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Use of Novel Parasites to Control Naïve North American Dreissenid Populations

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14. ABSTRACT
Reclamation has continued to collaborate with Molloy & Associates to search for novel hypervirulent parasites for the control of invasive dreissenid mussels in North America. The last three years has focused on expanding the field laboratory in Montenegro, identifying and collecting isolated dreissenid 'cousin' species and completing a proof-of-concept trials showing the transfer of parasites between a 'cousin' dreissenid and zebra and quagga mussels. The final report from Molloy & Associates (Appendix 1) details the scope, results, and future direction of the research. A new S&T proposal was submitted to continue this research until 2024. The identification of a biological control agent for dreissenid mussels is a long-term project, and the results from the last three years have continued to move this goal forward.

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Use of Novel Parasites to Control Naïve North American Dreissenid Populations

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Contents

Executive Summary

The Bureau of Reclamation began a collaboration with Molloy & Associates in 2017 to search for a host-specific biological control agent for invasive dreissenid mussels. The goal of this long-term project is to identify novel hypervirulent parasites from the dreissenid mussels' native range. Currently, there is no practical method for large-scale control of quagga and zebra mussels (*Dreissena rostriformis bugensis* and *Dreissena polymorpha*) once they have become established in a waterbody. There have only been two successful open-water eradication efforts on small bodies of water, both of which used muriate of potash (potassium chloride). There are several limitations to potash including costs, logistics, and holding period (requires a closed system). Instead of using chemical methods to treat a waterbody, identification of a hypervirulent parasite as an additional control method for ZM/QM in open water needs to be explored. The advantages of a biological control agent are that it is species specific, self-replicating and spreading. The use of a biological control agent will not result in the eradication of ZM/QM, but it will be a tool for the integrated pest management of the invasive mussels.

Over the last three years (2019-2021), Molloy & Associates have built and expanded a field laboratory in Montenegro where mussel dissections and long term transinfection studies can be performed (see cover photo of this report). Multiple sampling trips have been made throughout the Balkans and Turkey to seek out isolated populations of other members of the genus *Dreissena* (such as *D. carinata* and *D. anatolica*) for field laboratory study. Proof-of-concept studies have shown that it is possible to transfer parasites from congeneric 'cousin' dreissenid to uninfected zebra and quagga mussels. The final report submitted by Molloy & Associates (Appendix 1) provides details of research accomplishments and a summary table of the parasites discovered from mussel dissections.

Researchers from Reclamation's Ecological Research Laboratory (EcoLab) were actively involved in this project. Monthly phone calls were made with Molloy & Associates to discuss the status of the project and troubleshoot any issues. Reclamation researchers have presented the results of this project to a wide range of audiences, including participants of the Western Regional Panel and the International IPM Symposium, and a large network of European collaborators has been established. In 2021, Dr. Nathan Harms, USACE-ERDC, joined the team to provide his expertise in the regulatory process of biological control agents. Finally, Dr. Passamaneck, Reclamation, performed the molecular analysis to characterize dreissenid parasites identified in this project and these results are part of a manuscript (in preparation). In 2021, a new S&T proposal was submitted to continue this research until 2024. The collaboration between Molloy & Associates, Reclamation, and other collaborators has built a solid foundation for continuing the search and identification of candidate parasites to control invasive dreissenid mussels in North America.

1. Introduction

Two species of invasive mussels, *Dreissena polymorpha* (zebra mussel) and *Dreissena rostriformis "bugensis"* (quagga mussel) (ZM/QM), arrived in North America in the late 1980's (1). Except for the waterways of the Pacific Northwest, quagga and zebra mussels have established populations in freshwater lakes, reservoirs, and rivers throughout North America. In Reclamation's territory in the Western United States, quagga mussels were first detected in 2007 at Lake Mead, Nevada, and their impacts to facilities are a continuing issue for Reclamation researchers and managers.

There are several characteristics that make these invasive mussels an issue. First, both species are prolific breeders, so their populations can expand rapidly when conditions allow. Second, quagga and zebra mussels are filter feeders and can change the ecology of a waterbody. Finally, dreissenid mussels settle on hard surfaces such as water intakes, gates, diversion screens, hydropower equipment, pumps, pipelines, and boats. This behavior can lead to costly operational issues such as overheating and unplanned outages. Mussel fouling requires managers to implement control methods within the power plant such as filtration, chlorine, copper, or hydro-optic disinfection (HOD) ultraviolet (UV) light treatment. For example, Pucherelli et al. 2020 (2) reported that at Hoover Dam an estimated $2.6 million will be spent on installing HOD UV light treatment to prevent mussel settlement in the generator cooling water. Each year over $120,000 has been spent on maintenance ($88,000 for a single thrust bearing cooler replacement and for a dive team to remove mussels from water cooling inlets and outlets). Hoover Dam experiences 1-3 mussel related outages each year that cost anywhere from $44K-$80K. These are the costs for only one facility that has ZM/QM, and as more facilities are infested the costs will continue to increase. Finding ways to control and mitigate the impact of dreissenid mussels on Reclamation facilities and waters has been on ongoing challenge for over fifteen years.

The central goal of this project is to identify hypervirulent parasites that could reduce populations of quagga and zebra mussels in waterbodies. Currently, there is no viable method for controlling these invasive mussels in large bodies of water once they establish a reproducing population. There have only been two successful eradications of dreissenid mussels in North America, both were in small bodies of water using potassium chloride (muriate of potash). This treatment requires a long holding time and is only suited to closed systems, and treatment of a large body of water, such as Lake Mead, would not be financially feasible or environmentally suitable. Finding new novel and scalable methods of controlling dreissenid mussels is currently considered the best strategy for controlling established populations. The identification of a self-reproducing parasite that can impact both invasive mussel species is the central goal of this project.

Infectious disease caused by hypervirulent parasites is known to have significant and long-lasting impacts on plant and animal populations. Use of natural enemies as biological controls to suppress pest species populations has been done for decades (3). The process involves finding a potential biocontrol agent (insect, parasite, virus, etc.) that can has co-evolved with the target species in their native range and is therefore highly host-specific and will not cause harm to other species or the environment. Once potential biocontrol agents are identified they must undergo extensive host

specificity testing and risk analysis as part of the regulatory process prior to any release. This project is currently in the searching phase of developing a biocontrol agent.

Starting in 2017, collaboration between members of Reclamation's Ecological Research Lab (EcoLab) and Molloy & Associates began to investigate the use of hypervirulent parasites as a novel biocontrol method for invasive dreissenid mussels, (ST-2018-1625-01). During these first two years, a field laboratory in Montenegro was established and sampling trips were undertaken in the Balkans. The current S&T project was started to continue the research for an additional three years (2019-2021) and results are summarized in this report (ST-2022-19097-01). Over the last three years, the proof-of-concept studies showing that transinfection of parasites between ZM/QM and their cousins were accomplished. Because of the progress made during the last three years, it was decided to continue this research for an additional three years (2022-2024, Survey of 'Cousin' Dreissenid Species in Eurasia for Potential Biocontrol Agents to Control Invasive Quagga and Zebra Mussels in North America, Project ID 22005).

2. Methods and Results

This research effort is a collaboration between Molloy & Associates and members of the EcoLab. Currently, Reclamation researchers' roles in this project include project oversight, coordination, communication, identification of new team members, and molecular identification methods for both adult mussels and their parasites. A detailed summary of Molloy & Associates research and progress can be found in Appendix 1. Over the last three years, Molloy & Associates has continued the search for hypervirulent parasites in Eurasia including the Balkans and Turkey (Appendix 1, map of study area). A field laboratory with flow-through tanks for long term transinfection studies with different species of dreissenids has been established in Montenegro (see cover photo of this report). Proof-of-concept studies have shown that it is possible to infect zebra mussels and quagga mussels with ciliate parasites from their 'cousin' species.

Communication

Monthly phone calls are conducted with Reclamation researchers and Dr. Molloy to provide updates on the status of the research and discuss next steps and give input on planned experiments and tasks. These phone calls have allowed the research group to share results and discuss issues to maintain advancement and coordinate the evolution of the project. Dr. Molloy provides a written summary of calls to Reclamation researchers to document monthly progress. When necessary, additional emails and phone calls are made to discuss specific aspects of the project. Finally, Reclamation researchers have been able to give presentations about this project to a wide range of audiences, including Reclamation staff, the annual United States Army Corps of Engineers, Engineer Research and Development Center (USACE-ERDC) Research Meeting, the Western Regional Panel annual meeting, and the 10th International IPM Symposium.

New Team Members

This project was discussed for potential collaboration at the 2020 USACE-USBR Research Meeting. Dr. Nathan Harms, a Research Biologist with the United States Army Corps of Engineers Aquatic Ecology and Invasive Species Branch, Environmental Laboratory (USACE-ERDC), was able to join the team as a biocontrol expert. His expertise in developing biocontrol agents and moving them through the regulatory process will be important as this project continues to move forward and is currently in the process of putting together a white paper about the regulatory aspects of dreissenid mussels to provide a road map for open release once potential biological control agents are identified. ERDC was able to provide funds to cover Dr. Harms labor for participating in this project.

As part of the white paper, Dr. Harms has started the process of identifying key regulators (e.g., USEPA, USFWS) in the United States who would regulate the importation and release of a dreissenid biocontrol agent. Once identified, these agencies will be consulted on the information required to issue importation permits, including environmental data (e.g., non-target host use) from the European range, the design of the experiments to test the host-range of the parasite, and potential additional data that may be required for permitting. By seeking these agencies collaboration early in development, we will ensure that every experiment carried out helps build the case for specificity and efficacy of the potential biocontrol agent and that we provide sufficient information upon application for importation and release permits.

Molecular Analysis

To characterize the parasites used in the project, molecular analysis has been performed by Dr. Passamaneck in the Reclamation EcoLab. The goal of these molecular analyses is to identify unique species-specific DNA sequences, of barcodes, that may be used to reliably identify and distinguish morphologically similar parasites. During this project the focus of this work was on two different *Ophryoglena* species, parasites in the digestive gland of the dreissenid cousin *D. carinata*. Dr. Passamaneck has been able to establish methods for the DNA extraction and molecular analysis of the *cytochrome oxidase I* (*COI*) gene of the parasites (4). It was demonstrated that the two species of *Ophryoglena* in the *D. carinata* both had unique *COI* gene sequences that could be used to distinguish them from one another, and from related species found in *D. polymorpha*. The results of this analysis are part of a manuscript (in preparation) describing the morphology and molecular genetics of these two species.

3. Discussion and Next Steps

Despite the global pandemic, this research project has continued to move forward because of the dedication and diligence of Dr. Molloy, his team of technicians in Montenegro who have maintained the field laboratory and collected samples, and a network of European researchers who have participated in sample collection trips. Over the last five years, key steps have been made in the search for hypervirulent parasites. First, a field laboratory where long term transinfection studies

can be conducted has been built in Montenegro. Second, a network of researchers throughout Europe has been established to provide advice on sampling locations and assist in sampling trips. Third, proof-of-concept transinfection studies have shown that it is possible to transfer parasites between ZM/QM and their 'cousin' dreissenid's. Finally, Dr. Passamaneck of Reclamation has been able to establish the molecular methods for the characterization of the parasites.

A research proposal was submitted to the Research Office to continue this project for an additional three years, through 2024. This project will build on the last five years of research and continue the refine the search for host-specific hypervirulent parasites that will cause injury and mortality to ZM/QM as potential biocontrol candidates. During the next three years the following tasks will be performed in parallel:

►Continue to identify the best locations to collect 'cousin' dreissenid's by examining the scientific literature and seeking advice from collaborating Eurasian scientists, and to collect and transfer 'cousin' dreissenids to the field laboratory for dissection, identification of parasites, and to perform transinfection studies.

►Once a potential biocontrol candidate is identified, a series of experiments will be performed to answer the following questions:

- Are ZM/QM equally susceptible to the parasite?
- Are the life stages (juvenile and adult) mussel equally susceptible to the parasite?
- What stage of the parasite's life cycle initiates infection?
- What parasite intensity is required to achieve sufficient infection and death/debilitation for population-scale impacts?
- How do abiotic factors, such as temperature, pH, etc. affect lethality/debilitation?
- How host specific is the parasite? Can it be transferred to other species in its native Eurasian habitat, or have the potential to infect native North American species?

►Completion of a white paper describing the regulatory process for dreissenid biocontrol.

►Continued identification of both adult mussels and their parasites using molecular methods.

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[4] O. Folmer, M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek, "DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates," *Mol. Mar. Biol. Biotechnol.*, vol. 3, no. 5, pp. 294–299, 1994.

Appendix: Molloy & Associates Final Report

FINAL REPORT

PROJECT TITLE: Use of Novel Parasites to Control Naive North American Dreissenid Populations

PROJECT CONTRACT PURCHASE ORDER: 140R8118P0088

PROJECT PERIOD OF PERFORMANCE: September 2018 – March 2022

SUMMARY

- This document is a report of the research progress achieved by the above-listed Purchase Order awarded by Reclamation to Molloy & Associates, LLC (Dan Molloy, Contract Principal Investigator). The project made progress in investigating an "outside the box," unconventional research approach for the development of a live, biological agent to control the two pest *Dreissena* species (zebra and quagga mussel) that have invaded North America and which pose a significant fouling challenge to Reclamation infrastructures. In short, this project's unconventional research approach was to explore the question as to whether infectious organisms living inside "cousin" *Dreissena* spp. (i.e., mussels in the same genus as zebra and quagga mussels) had potential to also infect zebra and quagga mussels. If so, it was hypothesized that there was the potential that these parasites could possibly be so "novel" that they could either kill them or otherwise seriously debilitate them, and thus have potential as biocontrol agents.
- In viewing the entire project period, an enormous effort was applied to document mussel infection by dissection, with a total of 8,717 *Dreissena* dissections performed: 4,718 mussels dissected from samples collected from Eurasian waterbodies, 2,905 mussels dissected solely during transinfection tests, and 1,094 additional mussels dissected in other miscellaneous activities.
- The project began with a Phase 1 research period in which simple (more qualitative, than quantitative) "transinfection" tests were conducted at the Field Research Laboratory — a specialized research facility that was established in Montenegro and dedicated solely to this project. Exploration teams were sent out from this field laboratory to search in collaboration with home-country collaborating scientists throughout Eurasia to find cousin *Dreissena* populations that had infections with parasites. Such infected cousin species were then collected and brought back to the Montenegro field lab for use in transinfection tests against zebra mussels in the field lab's 14 aquaria that were custom designed and fabricated solely for such transinfection tests. These Phase 1 tests with ciliate protozoan parasites verified the project's hypothesis that parasites from cousin Eurasian *Dreissena* species could infect zebra mussels. In these Phase 1 tests, however, there was no evidence that any of the 5 transinfected ciliate protozoans caused any detectable injury to the zebra mussels. This was not surprising, however, since these ciliates showed more of an affinity to be commensal than pathogenic in their normal *Dreissena* host species. In addition, the research team is well aware that it will realistically take years of transinfection testing before luck and their intense effort rewards the project with the discovery of the hypervirulent parasite it seeks.
- About halfway through the contract period, the project's Phase 2 period was initiated with a major decision to significantly increase the physical capacity for transinfection tests at the Montenegro Field Research Laboratory. It was believed that if we greatly increased the number of transinfection aquaria, we could significantly accelerate research progress. Accordingly, the number of transinfection aquaria in the lab trailer was increased from 14 to 72, and another trailer was acquired where other aspects of the project (dissections, computer data entry, typical office activities, etc.) could occur. This decision proved very advantageous as the significant increase of the number of aquaria permitted: 1) successful multi-month-long transinfection tests against zebra and quagga mussels requiring dozens of aquaria in which the transinfection rates of parasites (i.e., prevalence and intensity) were highly quantified for the first time; 2) a successful highly-quantified, specialized test involving a dozen aquaria to evaluate the feasibility of a shell color-marking procedure and a springwater storage system – both of which may be valuable to use in future transinfection tests. In addition to these laboratory tests, the Phase 2 research period also included 2 successful transinfection field tests at Montenegro's Sasko Lake. As was observed in the above-mentioned Phase 1 transinfection tests, there was no evidence that of the transinfected ciliate protozoans in the Phase 2 lab and field trials showed any detectable injury to the zebra or quagga mussels. These results reinforced the research team's belief: 1) that this research project can be successful, but it will take continued patience and perseverance to successfully find the hypervirulent parasite that this project seeks and 2) that the team has developed excellent lab and field testing protocols in the search for it.

- The contributions of the following to the success of the project are gratefully acknowledged as all were critically important to the progress achieved in this project:
  - Mihailo Jovicevic (Lead Scientist at the Montenegro Field Research Laboratory);
  - 14 other scientists from 9 Eurasian countries (Albania, Bulgaria, Finland, France, Italy, Montenegro, North Macedonia, Serbia, and Turkey) who collaborated on this project;
  - the four technicians at the Montenegro Field Research Laboratory who took turns working weekends to keep the lab open and the mussels fed and the tests going 365 days a year.

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INTRODUCTION

This document is a report of the research progress achieved during a 3-year contract awarded by Reclamation to Molloy & Associates, LLC (Dr. Daniel Molloy, Contract Principal Investigator). During this period the project investigated an “outside the box,” unconventional research approach proposed by Dr. Molloy for the development of a live, biological agent to control zebra and quagga mussel populations throughout entire water bodies. The biocontrol agent this research project is dedicated to develop is aimed at having not only extraordinary selectivity in killing *Dreissena* mussels, but also an extremely low treatment cost. It would be a control method with unprecedented environmental safety and affordability that could be widely adopted for use by Reclamation, as well as by lake associations and other entities for control of dreissenids throughout North American water bodies, irrespective of their size.

PROJECT’S UNCONVENTIONAL RESEARCH PATH

Infectious diseases caused by hypervirulent (extremely lethal) parasites can have long-term, devastating impacts on animal populations. This is especially true when "naive" animal populations are exposed to "novel" parasites that they have been geographically separated from for millennia. Such naive host populations can be ravaged by the virulence of these novel parasites since they have not co-evolved with them and thus have little to no resistance to infection by them. In this research project, quagga and zebra mussels (*Dreissena rostriformis bugensis* and *D. polymorpha*) are being evaluated for their "naïveté" to parasites that normally infect only "cousin" *Dreissena* spp., i.e., other closely related species in the genus *Dreissena* whose evolution diverged from zebra and quagga mussels hundreds of thousands to millions of years ago. Such geographically isolated cousin *Dreissena* spp. (such as *D. caputlacus*, *D. anatolica*, *D. blanci,* and *D. carinata)* are present in Eurasia (i.e., Eastern Europe and Western Asia). Because zebra and quagga mussels have not been previously exposed to the parasites of their Eurasian cousins, infection by these latter parasites may prove hypervirulent. This project is designed to explore this latter possibility, and if successful, use one or more of the hypervirulent, novel parasites that the project has discovers to control zebra and quagga mussel populations in North America.

Eurasian region (encircled) where “cousin” *Dreissena* (species closely related to zebra and quagga mussels) exist

PHASE 1 RESEARCH ACCOMPLISHMENTS

Accomplishment #1 in Phase 1: Establishment of a field laboratory in Eurasia dedicated solely to the success of this Reclamation-funded project.

The trailer in Montenegro that was converted into the "Field Research Laboratory"

Early into this project, it was decided that an essential, key, initial objective was to establish a well-equipped, field laboratory in Eastern Europe solely dedicated to achieving the success of this Reclamation-funded project (see 3 photos on this page). Accordingly, a small trailer (~2 m x 5 m of floor space) was transformed into the project's "Field Research Laboratory" on the side of a mountain near Spuž, Montenegro. In hindsight (*now at the time of the writing of this Final Report in March 2022*), that decision has proven to have been the most significant and pivotal key factor propelling the project to its current level of success. Right from its inception, the Field Research Laboratory quickly became the hub of all Eurasian activity of the project. Besides the practicality of the laboratory being located right in the targeted field sampling area (i.e., Eurasia), it was custom designed with intense focus to carry out the project's following two primary research activities:

- To facilitate mussel dissections: From the start of the project, the Field Research Laboratory was equipped with enough bench space, microscopes, computers, monitors, cameras, high speed internet, refrigerators, etc. to allow up to 3 staff to work simultaneously dissecting cousin *Dreissena* and documenting their parasites (see progress in **Accomplishment #2** below).
- To facilitate the project's critically important transinfection testing: Its location on the side of a mountain allowed it to receive gravity-fed water from a spring located higher up on the mountain. That natural spring-water supply allowed the project to run transinfection tests in the lab's aquaria (custom made in Montenegro) to evaluate if the parasites of cousin *Dreissena* were capable of infecting and killing zebra and quagga mussels (see progress in **Accomplishment #3** below).

The research trailer at time of its purchase

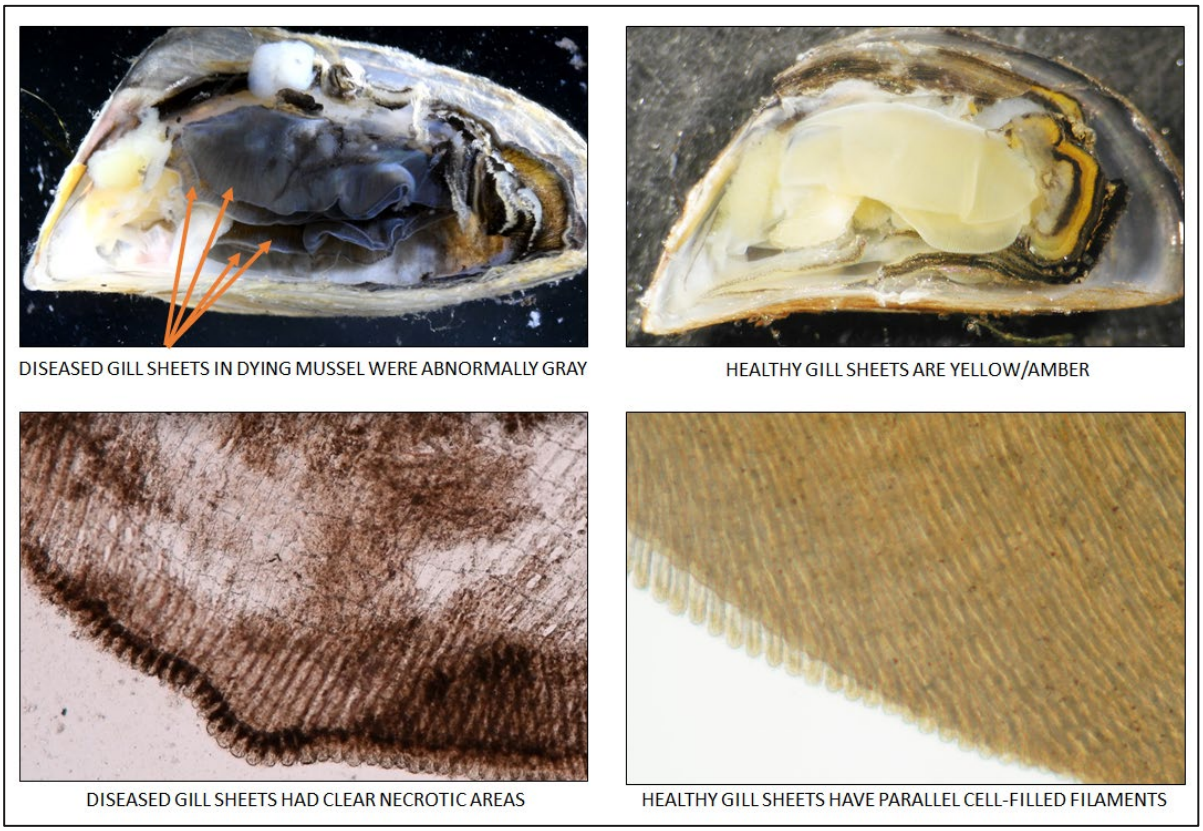
Exact same view within the research trailer during Phase 1 of the project, with 14 custom-made, flow-through testing aquaria installed across from a well equipped "dissection" bench

Accomplishment #2 in Phase 1: Finding infected "cousin" *Dreissena* in Eurasia for use in transinfection tests

In Phase 1, the project rapidly launched a rigorous Eurasian field sampling program to find parasites and any other evidence of infectious disease in cousin *Dreissena* spp. populations as well as the invasive *D. polymorpha* population that was present in Sasko Lake in Montenegro (see Table 1 at end of this report for a list of all project dissections). Dissections in Phase 1 of the project included:

- A total of 100 *D. polymorpha* dissected from Sasko Lake, with only the parasitic ciliate species *Ancistrumina limnica* observed.

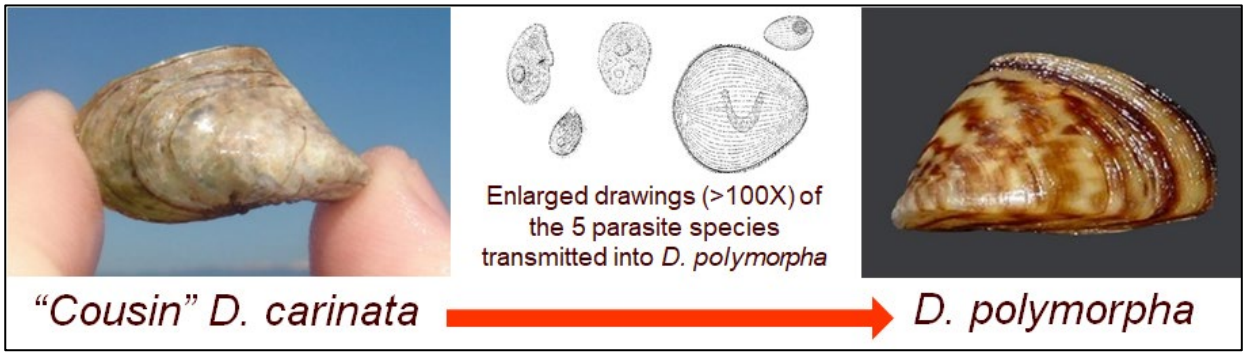
- A total of 2,372 cousin *Dreissena* spp. were dissected from 55 field samples collected in 3 Eurasian countries:
  - Turkey: 701 mussels – a mix of *D. anatolica and D. caputlacus* – were dissected from 5 samples.
  - Montenegro: 555 *D. carinata* were dissected from 7 samples.
  - North Macedonia: 1,116 *D. carinata* were dissected from 43 samples.
- As a result of these 2,372 dissections of cousin *Dreissena* spp., the following 12 species in 3 taxa (i.e., ciliates, trematodes, and mites) were observed inside the mussels:
  - 6 obligately-parasitic ciliate species (see list below) that are of special interest to the project since all are host specific to *Dreissena* – exactly the kind of potentially environmentally safe parasites from cousin *Dreissena* spp. that the projects seeks to evaluate for their possible lethality to zebra and quagga mussels:
    1. A new big *Ophryoglena* sp. that parasitizes digestive gland ducts.
    2. A new small *Ophryoglena* sp. that parasitizes digestive gland tubules.
    3. A new *Ophryoglena* sp. that parasitizes gills.
    4. The gill parasite *Conchophthirus acuminatus.*
    5. The gill parasite *Conchophthirus klimentinus.*
    6. The gill parasite *Hypocomagalma dreissenae*.
  - 1 parasitic ciliate species, *Ancistrumina limnica,* that is not of interest to the project since it is not obligately host specific to *Dreissena*, e.g., it has been reported from other molluscs.
  - 3 parasitic trematode species (*Phyllodistomum* sp., *Bucephalus* sp., and a metacercarial cyst of an unidentified species) that would never be considered as candidate *Dreissena* biocontrol agents since after infecting *Dreissena*, all 3 species parasitize other hosts in their complex life cycles.
  - 2 free-living mite species with no symbiotic association with *Dreissena* (i.e., they likely inadvertently entered into the mantle cavity through the inhalant siphon).
- In addition, one of the 389 dissected *D. anatolica* mussels from Lake Egirdir in Turkey was observed to be dying with symptoms of a gill disease. As illustrated in the 4 photos below, all 4 gill sheets (ctenidia) appeared very abnormal in color – very dark blue-gray (top left photo) rather than the normal yellow/amber (top right) and had regions of clear, necrotic, non-functional tissue (bottom left photo) rather than solid gill tissue (bottom right).



Finding such a diseased, dying *Dreissena* mussel is rare in nature, and this is exactly the kind of discovery that could be very beneficial to this project. But was this particular diseased mussel the breakthrough discovery that will probably lead to an infectious biocontrol agent useful to tame North American zebra and quagga mussels? Our analysis to date, suggests that it is not likely, as no parasites were visually detected.

Consequently, these necrotic, dark blue-gray gills are currently suspected to be more likely the result of either a non-infectious disease (e.g., gill damage from toxic algae) or a viral infection. The project has a preference not to develop a virus as a dreissenid biocontrol agent because of the relatively high risk of viral genetic mutations. The project, however, is still pursuing investigating the possibility that the causative agent of the necrosis might be a virus or some other microbe (i.e., pieces of mussel tissue that were placed in fixatives for subsequent analysis, including RNAlater, Bouin's, and ethanol still remain unprocessed).

<u>Accomplishment #3 in Phase 1</u>: Demonstrating proof of concept that infections can be passed from cousin *Dreissena* to zebra mussels: 5 ciliates from cousin *D. carinata* transmitted into *D. polymorpha.*



In Phase 1, 4 small-scale transinfection tests were conducted and these tests resulted in 5 obligate, *Dreissena*-specific ciliate parasites from cousin *D. carinata* being successfully transmitted into zebra mussels:

- The 5 ciliates transmitted into *D. polymorpha* from cousin *D. carinata* were:

Ciliate Species	Test #	Transinfection Details
Small digestive gland *Ophryoglena* n. sp	Test 1 (#2019-0010)	2 of the 5 *D. polymorpha* dissected during this test were observed to be infected with this species
Gill *Ophryoglena* n. sp.	Test 2 (#2019-0011)	3 of the 7 *D. polymorpha* dissected during this test were observed to be infected with this species
	Test 3 (#2019-0012)	3 of the 6 *D polymorpha* dissected during this test were observed to be infected with this species
Hypocomagalma dreissenae	Test 4 (#2019-0013)	1 of the 6 *D. polymorpha* dissected during this test was observed to be infected with this species
Conchophthirus acuminatus	Test 4 (#2019-0013)	4 of the 6 *D. polymorpha* dissected during this test were observed to be infected with this species
Conchophthirus klimentinus	Test 4 (#2019-0013)	2 of the 6 *D. polymorpha* dissected during this test were observed to be infected with this species

- These Phase 1 tests were more qualitative than quantitative and were primarily conducted to lay the groundwork for future highly-quantitative transinfection tests in Phase 2. They did demonstrate, however, that the establishment of the Field Research Laboratory with its custom-made aquaria were a tremendous (truly essential) asset to the project since they made these transinfection tests (which were of the highest priority of research effort to the project) relatively easy to conduct. Also, the results of these tests represented a milestone achievement for the project since a critically important project research objective was to test the parasites from cousin *Dreissena* to see if they could infect either quagga or zebra mussels. Thus, the entire research team took pride in that this was able to be accomplished in the initial Phase 1 of the project – virtually all because of the team's determination (in particular, Project Lead Scientist Mihailo Jovicevic) to locate a custom designer/builder of glass aquaria with inflow/outflow (flow-through) capabilities in Montenegro's capital city of Podgorica.
- The following are some details that shaped the experimental designs of the 4 above-mentioned transinfection tests:
    - As mentioned in <u>Accomplishment #2 in Phase 1</u>, the 2,372 dissections of cousin *Dreissena* spp. revealed the presence of 6 parasitic ciliate species that were obligately host specific to *Dreissena* (Table 1).
    - Because 5 of these 6 ciliate species were present in *D. carinata* populations in Montenegro, the 4 transinfection tests were carried out using these infected population of *D. carinata* from Montenegro. (It should be noted that quagga mussels were unavailable for transinfection trials in Phase 1).

- Before their dissection, the *D. polymorpha* were exposed to the parasites of *D. carinata* for 5 days in Test 1 and for ~3 months in Tests 2-4. At the time of their dissection, there was no clear evidence of any pathology in any of the *D. polymorpha* that were infected in any of the 4 transinfection tests; likewise infection with these 5 ciliate parasites in *D. carinata* appeared to have no significant negative impact.
- Zebra mussels used in the 4 transinfection tests in Phase 1 were collected from the single population that exists in Montenegro – a population that dissections during Phase 1 recorded no host-specific obligate ciliates, thus simplifying analysis of the test results, i.e., if any of the 5 ciliates species were found in the zebra mussels during the 4 Phase 1 tests, they had to come from the infected *D. carinata* in the acrylic tube that both species were placed in, e.g., as in the middle aquarium in the photo below.

In the transinfection tests using the custom-designed aquaria (Tests 2-4), one clear acrylic tube held both *D. carinata* and *D. polymorpha* (here in the center aquarium), and the other two aquaria served as controls, one with a tube of only *D. carinata* (here left) or *D. polymorpha* (here right)

Accomplishment #4 in Phase 1: Gaining insight into the life cycle of transinfected parasites: Reproduction tests were conducted at the Field Research Laboratory to better understand the life cycle of one of the ciliate parasites.

Two tests (#2019-0001 and #2019-0015-21) were conducted investigating asexual reproduction in the Gill *Ophryoglena* n. sp. -- one of the parasites that was successfully transmitted from *D. carinata* into *D. polymorpha* in two ~3-month-long transinfection tests (i.e., Test 2019-0011 and Test 2019-0012).
These reproduction tests both indicated that this ciliate species can rapidly (within ~2 days) asexually reproduce outside the mussel as follows:

- after the ciliate exits an infected mussel (following termination of its parasitic stage spent on the mussels gills), it attaches itself to a substrate and transforms itself into an encysted, non-feeding tomont stage within which tomites are produced by amitosis.
- at the completion of the series of amitotic divisions, the tomites leave the tomont and are now referred to as theronts that swim in search of a new mussel to infect.

The value of this above-mentioned life-cycle research was that we learned:

- that because of the rapid ~2-day asexual reproduction phase we observed in this ciliate species, it is likely that asexual reproduction did occur in this ciliate species in both of the ~3-month long transinfection tests
- that this ongoing reproduction of the ciliate thereby likely contributed to the level of transinfection recorded in *D. polymorpha* observed in both tests.

PHASE 2 RESEARCH ACCOMPLISHMENTS

Accomplishment #1 in Phase 2: Significant increase in testing equipment and infrastructure

The project's Phase 2 period began with a major decision to significantly increase the physical capacity for transinfection tests at the Montenegro Field Research Laboratory. It was believed that if we greatly increased the number of transinfection aquaria, we could significantly accelerate the research pace. Accordingly, the number of transinfection aquaria in the "Lab Trailer" (the trailer on right in image below) was increased from 14 to 72, and an "Office Trailer" was added (on left in image below) where other aspects of the project which had been previously done in the Lab Trailer could now occur (e.g., dissections, computer data entry, typical office activities, etc.).

Technician Milena Ikovic at the project's Field Research Laboratory in Spuz, Montenegro. An "Office Trailer" (L) was added during the project's Phase 2 period. This allowed the "Lab Trailer" (R) to be completely filled with 72 new customized testing aquaria

Project lead scientist Mihailo Jovićević and technicians Irma Muhović and Milena Iković (L to R) working in the Office Trailer

A view of one side of the Lab Trailer showing some of the 72 customized testing aquaria that were installed in the project's Phase 2 period

Accomplishment #2 in Phase 2: Increased testing capacity resulted in accelerated research progress

This "72-aquaria" decision proved very advantageous as the increase in the number of aquaria allowed the following comprehensive testing schedule during the Phase 2 research period:

Dual-purpose Laboratory test (Code #2021-002)
A successful highly-quantified, specialized dual-purpose laboratory test using a dozen aquaria was run to evaluate the following two factors:

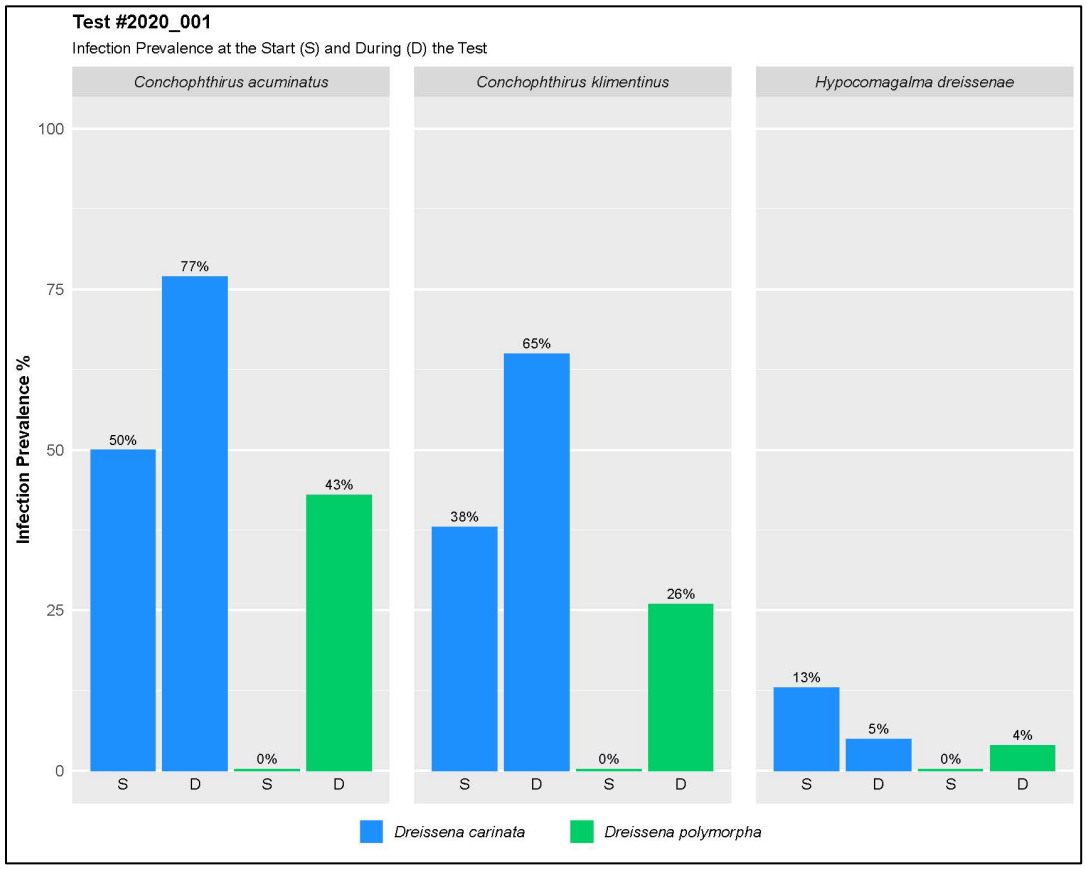
- Sometimes there is a need to store the springwater we use in our testing program (e.g., for use later during seasonal periods of naturally low springwater flow). If we temporarily stored the springwater that we use for our lab testing in a 10,000 L tank (that we had already purchased and buried below ground level) rather than using the springwater as we typically do (immediately using it as it flows down the mountain and into our Lab Trailer), would that have a disruptive, negative impact on the survival of the test mussels or their parasite prevalence or intensity?
  - Data Results: Analysis of the data at the end of this test indicated:
    - that there was no statistical difference in mean survival of mussels that were reared in our regular springwater or the springwater that had been reared in the springwater stored in the 10,000 L tank.
    - that there was no statistical difference in mean prevalence and intensity of endosymbionts in the mussels that were reared in our regular springwater or the springwater that had been stored in the 10,000 L tank.
  - Conclusions from the Results: There was no evidence that storing springwater in the 10,000 L tanks had a disruptive, negative impact on the survival of the test mussels or their parasite prevalence or intensity. That was good news for our testing program.
- Sometimes it is difficult to separate *Dreissena* species that we have mixed together in our transinfection tests due to the accumulation of mud naturally obscuring their shell morphological features during the months they are left in the acrylic tubes during our tests. Accordingly, we tested to evaluate if a shell color-marking procedure that we judged might be useful to mark mussels of a given species before their placement in an acrylic tube for use in a test would that have a disruptive, negative impact on the survival of the test mussels or their parasite prevalence or intensity.
  - Data Results: Analysis of the data at the end of this test indicated:
    - that there was no statistical difference in mean survival of mussels whether they were color-marked or not;
    - that there was no statistical difference in mean prevalence and intensity of parasites in the mussels whether they were color-marked or not.
  - Conclusions from the Results: There was no evidence that color-marking the mussels had a disruptive, negative impact on the survival of the these marked mussels or their parasite prevalence or intensity. That was good news for our testing program.

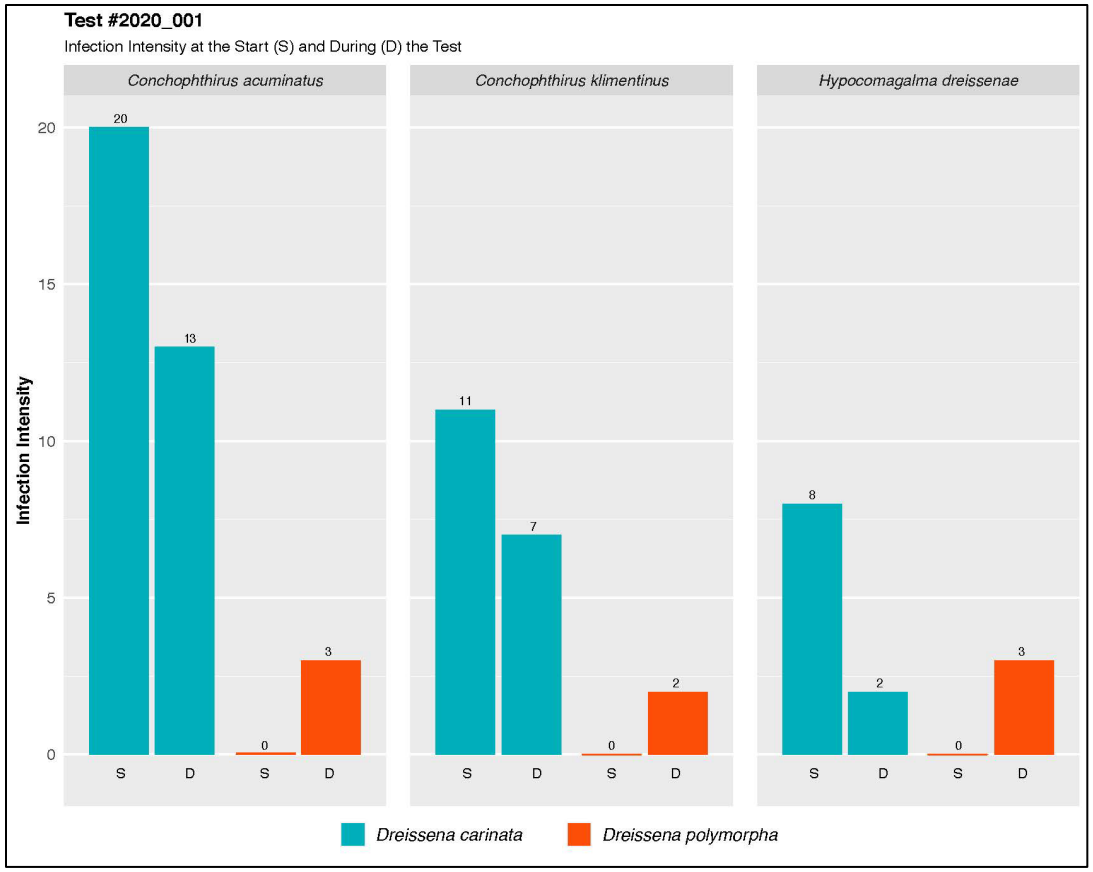
Transinfection Laboratory Test #1 (Test Code #2020-001) against zebra mussels and Transinfection Laboratory Test #2 (Test Code #2020-011) against quagga mussels (See results in bar graphs on pages 10 and 11.)
These two successful multi-month-long laboratory transinfection tests were conducted in which the transinfection rates of parasites (i.e., prevalence and intensity) were highly quantified for the first time in this project. In both these tests, the zebra mussels (*D. polymorpha*) and quagga mussels (*D. rostriformis*) had no infection at the start of the tests and both acquired infections (as displayed in the bar graphs both in terms of infection prevalence and infection intensity) from the cousin *D. carinata* that were reared with them in the aquaria.

Transinfection Field Test #1 (Test Code #2020-009) and Transinfection Field Test #2 (Test Code #2021-001) (See results in bar graphs on pages 12 through 14.)
Two successful field transinfection tests were caried out in Montenegro's Sasko Lake (these were the first project field tests ever conducted). In both these tests, the zebra mussels (*D. polymorpha*) had no infection at the start of the field tests and both acquired infections (as displayed in the bar graphs both in terms of infection prevalence and infection intensity) from the cousin *D. carinata* that were reared with them in the lake.

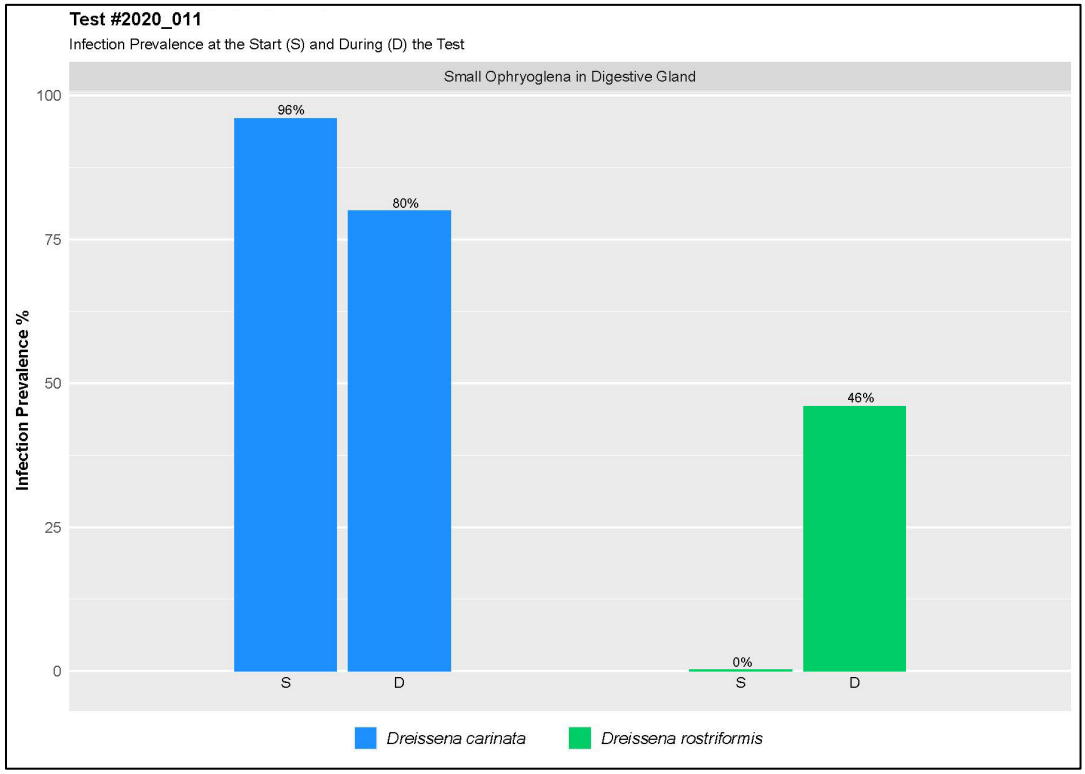
In summary, similarly to Phase 1 transinfection tests, there was no evidence that the above-mentioned transinfected ciliate protozoans in the Phase 2 lab and field trials caused any detectable injury to the zebra or quagga mussels. These results reinforced the research team's belief that it will take continued patience and perseverance to successfully find the hypervirulent parasite that this project seeks, but at least we have developed excellent lab and field testing protocols in the search for it.

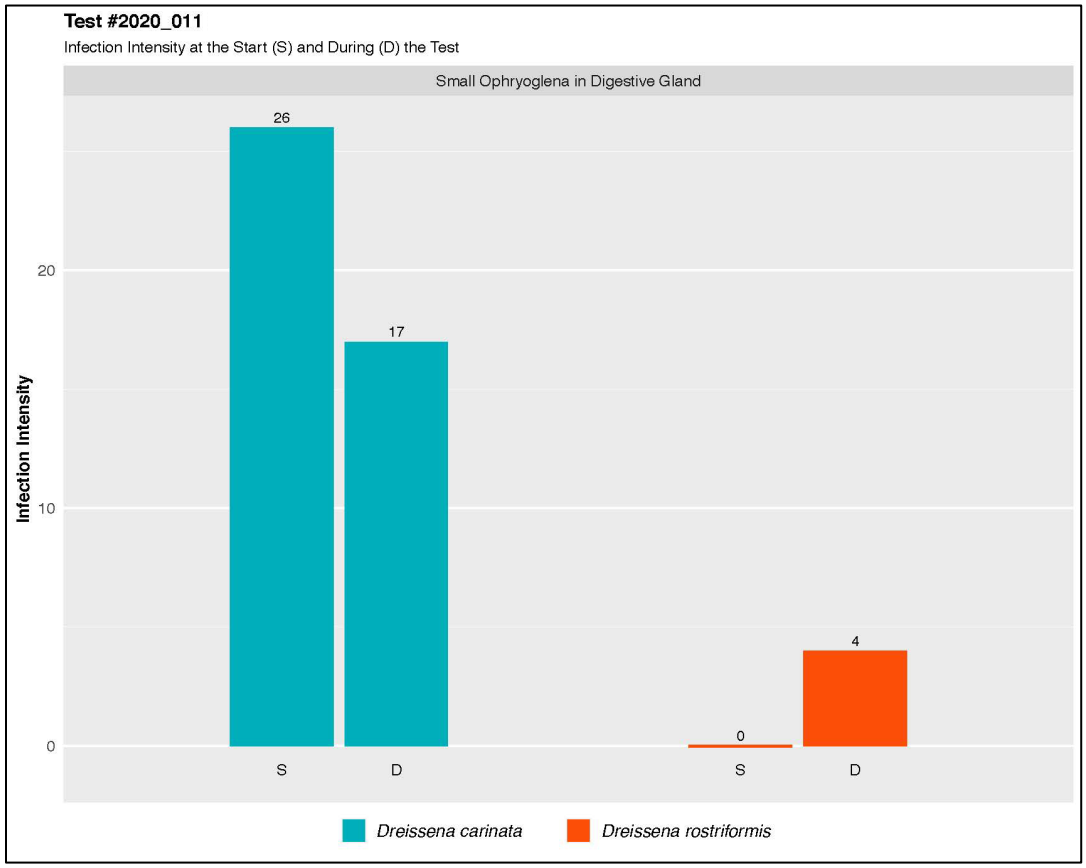
Transinfection Laboratory Test #1 (Test Code #2020-001) against *D. polymorpha* (Zebra Mussels)



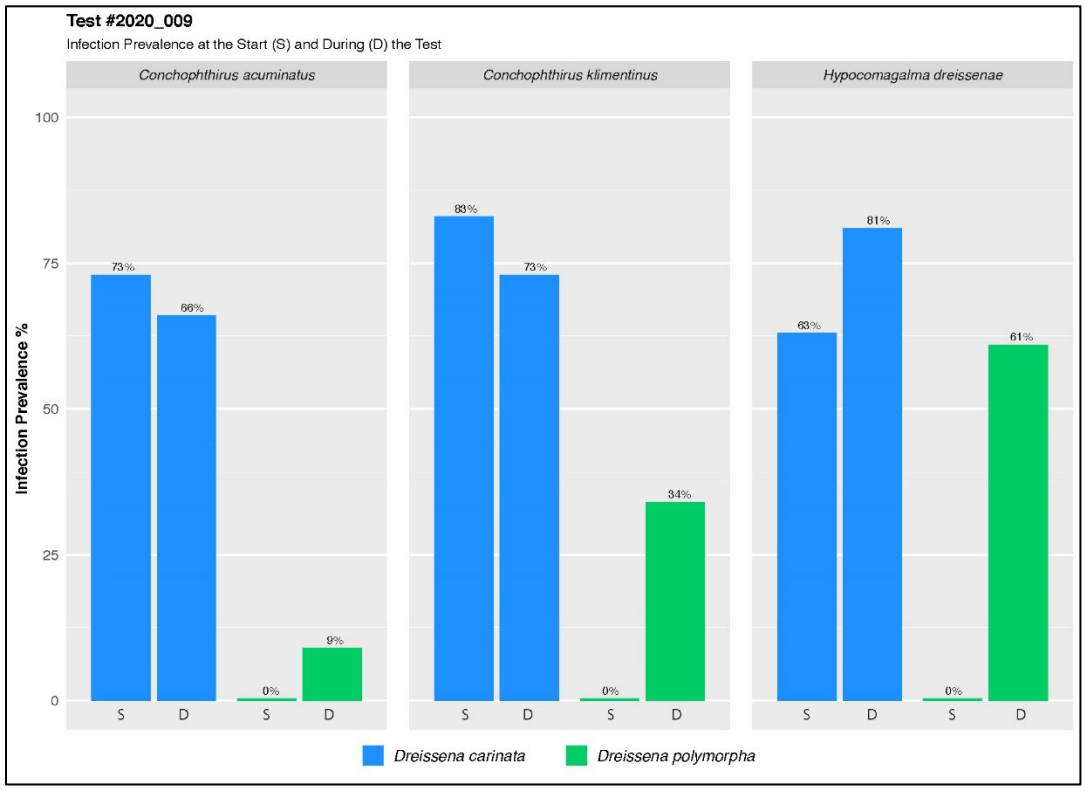


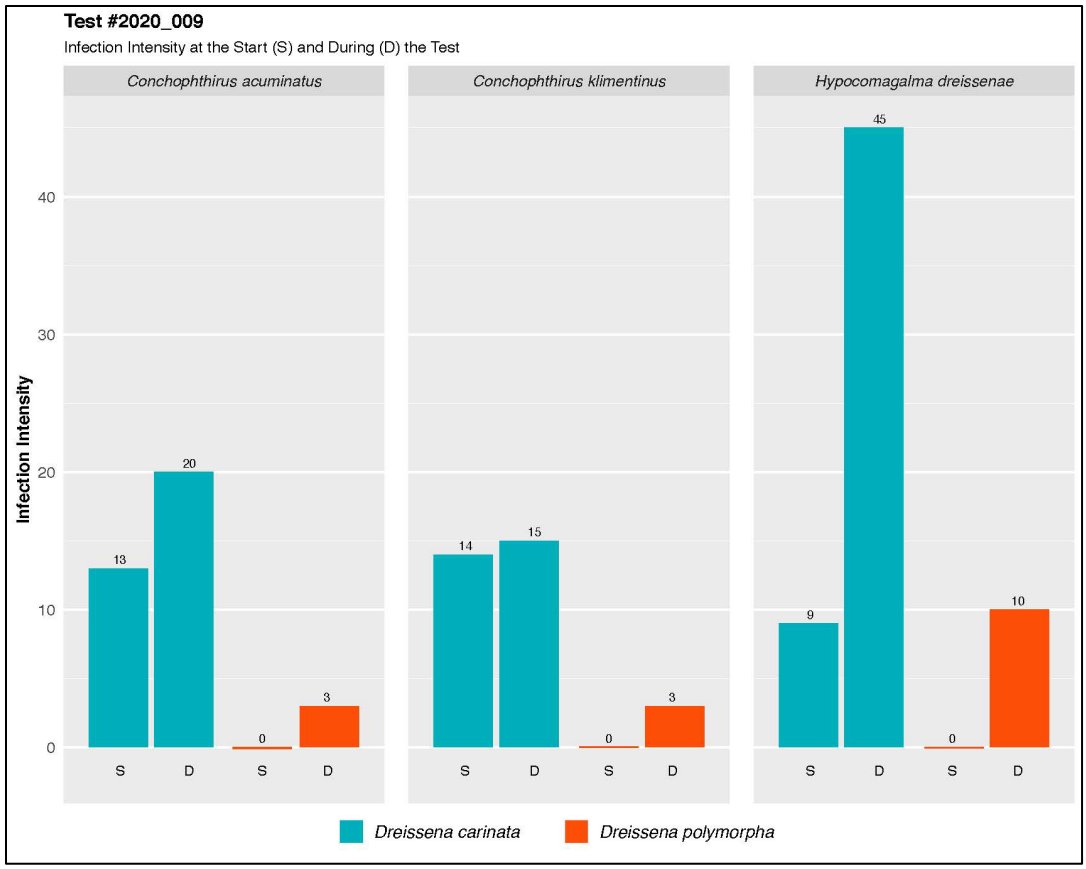
Transinfection Laboratory Test #2 (Test Code #2020-011) against *D. rostriformis* (Quagga Mussels)



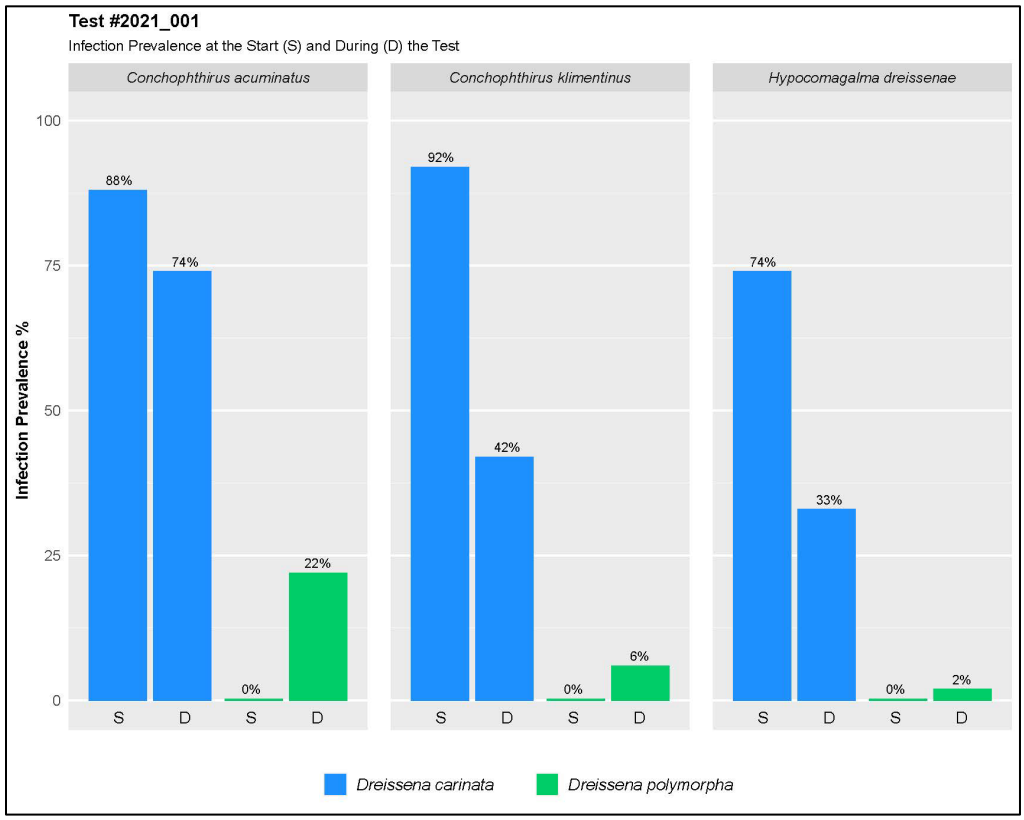


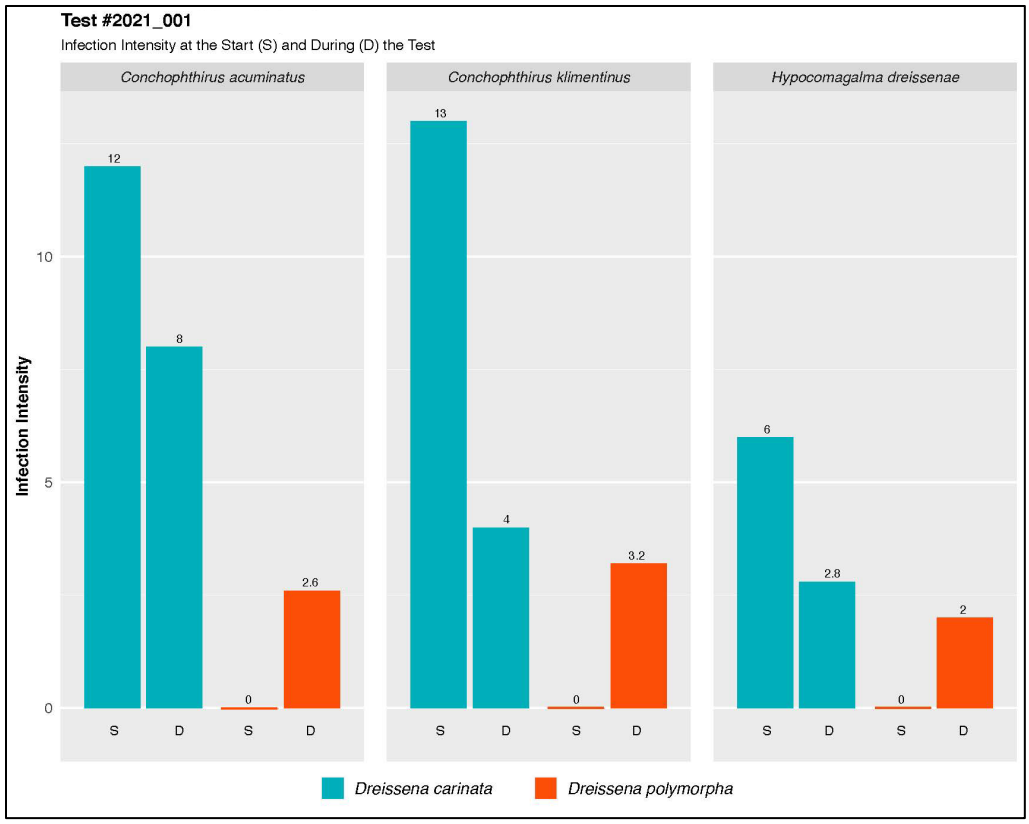
Transinfection Field Test #1 (Test Code #2020-009)



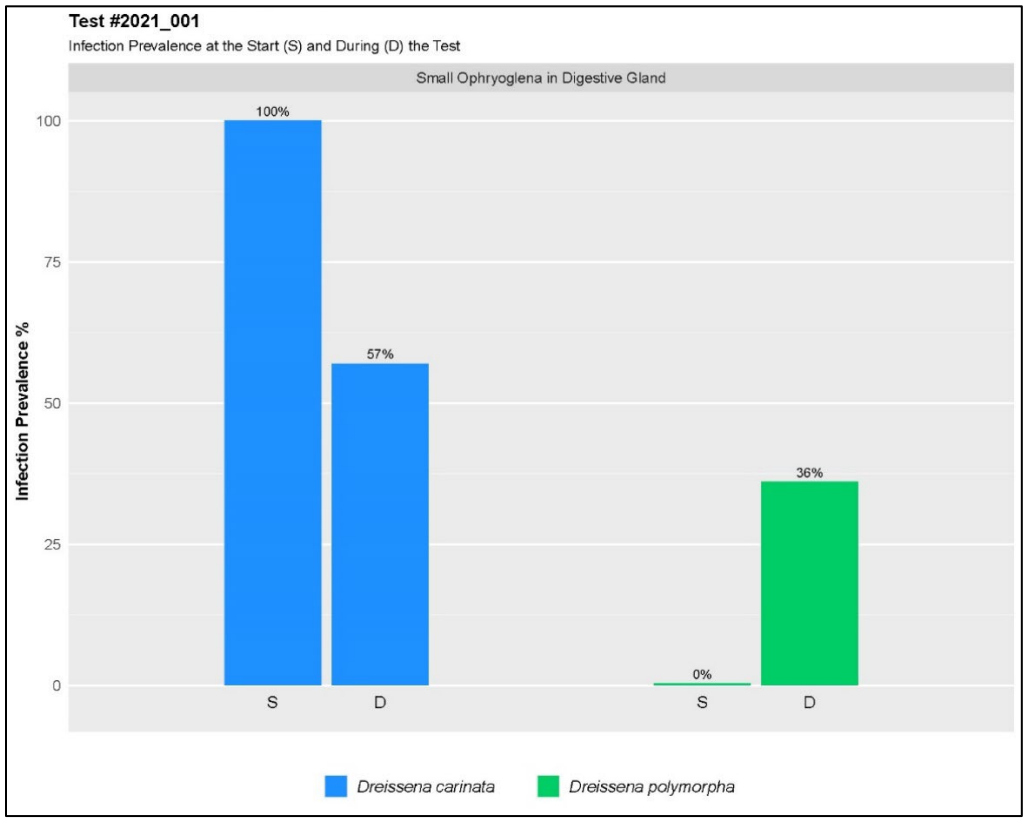


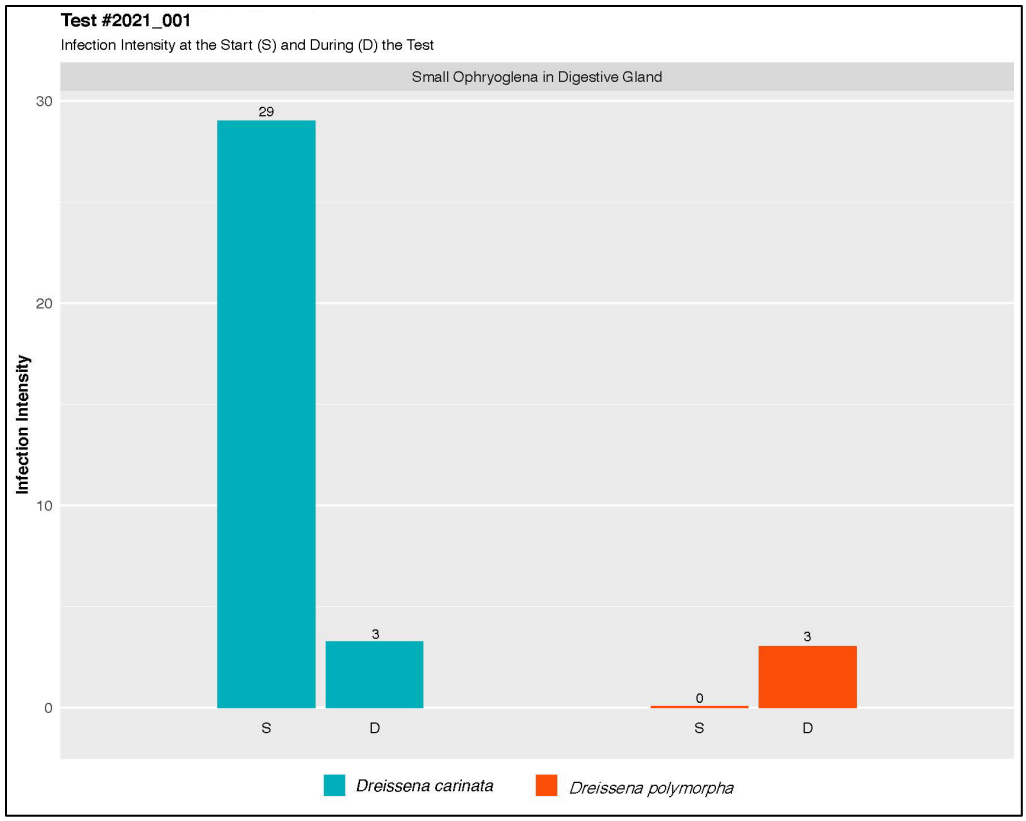
Transinfection Field Test #2 (Test Code #2021-001)





Transinfection Field Test #2 (Test Code #2021-001)





Accomplishment #3 in Phase 2: Advances in the molecular analysis reveal two new species of parasites in *D. carinata*.

A manuscript is now in preparation describing the morphology and molecular genetics of two new species of *Ophryoglena* that are parasites in the digestive gland of cousin *D. carinata.* The lead authors on the manuscript are molecular biologist Dr. Yale Passamaneck (Reclamation) and ciliate morphologist Dr. Sergei Fokin (University of Pisa).

A FINAL NOTE ON THE VALUE PLACED ON DISSECTIONS THROUGHOUT THIS ENTIRE PROJECT

In viewing the entire project period, an enormous effort was required to document mussel infection by dissection, with a total of 8,717 *Dreissena* dissections performed: 4,718 mussels dissected from samples collected from Eurasian waterbodies, 2,905 mussels dissected solely during transinfection tests, and 1,094 additional mussels dissected in other miscellaneous activities.

COLLABORATING EURASIAN SCIENTISTS IN THE PROJECT

The 15 scientists from 9 Eurasian countries (Albania, Bulgaria, Finland, France, Italy, Montenegro, North Macedonia, Serbia, and Turkey) listed below deserve enormous credit. Dr. Molloy enthusiastically sought out the assistance of expert Eurasian collaborators, and they responded with enthusiasm. The multiple significant achievements realized to date during this project are truly a reflection of the enormous international team effort put forth by these talented scientists (listed in alphabetical order):

	Dr. Sebnem Atasaral Expertise: Molecular Biology Institution: Karadeniz Technical University, Turkey	One of the project's two lead scientists for sampling in Turkey
	Gani Bego Expertise: Monitoring of Protected Areas Institution: Administration of the Protected Areas Korcha, Albania	Assisted in sampling and dissections in Albania
	Dr. Miodrag Djordjevic Expertise: Biostatistics Institution: University of Niš, Serbia	Provided statistical analysis of test data
	Dr. Sergei Fokin Expertise: Ciliate parasites Institution: University of Pisa, Italy	Described morphology of new ciliate species parasitic in *D. carinata*
	Dr. Laure Giamberini Expertise: *Dreissena* parasites Institution: University of Lorraine, France	Supplied zebra mussel parasites from France used for comparative sequencing versus Balkan parasites
	Mihailo Jovicevic Expertise: *Dreissena* parasites Institution: Pro Natura, Montenegro	The lead scientist at the Montenegro Field Laboratory overseeing all of its lab and fieldwork in Eurasia

	Dr. Wanying Liao Expertise: Ciliate biology and ecology Institution: University of Pisa, Italy	Made critically important permanent slides for type specimens of a new ciliate species
	Mahesh Nitla Expertise: Ciliate endosymbionts Institution: University of Pisa, Italy	Performed scanning electron micrographs of specimens of a new ciliate species
	Dr. Vladimir Pesic Expertise: Freshwater aquatic invertebrates Institution: University of Montenegro, Montenegro	Identified mites found inside *Dreissena* and provided general project guidance and support
	Dr. Spase Shumka Expertise: Freshwater aquatic organisms Institution: Agricultural University of Tirana, Albania	The project's lead scientist for sampling in in Albania
	Dr. Jouni Taskinen Expertise: Freshwater bivalve parasites Institution: University of Jyväskylä, Finland	Advised on general aspects of parasitology in freshwater bivalves
	Dr. Milco Todorov Expertise: Aquatic Invertebrates Institution: Biodiversity and Ecosystem Research, Bulgaria	Assisted in sampling in Albania
	Dr. Sasho Trajanovski Expertise: *Dreissena* parasites Institution: Hydrobiology Institute, North Macedonia	The project's lead scientist for sampling in North Macedonia
	Dr. Teodora Trichkova Expertise: *Dreissena* biology and ecology Institution: Biodiversity and Ecosystem Research, Bulgaria	The project's lead scientist for sampling in Bulgaria
	Dr. Mehmet Zeki Yildirim Expertise: Freshwater molluscs Institution: Mehmet Akif Ersoy University, Turkey	One of the project's two lead scientists for sampling in Turkey

MONTENEGRO FIELD RESEARCH LABORATORY TECHNICIANS

The key role these staff members played in the project's progress is gratefully acknowledged. They all, for example, took turns working weekends to keep the lab open, the mussels fed, and the tests going 365 days a year.


Milena Ikonić

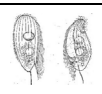
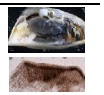
Jelena Jakišić

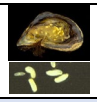
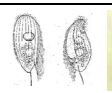
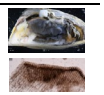


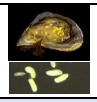
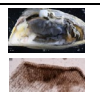
Irma Muhović

Andrijana Mićanović


Table 1. Parasites observed by dissection of field collected *Dreissena* during the entire project

Country	Sample #	Date of Collection MM/DD/YYYY	Water Body	Species	Comprehensive Dissections ~15 min per mussel using compound and stereomicroscope	Quick Dissections ~2 min per mussel typically using only stereomicroscope	Total Dissections	CILIATE Small *Ophryoglena* inside Digestive Gland Tubules	CILIATE Big *Ophryoglena* inside Digestive Gland Ducts	CILIATE Ophryoglena on Gill Surface	CILIATE Similar to *Conchophthirus acuminatus* on Mantle Cavity Tissues	CILIATE *Conchophthirus klimentinus* on Mantle Cavity Tissues	TREMATODE *Phyllodistomum* sp.	CILIATE *Hypocomagalma dreissenae* on Gills	TREMATODE *Bucephalus* trematodes inside gonads and other organs	TREMATODE Round transparent cysts (possibly *Leucochloridiomorpha constantiae)*	CILIATE *Ancistrumina limnica* on mantle cavity tissues	MITE On gill tissues	DARK BLUE-GRAY GILL DISEASE In live *D. anatolica* Turkish mussel
																			
								YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link
North Macedonia	M-018	9/17/2018	Lake Ohrid	carinata	20	0	20	●		●	●	●				NOT EXAMINED FOR IN THIS SAMPLE			
North Macedonia	M-019	9/17/2018	Lake Ohrid	carinata	20	0	20	●		●	●	●				NOT EXAMINED FOR IN THIS SAMPLE			
North Macedonia	M-020	10/17/2018	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				NOT EXAMINED FOR IN THIS SAMPLE			
North Macedonia	M-021	10/18/2018	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				NOT EXAMINED FOR IN THIS SAMPLE			
North Macedonia	M-022	11/16/2018	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				NOT EXAMINED FOR IN THIS SAMPLE			
North Macedonia	M-023	11/18/2018	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				NOT EXAMINED FOR IN THIS SAMPLE			
North Macedonia	M-024	12/17/2019	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				NOT EXAMINED FOR IN THIS SAMPLE			
North Macedonia	M-025	12/17/2018	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				NOT EXAMINED FOR IN THIS SAMPLE			
North Macedonia	M-027	1/17/2019	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				NOT EXAMINED FOR IN THIS SAMPLE			
North Macedonia	M-026-0.5	1/8/2019	Lake Ohrid	carinata	20	96	116	●	●	●	●	●				NOT EXAMINED FOR IN THIS SAMPLE			
North Macedonia	M-026-3.0	1/8/2019	Lake Ohrid	carinata	20	160	180	●	●	●	●		●			●			
North Macedonia	M-029-0.5	2/17/2019	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				●			
North Macedonia	M-029-3.0	2/19/2019	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				●			
North Macedonia	M-030-0.5	2/17/2019	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				●		●	
North Macedonia	M-030-3.0	2/19/2019	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				●			
North Macedonia	M-031-0.5	3/17/2019	Lake Ohrid	carinata	20	0	20	●	●	●	●					●			
North Macedonia	M-031-3.0	3/19/2019	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				●			
North Macedonia	M-032-0.5	3/17/2019	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				●			
North Macedonia	M-032-3.0	3/19/2019	Lake Ohrid	carinata	20	0	20	●	●	●	●	●	●			●			
North Macedonia	M-033-0.5	4/18/2019	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				●			
North Macedonia	M-033-3.0	4/17/2019	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				●			
North Macedonia	M-034-0.5	4/18/2019	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				●			
North Macedonia	M-034-3.0	4/17/2019	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				●			
North Macedonia	M-035-0.5	5/20/2019	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				●			
North Macedonia	M-035-3.0	5/21/2019	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				●			
North Macedonia	M-036-0.5	5/20/2019	Lake Ohrid	carinata	20	0	20	●	●		●	●				●			
North Macedonia	M-036-3.0	5/21/2019	Lake Ohrid	carinata	20	0	20	●	●	●						●			
North Macedonia	M-037-0.5	6/17/2019	Lake Ohrid	carinata	20	0	20	●	●		●	●				●			
North Macedonia	M-037-3.0	6/18/2019	Lake Ohrid	carinata	20	0	20	●	●		●					●			
North Macedonia	M-038-0.5	6/17/2019	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				●			
North Macedonia	M-038-3.0	6/18/2019	Lake Ohrid	carinata	20	0	20	●	●		●	●				●			
North Macedonia	M-039-0.5	7/17/2019	Lake Ohrid	carinata	20	0	20	●	●		●	●				●			
North Macedonia	M-039-3.0	7/18/2019	Lake Ohrid	carinata	20	0	20	●	●		●	●				●			

Country	Sample #	Date of Collection MM/DD/YYYY	Water Body	Species	Comprehensive Dissections ~15 min per mussel using compound and stereomicroscope	Quick Dissections ~2 min per mussel typically using only stereomicroscope	Total Dissections	CILIATE Small *Ophryoglena* inside Digestive Gland Tubules	CILIATE Big *Ophryoglena* inside Digestive Gland Ducts	CILIATE Ophryoglena on Gill Surface	CILIATE Similar to *Conchophthirus acuminatus* on Mantle Cavity Tissues	CILIATE *Conchophthirus klimentinus* on Mantle Cavity Tissues	TREMATODE *Phyllodistomum* sp.	CILIATE *Hypocomagalma dreissenae* on Gills	TREMATODE *Bucephalus* trematodes inside gonads and other organs	TREMATODE Round transparent cysts (possibly *Leucochloridiomorpha constantiae)*	CILIATE *Ancistrumina limnica* on mantle cavity tissues	MITE On gill tissues	DARK BLUE-GRAY GILL DISEASE In live *D. anatolica* Turkish mussel
																			
								YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link
North Macedonia	M-040-0.5	7/17/2019	Lake Ohrid	carinata	20	0	20	●	●	●	●		●			●			
North Macedonia	M-040-3.0	7/18/2019	Lake Ohrid	carinata	20	0	20	●			●		●			●			
North Macedonia	M-041-0.5	8/19/2019	Lake Ohrid	carinata	20	0	20	●	●		●	●				●			
North Macedonia	M-041-3.0	8/19/2019	Lake Ohrid	carinata	20	0	20	●	●		●	●				●			
North Macedonia	M-042-0.5	8/20/2019	Lake Ohrid	carinata	20	0	20	●			●	●				●			
North Macedonia	M-042-3.0	8/20/2019	Lake Ohrid	carinata	20	0	20	●			●	●				●			
North Macedonia	M-043-0.5	9/20/2019	Lake Ohrid	carinata	20	0	20	●		●	●	●				●			
North Macedonia	M-043-3.0	9/20/2019	Lake Ohrid	carinata	20	0	20	●			●	●				●			
North Macedonia	M-044-0.5	9/21/2019	Lake Ohrid	carinata	20	0	20	●	●		●	●				●			
North Macedonia	M-044-3.0	9/21/2019	Lake Ohrid	carinata	20	0	20	●	●	●	●	●				●			
				Subtotals	**860**	**256**	**1116**												
Montenegro	MON-023	5/25/2019	Šasko Lake	polymorpha	40	60	100										●		
Montenegro	MON-021	1/5/2019	Malo Blato	carinata	20	85	105	●		●	●	●		●					
Montenegro	MON-022	5/25/2019	Bojana River	carinata	40	60	100			●	●	●		●		●		●	
Montenegro	MON-029	6/22/2019	Bojana River	carinata	40	60	100			●	●	●	●	●		●			
Montenegro	MON-024	6/5/2019	Skadar Lake	carinata	40	0	40			●	●			●					
Montenegro	MON-026	6/13/2019	Skadar Lake	carinata	10	0	10			●	●			●					
Montenegro	MON-027	6/13/2019	Krupac Lake	carinata	40	60	100	●		●									
Montenegro	MON-031	7/15/2019	Krupac Lake	carinata	40	60	100	●		●									
Montenegro	MON-032	3/5/2020	Šasko Lake	polymorpha	100	100	200										●		●
Montenegro	MON-033	5/22/2020	Zeta River	carinata	200	100	300			●	●		●	●		●			
Montenegro	MON-035	5/29/2020	Bojana River	carinata	80	100	180	●	●	●	●	●		●		●			
Montenegro	MON-036	6/25/2020	Šasko Lake	polymorpha	50	0	50	●									●		
Montenegro	MON-037	6/25/2020	Bojana River	carinata	50	0	50	●	●	●	●	●		●		●			
Montenegro	MON-038	7/23/2020	Krupac Lake	carinata	114	0	114	●		●				●					
Montenegro	MON-039	7/27/2020	Šasko Lake	polymorpha	160	0	160										●		
Montenegro	MON-040	9/4/2020	Šasko Lake	polymorpha	40	0	40										●		
Montenegro	MON-041	10/3/2020	Bojana River	carinata	40	0	40		●	●	●	●		●		●			
Montenegro	MON-042	10/3/2020	Šasko Lake	polymorpha	40	0	40										●		
Montenegro	MON-043	10/8/2020	Šasko Lake	polymorpha	55	0	55										●		
Montenegro	MON-044	11/8/2020	Krupac Lake	carinata	50	0	50	●		●				●					
Montenegro	MON-045	11/10/2020	Bojana River	carinata	50	0	50			●	●	●		●		●			
Montenegro	MON-046	5/8/2021	Šasko Lake	polymorpha	50	0	50										●		
Montenegro	MON-047	5/8/2021	Šasko Lake	polymorpha	50	0	50	●									●		

Country	Sample #	Date of Collection MM/DD/YYYY	Water Body	Species	Comprehensive Dissections ~15 min per mussel using compound and stereomicroscope	Quick Dissections ~2 min per mussel typically using only stereomicroscope	Total Dissections	CILIATE Small *Ophryoglena* inside Digestive Gland Tubules	CILIATE Big *Ophryoglena* inside Digestive Gland Ducts	CILIATE Ophryoglena on Gill Surface	CILIATE Similar to *Conchophthirus acuminatus* on Mantle Cavity Tissues	CILIATE *Conchophthirus klimentinus* on Mantle Cavity Tissues	TREMATODE *Phyllodistomum* sp.	CILIATE *Hypocomagalma dreissenae* on Gills	TREMATODE *Bucephalus* trematodes inside gonads and other organs	TREMATODE Round transparent cysts (possibly *Leucochloridiomorpha constantiae)*	CILIATE *Ancistrumina limnica* on mantle cavity tissues	MITE On gill tissues	DARK BLUE-GRAY GILL DISEASE In live *D. anatolica* Turkish mussel
																			
								YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link	YouTube Video Link
Montenegro	MON-048	5/8/2021	Bojana River	carinata	50	0	50			●	●	●	●	●		●			
Montenegro	MON-049	5/9/2021	Krupac Lake	carinata	50	0	50	●		●				●					
Montenegro	MON-050	5/22/2021	Šasko Lake	polymorpha	50	0	50	●									●		
Montenegro	MON-051	8/29/2021	Bojana River	carinata	90	0	90	●		●	●	●	●	●		●			
Montenegro	MON-052	9/24/2021	Slano Lake	carinata	30	0	30	●		●				●		●			
Montenegro	MON-053	12/8/2021	Šasko Lake	polymorpha	50	0	50										●		
				Subtotals	**1719**	**685**	**2404**												
Turkey	TUR-001	5/31/2019	Eğirdir Lake	anatolica	42	60	102	●			●				●		●		
Turkey	TUR-002	5/31/2019	Beyşehir Lake	anatolica	40	60	100												
Turkey	TUR-003	9/11/2019	Eğirdir Lake	anatolica	40	247	287	●			●				●		●		●
Turkey	TUR-004	9/12/2019	Beyşehir Lake	anatolica	40	125	165								●				
Turkey	TUR-005	9/13/2019	Seyhan Reservoir	anatolica / caputlacus	40	7	47								●				
Turkey	TUR-007	9/8/2020	Eğirdir Lake	anatolica	100	100	200	●			●				●				
				Subtotals	**302**	**599**	**901**												
Albania	ALB-003	6/2/2018	Lake Ohrid	carinata	50	0	50	●	●		●	●				●			
Albania	ALB-004	11/7/2021	Lake Ohrid	carinata	57	0	57	●	●		●	●		●		●			
				Subtotals	**107**		**107**												
Bulgaria	BUL-001	10/26/2019	Ogosta Reservoir	rostriformis	20	0	20									●			
Bulgaria	BUL-002	10/26/2019	Ogosta Reservoir	rostriformis	20	0	20									●			
Bulgaria	BUL-003	10/29/2020	Ogosta Reservoir	rostriformis	100	0	100												
Bulgaria	BUL-004	11/15/2021	Ogosta Reservoir	rostriformis	50	0	50												
				Subtotals	**190**		**190**												
				TOTALS	**3,178**	**1,540**	**4,718**												

- **DATA - As part of your report, in the appropriate section, if there are any data sets with your research, please provide the following information:**
    - **Share Drive folder name and path where data are stored:** \\bor\do\TSC\Jobs\DO_NonFeature\Science and Technology\2019-PRG-Parasites to Control North American Dreissenid Populations
    - **Point of Contact name, email, and phone:** Jacque Keele, jkeele@usbr.gov, (720) 930-1056
    - **Short description of the data:** Final report
    - **Keywords:** Biocontrol, dreissenid mussels, quagga mussels, zebra mussels, novel associations biocontrol, parasite
    - **Approximate total size of all files:** 6 MB