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Evaluation of preservation methods for veliger detection field samples

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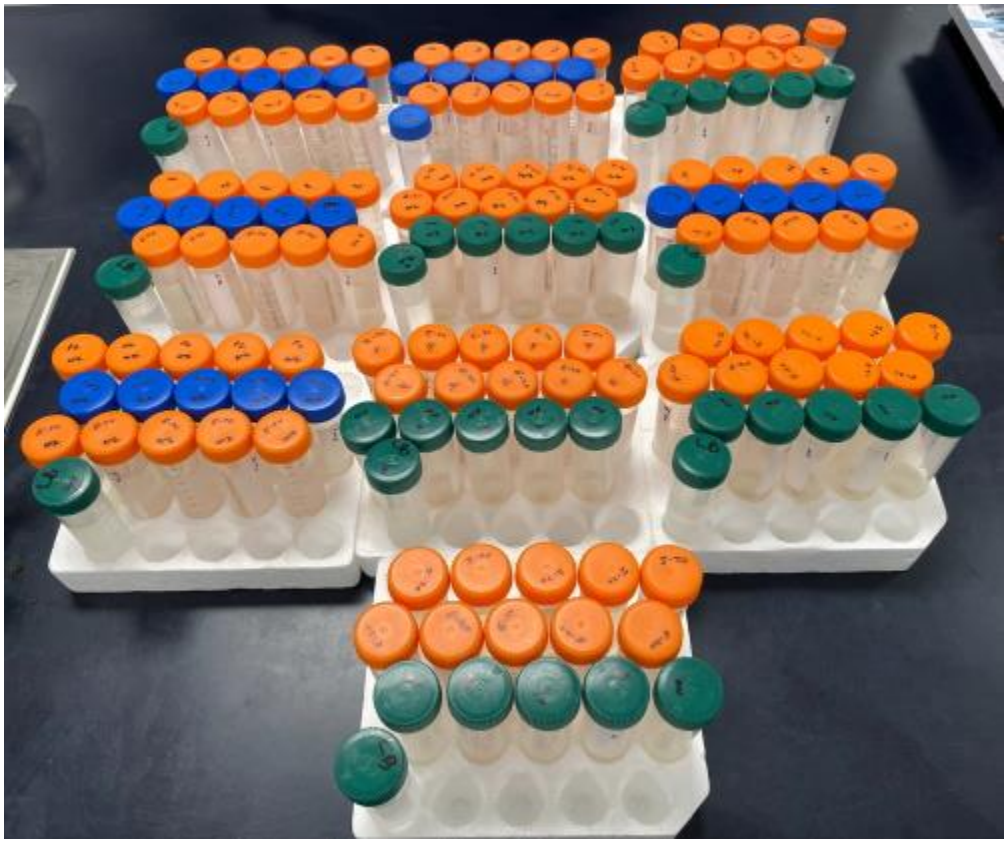

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14. ABSTRACT

Early detection of dreissenid mussels (quagga and zebra) provides the ability to plan for and limit the impact these invasive bivalves. Proper preservation of field samples is critical to the success of early detection efforts, as it is necessary to maintain sample integrity to ensure the reliability of sample analyses. The current study evaluated the impact of five preservatives: 20% ethanol, 70% ethanol, 70% isopropanol, Longmire's solution, and propylene glycol, on environmental DNA (eDNA) analyses for the detection of quagga mussel eDNA. All five preservatives successfully resulted in the detection of quagga mussel eDNA over 12-week time-course of the study. Results were further analyzed to calculate the starting quantity of target eDNA in the samples. 70% ethanol, 70% isopropanol, and propylene glycol all averaged similar starting quantities. Longmire's solution resulted in lower average starting quantities. Samples preserved with 20% ethanol had much higher calculated starting quantities, which was unexpected and runs counter to conventional wisdom on the necessity for high alcohol concentrations to prevent eDNA degradation. The reasons for this anomalous result are uncertain, although they may relate to the specific design of the current study.

15. SUBJECT TERMS
Invasive mussels, dreissenid mussel, quagga mussel, environmental DNA, polymerase chain reaction

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Evaluation of preservation methods for veliger detection field samples

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Acronyms and Abbreviations

Cq	Quantification cycle
EcoLab	Ecological Research Laboratory
eDNA	Environmental DNA
qPCR	Quantitative polymerase chain reaction
Reclamation	Bureau of Reclamation

Measurements

°C	degree Celsius
ml	milliliters

Contents

Executive Summary

Early detection of dreissenid mussels (quagga and zebra) provides the ability to plan for and limit the impact these invasive bivalves. Proper preservation of field samples is critical to the success of early detection efforts, as it is necessary to maintain sample integrity to ensure the reliability of sample analyses. For samples that will be evaluated for environmental DNA (eDNA) samples needed to be preserved in a manner that prevents the degradation of the target DNA of interest. Reclamation's Ecological Research Laboratory (EcoLab) collects aqueous samples with a tow-net, rather than filtering samples as is commonly performed for eDNA. Such aqueous samples require preservation that will prevent DNA degradation from microbial activity or hydrolysis prior to analysis. A variety of preservatives have been used for aqueous samples, but a comparative analysis had not previously been performed to evaluate the impacts of different reagents on the detection of dreissenid mussel DNA.

In the present study six preservation treatments (no preservative, 20% ethanol, 70% ethanol, 70% isopropanol, Longmire's solution, and propylene glycol) were tested in parallel. Samples were tested by qPCR for the presence of the quagga mussel COI gene across 12 weeks of storage following arrival of the samples in the laboratory. All the preservatives resulted in detection an 100% of samples for all timepoints, while the number of detections for samples without preservative decreased over the time-course. Calculation of starting quantities of the qPCR target was also performed to evaluate differences in the amount of target preserved in different treatments and across the time-course. None of the preservatives tested showed a measurable decrease in the starting quantity across the duration of the study. Samples preserved in 20% ethanol did have much higher starting quantities compared to the other preservatives. This result did not meet initial expectations and could be related to the specific parameters of the study rather than a general property of the preservative. Additional testing with samples having lower mussel densities and higher organic contents would help to resolve the relative advantages and limitations of the different preservatives evaluated.

1. Introduction

Dreissenid mussels ((quagga mussel (*Dreissena rostriformis bugensis*) and zebra mussel (*Dreissena polymorpha*)), are a major challenge for the Bureau of Reclamation and other water managers in the Western United States. These invasive freshwater mussels have a broad range of ecological and economic impacts. For Reclamation dreissenid mussels are particularly problematic, as their propensity to settle on hard substrates throughout infrastructure creates the potential for damage to equipment and interruptions to power generation and water delivery.

Since quagga mussels were first identified from Lake Mead in 2007, Reclamation has established an extensive early monitoring program, conducted primarily through the Technical Service Center's Ecological Research Laboratory (EcoLab). Early detection of dreissenid mussel populations is critical for mitigating the potential impacts of these invaders. In invasive species biology, it has been widely observed that most species undergo a sigmoidal pattern of population growth, referred to as the "invasion curve". Intervention efforts are generally considered to be most effective during the initial stages of the invasion, when population numbers are low. Data from the EcoLab suggest that consistent monitoring may be able to detect populations several years before the population has grown to the point where it may begin to impact Reclamation operations.

Critical to the success of early detection efforts is sample integrity. Samples are collected in the field and must be transported to the laboratory for analysis. The EcoLab analyzes for the presence of dreissenid mussels using both microscopy and environmental DNA (eDNA) methodologies. To facilitate sample collection by a variety of partners, EcoLab standard operating procedures specify collecting single samples that can be used for both microscopy and eDNA analysis. Collecting samples to be analyzed for both microscopy and eDNA requires that sample preservation of samples be optimized to meet the needs of both methods. This contrasts with most other labs which analyze for early detection of dreissenid mussels and only perform either microscopy or eDNA analysis. In such cases the sampling and preservation methods can be tailored to the single analysis method in use. For microscopy the primary objective is to maintain the integrity of mussel veliger shells, which require storage in a medium with a basic pH to prevent dissolution of the calcium carbonate shells. This is generally accomplished through the addition of a buffer (Tris or sodium bicarbonate [baking soda]) to maintain pH and alcohol (ethanol or isopropanol) to limit bacterial activity that can contribute to sample acidification. eDNA analysis relies on maintenance of DNA integrity in the sample. DNA degradation may be related to a variety of factors including temperature, pH, and bacterial activity in the sample. For aquatic eDNA studies, water samples are most often process by filtration though filters with small pore sizes. The integrity of DNA on the filter can then be maintained through desiccation or addition of a preservative such as ethanol, allowing long-term storage prior to DNA extraction and analysis.

The goal of the present study was to compare a range of preservation methods for plankton tow samples with respect to their impact on eDNA analysis for detection of dreissenid mussel eDNA. Six preservative treatments were tested: no preservative, 20 % and 70% ethanol, 70% isopropanol, Longmire's solution, and propylene glycol. There are advantages and disadvantages to each of the preservation reagents. Ethanol is widely used as a preservative for DNA in tissue samples and eDNA samples for long-term storage. Ethanol concentrations of 70% or higher are generally considered necessary to maintain DNA integrity in tissue and environmental samples. Such high

concentrations of ethanol can present challenges. A stock concentration of 95% or higher is required to achieve the final concentration of 70% once added to an environmental sample. The ethanol needs to be pure, as less expensive reagent grades contain denaturants that could interfere with lab analyses. Packages with samples containing 70% ethanol also require special labeling, as they cannot be shipped by air due to flammability. Isopropanol presents similar challenges with regards to flammability, but is readily accessible, with 91% isopropanol available from many pharmacies and grocery stores. Isopropanol is not widely used for sample preservation, and there is very little documentation of its value for preserving DNA relative to ethanol. Longmire's solution and propylene glycol are two novel preservatives that have shown promise for maintaining DNA integrity in samples prior to analysis. Longmire's solution is an aqueous solution composed of buffer, salt, and the surfactant SDS. It does not present the combustion risk of alcohol preservatives but may represent a toxicity risk due to the fact that it contains sodium azide. Propylene glycol is considered non-hazardous under OSHA classification, although it does have a low level of flammability.

Methods

Sample collection

Samples were collected from the outer dock of the Las Vegas Boat Harbor, in Lake Mead on April 6, 2022. Samples were collected using a plankton tow net with a 65-micron mesh size. Twenty-seven tows of 25 meters each were collected and combined. The combined sample was divided between six preservation treatments (no preservative, 20% ethanol, 70% ethanol, 70% isopropanol, Longmire's solution (Longmire et al, 1997), and propylene glycol). Details of the quantities for each preservative are listed in Table *1*. For each treatments samples were aliquoted for 5 time points (0, 2-, 4-, 8-, and 12-weeks post arrival in the laboratory). For each preservation method, and each time point, five replicate samples were collected, for a total of 150 samples, excluding field blanks. Samples were shipped to the EcoLab in Denver via ground shipment and were stored at 4°C until DNA extraction was performed.

Table 1. Quantities and percentages of preservatives tested

Preservative	**Stock %**	**Final %**	**Sample volume (ml)**	**Preservative volume (ml)**
No preservative	n/a	n/a	12	n/a
Ethanol	100	20	12	3
Ethanol	100	70	12	28
Isopropanol	91	70	12	30.8
Longmire's solution	100	25	12	4
Propylene glycol	100	50	12	12

Analysis

DNA was extracted using the *Quick*-DNA™ Fecal/Soil Microbe Microprep Kit (Zymo Research Corporation) following the EcoLab's standard operating procedures for DNA. Quantitative polymerase chain reaction assays (qPCR) were performed using PerfeCTa qPCR ToughMix (Quantbio) and the quagga mussel-specific primers and probe QMCOI (Sepulveda et al., 2019). Four replicate reactions were tested for each sample. Thermal cycling was performed using a BioRad CFX96, with Maestro software (Bio-Rad Laboratories, Inc.) used for instrument control and data analysis. A 2-step thermal cycling protocol was performed, with an initial denaturation step 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Samples were scored as positive if the Cq value was <= 38 cycles. Quantification was based on a gBlock synthetic oligonucleotide run in six reactions in a dilution series ranging from 2×10^0 to 2×10^5 copies per reaction. The gBlock dilution series was run on each qPCR plate to calibrate concentration for reaction in the same run. The dilution series was used to calculate a standard curve, from which

starting quantities of experimental samples were calculated. Standard curve and quantification calculation were performed in the Maestro software. Negative control samples (blanks) were generated at each step of sample collection and processing to test for potential contamination. There controls including field blanks, DNA extraction blanks, and qPCR reaction no-template controls. All controls were tested by qPCR. All reactions also included TaqMan Exogenous Internal Positive Control (IPC) reagent (Thermo Fisher Scientific) to test for any reaction inhibition. TaqMan Exogenous IPC was used at 1/5 of the manufacturer's recommended concentration. DNA concentration in eDNA extracts was tested with the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific).

Results

DNA extraction and controls

DNA was successfully extracted from all samples following the timeline in the project design. On Week 0 (week of sample arrival in the EcoLab) field blanks were also extracted for each preservation method. All controls performed expected, with no qPCR detections for any of the filed blanks, DNA extraction blanks, or qPCR no template controls. Internal positive controls also demonstrated no evidence of inhibition for any reactions.

Quagga mussel DNA detection and quantification

Samples were initially analyzed based on the criteria of detection/non-detection of quagga mussel DNA in the QMCOI qPCR assay. All qPCR results were initially scored for detection/non-detection, as this is the primary metric used by the EcoLab and reported to clients. Samples were scored as a positive detection if at least one qPCR replicate for the sample had a Cq value <= 38. For the five preservative treatments tested, all samples were scored as detections over the full 12-week course of the study (Figure *1*). For samples where no preservative was added, 5 of 5 samples were positive 0-, 2-, and 4-weeks post arrival in the EcoLab. By 8 weeks post arrival only 4 of the 5 samples tested positive, and by week 12 only 3 of the 5 samples tested positive. For each sample 4 qPCR technical replicates were tested, resulting in a total of 20 qPCR reactions per treatment for each time point. For the five preservative treatments tested, all reactions were scored as detections at each time point, with the exception of week 4 for the 70% isopropanol treatment, where 19 of the 20 qPCR reactions were positive (Figure *2*). For samples with no preservative added, all 20 reactions were positive in weeks 0 and 2. By week 4 only 16 of the 20 reactions were scored as positive. Eleven of 20 reactions were positive in week 8, and 9 of 20 reactions were positive in week 12.

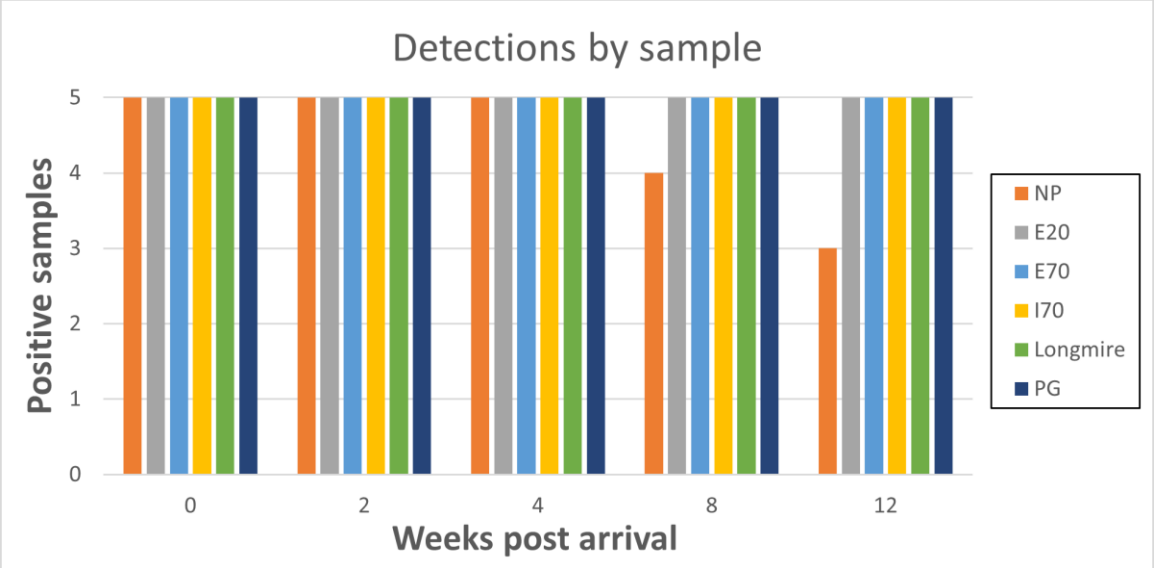

Figure 1. Chart of the number of samples scored as positive detections for quagga mussel DNA for each time point tested. Data are shown for samples with no preservative added (NP), and for samples preserved with 20% ethanol (E20), 70% ethanol (E70), 70% isopropanol (I70), Longmire's solution ((Longmire), or propylene glycol (PG).

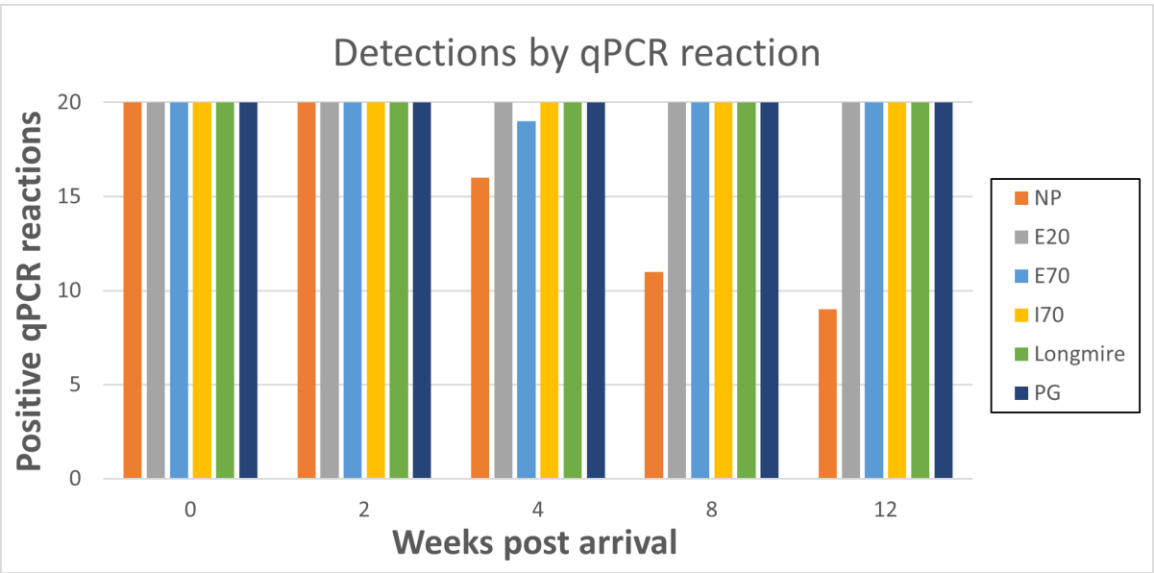

Figure 2. Chart of the number of qPCR reactions scored as positive detections for quagga mussel DNA for each time point tested. Data are shown for samples with no preservative added (NP), and for samples preserved with 20% ethanol (E20), 70% ethanol (E70), 70% isopropanol (I70), Longmire's solution ((Longmire), or propylene glycol (PG).

To analyze the results in more detail, the starting quantity of each sample was calculated based on the standard curve for the gBlock dilution series included in each reaction set. Quantification data for each preservation method are described below.

No preservative

Samples were transported and stored without the introduction of any preservative to measure the degradation of quagga mussel DNA in the absence of any intervention. No preservative samples extracted at 0 weeks post arrival had a mean calculated starting quantity of 1200 copies of the *COI* gene per reaction (Figure *3*). Samples from this initial timepoint showed a large standard deviation (±1883 copies per reaction), due largely to a single outlier sample with a calculated starting quantity of 4554 copies per reaction. Excluding this outlier, no preservative samples at week 0 had a mean calculated starting quantity of 362 copies per reaction. The mean calculated starting quantity of quagga DNA decreased progressively with storage time. By 2 weeks post arrival the calculated starting quantity was 197 copies per reaction, and for 4-, 8-, and 12-weeks post arrival the calculated starting quantities were all below 65 copies per reaction.

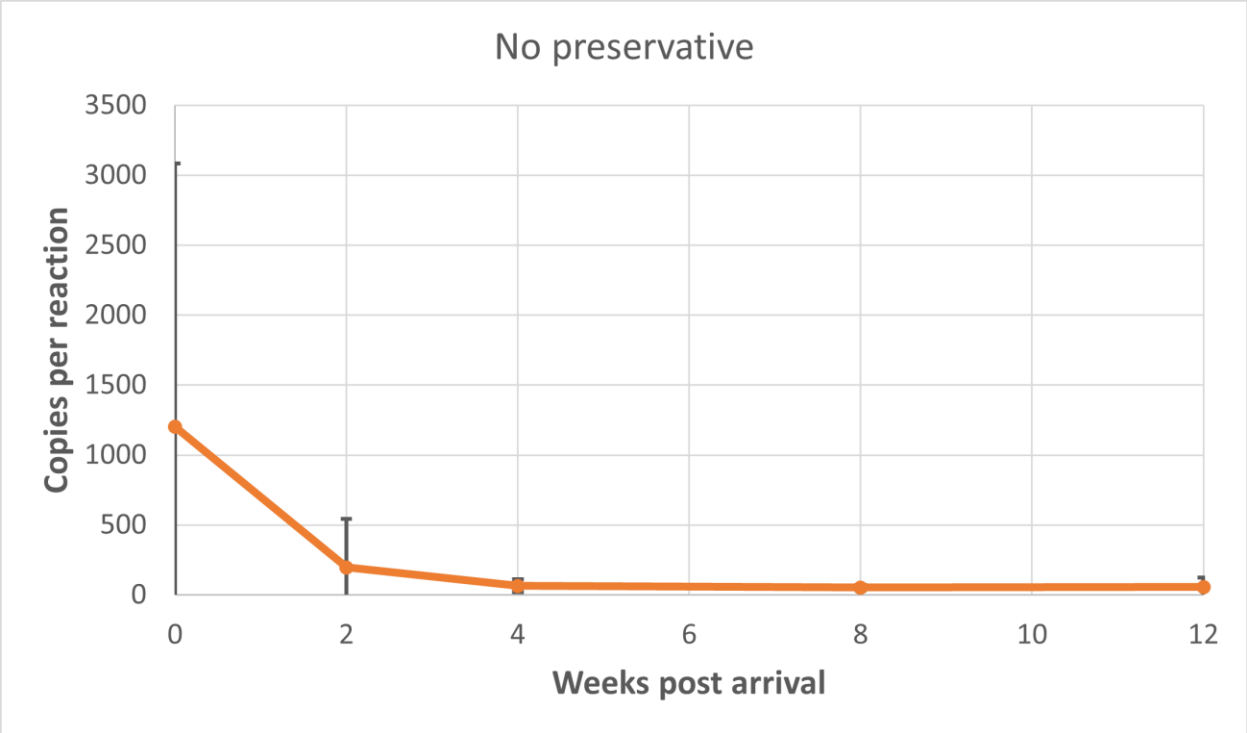

Figure 3. Graph of mean calculated starting quantities of the quagga mussel COI gene per reaction across the sampled timepoints for samples stored with no preservative added. Standard deviations from the mean are shown as bars above and below the mean for each sampled timepoint. Note that standard deviation values extending below 0 on the vertical axis are truncated.

20% ethanol

Samples preserved with 20% ethanol had a mean calculated starting quantity of 2642 copies per reaction at week 0 (Figure *4*). However, subsequent timepoints were found to have significantly higher concentrations of the quagga *COI* gene. Samples from 2-, 8-, and 12-weeks had mean starting quantities in excess of 19,000 copies per reaction.

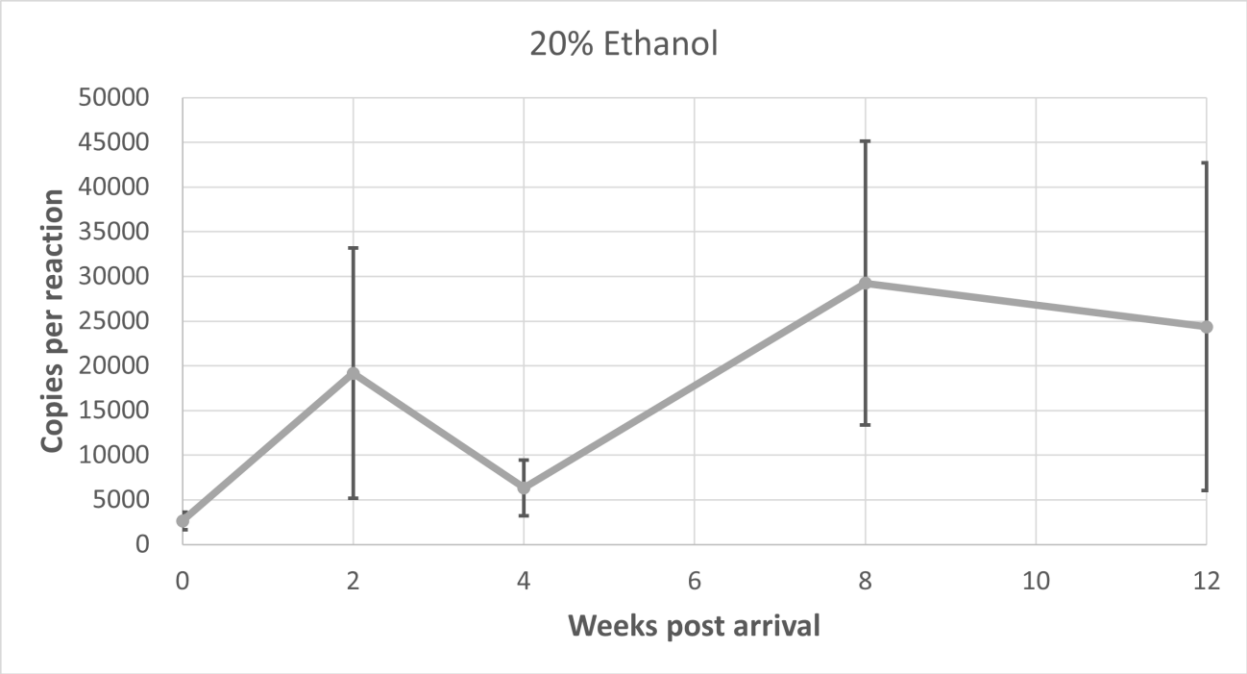

Figure 4. Graph of mean calculated starting quantities of the quagga mussel COI gene per reaction across the sampled timepoints for samples preserved with 20% ethanol. Standard deviations from the mean are shown as bars above and below the mean for each sampled timepoint.

70% ethanol

Samples preserved with 70% ethanol had a mean calculated starting quantity of 768 copies per reaction at week 0 (Figure 5). Subsequent timepoints had values ranging between 1744 and 3681 copies per reaction.

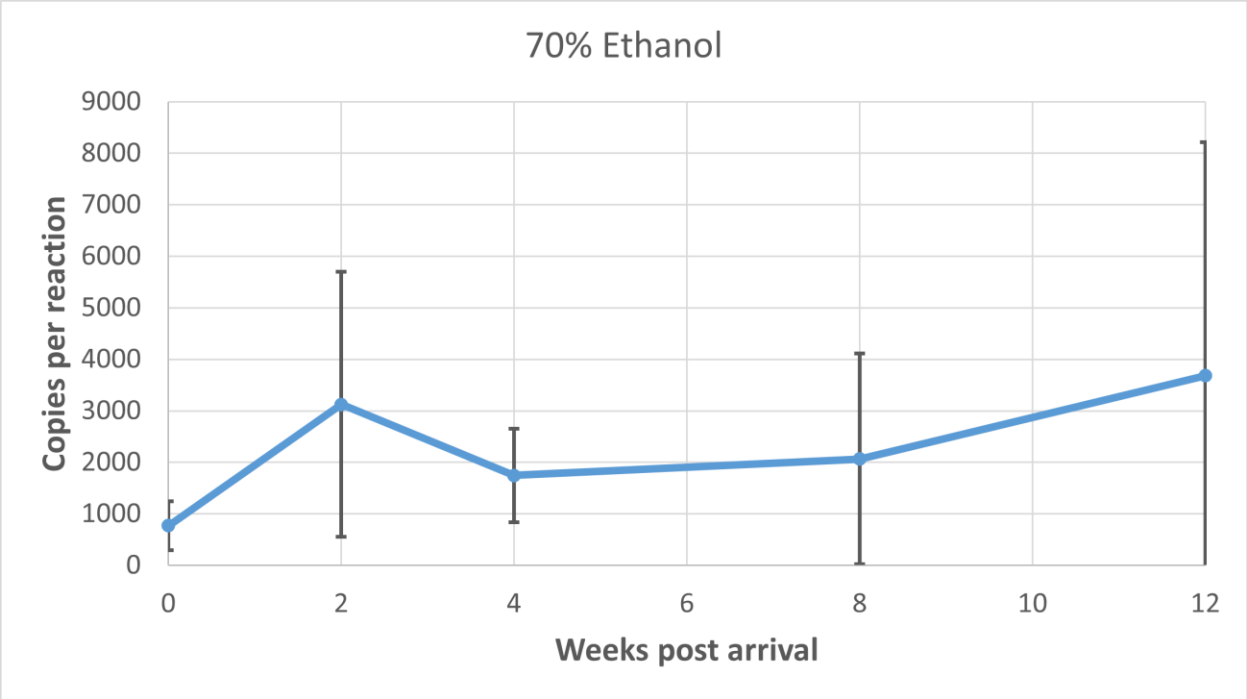

Figure 5. Graph of mean calculated starting quantities of the quagga mussel COI gene per reaction across the sampled timepoints for samples stored with 70% ethanol. Standard deviations from the mean are shown as bars above and below the mean for each sampled timepoint. Note that standard deviation values extending below 0 on the vertical axis are truncated.

70% isopropanol

Samples preserved with 70% isopropanol had a mean calculated starting quantity of 1824 copies per reaction at week 0 (Figure 6). Subsequent timepoints had values ranging between 400 and 5809 copies per reaction.

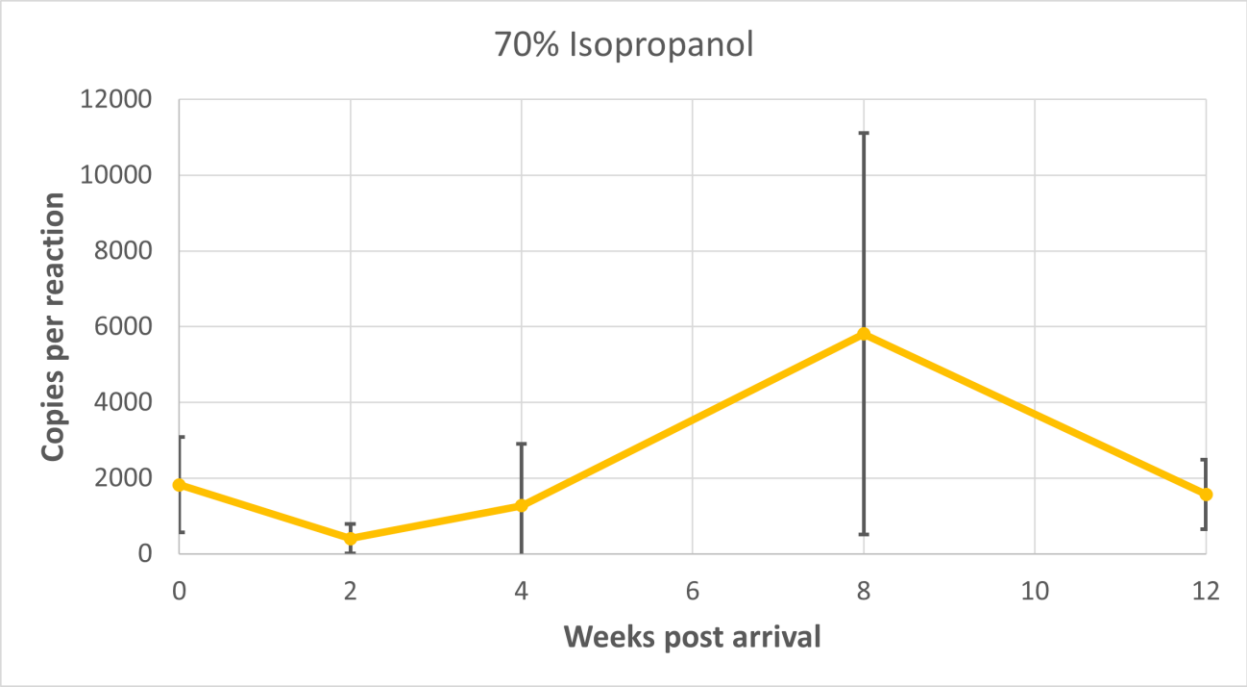

Figure 6. Graph of mean calculated starting quantities of the quagga mussel COI gene per reaction across the sampled timepoints for samples stored with 70% isopropanol. Standard deviations from the mean are shown as bars above and below the mean for each sampled timepoint. Note that standard deviation values extending below 0 on the vertical axis are truncated.

Longmire's solution

Samples preserved with Longmire's solution had a mean calculated starting quantity of 604 copies per reaction at week 0 (Figure 7). Subsequent timepoints had values ranging between 613 and 1384 copies per reaction.

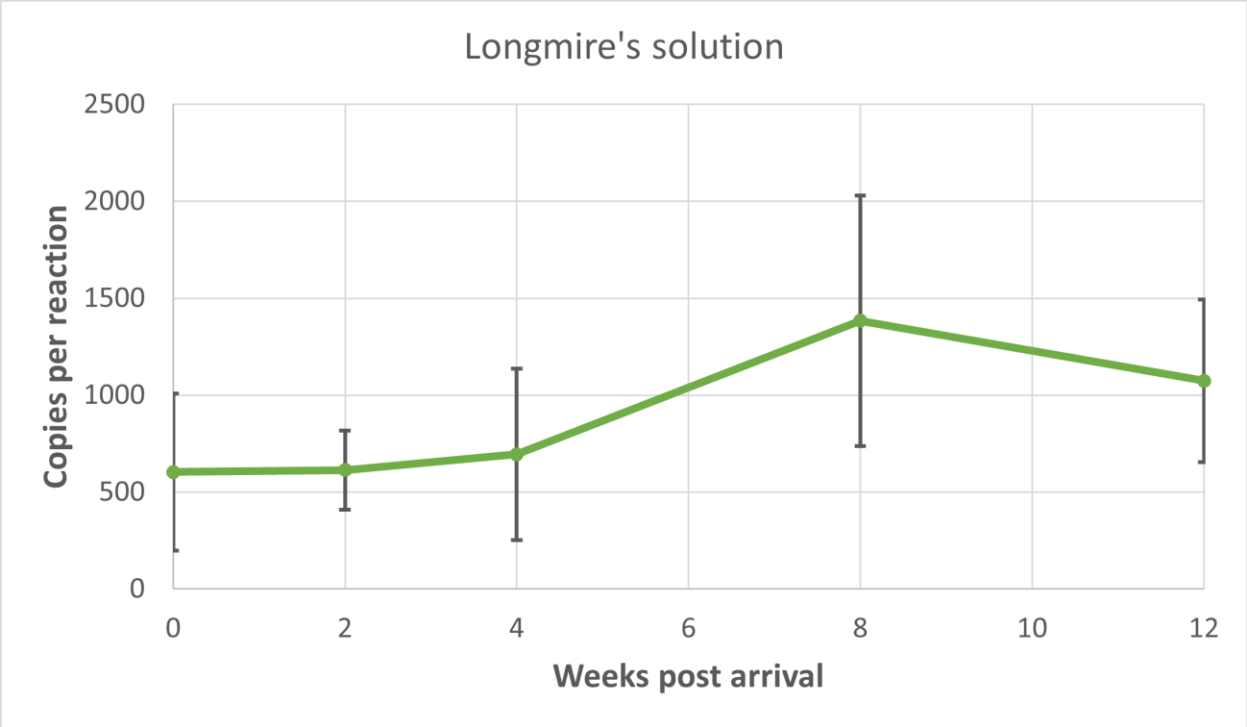

Figure 7. Graph of mean calculated starting quantities of the quagga mussel COI gene per reaction across the sampled timepoints for samples preserved with Longmire's solution. Standard deviations from the mean are shown as bars above and below the mean for each sampled timepoint.

Propylene glycol

Samples preserved with propylene glycol had a mean calculated starting quantity of 3054 copies per reaction at week 0 (Figure *8*). Subsequent timepoints had values ranging between 2353 and 2815 copies per reaction.

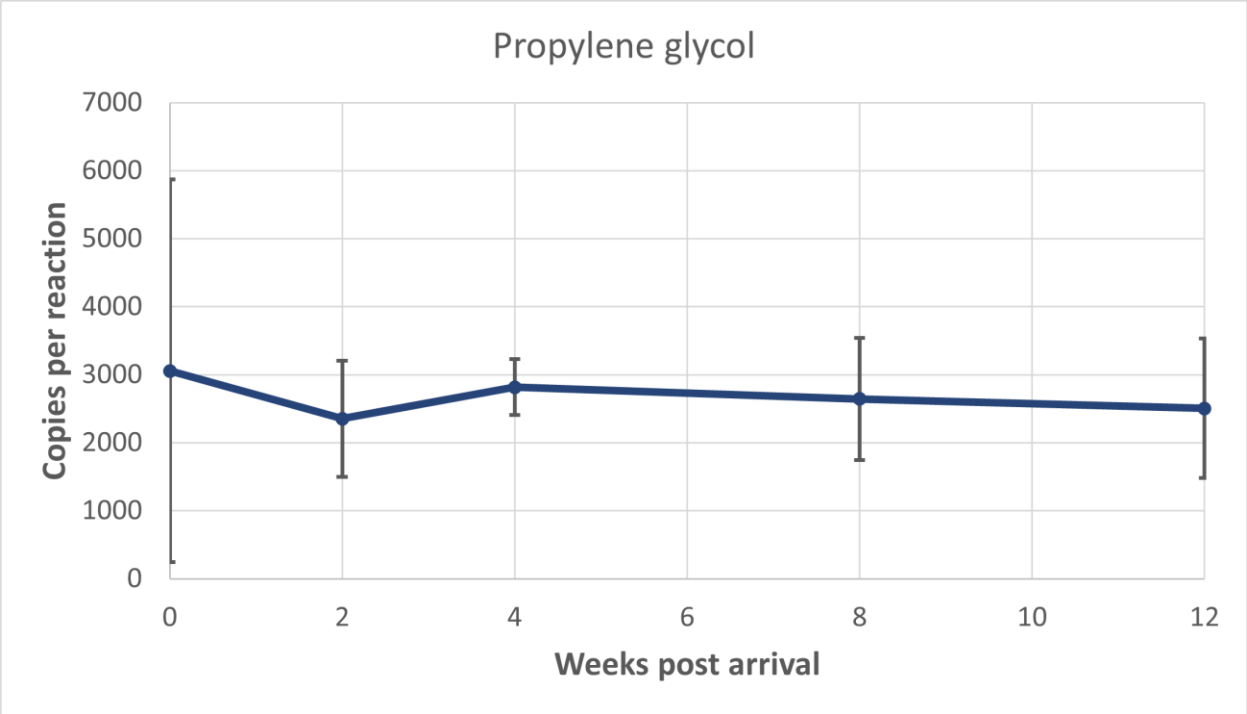

Figure 8. Graph of mean calculated starting quantities of the quagga mussel COI gene per reaction across the sampled timepoints for samples preserved with propylene glycol. Standard deviations from the mean are shown as bars above and below the mean for each sampled timepoint.

Comparison between treatments

Plotting data from all treatments on a single graph illustrates that the 20% ethanol treatment had much higher calculated starting quantities of the quagga COI gene at nearly all timepoints as compared to other treatments (Figure 9). At 2-, 8-, and 12-weeks post arrival the calculated starting quantities for the samples with 20% ethanol were nearly an order of magnitude higher than for other treatments. 70% ethanol, 70% isopropanol, and propylene glycol all resulted in comparable starting quantities across timepoints, although the 70% isopropanol data displayed more variability between timepoints than did the other two treatments. Longmire's solution generally had the lowest starting quantity of the preservatives tested.

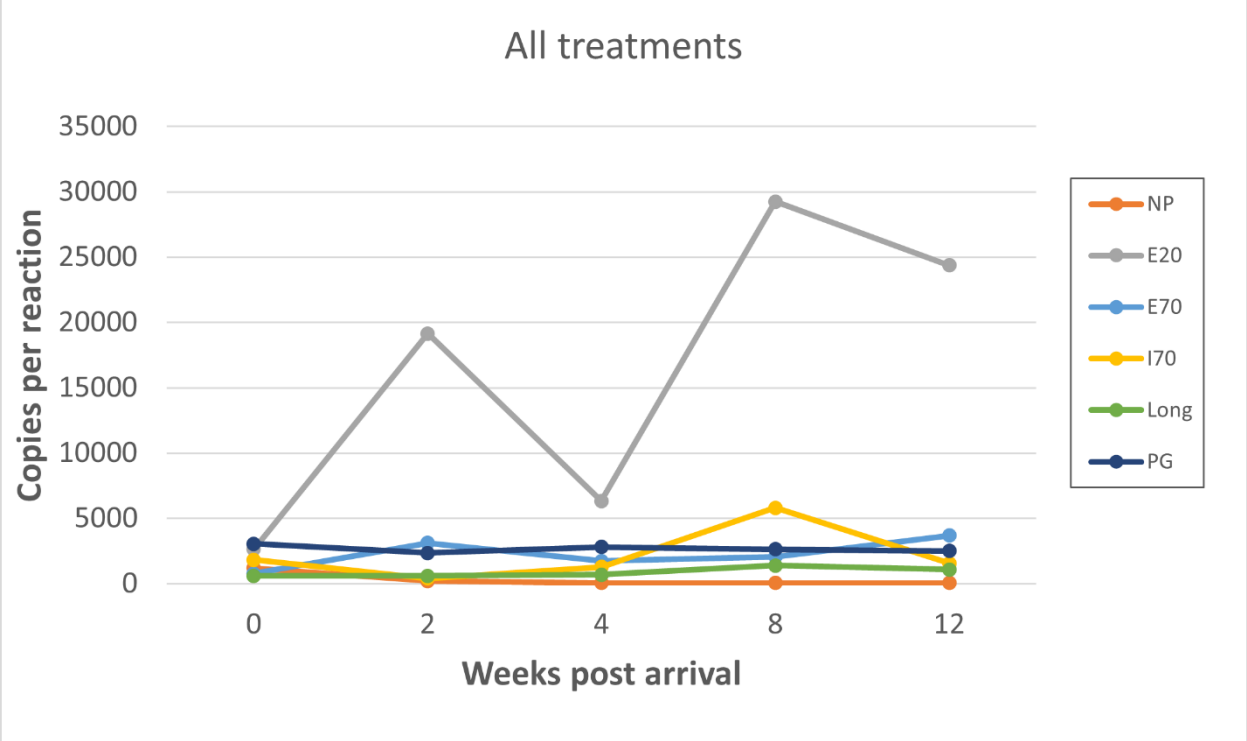

Figure 9. Graph of mean calculated starting quantities of the quagga mussel COI gene per reaction for the five preservations treatments and samples stored without preservative. Preservatives are abbreviated as follows in the legend: NP = no preservative; E20 = 20% ethanol; E70 = 70% ethanol; I70 = 70% ethanol, Long = Longmire's solution; PG = propylene glycol.

Discussion

Quagga mussel eDNA detection

All the preservatives tested were successful in resulting in returning detection across samples over the full time-course of the study. Detection/non-detection (also reported as positive or negative) of dreissenid DNA is the primary output that the EcoLab reports for eDNA analysis. In the context of the current experiments all of the preservatives tested performed successfully.

Quantification data

Quantification of starting quantity of DNA in each reaction (calculated as copies of the *COI* gene input to the reaction) were analyzed to provide more detail as to how the preservatives performed relative to one another. Higher copy numbers should represent better preservation, and also result in greater sensitivity. qPCR reactions for a given primer set have a limit of detection (LOD) and limit of quantification (LOQ) based upon the primer set, reagents, and amplification protocol in use. The LOD is the minimum concentration of the DNA target which can be reliably amplified and detected in 95% of samples, while the LOQ is calculated based the coefficient of variation for tested concentration (Kylmus et al., 2020). For the current study the relative starting quantities may relate to the final sensitivity of the assay. Early detections of dreissenid mussel are likely to occur in waterbodies with small populations of mussels, and as results low amounts of mussel eDNA in sampled waters. The more effective the preservation, the more likely that the eDNA in the processed and analyzed sample will be above the limit of detection of qPCR assay.

None of the preservatives tested showed a consistent decrease in the mean starting quantity of COI gene across the time points sampled. This result demonstrates that all of the preservatives are effective at preventing DNA degradation over the period of the study. In addition, 70% ethanol, 70% isopropanol, and propylene glycol had similar starting quantities across the study period, with mean values between 2100 and 2700 copies per reaction. Longmire's solution had a lower average starting quantity than the other preservatives, with an average of 874 copies per reaction across time points. 20% ethanol has much higher starting quantity than the other preservatives, with an average of 16,347 copies per reaction.

The very high starting quantities measured for 20% ethanol samples were unexpected and defy easy explanation. At face value this would seem to evidence that 20% ethanol in considerably better at preserving eDNA than the other preservatives tested. This is contrary to conventional wisdom that ethanol concentration should be at least 70% to inhibit DNA degradation caused by both microbial activity and hydrolysis in an aqueous environment. For longer term storage concentrations of ethanol in excess of 90% are generally recommended. Although not directly comparable, one recent study analyzed number of gene target copies from arthropod tissues storied in various concentrations of ethanol and found that copy number was significantly reduced in samples stored in lower concentrations of ethanol (Marquina et al., 2021). In some cases, there was a significant difference even between 70% and 95% ethanol. In the current study it is possible that the time series

was short enough that significant degradation was not occurring, however the dramatic decrease in the starting quantity in samples without preservative shows that this is not the case.

However, it could be that 20% ethanol was sufficient to limit microbial activity in the collected samples. Samples were collected from Lake Mead, which was considered oligotrophic-mesotrophic prior to the introduction of quagga mussels, and which has seen a reduction in phytoplankton biomass since their establishment. It could also be that the 20% ethanol carried with the sample during extraction is more compatible with the ZYMO extraction kit than the other reagents tested. This would mean that all the other reagents tested cause inhibition in the kit, resulting in lowered recovery of DNA, and that they all cause similar levels of inhibition. This seems unlikely but would need to be tested more directly with controlled inputs of DNA to be ruled out. An effort for correlation between the calculated starting copies of COI and the total DNA recovered from the ZYMO kit extraction was unsuccessful. In testing with the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific) none of the samples from week 8 that were preserved with 20% ethanol had measurable amounts of DNA above the limit of detection of the assay. This does suggest that the qPCR results may not be due to higher recovery of DNA from samples preserved with 20% ethanol.

Variability

High levels of variability in the calculated starting quantity of the COI gene were observed for all preservatives, as measured by the standard deviation within timepoints for a given preservative. This is not unexpected, as eDNA is likely to be spatially heterogenous. Particles containing target DNA may be distributed unevenly in the environment, resulting in difference in calculated starting quantities even when samples are collected in close spatial and temporal proximity. In the case of dreissenid mussels much of what is considered eDNA may in fact derive from intact veligers captured during sample collection. The number and size of veligers captured would thus have a large impact on the starting calculated starting quantity of COI gene. In the present data set largest standard deviations were observed at timepoints with the highest mean starting quantity, owing to the fact that individual samples at these timepoints had starting quantities sever times that of the other replicate samples. Propylene glycol generally had lower variability than other preservatives, both within and between timepoints. It is uncertain if this is due to some intrinsic characteristic of the preservative itself, or simply reflects a lower variability in the starting input of collected DNA. Low variability in the no preservative samples is likely due to high levels of DNA degradation across samples.

Conclusions

The most significant finding of the current study in that all the preservatives tested (20% ethanol, 70% ethanol, 70% isopropanol, Longmire's solution, and propylene glycol) resulted in qPCR detections for the quagga mussel COI gene across the time-course analyzed. Detection/non-detection by qPCR is the primary metric the EcoLab uses for evaluating the presence of quagga mussel eDNA in samples, and the data type that is reported to clients and partners. This study supports the use of 70% ethanol, which is the current standard in the EcoLab, as it produced results comparable to the other preservatives tested.

The results of this study are not without caveats. Chief among these is that fact that the samples collected and tested may not be representative of conditions where most first-time findings of quagga mussel eDNA occur. Tow net samples collected from Lake Mead have less planktonic biomass than do samples from many of the reservoirs and lakes in the Western United States that the EcoLab analyzes for early detection of dreissenid mussels. Higher biomass in samples could significantly impact the efficacy of preservatives and the results of eDNA analyses. Any adoption of other preservatives would require validation under conditions of high planktonic biomass and low veliger numbers. Such a scenario was not available for testing in the current study. In particular, the finding of high starting quantities with 20% ethanol would likely not hold up under other test conditions. In addition, the current study did not evaluate the impact of different preservatives on the ability to detect mussel veligers by microscopy. Without buffering samples tend to become acidic, resulting in the dissolution of calcium carbonate from the shells of veliger larvae. When the calcium carbonate dissolves it is no longer possible to identify veligers by cross-polarized light microscopy. The EcoLab has previously established that Tris buffer is effective samples preserved with either ethanol or isopropanol, maintaining the integrity of veliger shells. This has not yet been tested for either Longmire's solution or propylene glycol and would also need to be established before either could be used for sample preservation in the EcoLab.

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Data

Share Drive folder name and path where data are stored:

T:\Jobs\DO_NonFeature\Science and Technology\2019-PRG-Mussel Sample Preservation

Point of Contact name, email, and phone:

Yale Passamaneck, ypassamaneck@ubr.gov, 303-445-2480

Short description of the data:

Final report (PDF)
qPCR data (csv)

Keywords:

Quagga mussel, eDNA, qPCR

Approximate total size of all files:

1 MB