M.L. 2011, First Special Session, Chp. 2, Art.3, Sec. 2, Subd. 06a; M.L. 2014, Chapter 26, Section 2, Subdivision 19 Project Abstract
For the Period Ending June 30, 2015

PROJECT TITLE: Improved Detection of Harmful Microbes in Ballast Water
PROJECT MANAGER: Randall Hicks
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FUNDING SOURCE: Environment and Natural Resources Trust Fund

LEGAL CITATION: M.L. 2011, First Special Session, Chp. 2, Art.3, Sec. 2, Subd. 06a; M.L. 2014, Chapter 26, Section 2, Subdivision 19

APPROPRIATION AMOUNT: $250.000

Overall Project Outcomes and Results

While the Great Lakes face many threats, the presence of large and small invasive species threatens natural resources, people, and coastal economies. The objective of this project was to identify and evaluate the relative risk of potentially harmful bacterial groups and genes found in commercial ship ballast water that is discharged into the Duluth-Superior Harbor (DSH). Our ultimate goal was to establish a road map that can help direct future work towards higher risk ballast water microbial issues. To accomplish this goal, ballast water was collected from 16 commercial ships that ply the Great Lakes (i.e., "lakers") containing freshwater ballast water, 10 ocean-going ships (i.e., "salties") containing freshwater ballast water and 2 "salties" containing seawater ballast water in 2011 and 2012. Although there are nearly 1,000 vessel visits per year to this harbor, we collected almost three times as many ballast water samples as expected to create one of the largest repositories of ballast water microbial samples in the Great Lakes.

DNA from portions of these samples was extracted to identify different bacterial taxa while the remaining portions were frozen on membrane filters and stored as a sample repository for future studies. More than 170,000 partial bacterial 16S rDNA sequences were obtained for each sample. All sequence data were screened against two lists of bacterial genera that contain pathogenic bacterial strains. One list contained 20 genera of bacteria that include strains pathogenic to fish or wildlife, and the second list contained 57 genera of bacteria that are potentially pathogenic to humans. DNA from 15 of the 20 bacterial genera harboring fish or wildlife pathogens was detected in at least one ballast water sample. DNA from 37 of 57 bacterial genera that include human pathogens was detected in at least one ballast or harbor water sample. DNA sequences from a few of these bacterial taxa were often more common than DNA sequences from traditional indicator bacteria used for monitoring microbiological water safety.

Two genera containing bacterial strains pathogenic to fish and wildlife (i.e., *Tenacibaculum, Piscirickettsia*) and one genus containing a human pathogen (i.e., *Plesiomonas*) were evaluated further because all species within those genera were pathogenic indicating an elevated possibility of introducing a pathogen into the DSH environment. An example of this elevated risk is the bacterium *Piscirickettsia*, which causes "muskie pox" disease in muskellunge. DNA from this bacterium was found in 25% of the ships sampled, including ships transporting ballast water from Lake St. Clair where *Piscirickettsia* was found in dead muskellunge during a 2006 fish kill. It was interesting that DNA from *Renibacterium* species, the causative agent of bacterial kidney disease (BKD) in fish throughout the Great Lakes was not detected in any ballast water sample. Similarly, no DNA

sequences related to the ecologically harmful cyanobacerial genera *Anabaena* and *Microcystis* were detected in any ballast water or harbor water sample.

Microbes in ballast water may also modify native microbial populations by transferring genes for resistance the effects of antibiotics or the toxic effects of heavy metals. Six unique fosmid libraries containing bacterial metagenomic DNA were created for ship ballast water from Burns Harbor, IN, Hamilton, Ont., Cleveland OH, Detroit, MI and the Atlantic Ocean, and for Duluth-Superior Harbor water. Each fosmid library was screened for resistance to benzylpenicillin, cefotaxime and levofloxacin antibiotics and heavy metals, including cadmium, mercury, and zinc. Ballast water received from ports in larger, more densely populated cities (e.g., Cleveland, OH and Detroit, MI) usually had a larger proportions of microbial antibiotic and heavy metal resistance genes. Receiving ballast water form these harbors should cause greater concern for the spread of these genes to the Duluth-Superior Harbor than receiving ballast water from smaller metropolitan areas (e.g., Burns Harbor, IN). The information generated by this study provided the first step toward assessing the risks and potential impacts of microbial invasions in the Duluth-Superior Harbor and points to directions that warrant further research to develop methods to forecast future invasions.

Project Results Use and Dissemination

Information discovered by this project was disseminated in several ways. Preliminary results of this research were presented to the Duluth Harbor Technical Advisory Committee (HTAC), middle school teachers and students, Lake Superior Chapter of Muskies, Inc., and discussed with executives of the Lake Carriers' Association and the Great Lakes Maritime Research Institute. Ten research presentations were given to scientists at four regional and national scientific conferences, a Twin Ports Freshwater Folk meeting, and the U.S. EPA Mid-Continent Ecology Division in Duluth. Participants in the project also organized a scientific session on "Tools for Predicting and Managing Current and Future Invasions of Potentially Harmful Species in the Great Lakes" at the 2013 International Association of Great Lakes conference. DNA data housed at the University of Minnesota will be uploaded into national databases for searching and retrieval. This project provided training for a graduate student seeking a M.S. degree and a postdoctoral investigator. A M.S thesis and two scientific publications are being prepared from the results of this research.

Environment and Natural Resources Trust Fund (ENRTF)
M.L. 2011 Work Plan Final Report

Date of Status Update: 2/02/2016
Date of Next Status Update: Final Report
Date of Work Plan Approval: 6/23/2011
Project Completion Date: 6/30/2015

Project Title: Improved Detection of Harmful Microbes in Ballast Water

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Location:

Counties Impacted: St. Louis

Ecological Section Impacted: Northern Superior Uplands (212L)

Total ENRTF Project Budget:	**ENRTF Appropriation $:**	250,000
	Amount Spent $:	193,664
	Balance $:	56,336

Legal Citation: M.L. 2011, First Special Session, Chp. 2, Art.3, Sec. 2, Subd. 06a
M.L. 2014, Chapter 26, Section 2, Subdivision 19

Appropriation Language:
$125,000 the first year and $125,000 the second year are from the trust fund to the Board of Regents of the University of Minnesota for the University of Minnesota Duluth to identify and analyze potentially harmful bacteria transported into Lake Superior through ship ballast water discharge. This appropriation is available until June 30, 2014, by which time the project must be completed and final products delivered.

Carryforward: The availabiliity of the appropriations for the following projects are extended to June 30, 2015: (8) Laws 2011, First Special Session Chapter 2, article 3, section 2, subdivision 6, paragraph (a), Improved Detection of Harmful Microbes in Ballast Water;

I. PROJECT TITLE: Improved Detection of Harmful Microbes in Ballast Water

II. PROJECT SUMMARY:

While the Great Lakes face many threats, the presence of invasive species threatens not only Lake Superior but also Minnesota's people and coastal economies. The transport of organisms in the ballast water of ships is of global concern. The appearance of the fish virus VHS in the Great Lakes and the recent discovery of its DNA in parts of Lake Superior have led many to recognize that some microbes transported in the ballast water of commercial ships may be harmful invasive species, just like invasive species of plants and animals that threaten our natural resources. Our team will examine freshwater ('Lakers') and ocean-going ('Salties') commercial ships to identify harmful bacteria that are being transported in ballast water and discharged into Lake Superior. We will use state-of-the-art DNA sequencing techniques to identify harmful bacteria we should be most concerned about. The methods employed have the potential to detect rare microbes before they become common inhabitants in Lake Superior and nearby watersheds. Lastly, we will rank the most potentially harmful bacteria transported to the Duluth-Superior Harbor in the ballast water of commercial ships, which should be useful for developing guidelines for the microbiological safety of ballast water in the future.

III. PROJECT STATUS UPDATES:

Project Status as of *January 2012*: A postdoctoral associate and a graduate student were recruited to work on this project. They made progress towards the outcomes of all three activities in this project. Ballast water samples were collected from 19 commercial ships that visited the Duluth-Superior harbor and filtered for DNA extraction or bacteria were concentrated for developing fosmid libraries. DNA from three ballast and one harbor water sample were successfully amplified and these PCR products were submitted for sequencing on an Illumina sequencer. A supercomputer account was established and software was accessed to start analyzing DNA sequence data once it is obtained.

Project Status as of *July 2012*: The postdoctoral associate and the graduate student collected additional water samples and the sampling of ships that enter the Duluth-Superior harbor (DSH) continued during the spring and summer of 2012. There are currently samples of extracted bacterial DNA from the ballast water of 19 ships, 2 water samples from the DSH and 1 water sample from Silver Bay, MN. In addition, two paired ballast water-ballast tank sediment samples were collected and DNA was extracted. Three ballast and one harbor water sample were successfully sequenced on an Illumina sequencer and have been analyzed using the supercomputing capabilities of the University of Minnesota Supercomputing Institute. A second batch of 12 ballast water samples have been sequenced, the data returned and are currently being analyzed. A third batch of 8 samples has been submitted for sequencing.

Microbial cells in two of the ballast water samples collected in 2011 were pelleted and shipped to the Clemson University Genomic Institute (CUGI) in February 2011 for fosmid library creation. CUGI has constructed and shipped a fosmid library containing 36,000 clones for the Burns Harbor, IN sample. There was not enough DNA from cells in the second sample to create fosmid clones. Preliminary laboratory experiments have begun to perfect the methodology for screening of antibiotic and heavy metal resistance using bacteria isolated from water from the Duluth Superior Harbor.

Project Status as of *January 2013*: The postdoctoral associate and the graduate student collected 7 additional ballast water samples between the end of July 2012 and December 2012. There are now a total of 37 ballast water samples from 34 ships collected for this project. As of January 2013, DNA has been extracted from 25 ships. DNA from 24 ballast water samples have been successfully sequenced on an Illumina sequencer, sequence data was processed, sequence quality was checked and species lists were created. Another batch of sequences is currently being prepared for sequencing and analysis.

The graduate student used a QPix II instrument at the University of Minnesota to pick cell colonies from the Burns Harbor, IN fosmid library. The individual cell colonies containing fosmids were

placed in separate 384-well microplates. Both a master copy and a working copy of the fosmid libraries were created at the same time are kept in separate -80°C freezers at UMD. Microbial cells in two of the ballast water samples collected in 2012 along with microbial cells from a Duluth-Superior Harbor water sample were pelleted and shipped to Clemson University Genomic Institute (CUGI) in September 2012 for fosmid library creation. CUGI shipped the completed libraries back for the three water samples. Each constructed fosmid library contains at least 50,000 clones. The ballast water samples used for fosmid library construction originated from Detroit, Michigan and the Atlantic Ocean.

Project Status as of *July 2013*: Activity 1 has now been completed. Samples were collected from 29 ballast tanks of commercial ships, one ballast tank sediment as a part of a ballast water-ballast tank sediment paired sample, water at two sites (3 samples) in the Duluth-Superior Harbor and water from one site in the Silver Bay, MN Harbor. DNA has been extracted from all ship, sediment and harbor samples included in this project. Purified microbial DNA for all samples is in long-term storage at -80ºC in an ultracold freezer. The DNA extract repository is currently held at the Department of Biology at the University of Minnesota Duluth.

All DNA samples have been submitted for Illumina sequencing to the Biomedical Genomics Center of the University of Minnesota. The last batch of 14 samples is currently being sequenced. Pending the expected successful sequencing of this final batch of samples, this project will have generated bacterial DNA sequences for 29 ballast water samples, one sample of ballast tank sediment as a part of a ballast water-ballast tank sediment paired sample, 3 samples of Duluth-Superior Harbor water and a sample of Silver Bay Harbor water.

The graduate student used a QPix II instrument at the University of Minnesota to pick cell colonies from the Atlantic Ocean, Detroit, Michigan and the Duluth-Superior Harbor fosmid libraries. The individual cell colonies containing fosmids were placed in separate 384-well microplates. Both master and working copies of the fosmid libraries were created at the same time and are kept in separate -80°C freezers at UMD. Microbial cells in two ballast water samples from Hamilton, Ontario and Cleveland, OH collected in fall 2012 were pelleted and shipped to the Clemson University Genomic Institute (CUGI) in May 2013 for fosmid library creation. CUGI has completed the fosmid library for the Cleveland, OH sample but is still working on creating the Hamilton, Ontario library. Once fosmid libraries for these two remaining samples are created, there will be a total of 5 fosmid libraries for ballast water and one library for a site in the Duluth-Superior Harbor.

More than 50% of the 34 samples collected have been sequenced and analyzed including 15 ballast water samples. Analysis of the 14 remaining ballast water samples will start when the raw sequencing data arrives from the Biomedical Genomics Center at the University of Minnesota. The constituent communities of bacteria in many ballast and harbor water samples have been identified using the software package mothur. A list of 60 bacterial genera that contain potential human pathogenic species has been compiled. Similarly, a list of 19 bacterial genera that contain potential fish or wildlife pathogens has also been compiled. The ballast and harbor water bacterial community data have been cross-referenced with these lists. To date, we have identified 33 human pathogen-containing bacterial genera in the ballast water of commercial ships we have sampled, and 14 bacterial genera that contain fish or wildlife pathogens. These bacterial communities were also screened for the presence of potential environmentally disruptive bacteria such as harmful prokaryotic algae but none have been detected to date. A list of bacterial genera detected in the ballast water of ships but not detected in Lake Superior harbor water samples has also been created.

The graduate student is screening the Atlantic Ocean, Detroit, and Duluth-Superior Harbor fosmid libraries for antibiotic and heavy metal resistance to three antibiotics (benzylpenicillin, levofloxacin and cefotaxime) and three heavy metals (silver, cadmium and zinc) at minimum inhibitory concentrations published in peer reviewed journals for *Escherichia coli*. The student has completed screening the entire Burns Harbor fosmid library with these standard concentrations. Screening of the last remaining ballast water samples from Hamilton, Ontario and Cleveland, OH will commence once the fosmid libraries are received from Clemson University Genomic Institute.

Project Status as of *January 2014*: Activity 1 was completed in early 2013.

Activity 2 - Partial 16S rDNA sequences have now been obtained for all samples. The sequences from the remaining 14 samples were received from the UM Biomedical Genomics Center in mid-August 2013. Approximately, 200,000 sequences each were generated for the 29 ballast water samples, one ballast tank sediment sample, 3 samples of Duluth-Superior Harbor water and a sample of Silver Bay Harbor water. The sequence database has been completed and is stored on a supercomputer at the Minnesota Supercomputing institute.

Construction of one fosmid library (Hamilton, Ontario) was delayed three months. The Clemson University Genomic Institute (CUGI) requested additional pelleted cells from the Hamilton, Ontario ballast water sample in July and August 2013 because of difficulties creating a complete fosmid library. CUGI was able to create a library that consisted of approximately 12,000 clones for the Hamilton, Ontario ballast water sample and approximately 45,000 clones for the Cleveland, Ohio ballast water sample. These fosmid libraries were shipped to UMD in August 2013. The graduate student used a QPix II instrument at the University of Minnesota to pick cell colonies from the Hamilton, Ontario and Cleveland, Ohio fosmid libraries in September and October. This work was delayed about one month because the OPix II robotic instrument had been moved and not calibrated. This required a second trip to complete picking colonies for the final fosmid libraries. All fosmid libraries for the project have now been constructed. The individual cell colonies containing fosmids were placed in separate 384-well microplates. Both master and working copies of the fosmid libraries were created at the same time and are kept in separate -80°C freezers at UMD.

Activity 3 - Preliminary processing and analysis of DNA sequences from the V6 region of the 16S rRNA gene were completed for the 34 samples of ballast water, sediment, and harbor water samples, which include over 6 million individual sequences. Considering all samples, bacterial sequences were detected for 33 of 57 genera known to contain human pathogens and 14 of 20 genera known to contain fish and wildlife pathogens. A taxonomic classification of the 6 million sequences has been completed but a comparison of the bacterial compositions of different samples, statistical analyses, and phylogenetic trees remain to be completed. These analyses were delayed when the postdoctoral associate took a new position in September 2013. Further analyses of these sequences will be completed when a new postdoctoral associate is hired in early 2014.

Screening of the existing fosmid libraries was slowed in fall 2013 after learning that few clones in the initial libraries demonstrated resistance to the antibiotics and heavy metals. The graduate student worked to determine the minimum inhibitory concentration (MIC) specific to the *Escherichia coli* strain BL21DE3 used by CUGI as the host for the fosmids because initial screening of clones from three fosmid libraries indicated little resistance. The newly established MICs for the chosen antibiotics and heavy metals are now being used to rescreen the five fosmid libraries created by ballast water samples from Burns Harbor, Indiana, Atlantic Ocean, Detroit, Michigan, Cleveland, Ohio and Hamilton, Ontario and one fosmid library created from a Duluth-Superior Harbor water sample. This work requires that 1,260,000 clones are screened (6 fosmid libraries x 10,000 clones each x 7 antibiotics and heavy metals x 3 replicate measurements) before the results can be analyzed and functionally active genes identified.

Amendment Request (02/24/2014)

We are requesting a one-year extension to this project because of two unforeseen delays in obtaining and analyzing 16S rDNA sequences and fosmid libraries for ballast water samples. We are not proposing to shift funds between activities or between budget categories. Activity 1 has been completed while some outcomes of Activities 2 and 3 remain to be completed.

(1) Partial 16S rDNA sequences have now been obtained for all ballast water samples and are stored on a supercomputer at the Minnesota Supercomputing institute. Preliminary processing and analysis of DNA sequences from the V6 region of the 16S rRNA gene were completed for the 34 samples of ballast water, sediment, and harbor water samples, which include over 6 million individual sequences. However, complete statistical and phylogenetic analyses were delayed since the postdoctoral associate working on this project took a new position in September 2013. Remaining

analyses of these sequences will be completed when a new postdoctoral associate is hired in early 2014 and we now expect a new completion date of early 2015 for Activity 3-Outcome 1.

(2) Construction of fosmid libraries (Hamilton, Ontario) by the Clemson University Genomic Institute (CUGI) was delayed several months into mid-2013 because of difficulties encountered in creating complete fosmid libraries. Although all fosmid libraries have now been created for all samples, and the graduate student working on this project began picking cell colonies for these fosmid libraries in September and October, this work was delayed about one month because the robotic picking instrument had been moved and was not calibrated. This required a second trip to complete picking colonies for the final fosmid libraries. Screening of the existing fosmid libraries was slowed in fall 2013 after learning that few clones in the initial libraries demonstrated resistance to the antibiotics and heavy metals at the initial minimum inhibitory concentrations (MIC) chosen. This issue has been corrected. Newly established MICs for the chosen antibiotics and heavy metals are now being used to rescreen the five fosmid libraries created with ballast water samples from Burns Harbor, Indiana, Atlantic Ocean, Detroit, Michigan, Cleveland, Ohio and Hamilton, Ontario and one fosmid library created from a Duluth-Superior Harbor water sample. This work requires that 1,260,000 bacterial clones are screened (6 fosmid libraries x 10,000 bacterial clones each x 7 antibiotics and heavy metals x 3 replicate measurements) before the results can be analyzed and functionally active genes identified. We anticipate that this screening can be completed by late 2014 and statistical analyses completed in early 2015 (Activity 2-Outcome 2 and Activity 3-Outcome 2).

Amendment Approved: 05/09/14

Project Status as of *July 2014*: Activity 1 was completed in early 2013.

Activity 2 - All six fosmid libraries have been created and shipped by Clemson University Genomic Institute (CUGI) from pelleted ballast water bacterial cells and Duluth-Superior Harbor water bacterial cells collected by the graduate student. As of late 2013, the graduate student had used a QPix II instrument at the University of Minnesota to pick cell colonies for all the final fosmid libraries. The individual cell colonies containing fosmids were placed in separate 384-well microplates. Both master and working copies of the fosmid libraries are kept in separate -80°C freezers at UMD.

Activity 3 - The graduate student has continued to screen the five fosmid libraries created by ballast water sampled from Burns Harbor, Indiana, Atlantic Ocean, Detroit, Michigan, Cleveland, Ohio and Hamilton, Ontario and one fosmid library created from a Duluth-Superior Harbor water sample. As of July 2014, the graduate student had screened approximately 938,700 clones (44,700 clones each x 7 antibiotics and heavy metals x 3 replicate measurements) of the 1,260,000 clones required to analyze and identify functionally active antibiotic and heavy metal resistant genes.

Project Status as of *January 2015*: Activity 1 was completed in early 2013.

Activity 2 - All six fosmid libraries have been created. Fewer fosmids than expected were obtained for the Atllantic Ocean fosmid library. In the latter half of 2014, the graduate students tried on two occasions to use the qPIX II instrument at the University of Minnesota Biotechnology Institute to pick additional cell colonies to increase the size of this fosmid library. The QPix II instrument was damaged during a move in 2014 and has not been repaired. Additional attempts to pick more cell colonies for the Atlantic Ocean fosmid library with a second instrument was attempted in late 2014 and January 2015, but this instrument at the Biotechnology Institute was malfunctioning and could not be used successfully. At this time, it appears these instruments will not be repaired soon, so efforts were focused on screening the remaining fosmids in all libraries with the time remaining on the project.

Activity 3 - The graduate student has completed screening the four fosmid libraries created with ballast water sampled from Burns Harbor, Indiana, Detroit, Michigan, Cleveland, Ohio and Hamilton, Ontario and one fosmid library created from a Duluth-Superior Harbor water sample. The graduate student is continuing to screen the fosmid library created by ballast water sampled from the Atlantic Ocean. As of January 2015, the graduate student had screened approximately 1,116,108 clones (53,148 clones each x 7 antibiotics and heavy metals x 3 replicate measurements). Digitizing and statistical analysis of all fosmid library data was initiated and is continuing.

Overall Project Outcomes and Results:

While the Great Lakes face many threats, the presence of large and small invasive species threatens natural resources, people, and coastal economies. Ballast water from 28 freshwater and ocean-going commercial ships was sampled in the Duluth-Superior Harbor between 2009 and 2012 to identify potentially harmful bacteria. More than 170,000 partial bacterial 16S rDNA sequences were obtained for each sample. DNA sequences were detected from 15 and 37 bacterial genera containing fish & wildlife, and human pathogens, which were often more common than DNA sequences from traditional indicator bacteria for monitoring microbiological water safety. The bacterium *Piscirickettsia* was detected that causes "muskie pox" disease in muskellunge. DNA from this bacterium was found in 25% of the ships sampled, including ships transporting ballast water from Lake St. Clair where *Piscirickettsia* was found in dead muskellunge during a 2006 fish kill. Microbes in ballast water may also modify native microbial populations by transferring genes for antibiotic or heavy metal resistance. Six unique fosmid libraries containing bacterial metagenomic DNA were created for ship ballast water from the Burns Harbor, IN, Hamilton, Ont., Cleveland OH, Detroit, MI and the Atlantic Ocean, and water from the Duluth-Superior Harbor. Each fosmid library was screened for resistance to benzylpenicillin, cefotaxime and levofloxacin antibiotics and heavy metals, including cadmium, mercury, and zinc. Ballast water received from ports in larger, more densely populated cities (e.g., Cleveland, OH and Detroit, MI) usually had a larger proportion of microbial fosmids with antibiotic and heavy metal resistance genes and may cause greater concern for the spread of these genes to the Duluth-Superior Harbor than receiving ballast water from smaller metropolitan areas (e.g., Burns Harbor, IN). Identification of potentially harmful microbes and genes is the first step toward assessing the risks and impacts of microbial invasions and developing methods to forecast future invasions.

IV. PROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1: Collect Ballast Water from Commercial Ships and Extract DNA

Description:
Large volumes of ballast water will be collected from up to 10 commercial vessels in the Duluth-Superior harbor throughout the summers 2011 and 2012 as sampling opportunities arise with the Minnesota Pollution Control Agency (MPCA). Typically, ballast water samples will be collected by siphoning water using polyethylene tubing from a ballast tank, through a sounding tube off the side of the ship to the dock where it is captured. Ballasting history of the ballast water tanks will also be obtained through personnel interviews and access to ballast log records as determined by the ship officer present at the time of sampling. Each ballast water sample will be filtered onto a large Durapore membrane filter to concentrate bacterial cells. A portion of each membrane filter will be used to prepare DNA for sequence analysis of 16S rDNA and for the development of bacterial fosmid libraries. Total DNA will be extracted using MoBio PowerSoil® DNA extraction kits, eluted in nuclease-free water and then frozen (-80°C) until used for sequencing and constructing fosmid libraries.

Summary Budget Information for Activity 1:

ENRTF Budget: $ 37,874
Amount Spent: $ 36,274
Balance: $ 1,600

Activity Completion Date: November 2012

Outcome	Completion Date	Budget
1. *Collect ballast water from up to 10 commercial ships and establish ballast water collection*	October 2012	$ 18,790
2. *Extract microbial community DNA from water samples and develop a repository of purified microbial DNA from ballast water*	November 2012	$ 19,084

Activity Status as of *July 2012*: The postdoctoral associate and the graduate student collected additional water samples and the sampling of ships that enter the Duluth-Superior harbor (DSH) continued during the spring and summer of 2012. There are currently samples of extracted bacterial DNA from the ballast water of 19 ships, 2 water samples from the DSH and 1 water sample from Silver Bay, MN. In addition, two paired ballast water-ballast tank sediment samples were collected and DNA was extracted.

Activity Status as of *January 2013:* The postdoctoral associate and the graduate student collected 7 additional ballast water samples between the end of July 2012 and December 2012. There are now a total of 37 ballast water samples from 34 ships collected for this project. As of January 2013, DNA has been extracted from 25 ships.

Activity Status as of *July 2013*: Activity 1 has now been completed. Samples were collected from 29 ballast tanks of commercial ships, one ballast tank sediment as a part of a ballast water-ballast tank sediment paired sample, water at two sites (3 samples) in the Duluth-Superior Harbor and water from one site in the Silver Bay, MN Harbor. DNA has been extracted from all ship, sediment and harbor samples included in this project. Purified microbial DNA for all samples is in long-term storage at -80ºC in an ultracold freezer. The DNA extract repository is currently held at the Department of Biology at the University of Minnesota Duluth.

Activity Status as of *January 2014*: Activity 1 was completed in early 2013.

Activity Status as of *July 2014*: Activity 1 was completed in early 2013.

Activity Status as of *January 2015*: Activity 1 was completed in early 2013.

Final Report Summary:

Ballast and Harbor Water Sample Collection. Between July 2011 and the end of the shipping season in 2012, ballast water samples were collected from 24 commercial ships at nine dock locations in the DSH (Fig. 1, Table 1). Ballast water was collected either prior to or during active deballasting operations. Ballast water was collected from 16 commercial ships that ply the Great Lakes (i.e., “lakers”) containing freshwater ballast water, 10 ocean-going ships (i.e., “salties”) containing freshwater ballast water and 2 salties containing seawater ballast water (Table 1). Water samples were collected in pre-rinsed, 20 L plastic carboys from the ballast tank by using a length of 0.5” polyethylene siphon hose lowered through a sounding tube located on the deck of the ship when feasible or collected directly from the ballast tanks or from a ballast pump when the ballast water tank volume was too low to properly form a siphon on the deck. Water temperature and conductivity of ballast water samples were measured in the field with a YSI Model 85 handheld meter. In addition, three water samples were collected from various parts of the DSH and a single water sample was taken from Silver Bay, MN, which lies 55 miles northeast of the DSH. Ballast water from a laker with ballast water originating from Detroit, MI was sampled, the ballast was drained and then a sample of the remaining sediment in the ballast tank was collected with a sterile spoon and included in the analysis. All samples were transported to the lab at UMD within hours of collection, stored at 4°C and filtered within 24 hrs.

Subsamples (10 ml) of ballast water samples were preserved with 0.5 ml of 37% (w/v) formaldehyde (~2% final concentration) and stored in the dark at 4°C for up to 2 weeks before prokaryotic cells were counted with an epifluorescence microscope. A small portion (0.5-1 ml) of each preserved sample was placed on a black polycarbonate filter (25 mm dia., 0.22 µm pore; GE Water & Process Technologies) in a filter tower, and 200 µL 4’6-diamidino-2-phenylindole (DAPI; 10 µM final concentration) was added to stain DNA in microbial cells for 5 min. The stained prokaryotic cells were then filtered onto the membrane filter under low pressure (15 cm Hg), and counted using a Nikon Eclipse 80i epifluorescence microscope. Typically, there were fewer prokaryotic cells in ballast water (0.9-2.9 x 10^6 cells/ml) than in water from the Duluth-Superior harbor (3.7-5.3 x 10^6 cells/ml; Table 1).

Water Filtering. Water samples (4 to 34 liters) from ship ballast or natural waters were pressure filtered through 142 mm (0.2 µm pore) Duropore membrane filters. These filters were used for extracting DNA for 16S rDNA amplicon sequence analysis and for creating E. coli fosmid libraries for six samples. These filters were stored frozen (-80°C) until DNA was extracted unless a fosmid library was also created (see Concentrating Cells for Fosmid Libraries below).

DNA Extraction. Portions of filters containing microbes were extracted with a MoBio PowerSoil DNA extraction kit according to the manufacturer's instructions. DNA was eluted with nuclease-free water and stored at -80°C until PCR reactions. Purified microbial DNA and filtered microbial samples were kept in long-term storage at -80°C in an ultracold freezer at the Department of Biology at the University of Minnesota Duluth.

ACTIVITY 2: Sequence Bacterial Genes Found in Ship Ballast Water

Description:
Once extracted, DNA corresponding to the V5 and V6 hypervariable regions of the full-length 16S rDNA gene will be amplified by PCR using primers. The amplicons from multiple samples will be pooled together and the multiplexed amplicons will be sequenced on an Illumina/Solexa Sequencer at the National Center for Genomic Research (NCGR) in Santa Fe, New Mexico. In addition to phylogenetic information from 16S rDNA, our metagenomic analyses will also examine the functionality of microbial communities (i.e., genes conferring resistance to antibiotics and heavy metals) in up to ten samples of ship ballast water. To do this, we will send a portion of the extracted DNA samples (as described above) to the Clemson University Genome Institute for the construction of functional gene libraries. Library clones will be picked into 384 well microplates using a Qbot colony-picking robot. Functionally active fosmid clones will be sequenced at the Biomedical Genomics Center at the University of Minnesota and screened by the graduate student for functionally active genes involved in resistance to antibiotics and heavy metals.

Summary Budget Information for Activity 2:

ENRTF Budget:	**$ 109,103**
Amount Spent:	**$ 87,512**
Balance:	**$ 21,591**

Activity Completion Date: April 2013

Outcome	Completion Date	Budget
1. *Sequence bacterial 16S rRNA gene using the Illumina system and create a sequence database for ballast water bacteria*	April 2013	$ 68,869
2. *Construct fosmid libraries of function genes to detect harmful genes and processes*	April 2013	$ 40,234

Activity Status as of *July 2012*: DNA from three ballast and one harbor water sample were successfully sequenced on an Illumina sequencer, sequence data was processed and sequence quality was checked. Approximately 190,000 sequences were generated for each sample and initial analysis has been completed using the supercomputer at the University of Minnesota Supercomputing Institute. Twenty additional ballast water samples have been amplified, multiplexed and placed into the Illumina sequencing queue at the Biomedical Genomics Center at the University of Minnesota for Illumina sequencing and subsequent analysis. Twelve of those 20 samples have successfully been sequenced and the raw data returned to UMD.

Microbial cells in two of the ballast water samples collected in 2011 were pelleted and shipped to the Clemson University Genomic Institute (CUGI) in February 2011 for fosmid library creation. The sources of these ballast water samples were Monroe, MI and Burns Harbor, IN. CUGI has constructed

and shipped a fosmid library containing 36,000 clones for the Burns Harbor, IN sample. There was not enough DNA from cells in the Monroe, MI sample to create fosmid clones.

Activity Status as of *January 2013*: DNA from 24 ballast water samples have been successfully sequenced on an Illumina sequencer, sequence data was processed, sequence quality was checked and species lists were created.

The graduate student used a QPix II instrument at the University of Minnesota to pick cell colonies from the Burns Harbor, IN fosmid library. The individual cell colonies were placed in separate 384-well microplates. Both a master copy and a working copy of the fosmid libraries were created at the same time are kept in separate -80°C freezers at UMD. Microbial cells in two of the ballast water samples collected in 2012 along with microbial cells from a Duluth-Superior Harbor water sample were pelleted and shipped to Clemson University Genomic Institute (CUGI) in September 2012 for fosmid library creation. CUGI shipped the completed libraries back for the three water samples. Each constructed fosmid library contains at least 50,000 clones. The ballast water samples used for fosmid library construction originated from Detroit, Michigan and the Atlantic Ocean.

Activity Status as of *July 2013*: All DNA samples have been submitted for Illumina sequencing to the Biomedical Genomics Center of the University of Minnesota. The last batch of 14 samples is currently being sequenced. Pending the expected successful sequencing of this final batch of samples, this project will have generated bacterial DNA sequences for 29 ballast water samples, one sample of ballast tank sediment as a part of a ballast water-ballast tank sediment paired sample, 3 samples of Duluth-Superior Harbor water and a sample of Silver Bay Harbor water.

The graduate student used a QPix II instrument at the University of Minnesota to pick cell colonies from the Atlantic Ocean, Detroit, Michigan and the Duluth-Superior Harbor fosmid libraries. The individual cell colonies containing fosmids were placed in separate 384-well microplates. Both master and working copies of the fosmid libraries were created at the same time and are kept in separate -80°C freezers at UMD. Microbial cells in two ballast water samples from Hamilton, Ontario and Cleveland, OH collected in fall 2012 were pelleted and shipped to the Clemson University Genomic Institute (CUGI) in May 2013 for fosmid library creation. CUGI has completed the fosmid library for the Cleveland, OH sample but is still working on creating the Hamilton, Ontario library. Once fosmid libraries for these two remaining samples are created, there will be a total of 5 fosmid libraries for ballast water and one library for a site in the Duluth-Superior Harbor.

Activity Status as of *January 2014*: Partial 16S rDNA sequences have now been obtained for all samples. The sequences from the remaining 14 samples were received from the UM Biomedical Genomics Center in mid-August 2013. Approximately, 200,000 sequences each were generated for the 29 ballast water samples, one ballast tank sediment sample, 3 samples of Duluth-Superior Harbor water and a sample of Silver Bay Harbor water. The sequence database has been completed and is stored on a supercomputer at the Minnesota Supercomputing institute.

Construction of one fosmid library (Hamilton, Ontario) was delayed three months. The Clemson University Genomic Institute (CUGI) requested additional pelleted cells from the Hamilton, Ontario ballast water sample in July and August 2013 because of difficulties creating a complete fosmid library. CUGI was able to create a library that consisted of approximately 12,000 clones for the Hamilton, Ontario ballast water sample and approximately 45,000 clones for the Cleveland, Ohio ballast water sample. These fosmid libraries were shipped to UMD in August 2013. The graduate student used a QPix II instrument at the University of Minnesota to pick cell colonies from the Hamilton, Ontario and Cleveland, Ohio fosmid libraries in September and October. This work was delayed about one month because the OPix II robotic instrument had been moved and not calibrated. This required a second trip to complete picking colonies for the final fosmid libraries. The individual cell colonies containing fosmids were placed in separate 384-well microplates. Both master and working copies of the fosmid libraries were created at the same time and are kept in separate -80°C freezers at UMD.

Activity Status as of *July 2014*: All six fosmid libraries have been created and shipped by Clemson University Genomic Institute (CUGI) from pelleted ballast water bacterial cells and Duluth-Superior

Harbor water bacterial cells collected by the graduate student. As of late 2013, the graduate student had used a QPix II instrument at the University of Minnesota to pick cell colonies for all the final fosmid libraries. The individual cell colonies containing fosmids were placed in separate 384-well microplates. Both master and working copies of the fosmid libraries are kept in separate -80°C freezers at UMD.

Activity Status as of *January 2015*: All six fosmid libraries have been created. Fewer fosmids than expected were obtained for the Atlantic Ocean fosmid library. In the latter half of 2014, the graduate students tried on two occasions to use the qPIX II instrument at the University of Minnesota Biotechnology Institute to pick additional cell colonies to increase the size of this fosmid library. The QPix II instrument was damaged during a move in 2014 and has not been repaired. Additional attempts to pick more cell colonies for the Atlantic Ocean fosmid library with a second instrument was attempted in late 2014 and January 2015, but this instrument at the Biotechnology Institute was malfunctioning and could not be used successfully. At this time, it appears these instruments will not be repaired soon, so efforts were focused on screening the remaining fosmids in all libraries with the time remaining on the project.

Final Report Summary:

PCR Reactions. Bacterial DNA in the V6 hypervariable region of the 16S rRNA gene was amplified in triplicate with tagged PCR primers (967F and 1046R). 967F was in form of a cocktail of five modified primers (5'-CNACGCGAAGAACCTTANC, 5'- CAACGCGAAAAACCTTA, 5'-CAACGCGCAGAACCTTACC, 5'-ATACGCGAR¬GAACCTTACC, and 5'-CTAACCGANGAACCTYACC), which was described to increase the number of taxa that matched primer sequences. The 1046R reverse primer (5'-ID-CGACRRCCATGCANCACCT) had a barcoded Illumina adapter sequence (ID) that included a six-base multiplexing identification barcode which was unique to each sample (but the same for triplicate PCR reactions). The PCR amplification mixture (50 µl/reaction) contained: 1 µl 200 µM dNTPs (Promega, Madison, WI); 5 µl 10× PCR buffer (Denville Scientific, South Plainfield, NJ); 1 µl 5.0 units of Choice Taq DNA polymerase (Denville Scientific, South Plainfield, NJ); 2 µl 0.2 µM concentration of each PCR primer; and 11 µl nuclease-free water. Fifty ng of DNA from a ballast water sample were added to amplification mixtures as the template. PCR amplification was conducted in a BioRad DNA Engine Thermal Cycler using the calculated control method. After an initial denaturation at 95°C for 5 min, 25 cycles of PCR of 95°C denaturation for 30 sec, 55°C annealing for 30 sec, and 72°C extension for 30 sec were conducted. Finally, a 2 min extension at 72°C ended the PCR cycle. PCR products were cleaned using an Ultraclean PCR Clean-up DNA Purification Kit (MoBio Laboratories Inc., Carlsbad, CA). The concentration and quality of the PCR products were assessed on a NanoDrop 1000 spectrophotometer (Wilmington, DE).

***Illumina Sequencing of PCR Products*.** The triplicate PCR products for each sample (Table 1) were pooled to create an Illumina library for each ballast water sample, and then these samples were sent to University of Minnesota Biomedical Genomics Center for running paired-end multiplex sequencing on an Illumina MiSeq platform.

Concentrating Cells for Fosmid Libraries. The filters of ballast or harbor water samples selected for creating fosmid libraries were cut in half. Microbial cells were concentrated from one-half of each filter and the other half was stored frozen (-80 °C) until microbial DNA was extracted. Each filter portion designated for creating a fosmid library was placed in a 50 ml disposable centrifuge tube with 2 ml of buffer (0.1% sodium pyrophosphate at pH 7.0 with 0.2% Tween-20). The tube was agitated for three minutes using a flat padded vortex mixer. The solution from the conical tube was aliquoted into three 1.5 ml microcentrifuge tubes, which were centrifuged for 2 min at 15,000 rpm. The supernatants were removed and the three replicate pellets for each sample were stored frozen (-80 °C). Subsamples of these pellets were examined with an epifluorescent microscope to ensure intact prokaryotic cells were visible. Then, pellets were shipped overnight on dry ice to the Clemson University Genomics Institute (CUGI) for creating fosmid libraries.

***Creating Fosmid Libraries*.** Fosmid libraries were created for five ballast water samples and one water sample from the Duluth-Superior Harbor to estimate the relative abundance of prokaryotic genes coding for resistance to antibiotic or heavy metals. Four fosmid libraries were created from freshwater ballast water samples. These samples were selected base upon based upon invasive species "hot spots" in the Laurentian Great Lakes, Areas of Concern listed under the 1987 Great Lakes Water Quality Agreement between Canada and the United States, and the frequency of the port's ballast water discharge into the Duluth-Superior Harbor. Another fosmid library was created from microbial DNA extracted from the seawater ballast in an ocean-going ship to compare with fosmid libraries created from freshwater ballast. Finally, a Duluth-Superior Harbor water sample was selected for creating a fosmid library so that the frequency of antibiotic and heavy metal resistance in this harbor could be compared to the frequencies of resistance in the various ballast water samples.

Upon receiving the cell pellet samples, the Clemson University Genomics Institute extracted total metagenomic DNA from each cell pellet using an Epicentre™ metagenomic DNA isolation kit for water (Madison, WI) with the following modifications: incubation at 37□C was extended to one hour and metalysis and protein digestions was extended to 30 min at 65□C. The metagenomic DNA from each sample was randomly fragmented through the purification process with most DNA fragments being approximately 40 kb long. End-repair and phosphorylation was completed on the purified metagenomic DNA. DNA fragments 38 to 45 kb in size were separated by agarose gel electrophoresis (1% low melt agarose (Seaplaque), 20 V/cm for 15 hrs at 4□C). DNA fragments of the appropriate size (38 to 45 kb) were eluted from the gel by electroelution (model 422 Electro-Eluter, Biorad) and then concentrated by ethanol precipitation. The DNA fragments were ligated into the Eco72i site of the pCC2F0S vector (Epicentre®, Madison, WI). The ligation products were packaged in vitro into MaxPlax™ lambda phages (Epicentre®, Madison, WI). The lambda phages then infected the *E.coli* EPI300-TRR cells (Epicentre®, Madison, WI) with the ligation products. A final concentration of 20% glycerol was added to the transformation and the fosmids were stored frozen (-80°C) and shipped overnight on dry ice to the University of Minnesota Duluth.

A QPix II robotic sampler at the University of Minnesota Biotechnology Institute was used to pick *E. coli* cells containing fosmid inserts from the selected ballast water samples. The Qpix II placed portions of isolated cell colonies containing fomid inserts into 384-well NUNC microplates that contained Hogness Modified Freezing Media (HMFM) using JGI custom software to create the fosmid library for each sample. Between 8.500 and 11,000 *E. coli* cells containing inserted DNA fosmids were picked for each sample, except for the seawater ballast sample from the Atlantic Ocean (~4,000 fosmids) for a total of more than 54,000 cells. Both master and working copies of fosmid libraries were created. Once the NUNC microplates were inoculated with cells, then they were incubated for approximately 18 hrs at 37 °C and stored frozen (-80°C) until fosmids in individual libraries could be screened for antibiotic and heavy metal resistance genes.

ACTIVITY 3: Analyze Gene Sequences of Bacteria Found in Ships' Ballast Water

Description:
The 16S rDNA sequence data obtained will be compared to V5 and V6 region reference databases and the taxonomic classification of 16S rDNA PCR products will be assigned using reference databases and taxonomic classification tools and software. The taxonomic signature of microorganisms in each sample will be compared within and across samples and statistically analyzed. The resulting relationships that are identified will be tested by constructing bootstrapped phylogenetic trees. Comparisons of bacterial constituents in the ballast waters will be determined by examining the numbers and types of phyla (or operational taxonomic units) in each sample. When completed, these analyses will give us a comprehensive picture of the bacterial structure of ballast water. Additional analysis will be done to examine the fosmid libraries for functionally active genes that are involved in the resistance to antibiotics and heavy metals. Sequence data from functionally active fosmid clones

will be assembled into contigs, and analyzed by Blast and IMG-ACT software and websites. The DNA sequences in these fosmid clones will be annotated (identified) by the graduate student and postdoctoral associate and submitted to the IMG-ACT database.

Summary Budget Information for Activity 3:

ENRTF Budget: $ 103,023
Amount Spent: $ 69,878
Balance: $ 33,145

Activity Completion Date: June 2013

Outcome	Completion Date	Budget
1. *Analyze 16S rDNA sequences and construct phylogenetic trees to identify and rank the most common and the potentially harmful bacteria in ballast water*	June 2013	$ 70,439
2. *Annotate genes in fosmid libraries to identify harmful genes and processes*	June 2013	$ 32,584

Activity Status as of *January 2012*: Progress was made toward project outcomes listed in Activities 1, 2, and 3 during this period. Ballast water was collected from 15 commercial ships from late July to early December 2011. Fourteen of these samples were freshwater ballast and one was seawater ballast. These ships transported and then deposited ballast water into the Duluth-Superior harbor from harbors in Michigan, Ohio, Indiana, Ontario, Quebec, and the Atlantic Ocean. Sediment samples from ballast tanks were also collected from two ships. These ballast water samples complete Activity Outcome 1.1. DNA from these samples will soon be extracted for DNA sequence analysis. Bacteria were concentrated in five of these ballast water samples in preparation for developing fosmid libraries. Some progress was made towards the first outcome of the second Activity. DNA from bacteria in three ballast water samples and one harbor water sample previously collected was successfully amplified and submitted for Illumina sequencing (Activity Outcome 2.1). Analysis of these DNA gene sequences (Activity 3) will commence as soon as the data is received. We also set up an account at the UM Supercomputer Center to access software (e.g., mothur) to analyze the large DNA sequence databases (Activity Outcome 3.1). After a tutorial, we spent time practicing using this software to analyze the DNA sequences data that is being obtained in Activity 2. Work on Activity Outcomes 2.2 and 3.2 has not yet begun.

Activity Status as of *July 2012:* Community analysis of 3 ship ballast water samples and 1 harbor water sample has been completed using the supercomputing capabilities of the University of Minnesota Supercomputing Institute.. The constituent community of microorganisms has been identified for these samples using the Mothur software. Lists of potentially harmful genera of microorganisms detected in these samples and their frequency of detection have been created. The second batch of 12 samples submitted for Illumina sequencing has been sequenced and the raw data sent back to UMD. Community analysis of the second batch of Illumina samples is ongoing. The third batch consisting of 8 samples has been submitted for sequencing. When all sequence data has been generated, the microbial communities of microorganisms in each sample will then be compared within and across samples and statistically analyzed.

Preliminary laboratory experiments have begun to perfect the methodology for screening of antibiotic and heavy metal resistance genes in fosmid library clones using bacteria isolated from Duluth-Superior Harbor water. Levofloxazin, benzylpenicillin and cefotaxime are the three antibiotics and cadmium, silver, zinc and mercury are the four heavy metals chosen for screening resistance in the preliminary experiments as well as for the fosmid clone libraries.

Activity Status as of *January 2013*: There have been 24 ballast water samples successfully sequenced and analyzed using the supercomputing capabilities of the University of Minnesota Supercomputing Institute and another batch of sequences is being prepared for sequencing and analysis. The constituent community of microorganisms has been identified for these samples using the

software package mothur. Lists of potentially harmful genera of microorganisms detected in these samples and their frequency of detection have been created.

The graduate student used a QPix II instrument at the University of Minnesota to pick cell colonies from the Burns Harbor, IN fosmid library. The individual cell colonies were placed in separate 384-well microplates and duplicate copies were stored in different freezers at UMD.

Activity Status as of *July 2013:* More than 50% of the 34 samples collected have been sequenced and analyzed including 15 ballast water samples. Analysis of the 14 remaining ballast water samples will start when the raw sequencing data arrives from the Biomedical Genomics Center at the University of Minnesota. The constituent communities of bacteria in many ballast and harbor water samples have been identified using the software package mothur. A list of 60 bacterial genera that contain potential human pathogenic species has been compiled. Similarly, a list of 19 bacterial genera that contain potential fish or wildlife pathogens has also been compiled. The ballast and harbor water bacterial community data have been cross-referenced with these lists. To date, we have identified 33 human pathogen-containing bacterial genera in the ballast water of commercial ships we have sampled, and 14 bacterial genera that contain fish or wildlife pathogens. These bacterial communities were also screened for the presence of potential environmentally disruptive bacteria such as harmful prokaryotic algae but none have been detected to date. A list of bacterial genera detected in the ballast water of ships but not detected in Lake Superior harbor water samples has also been created.

The graduate student is screening the Atlantic Ocean, Detroit, and Duluth-Superior Harbor fosmid libraries for antibiotic and heavy metal resistance to three antibiotics (benzylpenicillin, levofloxacin and cefotaxime) and three heavy metals (silver, cadmium and zinc) at minimum inhibitory concentrations published in peer reviewed journals for *Escherichia coli*. The student has completed screening the entire Burns Harbor fosmid library with these standard concentrations. Screening of the last remaining ballast water samples from Hamilton, Ontario and Cleveland, OH will commence once the fosmid libraries are received from Clemson University Genomic Institute.

Activity Status as of *January 2014*: Preliminary processing and analysis of DNA sequences from the V6 region of the 16S rRNA gene were completed for the 34 samples of ballast water, sediment, and harbor water samples, which include over 6 million individual sequences. Considering all samples, bacterial sequences were detected for 33 of 57 genera known to contain human pathogens and 14 of 20 genera known to contain fish and wildlife pathogens. A taxonomic classification of the 6 million sequences has been completed but a comparison of the bacterial compositions of different samples, statistical analyses, and phylogenetic trees remain to be completed. These analyses were delayed when the postdoctoral associate took a new position in September 2013. Further analyses of these sequences will be completed when a new postdoctoral associate is hired in early 2014.

Screening of the existing fosmid libraries was slowed in fall 2013 after learning that few clones in the initial libraries demonstrated resistance to the antibiotics and heavy metals. The graduate student worked to determine the minimum inhibitory concentration (MIC) specific to the *Escherichia coli* strain BL21DE3 used by CUGI as the host for the fosmids because initial screening of clones from three fosmid libraries indicated little resistance. The newly established MICs for the chosen antibiotics and heavy metals are now being used to rescreen the five fosmid libraries created by ballast water samples from Burns Harbor, Indiana, Atlantic Ocean, Detroit, Michigan, Cleveland, Ohio and Hamilton, Ontario and one fosmid library created from a Duluth-Superior Harbor water sample. This work requires that 1,260,000 clones are screened (6 fosmid libraries x 10,000 clones each x 7 antibiotics and heavy metals x 3 replicate measurements) before the results can be analyzed and functionally active genes identified.

Activity Status as of *July 2014*: The graduate student has continued to screen the five fosmid libraries created by ballast water sampled from Burns Harbor, Indiana, Atlantic Ocean, Detroit, Michigan, Cleveland, Ohio and Hamilton, Ontario and one fosmid library created from a Duluth-Superior Harbor water sample. As of July 2014, the graduate student had screened approximately 938,700 clones (44,700 unique clones each x 7 antibiotics and heavy metals x 3 replicate measurements) of the

approximately 1,260,000 clones required to analyze and identify functionally active antibiotic and heavy metal resistant genes.

Activity Status as of *January 2015*: The graduate student has completed screening the four fosmid libraries created with ballast water sampled from Burns Harbor, Indiana, Detroit, Michigan, Cleveland, Ohio and Hamilton, Ontario and one fosmid library created from a Duluth-Superior Harbor water sample. The graduate student is continuing to screen the fosmid library created by ballast water sampled from the Atlantic Ocean. As of January 2015, the graduate student had screened approximately 1,116,108 clones (53,148 clones each x 7 antibiotics and heavy metals x 3 replicate measurements). Digitizing and statistical analysis of all fosmid library data was initiated and is continuing.

Fosmid Library	Number of Unique Clones Screened
Burns Harbor, IN	8,446
Atlantic Ocean	3,000
Detroit, MI	10,710
Cleveland, OH	11,128
Hamilton, ON	10,169
Duluth-Superior Harbor	9,695
Total	**53,148**

Final Report Summary:

Illumina Sequence Analysis. Partial 16S rDNA gene sequence reads were analyzed on the Minnesota Institute of Supercomputing server with mothur version 1.27.0. The uninformative and primer sequences were trimmed off from the original sequence reads, and different samples were sorted based on the barcodes specific to each ballast water sample. Sequences were aligned to the SILVA database, and then chimeras were also removed from sequences by using UCHIME. The processed sequences were then taxonomically classified into operational taxonomic units (OTUs) at the ≥ 97% sequence similarity level using furthest neighbor clustering using the Wang method and the Ribosomal Database Project (RDP) taxonomic database release 9. In an effort to reduce the possibility of false positive detections, we defined detection as the identification of at least three sequences in a particular genus in any single sample. If none of the samples contained at least three sequences belonging to a particular genus, then that genus was classified as not detected (ND).

All sample sequence data were screened against two lists of bacterial genera that contain potentially pathogenic species or strains, which were assembled from online resources and Bergey's Manual of Systematic Bacteriology. One list contained 57 genera of bacteria that are potentially pathogenic to humans and the second list contained 20 genera of bacteria that are potentially pathogenic to fish or wildlife. Additionally, sample sequence data was screened for two ecologically disruptive genera *Anabaena* and *Microcystis*.

Bacterial Community Compositions of Ship Ballast and Harbor Water. The ballast water samples collected from 28 commercial ships contained cells from many bacterial phyla (Fig. 2). The bacterial phyla most commonly encountered were not surprising because several bacterial phyla are large, quite diverse, and their cells are detected in freshwater from throughout the world. Bacterial DNA sequences were most commonly encountered from the following bacterial phyla and classes: Actinobacteria, Bacteroidetes, Alpha-Proteobacteria, and Beta-Proteobacteria (Fig. 2).

Potential Pathogen DNA. The DNA sequence data obtained from the samples in this study were screened against numerous bacterial genera containing pathogenic bacterial strains. Two genera containing bacterial strains pathogenic to fish and wildlife (i.e., *Tenacibaculum, Piscirickettsia*) and one genus containing a human pathogen (i.e., *Plesiomonas*) were evaluated further because all species

within those genera were pathogenic indicating an elevated possibility of introducing a pathogen into the DSH environment.

Potential Fish or Wildlife Pathogens - Fifteen of 20 bacterial genera evaluated that contain fish or wildlife pathogens were detected in at least one ballast water sample. These bacterial genera included: *Aeromonas, Acinetobacter, Clostridium, Flavobacterium, Francisella, Lactococcus, Mycobacterium, Pasteurella, Pasteuria, Piscirickettsia, Pseudomonas, Tenacibaculum, Streptococcus, Vibrio,* and *Yersinia.* Five bacterial genera containing bacterial strains that are fish or wildlife pathogens were not detected in any ballast or harbor water samples: *Actinobacillus, Flexibacter, Nocardia, Renibacterium* and *Xanthomonas.* It is interesting that DNA from *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD), was not detected in any ballast water sample because this bacterium was introduced to the Great Lakes in 1967. Now, the disease it causes occurs throughout Lake Ontario, Lake Huron, Lake Michigan, Lake Superior, and their tributaries.

Tenacibaculum is a bacterial genus within the Bacteroidetes phylum that contains a number of important saltwater fish pathogens. Detecting *Tenacibaculum* serves as a proof-of-concept that DNA sequences from potentially pathogenic bacteria can be taken up in a distant port, travel thousands of miles sealed in a ship ballast tank, and be successfully detected in ballast water being released into the DSH, even after a mid-ocean exchange of ballast water. Sequences assigned to the genus *Tenacibaculum* were detected in 9 of 16 (56.3%) lakers. Broken down by sampling year, *Tenacibaculum* sequences were detected in 5 of 6 lakers (83.3%) sampled in 2011 (as well as in the ballast tank sediment sample from 2011) and 4 of 9 lakers (44.4%) sampled in 2012. *Tenacibaculum* was not detected in the single laker sample from 2009. The ballast water of 10 salties with freshwater ballast was sampled in this study. Sequences from *Tenacibaculum* were detected in 3 of 10 (30.0%) salties with FW ballast. Broken down by sampling year, sequences from *Tenacibaculum* were detected in 1 of 1 salties with FW ballast in 2009, 1 of 6 (16.7%) in 2011 and 1 of 3 (33.3%) in 2012. The ballast water of 2 salties with saltwater ballast were sampled in this study (1 salty in 2009 and 1 salty in 2011). Sequences from *Tenacibaculum* were detected in both salties (100%) including a high number of sequences (2083) from the salty originating from Bilbao, Spain in 2009.

Piscirickettsia is a bacterial genus in which the only known species (*Piscirickettsia salmonis*) is pathogenic to salmonid and non-salmonid fish, including muskellunge or “muskies” (*Esox masquinongy*). Therefore, the detection of *Piscirickettsia* sequenes in ballast water is of special interest because of a high probability that DNA from a known pathogen was released into the DSH. Sequences assigned to the genus *Piscirickettsia* were detected in 1 of 9 lakers in 2012 (11.1%), 3 of 6 lakers in 2011 (50%), but was not detected in the single sample from 2009. In salties with FW ballast, *Piscirickettsia* sequences were detected in 0 of 3 samples from 2012, 1 of 6 (16.7%) samples from 2011, and was not detected in any sample from 2009. Sequences assigned to the genus *Piscirickettsia* were detected in both of the salties with saltwater ballast (1 from 2009 and 1 from 2011). DNA from the *Tenacibaculum* nor *Piscirickettsia* bacteria genera were not detected in any of the four water samples taken from the Duluth-Superior Harbor or Silver Bay. MN.

Potential Human Bacterial Pathogens - Thirty-seven of 57 bacterial genera evaluated that contain human pathogens were detected in at least one ballast or harbor water sample. These genera included: *Acinetobacter, Aeromonas, Bacillus, Bacteroides, Bordetella, Burkholderia, Clostridium, Corynebacterium, Edwardsiella, Enterococcus, Erysipelothrix, Escherichia/Shigella, Francisella, Helicobacter, Klebsiella, Lactobacillus, Legionella, Leptospira, Mobiluncus, Mycobacterium, Pasteurella, Peptostreptococcus, Plesiomonas, Prevotella, Propionibacterium, Proteus, Pseudomonas, Rhodococcus, Rickettsia, Salmonella, Staphylococcus, Streptococcus, Streptomyces, Treponema, Veillonella, Vibrio* and *Yersinia.* Twenty bacterial genera containing human pathogens were not detected in any sample. These genera included: *Actinobacillus, Actinomyces, Borrelia, Branhamella, Brucella, Campylobacter, Chlamydia* and *Chlamydophila, Citrobacter, Enterobacter, Fusobacterium, Haemophilus, Micrococcus, Morganella, Nocardia, Listeria, Mycoplasma, Neisseria, Porphyromonas, Providencia,* and *Serratia.*

The only species in the bacterial genus *Plesiomonas* is a human pathogen, so the detection of *Plesiomonas* DNA in ballast water discharged into the DSH indicated a higher probability of a human pathogen being introduced into the DSH. Sequences assigned to the genus *Plesiomonas* were detected in 2 of 9 (22%) laker ballast tanks sampled in 2012 (22.2%), 5 of 6 laker ballast tanks in 2011 (83%) and was also detected in the single laker sample taken in 2009. In salty ships with freshwater ballast, *Plesiomonas* sequences were not detected in ballast tanks sampled in 2012, but were detected in 2 of 6 (33.3%) ballast tanks sampled 2011 as well as in the single salty ship carrying freshwater ballast in 2009. *Plesiomonas* sequences were detected in the salty ship carrying saltwater ballast sampled in 2011 but not in a salty ship carrying saltwater ballast sampled in 2009. A single DNA sequence assigned to the *Pleisiomonas* genus was detected in water sample taken near the Midwest Energy dock in the DSH during 2011, but was not detected in other water samples from the DSH or Silver Bay, MN.

Potentially Harmful Cyanobacteria - No DNA sequences related to the ecologically harmful cyanobacerial genera *Anabaena* and *Microcystis* were detected in any ballast water or harbor water sample collected in this study.

Screening Fosmid Libraries for Antibiotic and Heavy Metal Resistance. In 2009, 64.2% of the antibiotic market in the United States was comprised of just three classes of antibiotics: cephalosporins, broad-spectrum penicillins, and fluoroquinolones. One antibiotic from each of these classes were selected to characterize the frequency of antibiotic resistance. Benzylpenicillin, from the penicillin group, and cefotaxime, from the cephalosporin group, act against Gram positive and Gram negative bacteria by inhibiting synthesis of bacterial cell walls. Levofloxacin is a type of fluoroquinolone antibiotic that is prescribed as a second line antibiotic to individuals who have life-threatening bacterial infections that are resistant to other general types of antibiotics.

Cadmium (Cd), mercury (Hg), and zinc (Zn) are heavy metals that were selected for measuring resistance because they have been associated with aquatic environmental degradation in several watersheds of the Great Lakes. Cadmium is toxic to bacteria at high concentrations, but little is understood about the mechanisms of uptake and resistance in bacteria. Mercury was the most toxic heavy metal tested in *E. coli* and is also toxic to humans. Zinc is also toxic at high concentrations, but is required in many proteins involving redox reactions. The minimum inhibitory concentrations of the three antibiotics and three heavy metals were determined utilizing control clone *E.coli* EPI300-TRR cells supplied by the CUGI. The control clone had the pcc2F0S vector DNA but no purified metagenomic DNA inserted. The minimum inhibitory concentrations varied between the antibiotics (benzylpenicillin [17.5 µg/ml], cefotaxime [0.6 µg/ml], levofloxacin [0.02 µg/ml]) and heavy metals (cadmium [1.55 mM], mercury [0.02 mM], zinc [2.2 mM]) chosen for evaluation.

LB medium was autoclaved and then cooled to 55°C for 30 min after which filter sterilized stock solutions of each antibiotic or heavy metal was added to the minimum inhibitor concentration. The LB media amended with individual antibiotics or heavy metals were poured into separate QTray polystyrene plates (20x20 cm; Molecular Devices), covered, and cooled overnight. The QTrays were stored at 4°C until inoculated with *E. coli* cells from the fosmid libraries. Each QTray containing an antibiotic or heavy metal was inoculated with cells from each fosmid library using an autoclaved 384 pin microplate replicator. Each QTray plate was stamped six times to screen approximately 5,900 fosmid clones. The replicator was sterilized between applications by dipped in 70% ethanol for 10 sec, then 10% bleach for 10 sec, and then again in 70% ethanol for 10 sec. Finally, the replicator was flamed twice. Plates were incubated at 37°C for 24 h and then were evaluated for cell growth. If cell colonies grew on the amended LB media, then this growth indicated the cells in those colonies demonstrated resistance above the minimum concentrations inhibitory to the control *E. coli* cell line due to the insertion of an antibiotic or heavy metal gene in the fosmid DNA. More than 50,000 individual *E. coli* cells containing fosmid inserts were screened for genes conferring resistance to the three antibiotics and three heavy metals. The frequency of resistance in each ballast or harbor water sample to each

antibiotic and heavy metal was determined by dividing the number of colonies growing on LB medium amended with a specific antibiotic or heavy metal divided by the number of colonies growing on LB medium with no antibiotic or heavy metal. These frequencies were expressed as percentages (Table 2). For each antibiotic and heavy metal, T-tests were performed to identify differences ($p<0.05$) in resistance between ballast or harbor waters from difference sources. Resistance to antibiotics and heavy metals was compared with the population size and density of metropolitan areas that were the sources of the ship ballast waters tested. Population sizes and population densities for cities surrounding these harbors were obtained from the internet.

Proportions of Microbial Fosmid Clones Demonstrating Resistance to Antibiotics and Heavy Metals. More than 257,000 clones were created for the six fosmid libraries of bacterial DNA from different ship ballast water. Of these clones, more than 50,000 were screened in triplicate for resistance to the three antibiotics and three heavy metals. In total, more than 956,000 individual resistance tests were completed.

The percentage of microbial fosmids that demonstrated resistance to antibiotics was different for each of the three antibiotics examined (Table 2). These differences in resistance were more pronounced for antibiotics than for heavy metals. The proportion of microbial fosmid clones resistant to benzylpenicillin ranged from about 20% to over 98%, the proportion resistant to cefotaxime ranged from 59% to 90%, while the proportion resistant to levofloxacin ranged from 26% to 56%. A smaller proportion of microbes in the seawater ballast water (Ship 30) were resistant to the benzylpenicillin than the proportion of microbial fosmids from Great Lakes ballast water (Table 2). Typically, microbial fosmids from the seawater ballast also had low resistant to the other antibiotics cefotaxime and levofloxacin. A similar proportion of microbial fosmid clones from the seawater ballast water were resistant the cadmium, mercury and zinc heavy metals when compared with microbial fosmid clones developed from ballast water coming from Great Lakes harbors (Table 2).

There were differences between the proportions of microbial fosmid clones showing resistance to the three antibiotics from different ballast waters that originated from within the Great Lakes (Table 2). A smaller proportion of fosmid clones from Burns Harbor, IN and Hamilton, Ont. were resistant to benzylpenicillin than fosmid clones from Duluth, MN, Cleveland, OH and Detroit, MI. The order of the increasing proportion of resistance to benzylpenicillin was: Burns Harbor, IN=Hamilton, Ont.<Duluth, MN=Cleveland, OH=Detroit, MI. A similar pattern of resistance relative to the sources of the ballast water was also seen for the other two antibiotics as well. The order of the increasing proportion of resistance to cefotaxime was: Burns Harbor, IN<Hamilton, Ont.=Duluth, MN<<Cleveland, OH=Detroit, MI. For the antibiotic levofloxacin, the order from less resistant to most resistant was: Burns Harbor, IN<<Detroit, MI=Duluth, MN=Hamilton, Ont.<Cleveland, OH.

Interestingly, these patterns of resistance to the three antibiotics appeared to be related to the population density of the urban areas adjacent to the Great Lakes harbors that were the sources of the ship ballast water (Figs. 3-5). Typically, ballast waters from Great Lakes cities with a population density less that 1,300 people/sq. mile had a smaller proportion of microbial fosmid clones resistant to benzylpenicillin, cefotaxime, and levofloxacin than microbial fosmids created from ballast water originating from harbors in larger metropolitan areas like Cleveland, IN and Detroit, MI. This pattern was not observed for resistance to most heavy metals; cadmium was the exception (Fig. 6-8). Still, there should probably be greater concern in Minnesota for importing microbial antibiotic resistance genes from ballast water originating from ports associated with larger, more urbanized cities such as Cleveland, OH and Detroit, MI than from harbors in smaller or less dense metropolitan areas like Burns, Harbor, IN.

In conclusion, ballast water received from ports in larger, more urbanized cities (Cleveland, OH and Detroit, MI) may contain larger proportions of microbes harboring antibiotic resistance genes than receiving ballast water from harbors in smaller or less dense metropolitan areas (Burns Harbor, IN and Hamilton, ON). This difference is cause for concern for the spread of antibiotic resistance among

native bacterial populations in the Duluth-Superior Harbor if large amounts of ballast water discharged into this harbor are from harbors near large and densely populated cities.

Work Proposed that was not Completed in Activity 3 and Remaining Project Balance. There was a substantial remaining balance when this project ended ($56,336). This remaining balance was due primarily to three unanticipated events. First, it took much longer than anticipated to obtain the fosmid libraries from the Clemson University Genomic Institute. Several samples also had to submitted a second time in order to obtain any fosmids or libraries with enough fosmids for our purposes. These problems delayed the start of screening over 50,000 unique fosmid clones. Second, the hand screening of these clones in triplicate for the six antibiotics and heavy metals (>956,000 individual analyses) took the graduate student much longer than we anticipated. In fact, this screening continued into the one-year extension period. Third, the graduate student working on this aspect of the project was offered a permanent position outside the university soon after finishing the screening of the fosmid libraries and left the project. Ultimately, the time on the project ran out before genes for antibiotic and heavy metal resistance could be sequenced and annotated in some of the fosmids libraries to evaluate these genes and possibly identify which bacterial taxa in the original ballast water samples contributed these genes to the fosmid libraries. The graduate student leaving the project early (for a permanent position) and the uncompleted work in Activity 3 were primarily responsible for balance remaining ($56,336) at the end of the project period. The University of Minnesota submitted a check for these remaining funds to the LCCMR, which in turn was forwarded it to the MN Management & Budget Office on September 30, 2015.

V. DISSEMINATION:

Description:
Our research results will be disseminated to several target audiences. First, we will periodically present and discuss the results of our investigation with our collaborators at the Minnesota Pollution Control Agency. We also intend to present our research results to our scientific peers at national and international scientific meetings, and develop manuscripts for scientific publications. We will upload the metagenomic data into national databases (e.g., Genbank and IMG-ACT) for searching and retrieval by researchers, regulatory agencies, and the public to better understand the diversity of microbes in ballast water. In addition, there are other target audiences we wish to reach; ship owners and agents, port authorities and other organizations such as the Great Lakes Maritime Task Force and the Great Lakes Maritime Research Institute. Data and results from our testing will be distributed (by email or personal visits) to dock owners, the Duluth Seaway Port Authority, and the Great Lakes Maritime Research Institute. We expect these existing networks will in turn disseminate information about this issue and our activities to other areas of the great lakes. At the discretion of Minnesota Pollution Control Agency, we will help disseminate information about this project to legislators and citizens.

Status as of *January 2012:* No research results were disseminated to target audiences during the first six-month period of this project.

Status as of *July 2012:* The postdoctoral associate gave a presentation to the Duluth Harbor Technical Advisory Committee (HTAC) on March 7, 2012:

A.J. Reed, J.B. Welch, C. Sloan and R.E. Hicks. (2012). Microbial Diversity of Ship Ballast Water Transported to the Duluth-Superior Harbor. Harbor Technical Advisory Committee (HTAC) meeting. March 7, 2012. HITC Conference Center, Superior, WI.

Status as of *January 2013:* No research results were disseminated to target audiences between July 2012 and January 2013. However, one PI and the postdoctoral associate proposed and are co-organized a special session at the 2013 IAGLR meeting on tools for predicting and managing current and future invasions of potentially harmful species in the great lakes. They and their colleagues

submitted an abstract to this meeting entitled, “Molecular Detection of Potentially Harmful Bacteria Discharged into the Duluth-Superior Harbor in the Ballast Water of Commercial Ships”.

Status as of *July 2013*: The postdoctoral associate presented an invited talk in May 2013 at the Environmental Protection Agency in Duluth, MN:

Reed, A.J., Badgley, J.B., Welch, J.B., Sloan, C.M., Sadowsky, M.J., and Hicks, R.E. Molecular Detection of Potentially Harmful Bacteria Discharged into the Duluth-Superior Harbor in the Ballast Water of Commercial Ships. USEPA ORD NHEERL Mid-Continent Ecology Division. May 29, 2013.

The postdoctoral associate gave a talk and co-chaired a special session with PI Hicks (Tools for Predicting and Managing Current and Future Invasions of Potentially Harmful Species in the Great Lakes) at the 2013 International Association of Great Lakes Research:

Reed, A.J., Badgley, J.B., Welch, J.B., Sloan, C.M., Sadowsky, M.J., and Hicks, R.E. Molecular Detection of Potentially Harmful Bacteria Discharged into the Duluth-Superior Harbor in the Ballast Water of Commercial Ships. 2013 International Association of Great Lakes Research (IAGLR) Annual Conference, June 2-6, 2013, West Lafayette, Indiana, USA.

The graduate student also presented preliminary results of the fosmid library data in a poster at the 2013 IAGLR meeting:

Sloan, C.M., Reed, A.J., Sadowsky, M.J. and Hicks, R.E. Identification of Antibiotic and Heavy Metal Resistant Bacteria from Commercial Ship Ballast Water Discharged into the Duluth-Superior Harbor. 2013 International Association of Great Lakes Research (IAGLR) Annual Conference June 2-6, 2013, West Lafayette, Indiana, USA.

While presenting this poster, she was approached by two middle school science teachers associated with Merrill Area Publix Schools about this research. She will present information from this project on antibiotic and heavy metal resistance in ballast water to a middle school science class this fall.

Randall Hicks discussed preliminary DNA sequence results of ballast water bacterial communities with officers of the Lake Carriers’ Association in a conference call on June 24, 2013, arranged by the Carol Wolosz from the Great Lakes Maritime Research Institute.

Status as of *January 2014*: No new research results were disseminated to target audiences between July and December 2013.

Status as of *July 2014*: Randall Hicks and graduate student working on this project presented research results at the 2014 Joint Aquatic Science Meeting (JASM) in Portland, Oregon in May 2014.

Hicks, R. E., A. J. Reed, B. D. Badgley, C. M. Sloan, and M. J. Sadowsky. Toward Early Detection of Ballast Water-Derived Microbial Invasions and Understanding their Impacts. 2014 Joint Aquatic Sciences Meeting, May 18-23, 2014, Portland, OR. (oral presentation)

Sloan, C.M., Reed, A.J., Sadowsky, M.J., and Hicks, R.E. Characterizing Antibiotic and Heavy Metal Resistant Genes from Bacteria in Commercial Ship Ballast Water Discharged into the Duluth-Superior Harbor. 2014 Joint Aquatic Science Meeting (JASM) May 18-23, 2014, Portland, OR. (poster presentation)

Randall Hicks and the graduate student submitted abstracts, which were accepted, to the 2014 Upper Midwest Invasive Species Conference that will be held in Duluth, MN from October 20-22, 2014.

Hicks, R. E., A. J. Reed, B. D. Badgley, C. M Sloan, and M. J. Sadowsky. Toward Forecasting Microbial Invasions in the Great Lakes. 2014 Upper Midwest Invasive Species Conference (UMISC) October 20-22, 2014, Duluth, MN. (oral presentation)

Sloan, C.M., A. J. Reed, M. J. Sadowsky, and R. E. Hicks. Bacterial Antibiotic and Heavy Metal Resistance Genes Discharged into the Duluth-Superior Harbor. 2014 Upper Midwest Invasive Species Conference (UMISC) October 20-22, 2014, Duluth, MN. (oral presentation)

Status as of *January 2015*: Randall Hicks and the graduate student working on this project presented research results at the 74th Annual Meeting of the North Central Branch of American Society for Microbiology, the 2014 Upper Midwest Invasive Species Conference, and at the Twin Ports Freshwater Folk meeting in November 2014.

Hicks, R. E. Toward Forecasting Microbial Invasions in the Great Lakes. October 10-11, 2014, ASM North Central Branch Annual Meeting, University of Wisconsin-Superior, Superior, WI. (Keynote Address)

Sloan, C.M., A.J. Reed, M. J. Sadowsky, and R. E. Hicks. Characterizing Antibiotic and Heavy Metal Resistant Genes from Bacteria in Commercial Ship Ballast Water Discharged into the Duluth-Superior Harbor. 2014 74th Annual Meeting of the North Central Branch of American Society for Microbiology, October 10-11, 2014, Superior, WI. (poster presentation; awarded 3rd place for best student poster)

Hicks, R. E., A. J. Reed, B. D. Badgley, C. M. Sloan, and M J. Sadowsky. Toward Forecasting Microbial Invasions in the Great Lakes. 2014 Upper Midwest Invasive Species Conference 2014, October 20-22, 2014, Duluth, MN. (oral presentation)

Sloan, C.M., A. J. Reed, M. J. Sadowsky, and R. E. Hicks. Bacterial Antibiotic and Heavy Metal Resistance Genes Discharged into the Duluth-Superior Harbor. 2014 Upper Midwest Invasive Species Conference 2014, October 20-22, 2014, Duluth, MN. (oral presentation)

Sloan, C.M., A. J. Reed, M. J. Sadowsky, and R. E. Hicks. Potential Transport of Harmful Bacteria and Genes to the Duluth-Superior Harbor. Twin Ports Freshwater Folk, November 5, 2014, Duluth, MN. (oral presentation)

Final Report Summary:

Information discovered by this project was disseminated in several ways (see detailed information above). Preliminary results of this research were presented to the Duluth Harbor Technical Advisory Committee (HTAC), middle school teachers and students, Lake Superior Chapter of Muskies, Inc., and discussed with executives of the Lake Carriers' Association and the Great Lakes Maritime Research Institute. Ten research presentations were given to scientists at four regional and national scientific conferences, a Twin Ports Freshwater Folk meeting, and the U.S. EPA Mid-Continent Ecology Division in Duluth. Participants in the project also organized a scientific session on "Tools for Predicting and Managing Current and Future Invasions of Potentially Harmful Species in the Great Lakes" at the 2013 International Association of Great Lakes conference. DNA data housed at the University of Minnesota will be uploaded into national databases for searching and retrieval. This project provided training for a graduate student seeking a M.S. degree and a postdoctoral investigator. A M.S thesis and two scientific publications are being prepared from the results of this research.

VI. PROJECT BUDGET SUMMARY:

A. ENRTF Budget:

Budget Category	$ Amount	Explanation
Personnel:	$ 200,445	One month of summer salary is requested each year for Dr. Hicks (8% FTE), who will participate in all aspects of this project and oversee the activities of the postdoctoral associate and graduate research assistant. No salary is requested for Dr Sadowsky. Salary is requested for Dr. Andrew Reed (100% FTE), a postdoctoral associate, who will be responsible for collecting ballast water samples, extracting DNA and preparing samples for gene sequence determinations, analysis of the 16S rDNA

		and fosmid library DNA sequence results, and help writing reports and publications. Salary is also requested for a graduate research assistant (33% FTE), who will work with the postdoctoral associate on all aspects of the project and develop a thesis on functional genes that are identified in the fosmid libraries.
Equipment/Tools/Supplies:	$48,055	Supply funds are requested for collecting and extracting DNA from ballast and harbor water samples, expendable laboratory materials, and purchasing a meter to measure water DO and conductivity. The majority of supply funds are required for Illumina sequencing of DNA from ballast water samples, and constructing and analysis of the fosmid libraries. Other funds are requested to disseminate the project results in scientific journals and other publications.
Travel Expenses in MN:	$1,500	Travel funds are requested to collect ballast water samples and for travel (including lodging and meals) between Dr. Hicks' and Dr. Sadowsky's laboratories in Duluth and St. Paul, respectively.
Other:	$	
TOTAL ENRTF BUDGET:	**$250,000**	

Explanation of Use of Classified Staff: N/A

Explanation of Capital Expenditures Greater Than $3,500: N/A

Number of Full-time Equivalent (FTE) funded with this ENRTF appropriation: 1.41

B. Other Funds:

Source of Funds	$ Amount Proposed	$ Amount Spent	Use of Other Funds
Non-state			
	$	$	
State			
UMD Biology Department	$13,613	$	Dr. Hicks salary
TOTAL OTHER FUNDS:	**$**	**$**	

VII. PROJECT STRATEGY:

A. Project Partners:

John Thomas and Jeff Stollenwerk, Minnesota Pollution Control Agency. These partners will not receive ENRTF funds from this appropriation.

B. Project Impact and Long-term Strategy:

While the Great Lakes face many threats, the presence of invasive species threatens not only Lake Superior but also Minnesota's people and coastal economies. The transport of organisms in the ballast water of ships is of global concern. Over 182 species of non-indigenous algae, invertebrates, fish, and plants have been identified in the Great Lakes, and it has been estimated that 65% of those species were introduced by the discharge of ballast water from ships. The appearance of the fish virus VHS in

the Great Lakes and the recent discovery of its DNA in parts of Lake Superior have led many to recognize that some microbes may be harmful invasive species, just like invasive species of plants and animals that threaten our natural resources. The ballast water of ships can be a vector for the global transport of aquatic microorganisms. Ship-mediated transport of bacteria is of particular concern due to their abundance, potential pathogenicity, and the ability of some bacterial species to form resting stages. In 2005, more than 5 billion gallons of ballast water was discharged into the Duluth-Superior harbor, the largest volume discharged in any harbor within the Great Lakes. This fact makes early detection of ballast-water derived invasive microbes an extremely important goal. Some of the bacteria being released into Lake Superior may cause ecological damage, impact local coastal economies, and even threaten human and aquatic animal health in other inland lakes in Minnesota. Yet, very little is known about the types of bacteria that are being transported by ships into Lake Superior, and their potential for causing irreparable harm.

C. Spending History:

Funding Source	FY 2010	FY 2011
Great Lakes Protection Fund grant (through Northeast-Midwest Institute)	$58,952	$89,322

VIII. ACQUISITION/RESTORATION LIST: N/A

IX. MAP(S): N/A

X. RESEARCH ADDENDUM: See Research Addendum

XI. REPORTING REQUIREMENTS:

Periodic work plan status update reports will be submitted not later than January 2012, July 2012, January 2013, July 2013, January 2014, July 2014, and January 2015. A final report and associated products will be submitted between June 30 and August 1, 2015 as requested by the LCCMR.

Attachment A: Budget Detail for M.L. 2011 (FY 2012-13) Environment and Natural Resources Trust Fund Projects

Project Title: *Improved Detection of Harmful Microbes in Ballast Water*

Legal Citation: M.L. 2011, First Special Session, Chp. 2, Art.3, Sec. 2, Subd. 06a; M.L. 2014, Chapter 226, Section 2, Subdivision 19

Project Manager: *Randall E. Hicks*

M.L. 2011 (FY 2012-13) ENRTF Appropriation: $ *250,000*

Project Length and Completion Date: June 30, 2015

Date of Update: February 2, 2016

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Activity 1 Budget	Amount Spent	Balance	Activity 2 Budget	Amount Spent	Balance	Activity 3 Budget	Amount Spent	Balance	TOTAL BUDGET	TOTAL BALANCE
BUDGET ITEM	***Collect Ballast Water and Extract DNA***			***Sequence Bacterial Genes found in Ballast***			***Analyze Gene Sequences of Bacteria***				
Personnel (Wages and Benefits)	32,169	32,169	0	68,053	58,067	9,986	100,223	68,178	32,045	200,445	42,031
Randall Hicks, Project Manager: $27,227 (2 mo @ 100% time + 33.3% fringe benefits)											
Postdoctoral Associate: $124,464 (24 mo @ 100% time + 20.22% fringe benefits)											
Graduate Research Assistant: $48,754 (7.9 mo@ 50%-											
Professional/Technical Contracts											
Service contracts											
Equipment/Tools/Supplies											
Ballast water sampling supplies	805	805	0							805	0
Portable temperature/DO/salinity meter	1,600		1,600							1,600	1,600
DNA extraction and PCR reagents	2,000	2,000	0	3,500	2,378	1,122				5,500	1,122
Fosmid library costs (10 libraries@ $1,550 ea)				15,000	10,506	4,494				15,000	4,494
Illumina sequencing costs (30 samples @ $5,000 per 10				15,000	8,522	6,478				15,000	6,478
Chemicals and expendable lab supplies	1,000	1,000	0	7,150	5,764	1,386	1,000		1,000	9,150	2,386
Publication costs							1,000	266	734	1,000	734
Capital equipment over $3,500											
Fee Title Acquisition											
Easement Acquisition											
Professional Services for Acquisition											
Printing											
Travel expenses in Minnesota (*Specify types of travel expenses, e.g., mileage, lodging, meals. Per diems are not allowed.)*	300	300	0	400	2,275	-1,875	800	1,434	-634	1,500	-2,509
Other *(Describe the activity and cost - be specific)*											
COLUMN TOTAL	**$37,874**	**$36,274**	**$1,600**	**$109,103**	**$87,512**	**$21,591**	**$103,023**	**$69,878**	**$33,145**	**$250,000**	**$56,336**

Table 1. Ballast water collected from commercial ships in the Duluth-Superior Harbor and harbor water samples collected in this harbor and in Lake Superior. Only samples collected as part of this LCCMR project in 2011 and 2012 are shown, even though four additional ballast water samples collected in 2009 were also analyzed.

Ship Number	Ship Type or Site	Date Sampled	DSH Dock	Ballast Type	Last Ballast Source	Temperature (°C)	Conductivity (µS)	Bacteria (10^9/L)
11	Laker	7-27-11	Murphy Oil	Freshwater	Monroe, MI	20.8	58	2.94
12	Laker	8-2011	Burlington Northern	Freshwater	Sorel, Quebec	23.5	200	2.85
13	Laker	8-10-11	Midwest Energy	Freshwater	St. Clair, MI	24.2	164	
14	Laker	8-31-11	CN/DMIR	Freshwater	Marquette, MI	18.9	138	0.91
15	Saltie	9-8-11	DSPA	Saltwater	Atlantic Ocean	22.5	34 ppt (salinity)	0.21
16	Laker	9-27-11	Burlington Northern	Freshwater	Montreal, Quebec			1.51
17	Saltie	9-30-11	CHS	Freshwater	Windsor, Ontario	15	1467	1.41
18	Laker	9-30-11	Murphy Oil	Freshwater	Windsor, Ontario	16.4	206	1.28
19	Saltie	10-28-11	General Mills - Duluth	Freshwater	Cleveland, OH	12.7	400	2.30
20	Saltie	10-31-11	CHS	Freshwater	Lake Superior (48 49.2°N, 018 09.0°W)	12.6	815	1.71
21	Saltie	11-7-11	CHS	Freshwater	Hamilton, Ontario	15.8	284	1.68
22	Laker	11-16-11	Burlington Northern	Freshwater	Burns Harbor, IN	11.1	210	1.25
23a	Saltie	11-16-11	CHS	Freshwater	Cleveland, OH	11.8	392	1.46
23b	Saltie	11-16-11	CHS	Freshwater	Burns Harbor,	10.8	287	1.62

					IN			
24a	Laker	12-8-11	Midwest Energy	Freshwater	Zug Island, Detroit, MI with some Lake Superior water	11.0	140	1.83
24b	Laker	12-8-11	Midwest Energy	Sediment				
25	Laker	1-8-12	Silver Bay	Freshwater	Burns Harbor, IN with Lake Superior water	11.9	91	1.09
26a	Saltie	6-21-12	CHS	Freshwater	Burns Harbor, IN	18.7	508	2.35
26b	Saltie	6-21-12	CHS	Freshwater	Cleveland, OH	18.7	751	2.87
27a	Saltie	7-25-12	Port Terminal	Freshwater	Detroit, MI	24.6	654	1.89
27b	Saltie	7-25-12	Port Terminal	Freshwater	Hamilton, Ontario	24.9	845	1.89
28	Laker	7-27-12	Fuel Station next to Port Terminal	Freshwater	Indiana Harbor, IN	22.5	380	2.45
29	Laker	8-20-12	Hallett Dock #5	Freshwater	Nanticoke, Ontario	23.2	505	4.56
30	Saltie	8-21-12	Port Terminal	Saltwater	Atlantic Ocean	20.9	414	1.40
31	Saltie	9-27-12	CHS	Freshwater	Milwaukee, WI			
32	Laker	8-21-12	Hallett Dock #5	Freshwater	Nanticoke, Ontario			
33	Laker	10-19-12		Freshwater	St. Clair, MI / Essexville, MI			
34	Saltie	10-29-12		Freshwater	Toledo, OH			
	DSH –	7-26-11	Near	Freshwater				3.73

	Site 3		Murphy Oil					
	DSH – Site 4	7-26-11	Near Midwest Energy	Freshwater				5.27
	DSH	8-20-12	Cutler Magner /Graymont	Freshwater		21.5	84.0	4.97
	Silver Bay	7-27-11	Silver Bay	Freshwater				0.48

Table 2. Resistance of microbial fosmids from different harbor or ship ballast water sources to antibiotics and heavy metals. Fosmid libraries were constructed from microbial metagenomic DNA extracted from Duluth-Superior Harbor water and ballast waters collected from five commercial ships that discharged ballast water into this harbor from different sources. Mean values are given with standard deviations shown in parentheses. Mean resistance values with the same superscript letter were not significantly different.

Ship Number	**Source of Harbor or Ballast Water**	**Resistance to Antibiotic or Heavy Metal (%)**					
		Benzylpenicillin	**Cefotaxime**	**Levofloxacin**	**Cadmium**	**Mercury**	**Zinc**
	Duluth-Superior Harbor, MN	96.6 [a,b] (0.9)	78.0 [a] (2.4)	54.2 [a,b] (2.0)	83.0 [a] (1.6)	91.5 [a,b,c] (3.7)	50.5 (0.7)
23b	Burns Harbor, IN	88.0 [c] (2.8)	58.9 (3.9)	25.9 (1.2)	80.2 [a,b] (2.0)	89.9 [a,d,e,f] (6.6)	71.9 (1.8)
27b	Hamilton, Ont.	92.6 [c] (1.4)	73.3 [a, b] (5.9)	55.9 [b,c] (4.1)	69.2 [b] (5.7)	81.8 [d] (3.9)	39.2 (2.5)
26b	Cleveland, OH	98.3 [b,d] (0.5)	86.7 [c] (2.2)	64.7 (0.7)	92.4 [c,d] (0.7)	99.7 [b,g] (0.4)	66.5 (2.0)
27a	Detroit, MI	98.6 [a,d] (0.3)	89.9 [c] (1.0)	52.9 [a,c] (4.2)	93.0 [d,e] (2.2)	90.7 [c,e] (3.8)	75.7 (1.3)
30	Atlantic Ocean	19.6 (0.6)	67.5 [b] (1.4)	34.8 (2.6)	88.3 [c,e] (2.3)	100.8 [f,g] (2.7)	58.6 (1.5)

Figure Legends

Figure 1. Map of the Duluth-Superior Harbor (DSH) showing the dock locations where ballast water from commercial ships was collected during the 2011 and 2012 shipping seasons. A, B, and C are locations were water samples were taken from the DSH. Details about the ships and the sources of the ballast waters they contained are given in Table 1.

Figure 2. Phylum-level composition of bacterial DNA found in ballast water from commercial ships discharging ballast water from different sources into the Duluth-Superior Habor from 2009 to 2012. The phylum-level composition of bacterial DNA from Duluth-Superior Harbor and Silver Bay, MN water samples collected during the same period are also shown for comparison.

Figure 3. The percentage of microbial fosmids from different ballast water sources demonstrating resistance to the benzylpenicillin antibiotic compared to the population density (people/sq. mile) of the urban areas adjacent to the Great Lakes harbors that were the sources of the ship ballast water.

Figure 4. The percentage of microbial fosmids from different ballast water sources demonstrating resistance to the cefotaxime antibiotic compared to the population density (people/sq. mile) of the urban areas adjacent to the Great Lakes harbors that were the sources of the ship ballast water.

Figure 5. The percentage of microbial fosmids from different ballast water sources demonstrating resistance to the levofloxacin antibiotic compared to the population density (people/sq. mile) of the urban areas adjacent to the Great Lakes harbors that were the sources of the ship ballast water.

Figure 6. The percentage of microbial fosmids from different ballast water sources demonstrating resistance to cadmium compared to the population density (people/sq. mile) of the urban areas adjacent to the Great Lakes harbors that were the sources of the ship ballast water.

Figure 7. The percentage of microbial fosmids from different ballast water sources demonstrating resistance to the mercury compared to the population density (people/sq. mile) of the urban areas adjacent to the Great Lakes harbors that were the sources of the ship ballast water.

Figure 8. The percentage of microbial fosmids from different ballast water sources demonstrating resistance to the zinc compared to the population density (people/sq. mile) of the urban areas adjacent to the Great Lakes harbors that were the sources of the ship ballast water.

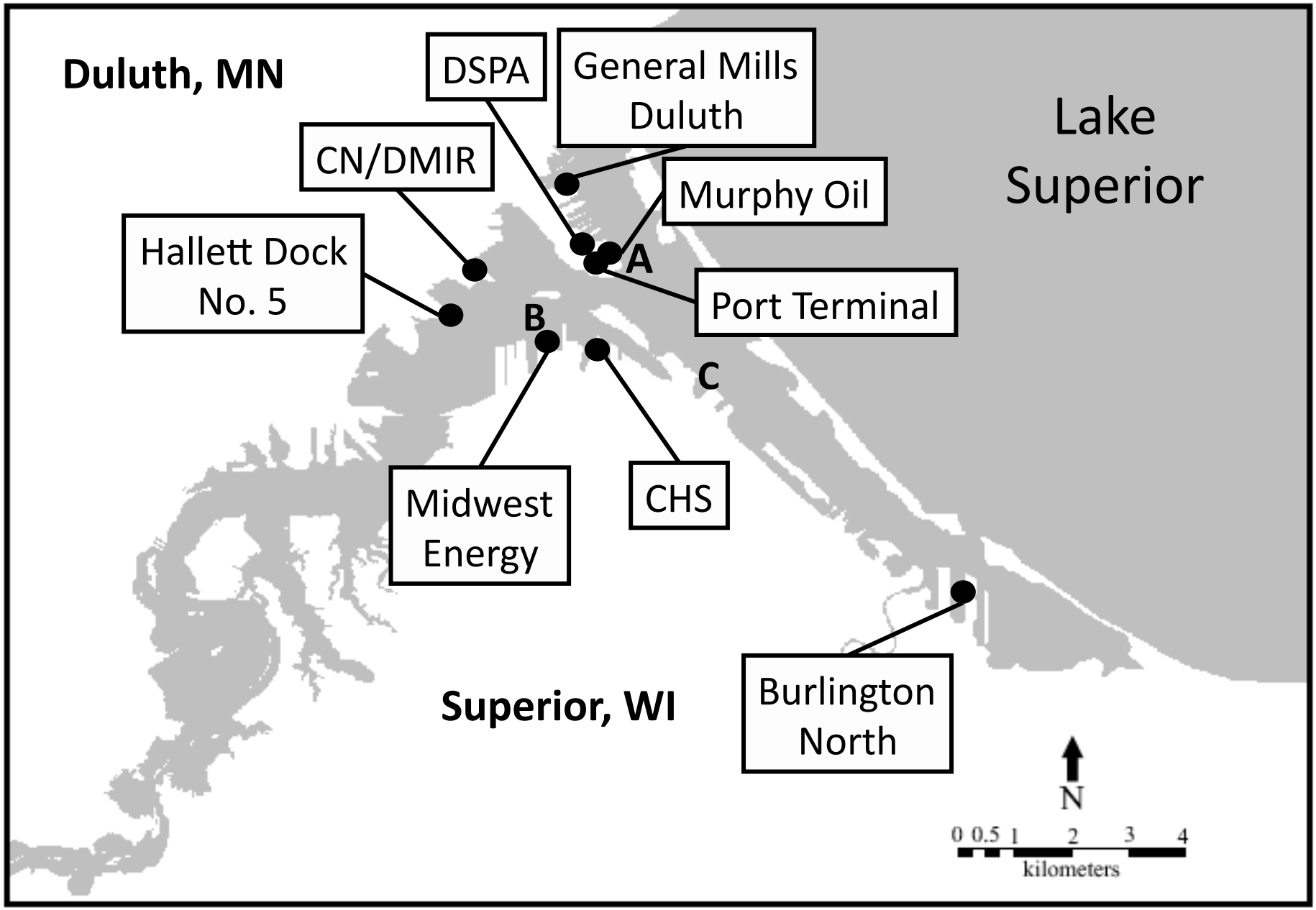
Duluth, MN
DSPA
General Mills Duluth
CN/DMIR
Murphy Oil
Lake Superior
Hallett Dock No. 5
A
Port Terminal
B
C
Midwest Energy
CHS
Superior, WI
Burlington North
N
0 0.5 1 2 3 4
kilometers

Figure 1

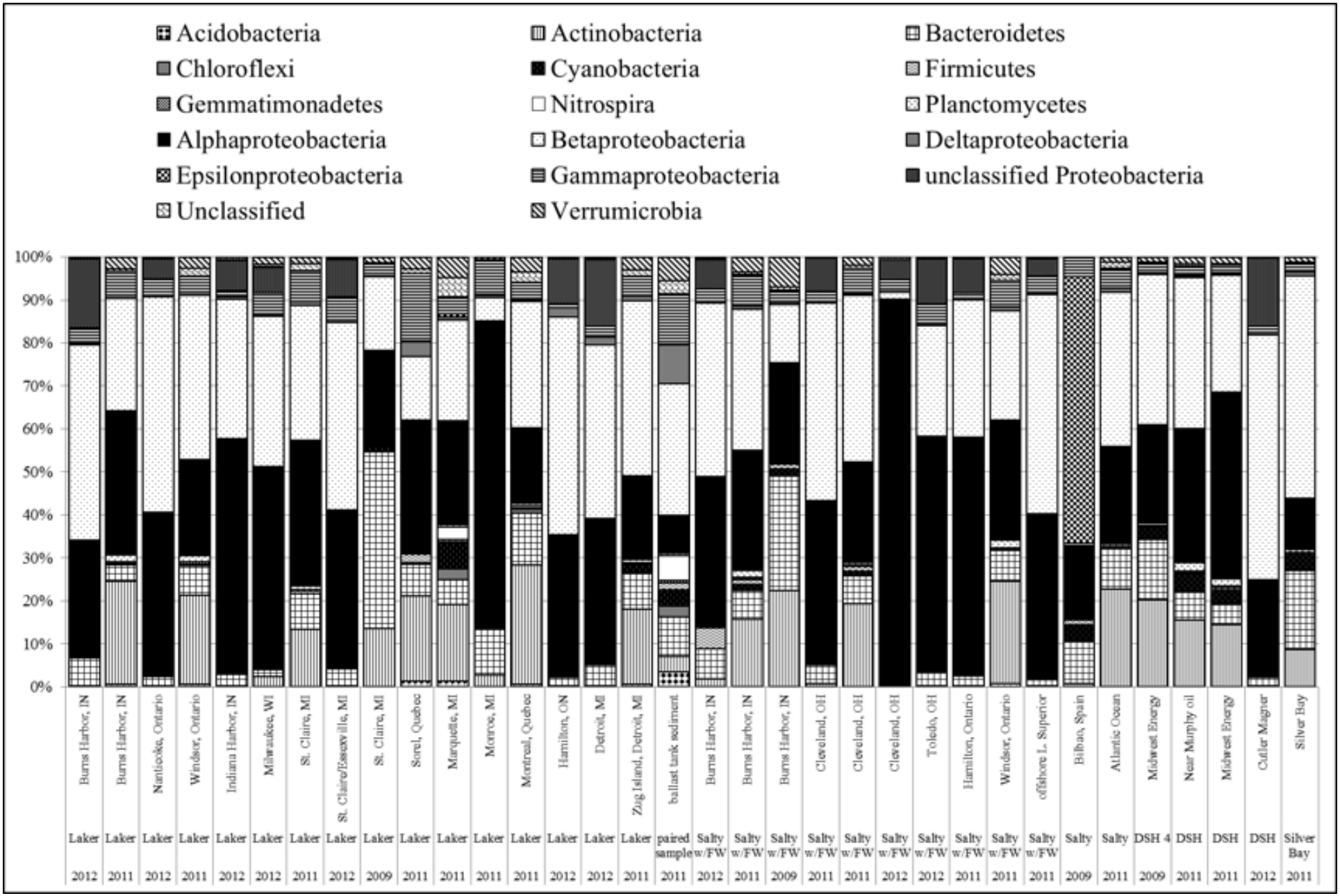
Acidobacteria
Actinobacteria
Bacteroidetes
Chloroflexi
Cyanobacteria
Firmicutes
Gemmatimonadetes
Nitrospira
Planctomycetes
Alphaproteobacteria
Betaproteobacteria
Deltaproteobacteria
Epsilonproteobacteria
Gammaproteobacteria
unclassified Proteobacteria
Unclassified
Verrumicrobia
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Burns Harbor, IN Laker 2012
Burns Harbor, IN Laker 2011
Nantucket, Ontario Laker 2012
Windsor, Ontario Laker 2011
Indiana Harbor, IN Laker 2012
Milwaukee, WI Laker 2012
St. Claire, MI Laker 2011
St. Claire/Essexville, MI Laker 2012
St. Claire, MI Laker 2009
Sorel, Quebec Laker 2011
Marquette, MI Laker 2011
Monroe, MI Laker 2011
Montreal, Quebec Laker 2011
Hamilton, ON Laker 2012
Detroit, MI Laker 2012
Zug Island, Detroit, MI Laker 2011
ballast tank sediment paired sample 2011
Burns Harbor, IN Salty w/FW 2012
Burns Harbor, IN Salty w/FW 2011
Burns Harbor, IN Salty w/FW 2009
Cleveland, OH Salty w/FW 2011
Cleveland, OH Salty w/FW 2011
Cleveland, OH Salty w/FW 2012
Toledo, OH Salty w/FW 2012
Hamilton, Ontario Salty w/FW 2011
Windsor, Ontario Salty w/FW 2011
offshore L. Superior Salty w/FW 2011
Bilbao, Spain Salty 2009
Atlantic Ocean Salty 2011
Midwest Energy DSH 4 2009
Near Murphy oil DSH 2011
Midwest Energy DSH 2011
Cutler Magner DSH 2012
Silver Bay Silver Bay 2011

Figure 2

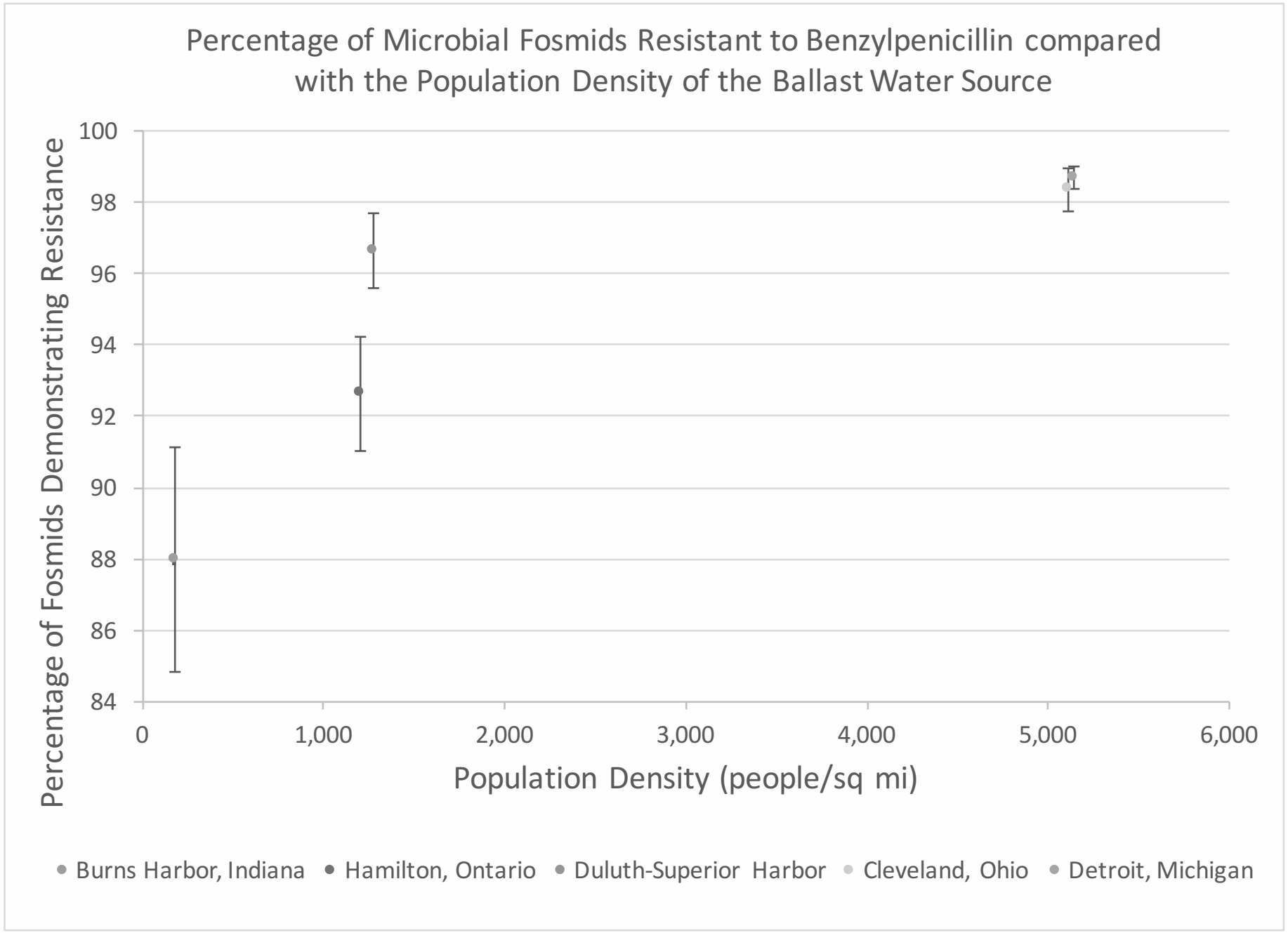
Percentage of Microbial Fosmids Resistant to Benzylpenicillin compared with the Population Density of the Ballast Water Source
Percentage of Fosmids Demonstrating Resistance
100
98
96
94
92
90
88
86
84
0
1,000
2,000
3,000
4,000
5,000
6,000
Population Density (people/sq mi)
Burns Harbor, Indiana
Hamilton, Ontario
Duluth-Superior Harbor
Cleveland, Ohio
Detroit, Michigan

Figure 3

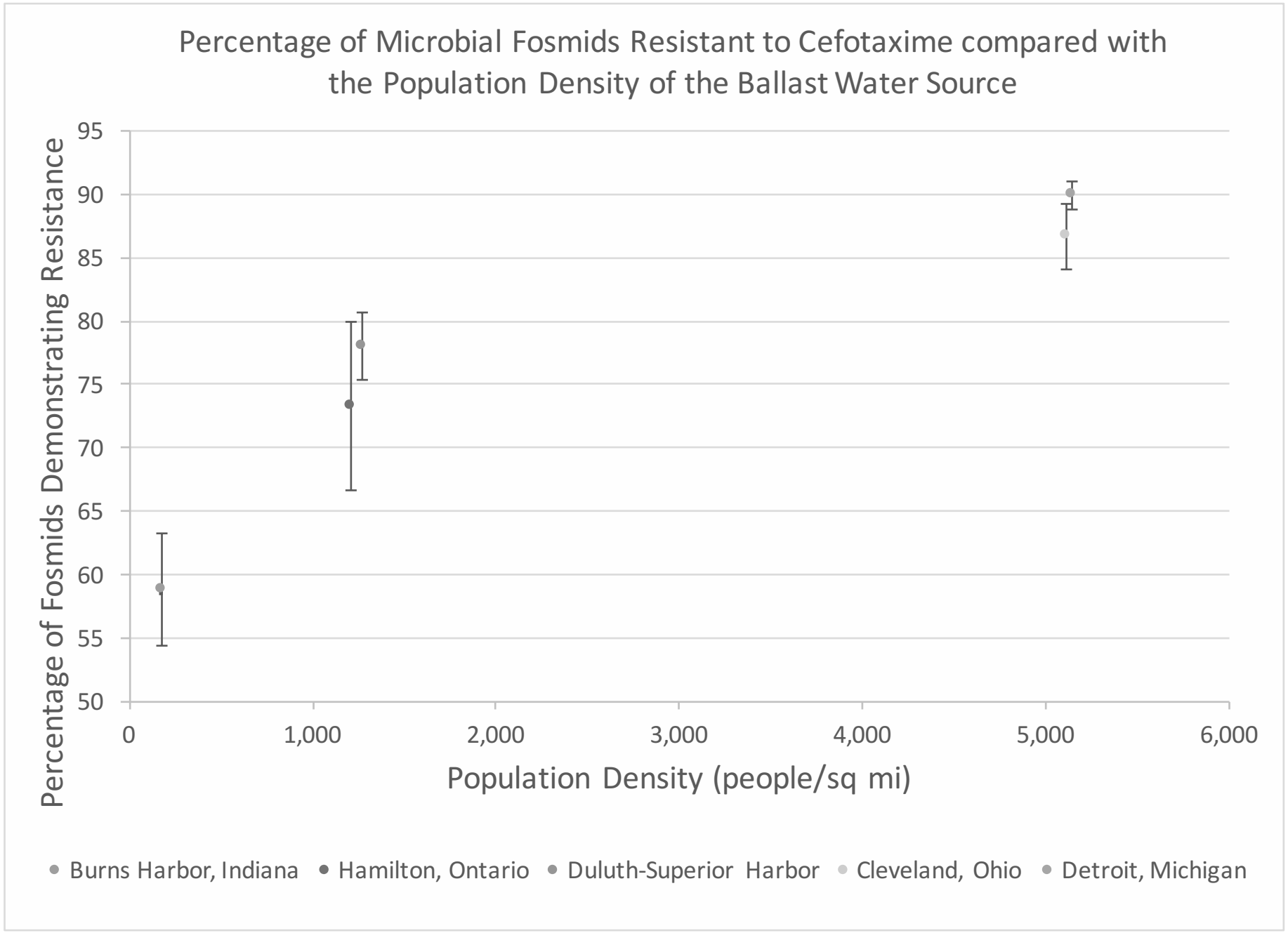
Percentage of Microbial Fosmids Resistant to Cefotaxime compared with the Population Density of the Ballast Water Source
Percentage of Fosmids Demonstrating Resistance
95
90
85
80
75
70
65
60
55
50
0
1,000
2,000
3,000
4,000
5,000
6,000
Population Density (people/sq mi)
Burns Harbor, Indiana
Hamilton, Ontario
Duluth-Superior Harbor
Cleveland, Ohio
Detroit, Michigan

Figure 4

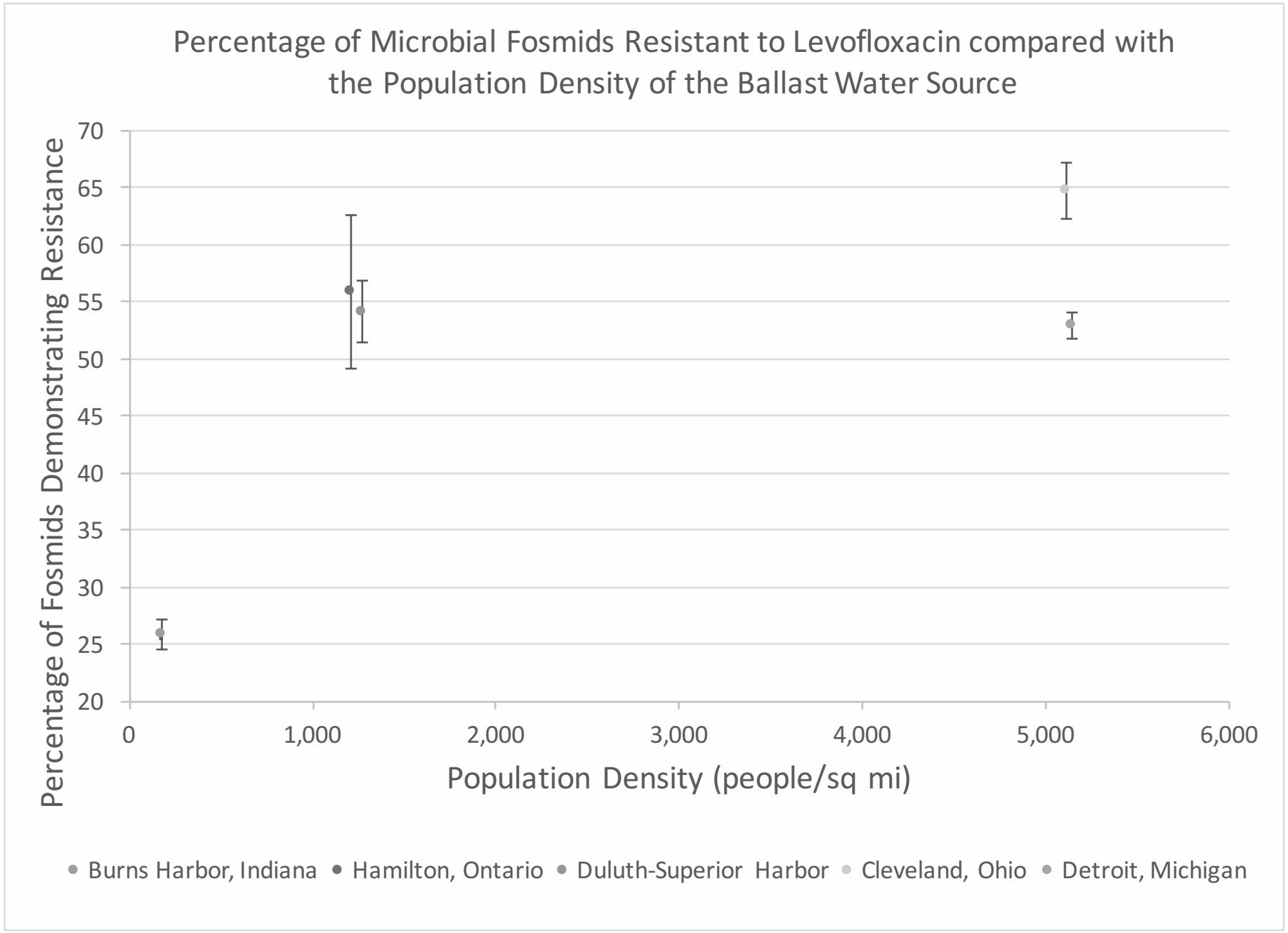
Percentage of Microbial Fosmids Resistant to Levofloxacin compared with the Population Density of the Ballast Water Source
Percentage of Fosmids Demonstrating Resistance
70
65
60
55
50
45
40
35
30
25
20
0
1,000
2,000
3,000
4,000
5,000
6,000
Population Density (people/sq mi)
Burns Harbor, Indiana
Hamilton, Ontario
Duluth-Superior Harbor
Cleveland, Ohio
Detroit, Michigan

Figure 5

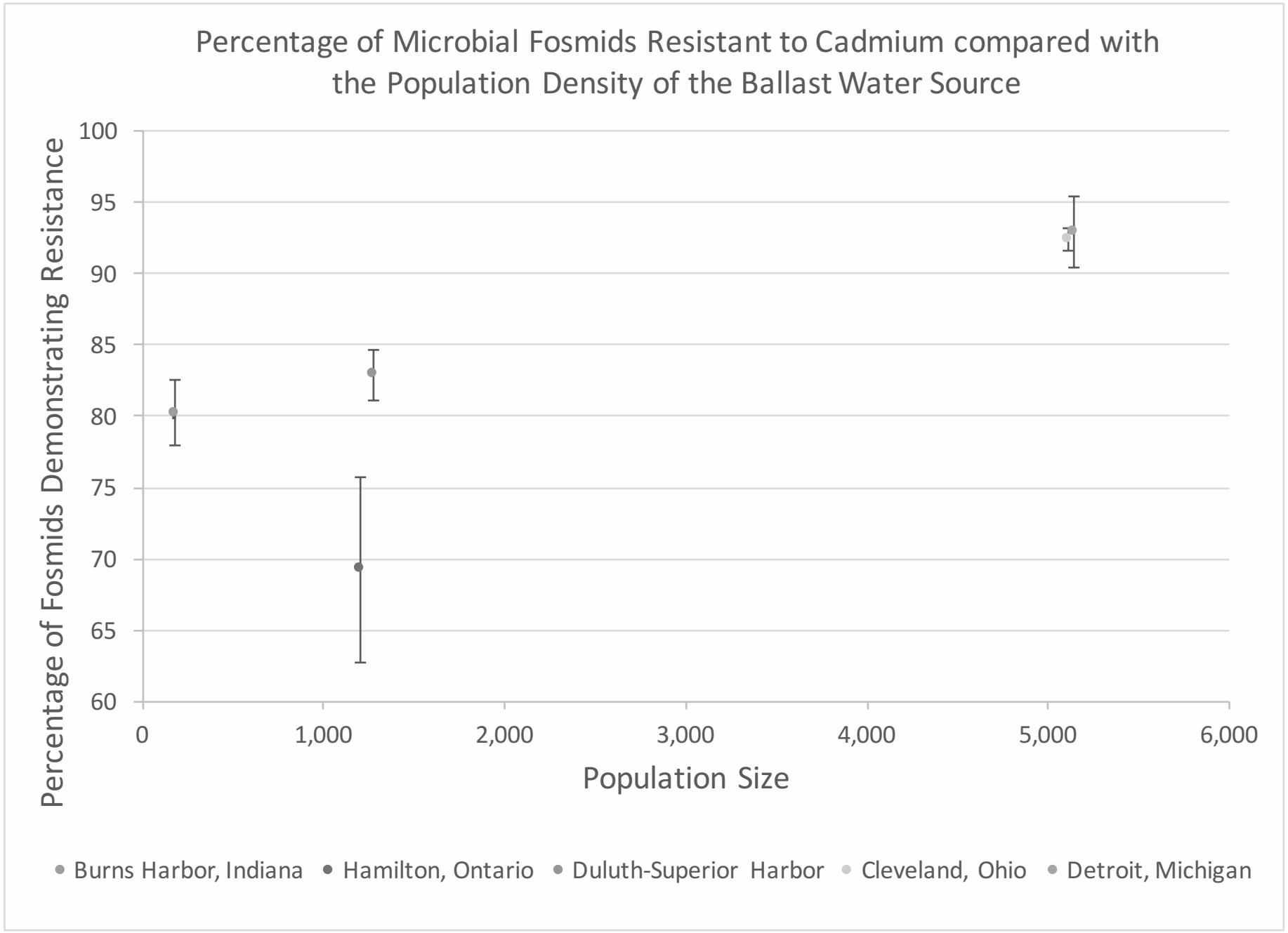
Percentage of Microbial Fosmids Resistant to Cadmium compared with the Population Density of the Ballast Water Source
Percentage of Fosmids Demonstrating Resistance
100
95
90
85
80
75
70
65
60
0
1,000
2,000
3,000
4,000
5,000
6,000
Population Size
Burns Harbor, Indiana
Hamilton, Ontario
Duluth-Superior Harbor
Cleveland, Ohio
Detroit, Michigan

Figure 6

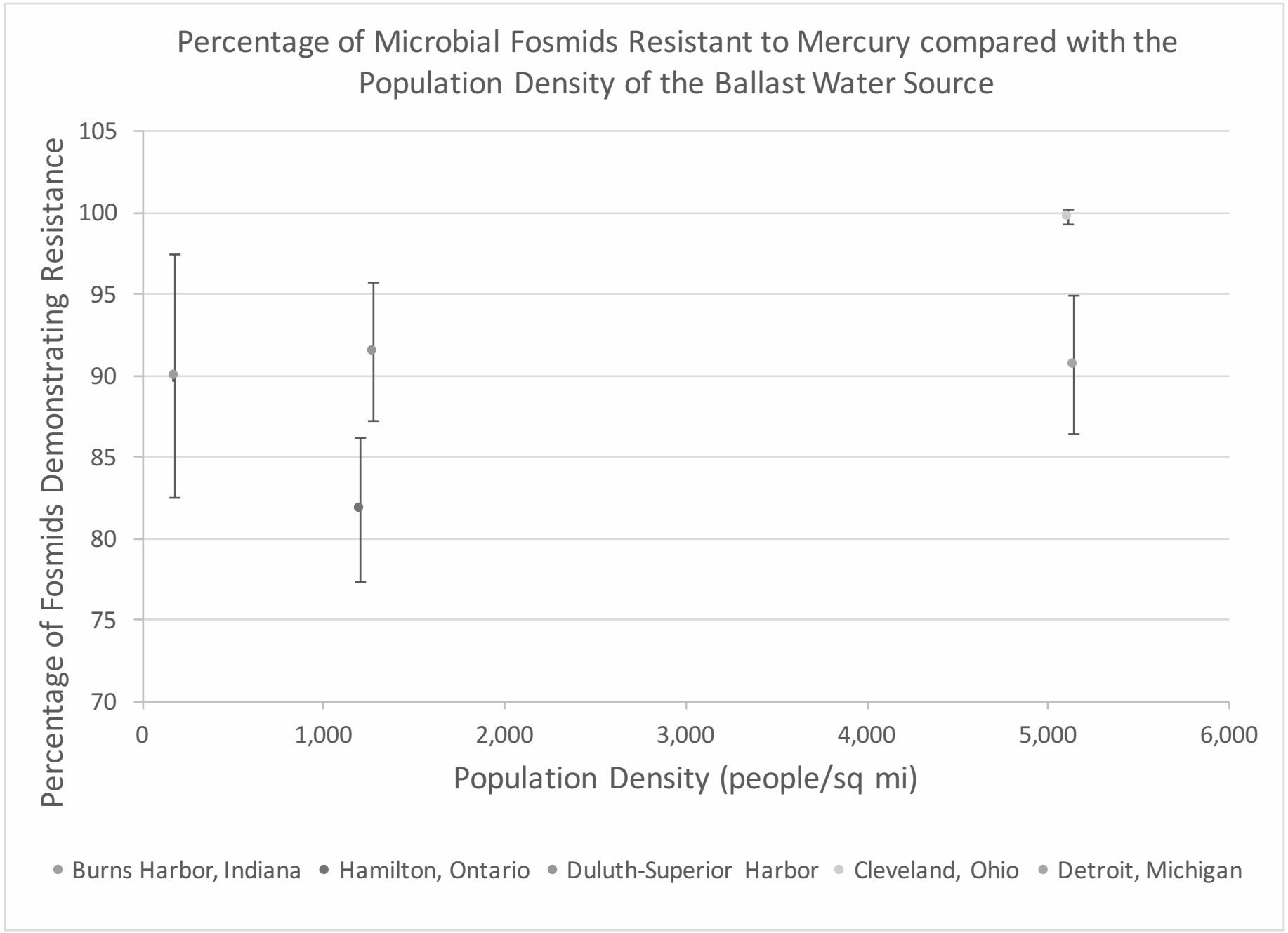
Percentage of Microbial Fosmids Resistant to Mercury compared with the Population Density of the Ballast Water Source
Percentage of Fosmids Demonstrating Resistance
105
100
95
90
85
80
75
70
0
1,000
2,000
3,000
4,000
5,000
6,000
Population Density (people/sq mi)
Burns Harbor, Indiana
Hamilton, Ontario
Duluth-Superior Harbor
Cleveland, Ohio
Detroit, Michigan

Figure 7

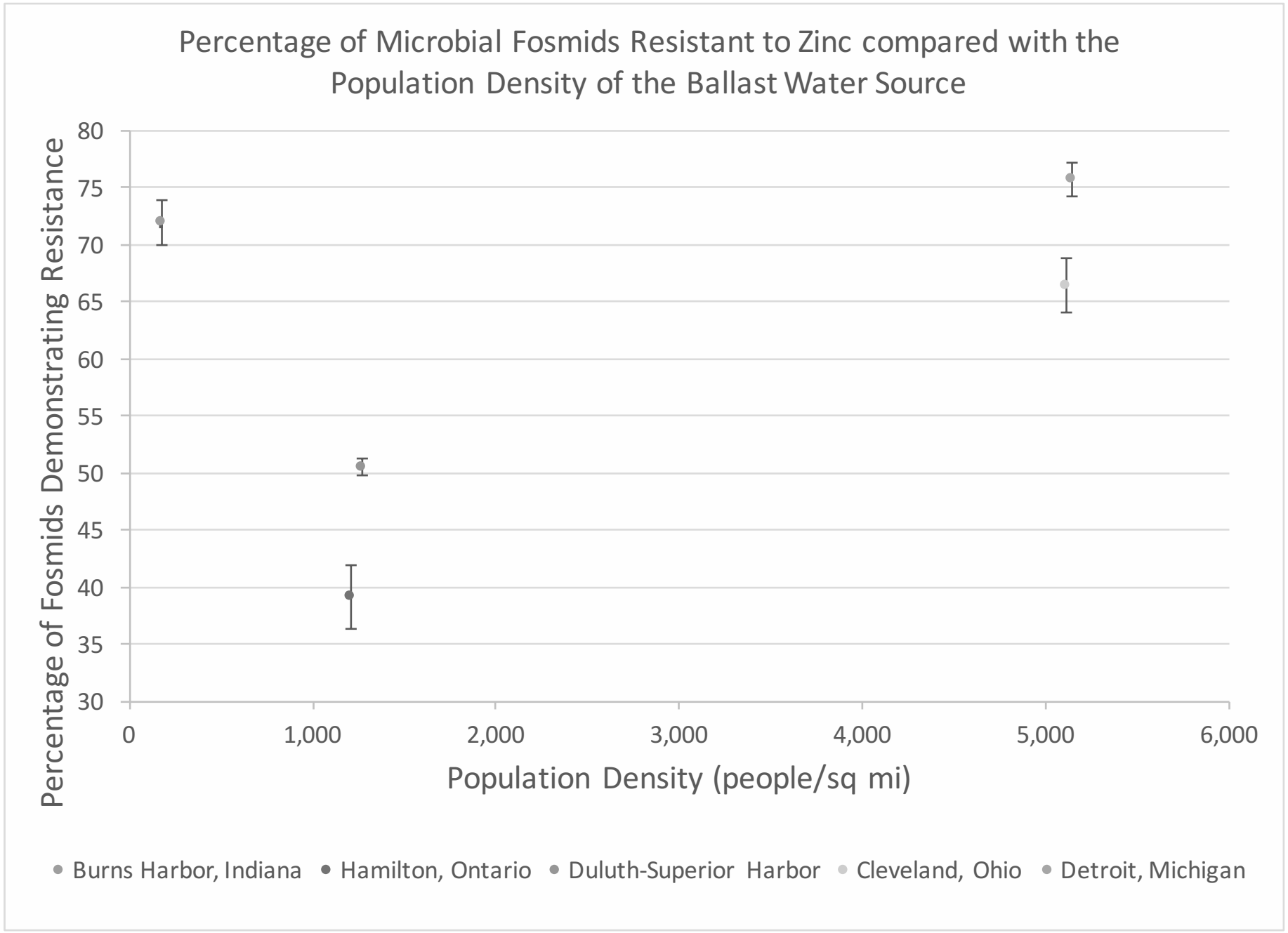
Percentage of Microbial Fosmids Resistant to Zinc compared with the Population Density of the Ballast Water Source
Percentage of Fosmids Demonstrating Resistance
80
75
70
65
60
55
50
45
40
35
30
0
1,000
2,000
3,000
4,000
5,000
6,000
Population Density (people/sq mi)
Burns Harbor, Indiana
Hamilton, Ontario
Duluth-Superior Harbor
Cleveland, Ohio
Detroit, Michigan

Figure 8