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Increasing Detection Sensitivity for Rare and Endangered Species in Kansas through Development of an Aquatic Environmental DNA Sampling Protocol

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INCREASING DETECTION SENSITIVITY FOR RARE AND ENDANGERED SPECIES IN KANSAS THROUGH DEVELOPMENT OF AN AQUATIC ENVIRONMENTAL DNA SAMPLING PROTOCOL.

being

A Thesis Presented to the Graduate Faculty

of Fort Hays State University in

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the Degree of Master of Science

by

Sarah Hallyburton

B.S., North Carolina State University

Date 4/18/22

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This Thesis for

The Master of Science Degree

By Sarah Hallyburton

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INTRODUCTION

Global climate change has widespread implications on natural communities; implications that will continue to amplify as climate projections progress as predicted. Shifts in climatic trends increases the frequency of extreme weather events (Yang et al. 2021), changes environmental characteristics including water chemistry (Huang et al. 2021), and alters community composition (Boukal et al. 2019). Effects of climate change on native species are exacerbated by human disturbance through changes in land use (Schrag et al. 2009), environmental degradation (Tyagi et al. 2014), introduction of invasive species (Clavero and García-Berthou 2005), and the fragmentation and destruction of habitat (Andrén 1994). Importantly, conservation of native species is critical in maintaining balance within an ecosystem and preserving ecosystem services that benefit human and wildlife populations alike. Large levels of disruption seen through the extinction of native species and the introduction of nonnative species have trophic wide repercussions that ultimately result in the loss of critical ecosystem services. These resulting extinctions leave gaps in ecological niches that are increasingly filled by invasive species and followed by the loss of ecosystem function (Hesselschwerdt and Wantzen 2018).

Across ecosystem types, freshwater ecosystems are at the highest risk for extinction due to climate change and human activity (Huang et al. 2021). Within freshwater systems in North America, freshwater mussels (Order: Unionida) are the most imperiled taxa. Globally, there are 840 species of Unionida, with an overwhelming 302 of those species located in North America (Bogan 2007; Graf and Cummings 2007). An estimated 72% of extant North American species are classified with some degree of conservation concern (Johnson et al. 2013). Freshwater mussels play an important role in maintaining healthy aquatic systems. These organisms filter

large quantities of water, removing suspended particles from the water column and depositing non-edible material into the substrate as pseudofeces. This feeding mechanism not only increases water quality but is an important process in nutrient cycling that promotes healthy aquatic vegetation growth (Spooner and Vaughn 2006). Having high-density mussel populations stabilizes riverbeds, thereby maintaining substrate stability during high flow events. This simultaneously protects habitat for that immediate community while preventing heavy sedimentation downstream (Vaughn 2018). These organisms provide invaluable ecosystem services for both aquatic and terrestrial organisms. Ecosystem services provided by mussels are not filled by other groups and are lost as populations and species diversity declines (Vaughn et al. 2015). Due in part to their natural history, freshwater mussels face many anthropogenic challenges that put them at elevated risk for extinctions. Threats include habitat degradation and destruction through pollution (Augspurger et al. 2007), damming (Dean et al. 2002), introduction of invasive species (Ricciardi et al. 1998), and historic exploitation (Anthony and Downing 2001). Even with conservation efforts, the current extinction rate of mussels in this century could be as high as 50% of remaining species (Ricciardi and Rasmussen, 1999).

The Cylindrical Papershell mussel (*Anodontoides ferrusacianus*) was listed as a Species in Need of Conservation (SINC) in Kansas in 1987 and the status was downgraded to endangered in 2019 (KDWP). Following the 2019 Kansas state statute, (K.S.A. 32-961(h)(2)), any species listed as endangered after 2016 must have an approved recovery plan within four years of listing; currently a recovery plan has been drafted for the Cylindrical Papershell but not yet approved. The historic range of the Cylindrical Papershell extended from the northeastern United States and southeastern reaches of Canada, through to Colorado in the West (Harrold and Guralnick

2010). The species occurred throughout the Kansas River Basin (Hoke 1997) but was last known to be restricted in Kansas to the Smoky Hill and Saline Rivers (Sowards et al. 2016; Karlin 2017). The most recent survey efforts in 2020 documented one surviving individual in the Smoky Hill River (Ryan Waters, KDWP, personal communication 2020). Like other freshwater mussel species, the Cylindrical Papershell might be temperature sensitive (Edgar 1965). Accordingly, declines might in part, be tied to rising summer water temperatures and the subsequent contraction of suitable habitat during extended periods of drought. Reintroduction efforts of the Cylindrical Papershell are essential to restore and maintain the historic distribution of this species. With the unpredictable effects of climate change, peripheral populations, such as those in Kansas, could be important to the overall survival of the species across the remaining distribution (Channell 2004). With reintroduction, an increase in monitoring will be required to evaluate success and changes in demographics as populations grow and disperse from the initial reintroduction sites.

Traditional sampling methods can be labor intensive and unsuccessful at locating rare species that are often highly dispersed throughout their habitat (Lor et al. 2020). As number of individuals declines, catch per unit effort declines, making the costs associated with traditional surveys alone rise. Methods such as hand grubbing take larger teams of technicians to physically manipulate the substrate searching for individuals. This method could have varying degrees of success under different flow conditions. Similarly, juvenile mussels are more difficult to locate and may be easily passed over by traditional sampling methods (Hornbach and Deneka 1996). Advances in molecular techniques are providing improved opportunities for species monitoring. Techniques like polymerase chain reaction (PCR) have been deployed rapidly since the

discovery and commercial production of the thermophilic Taq DNA Polymerase. Programmable thermocyclers and Taq DNA Polymerase have made the process of DNA amplification an automated experience requiring little time and manual labor. These advancements have made molecular techniques a viable addition to species monitoring plans because of the demonstrated success in amplifying degraded environmental DNA shed by organisms into the environment.

Because the life histories of freshwater organisms are intimately connected to filtering the aquatic medium, they are constantly shedding DNA into their environment. Therefore, molecular techniques are an appropriate method for surveying environmental samples for the detection of rare mussel species. Currier et al. (2017) demonstrated detection of freshwater pearly mussels in Ontario through environmental DNA (eDNA) and Jerde et al. (2011) used eDNA surveillance for early detection of invasive fish. Advancements in eDNA sampling techniques have proven to successfully detect aquatic organisms with a higher degree of sensitivity compared to traditional methods (Wilcox et al. 2013). Downstream transport of eDNA lowers detection probability of site-to-site presence, but this transport of DNA allows for larger stretches, in some cases up to 1.7 km of rivers and streams to be sampled at one time compared to quadrat sampling (Wacker et al. 2019). Positive eDNA results can be used to target traditional sampling efforts and optimize resource use. Under proper protocol, eDNA sampling can be a cost effective and reliable addition to species monitoring programs.

Although species with low and declining populations have the most to gain from incorporating eDNA surveys into conservation management plans and the success of eDNA surveys has historically been limited by low density populations (Wackert et al. 2019). The objective of this

project was to increase the detection sensitivity of eDNA sampling to produce reliable results when sampling for low density, endangered freshwater organisms. Increasing detection sensitivity and reliability are essential in conservation monitoring programs and will only gain increasing importance as we manage populations of aquatic species that are in decline as we progress through the Anthropocene.

METHODS

Primer Validation

To validate primers specific for the Cylindrical Papershell, DNA from mussel species with overlapping distributions with the Cylindrical Papershell in Kansas were examined. These primers targeted a mitochondrial DNA sequence on the NADH dehydrogenase subunit 1 (ND1) gene (Karlin 2017). Tissue samples were collected between May and August 2021 and stored in 70% ethanol. Small pieces of mantle tissue were removed using forceps and scissors sterilized with 50% bleach. All mussels were returned to their location in the river immediately following tissue sample collection. DNA was extracted from the tissue samples using the Qiagen Blood & Tissue Kit with no protocol modifications. Extraction nulls were included to monitor kit contamination. Null samples followed the same protocol except that tissue was not included in the initial reaction step. DNA was amplified by combining 22 µL Master Mix (Table 1), Cylindrical Papershell DNA primers (Table 3) and 3 µL DNA into a tube of Illustra PuReTaq™ Ready-To-Go™ PCR Beads. PCR nulls were subjected to the same protocol except the 3 µL of sample DNA was replaced with molecular grade water (VWR) to monitor samples for contamination. Samples were placed in the MiniAmp Plus Thermal Cycler and amplified on the mussel thermal cycling program (Table 2). Five µL of the resulting PCR product was inserted into a 2% Agarose E-Gel with 15 µL of E-Gel Sample Loading Buffer (Invitrogen). The product was subjected to electrophoresis for 15 minutes using the E-gel Power Snap (Invitrogen) for verification of successful amplification.

eDNA Collection

Water samples were collected to gather Cylindrical Papershell eDNA for protocol development. Samples were collected in 4 L Nalgene bottles. Bottles were decontaminated before and after collection with a 50% bleach solution (Goldberg et al. 2016). Samples were collected from three different locations. Two locations with confirmed Cylindrical Papershell presence and one unknown sample location were selected. For known positive locations, the Kansas Aquatic Biodiversity Center in Farlington, Kansas was selected because the species is being propagated for reintroduction efforts in tanks at this facility. The second positive sample was collected from a naturally occurring population in Taylor, Nebraska. Three live individuals were encountered within 10 m of the collection site. Lastly, a water sample was collected from one of the last known localities to support the species in Kansas, the Smoky Hill River near Hays, Kanas. This location was not surveyed for live individuals at the time of sample collection and no live individuals were observed.

Nalgene bottles were submerged to collect water from below the surface, closer to the substrate. Samples were collected between November 2021 and January 2022. Water samples were transported from the field on ice to prevent further degradation of DNA and refrigerated at 4 °C until filtering. Empty 2 L Nalgene bottles were carried into the field as collection nulls to monitor contamination. Null bottles were sanitized with 50% bleach before entering the field. Nulls were filled with tapwater and filtered upon returning to the lab.

DNA Capture

Smith-Root self-preserving eDNA filters were used as the primary DNA capture method to develop this protocol. The filter membrane is composed of polyethelsulfone (PES) and a pore

size of 0.45 μm was selected because this size has been shown to best capture DNA in natural waters (Wacker et al. 2019). The plastic tube from the filter pack was attached to the filter housing and two castrating bands were placed over the tube at the connection point with the filter housing to produce a tighter seal between the two pieces. The filter was connected to a 1 L filter flask and vacuum pump (Fisher Scientific Gast Vacuum Pump Model # 5KH33GN293KX). The filter tube was inserted directly into the Nalgene bottle before engaging the vacuum pump. After each liter of water was filtered, the filter flask was disconnected from the filter housing and emptied into a graduated cylinder before continuing; the filter tube was not removed from the Nalgene bottle. Up to 5 L of water were filtered through one filter. Following manufacturers' recommendations, the filter housing was cracked open and allowed to pull air through the filter for 20 seconds following the complete filtration of each sample.

A pre-filtering stage was added for samples from eutrophic waters to prevent premature clogging of the eDNA filters. A 1 μm cellulose filter (Sterlitech) was used with a 350 mL polysulfone analytical funnel and 4 L filter flask. This funnel and filter flask was connected to the vacuum pump to actively pull water through the filter. Pre-filtering removed larger organic material from the water prior to filtering through the smaller pore size. The filter funnel and filter flask were decontaminated using 50% bleach. The surfaces were rinsed clean of bleach and allowed to dry before contact with samples. The cellulose filter was inserted between the two funnel pieces and up to 350 mL of water was poured into the top container at a time. Once this filter began to clog it was removed from the filtering system, placed directly into ethanol, and replaced with a new filter. This process was repeated until the entire 4 L sample was processed. Pre-filtered water

was transferred back into the original Nalgene bottle and refrigerated until samples could be filtered with the Smith-Root eDNA filter.

e*DNA Extraction*

DNA extractions were performed on both 1 μm and 0.45 μm filters. Extractions were completed on 1 μm cellulose filter to test for eDNA retention that would affect eDNA capture on the 0.45 μm, PES eDNA filters. Filter extractions were performed using the Qiagen DNeasy PowerWater Extraction Kit with minor protocol modifications. Cellulose filters were removed from ethanol and allowed to dry overnight before extraction. eDNA filters were removed from the housing unit at the time of extraction. The entire cellulose filter was lightly rolled inward and inserted into the bead tube and 1000 μL PW1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 μg/ml RNaseA) was added to each tube. Due to the larger filter size, PES eDNA filters were cut in half using forceps and scissors sterilized with DNA Away™ Surface Decontaminant. Each half was lightly rolled inward and inserted into an individual bead tube, 1000 μL PW1 was added to each tube. A negative control with no filter was added to each extraction attempt to monitor for contamination. Bead tubes were attached to a Fisher Vortex Genie 2 using a 5 mL tube vortex adapter (Qiagen). Samples were vortexed at max speed for 10 minutes to mechanically lyse the filtered cells. Bead tubes were centrifuged for 1 minute at 6000 rpm to maximize supernatant yield. Supernatant from each bead tube was transferred to a separate, clean 2 mL tube and the remaining kit protocol was followed as outlined. Supernatants from the two filter halves were combined onto one MB Spin column and eluted with 100 μL EB (10 mM Tris-Cl, pH 8.5). DNA was amplified as indicated above. Samples that could be amplified and produced an appropriate

sized band on the agarose gel were quantified using the QuantusTM Fluorometer (Promega), methods outlined below.

To verify the resulting DNA represented the Cylindrical Papershell, gel extractions were performed on positive samples with no modifications to the QIAquick Gel Extraction Kit protocol. Extracted gel samples and associated forward and reverse primers were delivered to Azenta to be Sanger sequenced. Successful sequences were loaded into Geneious. The ends were trimmed to better target the DNA segments. Forward and reverse sequences were aligned, and BLAST was used to identify the sequences using the NCBI GenBank database.

Detection Limit

To estimate the lowest concentration, this filter and protocol could detect, the protocol above was used to extract and amplify Cylindrical Papershell DNA from tissue samples. PCR product was quantified using the nanodrop (NanoDrop^{TM} 2000 Spectrophotometer, ThermoFisher Scienctific). Known quantities of DNA (ng/µL) were diluted in 1 L of autoclaved reverse osmosis water and vortexed. Samples were filtered using the Smith-Root 0.45 µm eDNA filter. The filters were extracted following the extraction protocol outlined above and subjected to the same PCR protocols. Five µL of the PCR product was run on a 2% Agarose E-Gel for 15 minutes using the E-gel Power Snap (Invitrogen). Samples positive for eDNA were quantified, methods outlined below.

DNA Quantification

DNA was quantified using the Quantus™ Fluorometer (Promega). The Fluorometer was calibrated using a blank and standard sample. Two hundred µL of dsDNA dye solution was added to both calibrating samples in 0.5 mL tubes. One µL of Lambda DNA was added to the standard sample. µL of dsDNA dye solution and 1 µL of sample DNA was added to a 0.5 mL tube for each sample being quantified. Tubes were quickly vortexed and centrifuged before being covered by tin foil and incubated at room temperature for 10 minutes in the dark. The blank sample followed by the standard sample were inserted into the fluorometer and were calibrated under the One Sample settings. Remaining DNA samples were inserted and quantified immediately after.

RESULTS

Primer Validation

Cylindrical Papershell primers were species specific for this area of their distribution when using the mussel thermal cycling program (Table 2). Cylindrical Papershell primers amplified Cylindrical Papershell DNA. DNA from Fatmucket (*Lampsilis siliquoidea*), Paper Pondshell (*Utterbackia imbecillis*), Mapleleaf (*Quadrula quadrula*), Giant Floater (*Pyganodon grandis*), Pondhorn (*Uniomerus tetralasmus*), Fragile Papershell (*Leptodea fragilis*), Lilliput (*Toxolasma parvus*), Pink Papershell (*Potamilius ohiensis*), White Heelsplitter (*Lasmigona complanate*), and Pimpleback (*Cyclonaias pustulosa*) were not amplified using the DNA extraction protocol outlined above, Cylindrical Papershell primers, and the thermal cycling program.

DNA Extraction

This extraction protocol successfully extracted and amplified Cylindrical Papershell DNA from 5 L of water collected from the Kansas Aquatic Biodiversity Center rearing tank. DNA was successfully extracted and amplified from 3 L and 5 L of water collected from the naturally occurring population in Taylor, Nebraska. Sequencing results verified that positive samples were a match to Cylindrical Papershell sequences in NCBI GenBank. Extractions performed on the 1 µm cellulose filters used to pre-filter water samples were negative for Cylindrical Papershell DNA. Cylindrical Papershell DNA was not detected in the 4 L sample from Smoky Hill River near Hays, Kansas

Detection Limit

Quantities of 1 μL, 2 μL, and 5 μL of DNA (836.7 ng/μL) were diluted in 1 L H_2O. DNA dilutions were subjected to the above protocols and the 2 μL and 5 μL quantities produced clear bands. DNA was not detected in the 1 μL dilution. The detection limit of this filter and extraction protocol was 1673.4 ng/μL DNA in 1 L H_2O or approximately 1.7 ppb.

DISCUSSION

The objective of this project was to increase the detection sensitivity of rare and endangered aquatic organisms in Kansas using an eDNA protocol. The development of this protocol was directed at the Cylindrical Papershell mussel but can be adapted and applied to the monitoring of any freshwater species of interest so long as species-specific primers are developed. Collecting eDNA samples is an efficient and noninvasive method that can accurately detect aquatic organisms at low densities. When dealing with endangered species this method can aid in reducing stress to organisms in solely presence/absence studies. Notably, a single sample can be used to monitor multiple species making this a time efficient method for both large and small-scale monitoring efforts. This protocol should not be deployed as a stand-alone tactic but can be used to supplement traditional sampling methods to provide a more targeted approach when surveying large areas for rare and endangered aquatic organisms.

The contamination reducing and self-preserving design elements make the Smith-Root eDNA filter a suitable choice for field or lab filtering (Thomas et al. 2018). These aspects allow eDNA surveys to be implemented into sampling fulfilled by aquatic technicians and volunteer citizen scientists without requiring a molecular biology background. With the unpredictable changes in climatic patterns and projected rapid declines of aquatic organisms, the addition of widespread eDNA sampling could be a beneficial addition to increase conservation monitoring while remaining cost conscious.

When making conservation decisions informed by eDNA results, it is imperative that DNA from common species are not affecting the analysis. The negative specificity tests for the ten species

of freshwater mussels likely to occur in this study region provides high confidence that positive results indicate the presences of the target species. Regardless of this confidence, positive samples were extracted from the agarose gels and Sanger sequenced. Using BLAST to align sequences, samples were confirmed to be Cylindrical Papershell using reference material in NCBI GenBank. It is important to note that the use of these primers in another area of the Cylindrical Papershell distribution would need to be tested on any additional species where distributions overlap.

The addition of a pre-filtering stage increased the quantity of water that could be filtered through one Smith-Root eDNA filter. Without pre-filtering, highly eutrophic samples clogged the small pore sized PES filter membranes rapidly and limited filtrate to 100-300 mL of water before complete obstruction. This relatively small volume would not provide confidence in negative results when targeting rare species. I did not attempt to determine a minimum volume of raw sample in either of the positive sample locations. However, based on the estimated detection limit of 1673.4 ng/μL in 1 L (1.7 ppb) under ideal laboratory conditions, it seems detection would be unlikely in small, field collected, sample sizes of 1 L or less.

Many environmental factors influence eDNA quantity collected in a sample including sampling distance from the organism, water temperature, UV-B radiation, and stream velocity. Extraction from the filter is an additional constraint on small sample sizes due to eDNA retention in the filter membrane. Larger quantities of water can mitigate these variables by collecting larger quantities of DNA to meet detection requirements. I was able to document positive results with both 3 L and 5 L samples from natural waters. Accordingly, I recommend a minimum size of 4 L

be sampled to maintain confidence and streamline the field collection and extraction processes. I was able to combine multiple filters during the extraction stage to obtain a large enough water sample without pre-filtering, but due to the cost of materials, this would not be a cost or time efficient method to survey endangered species. Similarly, combining a large enough number of filter samples to reach a minimum detection limit threshold would increase the risk of human error and sample contamination during the extraction stage, decreasing the reliability of results. A 1 μm pore size was large enough to allow eDNA to pass through while removing the problematic larger organic material. One μm cellulose filters are inexpensive and can be replaced as needed when pre-filtering, making these filters a cost-efficient addition to the protocol.

During protocol development, the Qiagen Blood & Tissue Kit was originally tested with various modifications, including the addition of 'bead beating' (Smart et al. 2015). This extraction kit was not compatible with the PES filter and DNA was not detected in positive samples. The Qiagen PowerWater Extraction Kit was able to extract eDNA from the filtered water samples. Being able to release a suitable quantity of DNA captured in the filter was a limiting factor for this protocol and know dilutions of less than 1 ppb were not detected (836.7 ng /L of H_20).

Smith-Root eDNA filters are slightly larger than the size intended for the 5 mL bead tubes and success was inconsistent when whole filters were used in a single tube. Cutting the filter in half allowed for more movement between the filter, ceramic beads, and buffer while vortexing and likely increased the mechanical lysis of the filter membrane. The PES material was more difficult to homogenize than the cellulose material, therefore samples were vortexed for 10 minutes as opposed to the suggested 5 minutes in the kit protocol to adjust for the DNA holding

capacity of this material. Presumably more eDNA was released from the filter following this modification. “Bead beating” significantly increases DNA yield by releasing trapped DNA that could not be extracted chemically (Hundermark and Takahasi 2018) and was a beneficial component of this extraction kit. Even with the additional vortex time and increased buffer to membrane ratio after splitting filters in half, PES filters were not homogenized as completely as the cellulose filters. The alternative use of cellulose and other filter materials could be examined for eDNA yield in future studies involving the Smith-Root eDNA filter.

Other eDNA studies have stressed the importance of filtering water samples as immediately after collection as possible (Jerde et al. 2011; Hinlo et al. 2017), reporting rapid decay of DNA and loss of efficacy of stored samples (Goldberg et al. 2016). Known positive samples were stored refrigerated at 4 °C up to three months after collection and when quantified, were comparable to samples filtered two days after collection. Samples should be filtered as quickly as possible after collection to ensure high confidence in results in unknown samples. However, based on my results, this should not be a deterrent to adding eDNA surveys to monitoring efforts because samples can be effectively stored for longer periods of time and retain DNA yield.

Water samples in this study were collected during the cooler months and this might have contributed to the longevity of eDNA samples. Because warmer water temperature degrades eDNA at a more rapid rate than cooler temperatures (Tsuji et al. 2017), sampling during cooler seasons could be recommended when applicable. If the targeted organism remains active through the fall and winter seasons, collecting water samples during this time could increase the amount of water that can be filtered on one filter without the addition of pre-filtering. Similarly,

sampling during these seasons decreases the amount of time per filter due to the lowered productivity of the water. Quality of eDNA collected from higher and lower water temperatures were not examined in this study but samples collected during cooler seasons could theoretically have higher quality eDNA. When searching for species with low densities (e.g., endangered species) any factors that increase detection sensitivity should be considered. Future studies could investigate seasonal and diel changes in eDNA quantity in natural systems to identify a peak sampling window for the Cylindrical Papershell in Kansas.

Cylindrical Papershell conservation efforts in Kansas can be improved through the implementation of this protocol by increasing sampling efforts within targeted areas to locate remaining individuals. Remaining Cylindrical Papershell individuals from Kansas could be collected and relocated to the Kansas Aquatic Biodiversity Center to increase population size and to preserve remaining genetic variation. Maintaining genetic diversity is an important factor in the success of future reintroduction efforts. Preserving allelic diversity will provide Cylindrical Papershell populations the opportunity to adapt to future conditions. Similarly, this protocol can be implemented to monitor the success of reintroduction efforts by sampling upstream and downstream of reintroduction sites to evaluate natural movement of the species throughout the rivers as populations expand.

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TABLES

Table 1. Master Mix used with Illustra PuReTaq™ Ready-To-Go™ PCR Beads for standard PCR. 22 µL Master Mix was pipetted into the PCR Beads. PCR beads contained 200 µM of each dNTP in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, and 1.5 mM $MgCl_2$. Primers were diluted to a concentration of 10 µM.

Product	µL (per sample)
Forward Primer (CPF)	1 µL
Reverse Primer (CPR)	1 µL
H_2O	20 µL

Table 2. Mussel thermal cycling program. Thermal cycling program used to amplify freshwater mussel DNA during standard PCR.

STEP	TEMPERATURE	TIME
1.	95°C	3:00
2.	95°C	1:00
3.	55°C	1:00
4.	72°C	2:00
5.	Go To 2.	34X
6.	72°C	10:00
7.	4°C	∞

Table 3. Cylindrical Papershell species specific mtDNA forward and reverse primers for the ND1 gene. Amplicon length 106 bp. These primers were used to amplify eDNA extracted from filtered water and tissue samples. Purchased through www.sigma.com/oligos.

Oligo Name	**Sequence 5' to 3'**	**Scale (μmole)**	**Purification**	**Modifications**	**Comments**
CPF419-441	TCCCAGTTTATTAGGGCCTTTC	0.025	DESALT	NONE	L=22; TM=62; GC%=45.5
CPR502-525	CCTTGTCACGTACCTCCTAATTT	0.025	DESALT	NONE	L=23; TM=62; GC%=43.5

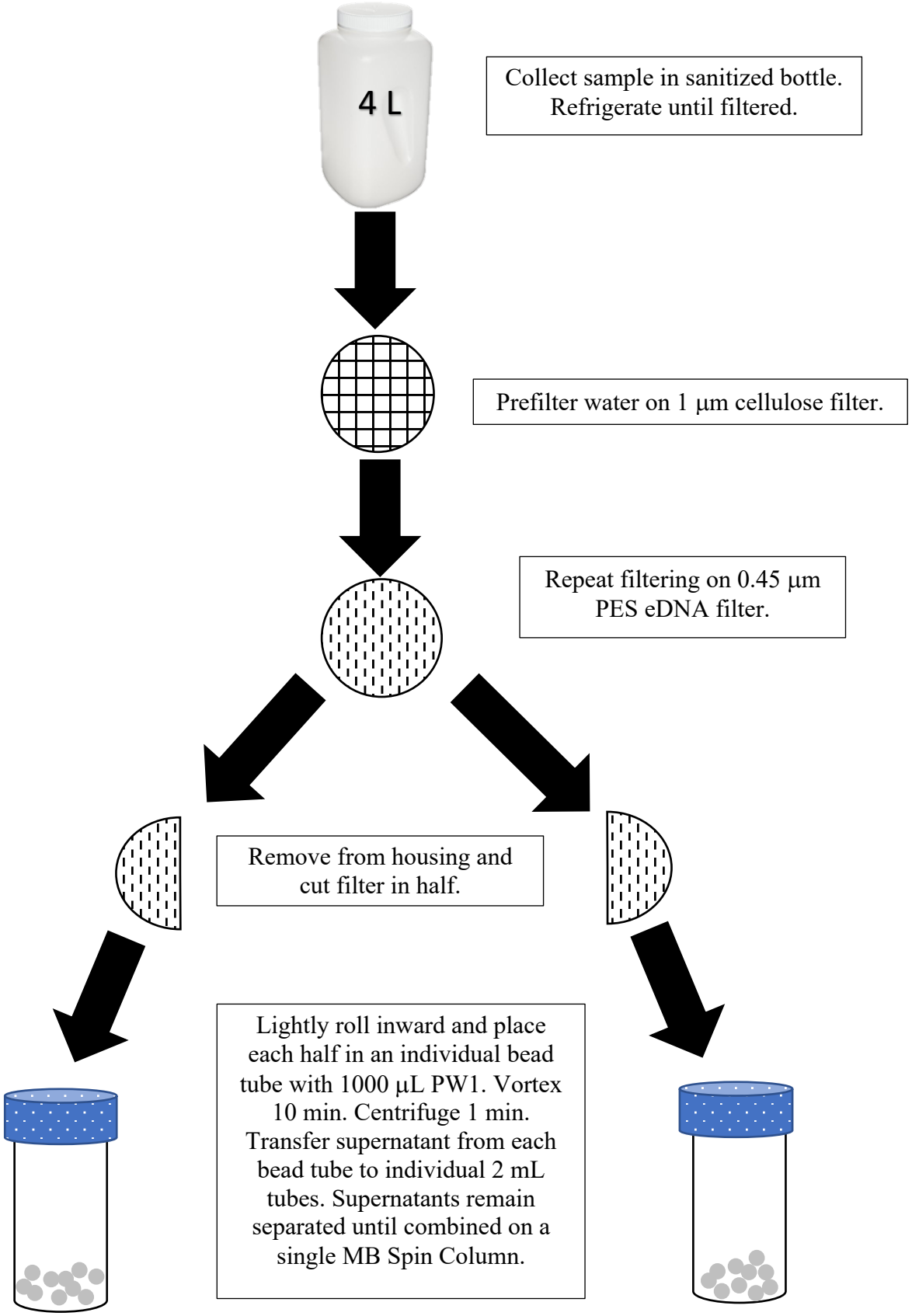
APPENDIX

Appendix A. Freshwater eDNA Sampling Protocol

1. Collect water samples in 4 L Nalgene bottles, sanitized with 50% bleach solution
2. Carry empty 2 L Nalgene bottles into field for null samples, fill with water and filter upon returning to the lab.
3. With a gloved hand, submerge 4 L Nalgene bottle to collect sample, change gloves between samples
4. Transport samples on ice and refrigerate until filtering
5. Prefilter eutrophic samples
    a. Insert 1 μm cellulose filter between two pieces of analytic filter funnel
    b. Pour up to 350 mL water through this filter at a time
    c. Replace filter as necessary when clogged
    d. Pour prefiltered water back into original Nalgene bottle and refrigerate until filtering on eDNA filter
6. Remove Smith-Root eDNA filter and tube from package, connecting the tube to filter
    a. Optional – place castration band over tube at base of filter housing for tighter seal
7. Connect vacuum pump to filter flask, filter flask to eDNA filter
8. Insert filter tube into Nalgene bottle and turn on vacuum pump (Fisher Scientific Gast Vacuum Pump Model # 5KH33GN293KX)
9. Cut filter in half using sterilized forceps and scissors
10. Roll and insert each half into a 5 mL PowerWater DNA Bead Tube
11. Add 1000 μL of Solution PW1 to each sample Bead Tube plus a negative control null Bead Tube
12. Vortex at max speed for 10 minutes
13. Centrifuge for 1 minute at 14.8 rpm (ThermoScientific Sorvall Legend Micro 21)
14. Insert pipette tip down into the beads and draw up supernatant (~600-650 μL), transfer supernatant from each bead tube into a clean 2 mL collection tube
15. Centrifuge for 1 minute at 14.8 rpms
16. Transfer supernatant to a clean 2 mL collection tube, being careful to avoid any remaining beads
17. Add 200 μL of Solution IRS to each sample
18. Vortex briefly to mix
19. Incubate at 4 °C for 5 minutes
20. Centrifuge for 1 minute
21. Transfer supernatant to a clean 2 mL collection tube
22. Add 650 μL of Solution PW3 and vortex briefly to mix
23. Load 650 μL of supernatant onto a MB Spin Column
24. Centrifuge for 1 minute at 14.8 rpm
25. Repeat until all supernatant from one filter (two halves) have been loaded onto a single MB Spin Column
26. Place MB Spin Column Filter into a clean 2 mL collection tube
27. Add 650 μL of Solution PW4 (shake before)
28. Centrifuge for 1 minute at 14.8 rpm
29. Discard flow through and add 650 μL of ethanol

30. Centrifuge for 1 minute at 14.8 rpm
31. Discard the flow through and centrifuge for 2 minutes at 14.8 rpm
32. Place MB Spin Column into a clean 2 mL collection tube with the hinged cap cut off
33. Add 100 µL of Solution EB to the center of the white filter membrane
34. Centrifuge for 1 minute at 14.8 rpm
35. Discard the MB Spin Column and cap the collection tube
36. Label PuReTaq™ Ready-To-Go™ PCR Beads and prepare Master Mix to cover all the extracted samples, a PCR null, and one extra
37. Add 22 µL Master Mix to each ready to go PCR bead tube
38. Add 3 µL DNA to each corresponding PCR bead tube
39. Add 3 µL H_2O to the PCR null tube
40. Briefly vortex tubes to ensure all DNA is in the bottom of tube with the reagents
41. Insert into PCR thermal cycler and start the chosen cycling program.
42. Load 2% Agarose (GP) E-Gel with SYBR Safe™ (Invitrogen) into E-gel Power Snap (Invitrogen)
43. Add 20 µL H_2O to lane 1 (and all lanes without sample DNA)
44. Add 10 µL DNA ladder and 10 µL loading buffer to lane 2
45. In lanes 3-12 add 5 µL PCR product and 15 µL loading buffer
46. Select E-Gel 0.8-2% program and adjust run time to 15 minutes

Appendix B. eDNA protocol illustrative diagram using Qiagen PowerWater Extraction Kit



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