Management of Biological Invasions (2019) Volume 10, Issue 1: 96–110

Research Article

Environmental DNA as a tool to help inform zebra mussel, *Dreissena polymorpha*, management in inland lakes

Jon J. Amberg[1,*], Christopher M. Merkes[1], Wendylee Stott[2], Christopher B. Rees[3] and Richard A. Erickson[1]

[1]*US Geological Survey, Upper Midwest Environmental Sciences Center, 2630 Fanta Reed Road, La Crosse, WI 54603, USA*
[2]*US Geological Survey, Great Lakes Science Center, 1451 Green Rd, Ann Arbor, MI 48105, USA*
[3]*US Fish and Wildlife Service, Northeast Fishery Center, 308 Washington Avenue, Lamar, PA 16848, USA*

Author e-mails: *jamberg@usgs.gov (JJA), cmerkes@usgs.gov (CMM), wstott@usgs.gov (WS), Christopher_Rees@fws.gov (CRB), rerickson@usgs.gov (RAE)*

**Corresponding author*

Citation: Amberg JJ, Merkes CM, Stott W, Rees CB, Erickson RA (2019) Environmental DNA as a tool to help inform zebra mussel, *Dreissena polymorpha*, management in inland lakes. *Management of Biological Invasions* 10(1): 96–110, https://doi.org/10.3391/mbi.2019.10.1.06

Received: 25 June 2018
Accepted: 6 September 2018
Published: 14 December 2018

Handling editor: David Wong
Thematic editor: Matthew Barnes

Abstract

Zebra mussels (*Dreissena polymorpha*) are an aquatic invasive species that plague much of North America and are difficult to impossible to eradicate once they become established. Therefore, prevention and monitoring are key elements in the control of these organisms. Traditional microscopy is commonly used in monitoring but requires the presence of larval veligers. This limits the times when resource managers can monitor for the presence in northern lakes. A new monitoring tool, environmental DNA (eDNA), may allow for a more efficient and cost-effective monitoring program for zebra mussels. We developed and tested an environmental DNA assay in the fall and spring for zebra mussels in two Minnesota lakes, one heavily infested and another newly infested. We found that DNA copy numbers tended to be higher near the lake bottom and DNA was more concentrated in softer substrates. We also found that the amount of zebra mussel DNA sampling in winter resulted in similar results to when sampled in fall. This suggests that one could collect and analyze eDNA for zebra mussels during winter months to help inform future efforts in monitoring and control.

Key words: zebra mussels, eDNA, dreissenid, monitoring

Introduction

The zebra mussel (*Dreissena polymorpha*) is an aquatic invasive species (AIS) that has plagued much of the Great Lakes Region. First discovered in the Great Lakes Basin in 1988 (Hebert et al. 1989), zebra mussels have spread throughout many of inland lakes within states bordering the Great Lakes. Zebra mussels currently threaten expansion into the northwestern portion of North America. Once established in a system, zebra mussels cause significant ecological and economic damage. Rapidly expanding populations of dreissenids have changed food webs (Holland 1993), primary productivity (Padilla et al. 1996a), benthic communities (Ricciardi et al. 1997), spawning habitat (Fitzsimons et al. 1995), nutrient cycling (Qualls et al. 2007), and food availability (Miehls et al. 2009) likely due to

their role as ecosystem engineers (Vanderploeg et al. 2002; Gutierrez et al. 2003; Sousa et al. 2009; Karatayev et al. 2007; Karatayev et al. 2015). These impacts threaten the health of native mussels and fish. Besides these ecological impacts, dreissenids have been estimated to cost the American economy billions of dollars annually (Pimentel et al. 2000, 2005).

Natural resource managers have had very little success eradicating zebra mussels once populations become established. Populations may be eradicated early in the colonization; therefore, early detection is a key element in their management. Unfortunately, the zebra mussel life-cycle allows them to easily invade new waterbodies. Zebra mussels can spawn at water temperatures above 11–12 °C and produce free-swimming veliger offspring (Garton and Haag 1993; Claxton and Mackie 1998). Veligers are microscopic larvae that spread through the water column. Zebra mussel veligers are easily transported from one water body to another without detection (Griffiths et al. 1991; Johnson et al. 2001). Traditional methods for early detection of zebra mussels use microscopy to detect the veliger in a water sample (Johnson 1995; Lucy 2006; Durán et al. 2010). This method is labor intensive (Hoy et al. 2010), and is constrained to the times veligers are present within the waterbody.

Environmental DNA (eDNA) has become a popular tool to detect the presence of an organism in aquatic and terrestrial systems. Researchers are continuing to develop eDNA methods to examine reproduction, movement, and abundance of an aquatic species (Erickson et al. 2016) and eDNA has become an integral component of some monitoring programs (Rees et al. 2014; Woldt et al. 2015). The use of eDNA, combined with quantitative PCR (qPCR), can provide results faster, at a lower cost, and potentially higher resolution (Peñarrubia et al. 2016). For example, it has been reported that eDNA was able to detect the presence of zebra mussels when microscopy failed in reservoirs in the Northern Iberian Peninsula (Peñarrubia et al. 2016; Ardura et al. 2017).

Environmental DNA has been previously used for detecting dreissenids. Multiple conventional PCR assays have been developed to detect zebra mussels (Lance and Carr 2012; Egan et al. 2015; Ardura et al. 2017) These initial eDNA assays require sequencing the amplicon to verify which species is present and as a result, at the time we conducted our study there remained a need for an assay specific for zebra mussels. Since, multiple dreissenid mussel quantitative PCR (qPCR) assays have been developed since (Gingera et al. 2017).

An eDNA assay for zebra mussels would allow resource managers to more efficiently monitor systems in the absence of veligers, which are only present a portion of the year. Veliger detection by microscopy alone limits their monitoring times when water temperatures exceed 12 °C. Environmental DNA may allow resource managers to detect the presence of zebra mussel

at times when water temperatures are below this critical threshold and help discover locations where additional samples could be collected to confirm the presence of veligers using microscopy. This would be particularly useful if one could simply sample waterbodies during winter while they were covered in ice. Alternatively, resource managers can use visual surveys to identify the presence of adult mussels. This can be very labor intensive and prone to false negatives than microscopy. Visual surveys rely on the detection of large mussels, and as such, usually indicate the presence of adults.

The goal of our study was to develop and evaluate the use of an eDNA survey to identify sites within a lake that have high numbers of zebra mussels. The specific objectives of our study were to: 1) design an eDNA assay for zebra mussels, 2) develop a standard sampling strategy, and 3) determine if a correlation exists between zebra mussel DNA and environmental parameters including depth, substrate type, and mussel biomass. The development of an eDNA protocol to inform control applications will improve management of zebra mussels and decrease the risk of spread into new waters.

Materials and methods

Assay design

Zebra mussel (*Dreissena polymorpha*) and quagga mussel (*D. rostriformis bugensis*) sequences that were deposited in GenBank (GenBank Accession numbers: U47650.1, AF096765.1, U47651.1, EU651840.1, EU604834.1, JQ435816.1, DQ840132.1, AF510504.1, EF080862.1, EF080861.1, JQ756298.1, JQ756297.1, AF510507.1, AF510506.1, AF510505.1, JX099437.1, U47653.1, EF414495.1, EF414494.1, HM210081.1, HM210080.1, HM210079.1, AF474404.1, DQ333702.1, DQ333701.1, KC429149.1, JQ435817.1, DQ840125.1, AF120663.1, AF510510.1, AF510509.1, AF510508.1, AM749000.1.) were used to design primers that targeted a small (< 200 base pairs) portion of the 5' end of the cytochrome *c* oxidase subunit I gene (*COI*). We targeted a region of *COI* where primers could be designed not to amplify native mussels but amplify both species of dreissenid. The sequences between the primers would have enough mismatches between zebra mussels and quagga mussels so that a probe could be designed to differentiate between species. Primer sequences for the dreissenid mussel *COI* were tested for specificity *in silico* using NCBI's Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and designed specifically to not.

To make the assay specific for zebra mussels, we then designed a Zebra Mussel amplicon-specific minor groove binder (MGB) probe to work with the dreissenid mussel primers. Oligonucleotide sequences are presented in Table 1. Assay specificity was tested against genomic DNA from zebra mussels and 27 non-target species (Table 2) common to waters of the Midwestern United States. Genomic DNA was tested in two replicate reactions each as described below.

Table 1. Oligonucleotide sequences of primers, probe and the region of the zebra mussel genome targeted for amplification and the efficiency of the assay, limit of detection, and limit of quantification.

Oligonucleotide	Sequence
Dre2-F	TGGGCACGGGTTTTAGTGTT
Dre2-R	CAAGCCCATGAGTGGTGACA
Dpo-Probe	6FAM-CGTCCTTGGTG
Dpo-gBlock	TGTGGGCTGGCCTTGTGGGCACGGGTTTTAGTGTTCTTATTCGTTTAGAGCTAAGGGCACCTG GAAGCGTCCTTGGTGATTGTCAATGATATAATGTAATTGTCACCACTCATGGGCTTGTTATAA TTGTTTGTCTAG
Assay Efficiency	99.25% (range 98–101%)
Limit of Detection	3.3 copies

Table 2. List of species whose genomic DNA was used to test for non-specific amplification of our zebra mussel assay. Positive symbol (+) indicates amplification and negative symbol (–) indicates no amplification.

Species	Result	Species	Result
Zebra mussel (*Dreissena polymorpha*)	+	Speckled dace (*Rhinichthys osculus*)	–
Plain pocketbook mussel (*Lampsilis cardium*)	–	Bluehead sucker (*Catostomus discobolus*)	–
Black sandshell mussel (*Ligumia recta*)	–	Channel catfish (*Ictalurus punctatus*)	–
Bighead carp (*Hypophthalmichthys nobilis*)	–	Largemouth bass (*Micropterus salmoides*)	–
Silver carp (*Hypophthalmichthys molitrix*)	–	Rainbow trout (*Oncorhynchus mykiss*)	–
Grass carp (*Ctenopharyngodon idella*)	–	Brown trout (*Salmo trutta*)	–
Black carp (*Mylopharyngodon piceus*)	–	Lake trout (*Salvelinus namaycush*)	–
Common carp (*Cyprinus carpio*)	–	Brook trout (*Salvelinus fontinalis*)	–
Gizzard shad (*Dorosoma cepedianum*)	–	Bluegill (*Lepomis macrochirus*)	–
Fathead minnow (*Pimephales promelas*)	–	Yellow perch (*Perca flavescens*)	–
Mosquitofish (*Gambusia affinis*)	–	Lake sturgeon (*Acipenser fulvescens*)	–
Emerald shiner (*Notropis atherinoides*)	–	Pallid sturgeon (*Scaphirhynchus albus*)	–
Golden shiner (*Notemigonus crysoleucus*)	–	Tilapia (*Oreochromis aureus* x *Oreochromis niloticus* hybrid)	–
Spotfin shiner (*Cyprinella spiloptera*)	–	Paddlefish (*Polyodon spathula)*	–

Developing sampling protocol

Lake Minnetonka was sampled in Minnesota during July 2014 to develop a sampling protocol. We chose Lake Minnetonka due to its location and high abundance of zebra mussels. Water was collected from the surface, mid-water and near the bottom directly above a known aggregation of zebra mussels. Ten 50-mL samples were collected from each water depth. Surface samples were collected by placing 50-mL sterile conical tubes just below the water surface to collect the surface film. Mid-water and bottom samples were collected using separate 2.2 L horizontal Van Dorn water sampler. Each water sampler was treated for at least 5 minutes with a 10% bleach solution to ensure sterility and to remove contaminating DNA. The water sampler was lowered to mid-depth or the bottom and sealed. Once each sample was collected it was capped and placed on ice and stored at −20 °C within six hours. After samples were collected they were transported to the US Geological Survey Upper Midwest Environmental Sciences Center in La Crosse, Wisconsin (UMESC) on wet ice. Immediately after receipt at UMESC, samples were stored at −80 °C until further processing. DNA was extracted from individual samples and quantified using the procedure mentioned below.

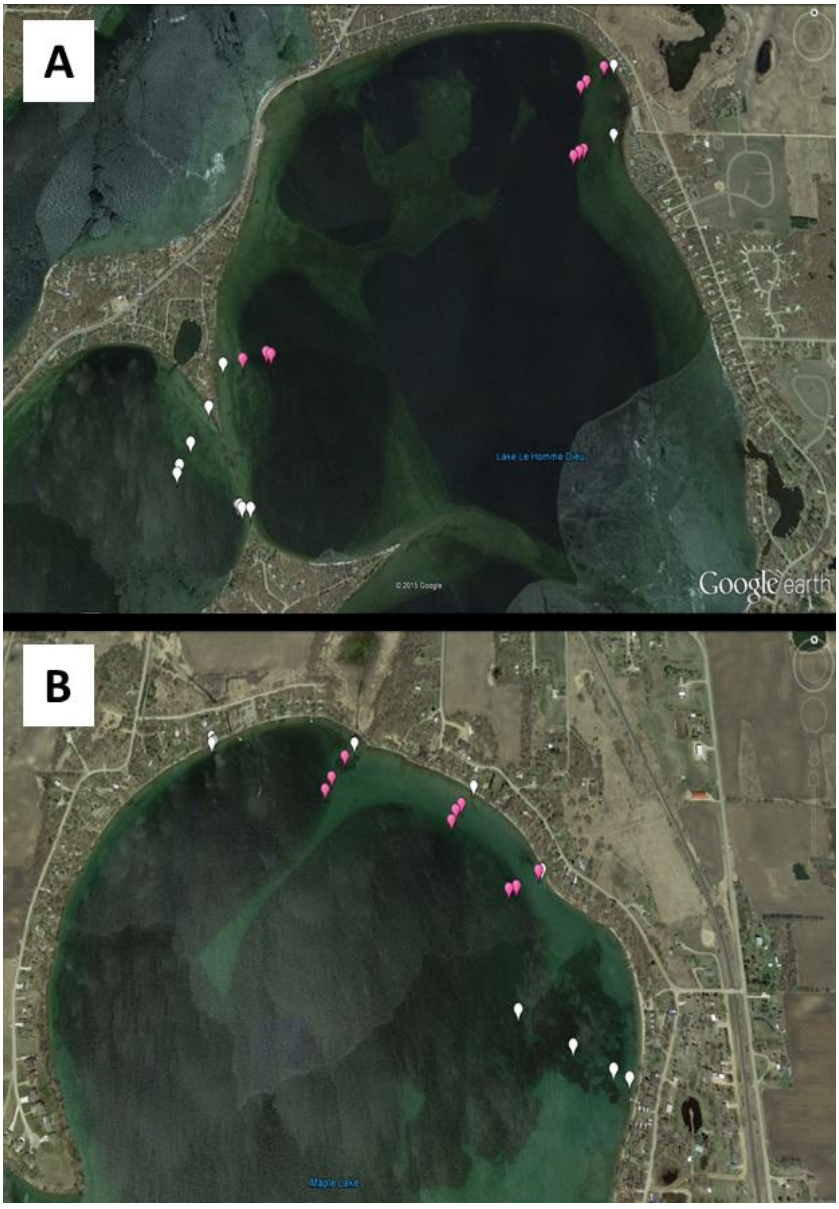

Figure 1. Sampling locations on Lake Le Homme Dieu (A) and Maple Lake (B) near Alexandria, Minnesota. All locations marked indicate where samples were collected. Pink and white pins indicate the locations where samples were collected in October 2014; We were only able to sample at the locations indicated with pink pins in March 2015 due to the depth of the ice.

Correlations among eDNA, biomass, and substrate type

To determine if a correlation exists between zebra mussel DNA and substrate type, density, and biomass, we sampled water from two lakes, Lake Le Homme Dieu and Maple Lake, near Alexandria, Minnesota (Figure 1). Lake Le Homme Dieu is approximately 728 ha with a maximum depth of 26 m. Zebra mussels were discovered in Lake Le Homme Dieu in 2009 and represents a lake with a well-established population. Maple Lake is 330 ha with a maximum depth of 24 m. Zebra mussels were discovered in Maple Lake in 2013 ("Infested Waters List: Minnesota DNR" available at https://www.dnr.state.mn.us/invasives/locations.html), represent a newly infested lake with an emerging population.

Table 3. Definitions of the six substrate types found in Lake La Homme Dieu and Maple Lake near Alexandria, Minnesota.

Type		Definition
Soft substrate	Flock	Extremely fine organic matter, without an established form
	Silt	Fine non-organic matter, can have an established form
	Sand	Granular and grains can be easily seen with naked eye
Hard substrate	Mixture	Organic matter containing larger organic material, including sticks
	Shell	Shell of dead zebra mussels
	Cobble	Stones larger than fingernail size

At each lake, we established four transects oriented perpendicular to shore that covered different substrate types from loose flocculent to cobble. Water samples were collected at depths of approximately 1, 2, 4 and 6 m along each transect using a 2.2 L horizontal Van Dorn water sampler in triplicate according to the method established above. Immediately following water sampling at each sample point, we placed a brick tied to a balloon marker and recorded GPS coordinates for further samplings. Each lake was sampled twice; first in late October 2014 and again under ice in early March 2015. During the March sampling event, we also collected water samples from the surface.

To verify the density of zebra mussels at each site, we used SCUBA divers to collect all the zebra mussels within triplicate 0.25 m^2 quadrats at each brick one day after eDNA sampling in October. Zebra mussels from each quadrat were brought to the surface and placed into separate plastic storage containers and placed on wet ice. All zebra mussel samples were frozen (−20 °C) within 4 h of collection. SCUBA divers also verified substrate (Table 3) at each sampling location. Albeit, the density of mussels at the brick may not representative of populations in the surrounding area for detection of zebra mussel DNA.

To estimate biomass, we calculated the ash-free dry weight (AFDW) for each Zebra Mussel sample. Each Zebra Mussel sample was weighed to determine total wet-weight. The moisture content was determined according to AOAC Official Method 934.01 (AOAC 2000) and subsequently ash-free dry weight was determined according to AOAC Official Method 942.05 (AOAC 2000). Analysis of moisture content and ash-free dry weight were conducted by the University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO). Ash-free dry weight was calculated using the following equation:

$$AFDW = S_a(s_a - m_a - a_a)/s_a)$$

Where S_a is the total mass (g) of the sample; s_a is the mass of the subsample used to determine AFDW; m_a is the mass (g) of moisture in the subsample; and, a_a is the mass (g) of ash in the subsample. AFDW was determined for each sampling quadrat.

Extraction and analysis of DNA

The exterior surface of each tube was cleaned with a 10% bleach solution prior to extraction to reduce cross contamination. Each 50-mL water sample was centrifuged at 5,000 × g for 30 minutes and the supernatant was decanted. We extracted DNA from the remaining pellet and residual water using the commercially available gMax mini genomic DNA extraction kit following the manufacturer's recommendations (IBI Scientific; Peosta, IA). We extracted 100 µL of deionized water as an extraction negative control with each extraction batch, and all samples had a final elution volume of 100 µL. We analyzed the DNA extracts in four replicate qPCRs with 1 µL of template in 20-µL reactions. Reactions contained 1x SensiFAST probe – no Rox master mix (Bioline; Taunton, MA), 200-nM forward and reverse primers, and 125-nM probe. Oligonucleotide sequences are listed in Table 1. We analyzed with the temperature profile of: 95 °C for 2 minutes; followed by 45 cycles of 95 °C for 30 seconds, 56 °C for 1 minute, 72 °C for 50 seconds; followed by 72 °C for 5 minutes; and a hold at 4 °C. We ran each plate on a Mastercycler Realplex 2 thermal cycler (Eppendorf North America; Hauppauge, NY) with four no template controls and two replicate standard curves. The standard curves contained gBlock gene fragment synthetic DNA of the target sequence (Integrated DNA Technologies; Coralville, IA) in a 6 order of magnitude, 5-fold dilution series from 31,250 copies down to 10 copies per reaction.

Data analysis

DNA copy number and ash free dry weights were log-transformed to account for over dispersion in distributions and to ensure normality of the distributions (Zar 2010). We used exploratory data analysis (EDA) before conducting confirmatory analyses to examine the data after collection and check for data entry error and gain insight into the data (Tukey 1977). Based upon the EDA, we did not include winter eDNA observations within the comparisons of fall DNA observations nor the ash-free dry weight because there were only 16 observations from the winter due to dangerous ice conditions that limited sampling. As part of the EDA, we examined the correlations among winter DNA, fall DNA, and ash-free dry weight. We also examined the relationships between DNA and environmental covariates including sediment type, lake, and depth.

We used structural equation (SE) modeling for our confirmatory analysis (Grace 2006; Grace et al. 2010) for each lake. Structural equation models are "Probabilistic models containing or specifying multiple causal pathways. SE models are characterized by (a) attempting to satisfy the criteria for drawing causal inferences and (b) permitting endogenous variables to be functions of other endogenous variables, thereby potentially containing indirect effects" (Grace et al. 2010). We specifically used a SE model to examine

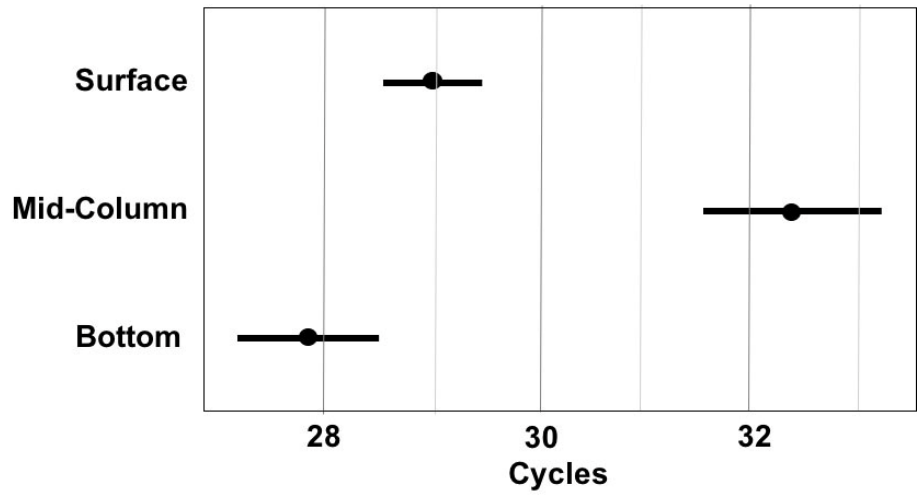

Figure 2. The mean number of cycles needed to detect DNA of zebra mussels from water samples collected at the surface, mid-column and bottom of Lake Minnetonka directly above a known zebra mussel population. A lower number of cycles indicates a greater amount of DNA. Bars represent the 95% confidence intervals.

the effects of soft (flock, silt, and sand) and hard (mixed, shell, and cobble) substrate, sample depth, lake, and log (AFDW) on log(eDNA):

$$AFDW \sim Soft + Hard + Depth + Lake$$

$$eDNA \sim AFDW + Soft + Hard + Depth + Lake.$$

All analyses were performed in R (R Core Team 2016) with a significance level of $\alpha \leq 0.05$. We are using the sem function from the laavan package in R to build our SEMs.

Results

Validation of molecular assay

We tested primer sequences (Table 1) for species-specificity *in silico* using NCBI's Primer-BLAST, and we found the primer sequences to be specific to the *COI* gene of zebra mussels with possible amplification from *Dreissena presbensis* or *D. stankovici* with four primer mismatches each. We validated the specificity of our assay against genomic DNA from zebra mussels and 27 non-target species listed in two replicate reactions. Our markers only detected DNA from zebra mussels and no detections were observed in any of the native mussels or fish species tested (Table 2). Therefore, we concluded that this marker was adequate for detection of the presence of zebra mussel DNA in these Minnesota water bodies.

Development of sampling protocol

Water samples collected near the bottom or at the surface had a 100% detection rate while mid-column samples had an 85% detection rate. Samples collected near the bottom had lower Ct-values, indicative of greater amounts of zebra mussel DNA, than those samples collected from the surface (Figure 2).

Table 4. Mean number of positive detections and copies of zebra mussel DNA, as well as ash-free dry weight (AFDW) of zebra mussels for six substrate types in Lake La Homme Dieu and Maple Lake near Alexandria, Minnesota. Number in parentheses represent standard deviations.

	Detections	DNA copies	AFDW (g)
Lake La Homme Dieu			
Flock	3.80 (0.41)	110.18 (200.95)	0.93 (1.80)
Silt	4.00 (< 0.01)	27.41 (33.11)	0.24 (0.44)
Sand	3.56 (0.53)	32.26 (82.71)	28.13 (32.76)
Mixture	3.44 (0.92)	45.72 (87.07)	1.39 (1.43)
Shell	3.17 (1.17)	6.96 (8.41)	0.19 (0.19)
Cobble	3.33 (0.82)	8.77 (4.40)	9.16 (14.90)
Maple Lake			
Flock	1.47 (1.73)	4.26 (7.09)	0.01 (0.01)
Silt	0.83 (1.60)	1.40 (3.38)	0.01 (0.02)
Sand	2.00 (1.00)	1.38 (.049)	0.22 (0.17)
Mixture	2.13 (1.51)	1.83 (2.05)	0.11 (0.17)
Shell	2.89 (1.76)	23.10 (26.39)	0.03 (0.04)
Cobble	1.92 (1.50)	1.54 (2.28)	1.06 (1.35)

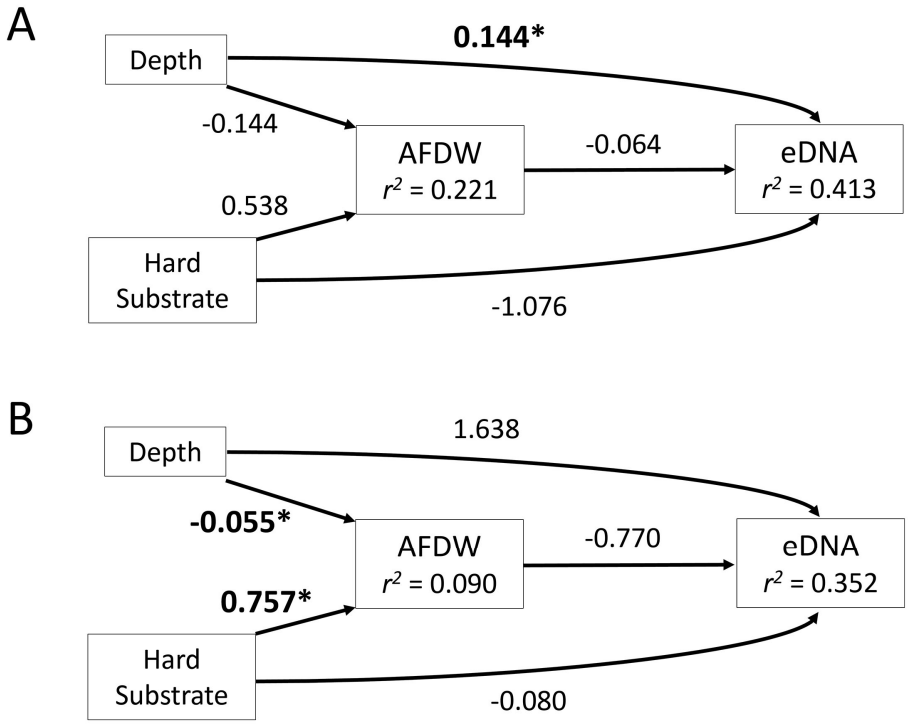

Figure 3. Structural Equation Model for zebra mussels in two lakes near Alexandria, Minnesota: Lake Le Homme Dieu (A) and Maple Lake (B). Nodes are environmental DNA copy numbers of zebra mussel DNA (eDNA), habitat, depth, lake and ash-free dry weight (AFDW). AFDW is log(AFDW + 0.1). eDNA is log(copy number eDNA + 0.1). Numbers next to a line between two nodes represents the correlation between the two nodes. The r^2 values in boxes correspond % variance of dependent variable explained by the independent variable. Values with an asterisk (*) indicate significant correlation between nodes. Our significance level was established at $\alpha \leq 0.05$.

Correlations among eDNA, biomass, and habitat

Density (AFDW) of zebra mussels and the amount of their DNA was greater in Lake La Homme Dieu than in Maple Lake (Table 4). SEM for both lakes is represented in Figure 3. Our models had Comparative Fit Index of 1.00 for both Maple Lake and Lake Le Homme Dieu. Likewise, the

Tucker-Lewis Index was 1.000 for both models as well. In Lake Le Homme Dieu, eDNA did not predict AFDW ($p = 0.52$). The number of copies of zebra mussel eDNA increased with depth ($p < 0.01$) and decreased with habitat ($p = 0.04$). Deeper water had less hard substrate than shallow sites in Lake Le Homme Dieu. The amount of DNA collected at a site during October did not correlate with that collected under ice in March at the same location ($p = 0.77$). In Maple Lake, eDNA was not correlated with AFDW ($p = 0.09$), depth ($p = 0.25$), or hard substrate ($p = 0.08$). The AFDW decreased with depth ($p < 0.01$) but increased with hard substrate ($p < 0.01$) in Maple Lake. Like Lake Le Homme Dieu, deeper water had less hard substrate than shallow sites in Maple Lake, but unlike Lake Le Homme Dieu, the amount of DNA collected at a site during October was similar with that collected under ice in March at the same location ($p = 0.01$).

Discussion

A thorough *in silico* analysis and then validation of the specificity of the marker against DNA from species expected to be present at the sampling sites is important prior to conducting the actual trial and can improve confidence in data interpretation (Goldberg et al. 2016). Our primary concern was the potential amplification of non-targeted DNA from species present in the test system. *In silico* analysis indicated our markers may cross amplify with *Dreissena presbensis* or *D. stankovici*. Both of these species live in the Baltic Region and have not been found outside of Europe. *Dreissena presbensis* is listed as near threatened on the IUCN Red List (http://www.iucnredlist.org/details/197089/0). Therefore, we concluded that neither of these two dreissenid species posed any risk in generating a false positive. Multiple markers for detection of zebra mussel DNA have been designed (Bronnenhuber and Wilson 2013; Egan et al. 2015; Peñarrubia et al. 2016; De Ventura et al. 2017), but none of these have been tested against DNA from species that are likely to be encountered in water samples collected from a lake within the Upper Midwestern United States and southern Canada. Our markers did not amplify genomic DNA from 27 species (Table 2), many of which are found in our test lakes. We did not test against all species common to our test lakes, because they had good representation in the public databases and *in silico* analysis suggested little to no chance of cross amplification. Cross-evaluation against genomic DNA from species likely to be encountered in the monitoring program ensures a high level of confidence in the specificity of the markers.

In addition to marker development, a standard sampling protocol is very important for collection of uncontaminated water samples. The primary source for DNA from carp is thought to be from their fecal material (Turner et al. 2014) and that carp DNA can be more concentrated in the sediments that at the water surface (Turner et al. 2015). This may likely be

true for eDNA from zebra mussels too. Sampling water at or near the surface works well to collect DNA shed for a targeted animal only if their fecal material floats. Fecal material from many aquatic animals, including zebra mussels, likely sinks and as such, their DNA likely will not accumulate at the surface and instead concentrate in the sediments. Zebra mussels produce pseudofeces, which may be a significant source of DNA, that also sinks. We expected to find more zebra mussel DNA near the bottom than at the surface, but this was not observed. Instead, there was no statistical difference between the number of copies of zebra mussel DNA from samples collected near the bottom or the surface (Figure 2). This discrepancy may be attributed to the time of the year this trial was conducted. We conducted this portion of our study in the summer of 2014. Lake Minnetonka has a well-established population of zebra mussels that are actively reproducing. We sampled in Robinson's Bay in about 3 meters of water. Robinson's Bay is under the influence of wave action due to a large fetch and parallel orientation with predominant winds. It is very likely the DNA, veligers, or both would have been uniformly or near-uniformly mixed in this area. Since veligers are planktonic, they can contribute to large amounts of DNA if collected in the water sample. We did not verify if our water samples contained veligers, but if they did the probability of detecting zebra mussel DNA in surface samples likely would be variable throughout the year; therefore, we decided to collect samples from the bottom. During the winter sampling, we did not detect zebra mussel DNA in any of the surface samples.

Sample storage is a vital component of a sampling protocol. Improper storage can result in differences in eDNA detection rates (Takahara et al. 2015). We froze our water samples prior to transporting to the lab for further processing. Takahara et al. (2015) found that freezing samples can negatively impact detections but did not affect copy numbers. All samples used in our study were treated identically, but we may have had slightly different detection rates if samples were not frozen.

In the heavily infested lake, Lake Le Homme Dieu we did not identify a correlation between AFDW of zebra mussels and substrate, or a positive correlation between zebra mussel DNA and substrate. Instead, we observed greater amounts of zebra mussel DNA in soft substrate and in deeper water. This is likely due to the extremely high abundance of zebra mussels in Lake Le Homme Dieu. Mussels use a wide range of substrates, including those that are not optimal, such as vegetation. The high density of mussels combined with wind-driven currents likely moved zebra mussel DNA into deeper deposition areas within Lake Le Homme Dieu; hence, we observed higher amounts of DNA from zebra mussels in deeper waters. These depositional areas are likely cold and protected from UV, both factors known to enhance the persistence of eDNA (Strickler et al. 2015). At high densities, zebra mussels likely shed DNA at a rate above the degradation

rate, which results in a saturation of zebra mussel DNA resulting in a limited ability for eDNA to help inform infested locations.

In Maple Lake, a lake with a newly established population of zebra mussels, AFDW correlated with habitat and depth but the amount of zebra mussel DNA did not correlate with these two factors. It was expected that both AFDW and zebra mussel DNA would be greater at sites with hard substrate that mussels could adhere, but only AFDW was correlated with habitat. This suggests that sampling over soft substrates and areas where zebra mussels are less likely to settle will work just as well as sampling over areas where the mussels would likely settle. If the DNA shed from an animal is primarily fecal in origin (Turner et al. 2014) and the feces of zebra mussels sinks and settles in deposition areas within the lake, then it would be likely that greater amounts of DNA would be detected in those deposition areas. These deposition areas within a lake are likely comprised of soft substrates and would not be classified as zebra mussel habitat. Much like what was observed in Lake Le Homme Dieu, the DNA concentrated in deposition zones or areas with softer substrates. Sampling over deposition zones would greatly simplify the strategy used in eDNA monitoring of zebra mussels.

We observed a correlation between sampling in the fall and in the late winter under the ice in Maple Lake. This suggests that sampling conducted in a newly infested lake does not have to target zebra mussel habitat, and sampling is equally effective under ice or in open water. An advantage for sampling under ice is that it allows for the DNA to accumulate by blocking UV and maintaining low temperatures, both factors known to degrade the DNA (Strickler et al. 2015) and there is less mixing. Winter sampling would enable a large number of northern lakes to be extensively surveyed by allowing access to sites throughout the lake without the need of a boat. Sampling in winter under ice would enable resource managers to then prioritize lake and locations for monitoring with SCUBA divers and/or microscopy in the spring and summer. Since zebra mussels are often transported into new lakes via boat traffic (Padilla et al. 1996b), observation of these microscopic zebra mussels is highly unlikely in a newly invaded lake. However, the use a zebra mussel eDNA survey at locations near boat launches could greatly improve detection and allow for control of the mussels. Additionally, monitoring for zebra mussels with eDNA under ice could help inform visual surveys.

In conclusion, we developed an eDNA assay for detecting zebra mussels in a lentic environment. Even though we could not correlate eDNA with AFDW, this method could detect new Zebra Mussel infestations. This assay or similar ones that detect dreissenid mussels (Andura et al. 2017; Gingera et al. 2017) may be useful in the early detection of zebra mussels and can be used during winter months. The use of eDNA may be a useful new tool for natural resource managers in their attempt to manage and control the spread of zebra mussels.

Acknowledgements

The authors would like to thank Pete Boma, Bridget Ladell, Jenna Malinauskas, and S. Grace McCalla (USGS – Upper Midwest Environmental Sciences Center) for assisting in the collection and processing of water samples. Finally, the authors thank the staff at the USGS Minnesota Water Sciences Center in their help in the collection of water samples during our winter sampling. This work was conducted under Legislative-Citizen Commission on Minnesota Resources, Environment and Natural Resources Trust Fund Grant M.L. 2013, Chp. 52, Sec. 2, Subd. 06f, and with funds from the Great Lakes Restoration Initiative template 674. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the US Government.

References

AOAC (2000) Official methods of analysis of AOAC. International 17th edition; Gaithersburg, MD, USA Association of Analytical Communities.

Ardura A, Zaiko A, Borrell YJ, Samuiloviene A, Garcie-Vazquez E (2017) Novel tools for early detection of a global aquatic invasive, the zebra mussel *Dreissena polymorpha*. *Aquatic Conservation: Marine and Freshwater Ecosystems* 27: 165–176, https://doi.org/10.1002/aqc.2655

Bronnenhuber JE, Wilson CC (2013) Combining species-specific COI primers with environmental DNA analysis for targeted detection of rare freshwater species. *Conservation Genetics Resources* 5: 971–975, https://doi.org/10.1007/s12686-013-9946-0

Claxton WT, Mackie GL (1998) Seasonal and depth variations in gametogenesis and spawning of *Dreissena polymorpha* and *Dreissena bugensis* in eastern Lake Erie. *Canadian Journal of Zoology* 76: 2010–2019, https://doi.org/10.1139/z98-150

De Ventura L, Kopp K, Seppälä K, Jokela J (2017) Tracing the quagga mussel invasion along the Rhine river system using eDNA markers: early detection and surveillance of invasive zebra and quagga mussels. *Management of Biological Invasions* 8: 101–112, https://doi.org/10.3391/mbi.2017.8.1.10

Durán C, Lanao M, Anadón A, Touyá V (2010) Management strategies for the zebra mussel invasion in the Ebro River basin. *Aquatic Invasions* 5: 309–16, https://doi.org/10.3391/ai.2010.5.3.09

Egan SP, Grey E, Olds B, Feder JL, Ruggiero ST, Tanner CE, Lodge DM (2015) Rapid molecular detection of invasive species in ballast and harbor water by integrating environmental DNA and light transmission spectroscopy. *Environmental Science & Technology* 49: 4113–4121, https://doi.org/10.1021/es5058659

Erickson RA, Rees CB, Coulter AA, Merkes CM, McCalla SG, Touzinsky KF, Walleser L, Goforth RR, Amberg JJ (2016) Detecting the movement and spawning activity of bigheaded carps with environmental DNA. *Molecular Ecology Resources* 16: 957–965, https://doi.org/10.1111/1755-0998.12533

Fitzsimons J, Leach J, Nepszy S, Cairns V (1995) Impacts of zebra mussel on walleye (*Stizostedion vitreum*) reproduction in western Lake Erie. *Canadian Journal of Fisheries and Aquatic Sciences* 52: 578–586, https://doi.org/10.1139/f95-058

Garton D, Haag W (1993) Seasonal reproductive cycles and settlement patterns of *Dreissena polymorpha* in western Lake Erie. In: T. Nalepa and D. Schloesser (eds), Zebra Mussels: Biology, Impacts, and Control Lewis Publishers, Boca Raton, FL, pp 111–128

Gingera TD, Bajno R, Docker MF, Reist JD (2017) Environmental DNA as a detection tool for zebra mussels *Dreissena polymorpha* (Pallas, 1771) at the forefront of an invasion event in Lake Winnipeg, Manitoba, Canada. *Management of Biological Invasions* 8: 287–300, https://doi.org/10.3391/mbi.2017.8.3.03

Goldberg CS, Turner CR, Deiner K, Klymus KE, Thomsen PF, Murphy MA, Spear SF, McKee A, Oyler-McCance SJ, Cornman RS (2016) Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution* 7: 1299–1307, https://doi.org/10.1111/2041-210X.12595

Grace JB (2006) Structural equation modeling and natural systems. New York, NY: Cambridge University Press, 365 pp

Grace JB, Anderson TM, Olff H, Scheiner SM (2010) On the specification of structural equation models for ecological systems. *Ecological Monographs* 80: 67–87, https://doi.org/10.1890/09-0464.1

Griffiths RW, Schloesser DW, Leach JH, Kovalak WP (1991) Distribution and dispersal of the zebra mussel (*Dreissena polymorpha*) in the Great Lakes region. *Canadian Journal of Fisheries and Aquatic Sciences* 48: 1381–1388, https://doi.org/10.1139/f91-165

Gutierrez JL, Jones CG, Strayer DL, Iribarne OO (2003) Mollusks as ecosystem engineers: the role of shell production in aquatic habitats. *Oikios* 101: 79–90, https://doi.org/10.1034/j.1600-0706.2003.12322.x

Hebert PDN, Muncaster BW, Mackie GL (1989) Ecological and genetic studies on *Dreissena polymorpha* (Pallas): a new mollusc in the Great Lakes. *Canadian Journal of Fisheries and Aquatic Sciences* 46: 1587–1591, https://doi.org/10.1139/f89-202

Holland RE (1993) Changes in planktonic diatoms and water transparency in Hatchery Bay, Bass Island area, western Lake Erie since the establishment of the zebra mussel. *Journal of Great Lakes Research* 19: 617–624, https://doi.org/10.1016/S0380-1330(93)71245-9

Hoy MS, Kelly K, Rodriguez RJ (2010) Development of a molecular diagnostic system to discriminate *Dreissena polymorpha* (zebra mussel) and *Dreissena bugensis* (quagga mussel). *Molecular Ecology Resources* 10: 190–192, https://doi.org/10.1111/j.1755-0998.2009.02727.x

Johnson LE (1995) Enhanced early detection and enumeration of zebra mussel (*Dreissena* spp.) veligers using cross-polarized light microscopy. *Hydrobiologia* 312: 139–146, https://doi.org/10.1007/BF00020769

Johnson LE, Ricciardi A, Carlton JT (2001) Overland dispersal of aquatic invasive species: a risk assessment of transient recreational boating. *Ecological Applications* 11: 1789–1799, https://doi.org/10.1890/1051-0761(2001)011[1789:ODOAIS]2.0.CO;2

Karatayev AY, Burlakova LE, Padilla DK (2015) Zebra versus quagga mussels: a review of their spread, population dynamics, and ecosystem impacts. *Hydrobiologia* 746: 97–112, https://doi.org/10.1007/s10750-014-1901-x

Karatayev AY, Padilla DK, Minchin D, Boltovskoy D, Burkalova LE (2007) Changes in global economics and trade: the potential spread of exotic freshwater bivalves. *Biological Invasions* 9: 161–180, https://doi.org/10.1007/s10530-006-9013-9

Lance RF, Carr MR (2012) Detecting eDNA of invasive dreissenid mussels, report on Capital Investment Project. ERDC Aquatic Nuisance Species Research Program Technical Note ANSRP-12-2

Lucy F (2006) Early life stages of *Dreissena polymorpha* (zebra mussel): the importance of long-term datasets in invasion ecology. *Aquatic Invasions* 1: 171–182, https://doi.org/10.3391/ai.2006.1.3.12

Miehls ALJ, Mason DM, Frank KA, Krause AE, Peacor SD, Taylor WW (2009) Invasive species impacts on ecosystem structure and function: A comparison of Oneida Lake, New York, USA, before and after zebra mussel invasion. *Ecological Modelling* 220: 3194–3209, https://doi.org/10.1016/j.ecolmodel.2009.07.020

Padilla DK, Adolph SC, Cottingham KL, Schneider DW (1996a) Predicting the consequences of dreissenid mussels on a pelagic food web. *Ecological Modelling* 85: 129–144, https://doi.org/10.1016/0304-3800(94)00185-5

Padilla DK, Chotkowski MA, Buchan LAJ (1996b) Predicting the spread of zebra mussels (*Dreissena polymorpha*) to inland waters using boater movement patterns. *Global Ecology and Biogeography Letters* 5: 353–359, https://doi.org/10.2307/2997590

Peñarrubia L, Alcaraz C, Vaate A bij de, Sanz N, Pla C, Vidal O, Viñas J (2016) Validated methodology for quantifying infestation levels of dreissenid mussels in environmental DNA (eDNA) samples. *Scientific Reports* 6: 39067, https://doi.org/10.1038/srep39067

Pimentel D, Lach L, Zuniga R, Morrison D (2000) Environmental and economic costs of nonindigenous species in the United States. *BioScience* 50: 53–65, https://doi.org/10.1641/0006-3568(2000)050[0053:EAECON]2.3.CO;2

Pimentel D, Zuniga R, Morrison D (2005) Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics* 52: 273–288, https://doi.org/10.1016/j.ecolecon.2004.10.002

Qualls TM, Dolan DM, Reed T, Zorn ME, Kennedy J (2007) Analysis of the impacts of the zebra mussel, *Dreissena polymorpha*, on nutrients, water clarity, and the chlorophyll-phosphorus relationship in lower Green Bay. *Journal of Great Lakes Research* 33: 617–626, https://doi.org/10.3394/0380-1330(2007)33[617:AOTIOT]2.0.CO;2

R Core Team (2016) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rees HC, Bishop K, Middleditch DJ, Patmore JRM, Maddison BC, Gough KC (2014) The application of eDNA for monitoring of the Great Crested Newt in the UK. *Ecology and Evolution* 4: 4023–4032, https://doi.org/10.1002/ece3.1272

Ricciardi A, Whoriskey FG, Rasmussen JB (1997) The role of the zebra mussel (*Dreissena polymorpha*) in structuring macroinvertebrate communities on hard substrata. *Canadian Journal of Fisheries and Aquatic Sciences* 54: 2596–2608, https://doi.org/10.1139/f97-174

Sousa R, Gutierrez JL, Aldridge DC (2009) Nonindigenous invasive bivalves as ecosystem engineers. *Biological Invasions* 11: 2367–2385, https://doi.org/10.1007/s10530-009-9422-7

Strickler KM, Fremier AK, Goldberg CS (2015) Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. *Biological Conservation* 183: 85–92, https://doi.org/10.1016/j.biocon.2014.11.038

Takahara T, Minamoto T, Doi H (2015) Effects of sample processing on the detection rate of environmental DNA from Common Carp (*Cyprinus carpio*). *Biological Conservation* 183: 64–69, https://doi.org/10.1016/j.biocon.2014.11.014

Tukey JW (1977) Exploratory data analysis. Reading, Mass: Addison-Wesley Pub. Co., 688 pp

Turner CR, Barnes MA, Xu CCY, Jones SE, Jerde CL, Lodge DM (2014) Particle size distribution and optimal capture of aqueous macrobial eDNA. *Methods in Ecology and Evolution* 5: 676–684, https://doi.org/10.1111/2041-210X.12206

Turner CR, Uy KL, Everhart RC (2015) Fish environmental DNA is more concentrated in aquatic sediments than surface water. *Biological Conservation* 183: 93–102, https://doi.org/10.1016/j.biocon.2014.11.017

Vanderploeg HA, Nalepa TF, Jude DJ, Mills EL, Holeck KT, Liebig JR, Grigorovich IA, Ojaveer H (2002) Dispersal and emerging ecological impacts of Ponto-Caspian species in the Laurentian Great Lakes. *Canadian Journal of Fisheries and Aquatic Sciences* 59: 1209–1228, https://doi.org/10.1139/f02-087

Woldt A, Baerwaldt K, Monroe E, Tuttle-Lau M, Grueneis N, Holey M, Brouder M, Yess S, Koproski S, Finney S, Finley J (2015) Quality Assurance Project Plan - eDNA Monitoring of Bighead and Silver Carps. U.S. Fish and Wildlife Service, USFWS Midwest Region. Bloomington, MN, 168 pp

Zar JH (2010) Biostatistical analysis (5th ed.). Upper Saddle River, N.J., Prentice-Hall/Pearson, 765 pp