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Assessing Ballast Water (BW) Management and Invasions in the Great Lakes: Site Selection and Draft Protocol for Shipboard Plankton Sampling at BW Sentinel Sites

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Mr. Alan Arsenault
Technical Director
United States Coast Guard
Research & Development Center
1 Chelsea Street
New London, CT 06320

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Ms. Gail Roderick, 860-271-2658, email: gail.e.roderick@uscg.mil.

16. Abstract (MAXIMUM 200 WORDS)

Commercial shipping is a major unintentional transport mechanism for aquatic organisms worldwide. Management measures, specifically for ballast water (BW), aim to remove or inactivate organisms prior to discharge in a new environment. This study identifies a BW sentinel site within the Great Lakes and aims to establish measurement methods to assess the concentration and diversity of BW biota prior to discharge. The team reviewed data from the National Ballast Information Clearinghouse and identified Duluth/Superior harbor as the initial Great Lakes BW sentinel site due to it being a location with high vessel traffic and BW discharge activity. In addition, the team established a working group with local collaborators to formulate effective sampling strategies, which use established laboratory protocols with modifications to address the working conditions on Laker vessels. Vessels transiting the Great Lakes carry unmanaged, exchanged and treated BW, and as such this study will assess all management levels. This study will sample vessels to obtain vital baseline information on the biota present in BW at a major port system within the Great Lakes. The team will continue to build on this study in outyears by increasing sample size at the sentinel site, with the potential to expand efforts to other sentinel sites.

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EXECUTIVE SUMMARY

Commercial shipping, primarily via ballast water and hull fouling, unintentionally spreads aquatic organisms across the globe through routine vessel operations. These organisms can have unintended, detrimental impacts on the receiving environment, and as such, it is necessary to mitigate the future spread of organisms via ships. This led to development and implementation of International Maritime Organization conventions and United States Coast Guard (USCG) regulations for ballast water management prior to discharge. Management techniques (i.e., ballast water exchange and onboard treatment) and their implementation aim to minimize future opportunities for organisms to spread. Studies which assess the efficacy of these management techniques are vital to quantify their success and inform the future management strategy.

This project will establish ballast water (BW) sentinel sites within the United States (US) Great Lakes in locations that receive high levels of vessel traffic and ballast water discharge. Once identified, a scientific sampling team will conduct baseline sampling on vessels to assess the diversity and concentration of biota present in ballast water in all management conditions (i.e., unmanaged, exchanged and treated).

The protocols described in this report are established sample collection and analysis methods already used to sample BW on vessels arriving at US coastal ports. The science team will collect ballast water samples directly from ballast tanks via a tank-access manhole. Samples will be returned to the laboratory and live analyses conducted on organisms ≥50µm (i.e., assessment of visible movement), ≥10 <50 µm (i.e., viability stains), and indicator microbes *Enterococci* and *E.coli* (i.e., using standard IDEXX® techniques). These analyses will enable the team to detect both the diversity of biota present in tanks, and also the concentration of live organisms, specifically in the three size classes outlined in the USCG Ballast Water Discharge Standard. These data will provide vital information regarding the efficiency of management methods, specifically, the expected reduction of invasion risk at increasing levels of management (i.e., unmanaged < exchanged < treated).

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ACRONYMS, ABBREVIATIONS, AND SYMBOLS

BW	Ballast Water
CMFDA	5-chloromethyl fluorescein diacetate
EPA	Environmental Protection Agency
FDA	Fluorescein diacetate
LSRI	Lake Superior Research Institute
MSU	Marine Safety Unit
NBIC	National Ballast Information Clearinghouse
NIS	Nonindigenous species
PPE	Personal protective equipment
SERC	Smithsonian Environmental Research Center
SOW	Statement of Work
US	United States
USCG	United States Coast Guard

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1 BACKGROUND AND OBJECTIVES

Commercial ships are a dominant source of biological invasions by nonindigenous species (NIS) in coastal waters, resulting from the unintentional delivery of aquatic organisms associated with the ballast water (BW) and outer hull surfaces of vessels. This has led to regulations by the United States Coast Guard (USCG) and United States Environmental Protection Agency (EPA) for ships to use management measures on their ballast water prior to discharge in United States (US) waters. The intent is to reduce the abundance and richness of NIS delivered, preventing new invasions and their associated impacts.

A key performance measure of the USCG BW Management Program is whether there is a reduction in concentration of organisms discharged in ballast water, and specifically whether vessels meet current specific discharge standards. The Smithsonian Environmental Research Center (SERC) Marine Invasions Research Lab, in cooperation with the USCG, has been sampling vessels' ballast water prior to discharge in US coastal waters at key (indicator) sentinel sites. Previous studies have focused on the concentration and diversity of biota in unmanaged and exchanged ballast water. Present studies quantify the concentration and diversity of live organisms in treated BW, with specific reference to the USCG Discharge Standards and the three regulated size classes.

The current project aims to establish one or more sentinel sites for BW sampling in the Great Lakes that complement several BW sentinel sites on the US Atlantic, Gulf, and Pacific coasts.

Specifically, this project will design, implement, and evaluate the concentration of organisms in BW arriving to one or more ports in the Great Lakes, using standardized measures to (a) assess whether vessels meet current discharge standards and (b) allow direct comparisons of measures at BW sentinel sites among US coastal regions that assess overall compliance and variation in both space and time.

In Year 1 of this project (2020), the two primary objectives (tasks) for BW surveys were:

Objective 1: **Synthesis and evaluation of commercial shipping and BW delivery/management patterns to US ports of the Great Lakes.** This identifies potential hotspots (ports) for NIS introductions by ships' BW to be considered as candidate sites for NIS field detection surveys and for shipboard BW sampling.

Objective 2: **Design, implement, and evaluate sampling protocols to measure plankton concentrations in ships' BW of vessels arriving to Sentinel Sites in the Great Lakes.** This establishes specific survey locations and methods for NIS detection, based on the Objective 1 evaluation, site visits and logistics, regional coordination with USCG and potential partners, and pilot measures/analyses.

For Objective 1, the team previously conducted in-depth analysis of past and current shipping and BW discharge in US ports of the Great Lakes, using detailed data from the National Ballast Information Clearinghouse (NBIC). A similar analysis was conducted for NIS distribution reported for the Great Lakes. This analysis indicated the Duluth/Superior region as a relative hotspot, in terms of both the magnitude of BW discharge (from both Laker and international arrivals) and the number of known NIS associated with BW as a possible vector.

For Objective 2, the team has already developed and implemented standard protocols for sampling biota in ships' BW at sentinel sites on the Atlantic, Gulf, and Pacific coasts. Unlike field surveys for detection of NIS that differ between freshwater and marine communities, the identical methods can be used for vessels arriving at both Great Lakes and coastal ports. The team also initiated a working group with local partners, using monthly meetings to coordinate regional shipboard sampling. Based on this work, this report provides a brief summary and overview of the draft protocol for the Great Lakes BW sentinel site surveys.

In general, this work focuses on vessel BW sampling and analysis, and on-going syntheses data from the National Ballast Information Clearinghouse to evaluate BW discharge in the Great Lakes. (A separate report addresses parallel measures underway to detect sentinel site NIS.)

2 APPROACH

2.1 Rationale for BW Sentinel Site Selection

To identify the first BW sentinel site, the team used NBIC data, specifically vessel arrival numbers and BW discharge volumes for a representative year (2019). The team looked for locations with high vessel arrival numbers and high volumes of BW discharge, see Figures 2.1.1 and 2.1.2.

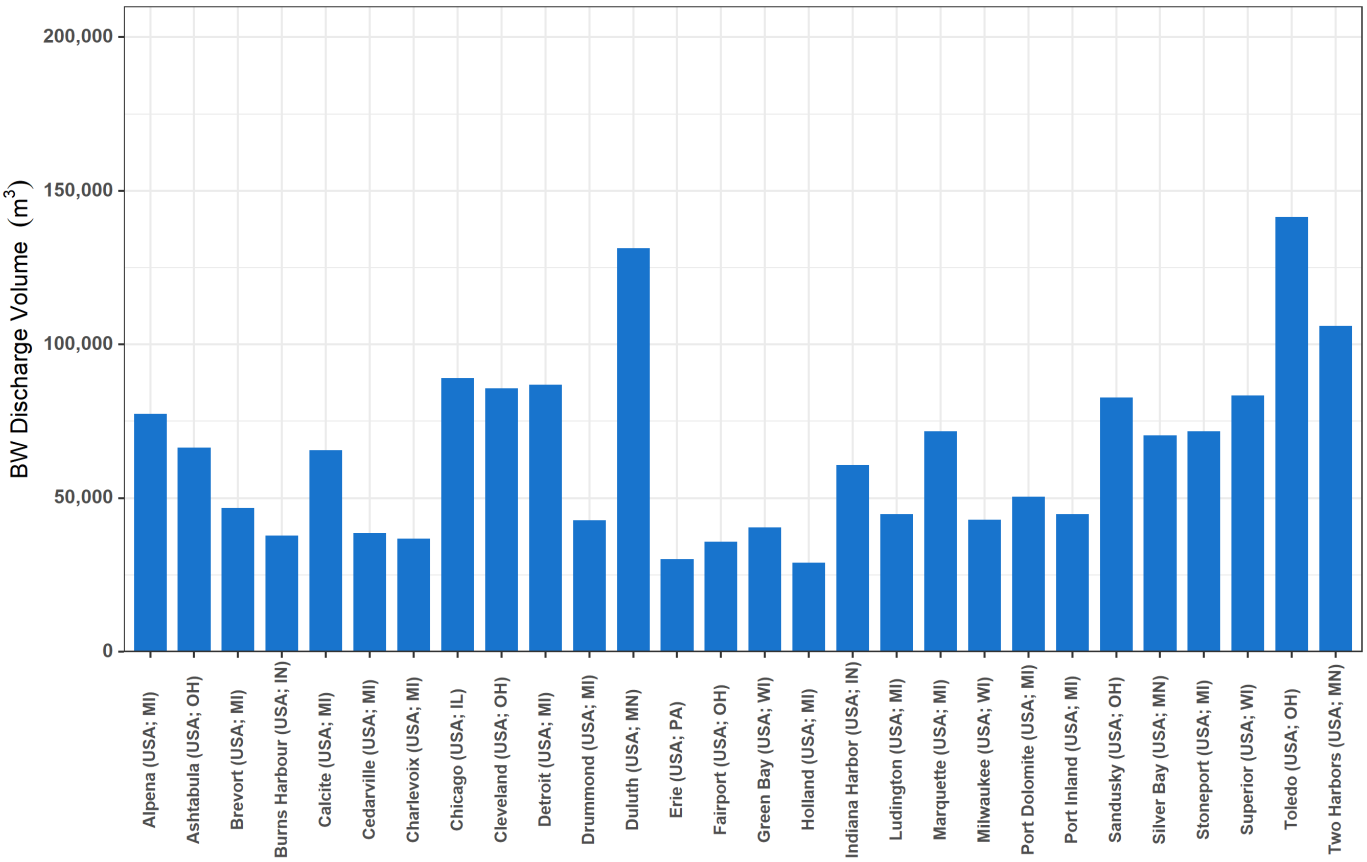

Figure 1. Total ballast water discharge volumes to ports across the Great Lakes in 2019. Only ports receiving > 25,000 m^3 included. (NBIC).

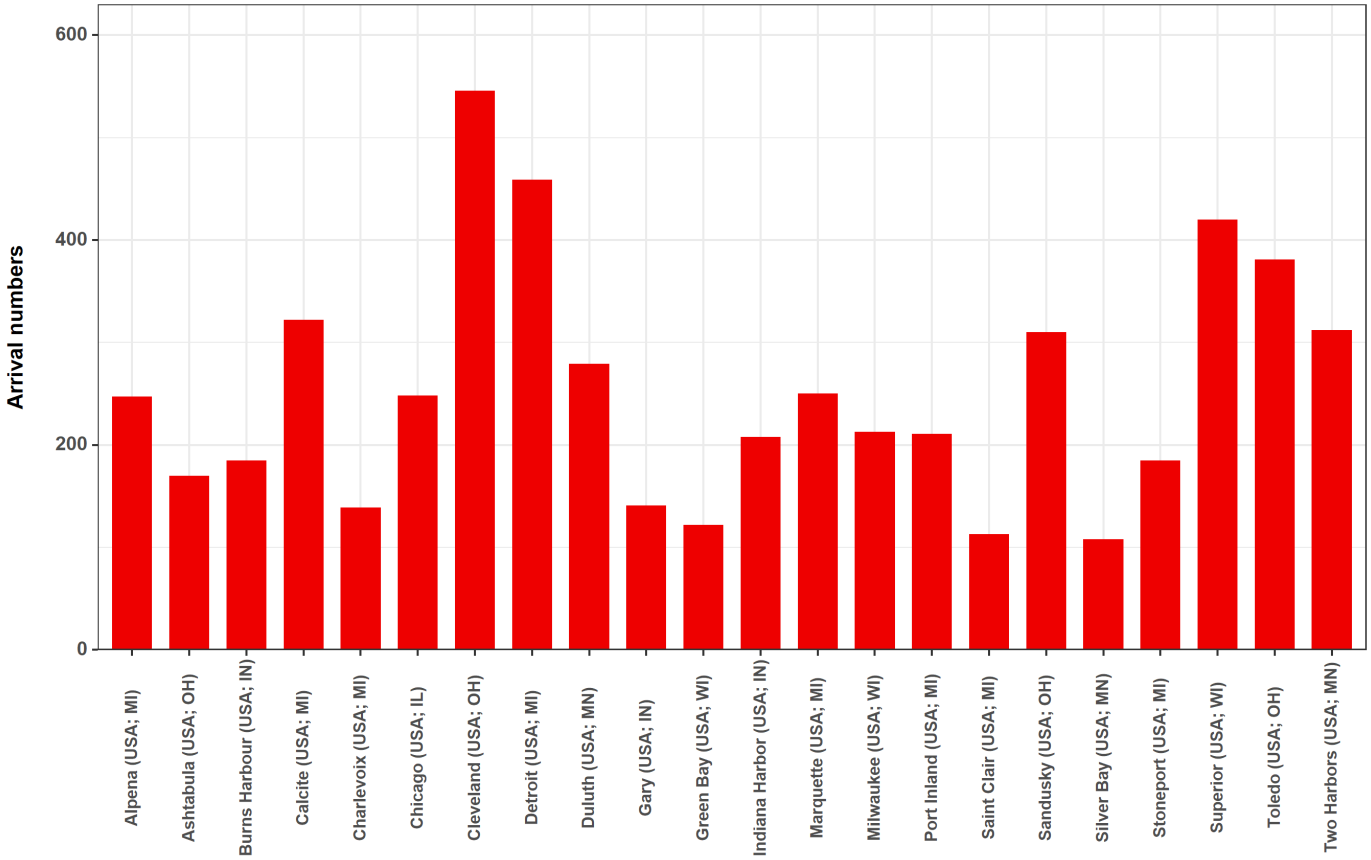

Figure 2. Total vessel arrival numbers to ports across the Great Lakes in 2019. Only ports receiving > 100 arrivals were included. (NBIC).

The counts in Figure 1 and Figure 2 represent discharge volumes and arrivals that include vessels arriving from outside the Great Lakes, and Lakers, whose trade (and in some instances, size) restricts them to the Great Lakes. Not all ports shown in Figure 1 appear in Figure 2.

The BW discharge volume and arrival number data highlighted Lake Superior, specifically the ports of Duluth and Superior, as a potential focal location for the first BW sentinel site. These ports combined receive the highest vessel arrival numbers and the highest volumes of BW discharge. There are also two other ports, Two Harbors and Silver Bay, within close proximity that offer potential sampling opportunities, but do not receive vessels from outside the Great Lakes. Figure 3 indicates BW discharge volumes by geographic area, highlighting the large quantities discharged near the western Lake Superior ports. Also note the multiple instances of mid-lake BW discharge.

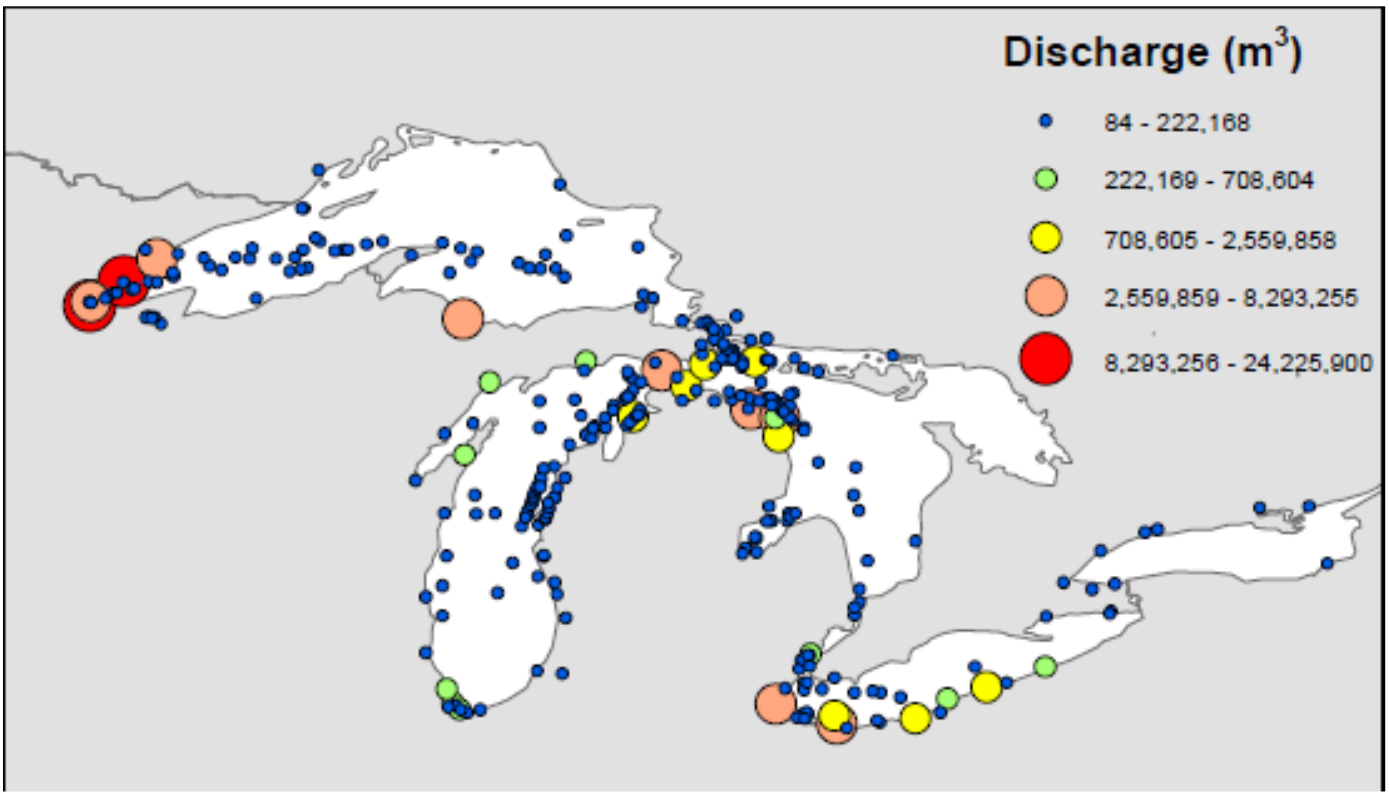

Figure 3. Cumulative volume of ballast water discharge reported by location in the Great Lakes for the two year period 2018 – 2019. (NBIC)

In addition, project collaborators (including EPA and Lake Superior Research Institute (LSRI)) are based in Duluth, which provides important laboratory and facilities access for the sampling team on the ground. Finally, USCG Marine Safety Unit (MSU) Duluth agreed to provide support for the sampling campaign, in terms of identifying potential target vessels, coordinating access with vessels and providing an escort for the sampling team.

2.2 Overall Sampling Design and Analyses

2.2.1 Overview of Sampling Activities

Shipboard sampling of vessels entering the Port of Duluth/Superior commenced in August/September 2021. The team intended to sample BW from at least ten vessels arriving to this sentinel site for 2021 (Project year 2). Sampling was performed on vessels carrying all types of BW (i.e., unmanaged, exchanged and treated), to give a full picture of biota in BW transported in the Great Lakes. All sample collection uses standard methods in summer-fall (warm season) to control for seasonal variation across years. Quantification of zooplankton and phytoplankton concentrations uses standard methods to evaluate size-based concentrations relevant to USCG discharge standards. Activities focus on bulk cargo vessels, both Lakers and arrivals from outside the St Lawrence Seaway.

2.2.2 Sample Collection

Vessels will be boarded by a sampling team on an opportunistic basis, aided by MSU Duluth or other collaborators, as applicable. Once onboard, the team members will collect the ballast water history for the tank to be sampled. This includes BW source location, age of BW, management status (none, exchanged, treated), type of management (if applicable), location of management (if applicable), and the volume of BW in tank.

a. Grab sample collection

The team will collect samples directly from the manhole or deck opening with assistance from the vessel's crew. Upon arrival, the accessible tank water depth will be measured, and a temperature and salinity profile of the tank at surface, mid and bottom of each tank will be collected using a handheld Yellow Spring Instruments (YSI®) conductivity, temperature and depth (CTD) sonde. Initially, grab samples will be collected directly from the tank using a bailer (or similar device) lowered to mid-tank depth for analysis of organisms 10-50 µm and indicator microbes (i.e., *E.coli* and *Enterococci*). Any samples required for environmental DNA (eDNA) or other method will be collected first using sterile collection equipment to limit any opportunity for contamination.

eDNA is DNA from specific organisms found in the environment. Using new molecular methods, researchers can sample and monitor traces of cellular material cast off by target organisms. This methodology can be used for the early detection of invasive species and identification of cryptogenic species (i.e., species of uncertain origin).

b. >50-µm sample collection

For unmanaged or exchanged ballast water tanks, plankton nets (35-µm or 80-µm) will be lowered vertically into the tanks for the full depth of the accessible water column. After vertically "towing," the net will be rinsed using pre-filtered BW and the sample will be collected. The team will perform two replicate vertical tows and collect each sample independently for subsequent analysis.

For BW treated using onboard ballast water treatment systems (or where net tows are not possible), the team will lower a submersible pump into the ballast tank to mid-depth, and pump >1000m^3 through a 35-µm plankton net. The net will be rinsed down using pre-filtered BW, and the sample will be collected.

Appendix A provides the detailed, standard shipboard procedures for this sampling effort.

2.2.3 Sample Analysis

Samples collected for indicator microbes, organisms 10-50 µm and organisms >50 µm will be analysed live. The team will use standard IDEXX® techniques for indicator microbes. Organisms 10-50 µm will be assessed using the fluorescein diacetate (FDA)/5-chloromethyl fluorescein diacetate (CMFDA) dual staining technique to quantify live individuals per mL. Organisms >50 µm will be assessed under a dissecting microscope using mobility to quantify live organisms per m^3. If organisms do not exhibit internal or external

mobility they will be 'poked' to try and induce mobility, if no movement is still observed the organism will be deemed dead.

2.2.4 Additional Opportunistic Sampling

The research team has sought collaborators to provide local expertise and if expanding the project scope of is warranted. The team is investigating the possibility of sampling in other parts of the Great Lakes, including Canada. In addition, the team is looking at the possibility of co-sampling vessels carrying treated BW with LSRI; sampling directly from the discharge line to compare with the in-tank sampling. Finally, the team is working on genetic sampling protocols to identify relevant measures that could add valuable information regarding the transport of non-native species in BW around the Great Lakes, such as identifying target species of concern and also community composition.

2.3 Statistical Analysis Plan

Using the resulting data, the team will conduct a standard array of statistical analyses to evaluate the concentrations of organisms discharged in BW, as a function of source and management. This will be reported each year and cumulatively, at the end of the project. As part of this project, the team will also characterize the volume of BW discharged to Duluth/Superior region and the Great Lakes more broadly as a function of source and treatment, based on data from the National Ballast Information Clearinghouse.

The biological measures will be compared directly to those available from the Atlantic, Gulf, and Pacific BW sentinel sites at the end of the project.

As possible, the team will also seek opportunities to (a) compare inline to open tank sampling, working with LSRI, and (b) evaluate community composition with collaborators, interested in leveraging the sampling effort.

Finally, the team will investigate opportunities to expand sampling of ships' BW to additional major Great Lakes ports, especially to ports that reflect different trading patterns.

3 CONCLUSIONS

This report establishes repeatable sampling and analyses processes for phytoplankton, zooplankton, and indicator microbes in BW aboard vessels that discharge in the Duluth/Superior Harbor. These procedures will provide consistent methodology over the next three years of sampling, allowing researchers to clearly note changes in BW NIS community presence, if changes are indeed occurring. Should this type of sampling program warrant expansion to other Great Lakes ports, research teams can follow the same procedures to arrive at sampling results and trends that could then be compared or contrasted to the Duluth/Superior results.

APPENDIX A. SOP: SHIPBOARD SAMPLING PROCEDURES

A.1 Equipment Required

A.1.1 General

- Minimum personal protective equipment (PPE) required: hard hat, steel toe footwear, safety glasses and high-visibility yellow vest.
- Ear plugs (for loud environments).
- Sampling datasheet, clipboard, pens, pencils, sharpie.
- USCG ship sampling letter.
- SERC BW Sample Collection Consent Form.
- Certificate of liability.
- Freshwater-calibrated (for lakers) or saltwater-calibrated (for salties) YSI CTD sondes.

Note: Team will email/submit a guest release form before boarding Interlake Steamship Company vessels.

A.1.2 Zooplankton (Including Genetic Samples)

- Plankton nets (2 of each) – 80-µm and 35-µm mesh, 30-cm diameter (cleaned/rinsed, checked for holes and repaired if needed). Nets should be stored in separate bags within the backpacks to keep clean until sampling.
- Cod end bucket *(80-µm and 35-µm mesh).*
- ¼ inch synthetic rope *(2 x vessel molded depth).*
- Transect tape *(weighted).*
- Spray washer.
- Rinse bottle.
- White tray.
- 7 µm sieve.
- 35-µm sieve.
- 80-µm sieve.
- Funnel.
- Submersible water pump.
- Battery and/or power converter.
- Hosing and hose clamps.
- Flat headed screwdriver.
- Bucket *(optional, for collecting rinse water to filter).*
- Zooplankton collection bottles (35-µm plankton net – live analysis): 2 x 125-mL, 2 x 250-mL and 1 x 500-mL Nalgene® bottles *(labelled).*
- 2 x 2-L Nalgene bottles *(rinse water).*
- Zooplankton collection bottles (35-µm and 80-µm plankton net – for genetics): 2 x 125-mL.
- Preprinted labels.

A.1.3 Phytoplankton, Indicator Microbes and eDNA Sample Collection

- Bleach-washed water bailer with line attached.
- Turner Aquafluor (2 chamber cells and solid standard).
- DI water.
- Kim wipes.
- Phytoplankton collection bottles: 3 x 500-mL Nalgene amber bottles.
- Indicator microbes collection bottles: 4 x 100-mL sterile IDEXX bottles (sodium thiosulphate) – includes spare.
- eDNA collection bottles: 2 x 500-mL Nalgene bottles.

A.1.4 Zooplankton Genetics Lab Equipment

- Ethanol (30 mL per sample).
- 35-µm sieve.
- 80-µm sieve.
- Funnel.
- Falcon tubes (50-mL).
- Preprinted labels.

A.1.5 Shoreside cleaning Equipment (For Cleaning the Water Bailer Before Each Event)

- 10% bleach.
- Bleach rinse bottle.
- Parafilm paper (to seal each end of the bailer after cleaning).

A.2 Methods

A.2.1 Ballast Water Metadata Collection

- To board the vessel wear appropriate PPE:
  - Minimum PPE required: hard hat, steel toe footwear, safety glasses, and high visibility yellow vest.
  - Carry earplugs in case the working environment onboard is loud.
  - Each team member should carry a flashlight or secure a headlamp to their hard hat prior to boarding.
- Upon boarding, meet with the crew, ideally Chief Mate, to determine which tank will be sampled.
- Collect the following information from the ballast water reporting form/log and record it on the sample collection datasheet:
  - Tank ID.
  - Tank capacity.
  - Ballast water age (i.e., number of days between the date of uptake into tank and the sampling date). In the case of exchanged tanks, the BW age will be the number of days between the date of BW exchange and the sampling date.
  - Volume of ballast water in the tank.
  - Type of management (i.e., unmanaged or exchanged).

- Ask a crew member to escort the sampling team to the ballast tank and confirm access through a manhole on deck. Crew will need to open the manhole to provide access.

Note: *Once the manhole is opened, eDNA samples are collected before doing anything else.*

A.2.2 eDNA Samples Collection

- Wear gloves.
- Attach the bleach-washed water bailer to a line and rinse three times with tank water.
- Lower the bailer into the ballast tank to approximately 0.5 m to collect water.
- Fill the 2-500-mL amber Nalgene® bottles after rinsing three times.
- Store on ice until dropping off to EPA for filtering.
- Once sampling is done, these samples should be dropped immediately at the EPA facilities (Chelsea).

A.2.3 Depth Profile Collection

- Samples will be collected using a submersible pump requiring a power source, so inform crew that you will need an electrical outlet.
- To begin the sampling process, first use the transect tape to measure the following:
  - Distance from manhole to the surface of the water.
  - Total depth from manhole to bottom of tank.
- Use the depth measurements to calculate the water depth and the mid-tank depth. Record both on the datasheet.
- Use the YSI sonde to collect salinity and temperature data at surface, mid-tank and bottom depths of the tank. (Surface = approx. 1 ft below surface, bottom = approx. 1 ft above bottom).
- Attach the water bailer to the line and lower three times (i.e., to each surface, mid-tank and bottom depths) to collect water for chlorophyll α measurement using the Turner Aquafluor. Record on the datasheet.

A.2.4 Zooplankton Pump Sampling (Live Analysis and Genetics)

- Set up the submersible pump and lower to mid-tank depth. Secure at this depth by tying off the rope.
- Using the 10-L carboy, start running water through the pump and record how long it takes to fill the carboy. Use this to calculate the flow rate.
- Calculate the time it will take to pump 1,000 L (genetics) and 1,600 L (live analysis):

$$Pump\ time\ (minutes) = \left(\frac{Number\ of\ seconds\ to\ pump\ 10\ L}{10}\right) * pump\ volume\ (L)/60$$

A.2.5 Genetics Sample Collection

- Use this method to collect 1,000L pump samples with (1) 35-µm net and (2) 80-µm net.
- Secure the 35-µm net in the tank, ideally so at least half of the net is in the water.
- Secure the hose from the pump at the top of the net so water will flow into the net.
- Start running the pump and collect 1,000 L through the net.
- Repeat this method to collect a sample with the 80-µm net.
- Handle samples in the same way as live analysis samples (Section 2.6).

A.2.6 Live Analysis Sample Collection

- Secure the 35-µm net in the tank, ideally so at least half of the net is in the water.
- Secure the hose from the pump at the top of the net so water will flow into the net.
- Start running the pump and collect 1,600 L through the net.
- Meanwhile, use the water in the 10L carboy to collect samples for IDEXX (indicator microbres) and 10-50 µm (phytoplankton):
    - 3 x 500-mL phytoplankton samples in 500-mL amber Nalgene bottles (rinse bottles 3 times prior to collection).
    - 4 x 100-mL bacteria samples in sterile 100-mL bottles.
- While the pump is collecting the zooplankton sample, make rinse water to fill 2-L bottles for hygiene tests, rinse bottle and spray washer. Stack the 35-µm and 7-µm filters on top of the spray washer. Pour any remaining water in the 10-L carboy through the filters. For additional water, lower the bailer or a bucket into the tank to collect water to filter. Ensure there is also enough rinse water to take back to the laboratory for the zooplankton analysis.
- Once the pump sample has been collected, turn off the pump and remove the hose from the net.
- Rinse the net using the spray washer to condense the sample in the cod end.
- Carefully pour the sample from the cod end into the labelled sample bottle using the funnel.
- Prior to taking another sample, thoroughly rinse the net and cod-end using the spray washer.

Note: As an alternative to using the pump, collect the zooplankton sample by vertical net tows. Use the height of the water column to determine how many plankton tows must be performed. To tow 1.6 m^3, 22 m (72.2 ft) of water must be towed through. After each tow, the net and cod-end must be rinsed and emptied into a sample bottle. Samples from the different tows are combined into one sample from each tank.

A.2.7 Zooplankton Genetics – Lab Processing

Note: After sampling, the zooplankton samples for genetics (i.e., 1 x 35-µm pump sample, 1 x 80-µm pump sample) will be processed under a fume hood in the Lake Superior Research Institute at the University of Wisconsin-Superior.

- Filter the sample using a 35-µm sieve to remove water.
- Rinse sample with 30 mL ethanol into a 50-mL falcon tube using a funnel.
- Repeat the process for the second replicate.
- Label falcon tube with the event ID, month/year, preservative, mesh size).

Net care: Rinse with fresh-water, dry, store and transport away from sharp or abrasive objects. Damage up to small holes may be fixed with a dab off silicon sealant.

Note: Bleach-wash the water bailer after each sampling event and use parafilm to seal both ends.