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Rapid Molecular Detection of Invasive Species in Ballast and Harbor Water by Integrating Environmental DNA and Light Transmission Spectroscopy

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Supporting Information

ABSTRACT: Invasive species introduced via the ballast water of commercial ships cause enormous environmental and economic damage worldwide. Accurate monitoring for these often microscopic and morphologically indistinguishable species is challenging but critical for mitigating damages. We apply eDNA sampling, which involves the filtering and subsequent DNA extraction of microscopic bits of tissue suspended in water, to ballast and harbor water sampled during a commercial ship's 1400 km voyage through the North American Great Lakes. Using a lab-based gel electrophoresis assay and a rapid, field-ready light transmission spectroscopy (LTS) assay, we test for the presence of two invasive species: quagga (*Dreissena bugensis*) and zebra (*D. polymorpha*) mussels. Furthermore, we spiked a set of uninfested ballast and harbor samples with zebra mussel tissue to further test each assay's detection capabilities. In unmanipulated samples, zebra mussel was not detected, while quagga mussel was detected in all samples at a rate of 85% for the gel assay and 100% for the LTS assay. In the spiked experimental samples, both assays detected zebra mussel in 94% of spiked samples and 0% of negative controls. Overall, these results demonstrate that eDNA sampling is effective for monitoring ballast-mediated invasions and that LTS has the potential for rapid, field-based detection.

INTRODUCTION

Invasive species have had a globally damaging effect on freshwater and marine ecosystems, adversely impacting biodiversity and human commerce.[1] In the United States alone, invasive species cause environmental damage and economic losses in excess of US $137 billion annually[2,3] and are now recognized as one of the leading global threats to native biodiversity and ecosystem functions.[4,5] Within the North American Great Lakes, there are over 180 aquatic invasive species that have significantly altered the ecosystem and are estimated to cost up to $800 million annually.[6−10] The majority of these aquatic invasive species (>70%) entered through transoceanic shipping routes, including the ballast water discharge of incoming ships.[11−14] A general lack of sensitive technologies for early detection can allow harmful invasions to be established to the point where it is too late to intercede for an increasing number of highly damaging species.[15]

The ecological and economic threats of aquatic invasions therefore require improved monitoring of the various anthropogenic pathways, such as ships' ballast water. Unfortunately, ballast water treatment technologies are still mainly in the research and development phase.[16,17] Consequently, there is an urgent need for rapid detection methods to identify harmful invasive species in ships' ballast prior to port entry to provide adequate time for initiating appropriate control actions. Even if ballast water treatment systems become widely adopted, the need will still exist for rapid inspection and enforcement tools, as a monitoring firewall against harmful invasives evading the first line of control.

Traditional microscope-based methods have been used to examine ballast samples for target organisms but are expensive and slow.[18] By the time results from microscopic examination are available, ships have long since reached their destinations, making it too late to intervene.[14] In addition, the most often encountered life stage in ballast tanks, larvae, are often not identifiable to the species level using morphological keys.[19]

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Accordingly, a rapid and sensitive on-board detection platform that can identify target species in large volume ballast samples requiring minimal user expertise would greatly facilitate inspection (voluntary or regulatory), enforcement, and control efforts.

Species detection also represents a fundamental tool for effective conservation and management. Without accurate, up-to-date knowledge of where rare endangered species are and where invasive propagules reside, cost-effective management is impossible. Environmental DNA (eDNA) sampling has become increasingly prevalent in such conservation and management efforts[20] and has recently experienced rapid development in their application to monitoring biodiversity.[21,22] In a management context, there are at least two major advantages of eDNA methods over traditional methods: (1) increased sensitivity[22,23] (i.e., increased probability of detecting a species if it is present) and (2) potentially lower costs, especially as eDNA methods increase in portability and automation.[24,25]

The overarching goal of our work is to enable accurate, sensitive, timely, and cost-effective detection of target species. In the present study, we merge two new, but proven technologies for the purpose of quickly and accurately detecting target species in ship's ballast and surrounding harbor waters. First, we use DNA filtering methods to isolate and purify field samples of environmental DNA (eDNA) from a ship's ballast water and the surrounding harbor area. In this regard, analysis of aquatic samples with traditional methods, such as microscopy and morphology, often poses insurmountable logistical challenges for detecting rare and taxonomically challenging organisms. However, species surveillance using eDNA exploits the fact that the aqueous environment often keeps microscopic bits of tissue from target invasive species in suspension in the water column, making it easier to sample DNA from even rare organisms that are present but invisible to traditional tools. We define eDNA here as all types of tissue samples collected by filtering water from an aquatic environment. These samples can include, but are not limited to, sloughed tissues or cells, larvae or adult organisms that are microscopic, milt, eggs, extracellular DNA from degraded tissues, scales, and invertebrate exoskeletons. The eDNA method has been successfully developed and applied to possible Asian carp invasions into the Great Lakes[23,26] and for other species surveillance questions.[22,27−31] The eDNA approach to species surveillance is rapidly developing into a burgeoning field of research in molecular ecology and application in conservation and invasive species management.[21,32]

Second, we combine the efficacy of eDNA sampling with a new field-based tool for target DNA detection, light transmission spectroscopy (LTS).[33,34] Typically, eDNA detection methods have relied on PCR or qPCR to amplify target DNA present in a sample, where amplification is equivalent to detection.[35] However, the LTS platform offers the possibility of field-based species detection because it is portable, rapid, and requires low electrical power to operate.[24] LTS determines the size, shape, and number of nanoparticles in suspension by measuring wavelength-dependent light transmittance through a sample containing nanoparticles in suspension.[33,34] LTS is applicable for species detection by measuring nanoparticles that specifically bind to target species DNA and therefore grow in size.[24,25,34] Specifically, carboxylated nanoparticles of a known size (typically around 200 nm in diameter) are bound with species-specific tags representing ∼18−26 base pair oligonucleotides that bind to a unique section of target DNA. When the tagged nanoparticles are in solution with the DNA of a target species, the tagged nanoparticles grow in diameter because the tags bind to PCR amplified DNA fragments. LTS can detect the size shift in particle size within seconds (Supplemental Figure 1, Supporting Information). The LTS device is portable and can run off a car's battery. Thus, LTS technology has promise for uses where speed and portability are paramount (e.g., real-time field surveys of ballast water, law enforcement inspections).

In the present study, we test the effectiveness of merging eDNA sampling with LTS for invasive species detection using two test species that are currently found in the ballast waters of ships from foreign ports entering the Great Lakes: (1) *Dreissena polymorpha* (zebra mussel) and (2) *Dreissena bugensis* (quagga mussel). Both invasive species are known to be in the Great Lakes with viable populations. We report that combining eDNA sampling with LTS can rapidly and accurately detect target invasive species in two types of environmental samples where the threat of aquatic invasion is the highest, within the ballast water of a large commercial shipping vessel and in the surrounding harbor where these ship-borne aquatic invasions usually begin. Moreover, these data support the continued development of the LTS portable real-time DNA detection system that could be used for onboard monitoring during a ship's passage prior to ballast discharge to dramatically reduce the impacts of future invasions.[12,14]

MATERIALS AND METHODS

Ballast and Harbor Sampling and eDNA Filtration. Ballast water was collected directly from the intake and discharge of a 1000 ft shipping vessel, the M/V *Indiana Harbor*, during its five-day, 1400 km voyage from the Indiana Harbor (41°39′57.1788″ N, 87°26′43.9038″ W) near Gary, Indiana, on Lake Michigan, to the Duluth-Superior Harbor near Duluth, MN (46°45′14.3136″ N, 92°5′59.1426″ W), on Lake Superior during mid-October 2012 (Supplemental Figure 2, Supporting Information). In parallel, we also collected water from the ballast intake source, Indiana Harbor, IN, and the destination port receiving the ballast water discharge, the Duluth-Superior Harbor, MN. All water samples were collected in a two-liter sterile Nalgene bottle as follows: Ballast intake samples (n = 20) were collected during ballast intake from a direct tap connected to the pumping system on October 17th, 2012. Source harbor samples (n = 5) were collected from the surface of the water directly next to the ship during ballast intake. Receiving harbor surface water samples (n = 3) were collected 5 days later (October 22nd, 2012) in the same manner just prior to ballast discharge. Ballast discharge samples (n = 13) were collected from the direct line to the pumping system from three separate tanks (designated tank #3 = 3 samples, tank #678 = 3 samples, and tank #5 = 5 samples). Each ballast tank is independently sealed from the other and, thus, can potentially harbor different contaminants (biotic or abiotic) in each.

For each of the ballast and harbor samples, a 250 mL aliquot was filtered through a 1.2 μm polycarbonate membrane filter (GE Water & Process Technologies) to screen for the aquatic invasive species, quagga mussel (*Dreissena bugensis*), expected to be present and active in the Great Lakes region at the time of sampling,[36−38] and zebra mussel (*Dreissena polymorpha*), also present but not expected to be as active as quagga mussels at

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Table 1. List of Species-Specific and Universal PCR Primers and Species-Specific Tags for LTS Used in the Present Study

oligonucleotide	sequence	ref.
Species-Specific PCR Primers		
Dreissena bugensis (quagga mussel) forward	5′-CCTTATTATCTGTTCGGCGTTTAG-3′	49
Dreissena bugensis (quagga mussel) reverse	5′-ACTTGTAACACCAATAGAAGTAC-3′	49
Dreissena polymorpha (zebra mussel) forward	5′-GGGATTCGGAAATTGATTGGTAC-3′	49
Dreissena polymorpha (zebra mussel) reverse	5′-GAATCTGGTCACACCAATAGATGTGC-3′	49
Universal Invertebrate PCR Primers		
universal invertebrate forward (LCO-1490)	5′-GGTCAACAAATCATAAAGATATTG-3′	39
universal invertebrate reverse (HCO-2198)	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	39
Species-Specific Tag for LTS		
Dreissena polymorpha (zebra mussel)	5′-GAATCTGGTCACACCAATAGATGTGC-3′	49
Dreissena bugensis (quagga mussel)	5′-ACAAGTTGGGGGTGGTTTAGGCGGGAGT-3′	49

this time of year. We will refer to these samples throughout as the *"standard"* ballast and harbor samples.

Spiked Experimental Ballast and Harbor Samples. To further test the effectiveness of eDNA sampling, we spiked a second 250 mL aliquot of ballast water samples with a small fragment of tissue (range in tissue amount: 66−520 μg) from the aquatic invasive species zebra mussel (*Dreissena polymorpha*). As discussed above, zebra mussels are present in the Great Lakes region but not present in the water column as temperatures cool during the fall, such as when we sampled in October of 2012.[37,38] Thus, the prediction is that the spiked samples should serve as a positive control for the negative result expected for zebra mussel from the field samples. All samples (n = 41) were screened for zebra mussels using eDNA, species-specific mitochondrial DNA (mtDNA) markers (Table 1) and gel electrophoresis prior to the spiking experiment, with 0% detection of zebra mussels. Each tissue sample was removed from a preserved adult using fine-tipped dissecting forceps and added to the eDNA filter to imitate the event that this target invasive species was collected during ballast water screening. We spiked 16 samples distributed equally across the four sampling categories. In addition, we generated eight control eDNA samples where no target zebra mussel tissue was added. The control eDNA extractions were done in parallel with the spiked samples. We will refer to these samples henceforth as the *"experimental"* ballast samples. All experimental ballast samples were filtered through a 1.2 μm polycarbonate membrane filter (GE Water & Process Technologies).

DNA Extraction from Filter and Polymerase Chain Reaction. eDNA was isolated from filters following the protocol described in Jerde et al.[23] and Egan et al.[24] In brief, the polycarbonate membrane filter from standard and experimental ballast and harbor samples was combined with 700 μL of CTAB buffer and 20 μL of Protinase K, vortexed for 15 s, and incubated at 63 °C for 1 h. After incubation, 700 μL of a 24:1 chloroform/isoamylalcohol solution was added to the solution, vortexed for 5 s, and centrifuged at 14 000 rpm for 10 min, and the supernatant was transferred to a new tube. Next, 1 mL of isopropanol was added; the sample was inverted gently to mix and incubated at −20 °C for ≥4 h. Samples were removed from −20 °C and centrifuged at 14 000 rpm for 20 min to form a pellet; the supernatant was poured off and washed twice with 500 μL of 70% ethanol, pouring off ethanol and maintaining the pellet each time. Pellets were dried for 10 min at 45 °C in a vacuum centrifuge and then resuspended in 50 μL of TE Buffer overnight at 4 °C in a refrigerator.

We measured the DNA concentration of each sample extracted using a Qubit 2.0 fluorometer (Life Technologies) with either the dsDNA Broad Range or High Sensitivity assay kits (Invitrogen). Sample DNA concentration was first measured with the Broad Range kit, and samples with undetectable DNA concentration were then measured again with the High Sensitivity kit. Two PCRs were performed on each sample. First, a species-specific primer was used in concert with gel-electrophoresis for a standard detection. Second, a universal primer, which amplifies the same section of the mtDNA in most invertebrates (Folmer et al.[39]), was used in concert with the LTS device to test this new, field-ready device.

The first PCRs were performed using species-specific primers listed in Table 1 that would amplify a section of the cytochrome c oxidase subunit I (COI) gene in the mtDNA for one of the target species only (quagga or zebra mussel). The PCRs were used to confirm the success of eDNA sampling from ballast and harbor water for species-specific detection of the two taxa. The species-specific mtDNA markers were developed for previous studies where they were tested for specificity, even against their closest related sympatric relative (i.e., quagga versus zebra and vice versa), and some fragments were sequence verified for additional verification (Li et al.,[34] Mahon et al.,[49] Mahon et al.,[25] Egan et al.[24]). Species-specific PCR was followed by standard gel electrophoresis detection analysis and included positive and negative controls. The lab-based gel detection offered a baseline for detection to which we could compare results from LTS detection. For each sample, nine replicate PCRs were run.

The second set of PCRs used universal invertebrate primers (Table 1), which amplify a ∼600 base pair section of the mtDNA COI gene for both target and nontarget invertebrate species. The long-term strategy for using universal primers is to develop a single PCR from which it would be possible to use LTS to detect the presence or absence of multiple species of interest using nanoparticles functionalized with different species-specific tags (i.e., single stranded oligonucleotides). The current LTS sequence tag is ∼340 bp downstream of the 5′ end of the ∼600 bp mtDNA fragment amplified by the universal primers. In effect, the universal primers would amplify all invertebrates present in a sample. Then, separate LTS analyses could be conducted on the single PCR reaction using a different species specific set of functionalized nanoparticles for each LTS trial to determine if a particular target species of invertebrate was present in the sample.[24,25,34]

PCRs with both primer sets were performed as follows: (i) 94 °C for 1 min, (ii) 94 °C for 30 s, (iii) 41 °C for 45 s, (iv) 72 °C for 1 min, (v) go to step 2 and repeat 30 times, and (vi) 72 °C for 8 min.[39] The total reaction volume was 25 μL and included: 1 μL of DNA template, 2.5 μL of 10× buffer, 2.5 μL

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of $MgCl_2$ (25 mM), 0.5 μL of forward primer (10 μM), 0.5 μL of reverse primer (10 μM), 0.5 μL of dNTPs (2.5 mM each; Invitrogen), 0.15 μL of Taq polymerase (5Prime), and 17.35 μL of ddH_2O.

Test of PCR Inhibition. We also tested for PCR inhibition in each sample. Abiotic substances, such as humic and fulvic acids, are known PCR inhibitors that are likely to be suspended or dissolved in ballast and port waters, where they are found in soils and sediments and get turned up a lot due to boat traffic and repeated dredging within the ports.[40] To test for PCR inhibition, we compared the observed and expected concentration of a positive control DNA sequence (Eurogentec Universal Exogenous qPCR Positive Control) using quantitative real-time PCR on an Eppendorf MasterCycler *realplex* machine. The master mix for each sample included 3.6 μL of H_2O, 10.0 μL of ABI TaqMan Environmental, 2.0 μL of positive control mix (Eurogentec), 0.4 μL of positive control DNA (Eurogentec), and 4.0 μL of template DNA.

Nanoparticle Preparation and Hybridization to eDNA Samples for LTS. To detect target species using LTS,[25,34] we first bound polystyrene beads with a carboxylated surface to a species-specific oligonucleotide tag that will only bind to DNA fragment amplified in the target species (see Table 1 for species specific tags). To generate these "functionalized" nanoparticles for species detection, we followed published protocols.[24,25,34] Briefly, we combined 10 μL of Polybead Carboxylate Microspheres (diameter = 209 nm; [C] = 5.68×10^{12} beads/mL; Polysciences, Inc.) with 1 mL of ddH_2O, 50 μL of Bead Coupling Buffer (Polysciences, Inc.), and 25 μL of an EDC coupling activating solution [EDC = 1-Ethyl-3-(3-(dimethylamino)propyl) carbodiimide HCl at 192 g/mol; solution = 1 mg EDC in 5 mL of ddH_2O]. Bead solution was mixed at 300 rpm on an orbital shaker for 15 min. Separately, 10 μL of each species-specific oligonucleotide tag ([C] = 100 ng/μL) was combined with 1 mL of ddH_2O and 50 μL of Bead Coupling Buffer (Polysciences, Inc.) and mixed for 1 min at 300 rpm on an orbital shaker. The bead solution and the tag solution were then combined and mixed constantly at 300 rpm for 2–4 h. For downstream LTS measurement, a "reference" solution containing all reagents except the beads and tags was also made, where bead and tag volume was replaced by equal amounts of ddH_2O.

PCR products (double stranded DNA from the amplified mtDNA COI region) from each sample was denatured by heating to 95 °C for 2 min, then immediately chilled on ice for 2 min. PCR product was combined with functionalized bead solution ([C] = 1.046109×10^9 beads/mL) in a 1:2 ratio at 48 °C for 1 min.[34] If target DNA was present, it would anneal (or bind) to the complementary species-specific tag and the nanoparticle would grow in diameter. We have previously shown that it does not grow when combined with nontarget DNA.[24,25,33,34] Each PCR-bead hybridization reaction was then placed on ice until LTS measurement.

Laser Transmission Spectroscopy (LTS) and Nanoparticle Measurement. LTS is used in the context of species detection to measure the size of nanoparticles in suspension. For the purpose of DNA diagnostics, LTS can detect the increase in size of the functionalized nanoparticles if the species specific tag has bound to the target species PCR product causing an increase in the diameter of the bead. LTS is based on measuring wavelength-dependent light transmittance through a sample containing nanoparticles in suspension. Briefly, the transmission of light through a sample cell containing particles plus suspension fluid is recorded along with that of a similar cell containing only the suspension fluid. The fundamental data-acquisition process involves measuring the wavelength-dependent transmission of light (quantified as extinction) through an aqueous suspension of nanoparticles. Here, the pertinent wavelength range is from ~300 to 1000 nm. Given the extinction information and the known wavelength-dependent properties of the beads, Mie theory[33] can be used to accurately determine the bead diameter. The extinction data are analyzed and inverted by a computer algorithm that outputs the particle size distribution. The sensitivity limit of LTS reported in Li et al.[33] for 1025 nm polystyrene spheres is ~3580 particles/mL (i.e., 3.5×10^{-17} molar). The precision, accuracy, sensitivity, and resolution of LTS using NIST traceable polystyrene particles are detailed in Li et al.,[33] where these properties are quantified for the size range important for DNA detection (~50–1000 nm). Further details of LTS theory and operation are detailed in Li et al.[33] and Li et al.[34]

RESULTS AND DISCUSSION

Species detection probabilities are typically low in aquatic environments, especially for the ballast of large commercial shipping vessels.[11−14] Yet, effective management of high-risk invasive species requires the early detection of an incipient invasion, which increases the feasibility of rapid responses to eradicate the species or at least contain its spread.[15,41] Here, we tested whether eDNA sampling can be an effective tool for species detection in heterogeneous ballast and harbor water samples, which contain a variety of aquatic species, their eDNA filtered tissues and cells, and a mixture of biotic and abiotic debris.

Amounts of eDNA Isolated from Harbor and Ballast Samples. First, the concentration of DNA isolated from ballast and harbor water samples was quantified using a Qubit 2.0 fluorometer (Life Technologies). Ballast intake and discharge were slightly less than the harbor samples, which is consistent with ballast representing a subset of available eDNA content in each harbor (mean ± SE: Indiana and Duluth harbors = 5.54 ± 1.32 μg/mL, Ballast intake and discharge = 3.28 ± 0.65 μg/mL; t test assuming unequal sample sizes: $t_{df=19.45} = 2.16$, $P = 0.04$). However, ballast intake and discharge did not differ significantly (Ballast intake = 3.67 ± 0.84 μg/mL, Ballast discharge = 2.68 ± 1.04 μg/mL; t test assuming unequal sample sizes: $t_{df=27.81} = 0.81$, $P = 0.43$), nor did samples from the two harbors (Indiana source harbor = 6.42 ± 0.95 μg/mL, Duluth destination harbor = 4.09 ± 1.17 μg/mL; t test assuming unequal sample sizes: $t_{df=2.29} = 1.24$, $P = 0.32$). In addition, the DNA concentrations did not differ between the spiked and control samples in our experiment (DNA [C] ± SE: spiked = 1.68 ± 0.21 μg/mL, control = 1.26 ± 0.29 μg/mL; $t_{df=22} = 1.21$, $P = 0.24$). These results are consistent with an expectation from real-world ballast and harbor water samples, where the DNA from many different organisms would be filtered and extracted from a sample.

Tests for PCR Inhibition. Prior to screening eDNA samples for invasive species with each technology, we tested for PCR inhibition since PCR inhibitors are likely present in ballast and harbor waters.[40] There was no evidence for systematic biotic or abiotic inhibition of PCRs in the study. We compared the observed and expected concentration of a positive control DNA sequence added to each sample using quantitative real-time PCR. The greater the difference generated between observed from expected concentrations

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Table 2. Results of eDNA Screens of the Standard and Experimental Ballast and Harbor Water Samples for the Gel Electrophoresis and LTS Assay Methods[a]

eDNA sample	type (number of samples)	DNA concentration (mean μg/mL ± sd)	gel assay 1	gel assay 2	LTS assay
standard	source harbor (5)	6.43 ± 1.01	100% (5/5)	89% (40/45)	100% (5/5)
	ballast intake (20)	3.86 ± 4.90	85% (17/20)	63% (113/180)	100% (20/20)
	receiving harbor (3)	4.01 ± 3.14	33% (1/3)	17% (3/18)	100% (3/3)
	ballast discharge (13)	2.68 ± 2.01	92% (12/13)	54% (63/117)	100% (13/13)
experimental	spiked (16)	1.68 ± 0.79	94% (15/16)	94% (45/48)	94% (15/16)
	control (8)	1.26 ± 1.09	0% (0/8)	0% (0/24)	0% (0/8)

[a]There were no positive hits in the standard samples for *D. polymorpha* using either of the two methods. PCR inhibition was also not observed in any of the samples. (Gel assay 1: % of three separate triplicate PCR reactions, where at least one positive reaction occurred out of the three; Gel assay 2: % of all nine total PCR reactions that was positive; LTS assay: % of samples with a clear double peak demonstrating a positive species detection.)

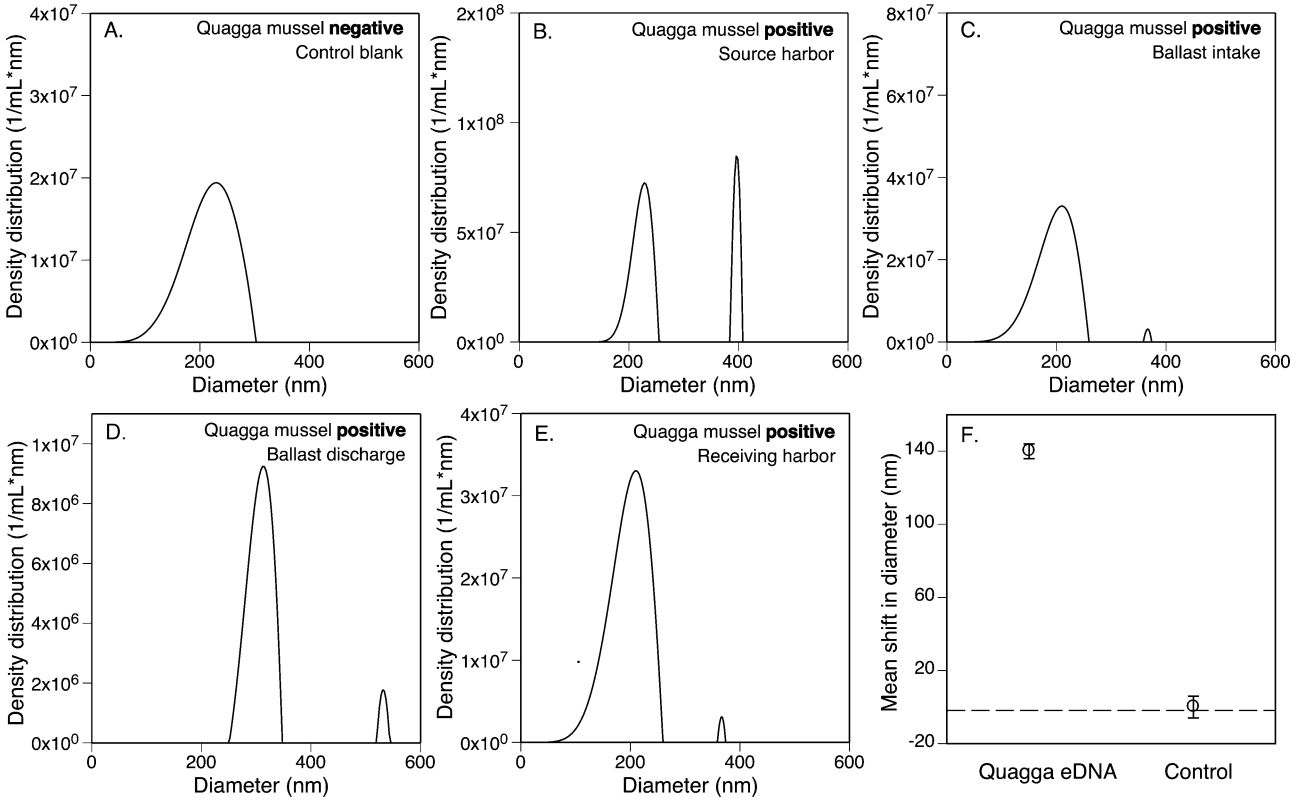

Figure 1. Naturally infested ballast and harbor water samples. Environmental DNA-based quagga mussel detection using LTS from naturally infested ballast water samples from the M/V *Indiana Harbor* and surrounding source and receiving harbor waters. Density distribution for one LTS measurement of (A) the control blank, where no eDNA was added; four positive quagga mussel detections from the (B) source harbor, (C) ballast intake, (D) ballast discharge, and (E) receiving harbor; (F) the average shift (±standard deviation) in the diameter of nanoparticles relative to the baseline beads attached with quagga-specific tags for all 41 standard samples (ballast and harbor). Dashed line in (F) marks the zero peak shift for nanoparticle and represents no detection of target.

suggests an increased chance of PCR inhibition. None of the 41 standard samples (ballast or harbor) or the 24 experimentally spiked samples (zebra or controls) showed any evidence of PCR inhibition (all values were less than 1.4; mean ± SE: 0.64 ± 0.01).

Standard Lab-Based eDNA Detection Using Gel Electrophoresis. Each standard ballast water sample from the field was screened for quagga and zebra mussel eDNA using species specific primers (Table 1) under the prediction that we should detect quagga mussels at a higher rate than zebra mussels in the Great Lakes during October because they are known to be more cold-tolerant.[36−38] On the basis of gel electrophoresis, quagga mussel was detected in 35/41 (85%) of the field samples, representing 17/20 of the ballast intake samples, 5/5 of the source harbor samples, 12/13 of the ballast discharge samples, and 1/3 of the destination harbor samples (Table 2). The six samples where quagga was not detected may represent: (1) a natural failure to capture quagga mussel in the samples, (2) a failure to isolate quagga DNA in samples in which it was present, but perhaps in low frequency, (3) PCR failure, or (4) a failure to detect DNA by gel electrophoresis in PCRs in which quagga amplification was low. The overall DNA concentration in six negative quagga samples did not, however, differ from the overall mean (failed quagga detection samples: 2.36 ± 1.12 μg/mL; one-sample *t* test: $t_{df=5} = -0.88$, $P = 0.42$). Thus, it was not the case that the negative quagga results for these six samples was due to little or no DNA captured and extracted from the filters.

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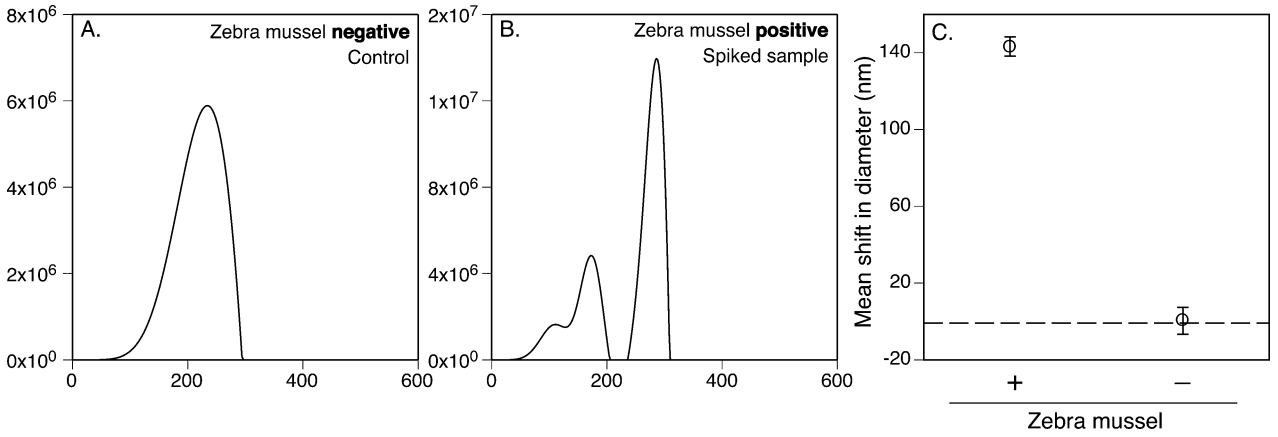

Figure 2. Experimentally spiked ballast and harbor water samples. Environmental DNA-based zebra mussel detection using LTS from experimentally spiked ballast water samples collected from the M/V *Indiana Harbor* and surrounding source and receiving harbor waters. (A) Density distribution of one LTS measurement of a control sample, where no zebra mussel tissue was added, (B) density distribution of one LTS measurement where zebra mussel tissue was added, and (C) the average shift (±standard deviation) in the diameter of nanoparticles for ballast samples where zebra mussel was added (+) relative to the samples were zebra mussel was not added (−). Dashed line in (C) marks the zero peak shift for nanoparticle and represents no detection of target.

Zebra mussel eDNA was not detected in any standard field sample using gel electrophoresis (0/41), as expected on the basis of their natural history. However, zebra mussels were detected in 15/16 of the spiked environmental samples compared to 0/8 control samples (Table 2). The one failed zebra detection had undetectable amounts of DNA present based on the Qubit analysis, suggesting that zebra mussel tissue was not captured on the filter in this eDNA sample or that the DNA extraction itself failed.

Experimental Field-Based eDNA Detection Using Light Transmission Spectroscopy. Prior to LTS species detection tests, nanoparticles were attached with species-specific oligonucleotide tags and measured to be 230 (±0.4) nm in diameter (Figure 1A). This LTS size distribution is larger than the original diameter of 209 nm for the carboxylated beads and represents the binding of the oligonucleotide tags to the beads.[24,34] Thus, 230 nm is the baseline diameter of beads used to test for size shifts associated with positive detection of target quagga and zebra mussel DNA.

LTS successfully detected quagga mussel eDNA in all 41 standard field samples, including the six samples where quagga mussel was not detected using gel electrophoresis. The LTS result suggests that the failure to detect quagga in the six cases by gel electrophoresis was due to a reduced sensitivity of electrophoresis versus LTS to detect target DNA at low concentration. We present LTS measurements from four representative positive quagga mussel detections from the source harbor, ballast intake, ballast discharge, and receiving harbor in Figure 1B−E, respectively. Positive detection is indicated by two peaks in the size distribution of nanoparticles. The peak having smaller diameter size represents functionalized nanoparticles that did not hybridize with target DNA in the reaction, while the peak of larger-size diameter represents instances when the oligonucleotide tag on the nanobead hybridized with target species DNA. Note that, in Figure 1B,C,E, the estimated diameters of the nanoparticles measured by LTS matched the predicted sizes of functionalized, nonhybridized nanoparticles (~230 nm) determined in control experiments, with positive detection causing a size shift from 140 to 160 nm for a subset of nanoparticles that bound target species DNA to mean diameters of 370 to 390 nm (see Figure 1F for summary statistics for all 41 positive quagga mussel results). In comparison, in Figure 1D, the two peaks were also present indicating a positive detection. However, both peaks were shifted larger than the mean diameters expected for nonhybridizing (230 nm) and hybrizing (~380 nm) nanoparticles. Variation from anticipated peak shifts was observed in 5 of the 57 positive results (8.8%) in the current study. The presence of the two peaks represented a positive detection in these 5 cases, since we have never observed a double peak in any negative control in this or any previous study.[24,25] However, it is possible that interactions between bound and nonbound nanoparticles may occasionally occur in the hybridization reaction to generate the greater than expected shifting of peaks in positive detections. Further work is needed to test this hypothesis. The average size shift (±standard deviation) in the diameter of nanoparticles relative to the baseline beads attached with quagga-specific tags for all 41 standard samples (ballast and harbor) was 139 ± 4.18 nm (Figure 1F).

In the experimentally manipulated ballast and harbor water samples, LTS successfully detected zebra mussel eDNA in 15/16 of the spiked samples (94%). The one sample where zebra mussel was not detected by gel electrophoresis coincided with the sample that zebra mussel was not detected by LTS. Thus, either DNA was not successfully isolated from this sample or the PCR failed, which is to be expected with real-world sampling of eDNA. As was the case for gel electrophoresis, all eight control samples were also negative for zebra mussel in LTS tests. Figure 2A shows the LTS measurement of one control sample where no target zebra mussel tissue was added; Figure 2B is the positive LTS result for one individual sample spiked with zebra mussel tissue, and Figure 2C is the average size shift of 141 ± 6.33 nm standard deviation observed in the diameter of the nanoparticles relative to the baseline beads for the 15 cases of positive LTS detection.

eDNA as an Effective Tool for Species Detection in Ballast and Habor Waters. In unmanipulated field samples, eDNA species detection matched expectations from the natural history of each species.[36−38] Zebra mussel, which is known to be less cold-tolerant that quagga mussels, was not detected, while quagga mussel was detected in most samples at a rate of

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85% for the gel assay and 100% for the LTS assay. In the spiked experimental samples, both assays detected zebra mussel in 94% of spiked samples and 0% of negative control samples. Overall, these results demonstrate that eDNA sampling is effective for monitoring ballast-mediated invasions and that LTS is a sensitive species-specific assay with the potential for rapid, field-based detection. Moreover, using our eDNA sampling and species detection methods, we found clear evidence of naturally occurring quagga mussel DNA in all four sampling localities (ballast and harbor water from the Indiana and Duluth harbors). Using traditional methods, it is common to find the dreissenid veligers (i.e., planktonic larval forms) in the ballast tanks of large ships; however, they are nearly impossible to determine morphologically to species based on standard microscopy.[42] Thus, in addition to detection, eDNA can offer greater taxonomic clarity.

One previous study has applied species-specific PCR to ballast water surveillance[43] in which standard PCR was used to screen for starfish larvae from bulk plankton samples, not eDNA. Deagle et al.[43] showed it worked with no false positives detected and could detect 1 larvae/200 mg plankton. In general, the eDNA approach has been applied successfully for species detection to multiple aquatic organisms (fish, amphibians, and invertebrates) worldwide[22,23,26,29−31] and is rapidly developing into a burgeoning field of research in molecular ecology and application in conservation and invasive species management.[21,32] Our results suggest that eDNA species detection from the ballast water of large transoceanic ships is a natural extension of the eDNA approach.

Next Steps: Moving into Rapid Field-Based Detection with LTS. We also demonstrate that the field-ready LTS is a very sensitive technique for rapid target species detection from ballast and harbor waters. Previous research regarding LTS as a DNA detection tool has shown that it can work effectively in species detection using pure lab samples with one species present,[34] when in the presence of the DNA of other closely related species,[25,33] at low concentrations,[25] and for real-world samples, where there are a variety of species present and the target DNA fragment is amplified with universal invertebrate primers and species specific LTS tagged nanobeads.[24] In fact, detection capabilities of this LTS system suggest potential detection to the picomolar (10−12) range.[25] The LTS device fits into a large briefcase and could run on a car battery in the field; thus, LTS offers the opportunity to become a rapid DNA detection method for real-world applications.[33,34] Our ultimate objective is to apply the technology for rapid point of sampling detection in nature. To this end, LTS technology is portable, fast, and relatively inexpensive; we have recently developed a beta-model LTS device that together with a field-based PCR unit fit in a "carry-on" sized suitcase. It can run for a day from a rechargeable 12-V battery, and following PCR amplification, it can measure a sample in less than 10 s. The next major phase of research is 2-fold: (1) to integrate already developed field DNA extraction techniques[44,45] and field PCR technologies[46,47] to be fully field-ready and (2) to conduct LTS testing on site and eventually to transfer the technology to personnel on the front line of invasive species detection in the field. In this regard, one extremely valuable application of LTS would be to begin rapidly screening the ballast tanks of transoceanic ships coming into the U.S. for invasive species. An urgent need exists for rapid detection methods like LTS to identify harmful invasive species in ballast prior to port entry to provide adequate time for implementing appropriate management actions.

The general purpose of our work is to enable improved environmental protection, which would benefit from timely data to determine if potentially invasive species are contained in particular ships entering the Great Lakes and other coastal regions of the world. Then the risks could be understood at the spatial and temporal scales needed to make appropriate decisions to reduce the potential for harm. We feel that the further development and implementation of the technology tested here could provide important information to guide decisions in the public and private sector, including those by government agencies, the shipping industry, insurers, and other stakeholders, as it will become possible to rapidly test for the presence of target organisms in specific ships. For example, the tools described here could enable regulators to ensure compliance in actionable time frames and could be used by ship operators to document compliance with various enacted and proposed ballast water standards.[48] Finally, ballast and harbor water monitoring is only one of many potential applications for the tested technology, including detection of invasive species in ports or other environments, threatened or endangered species, species of public health concern (e.g., pathogens, parasites, or indicator species), and species of concern for biosecurity.

■ ASSOCIATED CONTENT

Supporting Information

Schematic diagram of DNA detection using portable LTS; schematic diagram of nanobead preparation and binding of target DNA to functionalized beads; additional photos of voyage and data collection on the *M/V Indiana Harbor*. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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