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Assessing Ballast Water Management and Invasions in the Great Lakes: Recommendation of Site Selection and Draft Protocol for NIS Sentinel Sites

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16. Abstract (MAXIMUM 200 WORDS)
Nonindigenous species (NIS) can be a significant concern in U.S. waters causing extensive economic and ecological impacts. Ballast water discharge from commercial vessels is a major pathway in which nonindigenous species can be introduced. Although the U.S. Coast Guard has regulations for ballast water discharge, there are no standardized measurements currently established to assess the performance of these management programs in most geographic regions. This project aims to establish such measures at a sentinel site in the U.S. Great Lakes, to be conducted as repeated measures over multiple years. This report provides recommendations and rationale for site selection and sampling protocols for field-based detection measures, as specified in Year 1 of the project. The research team has selected Duluth-Superior harbor as a NIS sentinel site for this project, based on the relatively high volume of ballast water discharge, record of invasions, and partners with expertise at this focal site. The team is comprised of researchers from Smithsonian Environmental Research Center (SERC), USCG Research and Development Center (RDC), and collaborators from international, national, regional and local agencies. The team drafted protocols for field surveys to detect NIS and allow direct comparison to existing NIS sentinel sites in U.S. marine ports. The protocols detailed here involve sampling both the benthic and planktonic communities using a combination of habitat collectors, sediment grabs, and plankton pumps. This suite of sampling methods will ensure that repeatable, standardized data are collected, which will provide a comprehensive view of NIS presence within the areas sampled and how that relates to ballast water management.

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EXECUTIVE SUMMARY

Nonindigenous species (NIS) can be a significant problem in U.S. waters both in terms of their economic and ecological impacts if they become established in new areas. Commercial vessels are an important vector in the transport of new NIS through the discharge of ballast water and through the attachment of organisms on the ships' hulls. The U.S. Coast Guard and U.S. Environmental Protection Agency (EPA) have created regulations for ballast water discharge in U.S. waters, yet direct measurements are needed to assess the performance of the management programs. Specifically, baseline data in U.S. waters detailing the community composition are needed to effectively assess the performance (effects) of these regulatory programs on reducing invasions.

This report provides (1) a brief rationale for selection of a specific NIS sentinel site in the U.S. Great Lakes and (2) draft protocols that the research team, working with partners in other organizations, has developed to apply at the NIS sentinel site in the U.S. Great Lakes. The primary goal of the overall project design and implementation is to establish standardized baseline data on the detection of NIS, which can be used to provide a robust assessment of the performance of the ballast water management programs at one or more sites in the U.S. Great Lakes. In addition, the sampling protocols will allow for direct comparisons of NIS detection rates in other U.S. regions, especially coastal marine waters of the U.S., to examine changes in invasions across both space and time. Finally, the protocols in this report will provide data that will help improve and optimize future sampling designs in detecting NIS.

The protocols in this report are designed to specifically sample the annelid, crustacean, and molluscan communities within the benthos and the planktonic community. These are the taxa most likely to be detected and recognized as new (i.e., previously undetected) NIS in the U.S. Great Lakes, based on the historical record of invasions and the existing knowledge of taxonomy and global distribution of aquatic organisms. For benthic communities, both habitat crates filled with oyster shells and Hester-Dendy arrays will be deployed for six weeks to attract benthic organisms. These methods have been extensively used both in freshwater and marine systems to sample organisms and will likely be efficient in detecting many nonindigenous species present. In addition, soft-sediment grabs will be conducted to sample for additional species in soft-bottom communities that the habitat crates and Hester-Dendy arrays might not capture. To sample planktonic organisms in the water column, a plankton pump will be used to sample the zooplankton community, using standard plankton nets to filter out organisms present. A combination of morphological and genetic analyses is to be used to identify the organisms collected from these sampling methods and determine if NIS are present.

The NIS sentinel site selection and the specific protocols are designed explicitly to provide a high-quality statistical measure of NIS detection in selected key habitats. For the selected habitats, these intensive efforts should result in data with less variability and higher confidence in the resulting conclusions. In addition, this sampling design provides repeatable, standardized measures that are essential when evaluating the efficacy of ballast water management and creating a sentinel site.

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LIST OF ACRONYMS AND ABBREVIATIONS

°C	Degrees Celsius
BW	Ballast Water
$CaCO_3$	Calcium carbonate
cm	Centimeter
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DNE	Preservative that includes DMSO, NaCl, EDTA
EDTA	Ethylenediamine tetraacetic acid
EPA	Environmental Protection Agency
Gal	Gallon
HD	Hester-Dendy
L	Liter
m	Meter
m^2	Square Meter
m^3	Cubic Meter
mm^2	Square Millimeter
NaCl	Sodium chloride
NIS	Nonindigenous Species
SERC	Smithsonian Environmental Research Center
µm	micron
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 U.S. Coast Guard (USCG) and U.S. Environmental Protection Agency (EPA) for ships to undertake management practices on their ballast water prior to discharge in U.S. waters. These practices reduce the abundance and richness of NIS delivered, in order to prevent new invasions and their associated impacts.

A key performance measure of the USCG BW Management Program is whether there is a reduction in new ballast-mediated invasions, especially for locations with significant shipping activity. No national program using standardized, repeatable procedures to detect new invasions in U.S. waters currently exists; such a program is necessary to create baseline data to assess efficacy in reducing invasion rates. In part to address this gap, the Smithsonian Environmental Research Center (SERC)'s Marine Invasions Research Lab, in cooperation with the U.S. Coast Guard, has been establishing standardized performance measures for the USCG's Ballast Water Management program in coastal waters at key (indicator) sentinel sites in U.S. waters.

The current project aims to establish one or more sentinel sites for NIS detection in the Great Lakes that complement several NIS sentinel sites on the U.S. Atlantic, Gulf, and Pacific coasts. Specifically, this project will design, implement, and evaluate detection of NIS in the Great Lakes, using standardized and quantitative methods comparable to those established at coastal marine sites by the U.S. Coast Guard. This overall approach will (a) provide a robust assessment of effects (performance) of the BW Management Program on reducing new NIS invasions and (b) allow direct comparisons of NIS detection rate among U.S. coastal regions that assess changes in invasions in both space and time.

In Year 1 of this project, the two primary objectives (deliverables) for NIS sentinel site surveys were:

1) Provide a recommendation on selection of the NIS sentinel site(s) for this project. This recommendation includes a brief summary of the rationale for site selection.
2) Provide a draft protocol for survey methods for the recommended NIS sentinel sites. This will provide the rationale, strengths, and weaknesses of potential methods and outline the sampling plan to be implemented.

For Objective 1, the research team conducted in-depth analysis of past and current NIS biodiversity surveys in the Great Lakes, as well as current shipping and ballast water discharge levels for major U.S. ports. The team also convened a multi-institutional working group that met on a monthly basis to further evaluate existing approaches, methods, and coordination for establishing NIS sentinel site surveys. Based on this analysis, the team selected the Duluth/Superior region as the primary NIS sentinel site for sampling across Years 2-5. This location was selected because of the high number of known invasions in the region (e.g., Hoffman et al. 2011; NAS 2021) and the high volume of ship traffic and ballast water discharge from both lakers (i.e., U. S. and Canadian flag vessels whose trade routes are limited to the Great Lakes) and international vessels that transfer NIS (National Ballast Information Clearinghouse 2021). Selection of this site also promotes a collaborative approach with multiple groups actively focused on ship-mediated invasions and management at this location. The team details the rationale for selecting Duluth/Superior as a sentinel site in section 2.1

For Objective 2, the team conducted an extensive literature review and evaluated the application of methods used at existing NIS sentinel sites on marine coasts, for benthic and planktonic communities, where ballast-mediated invasions are common. The team also convened regular monthly meetings with a core working group that had extensive experience with both morphological and molecular methods of NIS detection, including both data workflows and statistical analyses. Based on this work to date, the team provides a brief summary and overview of the draft protocol for the Great Lakes NIS sentinel site surveys, in section 2.2 and subsequent sections of this document.

Based on this plan, the team implemented the protocol in summer of 2021, and results from implementation will be the subject of future reports. In general, this work will focus on field-based surveys and on-going literature syntheses, to detect new NIS invasions in the Duluth/Superior region. Other sites may be added, as opportunities allow. A parallel set of measures are also underway to evaluate biota present in ships' ballast water arriving to this sentinel site, and these will be addressed in a separate report.

2 APPROACH

Working with agency partners, the team designed measures to (a) assess the detection rate of new (previously unknown) NIS in benthic and planktonic communities in the Great Lakes associated with ships' BW and commercial shipping as a potential mechanism of introduction, and (b) provide statistical estimates and confidence for the total number of non-native species at the NIS sentinel site. In this respect, the previous review indicates that the Duluth/Superior region is an invasion and shipping hotspot in the Great Lakes, making this is an appropriate location to evaluate NIS invasion dynamics.

While ballast-mediated invasions can occur across a broad range of taxonomic groups, from viruses and bacteria to invertebrates and fish, it is the larger-sized organisms for which the taxonomy and biogeography are best resolved. In the Great Lakes, and elsewhere, most invasions associated with ships' ballast are invertebrates and fishes. While there are several existing programs focused on NIS fish detection in the Great Lakes, those for invertebrates are still very limited. Moreover, most of the known NIS in the Great Lakes associated with ballast water are invertebrates. For these reasons, this approach focuses primarily on detection of NIS for invertebrate taxa, especially those taxonomic groups with larger-body size that are relatively well-studied.

2.1 Rationale for Sampling Site

For a multitude of reasons, the team chose the Duluth/Superior harbor in Minnesota as the primary sentinel site for years 2-5. Based on the review and analyses of past NIS biodiversity surveys, the Duluth/Superior harbor has a history of NIS surveys and detections, providing a robust baseline as a sentinel site (Grigorovich et al. 2003, Trebitz et al. 2009, Hoffman et al. 2011; NAS 2021). Furthermore, the Duluth/Superior harbor experiences a significant volume of ship traffic and ballast water discharge, both of which are potential vectors for the introduction of NIS (National Ballast Information Clearinghouse 2021). Lastly, the multi-agency workshops include researchers from the EPA who have investigated NIS presence in the area. Not only have they surveyed the Duluth/Superior harbor in the past for NIS (Trebitz et al. 2009, Trebitz et al. 2010, Hoffman et al. 2011), but they also have the facilities and resources nearby, which greatly aid in establishing a sentinel site. Furthermore, they are actively engaged in leveraging and using the results of this work to enhance the understanding of invasion dynamics and risks to the region.

2.2 Overall Sampling Design

The general approach is to sample representative portions of the crustacean, molluscan, and annelid communities, both within the water column and benthos to measure the detection rates of NIS. As noted above, these are among the best resolved invertebrate taxa and also contribute strongly to ballast-mediated invasions, in both the Great Lakes and coastal marine sentinel sites (Fofonoff 2021; NAS 2021).

Using standardized methods comparable to the ones developed for marine systems, the team will evaluate the presence of NIS and its relationship with the USCG Ballast Water Management Program. As outlined in subsequent sections, these sampling methods include habitat (oyster shell) crates, Hester-Dendy (HD) arrays, soft-sediment grabs, and zooplankton pump samples. The team will use a variety of morphological and genetic techniques to identify the taxa, including barcoding voucher organisms and meta-barcoding (including environmental DNA (eDNA)) analysis for bulk samples.

In general, the sampling program is designed to address a variety of questions in the following topic areas:

- Detection of NIS and completeness of sampling:
  - How many NIS and what NIS are detected?
  - What is the total number of NIS estimated to be present, including unobserved species?
  - Are any new NIS present that were previously unknown to occur in (a) Duluth/Superior specifically or (b) the Great Lakes as a whole?
- Linking detected NIS to BW transport:
  - How many of the detected NIS can be linked to BW as a possible vector?
  - What proportion of NIS detected in field samples have been reported in BW samples from (a) Duluth/Superior specifically or (b) the Great Lakes as a whole?
- Comparison of sampling methods for NIS detection:
  - How many and which NIS are detected using each method?
  - Which sampling method or combination of methods is most effective at detecting NIS?
  - What is the overlap between species, particularly NIS, detected using meta-genetic methods and morphological taxonomy?
  - What is the efficacy of meta-genetic methods in detecting NIS?
- Environmental correlations with NIS detection:
  - How many and which NIS are detected in each area sampled?
  - How do NIS (and other species) vary with environmental variables in space and time?

2.3 Statistical Analysis Plan: Inferences & Limitations

Using the resulting data, the team will conduct a standard array of statistical analyses to address these questions. They will also use rarefaction to estimate the completeness of sampling for this level of sampling effort. This approach will be combined with richness estimators calculated from observations, especially the Chao2 and Jack estimators (e.g., Canning-Clode et al. 2008), to estimate the true NIS richness detected using each method in each habitat, and to generate confidence intervals for detection. The team will use multivariate generalized linear mixed models, distance-based multivariate analyses, and related techniques to assess community composition differences between sampling sites, including assessing the role of invasions in impacting community composition.

The chief advantages of the planned sampling program are that (1) it will efficiently generate statistically robust estimates of the number of NIS in the sampled areas, and (2) the standardized, systematic nature of the program makes it readily repeatable both in Duluth and in other locations for comparison across locations within the Great Lakes, and between the Great Lakes and coastal systems where the team has conducted extensive standardized surveys.

The standardized, systematic method does not capture species outside the sampling area or that occur largely in unsampled habitats. It may miss species that occur outside the temporal (seasonal) window of sampling, or which have life stages that are difficult to sample using the planned methods. In some cases, opportunistic sampling surrounding unusual environmental events can capture species that otherwise are difficult to capture. Nevertheless, the team believes that the suite of methods described here, which covers several major habitat types and includes both morphological taxonomic approaches and molecular methods, will capture many NIS associated with BW. Most importantly, these methods will provide a repeatable set of standardized measures for key habitats (at the sentinel site) needed to detect change and provide quantitative, statistical estimators.

3 SPATIAL DISPERSION AND REPLICATION

3.1 Sampling Sites

In general, the team selected 50 different sites within the Duluth/Superior Harbor, with the goal of sampling a standard set of habitats using a suite of sampling methods. These sites are selected within a 1-2m depth-range to control for depth. In addition, the team will stratify sampling sites by habitat type and select sites in a quasi-random fashion, ensuring broad coverage within the Duluth/Superior Harbor while still randomizing the selection process.

Figure 1 is a map of the St. Louis River Estuary[1] displaying the 50 sites used for surveying the benthic community. Different colors denote the blocks in which each site resides. The map is generated by overlaying a bathymetry map (shown in shades of blue) and a habitat map showing probability of submerged aquatic vegetation beds being present (show in shades of green).

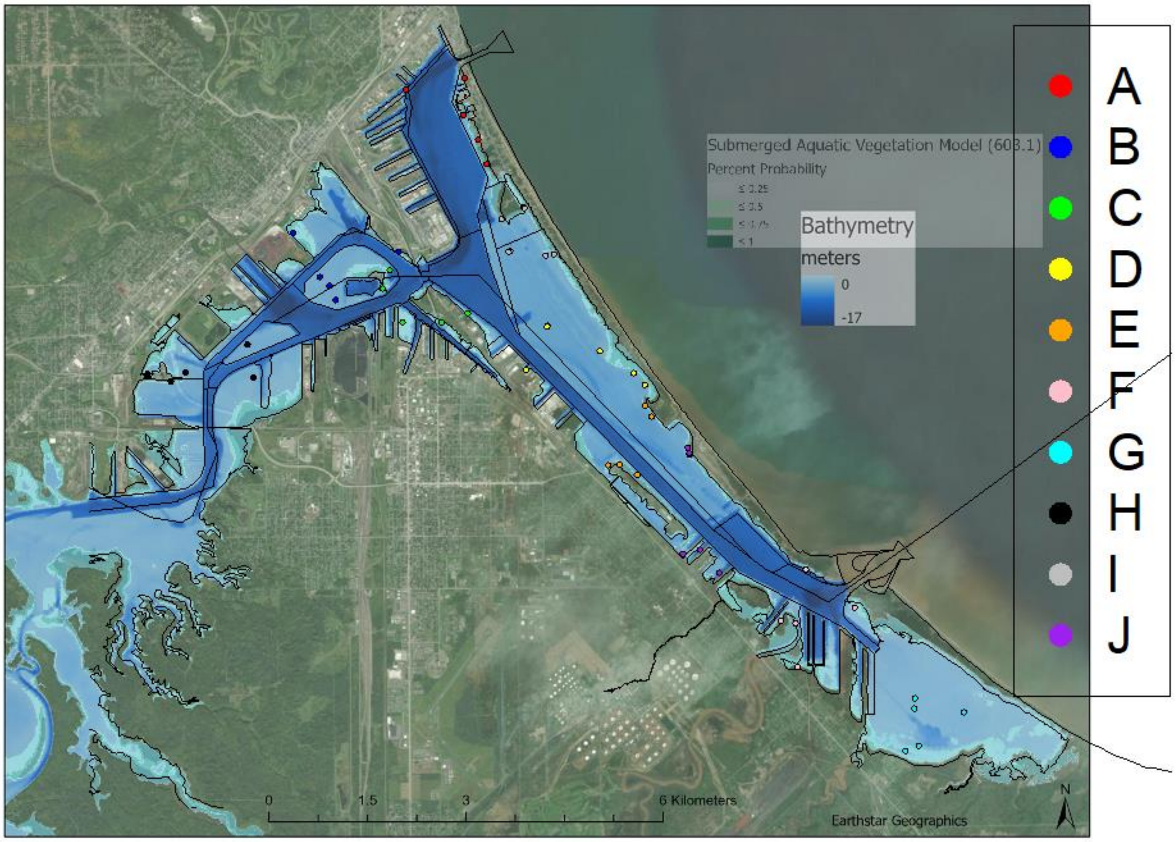

Figure 1. A map of the St. Louis River Estuary displaying the 50 benthic community survey sites.

Table 1 has the coordinates of each of the 50 sites used in sampling the benthic community. The "Block" column denotes which block the sampling site resides in (corresponding to the color-coding in Figure 1) and the "Site" column provides a unique identifier for each of the sampling sites. The "Habitat Deployed" column denotes whether both Hester-Dendys and oyster crates were used at the sampling site or whether just a particular habitat collector was deployed. In locations where the two habitat collectors were deployed separately, we used underwater chains to mark their locations rather than using surface buoys.

[1] Though Lake Superior and its tributaries (e.g., the St. Louis River) do not meet the traditional definition of "estuary," a location where the sea or tides meet freshwater outflow resulting in a brackish mix, the Estuary Restoration Act of 2000 included "*near coastal waters and wetlands of the Great Lakes that are similar in form and function to estuaries*." Since then, the term "freshwater estuary" has become accepted to describe freshwater ecosystems having characteristics similar to brackish estuaries, including mixing of river and lake water, freshwater "coastal" wetlands, and associated habitats.

Table 1. Geographic coordinates of each of the 50 sites used in sampling the benthic community.

Block	Site	Habitat Deployed	Latitude	Longitude
A	1	Both	46.7648	-92.08834
A	3	Both	46.76818	-92.09003
A	7	Both	46.7751	-92.10432
A	8	Both	46.7716	-92.09293
A	9	Both	46.77669	-92.09275
B	1	Both	46.74923	-92.12135
B	2	Both	46.74802	-92.11944
B	3	Both	46.74604	-92.11819
B	4	Both	46.75271	-92.10575
B	5	Both	46.75531	-92.12666
C	2	Both	46.74423	-92.0921
C	3	Both	46.74762	-92.10897
C	4	Both	46.74299	-92.09725
C	7	Both	46.74297	-92.10499
C	8	Both	46.75015	-92.10753
D	4	Oyster	46.74249	-92.07628
D	4	HD	46.74241	-92.07639
D	5	Both	46.73595	-92.059217
D	7	Both	46.73435	-92.057017
D	8	Both	46.7364	-92.08056
D	12	Both	46.73901	-92.06596
E	1	Both	46.7219167	-92.058567
E	3	Both	46.7233667	-92.062067
E	5	Both	46.7299333	-92.055717
E	7	Both	46.7314833	-92.056967
E	9	Both	46.72325	-92.064217
F	1	Both	46.70885	-92.025183
F	2	Both	46.7017833	-92.03005
F	6	Both	46.70145	-92.0272
F	7	Both	46.6954333	-92.026783

Table 1. Geographic coordinates of each of the 50 sites used in sampling the benthic community.

Block	Site	Habitat Deployed	Lat	Long
F	8	Both	46.7036167	-92.015517
G	1	Both	46.6896833	-92.003667
G	2	Both	46.6891667	-91.993917
G	3	Both	46.6845167	-92.002817
G	4	Both	46.6838333	-92.005483
G	5	Both	46.6910833	-92.003583
H	1	Both	46.7348333	-92.150833
H	2	Both	46.7360167	-92.147917
H	3	Both	46.7358667	-92.15545
H	4	Both	46.7399333	-92.135683
H	5	Both	46.7353333	-92.1345
I	2	Both	46.7522	-92.07665
I	3	Both	46.7572	-92.0854
I	5	Oyster	46.75277	-92.08371
I	5	HD	46.7527	-92.08398
I	6	Both	46.75237	-92.07503
I	11	Oyster	46.75883	-92.08107
I	11	HD	46.75873	-92.08086
J	1	HD	46.7247333	-92.048217
J	1	Oyster	46.7249	-92.048233
J	2	Both	46.7115833	-92.04615
J	3	Both	46.7084167	-92.042317
J	5	Both	46.71105	-92.0495
J	7	Both	46.7256667	-92.04855

3.2 Frequency

The team plans to sample the key habitats at least once during the summer or fall season in each of Years 2-4.

4 METHODS BY HABITAT TYPE

4.1 Habitat (Oyster Shell) Crates

4.1.1 Rationale

Habitat (or Oyster) crates (Figure 2) are complex habitats that serve as passive collectors for colonization and sampling of invertebrate communities. Oyster crates are chosen to sample the benthic community since they represent a standardized sampling method with prior studies showing that they are effective in sampling the benthic community (Roche et al. 2008; Fowler et al. 2013; Hewitt & McDonald 2013, Normant-Saremba et al. 2020). Oyster crates will provide a time-aggregated sample of the benthic community for a span of six weeks, increasing the likelihood of detecting rare NIS. Furthermore, because oyster crates are used by other researchers, this method will allow for cross-comparisons across different habitats (Roche et al. 2008). Although oyster shells are not a naturally-occurring substrate within the Great Lakes, they act more as a physical medium for invertebrates seeking refuge. Furthermore, the team will be pairing this method with other benthic samplers, ensuring that any bias with a particular method is minimized.

Figure 2. Oyster habitat crate being processed following retrieval from field site.

4.1.2 Design and Methods

The habitat crate consists of a 19.05cm x 15.87cm x 19.6cm plastic crate filled with oyster shells of varied sizes covered in ½’’ Vexar® mesh and will be used to sample the benthic community using both environmental Deoxyribonucleic Acid (eDNA) analysis and morphological identification. Habitat crates are retrieved with a net, ensuring that mobile organisms do not escape. The crates are then placed in 3.5-gal buckets and submerged in water from the site with the time of retrieval noted. The crates are transported to the wet lab for processing where water in the bucket is first filtered and frozen for eDNA. The team expects genetic material from the organisms within the habitat crate to leach out into the water during transport, and filtering the water should provide samples adequate for eDNA analysis. The water samples are filtered using a peristaltic pump with a Sterivex® filter. After 0.5L of water is filtered, the filter is then frozen at -20℃.

Crates are then processed for organisms. The crates are taken out of the buckets and placed on a sorting screen with a catchment basin beneath it. The crate is opened, any fish are removed, identified, and counted, and if native, returned to the water. The shells are then rinsed so that small organisms are washed into the catchment basin below. Water within the catchment basin is then run through a fine net and voucher organisms are placed in a sample jar with preservative for morphological identification or barcoding. Organisms not rinsed off will be removed from the shell with forceps and placed in the same jar. To standardize this effort, the team will use a stopwatch to keep a manual sort of the shells to 20 minutes with any unprocessed material preserved as bulk samples. Twenty minutes is enough time to look at a shell one time.

4.1.3 Spatial Dispersion and Replication

The team plans to deploy oyster crates at 50 sampling sites around the St. Louis River Estuary in Duluth, MN. These sites were chosen based on a standardized depth profile and will have a broad coverage of the area with sites that differ in their proximity to the harbor.

4.2 Hester-Dendy Arrays

4.2.1 Rationale

Hester-Dendy (HD) arrays (Figure 3) are chosen to sample the benthic community as a method to complement the oyster crates. Like the oyster crates, HD arrays will provide a time-aggregate sample of the benthic communities. HD arrays are commonly used in freshwater studies (Trebitz et al. 2010, Letovsky et al. 2012, Hoffman et al. 2016) and are effective in detecting NIS (Trebitz et al. 2010). Furthermore, the HD design is easily standardized and can be replicated across different sentinel sites. Prior studies show that while HD arrays can be effective in sampling a significant portion of the benthic community, certain taxa, such as annelids, might be harder to capture (Trebitz et al. 2010). To mitigate potential sampling biases, the team will also conduct sediment grabs to sample taxa not collected by either the oyster crates or HD arrays.

Figure 3. Hester Dendy (single unit).

4.2.2 Design and Methods

The Hester-Dendy array (Figure 4) consists of a flat metal rebar structure that will provide the base for three separate HD to be attached to it. Each HD is constructed out of fourteen 8 cm x 8 cm plates secured together with a bolt with space for colonizing invertebrates. One HD will be used for meta-barcoding analysis, a second for morphological analysis, and third as a reserve. HD arrays will also be retrieved with a net to minimize loss of organisms, and each HD structure will be stored separately in coolers with aerators and transported to the wet lab for processing. During processing, the HD plates are disassembled and organic matter within the plates are scraped into jars and preserved in either DNA solution for genetic identification or in 10% formalin for morphological identification. In addition, the coolers will be checked for any organisms that may have escaped from the HD during transport.

Figure 4. Hester Dendy (replicate units, as deployed in field).

4.2.3 Spatial Dispersion and Replication

The team plans to deploy an array consisting of three HDs at each of the 50 sampling sites around the St. Louis River Estuary in Duluth, MN. These sites are chosen based on a standardized depth profile and will have a broad coverage of the area with sites that differ in their proximity to the harbor. These are the same sites at which the team employs the other sampling methods. The HD arrays will be placed separately from the oyster crates, but both are attached by 5m of chain to the same buoy for easier retrieval.

4.3 Soft-Sediment Grabs

4.3.1 Rationale

Sediment grabs will also be used to sample the benthic community, since the taxa collected from sediment grabs should complement the communities sampled by both oyster crates and HD arrays. Because sediment grabs directly sample the substrate rather than colonizing invertebrates, the team is likely to sample taxa not normally found in collectors, such as annelids and benthic plankton (Trebitz et al. 2010). Furthermore, sediment grabs have been extensively used both in marine and freshwater systems, allowing the data to be comparable across sites. Sediment grabs may cause some variation in sampling due to the type of substrate (sand. clay, shell sand, gravel) at the sampling site. Since he team is more interested in species diversity than abundance, this variation in sampling should not substantially impact the results.

4.3.2 Design and Methods

At each sampling site where the passive collectors are deployed, the team will also use a petite-Ponar grab (Figure 5) to sample an area of ($0.1m^2$). Grab samples will be stored in a cooler filled with estuarine water at the sampling site and then transported to the wet lab for processing. Each sediment sample will be elutriated in a container where sediments are agitated with a stirring rod, causing mobile invertebrates to rise to the surface. Elutriation is a process for separating particles based on their size, shape and density, using a stream of liquid flowing in a direction opposite to the direction of sedimentation. The sample is then filtered through a $0.5mm^2$ sieve and preserved in formalin for morphological identification. The remaining bulk sample will also be preserved.

Figure 5. Ponar sampler to grab soft sediment samples.

4.3.3 Spatial Dispersion and Replication

The team will conduct Ponar grabs at each of the 50 sampling sites at which the team deploys oyster crates and HD arrays, which not only sample a particular site using a diversity of sampling methods but also allows for comparisons between the different methods to identify any potential sampling biases.

4.4 Zooplankton Pump Samples

4.4.1 Rationale

Plankton pump samples are chosen to sample the zooplankton community for the presence of NIS. Zooplankton sampling and community analysis is a standard method used in freshwater and marine salinities to detect NIS. The team has selected plankton pump sampling in particular, because pump sampling can be deployed in shallow water areas and can easily standardize the amount of water volume sampled. Unlike vertical plankton tows that sample the entire water column, plankton pumps can target specific depths to sample, providing additional information to where nonindigenous plankton are found. Plankton pumps have also been used in other sentinel sites, which allows for comparisons between sites. Plankton pumps may be prone to fouling if the water is filled with particles. Additional hardware is required to operate the pumps. These logistical challenges can be easily resolved with enough preparation.

4.4.2 Design and Methods

At each sampling site, the team filters ambient water through a 53-µm mesh sieve into wash bottles.

The team operates a modified trash pump (North Star® S106120 model; Honda® GX160 gasoline motor) coupled to a plankton net assembly (0.75m-diameter net; 80-µm mesh size) to filter 5m^3 of water drawn from 1m below the surface. The team will calculate the average flow rate of the pump based on time. To obtain 5m^3, the team operates the pump for 10 minutes.

After filtering through the plankton net, the net will be rinsed down the sides, and samples will be preserved for genetic identification in DNE (Dimethyl sulfoxide (DMSO), Sodium chloride (NaCL), and Ethylenediamine tetraacetic acid (EDTA)), the primary components of the preservative used for most meta-genetic samples.

This process is repeated for morphological identification, but samples are preserved in formalin.

4.4.3 Spatial Dispersion and Replication

The team plans to conduct two plankton pumps at each of the 50 sampling sites around the St. Louis River Estuary in Duluth, MN – one each for morphological and genetic analyses. These are the same sites, in which the team conducts benthic surveys. This allows for a comprehensive survey of the invertebrate community at each sampling site.

5 SAMPLING TIMELINE

The team assembled the various sampling units and supplies for sampling in late summer 2021, as indicated in Table 2.

Table 2. Timeline for the field sampling campaign.

	Trial deployments in SF and MD	Retrieve trial collectors and analyze samples	Travel to Duluth/Superior to assess sampling sites	Deploy collectors in Duluth/Superior	Conduct sediment grabs	Retrieve collectors/ Conduct benthic field sampling	Conduct zooplankton field sampling
May	Last week of May						
June			Week of June 21st				
July		First week of July		Week of July 26th			
August							
September					Week of September 7th	Week of September 7th	TBD

The team deployed habitat collectors in Duluth/Superior during late July, and retrieved and processed these collectors in the second week of September after Labor Day. This timeline allowed the collectors to be in the field for a minimum of six weeks. There were two field sampling teams – one each for zooplankton sampling and for benthic sampling. The zooplankton team includes three researchers. The benthic sampling team included 4 researchers from SERC, and collaborators from other institutions. A draft workflow for the field sampling is included in the Appendix.

6 CONCLUSIONS

This report spells out repeatable processes for benthic and zooplankton sampling in the Duluth/Superior Harbor environs. These procedures will provide consistent methodology over the next three years of sampling, allowing researchers to clearly note changes in 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.

Should sampling at other Great Lakes ports occur, while keeping with the benthic samples and those at 1-2m depth range, researchers will need to determine the individual sample locations based on a port's specific bathymetry, benthic habitat concentrations, tributary inflow, and circulation dynamics.

7 REFERENCES

Canning-Clode, J., Valdivia, N., Molis, M., Thomason, J.C., Wahl, M. (2008). Estimation of regional richness in marine benthic communities: quantifying the error. Limnol Ocean Methods 6: 580–590.

Fofonoff, P.W., Ruiz, G.M., Steves, B., Simkanin, C., Carlton, J.T. (2021). National Exotic Marine and Estuarine Species Information System. http://invasions.si.edu/nemesis. Access Date: 2021-05-30 Fofonoff

Fowler, A.E., Forsström, T., Numers, M. Von, Vesakoski, O. (2013). The North American mud crab Rhithropanopeus harrisii (Gould, 1841) in newly colonized Northern Baltic Sea distribution and ecology. Aquat. Invasions 8, 89–96.

Grigorovich, I.A., Korniushin, A. V, Gray, D.K., Duggan, I.C., Robert, I., Macisaac, H.J., 2003. Lake Superior: an invasion coldspot? Hydrobiologia 499, 191.

Hewitt, M.J., McDonald, J.I. (2013). The efficacy of crab condos in capturing small crab species and their use in invasive marine species monitoring. Manag. Biol. Invasions 4, 149–153. https://doi.org/10.3391/.2013.4.2.08

Hoffman, J.C., Kelly, J.R., Trebitz, A.S., Peterson, G.S., West, C.W. (2011). Effort and potential efficiencies for aquatic non-native species early detection. Can. J. Fish. Aquat. Sci. 68, 2064–2079. https://doi.org/10.1139/F2011-117

Hoffman, J.C., Schloesser, J., Trebitz, A.S., Peterson, G.S., Gutsch, M., Quinlan, H., Kelly, J.R. (2016). Sampling Design for Early Detection of Aquatic Invasive Species in Great Lakes Ports. Fisheries 41, 26–37.

Letovsky, E., Myers, I., Canepa, A., McCabe, D. (2012). Differences between kick sampling techniques and short-term Hester-Dendy sampling for stream macroinvertebrates. Bios 83, 47– 55.

NAS (Nonindigenous Aquatic Species. (2021). https://nas.er.usgs.gov/default.aspx.

National Ballast Information Clearinghouse (2021). NBIC Online Database. Electronic publication, Smithsonian Environmental Research Center & United States Coast Guard. Available from http://dx.doi.org/10.5479/data.serc.nbic; searched on 1 May 2021.

Normant-saremba, M., Hegele-drywa, J., Marszewska, L. (2020). Sampling native and non-native mobile epifauna with baited traps and habitat collectors - Port of Gdynia case study (southern Baltic Sea, Poland). Oceanol. Hydrobiol. Stud. 49. https://doi.org/10.1515/ohs-2020-0028

Roche, D.G., Torchin, M.E., Leung, B., Binning, S.A. (2008). Localized invasion of the North American Harris mud crab, Rhithropanopeus harrisii, in the Panama Canal: implications for eradication and spread. Biol. Invasions 11, 983–993. https://doi.org/10.1007/s10530-008-9310-6

Trebitz, A.S., Kelly, J.R., Hoffman, J.C., Peterson, G.S., West, C.W. (2009). Exploiting habitat and gear patterns for efficient detection of rare and non-native benthos and fish in Great Lakes coastal ecosystems. Aquat. Invasions 4, 651–667.

Trebitz, A.S., West, C.W., Hoffman, J.C., Kelly, J.R., Peterson, G.S., Grigorovich, I.A. (2010). Status of non-indigenous benthic invertebrates in the Duluth-Superior Harbor and the role of sampling methods in their detection. Journal of Great Lakes Research 36: 747-756

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APPENDIX A. DRAFT WORKFLOW FOR FIELD SURVEYS

A.1 Benthic Sampling & Processing

Two teams are envisioned working concurrently, including one for retrieval (sample collection) and another for processing, as outlined below.

- **Retrieval team (3 people – 1 to drive the boat and 2 to retrieve)**
  - Travel to the sampling site and identify the buoy marking the HD and habitat crates location.
  - Use Yellow Springs Instrument (YSI®) Pro2030 to measure water quality parameters, including conductivity, turbidity, dissolved oxygen (both mg/L and %), pH, and temperature.
  - Recover the habitat crates first. Use a boat hook to retrieve the attached rope, and a net to catch any mobile organisms that fall free of the habitat crates.
    - Place each habitat crate assembly in a dedicated bucket filled with at least 1L of water, ensuring that the habitat crates are submerged.
    - Place an aerator in the bucket.
    - Mark the time of habitat crate retrieval.
  - Recover the HD array. Use a boat hook to retrieve the attached rope, and a net to catch any mobile organisms that fall free of the habitat crates.
  - Remove each HD from the array and place each into a bag to prevent mobile organisms from escaping. Place the bags in a cooler.
- Use a Ponar grab sampler to grab 0.1m ^{2} of sediment. Place each sediment sample in a dedicated cooler with an aerator and water collected from the site.

- **Processing team (~8-12 people)**
  - **HD processing team (4-6 people processing HD). Expect 4 days to process 100 HD.**
    - Retrieve HDs from the boat team.
    - Randomly pick one HD for genetics and one for morphology.
      - If any HDs are missing, use the 3rd HD for backup.
    - Check buckets for any organisms that might have escaped.
    - Disassemble HD in a contained area to catch any fleeing organisms.
    - Rinse and scrape down HD.
    - Filter samples, then preserve respectively for genetics and morphology.
      - Given the low biomass, there is no need to pick specimens for voucher.
  - **Habitat crate processing team (4-6 people processing crates). Expect 4-5 additional days to process 50 crates.**
    - Remove habitat crates from 3.5-gal buckets and place them in a contained area.
    - Using a sieve, remove any organisms that might have escaped into the bucket.
      - Remove any fish; place native fish into bucket for return to lake.
    - Mark the time the habitat crate was placed in the contained area (to determine the crate's soak time in the bucket).
    - Filter the water remaining in the 3.5-gal bucket (which contains eDNA) and freeze the filter (2 people for eDNA extraction).

            - Using a peristaltic pump, filter 0.5L of the container DNE solution for genetic identification through a Sterivex® filter.
            - Once water has been filtered, store the filter at -20°C (minimum).
        - Disassemble habitat crates and begin sorting samples (2-4 people for oyster crates sorting).
            - Disassemble crates under a catchment basin.
            - Remove and identify any fish; place native fish in a bucket for return to lake.
            - Rinse the shells to dislodge any inverts into the catchment basin.
            - Select voucher taxa of interests (crustaceans, molluscans, and annelids – with an emphasis of annelids).
                - Standardize effort to 20 minutes.
            - Preserve the remaining bulk samples.
    - **Sediment processing team (2 people)**
        - Place sediments into the elutriation container (5-gallon bucket).
        - Slowly agitate the sample with a stirring device to promote live invertebrates to gather near the surface.
        - Slowly pour the water into a 500-micron sieve to catch the live invertebrates.
        - Refill the elutriation container and repeat this process until no invertebrates rise to the surface.
        - Place invertebrates into a jar and preserve with formalin.

Note: Transport team may be a subset of processing team and will focus on the following two tasks:
1) Transport containers with HD and oyster crates from dock to wet lab;
2) Transport any caught fish from wet lab to docks for release.

A.2 Zooplankton Sampling & Processing

- **Zooplankton team (3 people)**
    - Travel to the sampling site and identify the buoy marking the HD and habitat crates location.
    - Place the pump on a rubber mat at a forward location of the boat (near the bow). Ensure the pump is level and the exhaust isn't obstructed (to prevent overheating). The mat will prevent the pump from shifting and protect the bottom of the boat from damage.
    - Before attaching the output hose to the net & harness, run the pump for 1 minute outside of and away from the net, to flush the pump between replicates.
    - Submerge the intake hose 1-m below the water surface and secure it to the boat. The intake hose should be far from the net assembly (suction can easily entrap and damage the net) and any submerged aquatic vegetation (kelp, dock fouling, etc.). Ensure the intake is at least 1-m from the bottom to prevent collecting sediment.
    - Once flushed, secure the pump and attach the output hose to the net harness. Restart the pump and mark the time.
    - Run the pump for 10 minutes.
        - Use YSI® to measure water quality parameters during this time.
    - Secure the pump, then lift the net out of the water. Using the portable wash-down pump rinse any residual plankton (with ambient water) off the net and into the codend. Because the

wash-down hose does not filter water, rinse from the outside of the net only; do not use the hose inside the net.

- Once the net is rinsed-down, carefully pour the codend contents into a polyvinyl chloride (PVC) sieve (53-µm mesh; 5-inch diameter) and concentrate the raw plankton sample as much as possible. The goal is to have as little water as possible get into the Nalgene® sample bottle before preserving the sample. Keep the raw plankton sample below the 10% permanent marker line.
- Open the sample bottle, fit the funnel inside, and pour the concentrated PVC sieve contents into the bottle.
- Use squirt bottles containing filtered water to wash as much residual zooplankton as possible off the sieve and into the sample bottle. If the raw zooplankton sample goes over the 10% line, pour the excess into other sample bottles to ensure the water level is below the 10% line prior to adding preservative.
  - If sample bottles are pre-shot with full-strength buffered calcium carbonate ($CaCO_3$) formaldehyde, the volume of concentrated zooplankton will need to be estimated in the PVC sieve before adding it to the sample bottle, to determine if the sample needs to be split.
- Preserve replicate pump sample #1 in 10% DMSO salt solution.
- Preserve replicate pump sample #2 in 10% buffered formalin in filtered water.
- Before moving to the next collection site, rinse the sampling gear and pump thoroughly with fresh water. Dump any filtered water and make a new batch at the next collection site using that site's ambient water.

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