Reliability of Early Detection of *Dreissena* spp. Larvae by Cross Polarized Light Microscopy, Image Flow Cytometry, and Polymerase Chain Reaction Assays

Results of a Community Double-Blind Round Robin Study (Round Robin Study Phase II)

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10 Ocean Science Circle
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By

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Executive Summary

With funding provided by the U.S. Fish and Wildlife Service (USFWS) and coordinated through the USFWS Pacific Regional office (Paul Heimowitz, program manager), a method comparison double-blind study was undertaken to assess the current reliability of three different methods for detecting *Dreissena* spp. mussel larvae (veligers) in plankton net tow samples. The three methods examined were cross polarizing light microscopy (CPLM), imaging flow cytometry (IFC), and DNA-based polymerase chain reaction (PCR) assays. Reference samples consisting of concentrated plankton spiked with known numbers of *Dreissena* spp. larvae (0-27 range) were distributed to practicing analytical laboratories for analysis. For practical reasons, sampling was conducted in the winter of 2010, although it was recognized that it would have been more realistic to sample in warmer months typical of the periods when most routine monitoring occurs. This resulted in samples of lower complexity then routinely encountered by analytical laboratories and may have biased the study toward the underestimation of analytical errors. Eighteen independent laboratories participated in the study and analyzed 216 reference samples. Results indicated that CPLM was the most reliable of the methods, with an overall accuracy for presence/absence detection of 96.3%. IFC analysis was the second most reliable method, exhibiting a 91.7% accuracy rate. PCR was the least reliable method, exhibiting a 75.8% accuracy rate. The most prevalent type of error associated with all of the methods was false negatives, suggesting that all methods are more likely to fail to detect the presence of *Dreissena* spp. larvae rather than to falsely indicate their presence. Of the two microscopy-based methods (CPLM and IFC), IFC systematically underestimated the quantity of larvae in samples, while CPLM was more reliable with respect to larvae quantification. PCR methods are not yet quantitative, so they could not be evaluated in this regard. Based on this study, 10 specific recommendations for improving the reliability of dreissenid early detection monitoring programs are proposed.

1.0 Background and Project Rationale

Quagga mussels (*D. rostriformis bugensis*) were first detected west of the 100th meridian in Lake Mead, Nevada, at the Hoover Dam (Stokstad, 2007). Surveys conducted in the following months revealed the presence of both quagga and zebra (*Dreissena polymorpha*) mussels in many other Western United States water bodies (Quagga and zebra mussel control strategies workshop report, 2008). However, because there remain many western water bodies that have not been infested, a key issue for the management of these invasive mussels is an ongoing vigilant monitoring program. Monitoring and early detection of dreissenid mussels are key to minimizing the risks for the rest of the West from nearby potential seed populations. Early detection of the initial presence of the species makes it more likely that mitigation actions will slow down their spread and reduce their impact on the local ecology, as well as allow the implementation of effective control measures for the protection of water delivery infrastructure.

Because dreissenid mussel management efforts are dependent on accurate monitoring and efficient information dissemination, an important component of early detection monitoring programs is the ability to accurately detect, as early as possible, the introduction of these mussels into a pristine water body. The planktonic larval form (veliger) of *Dreissena* spp. mussels is a primary means of colonization in new water bodies and a harbinger of the presence of spawning adults. Detection of *Dreissena* spp. veligers is, therefore, a critical component of early detection monitoring programs. Available analytical techniques for the detection of *Dreissena* spp. larvae in plankton samples include two microscopy-based approaches including cross-polarized light microscopy (CPLM) and cross polarized light microscopy combined with imaging flow cytometry (IFC) (Johnson, 1995; FlowCAM® application note, 2008). A third approach involves the use of a polymerase chain reaction (PCR) assay to detect DNA specific to *Dreissena* spp. mussels in plankton samples (Frischer et al., 2002). Since management decisions are often based on the results derived from these analytical approaches, it is critical that the reliability of each of these assays can be accurately estimated and that continued efforts are made to improve and standardize each of these techniques. At present, the reliability of available veliger detection assays is not well understood, and the sources of analytical variability are speculative. This experiment was designed to quantify the relative reliability of currently used *Dreissena* spp. larvae early detection approaches.

1.1 Synopsis of Available Early Detection Methods

Cross polarized light microscopy is the most established among laboratory techniques (Johnson, 1995). Because all bivalve larvae exhibit distinct birefringent characteristics, this approach allows bivalve larvae to be easily

recognized under polarized light illumination, even in the presence of other plankton and material typically present in plankton net tow samples (Johnson, 1995). Under cross polarizing light, *Dreissena* spp. larvae exhibit a Maltese cross ("X" marks the larvae) and generally appear as bright white objects with a dark cross (90° angle), while other plankton species are very dim under cross polarized light and do not exhibit the characteristic Maltese cross. Standard dissecting stereoscopic microscopes are easily and economically modified with the necessary polarizing filters, or specialized microscopes can be purchased. In addition to the cross polarizing microscope, only minimal additional materials are required for microscopic analysis of plankton samples. Other supplies and equipment include appropriate fixatives (usually ethyl alcohol), counting chambers, and possibly additional settlement concentrators. It is also useful if the microscope is equipped with photographic capability so that images of larvae can be photographed, shared, and studied by other experts.

Although *Dreissena* spp. larvae are quite distinctive under a cross polarizing light microscope, it is possible that they can be confused for other bivalve species that may be in a sample including the Asian clam (*Corbicula fluminea*), the dark false mussel *Mytilopsis* sp., and possibly Unionidae larvae that may co-occur with *Dreissena* spp. species. Additionally, to a poorly trained eye, the ostracod crustaceans and even sediment particles may be mistaken for *Dreissena* spp. larvae. Therefore, it is of critical importance that any technician examining plankton tow samples for *Dreissena* spp. larvae be well trained, experienced, and familiar with other particles that may possibly be mistaken for *Dreissena* spp. larvae. To increase the likelihood of correct identification, the technician should be able to easily switch between cross polarized and non-polarized illumination to analyze further diagnostic characteristics. For instance, *Corbicula* veligers may look like D-shaped Dreissenid veligers, but *Corbicula* are much larger and their size ranges do not overlap. Ostracods have distinct appendages that can be identified under non-polarized light. In general, if an object exhibits a Maltese cross under cross polarized illumination, the object should also be examined under normal light to identify other diagnostic morphological features. In addition, high quality digital images should be taken of any suspected positives to be sent for outside opinions.

Additional analytical problems in detecting and identifying *Dreissena* spp. larvae can arise if the samples are poorly preserved or if they contain exceedingly high concentrations of non-dreissenid plankton. Poor preservation can result in the dissolution of veliger shells resulting in false negatives. Shell dissolution is common if the sample becomes even slightly acidic and is stored for any period of time. Some investigators have cautioned against the use of denatured alcohol (Wells, personal communication). Although there is not yet a consensus among experts regarding the optimal concentration of alcohol, anecdotally, concentrations from 25-95% ethanol have been reported to have been used successfully for the fixation of bivalve larvae. However, to our knowledge, there has never been a systematic study of this parameter. Extremely high plankton

concentrations in samples often obscure the detection of *Dreissena* spp. larvae even under cross polarizing illumination. When plankton concentrations are too high, it is necessary to dilute the sample (usually in distilled water), which greatly increases the amount of time required to examine the sample. Finally, it cannot be overemphasized that plankton communities vary between water bodies and with season; thus, microscopists must be well trained and familiar with plankton communities and plankton successional cycles to be able to examine and detect larvae in a diversity of plankton samples that they are likely to encounter.

By combining CPLM and IFC, the development of the FlowCAM® instrument can significantly decrease the labor involved in examining plankton samples for the presence of *Dreissena* spp. larvae (Spaulding et al., 2009). As described above, under cross polarizing light, the birefringence from the calcareous shells of zebra and quagga mussel veligers can also be imaged from a flowing stream. By applying automated pattern recognition algorithms including size aspect ratio and gray scale intensity, zebra and quagga mussel veligers may be distinguished from other particles and zooplankton. This technology is increasingly being adopted for the purpose of early detection of *Dreissena* spp. larvae in plankton samples.

In addition to an imaging flow cytometer, which costs $80,000 to $100,000 (U.S. dollars) (depending on how it is equipped), as with CPLM, only minimal additional materials are required for imaging flow cytometeric analysis of plankton samples. Other supplies and equipment include appropriate fixatives (usually ethyl alcohol) and, possibly, additional settlement concentrators.

PCR is a process that allows for the *in vivo* amplification of specific gene fragments. Since gene fragments unique to *Dreissena* spp. larvae can be targeted by a specific PCR assay, this approach can be utilized to detect the presence of *Dreissena* spp. larvae in a plankton sample, even if they are at very low concentrations. Theoretically, PCR assays provide several distinct advantages compared to cross polarized light detection strategies including: (1) increased sensitivity, (2) increased species specificity, and (3) the ability to process a much larger number of samples in the same amount of time needed to process microscopy samples. However, the realization of these advantages still requires considerable research and development.

PCR is a technique widely used in molecular biology. It derives its name from one of its key components, a DNA polymerase used to amplify a piece of DNA by *in vitro* enzymatic replication. As DNA replication in the PCR assay progresses, the DNA generated is used as a template for further rounds of replication. This initiates a chain reaction in which the DNA template is exponentially amplified. With PCR, it is possible to amplify a single or a few copies of a piece of DNA to generate millions or more copies of the DNA piece. This amplification makes it possible to simply detect the presence of a diagnostic genetic characteristic; for example, a gene unique to *Dreissena* spp. species. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for

amplification under specific thermal cycling conditions. In the case of *Dreissena* spp.-specific PCR assays, single stranded DNA primers (oligonucleotides) are designed that are specific for either *D. polymorpha* (zebra mussel), *D. bugensis* (quagga mussel), or assays that are capable of amplifying both species. To date, primers have been designed to target several different genes present in these species including the small and large subunit RNA genes (Frischer et al., 2002; Hoy et al., 2010), the Ribosomal Internal Transcribed Spacer sequences (unpublished), and the mitochondrial Cytochrome Oxidase I gene (Claxton and Boulding, 1998).

The lab infrastructure and materials required for PCR are more extensive (and expensive) than those required for cross polarized microscopy. However, PCR capability is currently available in a very large number of research, clinical, and commercial diagnostic laboratories, and the PCR technique is relatively easily learned and is regularly incorporated into classroom labs in undergraduate and even high school biology programs. Thus, there is a large availability of laboratory infrastructure and personnel capable of performing *Dreissena* spp.-specific PCR assays.

In practice, PCR detection of *Dreissena* spp. larvae in plankton samples is subject to many possible types of error that may result either in failure to detect larvae when they are present, or that indicate the presence of larvae when they are not actually present in a sample. One important factor that may result in failure to detect larvae when they are present or that reduces the detection sensitivity of PCR assays is that practicality requires that only a relatively small subsample of a plankton tow sample can be assayed, whereas it is generally feasible to examine an entire plankton tow sample microscopically. False negatives can also be obtained if substances that co-purify with DNA from the plankton sample inhibit PCR amplification of *Dreissena* spp. DNA. Several substances present in plankton tow samples including mucopolysaccharides, humics, and metals have been shown to be potent inhibitors of PCR (Wilson, 1997). PCR inhibitors seem to be a particular problem in samples from the Lower Colorado Basin (Kelly, personal observations). Finally, false negatives can be obtained due to poor preservation, resulting in the degradation of DNA containing tissues. Sample contamination is the most likely explanation for false positive detection by PCR. Contamination may occur at many different points of the analytical process including sample collection, DNA purification, and during PCR amplification.

PCR is a relatively new technique for the detection of zebra and quagga mussel larvae with a high potential for improving *Dreissena* spp. early detection monitoring programs. In fact, most of the *Dreissena* spp.-specific PCR assays used in this study have been developed only in the past few years, and most have not yet been published in the peer-reviewed literature and vetted by the research

and management communities. Thus, although interest in its use for *Dreissena* spp. veliger detection has increased in the Western United States, PCR is still largely a research-grade tool.

1.2 Comparison of Methods

For early detection, the efficacy of any single laboratory detection method can be highly variable; therefore, several western management organizations require that at least two independent detection methods be applied in order to verify results, particularly if it is the first detection of dreissenids in a water body (Anderson et al., 2009). However, since the discovery of quagga mussels in Lake Mead in early 2007 and widespread monitoring began, there have been many analytical discrepancies between results reported from different labs and based on the use of different detection technologies. For example, Frischer and Butler (2009) reported that out of 372 plankton samples collected from western waters in 2007 and 2008, and examined by both PCR and CPLM, there was a discrepancy in the results between 109 (29.3%) of the samples. In the majority of these cases (98), veligers were detected by microscopy but not by PCR. Identifying the sources of these discrepancies is a primary objective of the current study.

The reliability of veliger detection is dependent on many variables including the sample matrix and efficacy of the sampling technique, sample preservation, veliger abundance, method sensitivity and robustness, and analyst experience. In prelude to this study, in late 2008, a preliminary study was undertaken to determine the source of these discrepancies. This was the first of the so-called "Round Robin" studies and was designed to determine the amount of variability between microscopy and PCR results that could be attributed to the analytical procedures in the absence of confounding effects of analysis in a complex background of other plankton, detritus, and sediment. In this study, 12 participating microscopy and PCR labs analyzed sets of plankton-free lake water that had been spiked with known numbers of *D. polymorpha* veligers. Veliger concentrations ranged from 0 to >100 per 40 ml sample. With respect to presence/absence detection, 96% of the microscopic analyses agreed with expected results, while 85% of the PCR analyses matched expectations. In general, the Round Robin Phase I study illustrated and quantified analytical error associated with both microscopy and PCR approaches. Presence/absence detection by microscopy outperformed PCR by about 10% with respect to absolute accuracy. Inter-lab analytical variability of PCR analyses was about twice the rate of microscopy analyses. However, it was also evident that both approaches had the potential to be 100% accurate (Frischer and Butler, 2009).

1.3 Round Robin Phase II – Effect of Sample Matrix

Following on the results and basic experimental design of the Phase I study, Phase II focused on determining the analytical variability associated with more

realistic sample matrixes that included the presence of other plankton. The study also involved a greater number of laboratories and methodological approaches. Specifically, since the completion of the first study, several new PCR methods have been developed, and three new labs were routinely using IFC technology for the purpose of detecting *Dreissena* spp. larvae. Here we report the results of the Round Robin Phase II study.

2.0 Experimental Methods

2.1 Experimental Design Overview

In order to achieve the objectives of this study, to identify and quantify the reliability of currently available microscopy IFC- and PCR-based approaches for the early detection of *Dreissena* spp. larvae in plankton net tow samples, a double-blind, round robin study was designed and implemented. The approach taken was to identify and enlist as many laboratories as possible that are actively involved in early detection of Dreisenid mussels, provide them with a set of reference samples containing realistic planktonic communities spiked with known numbers of *Dreissena* spp. larvae, and synthesize the resulting data to assess assay reliability and identify future research directions for improving the ability to detect early introductions. In all cases, the participants and the person who distributed the sample were unaware of the contents of the reference samples until after all analyses were complete. This double-blind design eliminated the risk of prejudgment by the participants, which could distort the results.

2.2 Participating Laboratories

Eighteen independent laboratories participated in this study. Participating laboratories and contact information are provided in Appendix I. Of these laboratories, several were able to complete multiple types of analyses. Eleven laboratories were asked to analyze reference sample sets by CPLM, and nine completed the analyses. Five laboratories were asked to analyze reference sample sets by IFC using FlowCAM® instrumentation, and four completed the analyses. Twelve laboratories were asked to analyze reference samples by PCR-based analysis, and 11 completed the analyses. Two of the analytical laboratories that participated were also involved in the organization of the experiment (Skidaway Institute of Oceanography and the Bureau of Reclamation). However, neither the analysts nor the principals in these laboratories knew the status of the samples they received because they were prepared by a third party (Nierzwicki-Bauer, Darrin Fresh Water Institute), and their contents were not revealed until after the results were completed.

2.3 Collection of *Dreissena* spp.-Free Plankton Samples

In order to provide a realistic plankton matrix for the reference samples, it was necessary to collect *Dreissena* spp.-free concentrated plankton from a location where dreissenids were not present. Following consultation with several western invasive species managers including Larry Dalton (Utah) and Elizabeth Brown (Colorado), we identified Quail Creek Reservoir in Utah as a suitable site. Quail Creek Reservoir is a large (239 hectares) impoundment of Quail Creek and several tributaries in extreme southwestern Utah (latitude 37° 22 49N, longitude 113° 10 115W). The reservoir currently provides water for both municipal/industrial and agricultural users. To date, there have been no reports of *Dreissena* spp. larvae or adults in the reservoir, which is regularly monitored by personnel onsite at the Quail Creek Water Treatment Plant (QCWTP). However, shortly after the sampling for this project was completed, an adult quagga mussel was discovered in an adjacent and connecting water body, Sand Hollow Reservoir. The water chemistry of Quail Creek Reservoir would be supportive of *Dreissena* spp. mussels if they were to be introduced. Based on averaged STORET spring and summer data, calcium concentrations are 415 mg L^{-1}, pH is 8.1, and sufficient nutrients are present to support average total chlorophyll at levels of 1.7 µg L^{-1}. Primary production in the reservoir is believed to be phosphorus limited. Based on monitoring by the QCWTP in 2008-2010, water temperatures ranged from 6 to 28 °C, with temperatures permissive of *D. bugensis* spawning occurring from May through October. Plankton for this study was collected from the reservoir on January 18-19, 2010.

Sampling was accomplished using 63 µm, 8-inch and 12-inch vertically towed plankton nets from two sites in the reservoir. At the time of sampling, water temperature was 6 °C and chlorophyll concentrations were 1.3 to 1.5 µg L^{-1}. A total of 2.65 L of concentrated plankton was collected over the 2-day sampling period. After collection, plankton concentrates were pooled, examined microscopically for the presence of *Dreissena* spp. larvae, and ethanol preserved. Total chlorophyll in the concentrated plankton sample was 182 µg L^{-1} and *Dreissena* spp. larvae were not observed. Two sets of ethanol preserved samples were prepared. For microscopy analyses (CPLM and IFC), plankton samples were made 25% ethanol (final concentration), and for PCR analyses, samples were made 70% ethanol (final concentration). In each case, 1 L of concentrated plankton sample was diluted to 3.6 L so that the concentration of plankton in each sample type was the same. For microscopy samples, 1 L of 96.5% ethanol and 1.6 L of filtered Quail Reservoir water were added to 1 L of plankton concentrate. For PCR samples, 2.6 L of 96.5% ethanol were added to 1 L of plankton concentrate. Each sample set was distributed into four 1-L Nalgene bottles and shipped cold to the laboratories at the Darrin Fresh Water Institute in Bolton Landing, New York, where they were processed. Since we had difficulties shipping reagent grade ethanol in time for the planned sampling, we purchased and used a 96.5% neutral spirits grain alcohol (Spirytus Luksusowy vodka) from a

local liquor store in Nevada prior to our arrival in Utah. Previous experience with preservation of field samples using high proof grain alcohol intended for human consumption suggested that the use of this vodka would be suitable (Frischer, unpublished observations). Sampling and sample preparation were greatly assisted by Michelle Deras (QCWTP plant operator and biologist) and local Department of Conservation rangers Regan Wilson and Jonathan Allred.

Because of the timing of the study (funding and the availability of voluntary services from the participating analytical laboratories), sampling had to be conducted in the winter of 2010, although it was recognized that it would have been more realistic to sample in warmer months. As a result, the matrix of the blind samples may not be representative of plankton tow samples collected by various monitoring programs during warmer periods. Because the plankton were typically not as concentrated as those obtained during warmer months, it is possible that the study's results may be biased toward underestimating inaccuracy compared to more realistic samples. However, plankton samples included typical diversity of species including those that can be confused for *Dreissena* spp. larvae including ostracods, corbicula, and other native mussel larvae; therefore, it was concluded that the samples were appropriate for addressing the primary goals of this study.

2.4 Collection of *Dreissena* spp. Larvae

Quagga mussel (*D. bugenesis*) larvae were collected on January 20-21, 2010, from Lake Mohave near Katherine's Landing in northwestern Arizona (latitude 35° 13 05N, longitude 114° 33 58W). Lake Mohave is part of the Lower Colorado River Basin and managed by the National Park Service. Water conditions are optimal for the growth of *Dreissena* spp. mussels and support high densities of quagga mussels similar to Lake Mead, Nevada (Quagga and zebra mussel control strategies workshop report, 2008). Since 1950, when the lake was formed by the construction of the Davis Dam, water temperatures have ranged from 9 to 29 °C. *Dreissena* spp. veliger monitoring in Lake Mohave began in 2007, shortly after the detection of adult quagga mussels at a marina in the Border Basin of the Hoover Dam in Lake Mead in January, and indicate that spawning is continuous throughout the year in Lake Mohave with extended spawning peaks in the spring and fall (Holdren et al., 2010).

At the time of collection for this study, the water temperature was 10.9 °C, and *Dreissena* spp. larvae concentration was estimated to be 200 veligers per m^3 of lake water. Larval concentrations were estimated from three independent plankton tows at the site. Corbicula larvae and ostracods were present in some abundance in the plankton samples, though they were not quantified or included in the prepared reference samples. Total chlorophyll a concentrations were estimated to be 0.28 µg L^{-1} in surface waters. Chlorophyll a concentrations were estimated by acetone extraction as described by Parsons et al. (1984). Veligers were collected by a combination of vertical and horizontal plankton net tows

using 63 µm, mesh size plankton nets. Samples were collected from the first horizontal dock segment at "Marina 4," where veliger concentrations have been routinely monitored by the Bureau of Reclamation. The depth along this dock segment ranged from 6 to 7.5 m. All of the dock structures, lines, cables, and boats that were in the water were completely encrusted with quagga mussels. Only *D. bugenesis* was observed in fouling communities, although a systematic search for *D. polymorpha* was not conducted. There have been no reports of *D. polymorpha* from the site, so we were confident that zebra mussels were not present.

Over the 2-day sampling period (January 20-21, 2010), approximately 6,000 larvae were collected in 8.85 L of plankton tow material. After completing the plankton collection, the total volume of plankton tow material was reduced to 2 L by filtering though 63 µm mesh screening. As was done for the plankton samples from Quail Creek Reservoir, two sets of ethanol preserved samples were prepared. For microscopy analyses (CPLM and IFC), plankton samples were made 25% ethanol (final concentration), and for PCR analyses, samples were made 70% ethanol (final concentration). In each case, 1 L of concentrated plankton sample was diluted to 3.6 L so that the concentration of plankton in each sample type was the same. For microscopy samples, 1 L of 95% ethanol and 1.6 L of filtered Lake Mohave water were added to 1 L of plankton concentrate. For PCR samples, 2.6 L of 95% ethanol were added to 1 L of plankton concentrate. Each sample set was distributed into four 1-L Nalgene bottles and shipped cold to the laboratories at the Darrin Fresh Water Institute in Bolton Landing, New York, where they were processed. In addition to concentrated plankton samples, Lake Mohave water was also made 25% and 70% with respect to ethanol and shipped to our labs to use as a diluent when larvae were isolated. Since our reagent grade ethanol had arrived, it was used instead of the vodka used in the Quail Reservoir samples.

2.5 Sample Preparation and Distribution

Preserved samples arrived intact and still cold in New York approximately 1 week after being sent. The samples were sent by ground because they contained 70% ethanol preserved samples which, because they were flammable, had to be treated as hazardous materials. Additionally, from previous experience, we knew that ethanol containing water plankton samples can experience significant volume reduction due to the evaporation of ethanol when shipped by air. Reference sample sets for distribution to the analytical laboratories were prepared between February 11-15, 2010. Reference samples consisted of 25 ml of appropriate ethanol preserved plankton from Quail Creek Reservoir (25% for microscopy samples and 70% for PCR samples) spiked with a known number of larvae. Reference sample sets consisted of a total of nine samples; three 25 ml aliquots of Quail Creek Reservoir plankton without any larvae, three containing 2 to 4 larvae, and three containing 11 to 27 larvae. Individual reference samples were prepared by spiking Quail Creek Reservoir plankton with the appropriate number of larvae.

Larvae from Lake Mohave samples were first concentrated by settling, transferred to a Petri dish viewing slide under a dissecting stereomicroscope, and larvae individually transferred into Corning 50 ml polycarbonate conical centrifuge tubes with plug seal caps (cat No. 430290). These tubes were utilized because they minimize leakage due to the vaporization of ethanol during shipping. Individual larvae were transferred to sample tubes using a pipette with plastic tips. A total of 26 reference sample sets were prepared and assigned randomized numbers.

A master datasheet was kept at the Darrin Fresh Water Institute. The samples were then sent to the Bureau of Reclamation laboratories in Denver, Colorado, where they were distributed without specific knowledge of their contents to the participating analytical laboratories. Each participating laboratory was also sent a set of instructions (Appendix II). All samples were sent cold and by ground rather then air to minimize sample loss due to the evaporation of ethanol. Analysts were instructed to process the entire 25 ml volume of each sample, send results directly to the Darrin Fresh Water Institute, and provide a detailed description of the methodology employed to the Skidaway Institute of Oceanography. Methodological reporting was standardized for PCR laboratories to ensure consistent reporting of details. Specific information concerning the methodological approaches for concentrating plankton, purifying DNA, PCR amplification, quality control, and analysis was requested. All results were completed and compiled by August 2010.

2.6 Synthesis

Once all results were received, they were compared and matched against the master data sheet with the actual number of larvae in each sample and the identity of the analytical laboratory. For each type of analysis (CPLM, IFC, and PCR), observed results were compared to actual larval numbers to determine the accuracy of detection and, where possible, quantification. Regression analysis and Analysis of Variance statistical tests were used to determine the significance of relationships between actual and observed larval counts.

3.0 Results

3.1 Cross Polarized Light Microscopy

Nine laboratories completed analysis of veliger sample sets comprising 81 independent analyses. Of these laboratories, eight (88.8%) delivered perfect results with respect to presence or absence detection. A summary of these results is shown in Figure 1. Panel A presents the results obtained from all larval concentrations, panel B presents results from samples that did not contain larvae, panel C presents results from samples containing 2 to 4 larvae, and panel D

presents results from samples containing 11 to 27 larvae. Overall, the accuracy of detection was 96.3% (Figure 1A). Although error rates were low, the largest type of errors observed were false negatives (2.5%), while false positives accounted for 1.2% of the error. Both instances of false negative results occurred at the lowest veliger concentrations (Figure 1C).

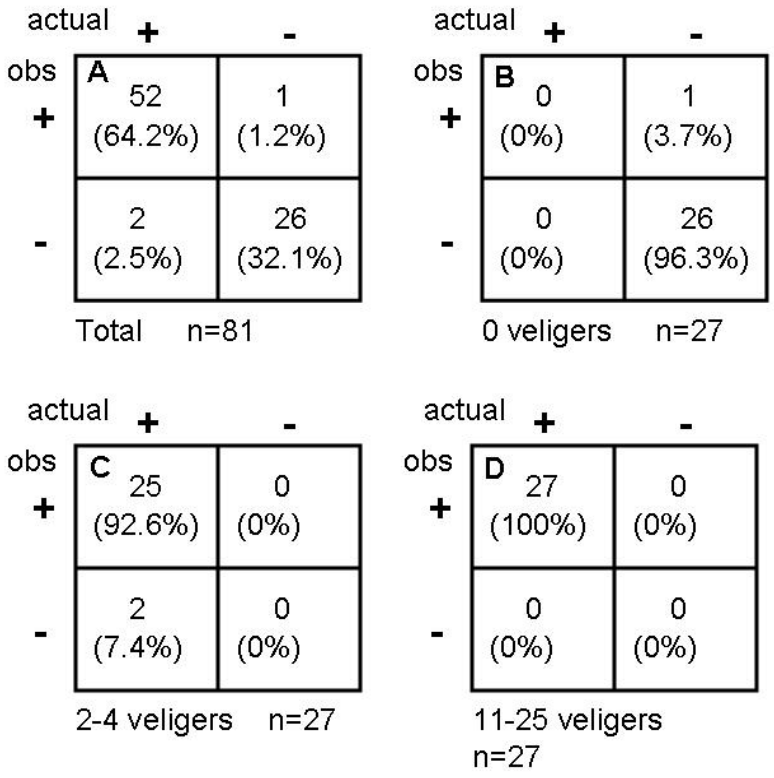

Figure 1. Comparison of actual to observed *Dreissena* spp. larval (veliger) counts by CPLM. (A) All samples, (B) Samples absent of larvae, (C) Samples containing 2 to 4 veligers, (D) samples containing 11 to 27 veligers.

Quantitatively, CPLM analyses resulted in excellent agreement between actual and observed veliger counts (Figure 2). Regression of actual versus observed veliger counts produced a slope of 0.99 ($r^2 = 0.89$) indicating a near perfect correspondence between the actual and observed numbers of veligers present in the samples. Of the 81 samples analyzed, 41 (51%) were in absolute correspondence. Of the remaining samples, 21 (26%) underestimated the actual number of veligers, and 19 (23%) overestimated the number of veligers actually present. If the results derived from samples not containing larvae were excluded from this analysis, approximately one-third each of the samples was in correspondence with actual counts, overestimates, and underestimates, respectively.

Overall, there was relatively little variability between the results produced by the different laboratories, indicating that CPLM is a relatively robust and mature

approach for detecting and quantifying *Dreissena* spp. larvae in plankton samples and that the available analysts are well trained. Between each of the labs, the primary difference in methodology was the approach used to concentrate plankton samples prior to microscopic examination. Of the nine labs, four initially concentrated the sample by gentle centrifugation, one concentrated by allowing the sample to settle by gravity, two collected larvae using fine meshed screening, and two labs did not concentrate samples. The single lab that did not achieve perfect results with respect to presence and absence detection did not concentrate the sample prior to microscopy.

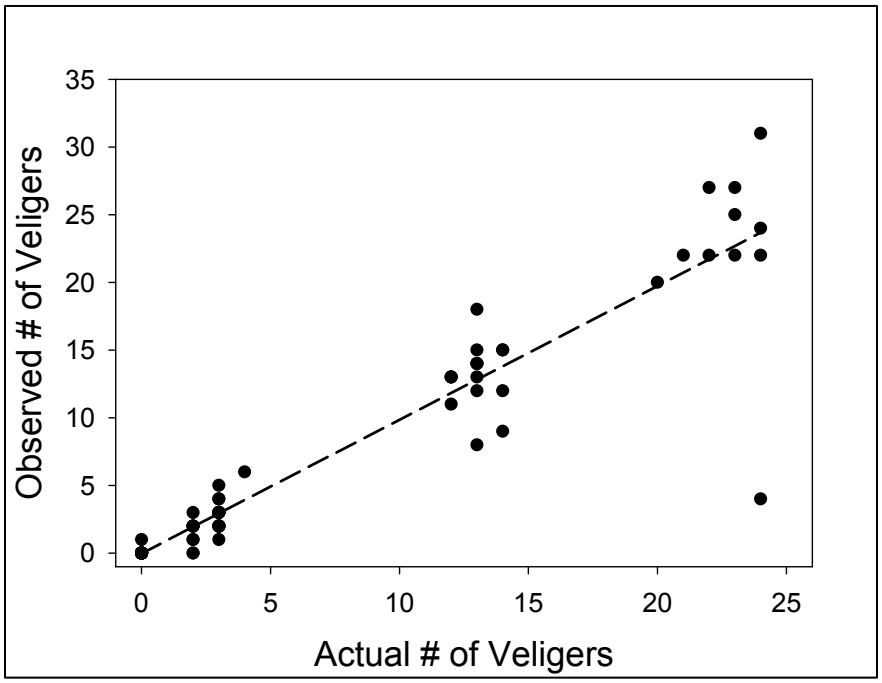

Figure 2. Comparison of actual versus observed *Dreissena* spp. veliger counts by CPLM. $r^2 = 0.89$, slope = 0.99.

The method of counting also varied with respect to whether a counting chamber was used. Three of the labs used counting chambers (Sedgewick-Raftner), while the others did not. With respect to counting accuracy, the use of counting chambers did not appear to make a large difference. Regardless of whether counting chambers were used, approximately half of all the counts were accurate. However, labs (3) using counting chambers typically reported a greater frequency of overestimated counts (41%), while labs (6) that did not use counting chambers reported a greater frequency of underestimated counts (31%). However, these errors may be attributable to technician variability, which was not explored during this study.

3.2 Image-Based Flow Cytometry

Four laboratories completed analysis of veliger sample sets comprising 36 independent analyses. Of these laboratories, two (50%) delivered perfect results with respect to presence or absence detection. A summary of these results is shown in Figure 3. Panel A reports results obtained from all larval concentrations, panel B presents results from samples that did not contain larvae, panel C presents results from samples containing 2 to 4 larvae, and panel D presents results from samples containing 11 to 27 larvae. Overall, the accuracy of detection was 91.7% (Figure 3A) and slightly lower than observed using standard CPLM. Of the three detection errors, two were false negatives and one was a false positive. Both instances of false negatives occurred at the lowest veliger concentrations tested (Figure 3C).

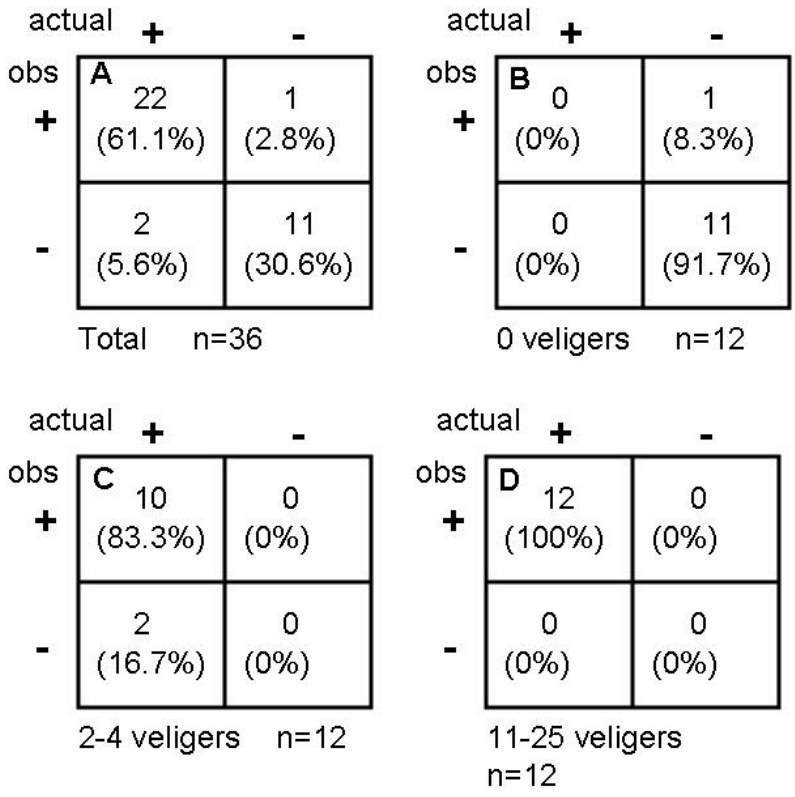

Figure 3. Comparison of actual to observed *Dreissena* spp. larvae (veliger) counts by IFC. (A) All samples, (B) Samples absent of larvae, (C) Samples containing 2 to 4 veligers, (D) samples containing 11 to 27 veligers.

Quantitatively, IFC did not perform as well as standard CPLM (Figure 4). Regression of actual versus observed veliger counts produced a slope of 0.75 ($r^2 = 0.82$), indicating a significant deviation ($p = 0.008$) from a slope of 1 had there been a perfect correspondence between actual and observed counts.

Excluding samples that did not contain veligers, in the majority of cases (17 out of 24), the number of veligers was underestimated by IFC. In 3 out of 24 cases, veliger abundance was overestimated, and in 4 out of 24 cases, veliger abundance was equal to the actual number of veligers in the sample. These results suggest that, at present, IFC systematically underestimates the abundance of *Dreissena* spp. larvae in plankton samples.

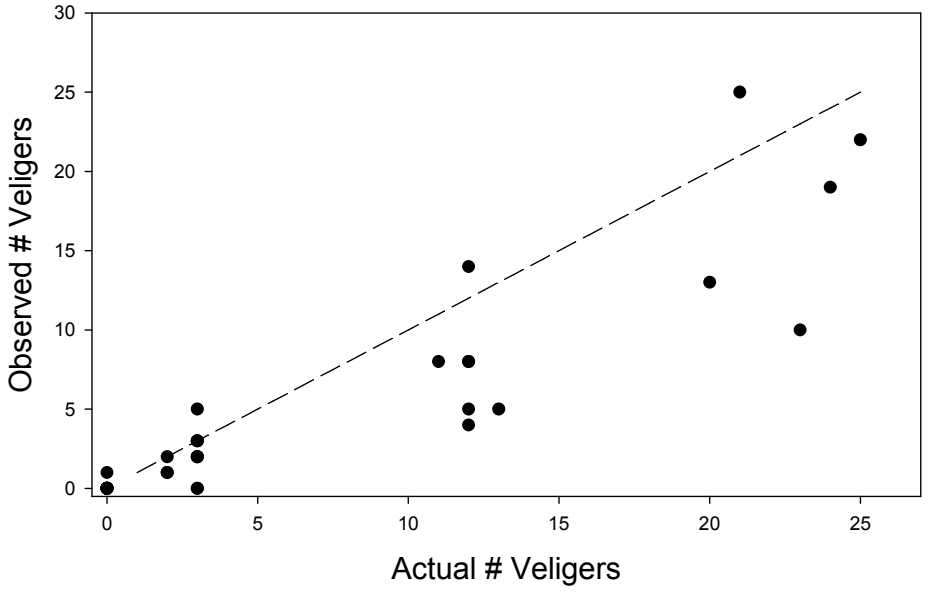

Figure 4. Comparison of actual versus observed *Dreissena* spp. veliger counts by IFC. r^2 = 0.82, slope = 0.75.

Isolating specific methodological variables that may have contributed to the variability in accuracy between labs and the systematic underestimation of larvae is difficult given the relatively large number of methodological variables involved in the method and the relatively small number of participating IFC laboratories. Methodological variables include the objective magnification, the size of the flow cell, the method to inject sample (settling or not), the size of the pre-screening filter used, the flow speed, image recognition parameters, and quality control procedures. Additional research is required to investigate the contribution of each of these parameters to the observed analytical variability associated with IFC.

3.3 Polymerase Chain Reaction

Eleven laboratories completed analysis of veliger sample sets comprising 99 independent analyses. Of these laboratories, none delivered perfect results with respect to presence or absence detection. A summary of these results is shown in Figure 5. Overall, the accuracy of detection was 75.8% (Figure 5A).

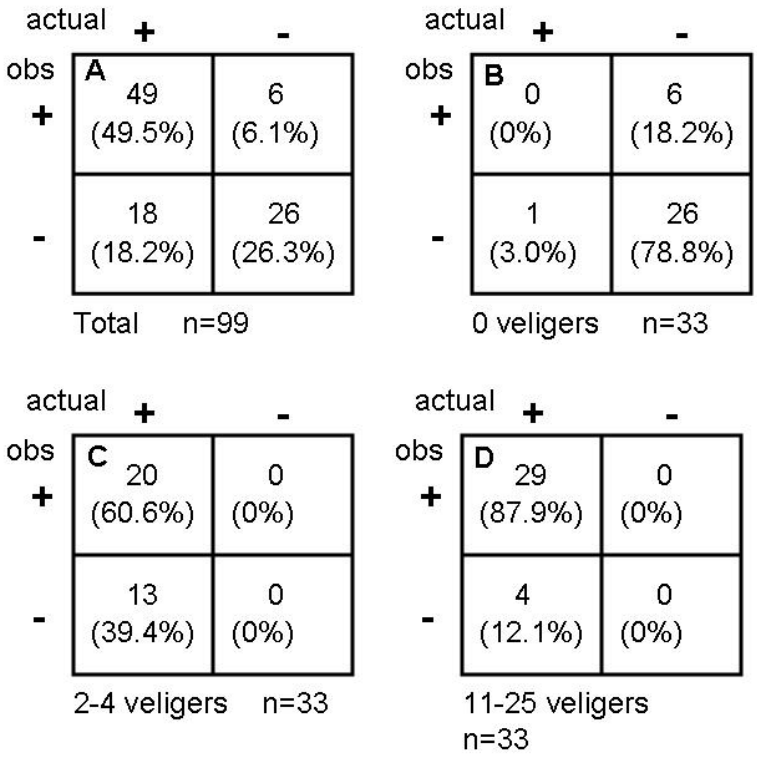

Figure 5. Comparison of actual to observed *Dreissena* spp. larvae (veliger) counts by PCR. (A) All samples, (B) Samples absent of larvae, (C) Samples containing 2 to 4 veligers, (D) samples containing 11-27 veligers.

The largest type of error was false negatives. In 18 (18.2%) out of the 99 samples, veligers were not detected when they were present. In the majority of these cases, veligers were missed in the samples containing the fewest number of veligers (Figure 5C), but even in samples containing 11 to 27 larvae, in 4 cases, veligers were not detected by PCR (Figure 5D). In 6 (6.1%) of the 99 analyses, veligers were detected by PCR in samples that did not contain them (false positives). These results demonstrate that significant variability associated with PCR can result in both false positive and negative detection and that even the most experienced laboratories may suffer from these problems. However, experience does seem to matter. Although rigorous data on laboratory experience is not available, a comparison of the results obtained by the laboratories that participated in both the first and second round of the study, compared to the newer laboratories that only participated in the second study (this study), the more experienced laboratories performed significantly better ($p = 0.016$). Of the five returning laboratories, four of them scored among the top performers (89.9% correct). Top performing labs were defined as those that misidentified no more than two of the nine reference samples. The average of this group was 86.8 ± 5%. Of the newer laboratories, none scored among the top, with an average performance of 62.4% ± 19% correct.

One potential important advantage of PCR-based methods, compared to microscopy-based ones, is the relative ease with which *Dreissena* spp. species can be distinguished based on genetic differences between the species. In this study, eight of the participating PCR laboratories utilized assays that allowed discrimination between quagga mussels (*D. bugensis*) and zebra mussels (*D. polymorpha*), and both species were detected by some of the laboratories. However, as described above, all the veligers utilized in this study were collected from Lake Mohave, Arizona, where the presence of *D. polymorpha* has never been reported, and inspection of adult populations in the vicinity of the larvae collection site at the time of collection did not reveal their presence. Thus, the detection of *D. polymorpha* in these samples seems likely to be erroneous.

An analysis of quantitative results is not possible because, in most cases, a quantitative PCR assay was not utilized during these studies. However, anecdotally, based on the labs that reported semi-quantitative results, there was little correlation between the reported relative larval concentration and the actual number of veligers present in the samples (data not shown). These results highlight the need for future improvement in several of the available PCR assays.

4.0 Conclusions and Recommendations

Based on the results of this study and the previous one (Frischer and Butler, 2009), it is apparent that, at the present time, CPLM is the most reliable of the available *Dreissena* spp. mussel detection assays. Impressively, eight of the nine participating laboratories returned perfect results with respect to presence and absence detection, and no systematic quantification errors were apparent. Thus, it can be concluded from this study that most practicing laboratories are sufficiently expert to conduct these analyses. CPLM is also the most mature of the approaches used for detecting and quantifying larvae. The approach has applied for the enumeration of *Dreissena* spp. larvae since the mid-1990s (Johnson, 1995). Consequently, the method has been relatively well standardized between laboratories, and most practitioners have considerable experience with the technology. However, there remains room for improvement. Specifically, in comparison of the detailed methods used by the different participating laboratories, the greatest variability involves the approach used for concentrating plankton samples prior to analysis and whether a counting chamber is utilized. Several different methods were used to concentrate plankton including centrifugation, settling, or filtration, and all seemed to work well. However, concentration, at least in the case of the reference sample set utilized in this study, seemed to be essential to the accuracy of the method since the one lab that did not detect veligers in the low concentration samples did not employ any type of concentration protocol. There was also some variability in whether a counting chamber was utilized. Based on the results of this study, it would seem prudent to recommend the use of counting chambers if quantification

was desired since those labs that did not use one tended to systematically underestimate the concentration of larvae present in a sample.

Imaging flow cytometry, facilitated by the FlowCAM® instrument developed by the company Fluid Imaging Technologies, performed somewhat less well than CPLM. In this study, four flow imaging cytometry laboratories participated, and two of them reported perfect results with respect to the detection of larvae in the prepared plankton samples. In the case where there were errors, false negatives were slightly more common than false positives, indicating that the IFC is more likely than CPLM to miss the presence of *Dreissena* spp. larvae. This result is supported by the observation that IFC systematically underestimated the abundance of larvae present in reference plankton samples. However, although the accuracy of IFC was not as high as CPLM, the results of this study suggest that this technology is capable of achieving similar accuracies as CPLM. The exact sources of increased errors by IFC are difficult to identify based on this study because the number of participating laboratories was small and the number of potential variables is relatively large. Discussions with practicing users of the FlowCAM® instrument suspect that a key issue is the initial concentration and delivery of plankton material into the flow stream (H. Nelson, personal communication).

The use of flow imaging technology for the purpose of *Dreissena* spp. larvae detection is relatively new, and the results of this study suggest that additional research would be beneficial to its improvement. The use of IFC technology for the purpose of detecting *Dreissena* spp. larvae in plankton samples was first reported by Farrell et al. (2006) and by the developers of the FlowCAM® instrument (FlowCAM® Application Note, 2008). To date, there are no reports of this technology published in the peer reviewed literature. However, several western laboratories involved in the monitoring of *Dreissena* spp. larvae have recently acquired FlowCAM® instruments and have been actively developing protocols and programs for *Dreissena* spp. larval detection utilizing this instrument. Thus, there is a high likelihood of accuracy improvements bringing this technology on par with the CPLM approach. Imaging flow cytometry is an important technology because it has the potential to significantly increase the throughput of samples, reducing cost per sample, and increasing the number and frequency of samples that can be examined. However, a systematic study of the effect of key analytical variables on the accuracy of IFC and the organization of specialized training programs to implement this technology in practicing veliger monitoring laboratories may help elevate this technology to the level of CPLM in the future.

Of the three technologies investigated in this study, PCR was the newest, most complicated, and the poorest performer. Of the 11 participating laboratories, none perfectly identified the presence and absence of *Dreissena* spp. larvae in the reference sample set. The most frequent type of errors was false negatives, indicating that PCR is more likely to miss the presence of larvae in a sample.

However, there were also examples where larvae were detected when they were not present (false positives). Pinpointing the specific sources of error is difficult because there were so many variables involved in the PCR assays utilized during this study. None of the 11 labs employed identical methods, and there are many variables associated with how the plankton sample was concentrated, how DNA was purified, the genes targeted by the assays, the oligonucleotide primers utilized, the size of gene fragments amplified, quality control procedures, and the experience level of the labs. Of the assays utilized, only three followed protocols that have been published in the peer-reviewed literature, and most have been developed within the past year or two. Methodological variation existed in all aspects of the assays including: (1) plankton concentration, (2) DNA isolation and purification, (3) PCR amplification, (4) quality control, and (5) analysis. A summary of the major PCR assay variables is shown in Table 1.

Table 1. PCR Performance Evaluation by Method

	Ranking (% correct)			
	88.9%	77.8%	55-67%	33.3%
No. of Laboratories	4	3	3	1
Methodological Procedure				
Plankton Concentration				
Centrifugation	4	2	1	1
Filtration	0	0	1	0
Gravity Settling	0	1	0	0
None	0	0	1	0
DNA Extraction				
Qiagen Mastermix	3	0	0	0
Qiagen Stool	0	1	0	0
MoBio Soil	1	0	0	0
Ampli Taq (Invitrogen)	0	0	0	0
Biolin	0	0	0	0
Platium Taq (Invitrogen)	0	0	0	0
Promega GoTaq	0	0	0	0
Promega Wizard	0	0	1	0
SYBR Green Super Mix	0	0	0	0
Switchbeads (Invitrogen)	0	0	1	0
Alkaline Lysis (no kit)	0	0	1	1
CTAB (no kit)	0	2	0	0
PCR Amplification (target gene[s])				
18S	1	0	0	0
coxI	1	1	1	0
28S and 28S	0	0	0	1
coxI and coxI	1	1	1	0
coxI and mt16S	0	0	1	0
ITS and coxI	1	1	0	0
Inhibitors and Amplification Enhancers				
GeneReleaser	2	0	0	0
T4 ssDNA binding protein	1	0	0	0
Bovin Serum Albumin	0	*1	0	0
Uracil	1	*1	0	0
Carrier DNA	0	*1	0	0
None	0	2	3	1

* Used together

To concentrate the plankton sample, most labs gently centrifuged the sample either once or twice to reduce the volume prior to DNA extraction. However, other concentration procedures, including settling and lyophilization, were utilized by core labs. Eight different DNA extraction procedures were used by the 11 laboratories including manual alkaline lysis and CTAB procedures, Qiagen's DNeasy tissue extraction kit, Qiagen's Stool kit, MoBio's ultra clean soil kit, Invitrogen's ChangeSwitch beads kit, and Promega's Wizard kit. These extraction procedures resulted in concentration factors from 50 to 450-fold relative to the original volume of the plankton sample (25 ml). Five different genes were targeted in the various assays including the 18S ribosomal RNA (rRNA) gene, the 28S rRNA gene, the mitochondrial Cytochrome Oxidase I (COI) gene, the mitochondrial 16S rRNA gene, and the nuclear Internal Transcribed Spacer (ITS) region. Seven of the laboratories utilized a multiplex assay format allowing the examination of multiple gene targets in a single assay. Most of the multiplex assays targeted two genes to allow the discrimination between *D. bugensis* and *D. polymorpha*, but one laboratory targeted four gene fragments allowing both the discrimination between *Dreissena* spp. species and to include internal redundancy in the assay. The size of the PCR amplicons targeted ranged from 363 bp to 700 bp, with the majority of assays targeting gene fragments in the smaller size range. Several laboratories utilized PCR reaction enhancers and/or protocols to remove inhibitors, but most did not. PCR enhancers that were used included T_4 gene product single stranded DNA binding protein, uracil-N-glycosylase, and Bovine Serum Albumin. Two laboratories utilized the PCR inhibitor remover GeneReleaser® in conjunction with their DNA purification procedures to enhance PCR amplification efficiency. With respect to assay quality control, all laboratories utilized at least one external positive and negative control. Generally, the positive control was DNA purified from an adult animal, and the negative control was a blank with no DNA. However, several laboratories employed multiple positive and negative controls. Secondary positive controls included purified DNA from verified *Dreissena* spp. larvae containing plankton samples, and purified target DNA derived from cloned gene fragments. Secondary negative controls included DNA from closely related organisms. All laboratories ran at least replicate PCR assays, and several ran triplicate assays. One laboratory also routinely performed replicate PCR assays utilizing different DNA template concentrations in their PCR reactions. Another laboratory routinely ran blind sample spikes as a measure of quality control. All laboratories detected the presence of *Dreissena* spp. DNA by visualization of the expected size PCR amplicon on electrophoretic agarose gels, and two laboratories routinely verified the identity of their PCR amplicons by sequencing.

As mentioned above, the use of PCR technology for the purpose of *Dreissena* spp. larvae detection is relatively new. The first reports of the utilization of DNA-based assays for the detection and identification of *Dreissena* spp. mussel larvae were published in the late 1990s and early 2000s (Claxton and Boulding, 1998; Frischer et al., 2002). These assays were developed in response to the invasion of *D. polymorpha* (zebra mussel) in the Great Lakes

and the Northeastern United States, but by the time they were developed, they were largely too late to be used for the purpose of early detection since the mussels had, by that time, already become well established. However, when *D. bugensis* (quagga mussel) was discovered beyond the 100th meridian in Lake Mead in January 2007, research to develop new and more accurate *Dreissena* spp. mussel PCR-based assays was initiated. This research was largely driven by the near consensus of experts and managers that it was necessary to utilize multiple (at least two) independent methods to detect the early presence of *Dreissena* spp. larvae in a body of water prior to implementing major management actions (Anderson et al., 2009; Frischer and Butler, 2009). As a result of the need for detection approaches independent of microscopy, at least five new PCR assays have been recently developed and, without much vetting or the benefit of peer review, are currently being utilized as independent verification methods for early *Dreissena* spp. mussel detection in active monitoring programs. The results of this study confirm that additional research and development efforts are required to improve the reliability of these DNA-based *Dreissena* spp. detection assays to bring them to the level of microscopy-based methods.

As with IFC, it is difficult to determine the exact methodological sources of the errors observed associated with PCR assays because of the relatively small number of participating laboratories relative to a large number of experimental variables. However, consistent among the highest performing laboratories was the use of centrifugation as a concentration method, DNA purification utilizing either the Qiagen DNeasy or MoBio ultra clean soil kits, assays targeting the 18S rRNA gene or the COI gene, and the use of either Qiagen's Mastermix or Invitrogen's AmpliTaq (Gold or Platinum) PCR assay reagents. Not surprisingly, there was a significant ($p = 0.012$) correlation between the experience level of the participating laboratories and their performance. All of the top performing laboratories were among the most experienced, while none of the newest laboratories were. However, this variable was only qualitatively evaluated based on surveys conducted by the Western Regional working group in the fall of 2010 (S. Phillips, 2010) and by assuming that all of the laboratories that participated in the 2009 Phase I study were experienced, while those that only participated in the 2010 study were not.

Because of the need for a second and independent method for verifying early invasion events, it is critical that the reliability of PCR-based *Dreissena* spp. detection assays be improved. During the first phase of this study, involving samples that consisted of essentially plankton-free lake water spiked with *Dreissena* spp. larvae, two out of the five PCR labs returned perfect detection results, indicating that PCR had the potential to be 100% accurate. In the current phase of the project, which involved incrementally more complex samples more closely resembling actual plankton net tow samples, those two laboratories were again among the top performing labs, but their results were not perfect, each misidentifying one of the nine samples examined.

The purpose of this study was to identify the reliability of CPLM, IFC, and PCR-based approaches for detecting low concentrations of *Dreissena* spp. mussels in plankton samples. Although the results of this study are insufficient for determining the exact sources of errors where they exist, and it was not designed to do so, the results do clearly demonstrate that, at the present time, CPLM is the most reliable method for detecting and enumerating *Dreissena* spp. larvae in plankton samples. Imaging flow cytometry also performs well but suffers from underestimation of larval concentrations to the extent that when larval densities are low, as would be expected in the case of early stages of a *Dreissena* spp. mussel invasion, there is a significant chance (50%) that larvae could be missed. PCR-based methods are currently the poorest performers especially, but not restricted to, when larval concentrations are low. A general conclusion of these studies is that the reliability of each method is directly proportional to the complexity of the assay.

5.0 Specific Recommendations

1. At the present time, CPLM analysis should be used as the primary approach for early detection of *Dreissena* spp. larvae. All potential detections should be documented with high quality photomicrographs.

2. Develop a comprehensive CPLM training program and expand current microscopy capacity and expertise.[1]

3. Establish a small group of certified experts who are capable of reliably identifying *Dreissena* spp. larvae from good quality micrographs.

4. Fund research to improve accuracy of IFC as a routine method for detecting and enumerating *Dreissena* spp. larvae, especially when larvae are at very low concentrations. Focus should be on sample concentration and delivery to flow stream protocols.

5. In association with laboratories currently utilizing IFC, routinely conduct CPLM analysis to facilitate cross-comparison between these methods.

6. Conduct technical workshops to discuss the results of this study and a broader review of PCR diagnostic methods. The product of such workshops should be the development of specific research recommendations.[1]

7. Fund research to identify the exact causes of variability in PCR methods. Conduct a methods "cook-off" study involving the most experienced PCR laboratories to evaluate all currently available methods.

[1] Projects are currently underway to address these recommendations.

8. Establish a laboratory certification standard for all types of detection methods for *Dreissena* spp. larvae.

9. Utilize certified laboratories to confirm findings by non-certified laboratories when they occur.

10. Because all methods have error associated with them, multiple (at least two) independent analyses should be used to confirm the presence of *Dreissena* spp. larvae, especially if it is the first report of dreissenids in a body of water.

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Appendix I

Participating Analytical Laboratories

Participant	Contact Person	Shipping Address	Methodology Group
* Fluid Imaging Technologies	Kent Peterson Harry Nelson	Benjamin Spaulding Laboratory Manager Fluid Imaging Technologies, Inc. 65 Forest Falls Drive Yarmouth, ME 04096 207-846-6100	FlowCAM
Clean Lakes	Leif Elgethun	Leif Elgethun Clean Lakes, Inc. 1770 West State Street, #125 Boise, ID 83702 208-301-2293	FlowCAM
Bureau of Reclamation	Kevin Kelly Denise Hosler	Kevin Kelly (86-68220) Bureau of Reclamation Denver Federal Center Building 67, Room 152 Denver, CO 80225 866-476-4550	FlowCAM
National Park Service	Erin Murchie	Erin Murchie Glen Canyon NRA 691 Scenic View Drive Page, AZ 86040 928-608-6268	FlowCAM
* Skidaway Institute of Oceanography	Marc Frischer	Tina Walters Skidaway Institute of Oceanography 10 Ocean Science Circle Savannah, GA 31411 912-598-2395 or 2418	PCR
*Bureau of Reclamation	Kevin Kelly	Kevin Kelly (86-68220) Bureau of Reclamation Denver Federal Center Building 67, Room 152 Denver, CO 80225 866-476-4550	PCR
* Metropolitan Water District	Paul Rochelle	Anne Johnson Metropolitan Water District of Southern California Water Quality Laboratory 700 Moreno Avenue La Verne, CA 91750 909-392-5019	PCR

Participant	Contact Person	Shipping Address	Methodology Group
* Pisces Molecular	John Wood	John Wood Pisces Molecular, LLC 2200 Central Avenue, Suite F Boulder, CO 80301 303-546-9300	PCR
* U.S. Geological Survey	Rusty Rodriguez	Rusty Rodriguez, Ph.D. Principal Investigator/Microbiologist U.S. Geological Survey 6505 NE 65th Seattle, WA 98115 206-526-6596	PCR
Wayne State University	Jeff Ram	Dr. Jeffrey L. Ram Department of Physiology Wayne State University 540 E. Canfield Street, Room 6112 Detroit, MI 48201 313-577-1558	PCR
California Department of Fish and Game	Jim Snider	Jim Snider Bodega Marine Laboratory Shellfish Health 2099 Westside Road Bodega Bay, CA 94923 707-875-2066	PCR
Scripps Institution of Oceanography	Ron Burton	Gary Moy Hubbs Hall, Room 2330 8750 Biological Grade Scripps Institution of Oceanography La Jolla, CA 92037 858-534-7827	PCR
University of New Mexico	Gavin Pickett	Gavin Pickett 915 Camino de Salud NE CRF Room 124 University of New Mexico Albuquerque, NM 87131 505-272-5564	PCR
U.S. Fish and Wildlife Service	Wade Wilson	Wade D. Wilson U.S. Fish and Wildlife Service Dexter National Fish Hatchery and Technology Center 7116 Hatchery Road Dexter, NM 88230 575-734-5910 ext. 41	CPLM
* Portland State University	Mark Sytsma	Steve Wells Portland State University Center for Lakes and Reservoirs 1719 SW 10th Avenue SB2 Room 246 Portland, OR 97201 503-725-9076	CPLM

Participant	Contact Person	Shipping Address	Methodology Group
* Bureau of Reclamation	Denise Hosler	Denise Hosler (86-68220) Bureau of Reclamation Denver Federal Center Building 67, Room 152 Denver, CO 80225 303-445-2195	CPLM
* U.S. Fish and Wildlife Service	David Britton	David K. Britton UT Arlington - Biology Life Science Building Room 337 501 South Nedderman Dr. Arlington, Texas 76019 817-272-3714	CPLM
East Bay Municipal Utility District	Dan Jackson	Dan Jackson EBMUC Laboratory 2020 Wake Avenue Oakland, CA 94607 510-287-1427	CPLM
Montana Department of Fish, Wildlife, and Parks	Eileen Ryce	Stacy Schmidt c/o Eileen Ryce 1420 East 6th Avenue Helena, MT 59620 406-444-2448 (office) 406-431-7134 (cell)	CPLM
California Department of Fish and Game	Jim Snider	Jim Snider Bodega Marine Laboratory Shellfish Health 2099 Westside Road Bodega Bay, CA 94923 707-875-2066	CPLM
EcoAnalysts, Inc.	Gary Lester	Shanda McGraw 1420 S. Blaine Street, Suite 14 Moscow, ID 83843 208-882-2588 ext. 21	CPLM
National Park Service	Erin Murchie	Erin Murchie Glen Canyon NRA 691 Scenic View Drive Page, AZ 86040 928-608-6268	CPLM

* = Returning Phase I laboratories

Appendix II

Dreissena Veliger Double-Blind Round Robin Study—Round Two: March 2010

Instruction Sheet

1. Please find enclosed a set of 9 x 25 ml natural water samples preserved either in 70% ethanol for PCR analysis or 25% ethanol for FlowCAM and microscopy analyses. Note samples are identified with Set # and Sample #. These are used for sample tracking.

2. Regardless of the method employed (PCR, FlowCAM, or microscopy), please analyze the entire 25 ml volume of each sample. Please record the volumes as given on the side wall of the sample tubes.

3. Using the data sheet attached here, please report results, volumes, and sample Set # via electronic mail to: nierzs@rpi.edu. For PCR, please indicate absence or presence for each species (zebra or quagga). For FlowCAM and microscope, please indicate absence or presence, along with veliger enumeration in each positive sample. Veliger enumeration can be reported simply as total number of veligers found (no need to calculate density in 25 ml). If additional data sheets are needed, please make photocopies of the one attached here.

4. Please provide, via electronic mail to marc.frischer@skio.usg.edu, a detailed description of your method employed, including a description of any sample preparation prior to analysis and the analysis itself. If appropriate, please provide any images of gels or sequence information (PCR) or micrographs (microscopy and FlowCAM).

5. Please return the cooler to:
   Kevin L. Kelly (86-68220)
   Bureau of Reclamation
   Denver Federal Center
   Building 67, Room 152
   Denver, CO 80225

6. Any questions or concerns, please contact:
   Kevin L. Kelly
   Bureau of Reclamation
   (866) 476-4550
   kkelly@usbr.gov

Thank you for your participation!

PCR Analysis Sample Set # _____

Sample ID #	Volume Received (mL)	Absence (−) or Presence (+)	
		Zebra Mussel	Quagga Mussel
1			
2			
3			
4			
5			
6			
7			
8			
9			

FlowCAM Analysis Sample Set # _____

Sample ID #	Volume Received (mL)	# Veligers Found (0 if absent)
1		
2		
3		
4		
5		
6		
7		
8		
9		

Microscopy (CPLM) Analysis Sample Set # _____

Sample ID #	Volume Received (mL)	# Veligers Found (0 if absent)
1		
2		
3		
4		
5		
6		
7		
8		
9		