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.

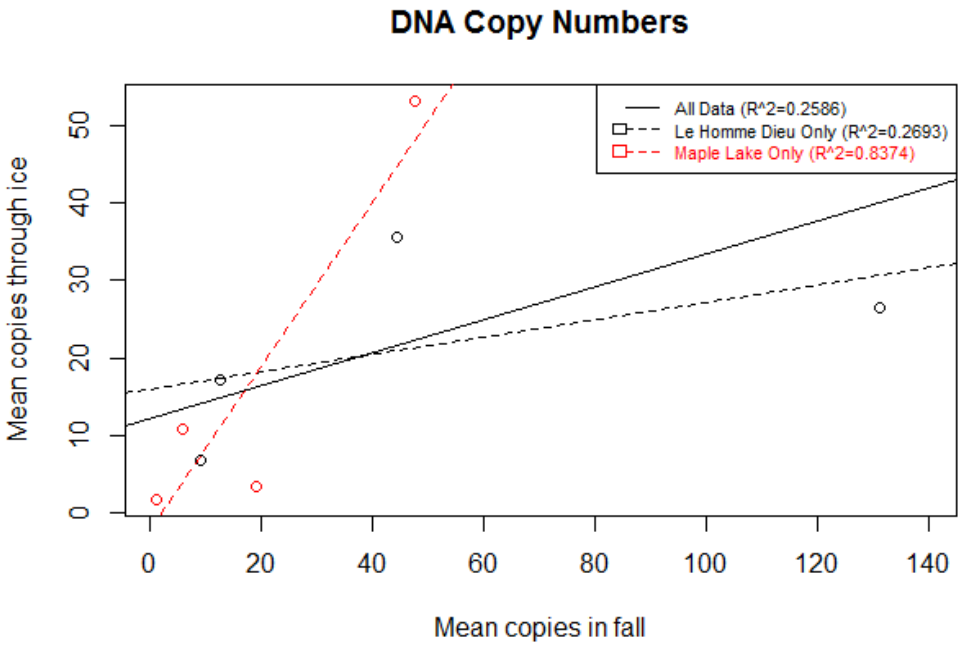
Outcome 3: Complete bathymetric surveys and data processing

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.

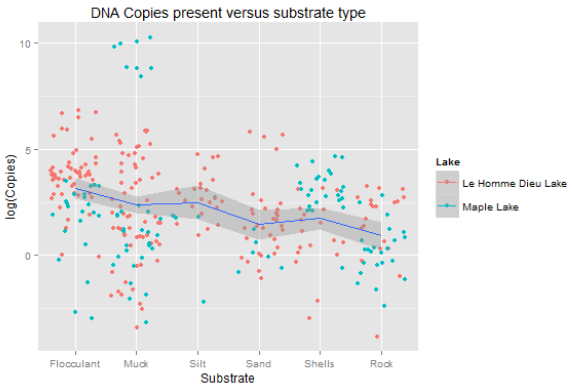
Outcome 4: Complete physical surveys and eDNA surveys and data processing

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.

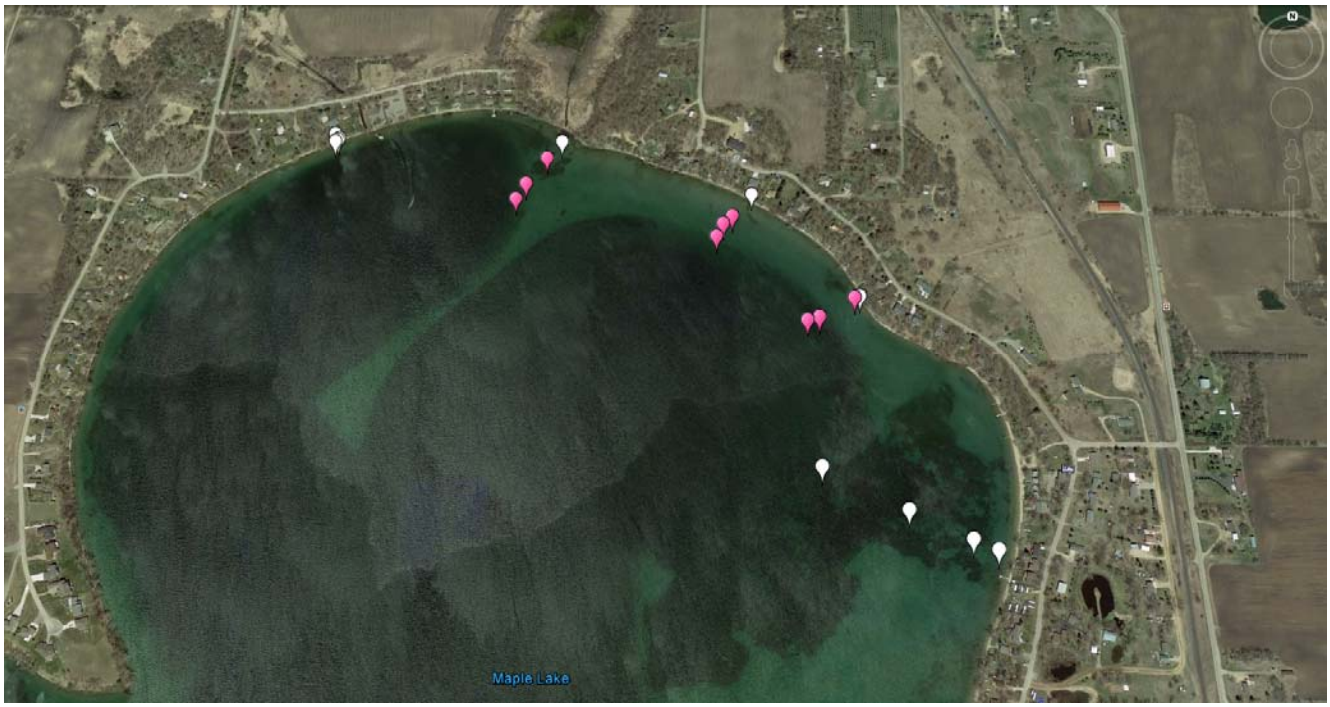
Outcome 5: Publish results

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:

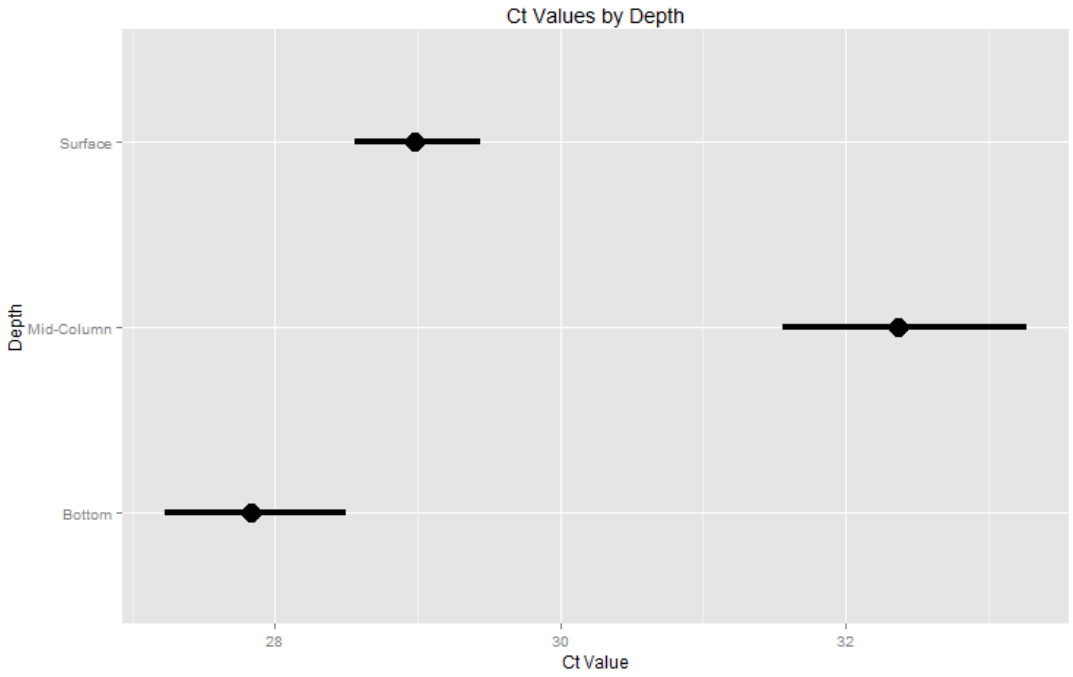
Outcome 1: Identify study lakes and sampling locations

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**