



# Montana Invasive Species Council

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## *Panel Report*

eDNA Science Advisory Panel: A discussion on eDNA technology use in invasive species management

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## Introduction

The Montana Invasive Species Council (MISC) created a Science Committee to identify areas where exploration into specific subjects could aid in addressing invasive species issues. Environmental DNA (eDNA) was identified as the first area for further exploration, specific to invasive dreissenid mussels. The use of eDNA as a tool to detect the presence of invasive species (or rare species) DNA in the environment holds both promise and uncertainty. eDNA technology is evolving rapidly and has been suggested to be a sensitive tool that may surpass traditional methods in the near future for efficiency and confidence. However, some natural resource managers have struggled with how best to utilize information provided from eDNA results in real-time management applications as well as having confidence in the method and results.

The MISC Science Committee determined that to better understand this technology and its potential use in Montana management situations, a scientific panel would be assembled to advise MISC in eDNA use in regard to monitoring for dreissenid mussels in Montana waters.

The members of the MISC Science Committee include: Tom Woolf, Montana Fish Wildlife & Parks; Adam Sepulveda, US Geological Survey; Erik Hanson, Confederated Kootenai Salish Tribes; Gordon Luikart University of Montana - Flathead Lake Biological Station; Andy Welch, Northwest Energy; David Brooks, Trout Unlimited; Lee Greenwood, The Nature Conservancy; Stephanie Hester, Montana Department of Natural Resources & Conservation and MISC Coordinator; and Kate Wilson, Montana Department of Natural resources & Conservation and Upper Columbia Conservation Commission Coordinator.

## Panel Purpose and Outcomes

To evaluate the use of environmental DNA for dreissenid mussel early detection and provide input and guidance to managers regarding its use.

- Review the state of the science for the use of eDNA for dreissenid mussel early detection.
- Identify gaps and challenges associated with current eDNA methods (both field and laboratory) related to dreissenid mussel early detection.
- Identify information / efforts that could help address those gaps / challenges.
- Provide input to management agencies on how to approach the use of eDNA for dreissenid early detection.

## Panelists

- 1) **Caren Goldberg**, Washington State University – Pullman, Assistant Professor
  - Caren is an ecologist and researcher focusing on detection of rare species using eDNA. She is one of the first biologists in the Pacific Northwest to take eDNA as a detection tool from demonstration experiments to practical applications.
- 2) **John Darling**, Environmental Protection Agency, National Exposure Research Laboratory, Senior Research Biologist

- John's research focuses primarily on applying genetic methods to understand the spread of aquatic invasive species in order to better inform risk analysis and the design of effective policy and management strategies.
- 3) **Jim Snider**, California Department of Fish and Wildlife, Research Scientist
    - Jim's work includes conducting studies on the accuracy and reliability of invasive mussel larvae detection, and larvae survivability under varying conditions.
  - 4) **Karen Vargas**, Nevada Department of Wildlife, AIS Coordinator (retired)
    - Karen has recently retired after developing and implementing Nevada's AIS program, which includes watercraft inspection efforts at Lake Mead. She has experience with the challenges of using eDNA as a detection tool and applying results to management decisions.
  - 5) **Jon Amberg**, US Geological Survey, Upper Midwest Environmental Sciences Center, Research Fish Biologist
    - Jon's work has included years of evaluating the use of eDNA for detection of Asian Carp, an invasive fish species. From this work emerged a quality action plan that identifies required protocols for eDNA sampling and processing, which have led to definitive test results regardless of the laboratory processing the sample.
  - 6) **Robert Bajno**, Fisheries and Oceans Canada, Biologist
    - Robert's research includes the development of eDNA protocols for the detection and monitoring of aquatic organisms of management concern. His current work is focused on the detection of zebra mussels in Manitoba, and at-risk and colonizing freshwater fish species in Canada's prairies and arctic regions.

Questions for panelists were compiled from input provided by members of the MISC and the Council's eDNA steering committee. The panelists were posed a series of questions which were answered prior to the face-to-face event. At the event, a discussion was organized around those questions and answers, and included opportunities for participant observers to pose additional questions to the panelists. At the event, discussion on those questions and answers identified the challenges, and recommendations of the use of eDNA for dreissenid mussel monitoring.



Figure 1. eDNA Science Panel, left to right: Karen Vargas, Robert Bajno, John Darling, Jim Snider, Jon Amberg, and Caren Goldberg.

## Key Challenges and Recommendations for Dreissenid Mussels by Panelists

### Challenges

- Lack of standardized protocols
  - Field collection
  - Lab analysis
  - Communication of results
  - Management response
- Balance of risk and uncertainty
  - Understand the costs of false negatives or false positives to assess risk tolerance
  - Perspective on terms false negatives and false positives
- Detection threshold of eDNA for false negatives is not known and varies with sampling/analysis methods
- A limited number of labs are conducting eDNA analysis for early detection of dreissenids and use different protocols
- No coordinated dreissenid eDNA group to help address gaps and encourage communication
- Few published peer reviewed studies for dreissenid eDNA
- Communicating what a “positive” eDNA sample means

## Recommendations

- Develop, refine, and agree upon method/standards with adaptive capacity
  - Decontamination protocols (utilize existing US Fish and Wildlife Service for Asian carp effort)
  - Field collection
  - Lab analysis including Quality Assurance/Quality Control standardization
  - Data reporting requirements and standards
- Develop consistent language (for both within lab and out)
- Develop a communication plan between managers and lab
  - Approach eDNA results as a link in a chain of evidence
  - Clearly define the steps to be taken following a detection. An eDNA detection could result in further sampling or directly lead to a management action, depending on these pre-defined steps
- Coordinate across western partners and cross-border partners via the suggested avenues
  - Coordination among managers: Utilize existing venue of Western Regional Panel on ANS and/or Western Governors Association
  - Coordination between managers and researchers: Establish forum to continue conversation
  - Coordination among researchers: Develop a system to share information
- Identify risk tolerance and map management actions for detection scenarios and trends
- Test assays with round robin process to assist with lab/manager confidence, identify areas for improvement in consistency and relationship building
- Gene sequence any positive result to confirm
- Optimal conditions for eDNA detection is during dreissenid spawning
- Use eDNA to contribute to the weight of evidence to determine presence of dreissenids
  - Develop/utilize a decision tree that incorporates monitoring results from different methods, likelihood of invasion, etc.

## Suggested parameters of a standard method<sup>1</sup>

- Grab samples thought to be better than plankton tow, but further evaluation is needed
- Surface water collection is preferred and less problematic
- Bleach is best for decontamination (50% solution)
- Minimize contamination with on-site processing
- Best to canvas waterbody with small samples
- Standardize assays using markers from different regions of the genome that are suited to answer question of study
- Use controls in the field and take replicate samples
- Use qPCR vs. conventional PCR

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<sup>1</sup> The suggested parameters are not based on empirical evaluation and therefore require rigorous evaluation.

## Session Questions and Discussion Highlights

For complete responses provided by the panelists, see Appendix A. A full transcript of the panel dialog is available upon request.

### Session One: State of the Science

- *What are some examples of using eDNA to successfully manage a biological problem?*
- *What are the biggest operational challenges associated with eDNA detection for fish species?*
- *Are there aspects of Asian carp eDNA research / methods that may be extrapolated to early detection of dreissenids?*
- *How were the political / public implications navigated through the Asian carp eDNA research / monitoring / detection process?*
- *Have eDNA methods been developed and utilized for non-dreissenid bivalve species? If so, has there been any specific challenges related to detecting bivalves?*
- *Why are there few peer reviewed publications related to the use of eDNA for dreissenid mussel early detection? Has there been sufficient peer review to justify the use of these methods to make management decisions related to new dreissenid detections?*
- *Why are there different methods for eDNA dreissenid early detection (lab and field collection methods)? Is there accepted standard methods for eDNA sample collection and analysis? If not, why isn't there an accepted standard method?*
- *Western states have not widely accepted the use of eDNA as an operational early detection tool for dreissenid mussels. What factors might influence the future use of eDNA as a detection tool?*

### Session one: Discussion

General points that were made by the panelists include:

eDNA results that suggest evidence of the presence of DNA which should prompt further exploration and sampling.

Over time, eDNA results can be important to indicate trends.

For multiple taxa and locations, eDNA tool has higher level of sensitivity than traditional methods. However, eDNA tool relative to traditional methods has not been rigorously evaluated for dreissenid mussels.

Refinement and standardization of the tool is needed both in the field and in the laboratory.

Sampling design will need to be tailored to that waterbody (i.e. captures parameters specific to the waterbody).

The method of eDNA analysis is a balance of risk versus uncertainty; further understanding the risk tolerance for false negatives and false positives is needed but is inherent to any tool that is utilized. A method to quantify uncertainties would assist in its further application.

Conforming methods are needed. Discussion on what entity would be best suited to develop conforming methods was explored. Environmental Protection Agency (EPA) could but would likely not develop standardized methods, US Geological Survey (USGS) is engaging in an effort to standardize within the agency. US Fish and Wildlife Service (USFWS) may be well-suited as they

currently house an eDNA laboratory. In the development of Asian carp methods, many federal agencies have participated in creating that standardization. The Western Regional Panel on ANS could potentially add minimum standards of eDNA for dreissens to the Quagga Zebra Mussel Action Plan.

A discussion explored the idea that eDNA methods are currently being held to a higher standard than other traditional methods of detection of aquatic species (e.g. microscopy), and that there are associated yet accepted errors with those traditional methods as well.

Field sampling and lab analysis workflow (including extraction methods and assays) should be developed that are repeatable, reproducible and incorporate environmental influences. However, different methods work better in different systems.

### Session Two: In the Field

- *Are there best practices for sampling location (e.g. shallow vs. deep, near access points, outlets)? Suggested depth to monitor?*
- *What, if any, standard field cleaning / decontamination protocols for field and lab exist to prevent DNA contamination?*
- *Can field / lab sample contamination be identified when there is a “positive” result? If yes, how?*

General points that were made by the panelists include:

Site specific and surface sampling is likely to capture DNA from living organisms; DNA will be found at depth but sediments can cause interference in the processing, and the optimal depth to sample will be site specific. Targeted sampling at locations like structures or access points to find DNA was suggested. Temporal sampling will be important to capture any potential trends.

Discussion on best timing for eDNA sampling and viability suggested that the viability of a sample will be dependent on the quality and quantity of the sample. A 1 to 2-week period is expected for DNA viability in the water. In general, 2000 mL is standard volume of collection. However, collecting a large quantity of samples can help to capture the patchy distribution of DNA.

50% bleach solution is recommended for decontamination of equipment. Look to the USFWS developed protocols for the Asian carp eDNA program (<http://www.asiancarp.us/documents/USACE-eDNA-QAPP.pdf>). To minimize contamination, single-use materials are often used. Communicate to the public that protocols are in place to increase confidence in the method.

Adequate training for all staff (field and laboratory) is required to maintain strict QA/QC standards. Field protocols should use duplicates to estimate the rate of false negatives and field controls to help detect contamination. Laboratory protocols using replicates and controls are required to strengthen confidence in results.

Establishing a forum to continue conversation among research laboratories could help build outward confidence in the use of eDNA. At this time there is limited communication among



those (researchers or private labs) working on dreissenid eDNA in North America. Further a communication plan is suggested at all levels of a process that utilizes eDNA results.

### Session Three: Analysis

- *Provide context for why there may be variation in molecular markers and how these differences may affect results?*
- *What molecular markers are currently being used with dreissenid eDNA analysis? Is there any consistency between labs for marker preference? Why or why not?*
- *Do labs have differing QA/QC methods / protocols for dreissenid mussel eDNA analysis? For qPCR dreissenid mussel eDNA analysis?*

### Session three: Discussion

General points that were made by the panelists at the event include:

Markers that are used in assays need to be validated where they will be used to avoid picking up non-target DNA (which is why there can be variation in markers). Build redundancy into the assays, so use markers from different regions of the genome (2-3 genes per assay). All potential interference should be addressed before analysis.

There is a lack of opportunity to publish information on the utility of assays and markers in a management setting, but sharing this information is critical for standardization. Sharing information among researchers and laboratories on assays and markers is being initiated via Washington State University here: <https://labs.wsu.edu/edna>; on twitter @eDNAresources; <https://eDNAresources> (live fall 2018); and at <https://www.monitoringresources.org/>

A discussion on lab comparisons study (i.e. round robin or blind study) was agreed to be beneficial. There are very few labs conducting the work, so the study would be relatively “small”. The following labs would be likely participants: USGS, Fisheries and Oceans Canada, USDA Forest Service, Bureau of Reclamation and private labs such as Flathead Lake Biological Station could also be included. Funding and agreement to participate would be needed to conduct the study.

There is a lack of standardized protocols currently across government/academic and private labs performing eDNA analysis. The main barrier for using private eDNA labs are possible concerns with transparency due to “intellectual property” or “proprietary information”.

USGS is currently working toward assay standardization within the agency.

Recommended protocols for labs to have a separate lab dedicated to low quality DNA-sample processing.

Probe-based qPCR is able to quantify the amount of DNA in a sample and is more sensitive than conventional PCR.

Determining chain of custody is an important aspect of eDNA use. The chain of custody provides structure for explaining results and predetermined procedures if faced with potential legal implications.

## Session Four: Interpreting Results

- *What are the criteria utilized to identify a sample “positive” for dreissenid DNA?*
- *Are there other species in MT waters that could produce positive results? Have all likely species that potentially could cause a false positive been evaluated (bivalves, other mollusks)?*
- *What, if any, biogeochemical factors can influence the results of eDNA?*
- *Is it possible for other species (blue-green algae, protists, other zooplankton) to cause a false positive?*
- *Using eDNA methods, what factors can produce false positives and how is that addressed? What additional steps are taken with samples to verify detections are not false positives?*
- *What is a “weak positive”, what does that mean and how should managers respond?*
- *Using eDNA methods, what factors can produce false negatives and how is that addressed?*

### Session four: Discussion

General points that were made by the panelists include:

Discussion on developing a tool to categorize and utilize results where detection patterns could be aligned with related management actions. Further consider including adaptive decision making capacity into determining those management decisions. Developing lines of evidence that could be matched to management actions would be helpful to managers.

Recommend the use of standardized language both within the laboratory and to the recipient of results. Utilizing different terminology to explain the results would be helpful where there is confusion and preconceived notions about words such as “positive” which does not provide adequate context to the information being reported.

A good standard protocol will help identify why/where you have contamination issue. The goal of quality assurance is to: 1) avoid contamination to the greatest extent possible; 2) enable identification of contamination when it does occur.

When things are rare in the environment there is an expectation that they will be difficult to detect and spottiness should be expected. Failed establishment of introduced species may explain “inconsistent” results particularly if we consider invasion 101 expectations. Positive detections that disappear are more desirable than multiple positives over time that eventually manifest into an infestation.

Although unlikely, it is possible for non-target organism DNA to produce a “positive” result. In these rare situations, follow-up investigation/analyses can reject these findings.

Gene sequencing is used to confirm a positive eDNA result.

## Session Five: Management Implications

- *With regards to the use of eDNA for early detection of dreissenids, what are the communication barriers between researchers and managers? What are the communication barriers between managers and the public? Do you have recommendations on ways to overcome those barriers?*

- *What key criteria do researchers need to demonstrate to managers to allow for eDNA to justifiably be accepted as an operational method for dreissenid early detection? Have these criteria been met with dreissenid eDNA?*
- *What responsibilities, if any, do researchers have when reporting/explaining results and how those may affect management issues/management implications?*
- *If a management agency receives “positive” eDNA result, is there a suggested management response to these results?*

#### Session five: Discussion

General points that were made by the panelists include:

At this time, if a positive result for dreissenid eDNA is received, the management response is additional samples are collected to verify the result.

In general, western invasive species managers are distrustful of eDNA results due to historic ambiguous results and poor communication.

Discussion on cost-effectiveness and efficiency suggested that eDNA is currently more expensive than microscopy processing, however results can be obtained more rapidly utilizing eDNA. However, multiple studies on other taxa (e.g. trout) have found the opposite; eDNA is more cost-effective than non-molecular methods. Further, there is a trend of decreasing costs for eDNA analysis, as well as cost-efficiency trend with high volume sampling. Once a protocol is established, the cost should significantly drop.

Managers could benefit from more education and information on the science of eDNA technology. This information would help in communicating with labs and communicating with the public. A variety of suggestions were made on how to provide that education, including a message of understanding what it means to sample for something rare in the environment.

Communication between the manager and the lab is critical before a project is initiated so that protocols, reporting results and expectations are clear for both parties.

Suggestions were made for better communication between managers and the public; for example, the challenges of looking for something that is rare in the environment, and that there is uncertainty involved in this work (cost of a false positive compared to a false negative), and eDNA detections could mean live or dead material.

A communication plan should be in place before sampling is initiated. This should outline how managers will proceed in the event of detections and how those results will be communicated.

#### Session Six: Next Steps

- *What further information is needed to increase the comfort level of managers to more broadly accept eDNA as a dreissenid early detection tool?*
- *Is it possible to have a national accreditation for eDNA labs? Is it possible to have a lab certification program for labs that conduct eDNA analysis?*

- *Could researchers develop criteria that could guide managers how to review and evaluate eDNA results? What would be included in a checklist guide that would ensure all factors are assessed and considered to ensure confidence in results?*

#### Session six: Discussion

General points that were made by the panelists include:

Lab accreditation is possible; however what level of accreditation is needed will be important to consider.

A Quality Assurance Project Plan was developed for eDNA and Asian carp, one could be developed similarly for dreissenids. Until then, there are questions that managers can ask labs to better understand their process and manager's confidence including; did the lab follow a Quality Assurance/Quality Control plan, if yes were there any QA/QC flags; does the lab perform gene sequencing on positive results; understand the quantification of the uncertainties (i.e. rate of expected false positives).

Identify pathways to address gaps.

## Conclusion and Next Steps

The MISC eDNA Science Advisory Panel was a successful step in better understanding the role for eDNA in the management of invasive species for the future. The management of invasive species, specifically dreissenids, presents unique management and political challenges. Clear acknowledgement of gaps and recommendations from the advisory panel provides a path forward for developing this technology into an operational tool that managers are comfortable using for dreissenid monitoring. Action on this issue will require international effort and include both managers and researchers to address gaps and needs in the development of this technology as an early detection tool. This is an issue that affects aquatic invasive species prevention and management beyond the boundaries of Montana, and steps forward will benefit agencies and stakeholders across jurisdictions. MISC will encourage action on these issues, but interested partners nation-wide will need to help push this effort forward.

MISC has identified the following steps to utilize the information from the panel:

- Make all information generated from the scientific advisory panel available to all interested parties
- Encourage the development of open dialog among eDNA dreissenid scientific community to promote further standardization of this tool
- Encourage the completion of a laboratory round-robin project among appropriate partners to promote further standardization of this tool
- Engage the Western Regional Panel on ANS and/or Western Governors Association to assist in the promotion/implementation of the next steps identified by the panelists
- Continue the discussion regarding the use of eDNA and promote coordination and cooperation as the development of this method moves forward



## Appendix A

### Answers to MISC Science Advisory Panel Questions

# Montana Invasive Species Council

## eDNA SCIENCE ADVISORY PANEL

### Answers to MISC Science Advisory Panel Questions

Questions were compiled from input provided by members of the Montana Invasive Species Council and the eDNA steering committee. All panelists received the following questions in preparation for the panel. All answers are “as is” provided by the panelist with the exception of simple formatting. The goal of the questions is to guide discussion during the panel event.

#### Session One: State of the Science

##### **What are some examples of using eDNA to successfully manage a biological problem?**

Amberg: Environmental DNA is typically used to detect the potential presence of a species. In the state of WI, a section of Black Earth Creek was found to have an isolated population of New Zealand Mud Snails (NZMS). During the development of an NZMS eDNA surveillance program, a second site was identified that was up stream of the original infestation. Due to this, the WI DNR developed communications to inform users of the potential to transport these species between waters. Also, some fisheries programs are using eDNA to help identify the upstream most point of an invader so they can target piscicide applications. They follow with an eDNA survey to determine if further control efforts are needed.

Bajno: [Much of the work on environmental DNA is based on evaluating the effectiveness of eDNA for detecting organisms and proof-of-concept through comparisons with conventional surveillance techniques. Studies have shown that eDNA analyses generally are comparable to conventional survey techniques but are much more efficient.](#)

- [Asian carp eDNA surveillance in the U.S. and Great Lakes.](#)
- [Davison et al. 2017 – Used eDNA to assess the efficacy of an eradication attempt of invasive \*P. parva\* \(topmouth gudgeon\) in seven ponds in England.](#)
- [Takahara et al. 2013 – Monitored the invasive bluegill sunfish in 70 ponds in Japan.](#)
- [Pawlowski et al. 2016 – Metabarcoding used to identify bioindicators of organic enrichment associated with salmon farming.](#)

Darling: In England, developers wanting to build on any wetland are required to conduct biological surveys for the great crested newt, a species of high conservation importance. In 2014, Natural England first started to accept eDNA methods (Biggs et al. 2015; Rees et al. 2014) to detect the presence and presumed absence of great crested newts in water bodies, assuming that those methods are conducted in accordance with published technical advice and by suitably trained personnel. These eDNA detections have legal force: developers are precluded from developing wetlands where there have been positive eDNA detections. Labs conducting eDNA analysis must participate in the proficiency testing scheme in order to be deemed competent (see [http://webarchive.nationalarchives.gov.uk/20140605154334/http://www.naturalengland.org.uk/Images/eps-news-april-2014\\_tcm6-37695.pdf](http://webarchive.nationalarchives.gov.uk/20140605154334/http://www.naturalengland.org.uk/Images/eps-news-april-2014_tcm6-37695.pdf) and <http://www.adas.uk/Service/edna-analysis-for-great-crested-newt>).

The US Environmental Protection Agency routinely employs PCR and qPCR methods for detecting *Enterococcus* as indicators of water quality, and adopts the results of monitoring using these methods to inform decisions about closure of water bodies. I include this example as a reminder that eDNA may be new to us, but it has been a standard approach for microbial researchers for some time.

Goldberg: [I work with the City of Bellevue, WA on early detection of New Zealand mudsnails for eradication. We've detected invasions up to 2 years before they were detected by visual surveys. They use the information to manage eradication efforts.](#)

Snider: My experience with eDNA extends only to quagga and zebra mussel detection and I know of no situation where eDNA has reliably predicted a mussel infestation. Assuming the result is accurate, a positive eDNA result is only an indication that a waterbody has been exposed to mussel DNA. Testing for eDNA is not probative of living mussel larval or adults, nor can eDNA be used to unambiguously indicate the presence of an established reproducing population of mussels.

Vargas: [not applicable](#)

### **What are the biggest operational challenges associated with eDNA detection for fish species?**

Amberg: Development of a standardized method. This goes from the sampling to the analysis. Rigorous validation of the assay that is to be used should be conducted. The AC group used the approach required in the development and acceptance of new analytical methods for chemical detections. One area that has continued to be a challenge has been the number of samples needed and the AC group has just begun to address this.

Bajno: [Challenges in developing species specific eDNA assays for indigenous species co-occurring with closely related organisms. Furthermore, introgression/hybridization of genomes can contribute to detecting false positives if the interaction of populations of closely related species is not well understood. Pinpointing the occurrence of rare species due to movement across large spatial scales. The resuspension of old sedimentary eDNA could produce false inferences of presence after organisms are gone. Contamination of eDNA samples during studies taking place in large waterbodies from sampling and fishing equipment. Sterilization of watercraft, nets, personal protective equipment, etc. is difficult, time consuming and most likely not effective. Difficulty in designing efficient but effective sampling strategies to correctly identify the presence/absence of targeted organisms due to variations in organism's biology and physiology \(e.g., life stage, seasonality\), biotic and abiotic environmental factors and fluid dynamics acting in the aquatic environment. Capacity of laboratory program to generate scientifically defensible outputs \(infrastructure and expert staff\).](#)

Darling: I imagine that the biggest operational challenges include development of sampling strategies that adequately target eDNA for detection of rare species (e.g. sampling design that minimizes false negatives) and translation of patterns of detections into either likelihood of fish presence or some kind of site occupancy model. Both of these challenges are related to uncertainty associated with the relationship between eDNA presence and the presence of live fish, which in turn depends on understanding of shedding patterns of eDNA, fate and transport or eDNA in the sampled system, and the possibility of exogenous sources of eDNA. My suspicion



is that for fish species these challenges are generally greater than those associated with things like appropriate design of molecular probes, methods, and QA/QC procedures. Other operational challenges are likely common to all eDNA monitoring efforts, e.g. sample handling, preservation, and processing methods that avoid loss of eDNA.

Goldberg: [Development of appropriate sampling protocols for different species and availability of processing labs.](#)

Snider: Not applicable to my experience with eDNA.

Vargas: [Not applicable](#)

### **Are there aspects of Asian carp eDNA research / methods that may be extrapolated to early detection of dreissenids?**

Amberg: Yes, several. There was a multiple lab validation study completed prior to establishing the program currently run by the USFWS. For the assay to be useful, labs should get consistent results. You need to have multiple markers so a positive can be validated and a detection is not solely based on a single sampling event. Use eDNA to direct further efforts, not trigger controls. And probably most importantly, have a communication plan ready. Initially, eDNA for AC was looked at as a snapshot, however, now the trend in data is what is evaluated.

Bajno: [I have not been involved in the Asian carp work in Canada over the last few years. Methodologies and QA/QC protocols developed in the Asian carp Quality Assurance Project Plan \(QAPP\) are transferrable to other eDNA studies.](#)

Darling: I think the most important lessons may be general, e.g. the importance of employing strict QA/QC protocols and establishing inter-laboratory repeatability of results to ensure confidence in the method, and the establishment of longitudinal monitoring to allow spatio-temporal patterns of detection to be used to infer target presence/absence. Another extremely important message, which I don't think has been well absorbed by all stakeholders: If the eDNA method is assumed to be much more sensitive than existing alternative methods, then asking for confirmation of target presence using those alternative methods may be unrealistic, even if it's possible to use eDNA detection patterns to narrow searches.

Goldberg: [Seems to me like a lot of the QAPP would be applicable to these large systems.](#)

Snider: Not applicable to my experience with eDNA.

Vargas: [Not applicable](#)

### **How were the political / public implications navigated through the Asian carp eDNA research / monitoring / detection process?**

Amberg: This is still working itself out. Asian carp DNA was detected in two areas, Chicago waterway and Upper Mississippi (MN waters). These detections were reported with no communication plan in place, with assays that were not thoroughly vetted (these were later

assessed by the interagency research group mentioned below). Failure to link a live fish capture with DNA detection caused hesitation with resource managers acceptance of eDNA and it is not being used to guide management actions in either IL or MN. Managers in these states do not link rapid response with DNA detection. Investments have been made toward better understanding eDNA by establishing a research group. This group was put together by the USACE and was comprised of researchers from the USACE, USFWS and USGS and tasked with evaluating how DNA can get into the system, establishing high-quality markers, and calibrating eDNA for Asian carp in general. Continued refinements in eDNA for AC and a much better understanding of how to use it has not been well accepted by resource managers. They continue to ask “what does it mean?”. Whereas the public sees it as a new tool that they should be using.

Bajno: [I cannot comment on this question as I do not have experience with this specific topic. I have not been involved in the Asian carp work in Canada over the last few years.](#)

Darling: There were two main challenges with the development of eDNA monitoring in the case of Asian carp. First, I suspect that few people expected early efforts to detect eDNA in the field to be as sensitive as they were—I certainly believe that nobody anticipated that these results might provide a very different picture of carp distribution than that assumed based on traditional methods. Unfortunately, I believe that this meant that researchers and managers alike were unprepared for the scrutiny leveled at the method, were unclear on the possible sources of uncertainty associated with it, and had not sufficiently considered how the eDNA information might be utilized in decision making. All of these things were learned on the fly, and in a situation that was fraught with external pressures of various sorts. If you were to design the process, you certainly wouldn't do it this way, and I would hesitate to use Asian carp as a model for how to navigate political/public perceptions; better probably to use it as a cautionary tale. The second challenge for Asian carp monitoring was the political climate. eDNA evidence was being considered in decisions with economic impacts measured in the billions of dollars. A large number of organizations with very different jurisdictions and approaches to decision making were engaged. In the absence of rigorous pre-planning for an eDNA monitoring program, I believe that this made the political situation more confusing as specific roles and responsibilities may have been ill defined and definitely evolved in the face of external pressures. I believe that once the potential importance of eDNA evidence became clear, people involved took the necessary steps to resolve uncertainties associated with eDNA detections, such as laboratory audits, support for research to understand the fate and transport of eDNA, and attempts to formalize inference from eDNA evidence. Unfortunately, I think that damage had already been done in the early, confusing stages of the process, and that this meant it was subsequently very difficult to build trust between all of the important stakeholders. I believe the lesson from the Asian carp situation is that steps should be taken PRIOR to implementation of a monitoring program to educate all stakeholders about the power and limitations of the approach, to clearly define roles and responsibilities, and to determine how eDNA evidence is to be used in the overall decision-making context.

Goldberg: [I'll leave this to the Asian carp experts.](#)

Snider: Not applicable to my experience with eDNA.

Vargas: [Not applicable](#)

**Have eDNA methods been developed and utilized for non-dreissenid bivalve species? If so, has there been any specific challenges related to detecting bivalves?**

Amberg: A few have been done, Asian clams and western pearly shell. In the Midwest, the only person that I know that has done work on developing an eDNA assay for freshwater bivalves is Wes Larson with the USGS Coop Unit at the University of Wisconsin, Stevens Point. The genetic information on native mussels is growing, but still is limited.

Bajno: [Currier et al. \(2018\). Validation of environmental DNA \(eDNA\) as a detection tool for at-risk freshwater pearly mussel species \(Bivalvia: Unionidae\). Aquatic Conservation: Marine and Freshwater Ecosystems, pp. 1-14.](#) Detection probabilities were higher for eDNA sampling than quadrat sampling, DNA concentrations did not predict population density, eDNA detection was not affected by sampling depth in shallow streams, high flows diluted but did not eliminate eDNA signals. [Cowart et al. \(2018\). Development and field validation of an environmental DNA \(eDNA\) assay for invasive clams of the genus Corbicula. Management of Biological Invasions, Volume 9, Issue 1, pp.27-37.](#) Failure of eDNA to detect organism expected to be present attributed to inhibitors in water samples (suspended sediments in water). No PCR reaction internal positive control was used to identify PCR inhibition. Dilution of possible inhibitors resulted in a single positive identified. Replicate sampling may have improved detection ability. Use of a non-species specific assay may have contributed to failure of a single species in the *Corbicula* genus. [Xia et al. \(2018\). Early detection of a highly invasive bivalve based on environmental DNA \(eDNA\). Biological Invasions, Volume 20, Issue 2, pp.437-447.](#) - *L. fortunei* (golden mussel) detection was higher in laboratory samples containing re-suspended matter from the substrate than that collected from the water surface. Detection success using eDNA varied depending on mussel abundance.

Darling: I am aware of eDNA methods having been developed for *Corbicula fluminea* (Clusa et al. 2017; Cowart et al. 2018), *Limnoperna fortunei* (Pie et al. 2017; Xia et al. 2017), *Mytilopsis leucophaeata* (Clusa et al. 2017), and *Sinandonta woodiana* (Clusa et al. 2017). I am not aware of any specific challenges that have been identified that are unique to detection of bivalves. It is possible that bivalves may present unique challenges in terms of DNA extraction in cases where whole organisms are captured (e.g. plankton tows for veliger larvae). I don't know if this is the case because I have done very little work personally with bivalves, and I am not intimately familiar with this literature.

Goldberg: [I had great detection of \*Corbicula fluminea\* on the Columbia River, but they were really common.](#)

Snider: In a comprehensive literature search, Roussel et al. (2015) found 35 species targeted in eDNA studies, where-in eight percent (n = 3) were mollusks. So, it seems there are not many eDNA bivalve studies. Any eDNA study would have challenges related to the target species' life history. Any detection protocol would have to be validated for the particular species under investigation. With bivalves challenges might include reproductive strategies, seasonality of spawning, and habitat preferences. All variables would have to be considered in the development and validation of any eDNA protocol.

Vargas: [Not applicable](#)

**Why are there few peer reviewed publications related to the use of eDNA for dreissenid mussel early detection? Has there been sufficient peer review to justify the use of these methods to make management decisions related to new dreissenid detections?**

Amberg: This is a new field of study, so there are only a few papers out there for each of the species of interest. Prior to deployment to guide management decisions, a thorough evaluation of the methodology needs to be conducted.

Bajno: [The application of eDNA techniques to conservation and management of aquatic resources has gained in popularity within the last ten years. Genomics technology has quickly evolved to become more sophisticated in detecting and characterizing genetic material. Until recently, conventional PCR \(cPCR\) markers were developed to identify dreissenids. Although cPCR markers have been widely used in eDNA studies, they are less sensitive than qPCR markers at detecting low quantity DNA concentrations expected during early detection studies. qPCR markers are currently the choice of research groups targeting specific organisms. The development of qPCR marker for dreissenids has occurred only in the last few years. Currently, the research community is continuing to develop and refine the methodologies to detect DNA in aquatic systems. Most studies are designed as proof-of concept or to ground-truth the utility of eDNA as a monitoring tool. Currently there are very few examples of eDNA studies initiated and utilized to generate management decisions. Studies investigating the utility of eDNA to detect dreissenids are quickly increasing and methods and techniques developed and refined in other eDNA studies can be transferrable to dreissenid detection research.](#)

Darling: One possible explanation is that managers are not so publication-centric as researchers, and so it is much more likely to see a publication describing the original development of a method than it is to see a publication describing its implementation in a decision-making setting. Unfortunately, this means that many (most?) of those originally published methods are not as rigorously tested in the peer reviewed literature as they perhaps should be. Much of the data on the utility of these methods comes not from the original manuscripts describing development (which will often be testing the tools on a rather constrained set of samples under perhaps idealized conditions) but rather from field applications (which will likely be looking at a much wider range of samples with varying challenge conditions). If this latter type of data is never published because the managerial community doesn't see publication as a priority, then the method may never get fully vetted by external peer review and it will be difficult for other research & development efforts to build on the lessons of those earlier methods. This may explain in part the proliferation of methods.

Goldberg: [I don't know.](#)

Snider: To my knowledge there are five articles related to dreissenid eDNA detection – all recently published; Ardura et al. (2017), Gingera et al. (2017), Hosler (2017), Ventura et al. (2017), and Peñarrubia et al. (2016). Each study should be evaluated on its own merits. Impact factors for respective journals might help to address the question of sufficient peer review. Perhaps the recentness of these studies signals an upswing in the attention the eDNA community will be giving to this taxon.

Vargas: Although publications exist regarding the use of eDNA for dreissenid mussel early detections, there hasn't been enough publications to justify the use of the methods to make management decisions based solely on those methods (e.g. positive dreissenid water). There have been far too many false positives without any physical evidence utilizing the current methods in the real world environment. Whether this is derived from field/lab contamination or laboratory analysis interference is unknown. However, false positives have made it very difficult for resource managers to make decisions based on the eDNA science alone.

**Why are there different methods for eDNA dreissenid early detection (lab and field collection methods)? Is there an accepted standard method for eDNA sample collection and analysis? If not, why isn't there an accepted standard method?**

Amberg: Again, this is a new technique. There generally isn't a standardized method in the initial stages of development. The collection of water can be dependent upon the system and the organism. Since the field is still in its infancy, many studies have overlapped so you have multiple markers and laboratory methods, but these methods could also be modified based on the question the researcher is trying to answer.

Bajno: Research groups are currently working on developing methods for early detection of dreissenids. Until standard approaches have been developed and adopted by research groups investigating dreissenid eDNA, methodologies will continue to be refined and optimized to create a tool that can be confidently implemented in monitoring programs. The rapid expansion of interest in eDNA and its application has resulted in generating an unprecedented number of protocols and methods to detect nucleic acid in the aquatic environment depending on applications, taxa, management needs, etc. Until there is a concerted effort by stakeholders to establish guidelines (e.g., Asian carp QAPP) ensuring QA/QC standards for regulatory monitoring programs are in place, individual research programs will continue to use an assortment of techniques to address research questions.

Darling: I believe there are a number of possible reasons for the proliferation of methods. First, it is possible that dreissenids present unique challenges to eDNA detection. I am not certain if this is the case, having limited direct experience with these methods. But it is possible that dreissenids are reluctant to shed sufficient quality DNA, or that for some reason it has proven challenging to design primers that achieve the sensitivity and specificity desired for management. Second, it seems clear that early methods assessed for their utility in early detection contexts performed quite poorly (Frischer et al. 2012). It is likely that these early results have led other researchers to attempt development of more reliable methods, and that these recently published methods have not yet had time to be applied widely enough to see an accepted method emerge. Third, my impression is that, unlike the Asian carp situation (which is coordinated by the Asian Carp Regional Coordinating Committee), dreissenid monitoring is being conducted by a diverse set of management groups often with non-overlapping jurisdictions and often with little coordination between them. I do not know the degree to which the Quagga/Zebra Mussel Action Plan of the ANSTF Western Regional Panel ([https://anstaskforce.gov/QZAP/QZAP\\_FINAL\\_Feb2010.pdf](https://anstaskforce.gov/QZAP/QZAP_FINAL_Feb2010.pdf)) has served as a coordinating framework for the development of standardized methods, although it certainly appears to be the most likely framework for such coordination. To the degree that such coordination may be lacking (either in principle or in practice due to limited funding), different jurisdictions will likely be developing their own protocols for lab and field collection methods.

Goldberg: Different labs have developed different workflows that work well in their systems. For example, researchers that work in systems with more particulates have been more concerned about filter type and pore size or even whether to use filters at all, while those that work in clear streams have focused on other issues. Different extraction methods that labs use work differently on different filter types, these must be paired for good results. Transportation of water before filtering can also be more of an issue for those working in the backcountry. So there are multiple pathways that work. We tried to get at the commonalities from labs across the world in this paper: <https://besjournals.onlinelibrary.wiley.com/doi/full/10.1111/2041-210X.12595>. Originally I thought we would land on one right way to do things- but it turns out to be more complicated.

Snider: I believe these are questions for the eDNA community to address. CDFW uses a sequential single-veliger analysis approach where microscopy and molecular methods are used supportively to confirm the identity of veliger larvae in a way that mitigates the uncertainty associated with stand-alone methods. Advocates may claim that eDNA monitoring is more sensitive and more efficient than traditional methods, but I question these claims. For example, in a round robin study (Frischer et al. 2012) demonstrated that microscopy (CPLM) was 96.3% accurate, while PCR (= stand-alone DNA testing) was 75.8% accurate.

Vargas: Consistent lab and field collection and analytical methods need to be one of the 1st steps in assessing the use of eDNA for dreissenid mussels for the methodology to be acceptable for management decisions. If consistent methods cannot be agreed upon then the methodology must be tested beyond that which is questionable for managers to use eDNA as an early detection methodology within itself. This should apply to both the analytical end and sampling protocol criteria.

Western Regional Panel is in the process of developing agreed upon sampling procedures, however, there is disagreement within the ranks on what those methods should consist of, for example, federal (BOR) sampling criteria is different than the sampling protocols many states are using. In addition, states also differ in how they sample. A standard protocol should be established but at the same time allowing for targeted and specific sampling protocols to be adjusted when the need exists.

**Western states have not widely accepted the use of eDNA as an operational early detection tool for dreissenid mussels. What factors might influence the future use of eDNA as a detection tool?**

Amberg: A thorough vetting of the DM eDNA protocol. In general, laboratory procedures can easily be standardized, but significant work needs to be done to get a better idea of detection probability in the field. This includes, how to best sample the water, where to sample, and how much water to sample to insure you don't lose DNA in the extraction process. Once the DNA is in a vial, it can easily be quantified and then the process standardized, but until then it is very difficult and is likely to be different from site to site.

Bajno: Currently there is no widely accepted and validated method for detecting dreissenids using eDNA. Until standardized and fully tested methods become available, managers face significant challenges when deciding management response to suspect positive detections. Management responses can be expensive; resulting in suboptimal resource allocation should detection be a false positive. Eradication efforts can be devastating to native species and

habitat. A lack of management action may lead to further spread of a nonindigenous species and exacerbate the problem and all decisions will require an assessment of the socio-, economic and environmental implication of the action

Darling: I think that the most significant change in the current situation would be the emergence of a single eDNA method (combination of primer sets and field and lab protocol) that has been determined to work in a range of challenge conditions and has received some sort of official license or adoption by a national or regional coordinating body or framework (e.g. USFWS, ANSTF WRP, QZAP, etc.). Until that happens I think that we will continue to see hesitancy and doubt about the utility of the method, and as each new jurisdiction begins to contemplate an early detection and monitoring program they will have to re-visit the same questions that MISC is currently asking themselves.

Of course, this answer avoids the more direct questions about what would need to happen to achieve that state where some coordinating body can support a single protocol. I think that the rest of the questions in this document address exactly that issue.

Another thing that would significantly influence adoption of eDNA as a detection tool would be demonstrations of its preventative value. For instance, Gingera et al. (2017) showed that eDNA is capable of detecting unexpected populations of zebra mussels at the invasion front. This in itself is a demonstration of the potential utility of the tool. What would really be useful is if early response to that invasion front population could be successful due to the early detection. It is unfortunately extremely challenging to demonstrate these sorts of results, but any case studies could be very compelling.

Goldberg: [An example of a state that is successful in using eDNA, in that early detections are caught without a lot of false alarms.](#)

Snider: Environmental DNA testing is high reward, high risk. A positive eDNA result in the earliest stages of an infestation, when it might not be possible to detect veligers or adults, is a high reward scenario. The risk is that resources might be allocated in response to a false positive eDNA result. Presently, indications are the risk outweighs the reward. There are examples of positive stand-alone DNA results in waterbodies where those results have not been indicative of mussel presence, even after years of continuous larvae monitoring in those same waterbodies. The correlation between the presence of mussel DNA and living larvae or adult animals has not been established. In the absence of such a correlation, the use of eDNA will have limitations.

Vargas: [Several factors are making it difficult to use eDNA as an early detection tool: Laboratory Expense, False Positives, False Negatives, Inadequate or non-agreed upon sampling and analytical methodology.](#)

[The states and federal agencies that have used eDNA as an early detection tool for mussels do not have a 100% proven track record \(positive detection with eventual mussel detections\), however, that is not to imply that eDNA shouldn't be used as an early detection tool; just the opposite, however, the methods \(lab/field\) need to be more developed and researched for managers to make decisions based on eDNA evidence alone. The scientific community needs to further define the methodology and potential laboratory interferences \(algae, sediment, organism/bio and physical attributes\) that can interfere with laboratory methods.](#)

## Session Two: In the Field

### **Are there best practices for sampling location (e.g. shallow vs. deep, near access points, outlets)? Suggested depth to monitor?**

Amberg: Short answer, no. This is something that should be done when developing a monitoring assay. Sampling is generally thought to be site specific and should be worked out prior.

Bajno: There is currently no standard for how or where eDNA samples are collected. There is considerable variation among research groups in the sampling methods used for collecting water samples for eDNA analysis. Collecting multiple biological replicates including negative controls with sterilized field equipment is mandatory.

Watercraft are a primary vector for the spread of zebra mussel, thus at minimum, sampling at and near access points (e.g., docks, boat launches, breakwaters, etc.) to waterbodies, especially in lake environments is recommended. These high traffic locations typically contain suitable substrate for zebra mussels to attach and are commonly situated in areas that provide sheltered sections relative to open water areas, thereby potentially minimizing dilution effects, improving chances to capture eDNA and increase likelihoods to identify sources of origin of eDNA material. Collecting water samples for eDNA analysis near these access points is easy to accomplish, minimizing site contamination since water sampling (and filtering) can be done without entering the waterbody. These lake access points are ideal candidates for monitoring points of invasion. Monitoring inlets to lakes provides information to assess whether eDNA originates from sources locally within the lake or is potentially transported from upstream locations. Monitoring outlets of lakes provides information on the risk of dreissenid invasion to downstream aquatic environments from infected areas.

The distribution of eDNA in lentic and lotic environments is not homogeneous. Location of sources of eDNA, persistence of eDNA in the environment and fluid dynamics in complex hydrological systems, support a sampling strategy of collecting samples at multiple sites within the waterbody to ensure suitable coverage to maximize capture of rare eDNA targets. Sampling strategies need to take into account risks to sample integrity from improper sampling efforts (e.g., incorrect sampling protocols off watercraft) so as to not compromise eDNA analyses. The cumulative property of eDNA in an aquatic environment may allow for greater chances to capture eDNA in downstream locations in flowing water or in areas where water movement is predictable (e.g., bottlenecks, eddies).

Environmental DNA samples should not be collected immediately after environmental events such as strong winds or rain which may compromise eDNA capture due to dispersal and dilution. Seasonality should be incorporated into eDNA sampling strategy design. Should the number of sampling opportunities at a waterbody be limited, water collection should occur when the biological processes and reproduction of the targeted organism is at the peak. For dreissenids, winter die-off and snow melt may decrease the concentration of eDNA present in a system. In aquatic environments, eDNA is routinely collected at the water surface or near the substrate. Collecting at the water surface requires less effort than collecting in the water column. Surface water is commonly collected using disposable supplies (e.g., bottles, analytical filter cups) whereas water column sampling typically uses reusable equipment (e.g., water samplers, veliger nets) requiring strict sterilization to prevent DNA contamination. Surface collection allows greater spatial sampling coverage than water column sampling with equal efforts. However, the increased exposure to ultraviolet light, higher temperatures and increased water agitation may



contribute to quicker degradation of surface eDNA. A benefit of collecting samples near the substrate is that DNA may persist longer in trapped in sediment than free in the water column, thus collecting at the sediment can be useful to identify whether an organism was ever present. However, sediments may contain higher levels of PCR inhibitors which can be collected with eDNA. These inhibitory materials frequently require samples to be further enriched in the laboratory which can contribute to the loss of low abundance DNA. Depending on the resources available for eDNA studies and the capacity of laboratory programs, sampling for eDNA at the water surface and near the substrate is recommended for low mobility organisms.

Darling: I have limited relevant experience with this issue and believe that these questions would be better handled by other participants. I assume that the question is specific to dreissenid monitoring, and not to eDNA monitoring in general.

Goldberg: It's best to be closest to the organisms. eDNA sinks, so although we often find it at the surface, if it's possible to sample lower down that could improve detection if that's where the organism is. However, sampling the sediment may indicate historical rather than current presence, which is not a problem for early detection but would be when there's been an eradication.

Snider: Sampling protocols have not been standardized. Sampling practices are often left to the discretion of field personnel. Field sampling conditions are highly variable and therefore it is difficult to make recommendations. Sometimes plankton tow samples, which are intended and designed to capture and collect veligers, are processed as eDNA samples. This practice has created confusion as to how eDNA testing is defined. There should be separate protocols for plankton tow collection, where the base analyte is the veliger, and eDNA sampling methods, where the base analyte is DNA, and more particularly DNA without any evidence of a biological source. A common practice seems to be the collection of 2 L water sample for eDNA analysis (= 2 L filtered). Yet there doesn't seem to be much discussion as to why 2 L is the preferred volume.

Vargas: Generally, for routine monitoring of mussels, access points and outlets (dam area) are used to collect samples for microscopy and eDNA analysis. Depth will depend on the organism being monitored. For quagga mussels they can be found relatively deep (40+ feet) and veligers aren't necessarily always found near the surface.

**What, if any, standard field cleaning / decontamination protocols for field and lab exist to prevent DNA contamination?**

Amberg: The USFWS does a great job in outlining the cleaning and field protocols they use and UMESC uses when conducting eDNA studies. This is outlined in appendix E of the QAPP (<https://www.google.com/search?q=Asian+carp+eDNA+QAPP&ie=utf-8&oe=utf-8&client=firefox-b>).

Bajno: Existing methods used in many AIS watercraft inspection/decontamination sites using hot water (50-60C) and high pressure methods are not sufficient to remove all traces of DNA. When not thoroughly sterilized, nucleic material can remain on equipment contributing to the risk of detecting nucleic acid during subsequent sampling events. We have found that veliger nets used in zebra mussel infected waterbodies in Manitoba but cleaned using hot water/high pressure

and allowed to dry, still contained eDNA after the cleaning process. Thus more stringent sterilization protocols for field equipment are required should eDNA techniques be integrated in surveillance projects using conventional methods. Bleach is an inexpensive but effective disinfectant most frequently used for sterilizing supplies and equipment used in eDNA programs. Sterilization protocols in various programs have commonly utilized bleach working solutions at a 10-50% concentration (commercial strength bleach diluted in water), however a 50% bleach solution is now generally recommended in decontamination protocols (Goldberg et al. 2016).

DFO zebra mussel eDNA field protocols used in Manitoba make use of disposable water collection/filtration supplies to minimize the risk of cross-contamination. These supplies (e.g., analytical filter assemblies, eDNA filter preservation tube) are assembled and packaged in a PCR cabinet with HEPA filtration and UV light sterilization functionality to minimize contamination. In the field, non-disposable equipment, supplies and field gear that come into contact with water are required to be sterilized using a 50% bleach solution for a minimum of 20 minutes (all surfaces in full contact with bleach solution) followed by rinses in distilled water. Other equipment (those unable to be submerged in bleach) are wiped down with 50% bleach solution and dried. Equipment sterilization is performed between sampling sites to prevent contaminating subsequent sampling locations with DNA and to prevent unintentional transport of alien organisms between sampling locations. Following field sampling, all equipment used in the field is required to be cleaned using the bleaching protocol followed by a soap and water wash.

Field sampling design is set-up to sample waterbodies in the direction of least probable to have zebra mussel present to those that are suspected to contain zebra mussels. Waterbodies known to be infected with zebra mussels are sampled on separate days than those only suspected or unknown. Sampling lotic environments is done in a downstream to upstream direction. Lentic environments are also sampled in a downstream to upstream direction if possible.

In the laboratory, field samples are wiped down with a 50% bleach solution before entering the analytical laboratory. Standard operating procedures are in place to ensure a clean working environment minimizing chances of nucleic acid and nuclease contamination. Bleach (25% working concentration) and commercial decontaminants (e.g., Decon Eliminate) are used to sterilize work surfaces, equipment and supplies. In addition, baking at 180C for 8 hours is used for certain lab materials to inactivate nucleases. eDNA samples are always handled and processed in PCR cabinets (HEPA and UV functions) to prevent sample contamination from outside environmental sources. After every use, PCR cabinet workstations are cleaned with Bleach or Eliminate and are subjected to UV sterilization. Before every use, PCR cabinet workstations are subjected to UV sterilization. The eDNA laboratories are under slight positive pressure ventilation. Ceiling mounted UV light systems can contribute to removing DNA contamination in laboratory spaces.

Darling: I have limited relevant experience with this issue and believe that these questions would be better handled by other participants. General field decontamination procedures have been described in the literature, but I'm not sure what standards might exist for dreissenid monitoring.

Goldberg: The QAPP is probably the gold standard. We summarized the issues in the critical considerations paper (<https://besjournals.onlinelibrary.wiley.com/doi/full/10.1111/2041-210X.12595>). Many other papers mention the same kinds of protocols (clean with bleach, take field negatives, etc.).

Snider: I would defer this question to the people who are doing eDNA testing. The potential for contamination is acknowledged by eDNA researchers and I believe most researchers are diligent in contamination prevention measures. However, contamination does occur, even when precautions are taken. Ideally, laboratories would willingly disclose contamination issues and implement meaningful corrective actions acceptable to AIS managers. As a practical consideration, I question whether the required level of decontamination can be maintained as part of a routine eDNA monitoring program.

Vargas: Western Regional Panel is also working on field cleaning and decontamination protocols based on mussel protocols previously developed by California Fish and Wildlife and other entities that routinely monitor for mussels. It must be remembered however, that for early detection most entities sampling are not performing research but looking for presence/absence and the methods developed must reflect the need rather than protocols too stringent for routine monitoring.

### **Can field / lab sample contamination be identified when there is a “positive” result? If yes, how?**

Amberg: Typically, if a negative control is found to be positive, then the whole batch is considered contaminated. But much can be learned from a positive negative control, especially since you have controls at various stages throughout the process. This would include field blanks to check to see if contamination occurred in the field, extraction controls to verify that you don't have contamination occurring during the extraction and run controls to evaluate if any of the mixtures used in the assay are have been contaminated. If extraction and/or assay controls are positive, then improvements in QA/QC for the lab is required and those samples should not be considered. If a field blank is contaminated, and all laboratory controls are negative, then the DNA entered into the process during field collection.

Bajno: The use of field blanks and controls are required to ensure that field samples are not contaminated from sources outside of the sampling location. Negative controls should be integrated into collection protocols at every sampling site to track contamination. Field kit blanks (e.g., cooler blanks, swabs of equipment) can be used to determine whether DNA is transported by sampling crews to subsequent sites.

In the laboratory, positive and negative controls should be used throughout the processing workflow to ensure QA/QC. Ideally, eDNA samples should be extracted in batches to ensure cross-contamination between sampling locations is minimized (i.e., eDNA samples from different waterbodies are not extracted alongside each other at the same time). Extraction positive control material should not be of the organism being monitored but will be subjected to a) PCR using genetic markers specific to the positive control material to determine whether the extraction process worked as expected and b) PCR using the genetic markers for the target organism of the eDNA study to determine whether cross-contamination occurred between samples during the extraction process.

For positive control material to be used in PCR, it is recommended that control standards are synthetic oligonucleotides (e.g., gBlocks) incorporating a DNA sequence insert that distinguishes it from wild type sequence (Wilson et al. 2016). Synthetic oligo controls aid in the identification of contamination (false positives) generated through laboratory processes.

It is mandatory to use 'No template controls' (NTC) throughout the entire eDNA workflow. When an NTC shows amplification, the analyses must be carefully scrutinized to identify sources or mechanisms of contamination.

Follow-up analysis on a separate day/time using additional technical replicates can provide insight whether spurious positive detections were observed initially.

Follow-up field sampling strengthens evidence for true positive detections.

Darling: The only way I can imagine doing this is through analysis of proper negative controls. Contamination of a sample with dreissenid genomic DNA would be impossible to distinguish from a true positive by sequencing analysis. It might be possible to design a method that would allow you to distinguish contamination by post-PCR DNA, but this would not cover contamination by genomic DNA. Application of appropriate controls would at least provide some assessment of the likelihood of a positive detection arising as the result of contamination.

Goldberg: It depends where the contamination comes from. Negative controls can indicate systemic contamination (e.g., grab bottles that were not decontaminated, contaminated reagents). Synthetic positive controls can include a marker that indicates their origin (and differs from the natural sequence). There is a great figure on sources and interpretation of error in Darling and Mahon 2011, From molecules to management... That paper provides a lot of great insight on many of the questions asked here.

Snider: In some cases, quality control measures, such as the use of travel blanks and negative controls, are sufficient to identify contamination issues. However, I have seen examples of obvious contamination where the source and cause of the contamination were very difficult to identify. Positive results must be interpreted with caution, especial if a stand-alone PCR result is used to make a management decision. The use of a sequential single-veliger approach, where the veliger is the base analyte, helps to mitigate contamination issues.

Vargas: This subject needs to be further discussed: are field and lab duplicates enough to detect contamination?

## Session Three: Analysis

**Provide context for why there may be variation in molecular markers and how these differences may affect results?**

Amberg: Each marker has its own kinetics which is based upon the sequence and optimization of the running conditions, as well as differences in the optics of the machines. There are typically only a few areas that are targeted within the 16,000 basepairs of the mitochondrial genome, but since markers are typically less than 200 bp, there are lots of potential markers within a single species. As an example, to identify the markers used by FWS for AC, a total of 15 different markers were compared in a blinded study that involved 3 laboratories that all had different instruments.

Bajno: When trying to detect rare species or free DNA in the aquatic environment, molecular markers are typically developed to target genes from mitochondria, ribosomes, or chloroplasts

due to the high copy number present in cells, thus making them the preferred option to single-copy DNA.

Markers are selected based on a high resolution to detect to the desired taxa level required to address eDNA research objectives (e.g., single species detection versus metagenomics). Genetic markers are designed to bind to conserved regions in targeted species but in genes/regions with sufficient variation to distinguish among species. Multiple genetic assays may be required if the DNA region being targeted is too similar in some taxa to distinguish to the required taxa level. Developing molecular markers in genes that have highly conserved DNA sequences across taxa can be problematic because DNA sequence polymorphisms required to develop genetic assays that differentiate species may not be present. On the other hand, markers developed in genes that contain a high degree of polymorphisms within a species may inadvertently not detect certain individuals/population because of PCR amplification failure due to oligo hybridization issues between genetic markers and template sequences. Depending on the spatial scale the eDNA assay will be applied, the sequence divergence between individuals, populations, stocks and evolutionary lineages need to be considered.

DNA is more stable than RNA in the environment. However, RNA is being investigated as an alternate approach to DNA in eDNA studies because it may better estimate the time since deposition of biological material in the aquatic environment. RNA is less stable and degrades faster than DNA, thus RNA may have the advantage of detecting living organisms, rather than detecting a historical record of all organisms, living or dead, that may have occurred at a given location (Pochon et al. 2017).

Assays are designed considering target specificity and PCR efficiency. Amplicon size, target sequence composition, thermodynamics between markers and target DNA, PCR reaction conditions and the presence of PCR inhibitors all can affect PCR efficiency (100% efficiency is the doubling of a PCR product with each cycle). These are a few factors that can influence the detection sensitivity of an eDNA assay.

Darling: Variation in molecular markers for species-specific detection consist primarily of 1) differences in the genomic region being targeted and 2) differences in the sequence of probes designed to bind that targeted region. Both types of variation are taken into account when attempting to design probes that will yield positive detections in the presence of any and all individuals of the target species, but not in the presence of any individual not belonging to that species. Variation in molecular markers therefore affects the likelihood of both false negative and positive results. In the case of the former, markers adopted may fail to bind to DNA of a target individual if that individual expresses variation at the marker locus that has not been anticipated in probe design (i.e. an unexpected mismatch with the probe sequence due to unknown individual variation at the target locus). In the case of false positives, markers may erroneously bind to DNA from a non-target individual if that individual expresses variation at the target locus that unexpectedly matches the probe sequence (i.e. the targeted locus is insufficiently variable to distinguish between targets and non-targets). Therefore, ideal markers exhibit sufficient variation at probe-binding regions that it is possible to exclude all non-targets, but not so much variation that it is impossible to design probes that are universal for all targets. This reasoning is similar to the reasoning behind DNA barcoding and the importance of the “barcode gap” to allow species-level identifications.

The adoption of different molecular markers for eDNA detection of a single species reflects researchers’ attempts to identify a target locus with optimal characteristics as described above. To the extent that these markers vary in their specificity and universality, we would expect them to exhibit different tendencies for false positive or false negative detections.

Goldberg: Some assays are more sensitive than others – sensitivity should be reported for each study (LOD). Assays targeting mitochondrial DNA are going to be more sensitive than those targeting nuclear DNA because the mtDNA is more abundant (many copies per cell).

Snider: Depending on varying rates of evolution, different markers can be used to resolve to different taxonomic levels. For example, the mt COI marker is the standard barcoding marker used to delineate metazoan species, while the mt 16sRNA gene might be better suited for the delineation of congeners (Hwang and Kim,1999). CDFW uses separate COI markers designed by Rochelle et al. (2010) for the confirmation of both quagga and zebra mussel veligers. Regardless of the marker, the assay must be validated.

Vargas: not applicable

**What molecular markers are currently being used with dreissenid eDNA analysis? Is there any consistency between labs for marker preference? Why or why not?**

Amberg: Markers are also developed for various reasons and approaches. Initially, markers were designed for conventional PCR, so they only consisted of a forward and reverse primer. These markers could be used in quantitative PCR using SYBRgreen, but I would highly recommend against that since SYBRgreen will bind and fluoresce in anything that is double stranded (there is lots of that in these types of samples). The current qPCR approach is to use probes, and some of those early markers are not suitable for adding a probe for a variety of reasons, including size, sequence, etc. Additionally, many studies have used only a single marker for their detections but having multiple markers in the analysis adds confidence in results. Currently UMESC uses a Dreissenid mussel marker that has been published (Gingera et al. 2017). If that is positive for Dreissenid DNA (in two separate runs), we then use a ZM-specific marker that was published in 2016 (Amberg and Merkes 2016) and a marker that is specific to Quagga Mussels (yet to be published). If either of the two species-specific markers are positive to confirm the Gingera assay, we sequence the result. If the sequence is for the targeted species, then we report it as a positive. Also, markers have been designed for various reasons and should be optimized for eDNA prior to use by the lab that is using them. Also, private companies have developed markers for Dreissenids and will not share their assay information stating that it is proprietary.

Bajno: Choice of genes/DNA sequences to develop eDNA assays is based in the ability to resolve genetic differences to the desired taxa level. Access to DNA sequences in DNA data banks such as GenBank and BOLD provide opportunities for researchers to perform extensive in-silico investigations in an effort to design effective genetic markers. Underrepresentation of DNA sequences for taxa which are to be targeted in eDNA analyses, and those that co-occur with the targeted organism, requires additional effort by research groups to construct DNA sequence reference libraries to be used in assay design.

Currently mitochondrial DNA gene sequences are the most available sequence data in publically accessible databases, and thus are good candidates for eDNA assay design. The cytochrome oxidase subunit 1 (COI) gene is one of the most represented sequences in these database and is the most frequently used gene for eukaryotic eDNA marker development.

Mitochondrial DNA markers are currently the preferred markers for designing eDNA assays for dreissenids. A few examples of assays being used that target zebra mussels include: Amberg and Merkes 2016 – qPCR (COI; mtDNA), Gingera et al. 2017 – multiplex qPCR (COI, 16S, CytB;

mtDNA), Williams et al 2017 – LAMP (COI; mtDNA), Penarrubia et al. 2016 – qPCR (histone H2B gene; nuclear DNA).

Darling: The literature reports numerous markers that have been used for dreissenid detection. I am not aware how many of them have been or are being currently applied in eDNA detection contexts. They are: COI, cyt b, and 16S (Gingera et al. 2017); 28S (Hoy et al. 2010); 18S (Frischer et al. 2002); COI (Baldwin et al. 1996); COI (Trevor Claxton and Boulding 1998); 16S (Ardura et al. 2017); COI (Mahon et al. 2011). My assumption is that there is little consistency across laboratories in marker preference, although I do not know that that is the case.

Goldberg: I don't know.

Snider: There are probably a number of markers that can be used successfully. I would guess a majority of protocols target the COI gene. I wouldn't expect much disagreement over marker selection options. It comes down to validation. Methods published within the context of a study are not necessarily tried-and-true (=validated). It seems researchers are often inclined to design their own primers (=marker), instead of using someone else's. Designating new primers may be out of necessity, depending on the PCR chemistry used. For example, smaller markers are preferred in qPCR assays as a means of achieving acceptable fidelity.

Vargas: Not applicable

### **Do labs have differing QA/QC methods / protocols for dreissenid mussel eDNA analysis? For qPCR dreissenid mussel eDNA analysis?**

Amberg: Yes, but movement toward ensuring adequate QA/QC has been suggested. Goldberg et al. (2016) provided some guidance on the minimum for publishing eDNA studies, but this is for publication purposes. Some have suggested a sort of accreditation for a lab so that resource managers can be confident in the results beyond what is needed for publication purposes. This would be similar to what is currently done for chemistry and forensic labs.

Bajno: Quality assurance and quality controls are the most important aspect of eDNA methods. DNA isolated from environmental samples is typically low-quality and low-quantity and are prone to contamination and/or degradation issues if not properly collected, preserved, stored and processed. Furthermore, error rates are of particular concern in eDNA studies focused on the detection of rare or cryptic organisms. Therefore, quality controls are in place to ensure integrity of methodology, analysis of scientific samples and interpretation of data. Research programs will have various QA/QC methods based on the eDNA application, methodology, personnel, budget, and laboratory infrastructure. Good laboratory practices are well established for laboratory programs to adopt, MIQE guidelines have been established to standardize qPCR experiment reporting, minimum information standards and recommendations have been developed (Goldberg et al. 2016), and peer-reviewed studies are quickly advancing our understanding of 'best-fit' methodologies for specific eDNA applications. There is currently no standard for qPCR analysis for eDNA studies targeting dreissenids. eDNA projects may use different: detection techniques (SYBR or hydrolysis probe qPCR, loop mediated isothermal amplification), PCR mastermix chemistries, fluorophore used, PCR cycles, volume of sample, inhibition enrichment methods, qPCR/dPCR instrumentation, plate mapping

arrangements, positive controls, number of NTCs, number of technical replicates and protocols for interpreting data, etc.

Validation standards for eDNA assay development can vary from lab to lab.

Darling: I am certain that labs have differing QA/QC methods for these analyses, although I do not know the details of such differences. QA/QC methods described in the papers cited above differ considerably, with some reports offering little in the way of prescriptive QA/QC protocols.

Goldberg: I think so.

Snider: I cannot address what other labs are doing. Recommended QA / QC protocols in laboratories doing molecular testing are typically consistent, although there's no guarantee recommendations are followed. In the absence of oversight, the veracity of a result often depends on the diligence of the analyst.

Vargas: Not applicable

## Session Four: Interpreting Results

### What are the criteria utilized to identify a sample “positive” for dreissenid DNA?

Amberg: That varies depending on the research question that is being asked and this varies from lab to lab. For UMESC in a monitoring situation, it is only determined positive if it is sequence verified. But before sequence verification, we first run a sample in quadruplicate (2 uL each), with an inhibition test in triplicate. If one shows a sign of amplification it is re-run in octet (2 uL each). If the sample show signs of inhibition, it is cleaned (zymogen) and re-run. If one of the octet for the sample shows amplification, then we test is the a ZM and a QM-specific marker in quadruplicate (2 uL). If one of those species-specific markers amplifies something, we then have the product sequenced and compare that sequence to our target. Only if there is a match do we call it a positive.

Bajno: Extraction: Positive extraction control was confirmed, no qPCR amplification was observed in the extraction negative controls (field, filtration, DNA extraction).

qPCR: Expected results observed in PCR positive controls (e.g., standards curves). qPCR run parameters, when a standard curve is used meet QA/QC standards (slope, efficiency, R2, etc.). All NTCs are negative showing no amplification. Internal positive controls show no inhibition (< 1 Ct difference from IPC in standards) of PCR in eDNA sample replicates.

Each eDNA biological replicate (each eDNA filter) is run, at minimum, at three technical replicates. Field negative controls are also run at three technical replicates. Amplification is considered to have occurred when an amplification plot (for at least one of the two assays) is observed above the run's baseline (multicomponent plot are analyzed to determine if fluorescence is an artifact not associated with template amplification). Suspect positives are identified when amplification occurs in at least one technical replicate of the biological replicates taken at the sampling site. Each biological replicate taken at the sampling site is then subsequently analyzed at a minimum of eight additional technical replicates to confirm presence of target DNA. A positive amplification observed at a technical replicate (both assays need to be amplified during qPCR analysis, but not necessary in the same technical replicate) at more than a single biological replicate to be considered a putative positive and identified for further



investigation. A third eDNA assay can be used to confirm target DNA presence. Sanger sequencing is attempted on the suspected positive samples to confirm presence of zebra mussel DNA. A sequence associated with zebra mussel must be generated to deem the sample positive. An eDNA sampled location is not identified as positive until, at minimum, a second and third sampling event corroborates the results from the first analysis. If partner agencies are involved in surveillance using eDNA, temporal sampling of a suspected positive waterbody is done by each agency on separate occasions to ensure mechanisms of contamination are minimized and to strengthen evidence in results (collection bias, etc.).

Darling: I believe that “positive” is a semantic designation that must take into account the correspondence between a particular outcome of a detection protocol and a particular management attitude. This means that the term should be defined in the context of overall risk management, with consideration for the decision-maker’s tolerance to various types of risk. Generally, managers with low tolerance for false negatives (failure to detect a target when it is present) may consider even a single result above threshold detection limits sufficient to announce a “positive” detection. Others with low tolerance for false positives (detection of a target when it is actually absent) may require, instead, multiple confirmatory detections above threshold limits. This may explain why, in the literature, multiple meanings accompany the term “positive” detection. General considerations for defining positive detections are therefore: the type of assay and possibility of defining numerical thresholds for single reactions (e.g. cycle number counts for qPCR or quantification of band density for standard PCR), the number of technical replicates performed for each sample, the expected rate of false positive and false negative reactions, as well as other information associated with the costs of various management actions (including inaction on a false negative).

Goldberg: [The lab I know of uses conventional PCR and sequences everything. I don’t know if just one well testing positive is enough to call a sample positive.](#)

Snider: At CDFW a positive eDNA results would not trigger “formal” action. Three elements define a positive veliger detection:

- 1) Microscopic observation of the veliger
- 2) Amplification of the veliger’s DNA using PCR to confirm the presence of quagga / zebra mussel DNA
- 3) Sequencing of the amplified DNA and matching the sequence to reliable (voucher) target sequences.

Vargas: [I’m unsure if this is referring to the lab or management decisions.](#)

**Are there other species in MT waters that could produce positive results? Have all likely species that potentially could cause a false positive been evaluated (bivalves, other mollusks)?**

Amberg: With a single marker, possibly. But with multiple markers, and sequence confirmation, highly unlikely.

Bajno: [If an eDNA assay is not carefully designed, it possible false positive results can be observed due to cross-amplification of organisms not incorporated in the development process.](#)

Darling: I do not have the expertise to answer this question.

Goldberg: [This has to be evaluated for every assay.](#)

Snider: Yes, it's possible. The potential for cross-reactivity would have to be considered for each waterbody tested using an eDNA approach. Not only would each method have to be validated, but methods might require validation for distinct waterbodies. A sequential single-veliger approach selective eliminates potential contaminants thus mitigating cross-reactivity.

Vargas: [This topic needs additional research if eDNA is to be used as a reliable and early detection tool: given some of the false positive results that have been evaluated over the years, my general consensus is that there are physical and/or biological interferences occurring in laboratory methods that are leading to false positives or false negatives. For example, samples collected and analyzed in the early spring or late fall vs during the middle of summer; whereby the summer samples results are believed to be false positives and the early spring and late fall samples results are negative utilizing eDNA. The area needs further research.](#)

### **What, if any, biogeochemicals factors can influence the results of eDNA?**

Amberg: There are a wide variety of PCR inhibitors. Inhibitors block amplification of the marker and should be tested for. UMESC tests for inhibition by running separate reactions for each sample (in triplicate) with spiking 100 copies of targeted DNA into each reaction. After analysis, those samples should indicate that at least 100 copies of DNA are present in that sample. If less, we know there is some inhibition.

Bajno: [Inhibitory substances include polyphenols \(e.g., Tannic acid\), polysaccharides, humic substances. Factors that may inhibit PCR can be detected using spectrophotometric methods to assess DNA extractions and PCR internal positive controls \(IPC\) during the PCR process.](#)

Darling: Factors such as water temperature, pH, UV exposure and others can influence the persistence of DNA in the environment, and therefore the likelihood of a monitoring program detecting that DNA. There is a considerable literature on this topic, e.g. (Barnes and Turner 2015; Barnes et al. 2014; Goldberg et al. 2016; Pilliod et al. 2014; Salter 2018; Song et al. 2017). The presence of inhibitors can also influence the efficiency of molecular detection methods such as PCR, resulting in false negative detections (Trombley Hall et al. 2013).

Goldberg: [Some factors may inhibit PCR and limit or prohibit the detection of eDNA. Inhibition should be tested for using a positive control and clean-up steps can be applied. However, this also causes a reduction in sensitivity. Acidity can increase DNA degradation rate and reduce detection probabilities.](#)

Snider: Ambient water chemistry defines habitat suitability (for quagga and zebra mussels) and habitat assessment is required to inform monitoring efforts. So water chemistry might be used to decide whether a waterbody is monitored...or not. Chemicals in the water might directly influence results by inhibiting DNA amplification. Water chemistry and temperature might also influence the persistence of DNA in the environment.

Vargas: [The laboratory scientists would be much better at answering this, however, the uprising of sediments and particles can interfere with water chemistry analysis and thus one can assume](#)

sediment can also interfere with eDNA analysis. In addition, sediment can many times act as a binding mechanism making eDNA unavailable for detection in the water column, however, when sediment is disturbed those particles can be released into the water column (Hg etc.).

**Is it possible for other species (blue-green algae, protists, other zooplankton) to cause a false positive?**

Amberg: If markers are not designed well and the assay is not optimized, one might get amplification of a marker. The probability of this occurring with a second marker would decrease. If you sequence product, you can verify that it is positive or if it is more likely something else. Additionally, eDNA should be used as one line of evidence and that trends in the data should be evaluated rather than simple snapshots.

Bajno: Depending on the specificity of an eDNA assay, there is always a chance that non-targeted organism are detected.

Input of large amounts of DNA by these abundant organisms into the eDNA workflow has the potential to saturate laboratory methods, possibly contributing to false negatives due to the loss in the ability to detect rare nucleic acids.

Darling: It is always possible for other species to cause a false positive. Phylogenetic relatedness is a guide to determining what species might have to be screened for possible cross-reactivity of probes, but it is not absolute. Convergent molecular evolution can result in distantly related species that possess probe binding sites similar enough to result in false positive reactions. In silico approaches can be used to more broadly determine if such species exist, but even these approaches are limited to existing sequence data. As a matter of principal it is probably a good idea to test probes on water samples similar to the target water body (i.e. likely possessing similar biotic communities) but known not to be invaded by the target species. In practice this approach may prove difficult, and it still does not guarantee the absence of any non-target species that might cross-react with the probe. Fortunately, this type of false positive error can be addressed often by sequencing amplification products.

Goldberg: Only if the assay is not designed and validated adequately.

Snider: It's possible. Specificity would be one component of method validation. The use of a sequential single-veliger approach mitigated specificity issues.

Vargas: As previously mentioned, this area needs additional research to be conducted to fully understand the interferences with eDNA detection of mussels and/or the laboratory methodology needs to be further researched.

**Using eDNA methods, what factors can produce false positives and how is that addressed? What additional steps are taken with samples to verify detections are not false positives?**

Amberg: a percentage of the positive amplifications should be sequenced.

Bajno: Low specificity of primers and probes. Investigations examining the specificity using in-silico, in-vitro and in-situ methods are required. Frequently, eDNA assays are designed to address local regional research objectives. Although of greater concern for indigenous species than alien species, the use of pre-existing assays for new applications, or more importantly in

new environments, may contribute to false positives. These assays must be revalidated to ensure that the presence of DNA sequences of organisms that may not have been assessed during initial assay design are not detected.

Risk of contamination through laboratory processes is of significant concern. Lab spaces are designed to compartmentalize and isolate the various procedures (e.g., clean room, extraction, PCR) in the eDNA processing workflow to minimize cross-contamination. Rigorous training in good laboratory practices are in place and standard operating procedures are strictly followed detailing instructions for storing, handling, and processing samples, maintaining a clean laboratory environment and equipment. Positive and negative controls are used throughout the eDNA workflow to ensure protocols are working as intended and contamination if present can be identified and tracked.

Cross-contamination during laboratory processes (see above section re: identification of field/lab contamination)

Allochthonous DNA and use of improperly sterilized field sampling gear can contribute to falsely detecting targeted organism when they have never been in the water system. In addition, the resuspension of old sedimentary eDNA can produce false inferences of presence after organisms are gone. Temporal field sampling design, increasing the number of biological replicates taken and additional spatial sampling can provide input into the validity of the false positive observation.

The use of laboratory qPCR technical replicates, detection at different assays (multiplex approach) and Sanger sequencing or metabarcoding techniques can strengthen the evidence for the presence of targeted DNA.

Darling: False positive detections can result from two possible sources. First, the molecular method may report a positive detection when, in fact, there is no dreissenid DNA in the sample. This can result from either contamination of the sample or from spurious amplification of non-target DNA. Second, it is possible for the sampling protocol to produce positive detections of dreissenid DNA despite the fact that there are no living dreissenids in the sampled system. This may result if there are alternative exogenous sources of DNA not associated with live mussels. I suspect this is considerably less likely for mussel monitoring than it is for monitoring of other target organisms (e.g. fish). It could result, for example, from contamination (i.e. sampling crews accidentally introduce DNA to the system), or possibly sources such as mobile fauna capable of excreting mussel DNA (e.g. fish and birds).

False positives of the first type can be sometimes ascertained through additional analysis, most specifically by sequencing amplification products if possible or by examining results of appropriate control reactions. False positives of the second type may be more difficult to identify; it is likely that the likelihood of their occurrence will have to be considered within the overall sampling scheme and interpretation of results.

(Darling and Mahon 2011) provides a useful typology of potential errors associated with DNA-based detection methods, and the various options available for addressing those errors.

Goldberg: False positives can be true detections of DNA that are not from live specimens (“zombie DNA”) or can be due to the introduction of DNA during the sampling process (contamination). There are extensive protocols for preventing contamination (e.g., see the QAPP). But interpreting a true detection of DNA that is not from live individuals is more difficult.

Snider: False positive can be attributed to the amplification of non-target DNA. False positives can also be attributed to contamination. Contamination might occur in the laboratory, in the

field (contaminated equipment), or in the environment if target DNA were somehow introduced. Method specificity is a component of method validation. QC and QA activities may or may not be able to prevent or detect contamination. In some cases, it might not be possible to differentiate a false positive from a true positive. Consider a scenario where dreissenid DNA present in the environment is detected, yet living veligers and adult mussels are absent. This would qualify as false positive, assuming the default hypothesis is the absence of living animals. The dilemma is that the eDNA assay performed as intended. This is another reason CDFW favors a sequential approach, using microscopy and PCR supportively.

Vargas: False positives are the weakness link to utilizing eDNA analysis: false positives lead to managers questioning the entire process from field sampling to laboratory analysis: why collect eDNA when false positives are assumed to be continually collected? In order for eDNA to be utilized as a truly early detection tool then both the laboratory (interferences/contamination) and field methodology (depth, timing, contamination) and protocols needs to be addressed. False positives are un-verified by additional sampling at a different time: increase in sampling sites and number of times: basically finding negative samples after finding positive samples. In addition, the use of microscopy and physical surveys of the suspect waterbody. However, the intent of utilizing eDNA is as an early detection tool rather than finding physical evidence of mussels.

#### **What is a “weak positive”, what does that mean and how should managers respond?**

Amberg: A positive is a positive, but much can be inferred from the number of replicated are positive and how many samples are positive. Some water samples can have all of the replicates amplified and all the water samples from that waterbody have positives. Other waterbodies may have one replicate amplify for a single water sample and all other water samples were found to be negative. Since “weak” is a relative term, one might consider the latter scenario a weak positive.

Bajno: Low proportions of laboratory technical replicates are considered positive. Biological replicates show inconsistency in detection of amplification (e.g., only a portion of field replicates yields positive PCR signals). Amplification plot shows up late in the thermocycling process (i.e., high Ct near or above 40 cycles) but above baseline. Detection of target DNA across temporal samples is reproducible but frequency of detection in technical replicates is inconsistent. Can be an indication that the abundance of the targeted organism is small and not well distributed in the water system (i.e., early stages of invasion). Results should be carefully interpreted. Follow-up temporal sampling at the site of detection is advised. Follow up with supplemental in-situ investigation sampling waterbodies of similar properties but know not to contain zebra mussel to provide possible evidence that the eDNA assay is not cross-amplifying a non-target organism. Given the challenges associated with contamination, PCR inhibition, species specificity, and cross-amplification of non-target organisms, it would be valuable to follow-up with an independent validation of test results at a different eDNA laboratory.

Darling: Please see above answer about “positive” detections. As these designations are semantic signals of management attitudes, I would recommend avoidance of terminology that is overly evocative or otherwise has the potential to confuse end-users or the public. I believe that

“weak” is an example of such terminology. Typically, “weak positives” would be considered results that are either barely above pre-determined detection thresholds, or are poorly replicable. However, it is also possible that a manager might consider a “weak positive” to include a strong signal detected only once in a water body and not confirmed by additional sampling. My recommendation would be to develop a tiered system that connects specific quantifiable endpoints of molecular methods with specified management attitudes, and to adopt neutral terminology to designate these tiers (e.g. a numbered or lettered system).

Goldberg: This means there was a very small amount of DNA of the target species in the sample. This should be considered a potential positive and additional testing (eDNA and/or field) should be conducted to confirm presence at the site.

Snider: Typically, “weak positive” refers to a faint DNA band on a gel, or perhaps a low DNA copy number using qPCR. “Faint” and “low” are subjective. Labs reporting results as “unconfirmed positive” or “positive with non-sequenceable DNA” are eluding to a weak positive. A result is either “positive” or “not detected” = present or absent. Labs should not be reporting “weak positive” results, as the language invites confusion and doubt. Labs should be confident in the results reported. However, sometimes there is ambiguity. In such situations labs should be working directly with managers to explore possible explanations.

Vargas: A weak positive should be addressed by additional sampling. I have a true dislike for the term “weak positive”. A “weak positive” should potentially be called a “weak negative” or “questionable result” not a “weak positive”. Managers are at a loss on how to deal with this term.

### **Using eDNA methods, what factors can produce false negatives and how is that addressed?**

Amberg: Inhibition (tested for), sensitivity of the markers (can be demonstrated) and sampling design and under sampling (needs research).

Bajno: eDNA field sampling design and field methodology QA/QC. Lack of appropriate number of replicates taken, volume of water sampled, contamination by nucleic acid degrading material can affect the success of capturing rare eDNA. Careful sampling design maximizing the number of samples taken for the targeted waterbody carefully considering contamination factors and mechanisms. Temporal sampling strategies increase chances of detection.

Assay design with low specificity, inconsistent PCR efficiency, and level of detection is high. The use of multiple markers (e.g., multiplex reactions) to increase robustness of eDNA assays can be implemented in laboratory methodology providing additional opportunities for detection should one marker fail.

Loss of DNA in laboratory processes. Strict QA/QC standards, rigorous training and appropriate infrastructure to carry out the work in a clean environment minimize possibilities of sample degradation. The use of positive controls throughout the eDNA workflow provides assurance in laboratory methods and provides the ability to identify procedural DNA loss.

Amplification of target DNA is impeded due to the presence of PCR inhibitors. Internal positive controls (IPC) should be utilized in PCR methods to identify possible inhibition. Alleviating inhibitory effects can be achieved by further processing samples using a variety of techniques.

Darling: As above for false positives, there are two types of false negatives. The first occurs when the molecular method fails to detect the presence of dreissenid DNA in a sample. This could occur if samples are poorly handled and DNA degrades or is lost prior to analysis (solution: proper sample handling and processing protocols), or if DNA occurs below the threshold of detection of the method (solution: develop more sensitive methods or design sampling strategies capable of capturing higher concentrations of target DNA). The second type of false negative occurs when there is a failure to sample target DNA despite the presence of live mussels in the sampled water body (solution: design sampling strategies with higher likelihood of capturing DNA from rare mussels).

Goldberg: eDNA is patchy and stays fairly close to organisms, so surveys should be designed to cover as much area as possible and be conducted at the right time seasonally to maximize detection of target organisms. Additionally, inhibitors can cause false negatives (these are tested for with internal controls). Lab methods need to be optimized to make sure the assay doesn't test negative on some genetic variant in the system and that extraction and amplification methods work. Positive controls are included for every reaction to confirm the success of the amplification. Assays validated with the three-step process detailed in the critical considerations paper should test positive when the DNA is in the sample.

Snider: False negatives can be attributed to the inability to detect target DNA that exists in a sample (e.g. sub-optimal DNA detection protocols), DNA degradation (short residency time), or ineffective field sampling. Given that "absence of evidence is not evidence of absence", false negative results can be intractable. CDFW reports results as "not detected" instead of "negative". To reduce the likelihood of a false negative result all aspects of the testing process, from field collection to the evaluation of amplified PCR product, need to be validated. Increased sampling frequencies can also mitigate the impact of false negatives. Everyone should be mindful that negative results, whether true negatives or false negatives, do have management implications.

Vargas: I'm assuming that biological and/or physical attributes in the water column can also create false negatives as I believe they have the potential to create "false positives".

## Session Five: Management Implications

**With regards to the use of eDNA for early detection of dreissenids, what are the communication barriers between researchers and managers? What are the communication barriers between managers and the public? Do you have recommendations on ways to overcome those barriers?**

Amberg: A clear communication plan should be established prior to sampling. It should state what management agency will do if a positive is detected. An example of one is in the FWS QAPP (link provided above)

Bajno: There is a need to develop consistent approaches to increase communication between researchers and managers so as to continually improve upon the interpretation of results and delivery of information and advice as the field grows. This will contribute to researchers

understanding managers' needs, managers understanding the fundamentals of the science and what it means, identifying communication channels and effective but efficient program delivery.

Darling: Researchers need to understand that managers are tasked with utilizing data to make decisions, and that such decisions will likely be costly and will therefore be scrutinized. Managers are therefore very keen on certainty, which is something that researchers may not even believe exists. Gaps in communication between these two groups thus often center on uncertainty. Researchers understand implicitly that there is uncertainty associated with their measurements, but may not understand the importance of being able to quantify that uncertainty clearly enough for managers to make decisions. Having observed a particular pattern of eDNA detections, it is not enough to identify the possible sources of uncertainty—it is necessary to determine in some way how those various sources of uncertainty affect the confidence that one has in the answer to the question everyone is asking: Is the target species present in the water body? So while managers must become comfortable dealing with information that is inherently and unavoidably uncertain, researchers must become comfortable with ways to quantify that uncertainty such that it can be communicated clearly to managers and other stakeholders.

I suspect that there is also some disconnect in terms of the focus on either false negative or false positive detections, with researchers being far more concerned with the former and managers more sensitive to the latter. This translates into a differential tolerance for error. This also becomes critically important in communication with the public. I suspect that the public will also be quite sensitive to false positive detections, as these threaten to impose burdens that seem avoidable. But the public must be made aware that burdens associated with false negatives may similarly be avoidable, and that the cost of false negatives may, in some cases, be considerably higher than the cost of false positives. Monitoring methods that are highly sensitive are likely to have higher false positive rates, and decisions to use such methods must be made in the context of risk management knowing the costs associated with management actions. Parallels are easily drawn from medical monitoring. Would you use a highly sensitive method to test for a disease if it meant a better chance at early intervention but also a higher likelihood of a false positive? It probably depends a whole lot on both the damage done by untreated disease and the damage done by treatment in the absence of disease. Generally, I believe that transparency is an antidote to suspicion and distrust. Clearly communicating uncertainties along with the risks (especially costs, if they are known) of error should help all stakeholders understand the value of particular monitoring tools.

Goldberg: [Others on the panel have much more experience in this area for invasive species. What I've experienced is that communicating uncertainty is key to acceptance of eDNA techniques. When managers expect results to be absolute, ambiguous results can be frustrating and may cause them to want to reject the whole method. It is important to communicate the level of evidence that eDNA results provide as to the presence of a species and to have plans for ambiguous or weak results.](#)

Snider: Researchers need to explain the limitations of eDNA monitoring to both managers and the public. Laboratories need to report results in a way that managers can interpret. Managers need to educate themselves as to the basics of how testing is done, so that they are in a position to ask important questions when positive results are reported. Managers need to communicate with testing laboratories routinely to make sure expectations are met.



Vargas: Communication barriers between researchers and managers:

- Understanding and using the term “weak positive”.
- Why can’t the field and laboratory methodology be agreed upon?
- Why can’t the laboratory analysis be “certified criteria” such as that utilized in water chemistry?
- Understanding that routine monitoring (collecting samples) for the utilization of eDNA cannot be such stringent criteria that entities are unable to collect samples.

Communication barriers between managers and the public?

Trying to explain to the public what eDNA “false positives and/or weak positives are” leads to a distrust for both the science and the agency: at this point in the eDNA research, I would not recommend that positive eDNA results be released to the public without evidence of a body and then only after verification has been performed with repeat sampling.

In my experience we have seen multiple samples collected and test positive utilizing both microscopy (veliger detection) and eDNA only to have multiple repeat sampling in a short time period performed with all results negative (microscopy/eDNA). Years later the results are still negative. If the samples were truly positive to begin with, the secondary set of samples should also have been positive (collected within a 10-day period following the 1st set of samples in relatively non-disturbed waters e.g. stable lakes). The only explanation is field and/or laboratory contamination of the samples.

Follow up verification and then additional follow up verification is the only way to truly address this topic. Results should not be released to the public until multiple verifications have been performed and never on just the eDNA results only. The implications of releasing initial positive results to the public without follow up sampling has resulted in numerous, strict and unnecessary watercraft decontaminations and a distrust by the public for science. A majority of the public wants to do what’s right, however, if they are led astray they will question the authority and the science behind it.

**What key criteria do researchers need to demonstrate to managers to allow for eDNA to justifiably be accepted as an operational method for dreissenid early detection? Have these criteria been met with dreissenid eDNA?**

Amberg: Demonstrate that eDNA is equal to or better than traditional methods for detection. Advancements in this area have been made over the past few years, but work is still needed.

Bajno: eDNA researchers need to demonstrate that eDNA methods are reliable and reproducible even when significant variation exists among studies across various geographical areas (e.g., physiological sources of eDNA production, movement of DNA in the environment, factors influencing eDNA persistence and degradation).

eDNA assays are validated and QA/QC programs are in place to ensure integrity of eDNA studies.

Laboratories have the capacity and expertise to perform the work and have the resources available to meet client and stakeholder needs.

Although not required, empirical evidence that eDNA was successfully used as the earliest detection tool to identify the presence of zebra mussel in the natural environment would help in building confidence in the tool.

Other than having facilities in place to perform eDNA work, the above criteria remain research objectives for dreissenids. However, a significant amount of research has been performed on eDNA science across applications and taxa, most of which are transferrable to dreissenid studies.

Darling: Criteria would include demonstration of standard forensic QA/QC protocols, communication of known uncertainties (e.g. false positive and false negative rates), comparisons in sensitivity and specificity with existing detection methods, comparisons of cost and analytical turnaround time with existing detection methods, and demonstration that tools work consistently under a variety of challenge conditions. I believe that these criteria have only partially been met with existing eDNA methods.

Goldberg: The total workflow would need to be validated – where and how many samples are collected, if water is pumped continuously or taken as grab samples, filter type, volume sampled, preservation, extraction method, analytical methods, data interpretation. This would need to be done in systems at very low levels of infestation to demonstrate the protocol effectiveness. I do not know the status of dreissenid eDNA monitoring programs with respect to this validation.

Snider: In addition to ensuring the veracity of a result through method validation and appropriate QA / QC measures, researchers also need to demonstrate a relationship between a positive eDNA result and the presence of living mussel larvae and adults. Currently this relationship has not been established.

Vargas: No, I don't believe that the criteria has been met for identifying a waterbody as positive based on eDNA results only. However, eDNA does seem to support the identification of a veliger identified utilizing microscopy.

**What responsibilities, if any, do researchers have when reporting/explaining results and how those may affect management issues/management implications?**

Amberg: Researchers should only report the results and clearly explain their QA/QC to management.

Bajno: When using eDNA for early detection, researchers need to carefully report the strengths and limitations and acknowledge the challenges of the method as a surveillance tool. Results should be transparent. All evidence should be reported so that a well-informed management decision can be made. Managers should be informed on project progress with results made available as soon as possible should suspected (not necessarily confirmed) detections be observed.

Darling: Results of eDNA detection methods should be quantifiable and as free from observer bias as possible—i.e. if standard PCR is adopted as a detection method, the presence of bands indicating detection should be determined not “by eye” but by image analysis that allows objective comparison of quantitative data across multiple tests. Preferable would be quantitative PCR approaches. More importantly, I think that results of eDNA detections should be delivered to managers only after they have been briefed extensively on both the capability and the limitations of eDNA protocols, including any quantified uncertainties (e.g. false positive

and false negative detection rates, if known), and after those managers have made the decision to consider eDNA detection evidence given those limitations. Communication of uncertainty after the fact is a recipe for confusion and mistrust. It is the responsibility of researchers to ensure that the communication has occurred and been effective prior to commencement of any eDNA detection program.

Failure of these communications is likely to result in conflicting views on the role of eDNA detections in any overall monitoring program, and may lead to rejection of eDNA detection as a useful tool by at least some managers. Such rejections are more likely in cases where there is a disconnect between managers' expectations and reality in terms of the tool's utility and associated limitations. The goal of briefing managers prior to implementation of a eDNA monitoring program is to limit (or eliminate) the opportunities for that disconnect to arise.

Goldberg: Researchers should explain sample results thoroughly, but after the workflow has been validated, there are only a few outcomes for a sample (e.g., negative, strongly positive, weakly positive). Quantitative results can be reported but they rarely impart meaning over these categories when the goal is early detection. Inhibition that was cleaned up or diluted should be reported because sensitivity may be reduced. Interpretation of what the results mean to management probably is best done on the agency side by someone that understands the potential sources of error as well as the implications.

Snider: There is difference between eDNA research and providing eDNA results to inform decision making. Currently, there seems to be a buyer beware relationship between laboratories providing results and managers using results to make decisions. Laboratories need to provide unambiguous results that are easy to interpret. Laboratories need to be honest about the limitations of the analysis. Laboratories need to be honest about any QC / QA issues, for example contamination. Laboratories also have an important role in quality control. Samples that do not meet standards must be rejected. However, lab personnel are not obligated to explain the potential implications of a decision to the people who are making the decision. Lab people aren't usually in a position to fully understand the implications of a management decision. And, managers too have a responsibility to drive the dialogue and facilitate communication.

Vargas: The short coming of utilizing eDNA need to be continually discussed between managers and researchers.

**If a management agency receives “positive” eDNA result, is there a suggested management response to these results?**

Amberg: No, this is a management decision and should be included in the communication plan.

Bajno: Only with careful validation and interpretation of results, should eDNA technology contribute significantly to management issues. Continued and expanded follow-up testing to monitor presence (including surveillance for physical specimen) within the waterbody. Perform risk assessments to identify and minimize (mitigate) risks for geographic spread (e.g., ensure decontamination stations are available and staffed, limit access to waterbody if possible).

Darling: As I stated earlier, I believe that management attitudes should be attached to particular outcomes of eDNA detection in a tiered system that recognizes both the weight of evidence for infestation associated with particular patterns of detection as well as the risk tolerance profile of the managers. Those management attitudes could include heightened follow-up monitoring efforts, quarantine of a water bodies, or even attempts at eradication. I don't feel comfortable recommending which of these attitudes ought to be associated with a particular pattern of eDNA detections, as I am not familiar with the approach to risk management in Montana.

Goldberg: [I'm going to leave this to those with more experience in this area.](#)

Snider: A positive eDNA result should not trigger regulatory action, nor should a positive eDNA result be met with dismissiveness. There should be follow-up sampling, assuming resources are available. All data, information, and best available science should be taken into consideration in an attempt to assess both the accuracy of the result and potential impacts. With regard to dreissenids in particular, ecological factors, such as ambient calcium concentration, should be given consideration.

Vargas: [As previously mentioned, repeat sampling and verification should be conducted. In addition, having another lab retest a positive result can also be helpful.](#)

## Session Six: Next Steps

### **What further information is needed to increase the comfort level of managers to more broadly accept eDNA as a dreissenid early detection tool?**

Amberg: Demonstrate that eDNA is equal to or better than traditional methods for detection. Advancements in this area have been made over the past few years, but work is still needed.

Bajno: [Most of the eDNA research done to date is of investigating the utility of eDNA as a supplementary tool to existing surveying methods in an attempt to generate evidence and develop confidence in the molecular tool. The evidence is considerable, that eDNA when used carefully, can be a valuable monitoring tool. Standardization of eDNA methods and inter-lab participation in validation studies, will build confidence in the use of eDNA as a tool for monitoring organisms of management concern. In its current state, eDNA should be integrated into existing monitoring programs to establish baseline datasets. Unfortunately, to decisively acknowledge that eDNA is the earliest method for aquatic invasive species detection, physical specimens would need be captured after eDNA detection. In geographic regions not invaded by dreissenids, intensive efforts using conventional surveying techniques should be executed to follow-up positive eDNA detections.](#)

Darling: Managers need to know that eDNA tools are reliable across various challenge conditions and that they are reproducible across laboratories. They need to know how to translate patterns of detection into likelihoods of the presence of live mussels; this requires information on the fate and transport of eDNA in the system of interest and information on the possibility of exogenous sources of eDNA, as well as knowledge of the uncertainties associated with the molecular tool (false positive and negative rates). They need to know how the sensitivity, specificity, analytical turnaround time and cost of eDNA tools compares to those for alternative tools.

Goldberg: [A plan of how to deal with uncertainty.](#)

Snider: Like any tool, eDNA monitoring has limitations. Managers have to be aware that an eDNA approach can only detect target DNA. A positive result does not necessarily mean that the target animal is present in the environment. A negative result does not necessarily mean that the target animal is absent. There is a risk of allocating resources in response to a positive eDNA result if the target animal is absent. Managers need to acknowledge these limitations. Researchers need to address the concerns regarding the accuracy of results. More specifically, there needs to be a clear connection established between a positive results and the presence of living mussels or larvae.

Vargas: [Additional research is needed for laboratory interferences. Standardized protocols for sampling, preservation, and laboratory testing.](#)

**Is it possible to have a national accreditation for eDNA labs? Is it possible to have a lab certification program for labs that conduct eDNA analysis?**

Amberg: This has been suggested before and it is possible, but the “who” does the accreditation has never been identified.

Bajno: [Given that variation in eDNA methodology can affect our ability to compare results and inferences among different laboratories, guidelines for validation and interpretation of eDNA data, and studies, should be developed. It is possible to have a national accreditation for eDNA labs \(e.g., ISO17025\) but most likely unnecessary. Purpose-built dedicated eDNA facilities, with consistent quality control protocols are already in service around the world \(e.g., Centre for Environmental Genomics Applications \(CEGA\), Newfoundland and Labrador; Spygen, France; Whitney Genetics Lab, Wisconsin\). Accredited labs working in regulatory programs are well staffed and have consistent resources to ensure QA/QC standards are met, a situation that smaller regional multidisciplinary facilities may not share. The mandatory use of accredited labs can be problematic in an environment where interest in eDNA research is increasing at an alarming rate but accreditation of laboratory facilities slow and complex. Thus laboratory certification programs could be valuable and would ensure that the work being performed in laboratory facility meets standards developed and approved by the expert community. Certification can allow flexibility of research programs to engage in a variety of eDNA studies.](#)

Darling: There is no reason this shouldn't be possible. In fact, I would think it should be possible to develop accreditation standards for laboratories that enable them to conduct eDNA analysis generally for any target organism. The QA/QC standards are likely not much different from existing standards for forensic DNA analysis of any environmental samples in any context. Guidelines based on those existing standards could probably already be determined, if they haven't already. As eDNA becomes a more widely adopted method I expect that we will eventually reach a critical mass at which such accreditation will become clearly desirable and probably necessary.

Goldberg: [Yes, but it would take funding to implement. Many research labs would not meet necessary criteria and certification would raise per-sample costs. But it is likely necessary for](#)

quality control if agencies don't have their own in-house lab. Even for those, it would be helpful to have a global list of guidelines, which we tried to do in the critical considerations paper.

Snider: I don't think accreditation and lab certification programs are feasible at this time. Such programs would require an agreed upon "gold standard" method, and currently no such standard method exists for the detection of dreissenid eDNA.

Vargas: *At some point in the future, this should be a requirement to assist managers in knowing that the samples were tested utilizing the best available methodology. However, we are not yet to that point in time.*

**Could researchers develop criteria that could guide managers how to review and evaluate eDNA results? What would be included in a checklist guide that would ensure all factors are assessed and considered to ensure confidence in results?**

Amberg: Goldberg et al. (2016) provides some guidance on the minimum for publishing eDNA studies and is a good starting point.

Bajno: *This is challenging activity requiring participation and development from both the research and manager groups. As an example, Goldberg et al. 2016 have developed a 'framework' for eDNA researchers which can be modified to include a management perspective.*

Darling: Yes, I believe it should be possible to do something like this. First, I believe that confidence in eDNA results derives from development and demonstration of rigorous QA/QC protocols and through transparency regarding the limitations of the method (e.g. clear communication of uncertainties like false positive and false negative rates). Moreover, I think researchers could help by developing tools that seek to interpret eDNA detection results in the context of other relevant information about a system. This is probably already done informally, but I have not seen it done formally. For instance, managers probably have some sense of the likelihood of a water body being contaminated based on habitat conditions and the connectivity of that water body to possible sources of invasion. For example: an unexpected detection of zebra mussel DNA in Canada occurred at a location likely at high risk of invasion based on recreational activity; both sources of information on the likelihood of zebra mussel presence were informally utilized to judge the location likely to be invaded, and adult mussels were later found at the site (Gingera et al. 2017). There may also be available evidence on prior sampling (either by eDNA or traditional means). I believe it may be possible to incorporate such information into a formal inference framework to help managers understand how a particular eDNA detection pattern translates into an overall likelihood that a particular water body is invaded.

Factors that should be considered in evaluating eDNA results should include: known uncertainties associated with the method (e.g. false positive and false negative rates) and the overall sampling effort (e.g. likelihood of exogenous eDNA, factors that may limit ability to sample existing eDNA); QA/QC protocols of responsible testing laboratories; information to contextualized observed detection patterns (e.g. results of previous monitoring, known or estimated invasion risk, etc.); any known variation in specificity or sensitivity associated with environmental conditions.

Goldberg: *Yes, but it might be fairly general unless it was for a certain system.*

Snider: There have been attempts to provide guideline. Any related checklist would have to cover every step of the analysis from sample collection to the reporting of results. The eDNA community should work with managers to construct such a checklist.

Vargas:

- a. Factors influencing bio/physical interferences in analysis would greatly assist the managers in making decisions.
- b. Additional sampling is a necessity to verify any initial positive results.
- c. Managers need to be able to trust the laboratories to perform adequate and proper eDNA analysis: this would occur when standardized laboratory and sampling methodologies have been developed and peer reviewed.

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#### **Additional feedback following the event:**

From John Darling

On my travels home I was thinking about our discussions, and I realized that there's a problem with the way a lot of people are using the term "false positive." I think that this may be contributing to confusion and mistrust among managers and the public. It seems to me that the tendency is to use the term "false positive" to describe any positive DNA-based detection that is not confirmed by other evidence (e.g. veliger or adult mussel detection). I strongly believe that this is incorrect. The term "false positive" should be reserved for cases in which there is a positive DNA detection when there are no living mussels present in the water body. You will notice that this is actually an extremely difficult state to ascertain, because it requires strong evidence of the absence of living mussels, and not just the presumption of their absence. Nevertheless, I believe it is possible to identify cases in which it is likely that there are

true false positives. Most of these will be encountered when there are known QA issues. For instance, in one of Karen's Nevada examples I think that there was reason to believe that contamination may have been the source of DNA since a field crew had moved directly from an infested water body. Or there may be a situation in which negative controls come back positive. In such cases, I think it is reasonable to talk about "presumed false positive" detections, based on the QA evidence. However, it would be wrong and very misleading to use the term "false positive" to describe cases where DNA-based detections cannot be confirmed by other means, assuming all QA standards have been met. This would be true even in cases where those detections are ephemeral, e.g. observed in one season and not observed again over the course of many years, or occur on water bodies presumed to be uninfested. (As an aside, I would say the same thing about microscopy evidence; assuming appropriate QA standards are met, a one-time single veliger detection should not be considered a "false positive.") I would much prefer to describe these cases as "unconfirmed positive detections," or something like that. I understand that in the recent past there has been so little trust in the DNA methodology that the tendency has been to think of any unconfirmed positive as a true false positive. However, there is an ENORMOUS difference between a "presumed false positive" (which implies that something went wrong with the method) and an "unconfirmed false positive" (which is simply recognizing the uncertainty associated with detections at low target densities). I think that most people, when they hear "false positive," are going to assume that the method has failed, and so continued use of this terminology to broadly refer to all unconfirmed detections could be disastrous for acceptance of these tools. If someone can develop protocols that we have reason to trust in the field, it's very important that we change the way all stakeholders think and talk about false positives.

On second thought, I also don't quite like the term "unconfirmed positive detection." That makes it sound like we don't really know if it's a positive DNA detection or not, which is not true. If I'm going to continue to dream of a world in which DNA evidence is sufficient to trigger management decisions, then I should advocate for language appropriate for such a world. If we trust the DNA method, then we shouldn't need to "confirm" a positive detection with alternative evidence.

So I guess I would propose the following language:

- False positive detection = QA flag has been thrown, and we can track down a specific source of error, e.g. we can determine that there was contamination of our samples or sequencing of a PCR product confirms non-target amplification
- Presumed false positive detection = QA flag thrown, but we can't track down the specific source of error, we just have some reason to not entirely trust the positive detection
- Positive detection = all QA standards have been met

Given these categories, I would classify many of the problematic detections that Karen described from Nevada as "presumed false positives" given the QA issues that she raised. If her theory is correct and there is non-target amplification, and if this were confirmed by sequencing, then these would change to "false positives."

Note that these categories imply something important about reporting results: It is absolutely essential that labs report any false positives AS false positives. In other words, if a lab runs some samples and a QA flag gets thrown that indicates the likelihood of false positives, the lab can't just throw those data out and not report them. Similarly, if a positive detection is later shown by sequencing to result from

non-target amplification, that should be reported as a false positive and NOT as a negative. This is the only way that we would be able to get a sense of the overall rate of false positives in the monitoring program. Maybe this is just stating the obvious, but I think it's important.

I'd be interested to hear anyone else's thoughts on this terminology. I'm not sure exactly how best to convey all of this, but I'm pretty sure that the way people use the term "false positive" now is eroding confidence in the method.