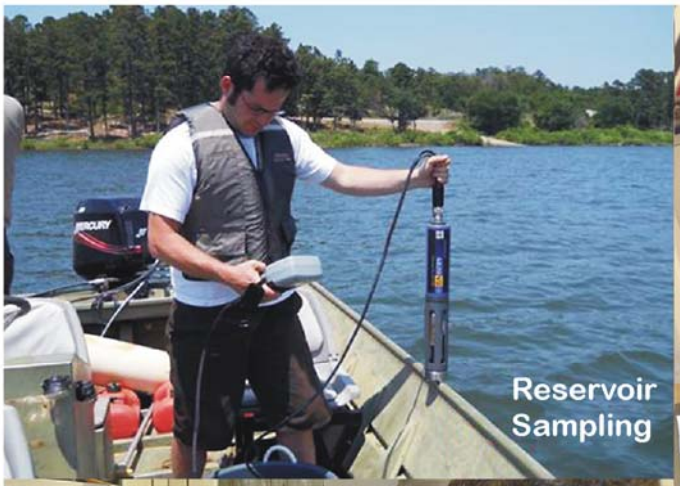
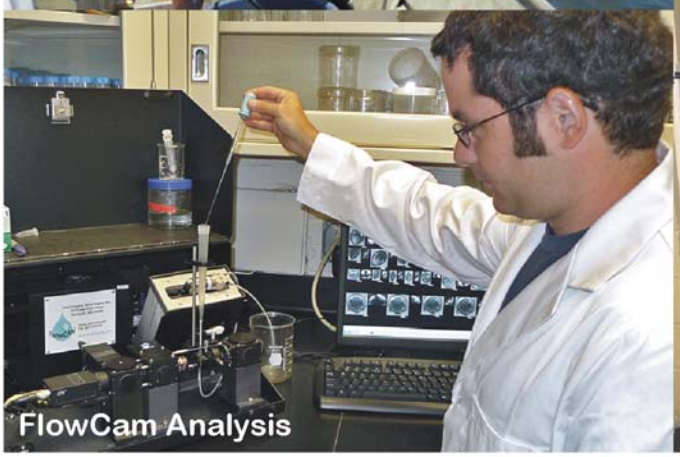
RECLAMATION

Managing Water in the West

Technical Memorandum No. 86-68220-13-10

Improving Accuracy in the Detection of Dreissenid Mussel Larvae





U.S. Department of the Interior
Bureau of Reclamation

August 2013

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.

The mission of the Reclamation Detection Laboratory for Invasive and Native Species is to collect, detect, inform: protecting the waters of the West.

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Improving Accuracy in the Detection of Dreissenid Mussel Larvae

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PEER REVIEW DOCUMENTATION

PROJECT AND DOCUMENT INFORMATION

Project Name: Reclamation Detection Laboratory for Invasive and Native Species
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Document Improving Accuracy in the Detection of Dreissenid Mussel Larvae

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Team Leader: Denise M. Hosler

Leadership Team: NA

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Document Author(s)/Preparer(s) Laboratory Lab Team

Peer Reviewer: Curt Brown, Director Research and Development

REVIEW REQUIREMENT

Part A: Document Does Not Require Peer Review

Explain: NA

Part B: Document Requires Peer Review: SCOPE OF PEER REVIEW

Peer Review restricted to the following Items/Section(s): Total document
Reviewer: Jamie Carmon, Kyle Rulli

REVIEW CERTIFICATION

Peer Reviewer - I have reviewed the assigned Items/Section(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: Jamie Carmon Review Date: August 28, 2013

Signature: Jamie Carmon

Reviewer: Kyle Rulli Review Date: August 28, 2013

Signature: Kyle Rulli

Preparer - I have discussed the above document and review requirements with the Peer Reviewer and believe that this review is completed, and that the document will meet the requirements of the project.

Team Lead: Denise M. Hosler Date: Sept 9, 2013
Signature

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ACRONYMS AND ABBREVIATIONS

CPLM	cross polarized light microscopy
DI	deionized
DNA	Deoxyribonucleic acid
LM	light microscopy
mL	milliliters
PCR	polymerase chain reaction
QA/QC	quality assurance and quality control
quagga mussel	*Dreissena rostriformes bugensis*
Reclamation Detection Laboratory	Reclamation Detection Laboratory for Invasive and Native Species, Denver, Colorado
SEM	scanning electron microscopy
SOP	Standard Operating Procedure
veliger	microscopic larvae
zebra mussel	*Dreissena polymorpha*

INTRODUCTION

History

The Reclamation Detection Laboratory for Invasive and Native Species (Reclamation Detection Laboratory) in Denver, Colorado, initially established in 1995, analyzed a few hundred samples a year for the presence of invasive dreissenid mussels. The American Recovery and Reinvestment Act of 2009 provided the small laboratory with the funds to grow, leading to the processing of thousands of samples a year. Currently, the laboratory not only specializes in invasive mussels, but has extended into species identification. The laboratory utilizes taxonomic and genetic testing to identify organisms, both native and invasive. The focus of this report is on quagga mussels, specifically the ongoing research that is conducted by the Reclamation Detection Laboratory.

Two species of dreissenid mussels are invasive in the United States; the *Dreissena rostriformes bugensis* (quagga mussel) and the *Dreissena polymorpha* (zebra mussel). Originally introduced into the Great Lakes region, *Driessena* have spread across the United States. Zebra mussel populations established quickly and spread down the east coast, whereas the spread west of the Mississippi River has been slower and predominately quagga mussels. Water quality parameters are thought to be one of the main reasons for the slow spread of mussels in the West, such as turbidity, temperature, pH, calcium content, and rapid drawdowns of reservoirs.

Visually there is little difference between these two invasive species of dreissenid mussel, both have striped shells and are small, about the size of a thumbnail. Quagga and zebra mussels are filter feeders. One adult is able to filter 1 liter of water a day resulting in the removal of phytoplankton, zooplankton, and nutrients from the water. Quagga and zebra mussels are also major biofoulers, clogging water intake structures that impact water treatment facilities and power-producing infrastructure. Dreissenid mussels are prolific breeders with a single female producing 1 million eggs per year. After the eggs are fertilized, microscopic larvae (veligers) develop in a few days. Free swimming veligers drift in currents between 1 to 2 months before settling on substrates.

Adult populations of dreissenid mussels are difficult to discover by normal field sampling procedures. They prefer subdued light and flowing water and the small size of the adult makes detection by divers difficult. The Reclamation Detection Laboratory has discovered that identifying the mussels in their veliger stage gives water managers an earlier warning about a possible infestation than waiting for adult populations.

Veligers

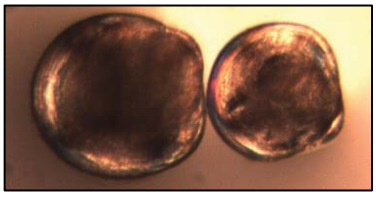
Veligers are the larval form of any bivalve. For the purpose of the early detection of dreissenid mussels, veliger refers to the larval form of the quagga or zebra mussel. Veligers are microscopic, between 97 and 492 microns; therefore, the Reclamation Detection Laboratory uses multiple testing methods to ensure that each sample result is confirmed. Sample handling in the field is the first step in determining the presence or absence of dreissenid mussels. Vertical tow samples are collected, by lowering a 64-micron plankton tow net to the bottom of a water body at several locations. Each sample is preserved with alcohol and buffered with baking soda to stabilize the pH until analysis is complete.

At the laboratory, the sample is analyzed by microscopy for the presence or absence of veligers. The primary testing method is cross polarized light microscopy (CPLM) as it outperforms light microscopy (LM) in detecting veligers in a raw water sample. Scanning electron microscopy (SEM) allows for an enhanced visual image of a veliger, which is later used by trained taxonomists to determine the identity of an organism. When a suspect veliger is found in the sample, a subsample is taken out for polymerase chain reaction (PCR) to determine the presence or absence of the quagga or zebra DNA. If the PCR result is positive, the DNA is sent to a commercial laboratory for gene sequencing. The result from the gene sequencing is matched to the known sequence and, if over 98 percent of the sequence matches, then the sample passes quality assurance and quality control parameters (QA/QC), and the raw water sample is classified as positive for dreissenid mussel DNA.

This report is to also provide quick reference material to the methods and procedures used in the ongoing research that is conducted by the Reclamation Detection Laboratory, such as:

- Testing Methods
- Veliger Degradation
- Decontamination Procedures
- Controlling Contamination
- Quagga Mussel Model Study (PCR)

Important Terms for Early Detection of Dreissenid Mussels



Dreissenid Mussel

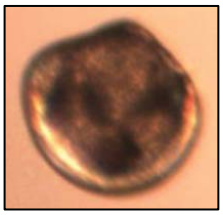
Two species infest the United States, *Dreissena rostriformes bugensis* (quagga mussel) and *Dreissena polymorpha* (zebra mussel).

Veliger

Larval form of any bivalve. (For the purpose of this report, veliger refers to the microscopic larval life stage of the quagga and zebra mussel).

CPLM (Cross Polarized Light Microscopy)

The primary testing method for veliger identification; the veliger will shine like a diamond on a black background, displaying a distinctive Maltese cross pattern on the shell.



LM (Light Microscopy)

Cross polarized filters are removed from the dissecting scope and the sample is analyzed under regular LM.

PCR (Polymerase Chain Reaction)

The Reclamation Detection Laboratory uses PCR to determine the presence or absence of dreissenid mussel DNA in raw water samples.

Buffer

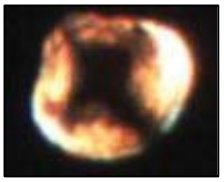
Usually baking soda, used to stabilize the pH and buffer a raw water sample. The Reclamation Detection Laboratory uses 0.2 gram of baking soda per 100 milliliters (mL) of a raw water sample.

Imhoff Cone With Passive Venoset System

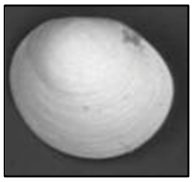
Settling cones used to settle raw water samples overnight.

TESTING METHODS

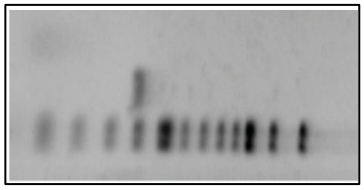
The Reclamation Detection Laboratory utilizes multiple testing methods when detecting the presence or absence of invasive dreissenid mussels. Microscopy is the most effective method of detecting veligers, and the Reclamation Detection Laboratory utilizes CPLM as the primary testing method for microscopic detection of veligers. When a suspect organism is found in a sample, the suspect is sent for SEM analysis. A subsample of the raw water sample is analyzed using PCR and the PCR result is confirmed by an outside laboratory by gene sequencing.



CPLM



SEM



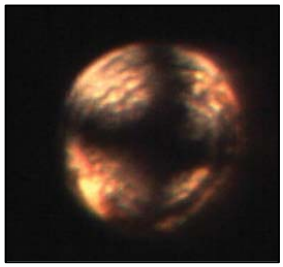
PCR

Gene Sequencing

One positive result does not mean a sustainable mussel population will develop. There are many water quality parameters that may inhibit infestation, such as calcium content, turbidity, rapid drawdown of the reservoir, temperature, and pH.

Microscopy

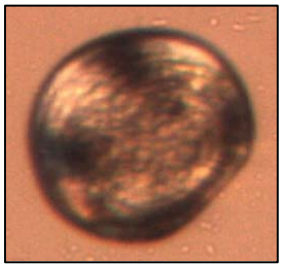
A dissecting microscope is equipped with cross polarized filters that create a black background and causes the calcium carbonate of the veliger shell to glow as light passes through the filters. Some portion of the shell will fall in line with the axis of the cross polarizing filters creating a distinctive Maltese cross pattern on the shell. The Maltese cross pattern is the main identifying feature for veliger identification using CPLM.



- Bright veligers are easy to detect against the black background.
- The distinctive cross on the shell is easy to identify amongst zooplankton and algae.
- Internal organs are not visible with CPLM.

LM does not utilize the cross polarizing filters on the dissecting scope and allows the technician to view all the organisms in the sample, including non-target organisms. Detection using LM is difficult as raw water samples are comprised predominately of zooplankton and algae.

- Difficult to detect amongst zooplankton and algae.
- No distinctive cross on the shell for easy detection.
- Internal organs and veliger integrity is visible with LM.



One veliger discovered in a raw water sample by CPLM or LM does not mean that a sustainable population will develop. However, it does indicate that an inoculation has occurred, potentially from a boater, and education efforts should be increased to amplify awareness of invasive mussels with the public.

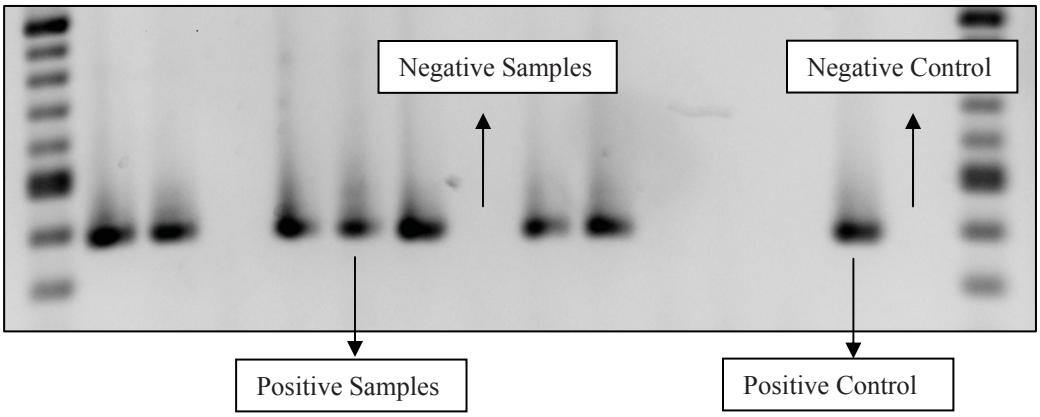
Polymerase Chain Reaction

Once a suspect is found by CPLM or LM, all subsequent raw water samples are subject to PCR testing.

The Reclamation Detection Laboratory uses PCR as a secondary testing method that determines the presence or absence of dreissenid mussel DNA. A subsample of the raw water sample is centrifuged for 30 minutes and DNA is extracted using a commercial kit that is optimized for extracting DNA from soil samples. This soil kit is used as it will break open the calcium carbonate veliger shell and optimizes detection of dreissenid DNA.

Species-specific primers are used to isolate and amplify the target gene used for the detection of dreissenid DNA. The amplified DNA is run on an electrophoresis gel and if the raw water sample contains dreissenid DNA, a band is produced on

the gel. When a band is produced on the gel, a portion of the amplified DNA is sent to an outside commercial laboratory where the gene sequence of the amplified DNA is matched to the known sequence. Only a gene sequencing result with a 98 percent or higher will pass QA/QC, and the raw water sample will be recorded as a positive result.



One positive PCR result does not mean a sustainable population will develop. A positive PCR result does mean that dreissenid DNA was in the sample and education efforts should be increased at the reservoir.

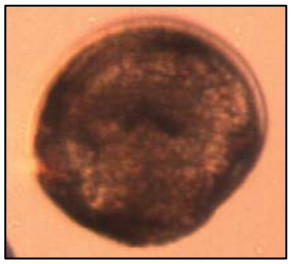
VELIGER DEGRADATION

What does pH have to do with it?

The Reclamation Detection Laboratory has determined that sample preservation is one of the most important factors in the detection of a veliger from a raw water sample. Samples with a pH below 7 degrade the veliger shell making the shell undetectable by CPLM. Raw water samples will become acidic over time, due to the addition of alcohol that euthanizes and preserves the sample. Buffering the sample with baking soda stabilizes the pH and preserves the veliger shell for easier detection by microscopy.

Sample Preservation Methods
1. Preserve sample by adding 20 percent alcohol to raw water sample volume.
2. Add 0.2 gram of baking soda per 100 mL of raw water sample.

Veliger in Buffered Sample



Day 7: pH 8

- Tissue is visible.
- Shell is whole.
- Detectable by CPLM.

Veliger in Unbuffered Sample

Day 7: pH 5

- Tissue is degraded.
- Shell is degraded.
- Not detectable by CPLM.

Acetic pH Versus Basic pH

To determine how important pH is on veliger shell integrity, 5 liters of deionized (DI) water was buffered using the Reclamation Detection Laboratory Standard Operating Procedure (SOP) for the field. Baking soda (0.2 gram of baking soda is added per 100 mL of DI water) was added and stirred with a magnetic stir bar until all the baking soda had dissolved into the water (pH 8). In a separate container, 5 liters of DI water was left unbuffered (pH 5). Each water type was aliquoted into 40-mL containers and 100, 50, and 25 quagga veligers were placed

in replicates of three for each water type and monitored for 42 days. No alcohol was added to the samples so veliger degradation could be monitored.

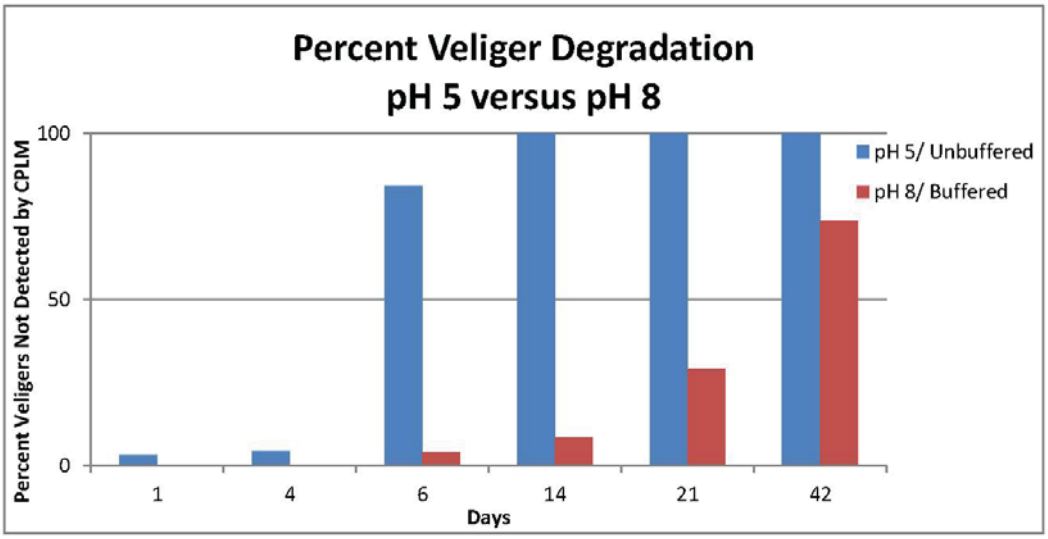


Figure 1.—Percent of veliger degradation (pH 5 versus pH 8).

All veligers in the DI water with pH 5 were undetected by CPLM by day 14, while the veligers in the DI water with pH 8 were detected by CPLM through the duration of the experiment. The pH of both sample types of water maintained consistent for the 42 days. Veliger detection by CPLM in the pH 8 water did decrease to less than 30 percent emphasizing the need to preserve samples with alcohol, as well as buffering the sample to raise the pH and stabilize the integrity of a veliger over time (figure 1).

Cross Polarized Light Microscopy Versus Light Microscopy

Baking soda increases and stabilizes the pH of raw water samples, which aid in preserving veliger recovery by CPLM over time.

The veligers in DI water with pH 5 were undetectable by CPLM by day 14. The veligers were still detected by LM (figure 2), which can create confusion because a degraded veliger is difficult to identify and is too fragile to be identified by SEM.

The veligers in the DI water with a pH 8 degraded much slower and were still detected by CPLM for the duration of the experiment (figure 3). Degraded veligers that are undetectable by CPLM may still be visible by LM, but with difficulty as zooplankton and algae impede and confuse visual confirmation.

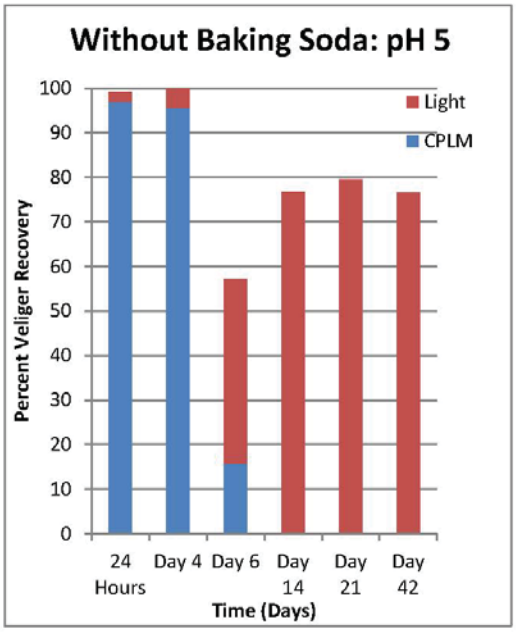


Figure 2.—Unbuffered sample without baking soda (pH 5).

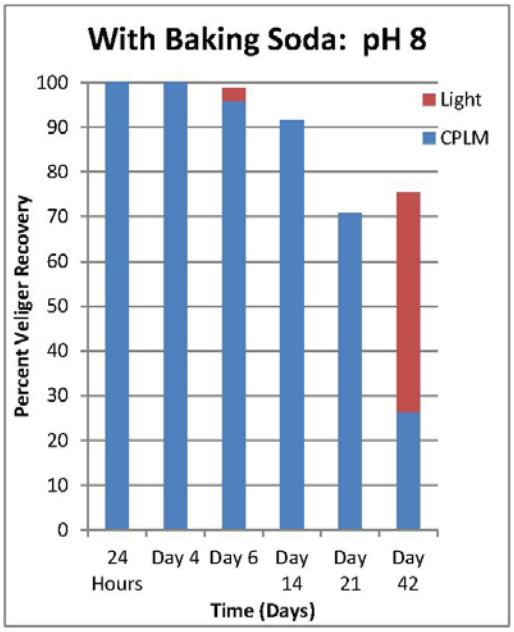
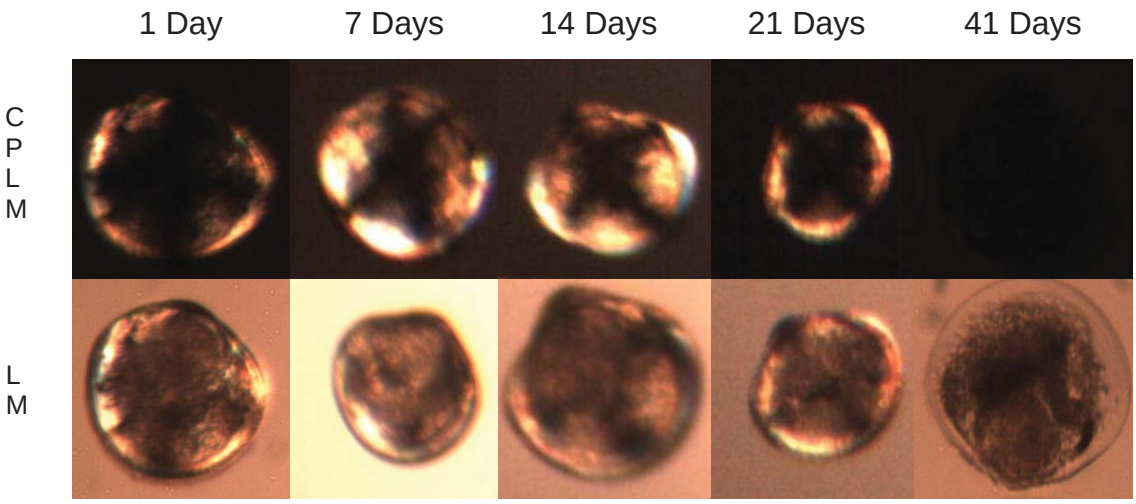


Figure 3.—Buffered sample with baking soda (pH 8).

Morphological Changes (pH 8)

Baking soda raises and stabilizes the pH, better preserving the veliger shell. The pictures below show quagga veligers in samples not preserved with alcohol and buffered with baking soda per the Reclamation Detection Laboratory SOP for the field. The procedure requires 0.2 gram of baking soda per 100 mL of raw water sample.

Veligers in DI water with pH 8 degrade and become undetected by CPLM, but the degradation is much slower than if the sample is unbuffered (pH 5). Adding 20 percent alcohol by volume to the sample will increase the integrity of the veliger over time.

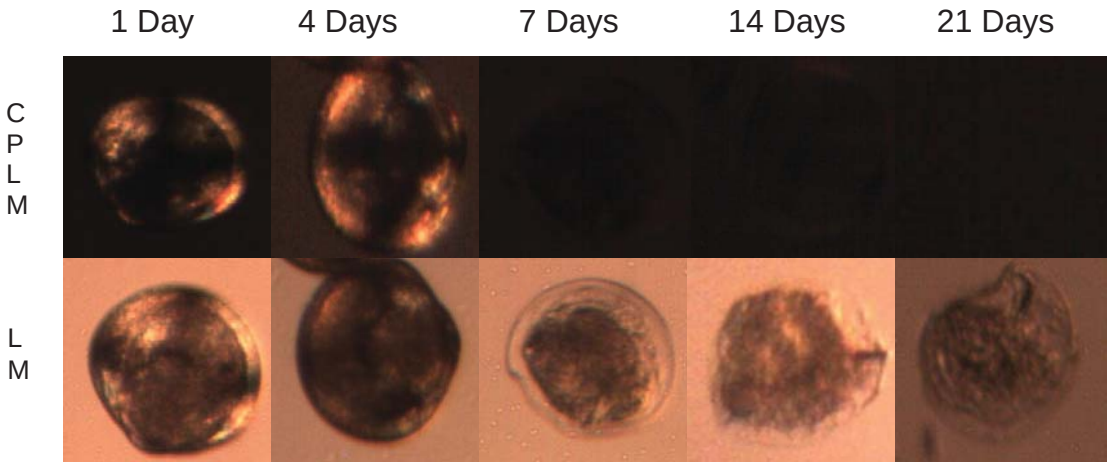


Morphological Changes (pH 5)

The pictures below show how quagga veligers degrade over time when DI water is unbuffered (pH 5) and no alcohol is added to the DI water.

Veligers are detected by CPLM at day 1 and day 4. By day 14, however, CPLM is ineffective for detection. Veligers are detectable by LM, but are difficult to identify due to degradation.

Buffering samples with baking soda and preserving the sample in alcohol is the most effective way to preserve samples over time.



Polymerase Chain Reaction Analysis

Samples where DI water was not preserved with alcohol or buffered with baking soda (pH 5) resulted in no quagga veligers being detected by CPLM by day 14. Since the veliger body is still detected by LM, PCR was run on these samples to determine if a positive PCR result could occur from degraded veligers.

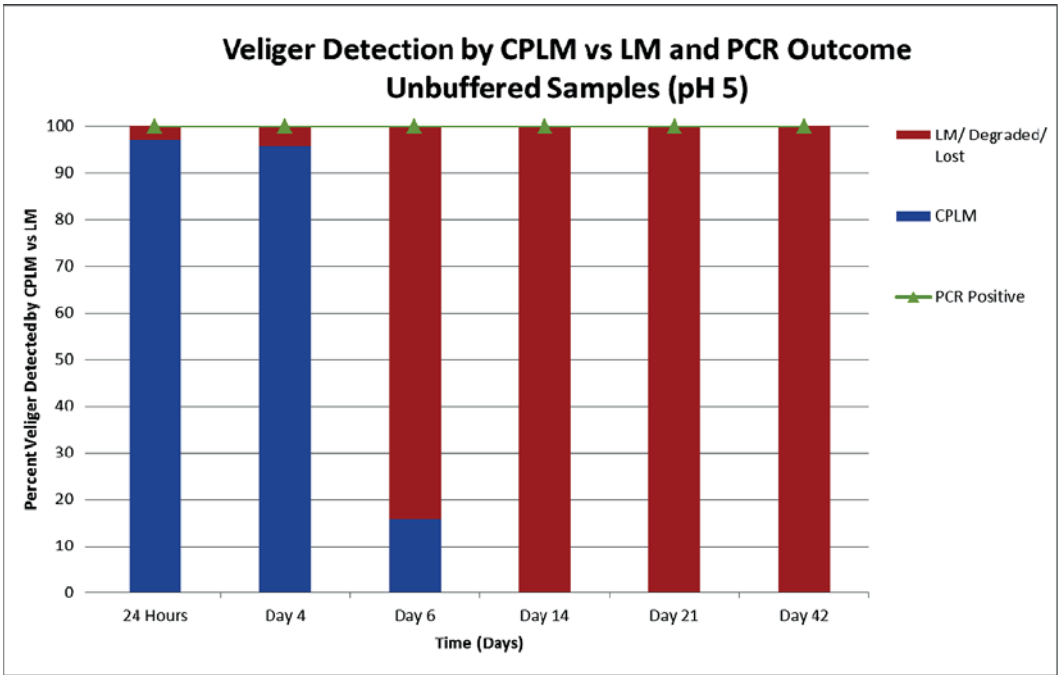


Figure 4.—Veliger detection by CPLM versus LM, and PCR outcome on unbuffered samples (pH 5).

Even samples with only 25 veligers still produced a positive PCR result when all the veligers were degraded and undetected by CPLM.

DECONTAMINATION PROCEDURES

Laboratory and Field

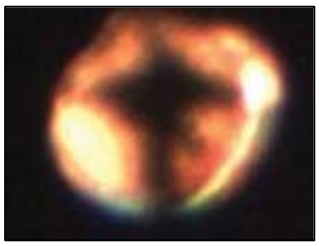
The Reclamation Detection Laboratory requires that all laboratory and field equipment be decontaminated in 5 percent acetic acid (vinegar). Vinegar (pH 2.5) degrades the shell and makes the shell undetectable by CPLM.

The Reclamation Detection Laboratory SOP for the field also requires that all laboratory and field equipment be decontaminated in bleach as bleach degrades residual DNA.

Below are how quagga veliger morphology changes in DI water, bleach, and vinegar:

DI Water: After 15 minutes,

- Shell is intact.
- Distinctive cross on the shell.
- Detectable by CPLM.

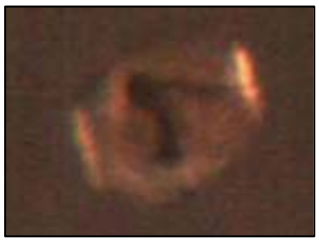


Bleach: After 15 minutes,

- Shell is bleached.
- Internal organs are gone.
- Shell is fragile and will break when touched.
- Distinctive cross on the shell.
- Detectable by CPLM.
- Could result in false positives.

Vinegar: After 15 minutes,

- Shell is degraded.
- Distinctive cross has disappeared on the shell.
- Not detectable by CPLM.
- May still be detectable by LM.

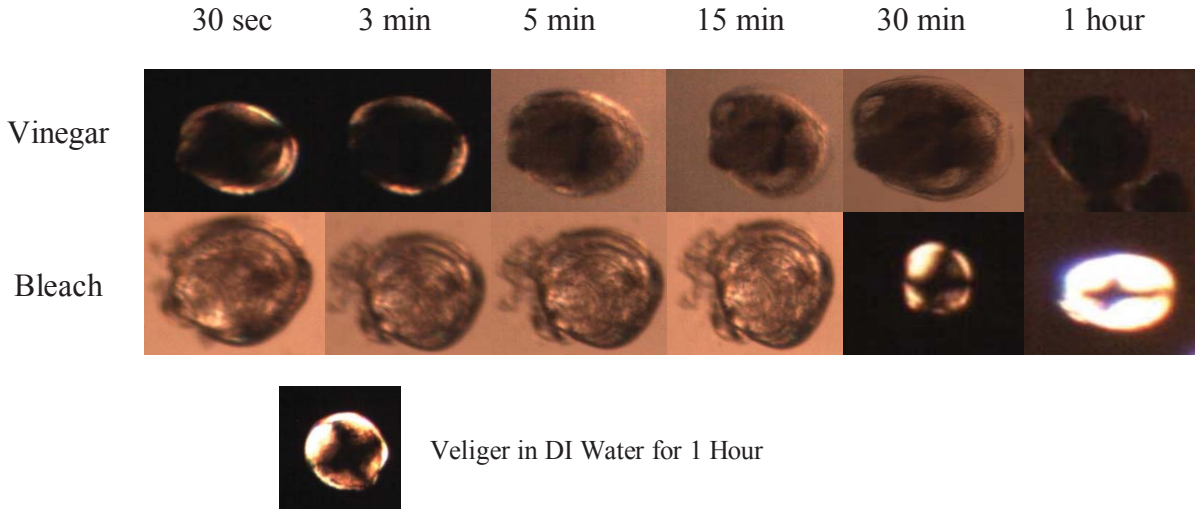


Effects of Vinegar and Bleach on Veliger Morphology—Microscopy

Laboratory and field equipment are soaked in vinegar (pH 2.5), which degrades the calcium carbonate shell making veligers unidentifiable with CPLM. A bleach rinse degrades any free-floating DNA that may still be present on any laboratory or field equipment.

A quagga veliger was covered in vinegar and monitored for 1 hour. After 3 minutes, the veliger cannot be identified under CPLM. The veliger is still detectable using LM; however, detecting veligers with LM is difficult in raw water samples.

A quagga veliger was covered in bleach and monitored for 1 hour. Immediately, the bleach penetrated the calcium carbonate shell causing the internal organs to leak out of the shell. After 3 minutes the initial release of internal organs is complete. Pictures at 30 minutes and 1 hour show that the veliger is still identifiable by CPLM. In fact, the veliger shell in bleach actually appears brighter under CPLM.



Veliger in DI Water for 1 Hour

Vinegar degrades the veliger shell quickly resulting in a negative result with CPLM. Veligers soaking in vinegar after 1 hour are still present in the water sample, but undetected by CPLM.

The use of bleach results in weaker shell integrity and slightest nudge will shatter the veliger body; however, the bleaching of the shell could result in some false positives by microscopy.

The Reclamation Detection Laboratory concludes that vinegar is the preferred method to degrade veliger morphology for microscopy. However, bleach degrades DNA and is useful to degrade any free-floating tissue that may be a cause of contamination.

Effects of Vinegar and Bleach on Veliger Detection—Polymerase Chain Reaction Analysis

Early detection for the presence of dreissenid mussels relies on both microscopy and genetic analysis of a raw water sample. All DNA is extracted from a subsample of a raw water sample. Species-specific primers of the target DNA are used for PCR. The amplified PCR product is analyzed for the presence or absence of the target DNA.

The Reclamation Detection Laboratory decontamination procedures require that all laboratory and field equipment be soaked in a 5-percent acetic acid solution (vinegar). The vinegar degrades the calcium carbonate shell resulting in a negative microscopy result. A bleach rinse is added to eliminate any free-floating tissue that may have a positive result by PCR.

Fifty quagga veligers were placed in DI water, vinegar, or bleach for 5, 10, 15, and 30 minutes in replicates of three. Each time point was analyzed for the presence or absence of quagga mussel DNA (figure 5).

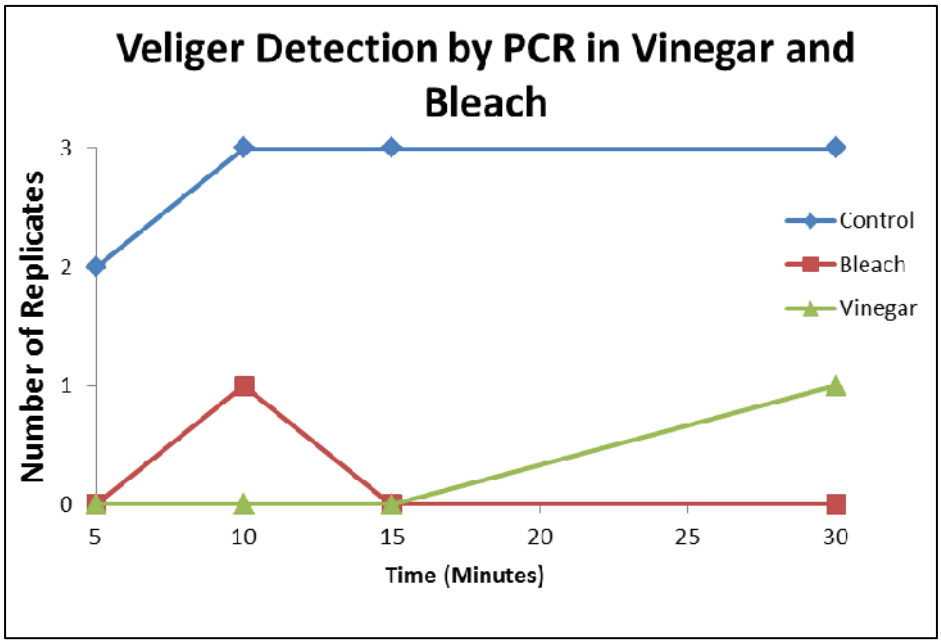


Figure 5.—Veliger detection by PCR in vinegar and bleach.

The positive PCR result for vinegar at 30 minutes shows that vinegar may not degrade all of the tissue in a sample. The positive PCR result for bleach at 10 minutes indicates that some veligers close tightly upon collection and the bleach cannot penetrate the shell to degrade the DNA in that short amount of time.

Microscopy analysis is completed first and would have shown the degraded and bleached veligers indicating that a cross contamination occurred. Laboratory and field equipment are soaked in acetic acid for hours after which time the acetic pH will have degraded the veliger body. In addition, a bleach rinse is used to remove any lingering DNA.

The Reclamation Detection Laboratory concludes that both a vinegar soak and a bleach rinse are necessary to remove all contamination from field and laboratory equipment.

CONTROLLING CONTAMINATION

Settling Cones

Microscopy

Imhoff cones modified with a passive venoset system are used to settle raw water samples overnight. After settling, 98 percent of veligers are recovered in the bottom 15 mL of the cone. To determine if veligers from a previous sample could contaminate the cones, 160 mL of quagga veliger-infested water was placed into three cones, settled overnight, and monitored at each decontamination step. All liquid in the cone was analyzed by CPLM and LM. DI water was used to rinse the cones and all scrubbing steps were skipped to avoid damage to the veliger shell, which can inhibit detection by microscopy.

The Reclamation Detection Laboratory requires an extensive decontamination procedure after every sample:

1. Scrub and rinse cone three times with DI water.
2. Soak cone in 250 mL of 5-percent acetic acid for 4 hours.
3. Scrub and rinse cone three times with DI water.
4. Rinse cone in bleach for 30 seconds.
5. Scrub and rinse cone twice with DI water.

No veligers were detected after the first acetic acid rinse. It is likely that scrubbing the cones with a brush would have removed the veligers from the cone before the acetic acid step.

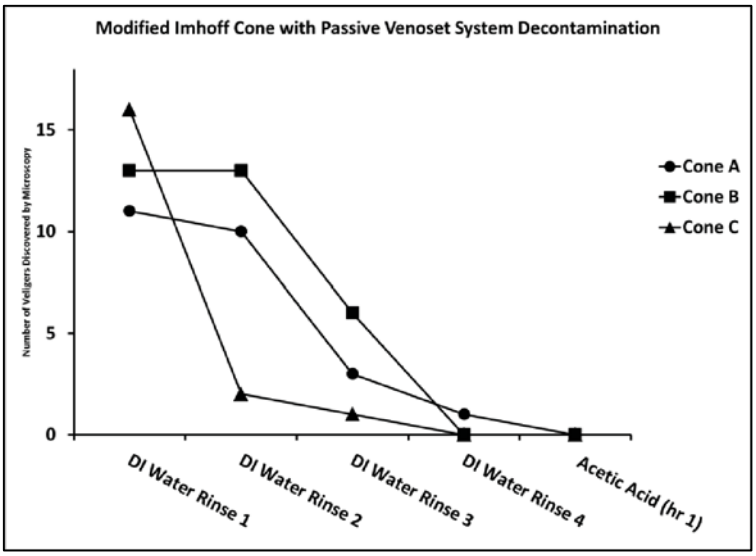


Figure 6.—Modified Imhoff cone with passive venoset system decontamination.

The Reclamation Detection Laboratory concludes that the protocol for decontamination of the settling cones is adequate to degrade the veliger body, eliminating the risk of cross contamination for microscopy.

Polymerase Chain Reaction

To determine the effectiveness of the Reclamation Detection Laboratory decontamination of the settling cones for dreissenid mussel DNA that may linger in the cone, the settling cone contamination study was duplicated and analyzed using PCR.

Repeating the settling cone contamination study, 160 mL of quagga veliger-infested water was placed into three Imhoff cones modified with a passive venoset system, and settled overnight. Then, 40-mL aliquots were taken at each decontamination step and analyzed by PCR for the presence or absence of dreissenid DNA. DI water was used to rinse the cones and all scrubbing steps were skipped to avoid damage to the veliger shell, which can inhibit detection by PCR.

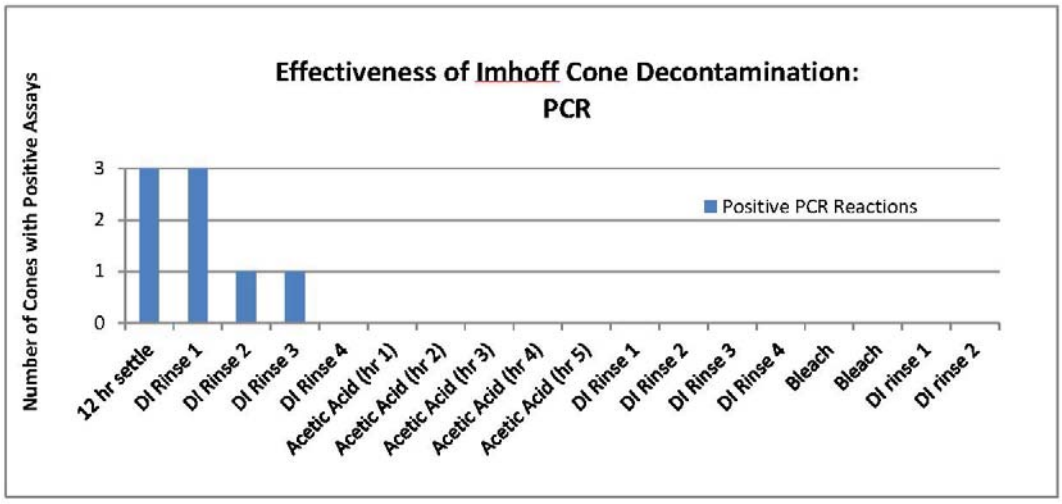


Figure 7.—Effectiveness of Imhoff cone decontamination (PCR).

All DNA is removed by rinsing the cones. Scrubbing the cones would aid in the removal of any veligers or any lingering DNA prior to the first acetic acid soak.

The Reclamation Detection Laboratory concludes that the protocol for decontamination of the settling cones is adequate to degrade the veliger body, eliminating the risk of cross contamination for both microscopy and PCR.

Wipe Tests for Polymerase Chain Reaction

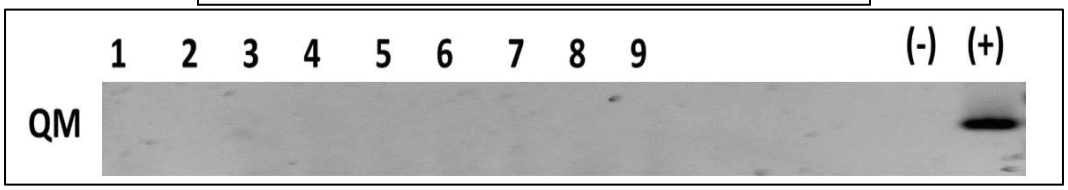
One way that the Reclamation Detection Laboratory determines the possibility of contamination is through wipe tests. These wipe tests analyze different areas on the laboratory to check for ambient dreissenid tissue and DNA. Wipe tests are routinely performed on the areas around the microscopes, centrifuges, and DNA/PCR/gel-running areas of the laboratory.

In addition, open tubes with DI water are set out in the DNA and PCR hoods to check for ambient DNA. The wipe test is either taken directly to PCR or the DNA is extracted. This enables detection of both ambient DNA contamination and any DNA from dreissenid tissue contamination.

Wipe tests have not yielded any areas of the laboratory testing positive for the presence of dreissenid tissue or DNA.

The Reclamation Detection Laboratory is continually aware of the possibility of cross contamination and takes steps to control for it. Dedicated equipment and disposable laboratory materials are used to decrease the risk of cross contamination. The Reclamation Detection Laboratory's continued vigilance will aid in controlling the risks of cross contamination in all aspects of early detection, from the field to the microscopy and PCR laboratories.

Common picture of the PCR result for a wipe test.



QUAGGA MUSSEL MODEL STUDY (PCR)

Is it possible to obtain viable DNA from a dried quagga mussel model?

Quagga mussel models are a valuable visual tool used to educate the public about the deleterious effects of dreissenid mussel infestation. What is the possibility that enough viable DNA will be present on the model to yield a positive result by PCR? For this study, a rope model incrusted with quagga mussels was used to obtain different types of samples. Three replicates of the following samples were collected for DNA analysis:

A) Agitated model and collected particulates that fell off the model.
B) One half shell rinsed with DI water.
C) Three half shells rinsed with DI water.
D) One closed shell, crushed.
E) Three closed shells, crushed.
F) Brushed wet paintbrush over model.
G) Wet net laid on model for 45 minutes.
H) Gloves swabbed with Q-tip after steps: A, C, E, F, and G.

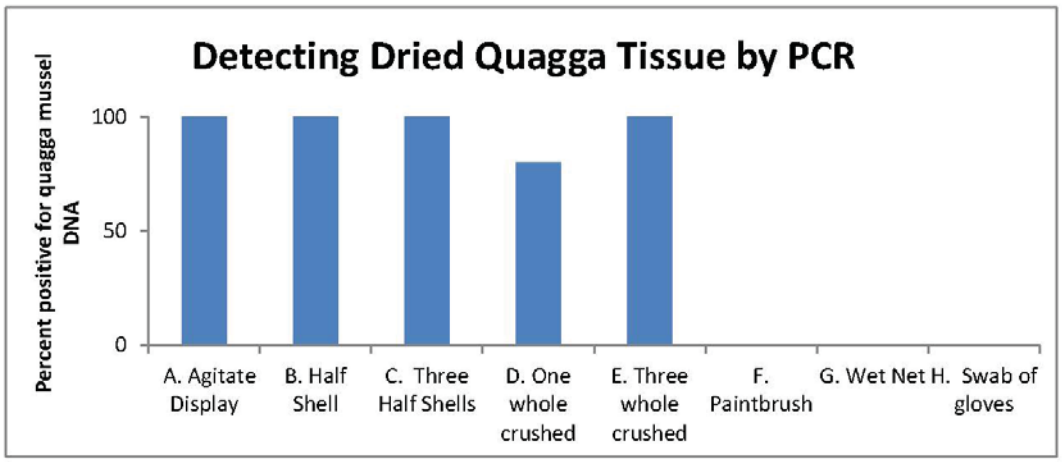


Figure 8.—Detecting dried quagga tissue by PCR.

Quagga mussel models contain tissue with viable DNA. While it is possible to get a positive DNA result from a dead and dried organism, some part of the mussel's tissue must be included in the sample to achieve a positive result.

The samples with the paintbrush, wet net, and glove swabs did not yield enough DNA to produce a positive PCR result. In addition, the one whole crushed shell did not yield 100-percent positive PCR results. Instead, one sample came back negative, showing that not all the shells on the model contained tissue with enough DNA for the assay to detect.