RECLAMATION

Managing Water in the West

eDNA Testing for Invasive and Endangered Species

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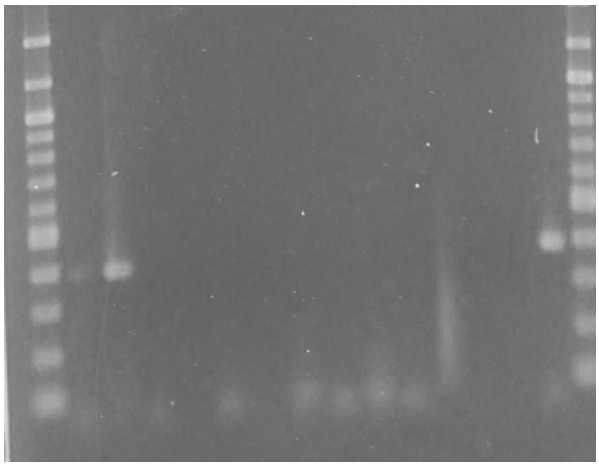

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Mission Statements

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.

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14. ABSTRACT ***(Maximum 200 words)***

The analysis of eDNA for the detection of invasive and endangered species is continuing to grow in the coming years. There are both advantages and disadvantages to performing eDNA analysis. This scooping project had several goals. First, to perform a literature search on current eDNA methods and techniques. Second to perform a time course study of the impact of inhibitors on PCR outcome. Inhibitors are a major issue for eDNA studies. Finally, to attend and present at the first annual eDNA workshop. While the impact of eDNA on environmental studies is growing as researchers realize the advantages of this method, it is also important to understand that there is still many areas of research that need to be addressed if this method is going to reach its full potential.

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Notices

None

Acronyms and Abbreviations

eDNA- environmental DNA
PCR- polymerase chain reaction
RDLES- Reclamation Detection Laboratory for Exotic Species
QM- quagga mussel
ZM- zebra mussel
NZMS- New Zealand mud snail

Executive Summary

Environmental DNA (eDNA) is emerging as an important tool for the detection of both invasive and endangered species. Reclamations Detection Laboratory for Exotic Species (RDLES) has used eDNA to test for the presence of Dreissenid mussel eDNA in samples from across the Western United States as part of the laboratories standard operating procedure for the early detection of both zebra and quagga mussels. This technique is important for researchers and it is important to understand the limitations and capabilities of eDNA as a tool for the detection of a wide range of organisms in the environment.

This project accomplished three goals. First, a short literature review giving background on eDNA and the ways that is it collected in the field. Second, a laboratory study on the impact of inhibitors on detectability of quagga mussel veligers over time. Inhibitors, such as humic acid, are of major concern when analyzing environmental sample because these compounds can inhibit a PCR reaction and lead to a false-negative result. This study was undertaken to understand the importance of polymerase chain reaction (PCR) inhibitors on the outcome of assay. Finally, the author attended and presented at a two day eDNA workshop on August 2 & 3, 2016. Research focused on different aspects of eDNA research that had been conducted by many different governmental agencies and universities was presented.

The use of eDNA, as a tool for the detection of invasive and endangered species, will continue to grow in importance as scientists continue expand their knowledge and understanding of this method. Several future directions for this research were identified during this study. First, the use of next generation DNA sequencing to perform meta-barcoding of whole communities instead of having to design species-specific PCR assays will streamline testing for novel study species, and provide ecologically relevant information on the complement of organisms in an environment. Second, modeling eDNA results can aid our understanding of the interplay between environmental conditions and findings of positive results. Finally, determining how to correlate positive findings with the number of organisms present at a sampling site would aid management decisions that incorporate eDNA data. The use of eDNA will continue to grow as methods continue to improve. In the coming years RDLES will continue to perform eDNA research and collaborate with researchers to create new eDNA assays for invasive and endangered species.

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Main Report

Introduction

Environmental DNA (eDNA) is an emerging tool for the detection of both invasive and endangered species. There are several steps in the eDNA process. First, samples are collected either by filtration onto a filter membrane, or by collection and preservation of a bulk sample. DNA is then extracted from the sample, and analyzed using species-specific PCR primers. If there is a positive PCR result that means that the DNA of the species of interest was present in the sample. The technology at this time is not able to tell researchers how many of a particular species were present in the sampled environment. This report is broken into three parts. First, a brief literature review of eDNA, second a study of the impact of humic acid on PCR outcome, and finally, attendance at an eDNA workshop.

Part I: Literature Review of eDNA

The use of environmental DNA to monitor for invasive and endangered species has been a growing area of research for the last few years. There are some important questions that need to be addressed when analyzing eDNA. What exactly is eDNA? Is it free floating DNA in the environment or is it bound within a cell or tissue matrix? It can actually be both of these things (Figure 1). When a bulk environmental sample is taken it is made up of both intraorganismal and extraorganismal eDNA. In bulk samples, there is a mixture of nuclear, mitochondrial, and chloroplast DNA present. For the purposes of this report, eDNA is defined as DNA isolated from an environmental sample, and could have been bound in tissue or free floating.

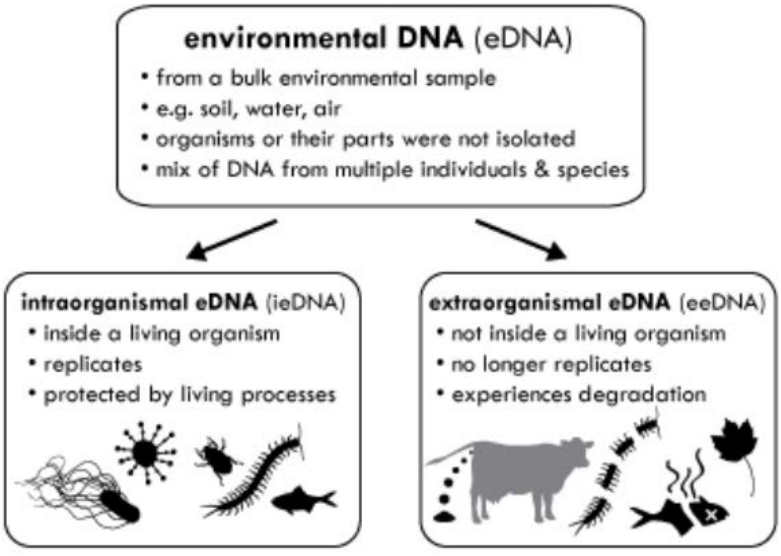

Figure 1: Sources of eDNA (from http://environmentaldna.com/, accessed 8/22/2016)

At RDLES, eDNA is used to test for the presence of invasive and endangered species in bulk water samples. The main invasive species that are monitored in the laboratory are *Dreissena bugensis* (quagga mussel) and *Dreissena polymorpha* (zebra mussel). For these assays, the DNA from a subsample of a bulk environmental sample is extracted and then polymerase chain reaction (PCR) is preformed to detect these two invasive mussels. This assay is a presence/absence test for mussel DNA. There are other uses for eDNA research that include diet analysis, inventory of biodiversity, and species distribution [1]–[4]. Determining the distribution of an invasive or endangered species is one of the key ways that eDNA analysis is being performed.

Many different species can be analyzed for by eDNA methods. One area of great interest has been for the analysis of aquatic organisms. Many of the initial eDNA assays were for frogs (American bullfrog) and fish (Asian Carp). These initial assays were performed to determine the presence or absence of these aquatic invasive species in a pond or lake [1]. Much research has also been done on the detection of endangered salamanders in streams. In addition to aquatic eDNA, fecal and soil samples can also be analyzed for eDNA. Fecal analysis can identify the organism and allow researchers to look at the diet. Soil samples can be analyzed for inhabitants that existed far into the past. The main focus of research at RDLES has been on aquatic eDNA.

One of the first eDNA tests was for the American bullfrog in French ponds in 2008 [1]. The Asian carp is another organism where eDNA has been used extensively to determine if it has spread into the Great Lakes [4]–[10]. Another area of eDNA detection has been for hellbender salamanders. These elusive organisms require intrusive field surveys to detect, however by using eDNA it is possible to detect these organisms without disrupting the environment [11].

Advantages and Disadvantages of eDNA

There are several advantages to using eDNA for the detection of species. For species that are rare or hard to detect, eDNA can be used to determine where the organism of interest might be. The cost of performing an eDNA survey when compared to traditional detection methods could be much less. At the same time, for organisms that are easy to find in the environment, eDNA testing could cost more. The relative cost, effort, and value of results of eDNA surveys, as compared with traditional survey techniques, have to be considered when deciding what detection methods to use.

Not every organism will readily be detected by eDNA. Organisms that live in small ponds can be easier to detect than those that live in rivers, lakes, or oceans. The scale of the water body impacts probability of detection. For an eDNA survey to be effective it is necessary for the organism to shed tissue, cells, and feces into the environment [4].

There are some disadvantages to eDNA analysis. First, detection of the target DNA does not mean that the organism of interest was in the environment at the time the sample was collected. A positive PCR result is indicative that the organism has been in proximity to the collection site, however, because eDNA has the potential to persist, it does not indicate if the organism is alive or dead, when the organism was present in the environment, or any population numbers. Determining how old the DNA is and how long it was present in the aquatic environment is difficult. There is ongoing research to overcome this issue that will hopefully allow researchers to create a better picture with positive PCR results.

Finally, in moving water, the eDNA signal can be diluted and moved away from the organism. So if there is a positive sample, the source DNA could be upstream from it. The distance that eDNA can travel from the source organism is an area where more research is needed.

Factors that Influence eDNA detection

There are many different factors in the environment that influence eDNA detection. First, the breakdown of eDNA in water can occur within a few days to a month [12], [13]. On the other hand, in soil eDNA can stay viable for decades. On one extreme there are even studies of soils collected from Greenland that have viable DNA that is 450-800 K years old [14]. In 2014, Pilliod et al., showed that it was possible to detect eDNA only for an hour after removing the organism of interest from running water [15]. There have been many different studies performed on the length of time that the eDNA is present in an aquatic system and detectible [16].

The degradation of eDNA in the environment occurs through several processes. The abiotic factors of temperature, UV radiation, amount of DNA present all impact the length of time that the eDNA stays in the environment [15]. Biotic factors influencing detection include bacterial degradation of eDNA. Once the sample is collected, it is important to remove PCR inhibitors during the DNA extraction process to improve detection probability.

Sample Collection Methods

eDNA samples can be collected in a variety of ways depending on the organism that is being test for. One of the major issues that researchers have encountered is cross-contamination in the field. This is one of the reasons that understanding contamination and preventing it is important. A second issue is that researchers need to have an understanding of where in the environment the organism resides. It would do no good to collect samples from a pond, when the organism is known to live in streams. By understanding the ecology, it helps to narrow down the potential sample sites.

There are two different ways that eDNA samples can be collected: filtration and precipitation [17]. For filtration methods, raw water is run through a filter

membrane, and then DNA is extracted from the membrane. Currently, there are four types of filters than are in use: glass fiber, cellulose nitrate, carbonate, and nylon. It is important that the filter pore size does not allow the DNA to pass through. With precipitation, water samples are collected and then sent to the laboratory for analysis.

The RDLES laboratory collects samples using plankton tow net with at 64 µm filter mesh. A bulk water sample is collected, baking soda is added to buffer the sample, and alcohol is added to preserve the sample. Samples are then shipped to the RDLES laboratory for analysis. Unlike other eDNA laboratories, RDLES performs both microscopy and PCR on bulk water samples for the detection of invasive dreissenid mussels. This necessitates the use of a much larger filter mesh size than is employed in most eDNA studies, as it is important to filter large quantities of water from the environment for microscopic identification of dreissenid veliger larvae.

It is important to understand the biology of the organism to determine the best sample collection sites and also if possible to sample during a breeding time to increase the odds of collecting DNA from the organism. The sample collection, either by filtration or precipitation, can affect the outcome. And should be carefully considered.

There are many reviews available that go into great detail on the use of eDNA. These reviews are listed here: [2], [3], [9], [17]–[23]

Future Directions

The use of eDNA analysis will continue to grow in the coming years as more research is performed and understanding of this method continues to grow. One area where there will be a great deal of growth is in the use of metabarcoding for the detection of multiple species of interest [24]. This technique will allow for many different organisms to be analyzed from a single sample at the same time. This will increase the amount of data that can collected and analyzed. There are still many unknowns about the use of positive eDNA findings for making management decisions.

Part II: Impact of inhibitors on eDNA detection

Humic Acid and DNA Detection

One of the limiting factors for environmental DNA analysis is the presence of inhibitors in water that can interfere with the PCR reaction. These chemicals can suppress the PCR reaction and lead to a false-negative in a sample where DNA from organism of interest is in fact present. One of the major environmental inhibitors is humic acid. This substance is produced during microbial degradation

of dead organic matter. It is a major component of soil and is also present dissolved in many aquatic environments. Humic acid is a complex mixture of acids that contain carboxyl and phenolate groups [25], and in raw water samples its presence can interfere with PCR analysis.

In an effort to understand the effects of humic acid on the outcome of PCR assays, a long-term study was performed to assess the impact of humic acid on the detection of quagga mussel veligers in water samples. For this study, veligers were placed in water collected from a local reservoir. The water was preserved with 20% alcohol and buffered according to RDLES field standard operating procedures. A known concentration of humic acid was spiked into half of the water samples. The concentration of humic acid that was used had in a previous study been shown to inhibit the PCR reaction. As a control, non-buffered and preserved water was analyzed side-by-side with the preserved samples. Microscopy and PCR was performed over twelve weeks on this water, to determine the impact of storage duration prior to analysis.

Materials and Methods

Sample Preparation

Water was collected at Chatfield Reservoir, Colorado in January 2016. The water was brought back to the RDLES Laboratory and filtered to remove zooplankton. The water was divided and processed in two different ways. In the first treatment, the reservoir water was buffered to pH 7.0 and preserved with 20% alcohol. Humic acid (1 ug/mL) was then added to the water. The tubes were set up in parallel with and without humic acid. These water samples where aliquoted into 50 mL conical tubes and stored at 4^{o}C until the veligers were added. In the second treatment, water was stored without any buffering or preservation. Following filtering to remove zooplankton, humic acid (1 ug/mL) was dissolved into the water and then this stock water was also aliquoted into 50 mL conical tubes.

Quagga mussel veligers were collected at Lake Mead, NV in January 2016 and preserved according to the RDLES Laboratory Field SOP. Baking soda and alcohol (20%) were added to preserve the sample prior to shipment to the RDLES Laboratory. Once the samples arrived, they were settled in settling cones overnight. The next day, the bottom 15 mL were collected from the settling cone and veligers were pipetted from the sample to create a veliger stock. Once the veligers were collected, 20 veligers were pipetted into the conical tubes.

Microscopy Analysis

In addition to DNA analysis, samples were also followed by microscopy. Over the twelve weeks the biofringence did not change for either the sample with humic acid or without humic acid.

DNA Extraction and PCR Analysis

Samples were analyzed according to the Polymerase Chain Reaction: Preparation and Analysis of Veliger Water Samples, PCR Laboratory Standard Operating Procedure (PCR SOP), version 4, RDLES Laboratory. Briefly, the 50 mL conical tubes were centrifuged for 30 minutes at 4500 x g. Following the centrifugation, the supernatant was poured off and the pellet was pipetted into the DNA extraction kit. The Mo Bio Ultra Soil DNA Kit (cat number) was used to extract the DNA.

After the DNA extraction was complete, samples were analyzed by PCR for the presence of quagga mussel DNA, according to RDLES PCR SOP for the detection of quagga mussels. The resulting PCR products were run on agarose gels and scored for positive or negative bands. Each sample was analyzed by PCR three times to get statistics on the number of positive results.

Results and Conclusions

The presence of humic acid in the preserved samples did not impact the outcome of most PCR analysis (Table 1 and Figure 2). Nearly all PCR reactions on buffered samples were positive, except at T=0 and 4, where some reactions on samples with humic acid did not produce positive bands. This could have been due to a pipetting error. In non-buffered samples no positive PCR results were found after week 6. Thus preserving the samples is key to maintaining the integrity of the DNA. The presence of humic acid in the non-buffered samples led to a quicker decrease in positive PCR outcome (week 4). Overall, however, humic acid at this concentration appears to have no impact on the ability of the PCR reaction to detect quagga mussel DNA. The soil DNA extraction kits are able to remove PCR inhibitors. When the sample is preserved correctly, the DNA remains intact and detectable by PCR.

Table 1: Results of humic acid study

Time (Weeks)	(-) HA	(+) HA	(-) HA, (-) Buffer	(+)HA, (-) Buffer
0	100	89	100	100
2	100	100	0	0
4	100	44	100	33
6	100	100	0	0
8	100	100	0	0
10	100	100	0	0
12	100	100	0	0

% Positive PCR Reactions

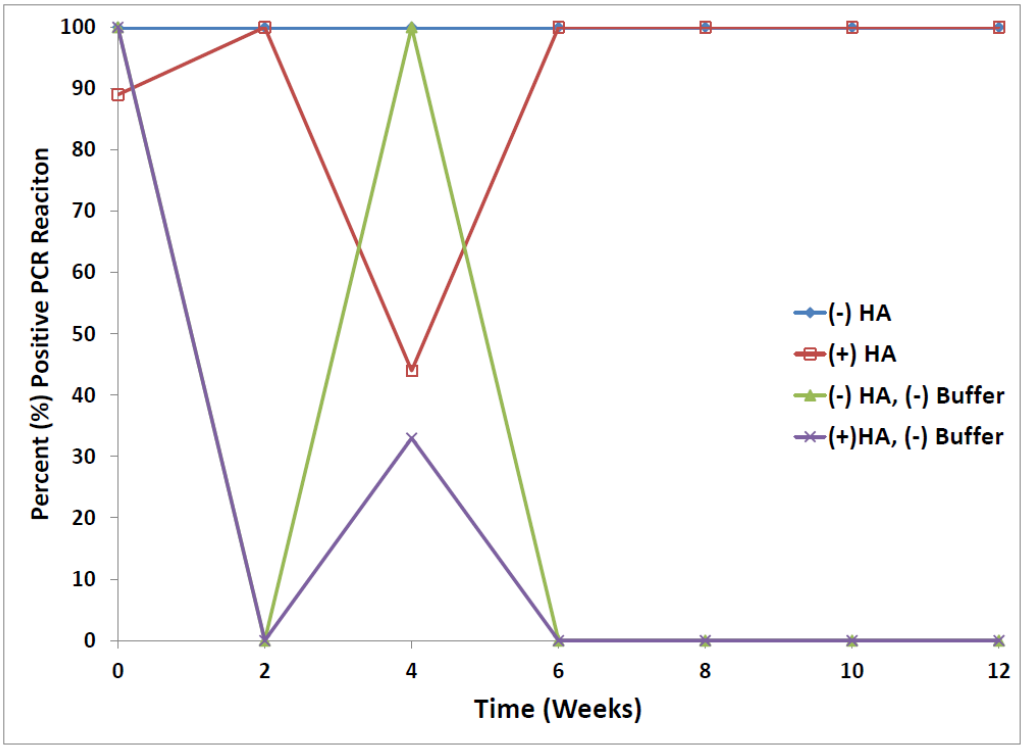

Figure 2: Percent positive PCR over twelve weeks

One of the factors that is known to impact the samples is pH. The pH of the preserved samples all started at 7 and over the course of the experiment remained around 7 for all the time points. The non-preserved samples pH started at 5.5 and remained that way throughout the entire experiment.

Part III: eDNA Workshop

The Bureau of Reclamation hosted the Inaugural Environmental DNA Training &Technical Exchange Workshop, August 2-3, 2016. Scientists from a wide range of federal agencies and also universities spent two days discussing eDNA research. There were six sessions that focused on different aspects of eDNA collection, processing, analysis, and data management. The author of this report was able to give one of the presentations. The presentation is attached in Appendix 1.

Summary

The use of eDNA to monitor rare or elusive organisms is going to continue to increase. Understanding the advantages and disadvantages of this technique is important to both researchers and managers. There are still many unknowns about eDNA, for example how to correlate the PCR result with a number of organisms

present in the environment. In addition, understanding how to overcome the impact of inhibitors, such as humic acid, so that false negatives can be avoided is another technical issue that has to be overcome. Over the last few years the RDLES Laboratory has developed robust techniques for the identification of eDNA from dreissenid mussels. The laboratory is continuing to refine this work, and is applying its expertise to eDNA analysis of other invasive and endangered species in Reclamation waters.

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Data sets that support the final report

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nted at the eDNA meeting