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Development of a Chimeric Biopesticide for the Treatment of Zebra and Quagga Mussels

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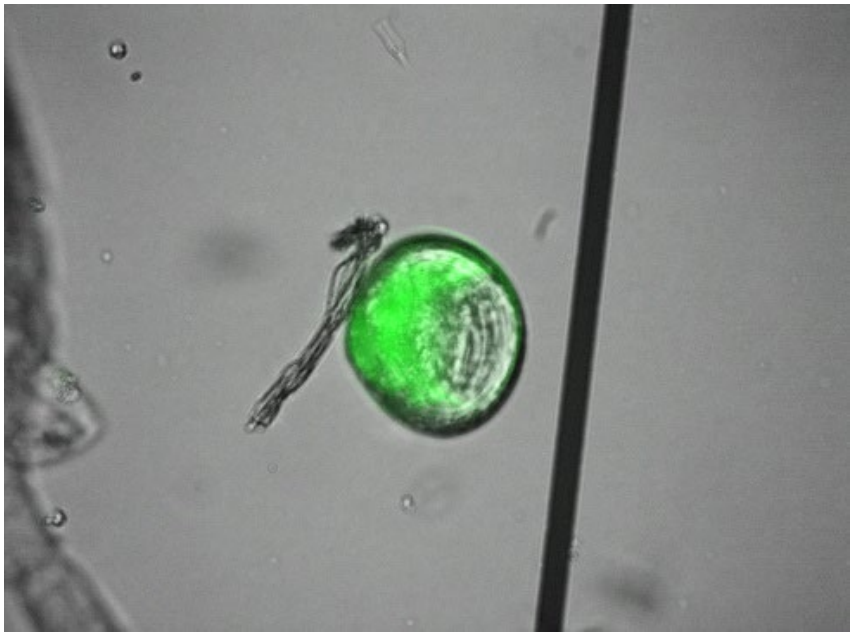
Interim Report No. ST-2021-19186-01



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14. ABSTRACT

The primary objective of this Phase I research project was to develop and determine the viability of a bioengineered, chimeric protein biopesticide for the treatment of zebra and quagga mussel infestations. To achieve this goal, Environmental Quality Operations (EQO) utilized previously developed monoclonal antibodies, specific to quagga mussels, as expressed by hybridoma cell lines. Antibody expressing hybridoma cell lines were developed by Reclamation and Metropolitan Water District of Southern California in an earlier research project (Final Report ST-2014-9640-01). Hybridoma ribonucleic acid (RNA) was isolated to determine the binding regions of the expressed antibodies. Once binding regions were successfully identified, biotherapeutic agents were constructed by combining the single-chain variable fragment (scFv) binding regions with a Pseudomonas exotoxin-A based backbone, connected by hinge sequences between the light and heavy chain, and between the scFv and the toxin backbone to avoid interactions between the subunits that could cause diminishment of the binding efficiency. Once chimeric biotherapeutic agents are created, a methodology for reliable transformation of a microalgae, for use as both a biotherapeutic production and delivery vectors, will be created and validated during Phase II of this research project.

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Acronyms and Abbreviations

ADP	adenosine diphosphate
CAP	Central Arizona Project
CRA	Colorado River Aqueduct
DNA	deoxyribonucleic acid
EF	elongation factor
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
EQO	Environmental Quality Operations
EUP	experimental use permit
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
HSP	heat shock protein
IP	intellectual property
K_D	dissociation constant
mAb	monoclonal antibody
MWD	Metropolitan Water District of Southern California
NSF	National Science Foundation
pAb	polyclonal antibody
PE	pseudomonas exotoxin
Reclamation	Bureau of Reclamation
RISE	Reclamation Information Sharing Environment
RNA	ribonucleic acid
S&T	Science and Technology
scFv	single-chain variable fragment

Measurements

ng/µL	nanogram per microliter
pM	picomolar (10^{-12} Molar)

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Executive Summary

The primary objective of this Phase I research project was to develop and determine the viability of a bioengineered, chimeric protein for the treatment of zebra and quagga mussel infestations. To achieve this goal, Environmental Quality Operations (EQO) utilized previously developed monoclonal antibodies, specific to quagga mussels and as expressed by hybridoma cell lines, that were discovered by the Bureau of Reclamation (Reclamation) and Metropolitan Water District of Southern California (MWD). Antibody expressing hybridoma cell lines were obtained by EQO and cultured for antibody expression. Expressed antibodies were purified and used in binding assays to confirm efficacy and reproduce results from the original work with these monoclonal antibodies performed by Reclamation and MWD. Additionally, hybridoma ribonucleic acid (RNA) was isolated to determine the binding regions of the expressed antibodies. Once binding regions were successfully identified, biotherapeutic agents were constructed by combining the single-chain variable fragment (scFv) binding regions with a Pseudomonas exotoxin-A based backbone, connected by hinge sequences between the light and heavy chain, and between the scFv and the toxin backbone to avoid interactions between the subunits that could cause diminishment of the binding efficiency. This was achieved by combining synthetic deoxyribonucleic acid (DNA) constructs through Gibson cloning, followed by subcloning of this construct into a production vector for microalgae.

Once chimeric biotherapeutic agents are created, a methodology for reliable transformation of a microalgae, for use as both a biotherapeutic production and delivery vectors, will be created and validated during Phase II of this research project. This will be achieved through experimentation with a cell wall deficient strain of *Chlamydomonas reinhardii* and the wild-type strain. Methods of transformation included chemical disruption followed by glass bead transformation and electroporation under a variety of conditions. Ultimately, a methodology using electroporation was adopted and showed consistent transformation with high efficiency. A Science and Technology (S&T) proposal for Phase II of this research project was submitted for FY22-FY24. Ultimately, to determine the effectiveness of the final products (chimeric biopesticide + microalgae production and transport vector) created as part of this project, biotherapeutic microalgae will be incubated with zebra and quagga mussel live veligers to determine their ability to kill the target species.

1. Introduction

Zebra and quagga mussels (*Dreissena* spp.) are aquatic, invasive, bivalve species that cause considerable damage to submerged infrastructure involved in the conveyance, treatment, storage, and use of water. Since their initial introduction into the United States in the 1980s, they have caused significant problems for utilities and industries in many Eastern and Central States, particularly in the Great Lakes region and along the Missouri and Mississippi Rivers. Quagga mussels (*Dreissena rostriformis bugensis*) were first detected in Lake Mead in 2007. Since then, the mussels have spread downstream into the Lower Colorado River region and have invaded the Central Arizona Project (CAP) and the Colorado River Aqueduct (CRA), including Lake Mathews, the terminal reservoir on the CRA.

Following initial introduction into an aquatic system, mussels attach to most submerged surfaces, with serious consequences for the drinking water and hydroelectric power industries, industrial cooling facilities, agricultural irrigation, and recreational use of water. Colonies of zebra and quagga mussels clog intake trashracks, pipes, valves, siphons, and irrigation and fire-suppression systems. Consequently, it is critically important to detect infestation in the early stages so that timely and cost-effective response plans and control strategies can be developed.

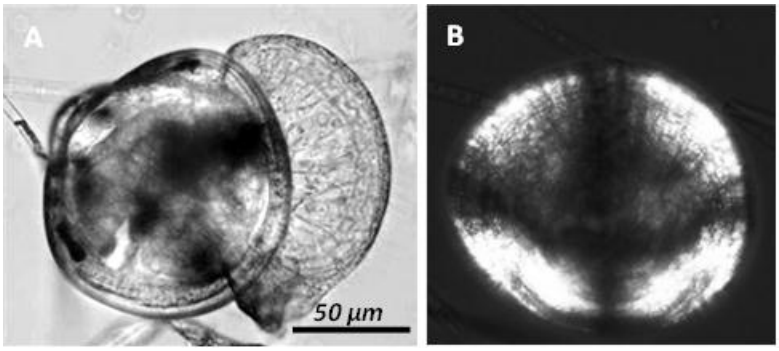

Figure 1 Quagga mussel veligers viewed by microscopy. (A) white light image of an umbonal veliger with vellum extended beyond the shell perimeter; (B) cross-polarized light image of a different umbonal veliger.

1.1 Project Background

To achieve a high degree of target specificity, immunotoxins utilize the antigen binding domain of monoclonal antibodies (mAbs) bound to toxin scaffolds by cross-linking chemistry, or through co-expression (Alewine 2015). The specificity provided by this approach in conjunction with the efficient method of action provided by the toxin scaffold's enzymatic activity have led to the development of successful immunotoxin-based therapeutics for various cancers, most notably leukemia and lymphomas (Wayne 2014). The work performed in this Phase I study will explore the

use of the small chain variable fragment (scFv) antigen binding domain of twenty previously discovered mAbs with specificity to *Dreissena* as the targeting domain in chimeric immunotoxins. In addition to the high specificity and binding affinity provided by using mAb binding domains, the cytotoxicity of the modified PE38 scaffold will result in a highly efficient biopesticide with low effective dosing relative to products currently on the market (Weldon 2011, Meehan 2014). Pseudomonas exotoxin-A (PE) is a highly cytotoxic protein that has evolved to exploit the intracellular trafficking and machinery of its eukaryotic host cells (Michalska 2015). Once bound to a cell, the PE protein functions by forcing internalization via clathrin coated pit formation, followed by exploitation of conserved retrograde transport pathways to reach the endoplasmic reticulum, where ADP-ribosylation enzymatic activity cleaves elongation factor-2 (EF2), a highly conserved enzyme, necessary for protein translation (Kreitman 1995; Kreitman 2009; Michalska 2015). Shutting down protein translation by ribosylation of EF2 or ribosomes themselves, as occurs with shigella-like toxin and ricin toxin, has been referred to as an "Achilles Heel" attack on the cell as this activity is highly effective at triggering irreversible apoptosis signaling pathways (Tumer 2012; Michalska 2015). The PE toxin is one of the most cytotoxic proteins produced from a long history of coevolution; it is also relatively easily controlled, as the wild-type targeting domain resides entirely in the N-terminal domain I, which acts independently of the enzymatic C-terminal domain III, and can be easily replaced with new targeting domains for the production of immunotoxins (Kreitman 2009; Kreitman 2011). As such, PE has been reduced to a PE38 subunit for human health applications and this scaffold has been further refined by the authors of this application for use in environmental applications as a highly targeted and highly efficient biopesticide (Higley 2011; Kreitman 2011). The use of a modified version of the PE38 protein is especially attractive for treatment of *Dreissena* due to the KDEL signaling sequence added to the c-terminus of this protein scaffold (Weldon 2011). Where wild type *Pseudomonas* exotoxin-A uses an endosome escape mechanism involving the toxin's Ia and II domains, the modified PE38 scaffold eliminates the Ia domain and a portion of domain II to remove normal cell targeting (Ogata 1990; Weldon 2011; Weldon 2013). The c-terminal sequence KDEL is a near universal targeting sequence for intracellular routing to the endoplasmic reticulum (ER), the required location for PE38 enzymatic activity, and has been bioengineered onto the PE38 scaffold to replace part of the wild-type routing mechanism lost with the removal of the N-terminal regions (Kreitman 1995; Tortorella 2012). Like other bivalves, it is reasonable to expect that *Dreissena* also rely heavily on the KDEL system for cellular regulation in response to environmental stressors (Wang 2013). This is achieved by increasing the production of heat shock proteins (HSP), a chaperone protein family that binds KDEL tagged proteins, naturally produced by the cell, and routes them to the ER to alter gene expression in response to stresses such as bacterial infection, change in pH, and change in temperature (Mayer 2005; Xu 2009). As such, it is unlikely that *Dreissena* will gain resistance to a modified PE38-based immunotoxin as normal stress and immunity reactions will facilitate its transport to the ER via the KDEL-HSP70 pathway, making the product more effective rather than rejecting it via hemocyte activity (Wang 2013).

In addition to high binding affinity, specificity, and efficient cell kill provided by this immunotoxin approach, the use of a recombinant system increases the production efficiency of the biopesticide by using a commercial production vector. *Pseudomonas* exotoxin-A 38kDa based immunotoxins have been production optimized in the biopharmaceutical industry resulting in a high quality, high yield product (Chandramohan 2017). One of the largest costs associated with the production of biopharmaceutical immunotoxins, is the requirement for the product to be purified thoroughly from the prokaryotic or eukaryotic production vector; however, these processes are not necessary in biopesticide production (Molloy 2013a). The production vectors of highest initial interest are *Chlamydomonas reinhardtii* and *Schizochytrium* based, transformed to produce a biopesticide via

bioaccumulation rather than excretion, and at a high intracellular concentration (Rasala 2010; Banuelos-Hernandez 2017). Additional production vectors may be explored as well over the course of the project, though it is likely that a bioaccumulating microalgae vector is preferable, as such a vector would not require purification, thereby decreasing production cost substantially, and act as an efficient delivery vector, as microalgae is a preferred food source of *Dreissena* (Nichols 1992; Berg 1996). For production scaling expertise, production regulatory assurances, and FIFRA regulatory navigation support, EQO has retained Exponent, a consultancy company with expertise in this field. Based on previous success with immunotoxins, it is expected that the biopesticide produced by this process will exhibit direct cell kill efficiency values (EC50) in the low pM concentration range (Higley 2011; Grant 2011; Luster 2012). As high efficiency filter feeders that readily clear microalgae from the water column, it is anticipated that a biopesticide produced in this fashion will be efficiently taken up by the target organisms, resulting in efficient and direct mortality (Berg 1996; Luster 2012; Karatayev 2014). At effective concentration doses in the low pM range and relatively high intracellular bioaccumulation of the biopesticide in the production vector, the effective number of digested *Chlamydomonas* or *Schizochytrium* attenuated cells resulting in target organism mortality is expected to be sufficiently low enough to achieve 100% mortality and production and delivery costs below $1 per acre foot of open water treated (Berg 1996; Luster 2012; Banuelos-Hernandez 2017).

1.2 Previous Work Performed

Final Report ST-2014-9640-01 described previous work completed that discovered monoclonal antibodies (mAbs) for the improved detection of quagga mussel larvae. This work was a collaboration between Reclamation and the Metropolitan Water District of Southern California (MWD). The goal of this project was to develop monoclonal antibodies (mAbs) that can be used to label veligers with fluorescent tags and purify veligers from complex samples using magnetic capture technology. Organism-specific antibodies have previously been used to improve detection and identification of many organisms in environmental waters, including mussel larvae. An antibody that recognizes quagga mussel veligers, coupled to magnetic beads or fluorescent tags, will greatly improve microscopic detection and identification of veligers in aquatic systems.

In this work, polyclonal antibodies were initially produced to assess the feasibility of antibody production using different preparations of veliger and adult mussel tissue. Antibodies were produced by QED Bioscience, Inc., in San Diego, California, using frozen laboratory-bred veligers, crude protein extracted from these veligers, and adult mussel mantle proteins prepared at MWD Water Quality Laboratory. Examination by epifluorescence microscopy demonstrated intense fluorescence in all stained samples, with the strongest fluorescent signals concentrated around the exposed vellum tissues near the opening of the bivalve veliger shell. Microscopic observation following immunomagnetic capture of veligers from Lake Havasu also showed localized binding of the pAb-magnetic bead complex to exposed veliger vellum tissue. Recovery efficiencies for pAb-mediated magnetic capture were 48 – 96%. However, pAbs can contain antibodies to multiple antigens that were present in the original immunogen, so they often lack specificity. Therefore, having demonstrated the feasibility of antibody production, the project focused on producing an anti-quagga mAb, which should have higher specificity because it is a single antibody recognizing a single antigen, rather than a mix of antibodies recognizing multiple antigens.

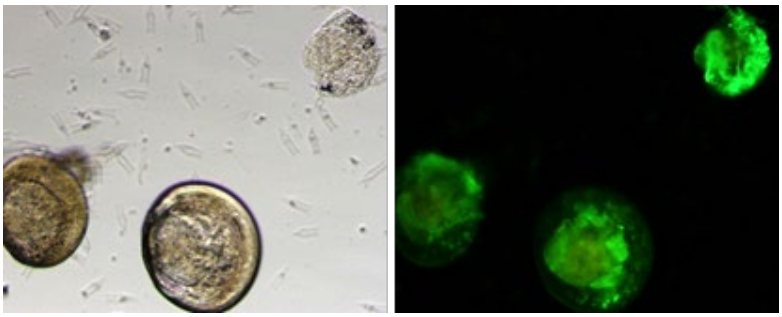

Figure 2 Stained veligers. D-shaped and umbonal veligers and a possible trochophore stained with cell culture supernatant from hybridoma subclone 2E10-1D6. Left panel shows white light microscopy, and right panel shows epifluorescence microscopy.

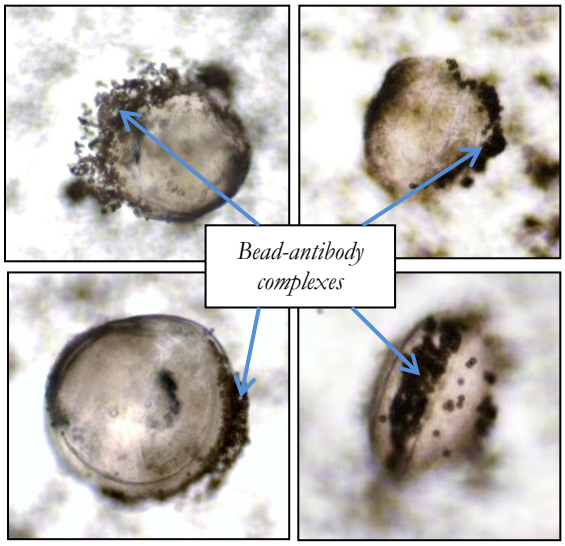

Figure 3 Paramagnetic bead-pR1000 polyclonal antibody conjugates bound to veligers. View by light microscopy.

To generate mAbs, protein was extracted from the mantle tissue of adult mussels harvested from Lake Havasu and the CRA. The protein was used as the antigen to elicit an initial antibody response. Protein extracted from 51 mussels covering four size classes and three locations was combined to produce a single heterogeneous immunogen. At the end of the 10-week immunization period, antibody-producing spleen cells were fused to each of two myeloma cell lines to produce fusion hybridomas, which were grown in 96-well plates using standard cell culture procedures. Producing a mAb requires multiple rounds of generating and screening many cell cultures. All the fusion products were screened against the original protein antigen using an enzyme-linked immunosorbent assay (ELISA) to determine whether they produced antibodies. Cell culture supernatants from the 10 hybridomas that displayed the strongest ELISA signal were also screened against whole quagga mussel veligers by indirect immunofluorescence microscopy. Based on the intensity of their fluorescence, six antibody-producing cell lines were subcloned to produce 20 cell lines. Cell culture supernatants containing unpurified antibody from 20 subcloned hybridoma cell lines were screened against protein extracted from adult quagga mussels and *Corbicula* using an ELISA assay. Most cell culture supernatants had relatively high absorbance values when assayed by ELISA against quagga

mussel protein compared to *Corbicula* protein, indicating a high level of specificity for quagga-specific antigen(s). All these antibodies were isotypes IgG1κ or IgG2bκ.

Twenty mAbs were produced as unpurified reagents, and eight were produced and purified on a larger scale, yielding milligram quantities of purified reagent grade mAb. When coupled with a secondary fluorescently labeled antibody, these quagga-specific antibodies-stained veligers, generated a variety of fluorescence staining patterns when observed by immunofluorescence microscopy. Some of the antibodies stained the entire internal veliger contents, generating intense green fluorescence, while others produced a speckled pattern of fluorescence with multiple foci of intense staining against an overall background of less intense staining. In other cases, staining was focused on the extruded vellum at the shell opening and around the periphery of veligers, while other subclones had intense staining that was localized to a single internal region of the veliger, which may represent a particular organ or part of an organ.

The antibodies produced by this project provided tools that could simplify microscopic detection and identification of veligers in water samples. In the Phase I research described here in this report, the antibody expressing hybridoma cell lines were provided and EQO utilized these previously developed mAbs, as expressed by the hybridoma cell lines developed by Reclamation and MWD, for the development of a chimeric biopesticide for the treatment of zebra and quagga mussels.

1.3 Research Partner

EQO is an Austin-based company in Texas specializing in the detection, monitoring, and eradication of aquatic invasive species. In June 2016, EQO launched a revolutionary biotechnology platform for the detection and quantitation of aquatic invasive species with unprecedented sensitivity and specificity. Since then, EQO has expanded its services and products to provide eradication and mitigation solutions for aquatic invasive species to water stakeholders responsible for watersheds, lakes, and rivers.

While Reclamation and Metropolitan Water District of Southern California (MWD) performed the original research leading to the discovery of source materials from which the best candidate mAbs were provided to EQO to start this research project, Phase I research on the development and testing of a chimeric biopesticide was conducted by EQO in their Austin-based biotechnology laboratories. Phase I is described here in this interim report.

As previously discussed with the Reclamation Research and Development Office on the protection of its resources, including antibodies, it is expected that EQO will file a patent to protect its intellectual properties (IP) generated from their biotechnology research in the development of a chimeric biopesticide for the treatment of zebra and quagga mussels. As such, methods and results are summarized here in this report and no data will be uploaded to the Reclamation Information Sharing Environment (RISE).

The patent filing will be completed prior to filing an experiment use permit (EUP) with the Environmental Protection Agency (EPA) under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) to conduct real world testing during Phase II, which was submitted as a new S&T proposal for FY22-FY24. During Phase II, we proposed to begin testing of the immunotoxin

biopesticide in both closed-water systems and open-water systems. As described in our S&T proposal for Phase II, this will be accomplished by developing a transport and production vector utilizing microalgae. Is it during this phase of the research that real world effectiveness of a chimeric biopesticide will be assessed. Data gained from this testing will also help with identifying and validating the best practice for a large-scale production system for the immunotoxin biopesticide. At the same, a regulatory and commercialization road map for product launch and scaling may be identified.

1.4 Objectives

During Phase I, the main objective was to develop and determine the viability of a bioengineered, chimeric protein for the treatment of zebra and quagga mussel infestations. The following objectives were accomplished during this phase:

1. Reproduce growth and binding confirmation of previously discovered mAbs.
2. Identification of the antigen binding scFv sequence in the twenty mAbs investigated.
3. Produced lead candidates for development as immunotoxin based biopesticides.
4. Determined binding affinity of immunotoxin lead candidates.
    a. Performed binding assays using fluorescently tagged antibodies to determine if the immunotoxins are effective at binding *D. polymorpha* larvae.
    b. Binding affinity was determined by calculation of the equilibrium dissociation constant (K_D) between the immunotoxin and mussel larvae protein. Binding assays resulting in a K_D value in the micromolar range or above were considered indicative of a poor binder unlikely to produce a final product that is marketable due to high effective dose.
5. Determined the potential of produced immunotoxins for effective eradication of Dreissenid mussels.
    a. Cell kill assays were performed using live populations of *Dreissena polymorpha* larvae and lab cultured adult mussels harvested from a locally infested source and cultured in EQO's ISO 8 clean room facility.
    b. Determination of effective cell kill was performed using an ATP production based luminescent assay with mussel larvae and by morphological examination of adults.
    c. Effective and efficient cell kill was accomplished. This was vitally important to the development of EQO's immunotoxin product to determine efficacy, subsequent direction of research, and marketability of the resulting product.
6. Determination of production vector capable of scaled production.
7. Determine next steps for Phase II research.
    a. The information provided by this Phase I research will allow researchers to make decisions regarding subsequent research and development. A description of the Phase II objectives and approaches are described in the S&T proposal submitted for FY22-FY24.

Methods and results from each of the objective listed above are summarized below.

2. Summary of Methods and Results

2.1 Methods

Objective 1: Reproduce growth and binding confirmation of previously discovered mAbs

Hybridoma cell lines were obtained from MWD and brought up from freeze down by the manufacturer's suggested protocol. Cells were passaged every 48-72 hours and split between 1:2 and 1:5 dependent on growth and confluency. Additional freeze downs were prepared in duplicate at the third passage, once growth stability had occurred. Cells were transitioned to a serum-free media for antibody harvesting. Antibody harvesting was performed multiple times to establish the optimal storage concentration to avoid protein aggregation. Purification protocol included upstream removal of large globular aggregates, followed by buffer exchange into FPLC loading buffer, and then FPLC purification using a protein G column.

Objective 2: Identify the antigen binding scFv sequence in the twenty mAbs being investigated

Antibody producing cells were lysed and RNA was extracted using industry standard techniques for RNA extraction from cell cultures. Concentrations were determined by absorbance at 260nm (A260) spectroscopy. RNA purity was determined by examination of A260/A280 and A260/absorbance at 230nm (A230). The ratio of A260/A280 should be greater than 1.6 for downstream applications, the ratio of A260/A230 should be greater than 1.8 for downstream applications. RNA samples were reverse transcribed to cDNA using a commercial reverse transcriptase. Resulting cDNA transcripts were amplified using a high-fidelity polymerase and primers for the conserved sequences 5' and 3' of the scFv sequence region. Amplification products were measured using A260 and checked for purity using A260/A280 and A260/A230 ratios as above. These amplification products were also migrated through an agarose gel via electrophoresis, probed with ethidium bromide, and imaged under UV excitation to verify purity of desired amplification product and anticipated size of amplified product. The PCR products were shipped to Functional Biosciences, a preferred vendor for Sanger sequencing analysis. Sequences provided were translated in silico to identify the antigen binding domain protein sequences of the analyzed mAbs. Consensus DNA sequences of light and heavy chains were included in a workflow document.

Objective 3: Produce lead candidates for development as immunotoxin based biopesticides

After the sequences of the antigen binding scFv regions were determined, DNA sequences for chimeric immunotoxins were generated via Gibson cloning assembly using the amplified scFv DNA regions and in-house, modified, PE38 scaffold DNA. Restriction enzyme sites were added during this process and the resulting construct was amplified using high-fidelity polymerase. This amplified DNA was spliced into a commercial production vector for co-expression. The resulting plasmids were sub-cloned into DNA expression competent cells, amplified, and individual colonies were grown according to manufacturer's instruction in a miniprep system. Extracted DNA was sent to Functional Biosciences for Sanger sequencing to verify DNA sequence.

Objective 4: Determine binding affinity of immunotoxin lead candidates

Immunotoxins produced during pursuit of objective 3, along with the negative control immunotoxin scaffold were used in the pursuit of this objective. Triplicate samples of *Dreissena polymorpha* larvae at a known concentration were incubated with serial dilutions of purified immunotoxins and negative control immunotoxin. The samples were then washed and subsequently probed with rabbit anti-*Pseudomonas* exotoxin-A as a primary antibody and fluorescently tagged goat anti-rabbit as a secondary antibody at the manufacturer's recommended saturation concentration. Fluorescence is detected using a multimode plate reader. Calculation of the equilibrium dissociation constant (K_D) was established by non-linear regression analysis performed in the Prism Graphpad software suite. Immunotoxins with K_D values calculated at micromolar or above were not considered for additional development. Assays were performed in at least triplicate to establish statistically relevant binding affinities. Statistical difference between treatment groups was determined using one-way ANOVA with a Tukey's post-test.

In addition to determination of K_D, immunotoxin binding was visualized as a quality control measure by incubating *Dreissena polymorpha* larvae with immunotoxin, washed, probed with fluorescently tagged secondary antibody, and visualized using fluorescent microscopy.

Objective 5: Determine the potential of produced immunotoxins for effective control of Dreissena polymorpha

This objective is in progress. Immunotoxins produced during pursuit of objective 3, along with the negative control immunotoxin scaffold will be used in the pursuit of this objective. Triplicate samples of living *Dreissena polymorpha* larvae at a known concentration are to be incubated with serial dilutions of final product microalgae expressing lead candidate toxin and negative control microalgae and incubated for 2, 4, 6, 12, 24, and 48 hours. Four replicate aliquots from each sample will then be transferred to 96 well plates and incubated with an ATP-based luminescent assay to determine viability following treatment with immunotoxins. Luminescence is detected using a multimode plate reader. Calculation of cell kill efficiency will be performed by calculating the concentration at which 50% of organism death occurs (CD50). This will be established using the non-linear regression analysis performed in the Prism Graphpad software suite. Candidates with CD50 values calculated at millimolar active ingredient or above will not be considered for additional development. Assays will be performed in at least triplicate to establish statistically relevant binding affinities. Statistical difference between treatment groups will be determined using one-way ANOVA with a Tukey's post-test.

Objective 6: Determination of production vector capable of scaled production

Chlamydomonas culturing for the purpose of recombinant protein production, is well studied and low cost compared to other microalgae systems (Rasala 2010; Davis 2016). Commercial production of transformed *Chlamydomonas* carries an estimated cost of $406-612 per ton (907kg) dry mass product depending on production pond size (2-10 acre) (Davis 2016). At a conservative estimate of an effective dose of 200g product per acre foot treated, the production cost in this type of system would range from $0.09 to $0.14 per acre foot effective dose at scale. For the purpose of this Phase I project, laboratory scale production of *Chlamydomonas* will be performed and analyzed for yield and cell kill activity.

For the completion of this objective, identified biopesticide candidates were cloned into wild type *Chlamydomonas* production vectors via electroporation, chemical treatment transformation, and glass

bead mediated transformation. An electroporation protocol has been successfully developed, resulting in reproducible transformation, growth, and QC protocols capable of producing product at scale. The first five batches have been successfully completed.

Objective 7: Determine next steps for research

The product will seek registration under FIFRA Sec.(c)(5) and perform the necessary testing in accordance with FIFRA and The Clean Water Act. In this process, the authors have entered into an agreement with Exponent, a consultancy firm with expertise in this process, and will pursue collaboration and testing with The Bureau of Reclamation, US-FWS, USGS, and independent, GLP-approved laboratories as necessary. A Phase II project will be pursued to solidify a scaled production protocol and to complete the requisite closed and open water system experimentation for approval under FIFRA in collaboration with Exponent. The authors anticipate successful approval by the EPA following completion of the required experimentation. In addition to pursuing Phase II funding through the National Science Foundation (NSF), the successful completion of this Phase I project adds significant value to EQO, putting the company in a strong position to fund large scale experimentation and commercialization of the resulting biopesticide product through venture network and angel funding. Further, the immunotoxin platform discussed herein can be targeted to alternate invasive species, e.g.: Asian carp, in the future.

2.2 Results

2.2.1 Antibody Production

Hybridoma cell lines were obtained from MWD and brought up from freeze down by the manufacturer's suggested protocol. Cells were passaged every 48-72 hours and split between 1:2 and 1:5 dependent on growth and confluency. Additional freeze downs were prepared in duplicate at the third passage, once growth stability had occurred. Cells were transitioned to a serum-free media for antibody harvesting. Antibody harvesting was performed multiple times to establish the optimal storage concentration to avoid protein aggregation. The purification protocol included upstream removal of large globular aggregates, followed by buffer exchange into FPLC loading buffer, and then by FPLC purification using a protein G column. Concentration was determined by Nanodrop A280 analysis for IgG (Table 1) and samples were ran on SDS-PAGE gels under native and denatured conditions (Figure 4).

Some artifacts were noted on Sample 1; however, it does look like it split into heavy and light chains based on banding. Sample 2 is slightly above 180kDa, which could mean that it is a glycosylated antibody. Samples 3, 4, 5, and 6 all have nice banding at the expected 156kDa molecular weight. To determine if the antibodies previously shown to bind specifically to quagga mussels were also capable of binding to zebra mussel veligers, the purified antibodies were incubated with freshly deceased zebra mussel veligers, stored in 50% ethanol. The procedure for antibody binding visualization used the primary antibodies produced by the hybridoma cell lines, and a secondary goat anti-mouse antibody with a FITC fluorescent tag and an anti-autofluorescence quencher (Figure 5). Zebra mussel binding was seen for all antibodies isolated, except for antibody #5-1B8-2E9.

Table 1 Antibody Concentrations.

Sample No.	Sample ID	Concentration (ng/µL)
1	2E10-1B10	179.9
2	2E10-1A8	32.5
3	1B8-2C12	158.1
4	3C4-2B11	166.6
5	1B8-2E9	205.3
6	3C4-2F5	142.6

ng/µL = nanograms per microliter

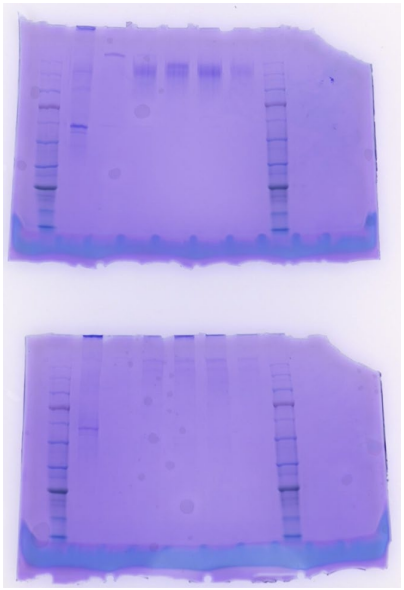

Figure 4 Antibody production gels.

Gel 1 (top): native protein.
Lane 1: 5ul Protein ladder, Lane 2: 20ul Sample #1 native protein, Lane 3: 20ul Sample #2 native protein, Lane 4: 20ul Sample #3 native protein, Lane 5: 20ul Sample #4 native protein, Lane 6: 20ul Sample #5 native protein, 20ul Sample #6 native protein, Lane 8: 5ul Protein ladder.

Gel 2 (bottom): 'linear protein' boiled at 100° C for 5 minutes. Lane 1: 5ul Protein ladder, Lane 2: 20ul Sample #1 linear protein, Lane 3: 20ul Sample #2 linear protein, Lane 4: 20ul Sample #3 linear protein, Lane 5: 20ul Sample #4 linear protein, Lane 6: 20ul Sample #5 linear protein, Lane 7: 20ul Sample #6 linear protein, Lane 8: 5ul Protein ladder.

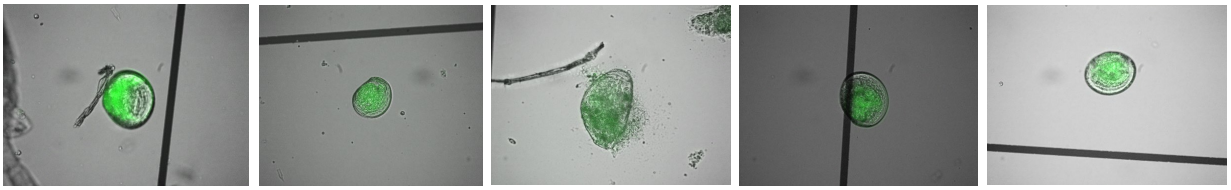

Figure 5 Zebra mussel binding visualization. Zebra mussels probed with antibody produced from Samples #1-#3, #4, and #6 (see Table 1).

2.2.2 Identification of Antigen Binding scFv Sequences

To identify the antigen-binding scFv sequence of six *Dreissena* (zebra and quagga mussel) antibodies, total RNA was extracted from mouse hybridoma cell lines expressing these antibodies. Antibody producing cells were lysed and RNA was extracted using industry standard techniques for RNA extraction from cell cultures (Table 2). cDNA was synthesized using the protocol from "A simplified workflow for monoclonal antibody sequencing" (Meyer 2019) utilizing a modified RT-PCR (reverse transcription polymerase chain reaction). This process uses an amplification process that relies on a process known as switching mechanism at 5' end of RNA transcript (SMART). The resultant cDNA was then sub-cloned into a DNA production vector and transformed into competent E. coli for growth. Transformed E. coli was plated on selection media and a minimum of 20 colonies per antibody of interest were amplified by colony PCR to confirm presence of target DNA. Colony PCR samples showing positive light and heavy chain amplification were grown overnight and plasmids purified. Ten plasmid sequences for both the light and heavy chain from each cell line were sent for Sanger sequencing at Functional Biosciences. This process was then repeated with ten additional plasmids at least once to determine consensus sequences (Figures 6 and 7). The resultant sequences were translated in silico to identify the antigen binding domain protein sequences. Once clear sequencing data for the scFv (light and heavy chain variable regions) of each cell line was obtained, a consensus sequence was identified and aligned to IgBLAST reference sequences using the IgBLAST alignment tool. IgBLAST identifies the top-matched germine V-genes. Full length consensus sequences of light and heavy chains were identified for all hybridoma cell lines with the exception of #5-1B8-2E9, which did not show a consensus sequence for the heavy chain variable region, indicating low expression of the antibody of interest. As such, this cell line was removed from further analysis or biotherapeutic development.

Table 2 RNA Concentrations.

Sample No.	Sample ID	RNA Concentration (ng/µL)
1	2E10-1B10	179.9
2	2E10-1A8	32.5
3	1B8-2C12	158.1
4	3C4-2B11	166.6
5	1B8-2E9	205.3
6	3C4-2F5	142.6

ng/µL = nanograms per microliter

Figure 6 Agarose gel of cDNA with Topo-Universal forward primer and nested PCR reverse of Kappa Light Chain. RNA samples isolated from cell lines 1-6 with DNA sizing ladders in lanes 1 and 8.

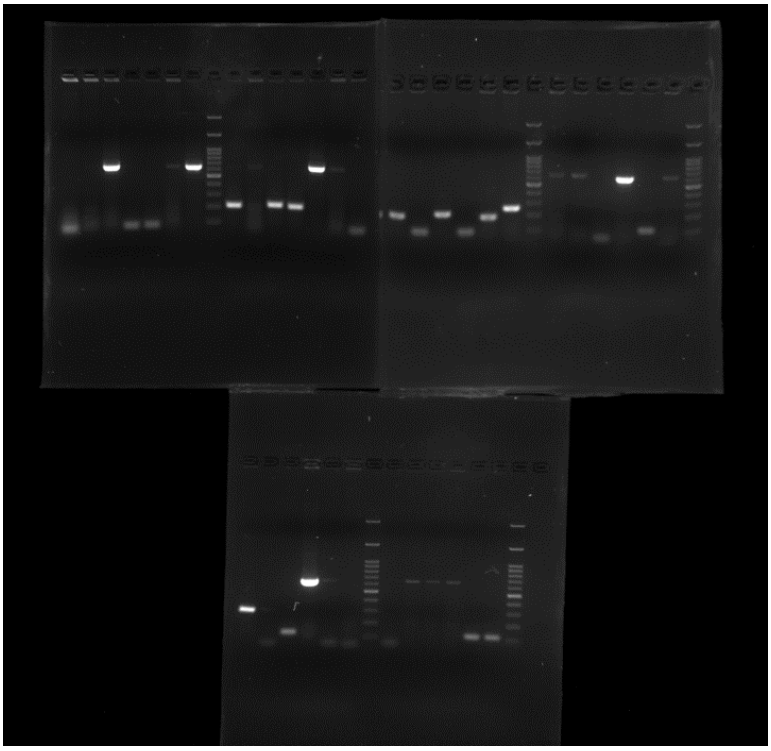

Figure 7 Examples of colony PCR quality control reactions. Samples with successful amplification of light and heavy chain sequences were grown, DNA was extracted from the cultures via miniprep and sent for sequencing.

2.2.3 Development of a Production and Delivery Vector

This objective is an initial effort to support Phase II of this research project (submitted as a separate S&T proposal for FY22-FY24). The goal is to develop a production and delivery vector capable of scaled production.

For the completion of this objective, a plasmid containing the *mCherry* gene as a reporter was cloned into wild type *Chlamydomonas* production vectors via electroporation and glass bead mediated transformation. The pBR_32 plasmid is a vector that encodes the red fluorescent protein mCherry which is regulated by the *C. reinhardtii* constitutive HSP70A-Rbc S2 promoter. HSP70A-Rbc S2 can be induced by both heat shock and light allowing it to be a tool for improved transgene expression. To test transformation efficiency, the successful accumulation of the fluorescent mCherry in the cytosol would provide faster visual detection of transformation than the typical algae colony PCR and confirm chloroplast localization. This plasmid also contains the ble2A expression cassette producing a zeocin-resistant *C. reinhardtii*. pBR_32 was linearized by the restriction enzyme ScaI and transformed into *C. reinhardtii* using various glass bead and electroporation transformation protocols. Glass bead transformations were performed by partially removing the cell wall using the enzyme subtilisin, which has been shown to be the most effective at a low concentration (0.3 Anson units/mL). Variables in this line of experimentation included bead size, concentration of subtilisin, and incubation temperature for cell wall depletion. 5mL of *C. reinhardtii* at 1.5 x 10^8 were suspended in subtilisin reaction buffer and incubated at various concentrations for one hour at 30°C. Cell wall depletion was verified by microscopy and microalgae was subsequently transformed with linearized and non-linearized pBR_32 plasmid DNA by buffer exchanging into nitrogen free TAP media with polyethylene glycol and 1mm glass beads. Samples were vortexed at max speed for times ranging from 15 seconds to 1 minute and plated on TAP media agar plates with Zeocin for selection of transformants. The efficiency of these approaches appeared to be low or non-existent and the resulting microalgae colonies were not stable. Electroporation variables included time of exposure, resistance, volts, and capacitance. A 250mL culture of wild-type *C. reinhardtii* was incubated to a concentration of 1 x 10^6 cells/mL. Algae was pelleted by centrifugation and the supernatant was removed, then the pellet was resuspended in transformation buffer and washed twice. The cell pellet was resuspended to a final concentration of 1.5 x 10^8 cells/L and 2-5µg of linearized pBR_32 vector was added to 250µL aliquots and transformed under various electroporation conditions. Transformants were incubated first in a high sucrose TAP media for 14 hours, then plated on TAP media plates with Zeocin for selection. Transformation efficiency along with the production of mCherry was assessed by spotting independent transformant colonies on the TAP-Zeocin plate by fluorescent microscopy. Under the tested conditions, non-transformed *Chlamydomonas* should emit very weak fluorescence in the TRITC channel used to detect mCherry. Due to autofluorescence and refraction of the agar plates, the fluorescent emission of wild-type and possible transformants showed similar emissions (Figure 8). However, after inoculating possible transformants into 1mL TAP-Zeocin media overnight they showed variability in fluorescent emission (Figure 9). This signal could be observed in the laboratory to increase over subsequent days of continued incubation with an elevated Zeocin concentration, indicating this effect was amplified and transformation efficiency increased.

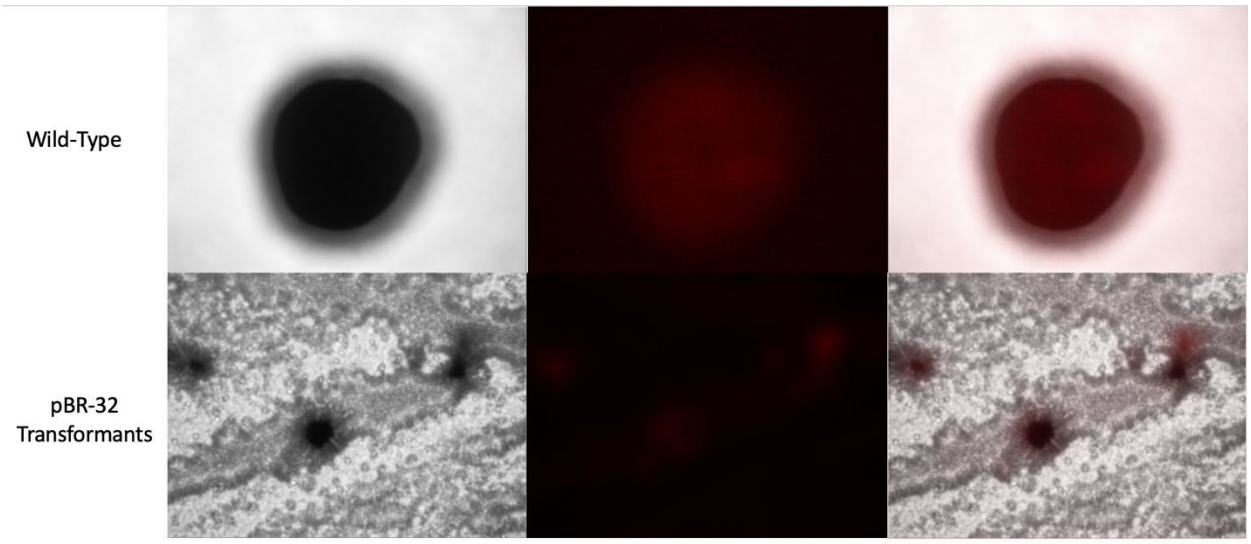

Figure 8 Fluorescence microscopy of algae colonies. Algae colonies on TAP media agar plates following transformation. An attempt to confirm transformation at an earlier stage of the process which was later refined.

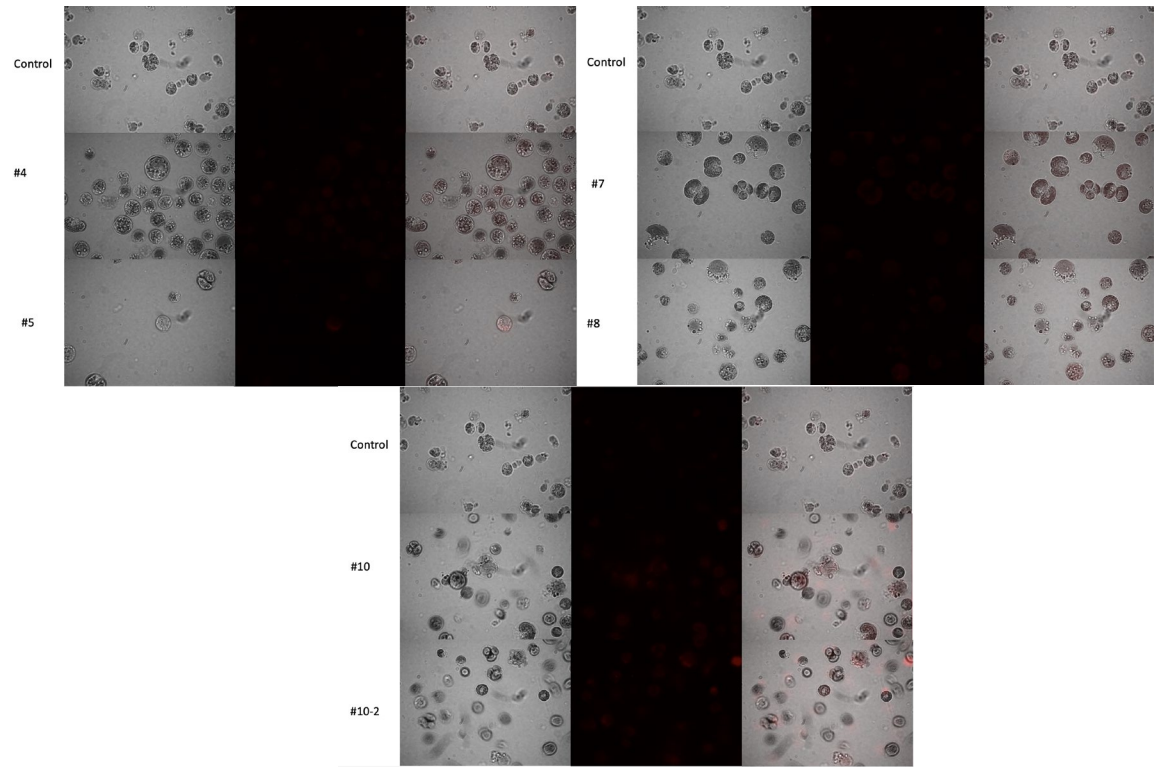

Figure 9 Fluorescent microscopy of microalgae transformants. In TAP media with Zeocin following three days growth. Left: bright field, middle: TRITC channel, right: image overlay. Frame 1: Non-transformed microalgae, microalgae colony #4 and #5 inoculants, Frame 2: Non-transformed microalgae, microalgae colony #7 and #8 inoculants. Frame 3: Non-transformed microalgae, microalgae colony #10 and a second image of #10 inoculant.

The electroporation protocol which consistently showed the highest stable transformation efficiency was Voltage: 500 V, Capacity 25 μF, Resistance: ∞Ω with a pulse time under 10ms (squared wave). This work was performed over the course of several months and required repeated iteration to determine the most effective methodology for reliable and high efficiency transformation. An electroporation protocol has been successfully developed, resulting in reproducible transformation, growth and quality control protocols capable of producing product at scale.

2.2.4 Biotherapeutic Production

After sequence verification three scFv binding domains were selected for biotherapeutic testing based on consistent frame, high recurrence consensus sequence and no stop codons present. Each scFv binding domain (EQO Binding Domain, EBD) and the PE38+ KDEL exotoxin (EQO-Toxin) was synthesized and cloned into pChlamy_4 vector using Gibson Assembly prior. Each plasmid was verified using Colony PCR and Restriction Enzyme digestion prior to sequencing (Figure 10). The verified plasmids were linearized by ScaI restriction digest and sub-cloned into microalgae using the aforementioned transformation protocol. Selection was performed on TAP media agar plates with 20μg/mL Zeocin. A minimum of twenty individual colonies were grown in 1mL of selection media and verified for construct inclusion via PCR. A minimum of three successful 1mL cultures were used to inoculate 250mL cultures in selection media and grown to confluency. Confluent cultures were lyophilized and stored at -20C for efficacy testing.

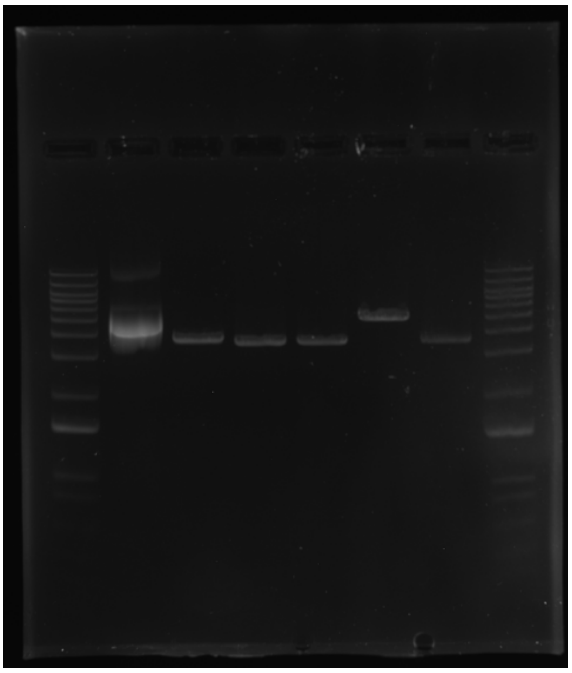

Figure 10 Verification of subcloning post-Gibson Assembly. Lane #1- 1Kb Ladder, Lane #2- pChlamy circular plasmid, Lane #3- pChalmy ScaI linearized plasmid, Lane #3, #4, #6- pChalmy Linearized Vector with No EBD+ EQO Toxin fragments Lane #5- pChalmy vector + EBD + EQO Toxin inserts, Lane #6- 1Kb Ladder

2.2.5 Efficacy Testing

Algae expressing the immunotoxin constructs developed in this project were used to determine mortality of target organisms. Early results show positive cell kill with minimal input algae. Additional work will need to be performed in phase II with adult organisms cultured in lab. Repeats of this experiment will need to be performed to show reproducibility of initial results.

Triplicate samples of living *Dreissena polymorpha* larvae at a known concentration were incubated with serial dilutions of final product microalgae expressing lead candidate toxin and negative control microalgae and incubated for 2, 4, 6, 12, 24, and 48 hours. Four replicate aliquots from each sample were transferred to 96 well plates and incubated with an ATP based luminescent assay to determine viability following treatment with immunotoxins. Luminescence is detected using a multimode plate reader. Calculation of kill efficiency was performed by calculating the concentration at which 50% of organism death occurs (CD50). This was established using the non-linear regression analysis performed in the Prism Graphpad software suite. Initial results suggest immunotoxin constructs one and two show efficiency in the low ng/L range. Prior to submission of a phase II application, these results will need to be confirmed with additional experimentation as variance was larger than anticipated and repeated measurements are needed to establish a verified CD50 value for the constructs. Additional experiments using larvae in culture, and adult mussels in culture will need to be performed to validate dose response and predict open water efficacy during phase II. Additionally, experiments with using off-target organisms, such as native mussels and fishes will need to be completed as part of the EPA approval process in phase II. The details of recommended future experimentation are included in the documents attached elsewhere in this report, as prepared by outside consultancy firm, Exponent.

3. Discussion

All key objectives of Phase I of this research project were completed, despite certain problems described below. Remaining research activities will continue during FY22-FY24 as Phase II focusing on the development of a transport and production vector for the chimeric biopesticide developed during Phase I.

3.1 Problems and Resolutions

3.1.1 Tissue Culture and Pandemic Related Work Stoppage

The hybridoma cell lines cultured for antibody production were acquired from MWD, where they were housed following the completion of earlier work funded by Reclamation. These cell lines had already undergone multiple passages by the time reserve freeze downs were prepared, as such, the number of passages remaining for successful production of antibody were limited. EQO was successful in creating back-up freeze downs of these cell lines and in harvesting antibody and RNA from these cell lines prior to the pandemic shut down that began in March of 2020. However, when a return to the lab was possible, it became apparent that during the shutdown, the liquid nitrogen dewar housing the back-up freeze downs had not been maintained by the facility during the

shutdown as expected and the cells were kept in suboptimal conditions. Once it became apparent that the vibrancy of the cells would not recover, additional cell lines from the LADWP facility were requested. This required additional time and the cell lines received were not capable of survival following five passages. EQO was able to obtain additional RNA samples from these cell lines to verify previous results, however, antibody production was significantly hindered. As time and budget would not allow for new cell lines to be produced by the original manufacturer, an alternate solution was required, and this impacted an antibody diagnostic objective. EQO decided it was best to complete the previous antibody goals using what remained of the stored antibody. The purpose of this experimentation was to calculate the binding efficiency of the previously discovered antibodies to complement the visualization of binding already completed. Antibody supply was limited and the ELISA protocol for calculation of binding efficiency was in the development. Once work began, it became apparent that this data was not critical to the project and it was more important to focus on scFv sequences, development of the transformation and production protocol, and the manufacturing of the end product. The calculation of dose response and kill efficiency was determined to take priority.

3.1.2 Ice Storm

An ice storm in Texas caused a lack of access to our lab for one week and the loss of power during this time caused the death of our microalgae cultures. We attempted to rescue the cultures but were ultimately not successful. As in house cultures were unable to be reestablished, EQO opted to purchase synthetic DNA versions of the scFv and toxin backbone sequences of interest. This allowed us to A) compress the timeline for reestablishment of microalgae cultures by allowing us to focus on transfection protocol improvement and B) obtain a more robust source for these sequences to avoid subsequent loss.

3.1.3 Underestimated Timeline

The amount of time required for the successful development of a transformation and microalgae growth protocol for production and application purposes was underestimated. The time for outgrowth of transformed *C. reinhadtii* and the requirement for subsequent selection pressure to improve transformation efficiency was not anticipated. The solution was to increase the degree to which experiments were ran in parallel. This required additional training of scientific staff by the P.I. in project management and experimental design.

4. Conclusions

Control of *Dreissena* infestation by chemical means is largely restricted to enclosed systems and requires additional remediation prior to water use. Biopesticides, or biotherapeutics, however, are considered a reduced risk treatment option by the EPA. EQO has developed three biopesticide candidates for production comprised of the enzymatic portion of a toxin for the mechanism of action and the binding domains from antibodies for targeting *Dreissena*. The EQO developed immunotoxin scaffold for use in environmental applications is based on a modified Pseudomonas

exotoxin-A 38kDa (PE38) protein. This bioengineered scaffold, when combined with a targeting domain, is well studied in human health applications, notably in oncology, but has not been previously applied in environmental applications. The bioengineered PE38 scaffold functions via forced internalization against target cells, intracellular routing following endocytosis, and direct cell kill by interruption of protein translation when combined with a targeting domain. The targeting domain for this scaffold has been provided by previous research by the Bureau of Reclamation, which produced twenty monoclonal antibodies (mAb) with molecular level targeted binding to *Dreissena*. The binding portion from six of the most promising of these mAbs, the Single-chain variable fragment (scFv) regions, were utilized as targeting domains for immunotoxin based biotherapeutic candidates developed in this Phase I project. The use of ScFv regions from mAbs, has become common place in pharmaceuticals like Herceptin® and MT-3724 and has been used here, resulting in a treatment product projected to exhibit high specificity, high efficiency, low production cost for necessary treatment doses, known mechanism of action, limited half-life, no long-term environmental impact, and limited to absent off-target impact.

Over the course of this project, the following objective necessary for development and identification of a production strategy for a biotherapeutic to address *Dreissena* infestation have been completed:

1. Reproduce growth and binding confirmation of previously discovered mAbs.
2. Identify the antigen binding scFv sequence in the twenty mAbs being investigated.
3. Development of a transformation and production protocol capable of scaled biotherapeutic production.
4. Produce lead candidates for development of immunotoxin based biopesticides.
5. Determine the potential of produced immunotoxins for effective control of *Dreissena polymorpha*.

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