United States Department of the Interior

U.S. Fish & Wildlife Service
UTA Box 19498
Arlington, Texas 76019

Tom Remington, Director
Colorado Division of Wildlife
6060 Broadway, Denver, CO 80216

Dear Dr. Remington:

Please find enclosed the final version of our report entitled "Zebra/Quagga Mussel Early Detection and Rapid Response: Blue Ribbon Panel Recommendations for the Colorado Division of Wildlife."

The other authors and I worked diligently to address the specific question posed to us by your invasive species biologists during the Blue Ribbon Panel meeting in September of 2008. We generated much discussion and have compiled our decisions and recommendations into the attached document.

In short, the Colorado Division of Wildlife's proactive strategy to prevent further invasions that is commendable. Your team of hard-working biologists is among the best I have had the opportunity to work with in all of the Western United States. In particular, the efforts of Elizabeth Brown and Vicki Milano to coordinate with regional partners to employ the most effective strategies for preventing and controlling aquatic invasive species will undoubtedly protect and conserve valuable natural biological resources within Colorado and the West as a whole. As a federal coordinator and biologist, I am thankful for all efforts to do a good job, but I especially appreciate coordinated strategies to employ the best information and methodologies available. For this, the Colorado Division of Wildlife should be recognized. I will undoubtedly highlight the Colorado Division of Wildlife as a recommended example for other state's wildlife agencies to follow.

Thank you for the opportunity to work with the State of Colorado and good luck with your prevention efforts.

Sincerely,

David K. Britton, Ph.D.
Assistant Aquatic Invasive Species Coordinator
Fisheries and Aquatic Resources Conservation
U.S. Fish & Wildlife Service, Southwest Region

Enclosures (1)

Zebra/Quagga Mussel Early Detection and Rapid Response: Blue Ribbon Panel Recommendations for the Colorado Division of Wildlife

FINAL

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1. Executive Summary

Given the potential for arrival and establishment of invasive quagga and zebra mussels in Colorado waterways, the Colorado Division of Wildlife (CDOW) convened a blue ribbon panel to review their current practices and policies aimed at controlling these pernicious organisms. The recommendations contained in this report are intended to ensure that CDOW employs current best practice to detect and prevent the spread of invasive mussel species, in a cost-effective and efficient manner. The panel represented a diversity of expertise in the detection of quagga and zebra mussels, their biology, and the methods and technology used to detect and identify these animals. The panel attended several days of presentations from agency representatives and parties active in Colorado and elsewhere who are currently engaged in monitoring and controlling invasive mussel infestations, and who described current practices of the CDOW and its agents. Focus of the meeting and this report is to answer several questions:

1. Are current monitoring activities adequate in scope and methodology to achieve the goal of timely detection of any quagga or zebra mussel introductions?

2. Do current practices for the accurate identification of invasive mussel species provide the requisite reliability and level of certainty needed to justify very expensive and disruptive emergency control and management responses?

Generally, the panel found the invasive mussel detection and management practices of the Colorado Division of Wildlife to be comprehensive, thoughtful, and effective. CDOW currently uses a sampling and monitoring scheme that is calibrated to perceived risk, with more intensive monitoring carried out in waters that are at highest risk for infestation. The panel recommended some refinement of the risk factors and indicators used to prioritize monitoring efforts (see Section 3, and Appendix, Table 1), but deemed the existing monitoring regime reasonable and cost-effective and in need of relatively minor tweaking to become a model program of its kind.

CDOW currently employs standard methods for sampling and detection, i.e., settlement substrates or traps which are regularly inspected for the presence of attached mussels, plankton tows which target larval stages present in the water column, and regular inspection of existing natural and man-made surfaces that provide suitable habitat in Colorado waters. While the panel does recommend CDOW investigate alternate methods of constructing and placing settlement traps, there was general agreement that effort and resources be directed primarily to plankton tows and water-sampling that targets veliger larvae. While useful as confirmation of an infestation, detection of settled mussels is indicative of an established, reproducing adult population, and hence not appropriate as a primary target if early detection is the main goal.

Much of the panel's discussion revolved around the question of the reliability of species identification methods. Early detection is key to controlling the spread of these organisms, and accurate identification of suspected quagga or zebra mussel invasions is critical, because the consequences of a false alarm are both expensive and highly disruptive. CDOW is currently adhering to an identification protocol that calls for three-fold verification of suspected invasive mussel infestations, in which the presence of quagga or zebra mussels must be confirmed by microscopy and by PCR performed in two independent laboratories. This discussion was prompted by CDOW's recent experience, in which some samples tested by different laboratories, or tested using the different methodologies for species identification, evinced contradictory results. However, upon closer investigation, it appears that discrepancies likely arise from the way samples are processed and distributed among the testing laboratories, rather than failure of one or another identification method. In order to ensure accurate species identification, the panel recommends:

- some modification to the way samples are currently being collected, and then processed subsequent to their acquisition. If implemented, these modified sampling procedures should prevent most of the difficulties encountered in previous testing.
- retention of the three-fold verification process, but with management response triggered even if only two of the three tests are positive for the presence of quagga or zebra mussels. In the event that only one laboratory should report a positive identification, this is grounds for increased monitoring, but does not warrant immediate implementation of containment and control measures. DNA extraction methods and PCR testing protocols should be standardized, with routine DNA sequencing of amplicons to verify species identification.
- it appears that the number of trained microscopists who can reliably identify dreissinid veliger larvae is quite limited, and we recommend CDOW make efforts to train additional technical support staff in the use of cross-polarized light methods of microscopy to enhance their screening abilities.

The panel also discussed alternative detection methods, those which are not currently employed by CDOW, and areas of detection and management of infestations that might be useful and/or warrant further research and investment. Details of these discussions and panel recommendations are provided below.

2. **Introduction**

Purpose

This expert panel was convened by the Colorado Division of Wildlife (CDOW) to review the State of Colorado's early detection program for zebra and quagga mussels. CDOW assembled this panel for the purpose of advising them regarding the best scientific methods for early detection and to ensure they are utilizing such methods to detect zebra and quagga mussels at the earliest stages of infestation. CDOW has organized this panel

to advise them, recognizing they have a tremendous responsibility to the people of the state and its partners to:

- utilize the best available methods, based on sound science, for early detection and rapid response,
- minimize the risk of invasive mussels spreading from known locations, and
- ensure allocated funding is spent efficiently on such efforts.

Roles and Responsibilities

Panel Members:

Panel members were assembled for the purpose of advising CDOW. The Panel operated independently of the presenters and observers. Feedback from the panel was open and knowing that the panel was free to criticize the current program, without risk of offense, for the purpose of making recommendations that will better the program. As much time as needed was allotted to review the current program, ask questions, hold discussions and make written recommendations to the state. The final product of the meeting is this written paper by the panel commenting and critiquing current detection methods and making specific recommendations on how to proceed in the future.

Presenters:

Presentations were provided in order to give the Panel a detailed overview of the early detection methods currently being utilized by CDOW and its partners. Presenters took the panel on a step by step journey through the process and protocols they follow, going into a fine level of detail. Presenters highlighted what has been working well and where they have found the challenges. Presenters walked the panelists through CDOW's early detection process from the moment a field tech goes to a water body to collect a sample for mussels, to the moment the ANS Coordinator gets the phone call or email stating Colorado is positive for zebra/quagga at that water body, to when a field response is initiated.

Observers:

CDOW recognizes the interdisciplinary nature of the impacts associated with zebra/quagga mussel infestations, as well as the management challenges on multi-jurisdictional western waters. CDOW also recognizes that a cooperative effort among all parties is required for a successful statewide early detection and rapid response program. No single entity is solely responsible for— or capable of— managing for zebra/quagga mussels in the State. Therefore, CDOW invited its partners to strengthen partnerships and benefit by learning from the panel. Observers were given an opportunity to ask questions and make comments at the conclusion of each topic.

Participants:

- Chris Holdren, US Bureau of Reclamation, Environmental Applications and Research Group
- Cort Anderson*, University of Idaho
- David Britton*, US Fish & Wildlife Service
- Dave Winters, US Forest Service

- Denise Holser**, US Bureau of Reclamation, Environmental Applications and Research Group
- Elizabeth Brown**, Colorado Division of Wildlife
- Greg Gerlich, Colorado Division of Wildlife
- Jaci Gould, US Bureau of Reclamation
- John Wood**, Pisces Molecular
- Larry Dalton, Utah Division of Wildlife Resources
- Marc Frischer*, Skidaway Institute of Oceanography
- Melanie Culver*, University of Arizona
- Mike Stone, Wyoming Game and Fish Department
- Paul Rochelle**, Metropolitan Water District of Southern California
- Renata Claudi*, RNT Consulting Inc.
- Vicki Milano**, Colorado Division of Wildlife
- Walt Donaldson, Utah Division of Wildlife Resources

* Panel Member
** Presenter

Overview of Zebra/Quagga Mussels

In terms of economic and ecological impacts, zebra and quagga mussels are among the worst aquatic nuisance species to have invaded North American waters. In general, these prolific, filter-feeding mussels threaten the foundations of natural food webs, discourage recreation, and impede the necessary flow of raw water in artificial conveyance systems and industrial piping. For example, zebra and quagga (dreissinid) mussels (genus *Dreissena*) threaten to out-compete native aquatic organisms, transform established trophic structure, and often blanket all available substrata in areas most conducive to their survival and reproduction. Large groups of dreissinid mussels weigh down buoys and floating docks. They damage boats by occluding through-hull fittings leading to damaged engines and pumps. They foul hulls and jam steering mechanisms. On a larger scale, zebra and quagga mussels jeopardize the sustained delivery of drinking water to the West by clogging intakes, straws, and water conveyance systems. Also compromised are industrial cooling systems, which frequently require mitigation for continued function after a zebra- or quagga-mussel infestation.

Although efforts to prevent the westward spread of zebra and quagga mussels have been ongoing, following their introduction to eastern North America in the late 1980s, quagga mussels were discovered in Lake Mead (Nevada/Arizona) in January of 2007. This was the first confirmed population of dreissinid mussels west of the continental divide. Spread to Lake Mead was likely the result of a houseboat moved from one of the Great Lakes to Lake Mead. However, it has not been confirmed that the Lake Mead population was a result of a single introduction. It is possible that multiple introductions have occurred. Several infested boats have been interdicted at Lake Mead in the past several years. Moreover, although the first reported sighting of quagga mussels in the West was at Lake Mead, it is possible that other reservoirs or lakes, such as Lake Havasu or Lake Mohave, could have been infested first. The population at Lake Mead, once discovered, was estimated to be at least three years old, based on age-class

analysis performed by Dr. Robert F. McMahon at the University of Texas at Arlington. After invasive mussels were first discovered in Lake Mead, they were also found in Lake Mohave and Havasu, both downstream of Lake Mead.

Lakes Mead, Mohave, and Havasu are part of the lower Colorado River system. This system is unique in that it supplies water, through artificial conveyance systems, to vast areas of southern Nevada, Arizona, and southern California. Millions of Americans depend on this system for irrigation and drinking water. Highlighted municipalities that rely on Colorado River water include Las Vegas, Phoenix, Tucson, Los Angeles, and San Diego. Metropolitan Water District of Southern California diverts substantial water from the lower Colorado River to southern California. Likewise, the Central Arizona Project diverts Colorado River water to Arizona. Thus, populations of invasive mussels established in the Lower Colorado River have supplied planktonic larvae to other waters connected through canals, pipes, and other artificial conveyances. Within a year, quagga mussels have been reported in multiple waters in southern California and in Arizona. Current technologies are inadequate to prevent unwanted downstream movement of zebra or quagga mussel larvae. Thus, continued invasion of connected waters was inevitable and expected.

Waters disconnected from the lower Colorado River, however, are also at risk. Under ideal conditions of high humidity and cool temperatures, adult zebra and quagga mussels can survive out of water for days—even weeks. This ability, along with their tendency to attach to hard substrates, provides a dangerous combination that allows these mussels to effectively "hitchhike" on trailered boats to new waters. Many of the newly infested waters and others not yet infested near the lower Colorado River are frequented by multitudes of recreational boaters. These boaters may endanger additional Western waters if precautions are not taken.

Importance of Early Detection and Rapid Response Program

The impacts of invasive species are costly, not only in terms of dollars (often measured in billions), but also in shortages of water, loss of recreational opportunities, lost business, unemployment, damage to goods and equipment, power interruption, and ecological degradation. Biological invasions are second only to habitat loss in driving the reduction of biological diversity and native species. The National Invasive Species Council has recognized that even the best prevention efforts cannot stop all unwanted species introductions. However, if incipient invasions are detected early, rapidly coordinated responses may help to eradicate or contain nuisance species before spread becomes unmanageable and control becomes technically and/or fiscally impossible or impractical. The primary objective of an early detection and rapid response program is to minimize the impacts of invasive species as quickly as possible. This cannot be accomplished unless an early detection and rapid response plan and required legislative authorities are already in place before an invasion occurs.

The State of Colorado, like many other western states, had initially demonstrated a reluctance to devote time and resources into early detection and preventative measures that could help decrease the likelihood of unwanted invasions. Typically, attitudes change once the presence of a notorious invader is confirmed within the state. Colorado has

followed this general pattern, fully recognizing the seriousness of the problem and initiating necessary actions only after the zebra/quagga-mussel invasion was underway within Colorado's jurisdiction. Understandably, authorities prefer to limit allocation of valuable resources unless it is absolutely clear that such resources are necessary. This strategy has sometimes proven successful for control and eradication of invasive weeds and other organisms. However, it is a mistake to assume that all invaders can be equally controlled. Many are much more insidious than others. Zebra and quagga mussels are a case in point. Once established, these species are practically impossible to eradicate with current technologies, except under extremely unusual circumstances. To date, only one successful eradication attempt has been confirmed in the United States. Meanwhile, the economic and ecological impacts of zebra and quagga mussels are severe and usually irreparable. Moreover, zebra and quagga mussels will not be the last devastating invaders.

The health of our aquatic resources is not unlike the health of our human bodies. As good stewards of our own health, we routinely take preventative, hygienic actions to avoid disease and monitor ourselves for signs of illness. It is widely recognized that such a strategy is prudent and essential to our continued survival and well being. Most of us realize that some diseases are troublesome, yet are easily remedied. We also understand that other diseases may be fatal if unnoticed or left untreated. Battles are often better fought if such potentially terminal diseases are detected early. In this context, invasive species are no different than diseases. Some can permanently alter or destroy the habitats that they invade. Thus, a strategy of prevention and early detection is as prudent and essential to the health of our public resources as it is to our own individual health.

The actions implemented by the State of Colorado since the initial discovery of dreissenid mussels in Lake Pueblo are exactly the type of actions that should have been initiated years ago. Now that the problem is well recognized, Colorado has joined the ranks of several other Western states that are employing the most appropriate tools and technologies to detect and monitor the status of their waters. Currently, cross-polarized light microscopy combined with DNA detection in plankton samples using PCR-based analyses are at the cutting edge of early dreissenid detection. The adoption of these tools in Colorado and other western states helps ensure the protection of non-invaded areas in the entire Western region. Moreover, Colorado's Division of Wildlife has become an active participant and leading proponent of preventative efforts including the 100th Meridian Initiative and other programs developed through the federal Aquatic Nuisance Species Task Force and its Western Regional Advisory Panel. By continuing to support and participate in these regional and national-level programs, the State of Colorado can help ensure that it remains at the cutting edge of current technologies and can benefit from knowledge and experience accumulated by other partners.

Management Responsibilities

Natural resource managers are generally charged with protecting and conserving such resources for the benefit of the people within their jurisdictions. Unfortunately, invasive species do not respect jurisdictional boundaries. Thus, the negligence, inaction, or indifference of natural resource managers in one jurisdiction could negatively impact many others. Colorado, for example, holds the headwaters that flow into 17 states. It is imperative that invasive species issues are seriously considered in such a keystone state.

Fortunately, none of us are alone. Cooperative partnerships like the 100th Meridian Initiative and the ANS Task Force with its Western Regional Advisory Panel have been developed to help coordinate invasive species prevention and control efforts at all jurisdictional levels. Managers must recognize that invasive species problems usually cannot be solved alone. They key is working together to protect all of our natural resources.

3. **Survey/Monitoring Program**

Colorado Division of Wildlife utilizes a prioritization strategy to rank water bodies within the state in terms of vulnerability to zebra/quagga mussel invasion. The prioritization strategy currently implemented involves evaluation of several parameters:

Number of marinas

Intuitively, a greater the number of marinas implies a greater the level of use for any particular water body. Additionally, more marinas means more slipped and moored boats stored on water. Such boats are especially problematic since these boats remain unmoved, in water for days, months, or even years. This provides ample time for zebra/quagga-mussel settlement in vast numbers and infiltration into areas that are difficult to decontaminate. In many instances, slipped or moored boats in one lake were previously slipped or moored in another. If the previous host lake was infested and the boat was not completely decontaminated before relocating to the new water body, then the new water body could be jeopardized. Adult mussels are more likely to survive overland transport than are recently settled juveniles. Additionally, dreissinid mussels tend to cluster together and, thus, would be in close proximity during spawning, increasing the likelihood of gametes finding corresponding partners for successful reproduction. Therefore, adults settled on slipped and moored boats reasonably could have higher success rates in terms of survival through translocation and subsequent reproduction. In contrast, planktonic larvae are in a relatively fragile life stage, less tolerant of temperature fluctuations and desiccation. Even if larvae do survive overland transport, they must settle, grow through reproductive maturity, and release gametes into open waters. Then, these gametes must find corresponding partners in order to produce viable zygotes before a new population can become established. Thus, veligers in bilge or other standing water may pose less of a threat than adults translocated through attachment to slipped or moored boats.

Level of out-of-state watercraft use

Boaters who travel from out of state often drive long distances to reach their destination. For this reason, these boaters are problematic. Such boaters could spread invasive mussels or other aquatic nuisance species to distant locations.

Type of boating

Although we are aware of no scientific studies that document differences between certain types of watercraft and risk of invasive mussel spread, it seems obvious that some types of watercraft are more prone to spread invasive mussels

than others. Large boats, such as house boats, and sailboats necessarily remain on water for extended durations. House boats, for example, are not easily trailered and require special (and expensive) equipment for removal. Sail boats are also more difficult to trailer than motorized pleasurecraft and angling boats. Sailboats also have complicated rigging that discourages repeated launching and removal. Extended contact with infested waters increases the likelihood of invasive mussel settlement on any suitable hard surface exposed to the infest water.

Slipped and moored boats, boats with vegetation and mud attached, and boats that anchor are all high risk. Anchor chains and lines in these boats should be inspected. However, if a boat is on the water for twenty-four hours or less, especially if it is moving during most of this time, there is very low risk of settlers. Boats such as pleasurecraft, angling boats, personal watercraft, and wakeboard boats are often launched, used almost continuously and retrieved the same day. This allows for less time for invasive mussels to settle on these types of watercraft.

It should be noted that any type of watercraft could carry invasive mussels. Mussels are often found on vegetation snagged by boats and trailers.

Presence or absence of boating and/or angling tournaments

Such tournaments attract boaters from afar, often in large numbers. Trailerable sailboats, in particular, can be risky because many of these boats have a retractable centerboard or keel that cannot be moved once the boat is on the trailer. This prevents successful decontamination in these areas.

Angling tournament organizers often have rules requiring clean, decontaminated boats. However, many participants arrive at tournament destinations to "prefish" (fish before the official tournament). There may not be any regulations for these activities.

Connectivity to other waters

This parameter is of primary importance. Any infested water body may supply viable larvae to any suitable habitat downstream. Even if an infested water body is marginal for the survival and reproduction of invasive mussels, downstream habitat may be more suitable.

Waters high in the watershed should be highly scrutinized and protected from invasion.

Substantial vulnerable infrastructure

Economic impacts will be worse on waters with considerable raw-water infrastructure such as power stations, hydro-electric dams, and water conveyance systems.

Suitability of habitat in terms of calcium concentrations

Calcium concentrations are known to affect invasive mussel reproduction, growth and survival. Calcium concentrations below 15 mg/L may limit colonization success; however there is no well-defined threshold. Necessary

calcium concentrations depend on many other environmental factors. Experts suggest that waters with calcium concentrations less than 12 mg per liter are at very low risk of dreissenid invasion, waters with 12–20 mg per liter have a low risk, waters with 20–28 mg per liter have a moderate risk, and waters with greater than 28 mg per liter have a high potential risk[1].

Other factors that should be considered for risk assessment. A list of criteria for assessing potential level of infestation is included in the appendix (Table 1). Specific factors that should be assessed include the following:

- Suitability of habitat in terms of dissolved oxygen concentration
  - Dreissenid mussels are relatively intolerant of low dissolved oxygen concentrations (< 25% of full air saturation levels, which at 20 °C is approximately 2.3 mg/L at sea level or 1.8 mg/L at an elevation of 7000 feet). Systems with less than 3 mg/L at 20 °C would have little chance of mussels surviving. Dissolved oxygen concentrations exceeding 50% saturation seems to be required for sustained, healthy populations. Habitats with dissolved oxygen concentrations near or below these levels should be less likely to sustain dreissenid mussel populations than habitats with persistently greater dissolved oxygen concentrations.

- Suitability of habitat in terms of ambient temperature
  - Zebra and quagga mussels are limited by extremely warm temperatures. The upper thermal limit for zebra mussels is near 30 °C (86 °F), while the upper thermal limit for quagga mussels is near 28 °C (82 °F). However, these limits may change over time as populations are exposed to selective pressures that annually remove thermally sensitive individuals.
  - Zebra and quagga mussels are also limited by freezing temperatures. Zero degrees C is the lower limit for both species. Invasive mussels are unlikely to persist in waters that annually freeze, unless waters at greater depths remain liquid and well oxygenated throughout the winter.

- Suitability of habitat in terms of pH and conductivity
  - Zebra mussels are more sensitive to acidic conditions than many other bivalves. Values of pH less than 6.5 or greater than 9.5 inhibit metabolic functions. Levels between 7.5 and 9.0 appear to be sufficient for survival, growth, and reproduction.

- Suitability of habitat in terms phosphorous and chlorophyll
  - High phosphorous and/or chlorophyll concentrations may indicate a plentiful food supply for filter-feeding dreissenid mussels. Thus, mesotrophic or eutrophic waters may be more at risk than oligotrophic waters. However, phosphorus levels over 35 mg/L has been shown to be

[1] Thomas R. Whittier, Paul L. Ringold, Alan T. Herlihy, Suzanne M. Pierson (2008) A calcium-based invasion risk assessment for zebra and quagga mussels (*Dreissena* spp). Frontiers in Ecology and the Environment: Vol. 6, No. 4, pp. 180-184.

associated with dreissenid mortalities possibly due to algal blooms clogging gills.

- Food quality (measured as the ratio of organic to inorganic suspended materials) may be a better indicator of zebra-mussel habitat suitability than food quantity. Suspended inorganic sediments in high concentrations may negatively impact zebra-mussel growth.

- The presence or absence of Asian clams, *Corbicula fluminea*
  - Asian clams have similar physiological requirements, thus, the presence of *C. fluminea* would indicate that the habitat is possibly suitable for invasive dreissenids.
  - If a water body does not have *C. fluminea* then the reason should be determined. It is important to distinguish whether the absence of Asian clams is because this species has not been introduced or because the habitat is not suitable.

Substrate Sampling

Substrate sampling has perhaps been the most common method of early detection employed until recently. Simple substrates are suspended on a rope or other type of line and are periodically examined for the presence of settled mussels. These substrates are typically made of PVC or ABS plastic, concrete, brick, or some other hard material. Sampling strategies have varied considerably among stakeholders. No national standard has yet been developed for deployment depth, number of substrates, type of substrate material, or interval between examinations. With substrate sampling, the presence of zebra or quagga mussels is indicated by one or more settled individuals on the substrate sampler (sometimes called a "trap").

The problem with this approach is that it has been difficult to demonstrate that this is an effective early detection methodology. In fact, in many cases where zebra or quagga mussels were first detected on artificial substrates, later inspections have discovered adult mussels large enough to have been there for multiple years. A case in point is the discovery of quagga mussels at Lake Mead. Artificial substrate samplers were deployed at Lake Mead and checked regularly (perhaps monthly or more often) by National Park Service volunteers prior to the discovery of quagga mussels in January of 2007. The first mussels found at Lake Mead were on a breakwater. After a closer examination of other areas within the reservoir, many large adult mussels, possibly up to three years old (according to Dr. Robert F. McMahon, The University of Texas at Arlington) were found. Thus, quagga mussels had been in Lake Mead for possibly three years before they were detected on a substrate that was already in place. The Lake Mead population of mussels had avoided the sampled substrate intended for detection for three years.

It is possible that the implementation of substrate samplers may be to blame for the difficulty of early detection. At Lake Mead, for example, substrate samplers were deployed within two meters of the surface, targeted at depths typical for zebra mussel populations. This seemed warranted because zebra mussels had proven much more successful at overland dispersal than quagga mussels in North America. The only

confirmed population of quagga mussels outside of the Great Lakes region was near St. Louis, Missouri. Nevertheless, it was quagga mussels that invaded Lake Mead. Since quagga mussels often inhabit depths greater than zebra mussels, it is possible that if the substrate samplers were deployed deeper, they may have been more successful at early detection.

Substrate sampling protocols used by CDOW utilize artificial substrates made of white PVC, based on Portland State University's original design, and a similar design modified by Bureau of Reclamation that uses a black corrugated plastic material. Although there is no national standard for substrate samplers, the Portland State University samplers are the most commonly used. CDOW Substrates have been deployed in spring or early summer and are hung from any structure with sufficient access. All samplers are mapped with GPS coordinates. At each deployment location, samplers are attached on a rope at ten foot intervals to the bottom of the water body. Areas for deployment are chosen to minimize human interference. Contact information is attached to the samplers in case a person removes one and has questions. Samplers at high priority lakes are monitored every two weeks using visual inspection with a magnifying glass. Technicians also feel the substrate for a sandpaper texture that might indicate recently settled juveniles. If anything is found, the technician reports to Vicki Milano (CDOW). Occasionally samplers have been cleaned to remove periphyton and debris that builds up between periodic checks.

The current substrate sampling program should be effective in detecting recently settled mussels. The current deployment times and monitoring intervals are appropriate. Deploying multiple samplers per rope line is recommended. A ten-foot interval between samplers is sufficient. However, deployment to depths below 100 feet is not necessary. Since zebra and quagga mussels tend to prefer settling on materials that have a biofilm, it is recommended that samplers not be cleaned between checks. Large amounts of debris, if present, may be removed, but removing the biofilm may actually render the samplers less useful.

It should be noted that visible mussels are not typically found until a population has become well established. Thus, substrate samplers are not the best tool for early detection. Nevertheless, these samplers—unlike plankton tows for veligers—offer the possibility of detecting mussels in a water body during times when spawning is not occurring. Historically, substrate samplers have not been very effective in detecting dreissenid mussels, but the reasons for this are still largely unknown. Many biologists have suggested that dark materials, such as those recommended by BOR may be more effective than lighter materials (e.g. white PVC) because mussels tend to avoid light. There is currently no published evidence to support this conclusion, but the idea seems reasonable.

Plankton Tow Sampling Program

Plankton tows followed by microscopic examination and/or PCR analyses are now becoming more utilized as early detection methods because these offer a considerable advantage over substrate sampling. These methods offer the possibility of detecting larvae before high densities of adult mussels establish. Even a few adult mussels can produce thousands or millions of veliger larvae. Thus, sampling plankton tows may provide the earliest detection possible at this time.

CDOW samples waters with a schedule reflective of perceived risk (determined with the prioritization strategy outlined above). Waters with high risk are sampled every two weeks when temperatures exceed 60 °F (15 °C). Vertical plankton tows are performed with a 63 µm mesh plankton net with a 500 mm diameter opening. Nets are lowered to within a foot from the bottom and slowly hauled vertically to the surface. Plankton sampling occurs at the same locations where substrate samplers are deployed. Distilled water is used to wash the plankton nets from outside toward the inside. Captured material collected in a cup at the base of the net is then transferred to a nalgene bottle where ethanol is added to a solution of 25% ethanol by volume. The bottles are marked before and after the addition of ethanol to indicate roughly the volume of each. Preserved plankton samples are then used for both light microscopic examination and for PCR analysis.

Although the basic procedure for obtaining plankton samples should be suitable for detecting veliger larvae, we recommend specific changes to the current protocols in order to improve effectiveness.

In many areas, zebra and quagga mussels have peak spawning periods in the spring and fall. Temperature, nutrient availability, and other factors may influence these peaks. If resources are limited, CDOW may want to focus sampling efforts during peak spawning times rather than spreading sampling efforts evenly throughout the spring, summer, and fall. This may increase the chances of detecting larvae while still at low densities. Nevertheless, more sampling is better than less sampling because early detection requires monitoring for a rare event. Moreover, it is more difficult to detect veliger larvae while densities are low (during the earliest stages of infestation) than it is to detect them when densities are higher (after the infestation is well established). Thus, increasing the frequency of monitoring will enhance the chances of detecting veligers early.

Another recommendation is that repeated plankton tows should be combined, mixed and then divided into separate containers if they are intended to go to different labs for examination. This could reduce the likelihood if conflicting results between labs. When monitoring for early detection (presence or absence) it is not important which plankton tow was successful in capturing veligers—only that veligers were detected.

A final recommendation for plankton tow samples is to take enough materials so that some may be retained for future analysis if necessary. In the case of conflicting results between labs, retained material may be used to resolve discrepancies.

Shoreline Sampling

Sometimes the initial discovery of zebra or quagga mussels comes from recreational water users who find these mussels along the shoreline or under rocks within a couple of meters from shore. Purposeful sampling along the shoreline may be helpful, but, in general, zebra and quagga mussels will not tend to accumulate along the shore until populations reach high densities. Further, reservoirs with fluctuating water levels may not have shoreline populations. There are better methods for detecting mussels early, including examining plankton samples under a microscope and PCR analysis. Both of these methods, however, are only useful after mussels have been introduced into the water body. Examination of boats and equipment coming to the water body may help prevent the problem before it ever occurs. The earliest type of detection would be

detecting mussels on a boat (or other equipment) planned to enter the water. A mandatory watercraft inspection and decontamination program may prevent irreversible impacts. Thus, inspecting incoming boats is probably more beneficial than inspecting shorelines. Routine shoreline monitoring could be left to local citizens and interest groups.

Tracking

CDOW is currently developing a database for keeping track of monitoring efforts within the state of Colorado. Additionally, there exists a need for regional-level cooperation with tracking zebra- and quagga-mussel monitoring information for other states and larger agencies who manage areas which cover more than one state. The U.S. Fish & Wildlife Service and the 100th Meridian Initiative should cooperate with Colorado on developing a monitoring database that will be useful to other state and federal agencies for the purpose of tracking monitoring efforts. A working group tasked specifically with this purpose will be proposed at the next national 100th Meridian Initiative meeting, scheduled for late January in Denver, Colorado.

4. **Microscopy**

Non-preserved Water Samples

Veliger larvae are approximately 100 to 450 microns in size. Thus, a microscope is required to visually identify them. Currently CDOW is not examining live plankton samples. If living plankton samples are available, veligers are not difficult to distinguish from other planktonic organisms. Preserved veliger larvae are often difficult to distinguish from other organisms under the microscope (even when using cross-polarized light). Thus, false positive are possible when other animals (e.g. ostracods) are mistaken for mussel larvae. A simple solution to this problem can be made by examining live plankton samples in the field before preservation. Veliger larvae have a unique organ called a velum that is practically unmistakable. The velum's beating cilia causes the veliger to move around in the water in a characteristic manner, making them easily recognized within the water. Therefore, it is less difficult to identify veligers when they are alive than it is after they have been preserved. Field technicians can be trained to examine samples on site and label any material that appears suspicious for close examination in the lab. It is recommended that any suspect samples be taken to a qualified lab immediately, without being preserved, for official determination. Veliger samples, if kept cool (< 50 °F) should survive for two or three days, offering sufficient time for laboratory evaluation. At low densities, a few veligers may be difficult to find under the microscope, but at higher densities veligers may be easily detected in unpreserved water samples. If samples have not been preserved, the technician may assess viability of the organism. This may be important, as living veligers may be a greater threat than non-viable veligers. Once samples are preserved, this information is lost. Pictures or video of suspected larvae is one of the best ways to confirm the presence of veligers because these can be shared with other technicians or experts for authentication.

In order to analyze live plankton samples it is important to keep the samples cool prior to examination. This may be done be refrigerating the samples or holding them in a cooler. Do not let samples directly touch ice or frozen gel packs. Under cool conditions,

mussel larvae should survive a couple of days if analysis cannot be performed immediately. Specifically, we recommend holding live plankton samples at a temperature of 40 °F (common for the average refrigerator). This should be sufficient to sustain living veligers in small samples (< 500 ml) for two or three days.

Cross-polarized Light

Mussel larvae can be distinguished from other planktonic objects in water samples with a microscopic technique that utilizes linear cross-polarized light. Only certain objects, including bivalve larvae, exhibit a birefringent characteristic that allows detection under cross-polarized light. When light is passed through two linear polarized filters that are aligned at 90° to each other, most light is obstructed and will not enter the microscope's objective lens. However, the crystalline structure of calcite in larval shells refracts light in two different directions. The refracted light passes through to the objective lens of the microscope and appears as a cross where birefringent material exists. This technique improves veliger larvae detection considerably by masking the background and all objects that do not exhibit this birefringent character. However, Asian clam (*Corbicula fluminea*) larvae and ostracods (planktonic crustaceans) also appear under cross-polarized light. Ostracods, for example, are fairly common in planktonic water samples. Thus, a trained and experienced technician is required to distinguish dreissenid larvae from other birefringent material. Improperly trained or inexperienced technicians may erroneously report the presence of zebra or quagga mussels in a water sample. Thus, ocular identification should not be used as a sole criterion for concluding that a water body is infested with zebra or quagga mussels. A second detection technique, such as PCR analysis should be implemented to confirm microscopic detection. Even if two independent labs using light microscopy report a positive hit for zebra or quagga mussels, it is still recommended that these reports be confirmed with PCR analysis. When possible photographs should be taken and circulated for further evaluation by other experts.

5. **Molecular Methods**

The panel heard multiple presentations which reviewed current practices using molecular methods to reliably detect and identify zebra and quagga mussels. Current practice is for plankton tow samples flagged as positive for zebra or quagga mussels after microscopy to be sent out for confirmation by molecular methods that incorporate some kind of PCR component. As CDOW administrative protocol is currently structured, EDRR [early detection, rapid response] is then triggered only when samples show positive by microscopy, and when that presence of quagga or zebra mussels is confirmed by two independent laboratories using PCR. Molecular methods are of maximum utility when applied to veliger larvae and, and discussion here thus reflects issues that arise from using PCR to identify invasive mussel species from plankton tow samples. Recent experience of CDOW and its collaborators in Bureau of Reclamation, Metropolitan Water District of Southern California and Pisces Molecular was reviewed, and some issues became apparent which need to be dealt with to ensure reliable and consistent molecular detection. These sort out into two general categories—1) sample collection, preservation, and processing; 2) PCR methodology.

Sample collection, preservation, and processing

Plankton tow samples typically result in a heterogeneous mixture, a complex stew of plankton, vegetation, and floating debris—essentially anything that is in the water column gets captured and concentrated. This material is then either used directly for microscopy and PCR assays without further treatment, or is preserved in the field with addition of ethanol to a final of concentration of 25 %. Upon arrival in the laboratory, samples are passed through a cone filtration apparatus, and aliquots examined by microscopy for presence of veliger larvae. If the larvae of invasive mussel species are observed, samples are then passed on to other laboratories for DNA analysis. Apparently contradictory results have been reported, instances in which PCR a) does not confirm the presence of quagga or zebra mussel larvae, despite these having been observed by microscopy, or b) different laboratories doing the PCR-based confirmation assays generate conflicting results. It seems likely that (most of) these discrepancies have to do with the way samples are collected and processed, rather than false or inadequate performance of one or another detection method. Specific recommendations are detailed below.

Sampling

In at least some instances, samples have been collected by duplicate plankton tows taken sequentially from the same location. In this case, the first towed sample may be delivered to the U.S. Bureau of Reclamation (BOR) for microscopic analysis and then later to the Metropolitan Water District of Southern California (MWD) for PCR analysis. Meanwhile, the second towed sample may be delivered to CDOW for microscopic analysis and then Pisces Molecular for PCR. Especially when veliger density is low, almost certainly the case in instances of early detection, it is entirely likely that one sample could have veliger larvae and the other not, leading to the problematic results described. For true validation, molecular assays need to be performed on exactly the same source material that is examined by microscopy. Additionally, and perhaps most importantly, it should be recognized that early detection requires discovery of a rare event. Capturing larvae in plankton tows may be improbable when sampling incipient populations. Consequently, even duplicate samples taken sequentially at the same location may not both capture veligers when these larvae are present at very low densities. Likewise, splitting a single sample into two sub-samples may produce one sub-sample that contains a single veliger and another sub-sample that contains no evidence of dreissinid presence. Contradictions such as those already experienced between laboratories are simply inherent when monitoring for rare events.

DNA extraction from heterogeneous samples

A further source of differing results between labs is the manner in which samples are processed to extract DNA. In most instances, only some fraction of the total sample volume will be used for DNA extraction, typically 0.5-1 mL. If this fraction contains the target organism, then its DNA will be present in the purified DNA extract, and will be detected with PCR. In cases of low veliger density however, it is again likely that extracting DNA from only a (small) portion of the available sample could result in a false negative finding, simply because the target organism is not present in that portion of sample from which DNA was purified. This could also account for discrepancies between laboratories doing PCR—if a plankton tow sample is split, with portions being sent to

different laboratories, there is no guarantee that each fraction will contain the same suite of organisms, and hence may not respond similarly in PCR trials. One way to avoid this would be to screen samples using cross-polarized microscopic detection methods for veligers and ensuring that suspected veligers are present in all sub-samples. Another approach would be to retain the original sample for later use if the analysis of one sub-sample was found to be incongruent with analysis of another. A third sub-sample could then be analyzed from the original sample to help address inconsistencies. However, in cases of extremely low veliger densities, it is possible that only one sub-sample could contain a single veliger detectable by PCR analysis while multiple other sub-samples contain no veligers, and thus reflect absence of dreissenids in the examined waters. For this reason, it is important to recognize that a positive identification from a single laboratory may not be in error even if other laboratories fail to confirm their results. Again, this is a problem inherent in early detection of rare events. Nevertheless, a single detection should warrant increased scrutiny of any water body until corroborating evidence can be obtained.

DNA inhibitors

Finally, sampling protocols that concentrate naturally occurring microorganisms—plankton tows--are famous for simultaneously concentrating naturally occurring PCR inhibitors. These are molecules present in the sample that generally interact with DNA directly or with enzymes needed for PCR amplification, preventing successful amplification of the target DNA. If sufficiently inhibited, dreissinid DNA in a water sample may not amplify as intended, and, thus, may lead to a false negative conclusion. Participants expressed well-founded concerns about the potential for false negatives in PCR-based assays due to the presence of these inhibitors. Anecdotally, PCR inhibition does not seem to be a problem for the PCR assays currently in use, but methodology does need to accommodate the possibility.

Several participants expressed concern about the possibility of PCR inhibition, in which naturally occurring PCR inhibitors could generate false negative results by poisoning the PCR reaction. Paul Rochelle (MWDSC) presented results of control experiments that looked at this possibility, and demonstrated that for the samples he was working with, using the DNA extracted according to his methods, there was no apparent PCR inhibition. These results notwithstanding, this is an issue of continuing concern, and warrants attention. It may well be that the samples processed by the MWDSC laboratory were atypical in not having a high concentration of inhibitors, a situation that may not obtain with other samples. Alternatively, it could indicate that his DNA extraction protocol is effective in removing PCR inhibitors.

PCR methodology

John Woods (Pisces Molecular) and Paul Rochelle (Metropolitan Water District of Southern California, MWDSC) reviewed existing PCR methods for detecting invasive mussels, and described primer sets that they have designed for this purpose, as well as extraction protocols for obtaining suitably clean DNA. All of the PCR assays described are valid and work when properly executed. However, those assays that have been developed more recently exploit the larger store of DNA sequences currently available in GenBank for zebra and quagga mussels, and consequently these more recent assays show

greater specificity for the species of interest. For maximal sensitivity, PCR primers should amplify a fragment of some high-copy number target sequence—by virtue of their higher frequency in the extracted DNA, PCR primers that target high copy number genes have an enhanced likelihood of encountering the target priming site(s), and are consequently more likely to initiate DNA synthesis. All of the primer combinations currently being used by CDOW and affiliated laboratories adhere to this principle, targeting either rDNA sequences, Intervening Transcribed Spacer (ITS) regions, or mitochondrial DNA sequences. [For the uninitiated, 18S, 28S rDNA, and ITS sequences are in nuclear DNA, and arrayed in tandemly repeated copies along the chromosome, with hundreds to thousands of copies being present in each diploid nucleus. Mitochondrial DNA derives from the mitochondrial genome present in mitochondria, and gets its high copy number because of the multiplicity of mitochondria in each cell, especially high in muscle tissue—typically several thousands of copies per cell.] Of the primer sets presented, those developed by Pisces Molecular (PM316/PM317, PM318/PM319) and the MWDSC (DBCytC189f/552r, zebCytC123f/zebCytC508r) laboratories are able to selectively amplify Quagga and Zebra mussels, and do not amplify other mussel taxa. The assay developed by Rodriguez at the USGS also can discriminate between Quagga and Zebra mussels, but requires double PCR reactions, and is reported to have higher frequency of ghost bands. It was noted that whenever the same DNA sample was tested by several of the methods currently in use, results were concordant, irrespective of the PCR method employed. It would appear that discrepancies arise when different laboratories are working from different DNA samples, again implicating how samples are processed, and how sampling is carried out.

Methods of DNA extraction were likewise subject to some discussion—different laboratories are using different methods of DNA extraction, all with apparent success, but doubtless with some variation in DNA concentration and purity. MWDSC uses an extraction procedure designed for soil samples (MOBIO UltraClean Soil DNA Isolation Kit), and as mentioned above, DNA extracted using this procedure does not appear to contain problematic concentrations of inhibitors. Other laboratories are using another commercially available DNA extraction kit from Qiagen. These also appear to extract DNA suitably free of inhibitors, although there were no results presented that directly demonstrated this, beyond the simple fact that many samples did amplify.

Recommendations for PCR-based identification of quagga and zebra mussels

1. Sampling procedures. Samples sent out for PCR testing need to be derived from the same plankton tow that is examined by microscopy for the presence of quagga and zebra mussels. Samples from separate plankton tows, even if these are taken at the same time and place, are not equivalent, and cannot be expected to always test identically by microscopy and PCR.

2. Plankton-tow sample handling. Plankton tow samples need to be processed to reduce bulk, remove extraneous debris, and to enrich for veliger larvae. CDOW should investigate methods of density centrifugation to achieve this end. We suggest trials of sucrose solution separation as described by Renate Claudi, but CDOW should also investigate simple density centrifugation methods involving percoll, and/or sucrose or glycerol step gradients, with the goal of concentrating

veliger larvae, and reducing extraneous organic debris and organisms that could impede DNA extraction and/or inhibit PCR reactions.

3. DNA handling. Once veliger larvae have been concentrated, to the extent that is possible, and microscopy completed, DNA should be extracted from the entire volume of veliger-containing liquid remaining, and this DNA extraction should take place at the laboratory that initially receives the samples from the field. Extracting DNA from the entire sample solves the problem of splitting the sample, with accompanying risk of a heterogeneous division of the sample. In the event that multiple extractions need to be carried out to accommodate the entire volume of material from a plankton tow, extracted DNAs should either a) be pooled and mixed to generate a homogeneous sample, or b) aliquots of extracted DNA from all extractions of material from a given plankton tow need to be tested by PCR. Extracting DNA at the initial receiving laboratory and subsequently providing aliquots to external laboratory or laboratories gets around the problem of different labs testing samples that are not true replicate samples. Further, CDOW should investigate commercially available DNA extraction kits and select one that provides suitably clean DNA, and which can be scaled up to accommodate larger volumes. There are many DNA extraction protocols which would serve, available in commercially available kit form, or which can be carried out in a laboratory without recourse to kits. However, we recommend use of one of the commercially available kits because these provide more consistent yield and quality of purified nucleic acids. Moreover, in the event that multiple laboratories need to extract DNA, using the same extraction procedures in all participating laboratories removes one source of potential variability in result.

4. PCR protocols. CDOW and affiliated laboratories should eschew the PCR protocols and primer sets that are not specific for zebra and quagga mussels. Of the primer sets and protocols presented during the August meeting, those developed by Pisces Molecular and the MWDSC appear to offer the best combination of performance and reliability—both specifically target quagga or zebra mussels, and amplify high-copy number regions. Because the amplicons generated by the Pisces Molecular primers are of different size, these primers could potentially be multiplexed, to test for presence of either or both quagga mussels and zebra mussels in a single reaction, although this would then need to be tested to ensure that there is no loss in sensitivity if multiplexing were to become routine. However, the labor and cost involved in PCR assays is mostly in DNA prep, hence the possibility of multiplexing is not sufficient grounds to preclude use of the MWDSC primer sets. Whichever primers are used, we suggest a standard set of controls, to provide some assurance as to experimental methods and performance of the reagents in these assays. Each assay should incorporate:
    a. Positive control to demonstrate that reagents and enzymes are functioning. Normally this would be a DNA sample of known provenance, demonstrated to contain the target organism's DNA in concentrations comparable to what one might expect in a plankton tow sample that contains some quagga and/or zebra mussel

b. Water only control, to test for contamination of reagents.
c. Some kind of control reaction to test for PCR inhibition. Reactions containing extracted DNA from plankton tows can be spiked with a fixed amount of some DNA of known provenance for which primers already exist. If these samples amplify readily, that is good evidence that the reaction is not seriously hindered by the presence of inhibitors. Alternatively, samples could be assayed for the presence of some ubiquitous DNA that will invariably occur in every sample. Some labs successfully use universal bacterial 16S primers for this purpose because bacteria are ubiquitous and available primers are robust across a variety of conditions. Other labs have been successful using universal 18S rRNA targeted primers for the same purpose. The advantage to this strategy is that no extra steps are required; the disadvantage is that there is no measure of the concentration of the targeted control DNA.

5. All positive PCR amplicons should be sequenced. Current practice is to view a PCR-based assay as positive for zebra or quagga mussel when a band of appropriate size shows up on an agarose gel. However, given the serious consequences entailed when a water body tests positive, it seems only prudent to spend a bit extra to make sure that this response is not triggered prematurely. Testing laboratories will surely make every effort to adhere to stringency requirements in their PCR reactions, but non-specific amplification does happen. Therefore, when a PCR product of appropriate size registers on a gel, that PCR product should be sequenced if it comes from a water source that has not already been confirmed positive for dreissenids. Sequencing may or may not be necessary for PCR products obtained from waters already confirmed positive for zebra or quagga mussels.

6. **Alternate Strategies for Early Detection**
   - Flow CAM: an automated process requiring specialized machinery to examine water samples for veligers. The process uses cross-polarized light microscopy combined with a computer visualization and analysis software that can be tailored to detect specific organisms in the sample. The efficacy of this technology has not been demonstrated, but BOR is working with Fluid Imaging Technologies to evaluate this tool as a possible early detection mechanism.
   - Scanning Electron Microscopy: detection would be similar to light microscopy, but finer detail available from a scanning electron microscope might help distinguish dreissenid veligers from other organisms. This method would also require specialized equipment, a qualified technician, and considerably more preparation compared to standard light microscopic techniques.
   - Acoustics for estimation of established populations – this methodology has promise for monitoring population density and tracking spread of adult mussel colonies—although promising, this technology needs further refinement and assessment before it will have broad application.

- Organized, local group of volunteers: first reports of dreissenid invasions often come from educated citizens who find them during routine activities. The usefulness of public outreach and education should not be underestimated with regard to early detection. Good training and experience is useful in finding these mussels.
- Drop a brick! Program: Concerned citizens living on Lake George, New York participate in a simple monitoring program that involves hanging bricks from their private docks. Presence and absence of zebra mussels is periodically reported to the Lake George Association.
- Diving: Bryan Moore of the National Park Service uses underwater cameras and trained divers to monitor for zebra and quagga mussels within the Lake Mead National Recreation Area. Experience is useful for divers. Unfamiliarity with living dreissenids *in situ* could lead to undetected mussels.

7. **Rapid Response Triggers**

Rapid response should be triggered if any of the following occur:

- an adult mussel is found (live or dead)
- at least one lab reports a finding from a microscopic examination and at least one lab indicates a positive detection using a PCR technique coupled with gene sequencing.

A limited initial response is warranted if a water body has been reported to be positive for zebra or quagga mussels by only one method (or one lab). Such a water body should be considered highly suspect. Immediately, a limited initial response should evaluate other factors including habitat suitability, potential for overland transport, at-risk infrastructure, potential for downstream transport, etc. The risk assessment currently employed by CDOW (and the additions suggested above) should be evaluated closely for any highly suspect water body.

Full scale response, however, should require dual confirmation. In this case, management decisions should be implemented immediately. If both microscopic examination and a PCR analysis indicate the presence of dreissenid mussels, or if two different PCR analyses indicate the presence of dreissenid mussels, CDOW should conclude that the suspect water body is positive for invasive mussels. For example, a single PCR test positive should initiate a limited response. Additional samples and information may be required. Dual confirmation should immediately be sought by sending extracted DNA to a second, independent lab for analysis. Alternatively or simultaneously, water samples should be examined by a qualified technician using light microscopy.

Veliger Density

There is no clear statistical relationship between veliger density in a sample to adult population density. The confirmed presence of veligers, regardless of density, should lead to management actions. Veliger density naturally changes over seasons. Thus, densities may spike in one season and may become undetectable in others. Changes in veliger density may not indicate how well a population is doing at a particular

location. However, multiple detections from a single water body should be considered indicative of an establishing population. On the other hand, a one time detection of veligers could occur even if the population does not successfully establish. It will be difficult to distinguish these scenarios in waters that are marginally suitable for zebra and/or quagga mussels.

8. **Management Implications**

Three-Tiered Approach

CDOW has suggested a three-tiered approach to identifying waters with zebra or quagga mussels. The first tier is microscopic analysis using cross polarized light. So far, this technology has been the most reliable—although this may change as PCR techniques specific for dreissenid mussels are improved. If a veliger is found with light microscopy, samples are sent to a molecular lab for confirmation using PCR analysis. This is the second tier. If PCR analysis confirms the presence of dreissenids, a second confirmation is sought by an independent molecular lab. This is the third tier. The panel's recommendation is that a three-tiered approach is too conservative.

Two-Tiered Approach

If veliger larvae are found using microscopic analysis, we recommend that managers employ a second technology, PCR analysis, for confirmation before concluding that a water body is positive for dreissenid mussels. Microscopic analysis can be very effective in identifying veliger larvae, but a false positive could result from an error due to an inexperienced technician or detection of non-dreissenid veligers or other birefringent organisms. All positive light-microscopy reports should be confirmed with PCR analysis. Confirmation should be sought immediately. Meanwhile, the source water body should be considered suspect and authorities should be notified that management actions may be necessary.

Full scale management actions (e.g. field watercraft inspection and decontamination) should require dual confirmation before implementation. If microscopic examination and a single PCR analysis both indicate the presence of dreissenid mussels, or if two independent PCR analyses indicate the presence of dreissenid mussels, management decisions should be implemented immediately. Additional samples and information may be required.

Lake Pueblo Case Example

Samples collected by a CDOW technician at Lake Pueblo indicated adult dreissenid mussels. Two adult mussels (in poor condition) were found attached to a substrate with byssal threads. Since dreissenid mussels are the only freshwater mussels in North America that adhere to substrata with byssal thread, this alone was strong evidence to suggest that Lake Pueblo has zebra or quagga mussels. Nevertheless, since the samples were in poor condition and covered in fungus, CDOW was reluctant to base a conclusion of this evidence alone. Water samples were also examined for the presence of veligers. BOR biologist, Denise Hosler found a veliger larva using cross-polarized light microscopy in a sample from Lake Pueblo. Confirmation was sought by PCR analysis. BOR, biologist Kevin Kelly confirmed the presence of dreissenid DNA by PCR analysis. Since two independent technologies (microscopy and PCR analysis) both indicated the

presence of dreissenid mussels at Lake Pueblo, it was appropriate for CDOW managers to conclude that this lake was positive for zebra or quagga mussels. Management actions initiated at that time were warranted. Subsequent examination of the extracted DNA revealed (by DNA sequencing) that the discovered DNA was that of *Dreissena polymorpha*, the zebra mussel.

Lake Granby Case Example

Bureau of Reclamation biologists retrieved plankton samples from Lake Granby and identified the presence of veligers with cross-polarized light microscopy. Samples were then tested by PCR analysis. BOR biologists were unable to confirm the presence of dreissenid DNA by PCR amplification. A second molecular lab, Pisces Molecular in Boulder, CO, also attempted to confirm dreissenids in water samples from Lake Granby. However, no dreissenids were detected. Samples were also sent to a lab at the Metropolitan Water District of Southern California (MWD). Results from MWD confirmed the presence of dreissenid DNA in the water sample. Early detection requires looking for veligers before population levels develop to high densities. As explained above, it is possible that multiple samples taken from a water body with an incipient population may not all contain veliger larvae. Thus, negative findings should be expected and may be common. At this point in the Lake Granby Case two different technologies (microscopy and PCR analysis) indicated that Lake Granby was positive for dreissenids. Thus, CDOW managers appropriately concluded that this water body contained zebra or quagga mussels. Another sample was later analyzed as positive for dreissenids by Pisces Molecular.

Summary

A positive detection of dreissenids either by light microscopic analysis or by PCR analysis warrants immediate further investigation. However, concluding that a water body contains zebra or quagga mussels should wait for corroborating evidence. Two independent labs must agree that dreissenids are present and *at least* one of these labs must use PCR analysis. If these criteria are met, it is appropriate to conclude that dreissenids are present. DNA sequencing is recommended and may be used as further evidence or to distinguish zebra from quagga mussels, but is not necessary before management actions are initiated.

9. **Future Research**

Future research ideas were only briefly discussed. However, several key areas were identified as promising:

- Immunostaining: a biochemical technique that utilizes antibodies that attach specifically to proteins unique to dreissenids. Such antibodies could be manufactured with stains that would allow quick detection of dreissind presence in water samples. These techniques have been implemented for years in other areas of biology but have not yet been applied to water samples to detect invasive dreissinids.
- Viability of veligers being transported on a boat or in a live well: Jason Geockler, Kansas State ANS Coordinator, has looked into this with a local university, however results have not yet been published in peer-reviewed

journals. The idea is to determine if veligers, one of the most fragile life stages of dreissenid mussels, are capable of surviving transport in live wells and other standing water areas on a boat. If not, then current management practices may need to be modified.

- Early detection methods in controlled environments: Under controlled conditions, can we assess how well various early detection methods provide the answers to which we seek? How do these various detection methods compare?
- Substrate efficacy: Are there substrates that are better for monitoring the presence of adult mussels? Several types of currently used substrates have proven to be ineffective in early detection. Are there better substrates that will do the job?
- Biocontrol: What potential biocontrol agents may exist for dreissenids? Dan Molloy (New York State Museum) and Marc Frisher have been looking into identifying parasites of dreissenids. Initial indications are that the North American dreissenids are remarkably parasite free.
- Alternative control methods: Control of zebra and quagga mussels in North America has proven to be difficult. Oxidizing chemicals are the control agents of choice by industrial raw-water users. Painstakingly difficult, manual scraping and power washing are also expensive and time consuming. Less expensive and environmentally destructive control measures should be sought.

10. **Conclusions**

The State of Colorado is commended for seeking forthright recommendations to improve their zebra/quagga mussel early detection and monitoring programs. Colorado waters are not only important to Coloradans, they are important to most of the Central Western United States because of flowing water connectivity. As such, management decisions and actions in Colorado could affect waters and individuals over a geographical area much larger than the State itself. Current practices that should be continued include the following.

- Outreach and education programs: The general public has been instrumental in early detection of zebra and quagga mussels in other states.
    - Individuals can only do their part if they know what to do.
    - The public are usefully for cleaning their own boats and for assisting with monitoring programs such as shoreline and artificial substrate sampling programs. Without the public's help, other efforts are rendered much less useful.
- Substrate sampling: Although artificial substrates have not proven to be the most effective tools for detecting zebra or quagga mussels early, recent improvements in design may help improve effectiveness. This is simple technology and it is relatively inexpensive. Recommended improvements follow.
    - Deploy samplers at multiple depths, but no more than 100 feet.
    - Do not clean samplers when checking them unless there is substantial debris.

  - At a minimum leave a biofilm on the samplers to improve effectiveness.
- Plankton Two samples: CDOW has been performing plankton tows using standard procedures. This should be continued. Recommended improvements follow.
  - Increased sampling frequencies during projected peak spawning times should increase the possibility of capturing veligers in plankton samples.
  - Sampling when spawning is unlikely to occur (in winter, for example), is not necessary.
  - Repeated plankton tows at a single location should be combined.
  - Some of the sample should be retained to resolved between-lab discrepancies if they arise.
  - If possible, plankton samples should be screened using cross-polarized microscopy to ensure that all samples intended for molecular analysis using PCR contain suspected veliger larvae.
  - Plankton tow samples should be processed to reduce bulk.
- Light Microscopic Analysis: This is a very useful step in early detection. Although it requires a trained and experienced technician, it utilizes only standard laboratory equipment. Recommended improvements follow:
  - Examine live plankton samples in the field, or
  - Return plankton samples without preservation to the lab. Unprocessed and unpreserved samples should be held below 50 °F for a maximum of three days.
  - Take photographs or video of all suspected veligers.
- DNA Extractions: Recommended improvements follow.
  - DNA should be extracted from the entire plankton sample.
  - All extracted DNA samples should be pooled to generate a homogenous sample.
- PCR Analysis: Recommended improvements follow.
  - Only PCR protocols specific for zebra and quagga mussels should be used.
  - Controls should be included in each PCR amplification, including a positive and a water-only
  - Some test for PCR inhibition should also be included.
- Sequencing: Recommended improvements follow.
  - All positive PCR amplicons should be sequenced if dreissenids have never been detected in the source water body previously.
  - Management actions should not wait for sequencing.
- Management Implications: Recommended improvements follow.
  - Detection of living adult mussels should trigger management actions immediately.
  - Management actions should be triggered if two independent labs report the presence of zebra or quagga mussel larvae in plankton samples from a suspect water body if at least one of the lab's

conclusions are based on DNA evidence (either PCR analysis or DNA sequencing or both).

- Two PCR-based positives are only necessary in the absence of evidence from light microscopic evaluation. With a microscope positive, only one PCR corroborating positive should be necessary to conclude that the suspect water is infested.
- PCR analysis, DNA sequencing, and microscopic examination for veligers are the currently recommended technologies for determining the presence of veligers.

The invited panel members sincerely thank the State of Colorado for requesting our input. Colorado is clearly dedicated to implementing effective, state-of-the-art programs for early detection and monitoring. We hope the State is successful in controlling the spread of all unwanted species within and from its jurisdiction, and we hope that Colorado will continue to work cooperatively with other agencies, both state, regional, and federal, in the future.

Appendix

Table 1. Criteria used in determining levels of infestation in temperate zone
Courtesy of Dr. Gerald Mackie, University of Guelph.

Parameter	None	Little	Moderate	High
Calcium mg/L	<10	<16	16-24	≥24
Alkalinity mg $CaCO_3$/L	<35	35-45	45-89	>90
Total Hardness mg $CaCO_3$/L	<40	40-44	45-90	≥90
pH	<7.2	7.2-7.5	7.5-8.0 or 8.7-9.0	8.0-8.6
Mean Summer Temperature °C	<18 or >28	18-20	20-22 or 25-28	22-24
Dissolved Oxygen mg/L (% saturation)	<6 (25%)	6-7 (25-50%)	7-8 (50-75%)	≥8 (>75%)
Conductivity µS/cm	<30	<30-37	37-84	≥85
Salinity mg/L (ppt)	>10	8-10 (<0.01)	5-10 (0.005-0.01)	<5 (<0.005)
Secchi depth m	<0.1	0.1-0.2 or >2.5	0.2-0.4	0.4-2.5
Chlorophyll *a* µ/L	5 or >25	2.0-2.5 or 20-25	8-20	2.5-8
Total phosphorous µg/L	<5 or >35	5-10 or 30-35	15-30	10-15
Total Nitrogen µg/L	<200	200-250	250-300	300-500