WESTERN REGIONAL PANEL
on Aquatic Nuisance Species

Laboratory Standards for Zebra and Quagga Mussel Veliger Analysis

Prepared by

ANS Laboratory Standards Workgroup

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This Lab Standards document for early detection of *Dressenids* (zebra and quagga mussels) was developed by a workgroup of the Western Regional Panel (WRP) on Aquatic Nuisance Species (ANS) as part of the Building Consensus in the West (BC) Workgroup. This document is an initial step towards understand best practices for laboratory personnel and is a precursor to ANS laboratory standards and quality assurance recommendations. The information described in this document were adapted from existing protocols developed by the members of this workgroup. Many thanks to the workgroup members that participated in the development of these standards!

2018 Lab Standards Workgroup Members (*Table 1)*

First	Last	Agency/ Company
Elizabeth	Brown	Chair, Western Regional Panel Colorado Parks and Wildlife
Christopher	Churchill	US Geologic Survey
Denise	Hosler	US Bureau of Reclamation
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List of Acronyms

AIS	aquatic invasive species
BMS	blind-matrix spiked samples
CPLM	cross-polarized light microscopy
DIC	differential interference contrast
eDNA	environmental DNA
PCR	polymerase chain reaction assay
QAQC	quality assurance quality control
Tris	tris(hydroxymethyl)aminomethane
WRP	Western Regional Panel

Introduction

Aquatic invasive species (AIS) alter habitat for fish, wildlife, and plants, and are becoming a dominant component of aquatic ecosystems (Sanderson, Barnas & Rub 2009). If established in areas they are not native, AIS such as zebra mussels (*Dreissena polymorpha*) and quagga mussels (*Dreissena rostriformis bugensis*), hereafter referred to as *Dreissena* mussels, could cause extensive economic and ecological impacts (Dermott and Kerec 1997; Mann, Radtke, Huppert, Hamilton, Hanna, Duffield & Netusil 2010; Ricciardi, Neves & Rasmussen 1998).

Dreissena mussels attach to hard submerged surfaces such as rock, concrete and steel using byssal threads, and this biofouling can create operational problems for hydroelectric and irrigation facilities (Boelman, Neilson, Dardeau & Cross 1997; Claudi and Mackie 1994; Jenner, Whitehouse, Taylor & Khalanski 1998; Neitzel, Johnson, Page, Young & Daling 1984). *Dreissena* can form large dense populations and through their collective filter feeding and deposition of feces and pseudofeces, they change the manner energy moves in an ecosystem, as well as increasing water clarity, light penetration, and the growth of rooted macrophytes (Bastviken, Caraco & Cole 1998; Botts, Patterson & Schloesser 1996; Burlakova 1995; Caraco, Cole & Strayer 2006; Effler and Siegfried 1998; Effler, Matthews, Brooks-Matthews, Perkins, Siegfried & Hassett 2004; Fahnenstiel, Lang, Nalepa & Johengen 1995; Horvath, Martin & Lamberti 1999; Strayer 2008).

Dreissena mussels were first introduced into North America in the Great Lakes region during the late 1980's and have spread throughout much of the Eastern United States and Canada. The 100th Meridian Initiative and the Western Regional Panel were formed to prevent the further westward spread of AIS such as *Dreissena* mussels. In 2007, established *Dreissena* mussel populations were first detected west of the Rocky Mountains in the lower Colorado River system and have since spread to multiple western water bodies including but not limited to Lake Powell in Utah, and Lake Winnepeg in the Canadian Providence of Manitoba.

Dreissena mussels have multiple dispersal mechanisms and can spread overland to new waterways as well as colonize hydrologically connected areas. Trailered watercraft with attached hitchhiking juvenile and adult mussels are a primary vector for the overland transport of *Dreissena* mussels and increases the risk of inter-basin introduction (Buchan and Padilla 1999, Johnson, Ricciardi & Carlton 2001). *Dreissena* mussel larvae, referred to as veligers, develop in the water column (Raven 1958). These planktonic veligers are dispersed downstream and to downwind areas as they develop in the water column. Additionally, the movement of water containing veligers may inadvertently transport *Dreissena* mussels such as in ballast water tanks and live fish wells. When developmentally ready, veligers actively settle out of the water column onto a variety of submerged substrates including man-made objects and attach using byssal threads and grow into juvenile and adult mussels (Ackerman, Sim, Nichols & Claudi 1994; Roe and MacIsaac 1997; Sprung 1993). The settled juvenile and adult *Dreissena* mussels tend to be gregarious and form clumps. Individual mussels, however, can translocate to other areas within water bodies by crawling across the substratum on their foot, drifting in the water column and by using other means. The risk posed to un-infested water bodies by both proximate and distant *Dreissena* populations is significant.

Monitoring and early detection of *Dreissena* mussels are key to minimizing the risks posed to un-infested waters by these potential seed populations. Invasions by non-native species that eventually become invasive typically include a period of slow population growth, followed by an exponential increase in coverage (Lockwood. Hoopes & Marchetti 2007). It is in the early stages of infestation, when population sizes are relatively small, that control and containment efforts can be most cost effective.

There are multiple monitoring methods for *Dreissena* mussels targeting the different mussel life stages. The planktonic veligers may be the first life stage to colonize a new area and indicate the presence of an incipient local population. Early detection monitoring for the veligers is done with plankton samples that are analyzed using microscopic and molecular techniques.

Veliger detection in plankton samples is confounded by the inherent rarity of a species at low density, the interfering matrix of exogenous organic and inorganic particles found in natural waters, and other factors such as the risks of contamination. Bias associated with veliger detection during analysis includes both false-negative (i.e., failing to detect veligers when they are present) and false-positive results (i.e., detecting veligers when they are absent).

Accurate and timely specimen detection and identification are paramount for producing reliable veliger data that managers are willing to use to guide actions. Incorrect and ambiguous results confuse policymakers and managers, complicate other agency efforts, and compromise trust in the scientific community.

Regional standardized protocols for the laboratory analysis of plankton samples for the presence of *Dreissena* veligers are lacking. Multiple agencies and organizations analyze veliger plankton samples, and each laboratory has developed their own protocols accordingly. The importance of quality sample analysis and standardized protocols has been recognized, and there have been numerous collaborative efforts to augment the quality and standardization of veliger sample collection and analysis including workshops (*Early Detection of Dreissena Mussel in the West Workshop*. 2009. Denver Colorado; *Dreissena Mussel Early Detection Monitoring Methods and Quality Assurance Workshops*. 2012. Fort Worth, Texas), reports and surveys (Kelly, Hosler and Wells 2009; Frischer and Butler 2009; Phillips 2010; Frischer, Nierzwicki-Bauer & Kelly 2011; Wells and Sytsma 2012) as well as efforts to collate and disseminate protocols such as www.westernais.org and www.pnamp.org.

The Building Consensus Committee of the Western Regional Panel (WRP) is a collaborative effort to protect western waters from *Dreissena* mussel infestation. The goals of WRP's Building Consensus Committee include increasing the economies and efficiencies for watercraft inspection and decontamination programs, increasing the consistency of the messaging and experiences for recreational boaters, and developing standard protocols and definitions for waterbody classification, monitoring and regulations among western states. The AIS Laboratory Standards Committee was formed at the bequest of WRP's Building Consensus Committee to improve the quality and standardization of the laboratory protocols

used for the analysis of plankton samples for the presence/ non-detect of *Dreissena* larvae, or veligers.

Objective

The AIS Laboratory Standards Subcommittee, hereafter referred to as Lab Standards Committee, was formed to improve the quality and standardization of protocols used by laboratories analyzing plankton samples for the early detection of *Dreissena* mussel veligers using cross-polarized light microscopy (CPLM). The Lab Standards Committee recognized the need to include other analytical techniques and considered protocols relevant to molecular approaches such as polymerase chain reaction assay (PCR) and environmental DNA (eDNA).

The Lab Standards Committee goals included the following:

- evaluate current AIS laboratory protocols,
- discuss American Fisheries Society laboratory certification program to use as a template for regional standardization, and
- develop best management practices for quality control.

Methods

The Lab Standards Committee was formed in 2016 by soliciting volunteer committee members from the laboratories analyzing veliger samples as well as agencies conducting and coordinating early detection monitoring (Table 1). The Lab Standards Committee was not comprehensive, but efforts were made to be representative of the different laboratories and analytical techniques employed for veliger plankton analysis.

The Lab Standards Committee communicated via email and telephone conference calls. Email was used to solicit committee members, share documents, communicate tasks and timelines, and to coordinate conference calls. Conference calls were organized by the Lab Standards Committee Chairperson, Steve Wells. The Lab Standards Committee goals were identified by the WRP's Building Consensus Committee. Lab subcommittee decisions were made during conference calls and were determined by a majority vote.

Table 1: Members of the Lab Standards Committee. Lab code is provided to reference the protocols defined in Table 2 and summarized in Tables 3, 4, 5, and 6.

First	Last	Agency/ Company	Lab code	Email
Elizabeth	Brown	Colorado Parks and Wildlife Chair, Western Regional Panel	N/A	Elizabeth.brown@state.co.us
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The Lab Standards Committee reviewed existing laboratory protocols provided by committee members to identify key parameters to address when developing a quality assurance project plan for a laboratory analyzing plankton samples for *Dreissena* veligers. The Lab Standards Committee was focused on protocols for CPLM analysis, but also evaluated sample preservation and handling as well as decontamination protocols used for PCR and eDNA. Committee members shared laboratory protocols (identified by Lab Code in Table 1) and the protocols were collated into a spreadsheet format organized into four general categories: sample preservation and handling; sample analysis; quality assurance quality control (QAQC); and equipment decontamination. The committee reviewed the protocols and identified the parameters where there were differences among the laboratories, and these differences were discussed to identify areas of potential convergence as well as areas needing additional research. The key parameters that were identified by the Lab Standards Committee are defined in Table 2.

Table 2: Parameter definitions and example answers for key parameters in a quality assurance project plan for Dreissena veliger CPLM laboratories. Parameters are grouped into four categories including Sample Preservation and Handling (No. 1 – 14), Sample Analysis (No. 15 - 26), Quality Assurance Quality Control (No. 27 - 37), and Equipment Decontamination (No. 38 - 45).

No.	Parameter	Definition	Example answer(s)
1	Preservative	Type of preservative added to plankton sample to maintain sample integrity.	• regular ethanol (non- denatured) • regular ethanol (denatured) • isopropyl
2	[Preservative]	Concentration of preservative in the final sample, e.g., 20% preservative concentration = 100-mL preservative + 400-mL lake water and plankton.	• 20% alcohol solution • 25% alcohol solution • 50% alcohol solution • 70% alcohol solution (preferred)
3	Holding temp	Temperature range samples maintained within laboratory prior analysis for given concentration of preservative.	• 0 – 4ºC (32 – 39ºF) for alcohol solutions < 70% • 0 - 23ºC (32 - 73ºF) for alcohol solutions ≥ 70%
4	Max holding time	Maximum amount of time veliger specimen integrity is assumed acceptable given preservation and handling methods.	• 2 WKS ([<70%] @ 0-4ºC) • 3 WKS ([<70%] @ 0-4ºC) • 12 WKS ([≥70%] @ 23ºC) • 48 WKS ([≥70%] @ 23ºC)

5	Analysis hold time	Average (or most common) time period between sample receipt into laboratory and analysis.	• ≤ 14 DAYS • ≤ 21 DAYS • ≤ 84 DAYS
6	Buffer agent	Type of buffering agent added to sample to maintain pH of preserved sample above pH 7.	• Sodium bicarbonate ($NaHCO_3$) • Tris(hydroxymethyl)-aminomethane (tris) • None
7	Field buffer	Method buffering agent is added to sample in the field or to stock preservative prior to field collection and lab receipt.	• 0.1-mL spoon • 4% solution, add 5% w.v. • micro spoon, 50-mg/50-mL total sample • 7 drops 4M Tris/500-mL total sample • not applicable
8	Lab buffer	Method buffering agent is added to sample in laboratory to maintain sample above pH 7.	• 0.1-mL spoon • 0.1-mL spatula • 4% solution add 5% w.v • 50-mg/50-mL total sample • drops 4M Tris solution as needed • none
9	pH measured	Is pH of preserved plankton samples measured in lab?	• Yes • No
10	pH frequency	A general description of how pH of samples is measured.	• Field and laboratory login • At time of analysis • Laboratory login, random subset each batch of samples
11	Shipping method	Type of shipping and carriers used (alcohol concentration).	• FedEx Overnight (20-50%) • FedEx Ground (≥ 70%) • USPS Surface Mail (≥ 70%) • hand delivery (20- ≥ 70%)
12	Sample storage	Samples stored in laboratory post analysis for specified time, if requested by the sample owner. Samples destroyed after storage period. Storage fees possible.	• Yes • No
13	Storage period and disposal	Average storage period samples are stored in laboratory post analysis?	• End of season • After two years • Not applicable

		After the storage period, the samples are destroyed.	
14	Container re-use	After samples are destroyed, sample containers are cleaned for re-use using brush, soapy water, acid and Alconox or bleach? Cleaning and shipping fees may apply.	• Yes • No
15	Filter use	Are samples filtered prior to analysis to remove large particulates and debris?	• Yes • No
16	Filter size	Mesh size of filter, if used to remove large particulates and debris?	• Not applicable • 280-µm • 750-µm
17	CPLM	Routine sample analysis via cross-polarized light microscopy? Veliger shells are birefringent and used for detection (Johnson 1995); other objects are birefringent.	• Yes • No
18	Microscope routine scan	Type of microscope used for routine scan of samples and specimen detection.	• Compound light • Stereo- or dissecting light • Both compound and dissecting
19	Magnification routine scan	Total magnification used for routine scan of samples and suspect detection.	• 10x – 60x • 6.7x – 90x • 6x – 40x
20	Microscope suspects	Type of microscope used for identification of suspect specimens.	• Compound light • Scanning electron • Differential interference contrast
21	Magnification suspects	Total magnification used for identification of suspect specimens.	• 200x • 100x – 1,000x • 100x – 400x

22	Cell routine scan	Preferred or most commonly used type of counting cell or chamber used for routine scan of plankton sample.	• Petri (glass petri dish) • SR cell (Sedgewick-Rafter cell)
23	Report percent done	Percentage of concentrated particulate in sample analyzed is reported to sample owner? Particulates are the pelleted portion of sample containing veligers and genetic material. Total sample volume reflects lake water and preservative as well as the particulates.	• Yes • No, results recorded in laboratory
24	Percent sample done	Average amount or range of concentrated particulate of veliger sample analyzed via microscopy during routine scan. Amount of sample analyzed is determined by sample owner and varies by project.	• ~ 20% • 100% • (20% – 100%) • (25% - 100%)
25	Report bivalves	Non-target bivalves such as unionid glochidia encountered during analysis are reported to sample owner.	• Yes • Yes, imaged, abundance or presence by project • Recorded, not reported • Recorded and imaged, not reported
26	Report ostracods	Non-target ostracods, birefringent organisms of similar size and shape as *Dreissena* veligers are reported to sample owner.	• Yes • Yes, imaged, abundance or presence by project • Recorded, not reported • Recorded and imaged, not reported

Table 2 continued

No.	Parameter	Definition	Example answer(s)
27	Previous Round Robin	Laboratory participated in the previous Round Robin Exercises done in 2008 and/or 2010 (Frischer et al. 2011).	• Yes • No
28	New Round Robin	Laboratory interested in participating in new/ annual Round Robin Exercise.	• Yes • No
29	New RR timing	Preferred time of year to conduct a new Round Robin Exercise?	• late fall or early spring • late winter • late winter or early spring • not applicable
30	New whole sample spikes	Laboratory interested in using whole sample spikes?	• Yes • No
31	Analyst performance testing	Do you conduct analyst performance testing, e.g., employees evaluate set of training samples and one sample with spiked *Dreissena* veligers?	• Yes • No
32	Analyst test acceptance	Criteria used for acceptance of analyst performance testing?	• 98% recovery • 97% recovery <50 veligers/slide and 95% recovery >50 veligers/slide • Not applicable
33	Analyst test frequency	Frequency of the analyst performance testing?	• Once • Semi-annual • Not applicable
34	Blind matrix spiked sample	Are BMS used during routine analysis, e.g., spiking sample with known amount of *Dreissena* veligers?	• Yes • No
35	BMS frequency	Frequency of the BMS samples?	• One BMS every 15 samples • One BMS annually • Not applicable

Table 2 continued

No.	Parameter	Definition	Example answer(s)
36	BMS acceptance	Criteria used for BMS acceptance?	• Detection • Not applicable
37	Action if fail BMS	Corrective steps taken if spiked veligers are not detected during routine analysis?	• Re-analysis of all samples in batch with new BMS; flag results if second BMS missed; increase analyst training. • Not applicable
38	Scrub with brush, soap and water	Equipment in contact with sample is cleaned by scrubbing with a brush (e.g., plastic bottle brush) in soap and water to physically remove veligers and other material?	• Yes • No
39	Bleach solution used	Equipment in contact with sample is cleaned by soaking in bleach solution and/ or spraying and wiping with disposable towels to destroy genetic material?	• Yes • No
40	[Bleach]	Concentration of store-bought bleach solution used for destroying genetic material, e.g., 10% bleach = 50mL store-bought bleach + 450mL water.	• 5-7% • 10% • Not applicable
41	Bleach contact time	Amount of time equipment is exposed to bleach solutions.	• Minutes • Two minutes • Five minutes • 10 minutes • 10 – 15 minutes • Not applicable
42	Acid solution used	Equipment cleaned by soaking in acetic acid solution to destroy veliger shells?	• Yes • No

Table 2 continued

No.	Parameter	Definition	Example answer(s)
43	[Acetic acid]	Concentration of acetic acid solution used for destroying veliger shell, e.g., store-bought white vinegar is 5-6% acetic acid.	• 5 - 6% • Not applicable
44	Acid contact time	Amount of time equipment is exposed to acid solutions.	• 2 hours (minimum) • 6 hours (minimum) • Not applicable
45	Other decon measures	Other steps used for laboratory equipment decontamination not previously mentioned.	• Lab dishwasher with Alconox • DNAse/RNAse on countertops routinely • 4% HCl acid soak, 4 hours minimum soak then rinse 5x water • None

Table 2 End

Results

The Lab Standards Committee determined that the standardization of laboratory protocols was beyond the initial capacity of the committee, and decided that additional expertise is needed on the committee to evaluate the different protocols and make recommendations for best management practices, (e.g., American Fisheries Society laboratory certification program).

Laboratories, for the most part, developed independent protocols deliberately to best preserve specimen integrity in a cost effective and safe manner. Laboratories modified protocols to meet project and/ or sample owner specifications. Standardization could impede this flexibility.

The Lab Standards Committee identified and collated the existing CPLM laboratory protocols for veliger sample preservation and handling in Table 3, sample analysis in Table 4, quality assurance quality control (QAQC) in Table 5, and equipment decontamination in Table 6.

When appropriate, participating laboratories modified protocols in response to the Lab Standards Committee discussions, e.g., Laboratory G increased the concentration of bleach used for equipment from 7% to 10% store-bought bleach.

In general, the protocols used for CPLM sample preservation and handling (Table 3) as well as equipment decontamination (Tables 6) are acceptable, or at least compatible with PCR. In other words, a properly handled veliger sample analyzed via CPLM can then be prepared and analyzed via PCR.

The protocols presented in Tables 3 – 6, however, are not suitable for eDNA analysis due to concerns regarding decontamination protocols and the risks of false positive results. For example, the concentration of bleach solutions discussed for eDNA equipment decontamination was 50-100% store-bought bleach, whereas the concentrations of bleach solutions used for PCR and CPLM equipment decontamination was approximately 10% store-bought bleach. It is recommended that the WRP consider establishing a separate committee related to eDNA.

Alcohol is the preservative used for veliger samples analyzed via CPLM (Table 3). For the moment, absolute ethanol is the preferred option for preservation. Avoid using denatured ethanol or isopropyl alcohol (rubbing alcohol) as these may interfere with analysis or pose a health risk to laboratory staff.

There are two general strategies for sample preservation and handling during the period between sample collection and analysis. The first strategy involves preserving samples in solutions of 20 – 50% alcohol and keeping samples refrigerated (0 – 4^{o}C) to arrest microbial activity prior to analysis. The second strategy preserves samples in solutions of 70% or greater alcohol, and the samples can be kept at room temperature or refrigerated. Alcohol solutions greater than or equal to 70% are considered a Class 3 Flammable Liquid, and shipping restrictions require these samples to be shipped via FedEx Ground or USPS Surface Mail only. The ORM-D exemption, valid through 2020, provides for shipping Class 3 Liquids without Hazmat certifications. Samples preserved in lower alcohol concentrations, such as 25% alcohol, can be shipped in airplanes, but these samples must be kept cold and are shipped overnight on blue ice, e.g., FedEx Overnight (Table 3).

The pH of veliger plankton samples must be maintained above 7.0, and hence buffering agents are added to most plankton samples at the time of collection as well as in the laboratory such as during sample login (Table 3). Baking soda, or sodium bicarbonate, is the predominant buffering agent used; however, tris(hydroxymethyl)aminomethane (Tris) is also used. Baking soda is typically added via micro spoons/ spatulas while Tris is added as liquid drops.

Tris buffer is a preferred buffer as it is better at buffering the solution and does not precipitate out of solution. However, if Tris buffer is not available, baking soda should be used. Only one of these buffers should be used in your samples. Do not use both Tris and baking soda.

- For 4M Tris pH7.5 buffer, use a 1 mL plastic transfer pipette to add 2 drops of Tris buffer per every 100 mL of sample.
- For baking soda, add 0.2 grams (0.2 mL or 2 level scoops with a 0.1-gram measuring spoon) per every 100 mL of sample in order to buffer the pH

    Source: Standard Operating Procedure: Field Sampling Methods for Invasive Mussel Early Detection, Bureau of Reclamation Ecological Research Laboratory, May 2020.

Sample analysis occurs within several weeks of laboratories receiving samples. In general, laboratories analyzed samples in 2 – 4 weeks or within 12 weeks of receipt (Table 3). Post

analysis, most laboratories offered sample storage services for time periods ranging from the end of the collection season up to two years (Table 3).

All participating laboratories used cross-polarized light microscopy (CPLM) for the routine scanning of samples using both compound and stereo-microscopes (Table 4). During routine scan, plankton samples are analyzed in glass petri dishes or Sedgewick-Rafter cells under total magnifications ranging from 6x to 90x (Table 4). If a suspect specimen is detected during routine analysis, identification is done using compound, differential interference contrast (DIC), as well as scanning electron microscopy with total magnifications ranging from 100x to 1,000x (Table 4). All laboratories recorded the amount of the concentrated particulate analyzed, which ranged from 20 to 100%, as well as the presence of extraneous bivalve larvae and juveniles and ostracods.

The participating CPLM laboratories addressed biases such as false negatives due to analyst error using analyst testing or blind matrix spiked (BMS) samples (Table 5). Analyst testing involved an analyst evaluating a set of training samples, and one or more of these samples contains a known amount of spiked *Dreissena* veligers. The acceptance criteria for analyst testing was ≥ 95% recovery of spiked veligers (Table 5). BMS samples involved spiking a known number of *Dreissena* veligers into a split of a random sample submitted with other samples for routine analysis. The acceptance criteria for BMS samples was detection of spiked veligers (Table 5).

False positive results such as from contaminated equipment are addressed by all laboratories with cleaning protocols. All equipment in contact with plankton samples is cleaned with a brush, e.g., plastic bottle brush and soap and water to physically remove extraneous material (Table 6). Most participating laboratories used bleach (10% store-bought bleach) and acid solutions (5% acetic acid) to destroy genetic material and dissolve the calcite crystalline veliger shell (Table 6). Other decontamination protocols included routinely wiping countertops with DNAse/RNAse, as well as cleaning equipment using high pressure water, Alconox in a dishwasher, and 4% HCl acid bath (Table 6).

Table 3: Protocols used by veliger laboratories for sample preservation and handling. Parameters defined in Table 2. Lab Codes identified in Table 1.

No	Parameter	Laboratory Code						
		A	**B**	**C**	**D**	**E**	**F**	**G**
1	Preservative	Regular ethanol (denatured)	Isopropyl	Regular ethanol (non-denatured)	Regular ethanol (non-denatured)	Regular ethanol (non-denatured)	Regular ethanol (denatured)	Isopropyl or regular ethanol (non-denatured)
2	[Preservative]	50%	20%	70%	20%	50%	80%	70%
3	Holding temp	0 – 4°C (32 – 39°F)	0 – 4°C (32 – 39°F)	21 – 23°C (70 – 73°F)	0 – 4°C (32 – 39°F)	3 – 4°C (37 – 39°F)	21 – 23°C (70 – 73°F)	21 – 23°C (70 – 73°F)
4	Max holding time	< 12 weeks	~ 2 – 3 weeks	2 weeks	~ 12 weeks	2 – 3 weeks	< 12 weeks	< 48 weeks @ 70% alcohol 23oC 2 weeks @ < 70% alcohol 0 – 4oC
5	Analysis hold time	≤ 12 weeks	~ 2 – 3 weeks	2 weeks	~ 12 weeks	2 – 3 weeks	≤ 12 weeks	2 – 4 weeks
6	Buffer agent	$NaHCO_3$	$NaHCO_3$	$NaHCO_3$	$NaHCO_3$	$NaHCO_3$	N/A	Tris
7	Field buffer	N/A	0.1-mL spoon	N/A	4% solution add 5% w.v.	Micro spoon, 50-mg/50-mL total sample	N/A/	N/A

No	Parameter	Laboratory Code						
		A	**B**	**C**	**D**	**E**	**F**	**G**
8	Lab buffer	0.1-mL spatula/ spoon	0.1-mL spatula/ spoon	0.1-mL spatula/ spoon	4% $NaHCO_3$ solution, add 5% w.v.	0.1-mL spatula/ spoon, 50-mg $NaHCO_3$/50-mL total sample	N/A	7 – 14 drops 4M Tris solution/ 500-mL total sample
9	pH measured	Yes	Yes	No	Yes	Yes	Yes	Yes
10	pH frequency	At time of analysis, all samples	Laboratory login, all samples	N/A	At time of analysis, all samples	At time of analysis, all samples	At time of analysis, all samples	Laboratory login, random subset/ batch
11	Shipping method	N/A	FedEx Overnight	FedEx Ground (70%) FedEx Overnight (20-50%)	Next day, various carriers	Next day, various carriers and hand delivery	Ground, Purolator	FedEx Ground/ USPS Surface Mail (70%) FedEx Overnight (20-50%)
12	Sample storage	Yes	Yes	Yes	No	Yes	No	Yes
13	Storage period and disposal	≥ 2 years	End of season	End of season	N/A	≥ 2 years	N/A	≥ 2 years
14	Container re-use	No	Yes		Sometimes	No	Yes	Yes

Table 3 End

Table 4: Protocols used by veliger laboratories for sample analysis. Parameters defined in Table 2. Lab Codes identified in Table 1.

No	Parameter	Laboratory (Laboratories are identified with Lab Codes in Table 1)						
		A	**B**	**C**	**D**	**E**	**F**	**G**
15	Filter use	No	No	No	No	No	Yes	Yes
16	Filter size	N/A	N/A	N/A	N/A	N/A	280-µm	750-µm
17	CPLM	Yes	Yes	Yes	Yes	Yes	Yes	Yes
18	Microscope routine scan	Compound and stereo	Compound and stereo	Compound and stereo	Compound and stereo	Compound and stereo	Compound and stereo	Compound and stereo
19	Magnification routine scan	20x – 40x		10x – 40x	10x – 60x	6.7x – 90x	6x – 40x	10x – 40x
20	Microscope suspects	Compound	Compound or SEM	Compound	Compound or DIC	Compound or DIC	Compound	Compound
21	Magnification suspects	100x – 200x	100x – 1,000x	100x – 200x	100x – 200x	100x – 400x	100x – 400x	100x – 400x
22	Cell routine scan	SR cell	Petri	Petri	Petri	Petri	SR cell	SR cell
23	Report percent done	Yes	No, record in lab	Yes	Yes	Yes	Yes	Yes
24	Percent sample done	~ 20%		100%	100%	100%	25 – 100%	20 – 100%
25	Report bivalves	No, recorded	Yes	Yes	No, recorded	No, recorded	Yes	Yes
26	Report ostracods	No, recorded	Yes	Yes	No, recorded	No, recorded	Yes	Yes

Table 5: Protocols used by veliger laboratories for quality assurance quality control. Parameters defined in Table 2. Lab code identified in Table 1.

No.	Parameter	Laboratory Code						
		A	**B**	**C**	**D**	**E**	**F**	**G**
27	Previous RR	No	Yes	Yes	Yes	No	Yes	Yes
28	New RR	Yes	Yes	Yes	Yes	Yes	Yes	Yes
29	New RR timing	Late winter or early spring	Late fall or early spring		Late winter or early spring	Late winter or early spring	Late winter or early spring	Late winter or early spring
30	New whole sample spike	Yes	No	Yes	Yes	Yes	Yes	Yes
31	Analyst performance testing	Yes	Yes	No	No	No	No	No
32	Analyst test acceptance	97% recovery <50 veligers/slide and 95% recovery >50 veligers/slide	98% recovery	N/A	N/A	N/A	N/A	N/A
33	Analyst test frequency	Semi-annual	once	N/A	N/A	N/A	N/A	N/A
34	BMS	No	No	Yes	No	No	No	Yes

Table 5 continued

No	Parameter	Laboratory Code						
		A	**B**	**C**	**D**	**E**	**F**	**G**
35	BMS frequency	N/A	N/A	1 BMS annually	N/A	N/A	N/A	1 BMS per 15 samples
36	BMS acceptance	N/A	N/A	Detection	N/A	N/A	N/A	Detection
37	Action if fail BMS	N/A	N/A		N/A	N/A	N/A	Re-analysis of all samples in batch with new BMS; flag results, re-train analyst, increase dilution factor with second miss
Table 5 End								

Table 6: Protocols used by veliger laboratories for equipment decontamination. Parameters defined in Table 2. Lab Code identified in Table 1.

No	Parameter	Laboratory (Laboratories are identified with Lab Codes in Table 1)						
		A	**B**	**C**	**D**	**E**	**F**	**G**
38	Scrub with brush, soap and water	Yes	Yes	Yes	Yes	Yes	Yes	Yes
39	Bleach solution used	No	Yes	No	Yes	Yes	Yes	Yes
40	[Bleach]	N/A	1%	N/A	10%	10%	5 – 7%	10%
41	Bleach contact time	N/A	minutes	N/A	2 minutes	10 minutes	5 minutes	10 – 15 minutes
42	Acid solution used	No	Yes	No	Yes	Yes	No	Yes
43	[Acetic acid]	N/A	5-6%	N/A	5-6%	5-6%	N/A	5-6%
44	Acid contact time	N/A		N/A		≥ 2 hours	N/A	≥ 6 hours
45	Other decon measures	High pressure water	Dishwasher with Alconox	None	None	DNAse/RNAse on countertops routinely	None	4% HCL acid soak, 4 hr. minimum and rinse 5x

End Table 6

Discussion

The handling and analysis of veliger samples may affect specimen integrity, detection and identification. O'Meara, Hosler, Brenimer and Pucherelli (2013) measured veliger birefringence loss over a 30-day period, and birefringence loss was highest in acidic pH conditions. Cold holding temperature (4^{o}C) was another important parameter for preserving veliger birefringence in samples preserved in alcohol solutions of 25% and less (O'Meara et al. 2013). All the laboratories participating in the Lab Standards Committee routinely monitor, record, and adjust as necessary, the veliger sample handling and analysis conditions such as sample pH and holding temperature.

The important parameters for CPLM laboratory quality management are defined in Table 2 and identified Tables 3, 4, 5, and 6. The list of parameters includes how plankton samples are preserved and handled as well as quality control measures and equipment decontamination protocols.

The analytical techniques used to detect and identify *Dreissena* veligers in a plankton sample can be important determinants of how the sample is preserved, handled, and equipment decontaminated. These protocols are acceptable for CPLM analysis. In general, the protocols for sample preservation and handling (Table 3) as well as equipment decontamination (Table 6) are also acceptable for PCR molecular analysis.

Veliger samples being analyzed via eDNA techniques should use different field collection and laboratory protocols as compared to CPLM and PCR. It appears that the concentration of bleach solution acceptable for equipment decontamination for PCR and CPLM (e.g., 10% bleach solution for minutes contact) is likely inadequate for eDNA techniques (50% - 100% store-bought bleach). This raises serious problems of potential contamination. *Dreissena* veligers are handled in CPLM laboratories, e.g., creation of BMS samples (Table 5). Laboratory dishware washed using 10% bleach could still have viable genetic material that triggers a positive eDNA result. A positive result could be real or the result of contamination. Controlling for biases such as false positive results from contamination is largely dependent upon effective equipment decontamination protocols, and this may include dedicated equipment and separate physical laboratories for different analytical techniques.

In most cases, there were valid reasons for divergent approaches CPLM laboratories used to address a parameter, and the overall outcomes were similar. For example, the two general strategies used for sample preservation and handling are largely determined by preservative costs and shipping restrictions for Class 3 Flammable Liquids. The different preservation methods presented in Table 3 were found to be acceptable given that veliger samples were properly buffered and maintained at the proper holding temperatures.

The documentation of the sample handling and analysis protocols is important, and the record-keeping is the responsibility of the laboratory. Many laboratories, however, are not the sample owners. Laboratory records of sample handling and analysis should be available to sample owners.

Many of the participating CPLM laboratories have existing quality assurance or quality control (QA/QC) measures used to address bias such as analyst error. For example, some laboratories evaluate an analyst's ability to detect and identify spiked *Dreissena* veligers in a set of training samples. Other laboratories use BMS samples to evaluate analyst's ability to detect and identify *Dreissena* veligers spiked into the sample stream during routine analysis. These QA/QC measures are notable and encouraged. All of the existing QA/QC measures, however, are internal actions.

The Lab Standards Committee also recognized the need for external QA/QC measures. There was unanimous interest in developing another Round Robin Exercise similar to that done in 2010 (Frischer et al. 2011). According to the Lab Standard Committee, the best time of year to conduct such an exercise is the late winter to early spring period.

The Lab Standards Committee also discussed whole-sample spikes (e.g., spiking known number of *Dreissena* veligers into random samples in the field). There are problems with Round Robin exercises and whole-sample spikes that must be addressed including but not limited to chain-of-custody protocols, equipment decontamination, communication plan between sample owner/ spiker and the laboratory when veligers are detected, and acceptance criteria and corrective actions taken if measure failed.

Next Steps/ Recommendations

- All laboratories should continually develop and modify laboratory protocols for veliger sample analysis to account for new knowledge. At minimum, laboratory protocols should be updated annually.
- Investigate preservation methods, including alternate methods to using absolute ethanol, which is currently the preferred option for preservation and report back to WRP.
- Investigate best ways to buffer pH of veliger samples given importance of maintaining sample above pH 7. What are the pros and cons of the existing buffering agents, the compatibility with different analytical techniques, solubilities in water and alcohol, and the best ways to measure pH in buffered veliger samples at alcohol solutions ranging from 25% to 70% alcohol?
- Collate existing laboratory protocols used for PCR, eDNA, and other molecular veliger sample techniques. Highlight protocols that are compatible with multiple analytical techniques.
- Develop plan for new Round Robin exercise done in late winter to early spring including funding, source of veligers, and protocols for creating spiked samples.
- Develop plan for whole-sample spiked samples including chain-of-custody protocols, preservation, handling, and decontamination protocols, and communication plan for when veligers are detected by a laboratory.

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