

Mission Statements

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The mission of the Bureau of Reclamation is to manage, develop, and protect water and related resources in an environmentally and economically sound manner in the interest of the American public.

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188		
T1. REPORT DATE September, 2014		T2. REPORT TYPE Research		T3. DATES COVERED 2014 FY	
T4. TITLE AND SUBTITLE Optimization of Early Detection of Quagga Mussels by Polymerase Chain Reaction			5a. CONTRACT NUMBER RY1541ZQ201413688		
			5b. GRANT NUMBER		
			5c. PROGRAM ELEMENT NUMBER 1541 (S&T)		
6. AUTHOR(S) Jacque Keele, jkeele@usbr.gov , 303-445-2187 Jamie Carmon Denise Hosler, dhosler@usbr.gov , 303-445-2195			5d. PROJECT NUMBER 3688		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER 86-68220		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Bureau of Reclamation, Technical Service Center, Environmental Applications and Research Group			8. PERFORMING ORGANIZATION REPORT NUMBER 86-68220-14-13		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) Research and Development Office U.S. Department of the Interior, Bureau of Reclamation, PO Box 25007, Denver CO 80225-0007			10. SPONSOR/MONITOR'S ACRONYM(S) R&D: Research and Development Office BOR/USBR: Bureau of Reclamation DOI: Department of the Interior		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S) 01		
12. DISTRIBUTION / AVAILABILITY STATEMENT Final report can be downloaded from Reclamation's website: https://www.usbr.gov/research/					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT (Maximum 200 words) The optimization of PCR for quagga mussel early detection is an ongoing process. Understanding the importance of a quality control/ quality assurance plan has been important to maintaining good laboratory practices. Many different aspects of the PCR process have been explored: determining the best sample storage conditions, optimizing the DNA extraction, and the PCR master mix, and primers used for amplification. Also, determining how humic acid affects the PCR reaction was assessed to determine the best DNA extraction kit to use with raw water samples. Additionally, a PCR assay for the golden mussel early detection was developed. The optimization of quagga mussel PCR is an ongoing process that has enabled researchers at RDLES to increase their understanding of this assay.					
15. SUBJECT TERMS Quagga Mussels, Zebra Mussels, Invasive Mussels, Early Detection, PCR					
16. SECURITY CLASSIFICATION OF: U			17. LIMITATION OF ABSTRACT U	18. NUMBER OF PAGES 41	19a. NAME OF RESPONSIBLE PERSON Jacque Keele
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER 303-445-2187

S Standard Form 298 (Rev. 8/98)
P Prescribed by ANSI Std. Z39-18

PEER REVIEW DOCUMENTATION

Project and Document Information

Project Name: Optimization of early detection of invasive mussels by polymerase chain reaction

WOID: X3688

Document: Optimization of early detection of invasive mussels by polymerase chain reaction

Document Author(s): Jacque Keele, Jamie Carmon, and Denise Hosler

Document date: September 2014

Peer Reviewer: Chris Holdren

Review Certification

Peer Reviewer: I have reviewed the assigned items/sections(s) noted for the above document and believe them to be in accordance with the project requirements, standards of the profession, and Reclamation policy.

Reviewer

g C Holdren
(Signature)

Date reviewed

12/9/14

Acknowledgements

The authors of this research would like to thank the Research and Development Office for supporting and providing funding for this study. Also, thank you to the reviewers of this report and to Sherri Pucherelli, Kevin Bloom, and Kyle Rulli for assisting with this research.

Executive Summary

The Reclamation Detection Laboratory for Exotic Species (RDLES) has made a coordinated effort to increase the understanding and best protocols for the early detection of quagga mussels by DNA analysis. The polymerase chain reaction assay (PCR) is a complex assay that involves three steps: the DNA extraction, PCR assay, and gel electrophoresis to detect PCR products. All of the steps in this process can be optimized to increase the reliability, reproducibility, and detection limit of the assay. Creating an atmosphere where quality control and quality assurance is central to the analysis being carried out has been important to maintaining good laboratory practices at RDLES. Determining the best sample preservation conditions (percent alcohol and buffer) for raw water samples that will be tested by PCR has been a central theme of the research performed at RDLES. The outcome of these studies has shown the importance of buffering and the presence of alcohol in the plankton tow samples for maintaining veliger integrity over time. Studies have also been conducted to determine the best DNA extraction methods for raw water samples. The presence of environmental inhibitors decreases PCR sensitivity and overcoming this issue has also been an area of research. These findings have influenced the PCR standard operating procedure used at RDLES for the analysis of samples for quagga mussels. The next steps in this research will be to continue to increase the understanding of both the limits and advantages of PCR for use at Reclamation.

Introduction

The Reclamation Detection Laboratory for Exotic Species (RDLES) at the Bureau of Reclamation, Technical Service Center, Denver CO, performs polymerase chain reaction (PCR) analysis on raw water samples for the early detection of (*Dreissena rostriformis bugensis*) quagga and (*Dreissena polymorpha*) zebra mussels. This molecular assay involves isolating DNA from a raw water sample, using PCR to amplify a specific gene from the mussels, and analyzing the amplified PCR product on an agarose gel to determine the presence or absence of the target gene in the water sample. Many issues can arise during this multistep process. Even if the PCR assay has been perfectly optimized, not understanding potential experimental issues will lead to poor or hard to interpret results.

Dreissenid Mussels

Invasive dreissenid mussels impact Reclamation facilities and waters. Quagga and zebra mussels cause economic and environmental impacts where infestations occur. The mussels are able to cause significant damage to infrastructure, water intakes, trash racks, and other facilities that are on the water. Early detection of these mussels focuses on the free floating microscopic larval (veliger) life stage in order to provide water managers time to prepare for large mussel populations that can impact facility functions. RDLES performs early detection testing on waters from across the western United States. Early detection testing includes two separate tests. The first test is to detect the veligers using cross polarized light microscopy, because quagga and zebra mussels have a distinctive Maltese cross pattern when under polarized light. The second test is the polymerase chain reaction test which detects the presence of mussel DNA.

Quagga mussels predominate in the western United States, damaging infrastructure, and impacting water delivery. The golden mussel (*Limnoperna fortunei*) is another invasive species of interest to Reclamation due to RDLES collaboration with scientists in Brazil to develop the necessary PCR assays for the detection of this invasive mussel. Golden mussels are quickly colonizing and threatening the rivers of South America and could potentially be transported to North America. The early detection effort provides an early warning to water managers allowing time to obtain the resources to deal with an adult population. Reclamation had adopted PCR as a tool to assist with the early detection effort.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a popular molecular technique that has revolutionized and advanced the field of molecular biology. Prior to PCR it was difficult, costly, and time consuming to study genes of interest. It is now possible to isolate DNA from an environmental sample or monotypic (single) organism,

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and to analyze the DNA using PCR and get results within several hours. The PCR master mix reagents and primers are used in conjunction with the PCR instrument to amplify a few copies of the gene of interest to billions of copies. The PCR process involves three steps: denaturation, annealing, and extension. Isolating and analyzing a monotypic sample when compared to a mixed environmental sample is always easier because the monotypic sample contains less interfering transcripts and environmental inhibitors that could interfere with the PCR reaction. This PCR technique enables a researcher to amplify a specific gene of interest, sequence the gene and compare the gene to other sequences in published databases to give a conclusive identification to the PCR product.

The PCR process begins with the DNA extraction. Three different DNA extraction kits have been used at RIDLES over the last few years. The first kit used was the Qiagen DNeasy Blood and Tissue kit (Qiagen 2013) with an additional DNA clean up step of Gene Releaser. The second kit used was a soil extraction kit: the Mo Bio Ultra Soil. In 2013 Mo Bio improved their DNA extraction methods and created the Power Soil Kit (Mo Bio, 2013), which is the current kit used at RDLES. As the methods and technology used in environmental DNA extractions improves RDLES will continue to seek new and better ways of extracting DNA.

Following the DNA isolation, PCR is performed to amplify a fragment of the cytochrome oxidase I (COI) gene from both zebra and quagga mussel. The PCR reaction is set up as a master mix of reagents. These reagents include water, forward and reverse primers, magnesium chloride, 10X buffer, dNTP's (dATP, dTTP, dCTP, and dGTP), and Taq polymerase. Each of these reagents is needed to have a successful PCR reaction. COI is present in the mitochondrial genome, so there are multiple copies of this gene in each cell. The central goal of PCR is to take a few copies of a gene and amplify the copies into billions of copies that can be visualized by gel electrophoresis. PCR has three stages. First, the double stranded DNA is denatured at a high temperature to separate the DNA strands. Then, during the annealing step the temperature is lowered, and the two primers (forward and reverse) find complimentary DNA template to bind to. The final stage is the extension is when the Taq polymerase extends the primer to form a DNA stand (Figure 1).

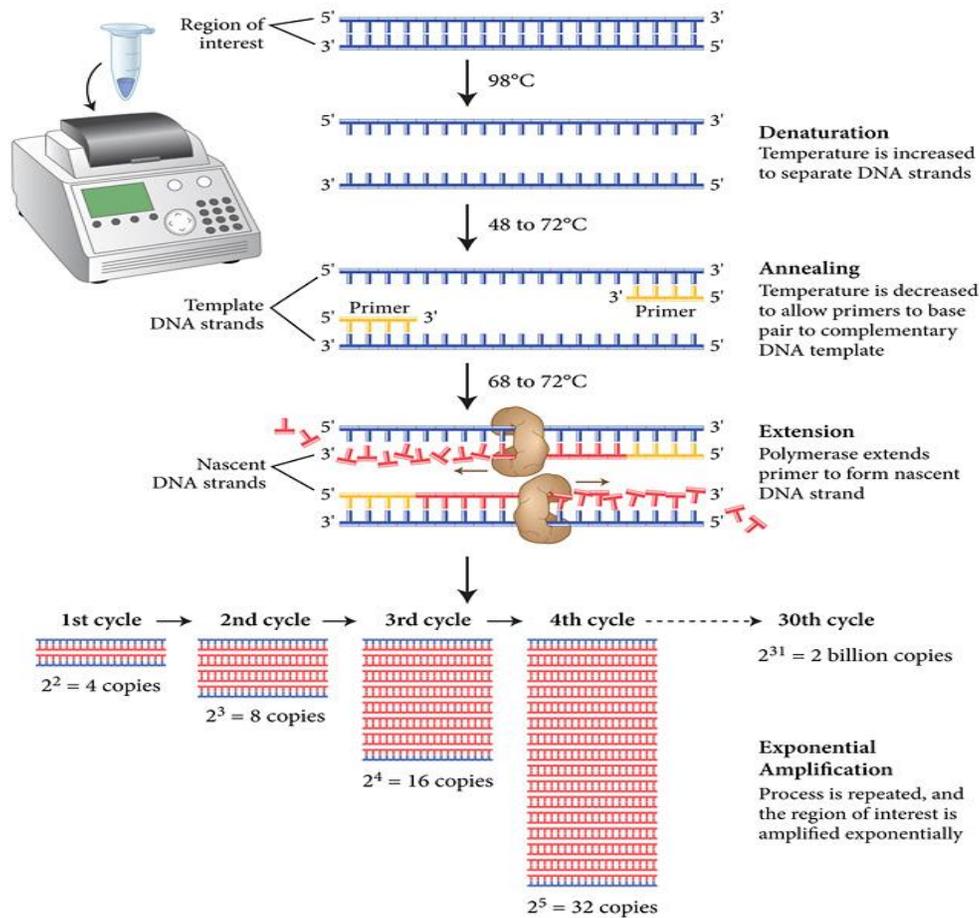


Figure 1: PCR method

(<https://www.neb.com/~media/NebUs/Page%20Images/Applications/DNA%20Amplification%20and%20PCR/pcr.jpg>)

The final step in the DNA analysis is to perform agarose gel electrophoresis. In this step, the PCR product is placed into a loading dye (New England BioLabs), and loaded onto an agarose gel. An electrical current is used to separate the DNA products on the gel by size. Smaller fragments move faster through the gel matrix than larger DNA fragments. A ladder of known base pair sizes is used to determine the size of the PCR products (New England BioLabs). In addition to a ladder, a known positive PCR reaction and negative PCR reaction are also analyzed to show that the PCR master mix is working correctly. If a PCR product gives a positive band then it is sent for DNA sequencing to determine the DNA sequence of the fragment. The sequencing information confirms the presence of dreissenid mussel DNA in a raw water sample.

PCR Research at RDLES

There is no standard method for the detection of the quagga mussel DNA because the labs performing early detection have not agreed on the best DNA extraction

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and PCR methods. Over the last two years, RDLES has performed many different experiments that have helped to increase the laboratories understanding of PCR and how best to use this tool for the early detection of quagga and zebra mussels. One of the issues has been determining the best way to analyze raw water samples which are a complex mixture of organisms, organic and inorganic materials, to get consistent clear results. Additionally, three large scale studies have been done at RDLES to look at the impact of sample preservation methods on the detection of veligers by microscopy and PCR. The focus of this effort was to optimize the PCR method for early detection of dreissenid mussels at RDLES.

Literature Review

The primary goal of this literature review is to summarize the methods that have been used for the early detection of zebra and quagga mussels from plankton tow collected water samples. The second section will be a review the current methods for the detection of golden mussels by PCR. Finally, microsatellite methods used to analyze different quagga and zebra mussel populations will be summarized.

Part I: Early Detection of Quagga Mussels from Raw Water Samples using PCR

Currently, there are only a handful of laboratories performing PCR analysis of raw water samples for the detection of quagga mussel larvae. Each lab uses slightly different DNA extraction and PCR methods that are either unpublished or adapted from published methods. Less than 30 publications have been written on quagga mussels and many of the publications focus on the mussel's biology and are not directly related to early detection. Published research uses adult quagga mussels (monotypic samples) to develop new primers for PCR detection. Once the PCR primers have been developed and tested with adult mussels, they can be tested on raw water samples containing veligers in order to determine specificity. These publications offer insight into how current PCR methods for early detection of quagga mussels have evolved.

Cytochrome Oxidase 1 (COI) Method Development

The PCR method developed by Claxton et al. (1997) is currently used for early detection of quagga mussels and is a starting place for development of additional COI assays. The method developed by Claxton et al. (1997) used non-specific PCR primers to amplify a 710 base pair fragment of the cytochrome oxidase I (COI) gene from adult quagga and zebra mussels. The primers used in this method were developed by Folmer et al. (1994). These primers have been useful for the amplification of the COI gene from many different invertebrates. The primers used by Claxton et al. (1997) were non-specific and were not able to differentiate between zebra and quagga mussels without additional analysis. In order to differentiate between the species the amplified product was analyzed by restriction length polymorphism (RFLP). A restriction enzyme was used to digest

the PCR product, and the differences in the sizes of the fragments enabled the researchers to differentiate between the quagga and zebra mussel. Although the method proposed by Claxton et al. (1997) was effective it required the extra RFLP step and the primers were not specific to mussels.

A modified method by Claxton et al. (1998) utilized the original 710 base pair fragment created with the Folmer et al. (1994) primers to develop new PCR primers that were designed to a region that was conserved between zebra and quagga mussels. These new primers decreased the size of the PCR product to 608 base pairs, but were still unable to differentiate between zebra and quagga mussels. RFLP was still needed to determine if the organism was a zebra or quagga mussel. Today, with the decrease in the cost of gene sequencing it is possible to analyze the PCR product by sequence rather than RFLP. The PCR method in the second publication is referred to as the 'Claxton' method.

In parallel with the development of the COI primers, Frischer et al (1997) began development of primers to the 18S ribosomal gene for the detection of zebra mussels with PCR. A second publication by Frischer et al (2002) tested the 18S primers with samples from different environments.

Currently, only one lab in California uses the Claxton primers for detecting quagga and zebra larvae from raw water samples. The two Claxton publications provided an early molecular test for both zebra and quagga mussels. In these two publications there is a clear evolution of the PCR assay. In the first paper, the researchers used non-specific primers to analyze adult mussels, and by the second publication a single primer pair that targeted both the zebra and quagga mussels. While this advances the research, the researchers did not design individual PCR assays for zebra and quagga mussels.

There are locations that are populated by both adult quagga and zebra mussels, and it is important to be able to distinguish between the two mussels. Although it is possible to determine identification based on shell morphology, there is enough phenotypic plasticity in the genus that identification is not always easy or clear. Using the Folmer DNA barcoding primers and methods to identify adult mussels can increase the accuracy of reporting (Folmer et al. 1994). DNA barcoding is a PCR method that uses primers that can amplify the cytochrome oxidase gene (COI) from a wide range of organisms.

Marescaux and Doninck (2013) recently utilized the standard Folmer DNA barcode (COI) primers to analyze the populations of quagga and zebra mussels in the Meuse River, Belgium. Adult mussels were collected from the Meuse River and DNA was extracted from 241 individuals using the DNeasy Blood and Tissue Kit (Qiagen). The universal Folmer DNA barcoding primers were used to amplify a 645 base pair (bp) fragment of the COI gene. The PCR products were sent for DNA sequencing and the results were used to perform a phylogenetic analysis to determine the relationships between the adult mussels.

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The research conducted by Marescaux and Doninck (2013) indicates that the universal Folmer primers successfully amplified the COI sequence from quagga and zebra mussels. The sequencing of COI revealed seven haplotypes (a group of genes inherited from one parent) among the 241 individuals analyzed. The results also showed that there were both zebra and quagga mussels present in the Meuse River. Two of the haplotypes clustered with quagga mussel, and the other five haplotypes clustered with zebra mussels. In addition, RFLP analysis showed that there were two restriction enzymes (*Hinf I* and *Scr FI*) that could be used to differentiate the two mussel species. The authors also studied two additional dreissenid species and found that the enzymes also produced unique patterns for *D. blanci* and *D. presbensis*. The use of COI to differentiate between multiple species of *Dreissena* mussels is important because it enables scientists to use molecular methods to determine which mussel species is present in a body of water.

Detection of Dreissenid DNA in Environmental Samples

Detecting quagga mussel veligers in raw water samples adds an additional challenge to the PCR method. Some of the first research was conducted by Ram et al. (2011) who used two different genes to analyze zebra and quagga mussel veligers in the Detroit River. Raw water samples were collected using plankton tow nets, the samples were centrifuged, the liquid decanted off, and the remaining pellet was homogenized in DNAzol (a product that lyses DNA in samples). The samples were treated with proteinase K and the Promega Wizard SV Genomic DNA purification system solutions. The DNA was extracted using the Promega Wizard kit's SV minicolumns (Ram et al. 2011).

Ram et al. (2011) designed and published primers on the mitochondrial 16S RNA gene for zebra mussels and COI gene for quagga mussel, and created a multiplex PCR reaction for the detection of both mussels in the same PCR tube. Multiplex PCR is where multiple genes are analyzed in the same PCR reaction. The 16S gives an amplified product of 236 bp and the COI primers give a 417 bp amplified product. According to Ram et al. (2011) the amplified products can be differentiated on an agarose gel.

RDLES Method for Detection of Dreissenid DNA in Environmental Samples

In the original method, RDLES used the Qiagen Blood and Tissue Kit to isolate DNA. Later, an additional step was added that, where after the DNA was isolated, a reagent called Gene Releaser (BioVentures, Inc. 2008) was added to help to remove PCR inhibitors from the DNA sample. This additional step was recommended by Pisces Molecular. This extra step increased both the time needed for DNA extraction and the cost, and seemed to dilute the sample. This caused RDLES to switch to the PowerSoil DNA Isolation Kit (Mo Bio) as the soil kit was designed to remove humic acids and other PCR inhibitors.

RDLES utilizes the COI gene to screen raw water samples for the presence of quagga and zebra mussels. Originally, RDLES used the Frischer PCR primers and method that gave a 712 bp PCR product. Over time a new method was introduced to RDLES. This method was developed by Paul Rochelle at the Metropolitan Water District (MWD). In the original assay designed by MWD, 510 bp of the COI gene from both zebra and quagga mussels was analyzed. Species specific primers to the COI gene were developed that could differentiate between quagga and zebra mussels. In 2010, the MWD shortened the 510 bp COI fragment to 383 bp and the new method was adapted by RDLES. The shorter fragment is easier to amplify but is still long enough for sequencing data to be obtained. In addition to the reduction of the COI fragment, the PCR master mix was manipulated and it was discovered that a slight increase in the magnesium chloride concentration increased PCR sensitivity. Each year, RIDLES updates the standard operating procedure to include new knowledge and understanding of the PCR process.

Part II: Golden Mussel Detection by PCR

In Brazil, the golden mussel (*Limnoperna fortunei*) was first detected in 1991 in the estuary of the Rio de La Plata and has now dispersed into the largest river systems of the Plata basin (Rio de la Plata, Rio Paraná, Rio Uruguay, and Rio Paraguay) (Boeger et al. 2007). These mussels have also spread into Lago Guaiba and the Lagoa do Patos (Boeger et al. 2007). Golden mussels are being transported inland at an estimated rate of 240 km per year (Darrigran et al. 2009). The golden mussel is morphologically and functional similar to zebra mussels (Boeger et al. 2007) and are known to cause biofouling of water intakes and impact hydroelectric dams by clogging pipes. They also displace native species and enable uncommon species to proliferate (Boeger et al. 2007).

There are only two published methods about the development of PCR primers and methods for the detection of golden mussels. Pie et al. (2006) used the primers developed by Folmer et al. (1994), to amplify and sequence a 298 base pair fragment of the COI gene from golden mussels. This DNA sequence was compared to four other mussel species to identify a region unique to golden mussels. Based on the unique sequence a primer pair was designed to be specific to the invasive golden mussel. Pie et al. (2006) also amplified a universal 18S as a positive control. Sensitivity tests were performed to determine the limit of detection of the primer pair, which was found to be successful. Boeger et al. (2007) successfully used the primers developed by Pie et al. (2006) to detect golden mussel veligers in raw water samples.

The primers developed by Pie et al. (2006) have been used by RDLES to develop a standard operating procedure for the detection of golden mussel DNA from raw water samples (Keele et al. 2014). The spread of golden mussels across South America is having both ecological and economic impacts on the people and environment. It is possible that golden mussels will be transported into North

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America and it is important that RDLES is prepared to analyze water samples for golden mussel presence. RLDES has taken the time to research the golden mussel to understand and raise awareness of this potential invasive organism that could impact the waters of North America.

Part III: Genetic studies of Quagga Mussels

Microsatellite Development

Prior to the advent of PCR microsatellite analysis, allozyme variation was used to determine if quagga mussels were present in North America. Allozymes are variant forms of enzymes that are coded for on different alleles. Spidle et al. (1994) used extracts made of the whole adult mussel, to determine the mussels identity based on variant forms of an enzyme. This assay relies on differences in the enzymes rather than DNA to determine identity. Mussels with known allele frequencies were placed next to the unknown mussels as a reference. Eleven gene loci (for example the enzymes: glucose-6-phosphate isomerase, isocitrate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase) were scored to determine the genetic characteristics of the individuals being analyzed. This study determined that the white “profunda” mussel found in the deep water of Lake Erie were actually quagga mussels and were able to confirm, for the first time that quagga mussels were present in North America.

Microsatellite analysis is used by a wide range of researchers. For example, microsatellite analysis is used by criminal forensics to identify an individual and by wildlife biologists to determine relationships within a population. Wilson et al. (1999) set out to design microsatellite markers for quagga mussels to help determine their patterns of colonization and diffusion across North America. Wilson et al. (1999) designed six polymorphic tri- and tetranucleotide microsatellite markers for quagga mussels. Using the genomic DNA extraction method from Claxton et al. (1997), DNA was isolated from adult mussels and then digested with *RsaI* and *HaeIII* and ran on a 1% agarose gel. Fragments of 300-1000 bp were cut from the gel and purified using a glass milk purification method. The size selected library was constructed by ligating the DNA into the vector pZErO, which was used to transfect electro competent *Escherichia coli* top 10F1 cells by electroporation.

After the transformation, the cells were allowed to grow, and a total of 50,000 colonies were screened using the oligonucleotides AAAT₆, AAT₁₀, GATA₇, AAC₉, and AAG₈ end labeled with [γ ³³P]-ATP using standard hybridization techniques. From 50,000 colonies there were 52 positive clones that were sequenced and 10 of these clones had primer sets designed for the sequence. Of the 10 primer sets developed, six showed reproducibility and were polymorphic within 36 individuals from Lake Erie. These six primers were called: Dbug1-6. These primers did not amplify a PCR product from zebra mussels. These six primer pairs showed high variability and because the primers are species specific

they can be used as tools to determine the gene flow between populations of quagga mussels.

The microsatellite primers developed by Wilson et al. (1999), were used by Therriault et al. (2005) to determine the gene flow of quagga mussel population in the Volga River system. These researchers amplified the six microsatellite loci (Dbug 1-6) from at least 32 individuals from 13 surveyed populations. Following PCR amplification, the DNA fragments were sized and analyzed for phylogenetic relationships. There were 179 alleles observed from the 6 loci that were studied. The data from this publication suggests that the surveyed populations are part of a larger metapopulation that is maintained by high gene flow. This suggests that there are multiple colonization events between the original population and sites where quagga mussels have invaded.

Additional tests for microsatellite loci have been designed. Feldheim et al. (2011) designed 14 new polymorphic microsatellite loci (8 were for zebra mussel and 6 were for quagga mussel). The new markers were compared to the Dbug 1-6 markers and were found to improve the resolution of the genetic diversity of quagga mussel populations.

There have been several studies using microsatellite loci to resolve the population dynamics, gene flow, and genetic diversity of the adult quagga mussel in populations in Europe and North America. The use of this technology has helped researchers to start to understand the population structure of quagga mussels. If there are multiple introductions of quagga mussels into a location, then determining the population structure of the adults will allow researchers to estimate the number of introductions that have taken place.

Summary

Currently, there is no standard method for the extraction or PCR reaction of quagga and zebra mussel DNA. There is also no standard method for the analysis of adult monotypic tissues. The laboratories doing these analyses are all using the COI gene, but the primers are not the same. There are currently three different primers being used: Claxton primers, MWD primers, and unpublished primers. This means that comparing results from one lab to another can be difficult. It is possible for one laboratory to get positive results while another is getting negative results from the same water sample or DNA extract. This does not mean that one lab is wrong and the other right. Differences in the laboratories DNA extraction methods and PCR master mix could lead to differences in the sensitivity of the PCR assay. The discrepancy could be due to the lottery of getting zebra or quagga mussel DNA into the extraction, and then into the PCR reaction. These divergent results are a major reason that RDLES continues to research and optimize extraction and PCR methods for the detection of invasive mussels.

RDLES Organization and Sample Handling

When conducting PCR analysis for the early detection of quagga and zebra mussels it is important to maintain good lab practices and standard operating procedures (SOP). Every year RDLES updates the PCR SOP to reflect new knowledge. Maintaining good laboratory practices and hygiene is critical for decreasing the risk of DNA cross contamination and false positives. RDLES has laboratory quality assurance/quality control (QA/QC) standards that help confirm positive results are not caused by laboratory contamination.

Lab hygiene is maintained by creating dedicated areas for DNA extraction, PCR set up, and gel analysis (Figure 2). This laboratory design ensures that the amplified PCR product is never in an area where DNA is being extracted or the PCR assay is being set up. In each area there are dedicated pipettes, sterile filter tips, consumable plastics, and reagents for each step of the analysis process. During all of these processes nitrile gloves are worn and changed on a regular basis.

In the DNA isolation room, commercial DNA extraction kits are used to extract DNA from complex raw water samples. All extractions are performed in the safety hood in the DNA prep area. Occasionally, known negative samples (DI water or soil) are analyzed to check for cross contamination within the DNA extraction and PCR analysis.

In the PCR preparation room there is a biological safety hood with a UV lamp where all PCR reactions are prepared. Aliquots are made of all reagents (10X buffer, primers, dNTPs, water, etc.) required for PCR. This ensures that if one of the reagents becomes contaminated the master stocks have not also been contaminated. Creating aliquots also allows for disposal if the control PCR reactions start to fail, or if the reagent becomes degraded.

In the gel preparation area, gels are run and analyzed. Every gel is loaded the same way to prevent confusion: the first (lane 1) and last (lane 16) lanes contain the DNA ladder, lanes 14 and 15 contain the negative (PCR master mix without DNA added) and positive control (adult mussel DNA), and lanes 2 through 13 contain the PCR products from the raw water samples.

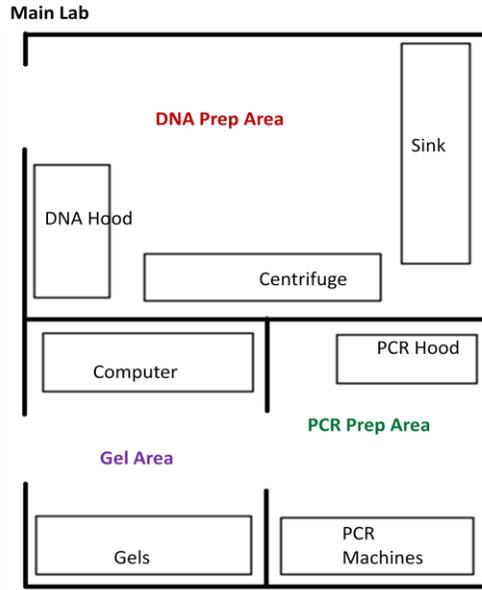


Figure 2: Map of the separate PCR areas of the RDLES lab.

After the PCR products are analyzed by gel electrophoresis, UV light is used to detect any bands present in the gel. Table 1 shows all of the possible outcomes associated with the PCR analysis. The RDLES QA/QC states that if either control fails the PCR reactions for the whole sample set have to be repeated. The ideal PCR outcome occurs when the positive and negative controls pass QA/QC (Figure 3).

Table 1: Possible outcomes of gel electrophoresis analysis

Positive Control	Positive	Negative	Positive
Negative Control	Negative	Negative	Positive
Outcome	Passes	Fails	Fails

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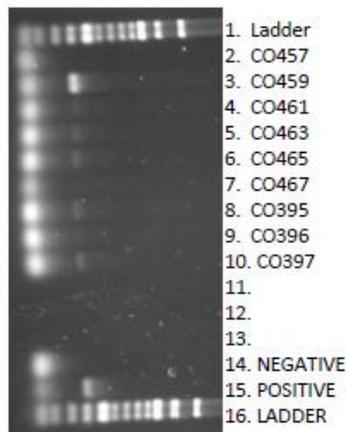


Figure 3: Ideal outcome for gel analysis. The negative control does not have any bands present in it, and the positive control has a single band of the correct size present. This indicates that both of the controls are working correctly. In addition, the ladder is clear and easy to read.

All laboratory areas are cleaned and decontaminated on a weekly basis. The amount of cleaning increases as the number of samples analyzed increases in order to decrease the risk of cross contamination. The DNA extraction hood, centrifuges, PCR set up hood, and gels areas are cleaned with 5% bleach. In addition, the PCR hood contains a UV light that is used for decontamination of the hood prior to setting up PCR reactions.

Assessment of Lab Contamination: Wipe Tests

Laboratory wipe tests are performed on a monthly basis to detect ambient quagga mussel DNA that could potentially contaminate water and DNA samples. This assay is done only for quagga mussel DNA because the majority of samples seen by RDLES contain quagga mussels. To perform this assay, Q-tips are used to take wipe samples of the microscopy area, DNA extraction hood, centrifuge, and PCR set up hood. Two wipe samples are taken from each area. One wipe test is directly analyzed for the presence of ambient DNA, and the second wipe test undergoes DNA extraction to detect the presence of mussel tissue or cells. RDLES has shown the effectiveness of using cotton swabs for wipe tests as it is possible to take a cotton swab, dip it into water containing quagga mussel veligers, and obtain a positive PCR result.

The monthly wipe tests indicate that quagga mussel DNA is not present in the areas analyzed and the availability of DNA and cells that could cross contaminate a sample is very low. These tests along with good lab practices help to ensure that when a positive sample is found it is a true positive and not the result of cross contamination from the laboratory.

Effectiveness of Laboratory Decontamination

RDLES uses Imhoff cones to settle raw water samples to reduce sample analysis time. Vinegar (5% acetic acid) and bleach are used to decontaminate the settling cones, but the effectiveness of this decontamination method had not been tested. One concern was that a water sample from an infested body of water would cause a false-positive in a negative water sample settled in the same cone if not properly decontaminated. The following study was designed to test the effectiveness of acetic acid and bleach at Imhoff cone decontamination.

Experimental Design

Three replicates of fifty veligers were exposed to acetic acid or bleach in Petri dishes for 5, 10, 15, and 30 minutes. The DNA from each treatment was isolated using the soil kit. The DNA was then analyzed by PCR for the quagga mussel COI gene. The resulting PCR products were analyzed by gel electrophoresis and scored for positive or negative outcome.

Results and Discussion

After 5 minutes, 67% of control replicates, which were not exposed to acetic acid or bleach, were positive, and after 10, 15, and 30 minutes 100% of control samples were PCR positive (Figure 4). All samples exposed to bleach were negative except for one sample that was positive after 10 minutes. All samples exposed to acetic acid were negative except for one sample at 30 minutes. It is important to note that these samples had the acetic acid or bleach added directly to them, and the samples were not shaken. When settling cones are cleaned a brush is used to scrub the cone. The positives in the acetic acid and bleach samples could be caused by tissue that is somehow protected from the degradation. Testing has shown that a combination of bleach and acetic acid is best for degrading the veliger DNA.

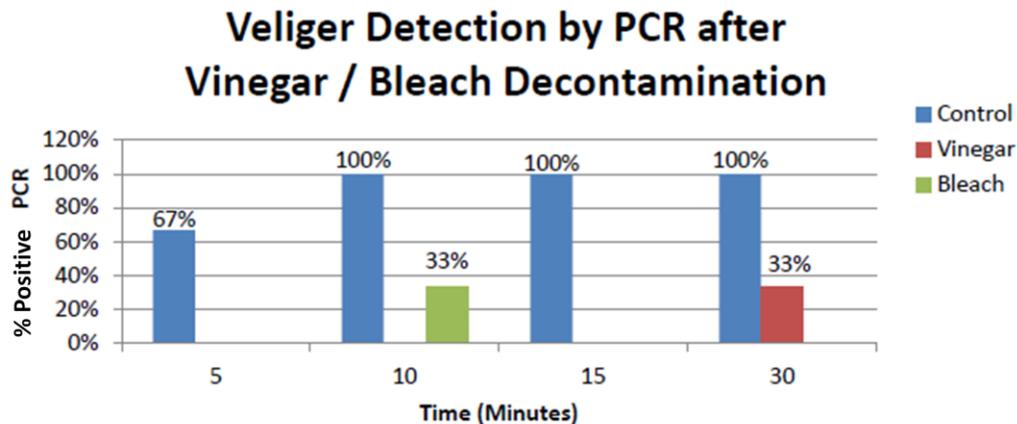


Figure 4: Percentage of positive PCR results from samples containing 50 veligers (3 replicates) in acetic acid and bleach for 5, 10, 15, and 30 min.

Preserving and Analyzing Complex Samples

The PCR samples analyzed at RDLES are collected using a 64- μ m plankton tow net, and they contain a variety of DNA. Samples come from reservoirs across the western United States, each with unique water conditions and chemistries. These samples are centrifuged to create a pellet and the organic and inorganic makeup of the sample influences the consistency of the pellet. Some pellets are high in algae and organic materials, while others are low in organic material and high in inorganic materials. One of the challenges in performing early detection of mussels has been to address the issues that arise when analyzing complex environmental samples. These challenges led to the series of studies described below.

Humic Acid Studies

Inhibitors to the PCR process are common in environmental samples. The major environmental inhibitor is humic acid, which are organic compounds that are the major component in soils (Matheson et al. 2010). These substances are created by degraded plants, and make up 5-7.63 mg/g of soil depending on the soil type (Matheson et al. 2010). Humic acid has been shown to inhibit Taq polymerase activity and template inhibition by restricting the primers from binding to the DNA template (Matheson et al. 2010). Overcoming this contamination is important if the PCR process is to work correctly.

Experimental Design

The goal of the first humic acid study was to determine how humic acid directly inhibits the PCR process. Humic acid was spiked directly into DNA extracts after the DNA had been extracted using the Power Soil kit and PCR was performed to test the impact of humic acid on the PCR outcome. Three stocks of humic acid were made at 0.1, 1, and 10 $\mu\text{g}/\text{mL}$. Adult quagga mussel DNA was added to each humic acid stock to make a 1:250 dilution of DNA. Three replicates of each stock were analyzed by PCR for the presence of quagga mussel COI.

Results and Discussion

COI was detected in samples without humic acid and in the 0.1 $\mu\text{g}/\text{mL}$ humic acid solution. COI was not detected in the 1 or 10 $\mu\text{g}/\text{mL}$ humic acid solutions (Figure 5). When there is no removal of humic acid from the DNA sample, the PCR assay can overcome 0.1 $\mu\text{g}/\text{mL}$ humic acid but once the humic acid levels are up to 1 $\mu\text{g}/\text{mL}$ concentration the PCR reaction is inhibited. This experiment did not evaluate the DNA extraction kits ability to remove humic acid.

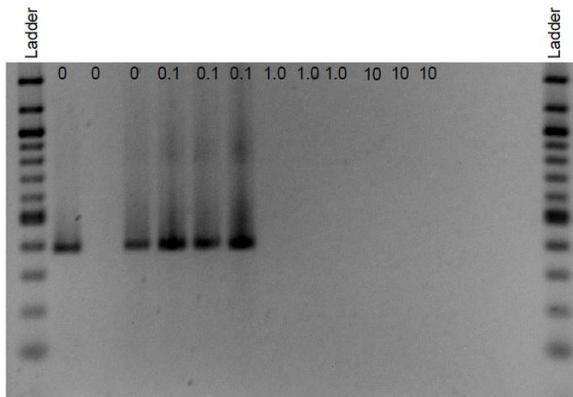


Figure 5: Titration of humic acid (0, 0.1, 1, and 10 $\mu\text{g}/\text{mL}$) directly into quagga mussel DNA. Each PCR reaction was performed in triplicate. Positive PCR results were obtained for the 0 and 0.1 $\mu\text{g}/\text{mL}$ titrations of humic acid. Increased concentrations (1.0 and 10 $\mu\text{g}/\text{mL}$) of humic acid inhibited the PCR reaction.

DNA Extraction Kits Ability to Overcome Humic Acid

Experimental Design

The goal of the second humic acid study was to test the performance of two DNA extraction kits with varying amounts of humic acid added to the DNA extract. To test the Power Soil Kit, 10 veligers were analyzed with either 250 μL of DI water as a positive control or humic acid at three concentrations (0.1, 1, 10 $\mu\text{g}/\text{mL}$). Each treatment was performed in triplicate. The DNA was extracted as per the manufactures instructions. To test the Qiagen kit 10 veligers were added to a 1.5-mL eppendorf tube with 250 μL of DI water or humic acid at one of three

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concentrations (0.1, 1, 10 $\mu\text{g}/\text{mL}$) and 5.5 μL of proteinase K and 50 μL of ATL buffer. Each treatment was also tested in triplicate. The samples were incubated at 56°C for 4 hours with frequent vortexing. The DNA extraction was then carried out according to the manufacturer's instructions. Three replicates of each treatment were tested.

Following the DNA extractions, 10 μL of DNA from the blood and tissue kit was added to 60 μL of Gene Releaser, heated at 85°C for 20 minutes, and centrifuged for 2 minutes at 10,000 rpm. This additional step was added to the blood and tissue DNA extraction process to determine the effectiveness of Gene Releaser at removing humic acids. PCR was then performed on the samples for the detection of quagga mussel COI following the RDLES SOP. Gel electrophoresis was used to determine the presence or absence of an amplified PCR product.

Results and Discussion

Each sample was analyzed for the presence or absence of a positive band. The soil kit produced positive PCR results for the majority of the samples analyzed (Figure 6). It was able to overcome a wide range of humic acid (from 0.1 $\mu\text{g}/\text{mL}$ to 10 $\mu\text{g}/\text{mL}$). The blood and tissue kit (Qiagen) did not perform as well as the soil kit (Figure 6). Across the range of humic acid concentrations the Qiagen kit only produced positive PCR results approximately 50% of the time. Even when the humic acid was not present, the Qiagen kit only produced 40% positive PCR reactions. The addition of gene releaser to the Qiagen kit did not increase DNA detection. It is important to note that the soil kit was designed to remove humic acid contamination. In order to prevent false negative results it is important to select the appropriate DNA extraction kit, especially if humic acid contamination is a concern.

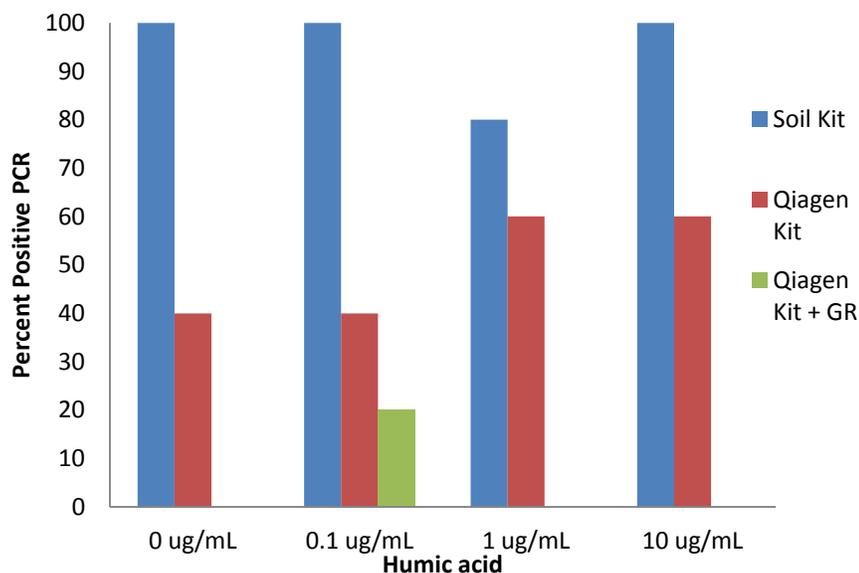


Figure 6: Three different DNA extraction methods (Soil Kit and Qiagen kit with and without Gene Releaser) were performed with 0, 0.1, 1, and 10 $\mu\text{g/mL}$ of humic acid with each sample containing 10 veligers. Following the DNA isolation, PCR was performed to assess how well each kit overcame the presence of humic acid. The soil kit outperformed the Qiagen kit.

Impact of Sample Preservation on DNA Detection

There has been a major effort at RDLES to understand the impact of sample preservation on sample integrity and detection. Over the last two years three major studies have been completed. The first study examined how pH (acidic or neutral) and alcohol concentration (0, 20, 50, and 70%) affect veliger shell and DNA integrity (Carmon et al. 2014a). This study showed that un-buffered samples lost birefringence, but were still detectable by PCR. The second study analyzed how the presence of organic inhibitors (zooplankton) in raw water affects veliger shell and DNA integrity when veligers are preserved with buffered vs. not buffered and with 0% or 20% alcohol (Pucherelli et al. 2014). This study showed that the addition of organic inhibitors affected the microscopy and PCR results over time. The third study assessed the impacts of veliger condition (degraded, broken, free-floating DNA) on PCR detection (Carmon et al. 2014b). This study showed that free floating DNA could be detected in a sample. All three of these studies have helped RDLES to understand the importance of proper sample preservation on maintaining veliger morphology and detectability by PCR.

Impact of Preservation with Different Alcohol Types on PCR

Sample preservation is important for maintaining veliger morphology and DNA. It is unknown if the type of alcohol used to preserve the raw water sample has an impact on the PCR result. To determine if the type of alcohol used to preserve the

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sample impacts the PCR outcome four different alcohols were tested: Everclear, reagent grade alcohol, denatured alcohol and iso-propanol.

Experimental Design

Tests to determine if alcohol type influences PCR results were set up by preserving water samples collected from Lake Mead, NV with 20% Everclear, reagent grade alcohol, denatured alcohol, or iso-propanol, and all were buffered. One replicate of each sample was prepared and analyzed at the time of arrival in the lab (time point 0) and at time points 2, 3, and 9 weeks. Half of the samples were held at room temperature and half were refrigerated over the time course. At each time point, DNA was isolated from the sample and analyzed for the presence of the quagga mussel COI gene by PCR as per the RDLES PCR SOP (Keele et al. 2013).

Results and Discussion

The type of alcohol used to preserve the sample did not affect the PCR outcome. In addition, positive PCR results were obtained for all of the samples 9 weeks after collection (Figure 7 and 8). Samples stored at room temperature and at 4°C were both positive overtime. While there is some variation in the intensity of the PCR bands on the gels, the number of quagga mussel veligers in each sample was not standardized across all samples which could account for the variation in band intensity. This study did not test the effects of alcohol on samples containing few numbers of veligers.

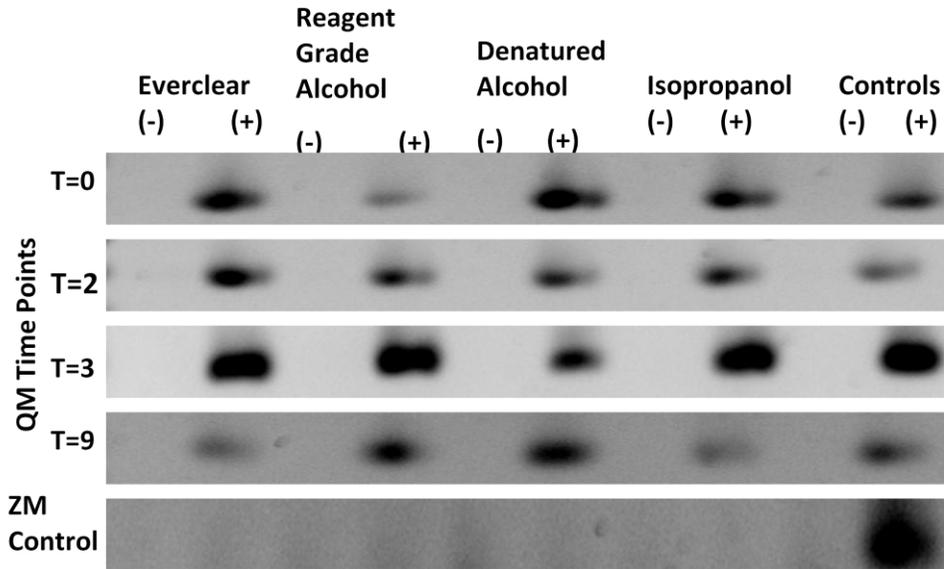


Figure 7: Gel showing quagga mussel positive bands of DNA from water samples from Lake Mead that were collected and stored in Everclear, reagent grade alcohol, denatured alcohol, and isopropanol at room temperature. DNA was isolated from the samples at t=0, 1, 2, 3, 9 weeks after collection, and then assessed by PCR for COI.

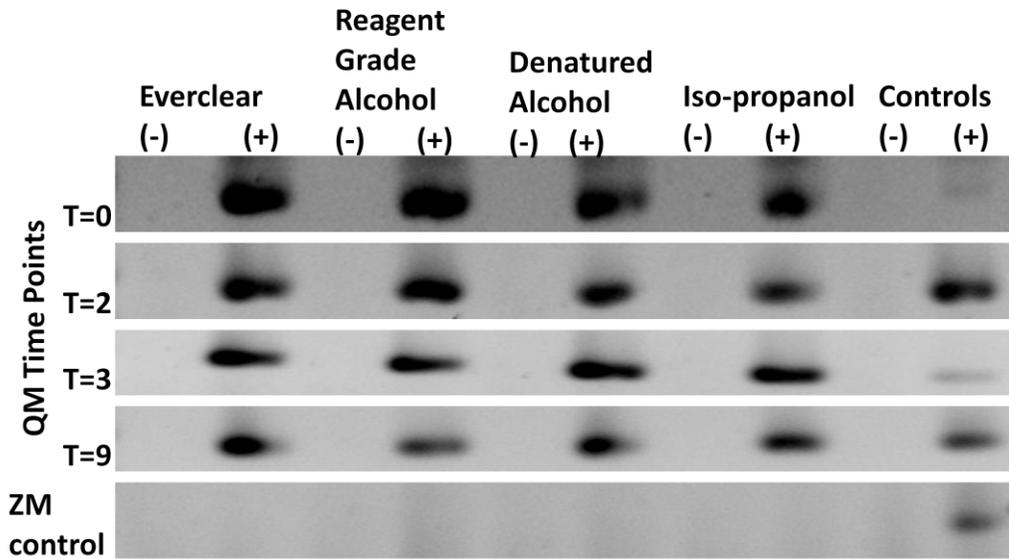


Figure 8: Gel showing quagga mussel positive bands for water samples from Lake Mead that were collected and stored in Everclear, reagent grade alcohol, denatured alcohol, and isopropanol at 4°C. The DNA was isolated from the samples at t=0, 2, 3, 9 weeks after collection, and then assessed by PCR.

PCR Method Optimization

Improving and making the PCR assay more sensitive for dreissenid mussels has been a major focus at RDLES. Optimization studies have been conducted to improve the following processes: DNA extraction, master mix optimization, comparison of DNA extraction kits, primer specificity, nested primers for quagga mussel COI, and gel electrophoresis. Unless otherwise noted all DNA extractions and PCR analysis was carried out using the RDLES PCR Laboratory SOP, version 4 (Keele et al. 2013).

PCR Master Mix Optimization: Magnesium Chloride

During PCR, magnesium chloride ($MgCl_2$) binds to the Taq polymerase enabling enzyme activity. Without $MgCl_2$ the PCR reaction would fail. Determining the optimal $MgCl_2$ concentration is important because having too little $MgCl_2$ inhibits the PCR reaction, while having too much $MgCl_2$ can cause non-specific PCR bands to be produced. In an effort to determine the optimal $MgCl_2$ concentration a series of dilution curves of quagga and zebra mussel DNA were created to determine the lowest concentration of DNA that would give a positive PCR result.

Experimental Design

The concentration of both adult quagga and zebra mussel DNA was determined by measuring the optical density (OD). The OD is measured with a spectrophotometer and the ratio of the reading at the wavelengths 260/280 is used to determine DNA concentrations. Serial dilutions of known concentrations were made for both quagga and zebra mussel DNA (starting at 80 ng/ μ L and going down to 0.04 ng/ μ L). Three different concentrations of $MgCl_2$ were used (2, 3, and 4 mM) in the PCR master mix. The PCR master mix already contains 1.5 mM $MgCl_2$ from the 10X buffer that is added. After the PCR analysis the resulting dilution curves were analyzed.

Results and Discussion

The original SOP for the detection of quagga mussel COI called for 2 mM of $MgCl_2$ to be added to the PCR reaction. By increasing the amount of $MgCl_2$ in the quagga mussel PCR reaction (from 2 to 3 mM) there is a several fold increase in the sensitivity of the PCR assay (Figure 9). The amount of $MgCl_2$ used in the SOP was increased to 3 mM improve the detection of quagga and zebra mussels. Increasing the $MgCl_2$ to much can lead to non-specific bands in the PCR outcome; this is why the concentration was not increased to 4 mM. Performing periodic checks of the PCR reagents to make sure that the optimal concentrations are being used is important to ensure the best PCR performance.

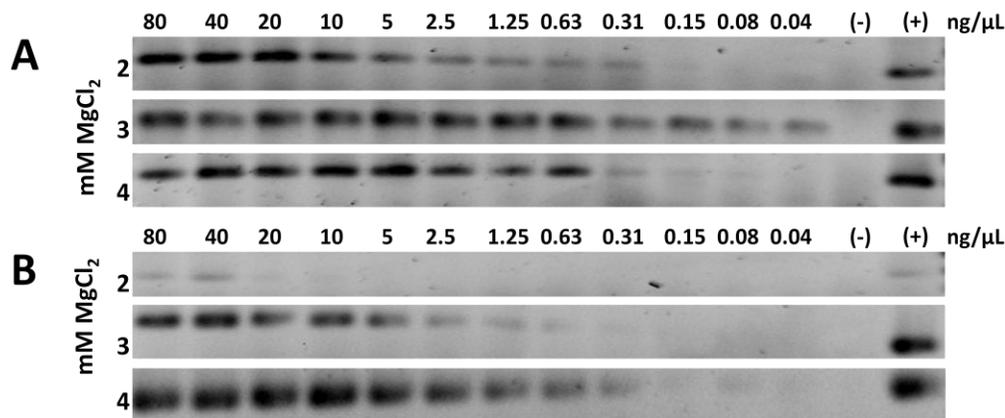


Figure 9: Dilution curves of COI PCR product from adult Zebra (ZM) (A) and Quagga (QM) (B) (ng/μL) using three different concentrations of magnesium chloride (2, 3, and 4 mM).

Comparison of Blood/Tissue and Soil Kits

The most important step in the analysis of water samples for quagga DNA is the DNA extraction. If this step fails the PCR will also fail. In 2012 RDLES changed to the MolBio soil kit. The main difference between this kit and the Qiagen blood and tissue kit that was originally used is the presence of a bead shaking step. The soil kit is designed to remove the inhibiting chemicals from the environment that interfere with PCR, such as humic acids. This new kit gives cleaner and more constant results. Initially, in addition to the DNA extraction step, Gene Releaser was also used to remove interfering compounds from the DNA extraction. To test the evidence that was building about the different kits and the addition of Gene Releaser each extraction process was tested to determine best performance.

Experimental Design

DNA was isolated using both the blood/tissue kit and the soil kit to test the limits of PCR detection. The samples used for this experiment were plankton tow samples from Lake Mead preserved as per the RDLES Field SOP (Carmon et al. 2013). The OD_{260/280} of these DNA extracts was determined, the concentrations of the DNA extracts were then calculated, and all of the samples were diluted to a concentration of 40 μg/μL. Gene releaser was used with both kits to determine the effects of this additional step. The DNA in four treatments was diluted in a serial dilution, and then analyzed by PCR to determine the detection limit with each extraction method. The original PCR SOP including the 2 mM MgCl₂ addition was used in this study.

The next experiment tested each extraction kit with and without Gene Releaser and two different MgCl₂ concentrations. The MgCl₂ concentrations used were derived from the original SOP for PCR (2 mM) and the new PCR method SOP (3 mM). For each DNA extraction method (Qiagen or soil kits), eighteen DNA

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isolations were done using Lake Mead plankton tow samples. After the DNA extractions were completed, Gene Releaser was used on 10 μ L of each of the extracts so that each sample was tested with and without Gene Releaser.

Results

The dilution curves comparing the two kits showed that the soil kit had a lower detection limit than the blood/tissue kit (Figure 10). This experiment also showed that the use of Gene Releaser did not increase the detection limits for either kit. Gene Releaser dilutes the DNA sample six-fold when it is used. This reagent is used with 10 μ L of DNA and 60 μ L of Gene Releaser. The addition of this reagent diluted the sample six fold and thus the number of copies of the COI gene that got into the PCR reaction was decreased.

The second experiment showed that samples containing a lot of mussel DNA (Lake Mead samples contain a lot of veligers) are not impacted by the different $MgCl_2$ concentrations (Figure 11). This experiment also showed that the blood/tissue kit required the use of Gene Releaser to give a positive PCR result. The soil kit was able to achieve 100% positive PCR without the use of Gene Releaser. This finding has prompted RDLES to update the PCR SOP to include use of the soil kit without Gene Releaser.

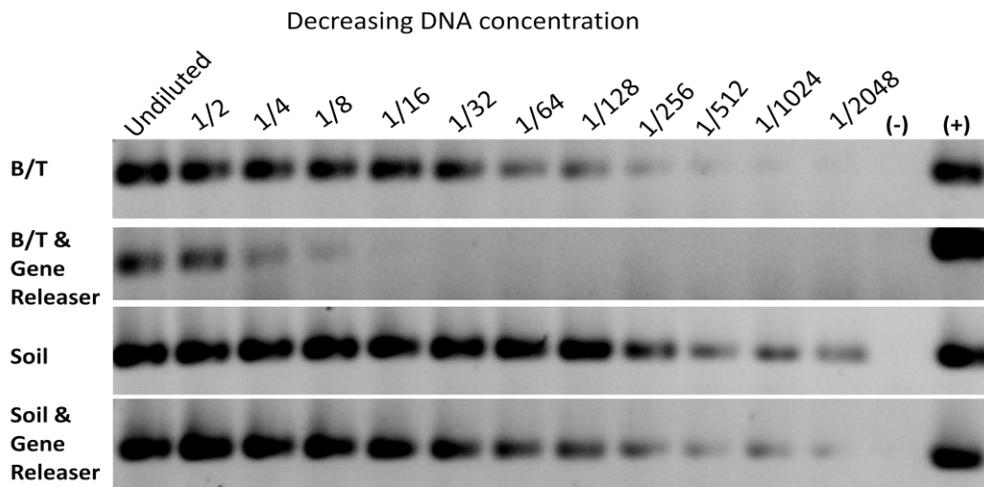


Figure 10: Gel containing PCR results of dilution curves from the total DNA isolated using the Blood and Tissue Kit (B/T) and Soil Kit with and without Gene Releaser. Visible bands on the gel indicate a positive PCR result.

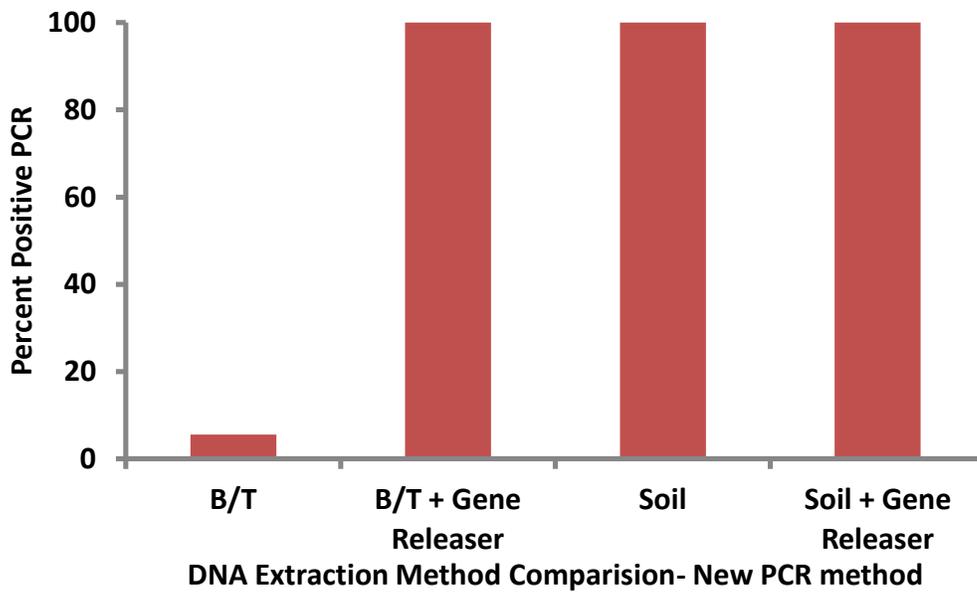
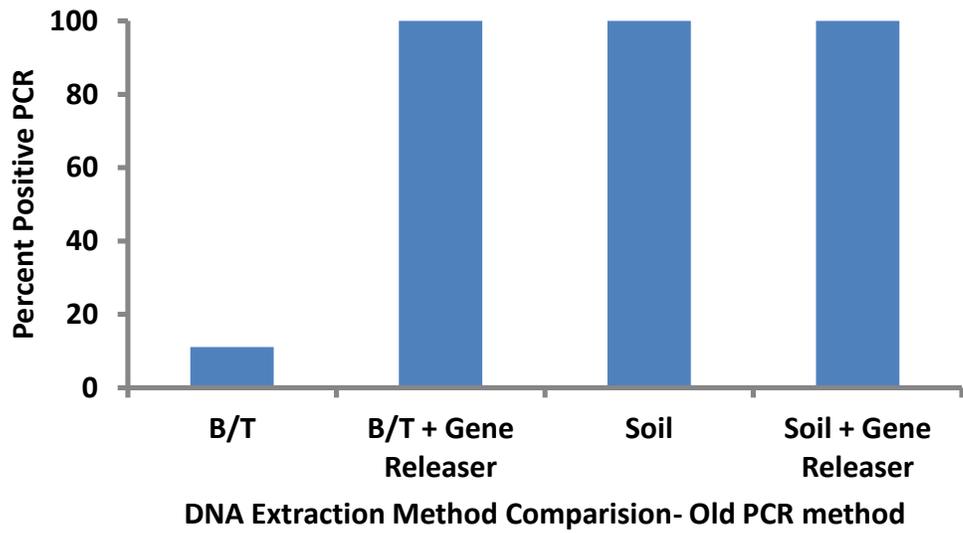


Figure 11: Percent of PCR positives from samples (18 samples each) extracted with the blood/tissue kit and soil kit with and without gene releaser.

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Primer Specificity for Quagga and Zebra Mussel

Experimental Design

The specificity of quagga and zebra mussel primers is important because cross reactivity could lead to the misidentification of the organisms in a sample. Two different experiments were performed to test this specificity. First, DNA from adult quagga and zebra mussels was isolated, and diluted to 1:100, 1:200, and 1:400 and analyzed by PCR. The DNA from both organisms was tested against the primers for both organisms. Secondly, DNA was isolated; using the soil kit, from different body parts of adult quagga and zebra mussels to determine what part of the adult could produce a positive PCR result. These parts include the mussel tendon, bisset threads, mussel gut, crushed whole adults, and crushed dried adults. In addition, quagga mussel veligers were tested. The goal of this experiment was to determine what parts of an adult mussel could give a positive PCR result.

Results

The first experiment showed that the primers for both quagga and zebra mussel do not cross react (Figure 12). This is important because it shows that it is possible to determine which dreissenid species is present in a raw water sample.

The second experiment shows that different parts of the adult mussel can produce positive PCR results (Figure 13). The only part of the mussel that did not give a positive PCR result was the bisset threads. This is not surprising because these threads are made of protein. There were several organisms whose identity could not be visually confirmed, these samples have a “?” beside their label in Figure 13.

There were several samples that were presumed to be from zebra mussels however, the PCR identified the samples as coming from quagga mussels. This means that the sample was misidentified. These results show that the PCR primers are specific and it is possible to differentiate between the two different mussel species.

Finally, the crushed whole adult and the dried whole adult quagga mussel samples both gave positive PCR results. These results show that it is possible to get positive PCR signals from single crushed whole quagga mussel adults that had been either been preserved in ethanol or dried. This is important because it shows that a closed mussel that has been dried still contains tissue with viable DNA.

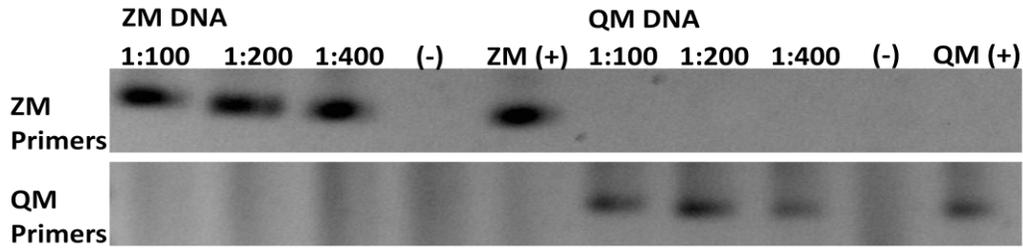


Figure 12: Gel showing primer specificity of zebra mussel (ZM) and quagga mussel (QM) primers. The primers do not show cross reactivity. The presence of a bar on the gel indicates a positive PCR result.

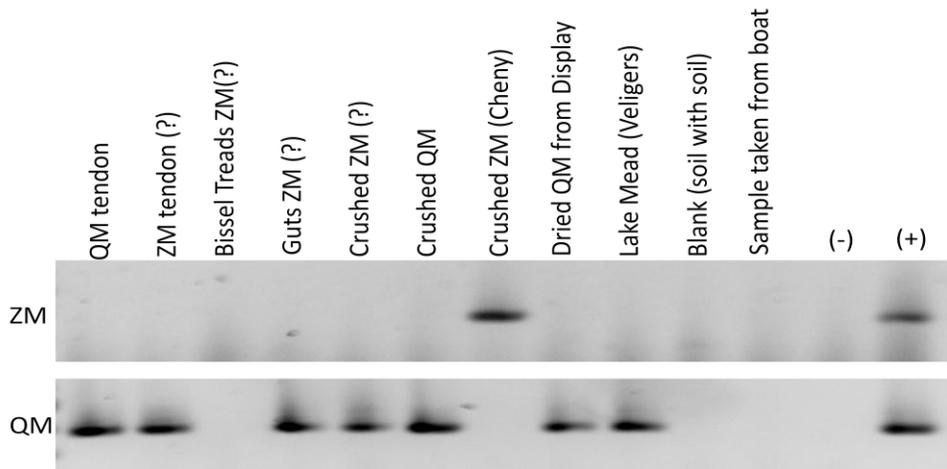


Figure 4: Gel showing PCR sample results tested with both quagga (QM) and zebra mussel (ZM) specific primers.

Quagga Mussel COI Nested PCR Primers

One of the major issues with early detection PCR is obtaining enough DNA for sequencing. One way to overcome this issue is to use nested PCR primers. With nested primers, a PCR product with a weak, non-sequencable band is re-amplified with primers that are internal to the original PCR primers. The primers are used by taking the original amplified PCR product and performing a second PCR reaction with the nested primers. This leads to a stronger PCR product signal that can be used for DNA sequencing. Figure 14 shows a diagram of how the nested primers work. In addition, the sequence from these bands are long enough to ensure that when analyzed with NCBI Blast there is enough sequence information to allow for an identification. The nested primer pair amplifies a ~250 bp fragment of the COI gene.

The nested primers were developed and are now included in the SOP (Keele et al. 2013). RDLES uses these nested primers to amplify enough DNA for sequencing when not enough DNA is provided in the original COI sequence. Nested primers were used four times in 2013 to obtain enough PCR amplicon to get good sequencing results.

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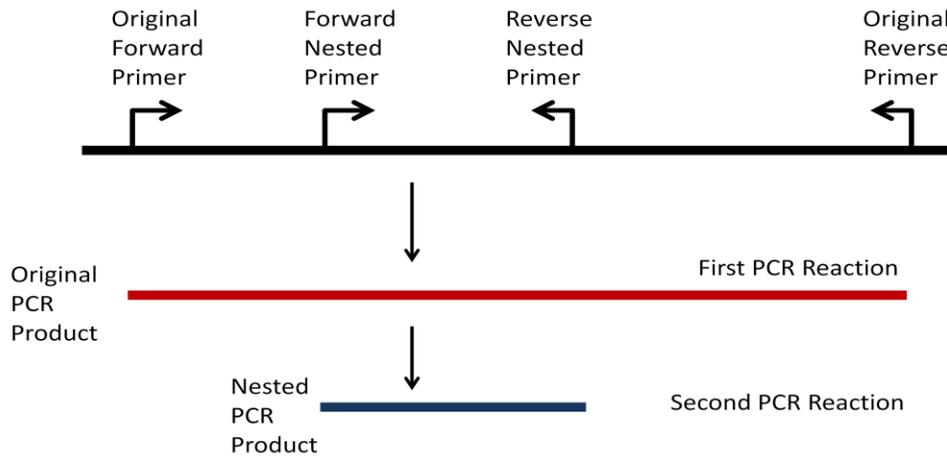


Figure 14: Diagram of Nested Primer

Gel Electrophoresis Optimization

Gel electrophoresis is the separation of DNA fragments on an agarose gel by electrical current. PCR product samples are loaded into wells on the gel, a current is applied, the PCR products separate by size, and GelStar is used to stain the gels. This is the method that RDLES uses to analyze the PCR products of quagga and zebra mussel detection as per the SOP. The intensity of the band on the gel can indicate if the sequencing will work or not. More intense bands show that there is more PCR product present in the PCR reaction. Weaker bands indicate that there is less PCR product present and thus the sequencing reaction might not be successful. Positive PCR products are sent to a commercial DNA sequencing company for analysis. This company checks the PCR products on an agarose gel and they use ethidium bromide (EtBr) to stain their gels.

Experimental Design

This experiment was performed to address multiple failed sequencing reactions that occurred at RDLES. A simple experiment was devised to compare the effectiveness of Gel Star (the stain that is used per the RDLES SOP) and EtBr (the stain that the commercial sequencing company uses). For this experiment, DNA was extracted from ten replicates of 10 veligers each as per the RDLES PCR SOP. Following the PCR amplification of the quagga mussel COI gene, the PCR product was loaded onto two separate gels. One gel was stained with Gel Star and the other with EtBr. For the gel stained with Gel Star, 5 μ L of Gel Star was added to 40 mL of TAE buffer and pored over the gel. The gel was stained for 30 minutes and the bands were visualized using the Gel Logic 200 Imaging System. Prior to pouring the second gel, 10 mg/mL of EtBr was added to the melted agarose. After the samples were ran on this gel, it was analyzed immediately with the Gel Imaging System.

Results and Discussion

The results show that Gel Star is able to stain a lower concentration of DNA than EtBr (Figure 15). The EtBr stain was able to detect the DNA but the bands look fainter and less robust. Based on these results it is likely that these samples would fail the QA/QC at the sequencing company and would require more DNA. Even though Gel Star makes the bands look very bright, there might not be enough DNA for sequencing in the reaction. The strength of the band helps to determine if the PCR product can be sequenced. Because of the issue, RDLES works directly with a scientist at the sequencing company to ensure that there is enough DNA for sequencing.

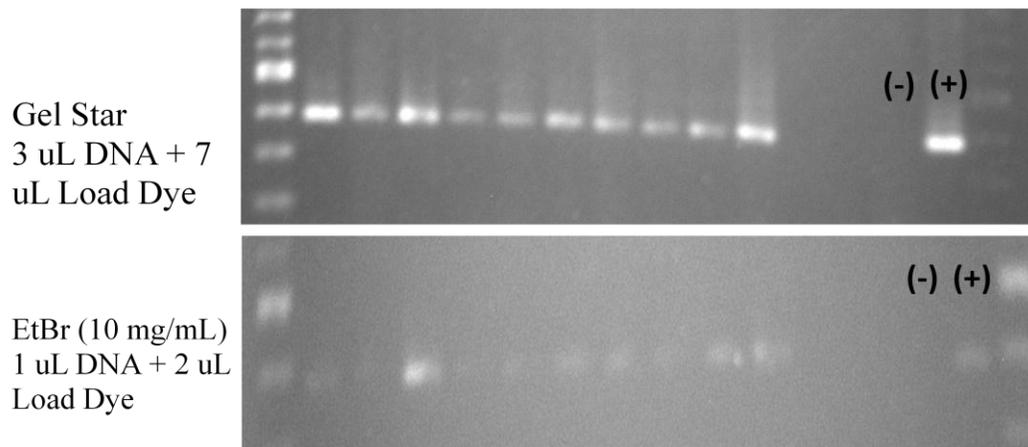


Figure 5: Comparison of Gel Star and EtBr staining.

Golden Mussel DNA Analysis

Limnoperna fortunei (golden mussel) are invasive bivalves from Asia that have caused both economic and environmental damage in South America. In 2013, RDLES worked with Brazilian researchers to create a PCR method for the detection of golden mussels in raw water samples. Published primers (Pie et al. 2006), and a primer developed by RDLES were tested to determine their detection limits and their specificity to golden mussel (Table 2). Both primer pairs are to the COI gene of golden mussel. To test the PCR primers three different experiments were performed: temperature gradient of the annealing temperature, dilution curves to determine limits of detection, and finally primer specificity were tested. Samples from Brazil were also analyzed to determine how well the PCR assay performs.

Methods

DNA from adult golden mussels was isolated and used in all of the experiments. The first experiment was designed to ensure the PCR program would amplify the target gene. To determine the best annealing temperature, a gradient from 57-62°

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was used in the PCR program. The PCR master mix and program used of quagga and zebra mussel detection was used as the starting place in optimizing the golden mussel PCR assay.

Next, dilution curves of golden mussel DNA were used to test the detection limits and specificity of the primers and assay. A serial dilution of golden mussel DNA was made and the PCR assay was performed. Included in this assay was quagga and zebra mussel DNA to determine if the golden mussel primers would be cross reactive with their DNA.

Finally, 20 samples containing known numbers of golden mussels, sent by Brazilian researchers, were analyzed to test the performance of the RDLES lab. Before each sample was analyzed by PCR they were first analyzed by microscopy to check for the presence of golden mussel veligers. The DNA extraction method and PCR assay used in this study are described in the RDLES Golden Mussel SOP (Keele et al. 2014). Any positive samples were sent for DNA sequencing to confirm that the DNA was from a golden mussel.

Table 2: Primers used to detect golden mussels. Set 342/343 was designed by RLDES, and Set 344/345 was designed by Pie et al. (2006).

Primer Name	Primer Sequence	PCR Product Size
342 F	AGGGACTGGTTGGACAGTTTATCC	250
343 R	ACCACCTAGAACTGGTAGGGAAAC	
344 F	TTTAGAGTTAGCACGTCCTGGTAGGTT	300
345 R	TCCAACCAGTCCCTACTCCACCCTCTA	

Results and Discussion

The temperature gradient showed that the golden mussel primers functioned at the same conditions as the quagga and zebra mussel COI PCR program and master mixes (Figure 16). All of the different annealing temperatures produced positive PCR results of the correct base pair size (Set 1: 250 bp and Set 2: 300 bp). The annealing temperature used in the COI quagga mussel PCR program is 59°, and this study indicates that the same temperature can be used for the golden mussel, allowing RDLES to be able to run all three assays (GM, ZM, and QM) using the same PCR programs.

The test of primer specificity showed that the golden mussel primers were specific and did not cross react with quagga or zebra mussel DNA. The dilution curve of golden mussel DNA showed that the PCR assay for both primer sets can detect a wide range of DNA concentrations (Figures 17 and 18).

The final test was to analyze a set of samples sent to RLDES by Brazilian researchers. Of the 20 samples analyzed only 2 samples did not pass the test. These samples gave a false positive result with the golden mussel primers. The

false positive results could have resulted from sample contamination, because some of the samples were leaking when they arrived at RDLES. So it is possible that cross contamination occurred in the transport of the samples to the laboratory. It is important to note that RDLES was successful at completing the round robin test that the Brazilians sent.

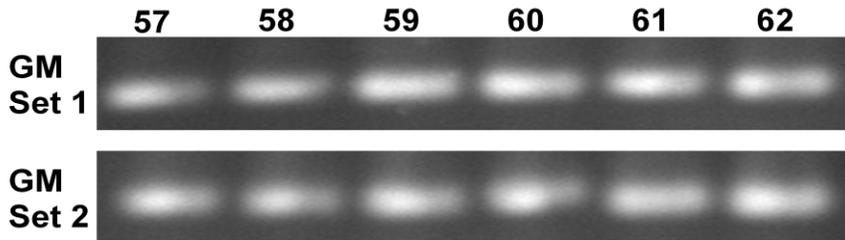


Figure 16: Temperature gradient of the two different golden mussel primers (Set 1=RDLES and Set 2=Pie et al.). The positive bands show that the primers are able to work across six different annealing temperature (57°-62°C).

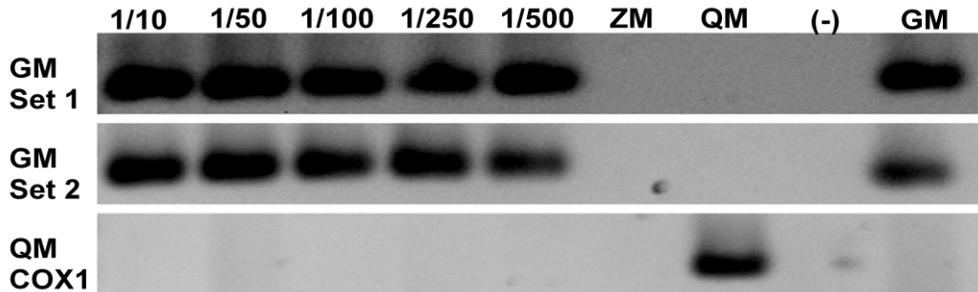


Figure 5: Dilution curve of golden mussel (GM) DNA concentration from two different golden mussel primers (Set 1=RDLES and Set 2=Pie et al.). Zebra (ZM) and quagga mussel (QM) DNA was not cross reactive with GM primers.

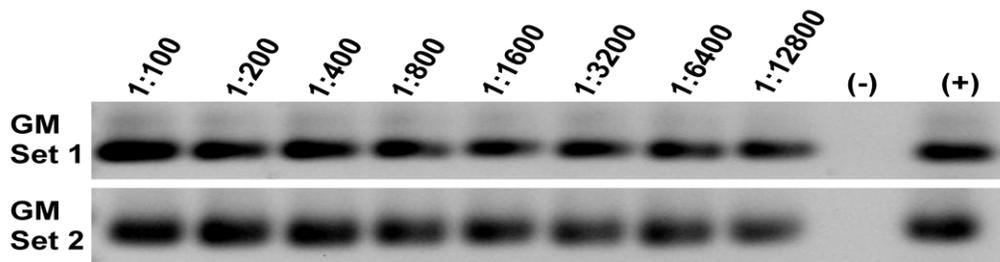


Figure 8: Dilution curve of golden mussel (GM) DNA from 1:100 to 1:12800 show the PCR primers are able to detect low DNA concentrations.

Summary

The studies presented in this document show the range of issues associated with

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PCR for the early detection of dreissenid mussels. The past two years of research into DNA and PCR methods for the early detection of quagga mussels have increased RDLES understanding of the best practices and methods for handling raw water samples.

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Share Drive folder name and path where data are stored:

H:/EnvRes Share/Mussel Samples/2014 Research Proposals/PCROPT

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