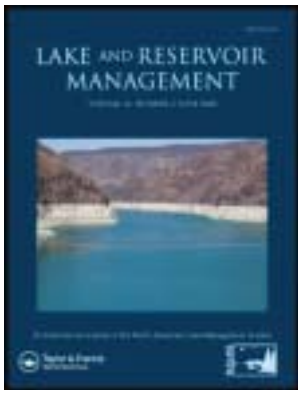

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Accuracy and reliability of Dreissena spp. larvae detection by cross-polarized light microscopy, imaging flow cytometry, and polymerase chain reaction assays

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Accuracy and reliability of *Dreissena* spp. larvae detection by cross-polarized light microscopy, imaging flow cytometry, and polymerase chain reaction assays

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Abstract

Frischer ME, Kelly KL, Nierzwicki-Bauer SA. 2012. Accuracy and reliability of *Dreissena* spp. larvae detection by cross-polarized light microscopy, imaging flow cytometry, and polymerase chain reaction assays. Lake Reserv Manage. 28:265–276.

The expansion of *Dreissena* spp. mussels into the western United States has generated an increased need for reliable early detection methods, especially for larvae (veligers), which are a primary transport vector and an indicator of spawning adults. Cross-polarized light microscopy (CPLM) currently provides the most reliable means for detecting quagga and zebra mussel (*Dreissena* spp.) larvae in plankton samples. In this study a double-blind experiment was undertaken to assess the current reliability of 3 different methods for detecting *Dreissena* spp. larvae in plankton samples. Methods included CPLM, imaging flow cytometry (IFC), and DNA-based polymerase chain reaction (PCR) assays. We distributed 216 reference samples consisting of concentrated plankton spiked with known numbers of *Dreissena* spp. larvae to 19 laboratories for analysis. Results indicated that presence/absence detection CPLM was the most reliable (96.3% accuracy), IFC analysis was next most reliable (91.7% accuracy), and PCR was the least reliable (75.8% accuracy). The most prevalent type of error associated with all the methods was false negatives, suggesting that all methods are more likely to fail to detect the presence of larvae rather than to falsely indicate their presence.

Key words: cross-polarized light microscopy, *Dreissena* spp., imaging flow cytometry, methods comparison, polymerase chain reaction (PCR)

Since the introduction of dreissenid mussels and their spread throughout the eastern United States, their eventual westward spread has been predicted (Bossenbroek et al. 2007). The first detection of quagga mussels (*Dreissena rostriformis bugensis*) west of the 100th meridian in the United States was in Lake Mead, Nevada (Stokstad 2007). They have since expanded their range and now threaten a large proportion of western US waterways, water bodies, and water infrastructure including dams and distribution systems (LAME 2007, Stokstad 2007, Hickey 2010, USGS 2011). Surveys conducted shortly after mussels were detected in Lake Mead revealed the presence of both quagga and zebra (*Dreissena polymorpha*) mussels in many water bodies in other western states (Benson 2010, Wong et al. 2012), possibly indicating their introduction prior to their discovery in Lake Mead. However, because many water bodies in this region have not yet been infested and many biological invasions are considered irreversible, a key issue for the management of the zebra and quagga mussel invasion in North America is implementing aggressive programs to prevent further spread. Limiting an invasive spread is especially important in the western United States where water delivery infrastructure is extensive and thus at high risk of severe economic impact. Early detection of a biological invasion increases the likelihood that mitigation actions will slow the spread of the invasive species, reduce the impact on local ecology, and allow the implementation of effective

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control measures (Vander Zanden and Olden 2008). A key component of prevention programs includes vigilant early detection and monitoring (Ruesink et al. 1995, Mack et al. 2000, Simberloff 2003, Vander Zanden and Olden 2008).

Dreissenid mussel management efforts designed to limit or prevent secondary invasions depend on accurate monitoring and efficient information dissemination. An important component of detection monitoring programs is the ability to accurately detect, as early as possible, the introduction of these mussels into a pristine water body (Frischer et al. 2002, Lucy 2006). Although human vectors are the primary means for new introductions (Bossenbroek et al. 2001, Johnson et al. 2001, Leung et al. 2006, Rothlisberger et al. 2010), once a population has been established, 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 (Johnson 1995, Lucy 2006, Wong et al. 2012). Detection of *Dreissena* spp. veligers is therefore a critical component of early detection monitoring programs and is routinely utilized in the Western United States (Anderson et al. 2009).

Available analytical techniques for the detection of *Dreissena* spp. larvae in plankton samples include 2 microscopy-based approaches: cross-polarized light microscopy (CPLM) (Johnson 1995) and CPLM combined with imaging flow cytometry (IFC; FlowCAM 2008). CPLM involves the manual inspection of plankton samples using a microscope equipped with cross-polarizing filters, and IFC automates the process of microscopic examination. Currently, FlowCAM, manufactured and distributed by Fluid Imaging Technologies, is the only IFC instrument on the market suitable for assessing the presence of bivalve larvae in plankton samples.

A third approach uses polymerase chain reaction (PCR)-based assays to detect DNA specific to *Dreissena* spp. larvae in plankton samples (Frischer et al. 2002, Hoy et al. 2010, Rochelle et al. 2010, Ram et al. 2011). PCR-based approaches extract total community DNA from the contents of a plankton net tow and use PCR amplification to detect genes unique to *Dreissena* spp. Compared to microscopy-based approaches, PCR-based methods are more recent and still largely in development (Bott et al. 2010). Because management decisions are often based on the results derived from these analytical approaches, it is critical that the reliability of each of these assays is accurately estimated and that continued efforts are made to improve and standardize each of these techniques. Although each of these approaches is regularly employed in current early detection programs, the reliability of each has not yet been well characterized, and when multiple techniques have been applied to the same plankton samples, conflicting results are often reported (Frischer and Butler 2009).

In this study we report the results of a community-wide, double-blind, round-robin experiment to assess the reliability of currently used approaches for the early detection of *Dreissena* spp. larvae in plankton samples.

Materials and methods

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 was to identify and enlist as many laboratories as possible that are actively involved in early detection of Dreissenid mussels, provide them with a set of double-blind reference samples containing realistic planktonic communities spiked with known numbers of *Dreissena* spp. larvae, and synthesize the resulting data to assess assay reliability. In all cases, the participants and the person who distributed the samples were unaware of the numbers of larvae in the samples until after all analyses were complete. This double-blind design eliminated the risk of prejudgment by the participants that could bias the results.

Participating laboratories

Samples were sent to 19 independent participating laboratories (Table 1), several of which completed multiple types of analyses. Nine laboratories analyzed reference sample sets using CPLM, 4 used IFC with FlowCAM instrumentation, and 11 used PCR-based methods.

Collection of Dreissena spp.-free plankton samples

To provide a realistic plankton matrix for the reference samples, *Dreissena* spp.-free concentrated plankton tow material was collected from a location where dreissenids were not present. Following consultation with several western invasive species managers, Quail Creek Reservoir in Utah was identified as a suitable site. Quail Creek Reservoir is a large (239 ha) impoundment of Quail Creek and several tributaries in southwestern Utah (37° 11′09.68″N, 113° 23′14.81″W). 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 on site at the Quail Creek Water Treatment Plant (QCWTP). However, *Dreissena* spp. DNA was detected in plankton samples from Quail Reservoir in August, September, and October 2010, but not in November or since (L. Dalton, Aquatic Invasive Species Coordinator, Utah Division of Wildlife Resources, March 2012, pers. comm.). Also, a single adult mussel was detected in a nearby reservoir (Sand Hollow) in May 2010.

Table 1.-Project participants.

Participating Laboratory	Contact	Method
1. Fluid Imaging Technologies	Harry Nelson	IFC
2. Clean Lakes, Inc.	Leif Elgethun	IFC
3. US Bureau of Reclamation	[1]Denise Hosler [2]Kevin Kelly	[1]IFC, [1]CPLM & [2]PCR
4. National Park Service	Erin Murchie-Janicki	CPLM & IFC
5. Skidaway Institute of Oceanography	Marc Frischer	PCR
6. Metropolitan Water District	Paul Rochelle	PCR
7. Pisces Molecular, LLC	John Wood	PCR
8. US Geological Survey	Rusty Rodriguez	PCR
9. Wayne State University	Jeffrey Ram	PCR
10. California Dept. of Fish and Game	James Snider	CPLM & PCR
11. Scripps Institute of Oceanography	Ron Burton	PCR
12. University of New Mexico	Gavin Pickett	PCR
13. University of Idaho	Cort Anderson	PCR
14. US Fish and Wildlife Service New Mexico	Wade Wilson	CPLM & PCR
15. Portland State University	Mark Sytsma Steve Wells	CPLM
16. US Fish and Wildlife Service Texas	David Britton	CPLM
17. East Bay Municipal Utility District, California	Dan Jackson	CPLM
18. Montana Dept. of Fish, Wildlife and Parks	Stacy Schmidt	CPLM
19. EcoAnalysts, Inc.	Gary Lester	CPLM

Water chemistry analysis indicates that Quail Creek Reservoir would favor *Dreissena* spp. mussels. Based on averaged STORET spring and summer data (1987–1997), the calcium concentration is 118 mg/L, pH is 8.0, and sufficient nutrients are present to support average total chlorophyll at levels of 1.7 μg/L. Primary production in the reservoir is believed to be phosphorus limited. Based on QCWTP monitoring in 2008–2010, water temperatures ranged from 6 to 28 C, with temperatures conducive to *D. bugensis* spawning occurring from May through October. Plankton for this study was collected from the reservoir on 18–19 January 2010.

Samples were collected from 2 sites in the reservoir using vertically towed, 63 μm mesh plankton nets with either an 8 or 12 inch diameter opening. At the time of sampling water temperature was 6 C and chlorophyll concentrations were 1.3–1.5 μg/L. Chlorophyll *a* concentrations were determined after acetone extraction as described by Parsons et al. (1984). Plankton samples contained a typical diversity of species including ostracods, *Corbicula*, and other native mussel larvae (*Unionidae glochidia*) that can be confused with *Dreissena* spp. veligers, but the absolute and relative abundances of each species were not quantified. 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 preserved in ethanol. Total chlorophyll *a* in the concentrated plankton sample was 182 μg/L, and *Dreissena* spp. larvae were not observed. Two sets of ethanol preserved samples were prepared for examination by microscopy-based or PCR methods. 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). For PCR samples, 2.6 L of 96.5% ethanol was added to 1 L of plankton concentrate. Each sample set was distributed into four 1 L Nalgene bottles and shipped with frozen freezer packs to keep the samples cool, but not frozen, to the laboratories at the Darrin Fresh Water Institute in Bolton Landing, New York, where they were processed.

Collection of Dreissena spp. Larvae

Quagga mussel (*D. bugenesis*) larvae were collected on 20–21 January 2010 from Lake Mohave near Katherine's Landing in northwestern Arizona (35°13′05″N, 114°33′58″W). Water conditions in Lake Mohave are favorable for *Dreissena* spp. mussel growth and support high densities of quagga mussels similar to Lake Mead, Nevada (Phillips 2010, Benson et al. 2007).

At the time of sample collection, the water temperature was 10.9 C, and the *Dreissena* spp. larvae concentration was approximately 200 veligers/m^3 of lake water. Larval concentrations were estimated by CPLM from 3 independent plankton tows at the site. The size range of larvae was not quantified, but larvae ranged from approximately 100 to 200 μm with smaller larvae being more numerous. Ostracods were present in some abundance in the plankton samples, although they were not quantified or included in the prepared reference samples. Total chlorophyll *a* concentration was 0.28 μg/L in surface waters. Veligers were collected

using a combination of vertical and horizontal 63 μm mesh plankton net tows from the first horizontal dock segment at "Marina 4," where veliger concentrations have been monitored routinely by the Bureau of Reclamation (C. Holdren, Supervisory Biologist, Bureau of Reclamation Environmental Applications and Research, Nov 2009, pers. comm.). The depth along this dock segment ranged from 6 to 7.5 m.

Over the 2 day sampling period (20–21 Jan 2010), approximately 6000 larvae were collected and concentrated to 2 L by filtering though 63 μm mesh screening. Following the procedure for plankton samples from Quail Creek Reservoir, 2 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). 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, filtered water from Lake Mohave was also made 25% and 70% with respect to ethanol and shipped to our laboratories to use as a diluent when larvae were isolated.

Sample preparation and distribution

Reference sample sets for distribution to the analytical laboratories were prepared during 11–15 February 2010 at the Darrin Fresh Water Institute, Bolton Landing, New York. Reference samples consisted of 25 mL of 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 9 samples: three 25 mL aliquots of plankton from Quail Creek Reservoir without any larvae; 3 containing 2–4 larvae; and 3 containing 11–27 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 and then were transferred to a Petri dish and viewed using a stereomicroscope equipped with cross-polarizing filters. Larvae were individually transferred into Corning 50 mL polycarbonate conical centrifuge tubes with plug seal caps (cat No. 430290) to minimize leakage due to the vaporization of ethanol during shipping by air. Individual larvae were transferred to sample tubes containing the larvae-free plankton (25 mL) from Quail Creek Reservoir using a pipette with plastic tips and the transfer verified. A total of 26 reference sample sets were prepared and assigned randomized numbers. Trip controls were not performed, but all laboratories confirmed that the tubes arrived intact without any volume loss.

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. 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 laboratories to ensure consistent reporting of details. Specific information was requested from all laboratories concerning the methodological approaches for concentrating plankton, enumeration, purifying DNA, PCR amplification, quality control, and analysis. All results were completed and compiled by August 2010.

Synthesis

Once all results were received, they were compiled and compared 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 numbers of larvae to determine the accuracy of detection and, where possible, quantified. For microscopy-based methods, regression analysis and analysis of variance statistical tests were used to determine the significance of relationships between actual and observed larval counts.

Results

Cross-polarized light microscopy

Nine laboratories completed analysis of veliger sample sets comprising 81 independent analyses. Of these laboratories, 8 (88.8%) delivered perfect results with respect to veliger presence or absence detection. Overall, the accuracy of detection was 96.3% (Fig. 1A). Although error rates were low, the largest type of errors observed were false negatives (2.5%), while false positives accounted for 1.2%. Both instances of false negative results occurred at the lowest veliger concentrations (Fig. 1C). Quantitatively, actual and observed veliger counts estimated by CPLM were highly correlated ($r = 0.94$, $p < 0.001$; Fig. 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 with the actual number of veligers. Of the remaining samples, 21 (26%) underestimated and 19 (23%) overestimated the number of veligers actually present in the samples. If the results derived from samples not containing larvae were excluded from this analysis, approximately one-third each of the samples was in complete correspondence with actual counts, overestimates, and underestimates.

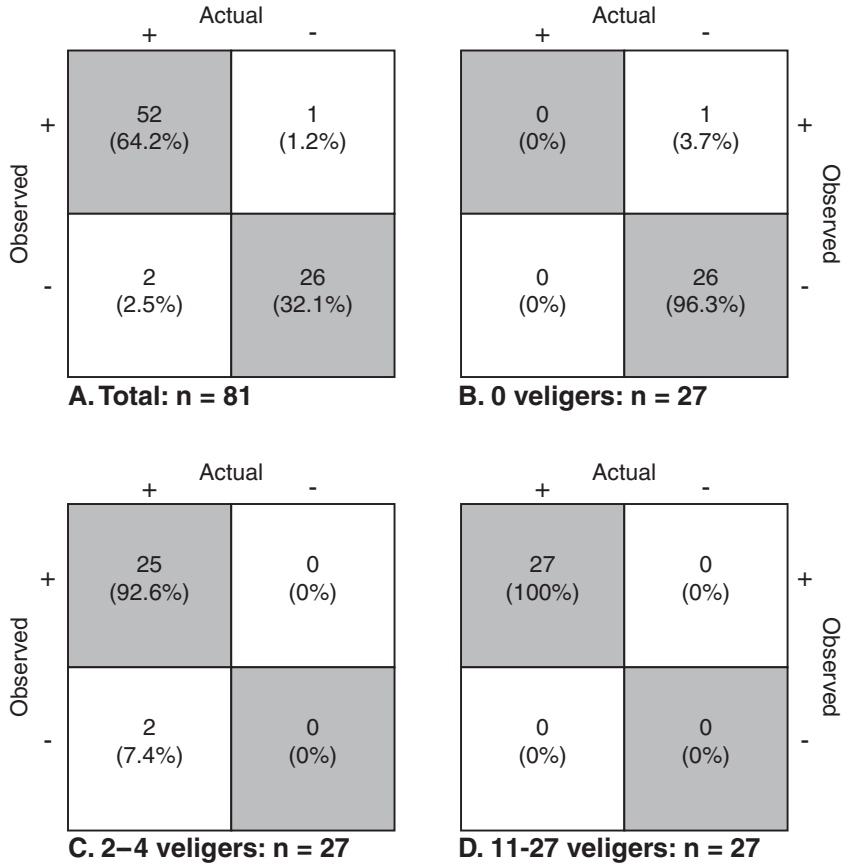

Figure 1.-Comparison of actual to observed *Dreissena* spp. larvae (veliger) counts by cross-polarized light microscopy. Results from 9 laboratories completing 81 independent analyses are shown. (A) All samples, (B) samples absent of larvae, (C) samples containing 2 to 4 veligers, (D) samples containing 11 to 27 veligers. The proportion of samples where actual and observed presence or absence was in agreement is shaded.

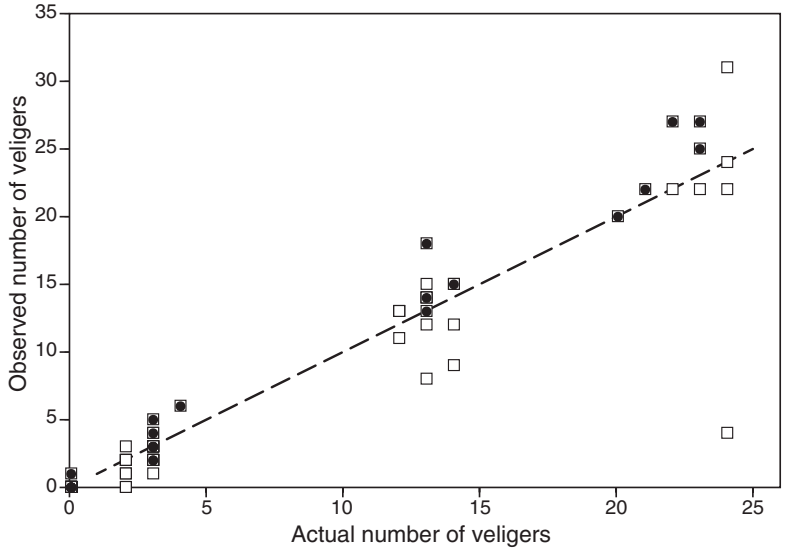

Figure 2.-Comparison of actual versus observed *Dreissena* spp. veliger counts by cross-polarized light microscopy. $r^2 = 0.89$, slope = 0.99. Samples counted using a counting chamber (■) or without (□) are indicated. Dashed line indicates the theoretical 1:1 correspondence line (slope = 1) between actual and observed veliger counts.

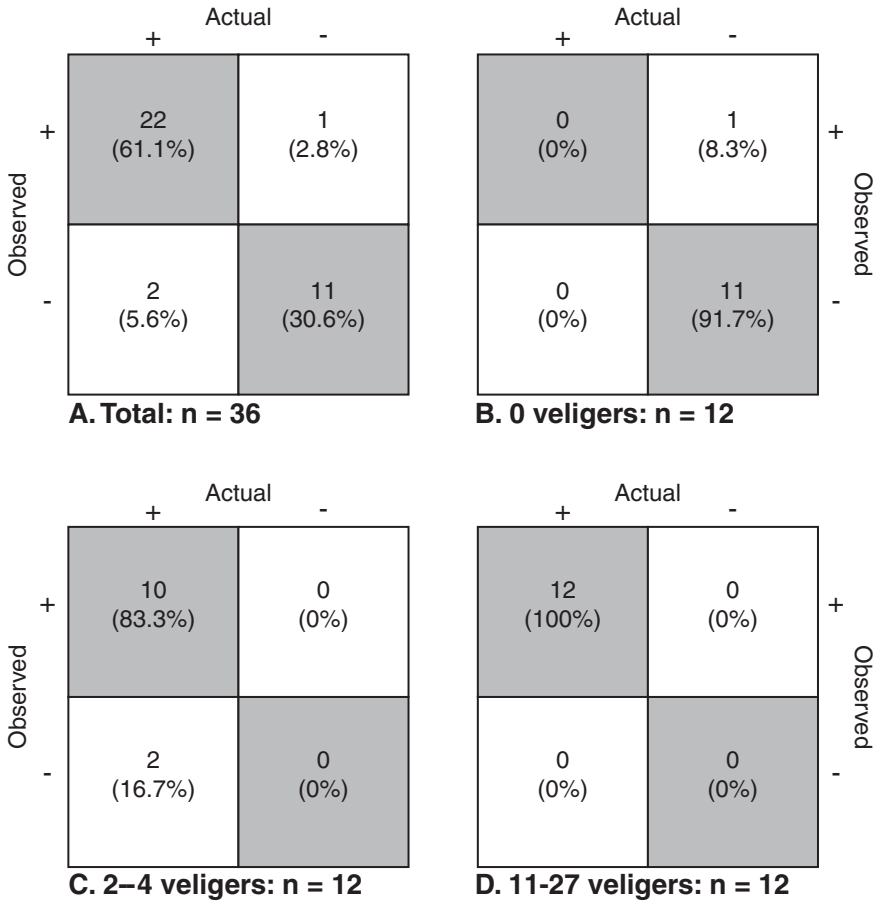

Figure 3.-Comparison of actual to observed *Dreissena* spp. larvae (veliger) counts by imaging flow cytometry. Results from 4 laboratories completing 36 independent analyses are shown. (A) All samples, (B) samples absent of larvae, (C) samples containing 2 to 4 veligers, (D) samples containing 11 to 27 veligers. The proportion of samples where actual and observed presence or absence were in agreement are shaded.

Imaging flow cytometry

Four laboratories completed analysis of veliger sample sets comprising 36 independent analyses. Of these laboratories, 2 (50%) delivered perfect results with respect to presence or absence detection (Fig. 3). Overall, the accuracy of detection was 91.7% (Fig. 3A), slightly lower than observed using standard CPLM. Of the 3 detection errors, 2 were false negatives and one was a false positive. As observed in CPLM analyses, both instances of false negatives occurred at the lowest veliger concentrations tested (Fig. 3C).

Quantitatively, IFC did not perform as well as standard CPLM (Fig. 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. By excluding samples that did not contain veligers, the number of veligers was underestimated by IFC in the majority of cases (17 of 24); in 3 of 24 cases, veliger abundance was overestimated, and in 4 of 24 cases, veliger abundance was equal to the actual number of veligers in the sample. Current results suggest that IFC systematically underestimates the abundance of *Dreissena* spp. larvae in plankton samples.

Polymerase chain reaction

Eleven laboratories completed PCR analysis of veliger sample sets comprising 99 independent analyses. Of these laboratories, none delivered perfect results with respect to presence or absence detection (Fig. 5). Overall, the accuracy of detection was 75.8% (Fig. 5A). The largest type of error was false negatives. In 17 (17.2%) 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 (Fig. 5C). Even in samples containing 11 to 27 larvae, in 4 cases veligers were not detected by PCR (Fig. 5D). In 7 (7.1%) of the 99 analyses, veligers were detected by PCR in samples that did not contain them (false positives).

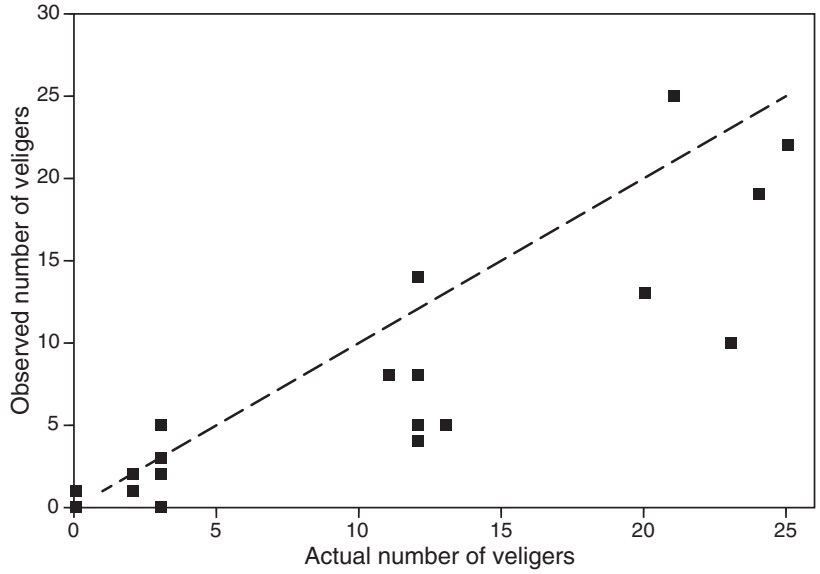

Figure 4.-Comparison of actual versus observed *Dreissena* spp. veliger counts by imaging flow cytometry. $r^2 = 0.82$, slope = 0.75. Dashed line indicates the theoretical 1:1 correspondence line (slope = 1) between actual and observed veliger counts.

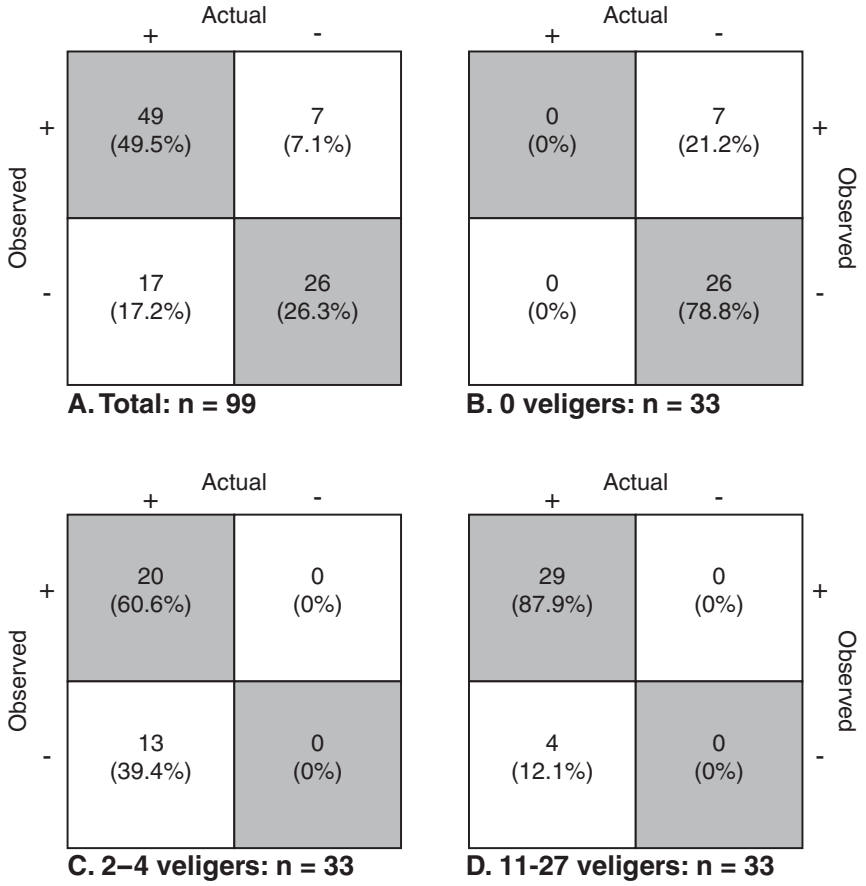

Figure 5.-Comparison of actual to observed *Dreissena* spp. larvae (veliger) counts by polymerase chain reaction assays. Results from 11 laboratories completing 99 independent analyses are shown. (A) All samples, (B) samples absent of larvae, (C) samples containing 2 to 4 veligers, (D) samples containing 11 to 27 veligers. The proportion of samples where actual and observed presence or absence were in agreement are shaded.

These results demonstrate that PCR analysis can result in both false positive and negative detection. Even the most experienced laboratories may suffer from these problems, but experience does seem to matter. Although rigorous data on laboratory experience are not available, laboratories that participated in a similar preliminary 2009 study and that had been involved for at least 3 years in PCR detection of *Dreissena* spp., and therefore considered to be experienced, outperformed less experienced laboratories ($p = 0.016$). Of the 5 experienced laboratories, 4 scored among the top performers (89.9% correct), misidentifying no more than one of the 9 reference samples. The average of the 5 experienced laboratories was 86.8 ± 5%. None of the less experienced laboratories scored among the top, with an average performance of 62.4% ± 19% correct.

Table 2.-CPLM performance evaluation by method.

RANKING (% Correct – Presence/Absence) 100% <100%		
# of laboratories **Methodological Procedure[1]**	**8**	**1**
Plankton Concentration		
Centrifugation	3	0
Sieving	2	0
Gravity Settling	1	1
None	1	0
Enumeration		
With counting chamber	3	0
Without counting chamber	4	1

[1] One laboratory did not provide methodological procedures.

Discussion

In this study the reliability of 3 different methodological approaches to detect *Dreissena* larvae at low concentrations in plankton net tow concentrates were examined. Currently, based on the results of this study and a previous one (Frischer and Butler 2009), CPLM is the most reliable of the available *Dreissena* spp. mussel larvae detection assays. Eight of the 9 participating CPLM laboratories returned perfect results with respect to presence and absence detection, and no systematic quantification errors were apparent. This study conclusively indicates that most practicing laboratories have sufficient expertise to conduct these analyses. As the first method to be applied for routine identification of *Dreissena* spp. larvae in plankton samples, CPLM is also the most mature of the approaches used to detect and quantify larvae. This approach has been applied for the enumeration of *Dreissena* spp. larvae since the mid-1990s (Johnson 1995). Consequently, CPLM has been relatively well standardized between laboratories, and most practitioners have considerable experience with the technology. Improvements are still needed, however. Specifically, comparing the detailed methods used by the different participating laboratories shows that the greatest source of analytical variability is derived from the approach used to concentrate plankton samples prior to analysis and the use of a counting chamber (Table 2). Several different methods were employed to concentrate plankton, including centrifugation, settling, or filtration. All seemed to work, but concentration, at least in the case of the reference sample set utilized in this study, seemed essential to the accuracy of the method. The one laboratory that failed to detect veligers in the lowest concentration samples did not employ any type of concentration protocol. Although statistically not significant, increased counting variability was associated with using a counting chamber. Results of this study indicate that is prudent to recommend the use of counting chambers if quantification is necessary. Laboratories that did not use counting chambers tended to systematically underestimate the concentration of larvae present in a sample (Fig. 2).

Imaging flow cytometry, facilitated by the FlowCAM instrument, performed less accurately than CPLM. Of the 4 flow imaging cytometry laboratories participating in this study, 2 reported perfect results with respect to the detection of larvae in the prepared plankton samples. In cases with errors, false negatives were slightly more common than false positives, indicating that IFC is more likely than CPLM to miss the presence of *Dreissena* spp. larvae. This conclusion 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. Sources of increased errors by IFC are difficult to identify based on this study because the number of participating laboratories was small (4) and the number of potential variables is relatively large (Table 3). Discussions with practicing users of the FlowCAM instrument suggest that a key issue is the initial concentration and delivery of plankton material into the flow stream (H. Nelson, Director of Sales, Fluid Imaging Technologies, Feb 2012, pers. comm.).

The use of flow imaging technology to detect *Dreissena* spp. larvae is relatively new, and results of this study suggest that additional research would be beneficial. Use of IFC technology to detect *Dreissena* spp. larvae in plankton samples was first reported by Farrell et al. (2006) and by the developers of the FlowCAM instrument (FlowCAM 2008, Spaulding et al. 2008). To date, reports of this technology published in the peer reviewed literature are nonexistent; however, several laboratories involved in the monitoring of *Dreissena* spp. larvae have recently acquired the FlowCAM

Table 3.-IFC performance evaluation by method.

RANKING (% Correct – Presence/Absence) 100% <100%		
# of laboratories		
Methodological Procedure	**2**	**2**
Plankton Concentration		
Gravity Settling	1	1
Not Specified	1	1
Pre-sieving Size		
300 μm	2	2
Magnification		
4×	2	2
Flow Tube (size and type)		
300 μm Field of View	2	2
Flow Speed		
Forward slow-5	1	1
Forward fast-5	1	1
Camera Settings		
As recommended by manufacturer with manual manipulation as needed	2	2

Table 4.-PCR performance evaluation by method.

RANKING (% Correct)				
# of laboratories				
Methodological Procedure	**88.9% 4**	**77.8% 3**	**55-67% 3**	**33.3% 1**
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 DNeasy tissue	3	0	0	0
Qiagen Stool	0	1	0	0
MoBio Soil	1	0	0	0
Promega Wizard	0	0	1	0
ChargeSwitch beads (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
COI	1	1	1	0
28S & 28S[1]	0	0	0	1
COI & COI	1	1	1	0
COI & mt16S	0	0	1	0
ITS & COI	1	1	0	0
PCR Amplification Reagents				
AmpliTaq Gold/Platinum & Invitrogen reagents	3	0	0	0
Qiagen Master Mix	1			
Bioline Taq and reagents	0	1	0	0
BioRad SYBR Green Supermix	0	0	1	0
Promega Go Taq & reagents	0	1	1	0
Not Specified	0	1	1	1
Inhibitor Removers & Amplification Enhancers				
GeneReleaser	2	0	0	0
T4 gene 32 ssDNA binding protein	1	0	0	0
Bovine Serum Albumin	0	[2]1	0	0
Carrier DNA	0	[2]1	0	0
None	0	2	3	1

[1] Different regions of the 28S rRNA gene targeted in a multiplex assay.
[2] Used together.

instrument, and programs for *Dreissena* spp. larval detection utilizing this instrument are actively being developed. Imaging flow cytometry is an important technology because of its potential to significantly increase the throughput of samples, reduce costs per sample, and increase the number and frequency of samples that can be examined. 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 3 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 error was false negatives, indicating that PCR, as with other methods, 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 due to the number of variables involved in the PCR assays utilized by different laboratories during this study; none of the 11 laboratories used identical methods. Furthermore, there were many variables associated with plankton sample concentration, DNA purification, 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 laboratories. Of the assays utilized, only 3 followed protocols that have been published in the peer-reviewed literature, and most have been developed within the past year or two (Table 4).

The majority of PCR protocols utilized in this study have not been published, and the development and optimization of new PCR protocols is being actively pursued by many groups; therefore, exploring the methods used in this study could help identify future improvements to PCR methodologies and allow more accurate and specific detection of the presence of *Dreissena* spp. larvae in plankton samples. To concentrate plankton samples, most laboratories gently centrifuged the sample either once or twice to reduce the volume prior to DNA extraction; however, other concentration procedures were also used, including settling and lyophilization. The 11 laboratories used 8 different DNA extraction procedures, 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 ChargeSwitch beads kit, and Promega's Wizard kit. These extraction procedures resulted in concentration factors 50–450-fold relative to the original volume of the plankton sample (25 mL).

The various assays targeted 5 different genes, 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 used a multiplex assay format allowing the examination of multiple gene targets in a single assay. Most of the multiplex assays targeted 2 genes or 2 different regions of the same gene to allow discrimination between *D. bugensis* and *D. polymorpha*. The size of the PCR amplicons targeted ranged from 363 to 700 bp, with the majority of assays targeting gene fragments in the smaller size range. Several laboratories used PCR reaction enhancers and/or protocols to remove inhibitors, but most did not. PCR enhancers used included T_4 gene 32 product single-stranded DNA binding protein and Bovine Serum Albumin. Two laboratories also included uracil-N-glycosylase in their PCR reactions to help prevent amplicon carryover contamination. Two laboratories used 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 used 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 using different DNA template concentrations in their PCR reactions. Another laboratory routinely included plankton samples spiked with veligers as a blind positive control and a general 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 2 laboratories routinely verified the identity of their PCR amplicons by sequencing.

As stated earlier, the use of PCR technology to detect *Dreissena* spp. larvae is relatively new. The first reports using DNA-based assays to detect and identify *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 they were mostly too late for early detection because the mussels had already become well established. In January 2007 when *D. bugensis* was discovered beyond the 100th meridian in Lake Mead, 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 multiple (at least 2) independent methods were needed to detect the early presence of *Dreissena* spp. larvae in a body of water prior to implementing major management actions (Anderson et al. 2009). At least 5 new PCR assays have been recently developed resulting from the need for detection approaches independent of microscopy. With neither careful vetting nor the benefit of peer review, these assays are currently being used 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 improve them to the level of microscopy-based methods.

As with IFC, it is difficult to determine the exact methodological sources of errors observed associated with PCR assays because of the relatively small number of participating laboratories relative to a large number of experimental variables. Consistent among the highest performing laboratories, however, was the use of centrifugation as a concentration method, DNA purification using 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. There was also a significant ($p = 0.016$) correlation between the experience level of the participating laboratories and their performance; all of the top performing laboratories were the most experienced with working with Dreissenid mussels. However, this variable was only qualitatively evaluated based on surveys

conducted by the Western Regional working group in fall 2010 (Phillips 2010) and by assuming that all the laboratories that participated in the 2009 Phase I study were experienced and those that only participated in the 2010 study were not. Nonetheless, these observations may suggest that perhaps even more than specific methodologies, experience and training are a large component of PCR performance variability.

Because of the need for a second and independent method to verify early invasion events, it is critical that the reliability of PCR-based *Dreissena* spp. detection assays be improved. During the 2009 study involving samples that consisted of essentially plankton-free lake water spiked with *Dreissena* spp. Larvae, 2 of the 5 PCR laboratories returned perfect detection results, indicating that PCR had the potential to be 100% accurate (Frischer and Butler 2009). In this study involving samples of incrementally greater complexity that more closely resembled actual plankton net tow samples, those 2 laboratories were again among the top performing laboratories. Only one type of plankton matrix was used in this study (Quail Creek Reservoir plankton collected in the winter). This plankton sample matrix was more complex than the one used in the earlier round-robin study (Frischer and Butler 2009), a variable that appeared to have impacted the measured accuracy of assays. Because lower reliability was observed in these studies when a single more complex sample matrix was used, the effect of sample matrix composition on the reliability of *Dreissena* spp. detection assays should be further explored. For example, commonly encountered samples with high algal and particulate loads are likely to negatively impact the accuracy of all detection and quantification methods. Thus, the results of this study may not be representative of all complex sample matrix types.

The purpose of this study was to identify the current reliability of CPLM, IFC, and PCR-based approaches for detecting low concentrations of *Dreissena* spp. mussels in plankton samples. Although the results are insufficient to determine the exact sources of errors where they exist (and the experiment was not designed to do so), they clearly demonstrate that CPLM is currently 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. However, continued development of PCR-based methods and accumulation of experience will likely lead to substantial improvement in the reliability of this approach. A general conclusion of these studies is that the reliability of each method is directly proportional to the complexity of the assay.

No standardized *Dreissena* spp. monitoring or quality assurance standards for detection are currently available, nor have responsible authorities for establishing standards been established. However, there is a growing consensus that laboratory certification and quality assurance programs would be useful for management communities responsible for monitoring and mitigating new invasions and spread of *Dreissena* spp. mussels (Frischer and Butler 2009). Studies such as this one that establish the reliability of analytical detection methods and the future establishment of quality assurance and technical certification programs will likely improve the possibility of mitigating the impact of the inevitable establishment of Dreissena mussel populations in the western United States during the next decade.

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