RECLAMATION

Managing Water in the West

Molecular Methods for the Ecological Research Laboratory

Research and Development Office
Science and Technology Program
(Final Report) ST-2019-1748-01

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14. ABSTRACT *(Maximum 200 words)* The field of molecular biology and of environmental DNA (eDNA) continues to advance at a rapid pace. This report can be divided into two main sections. The first section is a literature review of current environmental DNA research and the different amplification methods that are used to amplify DNA from eDNA samples. During the last three years new amplification methods using LAMP and qPCR methods for the invasive quagga and zebra mussels have been published by other researchers. The second section of this report is focused on comparing conventional PCR, LAMP assays, and qPCR for the detection of Dreissenid mussel DNA. LAMP is a newer amplification technique that has some advantages over the conventional PCR that is currently being performed. Understanding the limits and advantages of LAMP over conventional PCR is a central goal of this project. In the spring of 2019, a quantitative PCR (qPCR) instrument was purchased and comparing the results that can be obtained from this new instrument to conventional PCR was a major portion of the research conducted. Ensuring that the best eDNA methods are used to for the early detection of the invasive mussels is an ongoing process.

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Molecular Methods for the Ecological Research Laboratory

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Acronyms and Abbreviations

DNA- Deoxyribonucleic acid
eDNA- Environmental DNA
eRNA- Environmental RNA
LAMP-Loop mediated isothermal amplification
RNA-Ribonucleic acid
PCR- polymerase chain reaction
QM- Quagga mussel (*Dreissena rostriformis bugensis*)
qPCR-Quantitative polymerase chain reaction
ZM- Zebra mussel (*Dreissena polymorpha*)

Executive Summary

Both the quagga mussel (*Dreissena rostriformis bugensis)* and the zebra mussel (*Dreissena polymorpha)* are invasive freshwater bivalves that arrived in North America over thirty years ago. Quagga mussels (QM) were first found in Lake Mead in 2007 and have since spread throughout the lower Colorado River system and other waterbodies in the western United States. Zebra mussels (ZM) are predominantly in the eastern United States and are only present in a few sites west of the Mississippi. Both QM and ZM cause significant impacts to the environment, and to infrastructure such as dams, water intakes, and water treatment facilities. Both microscopy and environmental DNA (eDNA) methods are used by the Bureau of Reclamation's Ecological Research Laboratory to perform early detection for these invasive mussels. The field of molecular biology and of eDNA continues to advance at a rapid pace. This report is divided into two main sections. The first section is a literature review of current environmental DNA research and the different amplification methods that are used to amplify eDNA from samples. The second section of this report is focused on comparing conventional PCR, qPCR and LAMP assays for the detection of *Dreissenid* mussel DNA. In the coming years, qPCR will replace the conventional PCR method used by Reclamation researchers. Using LAMP assays for early detection of ZM and QM will not be pursued because the resulting amplification product cannot be sequenced to confirm the finding, which is part of the reporting process. The majority of eDNA detection programs have moved to using qPCR, so to keep current with standards in the field the Ecological Research Laboratory will move towards this technology. Ensuring that the best eDNA methods are used for the early detection of the invasive mussels is an ongoing process.

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Main Report

The quagga mussel (*Dreissena rostiformis bugensis)* and the zebra mussel (*Dreissena polymorpha)* are invasive freshwater bivalves that arrived in North America over twenty years ago. Quagga mussels (QM) were first found in Lake Mead in 2007 and have since spread throughout the lower Colorado River system and other waterbodies in the western United States. Zebra mussels (ZM) are predominantly in the eastern United States and are only present in a few sites west of the Mississippi. Both QM and ZM cause significant impacts to the environment, and to infrastructure such as dams, water intakes, and water treatment facilities. The Ecological Research Laboratory (formally known as Reclamation's Detection Laboratory for Exotic Species (RDLES)) has for over ten years been performing early detection for these two invasive species.

Plankton tow samples are collected in the field, preserved with ethanol and buffered with baking soda, and then sent to the laboratory for analysis. Samples come from Reclamation offices, states, and other clients. Cross polarized light microscopy (CPLM) is used to analyze samples for the presence of ZM and QM veligers, which is the larval life stage for both organisms. Once the CPLM is performed, environmental DNA (eDNA) is isolated from the bulk water sample and analyzed by polymerase chain reaction (PCR) in two separate tests for ZM and QM cytochrome oxidase I (COI) gene. Results are reported the clients to determine the best course of action if there is a positive finding. Understanding the best way to interpret and use eDNA results has been an ongoing issue for managers.

The field of molecular biology and of environmental DNA (eDNA) continues to advance at a rapid pace. This report is divided into two main sections. The first section is a literature review of current environmental DNA research and the different amplification methods that are used to amplify DNA from eDNA samples. Over the last three years, there have been several publications on the use of LAMP and qPCR for the detection of ZM and QM eDNA in water samples. The methods described in these publications, the amplification primers, were used in the experiments performed for this project.

The second section of this report is focused on comparing conventional PCR, LAMP assays, and qPCR for the detection of dreissenid mussel DNA. LAMP is a newer amplification technique that has some advantages over the conventional PCR that is currently being performed. Understanding the limits and advantages of LAMP over conventional PCR is a central goal of this project. Using LAMP assays for early detection of ZM and QM will not be pursued because the resulting amplification product cannot be sequenced to confirm the finding.

In the spring of 2019, a quantitative PCR (qPCR) instrument was purchased and comparing the results that can be obtained from this new instrument to conventional PCR was a major portion of the research conducted. In the coming years, qPCR will slowly replace the conventional PCR method used by Reclamation researchers. The majority of eDNA detection programs have moved to using qPCR, so to keep current with the field the Ecological Research Laboratory will move towards this technology.

Background

Environmental DNA

Environmental DNA (eDNA) for the last few years has grown in importance for the detection of invasive, endangered, and threaten species. Also, it has become a feature of monitoring programs for wide range of organisms. Some of the organisms that have been studied include: invasive pythons[1], crayfish [2], turtles [3], various fish species including Asian Carp [4][5][6], feral hogs [7], crested newt [8], hellbender salamander [9], and quagga/zebra mussels [10][11][12]. The incorporation of eDNA results into monitoring programs has not been without controversy, but as the science continues to advance the results are becoming more accepted. There are several advantages to using eDNA in monitoring programs. Some of these advantages include: higher chance of detecting a species, more cost efficient, accurate, and causes less disturbance to the environment [13].

There is still a great deal of research ongoing into overcoming the limitations of eDNA. There has been ongoing research into the environmental factors that degrade eDNA in the environment- sunlight, UV, pH, temperature all can affect the degradation rate of eDNA [14]. In addition, determining the length of time that eDNA persists in the environment and how far it can travel from its source is still being researched [15]. Computational methods are starting to be used to determine the number of eDNA samples that must be collected to have the best coverage for an organism of interest [16]. Metabarcoding methods are starting to be developed to test eDNA for the presence of multiple organisms simultaneously [17]. Finally, the biggest issue that is still being worked out is how to tie the eDNA results to actual biomass and the number of organisms of interest present in the environment [18]. eDNA does not currently inform researchers about the number of individuals present, it can only give a presence or absence result.

In the coming years, new eDNA extraction and amplification methods will most likely emerge that continue to change the perception of eDNA. As the cost of collection and analysis decrease, more monitoring will most likely be done using eDNA methods. Understanding this monitoring method is important for both researchers and managers. It will most likely emerge that for each target organism, specific DNA collection, processing, and amplification methods will have to be optimized to ensure the best detection method.

DNA Amplification Methods

There are several DNA amplification methods that are discussed in this report: conventional PCR, LAMP, and qPCR. Each of these methods has both advantages and disadvantages. Appendix A contains a table summarizing these three amplification methods. Below there is a summary of each method.

Conventional PCR

Conventional PCR is the method that is currently used by Reclamation researchers for the eDNA analysis of bulk water samples for the presence of ZM and QM DNA (https://www.usbr.gov/mussels/docs/PCRPreparationAnalysisVeligers.pdf). PCR uses primers to amplify a specific fragment of DNA [19]. For this method the PCR components (*Taq* polymerase, nucleotides (dNTPs), 10X buffer, magnesium chloride, forward and reverse primers, and DNA) are placed into a PCR reaction tube. There are three steps in the PCR reaction: denaturing, annealing, and extension (Figure 1). During the PCR reaction a specific strand of DNA is amplified to millions or even billions of copies that can then be visualized by gel electrophoresis and visualized using a DNA dye. PCR is used around the world in research, clinical, and forensic laboratories to analyze DNA to answer a wide range of questions. From the original PCR method, a wide range of new amplification methods and technologies have been developed.

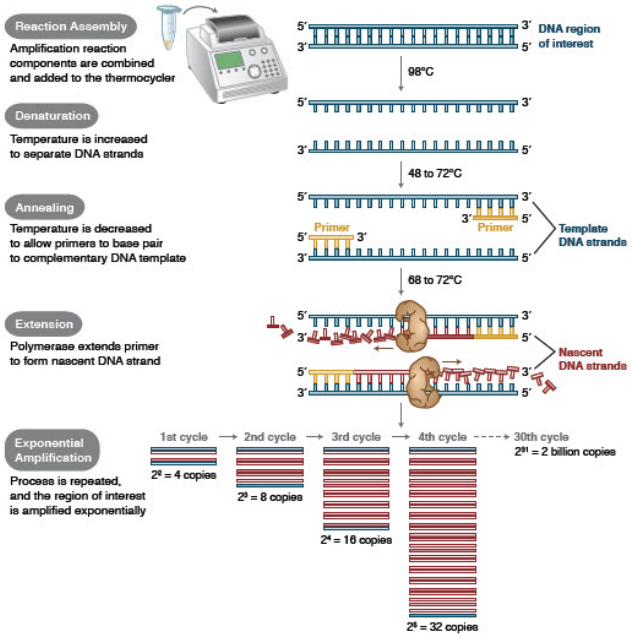

Figure 1: Conventional PCR components and process (NEB).

Quantitative PCR

Quantitative PCR (qPCR) is another amplification method that uses dyes or probes to detect the newly created PCR product (Figure 2). In 2019, the Ecological Research Laboratory was able to purchase its first qPCR instrument. One of the main functions for this instrument will be the analysis eDNA samples for the presence of invasive, endangered, and threated species. Unlike conventional PCR, for the applications that will be performed the qPCR, a master mix will be purchased. This master mix contains the *Taq* polymerase, dNTPs, magnesium chloride, 10X buffer and proprietary additives that help to overcome PCR inhibitors that are present in environmental samples. There are three primers (forward, reverse, and internal with a probe) that are used.

The advantage of qPCR is the presence of a third internal primer that contains a probe that when incorporated into the PCR product gives a light signal that the instrument can detect. This can also be done with a fluorescent dye. The qPCR instrument monitors the production of the amplification product in real time. Thus, the qPCR products do not have to be visualized by gel electrophoresis. qPCR can be more sensitive and have lower detection limits than conventional PCR. Using this technology will enable Reclamation researchers to continue to improve and expand the early detection method for ZM and QM, and other invasive species.

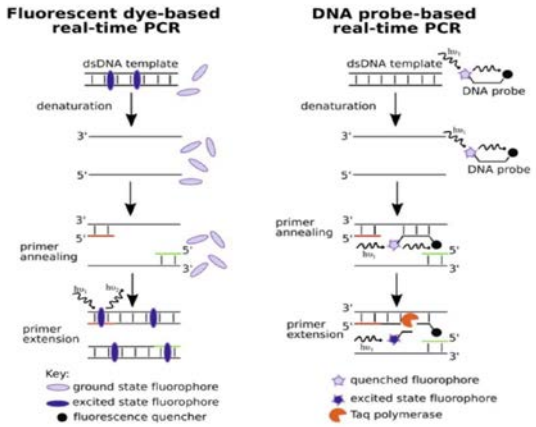

Figure 2: Comparison of qPCR methods- fluorescent dye based and DNA probe-based PCR (Qiagen).

Loop-Mediated Isothermal Amplification

Loop mediated isothermal amplification (LAMP) is an amplification method where a constant temperature is used to carry out the amplification [20] (Figure 3). Thus, the use of a thermal cycler is not needed, and the reactions can be carried out in a heat block, which lowers the cost of performing these assays. For these assays four to six primers are used: loop forward (LF), loop backward (LB), forward (F3), backward (B3), forward inner primer (FIP), and backward inner primer (BIP) (Figure 3). In each reaction mixture there are the primers, 10X buffer, dNTPs, and BST DNA polymerase. Other reagents can be added as needed to overcome inhibition. The isothermal reaction is carried out for an hour at 60-65ºC. The resulting product can be detected by turbidity, color change (blue to purple), or a fluorescent signal. The advantage of LAMP assays is that when a researcher is in an isolated place it is possible with only a heat block to get a positive or negative amplification result. For example, diagnostic assays for pathogens are being developed with LAMP [21][22]. The disadvantage of this method is that the results cannot be used in any further molecular tests such as cloning or DNA sequencing.

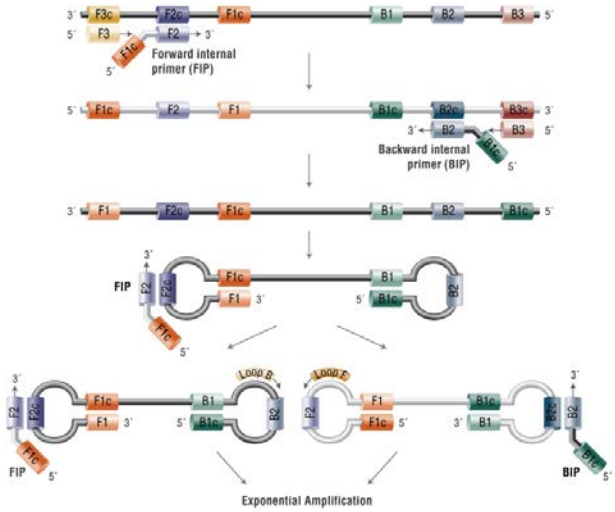

Figure 3: LAMP assay process showing the six different primers that are used (NEB).

QM and ZM eDNA analysis

The focus for Reclamation researchers has been on both *Dreissena rostiformis bugensis* (quagga mussel) and *Dreissena polymorpha* (zebra mussel) detection by molecular methods. For this reason, understanding the polymerase chain reaction (PCR) methods that have been developed and published in a peer review journal has been important. The last three years have seen the development of new amplification methods for these invasive mussels.

First, LAMP assays have been developed for both ZM and QM [23]. This method has been developed for deployment in the field so that rapid screening can be performed on suspect mussels [23]. This will allow personnel who are performing boat inspections to collect a sample from a boat, process the sample, and rapidly get results. The advantage of this is that it cuts down on the time required to receive conformation that an invasive mussel has been found. The primers from this publication were used by Reclamation researchers to investigate LAMP assays.

Second, there have been several publications on using qPCR to assess eDNA samples for QM and ZM presence [24], [25]. These publications have helped guide Reclamation researchers in determining which qPCR primers and reagents to use. In addition to qPCR, researchers have used high-throughput sequencing metabarcoding assays to study the genetic patterns of QM and ZM [10]. Finally, the ZM genome has been sequenced [26] and Reclamation researchers are in the process of sequencing the QM genome. Having access to both genomes will offer scientists the opportunity to investigate and hopefully find ways to control or even eradicate these invasive mussels.

Future technology and directions

The last few years have seen an expansion in the use of molecular biology at address ecological questions. Incorporating eDNA into the detection of invasive, endangered, and threatened species programs has started to show both researchers and managers the advantages and limitations of using this technology. New methods continue to be developed. Droplet digital PCR, metabarcoding using next generation DNA sequencing, and environmental RNA are all areas where research is currently being pursued by researchers around the world as methods that can be used for environmental monitoring. As new methods and techniques are developed it is important to continue to determine the best way these methods can be applied to projects that are performed by Reclamation researchers.

Methods

Several methods were used over the course of this project: conventional PCR, qPCR and LAMP assay. For all these experiments, DNA was extracted from adult ZM and QM, veligers, or bulk eDNA samples.

DNA Extractions

Adult zebra and quagga mussel DNA

Tissue samples were taken from adult ZM and QM that were preserved in ethanol. Each mussel was opened, the tissue was removed, and placed into an Eppendorf tube. The DNeasy Blood and Tissue kit (Qiagen 69504) was used to isolate DNA from the tissue samples following the manufactures protocol. Once the DNA was isolated it was stored at -20^{o}C until used in experiments.

DNA from tissue samples was also isolated using the PowerSoil kit (Qiagen 12888-100). Tissue samples were placed directly into the bead beating tube and then the DNA was isolated following the manufactures protocol. Once the DNA was isolated it was stored at -20^{o}C until used in experiments.

DNA from Individual Veligers

Method 1: Individual suspects are pipetted Eppendorf tubes. DNA from these individuals was isolated using the current standard operating procedure (PCR SOP) for the Ecological Research Laboratory (https://www.usbr.gov/mussels/docs/PCRPreparationAnalysisVeligers.pdf). The DNeasy Blood and Tissue Kit (Qiagen 69504) was used to isolate the DNA. Following the extraction, the DNA samples were stored at -20^{o}C until used.

Method 2: Individual veligers are pipetted into the PCR reaction tube, the master mix is added, and the amplification reaction is run. The resulting product is analyzed according to the amplification method being used.

eDNA from bulk water samples

eDNA from bulk water samples was isolated according to the current PCR SOP for the Ecological Research Laboratory. Briefly, from each bulk water samples, 40 mL was poured into at 50 mL conical tube. The tubes were centrifuged for 30 minutes at 4500 x g. Once the centrifugation was complete the supernatant was poured off without disturbing the pellet. From each pellet 250 µL was pipetted into the bead beating tube of the PowerSoil Kit (Qiagen 12888-100) and the DNA was isolated according to the manufactures protocol. Once the DNA was isolated it was stored at 4^{o}C during the experiments. After the experiments were completed the DNA extracts were then moved into a -80^{o}C freezer for long term storage.

Current eDNA methods

Using conventional PCR (cPCR) to analyze environmental samples from across the western United States has been a major focus of the work performed during the last three years. The

results obtained from the analysis of eDNA for the presence of both ZM and QM has been used by managers to make decisions regarding when to call a body of water positive for the presence of mussels. The analysis process of the samples is described below.

When samples arrive, the pH is taken and if it is below 7 baking soda is added to buffer the sample. The sample is then logged into the database and given a barcode label that allows the sample to be tracked throughout all the different steps. Samples are first placed in settling cones overnight and veligers which may be present then settle to the bottom 15 ml of the settling cone. The bottom 15 mL are then analyzed by microscopy for the presence of veliger bodies. Once the microscopy is completed, the 15 mL of sample that were analyzed are placed back into the original sample bottle.

Samples that are on the priority list are then analyzed for the presence of invasive mussel eDNA. The priority list consists of sites where a veliger or suspect was found at some time in the past. Samples are shaken and then 40 mL are poured into a 50 mL conical tube that is then centrifuged for 30 minutes. The supernatant is then poured off and the pellet is analyzed. A DNA soil extraction kit is used because of the presence of PCR inhibitors such as humic acid in the sample. Following the extraction samples are then analyzed with PCR primers (Appendix 1) to the cytochrome oxidase I (COI) gene that are specific to either ZM or QM.

Following the PCR reaction, the amplification products are analyzed by gel electrophoresis (Figure 4). Positive samples are repeated to show reproducibility. Positive PCR products are sent for DNA sequencing to verify that the fragment that was amplified is from either ZM or QM. Results are then uploaded into the mussel database and shared with clients.

When suspect veligers are found and after images have been taken, the suspects are analyzed by DNA methods to determine if they are either ZM or QM. A suspect is placed into an Eppendorf tube, and the DNA isolated using the DNeasy Blood and Tissue kit (Qiagen 69504). The DNA extract is then analyzed by the same PCR method used for the bulk water samples (PCR SOP). Using this method, it has been possible to get DNA results for individual suspects from samples where under the microscope there appeared to be no tissue left in the veliger.

Over the last three years (2017-2019), over a thousand samples have been analyzed with conventional PCR for the presence of invasive mussel eDNA. Most of the samples have been negative. In 2017, at total of 814 samples were analyzed. After 2017, a decision was made to purge the priority list of sites where there had been no suspect found in over 5 years. In 2018, a total of 494 samples were analyzed. The analysis of samples for 2019 is still ongoing because samples will be analyzed until November depending the samples that arrive in the laboratory.

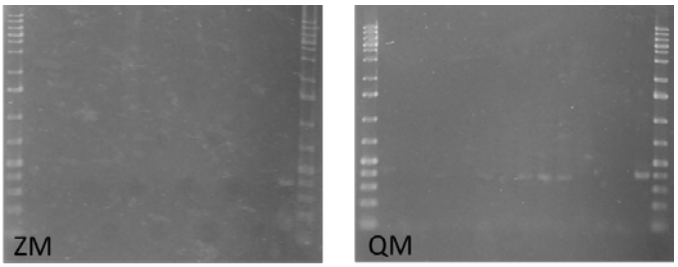

Figure 4: Example of ZM and QM gel.

QA/QC Protocols

All steps must pass quality assurance/quality control (QA/QC). All the PCR runs include both a negative (no template DNA) and a positive DNA control. For example, in Figure 3 the last two lanes of gel are where the negative and positive control are located. Both gels pass QA/QC. The QM gel contains positive COI results. If either of these two lanes fail, then the PCR reaction for the samples must be repeated. To control for contamination, dedicated pipettes and disposable filter pipette tips are used at each stage in the process. Nitrile gloves are worn and changed often. A commercial DNA extraction kit is used. Dedicated DNA extraction, PCR set up, and gel electrophoresis spaces are used. Controlling for contamination is an ongoing process in the laboratory where eDNA analysis is being performed. Separate areas for DNA extraction, PCR setup, and post-PCR analysis are used. All areas are cleaned using 20% bleach at least once a week. These physical controls help to decrease the risk of cross contamination. In addition, the positive and negative controls that are built into the assay must pass QA/QC.

Finally, once a month a wipe test is performed on areas of the laboratory that have the potential to cause cross contamination (Figure 5). The following samples are collected: veligers (positive sample) (lanes 1 & 2), DNA extraction hood (lanes 3 & 4), centrifuge used for DNA isolations (lanes 5 & 6), microscope use for sample analysis (lanes 7 & 8), PCR hood (lanes 9 & 10), and PCR instrument (lanes 11 & 12). For each area two samples are collected using a Q-tip wetted in DI water. The first sample set is directly analyzed by PCR. For the second sample set, the DNA is extracted using the Qiagen Blood and Tissue Kit (Qiagen 69504). Both sample sets are analyzed using the QM COI PCR assay. The ZM COI assay is not performed because when there is a positive finding in the laboratory it is usually from QM. The resulting PCR products are then analyzed by gel electrophoresis and recorded in the wipe test laboratory book. Over the last few years the results of the wipe tests only the positive veliger sample has been positive. These results indicate that the cleaning done in the laboratory is helping to ensure that the risk of cross contamination is minimized.

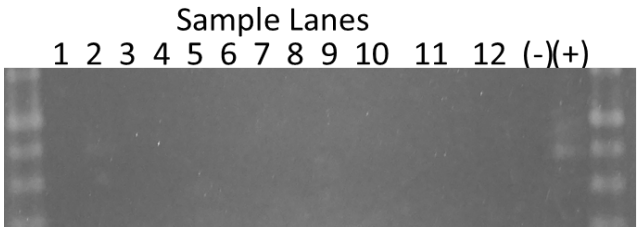

Figure 5: Example of wipe test gel. Each lane represents a different area of the lab that is analyzed.

qPCR Method Development

Introduction

The purchase of a qPCR instrument in spring 2019 enabled Reclamation researchers to start to optimize and validate use of the instrument for the detection of ZM and QM eDNA from bulk water samples. Several different experiments were performed. First, serial dilutions of ZM and QM were done to assess the limits of detection of the 16S dreissenid primers to both ZM and QM, and a COI primer set specific to QM. Second single veligers were analyzed because this is a method that is performed in the laboratory. Finally, samples from known positive water bodies were analyzed by both qPCR and conventional PCR. These samples were used to assess different master mixes that can be used.

Experimental Design

DNA isolated as described above was used in all the experiments. Single veligers were pipetted directly into the PCR strip tubes for analysis.

Two different master mixes, SsoAdvanced (Bio Rad 1725280) and iTaq (Bio Rad 1725132), were used with primers from [25] for the DNA dilution and single veliger experiments (Appendix 1). Bio-Rad CFX Maestro software was used to design the amplification program and control the qPCR instrument. The program was adjusted (temperatures and time at each step) to optimize the reactions. Each sample was analyzed in triplicate. Dilutions (1:10, 1:100, and 1:1000) of either adult QM or ZM DNA was used to create standard curves. For the bulk water sample analysis SsoAdvanced, iTaq, and PerfeCTa qPCR ToughMix (Quantabio 84241) mastermixes were all used. Each run included at least two negative controls where no DNA was added to the reaction. Following the amplification, results were assessed in the Bio-Rad CFX Maestro software and the Cq values were downloaded into an Excel spreadsheet.

Results

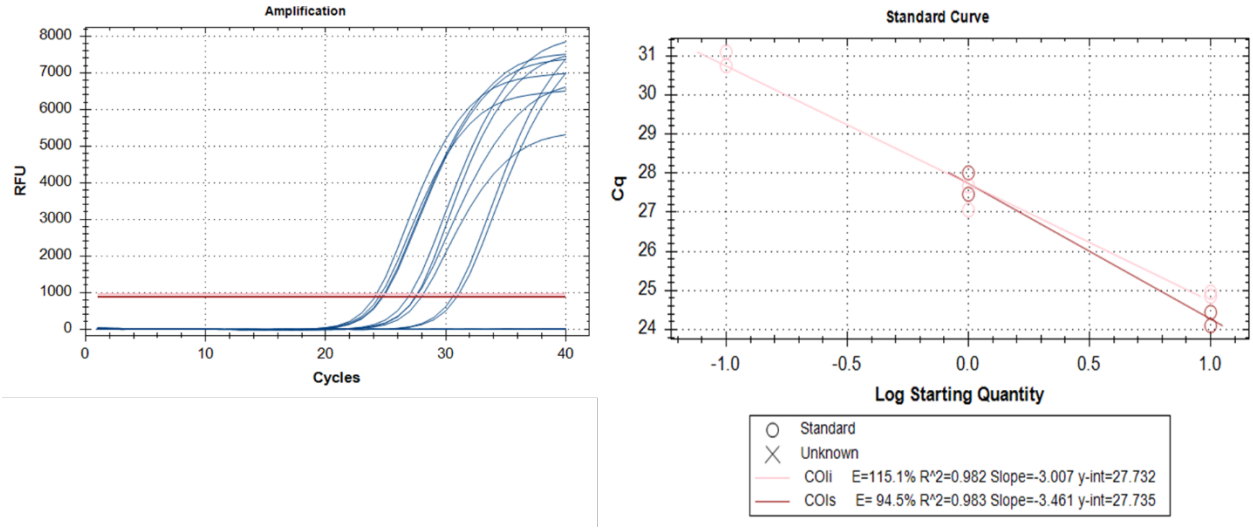

Figure 6: Comparison of SsoAdvanced and iTaq master mix with dilutions of QM DNA.

Three different master mixes have been assessed so far to determine which one will suit the needs of the QM and ZM eDNA analysis. For each of them multiple dilutions curves using ZM and QM DNA were performed. An example of the data that the instrument collects is in Figure 6. The qPCR program provides an amplification cycle graph. This shows where the instrument starts to detect the incorporated fluorescent primer. It also shows a standard curve graph. This graph gives the log starting quantity versus the Cq value. The Cq value is the PCR cycle where the amplification crosses a threshold value. Lower Cq values indicate that the threshold value was achieved with less amplification cycles. For example, in Figure 6 the threshold value is represented by the red line on the graph. This value can be adjusted as needed. The efficacy or E value should be between 90-110% and is defined as $E= -1 + 10^{(-1/slope)}$.

The graph also provides the R^2 and slope of the line for the dilution curve. Standard curves are used for each sample set that is analyzed so that it is possible to determine the copy number in a sample. For these experiments, DNA isolated from adult mussels was used. To get a more precise starting amount of material gBlocks gene fragments can be used. It is possible to order synthetic gBlock gene fragments for the target gene. Changes can be made in the synthetic gBlock gene such that if it contaminates a sample, it will then be possible to differentiate between the DNA from the eDNA sample and the synthetic DNA by DNA sequencing.

Table 1: Single veliger analysis by qPCR

Veliger	Cq iTaq	Cq SsoAdvanced
A	26.75	n/a
B	29.26	n/a
C	38.54	n/a
D	30.34	n/a
E	28.14	n/a
F	32.11	n/a

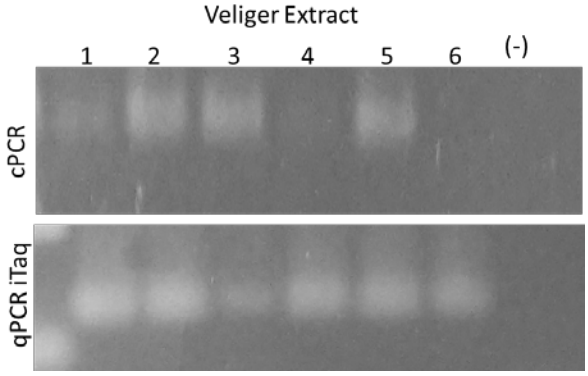

Figure 7: Direct analysis of individual QM veligers by cPCR and qPCR.

Single veligers were pipetted in to the reaction tubes directly to look at the variation in the amplification from individuals. DNA extractions were not performed as a way of limiting the loss of DNA in the sample. Positive Cq values were seen with the iTaq master mix (Table 1).

The SsoAdvanced master mix failed to have any positive Cq values. When the iTaq qPCR products were run out on a gel (Figure 7) and compared single veligers analyzed by cPCR, all six the iTaq reactions were positive. The cPCR had four out of the six veliger extracts give positive bands. Also, running the iTaq qPCR reactions on the gel revealed that there was enough PCR product present for DNA sequencing to be performed.

Table 2: Comparison of qPCR master mixes.

	iTaq				SSO Taq				PerfecTA ToughMix		
Sample	**Cq 1**	**Cq 2**	**Cq 3**		**Cq 1**	**Cq 2**	**Cq 3**		**Cq 1**	**Cq 2**	**Cq 3**
1	37.97	n/a	n/a		n/a	n/a	n/a		38.31	n/a	n/a
2	n/a	n/a	n/a		n/a	n/a	n/a		n/a	n/a	n/a
3	37.68	36.47	37.71		37.71	n/a	36.97		n/a	n/a	n/a
4	32.35	31.85	32.66		33.12	32.47	31.55		42.51	35.56	n/a
5	33.01	33.11	32.13		33.86	32.42	31.91		38.76	n/a	31.42
6	31.76	31.34	31.45		37.25	31.19	31.27		31.59	31.08	31.68
7	n/a	n/a	38.19		n/a	n/a	n/a		n/a	n/a	n/a
8	36.47	36.18	n/a		n/a	n/a	37.27		n/a	n/a	38.76
9	n/a	n/a	38.14		37.88	n/a	n/a		37.06	n/a	n/a
10	36.06	38.81	37.33		35.16	36.34	35.38		34.03	34.40	34.84
11	n/a	n/a	n/a		n/a	n/a	n/a		n/a	n/a	n/a
12	n/a	n/a	n/a		n/a	n/a	n/a		n/a	n/a	n/a

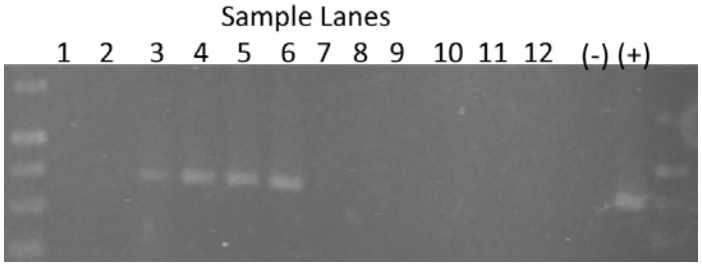

Figure 8: Analysis of the known positive samples with conventional PCR.

Bulk eDNA was isolated from twelve samples collected at locations where there are known QM populations. The samples were initially analyzed by microscopy and all were found to have either zero or only one veliger present. Thus, any positive QM results are from eDNA from sources other than intact veligers. The three different qPCR master mixes and conventional PCR was used to analyze each of the twelve samples (Table 2). For the conventional PCR (Figure 8), samples 3, 4, 5, and 6 all came back positive.

Using iTaq and SsoAdvanced mastermixes, samples were positive for all three reactions for samples 4, 5, and 6. PerfecTA qPCR Toughmix was positive for all three reactions for sample 6. In addition, all three master mixes showed positive results for sample 10 for all three reactions. Finally, all three had several reactions where one or two out of the three reactions were positive. The qPCR results were more sensitive than the conventional PCR in detecting samples that had QM eDNA present.

Summary

Determining the best way to perform qPCR assays for QM and ZM will be an ongoing process for the Ecological Research Laboratory. The sensitivity of this method looks like it will be greater than conventional PCR, which will improve the detection limits of the results that can be provided to managers and clients. How many reactions to do per samples, which controls should be included, and how many replicate assays should be performed on a sample before determining if it is positive will all have to be resolved. The work flow for analyzing bulk water samples by qPCR for invasive mussels will have to be validated to ensure that the results can be reproducible and understood by clients.

LAMP Assay Studies

Introduction

There have been at least two different publications by other groups for LAMP primers for QM, ZM, and both species (18S). For the studies performed for this report the method and primers developed by Williams et al [23] were used for QM and ZM analysis. By using published primers there is more certainty in the specificity and the ability of the primers to work. These primes were used with two different colorimetric detection methods were used, hydroxy naphthol blue (HNB) and WarmStart® LAMP 2X master mix.

Experimental Design

DNA isolated as described above was used in all the experiments from adult QM and ZM. Individual veligers were pipetted directly into PCR strip tubes and the reaction mixture added.

Amplification primers were used from Williams et al [23] (Appendix 1) for *Dreissena* sp. 18S rRNA, and cytochrome c oxidase (COI) gene for both QM and ZM. Each primer set consisted of six primers that were diluted to the recommended concentration and then a master mix of all six was made for use in the experiments. All the primers were ordered from Integrated DNA Technology, Inc. Molecular grade water was added to dilute each primer to the desired concentration.

Two different LAMP detection methods were used. The first, method was to use hydroxy naphthol blue (HNB) as the colorimetric indicator [27]. This method used *Bst* 2.0 Warmstart DNA polymerase (NEB M0538S). Each reaction mixture consisted of 1 µL (8000 U/mL) uM *Bst* polymerase, 2.5 µL 10X buffer, 3.5 µL 10 mM dNTP, 15 µL (100 mM) $MgSO_4$, 10.5 µL of molecular grade water, and 3 µL of DNA template. Using this method, negative results are pink and positive results turn yellow.

The second method used WarmStart® LAMP 2X master mix (NEB M1800S). Each reaction mixture consisted of 12.5 µL 2X master mix, 2.5 µL 10X primer mix, 1 µL target DNA, and 9 µL of molecular grade water. Using this method, negative results are purple and positive results turn blue.

For both methods, the reaction mixture was place in a PCR tube, then the target DNA was added. The samples were then place in the thermocycler for 60 minutes at 65°C. After the reaction was completed the PCR tube was removed from the thermocycler and the tube was then photographed to document any color change that occurred during the amplification.

Results

One goal of the project was to determine how LAMP assays would work with QM and ZM DNA. Dilution curves of QM and ZM DNA were used to assess three different LAMP primer sets: 18S *Dreissend* sp., ZM COI, and QM COI.

DNA from both adult ZM and QM was diluted 1:10, 1:100, and 1:1000 (Figure 9) using the Warmstart method. Each dilution was analyzed in duplicate. Specificity was also tested by using both ZM and QM DNA for each primer set. The 18S *Dreissend* sp. reactions were able to detect all three dilutions of both ZM and QM DNA and showed no reaction in the negative DNA control. The ZM COI reactions were able to detect the 1:10 and 1:100 dilutions of ZM DNA and showed no cross reactivity with the QM DNA. The QM COI reaction only detected the 1:10 dilution of QM DNA and showed no cross reactivity with ZM DNA. By comparison the conventional PCR analysis of dilutions of ZM and QM DNA from 1:50 to 1:1600 all were positive.

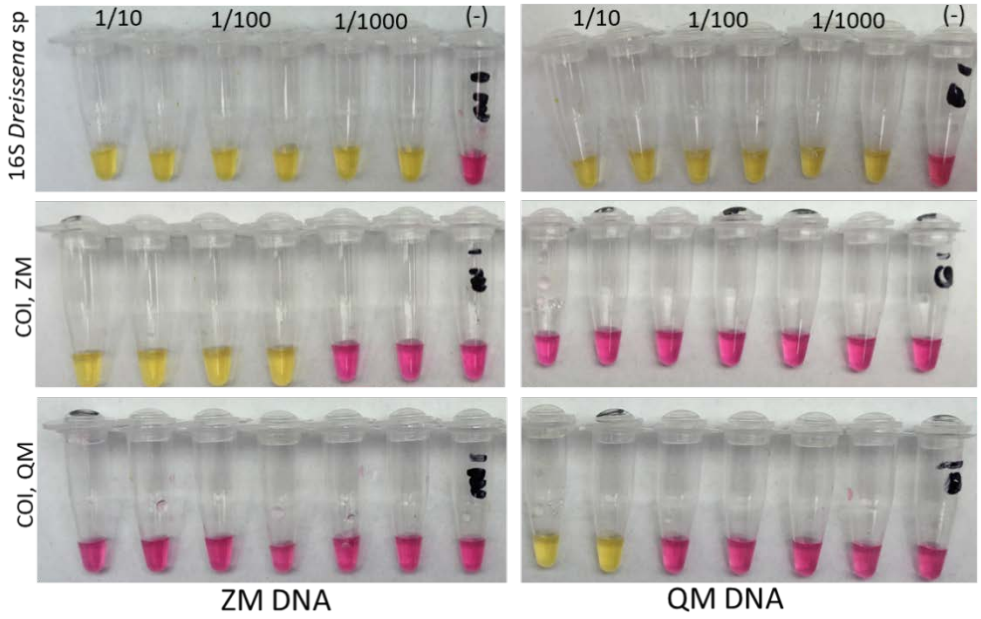

Figure 9: Colorimetric LAMP assay of ZM and QM dilution curves.

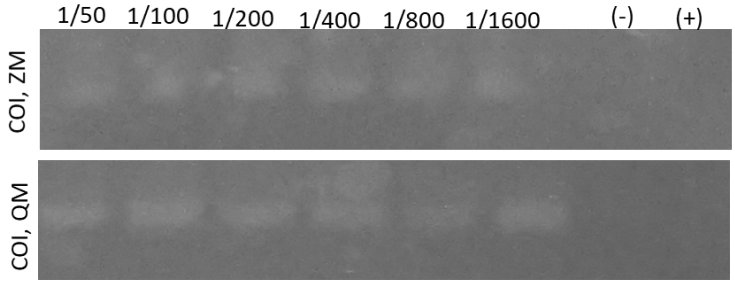

Figure 10: Conventional PCR serial dilution of ZM and QM DNA.

When single veligers were analyzed with the Warmstart method, none of the samples analyzed with the ZM primers were positive. Only one of the samples was positive for QM. For both ZM and QM the negative and positive controls passed QA/QC.

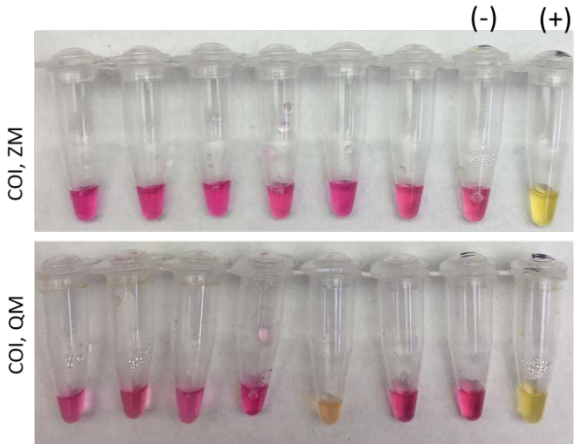

Figure 11: Single veliger DNA analyzed using the LAMP method.

The HNB method only worked for the 16S *Dreissend* sp primers. Each of the dilutions (1:10, 1:100, and 1:1000) was analyzed in duplicate. All the dilutions came back positive for either ZM or QM DNA. The assays for ZM and QM COI could not pass QA/QC. For both sets the negative and positive controls were not consistent. Also, the color change for this assay was difficult to see unless under a bright light. Out of the two LAMP assay methods used, the HNB method was not as stable as the Warmstart method.

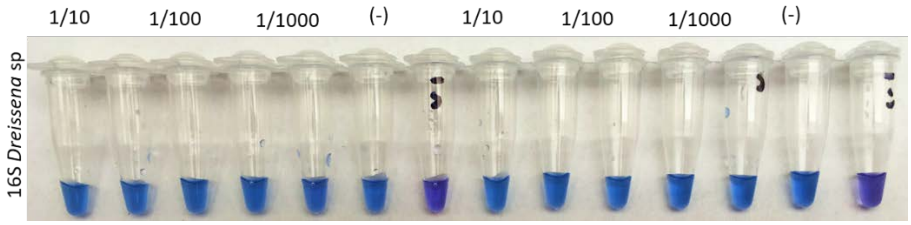

Figure 12: Colorimetric method with HNB dye.

Summary

There are several issues with the LAMP assay that make it less than ideal for using in early detection of invasive mussels. Once the amplification is complete a visual determination of positive or negative outcome is made based on a color change, which has the potential to be subjective. In addition, because the product of the reaction is not compatible with DNA sequencing it is not possible to validate that the product is specific to the target organism, versus being a false positive due to cross reaction with non-target DNA in the sample. Although the use of multiple primers in LAMP assays should ensure high specificity to the target organism, current reporting standards for state invasive species programs require DNA sequence confirmation for positive findings. The LAMP assay would not provide these results. Where this assay could be of use is in situations where suspect adults are found and a quick analysis to determine their species as either QM or ZM is needed. This would be done in the field. LAMP assays are supposed to enable researchers who are in isolated places the ability to analyze DNA samples without having to use a thermocycler.

Of the two different LAMP assays used the WarmStart method was far better overall. The color change was easy to determine and assess. All three primer sets could reliability detect DNA from both QM and ZM. The HNB method master mix consisted of multiple reagents that have to be mixed. Any mistakes would result in a negative or inconsistent result. For the Warmstart method the reaction mixture contained all the reagents needed, so there was less pipetting, and the results were more consistent. Finally, when compared to conventional PCR the LAMP assays did not perform as well. While an interesting amplification method, using LAMP assays for the detection of QM and ZM eDNA from bulk water samples will not be pursued by Reclamation researchers.

Outreach

While not for a main focus of this project, outreach was important to this project. For example, a presentation on eDNA was given to Reclamation employees to provide them background on the technology. Monthly participation in the Government eDNA Working Group (GEWEG) was very helpful for keeping contact with other eDNA practitioners. During these phone calls technical issues related to eDNA were discussed with government researchers from across a wide range of agencies. In November 2019, an in person eDNA meeting has been planned.

In addition, participation in the Western Regional Panel eDNA working group was also part of the project. This group was formed to look at the best practices for eDNA analysis of samples for ZM and QM. The group created a survey for managers and researchers to fill out to look at their views on eDNA. One of the members of the group gave a webinar about eDNA that was presented to a wide audience. In the coming year the eDNA working group will continue to give webinars about mussel eDNA issues. Hopefully the working group can work towards building consensus on the use of eDNA to detect invasive mussels.

Educating managers and the public about both the advantages and limitations of eDNA is an ongoing process. Participating in eDNA working groups and presenting about this research to diverse audiences is vital if these methods are to gain more acceptance. As the technology continues to move forward it will continue to be an important aspect of any research project related to eDNA.

Conclusions and Future Directions

The field of molecular biology continues to advance quickly with innovative methods and technology being developed for the detection of invasive, endangered, and threaten species. The conventional PCR assays have been studied and optimized for several years by Reclamation researchers. Moving from conventional PCR for the analysis of ZM and QM eDNA from bulk water samples to qPCR will be an ongoing process through the coming year. Ensuring that the qPCR assays are robust and can provide reproducible results will be important to using it for ZM and QM analysis. The use of LAMP analysis, while intriguing, currently is not an avenue of research that will continue to be pursued. Knowing and understanding LAMP assays is important because other researchers could start making used of them for the detection of invasive species.

It will be important to continue to monitor the literature for new PCR technologies and publications related to the invasive mussels. One area of interest is the analysis of water samples for the presence of environmental RNA (eRNA). Understanding how long eRNA lasts in the environment and what it can tell researchers about an organism of interest will be an ongoing are of investigation for researchers around the world. Molecular biology methods and technology continues to advance at a fast pace. Ensuring that the results obtained from these methods can help managers in their decision making will be an ongoing process.

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Appendix A – Amplification Primers for cPCR, qPCR, and LAMP Assays

Table 3: Conventional PCR primers for *Dreissena polymorpha* and *Dreissena rostriformis bugensis* cytochrome oxidase (COI) gene from https://www.usbr.gov/mussels/docs/PCRPreparationAnalysisVeligers.pdf

Species/Gene	Primer	Sequence (5'– 3')
Dreissena polymorpha/ cytochrome c oxidase (COI)	332	TGTCACCACTCATGGGCTTGTT
	333	TGCAGAACAAAGGGACCCGGTAAA
Dreissena bugensis/ cytochrome c oxidase (COI)	335	GAAACTGGTTGGTCCCGATA
	335	TAAGGCACCGGCTAAAACAG

Table 4: Quantitative PCR primers for Dreissenid sp. [24].

Species/Gene	Primer	Sequence (5'– 3')
Dreissend 16s rRNA (DRE16S)	Forward	TGGGGCAGTAAGAAGAAAAAAATAA
	Reverse	CATCGAGGTCGCAAACCG
	Probe	CCGTAGGGATAACAGC-MGBNFQ
Dreissena bugensis cytochrome c oxidase (COI) (DREQM)	Forward	CTCTTCATATCGGTGGAGCTTC
	Reverse	CAAAGGCACCCGATAAAACTG
	Probe	CCCGGCACGTATATTTCCTCATGTT

Table 5: LAMP assay primers for *Dreissena* sp. 18S rRNA and *Dreissena polymorpha* and *Dreissena rostriformis bugensis* cytochrome oxidase (COI) genes from [23].

Species/Gene	**Primer**	**Sequence (5'– 3')**
***Dreissena* sp. 18S rRNA**	FIP	TGAAAGATACGTCGCCGGCGAACTCGTGGTGACTCTGGAC
	BIP	TGC CTA CCA TGG TGA TAA CGG GTG TCT CAT GCT CCC TCT CC
	LF	GTGCGATCGGCACAAAGTT
	LB	TAACGGGGAATCAGGGTTCG
	F3	GTTAGCCCAGACCAACGC
	B3	CTTCCTTGGATGTGGTAGCC
***Dreissena polymorpha* cytochrome c oxidase (COI)**	FIP	AGAGACAGGTAAAACCCAAAAACTAATTGATTGGTACCAATAATACTGAG
	BIP	ATTTTGTTCAGCTTTTAGGGAAGGAAAAATCTATCGCAGGGCC
	LF	CGAGGGAAACCTATATCAGGAAGA
	LB	GGATTCGGGGGTGGTTGAACC
	F3	TAATGGGGGGATTCGGAA
	B3	GCTCCCCCAATATGAAGAG
***Dreissena bugensis* cytochrome c oxidase (COI)**	FIP	AAGAAGCTCCACCGATATGAAGAGCCACCGTTATCCAGGATT
	BIP	AGAACATGAGGAAATATACGTGCCCACCAATAGAAGTACAAAACAAAG
	LF	ATGGCTGGCCCTGAATGCC
	LB	GGGTGTCATCAGTTTTATCGGGT
	F3	ATTTGGTGGGGGTTGAAC
	B3	GGCTAAAACAGGTATTGCTAA

Data Sets that Support the Final Report

If there are any data sets with your research, please note:

- Share Drive folder name and path where data are stored: Z:\DO\TSC\Programs\Exotic Species Detection Laboratory\DATA\PROJECTS\Research Office\Molecular Methods - FA878
  - Point of Contact name, email, and phone: Jacque Keele, jkeele@usbr.gov, 303-445-2187
  - Short description of the data: Background literature collected for the project (PDF files). DNA amplification results for LAMP assays, qPCR assays, and PCR (Excel files and jpg files).
  - Keywords: LAMP, qPCR, PCR, eDNA
  - Approximate total size of all files: 176 MB