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Evaluation of eDNA qPCR monitoring as an early detection tool for a non-native mysid in Great Lakes Waters

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ABSTRACT

Early detection of aquatic invasive species (AIS) is vital to cost-effective prevention of their spread in the Great Lakes. Unfortunately, AIS surveillance has been generally too slow and geographically limited to support this purpose. Environmental DNA (eDNA) detection using quantitative polymerase chain reaction (qPCR) offers more rapid and affordable detection of likely AIS presence, but it does not directly discern live/dead status. Vital status verification using conventional surveys following positive eDNA qPCR detections could resolve this barrier, but only if the latter are adequately reliable and sensitive. Here we explore the reliability and sensitivity of eDNA qPCR monitoring for the bloody red shrimp (*Hemimysis anomala*), an AIS established in the southern Great Lakes but not yet widely distributed in Lake Superior, against conventional microscopy-based methods. We conducted this comparison using 1) harbor water from Muskegon Lake, MI where *H. anomala* is established, and 2) raw ballast water from ships transporting ballast from lower Lake Michigan to western Lake Superior. Our studies showed positive eDNA qPCR detections of *H. anomala* in all harbor and ballast samples for which conventional detection results were positive, and in some samples for which conventional results were negative. These results suggest that qPCR assays with adequate specificity could be an important tool in support of more effective and affordable early detection of target species in Great Lakes water, especially when combined with confirmatory conventional monitoring.

1. Introduction

Federal, state, and regional entities are working to more quickly detect target aquatic invasive species (AIS) within the Great Lakes basin to enable productive implementation of control measures (Great Lakes Commission, 2022; US FWS, 2019; U.S. Public Law 115-282, 2019). However, relatively slow and expensive conventional sample collection and analysis methods, such as plankton net sampling and microscopy, are obstacles to successful AIS early detection in the context of the expansiveness of the Great Lakes system and its harbors (Trebitz et al., 2017). As a result, environmental DNA (eDNA)-based monitoring methods such as species-specific quantitative polymerase chain reaction

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(qPCR) have been suggested as potentially less expensive and more reliable than conventional surveillance for rare organisms in harbors and vectors (*e.g.*, Darling and Frederick, 2018; Darling and Mahon, 2011; Egan et al., 2015; Feist and Lance, 2021; Ficetola et al., 2008; Hoffman et al., 2016; Jerde et al., 2011; Trebitz et al., 2017; Viard et al., 2016). Indeed, the prospect of using novel genetic detection tools for this purpose has been expressly noted in US federal statute and American and Canadian agency reports as an area of research interest for AIS early detection monitoring improvement (Environment and Climate Change Canada and the U.S. Environmental Protection Agency, 2022; U.S. Fish and Wildlife Service, 2019; U.S. Public Law 115-282, 2019). Molecular species detection also has been shown to be feasible and effective in the context of challenging water quality conditions such as those associated with Great Lakes harbors and ballast water (Egan et al., 2015).

As a stand-alone early detection surveillance tool, eDNA qPCR is limited by its inability to directly discern live/dead status or densities of detected target AIS, especially because trace eDNA may remain in the environment for variable periods after organism death depending upon the species and aquatic conditions (Dejean et al., 2011; Jo et al., 2019; Trebitz et al., 2017). Nonetheless, within the vast Great Lakes region, preliminary detections of organismal DNA would give valuable geographic focus to the more costly conventional methods required for confirmation of presence and vital status (Egan et al., 2015). Further, methods such as temporally repeated eDNA qPCR harbor surveys to determine eDNA signal longevity in the environment make even stand-alone eDNA detections informative of potential live/dead status (Barnes et al., 2014; Jo, 2023).

To explore the potential application of eDNA qPCR methods to early detection of a target AIS in Great Lakes water, we compared its capacity to detect presence/absence of a Great Lakes AIS, the bloody red shrimp (*Hemimysis anomala),* to that of conventional plankton net sampling and microscopic analysis. At the time of our field work (October 2015-November, 2015)*, H. anomala* was well established in the lower Great Lakes but not yet in Lake Superior, though after our field work was complete, individual specimens of the mysid were observed in Duluth-Superior Harbor (Kipp et al., 2022). In brief, we conducted paired sampling and analysis of water samples for *H. anomala* presence/absence using an *H. anomala*-specific eDNA qPCR assay and conventional methods, respectively, using:

- Surface water samples from Muskegon Lake in Lake Michigan, which was known at the time of this study to have established populations of *H. anomala*; and
- Untreated ballast water samples from U.S.-flag ships that travel only within the Great Lakes (hereafter, lakers) including uptake from a lower Lake Michigan harbor known to have established *H. anomala* populations present, and samples of subsequent ballast discharge to Duluth-Superior Harbor in Lake Superior.

We compared the results of the eDNA qPCR-based methods to those obtained using conventional methods. We hypothesized that the eDNA qPCR-based methods would be at least as sensitive as the conventional methods at detecting *H. anomala* presence such that positive eDNA qPCR detections would accompany positive microscopic detections for *H. anomala* across sample sites and events in both harbor and ballast water samples.

2. Methods

2.1. Hemimysis anomala qPCR assay

Our qPCR assay used a primer set developed to amplify a species-specific 148 bp fragment of the *Hemimysis anomala* COI gene: Hano_COI_F4 5′-CGGGTAACGTGTCACACATGG -3′ and Hano_COI_R4 5′-GGTATACTGTCAAACCCTATTCCTACA -3′ (Daniel Erickson, personal communications). This primer set was first tested for *H. anomala* specificity *in silico* against the all organisms in the NCBI nt database (https://www.ncbi.nlm.nih.gov/nucleotide/) with Primer-Blast and against all available freshwater mysid and *Hemimysis* COI sequences with multiple sequence alignment. The primer set specificity was then tested *in vitro* against tissue DNA from *Mysis diluviana* (the only other mysid in the Great Lakes), *Carcinus maenus* (green crab), *Dreissena bugensis* (quagga mussel), *Dreissena polymrpha* (zebra mussel), *Bythotrephes longmanus* (spiny waterflea), and *Cyprinus carpio* (common carp). To determine assay sensitivity and the relationship between qPCR Ct (cycle threshold) values and target DNA copy number, a 300 bp double stranded gBlock Gene Fragment (IDT, Newark, NJ,) containing the target sequence was synthesized based on *H. anomala* COI GenBank accession number EU029170. The gBlock stock was serially diluted ten-fold to achieve 3×10^9 to 3 copies per qPCR reaction and a standard curve (Ct value vs Log_{10} gBlock copy number) was constructed to determine PCR reaction efficiency and determine the limit of detection (LOD). LOD for the assay was defined as the lowest copy number that achieved a 95 % detection rate across all replicates (Klymus et al., 2019).

2.2. Comparison using harbor water

All activities in support of the harbor-based comparative analysis of *H. anomala* qPCR versus conventional detection methods took place in 2015–2016.

2.2.1. Site selection & sampling frequency

Muskegon Lake, Michigan is a location of known *H. anomala* occurrence with diverse habitat including sites deemed suitable to *H. anomala* colonization (Pothoven et al., 2007). Specific sampling sites were identified based on known habitat preferences of *H. anomala* (Pothoven et al., 2007), direct observation of *H. anomala* populations in Muskegon Lake (S. Pothoven, personal communications), and historic shipping berths (Fig. 1; Electronic Supplementary Material (ESM) Table S1). Three sampling events (October 2, October 26, and November 10, 2015) took place in which conventional and eDNA harbor sampling was conducted from a small boat or dock wall at the same time of day. Sampling for the two approaches was conducted in an immediately sequential manner in each sampling event. Water quality data were recorded (ESM Table S1). Over the course of the experiment, each site was sampled in this manner at least once and up to three times (ESM Table S1). Each paired sample-site/event supported an independent test of the two respective analysis method outcomes relative to *H. anomala* presence/absence.

2.2.2. Sample collection

Samples were retrieved at twilight or at night (ESM Table S1). In each sampling event, sampling for eDNA took place first, and just prior to conventional sampling. For each eDNA sampling event, wearing new gloves for each sample, we collected three 250 mL surface water grab samples from the harbor bank, pier, or a boat. If from a boat, two samples were taken from one side of the boat and one sample from the other side. Each 250 mL sample was filtered through a 25 mm diameter Whatman glass microfiber filter (pore size 0.45 μm) housed within a Swinnex filter housing (EMD Millipore) using a 50 mL syringe multiple times until the entire sample was filtered. Sterile forceps were used to transfer the filter into a labelled 1.5 mL microcentrifuge tube and stabilized with 750 μL of Longmire's buffer (Renshaw et al., 2015). eDNA negative control samples were prepared by filtering 250 mL of distilled water in the field at each site. All reusable sampling equipment was sterilized prior to each sampling event by soaking in 50 % bleach for 30 min, then rinsing in distilled water, and applying UV sterilization for 30 min on each side**.** Samples were stored at ambient temperature for 1–3 days as they were shipped to laboratory for further processing, and then stored at −20 °C until extraction.

Conventional sampling for *H. anomala* in harbor water was carried out immediately after eDNA sampling at each site consistent with peer-

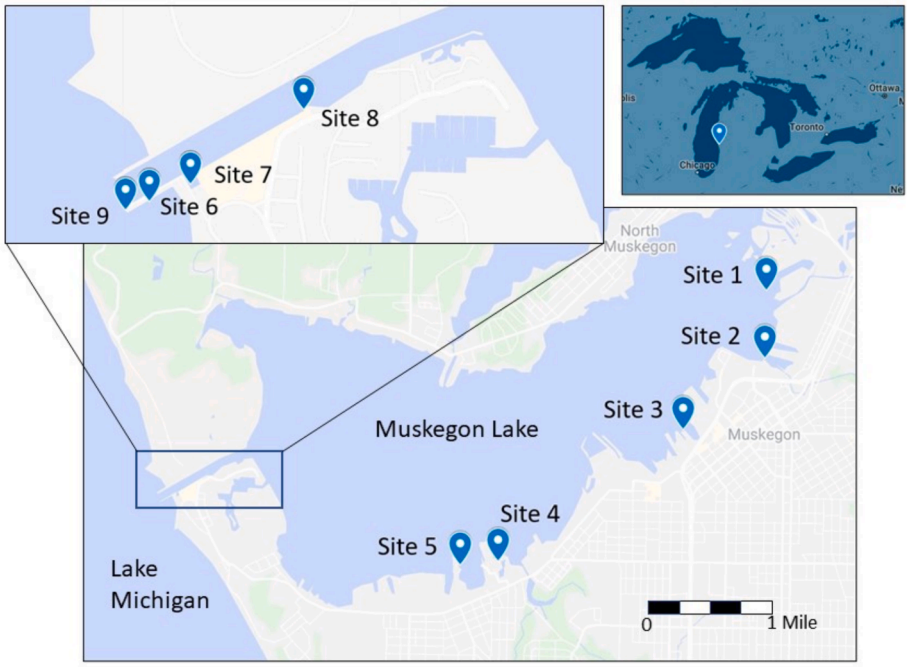

Fig. 1. Sampling sites in Muskegon Lake in western Michigan (site coordinates are listed in ESM Table S1) identified based on habitat preferences of H. anomala, direct observation of H. anomala populations in Muskegon Lake, and historic shipping berth locations.

reviewed methods for assessments of *H. anomala* densities (Borcherding et al., 2006; Marty et al., 2010; Walsh et al., 2010). We conducted two vertical net hauls using a 0.75 m diameter apparatus equipped with a 400 μm net mesh and 64 μm bucket mesh. The net was lowered to depths ranging from 1.27 to 3.9 m, left undisturbed for 1 to 3 min and then raised at a rate of ~0.3 $m \cdot s^{-1}$ (ESM Table S1). All net samples were preserved in 95 % EtOH.

2.2.3. Sample analysis

Microscopic analysis of conventional samples took place at the University of Wisconsin Superior's Lake Superior Research Institute (LSRI), and eDNA analysis took place at Governors State University (eDNA extraction) and the University of Notre Dame (qPCR) with no consultation between teams of analysts on test outcomes. For microscopic analysis, the entire volume of each sample was poured into a sieve (300 to 500 μm) to concentrate sample material. All fine materials and original preservative were reserved. The sample was rinsed with distilled water and a small volume of each prepared sample was placed in a gridded four- or six-inch petri dish and examined using a dissecting microscope. All mysids were removed from the sample, examined under higher power, and identified using characteristics described in Pothoven et al. (2007). After identification, all mysids were enumerated and placed in clean vials of 95 % EtOH. After the entire sample was processed, the sample material, including all organisms and preservative, was returned to the original sample container. Detection limits associated with each sample analysis were determined based on volume filtered given 0.75 m net diameter and depth of net tow to surface. The formula used to determine volume filtered for each tow was $V = \pi$ * net radius squared * depth of tow. Volumes filtered and organisms detected from the two replicate tows associated with each sample-site/event were combined into an overall sample volume and density estimate for that sample site/event. The detection limit for microscopic analysis was defined as 1 organism in the total volume of water sampled across the two net tows and expressed as $\#/m^3$.

Harbor water eDNA analysis began by extracting eDNA from filter membranes following a standard phenol–chloroform protocol detailed in Renshaw et al. (2015) in the Grey's PCR-free Environmental Genetics Laboratory at Governors State University. Briefly, the outsides of the tubes were wiped with 5 % bleach and 70 % ethanol to remove potential contaminants. Samples were incubated in a 65 °C water bath for 10 min, followed by addition of 900 μl of phenol:chloroform:isoamyl (25:24:1, Amresco), mixing by vortex mixer for 5 s, and centrifugation at 15,000 g for 5 min to separate the organic and aqueous phases after which 700 μl of the aqueous phase was transferred to a new tube. Next, 700 μl of chloroform:isoamyl (24:1, Amresco) were added and samples were vortexed for 5 s and centrifuged at 15,000 g for 5 min. A portion (500 μl) of the aqueous layer was transferred to a new tube, 1.25 mL of ice-cold ethanol and 20 μl of 5 M NaCL was added, and samples were precipitated overnight at −20 °C. Samples were then centrifuged at 15,000 g for 10 min to pellet the DNA, liquid was poured off, and pellets were left to dry at room temperature overnight. Dry DNA pellets were resuspended in 200 μl of 1X TE buffer and stored at −20 °C until use.

For each harbor water eDNA sample, three replicates were run on a Mastercycler® ep realplex (Eppendorf) at the Lodge Laboratory at the University of Notre Dame (South Bend, Indiana, USA) in the following 20 μl reactions: 4.85 μl of PCR-grade water, 4 μl of 5X Colorless GoTaq® Flexi Buffer (Promega), 0.4 μl of 10 mM dNTPs, 1.6 μl of 25 mM MgCl2, 1 μl of each 10 μM primer (forward and reverse), 0.15 μl of GoTaq® Flexi DNA Polymerase (Promega), 1 μl of EvaGreen (20X in water; Biotium), 2 μl of 4 μg/μl bovine serum albumin (Amresco), and 4 μl of eDNA extract. Thermocycling conditions were as follows: an initial denaturation at 95 °C for 3 min; 45 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 45 s, and extension at 72 °C for 1 min; followed by a melting curve analysis that transitioned from 60 °C to 95 °C over a span of 20 min. For each plate, positive (*H. anomala* tissue extract) and negative (PCR-grade water) controls were included, and samples were only considered positive if they had a positive Ct value and their melt curve peaks matched those of the positive controls. Positive hits were purified with ExoSAP-IT™ following manufacturer's instructions and then Sanger sequenced in the forward direction to confirm *H. anomala* identity on an Applied Biosystems 3730xl DNA Analyzer using manufacturer's recommendations. *H. anomala* identity was confirmed if there was ≥ 98 % sequence similarity to *H. anomola* COI accession EU029170. For context, the only other co-occurring mysid in the Great Lakes,

M. diluviana, has 92.6 % sequence similarity at this locus (ESM Fig. S1).

2.3. Comparison using ballast water

Our ballast water-based comparative analysis of *H. anomala* qPCR-based versus conventional methods of detection took place in 2017 using raw laker ballast water. Lakers are ships that ply largely dedicated trade routes in the upper four Great Lakes, hauling bulk cargoes generally from northern ports southward, and raw ballast water northward on the return voyages (Cangelosi et al., 2018). We analyzed samples collected from a single ship in laker trade on the Great Lakes which was subject to sampling for the Great Waters Research Collaborative's Great Lakes Ship Ballast Monitoring Project (GLSMP) (Cangelosi et al., 2018). The primary purpose of the GLSMP sampling was to understand trends associated with ballast-mediated organism movement within the Great Lakes system rather than to associate specific ships with specific organisms in ballast uptake or discharge (Cangelosi et al., 2018). Therefore, we do not report the name or owner of the ship from which the samples subject to examination in this experiment were collected. Dates of sample collection at specified ports are reported by month and week rather than by day.

2.3.1. Sample collection

Ballast water samples were collected for microscopy and eDNA qPCR analysis in parallel from fifteen laker ballasting events (four uptake and 11 discharge events) subject to monitoring in the GLSMP (Cangelosi et al., 2018). Methods of sample collection followed the sampling protocol for harbor water as closely as possible. To achieve this, we diverted a low flow stream of ballast water into a 19 L carboy while collecting conventional samples in a downstream plankton net apparatus (Cangelosi et al., 2018). For each sampling event, the carboy was well-mixed, and samples were filtered using a manifold vacuum filtration system. Otherwise, sample replication (three), filter membrane type (0.45 μm glass fiber), filters preservation (700 μl Longmire's buffer), blank field controls, and equipment sterilization followed the same protocols as those used for harbor water collection.

Conventional ballast water samples were collected by directing a large continuous stream of whole ballast water from the ballast main through a 35 μm plankton net, to produce a time-integrated sample of multiple cubic meters of ballast water. Sample water flow rate was controlled to deliver a target minimum sample volume of 2.0 m^3 of water. When possible, an additional larger-volume sample was also collected to more effectively collect *H. anomala.* In these instances, following collection of the sample, up to an additional 3.0 m^3 sample of ballast water was concentrated through a 400 μm plankton net. Collection of this second sample was only possible when a return port was installed in the vessel's ballast line, and enough time remained during cargo loading/unloading to allow for another ~60 min of sampling. Volumes collected and associated detection limits for microscopic analysis for *H. anomala* across trials are shown in ESM Table S2. Immediately following collection, club soda was added to the samples (5 mL per 100 mL sample) to narcotize the organisms (Makarewicz, 1991), and samples were placed on ice until preservation in 5 % neutral-buffered formalin solution.

2.3.2. Sample analysis

As with sample collection, ballast water sample analysis protocols were kept as similar as possible to those used in the harbor water study. Microscopic analysis was performed at LSRI and eDNA qPCR analysis at PSU-Behrend. There was no consultation between the laboratories during analysis, and samples were coded to obscure sample site/event origin. For microscopic analysis, the volume of raw ballast water filtered and examined as concentrated zooplankton samples ranged from 0.91 m (Trial 14) to 5.28 m (Trial 11). The microscopic detection limit associated with each sample analysis event was defined, respectively, as 1 organism in the equivalent volume of water sampled and analyzed.

For the eDNA qPCR analysis, 700 *u*L of Longmire's buffer was added to three sample tubes that were found to be dry upon receipt of samples at PSU-Behrend. Otherwise, ballast eDNA analysis was identical to that of the harbor water analysis, except that qPCR was run on a Step One System (ABI) machine and positive samples were initially checked by running the amplification products on a 1.75 % Seakem Agarose Gel (as opposed to a melt curve) using *H. anomala* genomic DNA qPCR product as a positive control to verify target amplicon size. Samples were only considered positive if they had a positive Ct value and the size of the amplification product matched that of the positive controls. The positive hits were then confirmed by purifying the amplification products and conducting bidirectional Sanger sequencing (as opposed to unidirectional sequencing for the harbor water positives) at Penn State's Huck Institute of the Life Sciences core facility.

2.4. Conventional and eDNA analysis outcome comparison

H. anomala detection outcomes using conventional methods (plankton net sample collection and microscopic analysis) and eDNA qPCR methods were compared within each sampling event and analyzed for percent-correspondence across events within each overall sampling context (*i.e.*, harbor or ballast water). Per our hypothesis, we expected that the eDNA qPCR method would detect *H. anomala* in all samples-site/events for which organisms were detected using microscopy-based methods. A positive eDNA qPCR result paired with a negative conventional result was deemed indeterminate relative to eDNA qPCR assay reliability, as this outcome could reflect either an eDNA qPCR detection true positive paired with a false negative conventional analysis outcome, or an eDNA qPCR false positive (which would not necessarily prohibit the tool's use for early detection survey purposes).

3. Results

3.1. Hemimysis anomala qPCR assay in silico and in vitro tests

Both *in silico* and *in vitro* testing of the primer set used in this study found it to be specific and sensitive to 148-bp region of the *H. anomala* COI gene. Primer-Blast against the full NCBI nucleotide database and a visual inspection of a multiple sequence alignment of COI sequences from 23 other mysid species failed to identify any species with fewer than 5 mismatches with either primer (ESM Fig. S1). Conversely, the primer set was found to match perfectly with 8 of 9 available *H. anomala* COI sequences and to have a single mismatch on the reverse primer of the one accession (EU029162.1). Because the available *H. anomala* COI accessions came from across this species range (Audzijonte et al., 2008), this result is good evidence that the current primer set is highly specific to *H. anomala* in freshwater eDNA samples in the Great Lakes and beyond. Analysis of our qPCR assays standard curve showed that Ct values were highly correlated with $\log_{10}$ target copy number ($R^2 > 0.99$) over a range of 3×10^9 to 3×10^0 copies per qPCR reaction and that both *in vitro* PCR efficiency (1.91 or 91 %) and LOD (16 copies per qPCR reaction) were very good (ESM Fig. S2).

3.2. Comparison using harbor water

In total, there were 19 Muskegon Lake sample-site/events comprising samples from 9 sites each sampled across 2 or 3 dates (ESM Table S1). Conventional analysis yielded 7 positive *H. anomala* detection sample-site/events among these 19 total sample-site/events, with abundance increasing through the fall sampling season (Table 1). *H. anomala* eDNA was detected by qPCR in 11 of the 19 sample-site/events (Table 1). There were no samples in which conventional methods detected *H. anomala* presence without an associated eDNA qPCR detection, but there were 4 instances of eDNA qPCR detection in the absence of conventional detections (Sites 4, 5, and 6 on October 2nd, and Site 5 on October 26th; Fig. 1; Table 1). There were no *H. anomala*

Table 1
Detection of *H. anomala* eDNA using qPCR and *H. anomala* specimens using conventional methods across Muskegon Lake sampling sites and events. eDNA detections are expressed as the number of positive qPCR reactions per nine replicates (3 biological replicates X 3 PCR replicates per biological replicates) and all positive results were confirmed by DNA sequencing. Microscopic detections are expressed as the average number of individuals/m^3 within calculated detection limits. Total density of *H. anomala* individuals was estimated in extremely dense samples. Shading indicates a positive eDNA detection in the absence of a paired microscopic detection. An "n.s." indicates site not sampled.

Sampling Site (Fig. 1)	Sampling Event	eDNA Detections (# positive/9 replicates)	Microscopic Detections	
			Average #/m^3 Observed	Detection Limit (#/m^3)
Site 1	10/2/15	0	0	≥0.6
	10/26/15	0	0	≥05
	11/10/15	n.s.	n.s.	
Site 2	10/2/15	0	0	≥0.6
	10/26/15	0	0	
	11/10/15	0	0	≥0.9
Site 3	10/2/15	0	0	≥0.6
	10/26/15	0	0	≥0.6
	11/10/15	0	0	≥0.4
Site 4	10/2/15	4	0	≥0.6
	10/26/15	5	45	≥0.5
	11/10/15	n.s.	n.s.	
Site 5	10/2/15	1	0	≥0.6
	10/26/15	1	0	≥0.5
	11/10/15	n.s.	n.s.	
Site 6	10/2/15	4	0	≥0.6
	10/26/15	5	8	≥0.6
	11/10/15	9	>1372	≥0.3
Site 7	10/2/15	n.s.	n.s.	
	10/26/15	2	12	≥0.3
	11/10/15	6	638	≥0.3
Site 8	10/2/15	n.s.	n.s.	
	10/26/15	n.s.	n.s.	
	11/10/15	4	>1372	≥0.3
Site 9	10/2/15	n.s.	n.s.	
	10/26/15	n.s.	n.s.	
	11/10/15	9	1741	≥0.4
Total Positive Detections		11	7	
Total Negative Detections		8	12	

eDNA qPCR detections in the negative control samples or laboratory blanks.

3.3. Comparison using ballast water

Ballast water sample analysis results are shown in Table 2. Microscopic sample volumes ranged from 0.91 m^3 to 5.28 m^3 due to variable ship operational constraints. Associated microscopic detection limits defined as the equivalent of 1 organism in the volume of water sampled were therefore variable (Table 2; ESM Table S2; Fig. 2; Cangelosi et al., 2018). eDNA sample volumes remained uniform (1 L) across events, however, each 1 L eDNA sample represented a well-mixed subsample of the larger volumes used for the microscopy-based survey.

H. anomala specimens were microscopically detected in 7 of 15 ballast sampling events: uptake samples from Trials 6, 11, 12 and 13, and discharge samples from Trials 10, 11 and 13 (Table 2). Estimated *H. anomala* specimen densities across these sampling events ranged from 0.2 to 3.3/m^3 (Table 2; Fig. 2; Cangelosi et al., 2018). Although not statistically significant across our limited sample size, there was a trend that lower *H. anomala* densities were microscopically detected only in higher volume ballast water samples (Fig. 2), suggesting volumes greater than ~2 L were needed to reliably detect *H. anomala* microscopically. In contrast, *H. anomala* eDNA was detected in 10 of the 15 ballast events sampled, irrespective of microscopic density estimate (Table 2). The ballast sampling events with positive eDNA qPCR detections comprised all the events with positive microscopic detections (7/7 microscope-positive events; Table 2) plus three sampling events (Trials 7, 14 and 15 discharges) in which eDNA alone was detected (3/8 microscope-negative events; Table 2). Blank samples for all sampling events were eDNA qPCR negative. Specimen detection limits for microscopic analysis across these *H. anomala* eDNA-positive but microscopic-negative ballast sampling events were ≥0.5 to ≥1.1/m^3 (Cangelosi et al., 2018; Table 2). Neither method detected *H. anomala* in discharge samples from Trials 6, 8, 9, 12, and 16 (Table 2).

Table 2
Detections of *H. anomala* eDNA using qPCR (whole water samples) and specimens using microscopy (net-filtered samples) across Great Lakes Ship Monitoring Project ballast sampling events. qPCR detections are expressed as # positive qPCR reactions per three replicates (1 biological sample X 3 PCR replicates per biological sample) and all positive results were confirmed by DNA sequencing. Microscopic detections are expressed as the average # individuals/m^3 within calculated detection limits. Shading indicates a positive eDNA detection in the absence of a paired microscopic detection.

GLSBMP Trial #	Ballast Operation (Month/Year)	eDNA Detections (# positive/3 replicates)	Microscopic Detections	
			#/m^3 Observed	Detection limit (#/m^3)
Trial 6	Uptake (09/2017)	3	0.4	≥0.4
	Discharge (09/2017)	0	0	≥0.5
Trial 7	Discharge (09/2017)	2	0	≥0.5
Trial 8	Discharge (09/2017)	0	0	≥0.5
Trial 9	Discharge (10/2017)	0	0	≥0.6
Trial 10	Discharge (10/2017)	3	3.3	≥0.2
Trial 11	Uptake (10/2017)	3	0.4	≥0.2
	Discharge (10/2017)	2	2.7	≥0.3
Trial 12	Uptake (10/2017)	3	0.2	≥0.2
	Discharge (10/2017)	0	0	≥0.2
Trail 13	Uptake (11/2017)	3	2.4	≥0.6
	Discharge (11/2017)	3	0.2	≥0.2
Trial 14	Discharge (11/2017)	1	0	≥1.1
Trial 15	Discharge (12/2017)	1	0	≥0.5
Trail 16	Discharge (12/2017)	0	0	≥0.2
Total Positive Detection Events		10	7	
Total Negative Detection Events		5	8	

4. Discussion

This study analyzed the reliability and sensitivity of eDNA qPCR survey methods relative to conventional microscopic survey methods for screening a Great Lakes harbor and ballast water for presence of a target

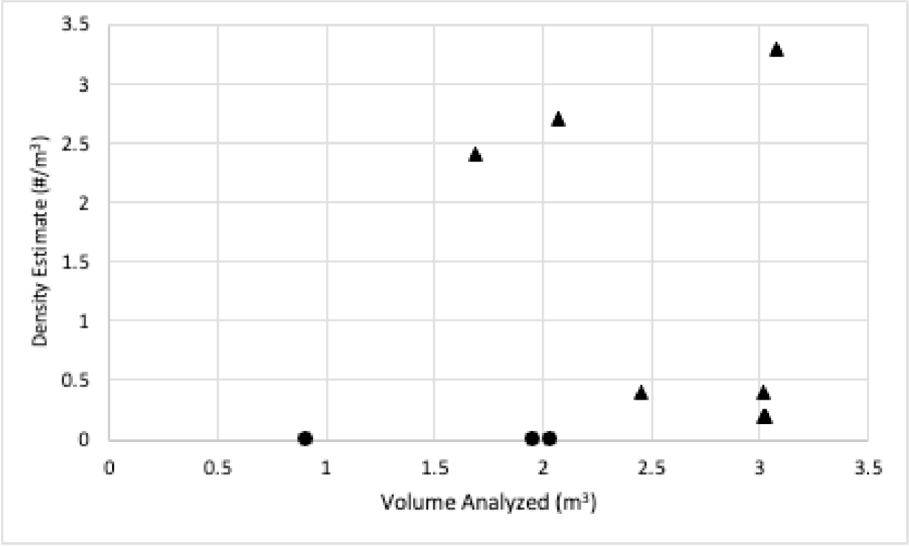

Fig. 2. Microscopic density estimates (# individuals/m^3) by net sample volumes for ballasting events with corresponding positive eDNA qPCR detections. There was eDNA/conventional method agreement across a range of microscopic density estimates high (filled triangles; 7 points). Samples for which there was a positive eDNA qPCR detection without microscopic detection of *H. anomala* tended to have lower volume net-filtered samples (filled circles; three points).

AIS, *H. anomala*. We found that negative eDNA qPCR results in both harbor and ballast water samples could be strong indicators of instances in which there would also be negative microscopic results; all eDNA qPCR negative samples, from both ballast and harbor waters, had negative conventional screening outcomes. Importantly, this trend persisted irrespective of microscopically-determined specimen density (*e.g.*, low target organism densities combined with high microscopic analysis volume, such as in the case of Trial 13 Discharge, Table 2; Fig. 2). This pattern of method agreement regarding AIS absence, even in the context of low target organism densities, suggests that eDNA qPCR methods can be useful for early detection of *H. anomala* in Great Lakes harbor and ballast water. It is worth noting, however, that although false negative occurrence did not appear to be an issue in this study, it can occur due to under-sampling water, laboratory error, and rapid degradation of eDNA. This liability underscores the potential value of temporally replicated survey design and positive field controls in eDNA-based early AIS detection surveys as a means of limiting false negative rates.

Our results also found strong concordance between positive eDNA qPCR detections and positive microscopy detections. Specifically, the only instances in which positive detections differed between the two methods were associated with eDNA qPCR-positive samples paired with microscope-negative ones. This pattern occurred in four harbor sampling site/events (including 2 sampling dates and 3 sampling sites, Table 1) and 3 of the 15 ballast water sampling events (Table 2). Given the study design, it was impossible to determine whether these stand-alone eDNA qPCR detections were true- or false-positives. However, there are grounds for presuming that most or all stand-alone eDNA-positives were accurate detections. First, all field and laboratory eDNA controls were negative for *H. anomala*, and positive qPCR detections were sequenced-confirmed, thus reducing the likelihood of contamination or non-specific amplification as a cause for false-positives, respectively. Second, the pattern of positive detections at a harbor site suggests that the post-summer reemergence of *H. anomala* into the water column (Nunn and Cowx, 2012) was detectable earlier using eDNA qPCR than through conventional methods. Specifically, no *H. anomala* specimens were encountered in the Muskegon Lake harbor survey using conventional methods across any sites during the first sampling event (October 2), though eDNA was detected at three sites sampled on that date (Sites 4, 5 and 6). *H. anomala* specimens were later microscopically detected at two of these sites (Sites 4 and 6 on October 26 and November 10; Table 1). The only harbor site (Site 5) with positive eDNA and negative microscopic detections across sampling events (October 2 and October 26), was not sampled in the final event (November 10) when *H. anomala* densities in the harbor were highest (Table 1). In the ballast water experiment, there is reason to think that stand-alone eDNA detections were true positives, as well. The subset of ballast sample events in which there were stand-alone *e*DNA-positives comprised instances with relatively coarse microscopic detection limits, i.e., only limited volumes of ballast water could be sampled and analyzed for *H. anomala* specimens (Table 2 and Fig. 2). Under such circumstances, the probability of microscopic detection of low densities of *H. anomala* was lowest, while sensitivity of the eDNA qPCR assay remained unchanged.

Nevertheless, while the occurrence of false eDNA qPCR negatives could call into question the utility of this approach for early detection monitoring of target AIS in the Great Lakes, false eDNA qPCR positives do not necessarily detract from that purpose provided their occurrence is relatively limited as it was in our experiments. Clearly, it is nonetheless essential to couple eDNA detection with subsequent confirmational steps, such as conventional surveys (e.g., LeBlanc et al., 2020) or spatially and temporally replicated eDNA qPCR surveys, prior to triggering significant remediation or other management actions. Still, doing so for the subset of harbors that yield positive eDNA qPCR detections would nonetheless afford far greater harbor/ballast water surveillance capacity than our conventional surveys alone.

While our harbor results show that eDNA qPCR is at least as effective as microscopy for detecting the presence of *H. anomala* Great Lakes water, a question remains whether eDNA qPCR surveys would be sensitive enough to detect newly discharged or small established populations. Unfortunately, our harbor study results cannot shed much light on this question because all our harbor-based microscopic density assessments were associated with relatively high *H. anomala* densities (190.5 to >2000 individuals/m^3). Results from the ballast water sample set, however, better show the potential of eDNA qPCR for detecting low *H. anomala* densities: positive eDNA detections were associated with microscopically observed densities ranging from 0 to 3.3/m^3 (with associated) microscopic detection limits as low as 0.2 organisms/m^3. However, it is important to note that our ballast water eDNA samples were obtained by sub-sampling a small volume of an integrated sample stream representing multiple cubic meters of raw harbor water taken into or discharged from ballast tanks. Depending on the patchiness of *H. anomala* distribution in a harbor, the probability of grab-sampling 1 L

of harbor water with trace *H. anomala* eDNA could be lower. Use of time-integrated pump sampling to increase spatial grain size in eDNA harbor sampling would help address this concern, as would temporally repeated sampling. In general, however, our findings are consistent with a recent meta-analysis of 535 papers which found that eDNA surveys of water bodies generally performed better or equal to traditional surveys (Fediajevaite et al., 2021).

Overall, our results suggest that eDNA qPCR surveys for *H. anomala* in samples of Great Lakes harbors and ballast water are at least as, and likely more, sensitive than those using microscopic methods for organism presence/absence in paired samples. This finding is consistent with that of several previous studies that have compared eDNA qPCR and traditional detection of freshwater AIS taxa including fish (Mahon et al., 2013), amphibians (Smart et al., 2015), mussels (Johansson et al., 2020), and crayfish (Tréguier et al., 2014). However, we note some studies have found traditional methods to be more sensitive than eDNA qPCR methods, such as for detection of semi-aquatic snakes (Rose et al., 2019) or the seasonally dynamic Great Lakes AIS zooplankton *Bythotrephes longimanus* (Walsh et al., 2019). Thus, although it appears that our eDNA qPCR survey was potentially more sensitive than traditional methods, there are important exceptions and testing eDNA qPCR survey sensitivity relative to conventional approaches on a species-by-species basis is warranted. Finally, considering the sensitivity of eDNA detection of *H. anomala* relative to conventional methods in our experiments, future comparisons of the reliability of eDNA detection *vis a vis* microscopic detection of a newly colonizing planktonic AIS (low densities) will benefit from similarly large sample volumes for microscopic analysis.

Is eDNA qPCR a cost-effective approach to support early detection for target invaders in the Great Lakes? While we cannot make broad generalizations given the many factors involved in survey optimization (Smart et al., 2016), we can conclude that for most AIS in the Great Lakes, the answer is likely yes. Indeed, for this study, eDNA collection and analysis was fast and inexpensive relative to that for the conventional methods, consistent with cost comparisons across approaches conducted by Fu et al. (2021). However, we also recognize that it was relatively easy for us to develop species-specific primers for *H. anomala* that did not require a more expensive hydrolysis probe. Only one other mysid shrimp is present in the Great Lakes region (*Mysis diluviana*; Kipp et al., 2022), the sequences and tissues of this cooccurring species (*H. anomala* and *M. diluviana*) were readily available, and we had access to molecular laboratories. In regions where there are several closely related species, or when sequences, tissue samples, and molecular capacity are less available, rigorous assay development may be more challenging and expensive (Langlois et al., 2021). Fortunately, improvements in species distribution and genetic sequence databases and a growing number of eDNA-focused laboratories are making species-specific qPCR assay development easier in general. For example, molecular data are increasingly available for barcoding genes of many common species in the Barcode of Life (Ratnasingham and Hebert, 2007) and GenBank (Benson et al., 2012) databases, and specific libraries are being compiled for invasive species in specific regions such as the Great Lakes (Daniel et al., 2021). Further, while qPCR assay development may require special expertise, once developed, resource managers anywhere, even in less well-equipped subregions, can readily collect and prepare samples for off-site analysis at regional centers of expertise. In summary, we believe eDNA qPCR surveys such as the one we tested could enable cost-effective and widespread early-detection monitoring for target AIS in the Great Lakes. Accompanying this capacity will be the opportunity for resource managers to prevent further spread of target invaders throughout the Great Lakes system.

5. Conclusion

Our results suggest that eDNA qPCR monitoring can be sensitive and reliable enough to serve as a means for the Great Lakes region to surveil harbors and ballast water for possible presence of *Hemimysis anomala* and potentially other planktonic AIS of concern. Any detections would need to be verified microscopically, but negative eDNA qPCR outcomes could be sufficiently reliable to signal AIS absence, especially in the context of a well-designed protocol with positive and negative controls and temporal replication. Because eDNA qPCR surveillance is relatively inexpensive to deploy broadly, the tool could make basin-wide AIS early detection in the Great Lakes feasible, and thereby better enable AIS spread-prevention.

CRediT authorship contribution statement

Allegra Cangelosi: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Mary Balcer:** Formal analysis, Investigation, Methodology. **Kelsey Prihoda:** Data curation, Methodology, Project administration, Validation. **Matthew Gruwell:** Formal analysis, Investigation, Methodology. **Matthew TenEyck:** Formal analysis, Investigation, Methodology. **Rebecca Aicher:** Investigation, Project administration. **Yuri Lopez-Camacho:** Conceptualization, Investigation. **Ivor T. Knight:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. **Erin K. Grey:** Conceptualization, Formal analysis, Resources, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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