Environmental DNA Mapping of Zebra Mussel Populations

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Abstract

Environmental DNA (eDNA) has become a popular tool for detecting aquatic invasive species, but advancements have made it possible to potentially answer other questions like reproduction, movement, and abundance of the targeted organism. In this study we developed a Zebra Mussel (*Dreissena polymorpha*) eDNA protocol. We then determined if this assay could be used to help determine Zebra Mussel biomass in a lake with a well-established population of Zebra Mussels and a lake with an emerging population of mussels. Our eDNA assay detected DNA of Zebra Mussels but not DNA from more than 20 other species of fish and mussels, many commonly found in Minnesota waters. Our assay did not predict biomass. We did find that DNA from Zebra Mussels accumulated in softer substrates in both lakes, even though the mussels were predominately on the harder substrates. Therefore, we concluded that eDNA may be useful to detect the presence of Zebra Mussels in these lakes but our assay/approach could not predict biomass.

Introduction

Environmental DNA (eDNA) is the detection of DNA shed from an organism from non-biological samples. It has been primarily used to determine the absence and presence of an aquatic invasive species (AIS). While becoming a popular tool for detecting AIS, continued development has advanced the utility of eDNA beyond detection to answer other questions such as reproduction, movement, and abundance of the targeted organism (Erickson et al. 2016).

The invasive Zebra Mussel (Dreissena polymorpha) has invaded much of the Great Lakes Region. Zebra Mussels were first discovered in the Great Lakes Basin in 1988 (Hebert et al. 1989). Since that time, rapidly expanding populations of dreissinid mussels have changed food webs (Holland 1993), primary productivity (Padilla et al. 1996), 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). These impacts threaten the health of native mussels and fish. Besides these ecological impacts, dreissenid mussels have been estimated to cost the US economy billions of dollars (Pimentel et al. 2000; Pimentel et al. 2005).

The Zebra Mussel life-cycle lends them to easily invade new bodies of water, and they have expanded their range to many inland lakes of the upper Midwest. Unfortunately, resource management agencies lack access to effective tools to control dreissenid mussel populations in open waters. There is a need for safe and effective control measures to reduce the environmental and economic impacts of dreissenid mussels. Advancements in eDNA research could lead to a Zebra Mussel eDNA survey tool that not only detects Zebra Mussels but can also be used to help identify sites for control applications.

The goal of our study was to develop and evaluate the use of an eDNA survey to indicate sites within a lake with high numbers of Zebra Mussels. The specific objectives of our study were to: 1) design an eDNA assay for Zebra Mussels, 2) determine an appropriate sampling strategy, and 3) determine if a correlation exists between Zebra Mussel eDNA and substrate, density, and biomass. The development of an eDNA protocol to help inform control applications could improve management of Zebra Mussels and decrease the risk of spread into new waters.

Materials and Methods

Validation of assay specificity

We tested primer sequences for species-specificity in silico using NCBI's Primer-BLAST (Ye et al., 2012), and we found the primer sequences to be specific to the cytochrome c oxidase subunit I gene (coi) of Zebra Mussels with possible amplification from D. presbensis or D. stankovici with 4 primer mismatches each. Both D. presbensis and D. stankovici are found only in the Balkan Region of Europe and have not been found in North America. We then designed a Zebra Mussel amplicon-specific minor groove binder probe with 2 mismatches to both species. Oligonucleotide sequences used are in Table 1. We also tested assay specificity in vitro against genomic DNA from Zebra Mussels and 27 non-target species (Table 2). Genomic DNA was tested in two replicate reactions each as described below.

Table 1. Oligonucleotide sequences of primers, probe and targeted region of the genomes.

Oligonucleotide	Sequence
Dre2-F	TGGGCACGGGTTTTAGTGTT
Dre2-R	CAAGCCCATGAGTGGTGACA
Dpo-Probe	6FAM-CGTCCTTGGTG
Dpo-gBlock	TGTGGGCTGGCCTTGTGGGCACGGGTTTTAGTGTTCTTATTC GTTTAGAGCTAAGGGCACCTGGAAGCGTCCTTGGTGATTG TCAATGATATAATGTAATTGTCACCACTCATGGGCTTGTTA TAATTGTTTGTCTAG

Table 2. Specificity of Zebra Mussel marker against genomic DNA from various aquatic species, many common to Minnesota waters. 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

We sampled Lake Minnetonka to develop a sampling protocol. Water was collected from the surface, mid-water and near the bottom directly above a known colony of Zebra Mussels. Ten 50-mL sterile conical tubes were placed just below the water surface to collect the surface film. Mid-water samples were collected using a 2.2 L horizontal Van Dorn water sampler. The water sampler was lowered to mid-depth and sealed. Ten 50 mL water samples were collected from the water sampler. The bottom samples were collected using a separate 2.2 L horizontal Van Dorn water sampler. This water sampler was lowered to 9 cm above the bottom where the water was collected and brought to the surface. Again, ten 50 mL water samples were collected from the sampler. Once each sample was collected it was capped and stored on ice. All samples were transported to the U.S. Geological Survey Upper Midwest Environmental Sciences Center in La Crosse, Wisconsin (UMESC) for further processing. DNA was extracted from individual samples and quantified using the procedure mentioned below.

Correlations among eDNA, biomass, and substrate type

To determine if a correlation exists between Zebra Mussel DNA and substrate, density, and biomass, we sampled water from two lakes, Lake Le Homme Dieu and Maple Lake, near Alexandria, Minnesota. Lake Le Homme Dieu is approximately 728 ha with a maximum depth of 26 m. This lake has had Zebra Mussels present since 2009 (Cha et al. 2013) and was chosen to represent a lake with a well-established population with a mean shell length of 0.82 ± 0.11 cm. Maple Lake is 330 ha with a maximum depth of 24 m. According the Minnesota Department of Natural Resources, Maple Lake was found to have Zebra Mussels in 2013 and was chosen to represent a lake with an emerging population with smaller mussels; 0.65 ± 0.14 cm mean shell length.

At each lake, we collected water from 9 cm above the bottom using a 2.2 L horizontal Van Dorn water sampler in triplicate according to the method established above. Samples were collected at depths of approximately 1, 2, 4 and 6 m along four transects in each lake. We tried to follow transects that covered different substrate types from loose flocculent to cobble in each lake. Immediately following water sampling at each sample point, we placed a brick, tied to a buoy, and recorded GPS coordinates for subsequent samplings at the same location. Each lake was sampled twice; first September 29 – 30, 2014 and again under ice March 9 – 10, 2015 (Figure 1).

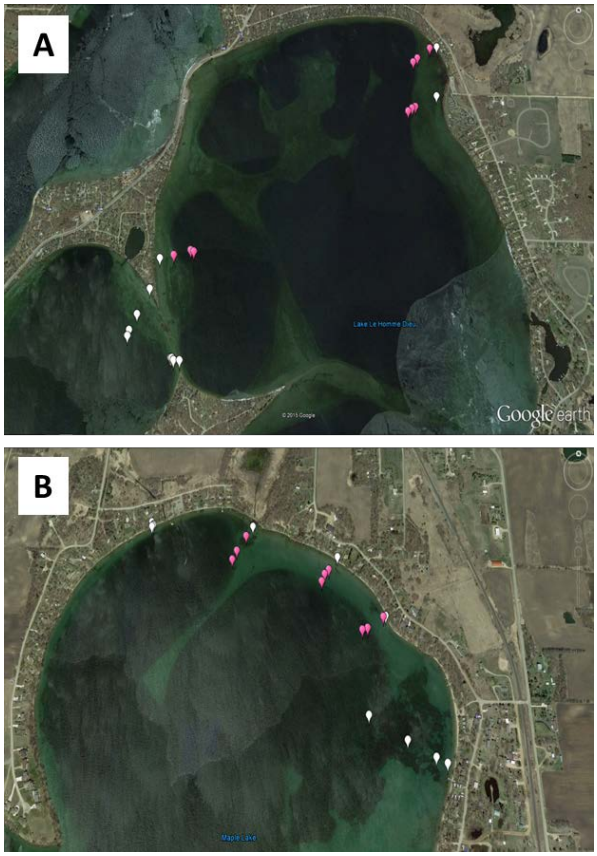

Figure 1. Sampling locations on Lake Le Homme Dieu (A) and Maple Lake (B) near Alexandria, Minnesota. All locations marked indicate where samples were collected. Locations marked in pink represent the locations where samples were also collected in March, whereas locations indicated white are sites where samples

The day following water sampling in September, we used SCUBA divers to collect all the Zebra Mussels in three 0.25 m2 quadrants near each brick. Zebra Mussels from each quadrant 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 at each sampling location.

To estimate biomass, we calculated the ash-free dry weight (AFDW) for each Zebra Mussel sample (Wetzel et al. 2005). Each Zebra Mussel sample was weighed to determine total wet-weight. The moisture content and dry weight (DW) was determined according to AOAC Official Method 934.01 and subsequently ash weight (AW) was determined according to AOAC Official Method 942.05. Both moisture content, dry weight and ash weight analyses were conducted by the University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO). AFDW was calculated subtracting AW from DW for the subsample and adjusting to the mass (wet-weight) of the whole sample.

DNA extraction and amplification

We centrifuged the 50 mL water samples at 5,000 x g for 30 minutes and decanted the supernatant. 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 5-fold dilution series from 31,250 copies down to 10 copies per reaction.

Analysis

We used Pearson product-moment correlation coefficient as a measure of the linear correlation between the following variables: depth, substrate type, detections, AFDW, fall DNA copies and winter DNA copies. Substrate type was divided into six categories based on the coarseness of the material: 1) flocculent, 2) silt, 3) muck mixture, 4) sand, 5) predominately shell and 6) cobble/stones. We compared each lake separately because of the known differences in Zebra Mussel populations. All analyses were performed using SigmaPlot® 13.0 (Systat Software, Inc., San Jose, CA USA) with a significance level of $\alpha \leq 0.05$.

Results and Discussion

Validation of molecular assay

Our primer-BLAST results predicted the sequences to specifically amplify a target region in the cytochrome c oxidase subunit I gene (*coi*) of Zebra Mussels with possible amplification from *D. presbensis* or *D. stankovici* with 4 primer mismatches each. Both *D. presbensis* and *D. stankovici* are found only in the Balkan Region of Europe and have not been found in North America. We validated the specificity of our assay against genomic DNA from Zebra Mussels and 27 non-target species listed in Table 2 with 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. Therefore, we concluded that this marker was adequate for detecting the presence of Zebra Mussel DNA in Minnesota waters.

Development of sampling protocol

We determined the amount of Zebra Mussel DNA in each of the 10 water samples from three depths in Lake Minnetonka: surface, mid-column, and benthic. 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 slightly lower C_t-values than those from samples collected from the surface (Figure 2). This indicates that benthic samples contained slightly more Zebra Mussel DNA. However, both surface and benthic samples had significantly lower C_t-values indicating significantly more Zebra Mussel DNA than mid-column samples (Figure 2).

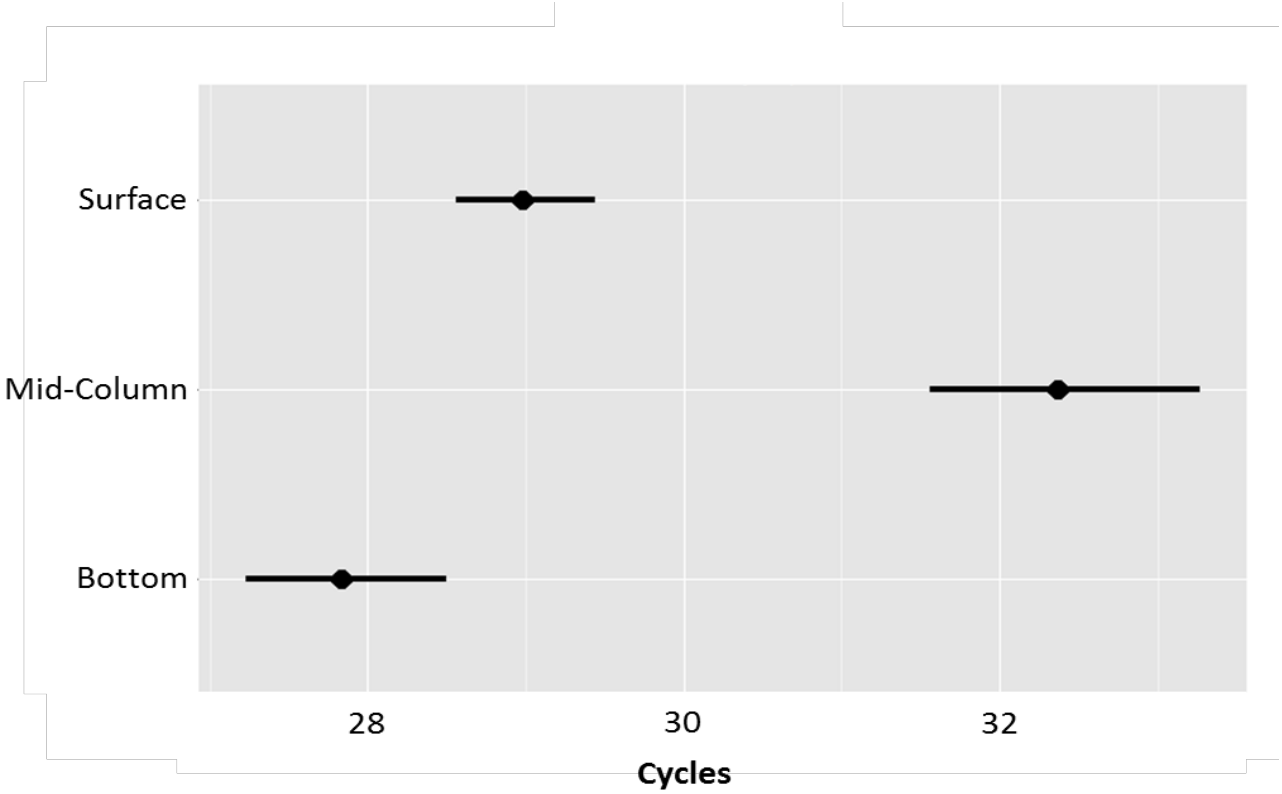

Figure 2. The mean number of cycles needed to detect DNA of Zebra Mussels from water samples collected at the surface, mid-column and bottom of Lake Minnetonka directly above a known Zebra Mussel population. The lower the number of cycles indicates a greater amount of DNA. Bars represent the 95% confidence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

Correlations among eDNA, biomass, and substrate type

Lake Le Homme Dieu (established population)

As expected, the coarser substrates (cobble) were generally found in the shallower waters, while finer substrates (flock) were located at the deeper sampling sites (Table 3). AFDW decreased with increased depth ($r = -0.266$, $p = 0.040$). No correlation between AFDW and substrate type was found ($r = 0.243$, $p = 0.061$) and there was no correlation between AFDW and the number of copies of Zebra

Mussel DNA for both fall and winter (Table 3). Thus, eDNA copy numbers were not found to accurately predict the biomass of Zebra Mussels in this lake that has an established population of Zebra Mussels. The number of positive detections was negatively correlated with substrate type ($r = -0.264$, $p = 0.041$). The results suggest that there is a higher probability of detecting Zebra Mussel DNA in areas that have softer substrates in lakes were the mussel population is well established but that eDNA copy counts do not correlate with the mass of animals within the area.

Table 3. Pearson product-moment correlation coefficients between depth, substrate type, detections, AFDW, fall DNA copies and winter DNA copies for Lake Le Homme Dieu. Values represent the correlation coefficient (top), P-value (middle), and number of samples (bottom) for each comparison.

	Substrate	Detections	Fall DNA copies	Winter DNA copies	AFDW
Depth	-0.458 <0.001 60	0.265 0.041 60	0.249 0.055 60	0.216 0.253 30	-0.266 0.040 60
Substrate		-0.264 0.041 60	-0.273 0.035 60	0.181 0.338 30	0.243 0.061 60
Detections			0.202 0.121 60	0.105 0.582 30	-0.157 0.230 60
Fall DNA copies				0.018 0.924 30	-0.119 0.366 60
Winter DNA copies					-0.119 0.530 30

Maple Lake (emerging population)

Substrate and depth in Maple Lake was similar to that of Lake La Homme Dieu (Table 4). AFDW decreased with increased depth ($r = -0.366$, $p = 0.041$). There was a significant correlation between AFDW and substrate type ($r = 0.424$, $p < 0.001$). Like Lake Le Homme Dieu, no correlation was determined between AFDW and the number of Zebra Mussel DNA copies (Table 4), which suggests that DNA copy numbers cannot accurately predict the biomass of Zebra Mussels in a lake. Unlike Lake Le Homme Dieu, no correlation was found between the number of detections for a sample and substrate type in Maple Lake ($r = 0.212$, $p = 0.104$). This indicates an equal probability of detecting Zebra Mussel DNA in areas with soft substrates as those with harder substrates.

Table 4. Pearson product-moment correlation coefficients between depth, substrate type, detections, AFDW, fall DNA copies and winter DNA copies for Maple Lake. Values represent the correlation coefficient (top), P-value (middle), and number of samples (bottom) for each comparison.

	Substrate	Detections	Fall DNA copies	Winter DNA copies	AFDW
Depth	-0.520 <0.001 60	-0.310 0.016 60	-0.019 0.884 60	-0.334 0.088 27	-0.366 0.004 60
Substrate		0.212 0.104 60	-0.030 0.822 60	0.208 0.297 27	0.424 <0.001 60
Detections			0.210 0.107 60	0.419 0.030 27	0.184 0.158 60
Fall DNA copies				0.642 <0.001 27	-0.057 0.668 60
Winter DNA copies					-0.077 0.702 27

Table 5. Mean number of positive detections and copies of Zebra Mussel DNA, as well as ash-free dry weight (AFDW) of Zebra Mussels for six sediment 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)
Muck mixture	3.44 (0.92)	45.72 (87.07)	1.39 (1.43)
Sand	3.56 (0.53)	32.26 (82.71)	28.13 (32.76)
Shells	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)
Muck mixture	2.13 (1.51)	1.83 (2.05)	0.11 (0.17)
Sand	2.00 (1.00)	1.38 (.049)	0.22 (0.17)
Shells	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)

Conclusion

We developed and validated a molecular assay that detects the presence of Zebra Mussels in a body of water. Zebra Mussel DNA did not correlate with biomass. DNA from Zebra Mussels accumulates in softer substrates in lakes as the mussels become established (Table 5). In lakes with an emerging population, sampling water near harder substrates will provide the greatest probability of detecting the presence of Zebra Mussels. In this study, we demonstrated that eDNA may be useful to detect the presence of Zebra Mussels in a lake but that currently available approaches are not able to correlate DNA copy number with biomass.

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