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Short Communication

Effect of pH, ethanol concentration, and temperature on detection of quagga mussel (*Dreissena bugensis*) birefringence

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Abstract

In the western United States, federal, state, and local agencies have engaged in extensive efforts to detect the presence of larval dreissenid mussels (veligers) in water bodies before such a presence becomes a full-scale infestation. Cross-polarized light microscopy (CPLM) is commonly used to detect the veliger's specific birefringence pattern, but the effect of sample preservation on dreissenid birefringence has not yet been determined. This study examined the effects of solution pH, ethanol concentration, and storage temperature on veliger birefringence loss. Birefringence loss was determined by examining veligers under CPLM at regular intervals over a 30 day period. Veliger birefringence loss was below five percent in all basic solutions, regardless of holding temperature or ethanol concentration. Veliger birefringence loss was also below five percent in acidic solutions that were refrigerated or contained 50–70 percent ethanol. Veligers in acidic solutions that were held at 25°C and 34°C with 0–25 percent ethanol had 7–25 percent veliger birefringence loss over a 30 day period. While ethanol addition and refrigeration are both important preservation methods, the results of this study indicate that samples collected for dreissenid veliger detection by CPLM must also be maintained at a basic pH in order to preserve veliger shell integrity and birefringence. Determining the best conditions for long-term preservation of veliger shells requires further investigation, and these results should not be assumed best for analyses other than CPLM.

Key words: dreissenid mussels; preservation; birefringence; detection, monitoring; cross-polarized microscopy; calcium carbonate shell

Introduction

Quagga mussels (*Dreissena rostriformis bugensis* Andrusov, 1897), a freshwater bivalve native to Ukraine, were found in Lake Mead, Nevada in January 2007 signaling the spread of invasive dreissenid mussels into the western United States. Mussel larvae (veligers) are thought to spread between water bodies by water currents, and through overland transportation by way of watercraft, trailers, fishing equipment, fish stocking activities, and other water-related activities (WRP 2010). The dreissenid invasion of North America has already had substantial effects, both ecologically (Higgins et al. 2011; Barbiero et al. 2006; McCabe et al. 2006; Noonburg et al. 2003; Ricciardi et al. 1998; Schloesser et al. 1998), and economically (WRP 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 veligers. There is a greater probability of detecting veligers as opposed to adults because hundreds of veligers can arise from just one pair of adults (Johnson 1995). With the continued threat of infestation, the development of standardized sampling and analytical methods for early detection of larval mussels is increasingly important in order to alert managing agencies and protect both the environment and infrastructure (WRP 2010).

Veliger samples are typically collected with a 64-µm plankton tow net, preserved, and sent to a lab where the sample is analyzed by cross-polarized light microscopy (CPLM). CPLM is

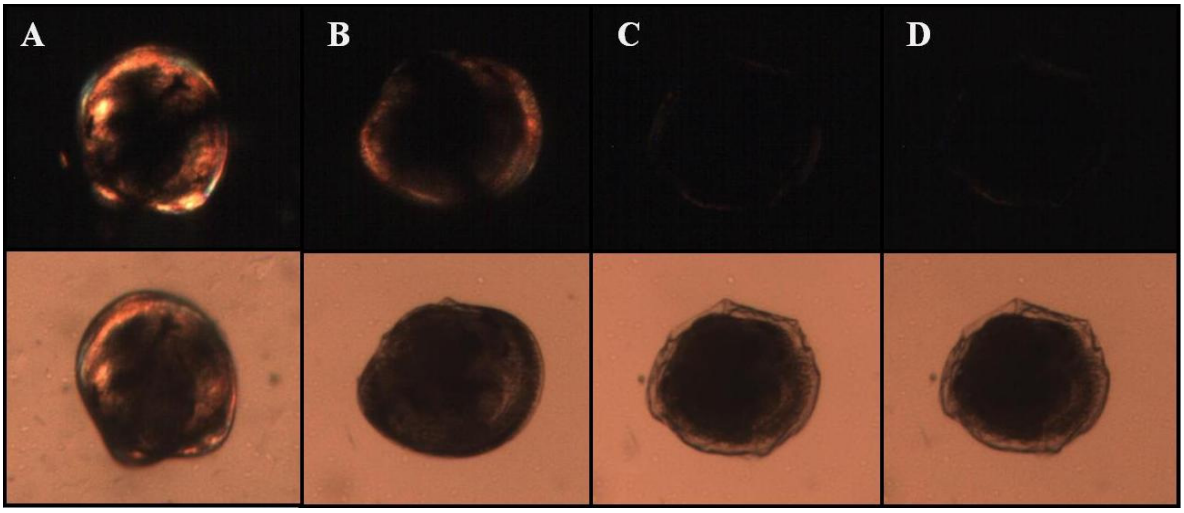

Figure 1. Quagga mussel veliger under cross-polarized light (top) and regular light (bottom) before and during exposure to five percent acetic acid solution (pH =2.5): A) prior to exposure B) immediately after exposure, C) after 5 minutes in solution, and D) after 10 minutes in solution. The mussel is approximately 240 μm in diameter. Photomicrographs by Suzanne Brenimer.

currently thought to be the most accurate and effective technique for initial veliger detection because the dreissenid veliger's shell has a specific molecular structure and organization that displays a distinct "Maltese cross" birefringence pattern when viewed under cross-polarized light (Johnson 1995). Therefore, if the veliger's shell is damaged or degraded in any way it is likely that the organism will not be detected by CPLM (Nichols and Black 1994). In the course of early detection sampling, lag time between dreissenid larvae collection and analysis can range from a few days to over a month, during which time veligers may suffer significant degradation if not properly preserved. Samples are thought to be properly preserved if they are refrigerated and alcohol (typically ethanol) is added, but it is unknown if and to what extent the percent of alcohol added to the sample and the temperature at which the sample is held directly impacts shell birefringence.

Other factors such as sample pH may also be critical for birefringence preservation. Veliger shells are made of calcium carbonate, which can be easily degraded when exposed to acidic pH. When veligers are exposed to acidic solution (five percent acetic acid, pH 2.5) they immediately display a notably duller birefringence signal, and after five minutes of exposure, veligers have warped and weakened shells with almost no indication of birefringence (Figure 1). After five minutes of exposure, the veligers are still visible under regular light, but are no longer birefringent (Figure 1). The loss of birefringence is likely due to dissolution of the calcium carbonate within the shell (Glover and Kidwell 1993). While it is unlikely that the pH of veliger samples would reach 2.5, it is possible for samples to become slightly acidic prior to analysis. The amount of veliger birefringence loss in slightly acidic samples is unknown.

Sample preservation and storage protocols are variable between agencies, indicating a need to define and standardize a proper sample preservation method. Accurate microscopic identification of veligers relies heavily on shell integrity, therefore it is critical to determine how sample preservation and storage influences shell degradation. The objective of this study was to determine the impact of pH, preservative concentration, and holding temperature on the percent loss of veliger birefringence. Understanding which factors affect birefringence loss will optimize detection by CPLM.

Methods

Birefringence loss was examined at acidic (pH 6-7) and basic pH (pH 7–9), and at five ethanol concentrations (0, 10, 25, 50, and 70 percent), and three storage temperatures (34°C, 25°C, and

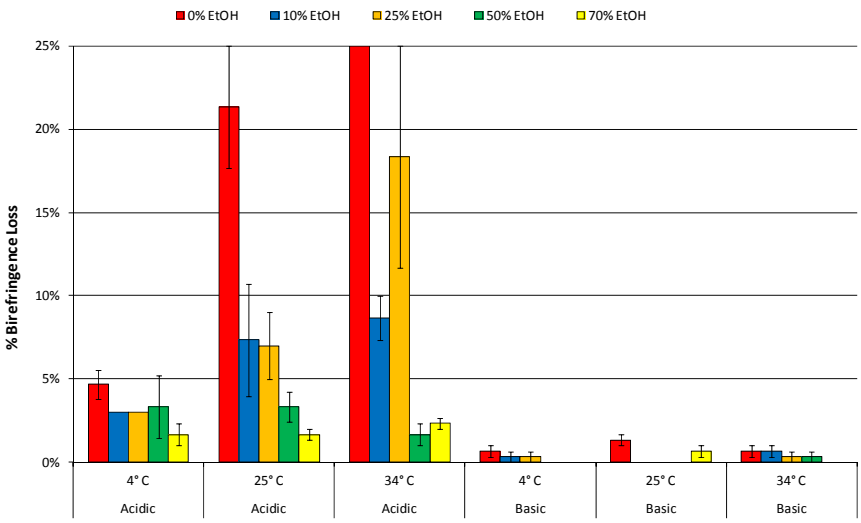

Figure 2. Means and standard errors of percent of quagga mussel veliger birefringence loss in acidic and basic samples with 0–70 percent alcohol concentration and at 4, 25, and 34°C after 30 days.

4°C). Each of the 30 treatments had 3 replicates resulting in a total of 90 experimental units. Sample solution stocks were created in 125-mL Nalgene containers. The water used for this study was collected from a pond on the Denver Federal Center campus. The pond water simulates a "real world sample" because it contains organic and inorganic materials, which can influence pH and preservation over time. This particular pond water was selected for this study because it had a basic pH (pH 8). Using a water source with a basic pH allowed for controlled acidification with the addition MES (2-(N-morpholino) ethanesulfonic acid). Ethanol was added as a preservative to each solution stock to achieve 0, 10, 25, 50, or 70 percent ethanol concentration by volume.

An average of 25 veligers (minimum= 16, maximum= 32) were added into each Petri dish containing 10 mL of the appropriate stock solution. The initial veliger count was recorded, and the Petri dish was covered with Parafilm and stored at one of three temperatures along with the corresponding solution stock bottle. The initial pH was recorded in each solution stock and was monitored during the 30 day period. Veliger birefringence was examined under a cross polarized microscope at 48-hour intervals over the 30 day period.

The veligers used for this study consisted of 80–90 percent straight hinged and umbonal larvae (97–230 µm) and 10–20 percent pediveliger larvae (230–462 µm), which were collected with a 64-µm plankton tow net from Lake Mead, Boulder City, Nevada. Because the tests were conducted in Colorado, the veligers were required to be euthanized and preserved prior to transport across state lines. Veligers were preserved in 20 percent ethanol and were stored at 4°C. Veligers were examined under cross polarized and regular light to detect any deterioration before they were included in the experiment. All veligers used in this study displayed birefringence at the beginning of the study and were transferred with a minimum of the original sample matrix.

Results and discussion

The percent of veligers with lost birefringence over a 30 day period was lowest (below 5 percent) in basic samples regardless of ethanol concentration or storage temperature (Figure 2). Veliger birefringence loss was also below 5 percent in acidic samples held at 4°C and acidic samples that were held at 25°C and 34°C with 50 percent or 70 percent ethanol concentration. Acidic samples held at 25°C and 34°C with ethanol concentrations between 0 and 25 percent had birefringence loss between 7-25 percent. The greatest loss of birefringence was seen in acidic samples held at 25°C and 34°C with 0 percent ethanol (Figure 2). Birefringence loss was variable within treatments because the pH shifted over the course of the experiment. The pH was variable over the 30 day period because a buffer

was not added, but the pH of the acidic group did stay acidic to neutral and the pH of the basic group stayed neutral to basic. The average pH of the acidic samples was 6.74, with a minimum pH of 6.20 and a maximum pH of 7.33. The average pH of the basic samples was 8.11, with a minimum pH of 7.33 and a maximum pH of 8.92.

Conclusions

The results of this study suggest that dreissenid veliger birefringence can be lost if samples become acidic. Even low levels of acidity (pH 6) can lead to birefringence loss. Refrigeration and addition of a higher ethanol concentration to acidic samples can reduce birefringence loss, but birefringence is best preserved in samples that are maintained at a basic pH. Since maintaining a basic pH appears to be critical for birefringence preservation, a buffer, such as baking soda (sodium bicarbonate), should be added to all veliger samples immediately after collection.

While the results of this study suggest birefringence loss in basic samples was not dramatically impacted by storage temperature or alcohol concentration it is still important to refrigerate and add ethanol to samples. Along with preserving the veliger's tissues, ethanol and refrigeration helps to euthanize, stabilize, and preserve all other organic material in the sample. Because of shipping regulations, samples for CPLM detection should only be preserved with 20 percent alcohol concentration.

Water managers depend on early detection results to respond to and reduce the impact of dreissenid invasion. Newly infested water bodies with low veliger populations require an extensive sampling effort to collect even one veliger. If that sample is not preserved with ethanol, buffered, and refrigerated it is possible that the veliger could be missed during analysis with CPLM, which reduces the amount of time the manager has to react to the invasion. While this study provides a basic idea as to how preservation affects birefringence loss, further knowledge is needed to determine how to best preserve veligers for other analysis methods such as PCR. A significant amount 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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