BUREAU OF RECLAMATION

Assessment of Laboratory Methods to Investigate Dreissenid Mussel Veliger Settling in Imhoff Cones Through Dense Organic Material

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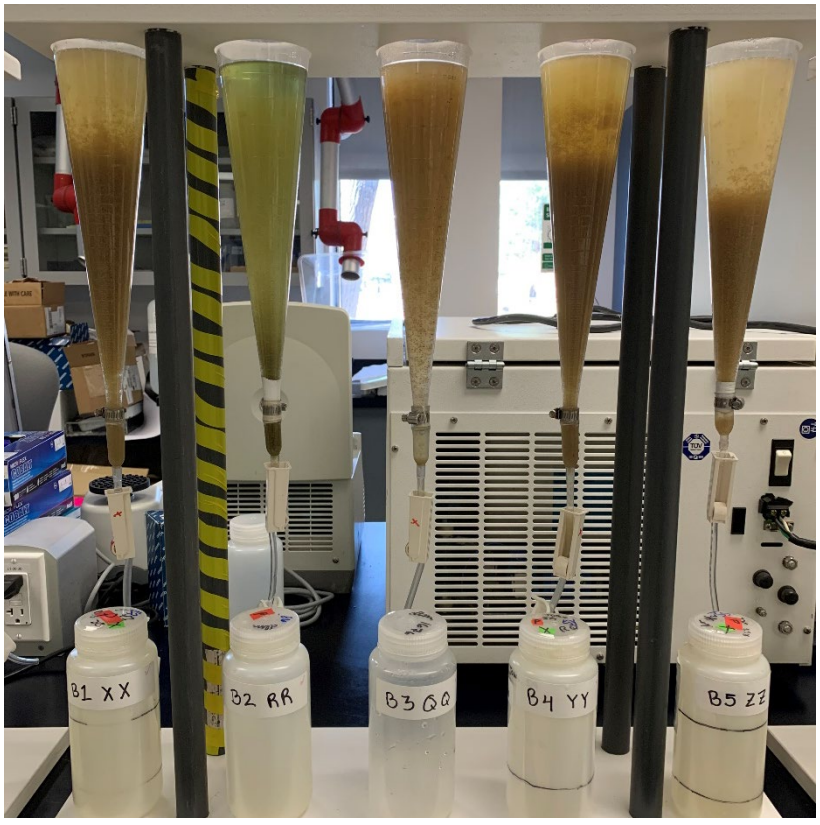
Research and Development Office
Final Report No. ST-2021-21083-01
EcoLab-X1083-2021-07



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14. ABSTRACT

The Bureau of Reclamation Ecological Research Lab utilizes Imhoff cones to settle water samples specifically collected to analyze for invasive dreissenid mussel larvae. Samples are settled, and the heaviest (bottom) 15 ml are collected and analyzed under a microscope looking for the larvae. Previous research claimed that veligers present in a water sample would settle at the bottom 15 ml. This study examined whether veligers would still settle in the bottom 15 ml through water samples that are thick with different types of suspended solids like zooplankton and algae. This study found that suspended solids, or organic material, and the amount of that material, in water samples does interfere with settlement of veligers in the heaviest 15 ml.

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Acronyms, Abbreviations and Definitions

CPLM	cross polarized light microscopy
DI Water	deionized water
Eco Lab	Ecological Research Laboratory
eDNA	environmental DNA
Reclamation	Bureau of Reclamation
TSC	Technical Service Center
TSS	total suspended solids
qPCR	quantitative polymerase chain reaction
SOP	standard operating procedure

Measurements

C	Celsius
g	gram
L	liter
mg	milligram
mL	milliliter
mM	millimeter
µM	micron/micrometer/one-thousandth of a millimeter

Contents

Page

Executive Summary

The Technical Service Center's Ecological Research Laboratory (Eco Lab) receives over 1500 plankton tow net samples each year as part of its early detection and monitoring program for invasive dreissenid mussels. Invasive dreissenid (quagga and zebra) mussels are harmful to Reclamation infrastructure and facilities, in addition to the ecosystems they inhabit, and recreation. Therefore, early detection and monitoring for their presence is important.

The analysis process outlined in the Eco Lab's Standard Operating Procedure (SOP) relies on the expectation that any mussel larvae (veligers) present in the plankton tow samples will settle in the heaviest/bottom 15 mL of an Imhoff settling cone (see Figure ES-1). Many of the samples received by the Eco Lab contain dense suspended solids including phytoplankton, zooplankton, and/or algae. The Eco Lab had conducted research in 2012/13 that concluded 98 percent of veligers in a sample would settle in the bottom 15 mL of an Imhoff cone. Details about this work are no longer available. It is unknown how many veligers were used in those test samples, or if the water samples used contained any sort of algae, zooplankton, phytoplankton, or other types of suspended solids. For the early detection of invasive mussels, finding a single veliger can initiate a rapid response to head off a larger issue of infestation, so it is crucial to know if the processes being utilized are effective for the types of samples that are widely received.

The research question for this project is: How is veliger settlement in Imhoff cones impacted when plankton tow samples contain high levels of total suspended solids (TSS)? It is hypothesized that elevated levels of TSS will affect the settlement of veligers in Imhoff cones.

To answer this question, samples received throughout the 2020 sampling season that had visibly high levels of organic material were analyzed and retained to re-use for this project. The TSS content of these samples were measured and then they were spiked with a known number of veligers, re-settled in the Imhoff cones and the bottom 15 mL, as well as the next 15 mL, were collected and analyzed under cross-polarized light microscopy (CPLM) to determine how many veligers were recovered. The rinse water from the cones was also collected and analyzed. Control samples that were made of deionized (DI) water, Tris buffer, and ethanol, were also spiked with veligers, settled, and analyzed.

Recovery rates varied widely from 3 percent to 87 percent, with the overall average being 43 percent veliger recovery. Veliger recovery was inconsistent, even in samples that were spiked with 100 or more veligers, and control samples with no TSS. Veligers were recovered in most samples, indicating that the method has some merit, but veligers were not always recovered in the bottom 15 mL and lower than expected recovery was achieved. The goal of early detection is to identify mussel presence when populations are small, and the results of this study indicate that it would be relatively easy to miss a single veliger amidst high amounts of material being observed. For samples from water bodies known to have dreissenid mussel infestations, this type of analysis may not provide accurate information regarding the total numbers of veligers present in a sample, without looking through the entire sample, which isn't feasible due to the amount of time that would be required.

This project has identified that the processes currently used by the Eco Lab for analysis of invasive mussel early detection samples may not be as effective as originally thought. Additional methods for veliger detection and enumeration should be investigated and current procedures should be optimized.

Figure ES-1: Four samples with varying levels of TSS set up in Imhoff cones

1. Introduction

1.1. Project Background

The early detection and monitoring program offered by the biologists in the Hydraulic Investigations and Laboratory Services group of the Technical Service Center (TSC) receives and analyzes 1500+ plankton tow water samples from various water bodies across the western US every year. Samples are analyzed for the presence of invasive dreissenid quagga (*Dreissena rostriformis bugensis*) and zebra (*Dreissena polymorpha*) mussels. These invasive mussels have already established in multiple Reclamation managed reservoirs including Lakes Mead, Powell, Mohave, and Havasu, in addition to other water bodies in AZ, CA, NE, KS, OK and TX. Dreissenid mussels are harmful to Reclamation structures and hydropower plants, the ecosystem and environment, and also have a negative impact to recreation. They are costly to address and nearly impossible to eradicate once established.

The analysis of early detection water samples relies on a process where the samples, which are typically 200-500 mL in volume, are settled in Imhoff cones for 24 hours (Figure 1). Any veligers present are expected to settle at the very bottom of the cone due to their dense calcium carbonate shell which is typically heavier than other material present in the water samples. The bottom (heaviest) 15 mL of the settled sample (Figure 2) is collected and screened under cross-polarized light microscopy in a petri dish (Figure 3) for the presence of the microscopic veligers.

This method saves time when compared to looking through the entire sample, which can be as much as 1 liter. Samples from waterbodies where a mussel veliger has previously been detected or are determined to be at increased risk for infestation are also tested for the presence of genetic evidence or environmental DNA (eDNA) using quantitative PCR (qPCR).

Many water samples that are collected are thick with phytoplankton, zooplankton, and other types of organic matter. There may also be sand, tree bark, juvenile fish, pollen, or any other number of materials present in the water column of freshwater reservoirs or lakes. This study is designed to determine if veligers still settle in the bottom 15 mL of an Imhoff cone even when thick, viscous material is present to ensure the process works for all types of samples that are received. This effort will evaluate if current standard operating procedures are effective. This project was conducted with materials on hand in the laboratory utilizing samples that have already been analyzed and would otherwise have been discarded.

In waterbodies with invasive mussel infestations, it's common to collect water samples with hundreds or even thousands of veligers present. On the other hand, only one to a very few veligers would be expected to be collected from early detection samples, since samples are being collected from water bodies where mussel populations have never been detected. Detection of a single veliger can initiate a rapid response chain of events, so it's crucial to be able to locate a single veliger in a water sample.

Plankton tow samples are typically collected by pulling or dragging a plankton tow net through the water column, up to five times, creating a sample that is concentrated with anything larger than

64 microns (µM). This is accomplished by using a net with a 64 µM pore size with a collection cup (cod end) at the base of the net. This allows water to flow through freely, but not the plankton or other material present that have been caught in the net and cod end. The samples are preserved in the field with ethanol, and a buffer is added to prevent degradation of the veliger shells. The samples are then sent to the Eco Lab for analysis. Samples are received from all Reclamation regions.

Figure 1: Plankton tow samples settled in Imhoff cones with high levels of organic material

Figure 2: A 15- mL test tube that contains part of a water sample filled with zooplankton

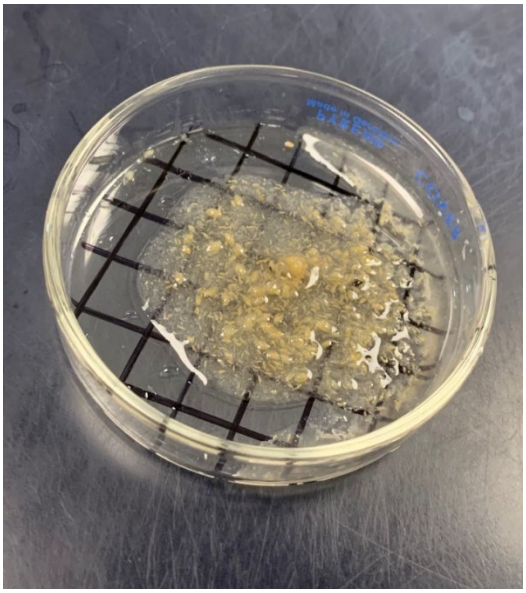
Figure 3: A petri dish with approximately 1 mL of a water sample that contains high amounts of zooplankton.

1.2. Previous Work & Study Question

The methods utilized by the Eco Lab, outlined in the most recent SOP, were designed by Lab staff in 2012/2013. At the time it was determined that 98 percent of veligers present in a sample would settle in the bottom/heaviest 15 mL in an Imhoff cone (Figure 1). The data produced by this research is no longer available and it's unknown if these tests were conducted with water samples

that were relatively clear/free of total suspended solids, or if samples with varying types of contents and quality were used.

This project was conducted to determine if water samples with high amounts of TSS like zooplankton and phytoplankton, will interfere with the settlement of veligers into the bottom 15 mL of a settled sample. Most of the samples received by the Eco Lab have high amounts of organic material. The hypothesized result is that elevated amounts of planktonic debris in plankton tow samples will interfere with veliger settlement in the heaviest 15 mL of a sample settled in an Imhoff cone.

2. Methods

A study plan was written and is available as Appendix A.

Twenty samples were selected to use, based on several criteria. Sample volumes were standardized to 300 mL, and the total suspended solids were measured to assess the amount of organic material and to rank the samples into 4 groups (A-D) with ascending levels of TSS in grams per 300 mL.

Samples were selected based on the following criteria:

- Previously analyzed and found to be negative for any indication of dreissenid mussels
- Varying (low, moderate, high) levels of visible organic material present in sample
- Did not contain organisms that appear similar to mussel veligers, such as ostracods, and did not contain baking soda or sand that might make it difficult to locate veligers
- A pH of 7 or higher
- A minimum sample volume of 350 mL

The TSS were measured by pouring a known amount of the sample through a pre-weighed filter with vacuum suction that would pull the water out but retain any material in the sample (Figure 4). The filter was then dried in a laboratory oven and then re-weighed twice (Figures 5 and 6). The difference between the filter and the filter plus sample indicated the amount of TSS for the volume of sample that was poured through the filter. This number was used to calculate the TSS for 300 mL. This procedure was based off the method for measuring Total Suspended Solids Dried at 103-105°C in the Standard Methods for the Examination of Water and Wastewater, 19th edition.

Samples were given new identifications based on their group (A-D) and then randomly assigned a number 1-5 with a second lettering that determined the amounts of veligers they would be spiked with (XX/10, QQ/20, ZZ/40, YY/100, RR/150).

Aliquots of veligers were made from a veliger stock and were carefully added to the samples. The aliquots were made from a stock of veligers from Canyon Reservoir in Arizona, which has an established population of quagga mussels. Veligers used for this project were whole and varying in size, but none larger than a pediveliger. Low retention micro-pipette tips were used to transfer veligers from a petri dish into 1 mL Eppendorf tubes. Micro-pipette tips were verified to be empty after each use to ensure all veligers were successfully transferred to the tubes, and tips were changed frequently. Each aliquot was preserved with ethanol to prevent degradation until they were added to the samples.

When aliquots were poured into prepared samples, the Eppendorf tubes were rinsed, and initial rinse water was also added to the sample. A second rinse of the Eppendorf tube was inspected under the microscope to ensure no veligers remained.

The spiked samples were then re-settled in the Imhoff cones for 24 hours and analyzed following the basic SOP for sample analysis. The significant differences from the SOP were that both the first and second bottom 15 mL of each sample were collected and analyzed under CPLM, as well as the rinse water used to rinse the cones after emptying the remainder of the samples back into their bottles.

The samples were photographed at various stages of the process (see Appendix B).

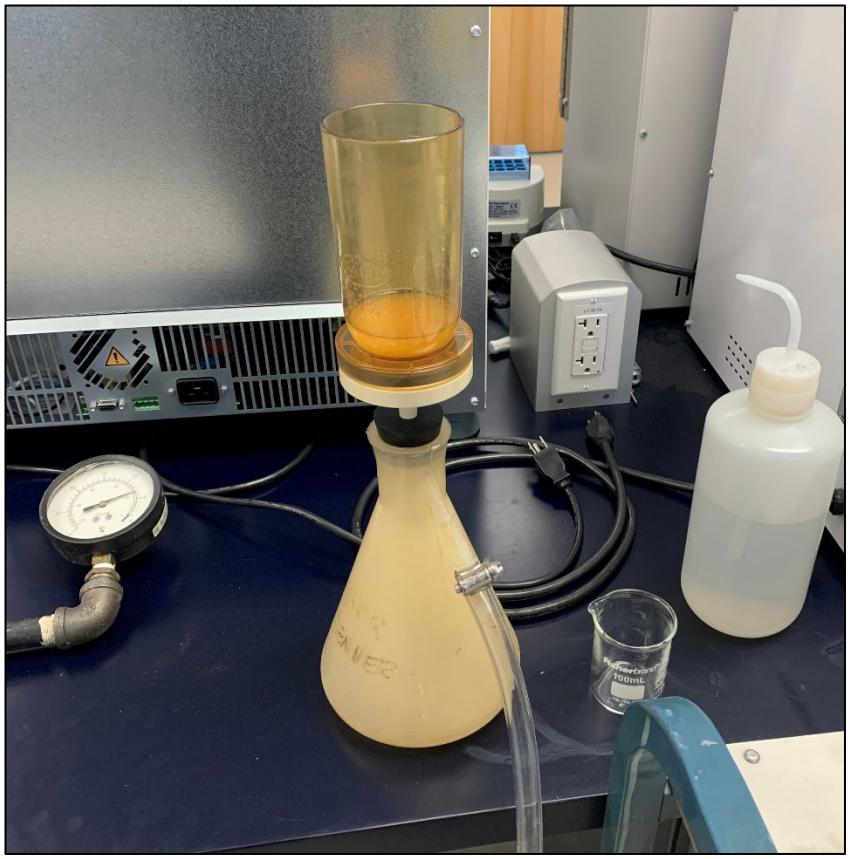

Figure 4: Vacuum filter apparatus used as part of the process of determining the total suspended solids in each sample

Figure 5: The total suspended solids test involved drying and weighing each filter to determine the amount of solid content in each sample

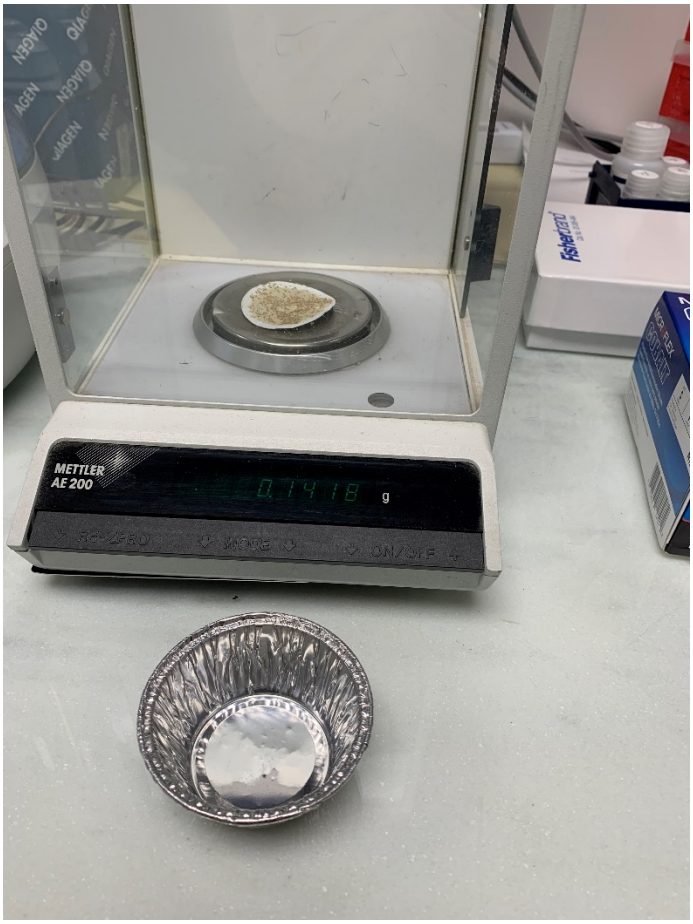

Figure 6: The filters with solids were weighed on a digital analytical balance scale

3. Results

The results of this study indicate that the presence and amount of TSS in a water sample impedes the recovery of veligers in the heaviest 15 mL. Higher amounts of TSS resulted in an overall reduced veliger recovery rate. Samples with high levels of TSS that consisted of zooplankton are noted to have reduced veliger recovery.

In addition to the heaviest 15 mL of settled sample, a second 15-mL sample was also collected and analyzed. Total recovery rates of 98 percent were not achieved for any samples tested, including the control samples, which contained no TSS and were made of deionized water, liquid buffer, and ethanol.

Results indicate that total suspended solids like zooplankton and algae in plankton tow samples interfere with the settlement of veligers in the bottom 15 mL of an Imhoff settling cone. Results also indicate that veligers are often not collected in the bottom 15 mL of a sample even when there are no TSS present. This could have been an error in study design, or indication of an issue with the cone design or collection methods.

Table 1 is displayed with the percentage of total veliger recovery in ascending order and shows the TSS as well as the contents of the samples. Table 2 is arranged in colored groupings for the amounts of TSS (lowest to highest).

Table 1: Samples sorted by ascending veliger recovery percentage, showing the contents of the TSS

Sample ID	TSS g/300mL	Contents	Veligers Spiked	Total Veligers Recovered	Percent of Veliger Recovery
CN5QQ	N/A	DI water, buffer, ethanol	20	0	0%
CN2 ZZ	N/A	DI water, buffer, ethanol	40	1	3%
C1 XX	1.4400	zooplankton	10	1	10%
CN 1XX	N/A	DI water, buffer, ethanol	10	1	10%
C2 ZZ	1.3500	zooplankton, algae (stringy, oily)	40	6	15%
D4 ZZ	1.6560	dense zooplankton	40	8	20%
B5 ZZ	0.9555	dense zooplankton	40	10	25%
B1 XX	1.2000	dense zooplankton	10	3	30%
D3 XX	1.9299	zooplankton	10	3	30%
C5 YY	1.4520	zooplankton	100	33	33%
B4 YY	1.2930	zooplankton	100	35	35%

Sample ID	TSS g/300mL	Contents	Veligers Spiked	Total Veligers Recovered	Percent of Veliger Recovery
C3 QQ	1.4010	zooplankton, (maybe) algae	20	7	35%
D1 QQ	1.7001	zooplankton	20	7	35%
C4 RR	1.3008	zooplankton	150	67	45%
CN3 YY	N/A	DI water, buffer, ethanol	100	48	48%
CN4 RR	N/A	DI water, buffer, ethanol	150	73	49%
D5 RR	1.6140	zooplankton, algae	150	95	63%
A1 XX	0.4410	zooplankton	10	7	70%
A3 ZZ	0.2580	light zooplankton	40	29	73%
A2 QQ	0.3432	zooplankton, algae	20	15	75%
B3 QQ	1.0959	zooplankton, algae, very oily	20	15	75%
A5 YY	0.4419	stringy algae	100	80	80%
A4 RR	0.0888	light zooplankton, algae	150	128	85%
B2 RR	0.5718	zooplankton, algae	150	132	87%
D2 YY	4.4880	zooplankton, algae	100	N/A	N/A

4. Data

Table 2: Sample TSS (ascending) and veliger recovery percentages

Sample ID	TSS g/300mL	Percent Recovery in 1st 15 mL	Percent of total Veliger Recovery
A4 RR	0.089	85%	85%
A3 ZZ	0.258	63%	73%
A2 QQ	0.343	70%	75%
A1 XX	0.441	30%	70%
A5 YY	0.442	72%	80%
AVERAGES	**0.31458**	**64%**	**77%**
B2 RR	0.572	84%	88%
B5 ZZ	0.956	5%	25%
B3 QQ	1.096	55%	75%
B1 XX	1.200	20%	30%
B4 YY	1.293	19%	35%
AVERAGES	**1.02324**	**37%**	**51%**
C4 RR	1.301	23%	45%
C2 ZZ	1.350	10%	15%
C3 QQ	1.401	25%	35%
C1 XX	1.440	0%	10%
C5 YY	1.452	15%	33%
AVERAGES	**1.38876**	**15%**	**28%**
D5 RR	1.614	51%	63%
D4 ZZ	1.656	10%	20%
D1 QQ	1.700	30%	35%
D3 XX	1.930	20%	30%
D2 YY**	4.488	N/A	N/A
AVERAGES	**2.2776**	**28%**	**37%**
CN 1XX	N/A	0%	10%
CN2 ZZ	N/A	3%	3%
CN3 YY	N/A	48%	48%
CN4 RR	N/A	49%	49%
CN5QQ	N/A	0%	0%
AVERAGES		**20%**	**22%**

***Sample D2YY was found not to be viable due to the quality of the algae present. It was not feasible to look through.*

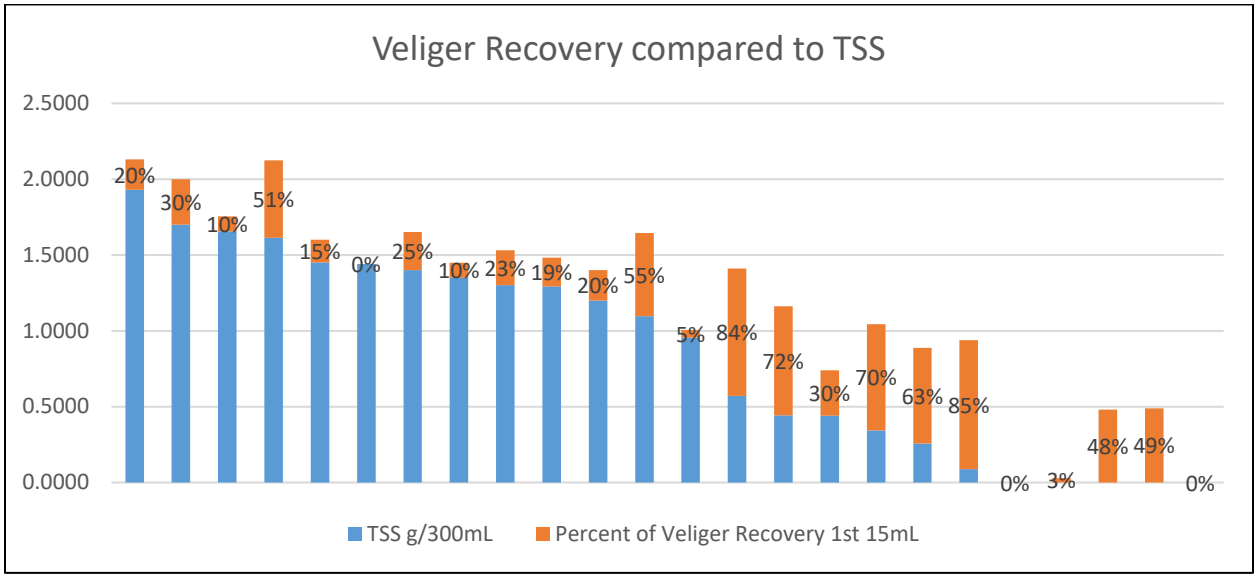

Figure 6: The relationship between total suspended solids (0-2.5g Per 300 mL) and the percentage of veliger recovery for the first 15 mL, including the five control samples

Figure 6 illustrates that veliger recovery tends to be higher in samples with lower TSS amounts. The chart shows the percentage of veliger recovery in the first 15 mL collected, which is consistent with current laboratory procedures. The Eco Lab receives samples with widely varying levels of TSS, though the majority received have high levels of organic material present, especially zooplankton.

5. Discussion

The settling cones do appear to be effective for detecting the presence of veligers for the majority of samples tested, meaning that if there are multiple veligers present, at least some will settle and be collected in the bottom 15 mL, but it is clear this method has limitations. The recommended course of action is to investigate the design of the settling cones and collection process to see if they can be modified to increase veliger recovery. A Reclamation Science and Technology sponsored research project has been submitted for approval for fiscal year 2022, which if approved, will allow further investigation of the cone's limitations, and identify better ways to analyze samples containing significant TSS.

It would be expected that the control samples which contain only DI water, 15 drops of Tris buffer, and ethanol, would have had high veliger recovery rates as there was no organic material or sediment to prevent the veligers from falling to the bottom of the Imhoff cone, but that was not observed in all the control samples. Considerations for this may include unanticipated veliger degradation, veliger retention in the sample bottles (though they were rinsed thoroughly), veliger retention along the sides of the cones (though rinse water was collected and analyzed for presence of veligers also), air bubbles that developed inside the veliger shells when the samples were shaken that might have prevented them from sinking, or other unknown reasons. Factors to consider in experimental samples might be the nature of the TSS themselves. Some types of algae or zooplankton can be "sticky" and veligers might get stuck elsewhere in the sample and be unable to sink through the contents down to the bottom 15 mL. The design of the cones may also be impeding the ability to

accurately collect the actual bottom 15 mL. Contents may be sitting in the shoulder of the apparatus added to the Imhoff cones to allow collection of the contents from the bottom. These are also relatively small sample numbers, with each grouping having only 5 samples.

The A group of samples had the lowest amount of TSS, and D group had the highest. The overall decline in veliger recovery as the TSS increased was expected. Three of the samples did not have any veligers recovered in the first 15 mL collected. One sample was from the C group and the other two were control samples. These samples had been spiked with either 10 or 20 veligers.

6. Next Steps

The Eco Lab has requested funding from the Reclamation Science and Technology Program for a 3-year study that will investigate multiple ways to optimize early detection of dreissenid mussels. Methods that will be investigated include re-design of the settling cones, on-site testing for dreissenid eDNA, using trained dogs to detect presence of veligers or eDNA by scent in preserved plankton tow samples, working with developers of an artificial intelligence program to teach it to enumerate veligers in samples from sites that have known mussel populations established, and reviewing the standard operating procedures of other labs that conduct similar work. Additional testing will also be conducted to determine why veliger recovery was low in control samples.

This report and report data will be stored in the following Bureau of Reclamation, Technical Service Center folder: Z:\DO\TSC\Jobs\DO_NonFeature\Science and Technology.

References

Bureau of Reclamation, Ecological Research Laboratory Standard Operating Procedure: Preparation and Analysis of Water Samples for Dreissenid Mussel Veliger Detection: Microscopy Document No. EcoLab-FA981-2020-04

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Glossary

Dreissenid (quagga/zebra) mussel. (*Dreissena rostriformis bugensis*) and zebra (*Dreissena polymorpha*) are freshwater bivalve mussels native to Eurasia. Established as an invasive species in the Great Lakes in the late 1980's and established (quagga mussels) in Lake Mead in 2007.

Imhoff cone. A clear, cone-shaped container marked with graduations, used by the Eco Lab to settle plankton tow samples as part of the process to analyze samples for early detection or enumeration of dreissenid mussel veligers.

Veliger. The microscopic, planktonic, larval stage of a dreissenid mussel.

Pediveliger. Veliger stage that has developed a foot and is ready to settle.

Plankton tow sample. Water sample collected by towing or pulling a net and cod end made of a very fine mesh that filters water and retains any planktonic organisms living in the water.

Appendix A: Study Plan

Settling Study Plan: To Determine if dreissenid mussel veligers will settle to the bottom 15 milliliters of a 200-500ml sample settled in an Imhoff cone.

To determine which samples will be used: Samples from reservoirs across the western US are received by Reclamation's Technical Service Center's Ecological Research Laboratory throughout the year. Most samples received are in 500-ml bottles. All samples received by the Eco Lab are analyzed by cross-polarized light microscopy (CPLM) by trained staff for the presence of dreissenid mussel veligers. Many are also analyzed for the presence of dreissenid eDNA. All samples considered for this study were determined not to have veligers or veliger DNA present.

Samples with visible and significant amounts of zooplankton and/ or phytoplankton were marked with green tape when they were initially settled in Imhoff cones as part of the standard process for sample analysis. Samples are poured into Imhoff cones and then the bottom 15 ml are collected after 24 hours by allowing the sample to pour down through plastic tubing connected to the bottom of the cone (Figure AA-1). Samples that became clogged in the tubing, requiring the physical assistance of a wire to poke through the debris, were marked with green tape as good candidates for this study.

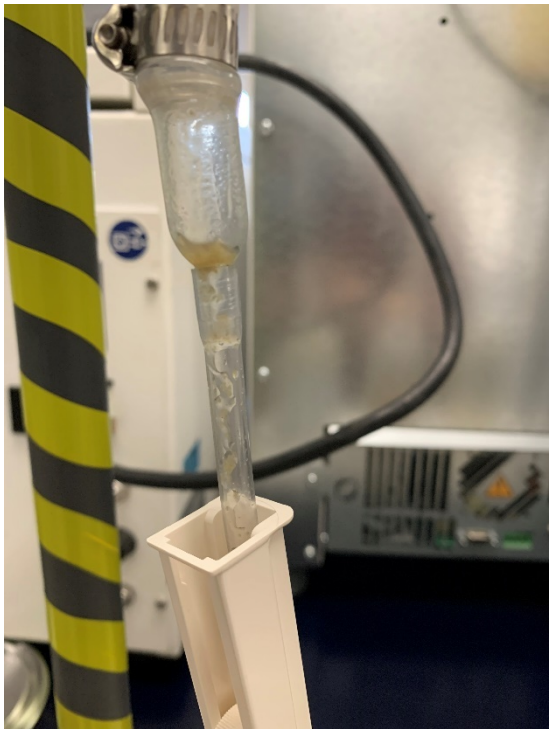

Figure AA-1 The tubing at the bottom of an Imhoff cone, and the top of a roller clamp in place to prevent liquid from releasing.

Samples were not included in the study if their contents would readily interfere with the ability to find veligers, such as sediment, high baking soda content, or the presence of another micro-

invertebrate organism called ostracods, which have similar birefringence, or "glow", to mussel veligers under CPLM.

Samples were selected from across multiple months and states to give a variety of conditions.

Sample pool was narrowed down to 20 samples, placed into categories based on how much organic material was present and settled at the bottom of the bottle. "Material" includes, but might not be limited to, phytoplankton and zooplankton. Samples collected in the field are preserved with a buffer (often TRIS), and ethanol (190 or 200 proof, or 70-91 percent isopropyl alcohol).

Control samples with 100 mL DI water, buffered with 15 drops of 4M Tris and preserved with 200 mL ethanol to be comparable to normal samples collected in the field will be used in each category. The ethanol and buffer are consistent with Eco Lab sampling procedures. Control samples will be spiked with the same veliger aliquots and have the same steps done, minus the assessment of total suspended solids.

Approximate total suspended solids (TSS) will be calculated for each sample by determining TSS for subsamples from each bottle prior to being spiked with veligers.

Samples will be categorized into four groups (A-D) with increasing TSS numbers.

Each sample will be photographed at various steps, and pictures will be in appendix to report

- Sample bottles with debris settled at bottom
- 1 mL under bright field microscope (will include dark field if determined valuable)
- Settled in cones

This study will use 20 samples that have already been processed by the Eco Lab as part of the early detection program. There will also be five control samples that will contain only DI water and preservatives of Tris buffer and ethanol.

Prep Work

1. From pool of prospective samples to use for study, narrow down to 20.
2. Measure the pH of all samples to ensure none are below pH of 7.
    - If a pH is below 7, add Tris and measure pH again, or use different sample.
3. Collect TSS data
4. Place 20 selected samples into 4 categories based on amount of TSS in each bottle, with Category A having the lowest and Category D having the highest:
    - Category A: least
    - Category B: < A
    - Category C: < B
    - Category D: highest
5. Prepare 5 control samples
    - Add 100 mL of DI water, 15 drops of Tris buffer, and 200 mL ethanol to a new 500 mL sample bottle
6. Using new sticker labels on front of each bottle, label sample bottles from table below

Table AA-1 Label identification information for samples used.

A 4: RR	A 3: ZZ	A 2: QQ	A 1: XX	A 5: YY
B 2: RR	B 5: ZZ	B 3: QQ	B 1: XX	B 4: YY
C 4: RR	C 2: ZZ	C 3: QQ	C 1: XX	C 5: YY
D 5: RR	D 4: ZZ	D 1: QQ	D 3: XX	D 2: YY
CN 4: RR	CN 2: ZZ	CN 5: QQ	CN 1: XX	CN 3: YY

7. Create crosswalk in MS Excel for 2020/2021 sample numbers (assigned by Eco Lab Mussels Database) with Settling Study labeled information using Veliger Label table above

Standardize Volume of Each Sample

1. Ensure that each sample bottle contains no more than 300 mL of sample (after collecting 50 mL to use for TSS test).
2. All sample bottles being used are standard 500 mL bottles
3. Using a clean 500 mL bottle, mark where 300 mL is
4. Invert sample bottles multiple times to ensure homogenized sample is being poured off, not just "water off the top"
5. Compare study samples and remove volume over 300 mL by slowly pouring excess into a clean beaker
6. If too much is poured out, slowly add back to sample bottle to get to 300 mL volume.

Conduct TSS Analysis

Adapted from Standard Methods for Examination of Water and Wastewater, 19th edition. 2540.D, Total Suspended Solids Dried at 103-105°C.

1. Label bottom of 20 aluminum weighing dishes with sample numbers (example: A 3:ZZ, D 1:QQ etc) with a Sharpie
2. Place one glass microfiber filter (round, 47 mM) in each dish
3. Record weight of each filter on analytical scale in grams [g]*, to the 4th decimal point
   *scale in Eco Lab measures in grams
4. Ensure each filter is kept specifically with labeled weighing dish to avoid mix ups between filters and dishes
5. Connect filtering funnel to vacuum pump, turn vacuum on
6. Remove top of funnel from collection flask and use tweezers to place filter
7. Put top of funnel back in place
8. Use DI water to wet filter
9. Invert sample bottle several times to homogenize contents
10. Quickly pour 50 mL of sample into a new 50-mL conical tube.
11. Pour contents of tube slowly into funnel, pausing when water is no longer being pulled through. This may only take 2-3 mL of sample.
12. Make note of how much sample was used.
13. Rinse down funnel with DI water to ensure all debris is rinsed onto filter
14. Wash filter and funnel with 25 mL DI water three separate times, allowing complete drainage between washings
15. Allow suction to continue for 3 minutes after final wash
16. Turn off suction to funnel and gently break suction by slightly lifting funnel off the collection flask and setting back in place

17. Remove funnel from flask
18. Discard contents of flask (as necessary)
19. Using tweezers, carefully remove filter and place into labeled weighing dish
20. Repeat until all 20 samples have been filtered
21. Carefully place all filtrates into lab oven and dry for 1 hour at 100°C ± 5°C
22. Carefully place all filtrates into desiccator for 15 minutes to allow cooling
23. Use tweezers to transfer filter onto analytical balance and record weight to the 4th decimal point
24. Repeat oven and desiccation process on each filtrate and record successive weights one additional time
25. If weight differences are more than .0005g, repeat drying, cooling, weighing process until they are lower than .0005g.
26. When weight differences are less than .0005g, calculate TSS for all samples using formula below and document on crosswalk.

Calculations:

- Convert all weight from g to mg (move decimal to the right 3 places):

2.3513g → 2351.3mg

$$\text{mg TSS per Liter:} = \frac{(A - B) \times 1000}{\text{Sample volume (ml)}}$$

Where A is weight (mg) of filtrate; B is weight (mg) of dry filter

Example, using 50ml volume:

A. Filtrate (filter + solids): 2351.3mg
B. Dry filter weight: 1939.7mg

(2351.3 – 1939.7) * 1000 / 50

1. 2351.3-1939.7 = 411.6
2. 411.6 * 1000 = 411,600
3. 411,600/50 = 8232mg total suspended solids per liter, or 8.232g/L

Assess Contents & Spike with Veliger Aliquots

1. Assess what type of material is in each sample prior to spiking/settling in cones:
    - Invert bottle several times to homogenize sample
    - Collect approximately 4 ml (two glass pipettes) into a petri dish and view under microscope to assess contents: zooplankton (copepods, daphnia), seeds, pollen, algae, sediment, etc.
    - Take photos of each sample under microscope using bright field view. Use CPLM if contents are easier to view in that setting.

2. Create and label veliger aliquots. Using a low-retention micropipette tip, veligers of varying sizes will be pulled from a stock solution and counted out into 2-mL Eppendorf tubes. There will be five tubes of each veliger quantity: 10, 20, 40, 100, 150.
    - Each set of aliquots will be tagged RR, ZZ, QQ, XX, YY to ensure that staff who are spiking the sample bottles do not know how many veligers go into each sample.
3. Spike one veliger aliquot from each quantity into one sample bottle from each TSS category using the table below*

*used https://www.randomizer.org/ to create 5 unique sets of ranges 1-5

- Rinse Eppendorf tubes with deionized water to ensure all veligers are washed into sample bottles by observing a final rinse content under CPLM

Table AA-2 How the samples are blindly labeled with the number of veligers

Veliger Tag*	**RR: 150**	**ZZ: 40**	**QQ: 20**	**XX: 10**	**YY: 100**
Label info:	A 4: RR	A 3: ZZ	A 2: QQ	A 1: XX	A 5: YY
	B 2: RR	B 5: ZZ	B 3: QQ	B 1: XX	B 4: YY
	C 4: RR	C 2: ZZ	C 3: QQ	C 1: XX	C 5: YY
	D 5: RR	D 4: ZZ	D 1: QQ	D 3: XX	D 2: YY
Control:	CN 4: RR	CN 2: ZZ	CN 5: QQ	CN 1: XX	CN 3: YY

4. Once lids are tight on bottle, invert bottle back and forth for 15 seconds to homogenize veligers throughout sample

Set Up & Take Down of Cones

1. Set up 25 decontaminated and dry Imhoff cones designated for use with water samples from waterbodies with known mussel populations (positive cones) as would be done for processing of normal Eco Lab samples, ensuring watch glasses are on covering the cones when not in use
2. Arrange samples by category A-D, 1-5 and Control (CN) 1-5 in front of cones.
3. Pouring samples into Imhoff cones used for mussel-positive waterbodies
    - Thoroughly shake sample prior to removing lid
    - Pour sample in to Imhoff cone while ensuring cones on either side have watch glass in place
    - Thoroughly rinse empty sample bottle and lid with DI water and pour into cone
4. Allow samples to settle for 1 hour.
5. Take note of each sample's color, quantity, and level of debris in cone (to the mL if possible)
6. Photograph each sample settled in cone
7. Allow samples to settle for 24 hours
8. Label two sets of 25 plastic test tubes (with caps) with sample & aliquot info and #1 or #2 for first or second set. Examples: A 4: RR #1; B 4: YY #2; D 1 QQ #2, etc.
9. Collect bottom 15 ml of each sample into plastic test tubes (set #1) with caps using standard laboratory procedures.
    - Ensure label info does not rub off, which happens if sample pours over label
    - Make note of which samples require use of wire to clear blockages in tubing
10. Collect second 15 mL of each sample in set of tubes labeled #2 following same procedures.
11. Allow the rest of sample to be drained back into sample bottle. DO NOT RINSE CONE YET.
12. Close tubing on cone with roller clamp.
13. Label 25 plastic test tubes with assigned sample names + RINSE. Example: "D 2: RINSE"
14. Rinse cone with DI water and collect rinse water into labeled plastic 15ml test tube.

Analysis of 3 sets of Subsamples

1. Analyze all 3 sets of subsamples (#1: bottom 15 mL, and #2: second bottom 15 mL, #3: rinse water) of spiked samples under CPLM in petri dishes with grid
    - Count all veligers (including broken ones that are greater than 50 percent intact)

- Make note of number of observed broken veligers
- Ensure no veligers remain in glass pipettes used to transfer sample from test tube to petri dish.

2. Once all 3 sets of subsamples/rinse have been analyzed under microscope, provide all data to Rheannan Quattlebaum.
3. Use Excel to determine percentage of veliger recovery for first 15 mL, second 15 mL, and rinse water.

Appendix B: Sample Photos

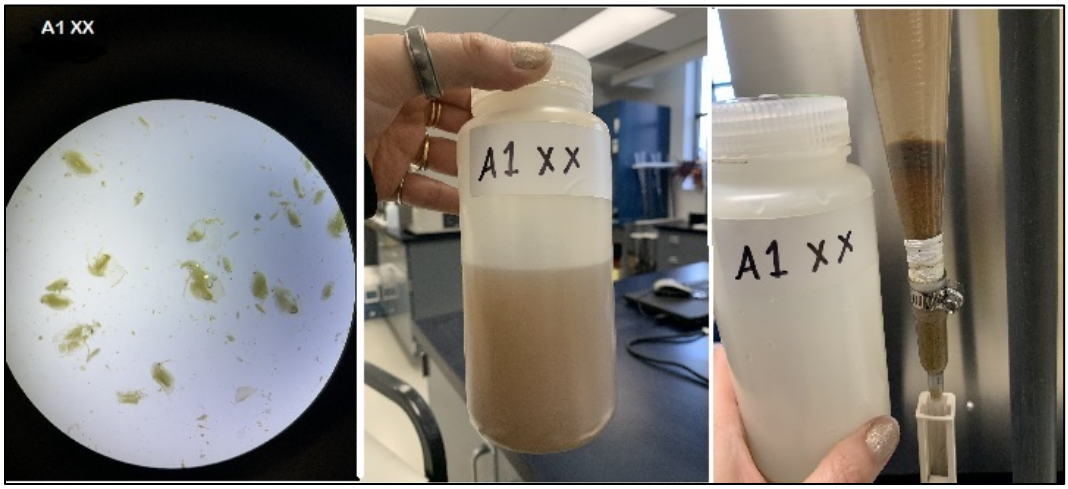

Figure AB-1 Photos of sample A1 XX in petri dish under a microscope, bottle, and Imhoff cone.

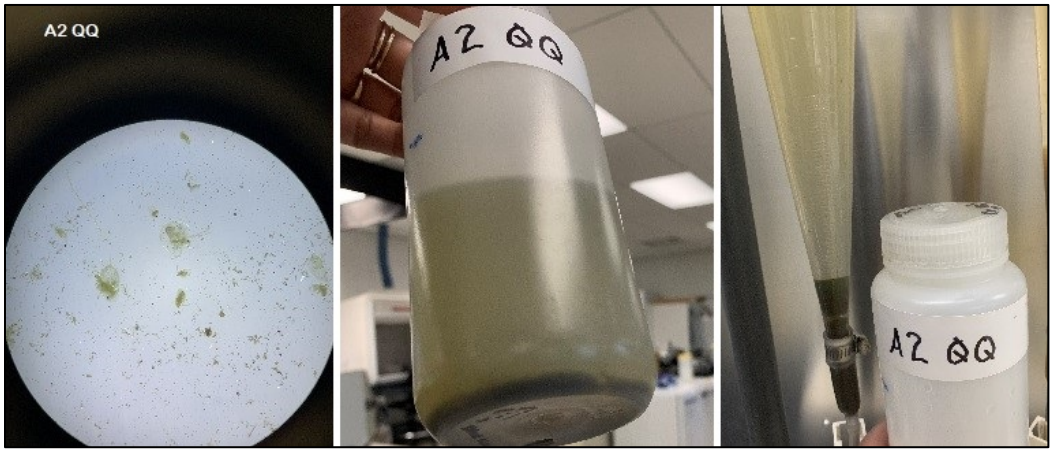

Figure AB-2 Photos of sample A2 QQ in petri dish under a microscope, bottle, and Imhoff cone.

Figure AB-3 Photos of sample A3 ZZ in petri dish under a microscope, bottle, and Imhoff cone.

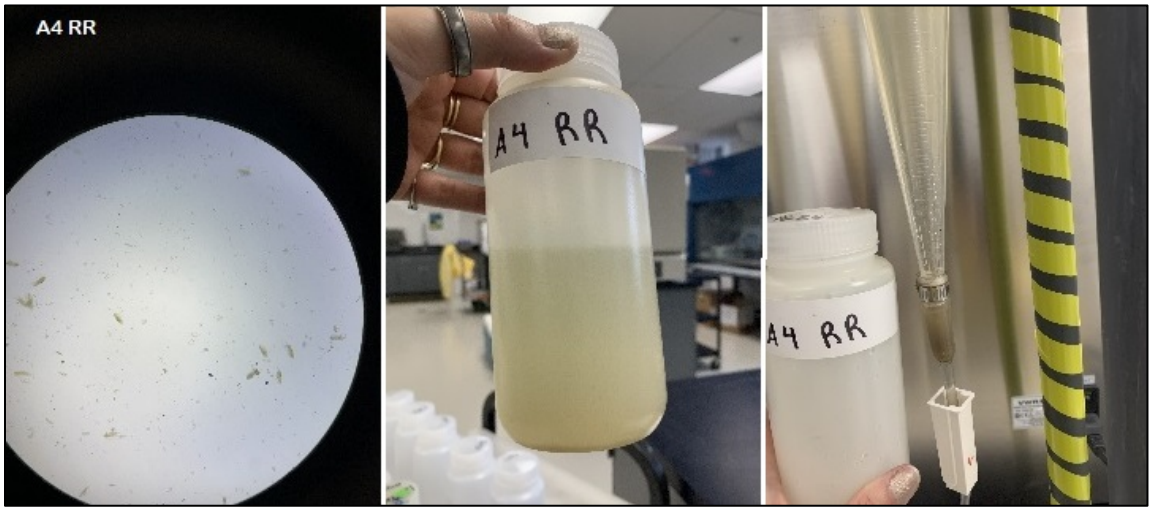

Figure AB-4 Photos of sample A4 RR in petri dish under a microscope, bottle, and Imhoff cone.

Figure AB-5 Photos of sample A5 YY in petri dish under a microscope, bottle, and Imhoff cone.

Figure AB-6 Photos of sample B1 XX in petri dish under a microscope, bottle, and Imhoff cone.

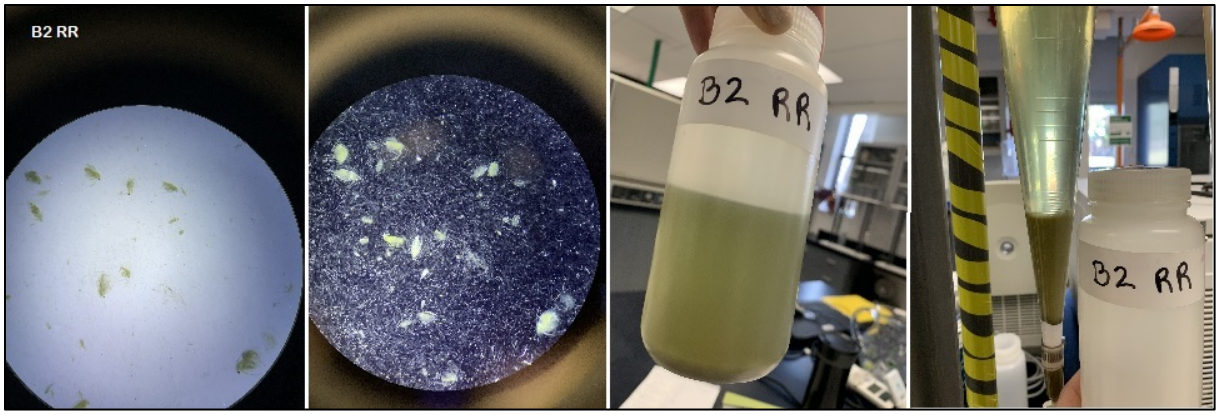

Figure AB-7 Photos of sample B2 RR in petri dish with both light and dark backgrounds under a microscope, bottle, and Imhoff cone.

Figure AB-8 Photos of sample B3 QQ in petri dish under a microscope, bottle, and Imhoff cone.

Figure AB-9 Photos of sample B4 YY in petri dish under a microscope, bottle, and Imhoff cone.

Figure AB-10 Photos of sample B5 ZZ in petri dish under a microscope, bottle, and Imhoff cone.

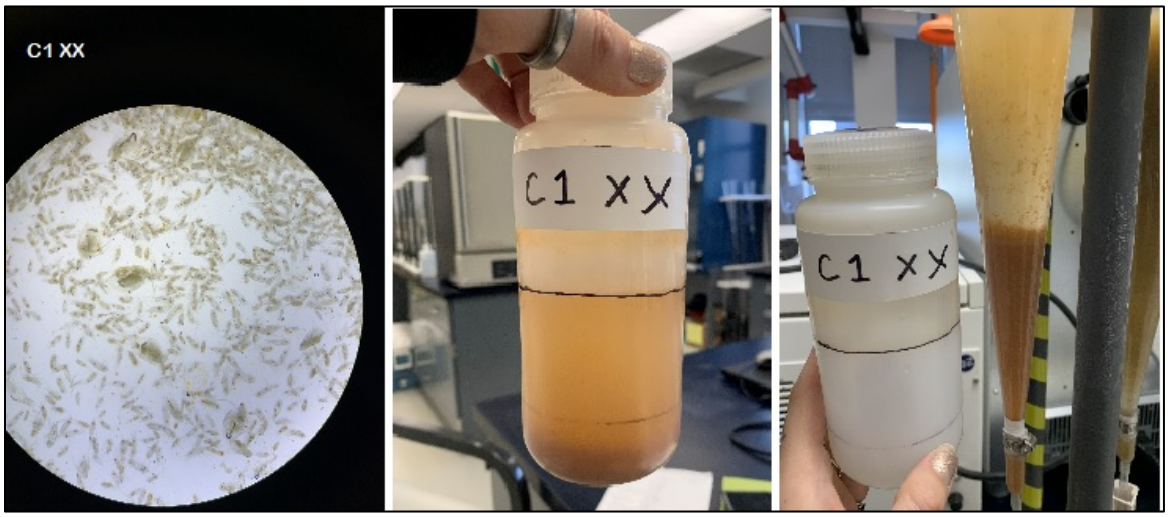

Figure AB-11 Photos of sample C1 XX in petri dish under a microscope, bottle, and Imhoff cone.

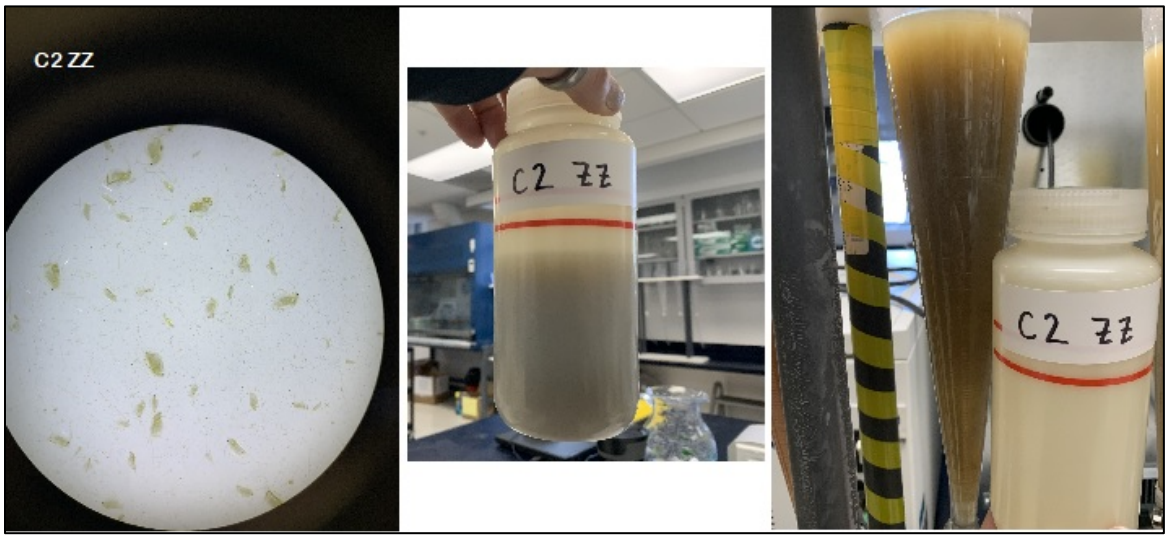

Figure AB-12 Photos of sample C2 ZZ in petri dish under a microscope, bottle, and Imhoff cone.

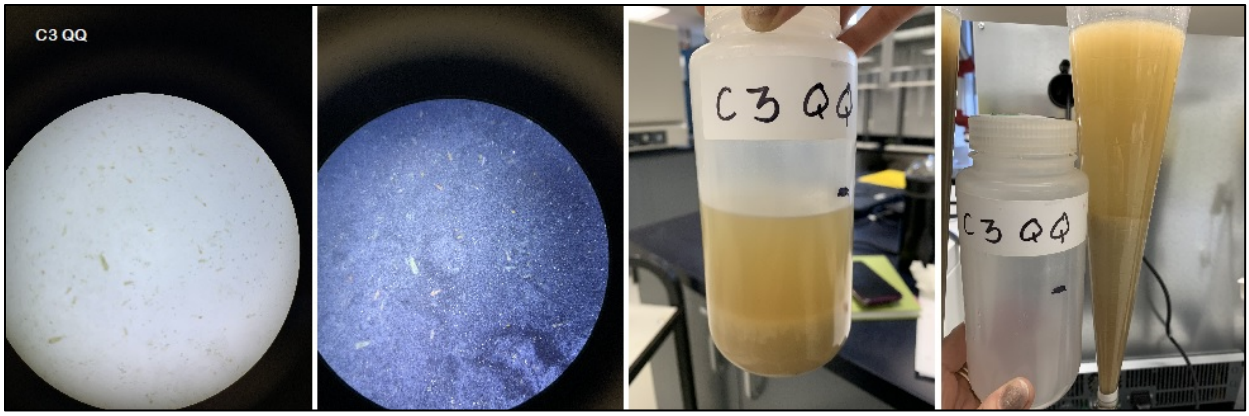

Figure AB-13 Photos of sample C3 QQ in petri dish with both light and dark backgrounds under a microscope, bottle, and Imhoff cone.

Figure AB-14 Photos of sample C4 RR in petri dish under a microscope, bottle, and Imhoff cone.

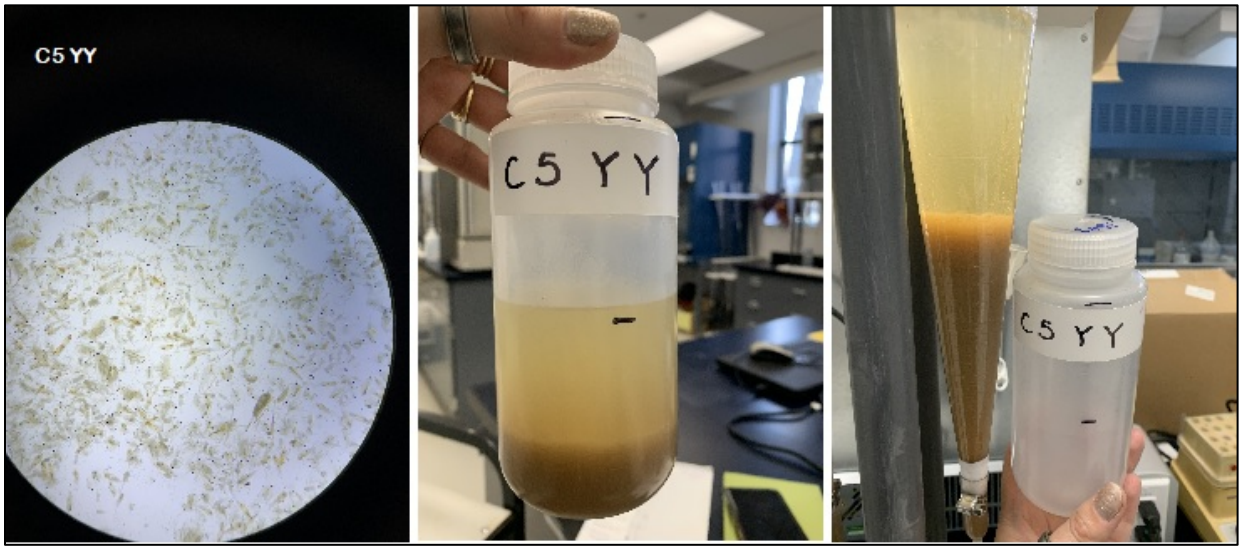

Figure AB-15 Photos of sample C4 RR in petri dish under a microscope, bottle, and Imhoff cone.

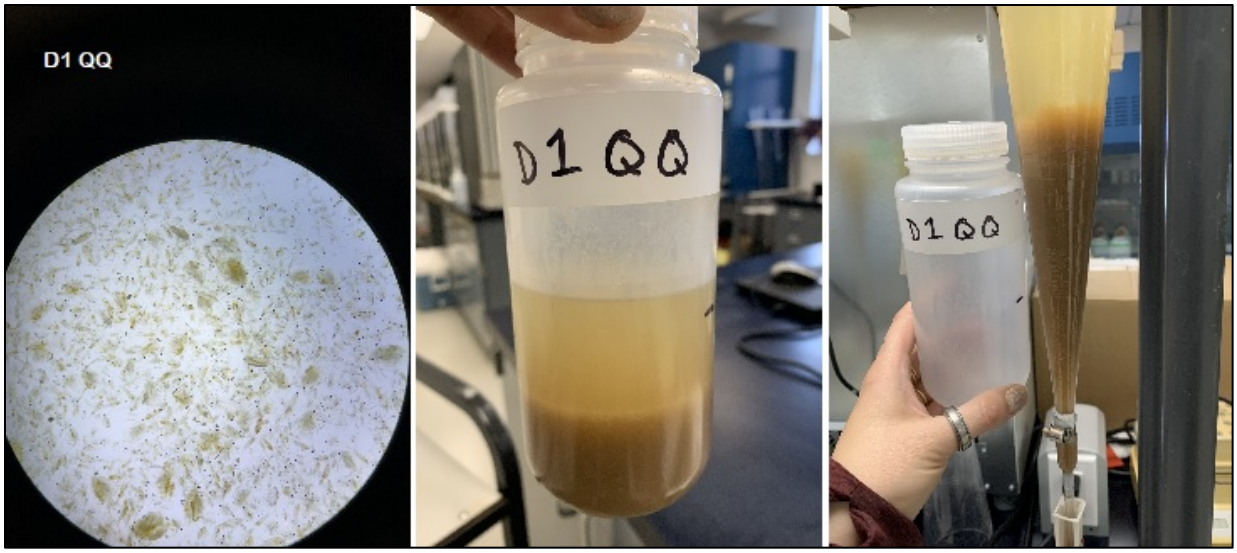

Figure AB-16 Photos of sample D1 QQ in petri dish under a microscope, bottle, and Imhoff cone.

Figure AB-17 Photos of sample D2 YY in petri dish with both light and dark backgrounds under a microscope, bottle, and Imhoff cone.

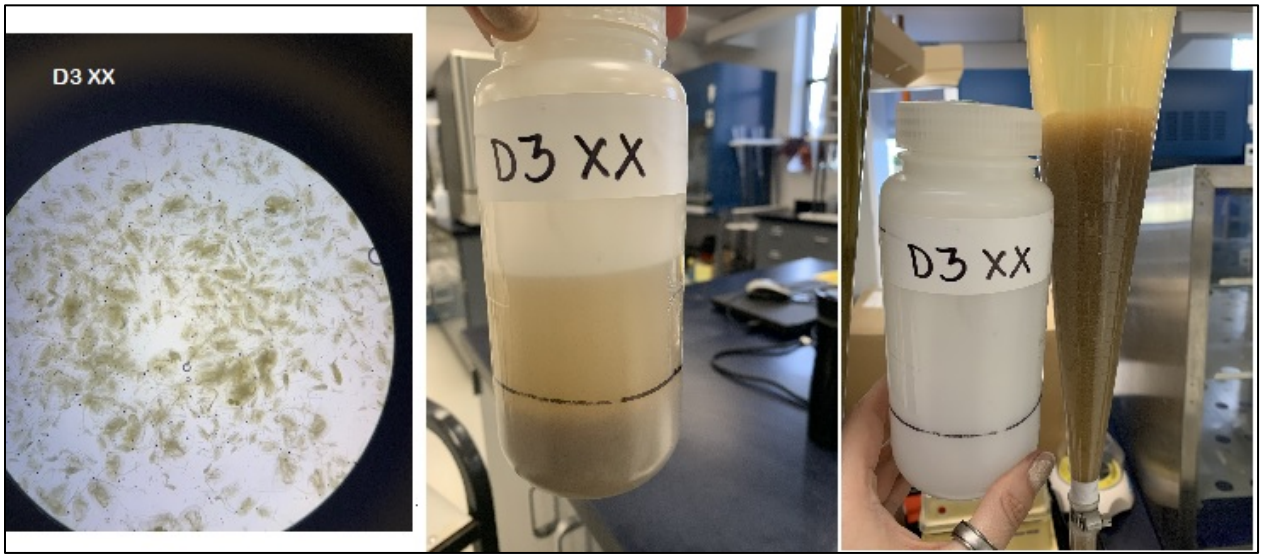

Figure AB-18 Photos of sample D3 XX in petri dish under a microscope, bottle, and Imhoff cone.

Figure AB-19 Photos of sample D4 ZZ in petri dish under a microscope, bottle, and Imhoff cone.

Figure AB-20 Photos of sample D4 ZZ in petri dish under a microscope, bottle, and Imhoff cone.