs, OH). The pH was determined with a Beckman Coulter® φ410 pH meter and probe (Beckman Coulter, Inc., Fullerton, CA). Temperature was measured with a Thermapen® digital thermometer (ThermoWorks, American Fork, UT). Water flow rates (mL/min) were measured daily in each tank. Hardness and alkalinity were measured from each tank at 1 h exposure; conductivity was measured from each tank at 1 h exposure and at 96 h post-exposure. Total hardness (mg/L as $CaCO_3$) was determined by titrimetric method with Manver Red indicator (USEPA 1983). Total alkalinity (mg/L $CaCO_3$) was determined by titrimetric method to a pH endpoint of 4.5 (APHA 1995). Conductivity was measured with a Fisher Accumet® conductivity meter (Fisher Scientific, Pittsburg PA).

At the conclusion of the 8 h exposure, the mesh bags containing 10 animals of each species were removed from each tank and animals were rinsed into a shallow pan to assess immediate mortality. Mortality was defined as lack of response to probing and light stimulation with a battery-wired forceps. Up to five live animals were indiscriminately selected from each bag and preserved in Davidson's fixative (Humason 1962) for histological examination. The remaining live animals were returned to the mesh bags and assessed at 24 h post-exposure following the same procedure. At 96 h post-exposure, test chambers (i.e., PVC chambers and dishpans) were individually removed from each tank and animals were rinsed into a shallow pan to assess survival. Each tank was also siphoned through a 600-µm bag filter to collect animals that had escaped from the test chambers. Three animals were indiscriminately selected from each tank and preserved in Davidson's fixative for histological examination. The remaining live animals were retained in 70% alcohol for length measurements. Total length of amphipods was measured from the base of the antennae to the tip of the third uropod along the curve of the dorsal surface. Total length of mayflies was measured from the base of the antennae to the last abdominal segment.

Histological processing

Animals that were processed for histology were preserved in Davidson's fixative for 24 h, rinsed with water, dehydrated in a graded series of ethanol or ethyl alcohol (50–100%) and embedded in Paraplast® using a tissue processor (Thermo Scientific Shandon Excelsior, ThermoFisher Scientific, Waltham, MA). Serial sections were cut (7–10 µm) with a rotary microtome (Leica RM2035, Leica Biosystems Inc., Buffalo Grove, IL), stained with hematoxylin and eosin, and examined with a compound microscope (≤1000×). Ten samples per species were indiscriminately selected from control and 100 mg/L treatments for histological examination. Because the mode of toxicity of *Pf*-CL145A in dreissenids is necrosis of the digestive epithelium, our histological examination focused on the digestive tract of both amphipods and mayflies. In addition, we examined the hepatopancreatic ceca, anterior dorsal and rectal ceca of *Gammarus* and fat bodies of *Hexagenia* since these organs are integral to nutrient digestion, absorption and metabolism. Photomicrographs of stained sections were made using light microscopy (Nikon Eclipse E600, DSFi1 digital camera, Nikon Instruments, Inc., Melville, NY). Histological descriptions and terminology for *Gammarus* followed that of Schmitz (1967) and Schmitz and Scherrey (1983); descriptions and terminology for *Hexagenia* were derived from Csoknya and Halász (1973), Saouter et al. (1991), Gaino et al. (1997), Harker (1999), Oliveira and Cruz-Landim (2003), and Liarte et al. (2014).

Zequanox analysis

Concentrations of Zequanox in the test tanks were determined by spectrophotometric comparison to a linear regression created from Zequanox standard solutions (Beckman UV/Vis Spectrophotometer, Model DU 800). Zequanox standards of 25, 50, 100, and 150 mg/L (A.I.) were prepared from dilutions of a 2,000 mg/L (A.I.) standard stock solution. A linear zero-intercept standard curve was prepared from the 25, 50, 100, and 150 mg/L dilution stocks using triplicate standard samples. The spectrophotometer was blanked using filtered (200 µm) pond water. Mid-column water samples were collected from each test tank for Zequanox concentration analysis at 0, 1, 2, 4, 6, and 8 h of exposure and at 1, 4, 8 and 16 h post-exposure.

Confirmatory post-test efficacy verification of Zequanox was completed at MBI. Efficacy trials were conducted on *D. rostriformis bugensis*, collected from Lake Havasu, AZ, at 20°C with three replicates of 20 animals exposed to 100 mg/L A.I. Zequanox. Mean mortality of mussels was 73.3% (standard deviation 12.6%) at 15 d post-exposure, which meets the quality control standards set forth by MBI for the product.

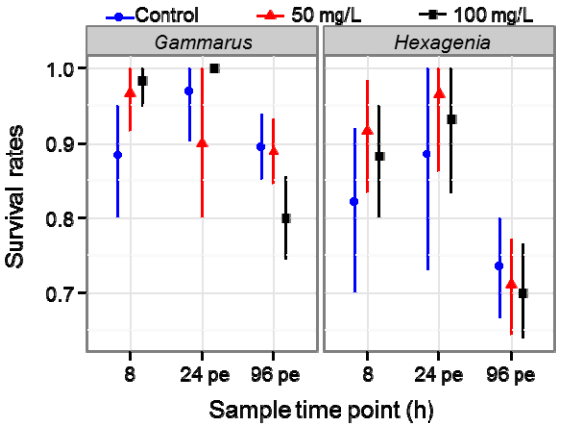

Figure 2. Survival rates of *Gammarus lacustris* and *Hexagenia* spp. in controls and Zequanox (mg/L active ingredient) treatments at each sampling time point. Exposure duration was 8 h. Survival rate (number survived/total) was assessed on a subsample of animals at 8 h (n = 10 per tank) and 24 h post-exposure (n = 5 per tank). Survival at 96 h post-exposure (pe) was assessed on 30 animals/tank. Bars represent 95% confidence limits.

Statistical analysis

Unrecovered animals were counted as mortalities in the analysis of survival. An outlier tank (control) of *Hexagenia* was removed at the 8 h time point because this tank had an extremely low recovery rate (<20%) when all other tanks had higher recovery rates (>75%). A generalized linear mixed effects model with a binomial error term (also known as a random-effect logistic regression) was used to analyze survival of each species by exposure concentration and time point (8 h exposure, 24 and 96 h post-exposure), while controlling for tank as a random effect (Bolker 2008). An interaction term was included between the two predictor variables. If the interaction was not significant, the model was re-parameterized without the interaction term. R (R Core Team 2015) was used to analyze the data. The glmmPQL function from the MASS package was used for the generalized linear mixed effects model (Venables and Ripley 2002). The ggplot2 package was used to plot the results (Wickham 2009).

Results

The results were similar for each species. Mean survival of *G. lacustris* exceeded 80% in all control and test concentrations at all three sampling time points (Figure 2). Mean survival of *Hexagenia* spp. ranged from 71% (control) to 91% (50 mg/L) at 8 h, 89–93% at 24 h post-exposure and 70–73% at 96 h post-exposure across all treatments. None of the interaction terms were significant (50 mg/L *Gammarus* $p = 0.8417$, *Hexagenia* $p = 0.7807$, $df = 15$; 100 mg/L *Gammarus* $p = 0.8684$, *Hexagenia* $p = 0.2070$, $df = 15$). In fact, the only significant terms from the analysis were the intercepts ($p<0.001$) and effect of the 96 h post-exposure time point ($p = 0.0045$, $df = 775{,}792$ *Gammarus* and *Hexagenia*, respectively). The number of organisms that died or were unrecovered was greatest at 96 h post-exposure but did not vary significantly among treatments. The contribution of unrecovered animals to total mortality in *Gammarus* was 9% in control and 50 mg/L treatments and 16% in the 100 mg/L treatment. The contribution of unrecovered animals to total mortality in *Hexagenia* was similar, ranging from 6% in control tanks to 12% in the 100 mg/L treatment tanks. In some cases, unrecovered animals had escaped from the test chamber and were later recovered when the contents of the test tank were drained. Mean recovery was not different among control and test treatments.

Concentrations of Zequanox were slightly higher than targeted in 50 mg/L tanks, averaging 54.3 mg/L at the onset of exposure and decreasing to a mean of 52.6 mg/L at 8 h (Figure 3). Concentrations in the tanks targeted for 100 mg/L treatments averaged 94.6 mg/L at 1 h and 82.3 mg/L at 8 h. The lowest mean concentration, 77.8 mg/L, occurred at 6 h. The inflection in mean Zequanox concentration from 6 to 8 h was attributed to variability in the depth of water sample collection between sampling times. After water flow was reestablished to the test tanks at the termination of the 8 h exposure, Zequanox concentrations decreased by 50% at 1 h post-exposure and were not detectable in the treatment tanks at 8-h post-exposure (Figure 3).

Water quality and chemistry were similar among tanks and treatments (Tables 1, 2). Dissolved oxygen remained >10 mg/L in all tanks at all sampling points. The pH was relatively high (>8.8) at the start of the exposure and decreased by 0.1–0.3 pH units in Zequanox treatments during the 8-h exposure; pH remained above 9 in the control tanks. During the post-exposure period, the pH remained above 8.3 and was similar among all tanks. Water temperature increased during the 8-h exposure from about 16 °C to 23.8 °C, but did not vary among tanks.

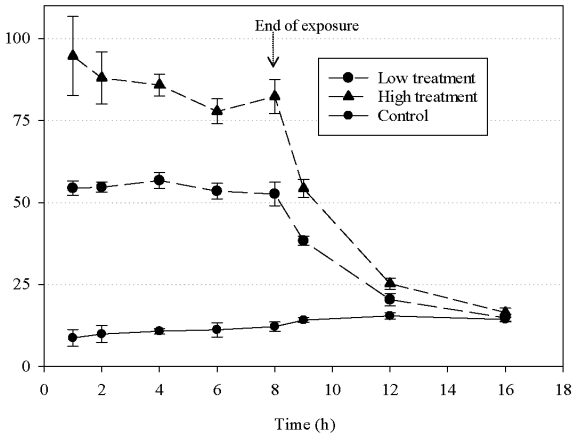

Figure 3. Mean Zequanox concentration (standard deviation) over time as determined by spectophotometry. The exposure was terminated at 8 h (arrow) and water flow resumed to the tanks.

Histology

There was no evidence of treatment-related histopathologic changes to the digestive tract or visceral organs of *G. lacutris* or *Hexagenia* spp. treated with 100 mg/L A.I. Zequanox.

Histology of amphipods: In the control animals, the midgut showed normal histological structure (Schmitz 1967), with an inner epithelium consisting of short columnar cells on a basement membrane, surrounded by circular muscle (Figure 4A). The dorsal median and rectal ceca each consisted of a monolayer of tall columnar cells, with non-vacuolated, basophilic cytoplasm (Figure 4A). The glandular epithelium of the hepatopancreatic ceca was columnar with large vacuoles near the luminal end and nuclei located near the basal end (Figure 4B). In amphipods treated with 100 mg/L Zequanox, the digestive epithelium of the stomach, midgut and hindgut were intact, with no evidence of sloughing or necrosis (Figure 4C). Cells of the hepatopancreatic ceca were comparable in appearance between control and treatment animals, with no signs of necrosis or inflammation (Figure 4D). There was no sign of hemocyte infiltration into the gut or ceca (Figure 4C, D). Furthermore, the lumen of both control and treated animals contained ingested material, indicating that the treatment did not cause purging of intestinal contents or cessation of feeding (Figures 4A, B, D).

Histology of mayflies: The midgut in control animals consisted of tall columnar epithelial cells, resting on a thin basement membrane, and underlying muscle fibers (Figure 5A, B). Using light microscopy, we could distinguish a distinct band along the apical

Table 1. Mean (standard deviation) dissolved oxygen and temperature, and pH range of each treatment group during the study period.

Water chemistry parameter	Treatment group (mg/L)	Pre-exposure[1]	Exposure period			Post-Exposure period			
			≤1 h	4 h	8 h	16 h	48 h	72 h	96 h
DO (mg/L)	0	10.42 (0.17)	11.50 (0.09)	13.52 (0.39)	16.13 (0.76)	11.55 (0.08)	13.47 (0.18)	12.70 (0.15)	11.47 (0.18)
	50	10.35 (0.08)	11.30 (0.09)	11.85 (0.19)	13.38 (0.75)	11.50 (0.06)	13.45 (0.18)	12.70 (0.09)	11.43 (0.18)
	100	10.60 (0.46)	11.25 (0.19)	11.33 (0.38)	11.38 (0.50)	11.40 (0.17)	13.30 (0.28)	12.62 (0.15)	11.38 (0.17)
Tempera-ture (°C)	0	16.00 (0.09)	17.22 (0.40)	20.62 (0.32)	22.87 (0.20)	16.57 (0.08)	16.57 (0.16)	17.77 (0.08)	18.40 (0.09)
	50	16.00 (0.09)	17.05 (0.28)	21.07 (0.80)	23.72 (0.83)	16.53 (0.05)	16.53 (0.12)	17.80 (0.11)	18.35 (0.12)
	100	16.02 (0.12)	17.28 (0.32)	21.40 (0.77)	23.87 (0.92)	16.58 (0.08)	16.63 (0.12)	17.77 (0.14)	18.38 (0.08)
pH	0	8.96-8.97	8.94-8.98	9.07-9.18	8.97-9.15	8.73-8.75	8.70-8.79	8.63-8.65	8.37-8.43
	50	8.94-8.96	8.81-8.90	8.88-8.92	8.79-8.92	8.73-8.75	8.73-8.78	8.64-8.67	8.33-8.42
	100	8.95-8.98	8.67-8.79	8.7-8.78	8.53-8.80	8.73-8.74	8.76-8.78	8.64-8.65	8.37-8.43

[1]Pre-exposure time points were measured approximately 1 h before application of Zequanox

Table 2. Mean (standard deviation) hardness, alkalinity and conductivity. Hardness and alkalinity were measured on water from each tank at 1 h; conductivity was measured on each tank at 1 h and 96 h (post-exposure).

Treatment group (mg/L)	Water chemistry parameter			
	Hardness (mg/L)[1]	Alkalinity (mg/L)[1]	Conductivity (µS/cm)[2]	
			1 h Exposure	Post-exposure
0	132.7 (1.6)	103.2 (1.2)	278 (3.6)	354 (2)
50	131.7 (0.8)	106.2 (2.5)	287 (4.0)	355 (2)
100	133.7 (0.8)	106.8 (1.5)	298 (2.7)	353 (2)

[1]Reported as milligrams per liter $CaCO_3$
[2]Temperature compensated to 25 °C

surface indicative of the brush border. The peritrophic membrane, an extracellular sheath, was visible in some sections. Fat bodies were seen throughout the body cavity, especially adjacent to the digestive tract and gonads (Figure 5A). Trophocyte cells in the fat bodies were large, ovoid, contained a large nucleus, and a cytoplasm filled with vacuoles (Figure 5A). Again, we saw no evidence of treatment-related histopathologic change in the midgut or fat bodies of treated *Hexagenia* nymphs (Figure 5C, D). The epithelium was intact and cell structure appeared normal (Figure 5D). The peritrophic membrane was observed between the gut contents and brush border of the epithelium (Figure 5C). The fat bodies were distinct with large vacuolated trophocytes, comparable to those of control animals (Figure 5C, D).

Discussion

Zequanox is composed primarily of organic particulates and produces a highly turbid suspension in the water column. Aquatic organisms may be negatively affected by a Zequanox suspension in several ways: (1) hypoxia may develop as the organic material degrades in a stagnant system (Whitledge et al. 2015), limiting gas exchange, (2) the particulate matter may cover or damage respiratory surfaces and interfere with gas exchange, and (3) the toxic component of *Pf*-CL145A may cause tissue damage and death. In our study, hypoxia was not a concern as dissolved oxygen concentrations remained >10 mg/L in all test tanks throughout the exposure period. High turbidity also did not appear to affect survival or recovery. In response to stressors, mayflies will often abandon their burrows (Fremling 1960, 1975). We could not directly observe the behavior of animals to determine whether Zequanox exposure triggered their escape from the test chambers. However, we found no difference in recovery of mayflies among control and test treatments, suggesting that Zequanox exposure did not elicit an escape response. Decreased recovery of *Gammarus* at 96 h post-exposure was partially attributed to cannibalism within the chambers (MacNeil et al. 2003; Dick 2009); however, recovery did not differ significantly among control and test treatments. Survival of test

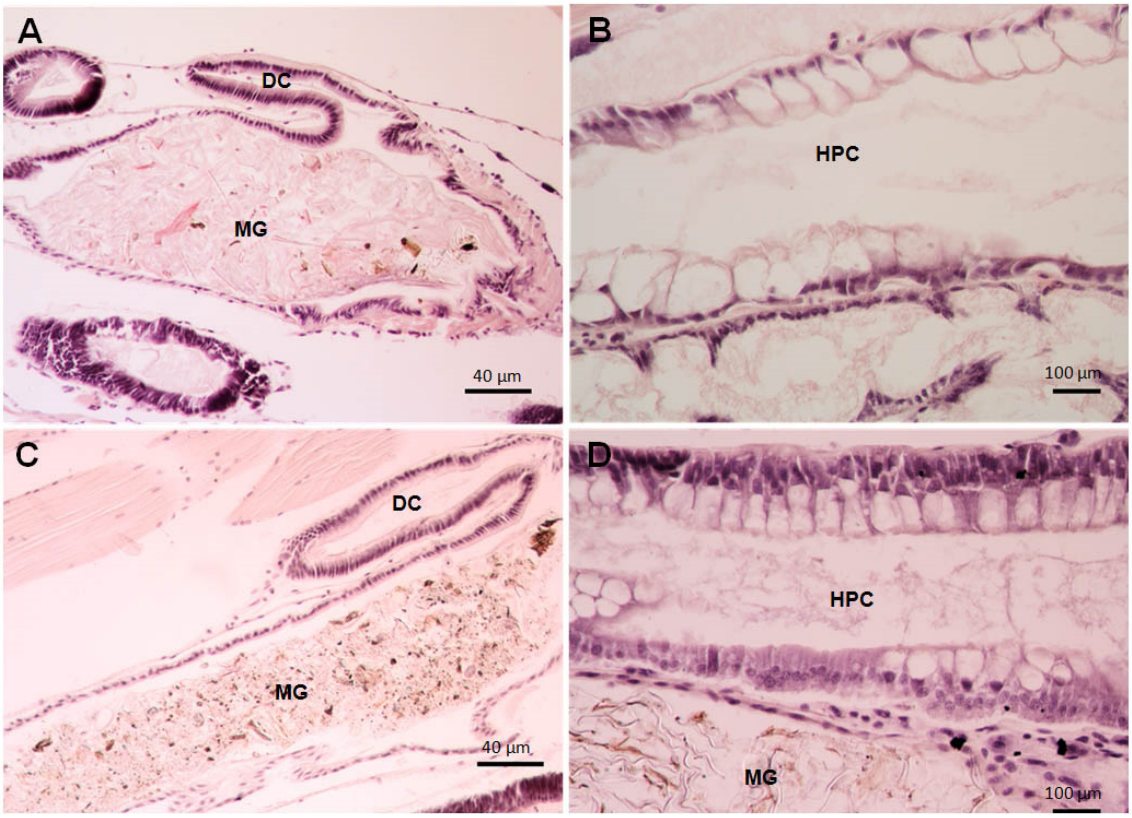

Figure 4. Photomicrographs of *Gammarus lacustris lacustris*, longitudinal sections. A) Control-midgut and dorsal cecum, B) Control-hepatopancreatic cecum, C)100 mg/L A.I. treatment-midgut and dorsal cecum, D) 100 mg/L A.I. treatment-hepatopancreatic cecum and midgut. DC=anterior dorsal cecum showing tall columnar epithelial cells without vacuoles, HPC=hepatopancreatic cecum; note basal location of nuclei and large vacuoles, MG=midgut, lined by short columnar epithelial cells. The gut lumen of animals in all treatments was filled with food.

organisms at 8 h and 24 h post-exposure was also unrelated to Zequanox treatment, suggesting that the toxic component of *Pf*-CL145A did not cause direct mortality of *Gammarus* and *Hexagenia* spp.

Ingestion of *Pf*-CL145A is necessary to produce mortality of dreissenids (Molloy et al. 2013a), as direct exposure (i.e., gills) is also not lethal. Most chemical molluscicides cause mussels to close and stop siphoning to reduce exposure to the toxin, but because of its high organic content, Zequanox is filtered out and ingested as a food source. Based on their modes of feeding, trophic uptake of Zequanox by both mayflies and amphipods was expected. *Hexagenia* nymphs are primarily detritus feeders that turn over the sediment with the forelegs (Zimmerman and Wissing 1980) and create water flow through the burrow by gill movements (Fremling 1960). They reportedly feed continuously and pass food through the short gut in 4–12 h (Zimmerman et al. 1975). As a result, mayflies could potentially ingest a significant amount of Zequanox. *Gammarus* are most likely to ingest Zequanox that has coated or been adsorbed to food items, such as the leaf litter provided in the test chambers. Histological examination of *Gammarus* and *Hexagenia* showed food in the digestive tract and confirmed that animals were eating during the trial and likely ingesting Zequanox particulates.

The toxicity of *Pf*-CL145A to dreissenids is not immediate, but is caused by gradual degradation of the digestive epithelium (Molloy et al. 2013a, b). Occurrences of mussel mortality may range from 3–21 days, depending on water temperature (Molloy et al. 2013a; Marrone Bio Innovations 2012b). Therefore, an extended post-exposure period is required to assess treatment mortality. However, Molloy et al. (2013b) reported signs of histopathology in dreissenids within 24–48 h of ingesting *Pf*-CL145A. Hemocyte infiltration was observed in the stomach lumina and digestive gland at 24 h and degradation of digestive

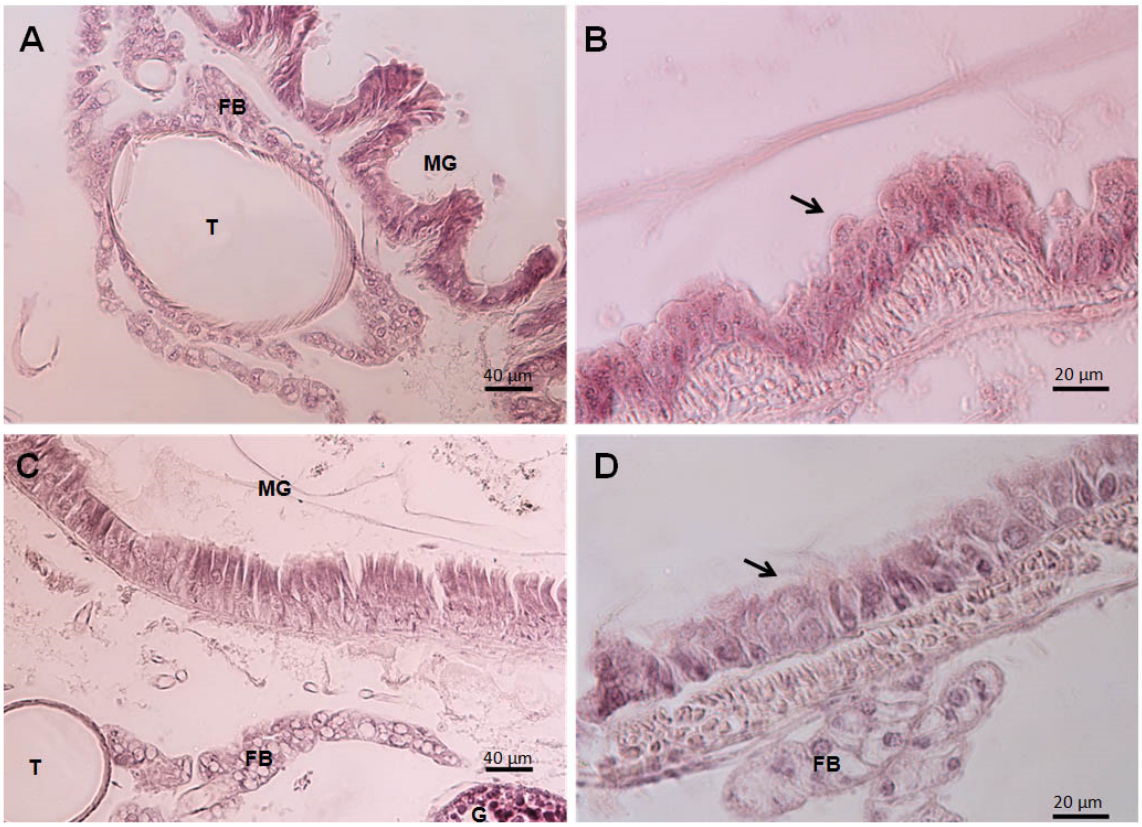

Figure 5. Photomicrographs of *Hexagenia* nymphs A) Control-midgut, tracheole and surrounding fat bodies, B) Control-gut epithelium (arrow) at 40×, C) 100 mg/L treatment-midgut, tracheole and surrounding fatbodies, D) 100 mg/L treatment-gut epithelium (arrow) at 40×. FB=fat bodies, G=gonad, MG=midgut, T=tracheole.

epithelium was evident at 48 h (Molloy et al. 2013b). An extended post-exposure period was impractical for test organisms in our study, particularly given the potential of cannibalism and escape. The histological exam was imperative for identifying tissue damage that could cause delayed mortality of the test animals. We saw no evidence in the digestive tract and associated viscera of *Gammarus* and *Hexagenia* that Zequanox exposure caused pathology similar to that reported in dreissenids. Cursory observation of other body tissues (i.e., gills, tracheoles, gonads, muscle) showed no other signs of pathology in either species. The presence of food and absence of of treatment-related histopathologic change in the digestive tract supports our conclusion that Zequanox does not cause significant mortality in *G. lacustris* and *Hexagenia* spp. nymphs.

Nontarget toxicity trials with Zequanox have been primarily conducted in indoor laboratories under controlled conditions (e.g., Molloy et al. 2013c; Luoma et al. 2015b). Mesocosm toxicity trials are a compromise between the highly controlled, but artificial environment of the laboratory and uncontrolled, natural conditions of a field application. Daily fluctuations in water temperature and pH in our mesocosm test system were greater than reported in most standard laboratory studies, but reflected commonly measured daily cycles in the earthen pond that provided source water for the test tanks. The variability in water temperature and pH did not appear to increase the sensitivity of mayflies or amphipods to Zequanox treatments. Moreover, the mesocosm environment provided a food supply and photoperiod for test organisms that more closely mimicked natural conditions. Results from mesocosm toxicity tests may better reflect the behavior of the test organism, degradation of the toxin, and fluctuations in water chemistry than laboratory-based tests (Mikó et al. 2015). However, loss of animals from cannibalism and escape from test chambers might have been reduced in a more controlled laboratory test system.

Desirable features of an agent for dreissenid mussel control include toxicity to all life stages, at a

concentration and duration of exposure that can be maintained in natural waters, safety to nontarget organisms, and rapid degradation in the environment. In addition to Zequanox, several chemical control agents that have been used for dreissenid control meet some, but not all of these features. Potassium chloride was successfully used to eradicate zebra mussels from a 12-acre quarry lake in Virginia (Fernald and Watson 2013). The quarry was isolated from other surface and groundwater connections and did not contain native species that are sensitive to potassium, such as unionid mussels. Potassium chloride (KCl) was applied for 3 weeks to achieve a concentration of 100 mg/L. Two years after application the concentration of KCl remained near 70 mg/L. Although KCl was an appropriate choice for this isolated body of water, its toxicity to other molluscs and persistence in the environment precludes its use in lakes and streams that contain native unionid mussels. The copper-based chemical, EarthTec Qz is the only molluscicide, other than Zequanox, registered by the USEPA for use in open water. Other copper-based chemicals have been used for eradication of dreissenid mussels in the United States under a Special Local Need Label issued by the USEPA. Most copper compounds can effectively kill adult dreissenids in 96 h of exposure (Watters et al. 2013; Claudi et al. 2014) and reduce veliger settling; however, copper-based products are toxic to a number of aquatic organisms including plants, fish, and other molluscs (USEPA 2008). Following application of copper sulfate to Lake Offutt in Nebraska to eradicate zebra mussels, significant mortality of fish was reported (URS 2009).

In most natural water bodies, dreissenid control efforts will require a balance between the negative effects of invasive mussels and the risk of the treatment to the native community. Amphipods and mayflies are of particular concern for resource managers because of their high value for the fishery and role as indicators of ecosystem health. Amphipods can comprise the major part of the diet for a variety of waterfowl and fish species (Anteau and Afton 2008; Pothoven and Madenjian 2008). *Hexagenia* spp. are preyed upon by a variety of fish, owing to their long-lives and multiple molts (Fremling 1960). Mayflies have routinely been considered indicators of water and sediment quality and are sensitive to point and nonpoint source pollution (Fremling 1970; Fremling and Mauck 1980; Resh and Jackson 1993; Barbour et al. 1999; Harwood et al. 2014). The existing database for nontarget tests with Zequanox indicates few negative effects of the product on the invertebrate species that have been tested.

The current study augments those data and provides evidence that ingesting *Pf*-CL145A under conditions simulating an actual application does not induce histopathologic change in tissues of the digestive tract in *Gammarus* and *Hexagenia* as it does in dreissenids. Comprehensive data on the effects of a control tool are imperative for decision-making by resource managers and for public support of control management plans. For example, anglers in Michigan were concerned that chemical treatments to control sea lamprey (*Petromyzon marinus* Linneaus, 1758) in a valued trout stream could cause mortality of mayflies and a concomitant reduction in the fishery. In order to reach agreement between anglers and managers on control application, the USFWS demonstrated that lampricide treatments did not negatively impact macroinvertebrates or fish in the stream (USFWS 2014). We recommend continued testing of Zequanox on additional taxa and life stages, especially if Zequanox becomes routinely used in a dreissenid control program. Further evaluation of indirect or latent effects (e.g., hypoxia, nutrient addition, increased turbidity) of treatments would further broaden our understanding of its effects on an ecosystem for informing decisions on dreissenid control strategies.

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Effects of Spray-Dried *Pseudomonas fluorescens*, Strain CL145A (Zequanox®) on Reproduction and Early Development of the Fathead Minnow (*Pimephales promelas)*

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Contents

Figures

Tables

Effects of Spray-Dried *Pseudomonas fluorescens*, Strain CL145A (Zequanox®) on Reproduction and Early Development of the Fathead Minnow (*Pimephales promelas)*

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Abstract

The biopesticide, Zequanox®, is registered for dreissenid mussel control in open water systems. Previous toxicity trials with nontarget organisms, including young-of-the year of several fish species and invertebrates, demonstrated selectivity of Zequanox for dreissenids. However, data are lacking on its safety to reproductive and early life stages of fish. The present study evaluated the effects of Zequanox on spawning and early life stages of the fathead minnow, *Pimephales promelas,* at the maximum approved concentration (100 mg Zequanox active ingredient /L) and exposure duration (8 h) for open water application. The results showed no significant effect of Zequanox on survival, condition, or cumulative egg deposition (21 d) in adult fathead minnow. Eggs (<24-h old) exposed to Zequanox developed to the eyed-stage at a similar rate to that of unexposed eggs. Additionally, Zequanox did not have a significant effect on survival and growth (90 d) of newly hatched fry (<24-h old). The results indicate that Zequanox treatment will not affect survival, spawning, and early life development of fathead minnows when applied at the recommended treatment regime.

Introduction

Zebra mussels *(Dreissena polymorpha* Pallas, 1771) and Quagga mussels, (*D.rostriformis bugensis* Andrusov, 1898) are nuisance invasive species that have expanded their range throughout the United States and into Canada since their arrival in 1985 (Mackie and Claudi 2009). These mussels have adversely affected aquatic communities on a number of levels. The high filtration capacity of dreissenids has caused a shift from a pelagic to a benthic food web, resulting in alterations in diet and concomitant reduction in condition of some fish species (Vanderploeg et al. 2002, Pothoven and Mathajian 2008, Nalepa et al. 2009). Settlement of dreissenids in high densities has caused degradation of fish spawning shoals (Marsden and Chotkowski 2001). Native mussels have declined and disappeared from habitats throughout the Great Lakes' region due to competition for food and colonization by dreissenids (e.g., Nalepa et al. 1996, Ricciardi et al. 1996, Schloesser et al. 1998, Strayer and Malcom 2007). Dreissenid infestations have altered fish management activities by compromising or eliminating sources of brood stock and eggs for aquaculture (OMNR 2005, Sykes 2009).

One potential molluscicide to control dreissenids in open water is the biopesticide,

[1] U.S. Geological Survey.

Zequanox®, a commercial formulation of a ubiquitous soil bacterium, *Pseudomonas fluorescens* strain CL145A. The product has shown specific toxicity to dreissenid mussels and low toxicity to a range of nontarget species (Marrone Bio Innovations [MBI] 2012a, Molloy et al. 2013a). Zequanox is registered by the US Environmental Protection Agency (USEPA) for treatment of dreissenids in closed and open water applications (MBI 2012b; registration number 84059-15). Toxicity tests with bluegill (*Lepomis macrochirus*), largemouth bass (*Micropterus salmoides*), and brown trout (*Salmo trutta*) showed no evidence of mortality from exposure to 100 mg Zequanox active ingredient (A.I.)/L for 72h (Molloy et al. 2013b). Luoma et al. (2015) tested fingerlings of a variety of warm, cool- and cold-water fish species in a continuous 24-h exposure to concentrations ranging from 50 to 300 mg Zequanox A.I./L. Significant differences in toxicity occurred among species; for example, rainbow trout (*S. gairdneri*) and lake sturgeon (*Acipenser fluvescens*) were highly sensitive (24-h LC50 = 19.2 mg A.I./L and 8.9 mg A.I./L, respectively), while the LC50 value exceeded 100 mg Zequanox A.I. /L for six other species. However, the exposure duration in their trials was three times longer than the expected exposure duration (i.e., 24-h continuous dosing). There are no published studies on the toxicity of Zequanox to fish in static exposures at the maximum approved open-water label concentration (100 mg Zequanox A.I./L) and exposure duration (8h). Additionally, previous trials with fish tested juvenile/fingerling life stages. Data are lacking on the safety of Zequanox to spawning adults, eggs, and early life stages of fish.

Fathead minnows are well-established test organisms for measuring toxicant effects on survival and reproductive fitness of a cyprinids and related species (Ankley et al. 2001; Jensen et al. 2001; Kahl et al. 2001). Reproductive maturity of males and females can be established by the appearance of secondary sex characteristics; males develop a dorsal pad and various sizes and numbers of nuptial tubercles. Fathead minnows are fractional spawners and females may produce clutches of 50-100 eggs every 3-5 days at 25°C (Gale and Buynak 1982; Jensen et al. 2001; Thorp et al. 2007). Fertilized eggs undergo cleavage to the blastula stage within 3-4 h of spawning and can readily be distinguished from unfertilized eggs by an opaque or clear appearance and a white spot where the yolk has precipitated (USEPA 2002). The eyed-stage develops within 48 to 72 h (USEPA 1996) and can be used to distinguish unfertilized or undeveloped eggs from developing larvae. Eggs hatch within about 96 h of fertilization (USEPA 1996; Thorpe et al. 2007). The resulting fry may be reproductively mature within 6 to 9 months, depending on rearing conditions.

The goal of the present study was to determine the nontarget effects of Zequanox on the survival and reproductive success of fathead minnows in simulated open-water applications. The specific objectives were to evaluate effects of 8-h exposure to 50 mg A.I./L and 100 mg A.I./L on: 1) survival of adult fathead minnows, 2) egg deposition, 3) egg hatchability, and 4) survival and growth of newly hatched fry.

Methods

Test System

Three separate tests were conducted in an outdoor mesocosm that consisted of 1000-L (total volume 980 L) circular tanks (high density polyethylene, 175 cm diameter x 64 cm height) located in two 0.004- hectare (ha) concrete ponds (Fig. 1). Daily and diurnal fluctuations in water temperature were minimized by filling the concrete ponds with well water to provide a thermal

jacket for the test tanks and by covering the ponds with black shade cloth. Pond water was pumped from a 0.1 ha earthen pond, passed through a 400 µm filter and delivered to a head-box system for distribution to nine test tanks per pond. Flow rate was adjusted to approximately 3.8 L/min per tank (~ 6 tank-exchanges/day). Aeration was supplied during the post-exposure rearing period through individual airstones in each tank that were connected to a regenerative blower. Aeration was not supplied during Zequanox exposure to simulate conditions in an open water application and assess treatment-related effects on dissolved oxygen concentrations.

Figure 1. Mesocosm test system consisting of nine 1000-L tanks within each of two 0.004 ha concrete ponds. Water was supplied to test tanks from a 0.25 ha earthen pond. Concrete ponds were filled with water and covered with shade cloth to moderate diurnal temperature fluctuation.

Zequanox Treatment and Concentration Verification

Two concentrations of Zequanox (50 and 100 mg Zequanox A.I./L) and an untreated control were tested at the expected environmental exposure duration (8h). The test material, Zequanox, was produced by Marrone Bio Innovations, Inc. (Davis, CA) and was a spray-dried powder formulation containing 50 % (w/w) active ingredient (*P. fluorescens,* strain CL145A). A dosing stock was prepared for each individual tank with water from the tank and the appropriate weight of dry Zequanox and added to the test tank within 5 minutes of preparation. Water flow to each tank was halted during the 8 h treatment. At the end of the exposure period, tanks were drained to half-volume and water flow was restored after the exposure.

The concentration of Zequanox (A.I.) in each test tank was determined by spectrophotometric comparison to a linear regression created from Zequanox standard solutions (Beckman UV/Vis Spectrophotometer, Model DU 800). Zequanox standards of 25, 50, 100, and 150 mg Zequanox A.I./L were prepared from serial dilutions of a 2,000 mg/L standard stock solution. A linear zero-intercept standard curve was prepared from the 25, 50, 100, and 150 mg Zequanox A.I.//L dilution stocks using triplicate standard samples. The spectrophotometer was blanked using filtered (200 µm) pond water. Mid-column water samples were collected from

each test tank for Zequanox concentration analysis at 0, 1, 2, 4, 6, and 8 h of exposure and at 1, 4, 8 and 16 h post-exposure. Confirmatory post-test efficacy verification of Zequanox was completed at MBI, Davis, CA. Results of post-efficacy assessment trials met the quality control standards set forth by MBI for the product.

Water Chemistry

Dissolved oxygen, temperature, and pH were measured daily in each tank during the pre- and post-exposure period and at 1 h and 8 h during the exposure period. Dissolved oxygen was measured with a YSI® 550A dissolved oxygen meter. The pH was determined with a Beckman Coulter® φ410 pH meter and probe. Temperature was measured with a Thermapen® digital thermometer. Water flow rates (mL/min) were measured daily in each tank. Total hardness (mg/L as $CaCO_3$) was determined by titrimetric method with Manver Red indicator (USEPA 1983). Total alkalinity (mg/L as $CaCO_3$) was determined by titrimetric method to a pH endpoint of 4.5 (APHA 1995). Conductivity was measured with a Fisher Accumet® conductivity meter. Hardness and alkalinity were measured from one replicate of each treatment before exposure and once during the exposure period. During the post-exposure period, hardness and alkalinity were measured weekly on the source water. Conductivity was measured in each tank before and once during the exposure period.

Adult Spawning Trial

Adult fathead minnows, 9 to 16 mo-old, were obtained in June 2014 from fish culture facilities at the Upper Midwest Environmental Sciences Center (UMESC). Fish were sedated with Aqui-S®20E (16 mg eugenol A.I./L), hand sorted by sex, and transferred into a partitioned raceway. Males were identified by the presence of tubercles on the head, a black spot on the dorsal fin and/or a dark band behind the head (Flickinger 1969). Females were identified by a lack of the aforementioned features and the appearance of the ovipositor (Flickinger 1969). Fish without defined sexual characteristics were omitted from the study. Forty female and 15 male fish were randomly distributed to nine test tanks (three replicate tanks per treatment). Mean total length of females was 62.8 mm (range 50.0-76.1 mm; SD=4.1) and of males was 70.1 mm (range 56.9- 85.0 mm; SD=5.3). Mean wet weight of females was 2.67 g (range 1.59-3.83 g; SD=0.48) and of males was 4.11 g (range 2.14-7.70 g; SD=0.89).

The following day, 10 spawning substrates were placed into each tank to monitor baseline egg production. Spawning substrates were constructed of a 15-cm length of 10-cm i.d. (inner diameter) polyvinyl chloride (PVC) pipe that was cut in half lengthwise (Fig. 2A). Substrates were observed daily for 5 days to verify that fish in each tank were in spawning condition and to provide an estimate of pre-exposure egg deposition. After the 5-d baseline spawning period, Zequanox was applied to the test tanks as described in the section *Zequanox treatment and concentration verification*. Water flow and aeration were halted to test tanks during treatment. Following Zequanox exposure, adult fish mortality and egg deposition were monitored daily for 21 days. Spawning substrates were removed from the tanks and examined; those with <50 eggs were cleaned and returned to the tank. All substrates with > 50 eggs were photographed for enumeration of eggs. The first ten substrates (1-10) with >50 eggs were transferred to a 1000-L rearing tank, corresponding to the adult treatment tank, in an adjacent concrete pond. When a substrate was removed from a test tank, a replacement substrate was placed into the tank to maintain a total of 10 substrates in the tank.

Spawning substrates 1-10 were incubated in the rearing tank in an upright position atop a mounted grill grate (57 cm diameter x 30 cm height) above a bubble wand (120-cm bubble wand) to maintain airflow over the eggs and reduce growth of fungus (Fig. 2B). Additionally, substrates were immersed in a fungicidal treatment (1667 mg/L formalin bath for 15 min) on three consecutive days (Schnick 1973). Substrates 1-10 were photographed again at 48 to 72 h to enumerate the number of eyed-eggs and assess fertilization and hatchability rates.

At the conclusion of the 21-day post-exposure spawning period, adult fish were euthanized in tricaine methanesulfonate (MS-222), sexed, measured and weighed. Unrecovered fish were counted as a mortality. The final count of females per tank (range =25 to 40, mean=33, SD=4) was used in analyses of egg production. Condition factor of adult fish was calculated as K = 100 (W/L^3) where K = condition, W = wet weight (g), L = total length (cm) (Nash et al. 2006). Cumulative egg deposition per female in a tank was enumerated over the 21-d post-exposure period. Percent eyed-eggs was determined for substrates 1-10 and was defined as: (number of eyed-eggs on substrate/number of eggs deposited on substrate) x 100.

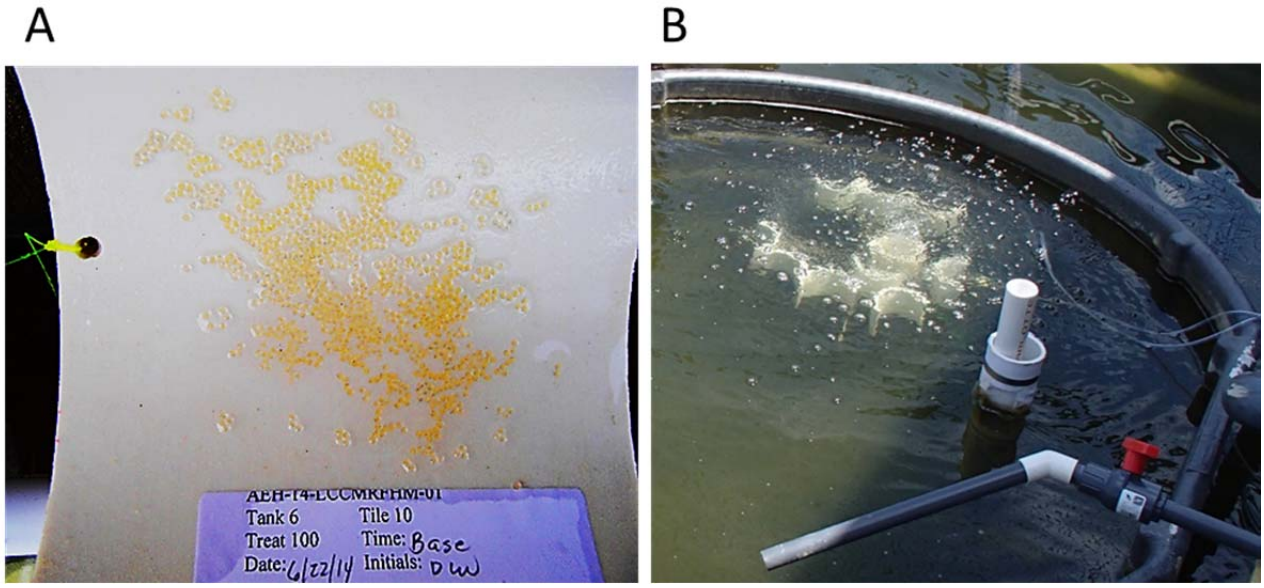

Figure 2. A) Spawning substrates with newly deposited eggs on the concave surface. A digital photograph was taken of substrates for enumeration of newly deposited eggs and eyed-eggs. B) Incubation of spawning substrates in the test tank atop a mounted bubble wand.

Egg Trial

Mature fathead minnows were transferred from indoor culture facilities into outdoor 0.04 ha concrete ponds in early June 2014. About 150-180 spawning substrates were placed into each pond and checked at about the same time each day for egg deposition. Substrates with >50 eggs (<24-h old) were placed into a cooler of pond water and immediately transferred to the mesocosm for distribution to test tanks. Ninety substrates were marked with an identification number and randomly distributed to one of nine test tanks (three replicate tanks per treatment) for a total of 10 substrates per tank. Within 4 h of initial substrate collection from the pond, Zequanox was applied to the test tanks (as described in the section *Zequanox Treatment and Concentration Verification).* At 1-h post-exposure, all substrates in a tank were removed and simultaneously immersed in a formalin bath, as described in the section *Adult Spawning Trial,* returned to the tank, and incubated on a grill grate with aeration (Fig. 2B). Substrates were

individually photographed on Day 0 and Day 3 for enumeration of initial egg deposition and number of eyed-eggs, respectively. Percent hatchability was defined as: (number of eyed-eggs on substrate/number of eggs deposited on substrate) x 100.

Fry from the adult spawning and egg trials were maintained in separate replicate treatment tanks and reared in the outdoor mesocosm until October 2014. Estimates of fry mortality were confounded by the occurrence of several disease outbreaks and potential nutritional deficiencies; therefore, data collection was terminated at 30 d post-exposure.

Fry Trial

Adult fathead minnows were transferred from indoor culture facilities at UMESC to two outdoor ponds in early June 2015. Spawning substrates were placed throughout the ponds and checked at the same time each day for newly deposited eggs. When sufficient eggs were found deposited on one day, substrates were placed into a cooler of pond water and transferred to a wet lab. Eggs were stripped from substrates by immersion in a de-adhesion solution of sodium sulfite (15 g/L) for approximately 3 minutes followed by immersion in a bucket of well water and collection of sloughed eggs. Eggs were transferred to a McDonald upwelling incubator jar for hatching. Water temperature was maintained at 23°C and flow rate was ~7.5L/min. Newly hatched fry (<24-h old) were collected on Day 4 of incubation and randomly distributed (n=300 fry per replicate) to each of 18 test tanks (6 replicates per treatment) in the outdoor mesocosm.

To observe fry during the exposure, a 100-L stainless steel vessel (35 cm x 60 cm x 60 cm, H x L x W) was used to hold fry during the exposure period. Fry were allowed to acclimate overnight and the following day, Zequanox was applied to the stainless steel vessel (as described in the section *Zequanox Treatment and Concentration Verification)*. At the end of the 8-h exposure, the contents of the stainless steel vessel were poured into the 1000-L tank; water flow was established and air stones were placed into each test tank. Daily observations of fry mortality were recorded in each tank for 90 days.

On days 44 and 90 post-exposure, condition factor was determined from a subsample of 20 fish per tank. Fish were euthanized in MS-222 (250 mg/L) and then weighed (wet weight, 0.01 g) and measured (total length, 0.1 mm). Total fish survival was determined on day 90. Each test tank was drained and fish were transferred to a 9.5 L bucket for euthanization in MS-222. The total number of fish was counted and a total wet weight was obtained for each test tank.

Data Analysis

In every analysis, the tank was treated as the experimental unit. All statistical analyses were performed using SAS Version 9.3 (SAS Institute, Inc.) and statistical significance was defined at $\alpha < 0.05$. Analysis of water chemistry (dissolved oxygen, pH, temperature, alkalinity, water hardness, and conductivity) and exposure concentration were summarized with simple descriptive statistics. Egg production per female (adult trial) was compared across treatments with Kruskal-Wallis nonparametric test (Proc nparway1). Percent eyed-eggs (adult and egg trial) and percent mortality at 90 days (fry trial) were analyzed with a generalized linear mixed model (Proc glimmix) with treatment as a fixed effect, tank as a random effect, and a binomial logistic regression (logit link function) with random intercepts. A scale parameter was added to the model using the random_residual_statement. Responses of each treatment group were individually compared to the control group using a two-sided least squares means (LSD) comparison test. Condition factor of adult fish (21 d post-exposure) and fry (44 and 90 d post-exposure) was modeled using a mixed effects model (Proc mixed) with treatment as a fixed

effect and tank as a random effect. Condition factor (by sex) of the control groups was compared to that of the treatment groups using a two-sided LSD comparison test.

Results

Water Quality and Zequanox Concentration

Mean measurements of dissolved oxygen, pH, and temperature were similar for the three trials (Table 1). Mean temperature ranged from 24.9°C to 26.2°C during the exposure and from 21.5°C to 22.3°C during post-exposure period of the three trials. A diurnal fluctuation in pH occurred in all tanks in the three trials that ranged from a morning low of about 8.10 to a peak of about 9.60 at late afternoon (Table 1). Dissolved oxygen concentrations decreased slightly during the 8-h exposure in the treatment tanks compared to the control tanks, but remained >7.9 mg/L in all tanks (Table 1).

The mean concentration of Zequanox was similar among replicates in the three trials (Table 2). Overall, the lowest mean concentrations occurred in the adult spawning trial and the highest occurred in the fry trial (Table 2). Differences in measured concentrations of Zequanox among trials are partly attributed differences in the volume of the test tanks between the adult/egg trials and fry trials and settling of the product during the 8-h exposure period.

Adult Spawning Trial

Baseline spawning (pre-exposure) occurred in every tank. The number of spawning substrates with eggs deposited ranged from 4 to 13 per tank and the total number of eggs ranged from 2156 to 8974 per tank. Mean number of eggs per substrate was 700, SE=163.
A spawning event occurred on every day of the 21-d post-exposure spawning period in at least one tank and spawning occurred consistently throughout the 21-d period, except for a slight downward trend in the last 3 days of the trial (Fig. 3). The total number of spawned substrates ranged from 18–32 per tank and was not related to treatment (chi-square=0.81, p=0.67, 2 df). Mean cumulative egg production per female ranged from 344 eggs, SE=77 (50 mg/L Zequanox) to 409 eggs, SE=49 (control) and did not differ significantly among treatments (chi-square=1.01, p=0.59, 2 df) (Fig. 4). Additionally, there was no significant effect of Zequanox treatment on fertilization and development to the eyed-stage of fathead minnow eggs on the first ten spawning substrates (p=0.11, F=2.26, 2 df) (Fig. 5). Although there was a trend downward in percent eyed-eggs in the 100 mg/L treatment, differences among treatments were not significant (p=0.11, F=2.26, 2 df).

Total mortality of adult fish at 21-d post exposure ranged from 0% to 12% (n=0 to 7 fish per tank). Mean mortality in the three treatments was 4% (controls), 3% (50 mg/L) and 6% (100 mg/L). Mean condition factor of fish at 21-d post-exposure did not differ (within sex) between control and treatments (females, p=0.93, F=0.93; males p=0.262, F=1.33, 2 df). Mean condition factor of females was 1.07, standard error (SE) =0.02 (control), 1.07, SE=0.01 (50 mg/L) and 1.08, SE=0.02 (100 mg/L). Mean condition factor of males was 1.15, SE=0.01 (control), 1.18, SE=0.02 (50 mg/L) and 1.22, SE=0.03(100 mg/L).

Table 1. Mean (SD) dissolved oxygen (DO) and temperature and pH range during Zequanox trials of fathead minnow adults, eggs, and newly hatched fry.

Water quality parameter	Treatment group	Adult trial	Egg trial	Fry trial
		Exposure		
DO (mg/L)	Control	9.32 (2.19)	9.23 (1.35)	9.02 (0.63)
	50 mg/L	8.13 (0.49)	8.84 (1.74)	8.74 (0.57)
	100 mg/L	7.91 (0.63)	8.56 (1.85)	8.71 (0.53)
pH range	Control	8.94 (8.85–9.10)	8.86 (8.54–9.22)	8.75 (8.39–9.26)
	50 mg/L	8.77 (8.64–8.88)	8.75 (8.57–8.93)	8.63 (8.31–9.25)
	100 mg/L	8.63 (8.33–8.87)	8.59 (8.55–8.64)	8.60 (8.02–9.25)
Temp (°C)	Control	26.2 (1.65)	26.0 (1.05)	24.9 (1.80)
	50 mg/L	26.3 (1.66)	26.0 (1.10)	24.7 (1.72)
	100 mg/L	26.3 (1.72)	26.1 (1.04)	24.9 (1.79)
		Post-exposure observation		
DO (mg/L)	Control	8.86 (2.20)	9.27 (1.03)	8.13 (0.87)
	50 mg/L	8.86 (2.22)	9.27 (0.98)	8.11 (0.88)
	100 mg/L	8.87 (2.13)	9.20 (0.97)	8.14 (0.89)
pH range	Control	8.78 (8.13–9.62)	8.89 (8.31–9.94)	8.53 (7.77–9.57)
	50 mg/L	8.79 (8.14–9.61)	8.90 (8.31–9.94)	8.55 (8.04–9.57)
	100 mg/L	8.78 (8.13–9.60)	8.89 (8.33–9.92)	8.53 (8.02–9.63)
Temp (°C)	Control	22.3 (1.48)	21.5 (1.56)	21.7 (2.37)
	50 mg/L	22.3 (1.49)	21.5 (1.57)	21.7 (2.37)
	100 mg/L	22.3 (1.47)	21.5 (1.56)	21.6 (2.36)

Table 2. Mean concentration (SD) of Zequanox during 8-h exposure period in three replicate tanks.

Treatment group	Adult	Egg	Fry
Control	ND[1]	ND	ND
50 mg/L	47.3 (4.2)	49.6 (5.6)	54.7 (2.2)
100 mg/L	87.7 (6.4)	93.9 (8.9)	100.7 (6.9)

[1]Not detected.

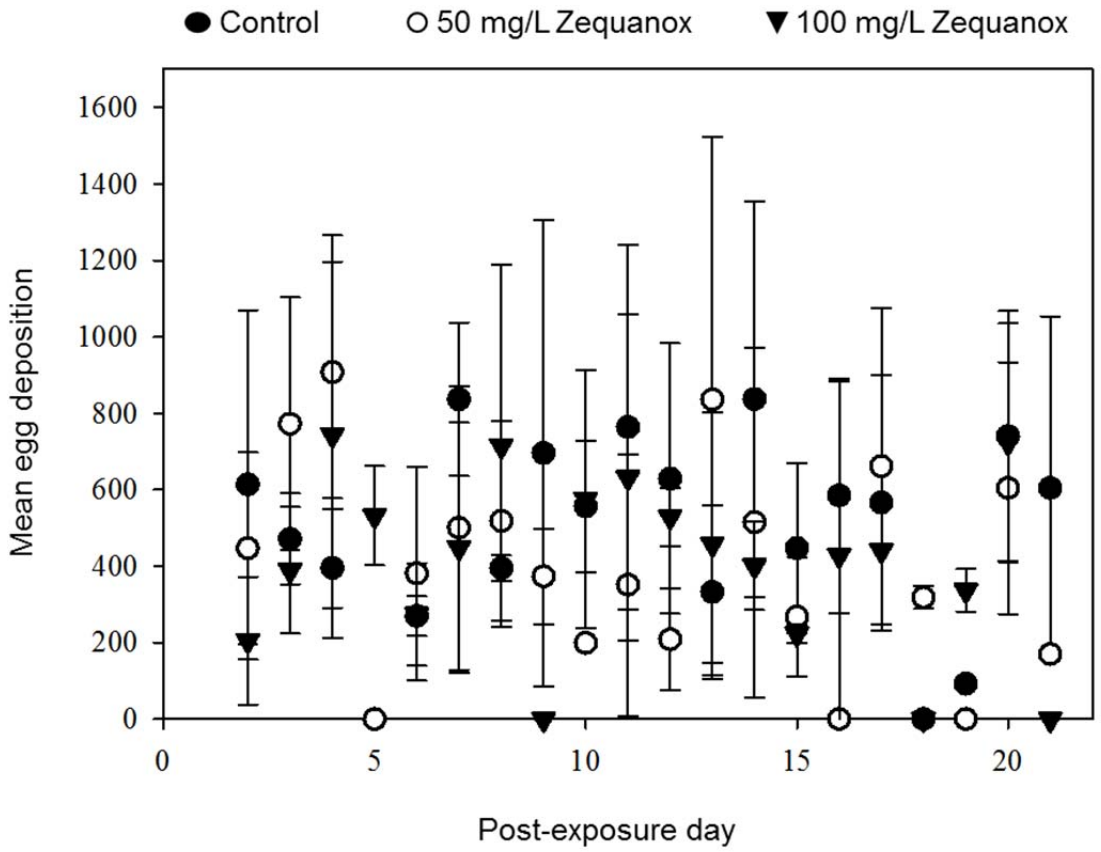

Figure 3. Adult trial: Mean (standard error, SE) daily egg production per female fathead minnow after Zequanox treatment.

Egg Trial

Zequanox treatment did not have a significant effect on development to the eyed-stage of deposited eggs (Fig.6). The percent of eggs that developed to the eyed-stage ranged from 92.6%, SE=0.02% (50 mg/L) to 94.8 %, SE=0.01% (control) and was not significantly different between the control and treatments (p=0.82, F=0.82, 2 df).

Fry Trial

Zequanox treatment did not have a significant effect on 90-d survival of newly hatched fathead minnow fry. Cumulative mean survival ranged from 83.7%, SE=0.02% (100 mg/L) to 81.4%, SE=0.01% (50 mg/L) (Fig. 7) and did not differ significantly between control and treatments (p=0.54, F=0.64, 2 df). Additionally, the 44-d (p=0.75, F=0.29, 2 df) and 90-d (p=0.30 f=1.21, 2 df) condition factor of fry was not significantly different among control and treatments (Fig. 8).

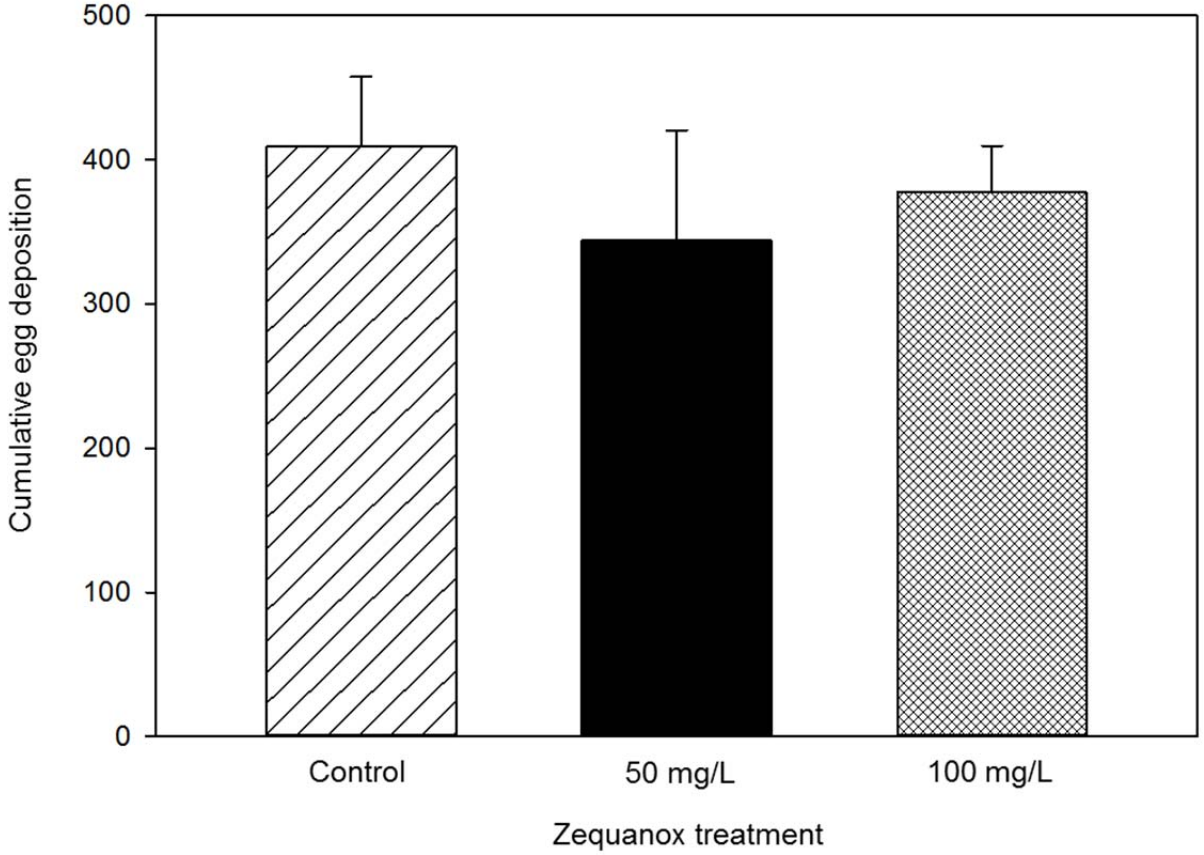

Figure 4. Adult trial: Mean (SE) 21-d cumulative egg deposition per female after Zequanox treatment.

Conclusions and Discussion

Our results indicate that Zequanox treatment at the maximum approved concentration and exposure duration is safe for adult and early life stages of fathead minnow. Mortality of adults and newly hatched fry was minimal in all treatments. The mode of action of Zequanox is through ingestion and degradation of the digestive epithelium (Molloy et al. 2013c). As a result, organisms that are susceptible to the toxic component in Zequanox may have reduced growth and condition rather than overt mortality. Luoma et al. (2015) reported reduced condition factor at 22 d post-exposure in several fish species for which the LC50 value was >100 mg/L. For example, the 24-h LC50 value for largemouth bass was 173.6 mg/L, but condition factor was significantly less in fish exposed to ≥75 mg/L Zequanox. In the present study, mean condition factors of adults and newly hatched fry were not significantly different among fish in the control and Zequanox treatments (Fig. 8).

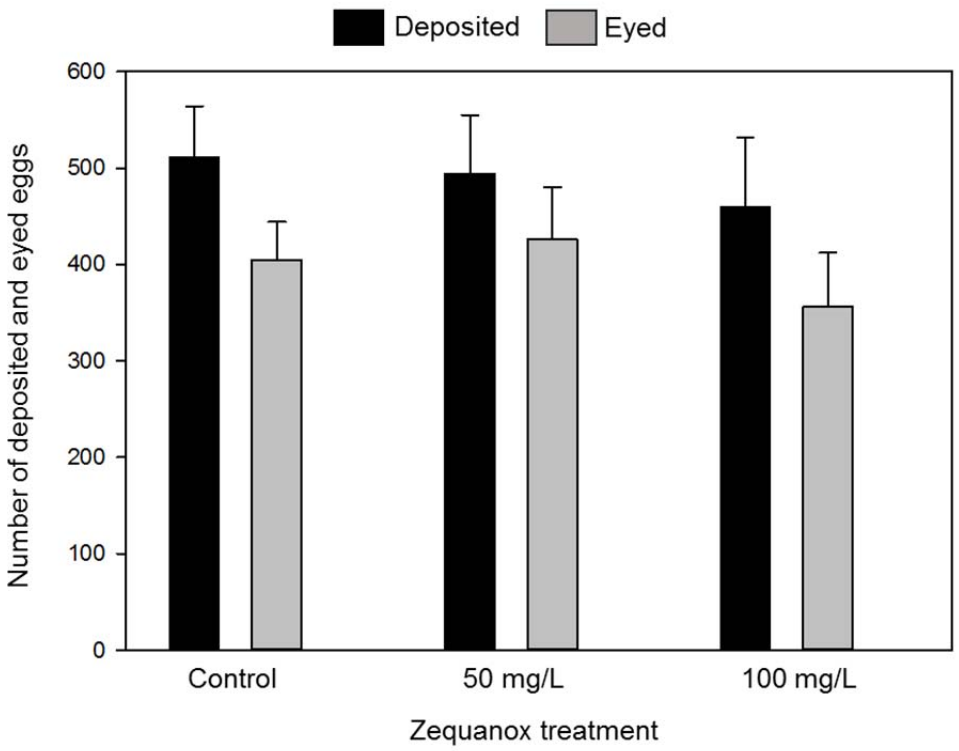

Figure 5. Adult trial: Mean (SE) egg deposition and development to eyed stage (Substrates 1-10) of fathead minnow eggs after Zequanox treatment.

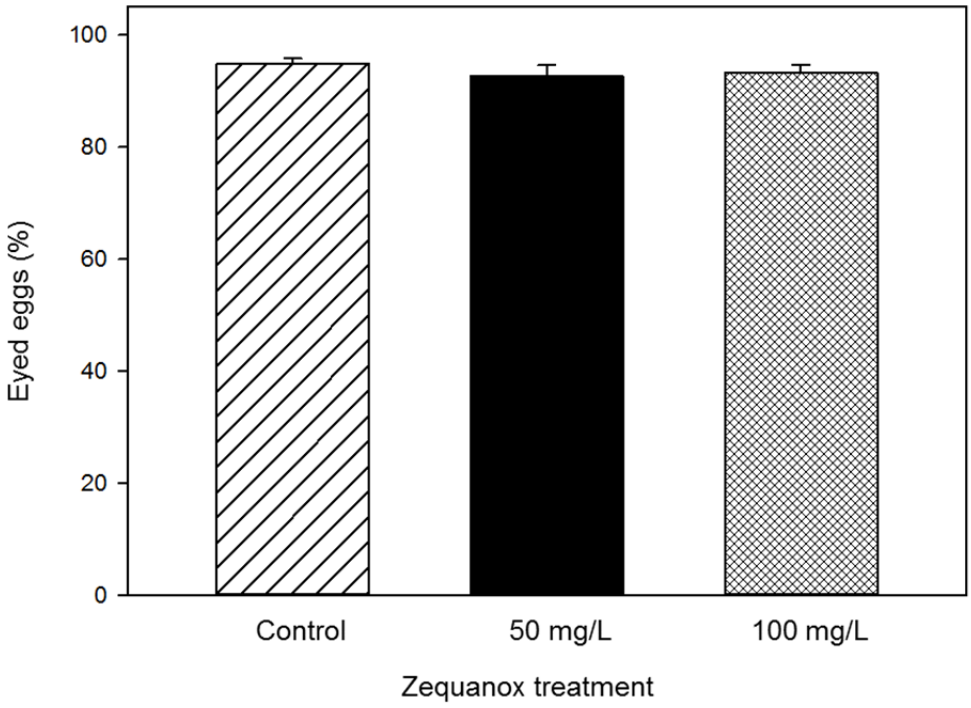

Figure 6. Egg trial: Mean percentage (SE) of fathead minnow eggs developing to eyed-stage after Zequanox treatment.

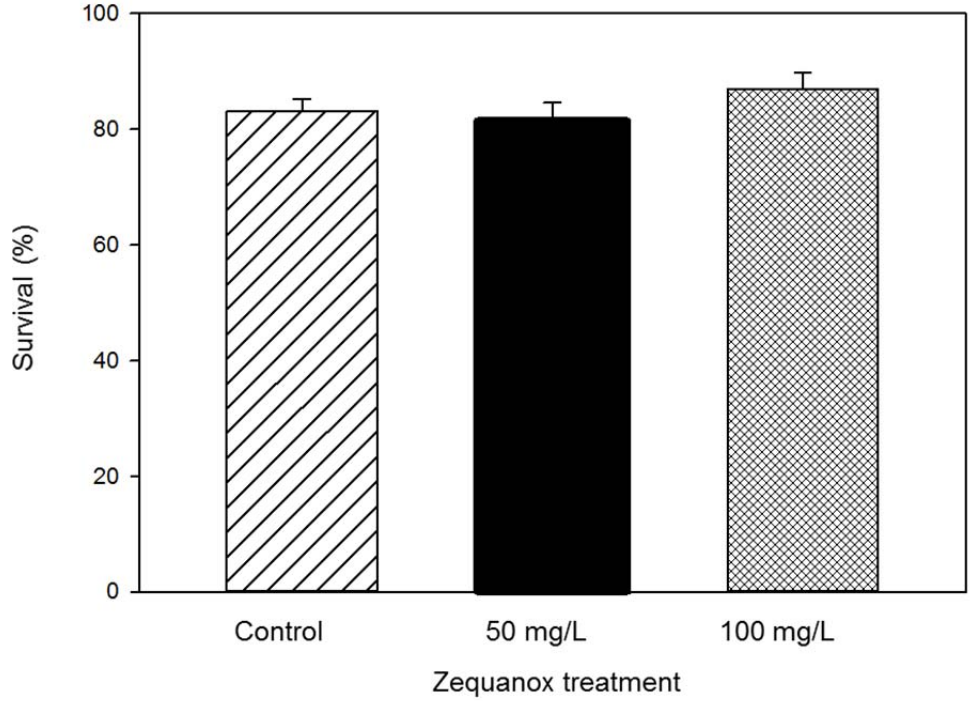

Figure 7. Fry trial: Mean percent survival (SE), 90-d post-exposure, of fathead minnow fry after Zequanox treatment.

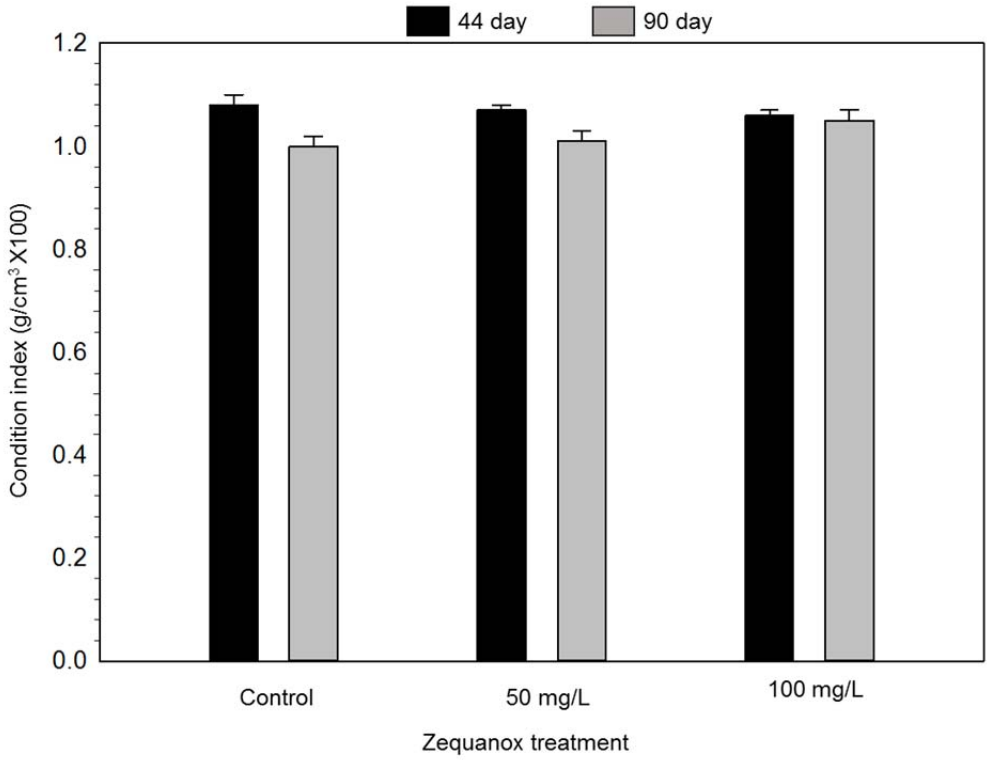

Figure 8. Fry trial: Mean (SE) condition factor of fathead minnow fry after Zequanox treatment.

By all measures used in the present study, Zequanox had no negative effect on spawning of fathead minnows during or after exposure (Figs. 3 and 4). Zequanox is composed primarily of organic particulates and produces a highly turbid suspension in the water column. Eggs were found on spawning substrates on Day 1 post-exposure indicating that turbidity did not prevent spawning or fertilization during the 8-h exposure (Fig. 3). There was no direct or lingering effect of Zequanox on egg deposition and fry development at the concentrations and exposure duration that were tested (Figs. 3-8). Females continued to deposit eggs during the post-exposure period and development of those eggs to the eyed-stage was similar in all treatments (Fig. 5).

The results suggest several potential applications for Zequanox in fishery management. The biopesticide may be used for removing dreissenid mussels from fish spawning shoals and reefs as part of a dreissenid integrated pest management program. Zequanox may be one alternative for killing dreissenid veligers and settlers in waters that contain fish or eggs, such as aquaculture ponds, when KCl-formalin combination or other salts are ineffective or unsafe for the species or life stage of fish.

However, the use of Zequanox in waters that contain fish will depend on the fish species. Some cool-water fish species may be more sensitive to Zequanox than fathead minnows and other warm-water species. For example, the survival and condition factors of fingerling lake trout (J. Luoma, personal communication) and rainbow trout (Luoma et al. 2015) were reduced after Zequanox treatment. Before large scale treatment of fish with Zequanox is conducted, trial exposures with the species and life stage of concern are recommended to verify its safety.

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Mapping of Two Minnesota Lakes Using Commercial Side-scanning Sonar to Characterize Substrate Hardness and Vegetated Habitat

By Richard L. Kiesling

Open-File Report 2016-XXX

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Contents

Figures

Abbreviations

eDNA	Environmental DNA
ft	feet
GIS	Geographic Information System
GPS	Global Positioning System
SD	Secure digital
Sonar	Sound Navigation and Ranging
USGS	United States Geological Survey

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Mapping of Two Minnesota lakes Using Commercial Side-scanning Sonar to Characterize Substrate Hardness and Vegetated Habitat

By Richard L. Kiesling

Abstract:

Sonar surveys of two Minnesota lakes were conducted in July and August of 2014 to provide bathymetric maps of relative substrate hardness and vegetation height and biovolume. The two lakes were chosen based on reported levels of zebra mussel (*Dreissena polymorpha*) infestation, and data were collected as part of a larger project to investigate potential management methods for control of zebra mussel infestations. Sonar data from surveys were processed using a commercially available post-processing software tool that produced same-day processing of transect data from the field using client software accessing a remote server. Processed data were used to produce geo-referenced maps by spatial analysis of the data using the kriging geo-statistical tool.

Description of Study Objective:

The objective of the study was to develop detailed bathymetric maps for selected areas of two Minnesota lakes with different levels of zebra mussel infestation. The objective was to complete a sonar survey of a high-density lake with a well-documented larval dispersal period, and then select a recently colonized, low density lake suitable for testing the limits of biochemical identification and detection assays. Both lakes were to be surveyed with a commercial sonar system in common use by State of Minnesota personnel, and the signal returns from the sonar unit were to be processed to and evaluated by a commercial software product capable of processing and analyzing the dual-beam transducer data. The overall goal of the project was to generate maps of bottom substrate hardness and vegetation height and density.

Approach and Scope:

Data were collected using a commercial sonar platform from Lowrance™ High Definition System (HDS®). An HDS® 10 unit equipped with a 200 kHz transducer operating with a 20 degree beam angle and a 455kHz side-scanning transducer for shallow vegetation characterization was used to cover predefined sampling grids. Raw data were saved on the sonar unit during collection and then processed using a commercial software product. The integrated software product combined a client software application with a remote server geo-spatial statistical program. Data were transmitted from the field for data processing wireless internet communication through a cellular communication port. The entire process followed is outlined in the following steps which led to the production of the final product

Bathymetric Mapping

1. Zebra mussel habitat mapping surveys were conducted using a commercial sonar unit equipped with high-resolution side-scanning sonar linked with real-time differential GPS data collection.
2. Data from each mapping area in Lake Le Homme Dieu, Alexandria, MN, or in Maple Lake, Forada, MN, were collected in transects spaced thirty meters apart on a rectangular grid. A single survey was conducted for each lake during mid-summer (July-August) to determine the substrate type, depth and vegetative cover during the zebra mussel larval (i.e. veliger) settling period.
3. Data from surveys were uploaded to a cloud server and processed using a commercially available post-processing software tool that allowed for near real-time uploading and concatenation of transect data from the field.
4. Processed data were to generate bathymetric maps of depth, relative substrate hardness, vegetation height above the recorded substrate, and plant volume as a percentage of the water column. Maps were generated using the kriging geo-statistical method.
5. Processed data from surveys for relative substrate hardness, vegetation height above the recorded substrate, and plant volume were imported into an ArcGIS geo-spatial database and subjected to kriging analysis to provide for an independent assessment of the methods used by the commercial software product.

The geo-spatial analysis using ArcGIS formed the basis of the USGS model archive for this data product.

Methods

Data Collection Platform Configuration

Data were collected using a commercial sonar platform from Lowrance™ High Definition System (HDS®) consumer echo-sounder (Contour Innovations LLC 2013). Transect data were collected using an HDS® 10 unit equipped with a 200 kHz transducer operating with a 20 degree beam angle and a 455kHz side-scanning transducer for vegetation characterization. The HDS® unit was configured using the shallow water setting which provides a constant 75% ping speed or 15 pings per second. Ping speed is the rate at which the transducer transmits a sound pulse into the water column and receives the response echo, controlling the along-transect coverage. During data collection, pings are combined into data ensembles, and ping values within an ensemble are averaged to produce a reported data value.

Data density in the field was controlled by the velocity of the boat as it traveled along the sampling transect during data collection. During sampling for this project, boat speed was maintained at five miles per hour providing a forward velocity of approximately seven feet per second. An estimate of the average sonar data density used for this project can be calculated by combining the forward velocity estimate with ping speed, resulting in an average sonar data density of approximately one sample every two feet of linear distance along a sampling transect.

Pulse width (i.e., band width) is not user controlled with the Lowrance™ system but is dynamic and varies depending on depth. Software algorithms for the ciBioBase application are optimized at 3200 bytes per second with a range window set to Auto on the HDS®-10 unit. This configuration of the unit provided the optimal range resolution of the sonar return signal for the range of depths available to the system.

Data Acquisition

Data were collected along transects spaced 30 meters apart using an eighteen-foot aluminum-hulled boat with a modified V hull configuration. Sonar transducers were mounted on opposite sides of the transom of the boat approximately 20 centimeters below the water line. Tracks for data collection were laid-out in the navigation display and followed at five miles per hour during data acquisition. The data feed from the transducers were continuously monitored on the heads-up display for signs of signal interference between the transducers or for problems with turbulence in the vicinity of the transducer mounts. In some instances, boat speed was reduced three miles per hour to reduce the frequency of sonar signal degradation.

Data ensembles were acquired at one second intervals. Feature data from pings that occurred between position reports were reported as an average value for each GPS geo-referenced data point. As a result, attribute data for a specific feature class including depth, bottom hardness, and plant height represents the average of 15 values from sonar returns during the sampling interval.

During acquisition, sonar data were simultaneously paired with global position (GPS) data using a built-in, time-referenced GPS unit on the HDS®-10. The GPS unit for the HDS®-10 was set to differentially correct the GPS satellite data using the Wide Area Augmentation System (WAAS) navigation system. Acoustic signal data and GPS position data were logged to SD data storage cards using the (.sl2) format for later upload to the Contour Innovations cloud server for post processing using the ciBioBase software tool.

Post Collection Data Processing

Unprocessed data files were uploaded to the Contour Innovations centralized servers using a client software program supplied as part of the ciBioBase GIS software system. Raw data files for GPS positional data and quality-assured sonar response data were processed using proprietary algorithms into estimates of bottom depth, plant height, plant bio-volume, and bottom hardness features. Within the ciBioBase software package, each data ensemble goes through a quality-assurance test to determine whether the feature data for the sampling period can be extracted and used for further analysis. If the data pass the

internal data filters, values are sent on to the respective feature detection algorithms. Data failing to meet the quality assurance tests are removed from consideration for summarization

Map Development

Processed data were used to generate geo-referenced, surface response maps for each feature class using kriging to generate a grid of equal-area cells referred to as a raster grid. Kriging is a geo-spatial analysis method that uses the actual statistical relationship of neighboring data points to make predictions in un-sampled locations. The following explanation from the ArcGIS online help manual helps explain the kriging process:

> "Kriging assumes that the distance or direction between sample points reflects a spatial correlation that can be used to explain variation in the surface. The Kriging tool fits a mathematical function to a specified number of points, or all points within a specified radius, to determine the output value for each location. Kriging is a multistep process; it includes exploratory statistical analysis of the data, variogram modeling, creating the surface, and (optionally) exploring a variance surface. Kriging is most appropriate when you know there is a spatially correlated distance or directional bias in the data." (http://desktop.arcgis.com/)

Once kriging had produced x,y,z grids of raster cells for each feature class, the data were used to project the data in three dimensions, producing a map of each attribute class for each survey. Maps were used to choose potential eDNA sampling sites prior to preliminary field data collection.

In order to be able to reproduce the statistical analysis used in the ciBioBase software, processed data files from the cloud server were incorporated into an ArcGIS project and subjected to kriging analysis following the reported analysis criteria from ciBioBase. This independent analysis of the feature class and GPS location data confirmed the proprietary mapping program included in the ciBioBase processing software. Once data were geo-referenced in ArcGIS, the results were combined with existing Minnesota DNR GIS base layers for lake bathymetry and satellite imagery to generate ArcGIS geo-spatial databases

and map products. The ArcMap data platform provides the end user with the option of changing the parameters used to generate the feature maps. For example, the buffer width surrounding the transect data can be changed during kriging analysis, providing for a more narrow or a wider map footprint.

Results and Discussion

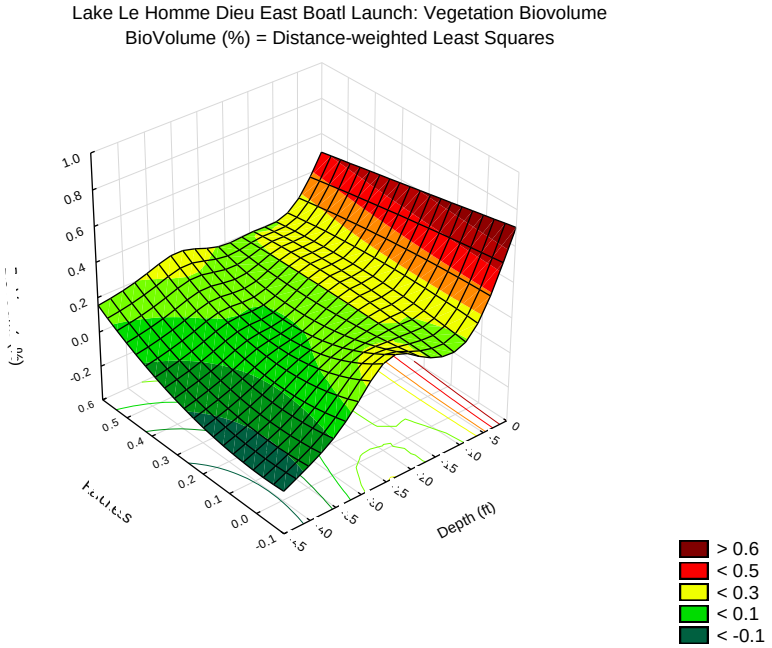
Bathymetry from the data collection effort provided one-foot contour resolution within the survey areas. This level of resolution was more than three times the available bathymetry resolution from MN DNR historical mapping efforts conducted prior to their use of the ciBioBase software (fig. 5). In addition to higher-resolution bathymetry, the data for substrate hardness and vegetation bio-volume in the water column provided important data on habitat quality for zebra mussels (figs. 6-13). Zebra mussels in Lake Le Homme Dieu and in the connected Lake Carlos have been observed to preferentially colonize hard substrates (Kiesling, unpublished video surveys). In addition to substrate hardness, observed zebra mussel density (> 10 individuals per plant) in vertical (>40centimeters) vegetation stands in Lake Le Homme Dieu suggest that vegetation biomass in the water column is a potential axis of zebra mussel distribution in the study lakes (fig. 1). The amount of plant biovolume at the survey sites is significant (e.g., figs. 2 - 4), accounting for more than half of the water column in many location in Lake Le Homme Dieu. The distribution of vegetation biovolume also varies between the sites within Lake Le Homme Dieu, as well as between the two lakes.

Maps generated by the ciBioBase software identify both Maple Lake and Lake Le Homme Dieu as having a wide range of available depths, substrate types, and vegetation height and biovolume cover (figs. 2-4). This range of feature-class values provided the eDNA sampling teams with a broad choice of habitat class combinations to guide their sampling plans. Sampling transects were located in or very near to sonar survey data collection areas. Maps from multiple areas in Lake Le Homme Dieu have also made it clear that feature characteristics such as vegetation bio-volume do not track depth or substrate hardness directly (e.g., east launch area maps) suggesting a need for follow-up analyses of the factors controlling the vegetation distribution. However, it is possible to

construct generalized response surfaces for understanding how more than one feature at a time can influence vegetation biovolume (fig. 1).

Development of an ArcGIS geo-spatial data product provides a common platform for future spatial analysis of the mapping datasets from this project. In addition to the GIS database, the ability to generate the same maps in ArcMap as were produced by the commercial software product (e.g., figs. 1-9) provides an important statistical data archive for the results of the kriging analysis in ciBioBase. Data from the project are also available through the Contour Innovation cloud server and can be incorporated into future map enhancements for the lakes by requesting permission to use the data.

a.



b.

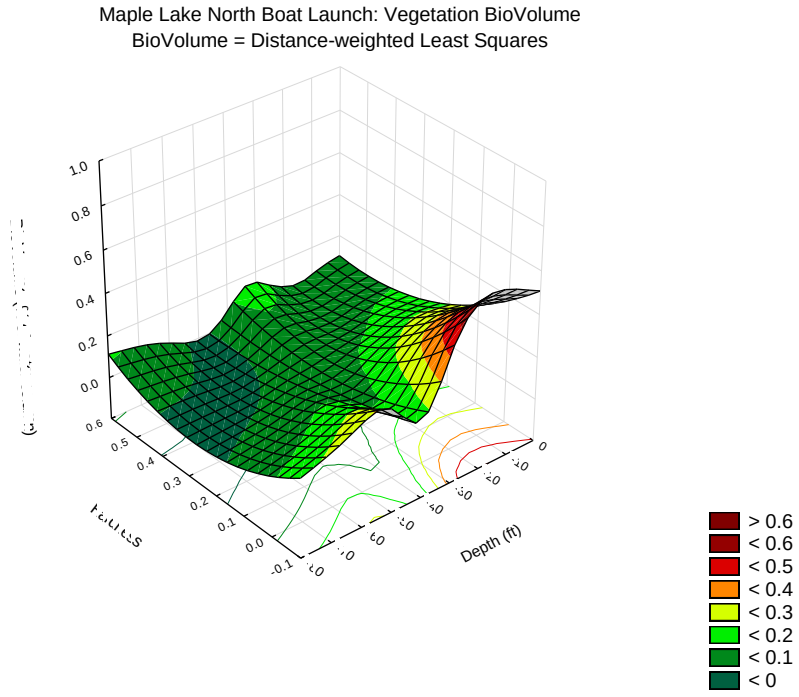
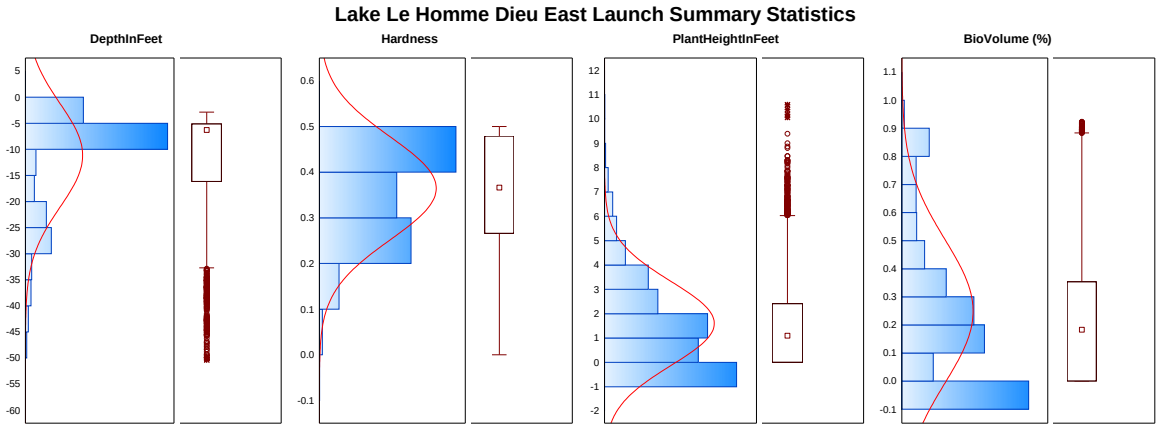


Figure 1. Plant biovolume as a function of water depth (ft) and hardness from (a) Lake Le Homme Dieu East Boat Launch site and (b) Maple Lake North Boat Launch site

a.



DepthInFeet		Hardness		PlantHeightInFeet		BioVolume (%)	
N:	5724	N:	4960	N:	5599	N:	5599
Mean:	-11.40	Mean:	0.362	Mean:	1.579	Mean:	0.247
Median:	-6.291	Median:	0.366	Median:	1.095	Median:	0.183
Min:	-50.33	Min:	0	Min:	0	Min:	0
Max:	-2.875	Max:	0.500	Max:	10.58	Max:	0.923
L-Qrt:	-16.17	L-Qrt:	0.266	L-Qrt:	0	L-Qrt:	0
U-Qrt:	-5.110	U-Qrt:	0.479	U-Qrt:	2.414	U-Qrt:	0.354
Variance:	97.41	Variance:	0.0126	Variance:	2.945	Variance:	0.0645
SD:	9.870	SD:	0.112	SD:	1.716	SD:	0.254
Std.Err:	0.130	Std.Err:	0.00159	Std.Err:	0.0229	Std.Err:	0.00339
Skw:	-1.503	Skw:	-0.332	Skw:	1.377	Skw:	1.077
Kurt:	1.230	Kurt:	-0.830	Kurt:	1.814	Kurt:	0.225
95% Conf SD		95% Conf SD		95% Conf SD		95% Conf SD	
Lower:	9.692	Lower:	0.110	Lower:	1.685	Lower:	0.249
Upper:	10.05	Upper:	0.115	Upper:	1.749	Upper:	0.259
95% Conf Mean		95% Conf Mean		95% Conf Mean		95% Conf Mean	
Lower:	-11.66	Lower:	0.359	Lower:	1.534	Lower:	0.240
Upper:	-11.14	Upper:	0.365	Upper:	1.624	Upper:	0.253

b. c. d.

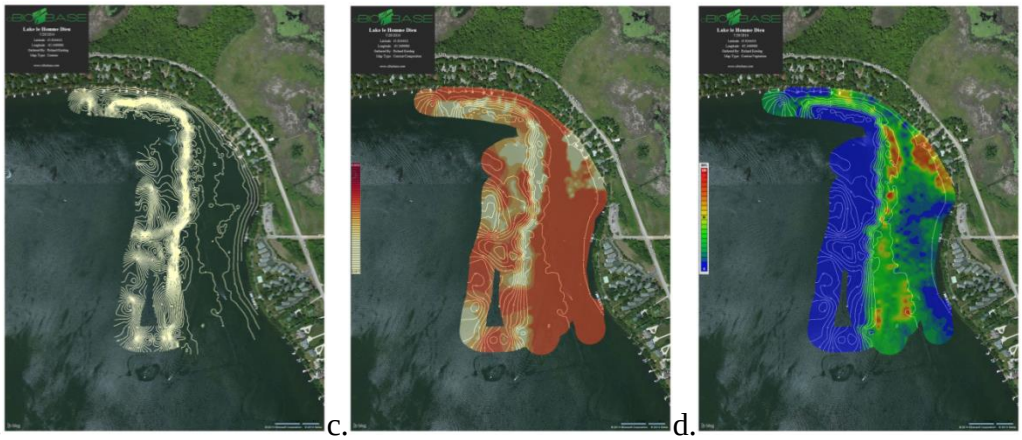
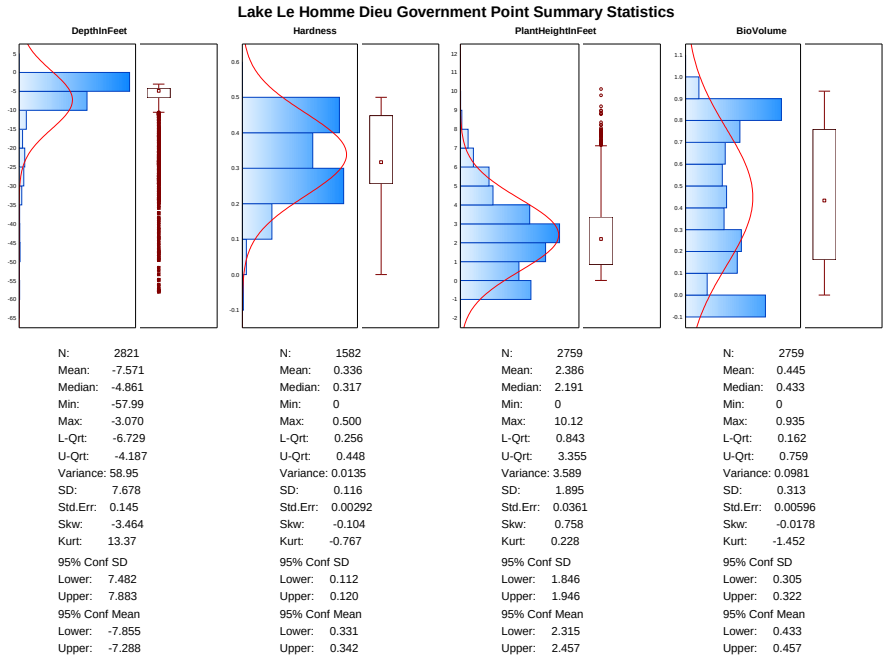


Figure 2. Statistical summary (a) for feature class data collected for East Boat Launch site from Lake Le Homme Dieu. Raster class data for depth (b), hardness (c) and plant biovolume (d) are displayed as color scale maps

a.



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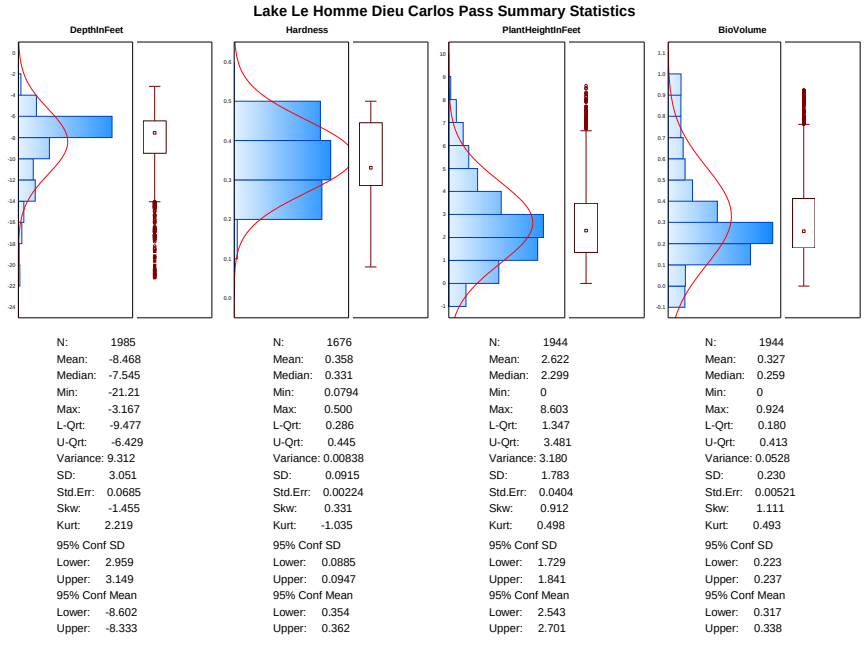
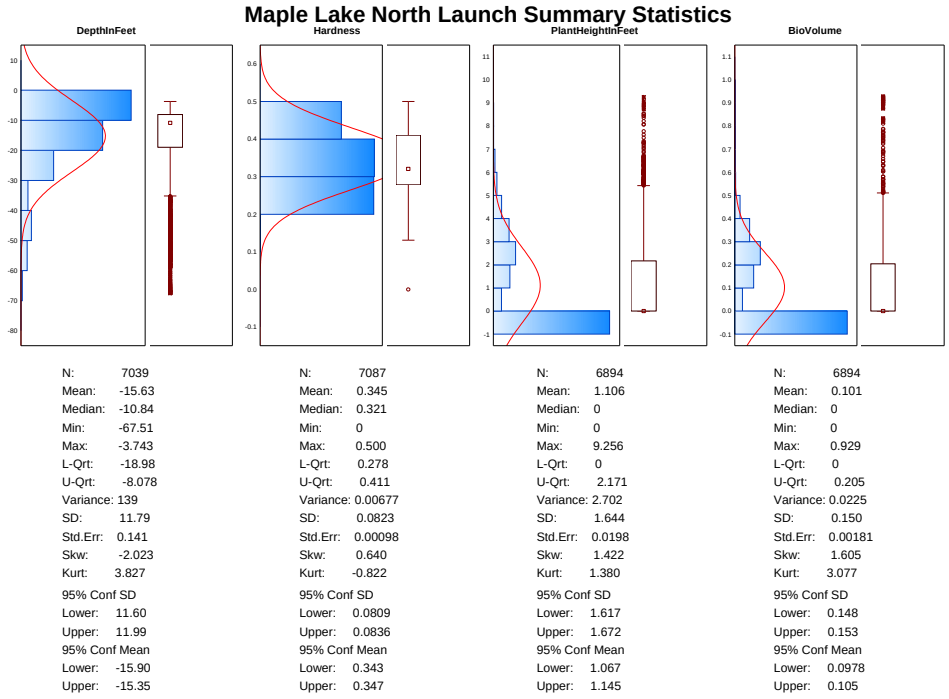


Figure 3. Statistical summary for feature class data collected for (a) Government Point site and (b) for Carlos Pass site from Lake Le Homme Dieu

a.



b.

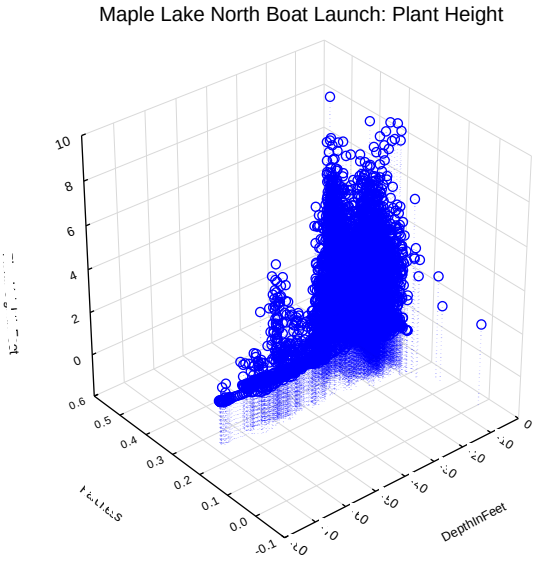


Figure 4. Statistical summary for (a) Maple Lake North Launch feature class data and (b) plant height data as a function of depth and substrate hardness

Figure 5. Lake Le Homme Dieu East Launch site one-foot contour data

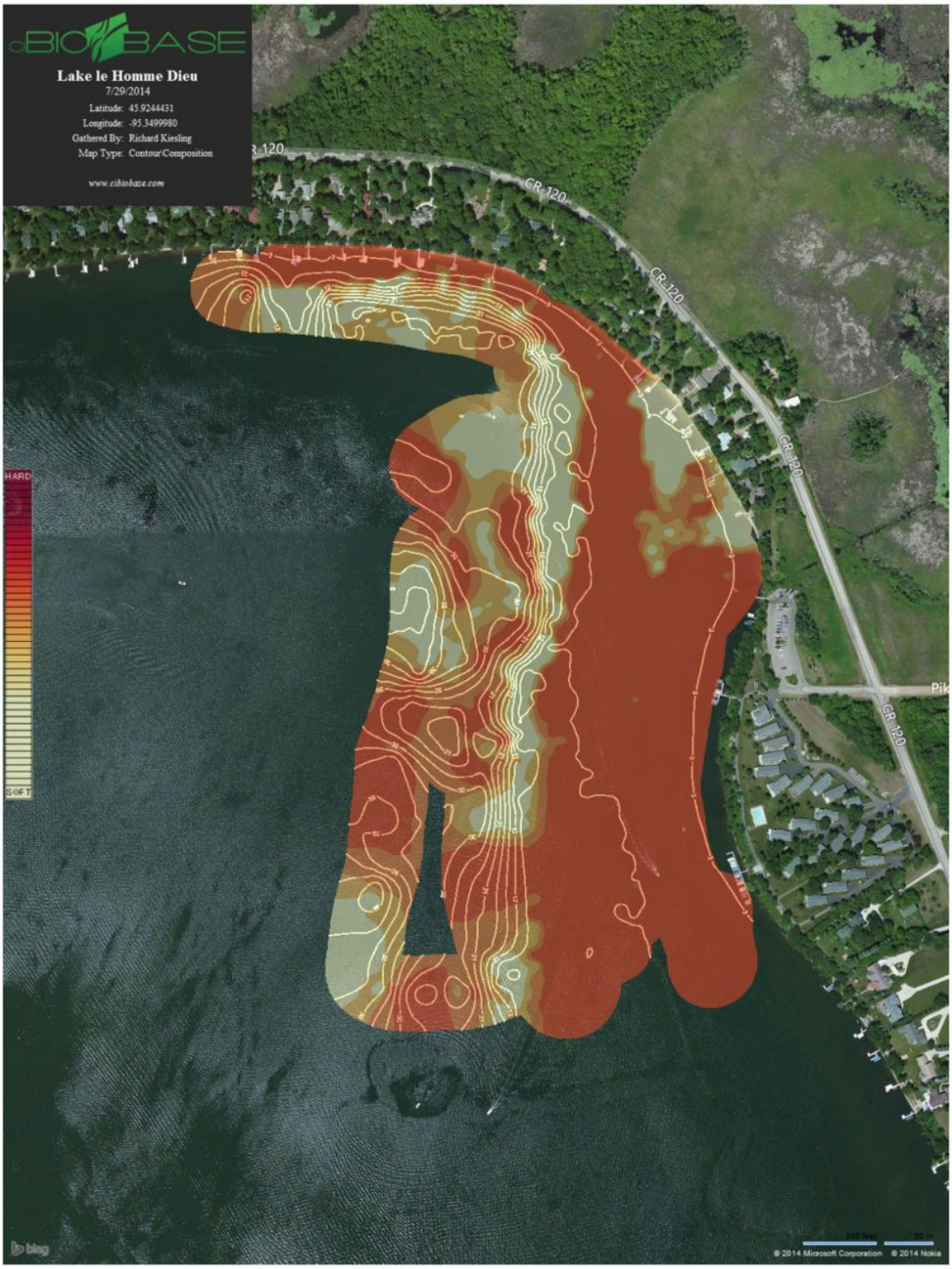


Figure 6. Lake Le Homme Dieu East Launch substrate hardness with three-foot depth contours

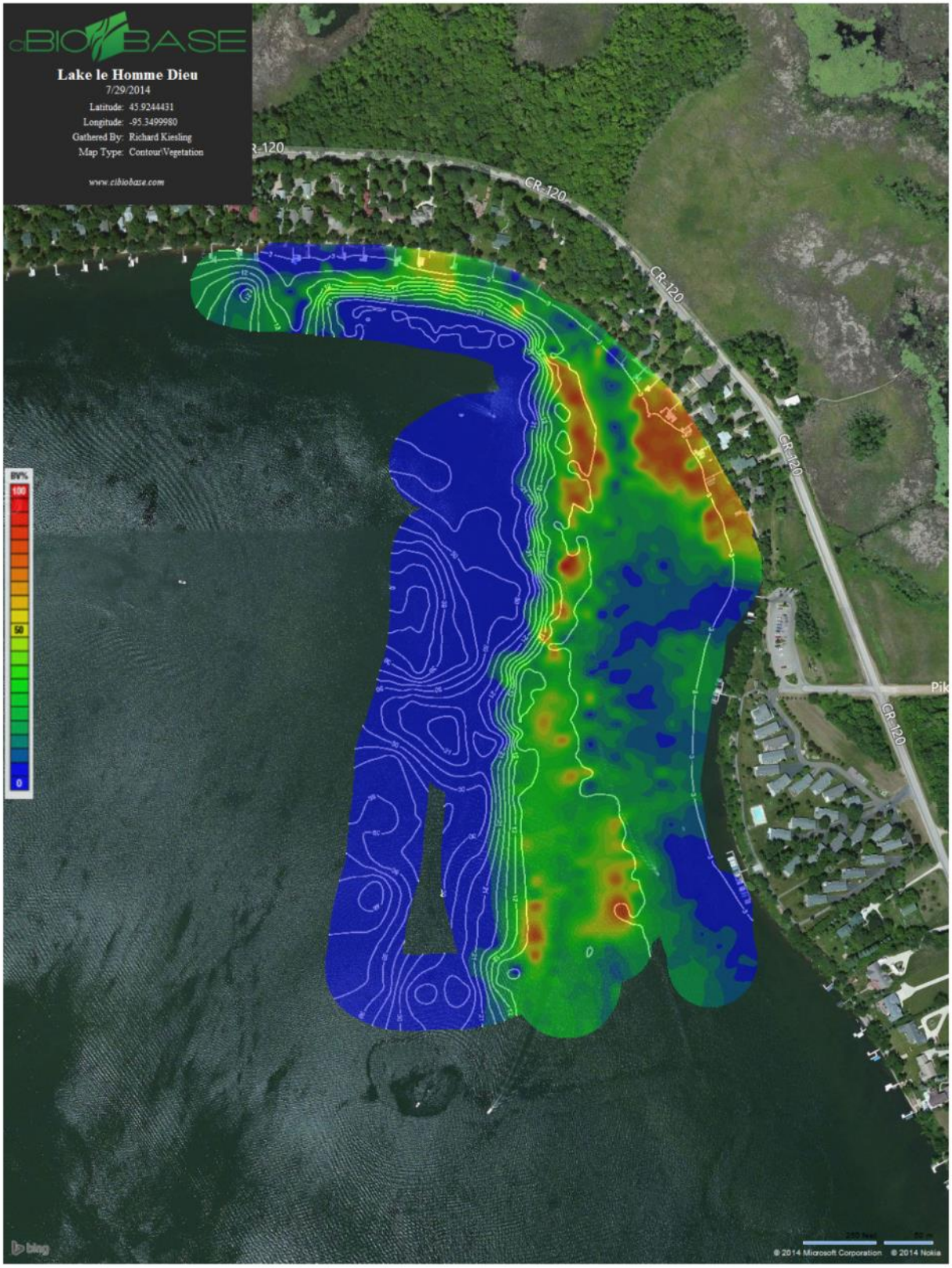


Figure 7. Lake Le Homme Dieu East Launch vegetation biovolume as % of water column

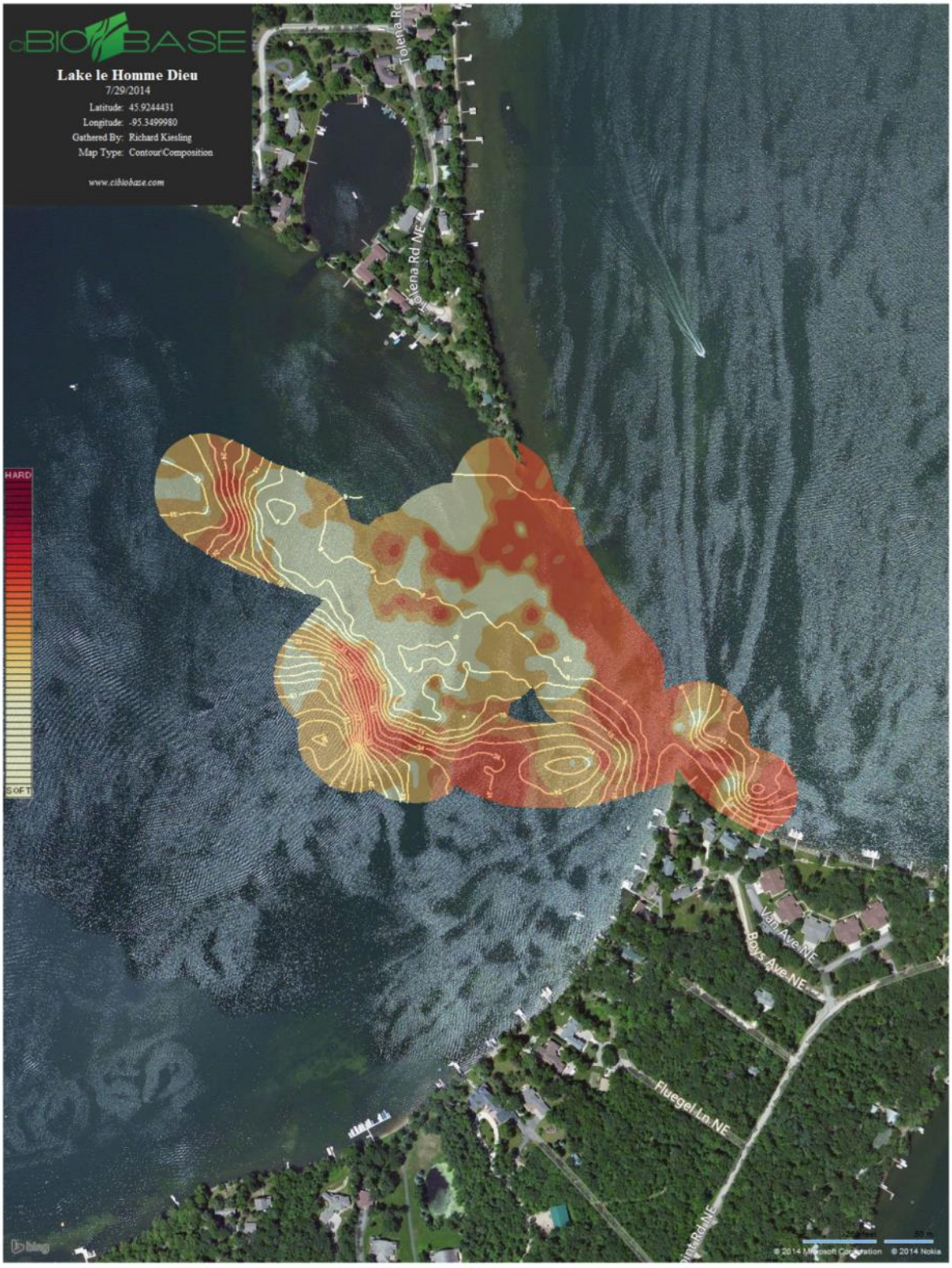


Figure 8. Lake Le Homme Dieu Government Point hardness with three-foot depth contours

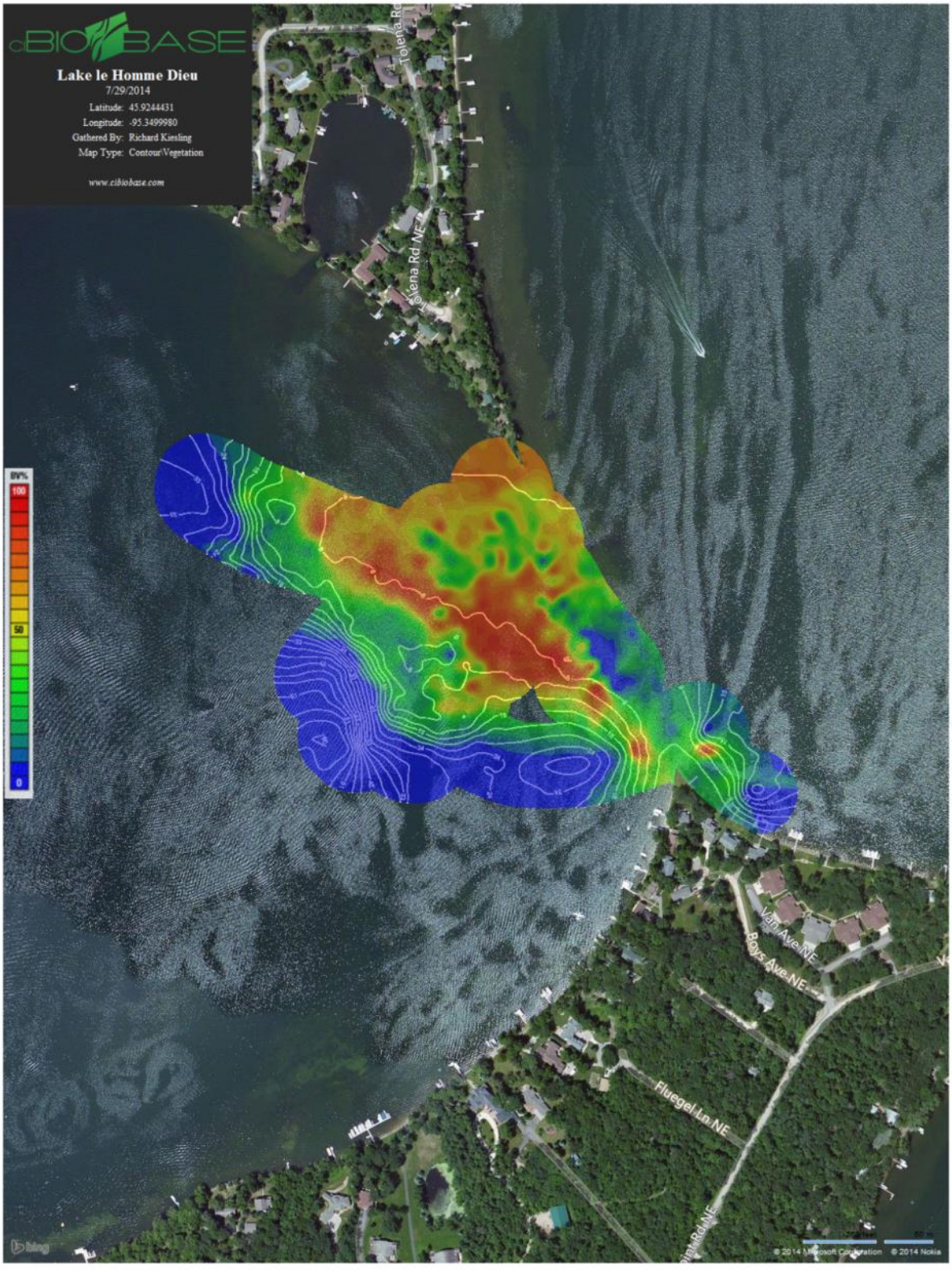


Figure 9. Lake Le Homme Dieu Government Point vegetation biovolume as % water column

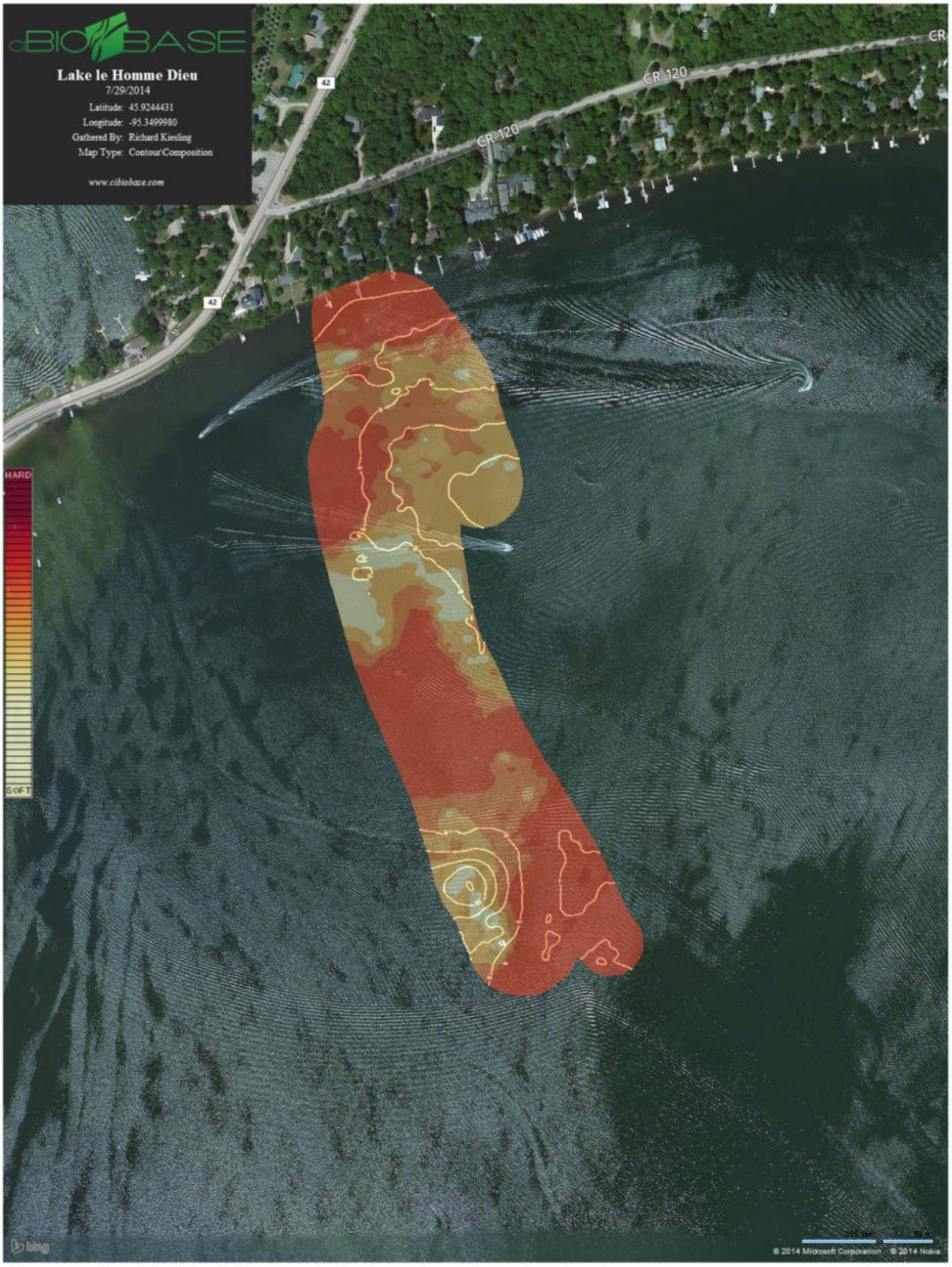


Figure 10. Lake Le Homme Dieu Carlos Pass hardness with three-foot depth contours

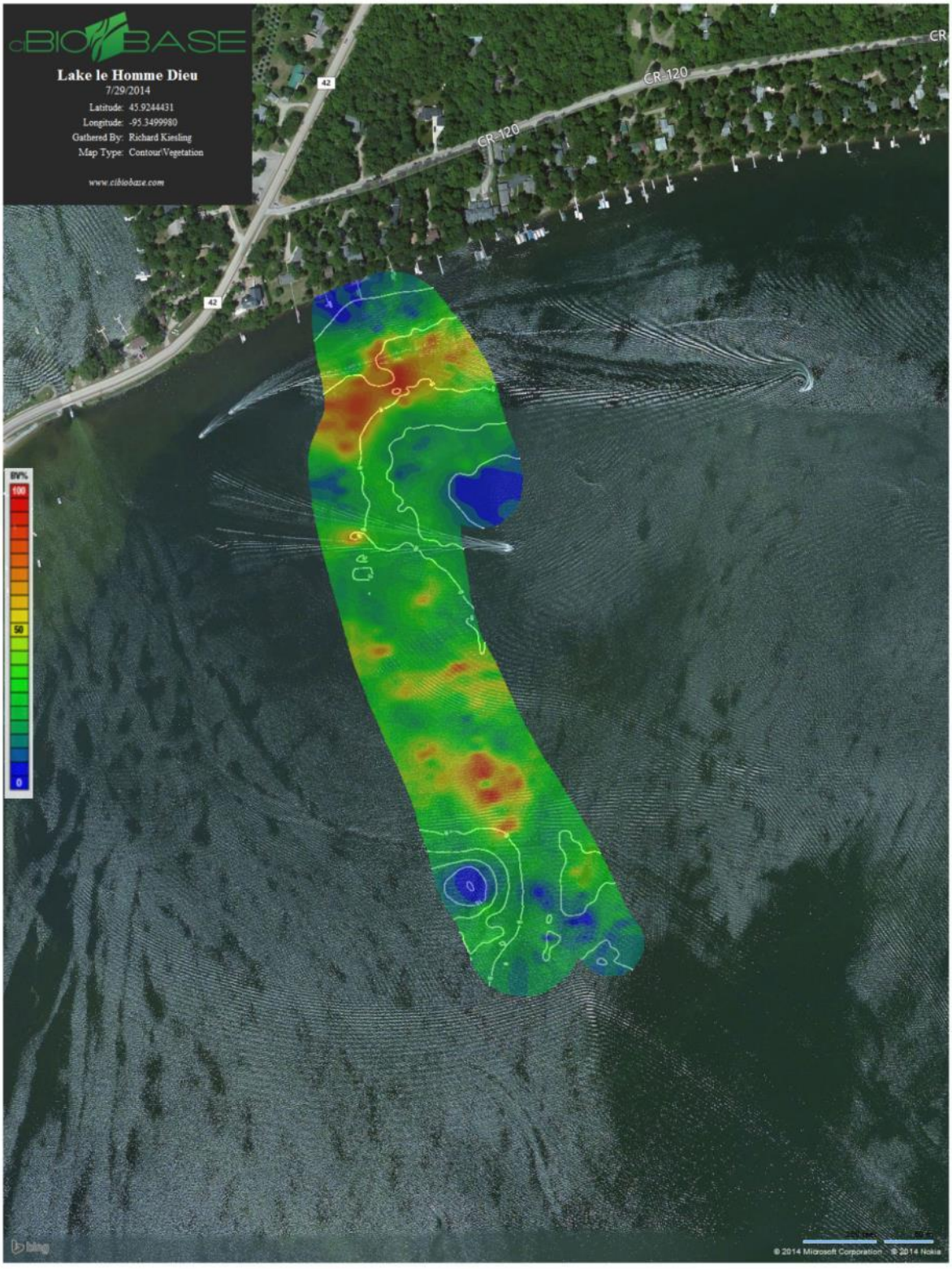


Figure 11. Lake Le Homme Dieu Carlos Pass vegetation biovolume as % water column

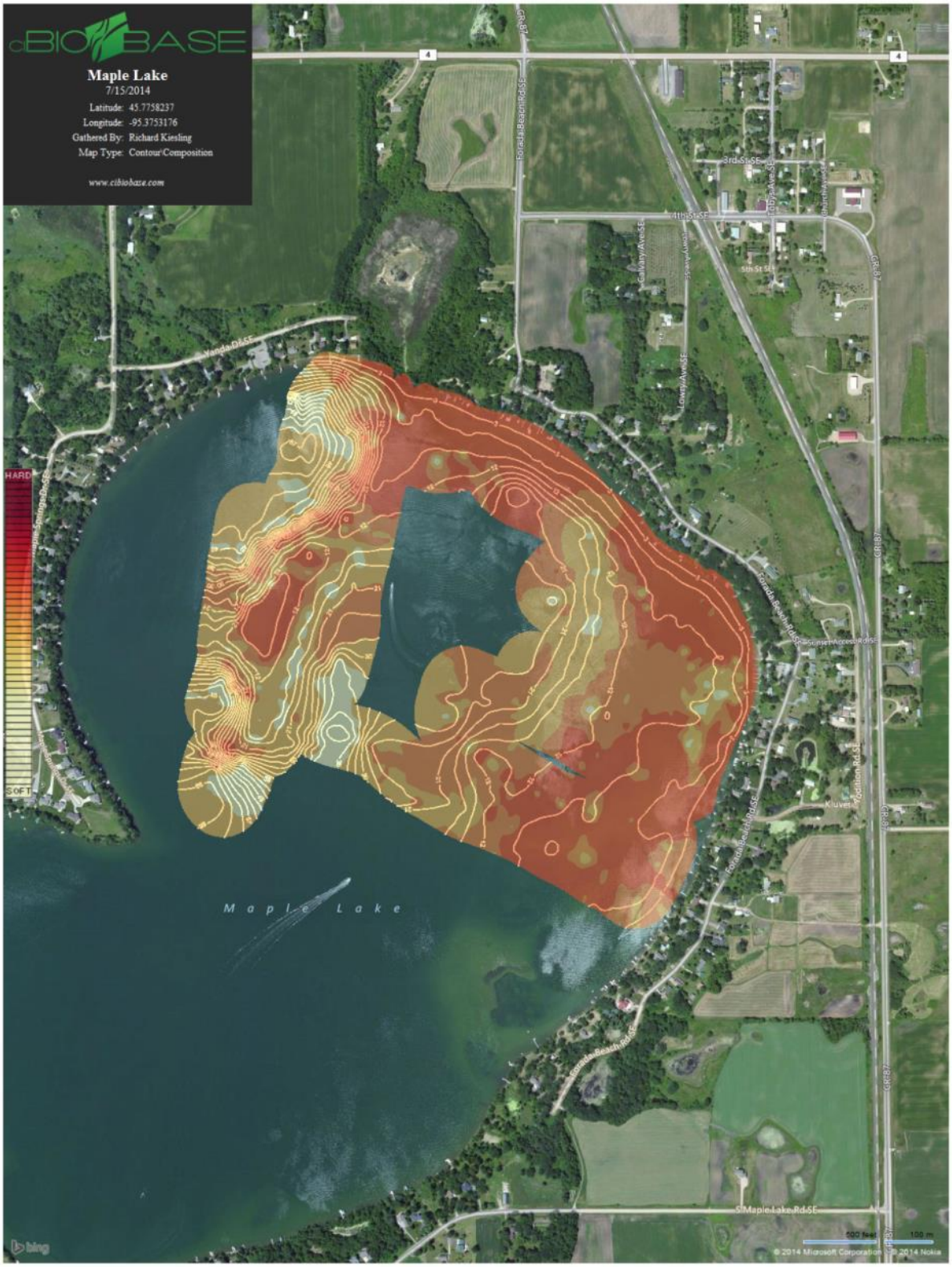


Figure 12. Maple Lake North Launch hardness with three-foot depth contours

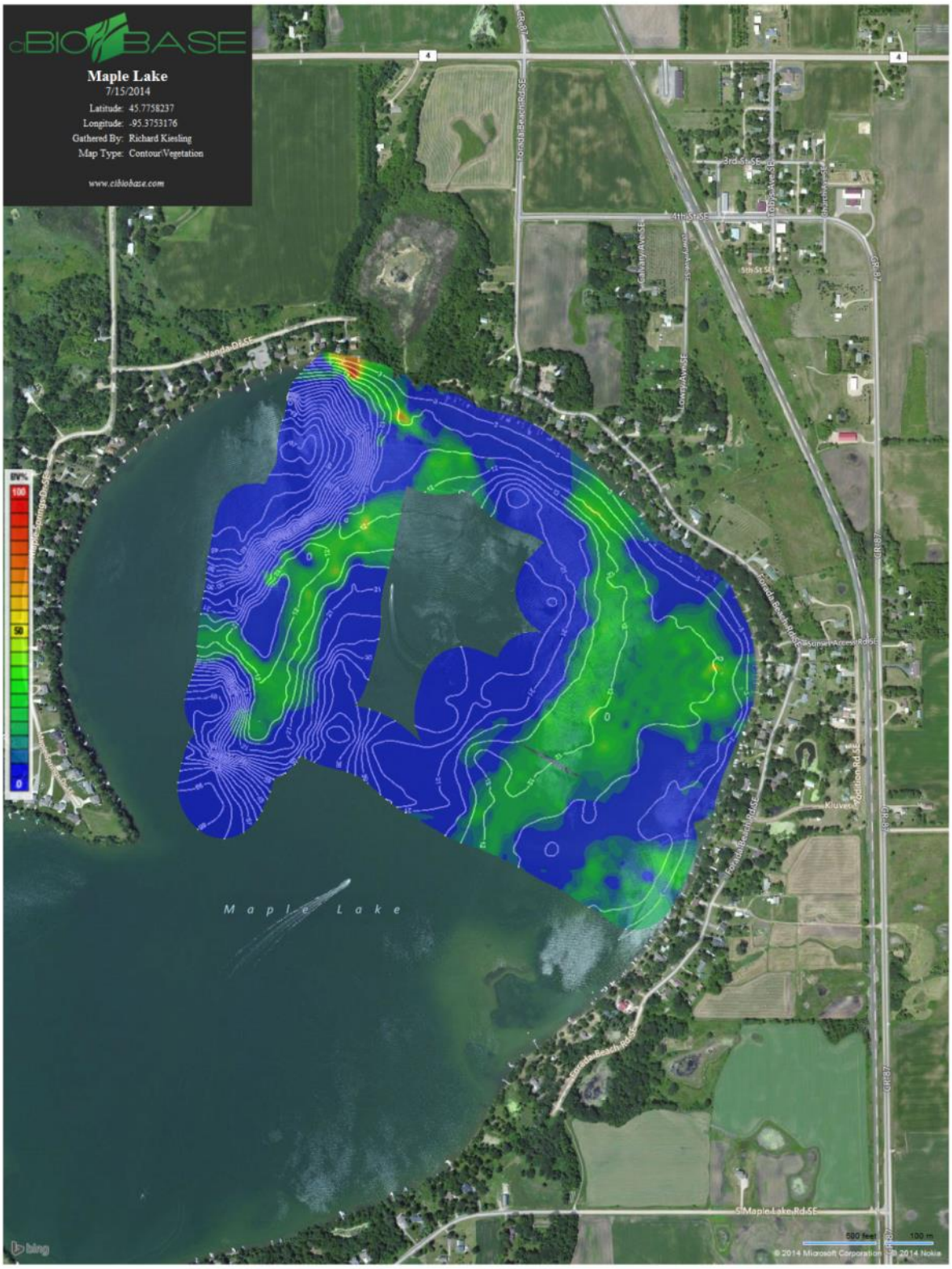


Figure 13. Maple Lake North Launch vegetation biovolume as % water column

Environmental DNA Mapping of Zebra Mussel Populations

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Contents

Figures

Tables

Environmental DNA Mapping of Zebra Mussel Populations

By Jon J. Amberg and Christopher M. Merkes

Abstract

Environmental DNA (eDNA) has become a popular tool for detecting aquatic invasive species, but advancements have made it possible to potentially answer other questions like reproduction, movement, and abundance of the targeted organism. In this study we developed a Zebra Mussel (*Dreissena polymorpha*) eDNA protocol. We then determined if this assay could be used to help determine Zebra Mussel biomass in a lake with a well-established population of Zebra Mussels and a lake with an emerging population of mussels. Our eDNA assay detected DNA of Zebra Mussels but not DNA from more than 20 other species of fish and mussels, many commonly found in Minnesota waters. Our assay did not predict biomass. We did find that DNA from Zebra Mussels accumulated in softer substrates in both lakes, even though the mussels were predominately on the harder substrates. Therefore, we concluded that eDNA may be useful to detect the presence of Zebra Mussels in these lakes but our assay/approach could not predict biomass.

Introduction

Environmental DNA (eDNA) is the detection of DNA shed from an organism from non-biological samples. It has been primarily used to determine the absence and presence of an aquatic invasive species (AIS). While becoming a popular tool for detecting AIS, continued development has advanced the utility of eDNA beyond detection to answer other questions such as reproduction, movement, and abundance of the targeted organism (Erickson et al. 2016).

The invasive Zebra Mussel (Dreissena polymorpha) has invaded much of the Great Lakes Region. Zebra Mussels were first discovered in the Great Lakes Basin in 1988 (Hebert et al. 1989). Since that time, rapidly expanding populations of dreissinid mussels have changed food webs (Holland 1993), primary productivity (Padilla et al. 1996), benthic communities (Ricciardi et al. 1997), spawning habitat (Fitzsimons et al. 1995), nutrient cycling (Qualls et al. 2007), and food availability (Miehls et al. 2009). These impacts threaten the health of native mussels and fish. Besides these ecological impacts, dreissenid mussels have been estimated to cost the US economy billions of dollars (Pimentel et al. 2000; Pimentel et al. 2005).

The Zebra Mussel life-cycle lends them to easily invade new bodies of water, and they have expanded their range to many inland lakes of the upper Midwest. Unfortunately, resource management agencies lack access to effective tools to control dreissenid mussel populations in open waters. There is a need for safe and effective control measures to reduce the environmental and economic impacts of dreissenid mussels. Advancements in eDNA research could lead to a Zebra Mussel eDNA survey tool that not only detects Zebra Mussels but can also be used to help identify sites for control applications.

The goal of our study was to develop and evaluate the use of an eDNA survey to indicate sites within a lake with high numbers of Zebra Mussels. The specific objectives of our study were to: 1) design an eDNA assay for Zebra Mussels, 2) determine an appropriate sampling strategy, and 3) determine if a correlation exists between Zebra Mussel eDNA and substrate, density, and biomass. The development of an eDNA protocol to help inform control applications could improve management of Zebra Mussels and decrease the risk of spread into new waters.

Materials and Methods

Validation of assay specificity

We tested primer sequences for species-specificity in silico using NCBI's Primer-BLAST (Ye et al., 2012), and we found the primer sequences to be specific to the cytochrome c oxidase subunit I gene (coi) of Zebra Mussels with possible amplification from D. presbensis or D. stankovici with 4 primer mismatches each. Both D. presbensis and D. stankovici are found only in the Balkan Region of Europe and have not been found in North America. We then designed a Zebra Mussel amplicon-specific minor groove binder probe with 2 mismatches to both species. Oligonucleotide sequences used are in Table 1. We also tested assay specificity in vitro against genomic DNA from Zebra Mussels and 27 non-target species (Table 2). Genomic DNA was tested in two replicate reactions each as described below.

Table 1. Oligonucleotide sequences of primers, probe and targeted region of the genomes.

Oligonucleotide	Sequence
Dre2-F	TGGGCACGGGTTTTAGTGTT
Dre2-R	CAAGCCCATGAGTGGTGACA
Dpo-Probe	6FAM-CGTCCTTGGTG
Dpo-gBlock	TGTGGGCTGGCCTTGTGGGCACGGGTTTTAGTGTTCTTATTC GTTTAGAGCTAAGGGCACCTGGAAGCGTCCTTGGTGATTG TCAATGATATAATGTAATTGTCACCACTCATGGGCTTGTTA TAATTGTTTGTCTAG

Table 2. Specificity of Zebra Mussel marker against genomic DNA from various aquatic species, many common to Minnesota waters. Positive symbol (+) indicates amplification and negative symbol (–) indicates no amplification.

Species	Result	Species	Result
Zebra Mussel (Dreissena polymorpha)	+	Speckled Dace (Rhinichthys osculus)	-
Plain pocketbook mussel (Lampsilis cardium)	-	Bluehead Sucker (Catostomus discobolus)	-
Black sandshell mussel (Ligumia recta)	-	Channel Catfish (Ictalurus punctatus)	-
Bighead Carp (Hypophthalmichthys nobilis)	-	Largemouth Bass (Micropterus salmoides)	-
Silver Carp (Hypophthalmichthys molitrix)	-	Rainbow Trout (Oncorhynchus mykiss)	-
Grass Carp (Ctenopharyngodon idella)	-	Brown Trout (Salmo trutta)	-
Black Carp (Mylopharyngodon piceus)	-	Lake Trout (Salvelinus namaycush)	-
Common Carp (Cyprinus carpio)	-	Brook Trout (Salvelinus fontinalis)	-
Gizzard Shad (Dorosoma cepedianum)	-	Bluegill (Lepomis macrochirus)	-
Fathead Minnow (Pimephales promelas)	-	Yellow Perch (Perca flavescens)	-
Mosquitofish (Gambusia affinis)	-	Lake Sturgeon (Acipenser fulvescens)	-
Emerald Shiner (Notropis atherinoides)	-	Pallid Sturgeon (Scaphirhynchus albus)	-
Golden Shiner (Notemigonus crysoleucus)	-	Tilapia (Oreochromis aureus x Oreochromis niloticus hybrid)	-
Spotfin Shiner (Cyprinella spiloptera)	-	Paddlefish (Polyodon spathula)	-

Developing sampling protocol

We sampled Lake Minnetonka to develop a sampling protocol. Water was collected from the surface, mid-water and near the bottom directly above a known colony of Zebra Mussels. Ten 50-mL sterile conical tubes were placed just below the water surface to collect the surface film. Mid-water samples were collected using a 2.2 L horizontal Van Dorn water sampler. The water sampler was lowered to mid-depth and sealed. Ten 50 mL water samples were collected from the water sampler. The bottom samples were collected using a separate 2.2 L horizontal Van Dorn water sampler. This water sampler was lowered to 9 cm above the bottom where the water was collected and brought to the surface. Again, ten 50 mL water samples were collected from the sampler. Once each sample was collected it was capped and stored on ice. All samples were transported to the U.S. Geological Survey Upper Midwest Environmental Sciences Center in La Crosse, Wisconsin (UMESC) for further processing. DNA was extracted from individual samples and quantified using the procedure mentioned below.

Correlations among eDNA, biomass, and substrate type

To determine if a correlation exists between Zebra Mussel DNA and substrate, density, and biomass, we sampled water from two lakes, Lake Le Homme Dieu and Maple Lake, near Alexandria, Minnesota. Lake Le Homme Dieu is approximately 728 ha with a maximum depth of 26 m. This lake has had Zebra Mussels present since 2009 (Cha et al. 2013) and was chosen to represent a lake with a well-established population with a mean shell length of 0.82 ± 0.11 cm. Maple Lake is 330 ha with a maximum depth of 24 m. According the Minnesota Department of Natural Resources, Maple Lake was found to have Zebra Mussels in 2013 and was chosen to represent a lake with an emerging population with smaller mussels; 0.65 ± 0.14 cm mean shell length.

At each lake, we collected water from 9 cm above the bottom using a 2.2 L horizontal Van Dorn water sampler in triplicate according to the method established above. Samples were collected at depths of approximately 1, 2, 4 and 6 m along four transects in each lake. We tried to follow transects that covered different substrate types from loose flocculent to cobble in each lake. Immediately following water sampling at each sample point, we placed a brick, tied to a buoy, and recorded GPS coordinates for subsequent samplings at the same location. Each lake was sampled twice; first September 29 – 30, 2014 and again under ice March 9 – 10, 2015 (Figure 1).

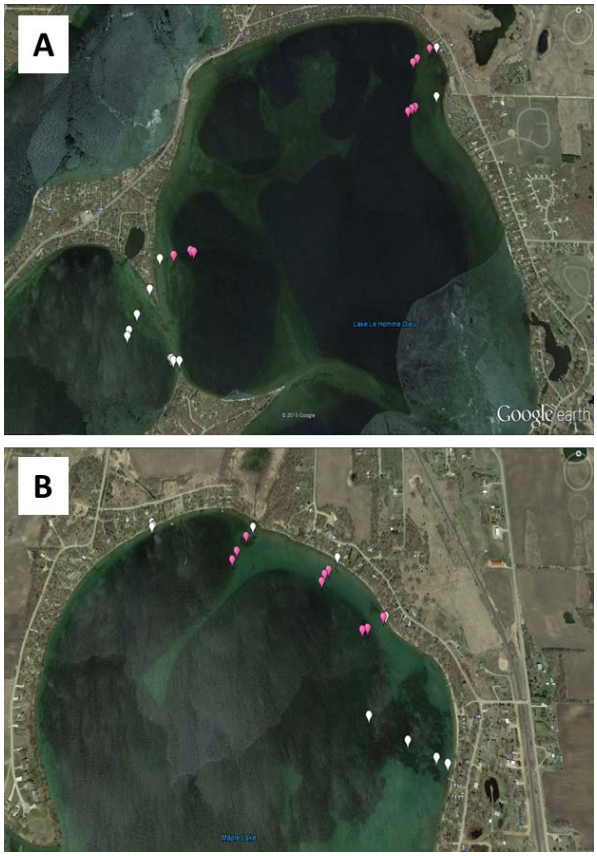

Figure 1. Sampling locations on Lake Le Homme Dieu (A) and Maple Lake (B) near Alexandria, Minnesota. All locations marked indicate where samples were collected. Locations marked in pink represent the locations where samples were also collected in March, whereas locations indicated white are sites where samples

The day following water sampling in September, we used SCUBA divers to collect all the Zebra Mussels in three 0.25 m2 quadrants near each brick. Zebra Mussels from each quadrant were brought to the surface and placed into separate plastic storage containers and placed on wet ice. All Zebra Mussel samples were frozen (-20°C) within 4 h of collection. SCUBA divers also verified substrate at each sampling location.

To estimate biomass, we calculated the ash-free dry weight (AFDW) for each Zebra Mussel sample (Wetzel et al. 2005). Each Zebra Mussel sample was weighed to determine total wet-weight. The moisture content and dry weight (DW) was determined according to AOAC Official Method 934.01 and subsequently ash weight (AW) was determined according to AOAC Official Method 942.05. Both moisture content, dry weight and ash weight analyses were conducted by the University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO). AFDW was calculated subtracting AW from DW for the subsample and adjusting to the mass (wet-weight) of the whole sample.

DNA extraction and amplification

We centrifuged the 50 mL water samples at 5,000 x g for 30 minutes and decanted the supernatant. We extracted DNA from the remaining pellet and residual water using the commercially available gMax mini genomic DNA extraction kit following the manufacturer's recommendations (IBI Scientific; Peosta, IA). We extracted 100 µL of deionized water as an extraction negative control with each extraction batch, and all samples had a final elution volume of 100 µL. We analyzed the DNA extracts in four replicate qPCRs with 1 µL of template in 20 µL reactions. Reactions contained 1x SensiFAST probe – no rox master mix (Bioline; Taunton, MA), 200 nM forward and reverse primers, and 125 nM probe. Oligonucleotide sequences are listed in Table 1. We analyzed with the temperature profile of: 95°C for 2 minutes; followed by 45 cycles of 95°C for 30 seconds, 56°C for 1 minute, 72°C for 50 seconds; followed by 72°C for 5 minutes; and a hold at 4°C. We ran each plate on a Mastercycler Realplex 2 thermal cycler (Eppendorf North America; Hauppauge, NY) with four no template controls and two replicate standard curves. The standard curves contained gBlock gene fragment synthetic DNA of the target sequence (Integrated DNA Technologies; Coralville, IA) in a 5-fold dilution series from 31,250 copies down to 10 copies per reaction.

Analysis

We used Pearson product-moment correlation coefficient as a measure of the linear correlation between the following variables: depth, substrate type, detections, AFDW, fall DNA copies and winter DNA copies. Substrate type was divided into six categories based on the coarseness of the material: 1) flocculent, 2) silt, 3) muck mixture, 4) sand, 5) predominately shell and 6) cobble/stones. We compared each lake separately because of the known differences in Zebra Mussel populations. All analyses were performed using SigmaPlot® 13.0 (Systat Software, Inc., San Jose, CA USA) with a significance level of $\alpha \leq 0.05$.

Results and Discussion

Validation of molecular assay

Our primer-BLAST results predicted the sequences to specifically amplify a target region in the cytochrome c oxidase subunit I gene (*coi*) of Zebra Mussels with possible amplification from *D. presbensis* or *D. stankovici* with 4 primer mismatches each. Both *D. presbensis* and *D. stankovici* are found only in the Balkan Region of Europe and have not been found in North America. We validated the specificity of our assay against genomic DNA from Zebra Mussels and 27 non-target species listed in Table 2 with two replicate reactions. Our markers only detected DNA from Zebra Mussels and no detections were observed in any of the native mussels or fish species tested. Therefore, we concluded that this marker was adequate for detecting the presence of Zebra Mussel DNA in Minnesota waters.

Development of sampling protocol

We determined the amount of Zebra Mussel DNA in each of the 10 water samples from three depths in Lake Minnetonka: surface, mid-column, and benthic. Water samples collected near the bottom or at the surface had a 100% detection rate while mid-column samples had an 85% detection rate. Samples collected near the bottom had slightly lower C_t-values than those from samples collected from the surface (Figure 2). This indicates that benthic samples contained slightly more Zebra Mussel DNA. However, both surface and benthic samples had significantly lower C_t-values indicating significantly more Zebra Mussel DNA than mid-column samples (Figure 2).

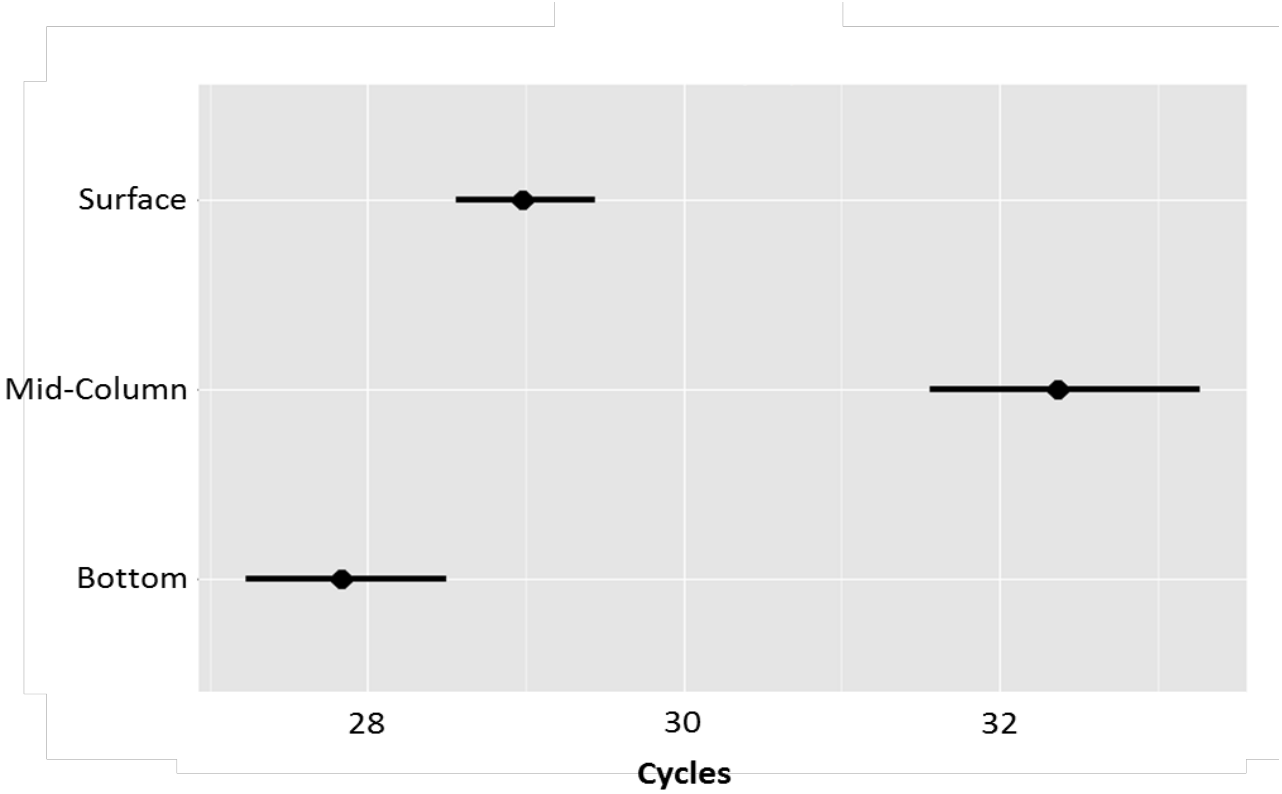

Figure 2. The mean number of cycles needed to detect DNA of Zebra Mussels from water samples collected at the surface, mid-column and bottom of Lake Minnetonka directly above a known Zebra Mussel population. The lower the number of cycles indicates a greater amount of DNA. Bars represent the 95% confidence intervals.

Correlations among eDNA, biomass, and substrate type

Lake Le Homme Dieu (established population)

As expected, the coarser substrates (cobble) were generally found in the shallower waters, while finer substrates (flock) were located at the deeper sampling sites (Table 3). AFDW decreased with increased depth ($r = -0.266$, $p = 0.040$). No correlation between AFDW and substrate type was found ($r = 0.243$, $p = 0.061$) and there was no correlation between AFDW and the number of copies of Zebra

Mussel DNA for both fall and winter (Table 3). Thus, eDNA copy numbers were not found to accurately predict the biomass of Zebra Mussels in this lake that has an established population of Zebra Mussels. The number of positive detections was negatively correlated with substrate type ($r = -0.264$, $p = 0.041$). The results suggest that there is a higher probability of detecting Zebra Mussel DNA in areas that have softer substrates in lakes were the mussel population is well established but that eDNA copy counts do not correlate with the mass of animals within the area.

Table 3. Pearson product-moment correlation coefficients between depth, substrate type, detections, AFDW, fall DNA copies and winter DNA copies for Lake Le Homme Dieu. Values represent the correlation coefficient (top), P-value (middle), and number of samples (bottom) for each comparison.

	Substrate	Detections	Fall DNA copies	Winter DNA copies	AFDW
Depth	-0.458 <0.001 60	0.265 0.041 60	0.249 0.055 60	0.216 0.253 30	-0.266 0.040 60
Substrate		-0.264 0.041 60	-0.273 0.035 60	0.181 0.338 30	0.243 0.061 60
Detections			0.202 0.121 60	0.105 0.582 30	-0.157 0.230 60
Fall DNA copies				0.018 0.924 30	-0.119 0.366 60
Winter DNA copies					-0.119 0.530 30

Maple Lake (emerging population)

Substrate and depth in Maple Lake was similar to that of Lake La Homme Dieu (Table 4). AFDW decreased with increased depth ($r = -0.366$, $p = 0.041$). There was a significant correlation between AFDW and substrate type ($r = 0.424$, $p < 0.001$). Like Lake Le Homme Dieu, no correlation was determined between AFDW and the number of Zebra Mussel DNA copies (Table 4), which suggests that DNA copy numbers cannot accurately predict the biomass of Zebra Mussels in a lake. Unlike Lake Le Homme Dieu, no correlation was found between the number of detections for a sample and substrate type in Maple Lake ($r = 0.212$, $p = 0.104$). This indicates an equal probability of detecting Zebra Mussel DNA in areas with soft substrates as those with harder substrates.

Table 4. Pearson product-moment correlation coefficients between depth, substrate type, detections, AFDW, fall DNA copies and winter DNA copies for Maple Lake. Values represent the correlation coefficient (top), P-value (middle), and number of samples (bottom) for each comparison.

	Substrate	Detections	Fall DNA copies	Winter DNA copies	AFDW
Depth	-0.520 <0.001 60	-0.310 0.016 60	-0.019 0.884 60	-0.334 0.088 27	-0.366 0.004 60
Substrate		0.212 0.104 60	-0.030 0.822 60	0.208 0.297 27	0.424 <0.001 60
Detections			0.210 0.107 60	0.419 0.030 27	0.184 0.158 60
Fall DNA copies				0.642 <0.001 27	-0.057 0.668 60
Winter DNA copies					-0.077 0.702 27

Table 5. Mean number of positive detections and copies of Zebra Mussel DNA, as well as ash-free dry weight (AFDW) of Zebra Mussels for six sediment types in Lake La Homme Dieu and Maple Lake near Alexandria, Minnesota. Number in parentheses represent standard deviations.

	Detections	DNA copies	AFDW (g)
Lake La Homme Dieu			
Flock	3.80 (0.41)	110.18 (200.95)	0.93 (1.80)
Silt	4.00 (< 0.01)	27.41 (33.11)	0.24 (0.44)
Muck mixture	3.44 (0.92)	45.72 (87.07)	1.39 (1.43)
Sand	3.56 (0.53)	32.26 (82.71)	28.13 (32.76)
Shells	3.17 (1.17)	6.96 (8.41)	0.19 (0.19)
Cobble	3.33 (0.82)	8.77 (4.40)	9.16 (14.90)
Maple Lake			
Flock	1.47 (1.73)	4.26 (7.09)	0.01 (0.01)
Silt	0.83 (1.60)	1.40 (3.38)	0.01 (0.02)
Muck mixture	2.13 (1.51)	1.83 (2.05)	0.11 (0.17)
Sand	2.00 (1.00)	1.38 (.049)	0.22 (0.17)
Shells	2.89 (1.76)	23.10 (26.39)	0.03 (0.04)
Cobble	1.92 (1.50)	1.54 (2.28)	1.06 (1.35)

Conclusion

We developed and validated a molecular assay that detects the presence of Zebra Mussels in a body of water. Zebra Mussel DNA did not correlate with biomass. DNA from Zebra Mussels accumulates in softer substrates in lakes as the mussels become established (Table 5). In lakes with an emerging population, sampling water near harder substrates will provide the greatest probability of detecting the presence of Zebra Mussels. In this study, we demonstrated that eDNA may be useful to detect the presence of Zebra Mussels in a lake but that currently available approaches are not able to correlate DNA copy number with biomass.

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Development of Targeted Delivery Techniques for Zequanox®

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Contents

Figures

Tables

Development of Targeted Delivery Techniques for Zequanox®

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Abstract

The effects of water temperature and concentration on the physical characteristics of Zequanox®, a dead-cell spray-dried powder formulation of *Pseudomonas fluorescens* (strain CL145A) used for controlling invasive dreissenid mussels (zebra mussel, *Dreissena polymorpha,* and quagga mussel, *Dreissena bugensis*), were investigated to determine optimal temperature-specific concentrations and delivery techniques for use during open-water subsurface Zequanox applications. Temperature-controlled laboratory tests evaluated viscosity, settling, stratification, and buoyancy of various concentrations of Zequanox suspension in water to select an optimal target viscosity for Zequanox applications. A two-step linear regression procedure was used to create a temperature-specific Zequanox prediction model from the viscosity data. The prediction model and subsurface application techniques were validated by conducting three independent outdoor pond trials at temperatures of ~9, 14, and 20°C. During these outdoor trials, subsurface applications of Zequanox at concentrations predicted by the model were performed and water samples were collected at varying depths and analyzed via spectroscopy to determine Zequanox concentration and dispersion. Although the predicted Zequanox concentrations and delivery techniques used resulted in successfully maintaining lethal Zequanox concentrations in the bottom 7.5 cm of the water column for the duration of the exposure, a revised prediction model is also provided for more accurately selecting temperature-specific Zequanox concentrations.

Introduction

Dreissenid mussels, including the zebra mussel, *Dreissena polymorpha*, and the quagga mussel, *Dreissena bugensis,* are bivalves native to the Black, Caspian, and Azov Seas of the Ponto-Caspian region of Eurasia (Spidle et al., 1994; Gollasch and Leppäkoski, 1999). Invasive zebra mussels and quagga mussels were introduced to North America in the mid to late 1980s (Roberts, 1990; Spidle et al., 1994), likely as free swimming veligers discharged in ballast waters of oceanic freight ships (Griffiths et al., 1991). Adult zebra mussels were first discovered in 1988 on Lake St. Clair (Hebert et al., 1989) and they have continued a rapid invasion throughout North American waterways. Dreissenid mussels are highly efficient invaders because of their high reproductive fecundity, planktonic larval dispersal, ability to attach to most surfaces by the use of byssal threads (Birnbaum, 2011), lack of major ecological constraints, and new infestations being aided by anthropogenic means (Gollasch and Leppäkoski, 1999; Ludyanskiy et al., 1993). Severe biofouling is an economically harmful characteristic of dreissenid

[1] U.S. Geological Survey.

mussels that has been responsible for significant financial costs to many industries located on infested waterways (Ludyanskiy et al., 1993). Epizoic colonization of already imperiled native unionid species is one of the greatest ecological effects of dreissenid mussels (Hebert et al., 1989; Schloesser et al., 1996). Currently, eradication of established populations of dreissenid mussels in large systems is not feasible, but implementation of an Integrated Pest Management (IPM) program that combines physical, mechanical, biological, chemical, and cultural control mechanisms could be used to decrease the ecological and economic impacts of this invader (Culver et al., 2013). One product that could be incorporated into an IPM program for controlling dreissenid mussels is Zequanox®, a dead-cell spray-dried powder formulation of *Pseudomonas fluorescens* (strain CL145A). The New York State Museum Field Research Laboratory first isolated *Pseudomonas fluorescens*, strain CL145A, and discovered that when ingested by dreissenid mussels, it induces mortality by degrading the epithelial cells within the mussel's digestive system (Molloy et al., 2013). Marrone Bio Innovations (Davis, CA) acquired the rights to *Pseudomonas fluorescens*, strain CL145A, and developed the product, Zequanox, which was registered by the U.S. Environmental Protection Agency for controlling dreissenid mussels in defined discharge water systems in 2012 and for open-water use in 2014 (EPA Reg. No. 84059-15). Previous research investigated the use of Zequanox in laboratory and field settings for dreissenid mussel control (Luoma et al., 2015a; Luoma et al., 2015c) and non-target animal exposure-related impacts (Luoma et al., 2015b; Luoma et al., 2015d). The work conducted by Luoma et al. (2015a) demonstrated the potential for subsurface Zequanox applications by conducting successful applications in 350-L tanks; however, the effects of temperature and concentration on the dispersion of Zequanox suspensions were not investigated in that study.

Subsurface Zequanox applications are desirable because they would (1) significantly reduce the amount of Zequanox required for treatment, subsequently reducing cost; (2) decrease the potential for exposure, reducing risk to non-target species; and (3) decrease nutrient input inherently related to the Zequanox product. Fundamental challenges related to subsurface Zequanox applications include the potential for premature Zequanox migration/dilution and the significant impact of water temperature on the viscosity of Zequanox suspensions. Therefore, the present study was conducted in an attempt to create a standardized procedure for selecting the appropriate concentration of Zequanox in suspensions created for subsurface applications over a range of water temperatures. This included conducting a series of temperature-controlled laboratory tests that evaluated viscosity, settling, stratification, and buoyancy of various concentrations of Zequanox at different water temperatures. Results of these laboratory tests were used to estimate the optimal Zequanox suspension viscosity for subsurface application at a range of environmental temperatures. The laboratory-derived, temperature-dependent Zequanox concentration selection protocol was then evaluated by conducting a series of outdoor tests at three different environmental temperatures.

Study Overview

Laboratory

Laboratory tests were conducted in an environment-controlled chamber at four temperatures (7, 12, 17, and 22°C). At each temperature, four Zequanox suspensions (ranging from 5–25% w/v) were evaluated to determine (1) the effects of temperature and (2) the effects of Zequanox concentration on the viscosity, settling, stratification, and buoyancy of Zequanox suspensions. The Zequanox suspensions were prepared in well water by mixing with a household immersion blender and the viscosity of the suspensions were measured using cup viscometers.

Zequanox suspensions were injected with 1-mL syringes into a series of twelve 100-mL graduated cylinders (four Zequanox suspensions X three replicates) and initial observations of air entrainment were followed by observations of settling and stratification throughout the 8-hour exposure period. The laboratory observations were used to determine a target Zequanox suspension viscosity for subsurface applications. A two-step linear regression procedure was used to predict temperature-concentration combinations that would result in the target Zequanox suspension viscosity at water temperatures ranging from 7 to 22°C.

Pond

The laboratory-derived, temperature-concentration prediction model was verified by conducting a series of replicated outdoor pond tests at three water temperatures (~9, 14, and 20°C). Zequanox suspension concentrations were selected from the prediction model and the prepared suspensions were applied to three replicated 9-m^2 test enclosures that were placed in 0.004-ha concrete ponds. Water samples were collected from three depths (7.5, 30, and 60 cm from pond bottom) in each enclosure 1, 2, 4, and 8 hours after Zequanox application and analyzed via spectroscopy to determine Zequanox concentrations. Zequanox concentrations were compared by sampling depth using a General Linear Model procedure in SAS version 9.3.

Materials and Methods

Test Article

The test article was Zequanox®, a commercially available spray-dried powder formulation of *Pseudomonas fluorescens*, strain CL145A (*Pf*-CL145A) produced by Marrone Bio Innovations (Davis, CA). Zequanox is formulated to contain 50% weight to weight *Pf*-CL145A as the active ingredient (A.I.). The test article, lot number 401P130918C, was expired for use in dreissenid mussel control applications; however, the physical characteristics of the test article are not affected by biological activity and therefore the test article was deemed acceptable for use in this study (Megan Weber, Zequanox Product Development Manager at MBI, pers. comm., 2014). Concentrations of test article in suspensions are reported as % weight to volume (w/v) and spectroscopy measurements were conducted and are reported based on mg A.I./L.

Test Systems

Laboratory

Laboratory tests were conducted within a 22-m^2 environmental chamber at the Upper Midwest Environmental Sciences Center (UMESC), which maintained the temperature ±1°C. Twelve 100-mL polymethylpentene graduated cylinders (Fisher Scientific, Hampton, NH, cat. no. 03-007-33) filled with temperature-appropriate well water were used as the observation system. The test article application system consisted of disposable 1-mL syringes (Becton, Dickinson and Co., Franklin Lakes, NJ, mfr. no. BD309628) held at a fixed height in the water column by positioning the syringe in an acrylic mounting block, which was placed on top of the graduated cylinders (Fig. 1).

Viscosities of Zequanox suspensions were measured with cup viscometers (Cole-Parmer Viscosity Cups #1, 3, 4, and 5, Cole-Parmer Instrument Co., Vernon Hills, IL; EZ Zahn Viscosity Cup #2, Gardco, Pompano Beach, FL).

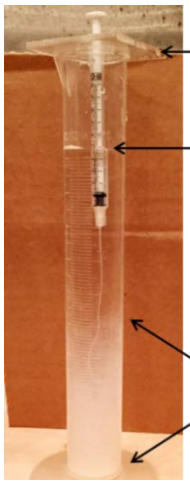

Figure 1. An example of laboratory test system replicate showing Zequanox stratification.

Pond

Pond tests were conducted in replicate 9-m^2 test enclosures positioned in independent 0.004-ha concrete ponds (Fig. 2a). The enclosures were assembled by interconnecting four welded aluminum frame panels (3.0 x 1.8 m, L x H) that were covered with an impermeable 30-mil ethylene propylene diene monomer (EPDM) pond liner membrane. Panels were constructed with 0.3-m EPDM bottom-sealing skirts that contained ballast chain and each assembled enclosure had multiple sand bags (n = 16, ~12 kg each) placed on the skirts to aid in creating the bottom seal.

Water sampling systems were constructed for each enclosure and consisted of peristaltic pumps fitted with dual channel pump heads (Masterflex Digi-Staltic pump drive, model 77310-01 and Easy-Load II, model 77202-60, respectively, Cole-Parmer Instrument Co., Vernon Hills, IL), peristaltic tubing (Masterflex Silicone Tubing L/S 16, item no. EW-96400-16, Cole-Parmer Instrument Co., Vernon Hills, IL), vinyl airline tubing (4.8-mm inside diameter [ID]), and positioning rods (3 vertical aluminum rods [0.95-cm diameter] welded to a horizontal 1.5-m piece of aluminum angle [5.1 x 5.1 cm]) that were used to secure the tubing at the desired sampling locations and depths (7.5, 30, and 60 cm from bottom) for a total of nine sampling locations within each pond (Fig. 2b).

Zequanox delivery systems, with similar construction to water sampling systems, were used to apply Zequanox to each enclosure replicate. Peristaltic pumps were calibrated to deliver 40 mL/minute/tube and used to deliver Zequanox to 16 locations in each enclosure (Fig. 2b). The Zequanox was pumped through tubing that terminated 90 cm from pond bottom with T-shaped hose barb fittings mounted to aluminum positioning rods (four stands, each constructed from four vertical aluminum rods welded to a horizontal 1.8-m length of aluminum angle).

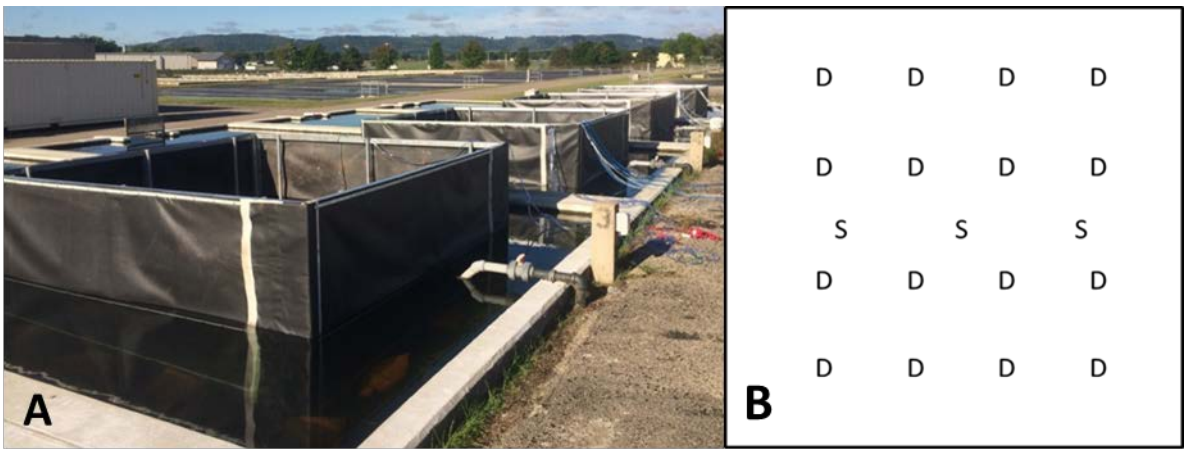

Figure 2. Test enclosures in outdoor concrete pond complex (A, left); plan view of treatment enclosure with delivery (D) and sampling (S) apparatus placement (B, right). Each delivery point terminates 90 cm from pond bottom, whereas each sampling point terminates 7.5, 30, and 60 cm from pond bottom.

Test Article Applications, Observations, and Data Analysis

Laboratory

At each of four test temperatures, a series of twelve 100-mL graduated cylinders were filled with 100 mL of temperature-acclimated well water and labeled by Zequanox concentration and replicate number (n = 4 Zequanox suspensions X 3 replicates). Four suspensions, with Zequanox concentrations ranging from 5 to 25% (w/v), were prepared in 500-mL batches by mixing Zequanox and well water with an immersion blender for one minute. Equivalent masses of Zequanox from each suspension (ranging in volume from 0.25 to 1.00 mL) were drawn into 1-mL syringes and placed on top of the graduated cylinders using an acrylic mounting block. The mounting blocks secured the syringes in a fixed position, with the tip of the syringe terminating at the 80-mL graduation mark. Syringe plungers were depressed at a consistent rate to administer Zequanox into the graduated cylinders.

Observations

Prior to application, viscosities of prepared Zequanox suspensions were measured in triplicate using an appropriate-sized cup viscometer to obtain efflux time in Zahn seconds, which was then converted to viscosity in centistokes (cSt) using conversion equations provided by the viscometer manufacturer. Buoyancy upon initial application, followed by settling and depth of stratification layer observations were also made 1, 2, 4, and 8 hours after application. Stratification layers were observed using mL graduations on the cylinders and were converted into distance with a digital caliper, in mm, and also into percentage of water column.

Water temperature, pH, hardness, and alkalinity were measured in triplicate on water samples collected prior to Zequanox application following internal UMESC protocols (UMESC SOPs AEH 186, 712, and 706). Data analyses for water chemistry were limited to simple summary statistics; comparative statistics were not generated. Stratification and settling observations were numerically ranked based on defined data ranges and were used to determine a target Zequanox suspension viscosity for subsurface applications.

Qualitative stratification and settling observations were numerically ranked (Table 1), assessed, and used, in combination with buoyancy observations, to estimate an acceptable target Zequanox suspension viscosity for subsurface applications of 180 cSt.

Two-Step Linear Regression Prediction Model

A two-step linear regression procedure was used to predict temperature-concentration combinations that would result in Zequanox suspensions with a viscosity of 180 cSt at water temperatures ranging from 7 to 22°C. The first step involved plotting viscosity data versus Zequanox suspension concentrations from each test temperature and then fitting linear regressions. With the exception of the 12°C trial, viscosity measurements ≥ 575 cSt were excluded when creating these temperature-specific linear regression models. Inclusion of a 575-cSt observation, from the 12°C test, was required to maintain at least three data points for the creation of each model. The second step involved selecting Zequanox concentrations corresponding to a viscosity of 180 cSt at each test temperature. These data were then plotted and a second linear regression model was created and used to predict the concentration of Zequanox, which would result in a suspension with a viscosity of 180 cSt for any water temperature ranging from 7 to 22°C.

Table 1. Numerical ranking of qualitative Zequanox settling observations and stratification layer data.

[Numerical rankings for settling: heavy (0), medium/heavy (1), medium (2), light (3), and very light (4); numerical rankings for stratification layer: 0 to 5% (0), 6 to 25% (1), 26 to 50% (2), 51 to 75% (3), 76 to 100% (4)]

Zequanox concentration (% w/v)	Mean numerical ranking		Zequanox concentration (% w/v)	Mean numerical ranking	
	Settling	Stratification layer		Settling	Stratification layer
	7ºC			17ºC	
5	1.0	2.0	5	3.0	2.8
7.5	0.0	0.4	10	0.0	1.8
10	0.0	0.0	15	0.0	1.2
12.5[1]	0.0	0.8	20[1]	0.0	0.0
	12ºC			22ºC	
5	2.0	2.2	10	4.0	3.0
10	0.0	0.0	15	1.0	2.6
15[1]	0.0	0.0	20[1]	0.0	2.6
20[1]	0.0	0.4	25[1]	0.0	1.0

[1]In all replicates, suspensions were observed to float upon application due to air entrainment; these concentrations were considered unsuitable when selecting optimal Zequanox concentration and viscosity.

Pond

Prior to initiation of pond trials, a non-replicated scale-up test of the pond delivery system was conducted in a 350-L laboratory test tank. During this testing, Zequanox suspensions previously observed to be non-buoyant during laboratory testing were found to be buoyant, and as a result, premature mixing within the water column was observed. Air entrainment during suspension preparation was speculated to be the cause for buoyancy. Therefore, a silicone-based aquaculture defoaming agent (Proline Foam Eliminator, Pentair Aquatic Eco-Systems Inc., Apopka, FL) was added to all prepared suspensions at 0.1% (v/v).

For each of the three outdoor pond trials, the ponds were filled with well water and allowed to acclimate to temperatures of ~9, 14, or 22°C for a minimum of 48 hours prior to Zequanox application. Three treatment ponds and one control pond were randomly assigned to the four enclosures for each temperature trial. On each trial day, water temperature was measured and the laboratory-derived temperature-concentration model was used to predict the concentration of Zequanox required for a suspension to achieve the target viscosity of 180 cSt. The predicted concentrations of Zequanox ranged from 7.8 (9°C) to 15.2% w/v (22°C). For each treated enclosure, the appropriate volume Zequanox suspension required to treat the water in the bottom 60 cm of the test enclosure at 100 mg A.I./L was prepared by mixing Zequanox into pond water with an immersion blender for ~90 seconds and approximately 15 minutes after preparation, the viscosity of each suspension was measured in triplicate as previously described. Zequanox suspensions were then applied to the test enclosures at a rate of 40 mL/minute through each of the 16 delivery tubes. Care was taken to bleed the application lines of air prior to beginning the Zequanox applications and to prevent air from entering the application lines during the application process. The time required for application ranged from ~15 to 28 minutes per enclosure because the volume of Zequanox suspension required to achieve the target concentration of 100 mg A.I./L varied for each temperature.

Concentration Verification and Water Chemistry

Water sampling tubing was flushed for several minutes immediately prior to sample collection. Water samples were drawn through all nine sampling tubes in an enclosure simultaneously, resulting in triplicate samples being collected from each depth (7.5, 30, and 60 cm from bottom). Samples were pooled by depth and then analyzed for concentration via spectroscopy by comparing the absorbances of samples to a linear regression created from known concentrations of Zequanox A.I. (50, 100, 200, and 300 mg A.I./L) using a Beckman DU Series 800 spectrophotometer at 660 nm. A General Linear Model (SAS Version 9.3, SAS Institute, Inc., Cary, NC) was used to compare Zequanox concentrations by temperature at various depth combinations (7.5 cm only; 7.5 and 30 cm combined; and 7.5, 30, and 60 cm combined).

Water temperature, pH, dissolved oxygen, hardness, and alkalinity were measured in each test enclosure prior to Zequanox application following internal UMESC protocols (UMESC SOP AEH 186, 304, 712, and 706). Surface water temperature was measured in each test enclosure at 1, 4, and 8 hours. To prevent mixing of the stratified Zequanox into the water column, pH and dissolved oxygen were measured near the bottom of the water column upon termination (8 hours). Data analyses for water chemistry were limited to simple summary statistics; comparative statistics were not generated.

Results and Discussion

Laboratory

Mean water quality parameters (pH, temperature, hardness, and alkalinity) for the laboratory trials are summarized in Table 2. For all four laboratory trials, water hardness and alkalinity ranged from 187 to 190 mg/L and 141 to 146 mg/L, respectively, and pH ranged from 7.80 to 8.01. Viscosities of the Zequanox suspensions are summarized by test temperature in Table 3. The first-step temperature-specific viscosity regression models and the second-step temperature-concentration prediction models are displayed in Figs. 3 and 4. The correlation coefficients of the first-step regression models ranged from 0.929 to 0.987 (Fig. 3) and the correlation coefficient of the second-step regression model was 0.83 (Fig. 4).

Table 2. Water quality parameters observed in the laboratory trials.

	Mean water chemistry parameter (SD)			
Target temperature (ºC)	Observed temperature (ºC)	pH[1] (standard units)	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)
7	7.2 (0.0)	7.90 (0.01)	189 (1)	144 (1)
12	12.2 (0.0)	7.94 (0.02)	187 (0)	144 (1)
17	17.0 (0.1)	7.80 (0.01)	188 (1)	141 (1)
22	21.6 (0.1)	8.00 (0.01)	189 (1)	146 (1)

[1]pH values were log transformed prior to calculating mean values; standard deviations were calculated from observed pH values.

Table 3. Mean viscosity of Zequanox suspensions prepared during the laboratory trials.

Zequanox concentration (% w/v)	Mean viscosity in centistokes (SD)	Zequanox concentration (% w/v)	Mean viscosity in centistokes (SD)
7ºC		17ºC	
5	10 (1)	5	4 (0)
7.5	110 (4)	10	83 (2)
10	255 (7)	15	316 (9)
12.5	1058 (23)	20	583 (27)
12ºC		22ºC	
5	8 (1)	10	13 (1)
10	170 (5)	15	58 (2)
15	583 (35)	20	236 (7)
20	>1725[1]	25	454 (9)

[1]Over range of #5 viscometer cup.

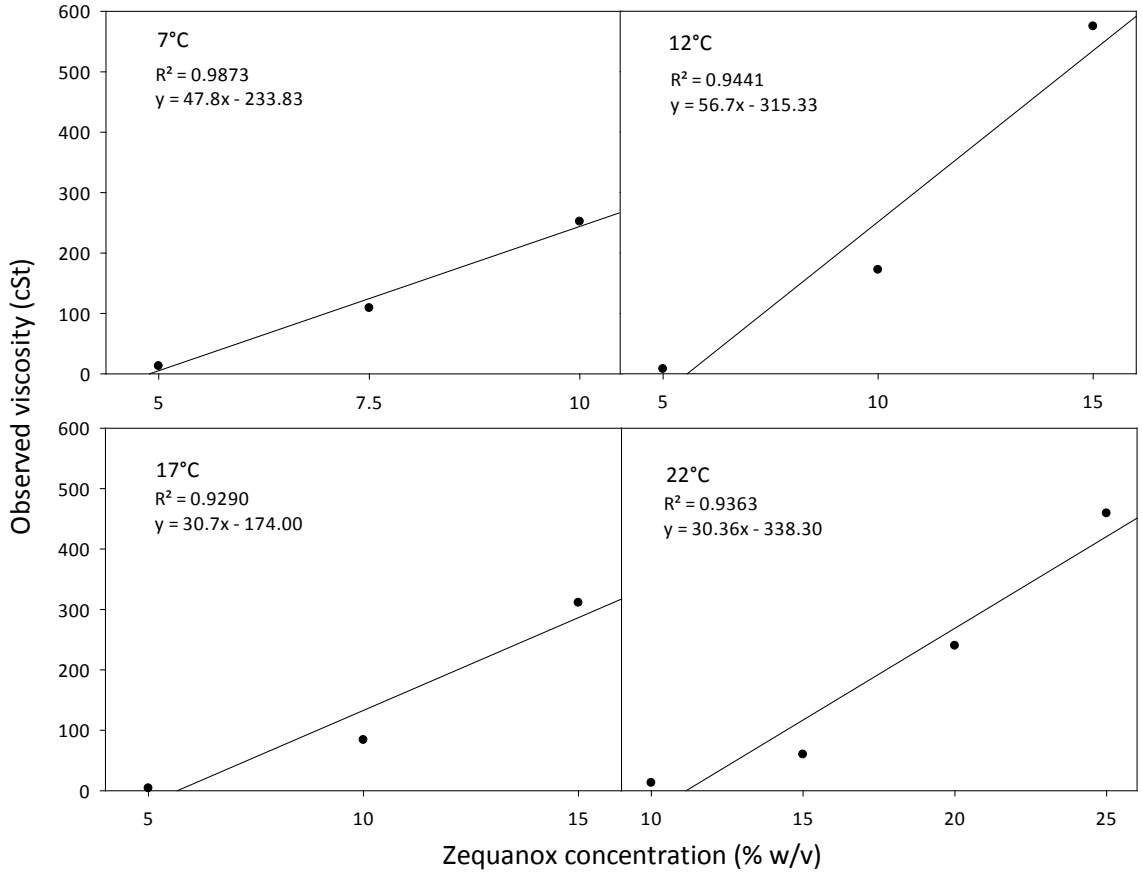

Figure 3. First-step linear regressions plotting Zequanox concentrations and viscosities from laboratory tests.

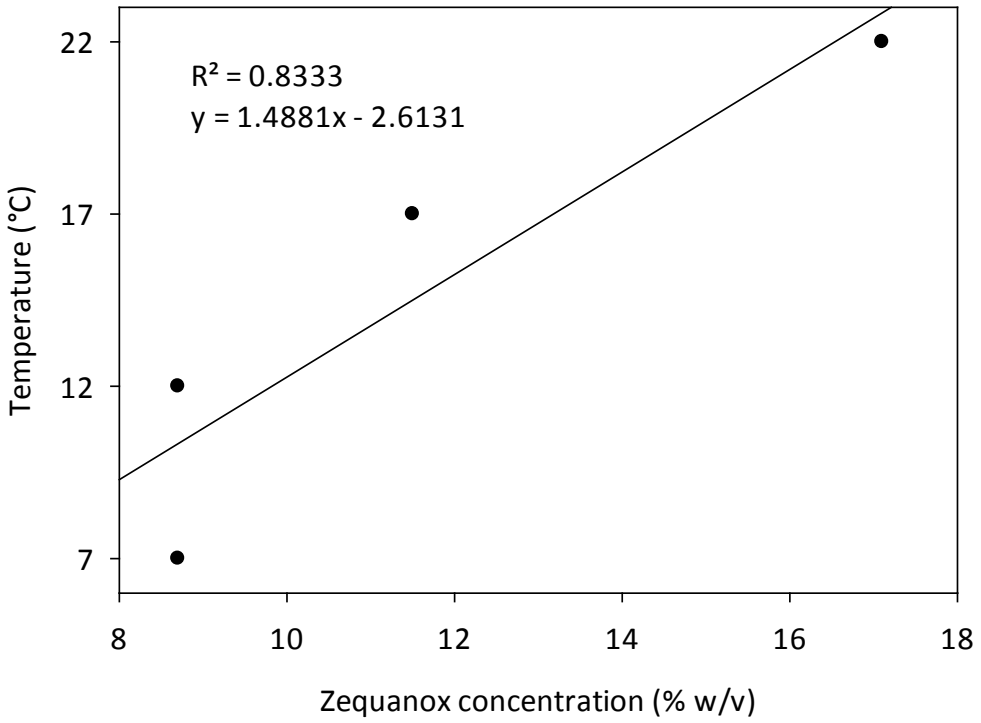

Figure 4. Second-step linear regression used to predict Zequanox concentrations at the target viscosity (180 cSt) for temperatures ranging from 7 to 22°C. This prediction regression was used to determine Zequanox concentrations in suspensions used for applications in outdoor pond tests.

Pond

Mean pre-exposure water quality parameters (pH, temperature, dissolved oxygen, hardness, and alkalinity) for the pond trials are summarized in Table 4. Individual pre-exposure hardness and alkalinity measurements in all three pond trials ranged from 178 to 194 and 137 to 145 mg/L, respectively; pH ranged from 7.87 to 8.08; and dissolved oxygen ranged from 7.79 to 10.58 mg/L. Water quality parameters during the exposure period (temperature [1-, 4-, and 8-hour]; pH and dissolved oxygen [8-hour only]) for the pond trials are summarized in Table 5. Individual exposure period dissolved oxygen and pH measurements in all three pond trials ranged from 7.84 to 8.09 and 7.49 to 10.27 mg/L, respectively. Application of Zequanox to the enclosures had no appreciable impact to dissolved oxygen and pH for the duration of the exposure period. Throughout the exposure period, the dissolved oxygen, pH, alkalinity, and hardness remained at acceptable levels for aquaculture according to Timmons and Ebeling (2013).

Table 4. Pre-exposure water quality parameters observed during the pond trials.

Target temperature (ºC)	Mean water chemistry parameter (SD)				
	Observed temperature (ºC)	pH[1] (standard units)	Dissolved oxygen (mg/L)	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)
9	8.6 (0.2)	7.97 (0.08)	10.32 (0.16)	179 (1)	138 (1)
14	13.8 (0.1)	8.05 (0.02)	8.07 (0.19)	193 (1)	145 (1)
20	19.7 (0.1)	7.96 (0.02)	8.43 (0.23)	181 (1)	139 (1)

[1]pH values were log transformed prior to calculating mean values; standard deviations were calculated from observed pH values.

Table 5. Exposure period water quality parameters observed during the pond trials.

Target temperature (ºC)	Mean water chemistry parameter (SD)				
	1-hour temperature (ºC)	4-hour temperature (ºC)	8-hour temperature (ºC)	8-hour pH[1] (standard units)	8-hour dissolved oxygen (mg/L)
9	8.4 (0.2)	8.5 (0.2)	8.6 (0.2)	8.01 (0.01)	10.09 (0.17)
14	14.1 (0.2)	15.3 (0.2)	15.4 (0.1)	7.95 (0.08)	8.92 (0.42)
20	20.1 (0.1)	20.7 (0.2)	20.2 (0.1)	7.98 (0.07)	8.15 (0.83)

[1]pH values were log transformed prior to calculating mean values; standard deviations were calculated from observed pH values.

Mean viscosities of Zequanox suspensions during the pond trials were 49, 133, and 275 cSt for 9, 14, and 20°C tests, respectively. The observed variance from the target of 180 cSt was likely influenced by the addition of defoaming agent, which was not used in the laboratory tests; error in the prediction regression model; and difficulty in obtaining precise viscosity measurements with cup viscometers. Although there was considerable variance in the observed viscosities of the applied Zequanox suspensions, each pond trial maintained concentrations near or above the target concentration of 100 mg A.I./L for the entire exposure duration near the water/substrate interface (7.5 cm). Observed mean Zequanox concentrations for all three pond tests were as follows: 7.5-cm samples ranged from 98.7 to 138.5 mg A.I./L, 30-cm samples ranged from 23.8 to 94.2 mg A.I./L, and 60-cm samples ranged from 1.9 to 30.5 mg A.I./L (Fig. 5). For the 7.5-cm samples, significantly higher Zequanox concentrations were detected in the 20°C trial compared to the 9 and 14°C trials ($P < 0.001$) and no difference was detected between the 9 and 14°C trials ($P = 0.58$). All trials maintained lethal levels of Zequanox at a depth of 7.5 cm for the duration of the exposure, and the increased concentrations observed in the 20°C trial correlates with the significantly higher viscosity observed in this trial. When comparing the results of this trial to the mortality of zebra mussels observed in a Zequanox study conducted by Luoma et al. (2015a), the concentration of Zequanox measured in the 30-cm samples remained above lethal levels in the 9°C trial for the duration of the exposure and for approximately 4 and 3 hours during the 14 and 20°C trials, respectively. All samples measured at the 60-cm depth were likely below lethal levels for the duration of the exposure period. Although the 9°C trial maintained lethal levels in a larger portion of the water column than did the 14 and 20°C trials, wave action and other disturbances to the Zequanox stratification layer in field applications would likely result in more

rapid Zequanox dispersion than was observed in our pond trials. Therefore, the observed mean viscosity of 49 cSt indicates that the Zequanox suspension applied during the 9°C pond trial was likely too diluted and should have been closer to the selected viscosity of 180 cSt.

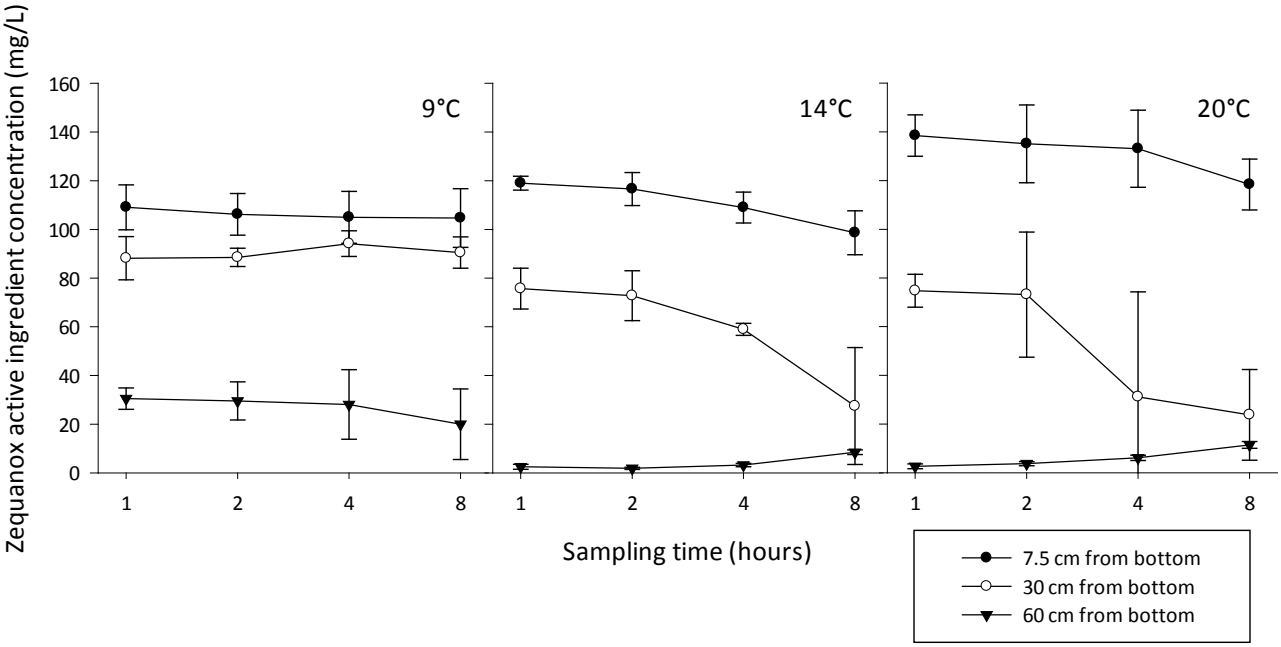

Figure 5. Mean Zequanox active ingredient concentrations observed by temperatures in outdoor pond trials.

Revised Prediction Model

At the coldest pond temperature evaluated (9ºC), the two-step linear regression model used in the study predicted a concentration of Zequanox that yielded a suspension that was too thin ($\bar{x}$ = 49 cSt), and at the warmest pond temperature evaluated (20ºC), it predicted a concentration of Zequanox that yielded a suspension that was too viscous ($\bar{x}$ = 269 cSt). A reexamination of the methods used to create the prediction model determined that creating first-step temperature-specific models using SigmaPlot (Version 13, Systat Software, Inc., San Jose, California) with 2-parameter power regression equations ($y = ax^b$) was superior than the linear method because it provided a better fit, as demonstrated by correlation coefficients > 0.99, while including all data points for each temperature (Fig. 6). The optimal viscosity of 180 cSt was entered into each new power model to determine optimal Zequanox concentration at each temperature and a new second-step logarithmic temperature-concentration prediction model ($y = a\ ln(x) + b$) was generated (Fig. 7). The Zequanox concentrations predicted to achieve the target viscosity of 180 cSt, using both the original linear and the revised logarithmic models, are presented in Table 6. The revised logarithmic model predicts Zequanox concentrations closer to the target viscosity of 180 cSt than the original linear model by increasing and decreasing the Zequanox concentration for the lower and higher temperatures, respectively. Additional multiple linear regressions were developed to model the relationships between viscosity, temperature, and Zequanox concentration using both logarithmic and square root transformations; however, these models failed to predict Zequanox concentrations as well as the revised two-step logarithmic temperature-concentration model at either the lower, higher, or both ends of the temperature spectrum evaluated and therefore these models were rejected.

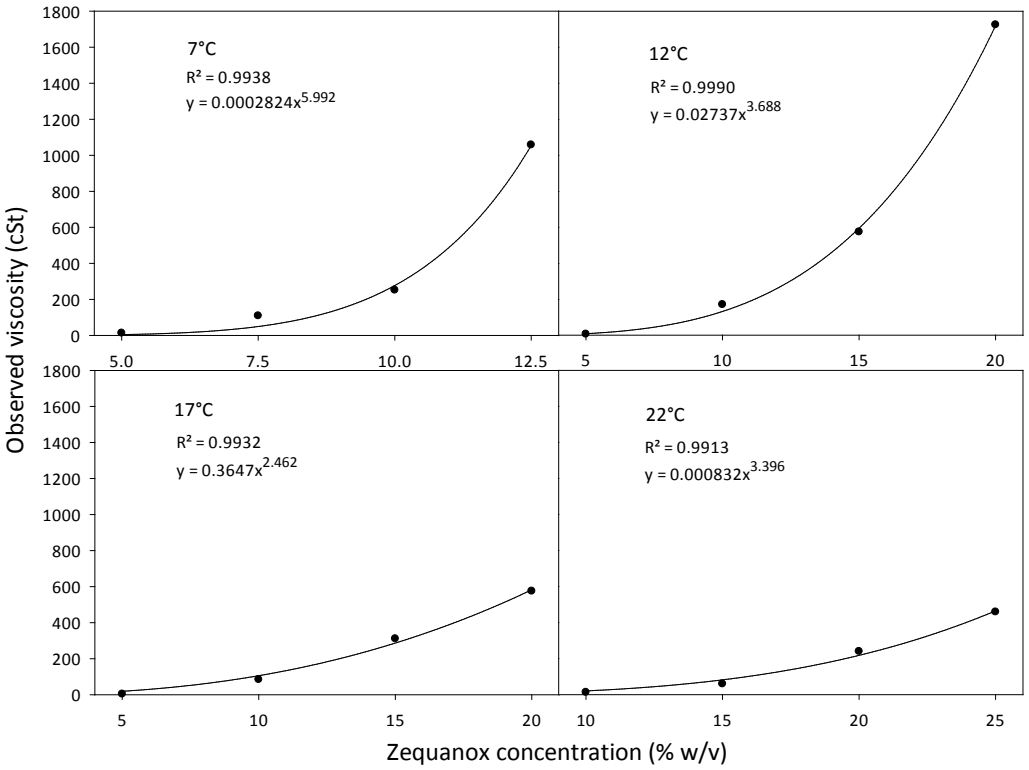

Figure 6. Revised first-step power regressions plotting Zequanox concentrations and viscosities from laboratory tests.

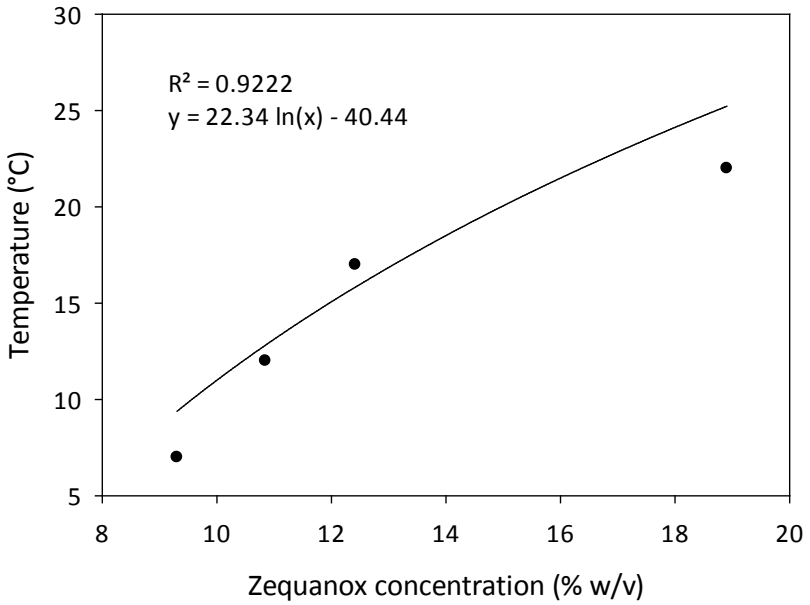

Figure 7. Revised second-step logarithmic regression derived from concentrations produced by revised first-step power regressions. This regression should predict Zequanox concentrations closer to the target viscosity (180 cSt) for temperatures ranging from 7 to 22°C.

Table 6. Predicted Zequanox concentrations required to achieve a suspension viscosity of 180 cSt for temperatures ranging from 7 to 22°C.

Water temperature (ºC)	Predicted Zequanox concentration (% w/v)		Water temperature (ºC)	Predicted Zequanox concentration (% w/v)	
	Linear[1]	Logarithmic[2]		Linear[1]	Logarithmic[2]
7	6.5	8.4	15	11.8	12.0
8	7.1	8.7	16	12.5	12.5
9	7.8	9.1	17	13.2	13.1
10	8.5	9.6	18	13.9	13.7
11	9.1	10.0	19	14.5	14.3
12	9.8	10.5	20	15.2	15.0
13	10.5	10.9	21	15.9	15.6
14	11.2	11.4	22	16.5	16.4

[1]Predicted Zequanox concentrations from the two-step linear model used in the study.

[2] Revised two-step power regression and logarithmic model predicted Zequanox concentrations.

Conclusion

The laboratory trials clearly demonstrated that temperature greatly impacts the viscosity of Zequanox suspensions, and achieving precise viscosity measurements of Zequanox suspensions is difficult with cup viscometers. The laboratory trials and preliminary scale-up tests showed the importance of eliminating air entrainment within Zequanox suspensions, which can result in Zequanox mixing throughout the water column. The use of a silicone-based aquaculture defoaming agent in the Zequanox suspensions appeared to reduce air entrainment, thereby increasing the potential for successful subsurface applications by allowing the use of more viscous suspensions. The results of this study, including the revised prediction model and the methods for subsurface delivery of Zequanox, will aid resource managers engaged in Zequanox applications. The use of subsurface Zequanox applications should be limited to quiescent waters in order to maintain a stratified Zequanox layer to achieve satisfactory dreissenid mussel control. This research provides foundational information for additional research related to subsurface Zequanox applications including refinement of the Zequanox concentration prediction procedure.

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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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Contents

Figures

Tables

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

By James A. Luoma and Todd J. Severson

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.

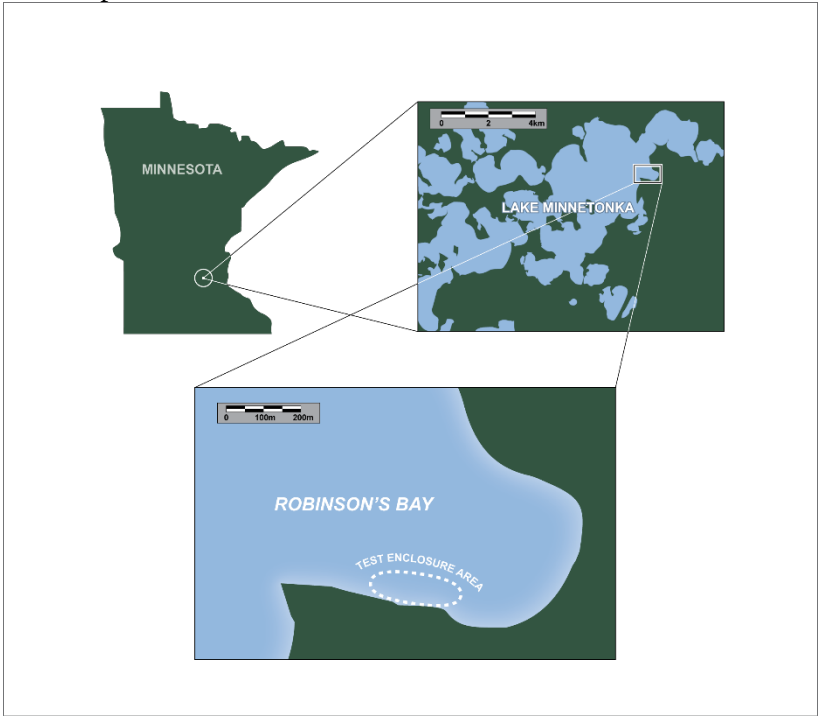

Figure 1. Location of test enclosures, Robinson's Bay, Lake Minnetonka, MN

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).

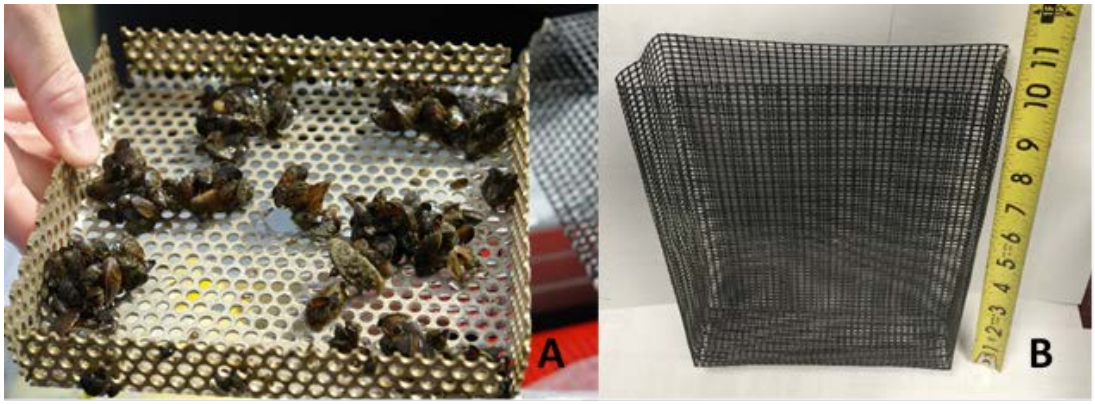

Figure 3. Example of type 2 sampler components consisting of a perforated aluminum tray with adhering zebra mussels (A, left) and a semi-rigid plastic mesh containment bag (B, right).

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.

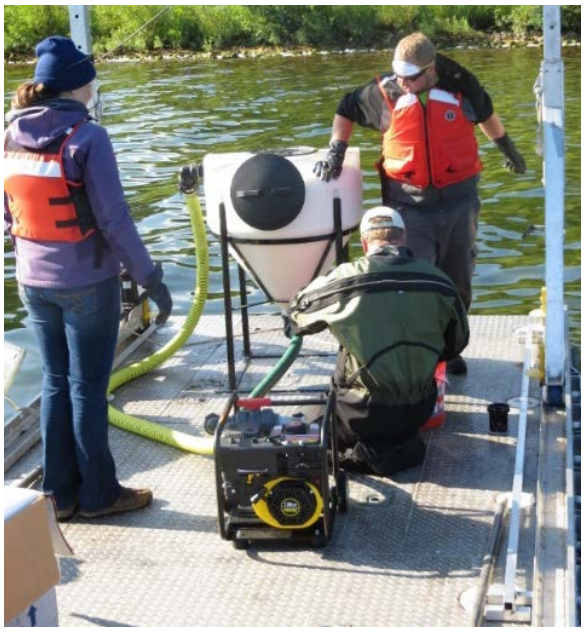

Figure 5. Zequanox suspension mixing system that was used to prepare the 5% (w/v) Zequanox suspensions for application to test enclosures in Robinson's Bay, Lake Minnetonka, MN.

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).

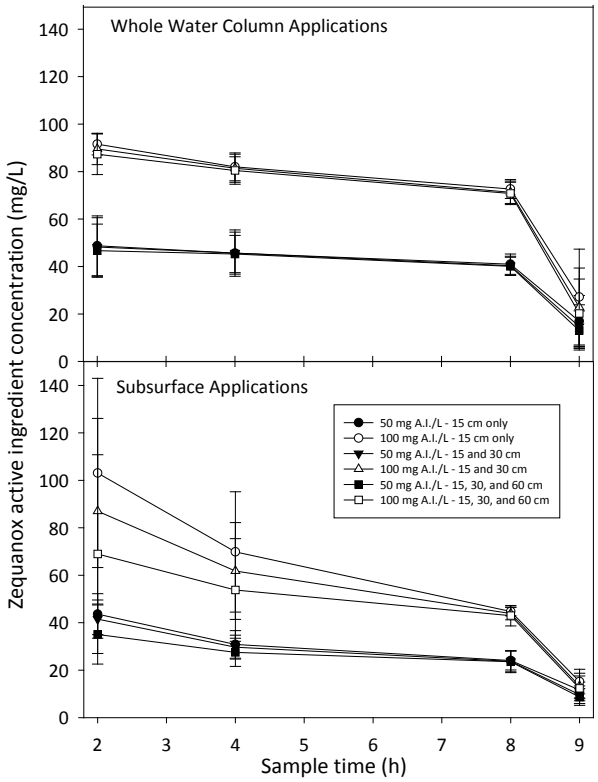

Figure 10. Mean Zequanox active ingredient concentrations (SD error bars; n = 3/sample depth/enclosure) measured in enclosures during the exposure period and 1-hour post exposure in the whole water column and subsurface applications conducted in Robinson's Bay, Lake Minnetonka, MN

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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ORIGINAL PAPER

Responses to elevated CO_2 exposure in a freshwater mussel, *Fusconaia flava*

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Abstract Freshwater mussels are some of the most imperiled species in North America and are particularly susceptible to environmental change. One environmental disturbance that mussels may encounter that remains understudied is an increase in the partial pressure of CO_2 (pCO_2). The present study quantified the impacts of acute (6 h) and chronic (up to 32 days) exposures to elevated pCO_2 on genes associated with shell formation (chitin synthase; *cs*) and the stress response (heat shock protein 70; *hsp70*) in *Fusconaia flava*. Oxygen consumption (MO_2) was also assessed over the chronic CO_2 exposure period. Although mussels exhibited an increase in *cs* following an acute exposure to elevated pCO_2, long-term exposure resulted in a decrease in *cs* mRNA abundance, suggesting that mussels may invest less in shell formation during chronic exposure to elevated pCO_2. In response to an acute elevation in pCO_2, mussels increased *hsp70* mRNA abundance in mantle and adductor muscle and a similar increase was observed in the gill and adductor muscle in response to a chronic elevation in pCO_2. A chronic elevation in pCO_2 also increased mussel MO_2. This overall increase in *hsp70* mRNA levels and MO_2 in *F. flava* indicates that exposure to elevated pCO_2 initiates activation of the general stress response and an increased energy demand. Together, the results of the present study suggest that freshwater mussels respond to elevated pCO_2 by increasing processes necessary to 'deal with' the stressor and, over the long-term, may reduce their investment in non-essential processes such as shell growth.

Keywords Chitin synthase · Heat shock protein 70 · Metabolic rate · Bivalve

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Introduction

Freshwater mussels have their highest abundance and diversity in North America, and provide many important ecological functions (Williams et al. 1993; Bogan 2008). For example, freshwater mussels filter large volumes of water daily, remove bacteria and particles from the water column, and generate nutrient-rich areas (Vaughn and Hakenkamp 2001; Hauer and Lamberti 2007). In addition, freshwater mussels provide an important resource as food for other aquatic and terrestrial animals (Vaughn and Hakenkamp 2001; Hauer and Lamberti 2007). Notably, freshwater mussel populations are on the decline, in both species richness and biomass (Williams et al. 1993; Lydeard et al. 2004; Regnier et al. 2009). Alterations in flow regimes, land-use changes, invasive species such as zebra mussels, and climate change are all thought to have contributed to these declines (Strayer et al. 2004; Vaughn 2010). With only a small percentage of stable freshwater mussel populations remaining (Williams et al. 1993) and continued degradation of freshwater ecosystems, there is an increased need to understand the vulnerabilities of these animals to environmental stressors, and the mechanisms underlying their physiological responses to these stressors (e.g., Jeffrey et al. 2015).

One environmental stressor that is currently understudied in the freshwater environment is the impact of elevations in the partial pressure of carbon dioxide (pCO_2). In the context

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of climate change and rising atmospheric CO_2, the impact of ocean acidification (i.e., elevated pCO_2) on marine calcifying organisms has been investigated to a large extent (reviewed by Fabry et al. 2008; Gazeau et al. 2013); however, virtually northing is known about the responses of freshwater bivalves to increased pCO_2 (Hasler et al. 2016). Upon entering freshwater, CO_2 results in a decrease in water pH due to the production of carbonic acid (H_2CO_3), leading to the release of H^+ and, thus, the weak acidification of water. Levels of freshwater pCO_2 can vary for a variety of reasons including, terrestrial productivity, precipitation, and local geology (Cole et al. 1994; Maberly 1996; Butman and Raymond 2011), resulting in CO_2 levels that can fluctuate both seasonally and daily, and that can exceed atmospheric levels (i.e., water bodies can be supersaturated with CO_2). River environments can thus experience a wide range of pCO_2 over the course of a year (from less than 100 to over 15,000 µatm), with higher values being observed in warmer, dryer periods (Cole and Caraco 2001). Although less well understood than for the marine environment, freshwater pCO_2 may increase as a result of increased atmospheric CO_2, greater terrestrial primary productivity, increased precipitation, and longer periods of dry conditions—although the magnitude of change is not known (Phillips et al. 2015; Hasler et al. 2016; Perga et al. 2016). Levels of freshwater CO_2 can also be intentionally elevated in the context of generating non-physical barriers to deter the movement of invasive fishes (Noatch and Suski 2012). The form that such a non-physical barrier may take has not yet been well defined, but CO_2 levels would likely dissipate as the distance from the CO_2 infusion site increases, thus mussels may be exposed to a gradient of CO_2 depending on their proximity to the barrier. Together, freshwater mussels may experience periods of elevated pCO_2 due to both natural and anthropogenic sources, and with pCO_2 expected to rise in the future, this necessitates a need for a better understanding of the consequences for freshwater mussels.

In the marine environment, a major consequence of exposure to elevated pCO_2 for bivalves is a reduction in both shell growth and biomineralization (reviewed by Gazeau et al. 2013). The mollusc shell provides an important external structure to support living tissues, protect against predators, and exclude mud and sand from the mantle cavity of burrowing species (Gazeau et al. 2013). Changes in the integrity of the shell have occurred due to exposure to conditions of ocean acidification, and dissolution of the shell as a result can have consequences for the health and survival of bivalves (reviewed by Gazeau et al. 2013). The mantle, a thin secretory epithelial tissue lining the inner surface of the shell, is responsible for mollusc shell formation, and shell calcification occurs in a small compartment (i.e., extrapallial cavity) located between the calcifying outer mantle and the shell (Wilbur and Saleuddin 1983). The shell is comprised of a mineral phase (95–99 % predominantly calcium carbonate; $CaCO_3$) and an organic matrix (1–5 %). Chitin is an insoluble polysaccharide that forms the highly structured organic framework of mollusc shells within which $CaCO_3$ minerals are deposited (Weiner et al. 1984; Levi-Kalisman et al. 2001; Weiss and Schonitzer 2006). The enzymes involved in chitin synthesis are important not only for mechanical strength and toughness of the shell, but also for coordination of mineralization processes and shell formation (Schonitzer and Weiss 2007). Chitin synthase (CS) is a key enzyme involved in the synthesis of chitin (Fang et al. 2011), and inhibition of CS during early development has been shown to negatively affect rates of shell development, solubility of the shell, and survival in *Mytilus galloprovincialis* larvae (Schonitzer and Weiss 2007). Furthermore, changes in the mRNA abundance of *cs* occurred in mantle of adult *Laternula elliptica*, a marine bivalve, in response to changes in environmental pCO_2 (and thus pH) (Cummings et al. 2011). Due to its importance in the biological control of shell formation and evidence of its regulation in response to situations of ocean acidification, *cs* provides a useful target to assess the impact of environmental stressors, such as elevated pCO_2, on shell formation in adult freshwater mussels.

The impacts of elevated pCO_2 on other cellular functions, such as mediators of cellular stress, have also been investigated to some extent in marine bivalves (e.g., Cummings et al. 2011). Heat shock proteins (HSPs) are among the most evolutionarily conserved proteins, and are induced by a number of factors beyond heat-stress that affect cell protein structure and functioning (Feder and Hofmann 1999; Sørensen et al. 2003). A key role of HSPs is to protect and repair cellular proteins damaged by exposure to stressors, and to minimize protein aggregation (Feder and Hofmann 1999). Heat shock protein 70 (HSP70) is the most abundant family of HSPs, and consists of the constitutively expressed HSC70 and inducible HSP70 that are ubiquitously distributed in eukaryotic cells (Feder and Hofmann 1999). The key role of HSPs in mechanisms of cellular protection renders them good markers of the stress status of an organism. In this way, HSPs provide information about the general condition and health of an organism as well as the sub-lethal effects (i.e., early warning signs) of a stressor, before more complex functions are compromised (reviewed by Fabbri et al. 2008). The inducible HSP70 is widely up-regulated in response to a variety of stressors in bivalves (e.g., Franzellitti and Fabbri 2005; Toyohara et al. 2005; Cellura et al. 2006; Cummings et al. 2011; Chen et al. 2014; Luo et al. 2014) and may represent a useful biomarker in examining elevated CO_2 as a potential stressor in freshwater mussels.

In addition to the impacts of elevated pCO_2 on cellular function, elevations in pCO_2 also have the potential

to affect whole-animal energetics and metabolism. In response to acute hypercapnia, metabolic depression is an adaptive response used by shelled molluscs to conserve energy, and is likely driven by decreased extracellular pH (reviewed by Pörtner et al. 2004; Gazeau et al. 2013). Acute responses to hypercapnia are time-dependent however, and long-term depressions in metabolic rate (rate of oxygen consumption; MO_2) due to chronic hypercapnia can be lethal (Gazeau et al. 2013). Interestingly, although some studies have observed decreases in metabolic rate due to exposure to elevated pCO_2 (e.g., Michaelidis et al. 2005; Fernández-Reiriz et al. 2011; Liu and He 2012; Navarro et al. 2013), results from other studies on marine bivalves have been mixed (reviewed by Gazeau et al. 2013). For instance, elevations in MO_2 in response to increased pCO_2 have been observed (e.g., Beniash et al. 2010; Lannig et al. 2010; Thomsen and Melzner 2010; Cummings et al. 2011; Parker et al. 2012) suggesting that shelled molluscs may be able to at least partially compensate for the energetic costs of acidosis (Wicks and Roberts 2012; Gazeau et al. 2013). Moderate elevations in pCO_2 below 1200 µatm (approximately three times current ocean pCO_2), on the other hand, have had minimal effects on MO_2 (e.g., Matoo et al. 2013). To date, no studies have quantified the impact of elevated pCO_2 on the MO_2 of freshwater bivalves, which is an important biomarker of the overall energetic status of an organism.

The goals of the present study were to define the impacts of elevated pCO_2 on the biological control of shell formation, as well as stress status at the cellular and whole-animal levels, in freshwater mussels. To accomplish these goals, adult Wabash pigtoe mussels (*Fusconaia flava*) were first exposed to a short-term elevation in pCO_2 (6 h) that was followed by a 6-h post-stressor period at ambient conditions. In this experiment, mussels were exposed to either ambient (300 µatm), 15,000 or 200,000 µatm pCO_2. In a second experiment, and to quantify the impacts of an extended exposure to elevated pCO_2, *F. flava* were exposed to either ambient (1000 µatm) or 20,000 µatm pCO_2 for 4, 8, or 32 days. In both experiments, *cs* mRNA levels and RNA:DNA ratio (as an indicator of total protein synthesis) were quantified in mantle tissue as indicators of shell formation, and *hsp70* mRNA levels were assessed across a number of tissues. In addition, MO_2 was assessed repeatedly in mussels exposed to the long-term CO_2 exposure.

Materials and methods

Experimental animals

Adult *F. flava* (wet mass, 27.0 ± 1.3 g; length, 50.2 ± 0.8 mm; mean ± standard error of the mean, SEM) were collected by benthic grab from Big Four Ditch, Paxton, IL. Mussels were transported in coolers (<1-h transport time) to the Aquatic Research Facility at the University of Illinois, Champaign-Urbana, IL, cleaned of epibionts, and individually tagged (Queen Marking Kit tags; The Bee Works, Orillia, ON, CA). Mussels were then placed into one of three 128.6 L recirculating systems, each consisting of three tanks with 5 cm of sand, supplied with water from a 0.04 ha naturalized, earthen-bottom pond, a UV Sterilizer (TMC Vecton 8 W, 11 L min^{-1} flow rate, Pentair, Apopka, FL, USA), a heater/chiller (TECO-US, Aquarium Specialty, Columbia, SC, USA), and a low pressure air blower (Sweetwater, SL24H Pentair, Apopka, FL, USA). Mussels were held for at least 1 week prior to the onset of experiments and were fed a commercial diet (*Nannochloropsis* sp. 1–2 microns and *Isochrysis*, *Pavlova*, *Thalassiosira*, and *Tertraselmis* spp. 5–12 microns; Instant Algae, Reed Mariculture Inc., Campbell, CA, USA) every other day for the duration of the study period, with the exception of the 48-h period prior to the onset of sampling. Dissolved oxygen (DO) and temperature were monitored daily with a portable meter (YSI 550A, Yellow Springs Instruments, Irvine, CA, USA) and averaged 8.0 ± 0.6 mg L^{-1} (mean ± SEM) and 17.5 ± 0.2 °C, respectively. Fifty percent water changes were performed weekly and no mussel mortality occurred throughout the experiment.

Short-term exposure to elevated pCO_2

Mussels ($N = 48$) were removed from recirculating systems and transferred to individual 0.71 l containers within a recirculating system. Each system consisted of a raceway with eight containers (i.e., held eight mussels at a time), and a central reservoir. Pond water was pumped from the central reservoir into individual containers and allowed to overflow into the raceway and return to the reservoir forming a closed system. Individual containers were supplied with an air stone to maintain DO levels. Temperature and DO were monitored (see above), and pH was measured using a handheld meter (WTW pH 3310 m, Germany), calibrated regularly throughout the study (Moran 2014). Free CO_2 and total alkalinity (TA) concentrations were measured using digital titration kits (Hach Company, Titrator model 16900, cat. no. 2272700 for CO_2 and cat. no. 2271900 for TA). Water temperature, TA, and pH values were then used to determine the pCO_2 in µatm using CO2calc where all default parameters were used with the exception that 'Set of constants' was set to 'Salinity = 0 (freshwater) K1; K2 from Millero 1979' (Robbins et al. 2010) (Table 1).

Following a 24-h period in individual containers, mussels were exposed to either ambient (273 ± 30 µatm), 15,000 µatm (14,772 ± 1685), or 200,000 µatm (188,114 ± 12,669) pCO_2 as described in Hannan et al.

Table 1 Water chemistry for short- and long-term pCO_2 exposures

Experiment	CO_2 level	Temperature (°C)	Dissolved O_2 (mg L^{-1})	pH	Total alkalinity (mg L^{-1})	Dissolved CO_2 (mg L^{-1})	pCO_2 (µatm)
Short term	Ambient (300 µatm)	17.8 ± 0.2	8.98 ± 0.12	8.65 ± 0.05	211 ± 5	13.5 ± 0.5	272.8 ± 30.2
	15,000 µatm	17.9 ± 0.25	8.76 ± 0.26	6.99 ± 0.02	202.6 ± 14.6	36.9 ± 4.7	14,772.6 ± 1685.3
	200,000 µatm	17.8 ± 0.2	8.56 ± 1.21	6.01 ± 0.01	268 ± 12	255.5 ± 0.5	188,114 ± 12,669.6
Long term	Ambient (1000 µatm)	17.6 ± 0.2	8.05 ± 0.04	8.42 ± 0.02	211.1 ± 2.3	16.2 ± 0.7	994.2 ± 61.8
	20,000 µatm	17.5 ± 0.2	7.99 ± 0.07	7.24 ± 0.06	255.1 ± 8.8	40.79 ± 3.1	22,712.0 ± 2482.5

Data are presented as mean ± SEM

(2016). A pCO_2 of 200,000 µatm was chosen as it represents a conservative target for a non-physical fish barrier due to its efficacy at deterring the movement of several juvenile fish species, including bigheaded carp (Kates et al. 2012). A second CO_2 level of 15,000 µatm was chosen as this level may be expected downstream of a CO_2 barrier and thus affect mussels not residing immediately within the CO_2 addition zone. A level of 15,000 µatm may also potentially arise in some freshwater systems as a result of climate change (Phillips et al. 2015; Hasler et al. 2016; Perga et al. 2016). Target CO_2 levels were achieved by the common method of bubbling compressed CO_2 gas (commercial grade, 99.9 % purity) into the central reservoir through an air stone (Riebesell et al. 2010). Levels of CO_2 were maintained within the central reservoir with a pH controller (PINPOINT®, American Marine Inc., CT, USA) that adds CO_2 if the pH rises above a target level (7.00 ± 0.10 and 6.00 ± 0.10 pH, for 15,000 and 200,000 µatm treatment, respectively) during the exposure period. Mussels were exposed to one of the three CO_2 treatments for 6 h and sampled either directly following the 6-h treatment, or after being held for an additional 6 h at ambient conditions (i.e., recovery period). Mussel wet mass (24.8 ± 1.5 g) and length (49.2 ± 1.0 mm), were measured as well as the dry mass of the soft tissue (1.05 ± 0.06 g); see 'Oxygen consumption') and no significant effect of CO_2 treatment or time-point were found (see Hannan et al. 2016). Mussels ($N = 8$) were sampled for mantle, gill, foot, and adductor muscle tissues that were placed in 1 ml of RNAlater Stabilization Solution (Ambion, cat. no. AM7020, Life Technologies, Carlsbad, CA, USA) and stored overnight at 4 °C prior to storage at −80 °C.

Long-term exposure to elevated pCO_2

Mussels ($N = 48$) were separated into one of two recirculating systems (as described above) and held at either ambient (994 ± 62 µatm) or 20,000 µatm (22,712 ± 2482 µatm) pCO_2 for 4, 8, and 32 days. Note that the difference in the ambient CO_2 levels between the short- and long-term exposures was due to natural fluctuations in the CO_2 levels of the pond water. Target CO_2 pressures were again achieved by bubbling CO_2 gas into the reservoir through an air stone, and maintained using a pH controller set to a pH of 7.20 ± 0.10, as described above. A level of 20,000 µatm was chosen as this may represent a level that mussels downstream of a CO_2 barrier may experience, and it was unknown whether mussels would survive long-term exposure to a higher level of CO_2 (i.e., like the upper level used in the 'short-term' experiment). Water quality measurements including temperature, DO, pH, alkalinity, concentration of CO_2, pCO_2 in µatm (calculated by CO2Calc) were monitored using the same methods as described in the short-term experiment (Table 1). Mussels ($N = 8$) were sampled as above after 4, 8, and 32 days of exposure to either ambient or elevated CO_2 conditions. No significant effect of CO_2 treatment or time-point were found for wet mass (25.4 ± 1.7 g) and length (49.7 ± 1.0 mm), as well as the dry mass of the soft tissue (1.02 ± 0.06 g) (see Hannan et al. 2016).

RNA and first-strand cDNA synthesis

Total RNA was extracted from 50 to 100 mg of tissue using TRI Reagent (Ambion, cat. no. AM9738, Life Technologies) according to the manufacturer's protocol. Tissues were disrupted and homogenized with a mechanical homogenizer (Tissue-Tearor®, Biospec Products Inc., model no. 935370, Bartlesville, OK, USA). Extracted RNA was quantified using a Nanodrop ND-1000 UV–Vis spectrophotometer (Peqlab, Erlangen, Germany) and 1 µg of RNA was treated with deoxyribonuclease I (Amplification Grade, DNase; cat. no. 18068015, Invitrogen, Life Technologies). To synthesize cDNA, MultiScribe Reverse Transcriptase, RNase inhibitor, and random primers were used according to the manufacturer's protocol (High-Capacity cDNA Reverse Transcription kit; Applied Biosystems, cat. no. 4374966, Life Technologies).

Gene sequences

For the purpose of developing primers for quantitative real-time RT-PCR (qPCR; see below), partial sequences were generated for *cs*, *hsp70*, and *18s* from cDNA synthesized from mantle tissue. Gene-specific primers (Table 2) were designed based on conserved regions of sequences from several bivalve species using Primer3plus (primer3plus.com). For *18s*, primer forward 2 was nested within the product from primers forward 1 and reverse 1 to extend the sequence; a single set of primers was sufficient to generate a partial sequence for *hsp70* and *cs*. Primers for *cs* were based on conserved regions in *Atrina rigida* (DQ081727), *L. elliptica* (HQ186262), *M. galloprovincialis* (EF535882), *Pinctada fucata* (AB290881), and *Septifer virgatus* (AB613818). Primers for *hsp70* were based on conserved regions in *Argopecten irradians* (AY485261), *A. purpuratus* (FJ839890), *Chlamys farreri* (AY206871), *Corbicula fluminea* (KJ461738), *Crassostrea ariakensis* (AY172024), *C. gigas* (AF144646), *C. hongkongensis* (FJ157365), *C. virginica* (AJ271444), *Cristaria plicata* (HQ148706), *Hyriopsis cumingii* (KJ123764), *L. elliptica* (EF198332), *Meretrix meretrix* (HQ256748), *Mizuhopecten yessoensis* (AY485262), *M. coruscus* (KF322135), *M. galloprovincialis* (AB180909), *P. fucata* (EU822509), *Paphia undulata* (JX885711), *Pteria penguin* (EF011060), *Ruditapes philippinarum* (KJ569079), *Sinonovacula constricta* (JF748730), and *Tegillarca granosa* (JN936877). Primers for *18s* were based on conserved regions in *Anodonta cygnea* (AM774476), *Elliptio complanata* (AF117738), *Lampsilis cardium* (AF120537), *Psilunio littoralis* (AF120536), and *Unio pictorum* (AM774477).

All PCR reactions were performed using an Eppendorf Mastercycler. Reaction compositions (total volume 25 µl) were as follows; 2 µl cDNA, 0.2 µM primer, and 5 µl Taq 5X Master Mix (cat. no. M0258L, New England BioLabs, Ipswich, MA, USA). In each case, cycling conditions were 95 °C (30 s), 55 °C (30 s), and 68 °C (30 s) for 38 cycles. Resulting amplicons were run on 1.5 % agarose gels with ethidium bromide and extracted using a QIAquick gel extraction kit (cat. no. 28704, QIAGEN, Valencia, CA, USA). Amplicons were cloned using a PCR cloning kit (cat. no. 231122, QIAGEN) and Sub-cloning Efficiency DH5α Competent Cells (Invitrogen, cat. no. 18265017, Life Technologies) following the manufacturers' protocols, with the exception that cloning reactions were scaled to 5 µl rather than 10 µl. Plasmids were extracted using a QIAprep Spin Miniprep Kit (cat. no. 27104, QIAGEN) and were sequenced by Core DNA Sequencing Facility (University of Illinois at Urbana-Champaign, Urbana, IL, USA) resulting in partial sequences: 712 bp for *cs* (KX342020), 958 bp for *hsp70* (KX342019), and 1240 bp for *18s* (KX342024). These partial sequences were sufficient to generate primers for qPCR (see below). Partial sequences were also generated for *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*; KX342023), *elongation factor 1-α* (*ef1-α*; KX342022), and *β-actin* (KX342021) for use as alternate normalization genes; however, *18s* was chosen as it varied the least across individuals and treatment groups when analyzed by qPCR.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was used to assess the relative abundance of *cs* and *hsp70* mRNA. Oligonucleotide primers were generated using Primer3plus (primer3plus.com) for the target genes as well as the reference gene *18s* (Table 2), and their specificity was verified by sequencing

Table 2 Oligonucleotide primer sets used for gene cloning and quantitative real-time RT-PCR (qPCR) in *Fusconaia flava*

Purpose	Gene	Primers (5′–3′)	Product size (bp)
Gene cloning	*cs*	Forward—TGT GCT ACA ATG TGG CAC GA	
		Reverse—TAC CAC ACC ATC GGA CCT GA	
	hsp70	Forward—CCA TTG CCT ATG GTC TGG A	
		Reverse—TTG CTG AGA CGA CCT TTG TC	
	18s	Forward 1—GGT TCC GCT GGT GAA TCT GA	
		Reverse 1—CAC CAC CCA CCG AAT CAA GA	
		Forward 2—CTT GGA TCG CCG TAA GAC GA	
		Reverse 2—CCT TCC GGG TAA GGG CAA AT	
qPCR	*cs*	Forward—GAG TCG ATT GGC CCA AGA CA	104
		Reverse—CCA CCT GTT CGT CGA GTT CA	
	hsp70	Forward—GAG CAT CAC CAG GGC AAG AT	103
		Reverse—TGG CTT GTC CAT CTT GGC AT	
	18s	Forward—GCT CGT AGT TGG ATC TCG GG	76
		Reverse—CCA GGA GGT AGG TCA GGA CA	

cs chitin synthase, *hsp70* heat shock protein 70

the product from each primer set. To optimize reaction compositions, standard curves were generated for each primer set using cDNA pooled from individuals across treatment groups (efficiencies were ≥0.85). Real-time PCR was carried out using RealMasterMix SYBR ROX (cat. no. 22008800, 5 Prime) and ABI 7900HT Fast Real-Time PCR System (Life Technologies) with the following cycling condition: 95 °C for 15 s and 60 °C for 60 s over 40 cycles. For all reactions, manufacturer's instructions were followed with the exceptions that reactions were scaled to 10 µl, of which 5 µl was the RealMasterMix SYBR ROX. For *cs* and *hsp70*, cDNA was diluted 20-fold, and 1000-fold for *18s*. Primer concentrations were 0.1 µM. The abundance of each gene was calculated relative to the 'control 6-h treatment' group for the short-term experiment and to the 'control 4 d' group for the long-term experiment using the modified delta–delta Ct method (Pfaffl 2001) with *18s* as the normalizing gene.

Ratio of RNA to DNA

The ratio of RNA:DNA has been used as a measure of protein synthesis and overall mussel health in previous studies (e.g., Norkko et al. 2006; Menge et al. 2007). In the present study, the ratio of RNA:DNA was assessed in the mantle tissue. Total RNA and DNA were extracted from the same piece of mantle tissue using the AllPrep DNA/RNA Mini Kit as in Tsangaris et al. (2010) following the manufacturer's protocol. Levels of RNA and DNA were then determined using a Qubit® 3.0 Fluoremeter (Fisher Scientific, Hanover Park, IL, USA) and expressed as the ratio of the RNA to DNA.

Oxygen consumption

An additional set of mussels ($N = 16$) from the 'long-term exposure to elevated pCO_2' experiment were assessed repeatedly for MO_2 at 4, 8, and 32 days of exposure. The MO_2 was determined using computerized intermittent-flow respirometry (Steffensen 1989). Briefly, the system consisted of four glass chambers (143 mm length × 45 mm diameter) that were each connected to two pumps, one for recirculation, and one for flushing ambient oxygenated water into the chamber. The total volume of the set-up, including the glass chamber and all associated tubing was 0.248 L. The MO_2 in each individual chamber was quantified within twelve 70 min cycles consisting of a 55 min measurement period, a 14 min flush period, and a 1 min wait period prior to commencing the subsequent cycle. The MO_2 (mg O_2 g^{-1} h^{-1}) for each mussel was calculated as:

$$MO_2 = \alpha\, V_{resp} \beta\, M_b^{-1}$$

where V_{resp} is the volume of each chamber minus the volume of the mussel (L), β is the oxygen solubility (adjusted daily for the barometric pressure and temperature), and M_b is the dry weight of the mussel (g; see below). For each trial, the coefficient of determination (r^2) for all slope measurements was >0.85. Calibration of the fiber optic oxygen probes with oxygen-free and fully saturated water was performed regularly throughout the experiments. Data were recorded using AutoResp software (Version 1.4), and background O_2 levels were collected and adjusted for during each trial (Steffensen 1989). At the completion of the 32 days repeated sampling period, mussel soft tissues were excised and dried at 99 °C (Widdows et al. 2002) for 24 h to determine dry weight.

Statistical analysis

The effects of CO_2 exposure on the mRNA abundance of *cs* and *hsp70* and the RNA:DNA ratio were assessed using a two-way analysis of variance (ANOVA) with pCO_2 level, sampling time, and their interaction (pCO_2 × sampling time) entered as fixed effects. If at least one of the main effects, or the interaction term, was significant, a Tukey–Kramer honestly significant difference (HSD) post hoc test was applied to separate means.

A general linear mixed effect model (GLMEM) was used to assess the impact of elevated pCO_2 on mussel MO_2 during long-term exposure. Main effects, including pCO_2 treatment, sampling time (4, 8, and 32 days), as well as the interactions of treatment × time, were treated as fixed effects, and mussel ID was treated as a random effect. The use of a random effect was necessary because multiple measurements were taken from each animal across trials, thus, each measurement was not independent (Laird and Ware 1982; Lindstrom and Bates 1990). The GLMEM was fit using 'lmer' from the R package lme4 (Bates 2010), and coefficients were estimated using restricted maximum likelihood. To define the importance of the fixed effects, the sim function ('arm' package in R) was used to generate $N = 1000$ posterior simulations of each fixed effect. The resulting posterior distribution of effect estimates were evaluated and those that did not overlap zero at the 95 %-level were considered significant.

For all statistical analyses, a visual analysis of fitted residuals using a normal probability plot (Anscombe and Tukey 1963) and a Shapiro–Wilk normality test were used to assess normality. A Levene's test, in combination with visual inspection of fitted residuals, was used to assess the homogeneity of variances. If either the assumption of normality or the homogeneity of variance were violated, data were ranked transformed and run with the same parametric model provided that the assumptions were met (Conover and Iman 1981; Iman et al. 1984; Potvin and Roff 1995).

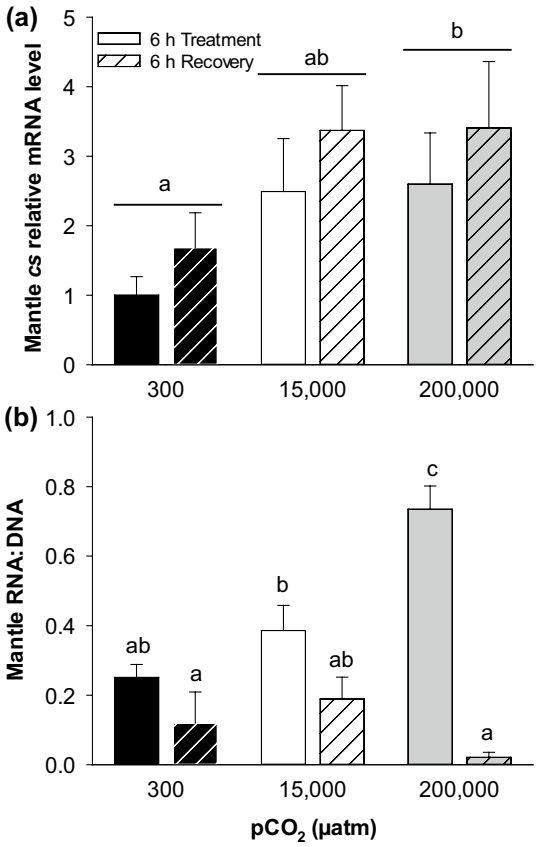

Fig. 1 Mantle (**a**) relative mRNA abundance of *chitin synthase* (*cs*) and **b** ratio of RNA to DNA in *Fusconaia flava* exposed to a short-term elevation in pCO_2. Mussels were exposed to one of three treatments: ambient (300 µatm), 15,000 or 200,000 µatm pCO_2 for 6 h followed by exposure to 6 h at ambient conditions (recovery). Data are presented as mean ± SEM ($N = 7$–8). All mRNA data were normalized to the mRNA abundance of *18s* and expressed relative to the 6-h ambient (300 µatm) treatment group. Treatment groups that do not share a letter are significantly different from one another. For **a**, only a significant effect of pCO_2 treatment occurred, with no significant effect of sampling time (two-way ANOVA; see Table 3)

Statistical analyses were performed using R version 3.2.2 and the level of significance (α) was 0.05.

Results

The effect of exposure to a short-term elevation in pCO_2

Mantle *cs* mRNA abundance was significantly increased by the exposure to a short-term elevation in pCO_2 relative to controls for those mussels exposed to the highest pCO_2 (200,000 µatm), with no significant effect of sampling time (Fig. 1a; Table 3). In mantle tissue, the RNA:DNA ratio was also significantly elevated by 6 h of exposure to the highest pCO_2 (200,000 µatm) relative to mussels held at ambient conditions, levels that returned to control levels following 6 h at ambient conditions (300 µatm; Fig. 1b; Table 3).

Mantle and adductor muscle *hsp70* mRNA levels were not affected following 6 h of exposure to 15,000 µatm pCO_2, but these levels were significantly elevated 6 h post-stressor (Fig. 2a, b; Table 3). Exposure to the highest pCO_2 (200,000 µatm) for 6 h resulted in a significant elevation in *hsp70* mRNA levels in the adductor muscle relative to mussels held at ambient pCO_2, which subsequently returned to control levels after an additional 6-h period at ambient conditions (300 µatm; Fig. 2b; Table 3). In the gill and foot, *hsp70* mRNA levels were largely unaffected by short-term exposure to elevated pCO_2 (Fig. 2c, d; Table 3), with the exception that exposure to 200,000 µatm pCO_2 caused a decrease in *hsp70* mRNA levels in gill that returned to control levels following 6 h at ambient conditions (300 µatm; Fig. 2c; Table 3).

The effect of exposure to a long-term elevation in pCO_2

Contrary to the impact of a short-term elevation in pCO_2, *cs* mRNA levels were significantly reduced during long-term exposure to 20,000 µatm pCO_2 in CO_2-treated mussels relative to control mussels (held at 1000 µatm), with no significant effect of sampling time (Fig. 3a; Table 4). Additionally, the ratio of RNA:DNA in mantle tissue was relatively unaffected by CO_2 treatment at 20,000 µatm and was only significantly elevated after 8 days of treatment compared to control mussels at 4 days, but not compared to control mussels held for the same duration (i.e., 8 days; Fig. 3b; Table 4).

Long-term exposure to 20,000 µatm pCO_2 resulted in an overall increase in *hsp70* mRNA levels in the gill of CO_2-treated mussels relative to mussels held at ambient conditions, with no significant effect of sampling time (Fig. 4c; Table 4). Similarly, *hsp70* mRNA levels were significantly elevated by CO_2-treatment at 4 and 8 days in the adductor muscle; however, after 32 days of exposure to elevated pCO_2 these levels were no longer different from mussels held at 1000 µatm for the same period of time (Fig. 4b; Table 4). Mantle and foot *hsp70* mRNA levels were not significantly affected by long-term pCO_2 treatment (Fig. 4a, d; Table 4).

Exposure to 20,000 µatm pCO_2 also resulted in an increase in MO_2 compared to mussels held at ambient conditions (1000 µatm), with no significant effects of sampling time (Fig. 5; Table 5).

Discussion

The present study provides evidence that exposure to elevations in pCO_2 may result in changes in shell formation

Table 3 Results of two-way ANOVA for the effects of short-term exposure to elevated pCO_2 in *Fusconaia flava*

Variable	Main effects	Degrees of freedom	Sum of squares	*F* value	*P*
Mantle *cs*	pCO_2	2	65.70	3.817	**0.030**
	Time	1	17.50	2.032	0.161
	pCO_2 × time	2	0.20	0.010	0.990
Mantle RNA:DNA	pCO_2	2	0.28	4.736	**0.014**
	Time	1	1.46	50.137	**<0.001**
	pCO_2 × time	2	0.80	13.806	**<0.001**
Mantle *hsp70*	pCO_2	2	7.13	4.216	**0.022**
	Time	1	1.25	1.484	0.230
	pCO_2 × time	2	6.43	3.802	**0.031**
Adductor hsp70	pCO_2	2	1916.40	13.415	**<0.001**
	Time	1	491.60	6.882	**0.012**
	pCO_2 × time	2	2396.3	16.773	**<0.001**
Gill *hsp70*	pCO_2	2	3.63	7.625	**0.002**
	Time	1	0.01	0.036	0.851
	pCO_2 × time	2	1.64	3.432	**0.042**
Foot hsp70	pCO_2	2	239.00	0.658	0.523
	Time	1	523.00	2.877	0.097
	pCO_2 × time	2	434.00	1.193	0.314

cs chitin synthase, *hsp70* heat shock protein 70

Significant *P* values are bolded and variables in italics are run on ranks

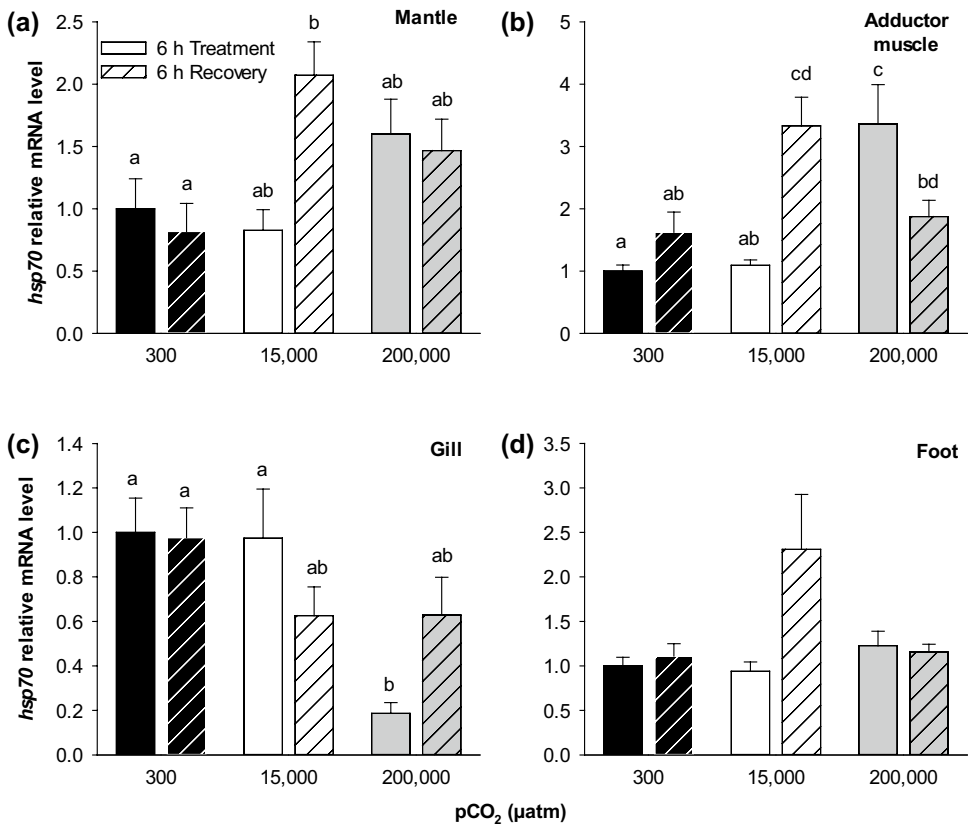

Fig. 2 Relative mRNA abundance of *heat shock protein 70* (*hsp70*) in the **a** mantle, **b** adductor muscle, **c** gill, and **d** foot of *Fusconaia flava* exposed to a short-term elevation in pCO_2. Mussels were exposed to one of three treatments: ambient (300 µatm), 15,000 or 200,000 µatm pCO_2 for 6 h followed by exposure to 6 h at ambient conditions (recovery). Data are presented as mean ± SEM ($N = 7$–8). All data were normalized to the mRNA abundance of *18s* and expressed relative to the 6-h ambient (300 µatm) treatment group. Treatment groups that do not share a letter are significantly different from one another. Neither pCO_2 treatment nor sampling time had a significant effect on the *hsp70* mRNA level in the foot (two-way ANOVA; see Table 3)

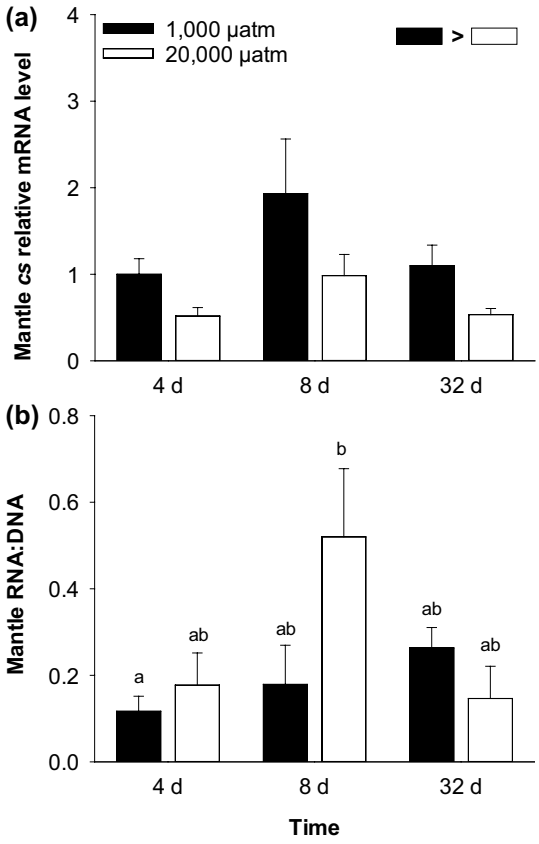

Fig. 3 Mantle (**a**) relative mRNA abundance of *chitin synthase* (*cs*) and **b** ratio of RNA to DNA in *Fusconaia flava* exposed to a long-term elevation in pCO_2. Mussels were exposed to either ambient (1000 µatm) or 20,000 µatm pCO_2 for 4, 8, or 32 days. Data are presented as mean ± SEM ($N = 7$–8). All mRNA data were normalized to the mRNA abundance of *18s* and expressed relative to the 4 days ambient (1000 µatm) treatment group. Treatment groups that do not share a letter are significantly different from one another. For **a** only a significant effect of pCO_2 treatment occurred, with no significant effect of sampling time (two-way ANOVA; see Table 4)

in freshwater mussels, particularly if the exposure is prolonged. Chitin plays an important structural role in the formation of mussel shells, and thus changes in the expression of *cs*, the enzyme that synthesizes chitin, may have consequences for shell formation (Weiss et al. 2006; Schonitzer and Weiss 2007). In the present study, short-term exposure to elevated pCO_2 resulted in an overall elevation in *cs* mRNA that was significant for mussels exposed to the highest pCO_2 (200,000 µatm). Previous work on the Antarctic bivalve, *L. elliptica* exposed to elevated pCO_2 (~800 µatm), but for a longer period of time (21 days), showed a similar increase in *cs* mRNA that was attributed to increased effort in the calcification of the shell (Cummings et al. 2011). Interestingly, in the present study, a longer exposure to elevated pCO_2 (up to 32 days) at 20,000 µatm, resulted in approximately a twofold decrease in *cs* mRNA levels compared to mussels held at ambient pCO_2 conditions (1000 µatm). It is possible that *F. flava* exposed to a relatively short elevation in pCO_2 (i.e., 6 h) may up-regulate processes to maintain normal shell formation in a compromising environment (i.e., elevated pCO_2 and concomitant decrease in pH), while prolonged exposure to pCO_2 may result in resources being diverted away from non-critical functions (e.g., shell formation) to other more vital functions (e.g., stress response). Additionally, as bivalves utilize $CaCO_3$ stores released from the shell to buffer against the acidosis experienced as a result of exposure to elevated pCO_2 (e.g., Crenshaw 1972; Michaelidis et al. 2005; Hannan et al. 2016), investing further in additional shell growth may be futile if shells are to be degraded to buffer acidosis. In addition to *cs*, the ratio of RNA to DNA in the mantle provides a measurement of total protein synthesis of processes likely to be associated with shell formation (Norkko et al. 2006). Similar to *cs* mRNA levels, the ratio of RNA to DNA in mantle was significantly elevated by short-term exposure to 200,000 µatm, but was largely unaffected by long-term exposure to 20,000 µatm pCO_2. Thus, processes associated with shell formation may be up-regulated during an acute exposure to elevated pCO_2, but not during an extended exposure to elevated pCO_2. Following a 6-h post-stressor period, mantle RNA:DNA fell to baseline levels, suggesting that this increase in protein synthesis in the mantle is transient. Though measurements of *cs* mRNA and mantle RNA:DNA are not direct assessments of calcification, changes in these factors may affect the structure and formation of the shell (Schonitzer and Weiss 2007; Cummings et al. 2011; Fang et al. 2011). The results of the present study provide evidence for regulation of the biological control of shell formation in response to changes in pCO_2, and provide potential biomarkers for further assessments of biomineralization in freshwater mussels.

The induction of HSPs by environmental stimuli has been widely documented, and the HSP70 family is frequently used as an indicator of the physiological mechanisms used by bivalves (and other animals) to cope with environmental disturbances (reviewed by Fabbri et al. 2008). The occurrence of the inducible *hsp70* mRNA under unstressed conditions is consistent with the ability of mussels to thrive in environments with fluctuating physical and chemical variables (Franzellitti and Fabbri 2005). In the present study, *hsp70* mRNA was present in *F. flava* held at control/ambient conditions in all of the tissues examined (e.g., gill, adductor muscle, mantle, and foot). This basal transcript level of *hsp70* is in agreement with the expression profiles of other bivalve species (e.g., Franzellitti and Fabbri 2005; Cellura et al. 2006; Cummings et al. 2011; Chen et al. 2014; Ivanina et al. 2014) and may help to minimize the effects of moderate environmental stressors. The synthesis of *hsp70* transcripts can be further increased in response to increased cellular stress, although this appears

Table 4 Results of two-way ANOVA for the effects of long-term exposure to elevated pCO_2 in *Fusconaia flava*

Variable	Main effects	Degrees of freedom	Sum of squares	*F* value	*P*
Mantle cs	pCO_2	1	1323	9.914	**0.003**
	Time	2	446	1.670	0.201
	pCO_2 × time	2	257	0.962	0.391
Mantle RNA:DNA	pCO_2	1	77	0.479	0.493
	Time	2	385	1.205	0.310
	pCO_2 × time	2	1624	5.081	**0.011**
Mantle hsp70	pCO_2	1	70	0.368	0.547
	Time	2	26	0.069	0.934
	pCO_2 × time	2	111	0.294	0.747
Adductor hsp70	pCO_2	1	4144	42.920	**<0.001**
	Time	2	248	1.284	0.288
	pCO_2 × time	2	765	3.960	**0.027**
Gill *hsp70*	pCO_2	1	15.245	44.204	**<0.001**
	Time	2	0.379	0.550	0.581
	pCO_2 × time	2	0.847	1.228	0.303
Foot hsp70	pCO_2	1	280	1.791	0.189
	Time	2	805	2.573	0.090
	pCO_2 × time	2	408	1.306	0.283

cs chitin synthase, *hsp70* heat shock protein 70

Significant *P* values are bolded and variables in italics are run on ranks

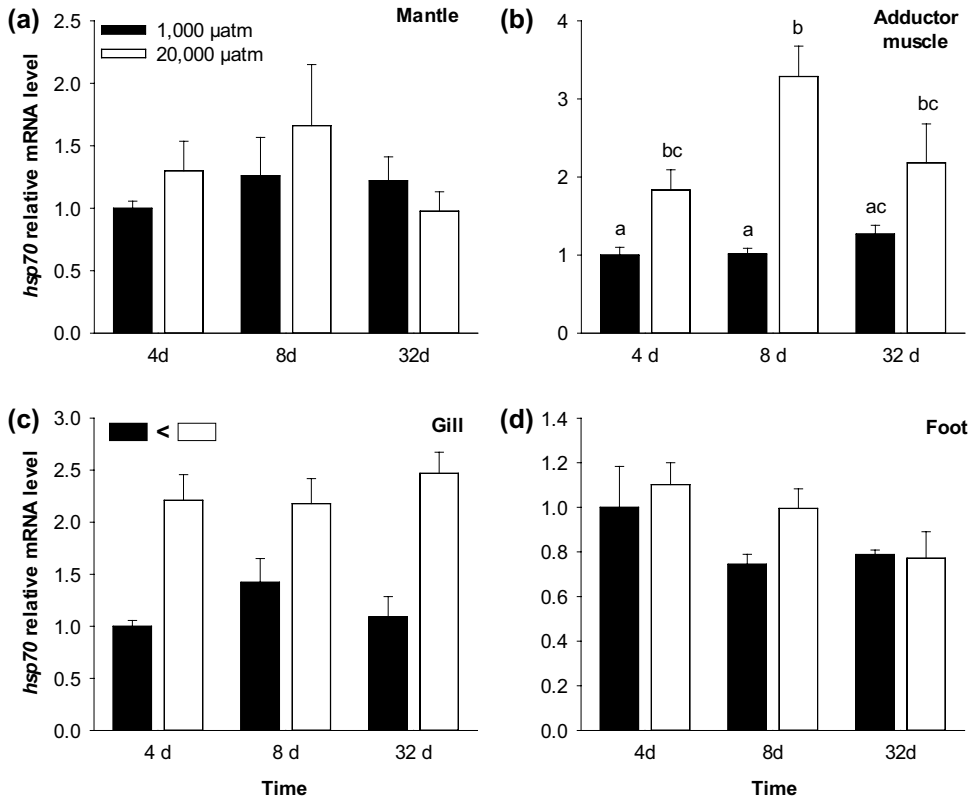

Fig. 4 Relative mRNA abundance of *heat shock protein 70* (*hsp70*) in the **a** mantle, **b** adductor muscle, **c** gill, and **d** foot in *Fusconaia flava* exposed to a long-term elevation in pCO_2. Mussels were exposed to either ambient (1000 µatm) or 20,000 µatm pCO_2 for 4, 8, or 32 days. Data are presented as mean ± SEM ($N = 8$). All data were normalized to the mRNA abundance of *18s* and expressed relative to the 4 days ambient (1000 µatm) treatment group. Treatment groups that do not share a letter are significantly different from one another. For **c**, only a significant effect of pCO_2 treatment occurred, with no significant effect of sampling time (two-way ANOVA; see Table 4). Neither pCO_2 treatment nor sampling time had a significant effect on the *hsp70* mRNA level in the mantle or foot (two-way ANOVA; see Table 4)

to occur in a tissue-, time-, and stressor-specific manner in bivalves (reviewed by Fabbri et al. 2008), as was observed in the present study.

The dynamics of *hsp70* mRNA expression were dependent on the severity of the CO_2 stressor during an acute exposure. An increase in *hsp70* mRNA levels in response

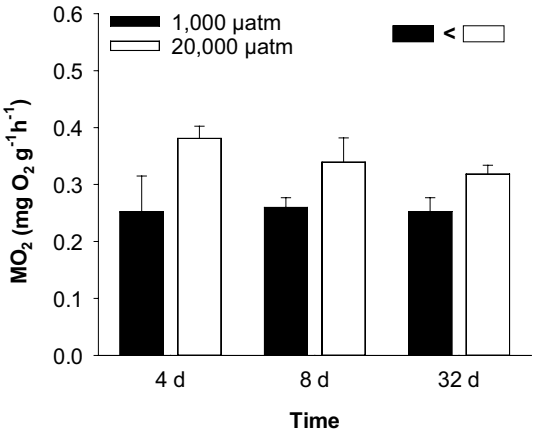

Fig. 5 Oxygen consumption (MO_2) of *Fusconaia flava* exposed to either ambient (1000 µatm) or 20,000 µatm pCO_2 for 4, 8, or 32 days. Mussels were repeatedly sampled for MO_2 over the course of the long-term exposure to elevated pCO_2. Data are presented as mean ± SEM ($N = 7$–8). Only a significant effect of pCO_2 occurred, with no significant effect of sampling time (general linear mixed effect model; see Table 5)

Table 5 Results of the general linear mixed effect model used to evaluate the effect of long-term pCO_2 exposure on oxygen consumption rate (MO_2) in *Fusconaia flava*

Parameter	Mean	95 % credible interval
Intercept (Ambient, 4 days)	0.25	0.18, 0.32
pCO_2 treatment	0.13	**0.02, 0.23**
8 days	0.01	−0.08, 0.11
32 days	0.00	−0.10, 0.10
pCO_2 × 8 days	−0.05	−0.19, 0.09
pCO_2 × 32 days	−0.06	−0.21, 0.08

to 6 h at 15,000 µatm pCO_2 was only evident in mantle and adductor muscle following an additional 6 h at ambient conditions (300 µatm). Thus, at moderate elevations in pCO_2 (15,000 µatm), a longer post-stressor period following an acute exposure may be necessary to visualize changes in *hsp70* mRNA levels and is in line with previous studies (e.g., Piano et al. 2004; Franzellitti and Fabbri 2005). Interestingly, although an increase in *hsp70* mRNA levels did not occur until 6 h post-stressor at 15,000 µatm, an increase in *hsp70* mRNA in the adductor muscle occurred following the 6-h CO_2 exposure at the highest CO_2 level of 200,000 µatm, and these levels began to return to pre-exposure levels following an additional 6 h at ambient conditions. The more rapid increase in *hsp70* mRNA levels in the adductor muscle (i.e., following the 6-h stressor) in response to 200,000 µatm compared to 15,000 µatm pCO_2 may reflect a response to a more severe stressor. In addition, in response to a higher level of CO_2 exposure (200,000 µatm), gill *hsp70* mRNA levels fell following the 6-h exposure period and began to increase to baseline levels 6 h post-stressor. A similar transient decrease in *hsp70* mRNA occurred in the digestive gland of *M. galloprovincialis* 1 h following an acute heat stressor, levels that subsequently increased above baseline 3 h post-stressor (Franzellitti and Fabbri 2005). It was suggested that the transient decrease in *hsp70* mRNA may reflect regulation of RNA metabolism during the heat shock response (Yost et al. 1990; Fabbri et al. 2008), and may help to explain the decrease in gill *hsp70* mRNA observed in the present study in response to the highest CO_2 exposure level (i.e., 200,000 µatm). The subsequent increase in *hsp70* mRNA to basal levels in the gill 6 h post-stressor may also reflect an increase in transcript production that may have exceeded control levels with a longer recovery period, as seen in previous studies (e.g., Piano et al. 2004; Franzellitti and Fabbri 2005). Together, these results suggest that acute exposure to elevated pCO_2 results in activation of the cellular stress response, and that this response is dependent on the tissue and severity of the stressor.

Chronic exposure to elevated pCO_2 also resulted in cellular responses to the stressor that were dependent on the tissue and duration of treatment. In gill, although *hsp70* mRNA levels were unaffected by short-term exposure to 15,000 µatm, they were significantly elevated throughout a 32-day exposure at 20,000 µatm. The delay in the increase of *hsp70* mRNA in response to elevations in pCO_2 further supports the idea that an extended exposure (or post-stressor period following a short-term stressor) may be necessary for this response to develop completely (reviewed by Fabbri et al. 2008). For the adductor muscle, a transient response was observed over the long-term exposure of *F. flava* to 20,000 µatm pCO_2, where *hsp70* mRNA levels were elevated at 4 and 8 days of CO_2 treatment but were no longer different from mussels held at ambient conditions for the same period of time at 32 days. A return to control levels of the mRNA abundance of *hsp70* at 32 days suggests that responses to a CO_2 stressor in the adductor muscle may be desensitized over a long-term exposure to elevated pCO_2. A similar transient response to Hg^{2+} exposure in *M. galloprovincialis* was observed, where *hsp70* mRNA in the digestive gland was elevated following 1 day of exposure but returned to basal levels by 6 days of exposure (Franzellitti and Fabbri 2005). In this study, Franzellitti and Fabbri (2005) found that mRNA abundances of the constitutive *hsc70* were inversely related to *hsp70*, and suggested that *hsp70* may be involved in the shorter-term response, whereas *hsc70* may be involved in longer-term cytoprotection (Franzellitti and Fabbri 2005). Although *hsc70* mRNA levels were not assessed in the present study, the potential short- and long-term roles of *hsp70* and *hsc70*, respectively, may also occur in the adductor muscle of *F. flava*

in response to elevations of pCO_2, though further investigation on this topic is required. Although mantle *hsp70* mRNA levels were elevated by short-term pCO_2 exposure at 15,000 µatm in *F. flava* (present study), and in the marine bivalve *L. elliptica* following 21 days at ~800 µatm (Cummings et al. 2011), exposure to 20,000 µatm for up to 32 days had no significant impact. Overall, exposure to elevated pCO_2 at any level or duration assessed in the present study had no effect on foot *hsp70* mRNA levels, indicating that the foot may be less affected, or is not responding in the same way as other tissues during CO_2 exposure. Together, the results of the present study suggest that *hsp70* responses to exposure to elevated pCO_2 are transient in some (e.g., mantle, adductor muscle) but not all tissues (e.g., gill), and some tissues may be more robust (e.g., foot) to changes in pCO_2. Although not consistent across all tissues and treatments, elevations in *hsp70* mRNA are indicative of an increase in the general stress response, a response that is likely to be energetically costly (Sørensen et al. 2003); however, an evaluation of variables beyond *hsp70* would be necessary to gain a more complete understanding of the stress status of mussels in response to elevated pCO_2 (e.g., glycogen stores, other HSPs, oxidative stress genes, etc.).

Whole-animal MO_2 was elevated in *F. flava* in response to prolonged exposure to elevated pCO_2. When exposed to 20,000 µatm pCO_2 for up to 32 days, mussels displayed an overall increase in MO_2, indicating that mussels were consuming more oxygen, and likely also expending more energy, to deal with CO_2 exposure. Previous work evaluating the consequences for elevated pCO_2 on marine bivalves found that increases in pCO_2 also caused elevations in MO_2 (e.g., Beniash et al. 2010; Lannig et al. 2010; Thomsen and Melzner 2010; Cummings et al. 2011; Parker et al. 2012). Increases in MO_2 are thought to occur due to a higher energy allocation to homeostasis (Beniash et al. 2010), and these increases may allow for quicker and more complete compensation of homeostatic disturbances induced by elevated pCO_2 (Parker et al. 2012). Increases in metabolic rate may be one of the mechanisms responsible for higher resilience to elevations in pCO_2 in some marine bivalves (e.g., oysters; Parker et al. 2012) and to support processes such as ion and acid–base regulation (e.g., Hannan et al. 2016), protein synthesis, and growth (Pörtner 2008). This increase in metabolic rate in *F. flava* may thus be adaptive, provided that food availability is not restricted, and metabolic rate is not elevated beyond a level that is sustainable. Interestingly, other studies assessing the consequences of pCO_2 exposure on marine bivalves found that increases in pCO_2 had either minimal effects on MO_2 levels (Matoo et al. 2013) or resulted in a decrease in MO_2 (e.g., Michaelidis et al. 2005; Fernández-Reiriz et al. 2011; Liu and He 2012; Navarro et al. 2013). The variation in the metabolic responses of marine bivalves may stem from differences in their resilience to changes in environmental pCO_2 (Parker et al. 2012). To our knowledge, the present study is the first to assess the impacts of elevated pCO_2 on MO_2 in a freshwater mussel. The increase in MO_2 observed in *F. flava* was in response to a much higher CO_2 level (i.e., 20,000 µatm, approximately 20 times the current mean pCO_2 in freshwater systems) (Cole et al. 2007) compared to previous exposures of marine bivalves. The capacity of *F. flava* to sustain increases in MO_2 over a period of at least 32 days suggests that freshwater mussels may be more robust to environmental changes in pCO_2 than marine mussels. This potential increase in CO_2 tolerance may have arisen in freshwater mussels due to their exposure to the natural fluctuations in pCO_2 that occur in freshwater systems (Maberly 1996; Hasler et al. 2016). The increase in MO_2 in *F. flava* during exposure to elevated pCO_2 may reflect an increase in the energy demand of processes involved in the stress response, that is also supported by the observed increases in *hsp70* mRNA levels, although the measurement of additional energetic parameters (e.g., metabolites) are necessary to support this hypothesis. Overall, the increase in MO_2 observed in *F. flava* suggests that freshwater mussels may be resilient to large elevations in pCO_2; however, it remains unclear whether mobilization of energy resources will be sufficient to support processes associated with the stress response necessary for survival, as well as growth and reproduction if exposures are prolonged (i.e., beyond 32 days), which may have population level consequences.

Results from the present study suggest that *F. flava* respond to both acute and extended elevations in pCO_2 by increasing processes related to the stress response. During short bouts of increased pCO_2, mussels attempt to maintain normal physiological functions such as shell formation. However, during extended exposure to elevated pCO_2, investing in processes such as shell formation may become less important, and mussels may divert limited resources away from non-vital functions to processes necessary for survival such as the stress response (present study) and acid–base balance (reviewed by Gazeau et al. 2013; e.g., Hannan et al. 2016). This diversion of limited resources may have long-term consequences for the survival and fitness of mussel populations that are already imperiled (reviewed by Pörtner et al. 2004). Encouragingly, the increase in MO_2 observed in *F. flava* in response to elevations in pCO_2 suggests that adult freshwater mussels may have the capacity to regulate in situations of elevated CO_2, at least in the short term (i.e., up to 32 days). The present study also provides potential targets for assessing the physiological status of mussels. For instance, the mantle tissue may provide a useful target for assessing the biological control of shell formation during both acute and chronic exposure to elevated pCO_2; although a broader picture of the mechanisms underlying this control would be gained by

assessing multiple genes (in addition to *cs*) associated with shell formation. In addition, although foot tissue provides a useful tissue for non-lethal sampling in assessments of physiological condition (e.g., Fritts et al. 2015), its use in assessing the impacts of elevated pCO_2 may be less informative compared to other tissues such as the gill and adductor muscle. Together, the data presented in the current study suggest that in future situations where pCO_2 elevations are expected to occur (e.g., due to increased atmospheric CO_2 or the deployment of a CO_2 fish barrier), mussels would be expected to have an increased energy demand, and respond in a way that is indicative of stress.

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