Characterizing Antibiotic and Heavy Metal Resistance Genes from Bacteria in Commercial Ship Ballast Water Discharged into the Duluth-Superior Harbor

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DEDICATION

This thesis is dedicated to my family. Thank you for all the support, patience, and encouragements.

ABSTRACT

Ballast water discharge is a powerful vector for introducing invasive species into aquatic ecosystems and microorganisms numerically dominate the discharge. Invasive bacteria may not only alter the diversity of native bacterial communities but also transfer genetic resistance to antibiotics and heavy metals into these communities. Antibiotic and heavy metal resistance was characterized for bacteria found in ballast water collected from commercial ships actively discharging ballast water into the Duluth-Superior Harbor during 2011 and 2012. Six fosmid libraries containing metagenomic DNA were constructed from ballast water and Duluth-Superior Harbor water. These libraries were screened for antibiotic resistance to benzylpenicillin, cefotaxime, and levofloxacin and heavy metal resistance to cadmium, zinc and mercury to determine resistance by bacteria in each water sample. There were differences between the proportions of microbial fosmids showing resistance to the three antibiotics from different ballast waters than originated from within the Great Lakes. The order of 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 seen for the other two antibiotics as well. The order of 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. 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. Typically, ballast waters from Great Lakes

cities with a population density less than 1,300 people per square 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, OH and Detroit, MI. The percentage of fosmids demonstrating resistance to heavy metals was less obvious between the three heavy metals compared to the three antibiotics. Ballast water received from ports in larger, more urbanized cities may include more bacteria with antibiotic resistance genes and cause greater concern for the spread of antibiotic resistance among native bacterial populations in the Duluth-Superior Harbor than ballast water received from harbors in smaller metropolitan areas.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Full Title	Abbreviations
Duluth-Superior Harbor	DSH
International Maritime Organization	IMO
Exclusive Economic Zone	EEZ
National Ballast Information Clearinghouse	NBIC
Nonindigenous Species	NIS
Code of Federal Regulation	CFR
United States Coast Guard	USCG
Horizontal Gene Transfer	HGT
Areas of Concern	AOC
Clemson University Genomic Institute	CUGI
Lysogeny Broth	LB

CHAPTER I

Introduction and Overview

For the past century and a half, ships have used ballast water for stability by taking on or discharging water at ports of call. This exchange makes ballast water a powerful vector for introducing invasive species into new ecosystems. Approximately 79 million metric tons per year of ballast water is discharged in United States waterways (Carlton et al. 1995), and in 2005 more than 5 billion gallons of ballast water was discharged in the Great Lakes alone with the Duluth-Superior Harbor (DSH) receiving the most discharged ballast water (MPCA 2008; Rup et al. 2010).

Ballast water can contain a diverse range of life, which is then discharged by a ship into a given harbor (Drake et al. 2002), including some microorganisms that are pathogenic (McCarthy and Crowder 2000). The Eurasian zebra mussels (*Dreissena polymorpha*; Hebert et al. 1989) and the sea lamprey (*Petromyzon marinus*; Mills et al. 1994) in the Great Lakes, *Vibrio cholerea* O1 in the United States originating from South America (McCarthy and Khambaty 1994), and the Japanese toxic dionoflagellate *Gymnodinium catenatum* in Australia (Hallegraeff 1998) are a few well known invasive species that have been introduced by ballast water discharge.

Approximately 180 nonindeginous aquatic species have become established in the Lauretian Great Lakes (Ricciardi 2006). Biological invaders are a leading cause of homogenization, species extinctions, and ecosystem distruptions worldwide (Ricciardi and MacIsaac 2000; Ricciardi 2001; Drake and Lodge 2004). After habitat destruction,

invasives species are the second leading cause of the decline in global biological diversity (Vitousek et al. 1997).

Current ballast water regulations from the International Maritime Organization (IMO) require commercial ships to complete an open-ocean exchange of ballast water prior to entering an exclusive economic zone (EEZ) within the United States. In this exchange, coastal water is removed from the ballast water tanks and is replaced with open-ocean water (IMO 2004). Open-ocean exchange is designed to reduce the threat of nonindigenous species through discharging the port water at sea, incoming oceanic species should be at a lower density, and the change of salinity may affect the survivability of the organisms (Bailey 2015). However, viruses and bacteria are able to survive ballast water passages and can be viable upon discharge allowing for population establishment (Ruiz et al. 2000). Existing ballast water monitoring programs are aimed at fisheries, water quality, and contaminants. Little work has been done on the impact of potentially invasive microorganisms in the Great Lakes basin (Bain et al. 2011; Litchman 2010).

The United States Coast Guard published a rulemaking docket in the *Federal Register* on March 23, 2012 that amended the 2004 ballast water regulations set by IMO. The amendments included a standard concentration of living organisms that can be discharged in ballast water (Table I-1). The regulations include concentrations for indicator microorganism (*Vibrio cholerae*, *E. coli* and intestinal enterococci) but not overall microorganism concentrations, which means that nonindigenous microorganisms still have the potential to be transported through ballast water and discharged into a new environment (33 C.F.R. § 151.1511, 2012). The 2004 ballast water management

Table I-1. Standard concentrations of living organisms that can be discharged in ballast water set by the United States Coast Guard (CFR 2012).

Organisms	Concentration
Size: ≥ 50 micrometers	Fewer than 10 living organisms per cubic meter of ballast water
Size: 50 – 10 micrometers	Fewer than 10 living organisms per cubic milliliter of ballast water
Indicator microorganism: *Vibrio cholerae* (serotypes O1 and O139)	Fewer than 1 colony forming unit (CFU) per 100 milliliters
Indicator microorganism: *Escherichia coli*	Fewer than 250 CFU per 100 milliliters
Indicator microorganism: intestinal enterococci	Fewer than 100 CFU per 100 milliliters

regulations only affected vessels that came into the EEZ and not vessels transferring ballast water interregionally. The updated ballast water management regulations include ships greater than 1,600 gross register tons that do not operate outside of the U.S. EEZ and take on and discharge ballast water (USCG 2012).

Microorganisms numerically dominate ballast water discharge. For example, Ruiz et al. (2000) estimated 10^{18} bacteria cells and 10^{19} viruses were discharged annually into the lower Chesapeake Bay region, which is one of the largest estuaries in the United States. In Lake Superior, Kim et al. (2015) found that a majority of the viral sequences from ballast and harbor waters from the Duluth-Superior harbor could not be assigned to any taxa associated with reference sequences, indicating the lack of knowledge on viruses in ballast and harbor waters. However, the assigned viruses were dominated by double-stranded DNA phages, and sequences associated with potentially emerging viral pathogens of fish and shrimp were detected with low amino acid similarity in both ballast and harbor waters (Kim et al. 2015).

High concentrations of bacteria, high rates of asexual reproduction, survival strategies such as cyst formation, and tolerances for environmental changes allow for invasive microorganisms to persist in a new environment (Ruiz et al. 2000). Microbial communities consist of both indigenous and widespread species, and the immigration into those communities by invasive microorganisms can lead to ecological disruptions of the environment (Vitousek et al. 1997). For example, nonindigenous bacteria can shift stoichiometric ratios within the environment, such as nitrogen and phosphorous ratios,

causing changes in the native biogeochemical cycles which could lead to alterations and/or breakdowns within the food web (Litchman 2010).

Another way invasive bacteria can alter native bacterial communities is by transferring genetic material in a process called horizontal gene transfer (HGT). In this process, genetic material is transferred via a plasmid from one bacterium to another, and the individual cell receiving the plasmid is not necessarily from the same bacterial species, genus, or domain. The rate of HGT plasmid acquisition has been documented to accelerate in heterogenous bacterial populations (Dionisio et al. 2002). Plasmids contain genetic material that encodes nonessential accessory genes that can be related to the transmission of the plasmid and the survivability of the organism in specific environments (Bergstrom et al. 2000; Read and Ussery 2006). HGT is one of the leading forces of evolution in single-cell organisms and is a main mechanism in the spread of antibiotic resistance in bacterial populations (Gogarten and Townsend 2005).

Worldwide, antibiotics are the primary strategy for managing bacterial infections (Hall 2004). The increased use of antibiotics for clinical and agricultural purposes has led to environmental selection pressures causing the development, expansion and persistence of antibiotic resistance in bacterial populations (Alonso et al. 2001; Neu 1993, Svara and Rankin 2011). As mentioned previously, HGT is a major mechanism in the spread of genes resistant to antibiotics; other mechanisms include integrons and mutations in the genetic code (Hall 2004; Alonoso et al. 2001).

Many bacteria have been able to develop resistance to a variety of antibiotic agents and this ability indicates the serious nature of bacterial antibiotic resistance

(Zasloff 2002). There have only been two novel antibiotics approved by the FDA in the past fifteen years, and major pharmaceutical companies have either downsized or completely halted antibiotic development (Singleton 2011). The increase in antibiotic resistance within bacterial populations coupled with the lack of further antibiotic development is creating a significant health issue for the general populace.

It is important to characterize the bacterial antibiotic resistance being discharged with ballast water into DSH due to the mobility and consequence of antibiotic resistant genes. In 2017, the DSH received the most ballast water discharged, over 16 million metric tonnes, compared to the other United States Great Lakes ports (NBIC 2018). Sandusky, Ohio received the second highest amount of ballast water discharge with only approximately 3.6 million metric tonnes (NBIC 2018). Because of the high frequency of ballast water discharged into the DSH, the harbor has a greater risk of invasion by nonindigenous bacteria with the potential of carrying a variety of antibiotic resistance genes that can be transferred to native populations. Characterizing the type and source of antibiotic resistance should be of value in developing ballast water management methods to prevent the further spread of antibiotic resistant bacteria.

Heavy metal resistance has been linked to antibiotic resistance on plasimds in *Staphylococcus aureus* strains and is an indicator of an environmental disruption (Abd et al. 2011). Of the 53 natural occurring heavy metals, 17 are of biological importance (Nies 1999). Heavy metals that are biologically important to bacteria are involved in a range of metabolic activities as protein stabilizers and cofactors (Silver and Phung 1996) or are involved in different redox reactions (Nies 1999). High concentration of heavy metals may result in free radicals which can disrupt cellular components and function (Halliwell

and Gutteridge 1984, 1985). It is important for bacteria to balance the assimilation of biologically essential heavy metals and their tolerance of toxic concentrations.

Heavy metals have been associated with runoff from mining and general urbanization, such as effluent from waste-water treatment plants and landfills, which is why they are indicative of pollution and environmental disturbances (Eisler, 1998). Nonpoint sources of heavy metals in the Great Lakes include industrial waste site runoff, landfills, and contaminated ground water, while industrial and municipal wastewater discharges are important point sources of heavy metals (EPA 2012).

CHAPTER II

Antibiotic and Heavy Metal Resistant Bacteria from Ballast Water Discharged into the Duluth-Superior Harbor

INTRODUCTION

Since the late 1870's, ballast water has been used in the shipping industry to regulate a vessel's stability, increase draft, and change the trim. The discharge of ballast water is considered one of the primary sources for the introduction of aquatic organisms (Bailey 2015). Existing ballast water monitoring systems are aimed at fisheries, water quality, and contaminants with little work aimed at the impact of potentially invasive microorganisms (Litchman 2010).

Invasive microorganisms can persist in new environments because of high rates of asexual reproduction, strategies such as cyst formation, and tolerances for environmental changes (Ruiz et al. 2000). Nonindigenous bacteria may change native bacterial communities by transferring genetic material that can alter the survivability of the organisms in specific environments. This process is an important evolutionary force in single-cell organisms and is a mechanism for the spread of antibiotic and heavy metal resistance (Gogarten and Townsend 2005).

The purpose of this study was to determine the antibiotic and heavy metal resistance of bacteria that were discharged with the ballast water of commercial ships in the DSH and compare the resistance of the discharged bacteria to resistance of the native

DSH bacterial community. This assessment allowed for the detection of novel or more abundant resistance genes that were being transported into the community.

The study focused on groups of therapeutic antibiotics that are most commonly prescribed to individuals. In 2009, 64.2% of the antibiotic market in the United States was comprised of just three groups of antibiotics: cephalosporins, broad-spectrum penicillin, and fluoroquinolones (Hamad 2010). This study tested one antibiotic from each group. Cefotaxime, from the cephalosporin group, and benzylpenicillin, from the penicillin group, act against Gram-positive and Gram-negative bacteria by inhibiting synthesis of bacterial cell walls (Young 2010). They are both natural derived antibiotics from the beta-lactam class. Levofloxacin is a synthetically derived fluoroquinolone antibiotic that is prescribed as a second line antibiotic to individuals that have life threatening bacterial infections that are resistant to other general types of antibiotics (Young 2010).

This study also investigated the resistance to three heavy metals: cadmium, zinc, and mercury. These are trace metals that have been associated with aquatic environmental degradation in several watersheds of the Great Lakes (EPA 2012). Cadmium is toxic to bacteria at high concentrations, but little is understood about the mechanisms of uptake and resistance (Nies 1999). It is generally considered to be a nonbiologically usable metal. Zinc is also toxic at high concentrations but is required in many proteins involving redox reactions (Coleman 1998). Mercury is considered one of the most toxic heavy metals in the environment (Kiyono & Hou 2006). It can be concentrated through the food chain and exposure can have adverse effects on human health (Nascimento & Souza 2003).

METHODS

Collection of Ballast Water from Commercial Ships and Water from the DSH

Between 60 and 120 L of ballast and DSH water was collected from 25 commercial ships docked in the DSH and three sites in the DSH throughout the 2011 and 2012 shipping season (Figure II-1; Table II-1). Ballast water was collected from ballast tanks by siphoning water using clean 0.5-inch polyethylene tubing or by collecting water directly from the ballast pumps. While sampling, the ship's ballasting history, including locations and dates of ballast water discharge and uptake, was obtained through crew interviews and ballast log records. DSH water was collected by using 20 L plastic carboys and dipping them just below the water surface. Prior to water collection, the carboys were rinsed with DSH sample water. Measurements of dissolved oxygen, conductivity, pH and temperature were recorded for most ballast water samples, including the DSH water samples for the fosmid libraries, using an YSI Model 85 Handheld (YSI Inc., Yellow Springs, OH).

Ballast and DSH water samples were immediately transported back to the laboratory and duplicate subsamples were filtered onto two 142 mm diameter Durapore membrane filters (0.22 µm pore size; EMD Millipore Corporation, Darmstadt, Germany) to concentrate bacterial cells. All filters were stored in Whirl-Pak® bags (Nasco, Fort Atkins, WI) at -80°C until further analysis.

Figure II-1. Map of the Duluth-Superior Harbor (DSH) identifying the dock locations where ballast water was collected from commercial ships during the 2011 and 2012 shipping seasons. Letters A through C show the approximate location where DSH was collected. Table II-1 contains details on the ships and the sources of ballast water.

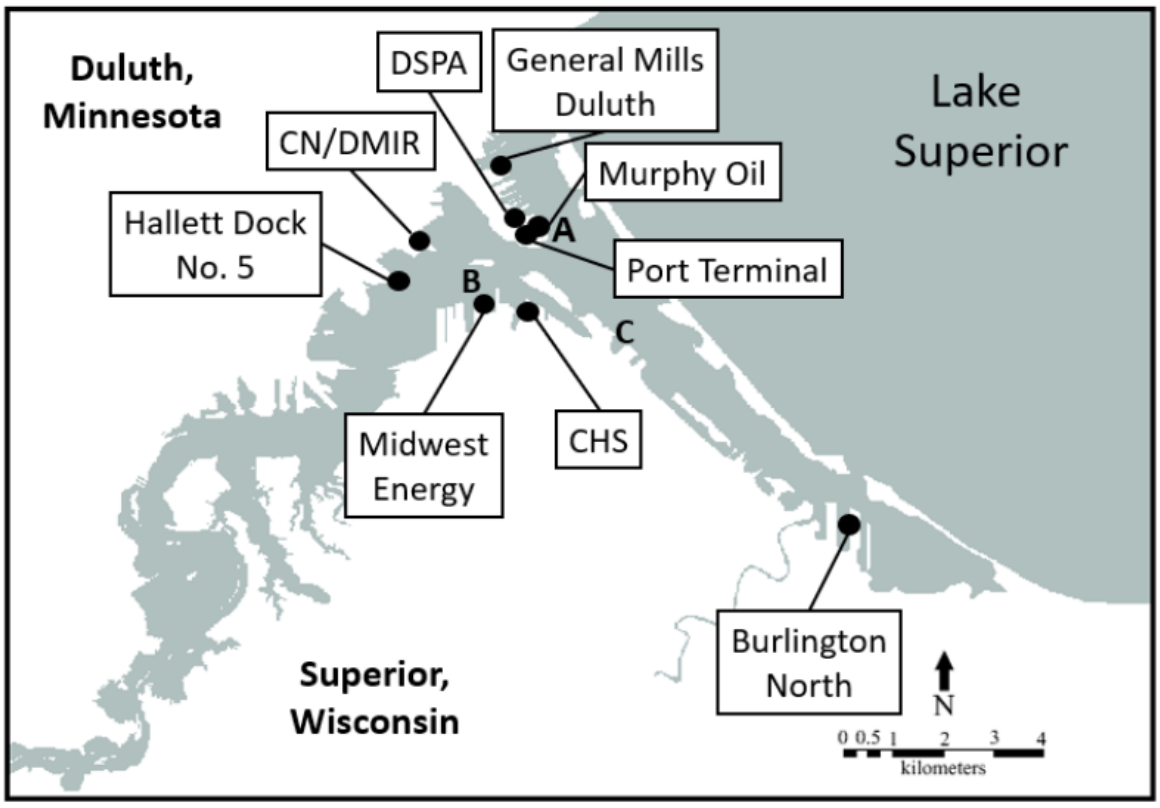

Duluth,
Minnesota
DSPA
General Mills
Duluth
Lake
Superior
CN/DMIR
Murphy Oil
Hallett Dock
No. 5
A
Port Terminal
B
C
Midwest
Energy
CHS
Burlington
North
Superior,
Wisconsin
N
0 0.5 1 2 3 4
kilometers

Table II-1. Collected ballast water from commercial ships in the Duluth-Superior Harbor (DSH) during the 2011 and 2012 shipping seasons and harbor water samples. Information was provided through in-person interviews and ballast log records provided by crew members during sampling. Ships were given a designator in place of the ship's name. A lowercase letter besides the ship designation indicates that more than one ballast water sample from a unique source was collected from the ship. Lakers are defined as cargo ships that are designed for and remain within the Great Lakes. Salties are ocean-going cargo ships that transport goods within the Great Lakes as well as the world oceans.

Ship/Site Designation	Ship Type or Site	Sample Date	DSH Dock	Ballast Type	Source of Ballast Water
1	Laker	7/27/11	Murphy Oil	Freshwater	Monroe, MI
2	Laker	8/2011	Burlington Northern	Freshwater	Sorel, QC
3	Laker	8/10/11	Midwest Energy	Freshwater	St. Clair, MI
4	Laker	8/31/11	CN/DMIR	Freshwater	Marquette, MI
5	Saltie	9/8/11	DSPA	Saltwater	Atlantic Ocean
6	Laker	9/27/11	Burlington Northern	Freshwater	Montreal, QC
7	Saltie	9/30/11	CHS	Freshwater	Windsor, ON
8	Laker	9/30/11	Murphy Oil	Freshwater	Windsor, ON
9	Saltie	10/28/11	General Mills – Duluth	Freshwater	Cleveland, OH
10	Saltie	10/31/11	CHS	Freshwater	Lake Superior (48 49.2°N, 018 09.0°W)
11	Saltie	11/7/11	CHS	Freshwater	Hamilton, ON
12	Laker	11/16/11	Burlington Northern	Freshwater	Burns Harbor, IN
13	Saltie	11/16/11	CHS	Freshwater	Cleveland, OH
14	Saltie	11/16/11	CHS	Freshwater	Burns Harbor, IN
15	Laker	12/8/11	Midwest Energy	Freshwater	Zug Island, Detroit, MI with some Lake Superior water
16	Laker	1/8/12	Silver Bay	Freshwater	Burns Harbor, IN with Lake Superior water
17a	Saltie	6/21/12	CHS	Freshwater	Burns Harbor, IN
17b	Saltie	6/21/12	CHS	Freshwater	Cleveland, OH
18a	Saltie	7/25/12	Port Terminal	Freshwater	Detroit, MI
18b	Saltie	7/25/12	Port Terminal	Freshwater	Hamilton, ON
19	Laker	7/27/12	Fuel Station next to Port Terminal	Freshwater	Indiana Harbor, IN
20	Laker	8/20/12	Hallett Dock #5	Freshwater	Nanticoke, ON
21	Saltie	8/21/12	Port Terminal	Saltwater	Atlantic Ocean
22	Laker	8/21/12	CHS	Freshwater	Milwaukee, WI
23	Saltie	9/27/12	Hallett Dock #5	Freshwater	Nanticoke, ON
24	Laker	10/19/12		Freshwater	St. Clair, MI / Essexville, MI
25	Saltie	10/29/12		Freshwater	Toledo, OH
A	DSH	7/26/11	Near Murphy Oil	Freshwater	
B	DSH	7/26/11	Midwest Energy	Freshwater	
C	DSH	8/20/12	Cutler Magner / Graymont	Freshwater	

Prokaryotic Cell Abundance

Total prokaryotic cells identified in each ballast and harbor water sample were counted using the DAPI staining method (Porter et al. 1980). From each water sample, 10 ml were preserved with 100 µL of 37% formaldehyde and the preserved samples were stored at 4° C for less than two weeks. Prokaryotic cells were concentrated on black polycarbonate membrane filters (Poretics; 25 mm diameter; 0.22 µm pore), stained with 4'6-diamidino-2-phenylindole (DAPI; 10 µM final concentration) and counted using a Nikon Eclipse E400 epifluorescence microscope. Cells in at least ten fields of view were counted for each replicate sample.

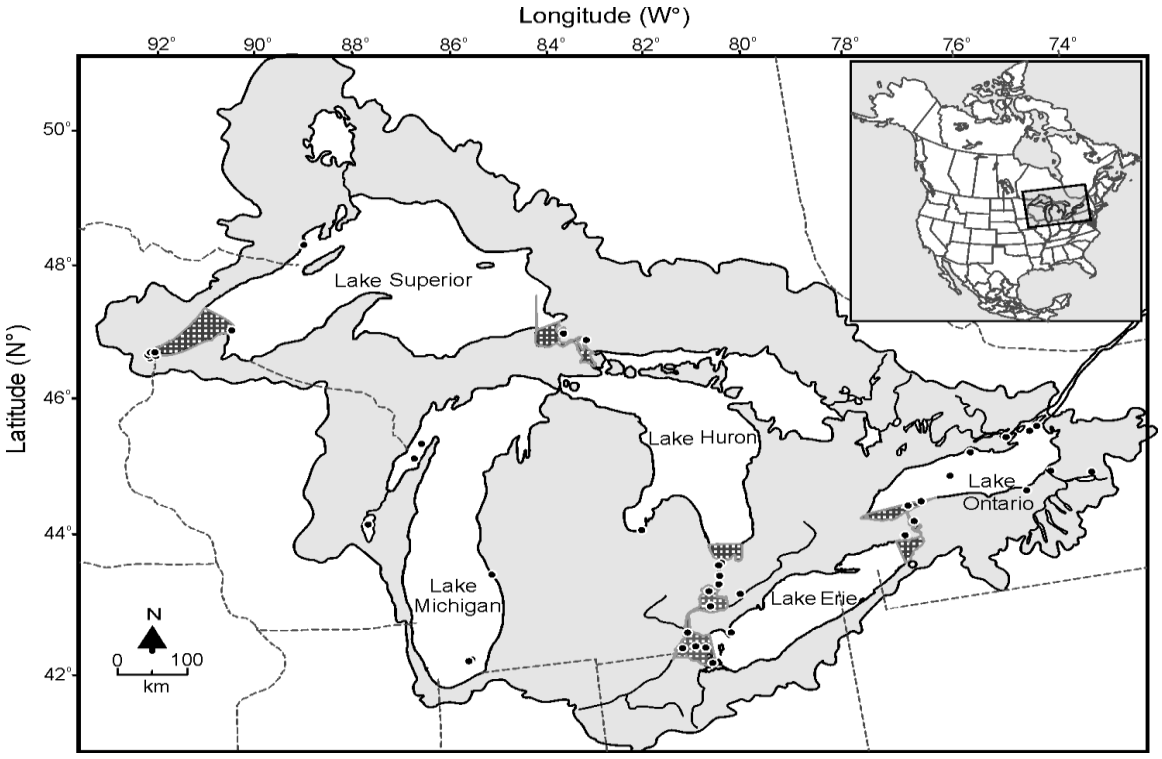
Selection of Ballast Water Samples for Fosmid Libraries

Fosmid libraries were created using DNA samples extracted from microbiota in the DSH and ballast water of five ships that visited the DSH between 2011 and 2012. One fosmid library was created from DSH water so the types and levels of antibiotic and heavy metal resistance could be characterized for the native microbial community. Another fosmid library was created from microbial DNA extracted from ballast water in an ocean-going ship carrying saltwater ballast to compare the type and level of antibiotic and heavy metal resistance of saltwater to freshwater ballast water discharge. The four remaining fosmid libraries were created from freshwater ballast water samples from ports of primary interest.

Ballast water from ports of primary interest were identified using three criteria: the port was in an invasive species "hot spot" (Grigorovich et. al 2003), Areas of Concern (AOC) listed under the 1987 Great Lakes Water Quality Agreement between Canada and

the United States, or by the frequency of the interested port's ballast water discharge into the DSH. Grigorovich et al. (2003) identified four invasion "hot spots," or corridors in the Laurentian Great Lakes that accounted for approximately 54% of established nonindigenous species in the Great Lakes; Lake Huron – Lake Erie corridor, Lake Erie – Lake Ontario corridor, Lake Superior – Lake Huron corridor and the western end of Lake Superior (Figure II-2). AOC are specific areas that have experienced environmental degradation that has impaired aquatic life in the area (EPA 2012). Environmental degradation could include, but is not limited to, mismanaged and previously unregulated heavy metals (such as mercury, chromium and lead), petroleum products, pesticides chlorinates and organic compounds into the water system. There are currently 31 AOCs listed for the United States and 12 areas listed for Canada. The frequency of ballast water discharge into the DSH was the last criterion for fosmid library creation. Ballast water from ports of primary interest within the Laurentian Great Lakes accounted for approximately 85% of all ballast water discharged in the DSH during the 2011 shipping season.

Figure II-2. A map of the Laurentian Great Lakes' "hot spots" (hatching marks) of nonindigenous species (•) signifies sites of established nonindigenous aquatic animals and protists and the gray areas on the map outside the Great Lakes indicate drainage basins in the region. Figure from Grigorovich et al. 2003.


Longitude (W°)
92°
90°
88°
86°
84°
82°
80°
78°
76°
74°
Latitude (N°)
50°
48°
46°
44°
42°
Lake Superior
Lake Huron
Lake Ontario
Lake Michigan
Lake Erie
N
0
100
km

Microbial Cell Preparation for Creating Fosmid Libraries

The selected ballast or harbor water filters were cut in half and microbial cells were concentrated from one-half of the filter. The other half of the filter was stored in a -80 °C freezer until microbial DNA could be extracted for sequencing. Each half filter was placed in separate 50 ml disposable centrifuge tubes with 2 mL of buffer (0.1% sodium pyrophosphate at pH 7.0 with 0.2% Tween-20). The samples were agitated for 3 min using a flat padded vortex (Scientific Industries, Inc., Bohemia, NY). Each solution from the conical tubes were aliquoted into three 1.5 ml microcentrifuge tubes, which were centrifuged for 2 min at 15,000 rpm (Eppendorf microfuge model 5424; Eppendorf AG, Hamburg, Germany). The supernatants were removed, and the cell pellets from duplicate subsamples were stored at -80 °C. Cell pellets were checked for visible bacterial cells with a Nikon Eclipse E400 epifluorescence microscope before being shipped to Clemson University Genomics Institute (CUGI) for fosmid library creation.

Fosmid Library Creation – Clemson University Genomic Institute

Upon receiving the cell pellet samples, personnel at CUGI extracted the total high molecular weight metagenomic DNA using an Epicentre™ metagenomic DNA isolation kit for water (Madison, WI) with the following modifications: incubation at 37^0C was extended to one hour and metalysis and protein digestions was extended to thirty minutes at 65^0C. The metagenomic DNA became randomly fragmented through the purification process with most of the DNA 40 kb long (EPILIT 2012). The 40 kb DNA fragments are comprised of both bacterial plasmid and chromosomal DNA since the average plasmid

size is approximately 78.9 kb and chromosomal DNA is approximately 3.87 (DiCenzo and Finan 2017).

End-repair and phosphorylation was completed on the purified metagenomic DNA. DNA fragments were size selected via agarose gel electrophoresis with the following conditions: 1% low melt agarose (Seaplaque) and 20 V/cm for fifteen hours at 4^0C. Fragments from 38 to 45 kb were purified from the gel by electroelution (model 422 Electro-Eluter; Biorad) and concentrated by ethanol precipitation. Since the size of plasmids can be less than 40 kb, not all plasmids from the water samples were selected. 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-TR^R cells (Epicentre®, Madison, WI) with the ligation products. A final concentration of 20% glycerol was added to the transformation and the fosmids were stored at -80^0C.

QPix II – University of Minnesota

The QPix II instrument (Molecular Devices, LLC., Sunnyvale, CA) at the University of Minnesota was used to pick *E. coli* cells containing fosmid inserts (created by CUGI) from the selected ballast water samples. The QPix II placed portions of isolated cell colonies into 384-well NUNC microplates (Thermo Fisher Scientific Inc., Minneapolis, MN) that contain Hogness Modified Freezing Media using Joint Genomic Institute custom software to create the fosmid library for each sample. Both a master copy and a working copy of the fosmid libraries were created at the same time. Once

the NUNC microplates were inoculated, then they were incubated overnight at 37 °C and stored at -80°C for future screening for antibiotic and heavy metal resistance genes.

Determining Minimum Inhibitory Antibiotic and Heavy Metal Concentrations

Lysogeny Broth (LB) was prepared and autoclaved, the LB medium was cooled at 55˚C for 30 minutes. Sterile filtered solutions of the antibiotics and heavy metals were added to the prepared media at standard minimum inhibitory concentrations (MIC) for *E. coli* (CLSI 2010, Richmond et al. 1976, and Spain 2003). The antibiotic or heavy metal amended media were poured into sterile disposable polystyrene Petri dishes (100 mmx 15 mm diameter; Thermo Fisher Scientific Inc., Minneapolis, MN) and cooled overnight in a fume hood. Sterile technique was used to streak the prepared plates with the control clone *E. coli* EPI300-TR^{R} cells supplied by CUGI. The control clone had the pcc2F0S vector with no purified metagenomic DNA. Inoculated plates were incubated at 37°C for 24 hours before being evaluated for cell growth. The antibiotic and heavy metal concentrations were adjusted until the MICs for the control clone was established (Table II-2).

Table II-2. Established minimum inhibitory antibiotic and heavy metal concentrations for the control clone E. coli EPI300-TR^{R} cells with the pcc2F0S vector.

Type of Treatment	Name	Concentration
Antibiotic	Cefotaxime	0.6 µg/ml
	Benzylpenicillin	17.5 µg/ml
	Levofloxacin	0.02 µg/ml
Heavy Metal	Cadmium	1.55 mM
	Zinc	2.2 mM
	Mercury	0.02 mM

Screening for Antibiotic and Heavy Metal Resistance

Lysogeny Broth (LB) media was prepared and autoclaved. The LB medium was cooled at 55°C for 30 minutes at which time sterile filtered stock solutions of the antibiotics or heavy metals were added at the established minimum inhibitory concentrations. The prepared media was poured into separate 20 cm x 20 cm Bioassay QTray polystyrene plates (approximately 250 ml per plate; Molecular Devices, LLC., Sunnyvale, CA) and cooled overnight in a fume hood. The QTrays were stored at 4°C until inoculated with *E. coli* cells from the fosmid libraries.

The QTrays containing antibiotics or heavy metals were inoculated with each fosmid library by using an autoclaved 384 pin microplate replicator (Boekel Scientific, Feasterville, PA). The replicator was dipped into the 384-well plates (each well contains one fosmid clone) for a specific fosmid library and then stamped onto the appropriate QTray. Each plate was stamped six times for a total of approximately 9,046 clones per fosmid library (Table II-3). Before the replicator was dipped into a new 384-well plate, it was first sanitized by being dipped into a 70% ethanol solution, then a 10% bleach solution and then back into the 70% ethanol solution for approximately ten seconds per solution. Finally, the replicator was flamed twice for approximately ten seconds. Plates were incubated at 37°C for 24 hours and then were evaluated for cell growth.

Table II-3. Average number of unique clones screened for each of the fosmid libraries from six different water sources. This study screened a total of 976,968 clones (54,276 total clones x three antibiotics x three heavy metals x three replicates).

Fosmid Library Water Source	Average number of unique clones
Duluth-Superior Harbor	9,695
Atlantic Ocean	4,129
Burns Harbor, IN	8,446
Cleveland, OH	11,128
Detroit, MI	10,710
Hamilton, ON	10.169
Total	**54,276**

Statistical Analysis

The variation of each fosmid library percent resistance to the three antibiotics and heavy metals were used to determine the confidence intervals with an alpha value of 0.05. T-tests were performed to determine the statistical difference of the six fosmid libraries to one another for a single antibiotic or heavy metal treatment. All analyses were performed using Microsoft Excel version 15 (Microsoft). Population density data was obtained using information available from the 2010 United States Census Bureau (available at https://factfinder.census.gov/faces/nav/jsf/pages/community_facts.xhtml) and the 2011 Statistics Canada Census Program (available at https://www12.statcan.gc.ca/census-recensement/index-eng.cfm).

RESULTS

Water Quality Measurements and Prokaryotic Cell Abundance

Temperature, dissolved oxygen, conductivity, and salinity water quality measurements were taken of the water source of the six fosmid libraries (Table II-4). Except for the Burns Harbor, IN ballast water sample, temperature measurements ranged from 18.7 ºC to 24.9 ºC. The Burns Harbor, IN ballast water sample temperature measurement was 10.8 ºC and it had a higher amount of dissolved oxygen compared to the other samples. The ballast water samples had higher conductivity compared to the DSH water sample. As expected, the Atlantic Ocean ballast water sample had the highest measured salinity value, 29.2 ppt, which is lower than the typical salinity value of between 34 ppt and 37 ppt for the Atlantic Ocean. The DSH water sample had the highest prokaryotic cells abundance of 4.9×10^6 cells per ml compared to the five ballast water samples, which ranged from 1.4×10^6 cells per ml, Atlantic Ocean, to 2.6×10^6 cells per ml, Cleveland, OH.

Table II-4. Water quality measurements and prokaryotic cell abundance for the six ballast water and Duluth-Superior Harbor water samples that used to create fosmid libraries for this study. Water quality measurements were taken in the field unless otherwise noted by an asterisk. The water quality measurements of water sources noted with an asterisk were made after ballast water was returned to the laboratory. Table includes ship/site designation listed in Table II-1.

Ship/Site Designation	Water Source	Temperature (°C)	Dissolved Oxygen (mg/L)	Conductivity (µS/m)	Salinity (ppt)	Cell Abundance (cells/mL)
B	Duluth-Superior Harbor*	21.5	2.8	84	0.0	4.9×10^6
21	Atlantic Ocean*	20.9	2.2	414.3	29.2	1.4×10^6
14	Burns Harbor, IN*	10.8	5.7	286.7	0.2	1.7×10^6
17b	Cleveland, OH	18.7	3.1	751.0	0.2	2.9×10^6
18a	Detroit, MI	24.6	3.4	654.0	0.3	2.0×10^6
18b	Hamilton, ON	24.9	0.1	845.0	0.1	1.9×10^6

Fosmids Demonstrating Resistance to Antibiotics and Heavy Metals

The percentage of fosmids demonstrating resistance to antibiotics was different for each of the three screened antibiotics, cefotaxime, benzylpenicillin, and levofloxacin (Table II-5). The percentage of fosmids resistant to cefotaxime ranged from 67.5% to 89.9%. A higher proportion of fosmids from Cleveland, OH (86.7%) and Detroit, MI (89.9%) were resistant to cefotaxime in comparison to the remaining ballast and harbor water fosmid libraries. Seventy-eight percent of DSH fosmids exhibited resistance to cefotaxime. These fosmids were not statistically different than the fosmids from Hamilton, ON ballast water, which demonstrated a 73.3% resistance to cefotaxime. The resistance to cefotaxime from the Atlantic Ocean ballast water fosmids were also not statistically different (67.5%). Fosmids generated from Burns Harbor, IN ballast water had the least resistance to cefotaxime compared to the other fosmid libraries. Fifty-eight percent of the Burns Harbor, IN fosmids demonstrated resistance.

Except for the Atlantic Ocean fosmids (19.6%), a large proportion of the other five fosmid libraries (88.0% - 98.6%) demonstrated a high resistance to benzylpenicillin (Table II-5). Fosmids from the Detroit, Cleveland, and DSH exhibited 98.6%, 98.3%, and 96.6% resistant to benzylpenicillin respectively. The resistance to this antibiotic for fosmids created from Hamilton (92.6%) and Burns Harbor (88.0%) ballast water samples, were not statistically different from one another.

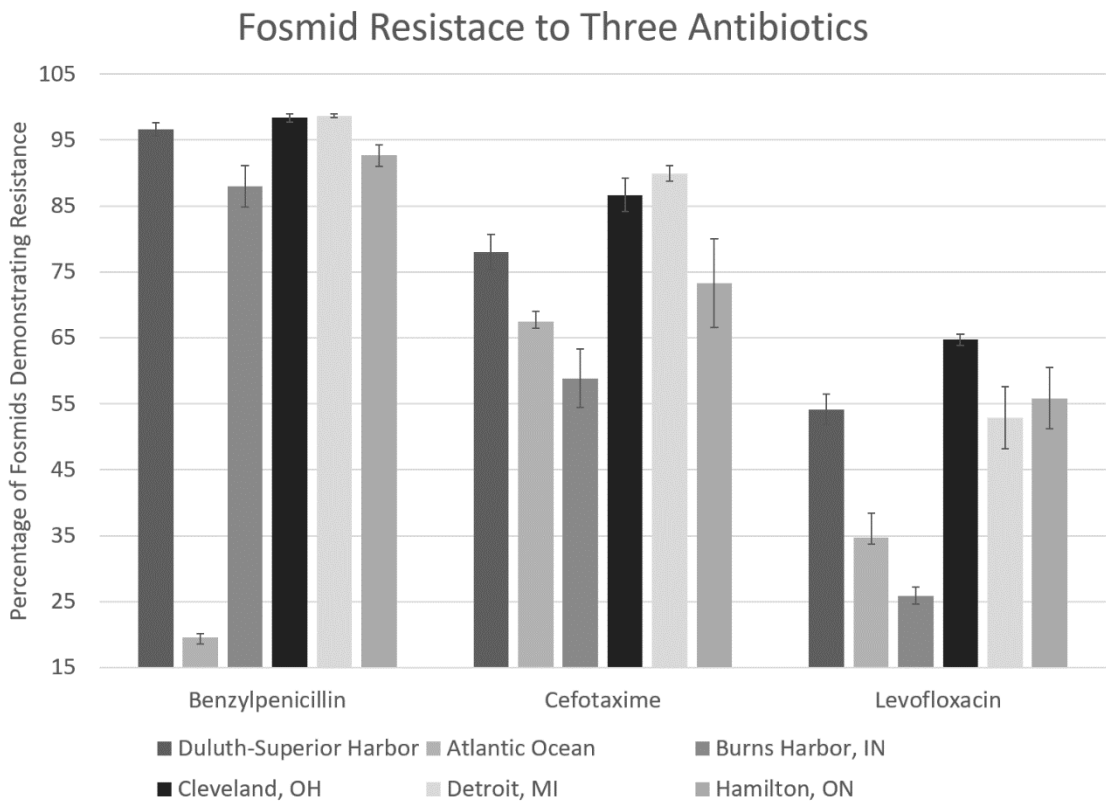
Table II-5. Resistance of fosmids from the harbor or ship ballast water sources to three antibiotics and three heavy metals. Mean percentage averages in the column are provided with standard deviations in parentheses. The Mean percentage averages with the same superscript in each treatment column are not statistically different ($p>0.05$). Table includes ship/site designation listed in Table II-1.

Ship/Site Designator	Water Source	Fosmids Resistance to Antibiotics and Heavy Metals (%)					
		Cefotaxime	Benzylpenicillin	Levofloxacin	Cadmium	Zinc	Mercury
B	Duluth-Superior Harbor	78.0 [a] (2.4)	96.6 [a,b] (0.9)	54.2 [a,b] (2.0)	83.0 [a] (1.6)	50.5 (0.7)	91.5 [a,b,c] (3.7)
21	Atlantic Ocean	67.5 [b] (1.4)	19.6 (0.6)	34.8 (2.6)	88.3 [c,e] (2.3)	58.6 (1.5)	99.5 [f,g] (0.8)
14	Burns Harbor, IN	58.9 (3.9)	88.0 [c] (2.8)	25.9 (1.2)	80.2 [a,b] (2.0)	71.9 (1.8)	89.9 [a,d,e,f] (6.6)
17b	Cleveland, OH	86.7 [c] (2.2)	98.3 [b,d] (0.5)	64.7 (0.7)	92.4 [c,d] (0.7)	66.5 (2.0)	99.7 [b,g] (0.4)
18a	Detroit, MI	89.9 [c] (1.0)	98.6 [a,d] (0.3)	52.9 [a,c] (4.2)	93.0 [d,e] (2.2)	75.7 (1.3)	90.7 [c,e] (3.8)
18b	Hamilton, ON	73.3 [a,b] (5.9)	92.6 [c] (1.4)	55.9 [b,c] (4.1)	69.2 [b] (5.7)	39.2 (2.5)	81.8 [d] (3.9)

Overall, a lower portion of fosmids were resistant to levofloxacin in comparison to cefotaxime and benzylpenicillin (Figure II-3). Resistance by the fosmids from the seawater ballast was also observed at a lower proportion for levofloxacin than most of the Great Lakes and DSH fosmids at 34.8%. Only the Burns Harbor fosmids demonstrated a lower resistance of 25.9% compared to the Atlantic Ocean fosmid library. The highest resistance to levofloxacin was observed from the Cleveland fosmid library at 64.7%. A little more than a half of the fosmids from Hamilton, Detroit, and DSH samples exhibited resistance to levofloxacin (Table II-5).

The percentage fosmids demonstrating resistance to heavy metals was less obvious between the three heavy metals compared to the three antibiotics. The percentage of fosmids resistant to cadmium ranged from 69.2% to 93.0%. Fosmids from the Detroit and Cleveland ballast water samples exhibited the highest resistance to cadmium at 93% and 92.4% respectively (Table II-5). Their resistance was not statistically different from one another nor the Atlantic Ocean ballast water fosmids, which displayed an 88.3% resistance to cadmium. Eighty-three percent of fosmids from the DSH ballast water sample displayed a resistance to cadmium, which was also not statistically different than the fosmids from the Burns Harbor ballast water sample, which resistance was 80.2%. The lowest observed resistance was from Hamilton, ON ballast water fosmids.

Figure II-3. Percentage of fosmids demonstrating resistance to the three antibiotics - benzylpenicillin, cefotaxime, and levofloxacin. The established minimum inhibitory concentrations for the control clone *E. coli* EPI300-TR^{R} with the pcc2F0S vector were used to determine the percent of fosmids resistant to the particular treatment. Concentrations used were 17.5 µg/ml of benzylpenicillin, 0.6µg/ml of cefotaxime, and 0.02 µg/ml of levofloxacin. Fosmids were from six libraries created from one harbor and five ballast water sources. Harbor water was collected from the Duluth-Superior Harbor. Ballast water sampled originated from the Atlantic Ocean, Burns Harbor, IN, Cleveland, OH, Detroit, MI, and Hamilton, ON. Bars represent the 95% confidence intervals.

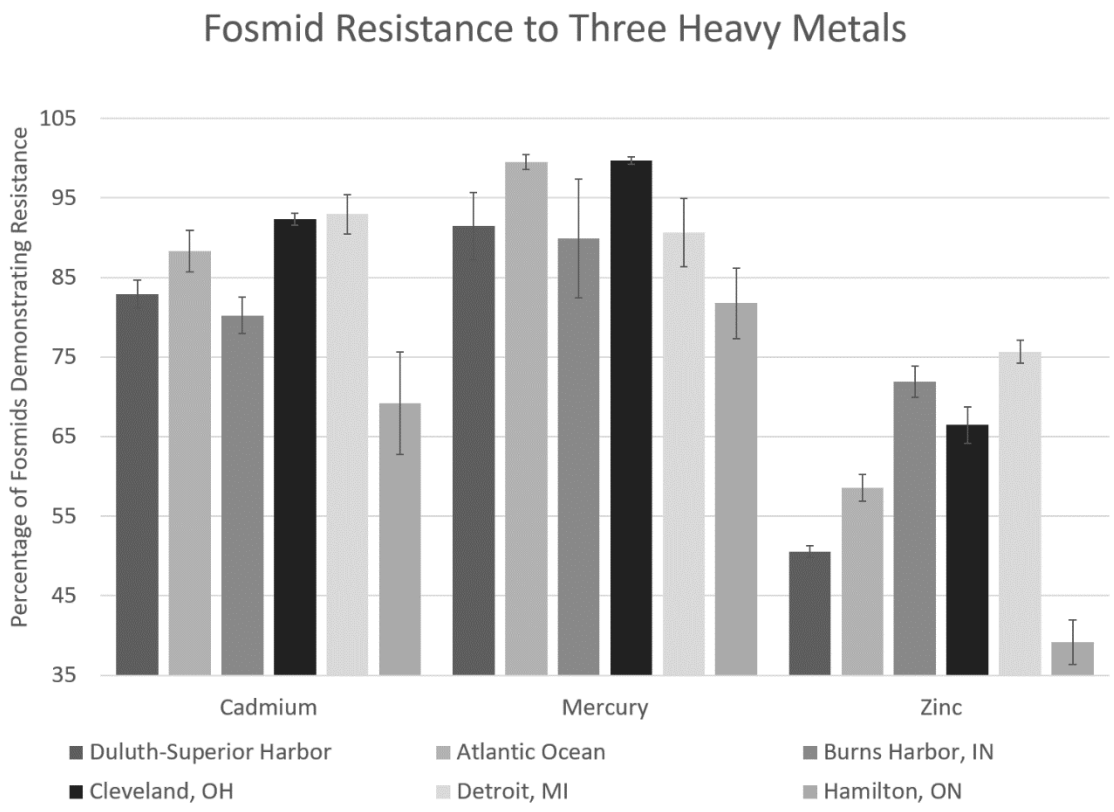

Fosmid Resistace to Three Antibiotics
Percentage of Fosmids Demonstrating Resistance
105
95
85
75
65
55
45
35
25
15
Benzylpenicillin
Cefotaxime
Levofloxacin
Duluth-Superior Harbor
Atlantic Ocean
Burns Harbor, IN
Cleveland, OH
Detroit, MI
Hamilton, ON

A lower proportion of fosmids were resistant to zinc compared to the other heavy metals, which ranged from 39.2% to 71.9% (Figure II-4). Each fosmid water sample was statistically different from one another. The fosmids from the Hamilton ballast water sample showed the lowest resistance to zinc, then the fosmids from DSH and Atlantic Ocean at 50.5% and 58.6% respectively. Sixty-six and one-half percent of Cleveland fosmids demonstrated resistance to zinc. Burns Harbor and Detroit ballast water fosmids exhibited the highest resistance to zinc at 71.9% and 75.7% respectively.

Resistance to the heavy metal mercury had the highest overall resistance range for the fosmid water samples of 81.8% to 100.8% (Table II-5). The fosmid water samples also demonstrated a wide variance of resistance to mercury and many of the libraries were not statistically different (Figure II-4). Hamilton fosmids demonstrated a resistance of 81.8% to mercury. Next, fosmids from the Burns Harbor library exhibited a resistance of 89.9%. Following Burns Harbor, Detroit (90.7%) and DSH (91.5%) had the next highest observed resistance to mercury. Cleveland ballast water fosmids demonstrated the second highest resistance at 99.7% and the Atlantic Ocean fosmid library exhibited the highest percentage resistance.

In general, a lower proportion of the Atlantic Ocean and Hamilton, ON fosmids were resistant to the antibiotics and heavy metals compared to the fosmids from the Great Lakes ballast and DSH water (Table II-5). A smaller proportion of fosmid clones from ballast water originating from Burns Harbor, IN and the Atlantic Ocean were less resistant to benzylpenicillin and levofloxacin compared to fosmids from DSH, Cleveland, OH, and Detroit, MI ballast water (Figure II-3). Fosmids from Hamilton, ON ballast water demonstrated a variety of antibiotic resistance in comparison to the other ballast

Figure II-4. Percentage of fosmids demonstrating resistance to the three heavy metals – cadmium, mercury, and zinc. The established minimum inhibitory antibiotic concentrations for the control clone E. coli EPI300-TRR with the pcc2F0S vector were used to determine the percent of fosmids resistant to the particular treatment. Concentrations used were 1.55 mM of cadmium, 0.02 mM of mercury, and 2.2 mM of Zinc. Fosmids were from six libraries created from one harbor and five ballast water sources. Harbor water was collected from the Duluth-Superior Harbor. Ballast water sampled originated from the Atlantic Ocean, Burns Harbor, IN, Cleveland, OH, Detroit, MI, and Hamilton, ON. Bars represent the 95% confidence intervals.


Fosmid Resistance to Three Heavy Metals
Percentage of Fosmids Demonstrating Resistance
105
95
85
75
65
55
45
35
Cadmium
Mercury
Zinc
Duluth-Superior Harbor
Atlantic Ocean
Burns Harbor, IN
Cleveland, OH
Detroit, MI
Hamilton, ON

and harbor fosmid libraries. The order of resistance to cefotaxime for the fosmids was similar to the other antibiotics. Burns Harbor, IN fosmids were less resistant compared to fosmids created with water samples from Atlantic Ocean, Hamilton, ON, and DSH. Cleveland, OH and Detroit, MI fosmids had a higher proportion of fosmid clones resistant to cefotaxime (Table II-5).

Discussion

Approximately 3,500 million tons of ballast water, and associated nonindigenous species (NIS) including invertabrates, phytoplankton, fish, and microbes, are transported each year by vessels around the world (Enderson et al. 2004). Carlton and Geller (1993) sampled ballast water from ships in Oregon's Coos Bay and identified plankton from 16 animal, three protist phyla, and three plant divisions with all major and minor phyla represented. Pathogenic bacteria have also been identified in ballast water including *Vibrio cholerea*, *Listeria monocytogenes, E. coli, Pseudomonas aeruginosa* (McCarthy and Crowder 2000; Burkholder et al. 2007). A variety of NIS have been introduced into the Laurentian Great Lakes with ballast water including, mollusks (*Dreissena polymorpha*), crustaceans, (*Bythotrephes longimanus)*, protozoans, (*Psammonobiotus commounis*), and fish (*Neogobius melanostomus)* (Sprules et al. 1990; Nicholls and MacIsaac 2004; Kotta et al. 2016).

This study is the first to identify the prevalence of antibiotic and heavy metal resistance genes from bacteria that are being actively discharged by vessels in the St. Louis River estuary near Duluth, MN. Similar studies have been conducted for ballast water transported outside of the DSH area as well as other aquatic environments. Thomson (2009) identified antibiotic resistant *Vibrio cholerae* cells isolated from ballast tanks of 17 commercial ships in the Great Lakes and Cheaspeake Bay. Resistance was detected for at least one of the 12 screened antibiotics in 79.9% of the isolates tested.

The Thomson study (2009) found that the most common resistance was to the beta-lactam class antibiotics, ampicillin and penicillin, with 67.2% of isolates demonstrating resistance to these two antibiotics. These antibiotics are in the same drug

class as benzylpenicillin and cefotaxime, which were used for screening in this study. The fosmids from Great Lakes harbor ballast water demonstrated an overall higher resistance to the beta-lactams antibiotics tested (Table II-5) compared to Thomson's findings. Only fosmids created from the Burns Harbor, IN ballast water resistance had a lower resistance to cefotaxime at 58.9% in comparison to Thomson's 67.2%. These results suggest that resistance to beta-lactam antibiotics are prevalent in ballast water from the Great Lakes and Chesapeake Bay and bacteria from ballast water sources in this study tended to be more resistant to the screened beta-lactams antibiotics compared to the *Vibrio cholerae* cells isolated by Thomson from commercial ships and the Chesapeake Bay.

Altug et al. (2012) isolated heterotrophic aerobic bacteria from ballast water in 21 different ships from 12 different regions of the world, including Southern China Sea, the Mediterranean, and the Black Sea, and found that all isolates were 100% resistant to the four antibiotics screened, including cefotaxime. The percentage of fosmids demonstrating resistance to cefotaxime in ballast water collected for this study ranged from 68% to 90% (Table II-5). Though cefotaxime resistance was prevalent in both studies, it appears that not as many cells (i.e. fosmids) may be resistant to cefotaxime in ballast water from the Laurentian Great Lakes than in ballast water from other regions of the world.

Mancuso et al. (2018) determined that all isolates from the Messina harbor in Turkey were very susceptible to benzylpenicillin, 20% of isolates were resistant to cefotaxime, and 6% were resistant against levofloxacin. This trend is similar to fosmids from the DSH, which demonstrated highest resistance for benzylpenicillin, then cefotaxime, and the least resistance to levofloxacin. The DSH and Messina harbor are

both important harbors for their respective regions with similar industries related to transporting goods and tourism. Because of these economic activities, both harbors experience similar anthropogenic pressures, such as municipal treated wastewater and industrial effluent discharges, that may impact the native bacterial communities and help explain the similar antibiotic resistance trends.

Two-hundred and fifty-five gram-negative bacterial isolates from the coast of the Eastern Mediterranean Sea were screened for antibiotic and heavy metal resistance by Matyar (2012). A couple of the antibiotics screened in the study were from penicillin and cephalosporin groups. Matyar (2012) found a high percentage of all of the isolates were resistant to ampicillin and cefazolin at 87.1% and 56.9% respectively. On average, isolates also demonstrated a 78.9% resistance to cadmium, which is within the range of fosmids demonstrating resistance to the heavy metal for this project (69.2% - 93%).

Matyar's (2012) collected sea water near important centers for tourism and industrial production. Iron and steel factories, a fertilizer factory, a refinery, and a coal-fired power plant discharge a high volume of processed and unprocessed waste at the sample sites. Hospital waste was also discharged near Matyar's sample sites. These types of industrial environmental pressures also occur in Great Lakes harbors where ballast waters originated from in this study, which might explain the high levels of heavy metal resistance observed in the fosmid libraries. The Hamilton, ON iron and steel industry began in the mid-19th century and accounts for a major part of Canada's national steel production (Encyclopaedia Britannica 2019). The highest concentration of steel mills are found in the Great Lakes region, including Indiana, Ohio, and Michigan (Energy 2019). Fosmids from the DSH water sample displayed similar resistance compared to the other

Great Lakes harbor samples. This similarity may be due to contaminants such as cadmium, mercury, and zinc originating from the pig iron, coking, and coal gasification plants as well as steel mills that were located at superfund sites (EPA 2017). Operations at these sites contaminated soil and underwater sediments with hazardous chemicals and heavy metals, which are still being cleaned up (EPA 2017).

One strength of the present study was the large number of replicate fosmids evaluated. A total of 54,276 unique fosmids from the six different fosmid libraries were screened, while previous studies screened 10 to a couple of hundred bacterial isolates for antibiotic resistance. The large replication in this study was possible by automating the picking of the *E. coli* cells containing fosmid inserts with the QPix II robot. Previous studies used the disk diffusion method to screen for antibiotic resistance and not all bacteria are culturable or grow at a rate that can be accurately tested by this method. Whereas, creating fosmid libraries uses *E. coli* cells, which can be easily cultured and replicate quickly to contain the metagenomic DNA for gene screening (Reller et al. 2009). Having a very large number of replicate measurements provides great confidence in the findings and allows detection of smaller differences between ballast water and harbor water samples.

The results presented here indicate that antibiotic resistance genes are prevalent in ballast water. Once ballast water is discharged, resistance genes in bacterial cells may be transferred to other prokaryotic cells in a process called horizontal gene transfer (HGT). HGT is one of the leading forces of evolution in single-cell organisms and is a main mechanism for the spread of antibiotic resistance in bacterial populations (Gogarten and Townsend 2005). During this process, the genetic material on a plasmid received by a

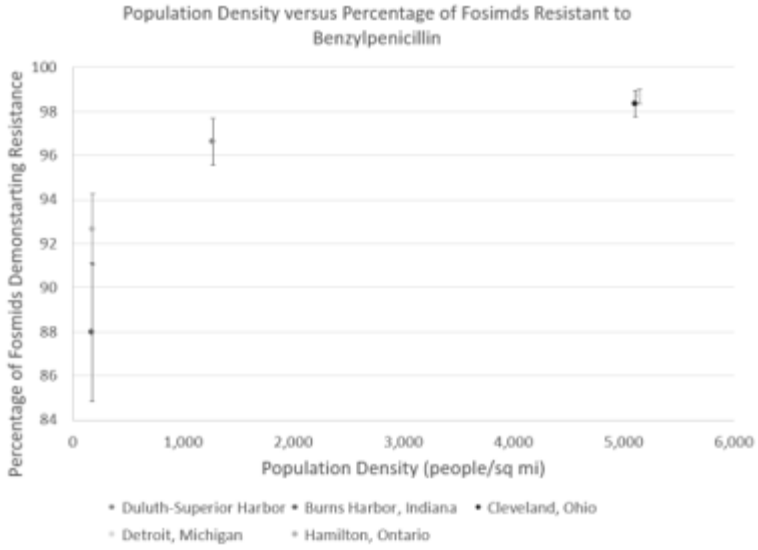
bacterium does not necessarily need to be from the same bacterial species, genus, or domain. Plasmids encoding antibiotic resistance can persist in environments even if selective pressure to retain these traits is absent (Smith and Bidochka 1998).

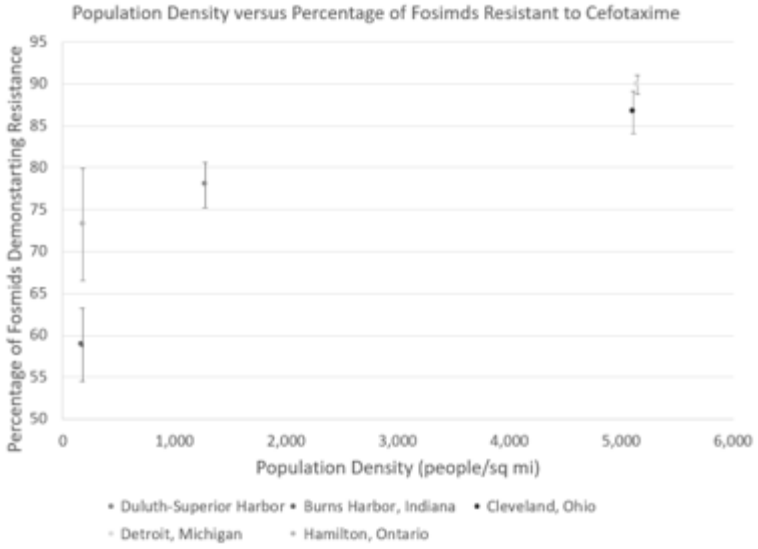
The spatial antibiotic resistance patterns observed did not appear to be related to the invasive species "hot spot" or corridors identified in the Laureation Great Lakes. The ports of Detroit, MI and Hamilton, ON are located within these invasive species corridors. Fosmids created with ballast water from these sites demonstrated the highest and lowest percent resistance to cefotaxime, the highest and second lowest to benzylpenicillin, and second lowest and second highest to levofloxacin (Table II-5). These results indicate that receiving ballast water from invasive species "hot spots" is not a good indicator of the risk of receiving antibiotic resistance genes via ballast water bacteria.

Instead, a spatial pattern of antibiotic resistance in this study appears to be related to the human population densities of urban areas adjacent to the Great Lakes harbors where the ballast water was obtained by the ships that were sampled (Figure II-5). Typically, fosmid libraries from port cities with a population density less than 1,300 people per square mile had a smaller portion of antibiotic resistance to the screened antibiotics compared with fosmids libraries created from cells in ballast water from larger urbanized Great Lakes cities such as Cleveland, OH and Detroit, MI. This pattern of where higher antibiotic resistance is found was also observed for resistance to the heavy metal cadmium but not for resistance to zinc and mercury (Figure II-6). Other factors besides human population density such as human population size of port cities where ballast water originated from, ballast water salinity, and distance of ballast water ports of

origin from the DSH were also evaluated. None of these factors, however, correlated with antibiotic or heavy metal resistance found in the ballast water bacterial fosmids (Appendix Figures A-1 through A-6).

Figure II-5. The percentage of fosmids from Duluth-Superior Harbor (DSH) and ballast water sources demonstrating resistance to the antibiotics benzylpenicillin, cefotaxime, and levofloxacin compared to the population density (people/square mile) of the urban areas adjacent to the Great Lakes harbors that were the sources of the ship ballast water. The percentage of fosmids demonstrating resistance to benzylpenicillin at the different sites were statistically different from one another except for DSH, Cleveland, OH, and Detroit, MI (upper graph). All sites were statistically different from one another for cefotaxime resistance except for DSH and Hamilton, ON as well as Cleveland, OH and Detroit, MI (middle graph). The percentage of fosmids from Burns Harbor, IN ballast water demonstrating resistance to levofloxacin was statistically different from the other four sites (lower graph). Cleveland, OH was statistically significant from DSH and Detroit, MI. Bars represent the 95% confidence intervals.


Population Density versus Percentage of Fosimds Resistant to Benzylpenicillin
Percentage of Fosmids Demonstarting 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)
Duluth-Superior Harbor
Burns Harbor, Indiana
Cleveland, Ohio
Detroit, Michigan
Hamilton, Ontario


Population Density versus Percentage of Fosimds Resistant to Cefotaxime
Percentage of Fosmids Demonstarting 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)
Duluth-Superior Harbor
Burns Harbor, Indiana
Cleveland, Ohio
Detroit, Michigan
Hamilton, Ontario

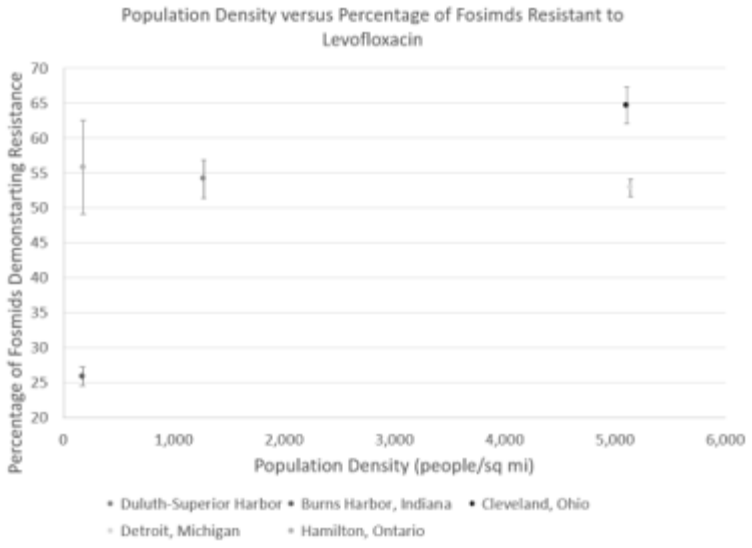
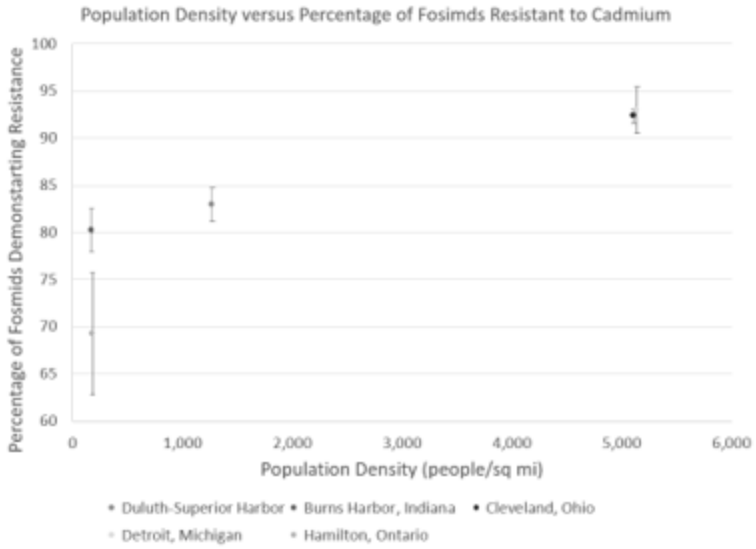
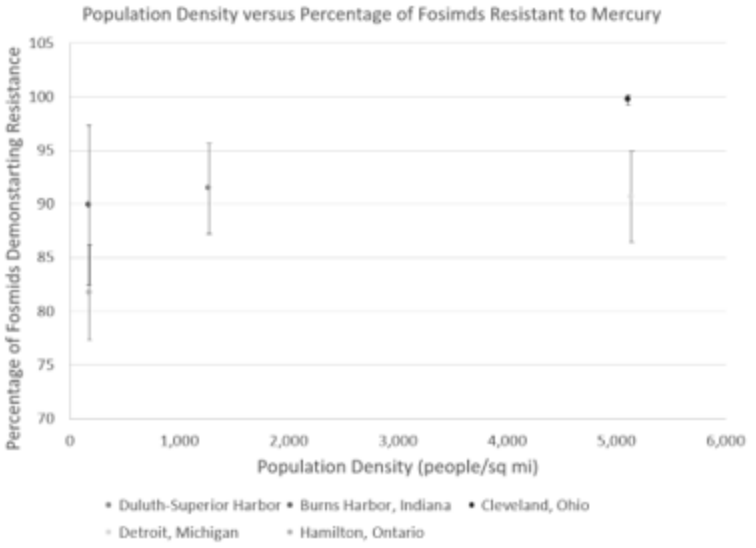

Population Density versus Percentage of Fosimds Resistant to Levofloxacin
Percentage of Fosmids Demonstarting Resistance
70
65
60
55
50
45
40
35
30
25
20
0
1,000
2,000
3,000
4,000
5,000
6,000
Population Density (people/sq mi)
Duluth-Superior Harbor
Burns Harbor, Indiana
Cleveland, Ohio
Detroit, Michigan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

Figure II-6. The percentage of fosmids from Duluth-Superior Harbor (DSH) and ballast water sources demonstrating resistance to the heavy metals cadmium, mercury, and zinc compared to the population density (people/square mile) of the urban areas adjacent to the Great Lakes harbors that were the sources of the ship ballast water. The percentage of DSH fosmids demonstrating resistance to cadmium was statistically different from the other harbor ballast water sources except for Burns Harbor, IN (upper graph). Burns Harbor, IN was statistically different from Cleveland, OH, and Detroit, MI. Cleveland, OH was statistically different from Hamilton, ON. The percentage of Hamilton, ON fosmids demonstrating resistance to mercury was statistically different from the mercury resistance found in the DSH, Cleveland, OH and Detroit, MI fosmid libraries (middle graph). All sites were statistically different from one another for the zinc treatment except for DSH and Detroit, MI (lower graph). Bars represent the 95% confidence intervals.


Population Density versus Percentage of Fosimds Resistant to Cadmium
Percentage of Fosmids Demonstarting Resistance
100
95
90
85
80
75
70
65
60
0
1,000
2,000
3,000
4,000
5,000
6,000
Population Density (people/sq mi)
Duluth-Superior Harbor
Burns Harbor, Indiana
Cleveland, Ohio
Detroit, Michigan
Hamilton, Ontario


Population Density versus Percentage of Fosimds Resistant to Mercury
Percentage of Fosmids Demonstarting 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)
Duluth-Superior Harbor
Burns Harbor, Indiana
Cleveland, Ohio
Detroit, Michigan
Hamilton, Ontario


Population Density versus Percentage of Fosimds Resistant to Zinc
Percentage of Fosmids Demonstarting 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)
Duluth-Superior Harbor
Burns Harbor, Indiana
Cleveland, Ohio
Detroit, Michigan
Hamilton, Ontario

Higher population density areas usually have more residential and industrial development, increased treated wastewater discharge and stormwater runoff, as well as higher levels of industrial discharge compared to areas with less human density. These factors lead to more frequent or larger discharges from these sources or higher concentrations of heavy metal and antibiotics pollutants in ports near areas with higher human population densities. LaPara et al. (2011) identified that tertiary-treated municipal wastewater released into the DSH was a source for antibiotic resistance genes. These pollutants lead to higher selective pressures for bacteria to maintain resistant genes, which then may be transported to other ports via ballast water exchange and discharge.

Understanding patterns of antibiotic and heavy metal resistance in bacterial populations worldwide could assist with revising or devising more impactful regulations for the Great Lakes. Current guidelines for vessels entering the Great Lakes require ships to complete an oceanic exchange of ballast water. Oceanic water exchange in ballast tanks is not effective at reducing the abundance or dispersion of bacterial cells. Seiden et al. (2010) collected ballast water samples during a trans-Pacific voyage from Japan to Canada and determined that there was no significant difference between bacterial abundance in ballast tanks that did or did not have a water exchange with oceanic water. Additional transoceanic voyage studies, such as Mimura et al. (2005) from Japan to Qatar, also concluded that the concentration of viable bacterial cells was not reduced as a result of the oceanic exchange. Drake et al. (2002) observed similar results on a transoceanic voyage from Israel to Baltimore. On Day 15 at the conclusion of the voyage, there was no significant difference in microorganism abundance and biomass between samples from ballast water tanks that completed an oceanic exchange and the control.

Thus, if prokaryotic abundance and biomass is not reduced by mid-ocean water exchanges, it becomes even more important to understand the levels of resistance genes harbored by bacteria in the different water sources used as ballast to create and support effective regulatory actions.

Regulators are looking into the efficacy of onboard ballast water treatment technologies because of the limitations of mid-ocean ballast water exchange. Effective treatments that meet the standards for living organisms discharged with ballast water set by the United States Coast Guard may only be achieved by wastewater-type treatment technologies installed on ships or by disposing of ballast at a land-based treatment facility (Bailey 2015). These treatment options are not always feasible and supplementary guidelines may be needed to minimize the impact of invasive species including non-indigenous microorganisms. Such guidelines might include the potential risk of taking ballast water from certain ports. This study demonstrated that there is a higher risk for transporting three different antibiotic and cadmium resistant genes from ports near areas of high human population density. By prioritizing risks for invasive microbial species or genes they contain and implementing new technologies, it may be feasible to reduce future NIS being introduced and their impacts on native populations and ecosystems.

In conclusion, this study determined that ballast water received from ports in larger, more urbanized cities (Cleveland, OH and Detroit, MI) may include larger proportions of bacteria with antibiotic and heavy metal resistance genes. Receiving ballast water from harbors near highly urbanized cities should be a greater concern for the spread of antibiotic and heavy metal resistance among native bacterial populations than receiving ballast water from harbors in smaller metropolitan areas, such as Burns Harbor,

IN and Hamilton, ON. Regulators might consider differences like these when developing or revising guidelines for what type and level of ballast treatment a commercial ship may need to reduce the risk of spreading antibiotic or heavy metal resistance genes in aquatic bacterial communities within the Laurentian Great Lakes. Stricter guidelines and better treatment might be required for ships that transport ballast water obtained from large urban areas than for ships that draw and discharge ballast water from ports near cities with lower human populations densities.

Industrial discharge, domestic waste, and effluents from wastewater treatment plants are known to be heavy metal and antibiotic resistant gene reservoirs that influence the bacterial community (Matyar et al. 2014, Stoll et al. 2012, LaPara et al. 2011). These anthropogenic pressures are also present in the DSH and probably pose higher risks for introducing resistant genes into native bacterial populations than ballast water being discharged into the DSH. This study demonstrated that a high percentage of fosmids from native DSH bacterial populations were already resistant to the antibiotics and heavy metals screened compared to some ballast water samples. Legislators should focus federal and state resources on addressing high risk sources for introducing resistance genes into waterways, including possible threats from commercial ship ballast water discharged into the DSH.

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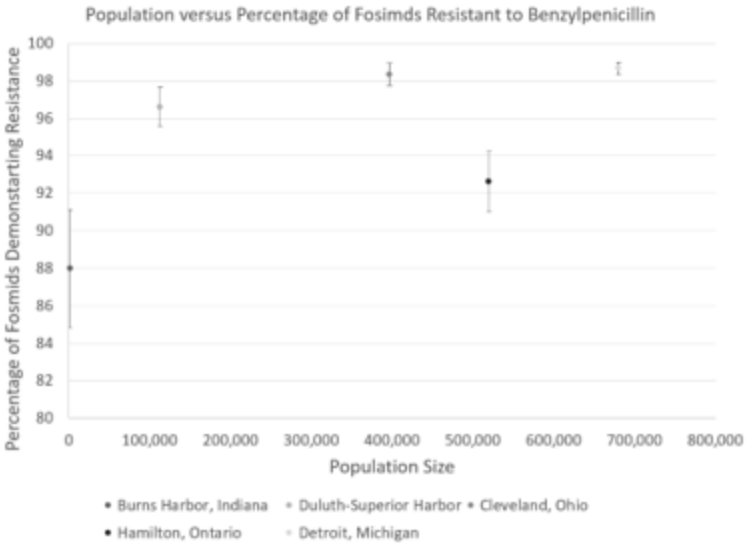
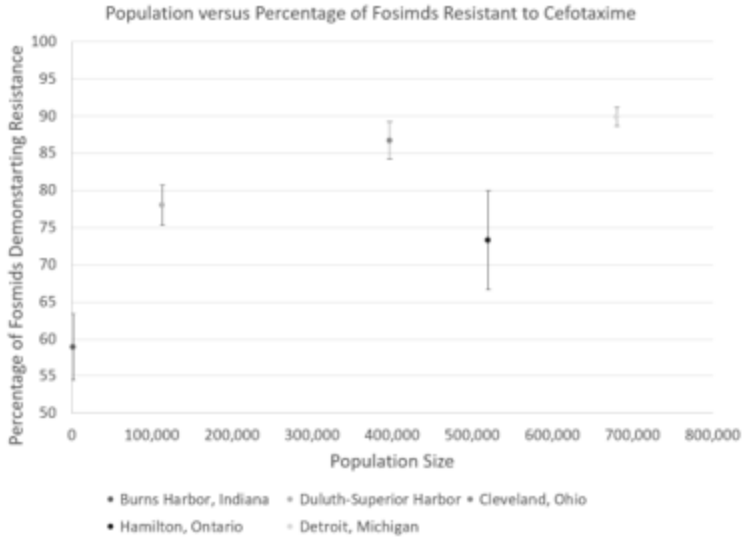
APPENDIX

Figure A-1. The percentage of fosmids from Duluth-Superior Harbor and ballast water sources demonstrating resistance to the antibiotics benzylpenicillin, cefotaxime, and levofloxacin compared to the population of the Great Lakes harbors that were the sources of the ship ballast water. Bars represents the 95% confidence intervals.


Population versus Percentage of Fosimds Resistant to Benzylpenicillin
Percentage of Fosmids Demonstarting Resistance
100
98
96
94
92
90
88
86
84
82
80
0
100,000
200,000
300,000
400,000
500,000
600,000
700,000
800,000
Population Size
Burns Harbor, Indiana
Duluth-Superior Harbor
Cleveland, Ohio
Hamilton, Ontario
Detroit, Michigan


Population versus Percentage of Fosimds Resistant to Cefotaxime
Percentage of Fosmids Demonstarting Resistance
100
95
90
85
80
75
70
65
60
55
50
0
100,000
200,000
300,000
400,000
500,000
600,000
700,000
800,000
Population Size
Burns Harbor, Indiana
Duluth-Superior Harbor
Cleveland, Ohio
Hamilton, Ontario
Detroit, Michigan

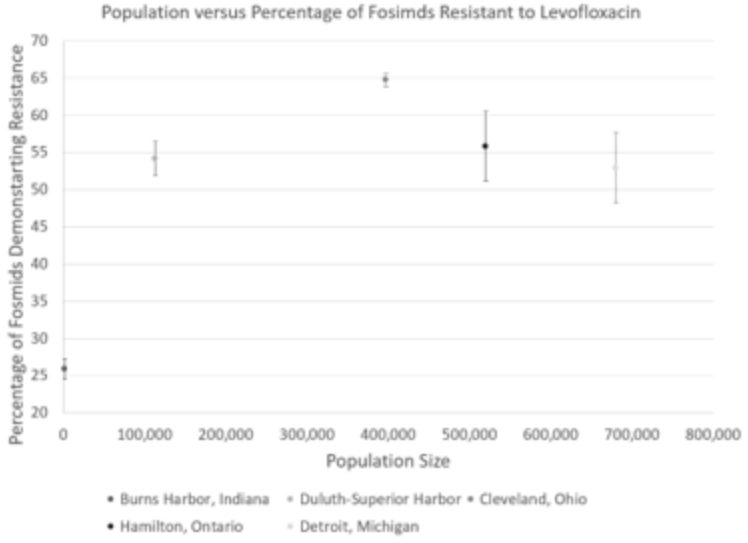
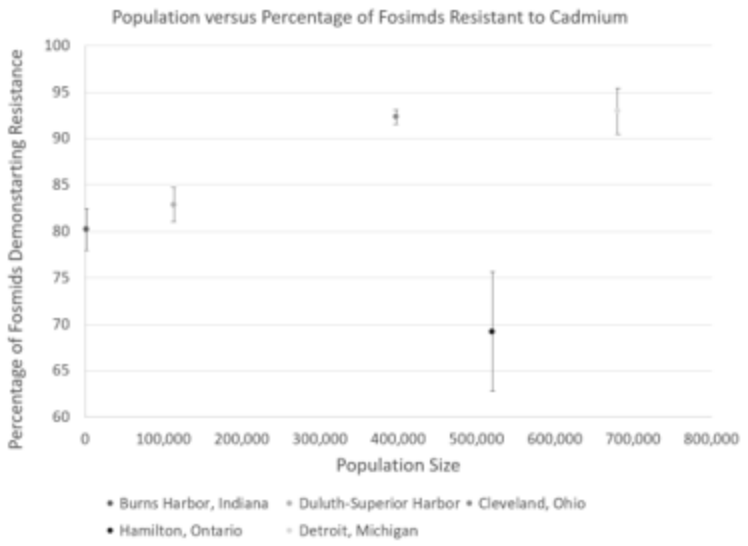
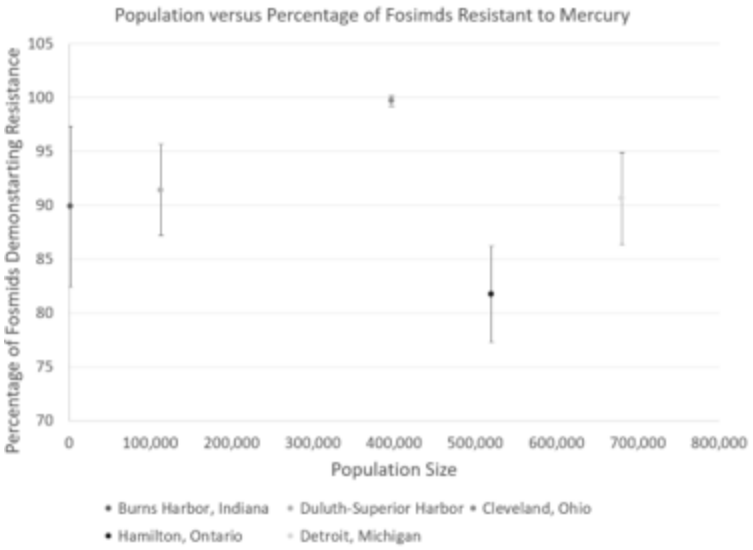

Population versus Percentage of Fosimds Resistant to Levofloxacin
Percentage of Fosmids Demonstarting Resistance
70
65
60
55
50
45
40
35
30
25
20
0
100,000
200,000
300,000
400,000
500,000
600,000
700,000
800,000
Population Size
Burns Harbor, Indiana
Duluth-Superior Harbor
Cleveland, Ohio
Hamilton, Ontario
Detroit, Michigan

Figure A-2. The percentage of fosmids from Duluth-Superior Harbor and ballast water sources demonstrating resistance to the heavy metals cadmium, mercury, and zinc compared to the population of the Great Lakes harbors that were the sources of the ship ballast water. Bars represents the 95% confidence intervals.


Population versus Percentage of Fosimds Resistant to Cadmium
Percentage of Fosmids Demonstarting Resistance
100
95
90
85
80
75
70
65
60
0
100,000
200,000
300,000
400,000
500,000
600,000
700,000
800,000
Population Size
Burns Harbor, Indiana
Duluth-Superior Harbor
Cleveland, Ohio
Hamilton, Ontario
Detroit, Michigan


Population versus Percentage of Fosimds Resistant to Mercury
Percentage of Fosmids Demonstarting Resistance
105
100
95
90
85
80
75
70
0
100,000
200,000
300,000
400,000
500,000
600,000
700,000
800,000
Population Size
Burns Harbor, Indiana
Duluth-Superior Harbor
Cleveland, Ohio
Hamilton, Ontario
Detroit, Michigan

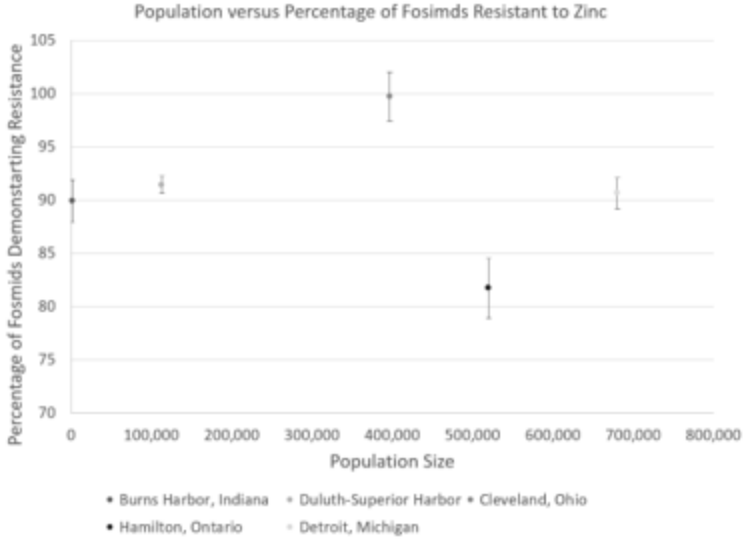
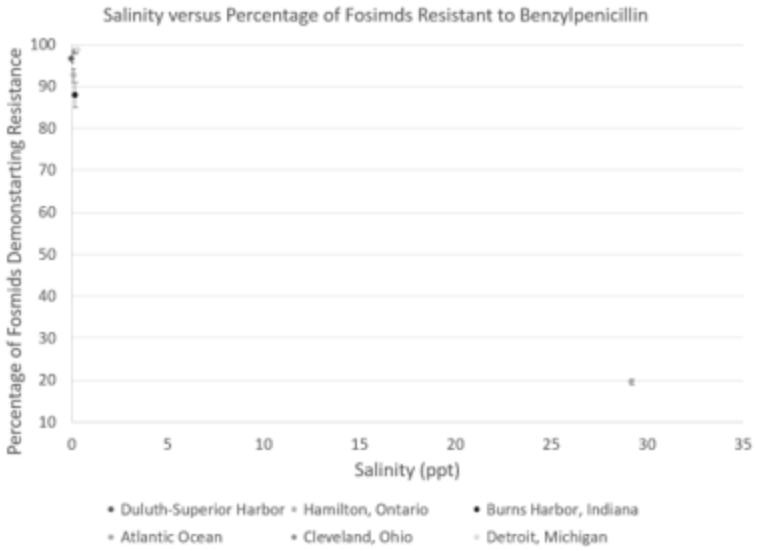
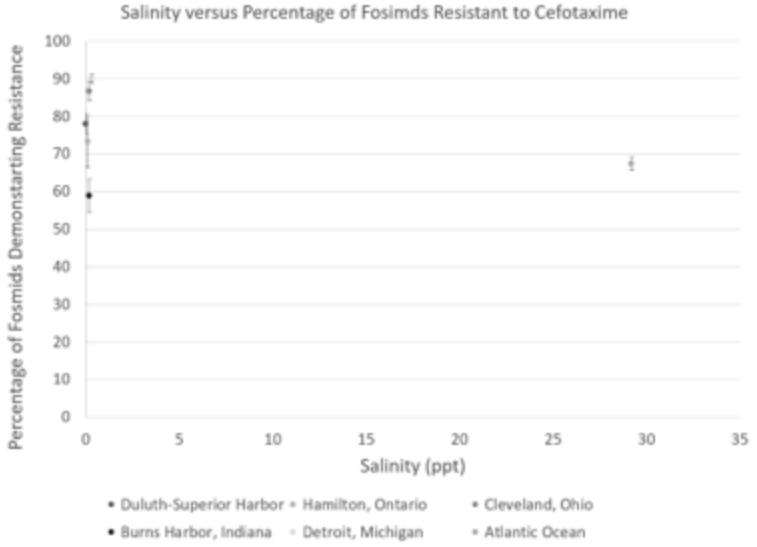

Population versus Percentage of Fosimds Resistant to Zinc
Percentage of Fosmids Demonstarting Resistance
105
100
95
90
85
80
75
70
0
100,000
200,000
300,000
400,000
500,000
600,000
700,000
800,000
Population Size
Burns Harbor, Indiana
Duluth-Superior Harbor
Cleveland, Ohio
Hamilton, Ontario
Detroit, Michigan

Figure A-3. The percentage of fosmids from Duluth-Superior Harbor and ballast water sources demonstrating resistance to the antibiotics benzylpenicillin, cefotaxime, and levofloxacin compared to the salinity of the water samples. Water quality measurements were taken in the field unless otherwise noted in Table II-4. Bars represents the 95% confidence intervals.


Salinity versus Percentage of Fosimds Resistant to Benzylpenicillin
Percentage of Fosmids Demonstarting Resistance
100
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
35
Salinity (ppt)
Duluth-Superior Harbor
Hamilton, Ontario
Burns Harbor, Indiana
Atlantic Ocean
Cleveland, Ohio
Detroit, Michigan


Salinity versus Percentage of Fosimds Resistant to Cefotaxime
Percentage of Fosmids Demonstarting Resistance
100
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
35
Salinity (ppt)
Duluth-Superior Harbor
Hamilton, Ontario
Cleveland, Ohio
Burns Harbor, Indiana
Detroit, Michigan
Atlantic Ocean

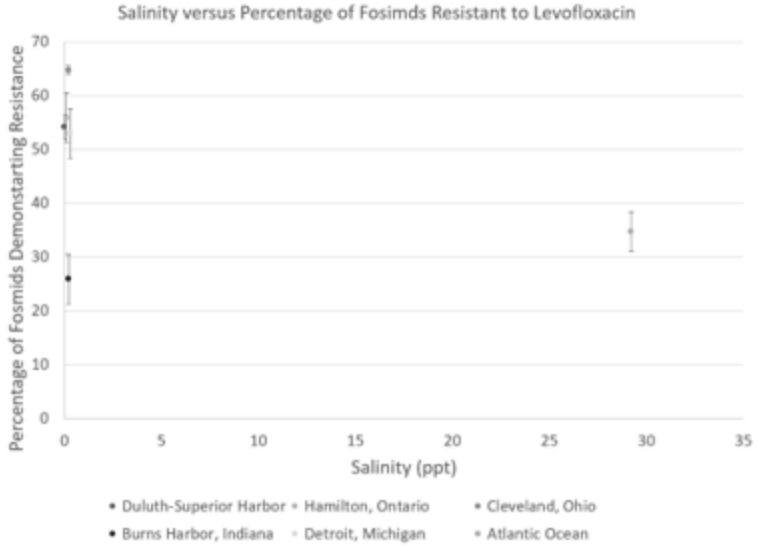
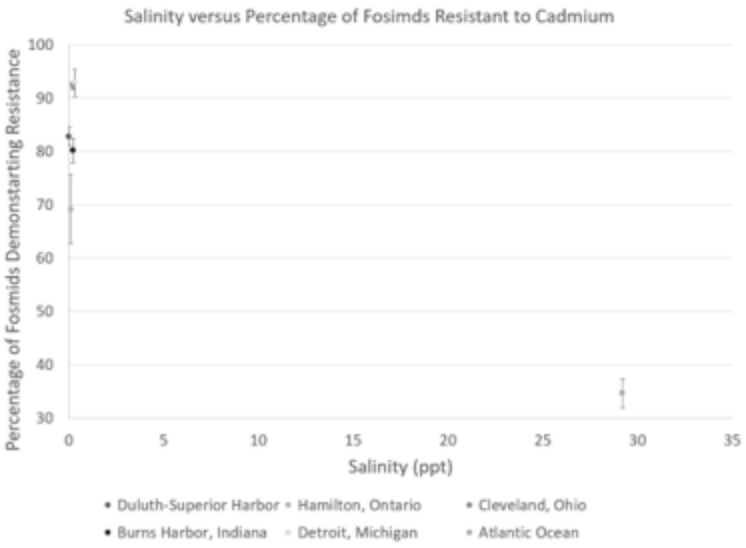
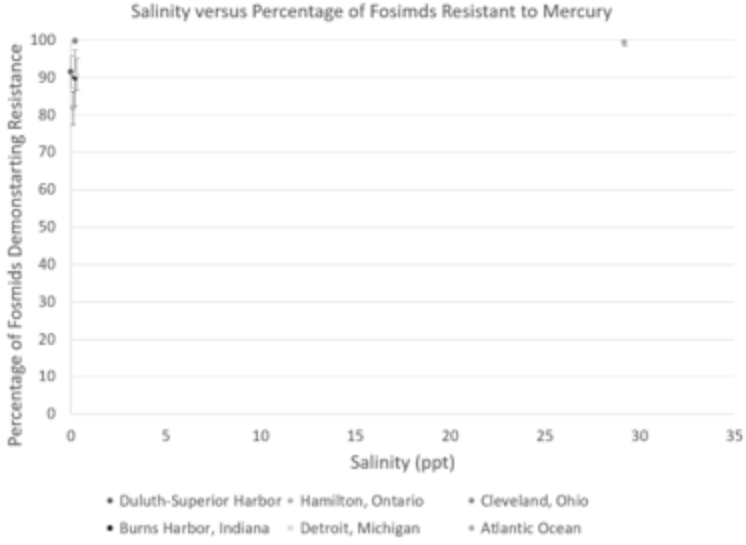

Salinity versus Percentage of Fosimds Resistant to Levofloxacin
Percentage of Fosmids Demonstarting Resistance
70
60
50
40
30
20
10
0
5
10
15
20
25
30
35
Salinity (ppt)
Duluth-Superior Harbor
Hamilton, Ontario
Cleveland, Ohio
Burns Harbor, Indiana
Detroit, Michigan
Atlantic Ocean

Figure A- 4. The percentage of fosmids from Duluth-Superior Harbor and ballast water sources demonstrating resistance to the heavy metals cadmium, mercury and zinc compared to the salinity of the water samples. Water quality measurements were taken in the field unless otherwise noted in Table II-4. Bars represents the 95% confidence intervals.


Salinity versus Percentage of Fosimds Resistant to Cadmium
Percentage of Fosmids Demonstarting Resistance
100
90
80
70
60
50
40
30
0
5
10
15
20
25
30
35
Salinity (ppt)
Duluth-Superior Harbor
Hamilton, Ontario
Cleveland, Ohio
Burns Harbor, Indiana
Detroit, Michigan
Atlantic Ocean


Salinity versus Percentage of Fosimds Resistant to Mercury
Percentage of Fosmids Demonstarting Resistance
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
Salinity (ppt)
Duluth-Superior Harbor
Hamilton, Ontario
Cleveland, Ohio
Burns Harbor, Indiana
Detroit, Michigan
Atlantic Ocean

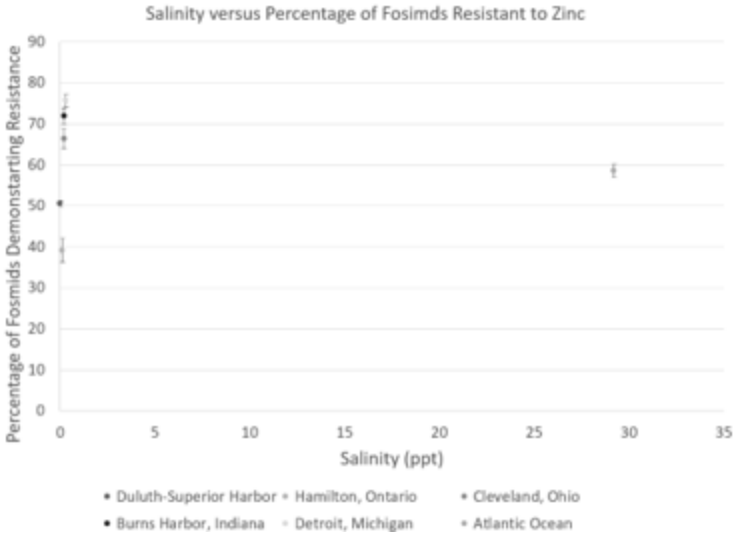
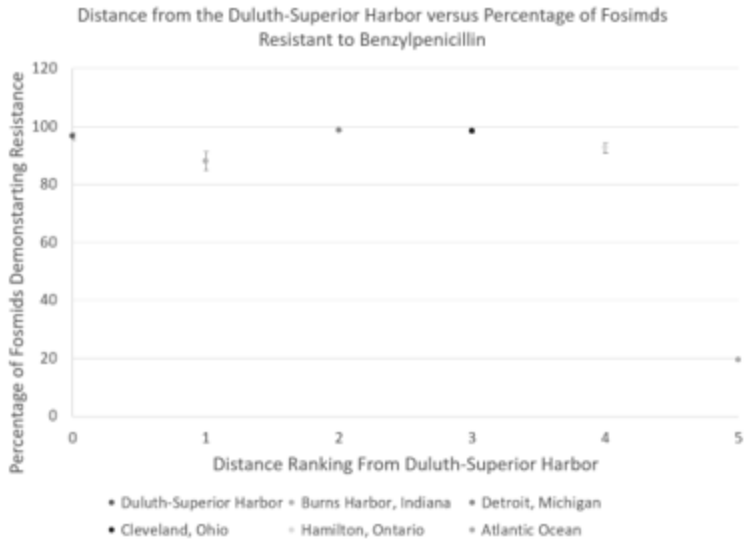
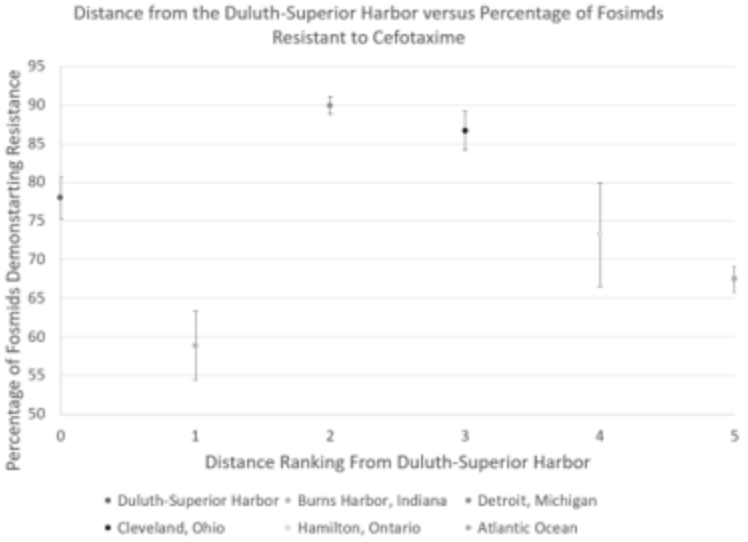

Salinity versus Percentage of Fosimds Resistant to Zinc
Percentage of Fosmids Demonstarting Resistance
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
Salinity (ppt)
Duluth-Superior Harbor
Hamilton, Ontario
Cleveland, Ohio
Burns Harbor, Indiana
Detroit, Michigan
Atlantic Ocean

Figure A- 5. The percentage of fosmids from Duluth-Superior Harbor (DSH) and ballast water sources demonstrating resistance to the antibiotics benzylpenicillin, cefotaxime, and levofloxacin compared to the distance from the DSH. Ballast water sample sites were ranked from one to five; one being the closet to DSH and five being the furthest away. Bars represents the 95% confidence intervals.


Distance from the Duluth-Superior Harbor versus Percentage of Fosimds Resistant to Benzylpenicillin
Percentage of Fosmids Demonstarting Resistance
120
100
80
60
40
20
0
0
1
2
3
4
5
Distance Ranking From Duluth-Superior Harbor
Duluth-Superior Harbor
Burns Harbor, Indiana
Detroit, Michigan
Cleveland, Ohio
Hamilton, Ontario
Atlantic Ocean


Distance from the Duluth-Superior Harbor versus Percentage of Fosimds Resistant to Cefotaxime
Percentage of Fosmids Demonstarting Resistance
95
90
85
80
75
70
65
60
55
50
0
1
2
3
4
5
Distance Ranking From Duluth-Superior Harbor
Duluth-Superior Harbor
Burns Harbor, Indiana
Detroit, Michigan
Cleveland, Ohio
Hamilton, Ontario
Atlantic Ocean

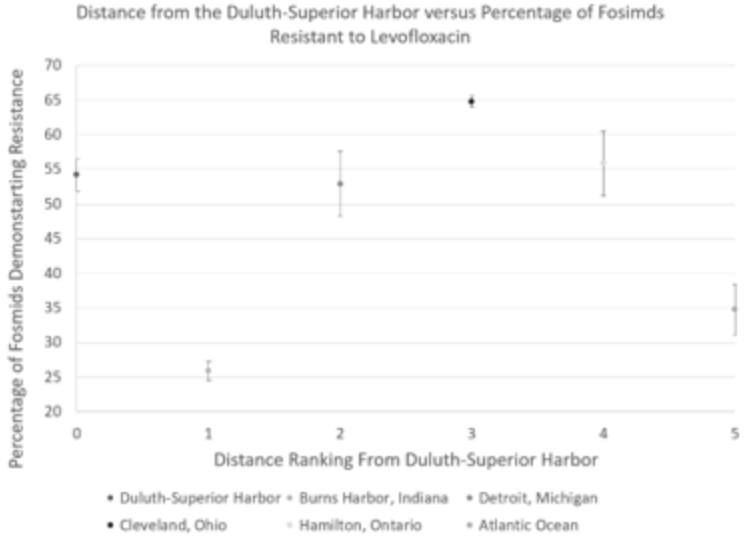
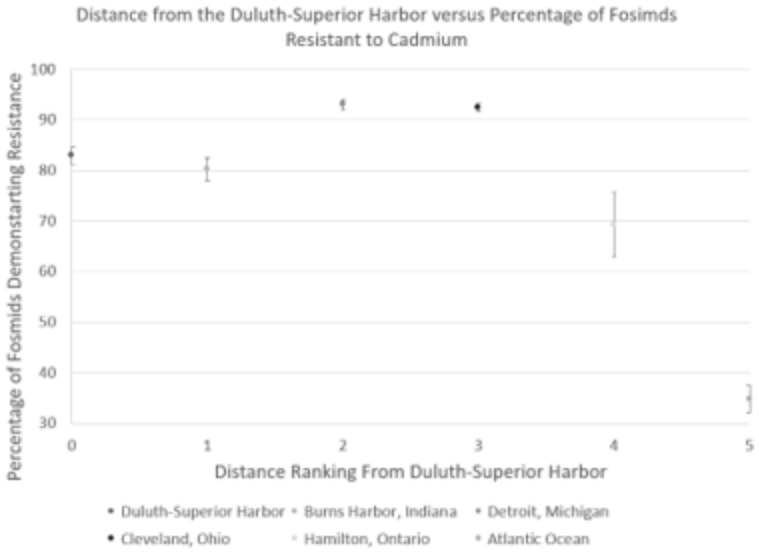
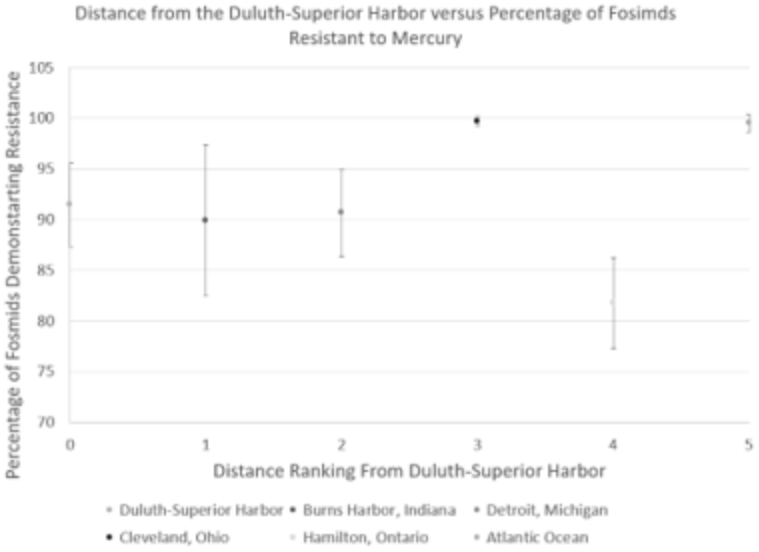
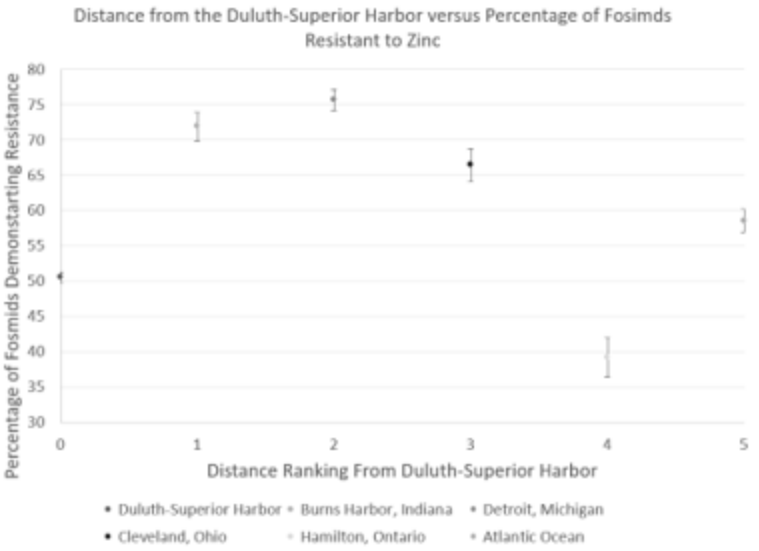

Distance from the Duluth-Superior Harbor versus Percentage of Fosimds Resistant to Levofloxacin
Percentage of Fosmids Demonstarting Resistance
70
65
60
55
50
45
40
35
30
25
20
0
1
2
3
4
5
Distance Ranking From Duluth-Superior Harbor
Duluth-Superior Harbor
Burns Harbor, Indiana
Detroit, Michigan
Cleveland, Ohio
Hamilton, Ontario
Atlantic Ocean

Figure A-6. The percentage of fosmids from Duluth-Superior Harbor (DSH) and ballast water sources demonstrating resistance to the heavy metals cadmium, mercury, and zinc compared to the distance from the DSH. Ballast water sample sites were ranked from one to five; one being the closet to DSH and five being the furthest away. Bars represents the 95% confidence intervals.


Distance from the Duluth-Superior Harbor versus Percentage of Fosimds Resistant to Cadmium
Percentage of Fosmids Demonstarting Resistance
100
90
80
70
60
50
40
30
0
1
2
3
4
5
Distance Ranking From Duluth-Superior Harbor
Duluth-Superior Harbor
Burns Harbor, Indiana
Detroit, Michigan
Cleveland, Ohio
Hamilton, Ontario
Atlantic Ocean


Distance from the Duluth-Superior Harbor versus Percentage of Fosimds Resistant to Mercury
Percentage of Fosmids Demonstarting Resistance
105
100
95
90
85
80
75
70
0
1
2
3
4
5
Distance Ranking From Duluth-Superior Harbor
Duluth-Superior Harbor
Burns Harbor, Indiana
Detroit, Michigan
Cleveland, Ohio
Hamilton, Ontario
Atlantic Ocean


Distance from the Duluth-Superior Harbor versus Percentage of Fosimds Resistant to Zinc
Percentage of Fosmids Demonstarting Resistance
80
75
70
65
60
55
50
45
40
35
30
0
1
2
3
4
5
Distance Ranking From Duluth-Superior Harbor
Duluth-Superior Harbor
Burns Harbor, Indiana
Detroit, Michigan
Cleveland, Ohio
Hamilton, Ontario
Atlantic Ocean