Using environmental DNA (eDNA) to detect the endangered Spectaclecase Mussel (*Margaritifera monodonta*)

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Abstract: *Margaritifera monodonta*, or the Spectaclecase Mussel, is a federally endangered freshwater mussel species that has experienced a 55% reduction in range and is currently concentrated in 3 rivers in the Midwest region of the United States (Gasconade and Meramec rivers, Missouri, and St Croix River, Wisconsin). The detection of new populations by traditional survey methods has been limited because these mussels tend to occur under large rocks and boulders. Environmental DNA (eDNA) technology has been used to detect invasive and rare species, but its use for detection of rare, benthic-dwelling species in large flowing systems has been limited. Here, we propose using eDNA to assess known populations of *M. monodonta*. We designed a *M. monodonta*-specific quantitative polymerase chain reaction (qPCR) assay and tested it using water samples from multiple *M. monodonta* housing tanks, water samples from 2 known mussel beds on the St Croix River, and water samples from 3 known mussel beds on the Mississippi River. We observed higher overall eDNA detection rates on the St Croix River (30.2%) compared to the upper Mississippi River (0.60%). We also observed higher eDNA detection rates (73.3–93.1%) in 2018 for samples collected during the larval release period in May compared to samples collected in August after the reproductive period had ended (55.6–70.8%) on the St Croix River. We tested samples collected at 3 distances downstream from the 2 mussel beds found in the St Croix River, but we did not observe a substantial effect of distance on our detection rates. However, we did observe greater detection rates for samples collected near the bottom compared to at the surface. Our results indicate that this novel qPCR assay can successfully detect *M. monodonta* eDNA and could be used to rapidly screen locations to guide intensive physical searches for populations in riverine systems.

Key words: unionids, qPCR, conservation, St Croix River, upper Mississippi River, freshwater mussels

Freshwater unionid mussels are one of the most imperiled faunal groups in the world (Ferreira-Rodríguez et al. 2019). Of the 298 native freshwater mussel species in North America, over 70% are ranked as endangered, threatened, or of special concern (Williams et al. 1993, 2017). The Spectaclecase Mussel, *Margaritifera monodonta* (Say, 1829), a federally endangered species (Federal Register 2012), was historically distributed in 44 streams from reaches of the upper Mississippi, Ohio, Cumberland, and Tennessee rivers in portions of 14 states (Alabama, Arkansas, Illinois, Indiana, Iowa, Kansas, Kentucky, Minnesota, Missouri, Ohio, Tennessee, Virginia, West Virginia, and Wisconsin) (Butler 2002, USFWS 2014). It is currently known from only 20 of those historical streams, a 55% reduction in its range. The 3 remaining strongholds of *M. monodonta* populations occur in the Meramec and Gasconade rivers in Missouri, and the St Croix River bordering Minnesota and Wisconsin. In 2012, a relatively large population was discovered on the Ouachita River in Arkansas, and an apparently reproducing population was found in the Green River in Kentucky. Extant populations of *M. monodonta* have been documented in at least 7 pools of the upper Mississippi River (UMR) but with low abundance. Occupancy records of *M. monodonta* from most UMR pools are 15 to 30 y old (Butler

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2002), except for the recent finding of a substantial and viable population in pool 15 of the Mississippi River located in Quad Cities, Iowa (H. Dunn, Ecological Specialists, personal communication, 2017).

Margaritifera monodonta generally inhabits medium to large rivers and is the only margaritiferid species known from the Mississippi River Basin. The species tends to aggregate under slab boulders, bedrock shelves, tree stumps, or in root masses, adjacent to but protected from swift current (Stansbery 1966, Buchanan 1980, Oesch 1984, Gordon and Layzer 1989, Parmalee and Bogan 1998, Baird 2000), making it difficult for traditional survey methods to detect new populations. As with any endangered species, information on present population size and location is critical for recovery of *M. monodonta*. Traditional sampling methods can be labor intensive, costly, and unsuccessful for locating rare and cryptic species (Obermeyer 1998, Baird 2000). Mussel surveys have been conducted every 4 to 5 y since 2004 in a reach of the St Croix River inhabited by *M. monodonta* (MCT 2019). The density of *M. monodonta* in quadrat surveys was estimated to be 0.01 to 0.02/m^2 (standard deviation [SD]: 0.08–0.25), whereas timed searches of targeted areas yielded relatively greater population estimates (15.75 catch/h; SD: 31.50) because of the aggregated nature of the species.

Environmental DNA (eDNA) is an emerging surveillance tool for detecting and monitoring imperiled species (Currier et al. 2018, Harper et al. 2018, Atkinson et al. 2019) and invasive species (Hunter et al. 2018, Fritts et al. 2019, Orzechowski et al. 2019) in aquatic systems. DNA that is shed from an organism into the environment can be collected, isolated, and identified using species-specific molecular markers (Thomsen and Willerslev 2015). Development of species-specific markers for rare and cryptic species, such as *M. monodonta*, can be used to augment and direct quantitative survey efforts that are often cost restricted in size and range.

The use of eDNA and quantitative PCR (qPCR) to detect species in aquatic systems has primarily focused on fishes, whereas use of these techniques for invertebrates and amphibians trails behind in the literature (Belle et al. 2019, Coble et al. 2019). Of these invertebrate eDNA studies, little focus has been on rare, threatened, or endangered species. The sensitivity of eDNA methods to detect low-density populations of benthic-dwelling organisms in a flowing system has only recently been demonstrated (Deiner and Altermatt 2014, Stoeckle et al. 2016, Carlsson et al. 2017, Dyer and Roderique 2017, Hu 2017, Currier et al. 2018, Roderique 2018, Schill and Galbraith 2019, Wacker et al. 2019). Relative to fish, mussels may shed less DNA because they can close their valves, are smaller in size and biomass, and generally have a restricted portion of soft tissue (i.e., the mantle and siphons) exposed to the water column (Takahara et al. 2012, Thomsen et al. 2012, Sassoubre et al. 2016, Sansom and Sassoubre 2017). DNA shedding may increase in association with filter feeding, burrowing, locomotor activity, reproduction, production of feces, and death (Geist et al. 2008, Sansom and Sassoubre 2017). The reproductive cycle of unionid mussels involves release of sperm into the water column by males and subsequent uptake of the sperm by females through water filtration. Eggs are fertilized and deposited in water chambers of the females' gills for development to the larval stage. After the larvae mature, females release larvae into the water column. The larvae attach to the gills of suitable host fishes for an obligatory parasitic stage and then excyst as transformed juveniles. Thus, detection of eDNA may be greater during the reproductive period of a mussel species. In the St Croix River, *M. monodonta* males release sperm and fertilization in early spring (April to early May, depending on water temperature). Females brood larvae for ~3 wk and larval conglutinates, or packets, are released in late May to early June (B. Sietman, Minnesota Department of Natural Resources, personal communication, 2016; M. Bradley, USFWS, personal communication, 2016).

The goal of this project was to establish an eDNA method for assessing freshwater mussels. In this study, we specifically focused on *M. monodonta*. Our 1st objective was to design and validate a qPCR assay specific to *M. monodonta*. Our 2nd objective was to determine the best sampling strategy (surface or benthic sampling) for capturing *M. monodonta* eDNA in water samples. Our 3rd objective was to evaluate the sensitivity of this method for detecting *M. monodonta* populations.

METHODS

We designed a qPCR assay to detect *M. monodonta* eDNA, and we verified the specificity against genomic DNA from both non-target freshwater mussels and fish species to address our project goals. We validated the assay using both laboratory and field-based sampling methods. In addition, we determined the sensitivity of the assay by generating the limit of detection and limit of quantification.

Assay design

We aligned DNA sequences from GenBank for *M. monodonta*, 3 congeners that do not overlap with *M. monodonta* distribution (Western Pearlshell, *Margaritifera falcata* [Gould, 1850]; Eastern Pearlshell, *Margaritifera margaritifera* [Linnaeus, 1758]; and Alabama Pearlshell, *Margaritifera marrianae* [R. I. Johnson, 1983]), and 4 species that co-occur with *M. monodonta* in the St Croix River (Mucket, *Ortmanniana ligamentina* [Lamarck, 1819]; Wabash Pigtoe, *Fusconaia flava* [Rafinesque, 1820]; Monkeyface, *Theliderma metanevra* [Rafinesque, 1820]; and Deertoe, *Truncilla truncata* [Rafinesque, 1820]) (Table S1). We used BioEdit version 7.2.0 (Hall 1999) to identify variable regions to fit primers

and probes that were conserved between *M. monodonta* sequences but divergent from other mussel species with at least 3 mismatches to non-targets. Based on this alignment, oligonucleotides were designed using *M. monodonta* sequences at the selected loci to have melting temperatures near 56°C for the primers and near 66°C for the probes with as close to 50% GC content as possible (Table 1). After primers and probes were designed, we checked the specificity of the primers with the National Center for Biotechnology Information (NCBI) Primer-BLAST tool (Ye et al. 2012) and found no non-target amplification was predicted.

Tissue collection

We evaluated the specificity of our assay by testing with genomic DNA extracted from *M. monodonta* and non-target fishes and freshwater mussel species inhabiting the St Croix River (Table S2 and S3). We obtained fish-fin clips during 2 separate electroshocking trips—the St Croix River on 12 June 2017 (collaborative effort by the United States Geological Survey-Upper Midwest Environmental Sciences Center [UMESC] and the United States Fish and Wildlife Service [USFWS]) and the UMR on 13 June 2016 (by the USFWS)—and from fishes available at the UMESC Fish Culture Facility. We extracted genomic DNA for *M. monodonta* and other non-target mussel species collected from the St Croix River from mantle biopsies or hemolymph. Additionally, we extracted DNA from conglutinates released from gravid *M. monodonta* mussels to verify that the mussels were indeed shedding detectable eDNA during spawning. We used a 25-gauge needle and 1-mL syringe to collect 1-mL hemolymph samples from the anterior adductor muscle from adult mussels retrieved by divers on the St Croix River on 31 May 2017. This method of hemolymph extraction from freshwater mussels is well established for DNA studies and does not harm the animals (Geist and Kuehn 2005). When we were unable to extract 1 mL of hemolymph from adult mussels, we collected mussel mantle biopsies to ensure that we would have enough DNA to complete both assay optimization and specificity testing. We placed mantle biopsies into 1-mL tubes containing absolute ethanol. We transported hemolymph and mantle samples on ice and stored them at −80°C at UMESC until DNA extraction. Divers also collected gravid *M. monodonta* mussels from the St Croix River and placed them into a cooler filled with river water to be transported to UMESC. The mussels were held in 1-L aerated containers of river water until larval conglutinates were released (<24 h). We collected the larvae from these containers with a wide-bore pipette and placed them into 95% non-denatured ethanol in 1.5-mL tubes for DNA extraction. We returned all adults to the collection site after conglutinates were released.

Aquarium testing

We collected water samples (50 mL) from *M. monodonta* rearing tanks at the Minnesota Department of Natural Resources, Lake City Mussel Culture Facility in Lake City, Minnesota, on 14 June 2018 to verify that the mussels shed enough eDNA for us to detect them. We collected 6 samples from a 30.3-L plastic tank containing an undetermined number of newly transformed juvenile *M. monodonta*, 6 samples each from three 3.8-L jars that each held

Table 1. Oligonucleotide sequences of *Margaritifera monodonta* ND1 assay primers, probe, and gBlock and the efficiency of the assay. Bold and gray-shaded sequences mark the primer and probe binding sites used in this study. Binding sites for an alternate COI marker 1 are bolded; binding sites for an alternate COI marker 2 and for an alternate ND1 marker are underlined.

Oligonucleotide	Sequence (5′–3′)
Cmon-ND1-F1	AGTGGGTGATACCWGTATCT
Cmon-ND1-R1	TACCCCTAGCACCATTTGAT
Cmon-ND1-Probe1	5HEX/TCTAGCCCT/ZEN/AAGACTATGACAACTTTTCC/3IABkFQ
Cmon-gBlock2	**ACCTTTGTCCGGAAATGTCTCT**CATTCTGGGGCTTCTGTGGATTTGGCTATTTTTTCTTTG CATCTTGCTGGTGCATCTTCTATTTTGGGGGCTATCAATTTTATCTCAACTGTTGGTAAT **ATGCGTTCTCCTGGCGTGGTGGCTGA**GCGGATTCCTTTGTTTGTGTGGGCTGTTACG**AT TACGGCAGTTTTGCTAGTGG**CGGCACTACCTGTTTTGGCTGGTGCTATTACTATGTTATT GACCGATCGTAATCTAGATCCATCTTTGTTTGATCCTACCGGTGGGGGTGA**AGTGGGTG ATACCAGTATCT**TCAAATAACTTTCCATTTATCTTAACACCCACATTAATACTAAT**TCTA GCCCTAAGACTATGACAACTTTTCC**CTTCCTTAAAACTAAGGT**ATCAAATGGTGCTAG GGGTA**CACTAACAGTATACACCACATTAATGGCCGGATGGGCCTCTAACTCAAAATACG CCCTTCTCGGAGCTATTCGCGCAATAGCCCAAACAATTTCATACGAGGTAACAATAGCCC TGATCATTCTATGTCACCTTCTTCTAAAAGGCCAAATAGACTGACTGAC
Assay efficiency	98.1% (range: 92.1–100)

a single adult *M. monodonta*, and 6 samples from the effluent of a 3785.4-L circular tank containing 8 Goldeye (*Hiodon alosoides* [Rafinesque, 1819]) that had been infested with *M. monodonta* larvae for a 1-wk period prior to sampling the tank. We stored water samples on ice and transported them to UMESC where we wiped the tubes with 10% bleach wipes and stored them at −80°C until DNA extraction. We extracted two 50-mL samples from each sampled tank and jar and tested them using the same thermal conditions and plate setup as for the field-collected eDNA samples described below.

eDNA field sampling

We validated the ability of our assay to detect *M. monodonta* in the field with water samples collected at 2 sites on the St Croix River and 3 sites on the Mississippi River immediately downstream from known *M. monodonta* populations as well as 2 sites on the Black River. To aid in the validation of our study, we targeted both a period of low DNA abundance (autumn) and a period of high DNA abundance from conglutinate release (spring). To determine the best sampling strategy (surface or benthic sampling) for capturing *M. monodonta* eDNA in water samples, we did surface water grabs and benthic samples. We collected surface water samples by submerging each 50-mL tube just below the water surface. For the benthic samples, we used a Van Dorn sampler to collect 1 L of water from near the river bottom, and then from that volume, we took triplicate 50-mL subsamples.

The *M. monodonta* population in the St Croix River is characterized as stable and relatively large (i.e., defined as 30–74 individuals observed in the most recent survey [T. Smith, USFWS, personal communication, 2018]). On the St Croix River, we collected water samples at 2 sites (A and B) immediately downstream from 2 mussel beds. Site A had a channel width of 85 to 100 m in the area of the mussel bed, whereas site B, which was 1500 m downstream from site A, had a narrower channel width (32–40 m). On 12 June 2017, we collected triplicate 50-mL surface grab samples at 8 points along a transect on both sides of the river at each of 5 distances downstream from the site B mussel bed: 0, 50, 100, 200, and 500 m (n = 120; Fig. 1). On 24 May 2018, we collected water samples from 5 randomly selected points at each of 2 distances (0 and 50 m) downstream from the mussel beds at sites A and B with a Van Dorn sampler (n = 60; Fig. 1). On 13 August 2018, we collected paired benthic and surface samples at 4 randomly selected points immediately downstream from the site A mussel bed and 3 randomly selected points immediately downstream from the site B mussel bed. At each randomly selected site, we collected triplicate 50-mL subsamples from 1-L grabs with the Van Dorn sampler held just below the surface and just above the bottom (n = 42; Fig. 1). Additionally, as a negative field control, we collected three 50-mL surface grab samples 150 m upstream from site A on both sampling trips in 2018. We predicted that this sampling location was going to yield negative detections because it was upstream from known *M. monodonta* mussel beds.

The *M. monodonta* populations in the Mississippi River occur in pools 15 and 16 located between Lock and Dam 14 near Hampton, Illinois, and Lock and Dam 16 near Muscatine, Iowa (USGS 2020a). The UMR navigation locks and dams are numbered sequentially in the downstream direction from Lock and Dam 1 in the Twin Cities, Minnesota, to Lock and Dam 26 near St Louis, Missouri. The term pool is used to describe the area between the navigation dams. The *M. monodonta* populations in pool 15 and pool 16 sites on the UMR were relocated from an interstate bridge construction site from 2016 to 2017 (K. Lundh, USFWS, personal communication, 2017). Mark recapture data on the 3 populations in the UMR suggest that populations at both sites 1 and 2 in pool 15 are persistent, whereas the pool 16 population might not be (H. Dunn and E. Grossman, Ecological Specialists, Inc., personal communication, 2020). We collected water samples immediately downstream from 2 mussel beds in pool 15 (sites 1 and 2) and 1 mussel bed in pool 16 on 18 August 2017. The 2 mussel beds in pool 15 are 660 m apart. The channel width at pool 15 site 1 was ~180 to 195 m, whereas site 2 had a width of 120 to 200 m. Pool 16 had a channel width of 110 to 130 m. We collected triplicate 50-mL surface grab samples at 6 random points along each side of the river at each of 3 distances downstream from the mussel beds: 0, 100, and 500 m (n = 162; Fig. 1).

We also collected water samples from 2 locations on the Black River in La Crosse, Wisconsin, that we expected to give negative results because there are no historical or current records of *M. monodonta* inhabiting these locations. We collected triplicate 50-mL surface grab samples on 31 October 2018 and benthic samples on 1 November 2018 from opposite sides of the Black River in La Crosse, Wisconsin. We collected benthic samples using subsamples from 1-L grabs with the Van Dorn sampler.

We recorded GPS coordinates at each sample location with a handheld Montana 650t GPS (Garmin, Olathe, Kansas). For the St Croix River sites, we measured water depth with a Humminbird Helix 10 depth finder (Johnson Outdoors, Racine, Wisconsin). We did not record water depth for the UMR sites and estimated the water depth for the Black River sites based on the string length of the Van Dorn sampler once it was just above the bottom at each sample location. We measured flow velocity with a Marsh McBirney Flo-Mate 2000 flow meter (Field Environmental Instruments, Pittsburgh, Pennsylvania) at the St Croix River sites (Table S4) to allow comparison among and between the St Croix sites. We did not record flow velocity data for the UMR sites, although we know that the UMR was

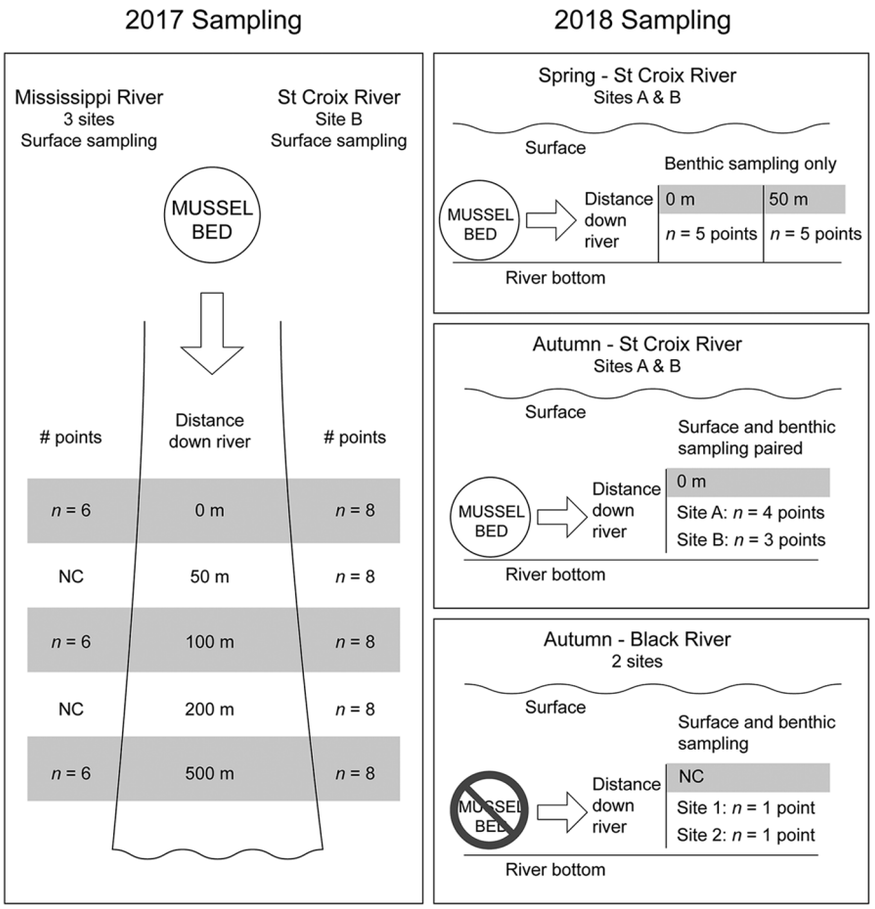

Figure 1. Schematic of the field sampling design used to collect water samples for *Margaritifera monodonta* environmental DNA detection. Sampling completed in 2017 is shown on the left and sampling in 2018 is shown on the right. In all cases, triplicate samples were collected at each sampling point. Only surface samples were collected in 2017, but surface and benthic samples were collected in 2018. St Croix River sites: A and B. Mississippi River sites: pool 15 sites 1 and 2 and pool 16. Black River sites: 1 and 2. NC indicates samples not collected at that distance.

discharging substantially more water (58,837 ft^3/s; USACE 2020) than the St Croix River (3560–4490 ft^3/s; USGS 2020b) during the sample period. We also did not record flow velocity at the Black River sites.

On all sampling trips, we collected 1 field blank at each site. These blanks consisted of tubes pre-filled with 50 mL of deionized water that we exposed to the air for 5 s before re-capping the tube and submerging it in the river water. We used aseptic procedures during all sample collections to prevent cross contamination. We used fresh gloves for each site and changed them if we inadvertently touched a surface that may have had DNA on it. We used separate Van Dorn samplers for each site and thoroughly rinsed the chamber with river water at each sample location within sites before collecting the sample. We placed samples on ice for transport to UMESC where we wiped the outsides of the tubes with 10% bleach wipes, then stored them at −80°C until further processing.

Molecular methods

Assay specificity testing We extracted genomic DNA from all target and non-target species with the extraction kit described below according to the manufacturer's instructions for tissue extractions. We then tested the DNA in duplicate using the assay conditions described below with 2 exceptions. We performed in-vitro testing using 1 μL of template DNA and Bullseye TaqProbe Mastermix (Midwest Scientific, Valley Park, Missouri) carried out in a total reaction volume of 20 μL. All qPCR products were run on 1.5% agarose gels at 80 volts for 1 h, and any band detected at the target band size was sent to the University of Wisconsin-Madison Biotechnology Center for Sanger sequencing. We confirmed the sequence identity as *M. monodonta* using NCBI's BLAST® (https://blast.ncbi.nlm.nih.gov). We also ran an annealing temperature gradient with this assay for *M. monodonta* and select non-target species replicates between 55 and 70°C to determine the optimal

annealing temperature. An annealing temperature of 64.5°C detected all replicates of *M. monodonta* without any loss of PCR efficiency and did not amplify any non-target species tested (Table S2 and S3).

eDNA samples We centrifuged water samples at 4°C for 30 min at 5000 × *g* using a high-performance centrifuge (model Avanti® J-26 XPI; Beckman Coulter, Indianapolis, Indiana). We decanted the supernatant and then extracted the residual water and remaining pellets using the gMax Genomic DNA Mini extraction kit (IBI Scientific, Dubuque, Iowa) according to the manufacturer's instructions. We included 1 extraction blank, which consisted of 100 μL of nuclease-free water in a 50-mL centrifuge tube, for each round of DNA extractions.

All qPCR assays were analyzed using a C1000 touch thermal cycler (model CFX96 Touch Real-Time Detection System; Bio-Rad Laboratories, Hercules, California). We analyzed samples in 25-μL reactions composed of the PerfeCTa qPCR ToughMix (Quantabio, Beverly, Massachusetts) with 2μL of template DNA, 1× master mix, 500 nM of each primer, and 125 nM of the probe using the thermal profile: 95°C for 30 s, 45 cycles of 95°C for 5 s, and 64.5°C for 30 s. On each plate, we ran standard curves made from serial dilutions of a synthetic DNA gBlock™ (Integrated DNA Technologies®, Coralville, Iowa) with 2 replicates at 10,000 and 1000 copies and 4 replicates at 100 and 10 copies. The gBlock consisted of concatenated sequences for multiple markers of *M. monodonta* that we considered (Table 1). We analyzed each sample in 4 replicates with an additional 3 replicates spiked with 100 copies of our gBlock DNA standard to test for inhibition. We also used 1 no-template control for each sample on the plate positioned between the analytical replicates and the spiked replicates. Standard efficiency of all qPCRs analyzed was 92.1 to 100.4%, no target DNA was detected in any no-template control, and no inhibition was detected.

Limit of detection and limit of quantification To determine the limit of detection (LOD) and limit of quantification (LOQ) of the assay, we ran the gBlock synthetic DNA standard using 48 replicates at 10,000, 1000, 100, 50, 10, 5, and 2 copies, and we analyzed the data with a publicly available R code (Merkes et al. 2019) using the settings of a best-fitting model for LOD, a 4th-order polynomial for the LOQ model, and a coefficient of variation (CV) threshold value of 35%. The script fit our detections for each standard to a logarithmic model to determine the lowest concentration of target DNA detected with 95% confidence in at least 1 of 4 replicates for our effective LOD following methods previously used (Forootan et al. 2017, Schloesser et al. 2018, Klymus et al. 2020). The script also determined the CVs of our quantitation cycle (C_q) values for each standard using the equation derived by Forootan et al. (2017) and fit them to a 4th-order polynomial model to estimate the lowest concentration of target DNA we could quantify within 35% CV precision. We selected the 2nd-best-fitting (4th-order polynomial) model for the LOQ analysis because the best-fitting (5th-order polynomial) model produced an artifactual CV rise above our 35% threshold between the 1000 and 10,000 copy standards, while the 4th-order polynomial model did not.

Statistical analysis and data availability

We fit the eDNA data to generalized linear mixed models in R (version 3.5.0; R Project for Statistical Computing, Vienna, Austria) using the glmer function with family set as binomial in the *lme4* package (Bates et al. 2015). We used detection (binary, whether the reaction detected or failed to detect target DNA) for our dependent variable with either site or river and either distance or depth as fixed effects and replicate sample (50-mL water sample) nested within sample location (1-L Van Dorn grab) as random effects, summarized by Eqs. 1 and 2.

$$\text{Detection} \sim \text{River} + \text{Distance} + (1|\ \text{Sample Location/Sample}) \quad \text{(Eq. 1)}$$

$$\text{Detection} \sim \text{Site} + \text{Depth} + (1|\text{Sample Location/Sample}) \quad \text{(Eq. 2)}$$

The data and R script are available at https://doi.org/10.5066/P9F0COLN with the exception that GPS coordinates are protected because of the endangered status of *M. monodonta*. The coordinates are available upon appropriate request.

RESULTS

Assay validation and limit of detection

Our assay successfully amplified *M. monodonta* DNA and was specific to the target species (Table S2 and S3). We determined that the effective LOD for our assay, based on using 4 replicates, was 18 copies of target DNA (95% confidence interval: 15.1–21.8) with an LOQ of 587 copies of target DNA.

Aquarium testing

We observed 100% positive detections for the water samples collected from the juvenile *M. monodonta* rearing tank and adult holding jars and 25% positive detections for the water samples from the Goldeye tank. All extraction blanks and negative controls resulted in non-detections.

eDNA field sampling

From our eDNA field sampling, we observed a much higher overall eDNA detection rate (number of detections/

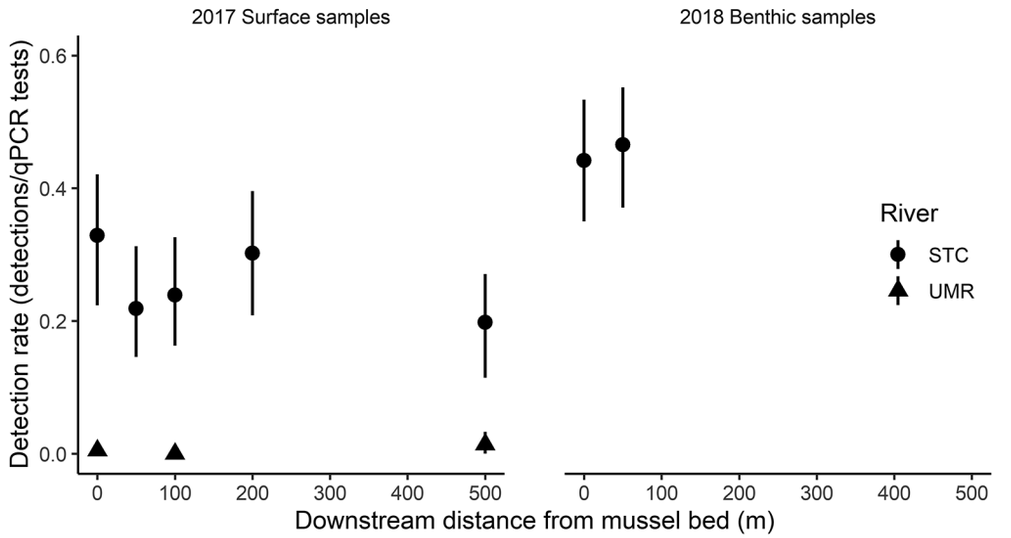

Figure 2. *Margaritifera monodonta* environmental DNA detection rates (detections/qPCR tests) across distances (m) downstream from the mussel bed. Circles indicate St Croix River (STC) sites, triangles indicate upper Mississippi River (UMR) sites, and the bars represent the 95% credible intervals. Results from surface samples collected in 2017 are shown on the left, and results from benthic samples collected in 2018 are shown on the right.

total no. of qPCR tests) on the St Croix River (30.2%) compared to the UMR (0.60%) ($p = 1.76 \times 10^{-13}$; Fig. 2). We also observed a slightly lower overall eDNA detection rate of 7.1% at 500 m downstream from the mussel bed for the St Croix River samples compared to 20.9% at 0 m, although there were overlapping 95% credible intervals for the St Croix River ($p = 0.466$; Fig. 2). The UMR had extremely low detection rates at all sampling distances (Fig. 2). In the St Croix River, we observed lower eDNA detection rates at site A (32.5%) compared to site B (58.6%) in the spring; however, the situation was reversed in the autumn (31.3 and 23.6% for sites A and B, respectively). While the site B detection rate was lower in the autumn, the credible intervals were largely overlapping and the effect of site was negligible in our generalized linear mixed model ($p = 0.3089$; Fig. 3). There was a trend toward higher detection rates in benthic samples for both sites A and B of the St Croix River, although credible intervals partially overlapped ($p = 0.0665$; Fig. 3). As expected, we did not detect any *M. monodonta* DNA in any water samples collected from the 2 Black River control sites.

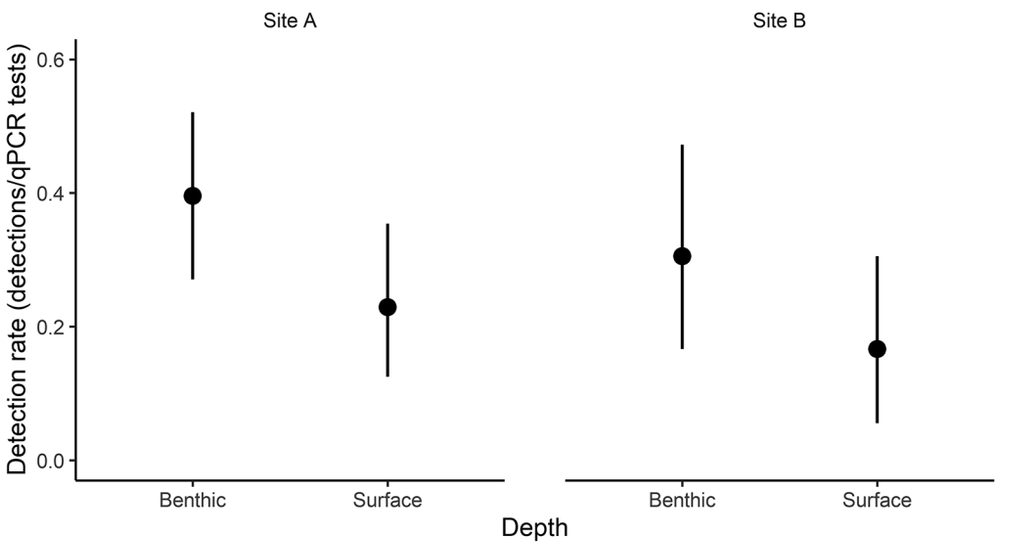

Figure 3. *Margaritifera monodonta* eDNA detection rates (detections/qPCR tests) for the St Croix River surface vs benthic sampling. Detection rates are separated by site in the left (Site A) and right panels (Site B). Depth indicates whether samples were collected near the bottom (benthic) or at the surface. Bars represent the 95% credible intervals.

DISCUSSION

The use of eDNA is a quick, non-invasive method of detecting target species of interest. The use of eDNA as a first step for detection followed by traditional survey methods for visual confirmation is a useful and powerful combination. We designed and validated the first reported qPCR assay with the capability of detecting *M. monodonta* by using eDNA. We also showed, for the first time, that freshwater mussel larval eDNA could be detected despite their small size and their being encapsulated on fish gills. We demonstrated that benthic water sampling may have greater potential for detecting mussel eDNA than surface water sampling.

Assay validation and limit of detection

Based on the LOD and LOQ values, our assay was slightly less sensitive than some other eDNA assays developed for other freshwater mussel species, which reported LOD values between 1 and 10 copies (Sansom and Sassoubre 2017, Dysthe et al. 2018). However, our assay was sensitive enough to detect *M. monodonta* eDNA at known sites and an unknown site with no previous dive survey data. When we compare the sensitivity of our assay to other eDNA assays in general, our LOD and LOQ values fell within reported ranges (Klymus et al. 2020). Based on the analysis of 36 qPCR assays by 7 independent laboratories, Klymus et al. (2020) reported that LOD values ranged from 2.19 to 260 copies/reaction, and LOQ values ranged from 6 to 839 copies/reaction.

Aquarium testing

Based on our aquarium testing results, we were confident that *M. monodonta* mussels shed enough eDNA that they would be detectable in water samples collected from the wild. We were surprised to get positive detections from the larvae encapsulated on the fish gills because we did not expect larvae to shed enough eDNA to be detectable. Despite collecting these samples opportunistically, we are confident that these detections were not due to contamination because the holding tanks were cleaned with a mild bleach solution, rinsed, and allowed to dry completely prior to adding the fish. To the best of our knowledge, this is the first study to report that freshwater mussel larval eDNA can be detected.

eDNA field sampling

The higher eDNA detection rate on the St Croix River compared to the UMR may have been due to differences in discharge between the 2 rivers. The UMR was discharging ~14× as much water as the St Croix River during the sample period; therefore, we suspect this difference in detection rates was a consequence of greater dilution in the UMR than in the St Croix River. This finding highlights one of the challenges of eDNA: the signal can be greatly influenced by flooding and fluctuations in flow rates (Wilcox et al. 2016). Interpretation of eDNA results can be further challenging because a positive detection of a target species does not necessarily mean that the species is present at the sampled location. A positive eDNA detection could be coming from a population upstream from the sampled location or even from a non-living source of DNA, such as dead animals or predator feces (Merkes et al. 2014). However, we do not think this situation applies in our study because we targeted sites of known mussel beds to validate our assay. Additionally, we do not believe that interference from an upstream population was an issue in our study because we did not notice an increase in *M. monodonta* eDNA detections at site B compared to site A for the St Croix River or at site 2 compared to site 1 for pool 15 of the UMR. Further research is needed to better understand eDNA shedding, degradation, and transport for *M. monodonta*.

While we did not observe a strong relationship between detection probability and distance, current literature shows that eDNA is diluted during transport and, therefore, has reduced detection probability farther downstream from the source (Deiner and Altermatt 2014, Jane et al. 2015, Sansom and Sassoubre 2017, Shogren et al. 2019). However, eDNA-transport studies report conflicting results on whether eDNA can be successfully detected downstream. Stoeckle et al. (2016) failed to detect *M. margaritifera* DNA 500 and 1000 m downstream from the mussel beds, whereas Deiner and Altermatt (2014) were able to successfully amplify Swollen River Mussel (*Unio tumidus* [Philipsson, 1788]) DNA 12 km downstream from the lake the species inhabited. Even though *M. monodonta* reside in sheltered habitats, it is possible that their eDNA is transported efficiently downstream in river systems, as has been suggested for *M. margaritifera* (Wacker et al. 2019). We observed only a marginal decrease in detections out to 500 m, but we suspect that if we had continued sampling farther downstream (e.g., 1–2 km) at the St Croix site B and the UMR pools 15 and 16 sites, then we likely would have observed a substantial distance effect with reduced detections at longer distances from the source population.

Margaritifera monodonta mussels tend to live in cracks and under rocks, which makes them difficult to find. Dozens of individuals could be living under a single rock left unturned by divers doing traditional surveys, so it is possible (and even likely) that the mussels may be more clustered or more dispersed than is known. Our detections dipped at 50 and 100 m at site B on the St Croix River but rose in the 200 m samples to be indistinguishable from the 0-m sample (Fig. 2). This pattern could be explained by our 0-m reference point actually being upstream from individuals not previously detected. We do not believe that

detections were increased at 200 m because of disturbance, given that the mussels are benthic, and we sampled from a boat and never came in contact with the substrate. Furthermore, pool 15 site 2 was 660 m downstream of pool 15 site 1, and we had 0 detections at site 2 despite having detections at site 1.

We expected to see higher overall detections in the spring than in the autumn because spring is a period of expected conglutinate release and high DNA abundance. We do not think that the reversal in detection rates between St Croix River sites A and B seen in the autumn was due to lower flow rates and water volumes (i.e., less dilution) because there was little difference between the mean flow rate and mean depth at sites A and B during the 2 collection periods (Table S4). For this same reason, we do not believe that eDNA from upstream mussel beds in site A affected detection at site B. Instead, the reversal in detection rates between sites A and B could point toward a representation of the mussel density at each site. Currier et al. (2018) found that eDNA copy estimates were positively correlated with quadrat-derived estimates of mussel density for Wavy-rayed Lampmussel, *Lampsilis fasciola* (Rafinesque, 1820); Kidneyshell, *Ptychobranchus fasciolaris* (Rafinesque, 1820); and Mapleleaf, *Quadrula quadrula* (Rafinesque, 1820).

Our results suggest that there may be a benefit of increased detection probability by collecting eDNA samples for this species near the stream bottom, as would be expected for a benthic-dwelling species, such as *M. monodonta*. However, our overall finding of a slight benefit of benthic sampling over surface sampling is not consistent with the findings of Currier et al. (2018). They evaluated whether sampling depth and mussel density of 4 at-risk unionid species—*L. fasciola*; Eastern Pondmussel, *Ligumia nasuta* (Say, 1817); *P. fasciolaris*; and *Q. quadrula*—influenced the probability of species detections in southern Ontario rivers. Despite sampling in depths <1 m, they determined there was no benefit of benthic sampling over surface sampling for any of the 4 mussel species, similar to our findings for the St Croix River samples (Table S4). However, a different study showed that Zebra Mussel, *Dreissena polymorpha* (Pallas, 1771) DNA concentrations were higher near the lake bottom (Amberg et al. 2019). We did not observe DNA concentrations high enough (above LOQ) to fully explore this relationship between depth and detection, but higher DNA concentrations would contribute to higher detection rates. We collected a small number of samples for comparison of depth and eDNA detections, and it seems likely that the relationship might have been stronger if we had collected more samples, leaving room for further study.

Perhaps the most substantial result from this study was our unexpected finding of *M. monodonta* eDNA in water samples upstream from the known populations in the St Croix River. We had collected these samples to serve as negative field controls within the same river system to provide a similar background sample matrix. However, we detected *M. monodonta* eDNA in these samples and again when we resampled the upstream site. We later confirmed on 23 August 2018 the presence of an adult *M. monodonta* near our upstream sampling location (D. Waller, United States Geological Survey, personal communication, 2018). The eDNA detection at the assumed control site, coupled with the collection of an adult *M. monodonta* mussel at this site, indicates the utility of using eDNA for identification of previously unknown populations.

Our work can help resource managers make more informed decisions on the conservation of this federally endangered species. As indicated with our control site data, eDNA positive detections can act as a guide for locating previously unknown mussel beds, even in a large river like the Mississippi River. Further research is needed to determine the persistence of *M. monodonta* eDNA downstream from the mussel beds and optimal sampling time and conditions for the greatest chance of detection.

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