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Research Article

Effects of buffer and isopropanol alcohol concentration on detection of quagga mussel (*Dreissena bugensis*) birefringence and DNA

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Abstract

The long term effects of sample preservation on dreissenid veliger detection by cross polarized light microscopy (CPLM) and polymerase chain reaction (PCR) is poorly understood. This study examined how buffer and isopropanol alcohol impact veliger morphology and detection. Veliger detection by CPLM was completely inhibited after 14 days in acidic deionized (DI) water without buffer or alcohol, but veligers were still detectable by PCR. Veliger detection by CPLM increased when the pH of samples was buffered with 0.2 grams of sodium bicarbonate per 100 mL of raw water sample, but long term deterioration was not completely prevented without the addition of isopropanol alcohol and buffer. After 36 days, 100% of veligers were detected by CPLM in samples preserved with 20, 50, and 70% isopropanol alcohol and 0.2 grams of sodium bicarbonate per 100 mL. This study indicates the significant role that sample preservation plays in the accurate identification of veligers in early detection sampling.

Key words: dreissenid mussels, preservation, cross polarized light microscopy, polymerase chain reaction, monitoring, calcium carbonate shell, early detection

Introduction

The spread of non-native zebra (*Dreissena polymorpha*, Pallas 1771) and quagga mussels (*D. bugensis*, Andrusov, 1897) has become a major threat to American waterways. Species in the genus *Dreissena* are highly polymorphic and prolific, with great potential for rapid adaptation (Mills et al. 1996). Originally introduced into the Great Lakes region, the mussels are now found across the United States. The dreissenid invasion of North America has already had substantial effects, both ecologically (Barbiero et al. 2006; Higgins et al. 2011; McCabe et al. 2006), and economically (Mackie and Claudi 2010). In the western states, the Bureau of Reclamation (Reclamation) and other federal, state, and local agencies have engaged in extensive efforts to detect the presence of dreissenid mussels in water bodies before full-scale infestations occur. The early detection effort is primarily focused on sampling for the larval (veliger) life stage because there is a greater probability of detecting veligers as opposed to adults. Veligers are distributed in the water column, and hundreds of veligers can arise from just one pair of adults (Johnson 1995). Early detection of veligers is difficult because only a small proportion of a water body is sampled and initial populations are small. Therefore, development of preservation methods that insure long term conservation of veliger integrity is of particular importance because every veliger collected provides critical data. With the continued threat of infestation, the development of standardized preservation methods for early detection of larval mussels is increasingly important in order to facilitate earlier detection of populations so that managing agencies have more time to implement controls.

Veliger samples are collected using a 64-µm plankton tow net. The Reclamation Detection Laboratory for Exotic Species (RDLES) field

standard operating procedure (SOP, Carmon and Hosler 2013) requires raw water samples to be preserved with 20% alcohol by volume and 0.2 grams of sodium bicarbonate (baking soda) per 100 mL. RDLES SOP does not specify the type of alcohol to be used, but has found that most people use isopropanol alcohol because it is easily accessible in the field. RDLES also suggests that raw water samples be stored in a cooler with ice to help preserve the sample by mitigating degradation caused by warm temperatures. Alcohol is added to the sample to euthanize and preserve all organisms and organic material, and RDLES requires the sample volume to be increased by 20% with alcohol because this is the maximum concentration allowable for shipping samples overnight by air, which allows samples to remain on ice during the entire shipping process. Baking soda (referred to as buffer throughout this paper) is added to samples as a buffer which raises and stabilizes the pH. RDLES measures the pH of all samples upon arrival in the lab, and has found that when buffer is added, the average pH upon arrival is 7.9. If a buffer has not been added, the average pH upon arrival is 4.9 (Hosler 2013). Although RDLES recommends these preservation methods to maximize short-term detection potential, little is known about how alcohol concentration and buffer affect long-term veliger preservation.

Veliger samples are analyzed with cross polarized light microscopy (CPLM) instead of light microscopy (LM) because veligers are birefringent (glow against a dark background) under CPLM, which makes them easier to detect amidst zooplankton, algae, and other debris. Under CPLM veligers display a unique 'Maltese Cross' pattern that is created because of the structure of their shell. The veliger's birefringent calcium carbonate shell is the primary identifiable characteristic under CPLM, and if this shell is damaged or degraded it is likely that the veliger will not be detected by the microscopist (Glover and Kidwell 1993; Nichols and Black 1994).

Laboratories may also conduct polymerase chain reaction (PCR) testing on the raw water sample from which a veliger is detected in order to validate microscopy findings. PCR testing provides evidence of dreissenid presence through amplification of dreissenid DNA that may be contained in the water sample. Positive PCR results can be validated by DNA sequencing. Accurate PCR detection requires mussel tissue to be present in the sample.

Mussel samples are commonly analyzed by microscopy and PCR, therefore it is critical to determine how sample preservation influences both shell and tissue (DNA) degradation over time. The first objective of this study was to determine how buffer influences the detection of veliger birefringence and DNA over time when samples lack alcohol. The second objective was to determine if the isopropanol alcohol concentration of buffered/basic samples affects veliger birefringence loss and ability of PCR detection. This study was also designed to determine if samples containing non-birefringent veligers, that are not detectable by cross-polarized microscopy, will test positive during PCR analysis. Understanding which factors affect veliger shell and tissue preservation over time will help to optimize detection by CPLM and validation by PCR analysis.

Methods

The veligers used for these studies were collected with a 64-µm plankton tow net from Lake Mead, Boulder City, Nevada. Tests were conducted in Colorado, which required veligers to be euthanized and preserved prior to transport across state lines. Veligers were preserved in 20% denatured alcohol with 0.2 grams of buffer per 100 mL, and were stored at 4°C for about 42 days prior to use. Before veligers were included in the study they were examined for shell, tissue and birefringence integrity. All veligers displayed birefringence prior to use.

Effect of buffer on veliger birefringence and PCR detection

Buffered and unbuffered solution stocks, without alcohol, were created in 1000-mL beakers. The pH 5, unbuffered, solution consisted of deionized water (DI) without sodium bicarbonate (baking soda). The pH 8 buffered solution consisted of DI water with 0.2 grams of baking soda per 100 mL of stock solution. Forty mL of each solution was transferred into 50-mL Falcon tubes. Falcon tubes received 100, 50, or 25 veligers. The Falcon tubes were stored at 4°C until observation. The pH of each solution was monitored (with pH strips) during each day of evaluation and was found to remain constant throughout the experiment. The pH was monitored with pH strips because this is how the pH of a sample is typically monitored at RDLES.

The percent of veligers with lost birefringence (veligers containing any birefringence were considered positive by CPLM) was examined in buffered (pH 8) and unbuffered (pH 5) samples without alcohol, after 1, 4, 6, 14, 21, and 42 days. RDLES sample processing occurs within 21 days of sample collection; therefore this experiment was extended to 42 days to determine longer term preservation. Each buffered and unbuffered sample set consisted of four replicates of three sample sizes (100, 50, and 25 veligers) for a single time point. The results from the 100 veliger sample size replicates were used to statistically analyze birefringence degradation; the 50 and 25 veliger replicates were included to test the sensitivity of the PCR analysis.

At examination, the content of the Falcon tube was poured into petri dishes and the tube was rinsed three times with the appropriate stock solution to recover any veligers remaining in the bottom or on the sides of the tube. The sample was first examined under CPLM, and the number of birefringent veligers was recorded. The sample was then examined under LM and the number of non-birefringent veligers was recorded. All birefringent and non-birefringent veligers were transferred into a 2-mL eppendorf tube for DNA extraction and PCR analysis. Birefringent and non-birefringent veligers were not assayed separately.

DNA Extraction

Veliger DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen 69506). The DNA was extracted using the Qiagen method. Modifications to the lysis step include the addition of 900 µL of ATL buffer and 100 µL of Proteinase K to each sample to assist in breaking down the veliger's calcium carbonate shell, because the Qiagen kit is optimized for blood and tissue samples. Samples underwent lysis for 4 hours at 56°C, and during each hour the samples were vortexed. After lysis, the Qiagen method was followed and the DNA analyzed by PCR as per the RDLES PCR SOP (Keele et al. 2012).

PCR Reaction

The samples were analyzed for the presence of the quagga mussel COX1 gene (Keele et al. 2012). The primers for quagga COX1 were F334 5'-GAAACTGGTTGGTCCCGATA-3' and R335 5'-TAAGGCACCGGCTAAAACAG-3'. PCR was performed using the following master mix: 9.8 µL molecular grade water, 2 µL 10x buffer, 1.6 µL dNTPs (2.5 mM), 1 µL each of the forward and reverse primers (1 µM), 2.4 µL of $MgCl_2$ (25 mM), 0.2 µL of Ampli Gold (Applied Biosystems N808-0242), and 2 µL of DNA template. The following PCR program was used: Pre-heat 95°C for 9 minutes, followed by 40 cycles of 95°C for 20 seconds, 59°C for 90 seconds, and 72°C for 90 seconds, followed by 72°C for 10 minutes, and then held at 4°C. After the PCR reaction, the samples were run on 1.5% agarose gels, stained with Gelstar (Lonza 1228822) and imaged using Gel Logic 200 Imaging System.

Effect of isopropanol alcohol concentration on birefringence and PCR detection in buffered solutions

Solution stocks were created in 1000-mL beakers with DI water, 20, 50, or 70% isopropanol alcohol, and 0.2 g of baking soda per 100 mL. Isopropanol was used in this study because it is commonly used in the field as a preservative because it is easily accessible. All solutions had a pH of 8, and were monitored during each day of evaluation. The pH remained constant throughout the experiment. Forty mL of the solutions were transferred into 50-mL Falcon tubes. Each Falcon tube received 100, 50, or 25 veligers. The samples were examined under CPLM and LM as described above, and using the same DNA extraction and PCR methods.

The percent of veligers with lost birefringence were examined in pH 8 buffered solutions containing 20, 50, or 70% isopropanol alcohol, after 6, 14, 21, and 36 days. Each alcohol concentration set consisted of four replicates of three sample sizes (100, 50, and 25 veligers) for a single time point. Different sample sizes were used to determine the sensitivity of the PCR testing.

Results

Effect of buffer on veliger birefringence and PCR detection

Statistical analysis was performed on the 100 veliger sample size only. Experimental data did not meet assumptions of normality or homogeneity of variance, likely due to relatively small sample sizes. The Kruskal-Wallis rank sum

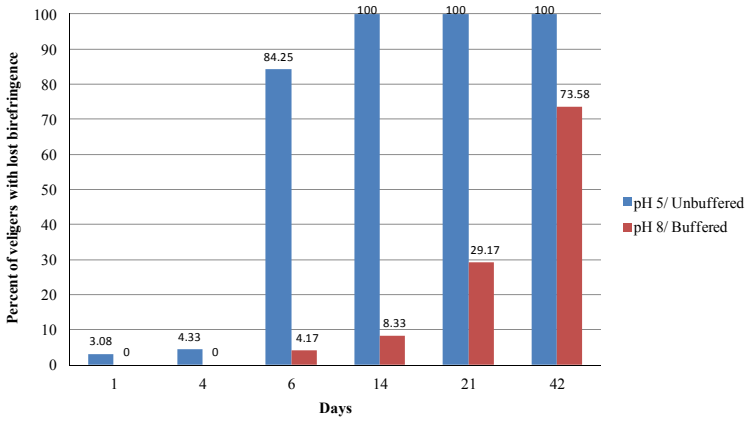

Figure 1. Comparison of the average % veliger birefringence loss in pH 5 (unbuffered) and pH 8 (buffered) solutions after 1, 4, 6, 14, 21, and 42 days.

test was used to determine significant differences between samples with ad-hoc comparisons using Mann-Whitney U tests with Benjamini and Hochberg p-value adjustments for multiple comparisons. Veligers in pH 5, DI water, without buffer or alcohol experienced more birefringence loss than veligers in pH 8, DI water, with buffer and without alcohol after six days (P= 0.02). After 14 days, 100% of veligers exposed to pH 5 solution had lost birefringence (Figure 1). Veliger's exposed to pH 8 solutions also experienced birefringence loss over time. Even though the pH remained basic (pH 8) for the duration of the experiment, the percent veliger birefringence loss on day 42 was comparable to loss seen in pH 5 samples at day 6 (P= 0.09).

Loss of birefringence does not reduce the ability of PCR tests to detect veliger DNA. PCR results were positive for unbuffered samples (at days 21 and 42) where 100% of veligers had lost birefringence (Table 1). In this study, sample size (25, 50 or 100 veligers) did not impact the ability of PCR detection, as positive DNA was detected in all sample sizes. PCR results were negative for buffered samples with a sample size of 25 after 42 days (Table 1). It is difficult to explain these negative results, but it is possible that the negative results were due to PCR processing error. PCR samples were not run on day 14 because of a laboratory error.

Effect of isopropanol alcohol concentration on birefringence and PCR detection in buffered solutions

There was 0% loss of birefringence in samples containing both isopropanol alcohol and buffer, even after a 36 day holding time (Table 2). The percent of isopropanol added to the sample did not appear to impact the ability of PCR tests to detect positive DNA. One of four PCR tests was negative on day six for samples preserved in 50% isopropanol at sample sizes 100, 50, and 25, and one of four PCR tests was negative on day six for a sample preserved in 70% isopropanol with 50 veliger sample size. These negative PCR results are difficult to explain, especially since most of the replicates were positive and samples at the longer time points are still positive. It is possible that the negative results were due to PCR processing error.

Discussion

Early detection of dreissenid mussels is critical in order to help prevent transport to other water bodies. Early detection sampling is conducted for many western water bodies in the United States, but sample preservation methods have not been standardized. The results of this study indicate that all veligers in a sample can lose their birefringence after 14 days if the sample is not preserved with isopropanol alcohol or maintained at a basic pH with a buffer. Birefringence loss can be significantly reduced by maintaining a basic pH with the addition of 0.2 grams of buffer per 100 mL of sample, but without alcohol, birefringence is still lost over time. Veliger birefringence loss was completely prevented for 36 days when samples were preserved with 20, 50, or 70% isopropanol alcohol by volume and 0.2 grams of buffer per 100 mL. Although the veligers used in this study were refrigerated and preserved for 42 days prior to use they did not lose birefringence

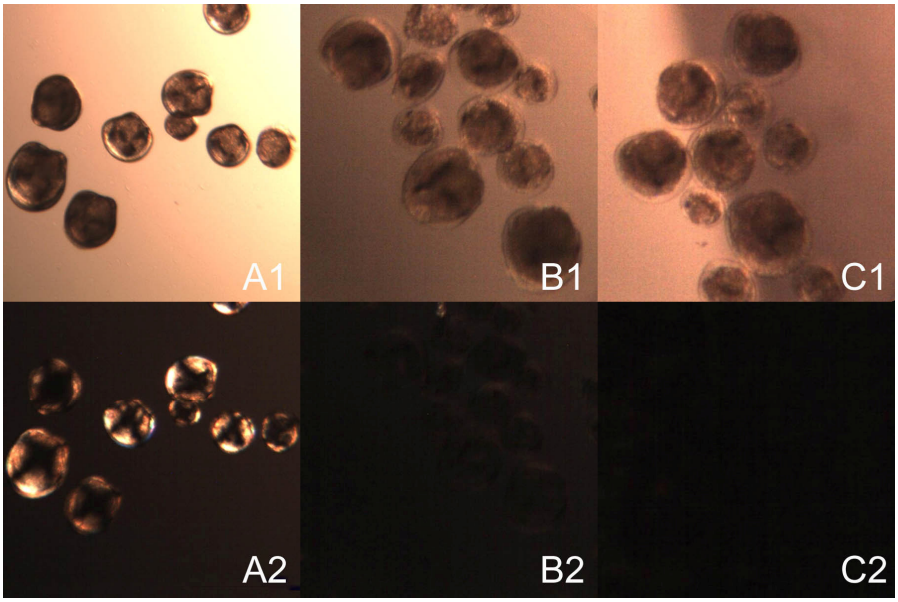

Figure 2. Comparison of veligers under light microscopy (top) and cross-polarized light microscopy (bottom) after 6 (A1 and A2), 14 (B1 and B2), and 21 (C1 and C2) days in pH 5 solution without alcohol or buffer (Photograph by Jamie Carmon).

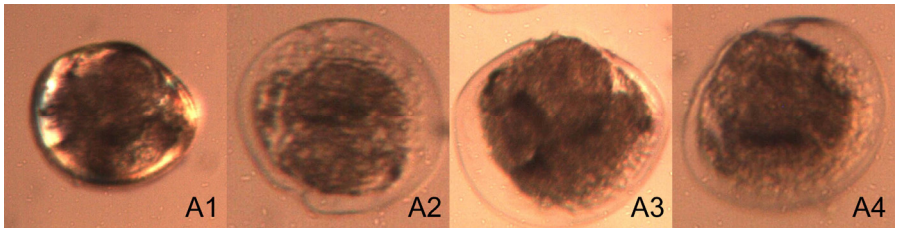

Figure 3. Macro view of veliger shell and organ deterioration under light microscopy after 1 (A1), 6 (A2), 21 (A3), and 42 (A4) days in pH 5 solution without alcohol or buffer (Photograph by Jamie Carmon).

Table 1. Average percent loss of birefringence, standard deviation, and number of samples (out of 4 replicates) with positive PCR results per sample size (100, 50, and 25) after 1, 4, 6, 14, 21, and 42 days in pH 5 (unbuffered) and pH8 (buffered) solutions. Statistical analysis completed for average percent loss of birefringence in the 100 veliger sample size group only. Means within a row with different letters are significantly different.

Sample Size		100			50			25		
Day	pH/buffer	Average loss birefringence	SD	PCR+	Average loss birefringence	SD	PCR+	Average loss birefringence	SD	PCR+
1	pH5 no buffer	-2.25% ^{AB}	0.03	4/4	-4.00%	0.03	4/4	-3.00%	0.04	4/4
4	pH5 no buffer	0.00% ^{A}	0.00	4/4	-9.00%	0.11	4/4	-4.00%	0.03	4/4
6	pH5 no buffer	-70.75% ^{C}	0.06	4/4	-90.00%	0.05	4/4	-92.00%	0.03	4/4
14	pH5 no buffer	-100.00% ^{D}	0.00	NA*	-100.00%	0.00	NA*	-100.00%	0.00	NA*
21	pH5 no buffer	-100.00% ^{D}	0.00	4/4	-100.00%	0.00	4/4	-100.00%	0.00	4/4
42	pH5 no buffer	-100.00% ^{D}	0.00	4/4	-100.00%	0.00	4/4	-100.00%	0.00	4/4
1	pH8 buffered	0.00% ^{A}	0.00	4/4	0.00%	0.00	4/4	0.00%	0.00	4/4
4	pH8 buffered	0.00% ^{A}	0.00	4/4	0.00%	0.00	4/4	0.00%	0.00	4/4
6	pH8 buffered	-0.50% ^{A}	0.01	4/4	-5.00%	0.04	4/4	-7.00%	0.09	4/4
14	pH8 buffered	-9.00% ^{EB}	0.06	NA*	-15.00%	0.14	NA*	-1.00%	0.02	NA*
21	pH8 buffered	-18.00% ^{E}	0.05	4/4	-37.50%	0.08	4/4	-32.00%	0.15	4/4
42	pH8 buffered	-50.75% ^{C}	0.14	4/4	-76.00%	0.13	4/4	-94.00%	0.07	0/4

*Due to laboratory error, the PCR samples for day 14 were not completed.

Table 2. Average percent birefringence loss for all sample sizes, per day, per isopropanol alcohol concentration, and the number of samples (out of four replicates) with positive PCR results per sample size.

Day	% Isopropanol	Average % lost birefringence	# of + PCR replicates out of 4, per sample size		
			100	50	25
6	20%	0%	4/4	4/4	4/4
14	20%	0%	4/4	4/4	4/4
21	20%	0%	4/4	4/4	4/4
36	20%	0%	4/4	4/4	4/4
6	50%	0%	3/4	3/4	3/4
14	50%	0%	4/4	4/4	4/4
21	50%	0%	4/4	4/4	4/4
36	50%	0%	4/4	4/4	4/4
6	70%	0%	4/4	3/4	4/4
14	70%	0%	4/4	4/4	4/4
21	70%	0%	4/4	4/4	4/4
36	70%	0%	4/4	4/4	4/4

when they were tested in isopropanol alcohol and buffer for an additional 36 days. The lack of tissue degradation or birefringence loss after the 42 day holding time and after the additional 36 days suggests that the veliger birefringence loss seen in the unpreserved buffer only samples are most likely a result of the treatment and not of deterioration from the 42 day holding time.

When a veliger's calcium carbonate shell begins to lose its integrity it is no longer visible under CPLM, but non-birefringent veligers are still visible under LM because their shell and tissues remain intact (Figure 2). It is common practice for microscopists to only scan mussel samples under CPLM because most water samples contain large amounts of organic and inorganic materials, making veligers difficult to detect under LM.

Preservation of birefringence is critical for detection of veligers by CPLM, but tissue (DNA) presence is most important for positive detection by PCR. This study demonstrates that samples with complete birefringence loss will still produce positive PCR results, because non-birefringent veligers can still contain tissue. Veliger DNA was detected in samples that were not preserved with alcohol and in acidic conditions for 42 days. Therefore, it is possible for samples containing non-birefringent veligers to be called negative by microscopy and positive by PCR because the DNA is still present. When isopropanol alcohol was added, the percent of alcohol added did not impact the ability of PCR to detect mussel DNA. When veligers are not preserved with isopropanol alcohol or buffer the internal organs begin to degrade and become dispersed throughout the shell instead of remaining compartmentalized (Figure 3). Overtime, it is possible that this tissue degradation could lead to negative PCR results.

This study did not test how alcohol without buffer influences veliger birefringence. Future research should determine if veliger birefringence is lost in acidic samples preserved with only alcohol. It would also be beneficial to determine how alcohol and buffer preservation impacts veligers in raw water samples that contain varying degrees of organic and inorganic materials. Early detection samples are collected from water bodies with dramatically variable water quality that would be difficult to simulate in the lab. However, it is clear that raw water samples include a variety of organic and inorganic materials that can influence veliger preservation. DI water was used in these experiments so that the effects of buffer and alcohol concentration on birefringence loss could be tested without the influence of additional water quality parameters. While our results show the true effect of buffer and alcohol concentration, the results may not be completely applicable to raw water samples.

Overall this study shows the importance of adding both alcohol and buffer to dreissenid veliger samples for long term preservation. This study suggests that veliger shell degradation does not affect tissue, as PCR can detect tissue past 41 days. This could result in cases where microscopy results are negative, while PCR results are positive. An explanation for this discrepancy could be due to degraded veliger morphology. A lot of time and money is spent on early detection of dreissenid veligers, but in order for this effort to be worthwhile proper sample preservation is critical.

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