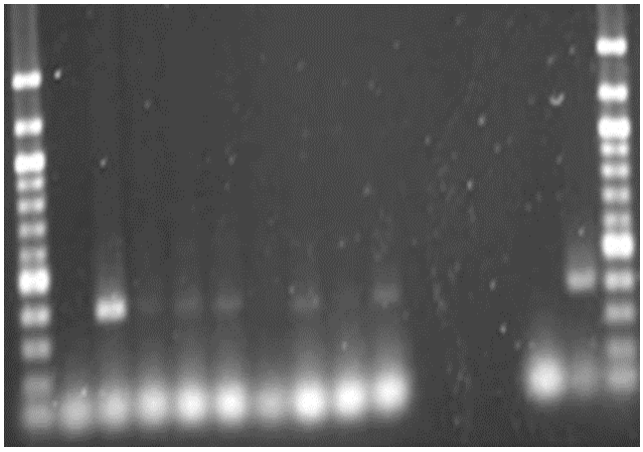
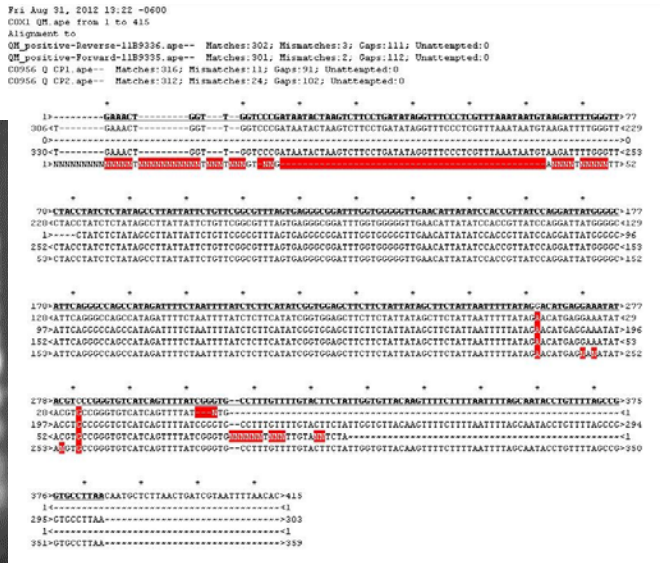
RECLAMATION

Managing Water in the West

Development of Standard Operating Procedures for the Detection of Invasive Species of Emerging Concern

Research and Development Office
Science and Technology Program
Final Report ST-2016-1248-1





U.S. Department of the Interior
Bureau of Reclamation
Research and Development Office

September 2016

Mission Statements

The U.S. Department of the Interior protects America's natural resources and heritage, honors our cultures and tribal communities, and supplies the energy to power our future.

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 09 2016	**T2. REPORT TYPE** Research	**T3. DATES COVERED** 2016 FY
T4. TITLE AND SUBTITLE Development of Standard Operating Procedures for the Detection of Invasive Species of Emerging Concern		**5a. CONTRACT NUMBER** RY1541ZQ201611248
		5b. GRANT NUMBER
		5c. PROGRAM ELEMENT NUMBER 1541 (S&T)
6. AUTHOR(S) Jacque Keele, jkeele@usbr.gov, 720-930-1056 Denise Hosler, dhosler@usbr.gov, 303-250-9166		**5d. PROJECT NUMBER** 1248
		5e. TASK NUMBER
		5f. WORK UNIT NUMBER 86-68560
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Bureau of Reclamation, Technical Service Center, Hydraulic Investigations & Lab Services		**8. PERFORMING ORGANIZATION REPORT NUMBER**
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) 2016-1248-1

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 development of standard operating procedures (SOP) for the detection of invasive aquatic organisms is an ongoing process at Reclamation's Detection Laboratory for Exotic Species (RDLES). Over the last year three organisms of concern (corbicula, spiny water flea, and New Zealand mud snail) were targeted for primer design and testing. This scooping project involved a literature search, and polymerase chain reaction analysis. Primers were designed and tested against positive tissue samples. Not all of the assays were able to give consistent results which means that it will be necessary to redesign and test new primers for the organisms. The lessons learned in this project will be applied to future endeavors as PCR assays are designed and tested for new organisms on interest.

15. SUBJECT TERMS: Polymerase chain reaction, environmental DNA, invasive species

16. SECURITY CLASSIFICATION OF: U			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** Jacque Keele
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	U	25	**19b. TELEPHONE NUMBER** 720-930-1056

PEER REVIEW DOCUMENTATION

Project and Document Information

Project Name Development of Standard Operating Procedures for the Detection of Invasive Species of Emerging Concern _WOID Z1248____
Document Development of Standard Operating Procedures for the Detection of Invasive Species of Emerging Concern
Document Author(s) Jacque Keele and Denise Hosler ____Document date Sept. 2016
Peer Reviewer Yale Passamaneck

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 _____ Date reviewed 9/16/2016
(Signature)

Disclaimer

Acknowledgements

The authors of this research would like to thank the Research and Development Office for supporting and providing funding for this study. We also want to thank our reviewer, Yale Passamaneck.

Notices

Acronyms and Abbreviations

COI- cytochrome oxidase I
eDNA- environmental DNA
NZMS- New Zealand Mud Snail
PCR- polymerase chain reaction
RDLES- Reclamation Detection Laboratory for Exotic Species
SWF- spiny waterflea

Executive Summary

Reclamation Detection Laboratory for Exotic Species (RDLES) in an effort to identify methods for the detection of invasive species over the last year worked to create molecular methods for the detection of three different species of concern. The first step in this process was to compile a list of invasive species that range from invertebrates to plants (Appendix 1). This table contains information on the availability of DNA sequences for primer design, and also if there are any known published molecular methods for the detection of the organisms DNA.

From this list three species were selected to move forward to PCR assay design at RDLES. They are the spiny water flea (*Bythotrephes longimanus),* Asian clam (*Corbicula fluminea*), and New Zealand mud snail (*Potamopyrgus antipodarum*). All three of these organisms are invasive aquatic invertebrates. *Corbicula* and New Zealand mud snails are already present in the Western United States. The spiny water flea is mainly present in the Eastern United States.

A literature search showed that there were published molecular methods for *Corbicula* and New Zealand Mud snails. The spiny water flea had many sequences present in online databases that could be used for primer design. The first step in this project was to design primers to the cytochrome oxidase I gene of the spiny water flea and to also obtain the published primers for the other two organisms. Once these primers were obtained, positive control tissue was extracted from samples that had been obtained of each of the organisms. The positive control tissue was used to test the PCR primers.

The results of these test showed that there was success the PCR assay for the spiny water flea. The assay was reproducible and could be multiplexed with the primers to the COI gene of *Dreissena bugensis* (quagga mussel). This assay will continue to be refined and validated in the RDLES Laboratory. On the other hand, the published primers for *Corbicula* and New Zealand mud snail that were obtained, did not perform as expected. These primers were not robust, with inconsistent PCR results. Further research will be needed to assess why the primers did not perform as expected and also to design additional PCR primers for these two organisms.

A major lesson that was learned is that not all published methods may be applied for the detection of eDNA from bulk water samples. The next step for this project is to continue to test and validate primers for all three of these organisms. This project allowed the RDLES Laboratory to gain valuable experience developing and working on the validation of new PCR assays.

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Main Report

Introduction

Over the last year, the Reclamation Detection Laboratory for Exotic Species (RDLES) at the Bureau of Reclamation, Technical Services Center, Denver CO, has worked on creating additional polymerase chain reaction (PCR) tests for the analysis of raw water samples for the early detection of spiny water flea, *Corbicula* (Asian clam), and New Zealand Mud Snails. The development of these assays involved isolating DNA from a known positive sample, designing and validating PCR primers, and testing the new PCR assay with spiked water samples.

Detection Methods

There are two main detection methods employed by RDLES: visual identification with microscopy and molecular detection of DNA using PCR. Visual detection involves using taxonomic keys and expertise to identify an organism under the microscope. Molecular detection involves using polymerase chain reaction (PCR) to identify the organism of concern (Figure 1). This detection method involves isolating DNA using a commercial kit, then using PCR primers to amplify a fragment of a gene from the organism. There are two different types of PCR reactions that can be performed. The first assay can be performed on a tissue sample or isolated organism. DNA barcoding is done using non-specific primers that can amplify a ~700 bp fragment of the cytochrome oxidase 1 (COI) gene that is then sequenced and analyzed to determine the identity of the organism. The second assay can be performed on bulk samples and involves creating species specific primers that can amplify a fragment of DNA from the organism of concern. When designing species specific assays it is important to ensure that the primers are specific to the organism of interest. The species specific assay can be used on both tissue samples and also on raw water samples.

With PCR there are several possible outcomes (Table 1). Conventional PCR gives a positive or negative result. It is important to note that false negatives can occur. This can happen when the DNA of the organism of interest is present, but for some reason does not amplify in the PCR reaction. Quality control and quality assurance is followed for all PCR assays. When issues do occur, such as a positive control fails, then the whole PCR reaction is repeated.

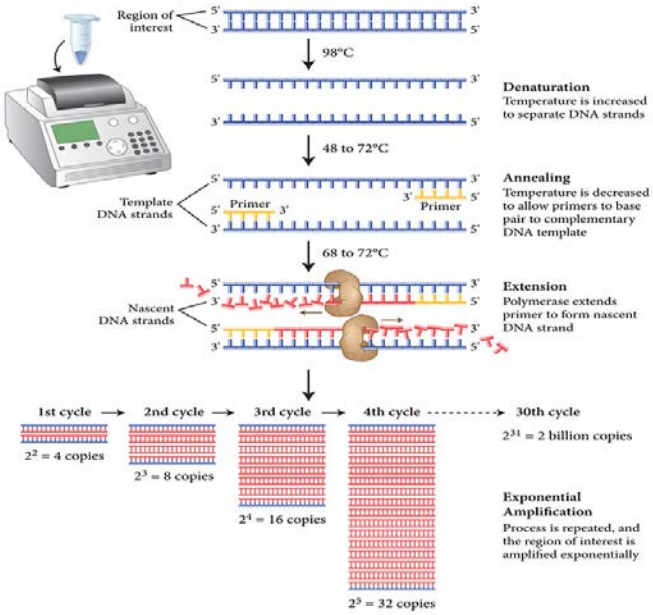

Figure 1: PCR method, (From: https://www.neb.com/~/media/NebUs/Page%20Images/Applications/DNA%20Amplification%20and%20PCR/pcr.jpg, accessed 9/15/2016)

Table 1: Possible outcomes of gel electrophoresis analysis

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

Organisms of Concern

There are many aquatic organisms of concern because of the wide range of negative impacts that these organisms can have on the environment. (Appendix 1). The first step in this project was to compile a table of organisms of concern. The organisms include: invertebrates (both microscopic and macroscopic, vertebrates (amphibians, reptiles, mammals, and fish) and plants. All of the organisms in the table have gene sequences in either the NCBI or DNA BOLD databases. The availability of cytochrome oxidase I (COI) sequences for many of these organisms will facilitate the design of PCR primers.

Three organisms were selected for standard operation procedure (SOP) development. They are the spiny water flea (*Bythotrephes longimanus)*, Asian clam (*Corbicula fluminea*), and New Zealand mud snail (*Potamopyrgus antipodarum*). All three of these organisms are aquatic invertebrates. The spiny water flea is a recent arrival in western waters and when fish consume this water flea it can damage the fish's stomach. The Asian clam is seen throughout waters

in the west, and often co-occurs with zebra (*Dreissena polymorpha)* or quagga (*Dreissena rostriformis bugensis)* mussels. Differentiating between the veliger forms of Asian clam and the Dreissenid mussels is important for the early detection of the invasive mussels. Finally, New Zealand mud snails (*Potamopyrgus antipodarum*) are a highly prolific invasive snail that are only in a few locations currently in the west, but because of their high fecundity it is important to be aware of their presence.

Assay Design

One of the goals for these new assays is that they will complement the current quagga and zebra mussel COI assays that are performed at RDLES. For this there are several considerations. First, it is important the assay produces a robust and reproducible PCR product with the positive control DNA. If this cannot be acheived then it might be necessary to design new PCR primers. Also, if possible, the new PCR assays should be able to be multiplexed with either the ZM or QM COI assay. This will allow for the simultaneous detection of two organisms using the sample PCR reaction. For multiplexing to happen it is necessary that the new assays have PCR products that are either smaller or larger than the band that the ZM and QM COI assay produces. These are the ideal parameters that went into the design of the new assays.

For the majority of the assays the cytochrome oxidase I (COI) gene is used. There are several reasons for this. First, because COI is a mitochondrial gene there are multiple copies of it in each cell. Mitochondrial genes are preferred over nuclear genes because of the higher copy number per cell. Second, the DNA barcoding community is using COI as the gene for the identification of organisms. So there is a high likelihood that for many of the organisms of concern there will be a COI sequence available. Also, if there is no known sequence for the organism then the DNA barcoding primers can be used to amplify a large fragment of DNA from the organism and then use that DNA sequence design species specific primers.

Some general references on PCR assay design: [1]–[4] . These publications go into detail on the challenges of PCR primer and assay design.

Assay Development

Water Fleas

There are three species of water flea that are of concern. *Daphnia lumholtzi* (Figure 3), *Bythotrephes longimanus* (spiny), and *Ceropagis pengoi* (fishhook) (Figure 2) are all aquatic zooplankton crustaceans. All three of these organisms are of concern because of how they impact the food web.

It was decided to not look at *Daphnia lumholtzi* for this project. A preliminary analysis of one of the COI sequences for this organisms showed that there are many closely related organisms. Thus, designing an assay for this *D. lumholtzi* will be a challenge that can be performed at a later time.

Background information on the spiny and fishhook waterfleas can be found in the following publications: [5], [6], [7]. These are invasive organisms from the same region as the quagga and zebra mussels. They also made their way to North American in the ballast water of transoceanic ships and were first seen in the Great Lakes. Once established there, these two species of waterfleas have slowly made their way across North America impacting fish species and zooplankton communities.

Spiny water flea (*Bythotrephes longimanus)*
Fishhook water flea (*Ceropagis pengoi*)

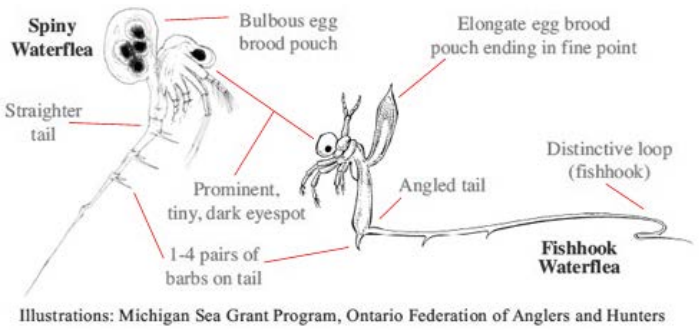

Figure 2: Illustration of the spiny waterflea and the fishhook waterflea (From: http://www.seagrant.umn.edu/ais/img/profile_identity_waterfleas.jpg, accessed 9/1/2016)

Figure 3: Image of *Daphnia lumholtzi* (From http://cfb.unh.edu/cfbkey/html/Organisms/CCladocera/FDaphnidae/GDaphnia/Daphnia_lumholtzi/Daphnia_lumholtzi_02_600x600.jpg, accessed 9/1/2016)

This year the focus was to design PCR primers and test the primers for the spiny water flea (Table 2). For the fishhook waterflea it was not possible to obtain any positive control tissue this year to test the PCR primers against. The primers were designed and are ready for when tissue sample can be obtained (Table 3).

Primer Design and Results:
Three primer pairs were designed and tested using positive control DNA (Table 2). The primers were designed to the COI gene. Spiny water flea (SWF) samples

were obtained from a collaborator and DNA was extracted according to the RDLES Laboratory SOP. Each primer set was tested with positive control DNA. As a way to test the specificity of the assay the SWF primers were tested against the positive control DNA of both QM/ZM. Primer set 3 gave the most consistent and robust results. Finally, it was possible to multiplex the SWF assay primer set 3 with the QM and ZM COI assays.

Table 2: Primers designed for spiny water flea PCR assay

Primer Set	**Primer Length**	**GC content**	**Tm (°C)**	**Size (base pairs)**
Forward 1	21	52%	55.1	404
Reverse 1	22	45%	54.6	
Forward 2	23	43%	53.9	345
Reverse 2	21	47%	54.5	
Forward 3	22	50%	55.4	297
Reverse 3	21	52%	55.7	

Table 3: Primers designed for fishhook water flea

Primer Set	**Primer Length**	**GC content**	**Tm (°C)**	**Size (base pairs)**
Forward 3	22	47.8%	54.9	251
Reverse 3	23	43.5%	54.5	
Forward 4	23	43.5%	54.4	214
Reverse 4	23	45%	54.3	
Forward 5	23	50%	54.6	259
Reverse 5	22	52.6%	54.9	

Primer set 3 for spiny water flea performed the best out of the three primers pairs that were designed and ordered. This primer set was then multiplexed with QM COI primers. The multiplexing was successful (Figure 3). In this experiment, both SW and QM primers were used together. Then DNA from SW, QM, and ZM was used to determine the specificity of the primer cocktail. The ZM DNA did not cross react with the QM primers. Instead the SW band is the only one seen in that lane with SW+ZM.

One issue is that the separation between QM and SW bands is very small and could be hard to detect if the gel does not give a clean result. This issue is seen when all three (SW+QM+ZM) were present in the sample, the SW band is hard to detect. Redesigning the SW primers be smaller would be useful so that there could be clearer separation of the QM and SW bands on the gel. Further work is needed to continue to validate these primers. One thing that needs to be done is to obtain water samples from where this organism is present to test the primers against.

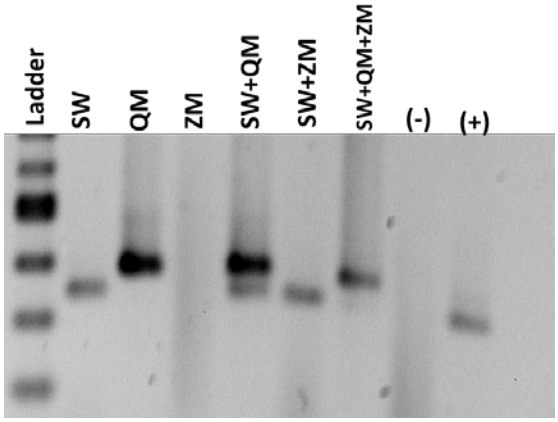

Figure 4: Multiplexing result of SW and QM PCR primers

Asian Clam (*Corbicula*) (*Corbicula fluminea)*

When samples are analyzed for the presence of QM and ZM veligers, *Corbicula* are often detected by microscopy by RDLES researchers. Having a genetic method to differentiate between COR and ZM/QM would be very helpful for the RDLES Laboratory.

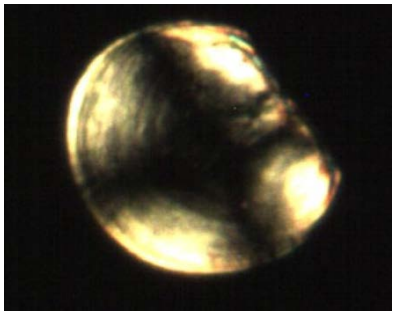

Figure 5: *Corbicula* veliger image, from Early Detection: Taxonomic Guide for Identification of Invasive Mussels, Jamie Carmon and Denise Hosler

A literature search revealed one publication where primers were designed for the detection of *Corbicula* veligers in raw water samples. The primers that Ludwig et al [8] designed were ordered from Integrated DNA Technology (IDT) and tested for use in the RDLES Laboratory. These primers target the cytochrome oxidase I (COI) gene and produce a 400 bp PCR product.

Primer Design and Results:

The primers are CorbF2 (5'-GCTATTCCAGGGACTTTA-3') and CorbR2 (5'-GCTCCAGGACGCATACAA-3'). From Ludwig et al, [8].

Corbicula were isolated from water samples and the DNA according to the RDLES Laboratory SOP. Once the DNA was isolated, a serial dilution of 1:50, 1:100, and 1:200 was created using the DNA. The preliminary PCR results showed that the primers were specific to corbicula and did not cross react with ZM or QM DNA. The next experiment was to optimize the primer concentration

and try to multiplex the reaction. The multiplexing did not work, and a positive band could not be obtained. Finally, it was possible to test these primers against real world samples that were known to contain corbicula.

The results were mixed. Some of the samples gave a positive band, while others that were known to contain *Corbicula* gave a negative band. Overall, the primers were not as robust and did not give consistent reproducible results. For example, repeating some of the experiment was difficult because the positive control DNA was did not always give a positive result. Additional *Corbicula* will have to be isolated to create new positive control DNA. While these primers might undergo further testing, in all likelihood new primers for this organism will be designed by RDLES researchers.

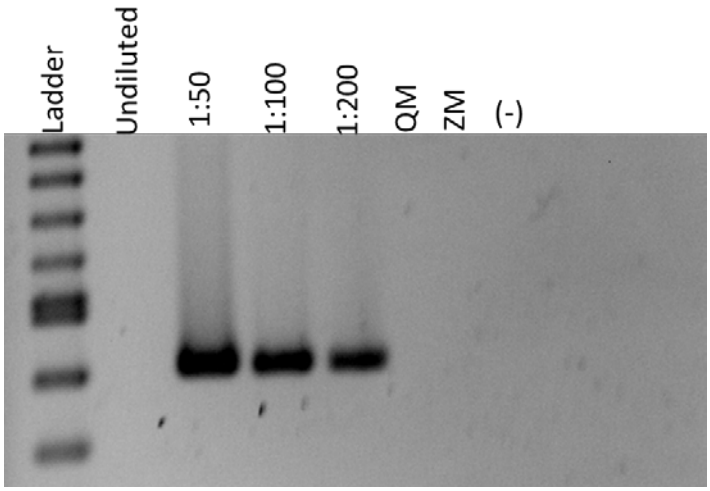

Figure 6: Preliminary corbicula PCR assay

New Zealand Mud Snail (*Potamopyrgus antipodarum)* (NZMS)
This invasive snail is of concern because of the large densities (200,000 m^2) in which they can occur [9]. It can impact native snails by competing for common food sources.


Figure 7: New Zealand Mud Snails (From http://nas.er.usgs.gov/XIMAGESERVERX/2011/20110314095956.jpg, accessed 9/1/2016)

Primer Design and Results:
For this organism it was also possible to find primers that had been published [10]. After extracting DNA from NZMS the positive control DNA was used to test the primers. After following the protocol from the published primers there was not successful. The resulting gels did not produce any band, making it difficult to determine the cause of the PCR failure.

When this happens it is difficult to determine the cause. There could be several possible causes for this. First, the tissue was not fully removed from the shell and the DNA was not extracted. Second, the reagents that were used here did not exactly match the ones used in the publication. The concentrations of the PCR reagents could have been different. It was not possible to obtain a positive band after several trials.

One of the issues with the NZMS are their shells. Determining the best way of removing the tissue from the shell for the DNA extraction is an issue. Also, if it is possible to remove the tissue, then taking a clean tissue sample and performing DNA barcoding would be possible. This eliminates the need for species specific primers. Designing new PCR primers for this organism will be an ongoing project.

Future Directions and Lessons Learns
Developing and validating PCR primers for a wide range of organisms will be an ongoing activity at RDLES. Over the last year there have been several lessons learned. First, primers that are published will not always be useful for analyzing the eDNA from bulk water samples. In that case it is important to design primers "in house" for the organism of interest. This will mean having to mine databases for DNA sequences of both the organism of interest and closely related species to design primer from. It is better to do this process in collaboration with another researcher to check and help each other. Second, obtaining enough positive control samples or tissue to extract DNA from and then have as positive control DNA. Having enough of the organism to make multiple DNA extractions as needed is important because the DNA stocks can degrade over time.

The future direction for this project is to continue to work on designing and testing PCR primers for a wide range of invasive species. This is important because the next invasive species of concern that could impact Reclamation facilities and waters is unknown. The rapid identification of an invasive organism is important determining the best methods for control or containment. A major method is the use of DNA tools for the molecular identification. Having those tools ready and waiting is can assist RDLES in determining the identification of an invasive species.

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Appendix 1: Aquatic Invasive Species and Organisms of Concern