ML 2016 Project Abstract
For the Period Ending June 30, 2021

PROJECT TITLE: Advancing Microbial Invasive Species Monitoring from Ballast Discharge
PROJECT MANAGER: Randall E. Hicks
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FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: M.L. 2016, Chp. 186, Sec. 2, Subd. 06c as extended by M.L. 2019, First Special Session, Chp. 4, Art. 2, Sec. 2, Subd. 19 as extended by M.L. 2020, First Special Session, Chp. 4, Sec. 2

APPROPRIATION AMOUNT: $368,000
AMOUNT SPENT: $311,862
AMOUNT REMAINING: $56,138

Sound bite of Project Outcomes and Results

Bacterial communities and pathogen-containing bacterial genera were characterized in ship ballast water, throughout the St. Louis River estuary including commercial dock areas and muskellunge habitats to better understand the risk of discharging ballast water from commercial ships into this estuary.

Overall Project Outcome and Results

While culture-based methods to detect indicator bacteria reduce the cost and complexity to monitor ballast and harbor waters, caution should be used when monitoring based on these indicators alone because their fates are not necessarily representative of bacterial cells in some pathogen-containing genera. Both UV-treatment and chlorination resulted in >99% removal of culturable indicator bacteria, however, each indicator responded differently with no regrowth of *Enterococcus*, moderate regrowth of *E. coli* for chlorine treated samples, and major regrowth of total bacteria after treatment. There were shifts in overall bacterial community composition after treatment including regrowth of cells from genera that harbor pathogens (particularly *Acinetobacter, Flavobacterium,* and *Pseudomonas*). Initially, *P. salmonis* DNA appeared to be present in the surface water of the St. Louis River estuary, but this result proved to be incorrect. This finding was confirmed by sequencing bacterial DNA at various sites in 2017 and 2019, which did not detect the presence of *Piscirickettsia* DNA. Bacterial communities and the pathogen-containing bacterial genera (PCGs) subset in water and sediments at four commercial docks in the Duluth-Superior harbor were different from other sites in the St. Louis River estuary. Higher relative abundances of PCGs were found in commercial dock sediments compared to the rest of the estuary. While there were only minor differences in the relative abundance of PCGs in surface water throughout the estuary, DNA from the *Flavobacterium* genus was more abundant at docks than other areas. Discharge of ballast water may affect the prevalence of PCGs in the Duluth-Superior harbor. Treatment of ballast water prior to discharge may reduce any human and wildlife pathogen load. In addition, care should be taken when dredging dock areas because disturbing sediment may temporarily increase the chances of exposing recreational users to pathogenic bacterial strains.

Project Results Use and Dissemination

In total during this project, three graduate students were trained, two M.S. degrees will be completed, four poster and four oral presentations were made at regional and national scientific conferences and venues, and project results were disseminated to collaborators and colleagues at the U.S. EPA Mid-Continent Ecology

Division and the Duluth Seaway Port Authority in Duluth, MN. One M.S. thesis will be appended to this project final report and the other will be forwarded when it is completed.

Environment and Natural Resources Trust Fund (ENRTF)
M.L. 2016 Work Plan Final Report

Date of Report: August 16 2021

Final Report

Date of Work Plan Approval: June 7, 2016

Project Completion Date: June 30, 2021

PROJECT TITLE: Advancing Microbial Invasive Species Monitoring from Ballast Discharge

Project Manager: Randall E. Hicks

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Total ENRTF Project Budget:		
	ENRTF Appropriation:	**$368,000**
	Amount Spent:	**$311,862**
	Balance:	**$56,138**

Legal Citation: M.L. 2016, Chp. 186, Sec. 2, Subd. 06c as extended by M.L. 2019, First Special Session, Chp. 4, Art. 2, Sec. 2, Subd. 19 as extended by M.L. 2020, First Special Session, Chp. 4, Sec. 2

Appropriation Language:
$368,000 the second year is from the trust fund to the Board of Regents of the University of Minnesota to identify bacteria in ship ballast water and St. Louis River estuary sediments, assess the risks posed by invasive bacteria, and evaluate treatment techniques for effectiveness at removing the bacteria from ballast water. This appropriation is available until June 30, 2019, by which time the project must be completed and final products delivered.

M.L. 2019 - Carryforward; Extension (a) The availability of the appropriations for the following projects is extended to June 30, 2020: (9) Laws 2016, chapter 186, section 2, subdivision 6, paragraph (c), Advancing Microbial Invasive Species Monitoring from Ballast Discharge;

M.L. 2020 - Sec. 2. ENVIRONMENT AND NATURAL RESOURCES TRUST FUND; EXTENSIONS. [to June 30, 2021]

I. PROJECT TITLE: Advancing Microbial Invasive Species Monitoring from Ballast Discharge

II. PROJECT STATEMENT:

The movement of aquatic invasive species via ship ballast water associated with maritime commerce is a long-standing concern. Ballast water can be a primary source of invasive species and a vector for microbial introductions and movements. In fact, the Duluth-Superior Harbor (DSH) receives twice the ship ballast water discharge volume and events of any other Great Lakes port and is one of four invasion "hotspots" within the Great Lakes based on initial discoveries of non-indigenous species. Some of the microbes being released into the DSH may threaten human and aquatic animal health, cause ecological damage, and impact local coastal economies. This makes early detection of potentially harmful microbes, assessing the risk posed, and development of mitigation strategies extremely important goals.

It is well known that ballast water can transport a wide array of invasive species including potentially harmful bacteria. The introduction and rapid spread of the VHS virus in fish throughout the Great Lakes led many to recognize that some microbes can be viewed as harmful invasive species, just like their invasive animal and plant counterparts. The potential for this threat to cause real human or environmental hazards in Lake Superior waters has not been extensively evaluated. A recent conceptual analysis underscored the importance of hazards from fish and wildlife pathogens. Recently completed research funded by the Minnesota ENRTF confirmed our concern about the potential for microbial invasions in the DSH because DNA sequences from 33 and 14 bacterial genera containing human, and fish and wildlife pathogens, respectively, were found in ship ballast waters. These bacterial DNA sequences were often more common than those of indicator bacteria recommended by the International Maritime Organization for monitoring microbiological safety of ballast and recreational waters. In this project, we will expand a database of bacterial diversity and potentially harmful microbes collected from commercial ship ballast water discharged in the Duluth-Superior Harbor. Common and rare bacterial taxa will also be identified in water and sediments of the St. Louis River estuary and compared with bacteria discharged with ballast water to predict the risk different bacterial groups pose on sustainable fisheries, human health, and a functional St. Louis River Estuary ecosystem.

The federally required ballast water treatment systems in ocean-going vessels, and best management practices in Great Lakes vessels can be evaluated prior to their installation for their capacity to significantly reduce the importation and redistribution of larger invasive organisms. However, little is known about the effectiveness of these treatment systems to remove or inactivate many bacterial pathogens. Thus, the University of Wisconsin-Superior Lake Superior Research Institute (UWS-LSRI) and the University of Minnesota will conduct bench scale experiments with mock ballast water treatment processes to contrast changes to the composition and relative abundance of fecal bacteria and indicator organisms using standard methods, versus non-conventional potentially harmful bacteria concentrations using genetic detection probes.

The University of Minnesota and Northeast-Midwest Institute (NEMWI) will address these issues in cooperation with the MPCA. The overall goals are to build an empirical database for assessing the actual hazard, if any, and evaluate ballast water treatment technology effectiveness for attenuating such a hazard. Addressing these issues will inform management strategies that mitigate the risk of inadvertently introducing potentially harmful bacteria.

III. OVERALL PROJECT STATUS UPDATES:

Project Status as of January 31, 2017: Progress was made towards the outcomes of all three activities in this project. A graduate student, Jennifer Knack, was recruited and started work on this project in August 2016. A search for a Postdoctoral Associate was started in July 2016 at the University of Minnesota Duluth. This search is still in progress. A subcontract to the Northeast-Midwest Institute (NEMWI) from the University of Minnesota was developed ~~and accepted~~ to assist with parts of Activity 2. Ballast from one commercial ship in the Duluth-Superior Harbor and surface water from 18 sites in the St. Louis River Estuary (SLRE) were sampled in August and

September 2016. Replicates of these water samples were filtered, and stored frozen at -80°C until DNA can be extracted. Two DNA extraction protocols are being tested using ship ballast samples collected in 2011 and 2012 to identify the method which provides DNA to obtain the best Illumina sequencing results. DNA from this test was extracted in October and November 2016 and submitted for Illumina sequencing of the V4 region of the bacterial 16S rRNA gene in early December 2016. The DNA sequence results recently obtained in early January 2017 are now being analyzed.

Amendment Request (07/28/2017): We are requesting permission to pursue a subcontract for services in Activities 1 and 2 with the Lake Superior Research Institute at the University of Wisconsin-Superior (UWS-LSRI) instead of the Northeast-Midwest Institute (NEMWI), as originally planned. This change is at the request of the NEMWI and preferred by the UWS-LSRI. Both the scope of Activities 1 and 2 and the funds for this subcontract for professional services from UWS-LSRI are unchanged. We are only proposing to shift subcontract funds from the NEWI to the UWS-LSRI. No funds are being shifted between activities or between budget categories. Amendment Approved 0[8/04/2017]

Project Status as of July 31, 2017: DNA sequence data from ballast water samples collected in 2011-2012 and extracted using two protocols were analyzed to identify the best DNA extraction procedure to use for ballast, water, and sediment samples collected during the project. Analysis of the 16S sequences from these samples showed that the Levar-Sheik DNA extraction protocol provided superior DNA sequences compared to extracting microbial DNA with a MoBio PowerSoil extraction kit. An undergraduate student, Alex Carlbom, was recruited to primarily work on Activity 2 for UMD. Dr. Hicks, Dr. TenEyck, Jennifer Knack (graduate student), and Alex Carlbom met in early May to discuss the set-up and ballast treatment options for the bench-scale experiments to evaluate common ballast water treatment technologies. The NEMWI and the UWS-LSRI requested that a subcontract for services in Activities 1 and 2 be developed with the Lake Superior Research Institute at the University of Wisconsin-Superior (UWS-LSRI) instead of the Northeast-Midwest Institute (NEMWI), as originally planned. A new subcontract was drafted at the University of Minnesota and an amendment request for this subcontract change to the project work plan was written and submitted to the LCCMR. Subsurface water samples were collected from 22 randomly selected sites in the St. Louis River Estuary on April 21 and 22, 2017. Three replicates of each water sample were filtered onto Duropore (0.2 µm-pore) membrane filters, and the filters frozen at -80°C until DNA was extracted. We used Levar-Sheik protocol to extract DNA from 48 replicate water samples collected in September 2016 and April 2017. Sites were selected and boat reservations were made to sample subsurface water again at 15 sites and sediment at 30 sites in the St. Louis River Estuary in early August 2017.

Project Status as of January 31, 2018: Analysis of the partial 16S sequences generated from the 2011-2012 ballast samples was continued (Activity 1). While there was no clear bacterial community composition signature due to the origin these ballast water samples, bacterial community structure was strongly correlated with temperature of the ballast water when it was harvested. A preliminary water treatment experiment, using UV and chlorine treatments, was conducted in September 2017 to test whether our DNA extraction protocol would generate sufficient high-quality DNA for all analyses (Activity 2). We learned that only 0.5 to 1 liter of water needed to be filtered to obtain enough bacterial DNA from each replicate and that an additional clean-up step in the DNA extraction process was necessary to obtain high-quality DNA from chlorine-treated water, probably due to a high salt content in this treatment. Sampling of water and sediment from the St. Louis River Estuary was completed on November 1, 2017 (Activity 3). Replicate (n=3) water samples were collected in early August (Aug. 1-3) and early November (Oct. 31-Nov. 1), 2017 from 20 to 22 randomly selected sites. Sediment was collected from 30 randomly selected sites in early August 2017. A total of 286 samples from 86 unique sites have now been collected and filtered and stored. DNA has been extracted from approximately half the samples, and partial 16S rRNA sequences have been acquired and initially analyzed for about a third of those samples. Preliminary analyses of partial 16S rRNA sequences of bacteria from these water samples indicated that water temperature appears to be a strong predictor of microbial community composition in the St. Louis River estuary.

Although further analysis of the partial 16S rRNA gene sequences is planned, we have completed sampling of water and sediment in the St. Louis River estuary (Activity 3, Outcome 1).

Amendment Request (07/31/2018): We are requesting that the project completion date be extended from December 30, 2018 to June 30, 2019. The main reasons for this request are that project work was slowed when the project manager was on a 5-month medical leave in 2016-17, we were unable to hire a postdoctoral associate, and new ballast water sampling was slowed because we are still seeking permission from ship owners to sample ballast water on their commercial ships, which has become more challenging than anticipated. No funds are being shifted between activities or between budget categories as part of this amendment request.

Project Status as of July 31, 2018: The analysis of pathogen-containing bacterial genera (PCG) in the 2011-2012 ballast water samples was completed (Activity 1). Between 0.47% and 8.05% of all DNA sequences in 21 ballast water samples collected in 2011-12 were from PCGs. Of the 70 genera containing potential fish and wildlife or human pathogens that we screened, DNA sequences from only six PCG were present in all ballast water samples we analyzed; *Acintobacter, Aeromonas, Flavobacteria, Legionella, Mycobacterium,* and *Pseudomonas*. The bacterial genera *Aeromonas, Flavobacteria* and *Pseudomonas* contain known fish pathogens while some species in the genera *Legionella* and *Mycobacterium* are human pathogens. No DNA sequences were detected from the PCG *Renibacterium* (*R. salmoninarum* causes Bacterial Kidney Disease in fish) or *Piscirickettsia* (*P. salmonis* strains cause piscirickettsiosis in trout and salmonid fish or Muskie Pox disease in muskellunge) in any of the 21 ballast water samples. Interestingly, no DNA sequences from indicator bacterial genera *Escherichia* and *Enterococcus* were detected and only 24% of the 21 ballast water samples contained sequences from the bacterial genus *Vibrio* but only at low frequency. These findings suggest that these indicator bacterial taxa may not be good indicators for the presence of many bacterial genera containing pathogenic strains that affect fish and wildlife.

A full-scale water treatment experiment was successfully conducted on May 14, 2018 to determine the efficacy of UV light and chlorine treatment to remove total bacteria, indicator bacteria, and pathogen-containing bacterial genera (PCG) from harbor water (Activity 2). While only 20% of intact prokaryotic cells were removed by the UV treatment and 31% by the chlorine treatment, culturable heterotrophic bacteria, total coliforms, *E. coli*, and *Enterococcus* spp. were reduced by greater than 99% in both the UV and chlorine treatments compared to the no-treatment control. DNA from all replicate samples of each treatment was extracted and is being prepared for 16S rDNA sequence and qPCR analyses.

There were distinct bacterial communities in water from three zones of the St. Louis River Estuary (SLRE) in both fall 2016 and spring 2017, but these differences were stronger in the fall (Activity 3). A random forest model analysis uncovered that different bacterial taxa were responsible for driving spatial variability in fall 2016 than in spring 2017. In fall, the relative abundance of members of the gamma-Proteobacteria, alpha-Proteobacteria, and Bacteroides were responsible for differences seen in communities in the SLRE, while in spring the relative abundances of members of the Verrucomicrobia, Actinobacteria, and Bacteriodes were responsible for these differences. Two new graduate students, Julia Zimmer and Lisa DeGuire, joined this project in mid-June 2018. They have been extracting microbial DNA from the remaining ~70 water samples collected from the SLRE in summer and fall 2017. Further analysis of these DNA sequence libraries for pathogen-containing bacterial genera (PCG) will be completed when 16S rDNA sequences have been obtained for all water and sediment samples. Data from Activities 1 and 3 were presented at two scientific conferences.

Amendment Request (01/11/2019): We are requesting that the project completion date be extended from June 30, 2019 to June 30, 2020. Our progress was hindered because of two delays beyond our control. First, the Project Manager's health problem required a 5-month medical leave during the first year of the project and delayed hiring new personnel needed to complete this project. This issue has been resolved, but the analysis of project samples is now behind our original timelines due to this delay. With new personnel now in place, we are making very good progress in finishing the preparation and analysis of many project samples but it is unlikely we can completely analyze and evaluate all sample data before the scheduled end of the project (June 30, 2019) without an extension. These analyses are needed to properly evaluate the role of ballast water in introducing

potentially harmful microbes to Minnesota waters. Second, we also experienced a delay in obtaining ballast water samples that are needed to complete two project objectives. We and our collaborators have been diligently communicating with ship owners and shipping associations to obtain their permission to take ballast water samples from their ships. However, obtaining their permission was slowed while they waited to learn of the outcome of pending federal legislation (VIDA bill - Vessel Incidental Discharge Act) that clearly defined whether states or federal agencies would implement and monitor compliance with ballast water regulations in the Great Lakes. The VIDA act was passed and signed by the President in mid-December 2018, so we are optimistic that these shipping companies will now assist us in obtaining ballast water in early to mid-2019 after shipping resumes on the Great Lakes in March 2019. We need additional ballast water samples to complete Activities 1 and 2, but have alternative plans in case we are unable to obtain ballast water samples again in 2019:

- Activity 1: If we obtain additional ballast water samples by August 30, 2019, then we anticipate Activity 1 can be completed by December 30, 2019 and we will proceed to evaluate the role of ballast water in introducing potentially harmful bacteria into the Duluth-Superior Harbor (Activity 3-Outcome 3). If we are unable to obtain more ballast water samples by August 30, 2019, then our alternative plan is to use new DNA sequence analyses for ballast water samples collected during 2011-12 to complete a comparison of ballast water and harbor water bacterial community DNA by the end of the project to assess the risks of ballast water introducing potential harmful microbes from different bacterial taxa (Activity 3-Outcome 3).
- Activity 2: If we cannot obtain a single ballast water sample by July 31, 2019, then our alternative plan is to conduct a second bench-scale experiment during August 2019 (Activity 3-Outcome 1) using water from the Duluth-Superior Harbor near where commercial ships berth instead of an authentic ballast water to evaluate the efficacy of UV light and chlorine treatments to remove total bacteria, indicator bacteria, and pathogen-containing bacterial genera (PCG).

Amendment Request signed into law 5/31/19

Project Status as of January 31, 2019: We made incremental progress on each Activity but most progress was related to Activities 2 and 3 during this period. First, the Project Manager (R. Hicks) applied for and was granted a one-semester sabbatical leave from the University of Minnesota starting in January 2019 to spend additional time managing this project and bringing it to a successful conclusion.

From April to December 2018, the Project Manager and Dr. Matthew TenEyk from the University of Wisconsin-Superior LSRI made multiple contacts with owners of various shipping companies in the Great Lakes fleet ("lakers"), agents for ocean-going ships arriving in the Duluth-Superior Harbor, and personnel from the Duluth Seaway Port Authority and MPCA seeking their help obtaining new ballast water samples. Unfortunately, no new ballast water samples were obtained in 2018 but we are continuing to communicate with these groups. Some changes during the past month make us optimistic that we will be able to collect ballast water samples again in 2019. We will need additional time to use these water samples in experiments and for microbiological analyses outlined in our project work plan.

We designed a second bench-scale treatment experiment using authentic ballast water for Activity 2 and discussed this experimental design with personnel from the subcontractor (UWS-LSRI) who will conduct this experiment. Starting this experiment was delayed because we were unable to obtain a ballast water sample in 2018 (see Activity 2). DNA was extracted from experimental water samples for the first bench-scale water treatment experiment conducted in May 2018 using harbor water. DNA sequences for these samples were returned from the UM Biomedical Genomics Center in October 2018 and are being processed and evaluated in early 2019. *E. coli* and *Enterococcus faecium* cultures were grown, cellular DNA was extracted to obtain authentic DNA for standards, and appropriate qPCR methods were selected and primers obtained to quantify the abundances of these indicator bacteria in samples from bench-scale experiments mentioned above.

In Activity 3, DNA was extracted from the remaining 47 water samples collected from various sites in the St. Louis River Estuary (SLRE) during summer and fall 2017. So, Activity 3-Outcome 1 has now been completed. All remaining DNA from SLRE water samples will be submitted for sequencing in January 2019. We designed and conducted a short experiment to determine DNA losses during our DNA extraction protocol to improve qPCR

method results in Activities 2 and 3. The range of DNA recovered from water using our extraction protocol ranged from 33 to 41% + 1.3-2.1%, recoveries which are typical of most extraction protocols. Finally, we researched different methods to quantify the presence of DNA from a fish pathogen, *Piscirickettsia salmonis*, in ballast and harbor water samples and tested a qPCR method that will be applied to ballast and harbor water samples (see additional details in status of Activity 3 below).

Project Status as of July 31, 2019: After waiting a month for their response, the Lake Carriers Association finally informed us in early April they would not help us obtain ballast water samples we needed for Activities 1 and 2 ("At this time we do not feel that we are able to facilitate another shipboard project for our members"). Our subcontractor, Dr. TenEyk, also contacted the Maritime Administration to seek their help. He reached out to other ship owners at meetings in spring 2019. Similarly, responses to his inquiries were slow and non-committal or went unanswered. We decided to proceed to complete Activity 1 with ballast water samples we have collected previously.

E. coli qPCR analyses were completed for DNA samples from the May 2018 bench-scale water treatment experiment to determine the efficacy of UV light and chlorine treatment to remove this indicator bacterium. Initial qPCR results showed that greater than 99% of *E. coli* were removed from unspiked and spiked samples in the UV and chlorine treatments. The only except was *E. coli* removal in the spiked UV treatment, which was much lower (68% removal). These results are similar to previous results for the removal of culturable *E. coli* cells in this experiment. While only 20% of intact prokaryotic cells were removed by the UV treatment and 31% by the chlorine treatment, culturable heterotrophic bacteria, total coliforms, culturable *E. coli* and *Enterococcus* spp. were reduced by greater than 99% in both the UV and chlorine treatments compared to the no-treatment control. An experimental design for the final bench-scale water treatment experiment was designed and revised in spring 2019. It became clear by June 2019 that we would be unlikely to obtain a ballast water sample for this experiment. So, we met with the University of Wisconsin-Superior subcontractor in mid-July 2019 to finalize the design of a bench-scale water treatment experiment using an alternative water sample - water from a dock area in the Duluth-Superior harbor where commercial ships actively discharge ballast water. Plans were made to collect this water by boat and conduct the experiment on August 7-8, 2019.

DNA sequences from the preliminary sediment samples submitted previously for partial 16S rRNA gene sequencing indicated that further DNA purification was not necessary to obtain good sequence data. DNA from all the sediment samples collected in the Saint Louis River Estuary (SLRE) and DNA from some water samples needing to be re-sequenced were submitted to the UM Genomics by Jennifer Knack for 16S rRNA V4 region sequencing by May 20th, 2019. 198 of 279 total water and sediment samples (~71%) have been sequenced. We are currently waiting for the last DNA sequence results to finish all further analyses. A hierarchical clustering analysis on the bacterial community DNA sequences from water that had already been received (Fall 2016 and Spring 2017 samples only) indicated that three previously established estuary zones (lower, middle, and upper) may not capture all the differences between bacterial communities in SLRE water during all seasons. In Fall 2016, the lower estuary zone was dominated by a single bacterial community type (which did not include Allouez Bay or the Nemadji River), but above this region two distinct bacterial communities were detected in both the middle and upper estuary areas. In Spring 2017 like Fall 2016, the lower estuary zone was also dominated by a single bacterial community type (which again did not include Allouez Bay) but there were different bacterial communities in the middle and upper estuary areas (but different from communities found in Pokegama and Kimballs Bay).

A qPCR assay targeting the ITS spacer region of ribosomal RNA in *Piscirickettisa salmonis* was optimized to measure this bacterium's abundance in environmental samples. *P. salmonis* is a bacterium that causes the disease "muskie pox" in muskellunge fish. DNA from several ballast water samples collected in 2011 and 2012 indicated the presence *P. salmonis* DNA using this assay. PCR products from these samples are being prepared for more detailed Sanger sequencing to help verify that the DNA amplified was indeed from *P. salmonis* before completing qPCR analyses for the remaining ballast water and additional harbor water samples. Additional water and sediment samples were collected in July 2019 from 50 sites in the SLRE to compare the abundance of *P. salmonis* DNA at locations in the SLRE heavily travelled by muskellunge and commercial docks where ship ballast water is commonly discharged. Water samples were filtered in triplicate within 48 hours and were stored

frozen at -80°C along with aliquots of sediment samples. DNA extractions of the sediment samples were started. In addition, 18 separate water samples were collected throughout the estuary in mid-July for cation analysis to determine the influence of the Lake Superior on the harbor bacterial communities. These samples were preserved in 0.5% nitric acid at 4°C until cation analysis can be performed. We also received a dozen fin tissue clips that had been collected in 2017-18 from muskellunge in the SLRE. DNA extracted from these fin tissue clips is now in our que for *P. salmonis* qPCR analysis. We also obtained the carcass of an adult muskellunge from the MN-DNR Fisheries Research Unit (French River). Tissues from this single fish will be dissected and *P. salmonis* qPCR assays are then planned for DNA from these tissues.

Project Status as of January 31, 2020: In early August of 2019, a bench-scale experiment was conducted with the University of Wisconsin-Superior's Lake Superior Research Institute (LSRI) to determine the efficacy of ballast treatment techniques (UV light and chlorination) on bacterial communities (Activity 2). Similar to the initial benchtop experiment in spring of 2018, half of the samples were spiked with cultures of *E. coli* and *Enterococcus faecium* to ensure the presence of indicator bacteria. UV and chlorine treatments were applied resulting in five replicates for all six treatment categories (control-no treatment, UV, and chlorine; with and without indicator bacteria spiking). Culturable *E. coli*, *Enterococcus* spp, total coliforms and total heterotrophic bacteria abundances were measured in water from all six treatments. The water remaining from the replicates of each treatment was split for collecting microbial cells for DNA extraction and another portion was preserved for microscopic direct counts of prokaryotic cell abundances, which were completed in fall 2019.

While results from a one-way ANOVA confirmed that total prokaryotic cell abundance did not show a significant difference in the number of intact cells between treatment categories ($p=0.339$), plate counts revealed greater than a 99% reduction in all culturable indicator bacteria (*E. coli*, *Enterococcus*, total coliforms and heterotrophic bacteria) in the UV and chlorine treated samples. From qPCR analysis, the percent reductions of *E. coli* in unspiked samples was 67.5% and 96.8% for the UV and chlorine treatments, respectively, and 91.8% and 99.9% in spiked samples (Fig. 2). Analysis of variance and Tukey's comparison of means (Tukey's HSD) tests were performed on culturable bacterial and the *E. coli* qPCR results and confirmed the significance of the bacterial removal for both treatment techniques ($p<0.00001$). The small differences in the results between each method of quantification were anticipated and probably reflected differences between what culturing versus molecular biology methods measure. Other portions of the DNA extracted from each sample from the experimental treatments were submitted to University of Minnesota Biomedical Genomics Center for 16S ribosomal RNA gene next generation sequencing (NGS) in late December 2019 and the remaining portions were reserved for additional qPCR analyses.

Additional water and sediments were collected from commercial dock areas in the Duluth-Superior harbor in early August 2019 (Activity 3). DNA was extracted from all the 79 water samples and 78 sediment samples taken from the St. Louis River Estuary during the summer of 2019. Conventional PCR was used to determine the quality of the extracted DNA from water and sediment samples. The DNA from water samples amplified well using bacterial primers, so these samples were sent to the University of Minnesota Biomedical Genomics Center for next generation DNA sequencing in late December 2019. We are still awaiting these DNA sequence results. The sediment samples did not amplify well, so those DNA samples are currently being cleaned to prepare them for DNA sequence analysis. DNA from fish tissue samples collected in 2017-18 were also extracted and will be sent for bacterial DNA sequence analysis with the remaining DNA from sediment samples. The ongoing *P. salmonis* qPCR analysis will allow us to predict *Piscirickettsia salmonis* presence at sites in the St. Louis River estuary. After qPCR analysis, DNA samples that indicate the presence of *P. salmonis* will be more fully sequenced to confirm that the amplified target sequence is from this microorganism of interest.

DNA from the remaining 81 water and sediment samples collected in summer and fall 2017 was submitted for bacterial DNA sequencing in May 2019 and raw sequence data was received in late August 2019 (Activity 3). During the fall, the initial processing of hundreds of thousand DNA sequences was completed including trimming primer sequences, quality control, removal of poor-quality sequences, alignment of sequences, and then classifying the bacterial origin of individual sequences from each sample. These processed sequences are being prepared for hierarchical clustering analysis to finish geographic and seasonal comparisons of bacterial

community composition of the water and sediments in the SLRE, including an evaluation of bacterial genera that contain pathogens for comparison with similar information about bacterial communities from ship ballast water.

Project extended to June 30, 2021 by LCCMR 6/18/20 as a result of M.L. 2020, First Special Session, Chp. 4, Sec. 2, legislative extension criteria being met.

Project Status as of July 31, 2020: [NOTE: The Covid-19 pandemic caused research laboratories at the University of Minnesota Duluth to be closed from early March to early June 2020. Even so, we made good progress analyzing DNA sequence data that was returned and quickly made good progress on lab analyses once research labs were reopened in June.]

In early August of 2019, a bench-scale experiment was conducted with the University of Wisconsin-Superior's Lake Superior Research Institute (LSRI) to determine the efficacy of ballast treatment techniques (UV light and chlorination) on bacterial communities. Quantitative PCR results indicated fecal indicator bacteria removal ranged from 67% to 100% (but typically greater than 92% for treatments spiked with these indicator bacteria) following chlorination or UV treatments of water. Chlorination appeared to be a slightly more effective treatment method than UV light for removing these fecal indicator bacteria from St. Louis River Estuary water. Cultured plate counts of the same samples revealed greater than a 99% reduction in all culturable indicator bacteria (*E. coli*, *Enterococcus*, total coliforms and heterotrophic bacteria) in the UV and chlorine treated samples. The small differences in the results between each method of quantification were anticipated and probably reflect differences between what culturing versus molecular biology methods measure.

Pathogen-containing bacteria genera identified in water samples after chlorine or UV treatments showed that DNA sequences from the *Flavobacterium* bacterial genus were extremely common in the control water sample and water samples after UV treatment (in water both spiked and unspiked with indicator bacteria). 16S rDNA sequences from the *Legionella* and *Mycobacterium* bacterial genera were responsible for relatively larger portions of bacterial sequences in the harbor water after the chlorine treatment. This makes sense as both of these genera have been shown to be particularly resistant to chlorine disinfection. Water samples treated with chlorine also had a larger proportion of bacteria DNA sequences from the Planctomycetes phylum, which contain many species that are found in marine and brackish environments. Bacterial community diversity, as measured by OTU richness, was greatest in the control samples, followed by UV-treated samples, and then chlorinated samples.

Ms. Knack finished a preliminary comparison of the relative abundances of pathogen-containing bacterial genera in St. Louis River Estuary (SLRE) water and sediment samples collected in 2016-17 to the same bacterial genera detected in commercial ship ballast water from 2011-12. Eleven pathogen-containing general, including nine genera that contain human pathogens, were found to be statistically higher in relative abundance in ballast water than in the estuary water or sediment. Five pathogen-containing genera, four of which include human pathogens, were detected in ballast water but not in the estuary water or sediment. Two bacterial genera (i.e., *Escherichia. Enterococcus)* that contain bacterial species (i.e., *E. coli*, *Enterococcus* spp.) used as indicators of water quality were present in ballast water, but their relative abundances were not statistically different from those in estuary water and sediment.

Ms. Zimmer prepared all 79 surface water samples and 78 sediment samples collected throughout the St. Louis River Estuary during summer 2019 for bacterial DNA analysis by performing DNA extractions on each sample. DNA from water samples was sent to the UM Genomics Center for sequencing in late December 2019 and raw sequence data results were returned in February 2020. DNA from sediment samples were sent in March 2020 and raw sequence data was returned in May 2020. These partial 16S rDNA sequences are currently being analyzed to compare the bacterial community diversity in water and sediments from throughout the St. Louis River Estuary and determine if different bacterial communities are found in the part of the estuary with active shipping docks or regions frequented by muskellunge fish compared to other regions of the St. Louis River Estuary. An analysis of pathogen-containing bacterial genera is also being performed for these water and sediment samples to compare with Ms. Knacks results for water and sediment samples collected in 2016-17. Ms. Zimmer also started quantitative PCR (qPCR) analyses of an ITS spacer region in the ribosomal RNA gene to quantify the relative abundance of the fish pathogen, *Piscirickettsia salmonis,* in DNA extracted from the 79

surface water samples and 78 sediment samples. The results of this analysis should help not only determine if this pathogenic bacterium responsible for "Muskie Pox" disease is present in the SLRE, but also where it is most commonly in the estuary waters and sediments.

Finally, a poster presentation of project research results was made at a regional conference on the St. Louis River Estuary in early March 2020. Six abstracts describing project results were submitted to give presentations at three different scientific conferences in 2020. Unfortunately, two of these conferences were cancelled due to the COVID-19 pandemic, but two presentations will be made at an online conference now scheduled for early November 2020.

Project Status as of January 31, 2021: Activity 1 -Ms. Zimmer complete quantitative PCR analyses targeting the ITS region of *P. salmonis* DNA in ballast water samples collected in 2011-2012. No detectable amounts of *P. salmonis* DNA were found in any of the ballast water samples using this *P. salmonis* specific assay targeting the ITS region of this species' DNA.

Activity 2 - In early August of 2019, a bench-scale experiment was conducted with the University of Wisconsin-Superior's Lake Superior Research Institute (LSRI) to determine the efficacy of ballast treatment techniques (UV light and chlorination) on bacterial communities. Ms. DeGuire continued analysis of the DNA sequence data to determine the proportions of the pathogen containing genera (PCG) in the bacterial communities of each ballast water treatment category of this bench-scale experiment. As with the overall bacterial communities, the relative abundance of the pathogen containing genera for the untreated and UV-treated samples was very similar. About 1% of the bacterial communities of unspiked (with indicator bacteria) and not treated, and unspiked UV-treated samples consisted of DNA sequences from PCGs. About 8% of the total sequences in spiked non-treated and UV-treated samples consisted of PCGs, and PCGs accounted for 5-6% of all sequences in samples treated with chlorine (both spiked and unspiked).

Activity 3 - Ms. Zimmer completed quantitative PCR analyses targeting the ITS region of *P. salmonis* DNA for water and sediments collected in the St. Louis River Estuary in 2019. *P. salmonis* DNA was detected in low quantities in water collected at many sites in the St. Louis River Estuary. The levels detected were so low that Ms. Zimmer has started to sequence these PCR products to confirm that the DNA was truly amplified from *P. salmonis* cells. These qPCR results are at odds with results from Illumina sequencing of DNA in water at the various sites, which did not detect the presence of DNA from the *Piscirickettsia* genus. This difference, if true, suggests that that if *P. salmonis* DNA is present in the estuary, it may be at concentrations too low to detect using Illumina DNA sequencing techniques. No *P. salmonis* DNA was present in detectable quantities using the qPCR assay in any sediment samples taken at the various St. Louis River estuary sites.

Sparse principle components analysis (sPCA) of the partial 16S rDNA sequences revealed clear differences between bacterial communities in the surface water at the commercial docks and muskellunge habitats or the rest of the St. Louis River estuary. When the proportions of pathogen-containing genera (PCGs) were considered alone, these taxa were responsible for about 3% of the total bacterial sequences in water within each site category. The commercial dock PCGs were dominated by DNA sequences from *Flavobacterium* genus, a genus commonly associated with ballast water discharge. *Escherichia-Shigella* and *Bacteroides,* genera commonly associated with gut microbiomes, were in the greatest relative abundance within the muskellunge habitats. Low abundances (>0.05%) of *Legionella* DNA sequences were found in water throughout the entire estuary. This genus contains several bacterial species responsible for Legionnaire's disease. *Legionella* species can be resistant to ballast treatment methods because their tendency to produce biofilms that can be protective against various chemical and physical disturbances. No *Piscirickettsia spp.* 16S rDNA sequences were detected in water at any estuary sites. Based on these findings, further quantitative analysis of *Legionella* and *Flavobacterium* species found in water may be warranted to determine which species are present and the sources and risks of potential pathogen strains.

Using SourceTracker analysis, Ms. Zimmer estimated the influence of different docks on the composition of bacterial communities at several locations in the St. Louis River estuary. The surface waters at Hallett Dock 5 and the CN dock appeared to have greater influences on the overall composition of bacterial communities in water at several sites in the estuary than water from other docks. When only sequences from PCGs were consider, PCGs from different docks contributed more equally to the composition of PCGs communities in water. This

finding may be an outcome of ballast water discharged at these docks. [A similar source analysis is being conducted for bacterial communities found in sediments but it is not yet completed.]

The results of sparse principle components analysis (sPCA) for sediment bacterial communities were not as revealing as the similar analyses of bacterial communities in surface waters at the commercial docks, muskellunge habitats, and the rest of the estuary. The total compositions of sediment bacterial communities at muskellunge habitats and other SLRE sites were indistinguishable from each other, as found for surface water bacterial communities. Unlike bacterial communities found in water, the compositions of total bacterial communities in sediment at commercial docks overlapped with the compositions of bacterial communities at muskellunge habitat sites or at sites randomly selected in other regions of the St. Louis River estuary.

When the proportions of pathogen-containing genera (PCGs) were considered alone, these taxa stood out more in the sediment at commercial docks compared to muskellunge habitats or other sites in the SLRE. DNA sequences from PCGs were responsible for more than 15% of the composition of the sediment total bacterial communities at commercial docks examined, while PCG sequences were less than 2.5% of the sequences found in sediment communities at muskellunge habitats and other SLRE sites. DNA sequences from only the *Flavobacterium* genus accounted for greater than 0.05% of all bacterial sequences found in sediment at sites in each category (docks, muskellunge habitat, other SLRE sites). DNA sequences from the *Streptococcus* genus were the most common PCGs bacterial sequences found in sediment at the commercial docks. The *Streptococcus* genus contains many species, including pathogenic strains known to infect not only humans but also fish and wildlife. Further investigation of *Streptococcus* species in sediment at commercial docks is probably warranted to determine the sources and risk of bacterial strains in this genus.

Overall Project Outcomes and Results:

While culture-based methods to detect indicator bacteria reduce the cost and complexity to monitor ballast and harbor waters, caution should be used when monitoring based on these indicators alone because their fates are not necessarily representative of bacterial cells in some pathogen-containing genera. Both UV-treatment and chlorination resulted in >99% removal of culturable indicator bacteria, however, each indicator responded differently with no regrowth of *Enterococcus*, moderate regrowth of *E. coli* for chlorine treated samples, and major regrowth of total bacteria after treatment. There were shifts in overall bacterial community composition after treatment including regrowth of cells from genera that harbor pathogens (particularly *Acinetobacter, Flavobacterium,* and *Pseudomonas*). Initially, *P. salmonis* DNA appeared to be present in the surface water of the St. Louis River estuary, but this result proved to be incorrect. This finding was confirmed by sequencing bacterial DNA at various sites in 2017 and 2019, which did not detect the presence of *Piscirickettsia* DNA. Bacterial communities and the pathogen-containing bacterial genera (PCGs) subset in water and sediments at four commercial docks in the Duluth-Superior harbor were different from other sites in the St. Louis River estuary. Higher relative abundances of PCGs were found in commercial dock sediments compared to the rest of the estuary. While there were only minor differences in the relative abundance of PCGs in surface water throughout the estuary, DNA from the *Flavobacterium* genus was more abundant at docks than other areas. Discharge of ballast water may affect the prevalence of PCGs in the Duluth-Superior harbor. Treatment of ballast water prior to discharge may reduce any human and wildlife pathogen load. In addition, care should be taken when dredging dock areas because disturbing sediment may temporarily increase the chances of exposing recreational users to pathogenic bacterial strains.

IV. PROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1: Collect Ballast Water from Commercial Ships
Description:
Ballast water will be sampled from at least five commercial freshwater and ocean-going ships to identify potentially harmful microbes and test ballast water treatment technologies (see Activity 2). We will work closely with the Minnesota Pollution Control Agency and the Wisconsin Department of Natural Resources to collect ballast water from commercial ships arriving in the Duluth-Superior Harbor, and with the NEMWI and UWS-LSRI

to collect ballast samples from other ships. DNA will be extracted from ballast water samples and bacterial community DNA sequenced to expand a database of bacterial diversity and potentially harmful microbes in commercial ship ballast water discharged in Minnesota.

Summary Budget Information for Activity 1:

ENRTF Budget: $ 103,983
Amount Spent: $ 78,451
Balance: $ 25,532

Outcome	Completion Date
1. Collect and expand repository of microbial DNA from commercial ship ballast water	November 2017
2. Expand a database of microbial diversity and bacterial pathogens in a variety of ship ballast waters	November 2018

Activity 1 Status as of January 31, 2017: A graduate student, Jennifer Knack, was recruited to work on the project and started work in August 2016. Mr. Michael Goettel of the Wisconsin DNR provided advice regarding how to gain access to commercial ships. The graduate student acquired a Transportation Worker Identification Credential (TWIC) for access to the international docks in Duluth-Superior Harbor. She and another graduate student sampled ballast water from one commercial ship docked in Superior, WI on August 2, 2016 with Mr. Goettel. Replicate samples of the ballast water were filtered, and the filters were stored at -80°C for future DNA extraction and sequencing. Representatives from the Minnesota Sea Grant Program and the Duluth Seaway Port Authority were contacted to help us gain access to additional commercial ships to sample. We are currently developing a letter to present to ship captains or owners in 2017 that describes our project and asks for their consent and assistance to sample ballast water from their ships.

Two DNA extraction protocols were tested in fall 2016 using 28 ship ballast water samples collected from 2011 to 2012 to determine which extraction method yields the best DNA sequence data. The V4 region of the 16S rRNA gene in the DNA from these samples was sequenced on an Illumina DNA sequencer at the University of Minnesota Biomedical Genomics Center. Sequence results were returned in early January 2017 and analysis of the DNA sequence data has just begun. Once analysis of these DNA data sets is completed, we will choose a DNA extraction protocol for extracting the ship ballast water, water, and sediment samples collected from the St. Louis River Estuary (See Activity 3 below) for remainder of this project.

Activity 1 Status as of July 31, 2017: Dr. Matthw TenEyck (UWS-LSRI) offered to supply filtered ballast samples from ships under GSI monitoring. DNA sequence data from ballast water samples collected in 2011-2012 and extracted using two protocols were analyzed to identify the best DNA extraction procedure to use for ballast, water, and sediment samples collected during the project. Analysis of the 16S sequences from these samples showed that the Levar-Sheik DNA extraction protocol provided superior DNA sequences compared to extracting microbial DNA with a MoBio PowerSoil kit. One of the bacterial groups identified was a *Tenacibaculum* species that may be a possible fish pathogen, and which was present in only one of these ballast water samples. Dr. Hicks is working on an explanatory letter to provide ship captains when we request to board their ships in the future to sample ballast water.

Activity 1 Status as of January 31, 2018: Jennifer Knack, the graduate student on this project, continued analysis of the partial 16S sequences generated from the 2011-2012 ballast samples. While there was no clear bacterial community composition signature due to the origin these ballast water samples, bacterial community structure was strongly correlated with temperature of the ballast water when it was harvested. We intend to collect additional ballast water samples this year, especially during the spring because all the 2011-2012 ballast samples were obtained during late summer and early fall, when water temperatures are warmest.

Activity 1 Status as of July 31, 2018: Starting in spring 2018, we continued to seek sources of ballast water working with Michael Goettel of the Wisconsin DNR and Dr. Matthew TenEyk from the University of Wisconsin-Superior. So far, neither of these contacts have been able to help us acquire ballast water samples in 2017 or

2018. We finished an explanatory letter and our written ballast water sampling protocol for ships owners and to provide ship captains when we board their ships to sample ballast water. In April 2018, we started directly contacting owners of various shipping companies in the Great Lakes fleet ("lakers") and agents for ocean-going ships arriving in the Duluth-Superior Harbor. Most of these owners did not responded to our initial requests, so we are continuing to seek their approval to sample ballast water from commercial ships in their fleet when they visit the Duluth-Superior Harbor.

Jennifer Knack finished the pathogen analysis of partial 16S rDNA sequences from 2011-2012 ballast samples (see attached Table 1). It is important to note that this analysis is only able to detect bacterial genera that contain pathogenic bacterial species or strains but it does not have the resolution to distinguish between pathogenic and non-pathogenic species/strains within each genus. Therefore, we use the term "pathogen-containing genera" (PCG) to refer to bacterial genera that contain some species or strains that are potentially harmful to fish and wildlife, or humans. We found the composition of PCG communities was not significantly different in freshwater ballast water from "laker" and "salty"-type ships. Similarly, the relative abundances of PCG were not significantly different in "laker" and "salty"-type ships. The origin of the ballast water was the best predictor of the composition and relative abundance of PCG.

Between 0.47% and 8.05% of all DNA sequences in 21 ballast water samples collected in 2011-12 were from PCGs. Similarly, between 0.63% and 9.03% of all DNA sequences from four harbor water samples (either the Duluth-Superior Harbor or Silver Bay Harbor) were classified to PCG. Of the 70 genera containing potential fish and wildlife or human pathogens that we screened, DNA sequences from only six PCG were present in all ballast water samples we analyzed; *Acintobacter, Aeromonas, Flavobacteria, Legionella, Mycobacterium,* and *Pseudomonas* (see attached table). The genera *Aeromonas, Flavobacteria* and *Pseudomonas* contain known fish pathogens while some species in the genera *Legionella* and *Mycobacterium* are human pathogens. No DNA sequences were detected from the PCG *Renibacterium* (*R. salmoninarum* causes Bacterial Kidney Disease in fish) or *Piscirickettsia* (*P. salmonis* strains cause piscirickettsiosis in trout and salmonid fish or Muskie Pox disease in muskellunge) in any of the 21 ballast water samples. DNA sequences from the bacterial genus *Tenacibaculum* were found in 24% of the ballast water samples but at very low levels (<0.05% of total sequences) and only in "salty" ships containing freshwater ballast water. *T. maritimum* causes an ulcerative disease known as tenacibaculosis, which affects a large number of marine fish species in the world but it is a marine bacterium that would not likely survive in freshwater habitats. Interestingly, no DNA sequences from indicator bacterial genera *Escherichia* and *Enterococcus* were detected in any of the 21 ballast water samples. DNA sequences from the indicator genus *Vibrio* were detected in 24% of the ballast water samples examined but only at low frequency (<0.01% of total sequences). These findings suggest that these indicator bacterial taxa, which are recommended in the International Maritime Organization's guidelines to evaluate and monitor the microbial safety of ballast water, may not be good indicators for the presence of many genera containing pathogenic bacterial strains that affect fish and wildlife. DNA sequence data from these ballast water samples are being further evaluated and will later be related to bacterial DNA sequence data obtained for the St. Louis River estuary water and sediment samples.

Activity 1 Status as of January 31, 2019: In spring 2018, we continued to seek sources of ballast water working with Michael Goettel of the Wisconsin DNR but were unable to take samples with him without the permission of ship owners. Since then, we and our subcontractor have been diligently communicating with ship owners and shipping associations to obtain their permission to take ballast water samples from their ships. From April to December 2018, the Project Manager and Dr. Matthew TenEyk from the University of Wisconsin-Superior LSRI made multiple contacts with owners of various shipping companies in the Great Lakes fleet ("lakers"), agents for ocean-going ships arriving in the Duluth-Superior Harbor, and personnel from the Duluth Seaway Port Authority and MPCA seeking their help. Obtaining permission to sample ballast water was delayed - most shipping companies did not respond to our initial inquiries. Our subsequent contacts with the Lake Carriers Association revealed that these owners were waiting for two reasons – a request from the Minnesota MPCA for them to respond to a ballast water inspection and management plan and the outcome of pending federal legislation (VIDA bill - Vessel Incidental Discharge Act) that clearly defined whether states or federal agencies would implement and monitor compliance with ballast water regulations in the Great Lakes. The VIDA act was passed

and signed by the President in mid-December 2018, so we are optimistic that these shipping companies will now assist us in obtaining ballast water in early to mid-2019 after shipping resumes on the Great Lakes in March 2019. We will need additional time to use these water samples in experiments and for microbiological analyses outlined in our project work plan.

We researched different methods to quantify the presence of DNA from a fish pathogen, *Piscirickettsia salmonis*, in ballast and harbor water samples. Graduate student J. Zimmer is developing a qPCR method that will be applied to existing and new ballast water samples (see additional details in status of Activity 3 below).

Activity 1 Status as of July 31, 2019: In early February, Dr. Hicks visited Jeff Stollenwerk at the Duluth Seaway Port Authority to update him about this project, explain our need for new ballast water samples, and asked for his positive reference about our project if contacted by ship owners. We followed up by contacting the Lake Carriers Association again in late February 2019 to ask for their assistance in obtaining permission to sample ballast water from their member's ships in 2019. After waiting a month for their response, the Lake Carriers Association finally informed us in early April they would not help us obtain ballast water samples that ("At this time we do not feel that we are able to facilitate another shipboard project for our members"). Dr. TenEyk contacted the Maritime Administration to seek their help. He also reached out to other ship owners at meetings in spring 2019. Similarly, responses to his inquiries were slow and non-committal or went unanswered. We also reached out again to Mike Goettel at the Wisconsin DNR and learned that he was conducting few if any ship inspections now that the federal VIDA act passed in late 2019. He indicated similar frustration with the Great Lakes shipping industry this spring - his inquires to inspect ships sometimes went unanswered or responses were slow. We decided to proceed to complete this Activity with ballast water samples we have collected previously. Ms. Knack completed additional analyses of the ballast water DNA sequences that were necessary to create figures and tables in spring 2019 and we started drafting a manuscript about ballast water bacteria and potential pathogens in May 2019.

Activity 1 Status as of January 31, 2020: Ms. Knack and Dr. Hicks continued to revise the text, figures, and tables for a manuscript about ballast water bacteria and potential pathogens from the project results in summer and early fall 2020. The manuscript is still in a draft stage.

Activity 1 Status as of July 31, 2020: Ms. Knack completed the analysis of bacteria communities and pathogen-containing genera for the ballast water samples and finished a preliminary analysis comparing them to the types of pathogen-containing genera found in water and sediment from the St. Louis River Estuary (see Activity 3 below for these results).

Activity 1 Status as of January 31, 2021: Ms. Zimmer complete quantitative PCR analyses targeting the ITS region of *P. salmonis* DNA in ballast water samples collected in 2011-2012. No detectable amounts of *P. salmonis* DNA were found in any of the ballast water samples using this *P. salmonis* specific assay targeting the ITS region of this species' DNA. Bacterial DNA community analysis of surface water and sediment taken at four docks in the Duluth-Superior Harbor was performed and compared to sites throughout of the St. Louis River estuary. A subset of these sequences, containing only pathogen-containing bacterial genera, was also used to predict the influence of ballast water discharge on pathogen communities in waters of the estuary (see Activity 3 below for these results)

Final Report Summary: Only one ballast water sample from a single commercial ship was collected in fall 2016. We were unable to obtain any additional ballast water samples from commercial ships despite repeated contacts and attempts during the 2017, 2018, and 2019 shipping seasons. We decided to proceed to complete this Activity with ballast water samples we had collected previously in 2011-12.

Jennifer Knack finished the pathogen analysis of partial 16S rDNA sequences from 2011-2012 ballast samples. It is important to note that this analysis is only able to detect bacterial genera that contain pathogenic bacterial species or strains but it does not have the resolution to distinguish between pathogenic and non-pathogenic species/strains within each genus. Therefore, we use the term "pathogen-containing genera" (PCG)

to refer to bacterial genera that contain some species or strains that are potentially harmful to fish and wildlife, or humans. We found the composition of PCG communities was not significantly different in freshwater ballast water from "laker" and "salty"-type ships. Similarly, the relative abundances of PCG were not significantly different in "laker" and "salty"-type ships. The origin of the ballast water was the best predictor of the composition and relative abundance of PCG.

Between 0.47% and 8.05% of all DNA sequences in 21 ballast water samples collected in 2011-12 were from PCGs. Similarly, between 0.63% and 9.03% of all DNA sequences from four harbor water samples (either the Duluth-Superior Harbor or Silver Bay Harbor) were classified to PCG. Of the 70 genera containing potential fish and wildlife or human pathogens that we screened, DNA sequences from only six PCG were present in all ballast water samples we analyzed; *Acintobacter, Aeromonas, Flavobacteria, Legionella, Mycobacterium,* and *Pseudomonas*. The genera *Aeromonas, Flavobacteria* and *Pseudomonas* contain known fish pathogens while some species in the genera *Legionella* and *Mycobacterium* are human pathogens. No DNA sequences were detected from the PCG *Renibacterium* (*R. salmoninarum* causes Bacterial Kidney Disease in fish) or *Piscirickettsia* (*P. salmonis* strains cause piscirickettsiosis in trout and salmonid fish or Muskie Pox disease in muskellunge) in any of the 21 ballast water samples. DNA sequences from the bacterial genus *Tenacibaculum* were found in 24% of the ballast water samples but at very low levels (<0.05% of total sequences) and only in "salty" ships containing freshwater ballast water. *T. maritimum* causes an ulcerative disease known as tenacibaculosis, which affects a large number of marine fish species in the world but it is a marine bacterium that would not likely survive in freshwater habitats. Interestingly, no DNA sequences from indicator bacterial genera *Escherichia* and *Enterococcus* were detected in any of the 21 ballast water samples. DNA sequences from the indicator genus *Vibrio* were detected in 24% of the ballast water samples examined but only at low frequency (<0.01% of total sequences). These findings suggest that these indicator bacterial taxa, which are recommended in the International Maritime Organization's guidelines to evaluate and monitor the microbial safety of ballast water, may not be good indicators for the presence of many genera containing pathogenic bacterial strains that affect fish and wildlife.

Ms. Knack completed the analysis of bacteria communities and pathogen-containing genera for the ballast water samples and finished a preliminary analysis comparing them to the types of pathogen-containing genera found in water and sediment from the St. Louis River Estuary (see "Activity 3 Status as of July 31, 2020" below for these results).

ACTIVITY 2: Comparison of Standard Versus Non-Standard Approaches for Assessing Current Ballast Water Treatment Technology Effectiveness for Neutralizing Potentially Harmful Bacteria

Description:

Our goal is to determine if neutralizing potentially harmful bacteria in ballast water can be most effectively assessed using standard microbial assessment tools for fecal indicator bacteria (standard approach), or genetic detection alternative methods. This objective will test whether BWMS induced changes in microbial composition of ballast water could be more readily and cheaply indicated by monitoring fecal indicator organisms, or genetic signals for potential microbial invaders. These bench-scale tests will be conducted on two ballast water samples at the Lake Superior Research Institute (LSRI) at the University of Wisconsin-Superior in Superior, WI. In addition to UV radiation, other conventional and treatment methods in development are also being considered for evaluation. LSRI will conduct standard fecal indicator bacteria analyses while the University of Minnesota will complete genetic detection analyses. The results will be applicability to in-line, in-tank, and on-shore treatment systems.

Summary Budget Information for Activity 2:	**ENRTF Budget:**	**$ 124,276**
	Amount Spent:	**$ 112,592**
	Balance:	**$ 11,684**

Outcome	Completion Date
1. Conduct bench-scale experiments to evaluate indicator and potentially harmful bacteria removal using common ballast water treatment technologies	November 2017

2. Correlate removal and inactivation of culturable bacterial indicators with DNA-based molecular methods to detect removal of bacterial indicators and potential pathogens	February 2018

Activity 2 Status as of January 31, 2017: A subcontract to the Northeast-Midwest Institute (NEMWI) from the University of Minnesota was developed ~~and accepted~~ to assist with parts of Activity 2. The Project Manager and graduate student met on July 6, 2016 with Ms. Allegra Cangelosi from the NEMWI and Dr. Matthew TenEyck from the Lake Superior Research Institute at the University of Wisconsin-Superior to discuss the project and make preliminary plans for bench-scale experiments and analyses they will conduct. We decided to start these bench scale experiments in 2017 because of their busy schedule with other projects in 2016. We plan to meet with them again in spring 2017 to design the initiate bench scale experiments that will be conducted.

Activity 2 Status as of July 31, 2017: An undergraduate student, Alex Carlbom, was recruited to work primarily on this activity for UMD. Dr. Hicks, Dr. TenEyck, Jennifer Knack (graduate student), and Alex Carlbom met in early May to discuss the set-up and ballast treatment options for the bench-scale experiments to evaluate common ballast water treatment technologies. Subsequently, Mr. Carlbom drafted an experimental protocol to determine how much water would be required in each ballast treatment to recover enough DNA for sequencing in future experiments.

The NEMWI and the UWS-LSRI requested that a subcontract for services in Activities 1 and 2 be developed with the Lake Superior Research Institute at the University of Wisconsin-Superior (UWS-LSRI) instead of the Northeast-Midwest Institute (NEMWI), as originally planned. A new subcontract was drafted at the University of Minnesota and an amendment request for this subcontract change to the project work plan was written and submitted to the LCCMR.

Activity 2 Status as of January 31, 2018: A preliminary water treatment experiment, using UV and chlorine treatments, was conducted in September 2017 to test whether our DNA extraction protocol would generate sufficient high-quality DNA for all analyses. Samples from this experiment were extracted and the DNA cleaned and amplified by PCR in October to December 2017. We learned that only 0.5 to 1 liter of water needed to be filtered to obtain enough bacterial DNA from each replicate and that an additional clean-up step in the DNA extraction process was necessary to obtain high-quality DNA from chlorine-treated water, probably due to a high salt content in this treatment. We plan to design the main experiment for this activity with Dr. TenEyck at UW-Superior soon, and anticipate conducting this experiment in spring and summer 2018.

Activity 2 Status as of July 31, 2018: A bench-scale water treatment experiment was successfully conducted on May 14, 2018 to determine the efficacy of UV light and chlorine treatment to remove total bacteria, indicator bacteria, and pathogen-containing bacterial genera (PCG) from harbor water. One set of samples was spiked with *E. coli* and *Enterococcus faecium* to ensure accurate removal efficiency measurements could be made. Six replicates of each spiked and unspiked experimental treatment and control were constructed. After appropriate exposure times, these replicates were sampled for microscopic counts of prokaryotic cells, counts of culturable bacterial tests, and microbial DNA extraction. While only 20% of intact prokaryotic cells were removed by the UV treatment and 31% by the chlorine treatment, culturable heterotrophic bacteria, total coliforms, *E. coli*, and *Enterococcus* spp. were reduced by greater than 99% in both the UV and chlorine treatments compared to the no-treatment control. DNA from all replicates of each treatment was extracted and is being prepared for sequencing the V4 region of the 16S rDNA gene to identify bacterial taxa and estimate the removal of different pathogen-containing bacterial genera. A new graduate student, Lisa DeGuire, started work in mid-June 2018 and will take the lead for qPCR analyses of *E. coli* and *Enterococcus* spp. and analysis of DNA sequences to compare with culturable counts. She will also develop an experimental design to repeat the water treatment experiment using ballast water as a matrix instead of Duluth-Superior Harbor water.

Activity 2 Status as of January 31, 2019: DNA was extracted from experimental water samples for the bench-scale water treatment experiment conducted in May 2018 and then submitted for 16S rDNA sequencing in August 2018. DNA sequences for these samples were returned from the UM Biomedical Genomics Center in

October 2018, and J. Knack is instructing L. DeGuire on bioinformatics techniques to analyze and evaluate these sequences.

We designed a second bench-scale treatment experiment using authentic ballast water and discussed this experimental design for with personnel from the subcontractor (UWS-LSRI) who will conduct this experiment. Starting this experiment was delayed because we were unable to obtain a ballast water sample in 2018 (see update about this in Activity 1). This experiment will be conducted as soon as a suitable ballast water sample becomes available in 2019. If we obtain a one-year no cost extension but a ballast water sample has not been obtained by July 31, 2019, then our alternative plan is to repeat this bench-scale experiment during August 2019 using Duluth-Superior Harbor water collected near where commercial ships berth instead of an authentic ballast water to evaluate the efficacy of UV light and chlorine treatments to remove total bacteria, indicator bacteria, and pathogen-containing bacterial genera (PCG).

L. Deguire acquired *E. coli* and *Enterococcus faecium* cultures, subcultured these samples, and then extracted DNA from the cell suspensions to obtain authentic DNA \for molecular analyses targeting these indicator bacteria for this activity. She researched and then selected appropriate qPCR methods to quantitatively estimate the abundance of each of these indictor bacteria, had the appropriate PCR primers synthesized, and obtained qPCR supplies to develop standard curves for these qPCR methods. Analyses of water samples using these qPCR methods will be conducted in winter and spring 2019 for the first bench-scale experiment samples.

Activity 2 Status as of July 31, 2019: L. DeGuire optimized quantitative polymerase chain reaction (qPCR) assays to quantify the abundance of the target indicator bacteria, *E. coli and Enterococcus faecium*, during spring 2019. A detection limit of 100 *E. coli* gene copies/100 ml of water were possible with the *E. coli* qPCR assay. *E. coli* qPCR analyses were completed for DNA samples from the May 2018 bench-scale water treatment experiment to determine the efficacy of UV light and chlorine treatment to remove this indicator bacterium. Initial qPCR results showed that greater than 99% of *E. coli* were removed from unspiked and spiked samples in the UV and chlorine treatments. The only except was *E. coli* removal in the spiked UV treatment, which was much lower (68% removal). These results are similar to previous results for the removal of culturable *E. coli* cells in this experiment. While only 20% of intact prokaryotic cells were removed by the UV treatment and 31% by the chlorine treatment, culturable heterotrophic bacteria, total coliforms, culturable *E. coli* and *Enterococcus* spp. were reduced by greater than 99% in both the UV and chlorine treatments compared to the no-treatment control. J. Knack and L. DeGuire are continuing to analyze the bacterial community and indicator bacterial DNA sequences for bench-scale water treatment experiment conducted in May 2018.

An experimental design for the final bench-scale water treatment experiment was designed and revised in spring 2019. It became clear by June 2019 that we would be unlikely to obtain a ballast water sample for this experiment. So, we met with the University of Wisconsin-Superior subcontractor in mid-July 2019 to finalize the design of a bench-scale water treatment experiment using an alternative water sample - water from a dock area in the Duluth-Superior harbor where commercial ships actively discharge ballast water. Plans were made to collect this water by boat and conduct the experiment on August 7-8, 2019.

Activity 2 Status as of January 31, 2020: In early August of 2019, a bench-scale experiment was conducted with the University of Wisconsin-Superior's Lake Superior Research Institute (LSRI) to determine the efficacy of ballast treatment techniques (UV light and chlorination) on bacterial communities. On August 7th, L. DeGuire and J. Zimmer collected water samples alongside a recently docked "laker" ship in the Duluth-Superior harbor to serve as the experimental water. Supplemental water quality data was also collected (i.e., pH, dissolved oxygen, conductivity, temperature, and water turbidity). Similar to the initial benchtop experiment in spring of 2018, half of the samples were spiked with cultures of *E. coli* and *Enterococcus faecium* to ensure the presence of indicator bacteria. UV and chlorine treatments were applied resulting in five replicates for all six treatment categories (control-no treatment, UV, and chlorine; with and without indicator bacteria spiking). Culturable *E. coli*, *Enterococcus* spp, total coliforms and total heterotrophic bacteria abundances were measured in water from all six treatments (Fig. 1). The water remaining from the replicates of each treatment was split for collecting

microbial cells for DNA extraction and another portion was preserved for microscopic direct counts of prokaryotic cell abundances, which were completed in fall 2019.

While results from a one-way ANOVA confirmed that total prokaryotic cell abundance did not show a significant difference in the number of intact cells between treatment categories (p=0.339), plate counts revealed greater than a 99% reduction in all culturable indicator bacteria (*E. coli*, *Enterococcus*, total coliforms and heterotrophic bacteria) in the UV and chlorine treated samples (Fig. 1). From qPCR analysis, the percent reductions of *E. coli* in unspiked samples was 67.5% and 96.8% for the UV and chlorine treatments, respectively, and 91.8% and 99.9% in spiked samples (Fig. 2). Analysis of variance and Tukey's comparison of means (Tukey's HSD) tests were performed on culturable bacterial and the *E. coli* qPCR results and confirmed the significance of the bacterial removal for both treatment techniques (p<0.00001). The small differences in the results between each method of quantification were anticipated and probably reflected differences between what culturing versus molecular biology methods measure.

Other portions of the DNA extracted from each sample from the experimental treatments were submitted to University of Minnesota Biomedical Genomics Center for 16S ribosomal RNA gene next generation sequencing (NGS) in late December 2019 and the remaining portions were reserved for additional qPCR analyses. Bioinformatics processing pathways for the partial 16S rDNA sequences we anticipate receiving by March 2020 are currently being established.

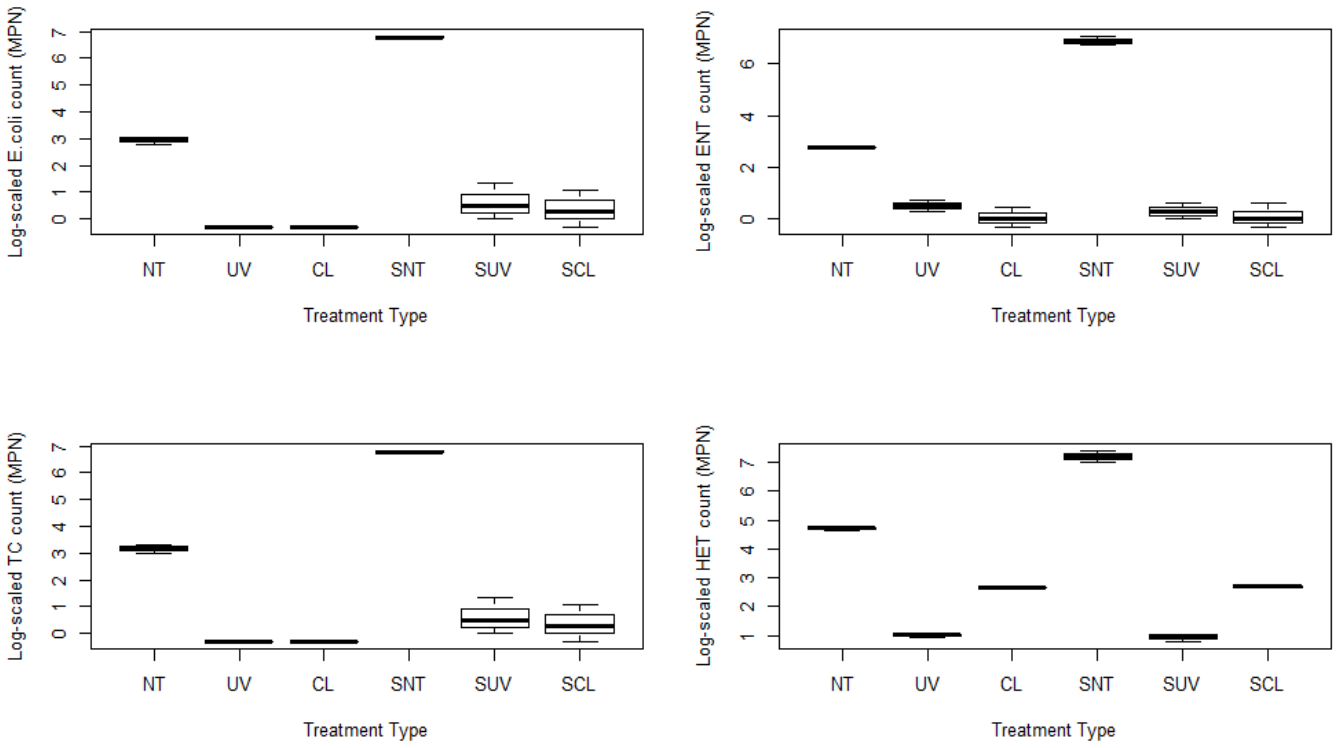


Figure 1. Boxplots of culturable bacteria counts (MPN) from the 2019 ballast experiment for target bacteria: *E. coli* (top left), *Enterococcus* spp (top right), total coliform (bottom left) and total heterotrophic bacteria (bottom left). Treatment categories included: no treatment (NT), UV light (UV), chlorine (CL), spiked no treatment (SNT), spiked-UV (SUV), and spiked-chlorine (SCL).

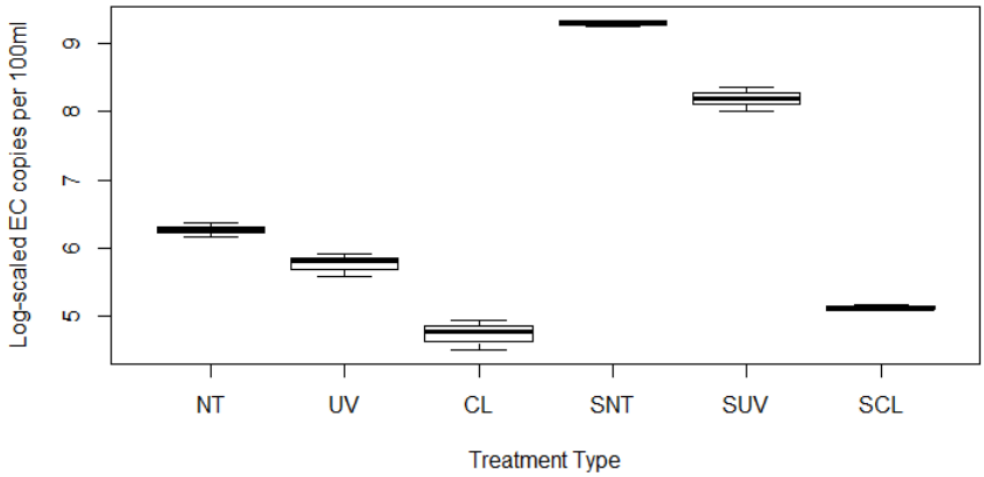


Figure 2. Log-scaled boxplot of *E. coli* qPCR results (gene copies per 100 ml) from the 2019 ballast experiment for target bacteria. Treatment categories

Activity 2 Status as of July 31, 2020: In early August of 2019, a bench-scale experiment was conducted with the University of Wisconsin-Superior's Lake Superior Research Institute (LSRI) to determine the efficacy of ballast treatment techniques (UV light and chlorination) on bacterial communities. In June 2020, when research labs at UMD were reopened after the COVID-19 shutdown, qPCR analysis for *Enterococcus* spp. (fecal indicator bacteria) and 16S rRNA gene (total bacteria) were completed (Table 1).

Target	Unspiked UV-treated	Spiked UV-treated	Unspiked Chlorination	Spiked Chlorination
Escherichia coli	67.45	91.78	96.84	99.99
Enterococcus faecium	77.22	94.99	99.24	100.00
16s rRNA (total bacteria)	99.08	99.55	99.81	99.94

These qPCR results showed fecal indicator bacteria removal ranged from 67% to 100% (but typically greater than 92% for treatments spiked with these indicator bacteria) following chlorination or UV treatment of water. Chlorination appeared to be a slightly more effective treatment method for removing these fecal indicator bacteria from St. Louis River Estuary water. Cultured plate counts of the same samples revealed greater than a 99% reduction in all culturable indicator bacteria (*E. coli*, *Enterococcus*, total coliforms and heterotrophic bacteria) in the UV and chlorine treated samples. The small differences in the results between each method of quantification were anticipated and probably reflect differences between what culturing versus molecular biology methods measure.

Another portion of DNA extracted from each sample from the experimental treatments were submitted to University of Minnesota Biomedical Genomics Center for 16S ribosomal RNA gene next-generation sequencing (NGS) in late December 2019 and the raw sequence data was returned in February 2020. The mothur software package and MiSeq SOP were used to sort, trim and align the sequence data to the Silva 132 taxonomic database. This data was then imported and analyzed in the statistical software R (version 3.5.1) using the phyloseq package. Pathogen analysis was performed using a list of 136 pathogen-containing genera (Fig. 1). The results of the pathogen analysis showed that DNA sequences from the *Flavobacterium* bacterial genus were extremely common in the control water sample and water samples after UV treatment (in water both spiked and unspiked with indicator bacteria). 16S rDNA sequences from the *Legionella* and *Mycobacterium* bacterial genera were responsible for relatively larger portions bacterial sequences in the harbor water after the chlorine treatment. This makes sense as both of these genera have been shown to be particularly resistant to chlorine

disinfection. Water samples treated with chlorine also had a larger proportion of bacteria DNA sequences from the Planctomycetes phylum, which contain many species that are found in marine and brackish environments (Fig. 2). Bacterial community diversity, as measured by OTU richness, was greatest in the control samples, followed by UV-treated samples, and then chlorinated samples.

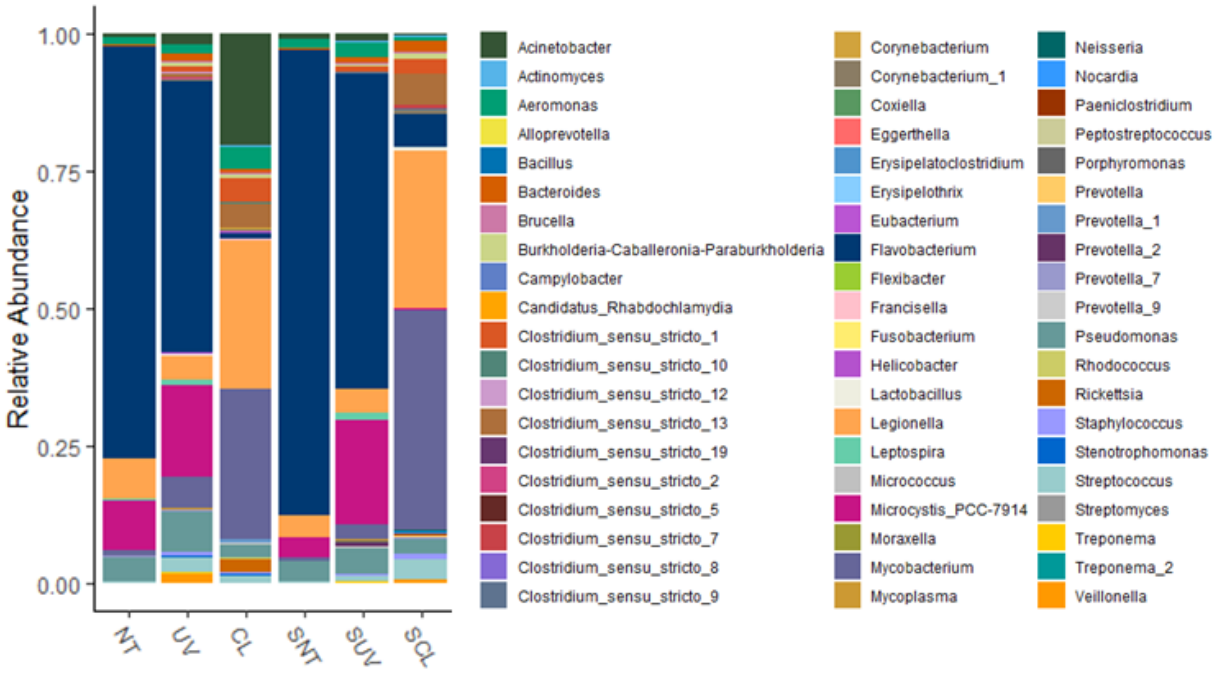


Fig. 1**.** Relative abundance of pathogen-containing genera across ballast treatment categories: no treatment (NT), UV light (UV), chlorine (CL), spiked no treatment (SNT), spiked-UV (SUV), and spiked-chlorine (SCL)

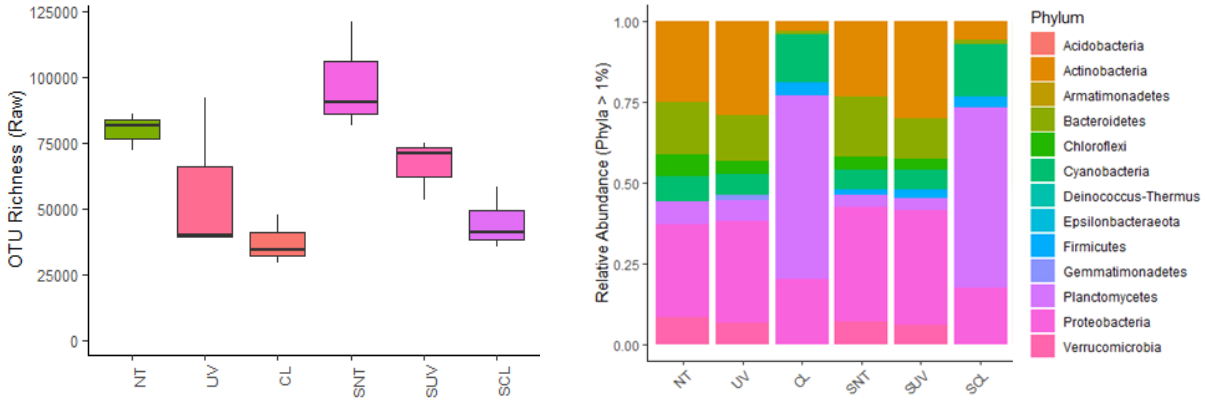


Fig. 2. (a) OTU Richness across treatment categories. (b) Relative abundance of major phyla across treatment categories.

Activity 2 Status as of January 31, 2021: Lisa DeGuire continued analysis of the DNA sequence data to determine the proportions of the pathogen containing genera (PCG) in the bacterial communities of each treatment category (Figure 1). As with the overall bacterial communities, the relative abundance of the pathogen containing genera for the untreated and UV-treated samples was very similar. About 1% of the bacterial communities of unspiked (with indicator bacteria) and not treated, and unspiked UV-treated samples consisted of DNA sequences from PCGs. About 8% of the total sequences in spiked non-treated and UV-treated samples consisted of PCGs, and PCGs accounted for 5-6% of all sequences in samples treated with chlorine (both spiked and unspiked).

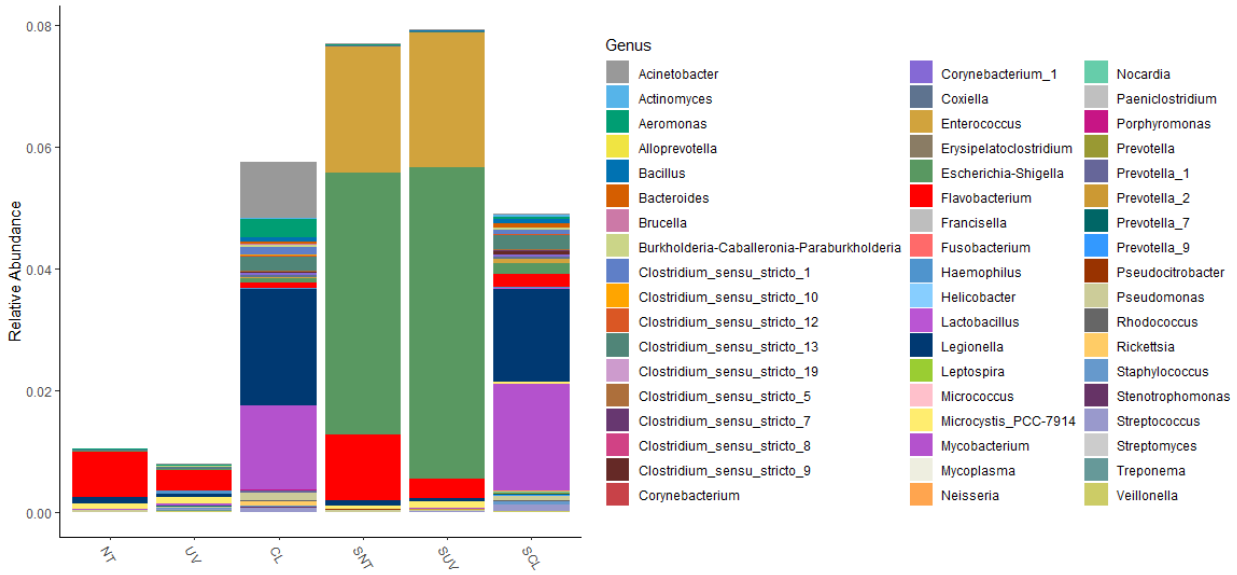


Figure 1. The relative abundance of the pathogen containing genera in each treatment category: non-treated (NT), UV-treated (UV), chlorinated (CL) and spiked counterparts (SNT, SUV, SCL).

Final Report Summary:

Overall, culture-based quantification, qPCR, and DNA sequencing demonstrated that both UV-radiation and chlorination effectively reduced indicator bacteria in the sample water. The differences in the percent removal results between each method of quantification (culture-based vs. molecular) likely reflect the differences between the variables quantified by each technique (live, culturable bacteria vs. amplifiable genetic information). The removal of *E. coli* and *Enterococcus* between the two techniques was more similar (and effective) for spiked samples than for the unspiked samples, which showed lower removal through qPCR (Table 1). The reduced efficacy may be related to the low initial abundance of indicator bacteria in the unspiked harbor water. While culturable methods may be better for instances where quantifying live indicator bacteria with easy and established protocols is preferred, molecular methods are able to account for viable-but-nonculturable cells and can provide information on wider range of microorganisms and potential pathogens.

Table 1. Percent removal (%) of indicator and total bacteria in different treatments, quantified through qPCR analysis and microscopy. Percent removal was calculated using the average abundance of triplicate samples from each treatment category.

Method	Target	Unspiked UV	Spiked UV	Unspiked Chlorine	Spiked Chlorine
qPCR	*Escherichia coli*	67.5	91.8	96.8	100.0
	Enterococcus faecium	77.2	95.0	99.2	100.0
	16S rRNA (total bacteria)	99.1	99.6	99.8	99.9
DAPI	Prokaryotic cells	31.1	19.4	36.4	7.9

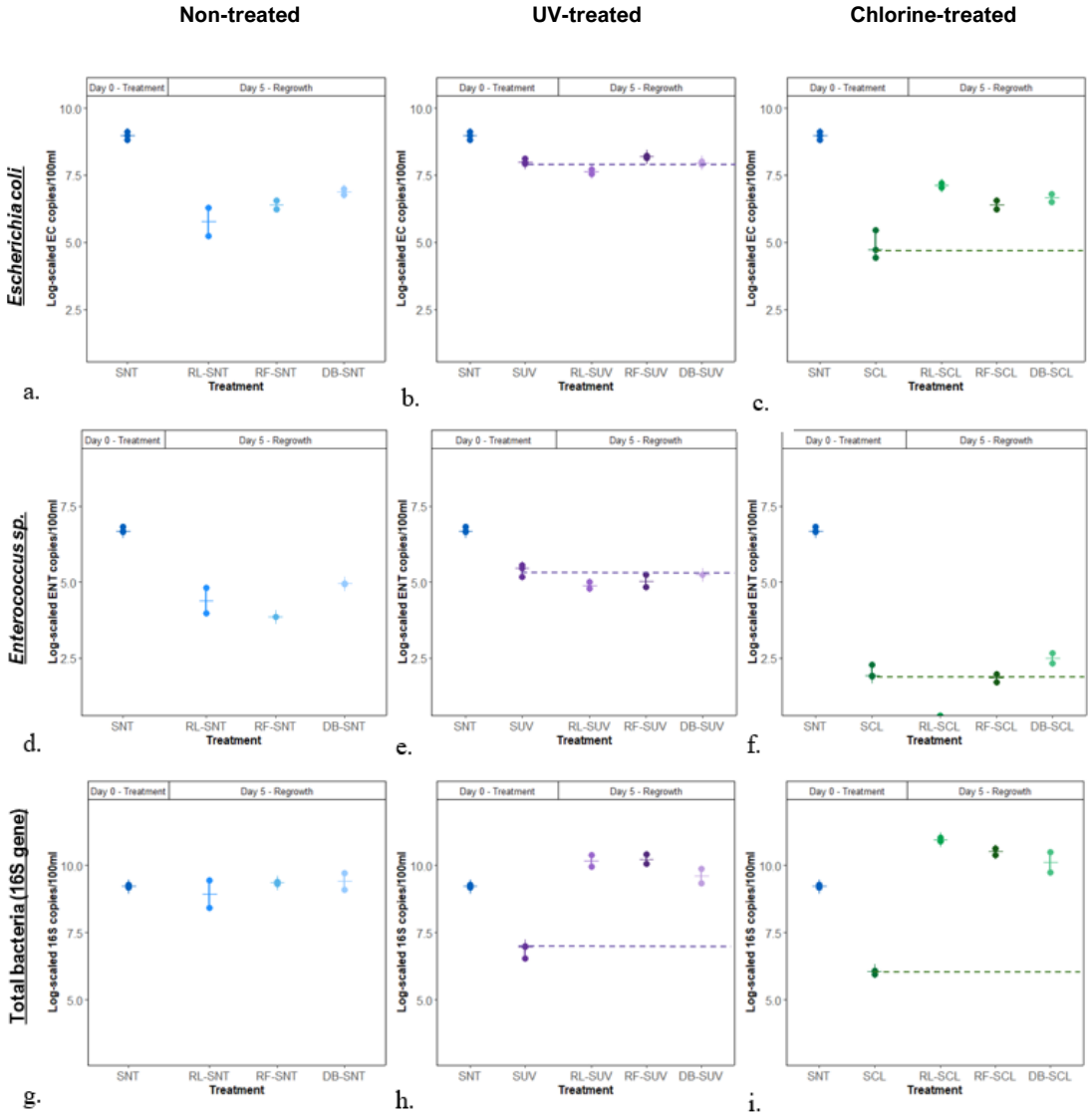
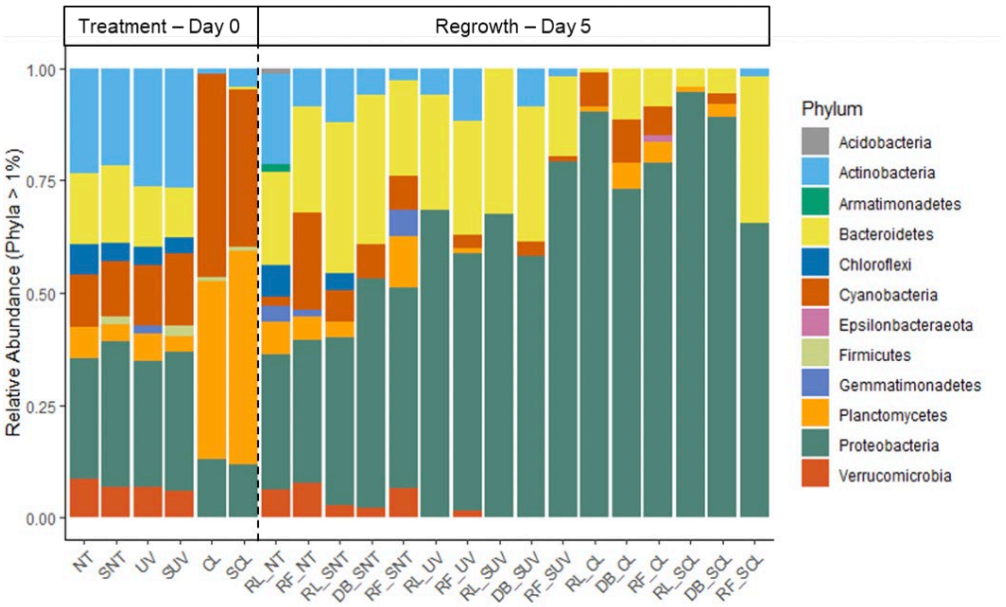


Figure 1. Abundance of target bacteria in spiked samples determined by qPCR analysis. Log-scaled abundance of *E. coli* (target gene copies/100 ml) (Figures 2a-c), log-scaled abundance of *Enterococcus sp.* (target gene copies/100ml) (Figures 2d-f), (c) log-scaled abundance of total bacteria (16S rRNA gene copies/100ml) (Figures 2g-i). Dashed line represents the median abundance immediately after treatment (Day 0). Day 5 regrowth samples include three incubation environments: lab incubation (RL), Nalgene field incubations (RF), dialysis bag field incubations (DB). Treatment categories include spiked non-treated samples (SNT), spiked and UV-treated (SUV), and spiked and chlorinated (SCL).

Despite the removal of over 99% of total bacteria seen in culturable and molecular methods (Fig. 1, Table 1), the composition of the bacterial communities in the non-treated and UV-treated samples was similar (Fig. 2). This result indicates proportional disinfection across bacterial taxa when UV light is used as a disinfectant. Conversely, bacterial communities in the chlorinated samples were different from the non-treated samples, indicating that some bacterial taxa were more resistant to chlorine disinfection than other taxa. The genus *Mycobacterium* was one of the most prevalent pathogen-containing genera (PCGs) in the chlorine-treated samples (Fig. 3) and this genus is known for its chlorine resistance. Strains of *Mycobacterium avium* have been found to be at least 500 times more resistant to chlorine than *Escherichia coli.* Planctomycetes and Cyanobacteria, the phyla with the largest relative abundance after chlorination (Fig. 2), also include representatives that have exhibited chlorine resistance.

A.



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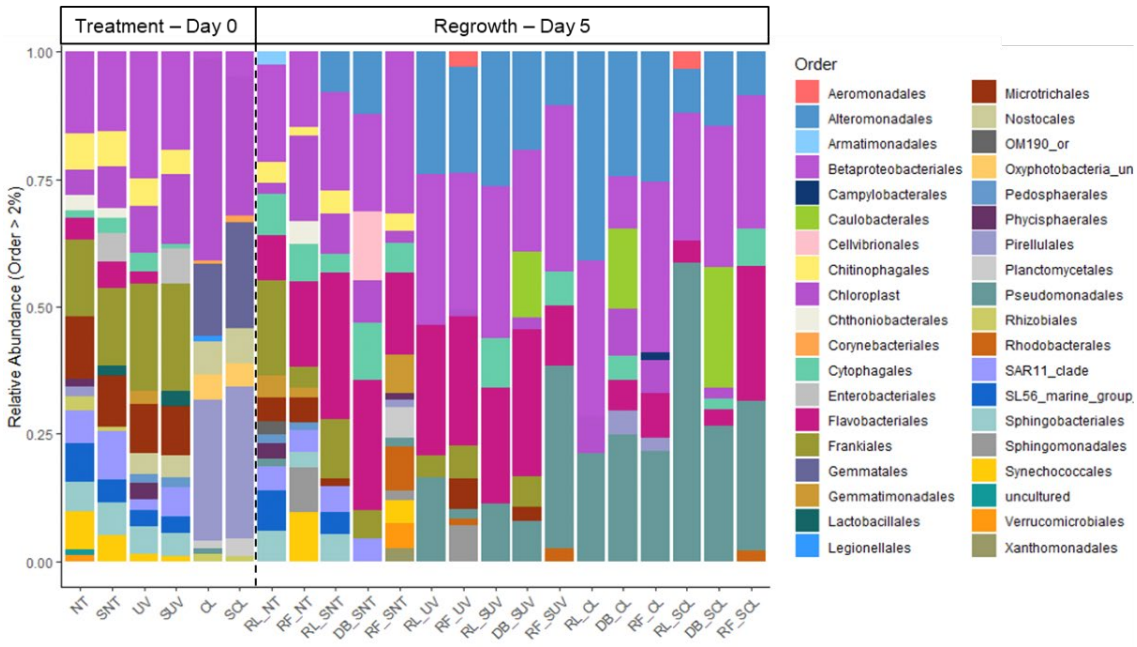

Figure 2. Relative abundance (%) of partial 16S rRNA sequences from bacterial communities at the (A) Phylum level and (B) Order level. Same day treatment samples (Day 0) on the left include unspiked non-treated (NT), UV-treated (UV), and chlorine-treated (CL) samples, as well as their spiked counterparts (SNT, SUV, SCL). The regrowth samples (Day 5) on the right include samples from three different incubations: a lab incubation (RL), a field incubation in dialysis bags (DB), and a field incubation in Nalgene bottles (RF).

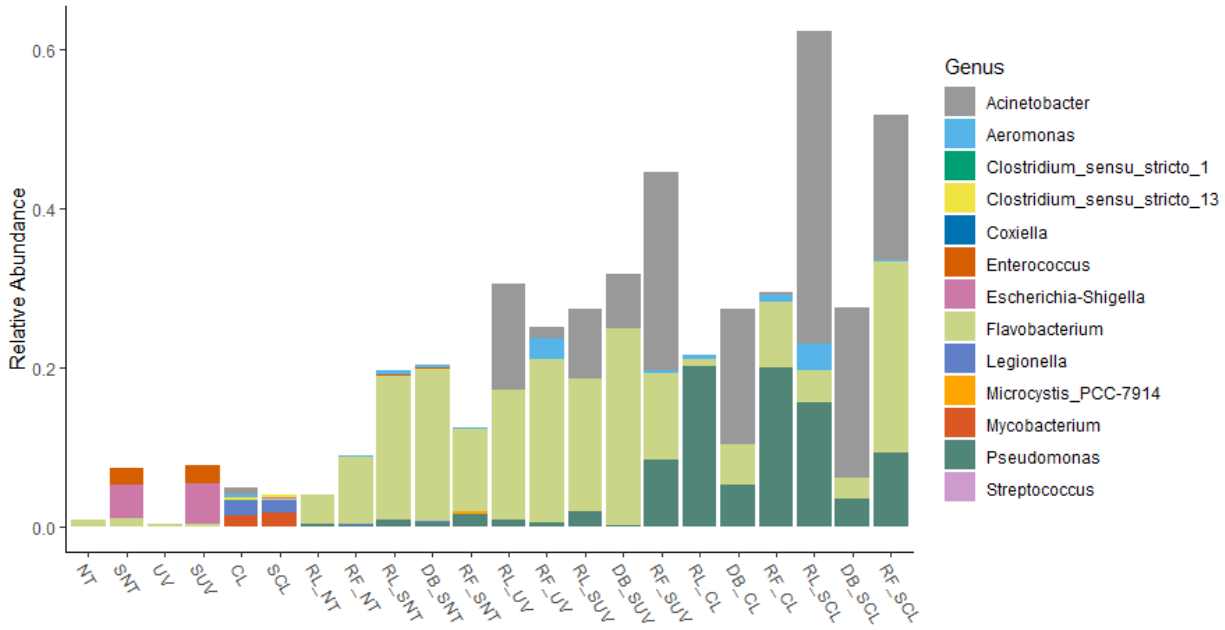

Figure 3. Relative abundance of pathogen containing genera determined from 16S rRNA sequences in the different treatments at the conclusion of the removal experiment (lab – left of vertical dashed line), and in different conditions within various regrowth incubation environments at the end of the five-day regrowth experiment (lab and field results – right of vertical dashed line). Dashed line separates samples from the different treatments at the end of the lab removal experiment (Day 0), on the left, from the samples from these water treatments in the three regrowth environments, on the right (Day 5). The regrowth experiment included water samples from three different types of incubation environments: a lab incubation (RL), a field incubation in dialysis bags (DB), and a field incubation in Nalgene bottles (RF).

Bacterial regrowth can occur when bacteria that remain viable after treatment reproduce, when cells are resuscitated from a viable-but-nonculturable state, or when DNA damage is repaired. Presumably, the cellular debris of the killed bacteria may provide nutrient sources to stimulate the regrowth of other cells in the communities after the UV and chlorine treatments; in these experiments, particularly members of the *Psuedomonas* and *Actinobacteria* genera (Fig. 3). Some species in the *Pseudomonas* and *Acinetobacter* genera, the two PCGs with the largest relative abundance in the chlorinated samples, are chlorine resistant. However, unlike the chlorine-resistant *Mycobacterium*, cells within the *Pseudomonas* and *Acinetobacter* genera are likely fast-growing, opportunistic microorganisms that thrive in low competition environments.

The most specific taxonomic classification possible for the partial 16S rRNA sequence analysis used here was the genus-level because of the high 16S rRNA gene similarity between closely related species. Therefore, the pathogenicity of the species and strains that occurred within the PCGs detected in these experiments could not be determined. Both the *Pseudomonas* and *Acinetobacter* bacterial genera, however, contain opportunistic human pathogens and the *Flavobacterium* genus accounts for 13% of total bacterial fish pathogens. Although these experiments demonstrated that some members of pathogen-containing genera can escape disinfection with UV light or chlorine, and even regrow and flourish after treatment, the public health and ecological risks of pathogen exposure via ballast discharge require further research, especially for members of the *Pseudomonas*, *Actinobacter*, and *Flavobacterium* genera in this harbor.

It is important to note differences in the application of disinfection techniques that may be used in a shipboard setting. Despite the diversity in ballast management systems, chlorination is common and usually only applied once while UV-radiation treatments often occur during both intake and discharge of ballast water. This double treatment provides any bacteria cells that may regrow with a second exposure immediately before entering the receiving waters. Thus, the results reported here support that conclusion that the timing of

disinfection plays a crucial role in bacterial abundance of post-treated ballast water. To maximize the impact of disinfection, treatment should occur just prior to discharge.

ACTIVITY 3: Taxonomically Identify the Common and Rare Bacteria in the SLRE

Description:

Sediments and water will be collected from multiple sites across the SLRE during the ice-free season (see attached map). Final sites selected for water and sediment samples will be determined after consultation with personnel from the Lake Superior National Estuarine Research Reserve, the U.S. EPA, and universities who have previously characterized the distributions of larger invasive species and chemical concentrations in this harbor. Water and sediment samples will be collected from research vessels using standard limnological and microbiological techniques. Microbial DNA will be extracted from the samples and bacterial 16S rDNA gene amplicons will be sequenced to identify common and rare bacterial taxa. This dataset will be compared with new and existing data on the bacterial compositions of ship ballast water and treated wastewater. This activity will focus on forecasting the risk that different ballast water bacteria pose on the sustainability of recreational fisheries, human health, and a functional SLRE ecosystem and help develop management strategies that prevent the unintended introduction of potentially harmful microbial species

Summary Budget Information for Activity 3:	**ENRTF Budget:**	**$ 139,741**
	Amount Spent:	**$ 120,819**
	Balance:	**$ 18,922**

Outcome	Completion Date
1. Collect sediments and water from the SLRE and extract microbial DNA	July 2018
2. Create a 16S rDNA sequence database of bacteria in SLRE sediments and water	September 2018
3. Identify potentially harmful bacteria in SLRE sediments and water to compare with new and existing datasets to identify potential sources of pathogens and estimate risk	December 2018

Activity 3 Status as of January 31, 2017: A search was started at the University of Minnesota Duluth in July 2016 to hire a Postdoctoral Associate to work on this project. This search is still in progress. The Project Manager and graduate student met separately with Dr. Anett Trebitz at the U.S. EPA Mid-Continent Ecology Division in Duluth, MN and Dr. Schon Schooler at the Lake Superior National Estuarine Research Reserve (NERR) to discuss their experiences collecting water and sediment samples representative of the St. Louis River Estuary (SLRE) system. Dr. Trebitz provided scientific papers they published and a random sampling scheme she and other USEPA personnel have used to take samples representative of the different regions of the SLRE. This sampling scheme was slightly modified to match our anticipated level of sampling (with advice from Dr. Schooler). It was used to randomly select 18 sites in the SLRE that would be sampled in fall 2016. Surface water samples were collected from these 18 sites on September 26, 2016 using the R/V Kingfisher from the Large Lakes Observatory. Replicate water samples from each site were returned to our lab at UMD, filtered onto Duropore membrane filters (0.2 µm pore size), and then the filters were frozen and stored at -80°C until DNA can be extracted for 16S rRNA gene sequencing analyses.

Activity 3 Status as of July 31, 2017: We randomly selected 22 sites in the St. Louis River Estuary to sample in April 2017. Three of these sites had been sampled the previous fall, while 19 sites had not been sampled previously. Sub-surface water samples were taken from each these sites on April 21 and 22, 2017 using the University of Minnesota R/V Kingfisher for sites > 1 m deep and a low-draft Jon boat for sites < 1 m deep. Mr. Tom Frantti piloted the R/V Kingfisher and Dr. Shon Schooler (Lake Superior National Estuarine Research Reserve) piloted the Jon boat. Three replicates of each water sample were filtered onto Duropore (0.2 µm-pore) membrane filters, and the filters frozen at -80°C until DNA was extracted. Analysis of the 16S sequences from 2011-2012 ballast water samples showed that the Levar-Sheik DNA extraction protocol was superior to a MoBio PowerSoil extraction kit. We used Levar-Sheik protocol to extract DNA from 48 replicate water samples collected in September 2016 and April 2017. Extraction of additional replicate water samples will be completed by early

fall. Sites were selected and boat reservations were made to sample subsurface water again at 15 sites and sediment at 30 sites in the St. Louis River Estuary in early August 2017.

Activity 3 Status as of January 31, 2018: Sampling of SLRE water and sediment was completed on November 1, 2017. Replicate (n=3) water samples were collected in early August (Aug. 1-3) and early November (Oct. 31-Nov. 1), 2017 from 20 to 22 randomly selected sites in the St. Louis River Estuary. Water has now been collected four times from the SLRE during three seasons; fall 2016, spring 2017, summer 2017, and fall 2017. Sediment was collected from 30 randomly selected sites in early August 2017. The R/V Kingfisher (Large Lakes Observatory), the R/D Browne (LSNERR), and the R2512 (LSNERR) were used to collect samples. Water samples were stored at 4°C in the dark and triplicate subsamples filtered onto 47 mm diameter Durapore (0.2 µm-pore) filters within one week of collection. These filters were frozen and stored at -80°C. Sediment samples were split; one subsample was frozen at -80°C to maintain community composition, and the remainder was stored at 4°C to maintain physical properties of each sediment sample. We have now achieved Outcome 1 in Activity 3.

DNA has been successfully extracted from approximately half of all samples taken so far. These DNA samples have been stored at -80°C until used for molecular analyses. We plan to extract DNA from the remaining water and sediment samples in winter and spring 2018 and submit them for illumina sequencing soon thereafter. Partial 16S rRNA gene sequences have been acquired for a single replicate from each of the water samples taken during fall 2016 and spring 2017. A preliminary analysis of these sequences showed a marked difference in the bacterial community compositions between the fall and spring samples, which strongly correlates with temperature. This result was not unexpected. Further analysis of these sequence libraries for genera harboring potential pathogenic bacterial strains will be completed when DNA sequences have been obtained for all water and sediment samples.

Activity 3 Status as of July 31, 2018: Jennifer Knack extracted DNA from sediment samples collected in summer 2017 and continued the preliminary analysis of the partial 16S rRNA sequences from water samples collected in fall 2016 and spring 2017. This further analysis led to the development of posters and a conference talk (see section V. Dissemination below for details). There were distinct bacterial communities in water from three zones of the St. Louis River Estuary (SLRE) in both fall 2016 and spring 2017, but these differences were stronger in the fall. Bacterial taxa responsible for driving the spatial differences in estuary water microbial communities were identified using a random forest model. This analysis uncovered that different bacterial taxa were responsible for driving spatial variability in fall 2016 than in spring 2017. In fall, the relative abundance of members of the gamma-Proteobacteria, alpha-Proteobacteria, and Bacteroides were responsible for differences seen in communities in the SLRE, while in spring the relative abundances of members of the Verrucomicrobia, Actinobacteria, and Bacteriodes were responsible for these differences. Further analysis of these sequence libraries for pathogen-containing bacterial genera (PCG) will be completed when DNA sequences have been obtained for all water and sediment samples.

Two new graduate students, Julia Zimmer and Lisa DeGuire, joined this project in mid-June 2018. They have been extracting microbial DNA from the remaining ~70 water samples collected from the SLRE in summer and fall 2017 that will be subsequently used for 16S rDNA sequence and qPCR analyses. Each of these new students will develop part of her M.S. theses using data from this project. Ms. DeGuire will be comparing the removal of indicator bacteria and pathogen-containing genera in UV and chlorine treatments (Activity 2) using both culturing and molecular biology-based approaches. Ms. Zimmer will be using qPCR molecular biology assays to determine the abundance of several pathogenic bacterial species in SLRE and ship ballast waters (Activity 1 and 3).

Activity 3 Status as of January 31, 2019: DNA was extracted from the remaining 47 water samples collected from various sites in the St. Louis River Estuary (SLRE) during summer and fall 2017. DNA has been extracted from all 83 water samples (in triplicate) and all 30 sediment samples collected from the St. Louis River Estuary, so Activity 3-Outcome 1 has now been completed. Five sediment DNA samples were submitted for preliminary 16S rDNA sequencing at the end of December 2018 to evaluate whether sediment DNA samples will require further purification to obtain good DNA sequence data. If our sediment DNA does not require further

purification after examining these preliminary sequencing results, then the DNA from the remaining sediment samples will be submitted for sequencing soon afterward. All DNA from SLRE water samples will be submitted for sequencing in January 2019. We anticipate that all remaining DNA samples will have been submitted for 16S rDNA sequencing by the end of March 2019.

We designed and conducted a short experiment to determine DNA losses during our DNA extraction protocol so that qPCR results in all activities can be corrected to be more quantitative. Purified harbor DNA was re-extracted and concentrations quantified by each person on our team who extracts DNA samples (J. Knack, L. DeGuire, J. Zimmer). The range of DNA recovered from water using our extraction protocol for all individuals ranged from 33% to 41% + 1.3-2.1%, recoveries which are typical of most extraction protocols. These extraction recovery estimates will be used for qPCR data collected in both Activities 2 and 3.

We researched different methods to quantify the presence of DNA from a fish pathogen, *Piscirickettsia salmonis*, in ballast and harbor water samples. J. Zimmer obtained a reference *P. salmonis* culture from Esteban Soto's lab at the University of California – Davis, and extracted DNA from this sample. Subsequently, she tested two PCR protocols for amplifying *P. salmonis* DNA using PCR primers that target bacterial 16S rDNA and the internal transcribed spacer (ITS) region of the ribosomal RNA operon. The ITS targeted primer approach provided better results and will be used in 2019 to quantify the abundance of *P. salmonis* DNA in ballast water and harbor samples.

Activity 3 Status as of July 31, 2019: DNA sequences from the preliminary sediment samples submitted previously for partial 16S rRNA gene sequencing indicated that further DNA purification was not necessary to obtain good sequence data. DNA from all the sediment samples collected in the Saint Louis River Estuary (SLRE) and DNA from some water samples needing to be re-sequenced were submitted to the UM Genomics by Jennifer Knack for 16S rRNA V4 region sequencing by May 20th, 2019. 198 of 279 total water and sediment samples (~71%) have been sequenced. We are currently waiting for the last DNA sequence results to finish all further analyses.

J. Knack performed a hierarchical clustering analysis, based on Bray-Curtis distances, on the bacterial community DNA sequences from water that had already been received (Fall 2016 and Spring 2017 samples). This analysis grouped sample points together based on how similar their bacterial communities were to each other. Similar bacterial communities were then located a map of the SLRE and zones of similar bacterial communities were delineated within the estuary using ArcGIS. This preliminary analysis indicated that three previously established estuary zones (lower, middle, and upper) may not capture all the differences between bacterial communities in water during all seasons. In Fall 2016, the lower estuary zone was dominated by a single bacterial community type (which did not include Allouez Bay or the Nemadji River), but above this region two distinct bacterial communities were detected in both the middle and upper estuary areas. In Spring 2017 like Fall 2016, the lower estuary zone was also dominated by a single bacterial community type (which again did not include Allouez Bay) but there were different bacterial communities in the middle and upper estuary areas (but different from communities found in Pokegama and Kimball Bay).

J. Zimmer finished optimized a qPCR assay, targeting the ITS spacer region of ribosomal RNA in *Piscirickettisa salmonis*, to measure this bacterium's abundance in environmental samples. *P. salmonis* is a bacterium that causes the disease "muskie pox" in muskellunge fish. DNA from several ballast water samples collected in 2011 and 2012 indicated the presence *P. salmonis* DNA using this assay. PCR products from these samples are being prepared for more detailed Sanger sequencing to help verify that the DNA amplified was indeed from *P. salmonis* before completing qPCR analyses for the remaining ballast water and additional harbor water samples.

Additional water and sediment samples were collected in July 2019 from the SLRE to compare the abundance of *P. salmonis* DNA at locations in the SLRE heavily travelled by muskellunge and commercial docks where ship ballast water is commonly discharged. The muskellunge locations were identified using acoustic tracking data for adult muskellunge taken by Erin Schaeffer (University of Minnesota) during summer 2017. Three site categories were identified – "muskie sites" (n=25), highly active docks (n=25), and sites randomly chosen throughout the estuary as a method of control (n=25). Surface water and sediment samples were taken from each 'muskie' and randomly chosen site in mid-July and refrigerated in the dark until processed further. Water samples were filtered in triplicate within 48 hours and were stored frozen at -80°C along with aliquots of

sediment samples. DNA extractions of the sediment samples were started. Water and sediment samples from commercial docks in the Duluth-Superior harbor will be collected in early August 2019 for comparison with samples taken the "muskie" and randomly chosen estuary sites. In addition, 18 separate water samples were collected throughout the estuary in mid-July for cation analysis to determine the influence of the Lake Superior on the harbor bacterial communities. These samples were preserved in 0.5% nitric acid at 4°C until cation analysis can be performed.

J. Zimmer also received a dozen fin tissue clips that had been collected in 2017-18 from muskellunge in the SLRE. DNA extracted from these fin tissue clips is now in our que for *P. salmonis* qPCR analysis. She also was fortunate to obtain the carcass of an adult muskellunge from the MN-DNR Fisheries Research Unit (French River) that had died during a gill net survey in the SLRE. Tissues from this single fish will be dissected and *P. salmonis* qPCR assays are then planned for DNA from these tissues.

Activity 3 Status as of January 31, 2020: In late August, J. Knack received bacterial DNA sequence data for the remaining 81 water and sediment samples collected in summer and fall 2017 that had been submitted for DNA sequence analysis in late May 2019. During the fall, she finished the initial processing of hundreds of thousand DNA sequences including trimming primer sequences, quality control, removal of poor-quality sequences, alignment of sequences, and then classifying the bacterial origin of individual sequences from each sample. She is preparing this data for hierarchical clustering analysis to finish geographic and seasonal comparisons of bacterial community composition of the water and sediments in the SLRE, including an evaluation of bacterial genera that contain pathogens for comparison with similar information about bacterial communities from ship ballast water.

Additional water and sediments were collected from commercial dock areas in the Duluth-Superior harbor in early August 2019. DNA was extracted from all the 79 water samples and 78 sediment samples taken from the St. Louis River Estuary during the summer of 2019. These extractions provided ample quantities of DNA to perform partial 16S rRNA gene sequencing using a next generation sequencing method to compare bacterial communities throughout the SLRE. Conventional PCR was used to determine the quality of the extracted DNA from water and sediment samples. The DNA from water samples amplified well using bacterial primers, so these samples were sent to the University of Minnesota Biomedical Genomics Center for next generation DNA sequencing in late December 2019. We are still awaiting these DNA sequence results. The sediment samples did not amplify well, so those DNA samples are currently being cleaned to prepare them for DNA sequence analysis. DNA from fish tissue samples collected in 2017-18 were also extracted and will be sent for bacterial DNA sequence analysis with the remaining DNA from sediment samples.

Cation analysis of 18 water samples (6 sites, 3 replicates each) collected in the St. Louis River estuary was completed and will be used in order to determine the influence of Lake Superior and the St. Louis River on estuarine bacterial communities. This analysis, along with the ongoing *P. salmonis* qPCR analysis, will allow us to better predict *Piscirickettsia salmonis* presence at sites in this estuary. After qPCR analysis, DNA samples that indicate the presence of *P. salmonis* will be more fully sequenced to confirm that the amplified target sequence is from this microorganism of interest.

Activity 3 Status as of July 31, 2020: Ms. J. Knack finished a preliminary comparison of the relative abundance of pathogen-containing bacterial general in St. Louis River Estuary (SLRE) water and sediment samples collected in 2016-17 to the same bacterial genera detected in commercial ship ballast water from 2011-12 (Activity 1). Eleven pathogen-containing general, including nine genera that contain human pathogens, were found to be statistically higher in relative abundance in ballast water than in the estuary water or sediment (Table 1). Five pathogen-containing genera, four of which include human pathogens, were detected in ballast water but not in the estuary water or sediment. Two bacterial genera (i.e., *Escherichia. Enterococcus)* that contain bacterial species (i.e., *E. coli*, *Enterococcus* spp.) used as indicators of water quality were present in ballast water, but their relative abundances were not statistically different from those in estuary water and sediment.

Table 1. Relative abundance of partial 16S rDNA sequences from pathogen-containing bacterial genera that were more common in commercial ship ballast water (2011-12) than water or sediment from the St. Louis River Estuary (2016-17).

Genus	Pathogen Group	Mean Relative Abundance ± Standard Deviation		
		Ballast	Estuary Water	Estuary Sediment
Bordetella	Human	0.0006% ± 0.0018%	0.0002% ± 0.0008%	nd
Leptospira	Human	0.0170% ± 0.0346%	0.0050% ± 0.0101%	0.0017% ± 0.0040%
Mobiluncus	Human	0.0001% ± 0.0006%	nd	nd
Moraxella	Human	0.00002% ± 0.0001%	nd	nd
Rhodococcus	Human	0.0082% ± 0.0330%	0.0004% ± 0.0014%	0.0006% ± 0.0011%
Stenotrophomonas	Human	0.0271% ± 0.0916%	0.0054% ± 0.0233%	0.0134% ± 0.0704%
Streptomyces	Human	0.0021% ± 0.0036%	0.0002% ± 0.0007%	0.0007% ± 0.0018%
Acinetobacter	Human and Fish/Wildlife	0.0832% ± 0.1194%	0.0251% ± 0.0392%	0.0170% ± 0.0751%
Actinomyces	Human and Fish/Wildlife	0.0096% ± 0.0310%	0.0001% ± 0.0006%	nd
Chlamydia	Human and Fish/Wildlife	0.00002% ± 0.0001%	nd	nd
Mycobacterium	Human and Fish/Wildlife	0.3529% ± 0.8131%	0.0206% ± 0.0346%	0.0108% ± 0.0156%
Simkania	Human and Fish/Wildlife	0.0010% ± 0.0042%	nd	nd
Vibrio	Human and Fish/Wildlife	0.0002% ± 0.0006%	0.00004% ± 0.0003%	nd
"*Candidatus* Xenohaliotis"	Fish/Wildlife	0.0002% ± 0.0005%	0.0001% ± 0.0004%	nd
Tenacibaculum	Fish/Wildlife	0.0020% ± 0.0090%	nd	nd
Microcystis	Ecologically or Economically Disruptive	0.2014% ± 0.5046%	0.0368% ± 0.0516%	0.0005% ± 0.0010%

nd: not detected

Ms. Julia Zimmer prepared all 79 surface water samples and 78 sediment samples collected throughout the St. Louis River Estuary during summer 2019 for bacterial DNA analysis by performing DNA extractions on each sample. Twenty-six of the 79 sample sites were located at four of the top "outgoing" docks that we consider to be the most influenced by ballast water discharge. DNA from water samples was sent to the UM Genomics Center for DNA sequencing in late December 2019 and raw sequence data results were returned in February 2020. DNA from sediment samples were sent in March 2020 and raw sequence data was returned in May 2020. These partial 16S rDNA sequences are currently being analyzed to compare the bacterial community diversity in water and sediments from throughout the St. Louis River Estuary and determine if different bacterial communities are found in the part of the estuary with active shipping docks or regions frequented by muskellunge fish compared to other regions of the St. Louis River Estuary. An analysis of pathogen-containing bacterial genera is also being performed for these water and sediment samples to compare with Ms. Knacks results for water and sediment samples collected in 2016-17.

Ms. Zimmer started quantitative PCR (qPCR) analyses of an ITS spacer region in ribosomal RNA gene to quantify the relative abundance of the fish pathogen, *Piscirickettsia salmonis,* in DNA extracted from the 79 surface water samples and 78 sediment samples. The results of this analysis should help not only determine if

this pathogenic bacterium responsible for "Muskie Pox" disease is present in the SLRE, but also where it is most commonly in the estuary waters and sediments.

Activity 3 Status as of January 31, 2021: Ms. Zimmer completed quantitative PCR analyses targeting the ITS region of *P. salmonis* DNA for water and sediments collected in the St. Louis River Estuary in 2019. *P. salmonis* DNA was detected in low quantities in water collected at many sites in the St. Louis River Estuary. The levels detected were so low that Ms. Zimmer has started to sequence these PCR products to confirm that the DNA was truly amplified from *P. salmonis* cells.

If the qPCR amplified DNA is confirmed to be from this pathogen, then concentrations of DNA from *P. salmonis* appear to increase from sites in the upper estuary (e.g,, St. Louis River, Spirit Lake) to sites near the mouth of the estuary (i.e., BNSF Dock 5, Allouez Bay). No *P. salmonis* DNA was detected by qPCR in water at Hallett Dock 5 (Figure 1). The location with the greatest concentration was BNSF Dock 5, where a cargo ship was actively discharging ballast when a water sample was collected. Other than these differences, there were no notable differences in the concentration of *P. salmonis* DNA in water at the various sites (i.e., commercial docks, muskellunge habitats, and general SLRE sites). These qPCR results are at odds with results from Illumina sequencing of DNA in water at the various sites, which did not detect the presence of DNA from the *Piscirickettsia* genus. This difference, if true, suggests that that if *P. salmonis* DNA is present in the estuary, it may be at concentrations too low to detect using Illumina DNA sequencing techniques. No *P. salmonis* DNA was present in detectable quantities using the qPCR assay in any sediment samples taken at the various St. Louis River estuary sites.

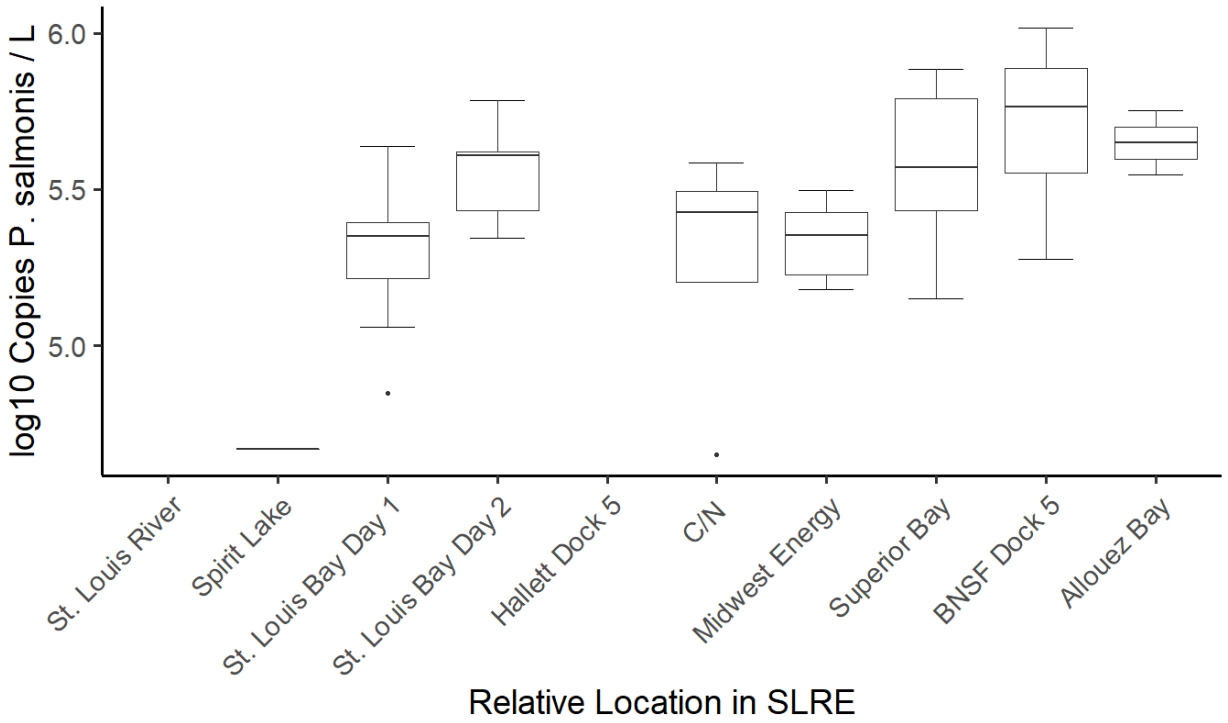

Figure 1. Log-transformed abundance of (suspected) *P. salmonis* DNA at various locations in the SLRE.

Sparse principle components analysis (sPCA) of the partial 16S rDNA sequences revealed clear differences between bacterial communities in the surface water at the commercial docks and muskellunge habitats or the rest of the St. Louis River estuary (Figure 2a). When the proportions of pathogen-containing genera (PCGs) were considered alone, these taxa were responsible for about 3% of the total bacterial sequences in water within each site category (Figure 2b). The commercial dock PCGs were dominated by DNA sequences from *Flavobacterium* genus, a genus commonly associated with ballast water discharge. *Escherichia-Shigella* and *Bacteroides,* genera commonly associated with gut microbiomes, were in the greatest relative abundance within the muskellunge habitats. Low abundances (>0.05%) of *Legionella* DNA sequences were found in water

throughout the entire estuary. This genus contains several bacterial species responsible for Legionnaire's disease. *Legionella* species can be resistant to ballast treatment methods because their tendency to produce biofilms that can be protective against various chemical and physical disturbances. No *Piscirickettsia spp.* 16S rDNA sequences were detected in water at any estuary sites. Based on these findings, further quantitative analysis of *Legionella* and *Flavobacterium* species found in water may be warranted to determine which species are present and the sources and risks of potential pathogen strains.

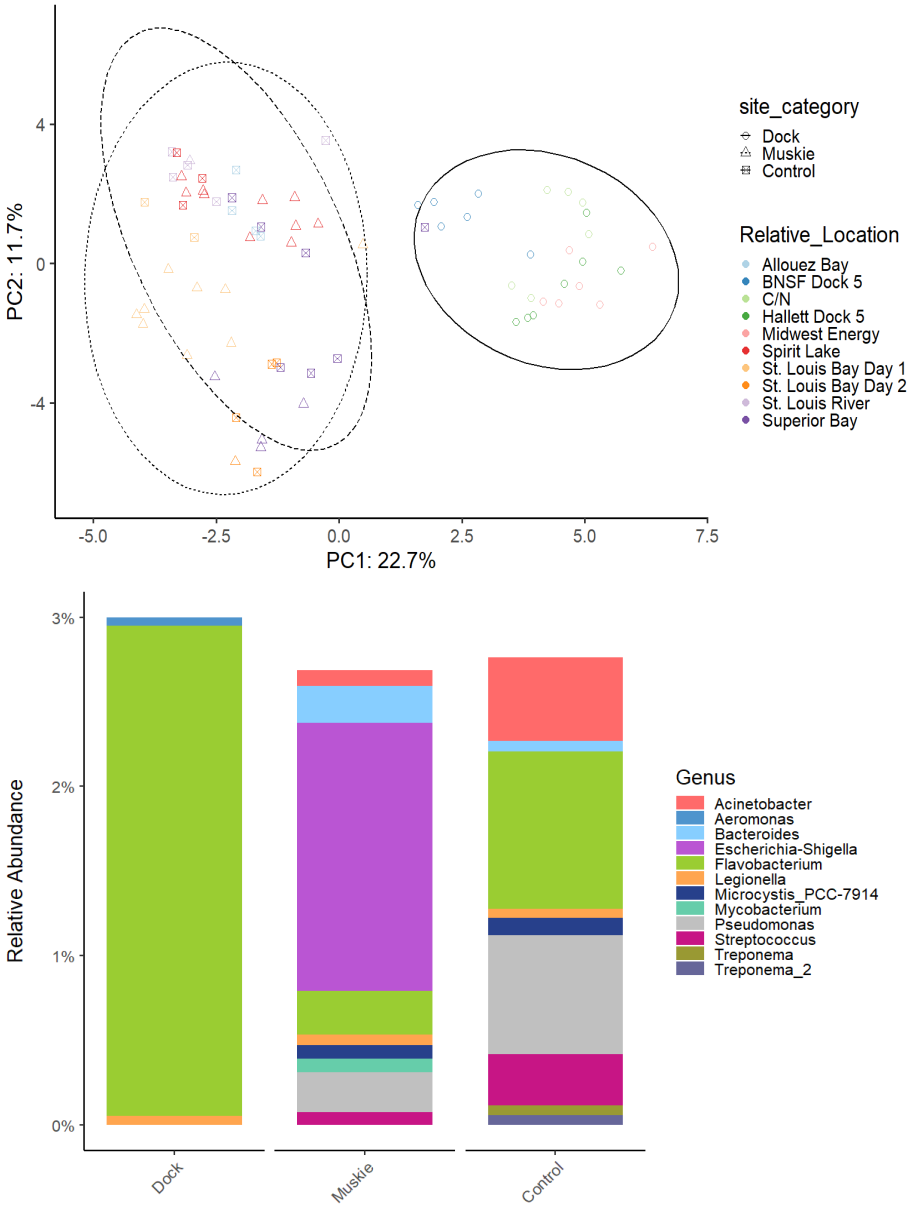

Figure 2. (a) Sparse principal component analysis (sPCA) of surface water pathogen-containing communities (upper panel). (b) Relative abundance of pathogen-containing genera (abundance > 0.05%) by site category (lower panel).

Using SourceTracker analysis, Ms. Zimmer estimated the influence of different docks on the composition of bacterial communities at several locations in the St. Louis River estuary. The surface waters at Hallett Dock 5 and the CN dock appeared to have greater influences on the overall composition of bacterial communities in water at several sites in the estuary than water from other docks (Figure 3.a). Only in Allouez Bay and the second day the St. Louis River was sampled was the composition of bacteria communities in water influenced by unknown sources of bacteria. However, the proportion of these unknown sources did not exceed the combined influence

of Hallett Dock 5 and CN. When only sequences from PCGs were considered, PCGs from different docks contributed more equally to the composition of PCGs communities in water (Figure 3.b). This finding may be an outcome of ballast water discharged at these docks. [A similar source analysis is being conducted for bacterial communities found in sediments but it is not yet completed.]

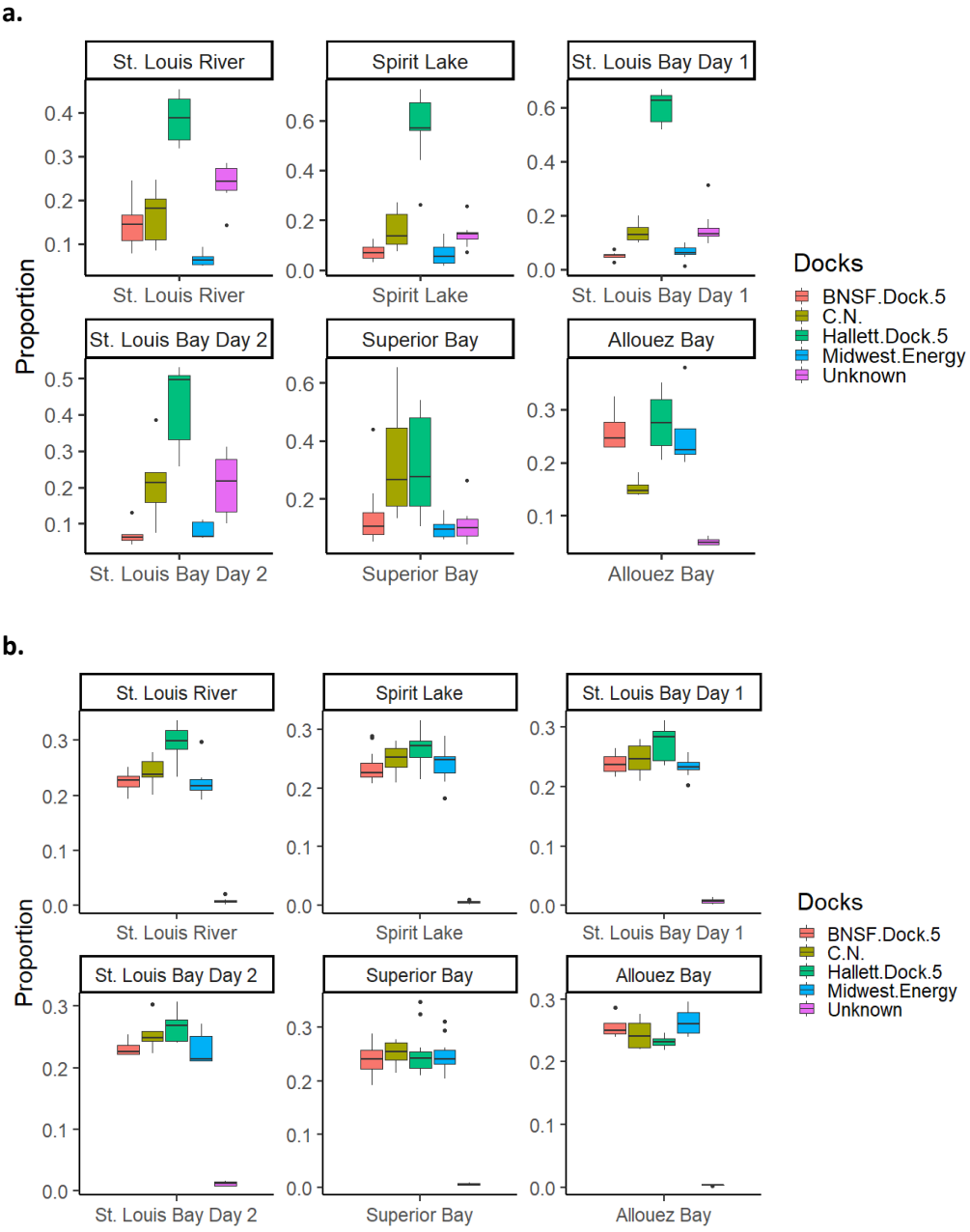

Figure 3. Sources (docks or unknown sites; bars) of bacteria in water at different sites (individual graphs in each panel) in the St. Louis River estuary in 2019 estimated using SourceTracker analysis. (a) Results for the composition of total bacterial communities in water (upper panel), and (b) results for only the pathogen-containing genera (PCG) found in water at the various sites (lower panel).

The results of sparse principle components analysis (sPCA) for sediment bacterial communities were not as revealing as the similar analyses of bacterial communities in surface waters at the commercial docks, muskellunge habitats, and the rest of the estuary (Figure 4.a). The total compositions of sediment bacterial communities at muskellunge habitats and other SLRE sites were indistinguishable from each other (Figure 4.a), as found for surface water bacterial communities (Figure 2.a). Unlike bacterial communities found in water, the

compositions of total bacterial communities in sediment at commercial docks overlapped with the compositions of bacterial communities at muskellunge habitat sites or at sites randomly selected in other regions of the St. Louis River estuary.

When the proportions of pathogen-containing genera (PCGs) were considered alone, these taxa stood out more in the sediment at commercial docks compared to muskellunge habitats or other sites in the SLRE (Figure 4.b). DNA sequences from PCGs were responsible for more than 15% of the composition of the sediment total bacterial communities at commercial docks examined, while PCG sequences were less than 2.5% of the sequences found in sediment communities at muskellunge habitats and other SLRE sites (Figure 4.b). DNA sequences from only the *Flavobacterium* genus accounted for greater than 0.05% of total bacterial sequences found in sediment at sites in each category (docks, muskellunge habitat, other SLRE sites). DNA sequences from the *Streptococcus* genus were the most common PCGs bacterial sequences found in sediment at the commercial docks. The *Streptococcus* genus contains many species, including pathogenic strains known to infect not only humans but also fish and wildlife. Further investigation of *Streptococcus* species in sediment at commercial docks is probably warranted to determine the sources and risk of bacterial strains in this genus.

a.

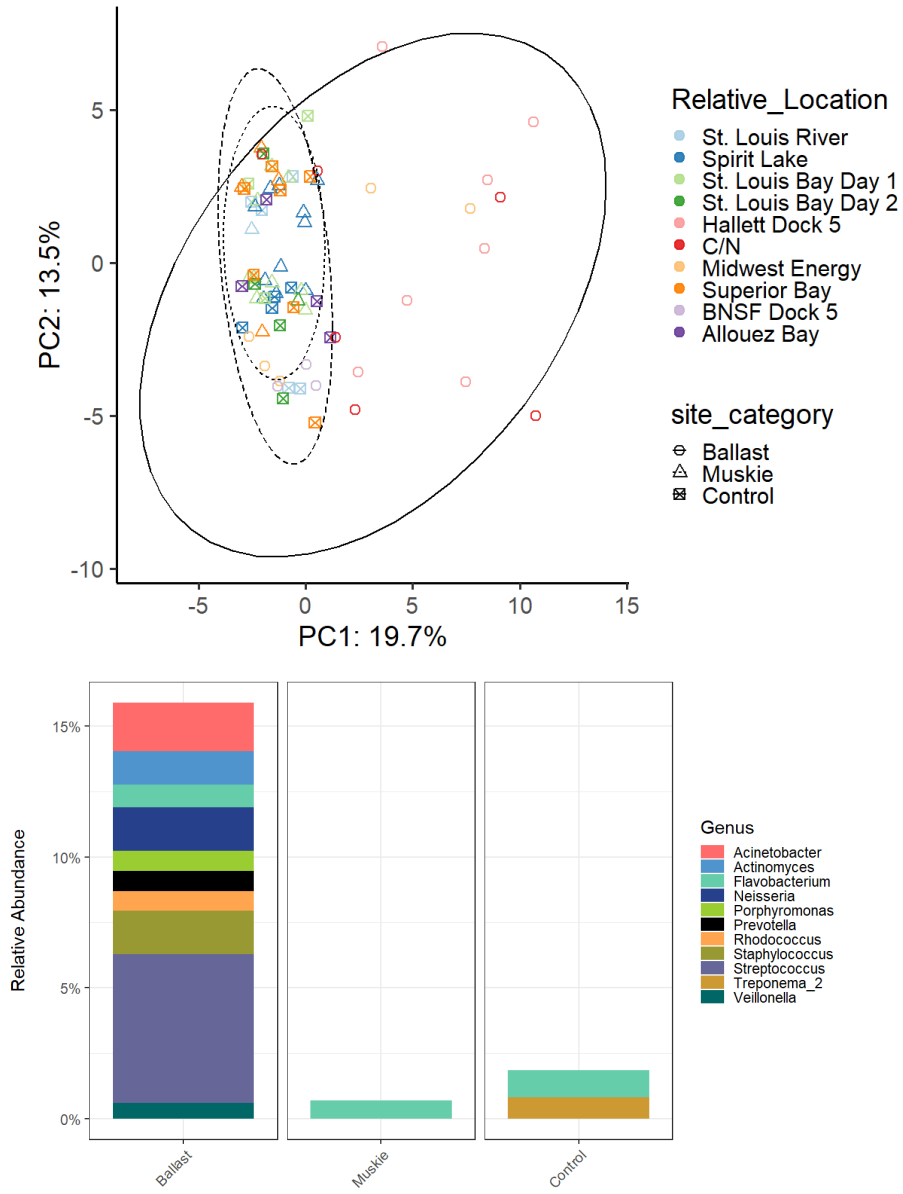

Figure 4. (a) Sparse principal component analysis (sPCA) of sediment bacterial communities at various sites and docks in the St. Louis River estuary (upper graph). (b) Relative abundance of DNA sequences from pathogen-containing bacteria in sediment at three categories of sites (docks (i.e., Ballast), muskellunge habitat (i.e., Muskie), and other SLRE sites (i.e., Control) (lower graph).

Final Report Summary:

Water samples were collected from 83 sites randomly chosen within the St. Louis River estuary from Fall 2016 to Fall 2017 and microbial DNA extracted. Thirty sediment samples from randomly chosen sites were collected in early August 2017 and microbial DNA extracted. Illumina DNA sequences were obtained for the partial bacterial 16S rRNA gene. Results from the analysis of these bacterial DNA sequences from water and sediments in the St. Louis River estuary and commercial ship ballast water were reported in the July 2019 and July 2020 status reports for Activity 3 (see above).

Water and sediment samples were also collected over 3 days in July 2019 from 79 sites including muskellunge habitats, commercial dock areas, and control sites throughout the St. Louis River Estuary for *Piscirickettsia salmonis* qPCR and bacterial 16S rRNA gene sequence analyses (see January 31, 2021 status report for Activity 3 above). Even though it initially appeared that *P. salmonis* DNA was present in the surface water of the estuary using this qPCR analysis (see January 2021 status report, above), a follow up analysis determined this observation was incorrect. The extracted bacterial DNA from the surface water samples underwent conventional PCR amplification using the *P. salmonis* specific primers and the PCR products were sent to the University of Minnesota Genomic Center for Sanger sequencing. Sanger sequences of the amplified DNA provides a way to confirm whether the qPCR amplification product was actually from *P. salmonis* DNA. The sequences from the Sanger sequencing did not match the sequence of *P. salmonis* or any other known microorganism. Therefore, it must be concluded that the amplification observed in the qPCR assay was non-specific amplification. This result was confirmed by the results from the Illumina sequencing of DNA from water and sediment samples at the various sites in the St. Louis River estuary in 2017 and 2019, which did not detect the presence of DNA from the *Piscirickettsia* genus. These results indicate either that this pathogenic microorganism was not present in water or sediments of this estuary during these times, or that it was too low in concentration to be detected by these molecular techniques.

Bacterial communities and the pathogen-containing bacterial genera (PCGs) subset in water and sediments at four commercial docks in the Duluth-Superior harbor were different from other sites in the St. Louis River estuary. Higher relative abundances of PCGs were found in commercial dock sediments compared to the rest of the estuary. While there were only minor differences in the relative abundance of PCGs in surface water throughout the estuary, DNA from the *Flavobacterium* genus was more abundant at docks than other areas. Discharge of ballast water may affect the prevalence of PCGs in the Duluth-Superior harbor. Treatment of ballast water prior to discharge may reduce any human and wildlife pathogen load. In addition, care should be taken when dredging dock areas because disturbing sediment may temporarily increase the chances of exposing recreational users to pathogenic bacterial strains.

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 genetic data into national databases 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 discusses with our collaborators at the Minnesota Pollution Control Agency, other interested Minnesota and Wisconsin agencies, 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. In addition, we intend to provide updates through public presentations at venues such as the Great Lakes Aquarium.

Status as of January 31, 2017: The Project Manager and graduate student had initial meetings and conversations familiarizing representatives from the Wisconsin DNR, Minnesota Sea Grant Program, Lake Superior NERR, U.S. EPA Mid-Continent Ecology Division, and the Duluth Seaway Port Authority with the goals and objectives of our study. Colleagues at several of these organizations offered to guide us how to gain access to sample ballast water in commercial ships, or provided input about our SLRE sampling design. As suggested by individuals from these groups, we are now creating a small handout intended for ship captains and operators of commercial ships we wish to sample ballast water from. This handout will state the purpose and goals of this research project, what samples and data will be collected, and how this data will be stored and used. This handout will be used to help us gain permission to collect ballast water samples from various commercial ships during the next two years.

Status as of July 31, 2017: We started drafting figures and tables for a manuscript describing the results from sequencing the 2011-2012 ballast samples using two DNA extraction procedures. We anticipate drafting a manuscript and submitting it to peer-reviewed journal for publication by the end of 2017.

Status as of January 31, 2018: Jennifer Knack, graduate student on this project, used the environmental estuary data in a geographic information system class project during the fall 2017 semester at the University of Minnesota Duluth. Ms. Knack is preparing an abstract to present some of the SLRE bacterial community results at the St. Louis River Summit in Superior, WI in March 2018. She will also submit an abstract to present a poster at the Association for the Sciences of Limnology and Oceanography summer meeting in June 2018. We are also starting to develop a manuscript for submission to a peer-reviewed journal that will describe the bacterial communities and evaluate bacterial genera containing potentially harmful strains found in the 2011-2012 ballast communities.

Status as of July 31, 2018: Jennifer Knack, a graduate student, presented two posters reporting project data at the St. Louis River Summit in March 2018, and gave a talk at the Association for the Sciences of Limnology and Oceanography summer meeting in June 2018 (see citations below). Work is continuing on a manuscript that will describe and evaluate pathogen-containing bacterial genera found in the 2011-12 ballast water communities.

Knack, J. J. and R. E. Hicks. Microbial Community Composition of Ballast from Commercial Ships in the Duluth-Superior Harbor. St. Louis River Summit. March 13-14, 2018. University of Wisconsin-Superior, Superior, WI. [poster presentation]

Knack, J. J. and R. E. Hicks. A Survey of the Bacterial Communities in the St. Louis River Estuary. St. Louis River Summit. March 13-14, 2018. University of Wisconsin-Superior, Superior, WI. [Poster presentation]

Knack, J. J. and R. E. Hicks. Environmental Gradients Can Predict Planktonic Bacterial Community Similarity in the St. Louis River Estuary. Association for the Sciences of Limnology and Oceanography Summer Meeting. June 10-15, 2018. Victoria, BC. [Oral presentation]

Status as of January 31, 2019: No additional project results were disseminated during this period. R. Hicks and J. Knack continued to prepare figures, tables, and text for a manuscript about potentially harmful bacteria in ballast water samples collected in 2011-12 that were re-sequenced during this project. We anticipate submitting this manuscript for publication by mid-2019.

Status as of July 31, 2019: R. Hicks presented preliminary project results and discussed them with Jeff Stollenwerk at the Duluth Seaway Port Authority on February 11, 2019. R. Hicks and J. Knack finished preparing figures and tables and started to draft a written manuscript about potentially harmful bacteria in ballast water for submission to a peer-reviewed scientific journal. No other project results were disseminated during this period.

Status as of January 31, 2020: No results from this project were disseminated to collaborators, colleagues, or the public during this period.

Status as of July 31, 2020: Ms. J. Knack submitted an abstract to present her research results from Activity 1 and 3 at the Association for the Sciences of Limnology and Oceanography – Society for Freshwater Sciences joint meeting in Madison, WI from June 7-12, 2020, but this meeting was cancelled due to COVID-19. The title of the presentation was "Potentially harmful bacteria in commercial ship ballast water entering the Duluth-Superior Harbor."

Lisa DeGuire and Julia Zimmer presented a poster reporting some project results at the St. Louis River Summit in March, 2020. Ms. DeGuire and Ms. Zimmer both submitted abstracts for presentations at the Association for the Sciences of Limnology and Oceanography summer meeting in 2020, but the meeting was cancelled due to the COVID-19 pandemic. Ms. Zimmer also submitted an abstract for an oral presentation at the International Symposium on Microbial Ecology August 2020 meeting, but that meeting was also cancelled due to the COVID-19 pandemic. Both Ms. DeGuire and Ms. Zimmer submitted abstracts to give presentations at the Upper Midwest Invasive Species Conference to be held online from November 2-6, 2020.

DeGuire, L., Zimmer, J.A., Hicks, R. E., TenEyck, M., Chun, C. L., Polkinghorne, C., Saillard, H., and Prihoda, K. Bench-Scale and Molecular Analysis Approaches to Evaluate the Risks of Pathogen Introductions into the St. Louis River Estuary. St. Louis River Summit. March 3-4, 2020. Duluth, MN. [poster presentation]

Status as of January 31, 2021: On November 4, 2020, Ms. DeGuire and Ms. Zimmer gave oral presentations at the Upper Midwest Invasive Species Conference (UMISC) as a part of the "Ballast Water: New Policies, Actions & Findings" special session.

DeGuire, L., M. TenEyck , C. Polkinghorne, H. Saillard, C.L. Chun, and R.E. Hicks. Bench-scale Evaluation of Ballast Water Treatment for Bacterial Communities. Upper Midwest Invasive Species Conference. Nov. 2-6, 2020. [Online oral presentation]

Zimmer, J.A., C. L. Chun, and R. E. Hicks. Predicting the Risk of Pathogen Introduction from Ballast Water Discharge into the St. Louis River Estuary, Minnesota. Upper Midwest Invasive Species Conference. November 2-6, 2020. [Online oral presentation]

Final Report Summary: Since the last work plan update, Ms. DeGuire presented a poster of some project results at the 2021 St. Louis River Summit and gave a talk to the Twin Ports Freshwater Folk organization:

DeGuire, L., M. TenEyck , C. Polkinghorne, H. Saillard, C.L. Chun, and R.E. Hicks. Bench-scale Evaluation of Ballast Water Treatment for Bacterial Communities. St. Louis River Summit. March 1-3, 2021. Online Conference. [poster presentation]

DeGuire, L. From Shipping to Swimming: Bacterial Monitoring in Ballast Water Treatment Systems and Recreational Waters. Twin Ports Freshwater Folk meeting. August 4, 2021. [Online oral presentation]

In total during this project, three graduate students were trained, two M.S. degrees will be completed, four poster and four oral presentations were made at regional and national scientific conferences, and project results were disseminated to collaborators and colleagues at the U.S. EPA Mid-Continent Ecology Division and the Duluth Seaway Port Authority in Duluth, MN. One M.S. thesis will be appended to this project final report and the other will be forwarded when it is completed.

VI. PROJECT BUDGET SUMMARY:

A. ENRTF Budget Overview:

Budget Category	$ Amount	Overview Explanation
Personnel:	$ 252,805	Salary support for the project manager (24 months @ 8.3% time), a postdoctoral investigator (27 months @ 100% time), and a

		graduate research assistant (26 months @ 50%-time)
Professional/Technical/Service Contracts:	$ 44,500	The Northeast-Midwest Institute and the Lake Superior Research Institute will provide professional services to test ballast water treatment process effectiveness using conventional microbial assessment methods. ~~It~~ They will also supply ship ballast water samples for DNA analysis. The funds include partial salaries for UWS-LSRI staff and supplies for laboratory analyses, and ship sample collection.
Equipment/Tools/Supplies:	$ 52,042	Supplies for water and sediment sampling ($3,650), DNA extraction and PCR reagents for assays ($13,922), DNA sequencing and data storage and analysis costs for DNA analyses ($20,270), chemical and expendable lab supplies to support field sample analyses and laboratory experiments ($14,200)
Travel Expenses in MN:	$ 3,108	Sampling trips to the St. Louis River estuary and GSI facility (30 trips, $600) and travel support for project personnel to disseminate project results at public venues, scientific conferences, and outreach events with target organizations ($2,508). Matching conference travel funding will be sought from the University of Minnesota.
Other:	$ 15,545	Publication costs for journal articles ($2,045), R/V Blue Heron ship time (1 day @ $8,500/day), small boat rental (10 days @ $500/day)
TOTAL ENRTF BUDGET:	**$368,000**	

Explanation of Use of Classified Staff: N/A

Explanation of Capital Expenditures Greater Than $5,000: N/A

Number of Full-time Equivalents (FTE) Directly Funded with this ENRTF Appropriation: 3.58

Number of Full-time Equivalents (FTE) Estimated to Be Funded through Contracts with this ENRTF Appropriation: ~~0.03 (NEMWI) +~~ 0.23 (UWS)

B. Other Funds:

Source of Funds	$ Amount Proposed	$ Amount Spent	Use of Other Funds
Non-state			
~~NEMWI~~ UWS-LSRI F&A Match	$10,112	$	Indirect Cost Match
State			
UM F&A Match	$163,939	$	Indirect Cost Match
TOTAL OTHER FUNDS:	**$174,051**	**$**	

VII. PROJECT STRATEGY:

A. Project Partners:

Great Ships Initiative, Northeast-Midwest Institute, Lake Superior Research Institute
Ms. Allegra Cangelosi will lead the GSI-based contribution to the project, namely, the collection of ballast water samples (Activity 1). She and Dr. Matthew TenEyck will also provide review of all project objectives and their outcomes. Dr. Matthew TenEyck, University of Wisconsin-Superior will conduct the UWS-based bench-scale experiments (Activity 2), using standard indicator bacteria assays for comparison with genetic detection approaches implemented by Dr. Randall Hicks. Research activities will take place in Duluth-Superior Harbor, and at the University of Wisconsin Superior's Lake Superior Research Institute in Superior, WI. The UWS-LSRI will receive ENRTF funds in a subcontract from the University of Minnesota.

Jeff Stollenwerk, Minnesota Pollution Control Agency. This partner will provide guidance and assistance in collecting ballast water samples from commercial ships entering the Duluth-Superior Harbor. He will not receive ENRTF funds from this appropriation.

B. Project Impact and Long-term Strategy:
This project will identify the prevalence of potentially harmful bacteria within the SLRE and compare these findings with new and existing data about the sources of these microbes. This information can be used to forecast the potential risks of introducing harmful microbes on the ecological and economic sustainability of the St. Louis River Estuary and the ballast water treatment activity will provide management strategies to mitigate the risk of unintentionally introducing new bacterial invasive species.

C. Funding History:

Funding Source and Use of Funds	Funding Timeframe	$ Amount
Environmental and Natural Resources Trust Fund: M.L. 2011, First Special Session, Chp. 2, Art.3, Sec. 2, Subd. 06a; M.L. 2014, Chapter 26, Section 2, Subdivision 19	7/1/2011 to 6/30/2015	$250,000

VIII. FEE TITLE ACQUISITION/CONSERVATION EASEMENT/RESTORATION REQUIREMENTS:

A. Parcel List: N/A

B. Acquisition/Restoration Information: N/A

IX. VISUAL COMPONENT or MAP(S): See attached map

X. RESEARCH ADDENDUM: A research addendum was peer reviewed for the parent project earlier in 2011 - M.L. 2011, First Special Session, Chp. 2, Art.3, Sec. 2, Subd. 06a. A research addendum was not requested for this continuing project.

XI. REPORTING REQUIREMENTS:
Periodic work plan status update reports will be submitted no later than January 2017, July 2017, January 2018, July 2018, July 2020 and January 2021. A final report and associated products will be submitted between June 30 and August 16, 2021.

Environment and Natural Resources Trust Fund
M.L. 2016 Final Project Budget

Project Title: Advancing Microbial Invasive Species Monitoring from Ballast Discharge
Legal Citation: M.L. 2016, Chp. 186, Sec. 2, Subd. 06c
Project Manager: Randall E. Hicks
Organization: University of Minnesota Duluth
M.L. 2016 ENRTF Appropriation: $368,000
Project Length and Completion Date: 5.0 years, June 30, 2021
Date of Report: August 16, 2021

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Activity 1 Budget	Amount Spent	Activity 1 Balance	Revised Activity 2 Budget 07/28/2017	Amount Spent	Activity 2 Balance	Activity 3 Budget	Amount Spent	Activity 3 Balance	TOTAL BUDGET	TOTAL BALANCE
BUDGET ITEM	***Collect Ballast Water from Commercial***						***Taxonomically Identify the Common and***				
Personnel (Wages and Benefits)	$86,530	$72,431	$14,099	$65,855	$57,284	$8,571	$100,420	$98,847	$1,573	$252,805	$24,243
Project Manager (Randall Hicks; $29,939; 74.8% salary+25.2% fringe; July 1, 2016-June 30, 2018; 8.4% FTE)											
Postdoctoral Associate (1 person to be announced; $138,892; 81.7% salary+18.3% fringe; July 1, 2016-Sept 20, 2018; 100% FTE)											
Graduate Research Assistant (1 person to be announced; $83,974; 55.0% salary+45.0% fringe; July 1, 2016-Aug 31, 2018; 50% FTE)											
Professional/Technical/Service Contracts											
~~*Northeast-Midwest Institute subcontract to design, set up, run bench-scale experiments and evaluate standard microbial assessment tools for fecal indicator bacteria.*~~											
University of Wisconsin Superior-Lake Superior Research Institute subcontract to design, set up, run bench-scale experiments and evaluate standard microbial assessment tools for fecal indicator bacteria. (07/28/2017)				$44,500	$44,500	$0				$44,500	$0
Equipment/Tools/Supplies	$15,553	$6,020	$9,533	$12,568	$10,792	$1,776	$23,921	$18,897	$5,024	$52,042	$16,333
Water and sediment sampling supplies ($3,650)											
DNA extraction and PCR reagents for assays($13,922)											
Illumina sequencing and data storage costs for DNA analyses$20,270)											
Chemicals and expendable lab supplies($14,200)											
Travel expenses in Minnesota											
Sampling trips to the St. Louis River estuary (20 trips) and GSI facility (10 trips) - $600; Travel support to disseminate project results at public venues, scientific conferences, and outreach events with target organizations - $2,508. Matching conference travel funding will be sought from the University of Minnesota.	$1,200		$1,200	$708	$16	$692	$1,200		$1,200	$3,108	$3,092
Other											
Publication costs for journal articles	$700		$700	$645		$645	$700		$700	$2,045	$2,045
R/V Blue Heron ship time			$0			$0	$8,500		$8,500	$8,500	$8,500
Small boat rental (10 days @ $500/day)			$0			$0	$5,000	$3,075	$1,925	$5,000	$1,925
COLUMN TOTAL	**$103,983**	**$78,451**	**$25,532**	**$124,276**	**$112,592**	**$11,684**	**$139,741**	**$120,819**	**$18,922**	$368,000	**$56,138**