Evaluation of a biopesticide against invasive species for native species restoration

Denise Ann Mayer  
*University at Albany, State University of New York*, dmayer@mail.nysed.gov

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EVALUATION OF A BIOPESTICIDE AGAINST INVASIVE SPECIES
FOR NATIVE SPECIES RESTORATION

by

Denise A. Mayer

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ABSTRACT

Evaluation of a Biopesticide against Invasive Species for Native Species Restoration

By

Denise A. Mayer

Since their introduction in the 1980s, dreissenid mussels (Dreissena polymorpha and D. rostriformis bugensis) rapidly spread throughout North America and have had severe impacts on the ecology of freshwater ecosystems. A project was initiated in the early 1990s at the New York State Museum’s Field Research Laboratory to discover and develop natural products to control industrial biofouling by dreissenid mussels. A strain of Pseudomonas fluorescens (Pf-CL145A), isolated from the sediment of a North American river, was found to be effective at killing dreissenids. The strain was patented for this use.

During the development of Pf-CL145A, the safety of the product to non-target organisms was evaluated. Ecotoxicological trials exposing the freshwater ciliate Colpidium colpoda, the freshwater zooplankton Daphnia magna, the freshwater amphipod Hyalella azteca, three fish species (Pimephales promelas, Salmo trutta, and Lepomis macrochirus), and seven bivalve species (Mytilus edulis, Pyganodon grandis, Pyganodon cataracta, Lasmigona compressa, Strophitus undulatus, Lampsilis radiata, and Elliptio complanata) suggest that Pf-CL145A could provide an effective and safe option for dreissenid management.

Zebra and quagga mussels thrive across a breadth of environmental conditions that are characteristic of many of North America’s lakes and streams. The limitations of Pf-CL145A due to four environmental factors that can be key parameters for the establishment of mass infestations of dreissenids (dissolved oxygen, temperature, water hardness, and suspended particles) were evaluated in laboratory trials. Pf-CL145A was highly effective under all conditions tested, indicating that the utility of this dreissenid-control product will likely not be limited by dissolved oxygen, temperature, water hardness, or suspended particle load.

Pf-CL145A’s safety to non-target organisms and utility across a wide range of environmental conditions have led to its consideration for limited use in open waters. The prospective application of Pf-CL145A as an aid in the restoration of North America’s native freshwater mussel species, 70% of which are extinct, endangered or imperiled, holds promise as a dreissenid management tool to enhance the success of native mussel recovery efforts.
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I am extremely grateful to many colleagues who provided assistance and inspiration for completing this dissertation: D. Molloy, for securing funding to support this research at the NYS Museum’s Field Research Laboratory; L. Burlakova, M. Gaylo, A. Karatayev, J. Morse, K. Presti, P. Sawyko, and E. Paul for assistance with non-target and environmental factor studies. They will be co-authors on manuscripts pertaining to these studies. I am grateful to D. Strayer for assistance with collection and identification of unionids. I extend my gratitude to P. Bolton and P. Sprague for their administrative and technical support. I am thankful to D. Molloy for his support and friendship as my major advisor, to G. Robinson for his encouragement and guidance as my academic advisor and my committee members D. Shub and D. Strayer for their support, advice, and suggestions that greatly improved this work and my educational experience. I extend gratitude to my collaborators at the Genoa National Fish Hatchery and Upper Midwest Environmental Science Center for their expertise and inspiration in the battle to help restore native mussels. Financial support for this work is gratefully acknowledged from the U.S. Department of Energy National Energy Technology Laboratory (Award Nos. DE-FC26-00NT40751 and DE-FC26-03NT41909), the U.S. Fish and Wildlife Service (Award No. 98210-0-G720), the National Science Foundation, the National Oceanic and Atmospheric Administration (Award No. NA86RG0056 to the Research Foundation of the State of New York for New York Sea Grant), the National Science Foundation SBIR/STTR (Award No. 0750549), the U.S. Environmental Protection Agency Great Lakes Restoration Initiative (Award No. GL-97229301-0), and the New York State Museum.
PREFACE

Since their introduction in the 1980s, dreissenid mussels (*Dreissena polymorpha* and *D. rostriformis bugensis*) rapidly spread throughout North America and have had severe impacts on the ecology of freshwater ecosystems. Dreissenid impacts stem from their efficient rates of filtration and ability to attach to hard surfaces with byssal threads. Once introduced into a natural water body, they are virtually impossible to eradicate and their populations often dominate the benthic community.

A project was initiated in the early 1990s at the New York State Museum’s Field Research Laboratory to discover and develop natural products to control industrial biofouling by dreissenid mussels. A strain of *Pseudomonas fluorescens* (*Pf*-CL145A) was isolated from a sediment sample from a North American river, that was effective at killing dreissenids was patented for this use. The main initiative for the project was to reduce the use of broad spectrum biocides that are currently being employed by industrial users to remediate fouling of their raw water dependent infrastructures by dreissenid mussels. In 2009 *Pf*-CL145A was licensed to Marrone Bio Innovations to develop the commercial product, Zequanox™, and formulations were developed for industrial applications such as to hydroelectric power plant cooling or service water systems.

Some additional background information about *Pf*-CL145A that was discovered during the research and development of the strain will be helpful in reading this dissertation, but is not discussed in detail within the text. Much of this background information is being prepared as a manuscript, Molloy et al., 2012 (in prep), to be submitted to the Journal of Invertebrate Pathology. While strain *Pf*-CL145A occurs
naturally, it is not present in high enough concentrations to kill dreissenids. The strain must be cultured under highly controlled conditions, which were developed at the NYSM, and then applied at much higher concentrations than found in nature. The toxic activity of Pf-CL145A is associated with the bacterial cells (i.e., endotoxin), as opposed to being released from the cells into the liquid fraction (supernatant) of the culture (i.e., exotoxin). Therefore, the particulate fraction of a culture, the cells, is harvested through centrifugation and the cell pellet is resuspended in water for application to dreissenids. The toxic activity of Pf-CL145A was not observed in nine other strains of Pseudomonas fluorescens that were tested. The toxic activity of Pf-CL145A is inactivated at high temperatures (i.e., >50°C for 30 min) and is relatively short-lived in natural conditions (i.e., cells become non-toxic after 24-30 hr when applied at 200 ppm at 20°C with aeration, Figure 3). Dead Pf-CL145A cells are as effective as live cells at killing dreissenids, indicating that the toxic effect is from intoxication, rather than infection. Therefore, commercial applications of the product will consist of killed Pf-CL145A cells.

Throughout the research and development of Pf-CL145A, the indication of the strain’s safety to non-target organisms was a driving force in obtaining funding for continued strain development. In Chapter 1, I describe ecotoxicological trials that were conducted with Pf-CL145A at the NYSM. Some of the non-target trials represent initial acute non-target screening trials and served as preliminary trials before more intensive toxicological studies, while other studies, i.e., the amphipod Hyalella azteca, the zooplankton Daphnia magna, and the native unionids Elliptio complanata and Pyganodon cataracta, were more thorough. All tests demonstrated Pf-CL145A’s promise for safety to non-target organisms. A manuscript describing results from this
chapter will be co-authored by colleagues who were instrumental to the work: D.P. Molloy, M.J. Gaylo, L.E. Burlakova, A.Y. Karatayev, K.T. Presti, P.M. Sawyko, E.A. Paul, and J.T. Morse.

While developing Pf-CL145A for industrial use, the original objective for this biocontrol project, we tested how various environmental factors might impact the efficacy of the Pf-CL145A product to dreissenids within industrial infrastructures, i.e., a pipe, in laboratory trials. We tested the impacts of dissolved oxygen level, temperature, pH (water hardness), and turbidity (suspended particle load). In Chapter 2, I describe these tests and consider the results in the context of these environmental conditions in open waters, i.e., lakes and rivers. The utility of a dreissenid management tool, such as Pf-CL145A, will be stronger if it is efficacious across a wide range of conditions.

Considering Pf-CL145A’s apparent non-target safety and utility across a range of environmental conditions, the strain is a good candidate for use in open-water ecosystems. In Chapter 3, I discuss potential specific uses for Pf-CL145A that are currently under development at the NYSM with collaborators from federal institutions in the Midwest. This work represents a new direction in Pf-CL145A research at the NYSM -- to evaluate and develop methods to use the Pf-CL145A product as an aid in the remediation and restoration of native species and/or ecosystems. In Chapter 3 I discuss an approach to evaluate whether Pf-CL145A might be useful in the protection and restoration of native unionid mussel populations.
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CHAPTER 1

Aquatic Ecotoxicology of *Pseudomonas fluorescens* strain CL145A:
a Selective Biopesticide for Controlling Zebra and Quagga Mussels (Dreissenidae)

Abstract

Since their introduction in the 1980s, zebra and quagga mussels (*Dreissena polymorpha* and *D. rostriformis bugensis*) have invaded many North American freshwater lakes and rivers. Due to limited control options, many infested facilities have had to rely on broad-spectrum, chemical biocides to manage pipe infestations. Chlorination, currently the most common chemical control method, is widely regarded as an unacceptable long-term solution since there is concern over its environmental impact. A novel strain of the environmental bacterial isolate, *Pseudomonas fluorescens* strain CL145A (*Pf*-CL145A), that was discovered and developed by a team of scientists at the New York State Museum, holds promise as an effective and safe biopesticide against dreissenids. Non-target trials with *Pf*-CL145A demonstrate a high level of specificity to *Dreissena*. In applications of *Pf*-CL145A achieving high kill to *D. polymorpha*, no mortality has been observed among any of the following non-target organisms: the freshwater ciliate *Colpidium colpoda*, the freshwater zooplankton *Daphnia magna*, the freshwater amphipod *Hyalella azteca*, three fish species (*Pimephales promelas*, *Salmo trutta*, and *Lepomis macrochirus*), and seven bivalve species (*Mytilus edulis*, *Pyganodon grandis*, *Pyganodon cataracta*, *Lasmigona compressa*, *Strophitus undulatus*, *Lampsilis radiata*, and *Elliptio complanata*). The results of our aquatic non-target toxicity trials
indicate that Pf-CL145A appears to be a safe alternative to chemical control, and suggest that its development could provide an effective and safe option for dreissenid management.

Introduction

Zebra and quagga mussels (*Dreissena polymorpha* and *D. rostriformis bugensis*) were first reported in North America in the mid-1980s and their impacts on industrial infrastructures are valued at billions of dollars (O’Neill, 2008). The environmental impacts of dreissenids are well documented and result mainly from their particle filtering and fouling of biotic and abiotic substrates (Ricciardi et al., 1998b; Karatayev et al., 2002). These activities increase water clarity, lead to the suffocation and population declines of organisms such as native freshwater mussels (unionids) (Ricciardi et al., 1998a; Vanderploeg et al., 2002), and create vastly modified benthic structures and nutrient pathways (Karatayev et al., 2002). Industrial systems suffer from reductions in efficiency and additional maintenance caused by the fouling of infrastructure by attached dreissenids. Fouling of pipes and infrastructure by dreissenids is typically remediated by flushing with broad-spectrum biocides, commonly chlorinated compounds (Mackie and Claudi, 2009). The free chlorine from these biocides can react with dissolved organics upon release into aquatic ecosystems to form carcinogenic compounds such as trihalomethanes and dioxins (Rook, 1976; Mackie and Claudi, 2009), and there is interest in developing methods to reduce the amount of chlorine released into natural systems (Volk et al., 2002). Discovering a biopesticide that could help reduce these impacts of
dreissenids by developing an environmentally benign alternative for industrial applications was one of the main goals of this biocontrol project by the NYS Museum.

A novel strain of the environmental bacterial isolate, *Pseudomonas fluorescens*, strain CL145A (Pf-CL145A) that was discovered and developed by a team of scientists at the New York State Museum holds promise as an effective biopesticide against dreissenids, particularly for use in industrial infrastructures (Molloy, 2001). This North American bacterial strain produces a cell-associated toxin whose activity can be preserved even after cells harvested from culture have been killed by gamma irradiation (Gammator, Isomedix, Inc. model number M-38-1; NY State Department of Health - Axelrod Institute, Albany, NY), resulting in a seven log reduction in the number of colony forming units of a cell suspension (i.e., from $1.7 \times 10^{10}$ to $2.0 \times 10^3$). In experiments in which dreissenids were exposed to either live Pf-CL145A cell material or irradiated cell material, there was no significant difference in mean mortalities observed (94.7 ± 6.1% versus 98.7 ± 2.3%, mean ± SD percent mortality, respectively; p=0.407). Product applications rely on the ingestion of killed cell particles with associated biotoxin as opposed to infection, and the mode of action of the biotoxin involves compromising the integrity of the epithelial lining of the digestive gland tubules within exposed *Dreissena* spp. (Figure 2, Molloy et al., in prep.). This method, therefore, contrasts with classical biological control methods which make use of the natural enemies of a pest species, typically from its home range. The toxic activity of Pf-CL145A, a strain discovered in a North American river that had not been invaded by dreissenids, relies on a toxin that fortuitously has a deadly impact on dreissenid mussels.
In this chapter, I report on initial, acute toxicity trials of Pf-CL145A to non-target aquatic organisms. Tests were conducted on the following non-target organisms: the freshwater ciliate *Colpidium colpoda*, the freshwater zooplankton *Daphnia magna*, the freshwater amphipod *Hyalella azteca*, three fish species (*Pimephales promelas*, *Salmo trutta*, and *Lepomis macrochirus*), and seven bivalve species (*Mytilus edulis*, *Pyganodon grandis*, *Pyganodon cataracta*, *Lasmigona compressa*, *Strophitus undulatus*, *Lampsilis radiata*, and *Elliptio complanata*). Results from these studies will help guide continued assessment of non-target safety of Pf-CL145A in dreissenid control and contribute to risk assessments by environmental management teams.

**Materials and Methods**

Throughout the development of Pf-CL145A, the product specificity was tested against a range of non-target organisms, as presented in Table 1. Organisms were selected in response to requests by funding agencies or in anticipation of requirements for product registration with the U.S. Environmental Protection Agency. Unless otherwise indicated, all testing was conducted at the New York State Museum Field Research Laboratory in Cambridge, NY (NYSM).

**Test material - Pf-CL145A cell suspension:**

*Pf*-CL145A cell-based inoculum was produced at the NYSM in static flasks following Molloy (2001). Cell pellets were harvested from final whole cultures by centrifugation and resuspended in dilution water (80 ppm KH₂PO₄, 405 ppm MgCl₂·6H₂O in deionized water that was pH adjusted to 7.2 with NaOH). To produce
larger quantities of *Pf*-CL145A, cells were produced in fermentation units at the Center for Biocatalysis and Bioprocessing at the University of Iowa (Iowa City, Iowa) and stored at -80°C prior to being thawed and suspended in dilution water immediately prior to use. *Pf*-CL145A cells were exposed to ionizing radiation (gamma or electron beam) to kill the cells by irradiation without losing efficacy against *Dreissena* (Molloy et al., in prep.). Irradiated cell suspensions were referred to as “killed” cell treatments. Standard plate counts of irradiated “killed” cell treatments on tryptic soy agar (Difco) resulted in a six to eight log reduction in colony forming units (i.e., from $10^{10}$ cfu/ml reduced to $10^1$ to $10^3$ cfu/ml). As an additional control, to assess whether sensitivity was likely due to the dreissenid-killing activity of the treatments or the addition of particulate matter and water quality issues, *Pf*-CL145A cells were exposed to heat (50 to 70°C) in a water bath or autoclaved for 30 to 60 min to inactivate the *Dreissena*-killing toxin to produce an inactive cell suspension (with low to no activity to dreissenids), referred to as “heat-inactivated” cell treatments. The “heat-inactivated” treatments were used to evaluate potential effects of suspended cell material on some of the non-target organisms tested, as specified in the methods where applicable.

**Confirmation of *Pf*-CL145A product efficacy:**

Standard bioassay methods were developed at the NYSM Field Research Laboratory to assess the efficacy of *Pf*-CL145A against zebra and quagga mussels. The general protocol was modified as necessary to adapt to specific requirements of a given experiment. In general, mussels (5 to 15 mm in length) were field-collected from local water bodies, including the Mohawk River (Halfmoon, NY), Lake Ontario (Rochester,
NY), and Hedges Lake (Washington County, NY), transported to the Field Research Laboratory and maintained in unchlorinated tap water in aquaria. One day prior to the treatment, a defined number of mussels, typically 25 to 100, was placed in one liter glass testing jars containing oxygen-saturated, synthetic, hard fresh water (192 ppm NaHCO₃, 120 ppm CaSO₄·2H₂O, 120 ppm MgSO₄, 8 ppm KCl) (USEPA, 2002). To ensure that only live individuals were present in each jar at the beginning of an experiment, only mussels that had attached to the jar were used in the test. At least one hour before treatment, 500 ml of oxygen-saturated hard water was added to the glass testing jars and supplied with gentle aeration. *Pf*-CL145A cell-based product was applied at 100 to 200 ppm (dry weight cells per unit volume) and exposure continued for 24 to 48 hours. At the end of the exposure period, treated water was poured off and mussels were rinsed and placed in clean plastic dishes with oxygen-saturated hard water. Dead mussels were identified as those having gaping shells that did not respond to a gentle touch with forceps. For tests conducted at 20 to 23°C, mortality was assessed and water changed daily for an additional 8 or 9 days (to achieve a total ten-day test period) at which time final mortality was calculated. Cooler temperatures required a longer observation period as indicated in the methods for those sections.

**Non-target organism testing:**

In most cases, zebra and/or quagga mussels were exposed to the treatment simultaneously in separate testing chambers to demonstrate efficacy of the product. The methods used for mussel exposure are similar in all cases and are, therefore, not reiterated for each non-target test organism. Unless otherwise specified, water quality parameters
were within the optimal zebra and quagga mussels, i.e., dissolved oxygen > 6 ppm, pH range 7.5 – 8.5 (moderate to very hard water). These conditions were also within the standard range for each of the non-target organisms tested.

Freshwater ciliate - *Colpidium colpoda*:

*Colpidium colpoda* were collected from a water pool at the NYSM Field Research Laboratory and cultured on rice seeds in stream water at 10 to 17°C in glass Petri dishes. Tests were conducted in 30-mm glass embryo dishes containing one ciliate in 0.5 ml stream water at 23°C. Immediately prior to treatment, water was removed by aspiration and replaced with aerated untreated or treated stream water to achieve untreated control and 100 ppm live *Pf*-CL145A cell treatments, respectively, with six replicates each. The total number of ciliates in each treatment was assessed with direct microscopic counts each day for a total of three days to assess mortality.

Cladoceran - *Daphnia magna*:

*Culturing and maintenance*:

*Daphnia magna* maintenance and testing were conducted according to USEPA (2002) guidelines for aquatic effluent acute toxicity testing. *D. magna* were obtained from the NYS Department of Environmental Conservation Bureau of Environmental Protection (Avon, NY) and cultured at the NYSM Field Research Laboratory and cultured in polystyrene cups containing 20 ml of oxygen-saturated, reconstituted, moderately hard water (96 ppm NaHCO₃, 63 ppm CaSO₄·2H₂O, 66 ppm CaCl₂·2H₂O, 30 ppm MgSO₄, 4 ppm KCl). The colony was maintained at 21 to 23°C on a 16/8 hour
light/dark cycle at a light intensity of 525 to 1075 lux. Each day adult *D. magna* were transferred to cups containing fresh hard water and fed YCT (yeast, Cerophyll®, and digested trout starter food) and *Selenastrum capricornutum* algae. Neonates were discarded during each water change unless required for an experiment. Cultures were renewed every three weeks with neonates that were less than 24-hour old.

*Treatment of D. magna with Pf-CL145A:*

Five neonates, less than 24 hour old, were transferred to eight Petri dishes each containing 25 ml of oxygen-saturated, reconstituted, moderately hard water. Four replicate dishes were treated at 200 ppm with *Pf*-CL145A killed-cell suspension, and four served as untreated controls. Neonates were fed at the start of the test and again at 24 hours. The dishes were placed in a constant temperature incubator at 23°C, and dissolved oxygen levels remained at or above the USEPA (2002) suggested minimum of 5.0 ppm. After 48 hours, the treated *D. magna* were transferred to new Petri dishes containing 25 ml of fresh, oxygen-saturated hard water and fed YCT and algal concentrate. Daily water renewals and mortality assessments were performed for an additional eight days.

*Amphipod - Hyalella azteca:*

Testing protocols were modified from United State Environmental Protection Agency freshwater sediment invertebrate toxicity testing methods (USEPA, 2000). Juvenile *Hyalella azteca* were obtained from Chesapeake Cultures (Hayes, VA) and were seven to 14 days old at initiation of the test. Organisms were held and tested in oxygen-saturated, synthetic, hard fresh water (USEPA, 2002) (192 ppm NaHCO₃, 120 ppm CaSO₄·2H₂O, 120 ppm MgSO₄, 8 ppm KCl) at 23°C (±2°C) and a 16/8-hour light/dark
cycle. Treatments of 0, 25, 50, 100, and 200 ppm of killed *Pf*-CL145A cell suspension in 200 ml were applied and exposures were 24 or 48 hours with ten organisms in each of three replicates per treatment. Following treatment exposure, organisms were transferred to clean glass containers with clean water. YCT (yeast, Cerophyll®, and digested trout starter food) was fed daily after the treatment period and water was renewed approximately every other day. Daily post-treatment observations and survival assessment continued for 12 to 13 days.

In addition to the standard untreated control, an additional experimental control was included to aid in analysis of the results. A *Pf*-CL145A killed cell suspension that was inactivated with heat treatment and demonstrated to have low to no toxicity to *Dreissena* was applied to *H. azteca* at concentrations of 100 and 200 ppm to assess impacts due to high nutrient or particle load. KCl reference toxicity tests, over a range of concentrations from 0 to 0.55 g/L, were also conducted on each group of *H. azteca*. The toxicity of *H. azteca* to KCl concentrations over a period of 96 hours was monitored as an indication of the relative health of the organisms used in each treatment exposure.

**Fathead minnow - *Pimephales promelas***:

*Static testing methods:*

Three-month-old fathead minnows (*P. promelas*) were obtained from Cosper Environmental Services, Inc. (Bohemia, NY) and placed in aquaria containing aerated unchlorinated tap water with filtration at 23°C (±1°C). Fish were fed every other day, alternating between flake fish food (Tetris) and frozen brine shrimp until 24 hours before treatment exposure, and then feeding resumed after the three-day exposure. One day
prior to treatment, 25 fish were transferred to 20-L aquaria containing 12 L of unchlorinated tap water. Live \( Pf-CL145A \) cells were applied to the treated aquaria at 100 ppm in triplicate for a 3 day treatment exposure. After treatment, fish were rinsed twice before being transferred to clean aquaria which contained unchlorinated tap water with aeration and filtration. Mortality assessments were performed immediately prior to a water change every other day for an additional 17 days.

*Flow-through testing methods:*

Fathead minnows were exposed to \( Pf-CL145A \) killed cells under flow-through conditions at the Rochester Gas and Electric coal-fired power station on Lake Ontario (Rochester, NY) in two separate tests. Flow-through treatments were conducted in sidestream monitoring aquaria, “bioboxes” (described in Mackie and Claudi, 2009), that were connected to the service water flow of the power station. Mean temperatures of the tests were 10°C and 21°C (± 1°C), respectively. Fathead minnows (35 to 55 mm in length) were supplied by the New York State Department of Environmental Conservation, Bureau of Environmental Protection (Avon, NY). Two aluminum fish baskets, each containing 50 fathead minnows were placed in each of two 80-L troughs receiving once-through service water at five liters per minute. A metered injection pump (Pulsafeeder, Inc.) was calibrated to deliver \( Pf-CL145A \) killed cells into the flowing service water of the treated biobox to result in a 50 ppm treatment concentration. The treatment concentration in the biobox was monitored throughout the 24-hour treatment period by relating optical density readings from the flow-through system to optical density readings from a quantified volume container treated at the desired target concentration. Following the 24-hour exposure, fish continued to be held under once-
through conditions in the power plant for 41 days. Fish were fed trout chow pellets, and
dead organisms, if observed, were recorded and removed daily.

**Bluegill - *Lepomis macrochirus***:

Young-of-the-year bluegill were obtained from Hopper-Stephens Hatcheries, Inc.
(Lonoke, AR) and upon receipt at the NYSM Field Research Laboratory were transferred
to 75-L aquaria containing aerated unchlorinated tap water at 12°C and then warmed
slowly and maintained at ambient temperature (20°C). Sunfish were fed fish chow
pellets approximately every other day until 24 hours pretreatment. One day prior to
treatment, 15 juvenile sunfish (mean (±SD) length 50.5 (±2.9) mm) were placed in each
of six 19-L glass aquaria containing 12 L of stream water with aeration at 20°C and
treated in triplicate with killed *Pf*-CL145A cells at 100 ppm (dry mass bacterial cell
weight per unit volume) or no treatment (untreated controls). After 72 hours of exposure
to the single-dose, fish were rinsed twice and transferred to clean 19-L glass aquaria with
aeration and filtration. Water was changed and mortality recorded each day for an
additional 11 days.

**Brown trout - *Salmo trutta***:

Young-of-the-year brown trout (*S. trutta*) were supplied by the New York State
Department of Environmental Conservation (NYS-DEC) fish hatchery in Rome, NY.
Fish were maintained in 5 to 7°C stream water on flow-through at the NYSM Field
Research Laboratory and were fed trout starter food daily until 24 hours prior to
treatment. One day prior to treatment, 25 brown trout were placed in each of nine plastic
containers (32 x 20 x 18 cm) each containing two liters of aerated stream water maintained at 5 to 7°C. Containers with fish were treated in triplicate with killed Pf-CL145A cells at 100 ppm (dry mass bacterial cell weight per unit volume). After 72 hours of exposure, trout were transferred by net to clean plastic containers with screened lids and held under flow-through conditions in stream water at 5 to 7°C for 39 days. During the post-treatment period, trout were fed starter food pellets and checked for mortality daily.

Blue mussel - *Mytilus edulis*:

Blue mussels were obtained from the Long Island Power Authority (formerly Long Island Lighting Company, Melville, NY). Blue mussels were maintained in 19-L aquaria in a solution of 2.8% (w/v) Instant Ocean® in unchlorinated tap water at ambient laboratory temperatures (20-23°C) with aeration and filtration (Tetra Whisper® aquarium filters). Blue mussels were selected based on size between 12 and 19 mm (mean length 15.0 ± 1.8 mm), and 25 individuals were placed in each of four one-liter testing jars with Instant Ocean® water and aeration and held overnight. The following morning, 100 ppm of Pf-CL145A live cell solution was applied to triplicate testing jars containing blue mussels. After 5 days of exposure to Pf-CL145A the treated water was poured off, and mussels were transferred to clean testing jars with aerated Instant Ocean® water. Mortality was checked, dead mussels removed and water changed each day for an additional 14 days.

_Laboratory trials to mixed species in aquaria:_

Unionids were collected from three sites for static laboratory experiments: the Moseskill River (Fort Edward, NY) where the collection included specimens of *Pyganodon grandis*, *Lasmigona compressa*, and *Strophitus undulatus*; the Webatuck Creek (Wassaic, NY) where the collection included only *Elliptio complanata*; and Lake Champlain (Ausable Point State Park, NY) for collection of *Lampsilis radiata*.

Following collection, unionids were placed in aquaria containing unchlorinated tap water with aeration at the NYSM laboratory (Cambridge, NY) and acclimated slowly to ambient temperature (16°C). Upon completion of the test unionids were identified using the key, *The Pearly Mussels of New York State* (Strayer and Jirka, 1997), and then confirmed by David Strayer at the Cary Institute for Ecosystem Studies (Millbrook, NY). The collection from the Moseskill River was composed of mixed species of unionids.

The following treatments were conducted:

_Moseskill River collection - mixed species_

The species of unionids collected from the Moseskill River were mixed and unidentified until the end of the test; therefore, the number of individuals of each species in a given treatment was variable. Five unionids were placed in each of four 38-L aquaria containing 12 L of unchlorinated tap water with high aeration. A single dose of *Pf*-CL145A live CF was applied at 100 ppm, and the unionids were exposed for three days. Following exposure, all unionids were rinsed and transferred to clean aquaria containing unchlorinated tap water with aeration. For an additional 28 days mortality
among unionids was checked daily. Mean (±SD) length of the mixed species was 48.8 (±11.5) mm. At the end of the test, unionids were sacrificed for identification and the number of individuals from each species in each treatment defined: *P. grandis* – one in untreated control, two treated with 100 ppm *Pf*-CL145A, *L. compressa* – three treated with 100 ppm *Pf*-CL145A, *S. undulatus* – four in untreated control, ten treated with 100 ppm *Pf*-CL145A.

*Webatuck Creek collection - Elliptio complanata:*

Fifteen *E. complanata* were placed in each of four 38-L aquaria containing 28 L of unchlorinated tap water with aeration. A single dose of *Pf*-CL145A live CF was applied to three of the aquaria at 100 ppm and the *E. complanata* were exposed for three days. Following exposure, the unionids were rinsed and transferred to clean 115-L aquaria filled with unchlorinated tap water with aeration and filtration (Whisper filters). Water was changed in the aquaria each week, and unionids were fed *Chlorella algae* three times each week. Unionids were checked each day for mortality for an additional 27 days after the treatment period.

*Lake Champlain collection - Lampsilis radiata:*

Fifteen *L. radiata* (mean (±SD) length 76.3 (±7.8) mm) were placed in each of four 38-L aquaria containing 28 L of unchlorinated tap water with aeration. A single dose of *Pf*-CL145A live CF was applied to three of the aquaria at 100 ppm and the *L. radiata* were exposed for three days. Following exposure the unionids were rinsed and transferred to clean 115-L aquaria filled with unchlorinated tap water with aeration and filtration (Whisper filters). Water was changed in the aquaria each week and unionids
were fed *Chlorella* algae three times each week. Unionids were checked each day for mortality for an additional 27 days after the treatment period.

**Laboratory trials in four-liter bottles:**

*Elliptio complanata* and *P. cataracta* were collected from Lake Cossayuna (Washington County, NY) and transported to the NYSM Field Research Laboratory in coolers to maintain temperature. The unionids were transferred to aquaria containing aerated stream water at the same temperature as the field collection and filtration was applied (Whisper filters). One day prior to treatment, a single unionid was placed in each 4-L glass testing bottle containing two liters reconstituted hard water with aeration at 23°C (±1°C). A single dose of *Pf-CL145A* live cells was applied at 100 and 200 ppm to ten replicates of each treatment of each species for 24 and 48 hours. At the end of the exposure, unionids were removed from the treated water, rinsed, and placed in 38-L aquaria containing 23°C stream water with aeration and filtration. Water was refreshed every two days. Unionids were not fed and were mortality was monitored daily for the total test duration, 34 days. Mean (±SD) length of *E. complanata* and *P. cataracta* used in the tests was 80.6 (±7.3) mm and 73.0 (±7.0) mm, respectively.

**Mesocosm trial:**

Mesocosms were constructed of open-ended transparent fiberglass cylinders, 30 cm in diameter x 30 cm in height, that were pushed approximately 5 cm into a sand-bottomed cement pond with flowing stream water at the NYSM Field Research Laboratory. Aeration was supplied to each mesocosm to ensure adequate oxygenation. The volume of water isolated by each mesocosm was approximately 15 L. One day before treatment application, *P. cataracta* were collected from Lake Cossayuna
(Washington County, NY), transported to the laboratory in coolers and ten unionids were placed directly into each of four mesocosms on top of the sand sediment at 21ºC (±3ºC). Live Pf-CL145A cells were applied, with mixing, at 200 ppm to the three replicate treated mesocosms for 48 hours. At the end of the treatment period, the fiberglass enclosures were removed and replaced with mesh cages having 1.5 cm openings. Unionids remained in the mesh-sided cages for an additional 28 days and mortality was assessed.

Statistics:

All binomial mortality data were prepared for parametric analysis by angular transformation (Sokal and Rohlf, 1995) and then tested for significance using t-tests, assuming unequal variances, if one treatment was included in the test design. For tests in which multiple comparisons were included, transformed data were tested for significance (p<0.05) by ANOVA and then differences between cases were assessed by Tukey's HSD multiple comparisons test. P-values are included in Table 1 where appropriate, i.e., for tests that included replication in untreated controls and where mean mortality was not higher in the untreated control than those treated with Pf-CL145A.

Results

Non-target trials with Pf-CL145A demonstrate a high level of specificity to Dreisena spp. The susceptibility of aquatic non-target organisms exposed to Pf-CL145A is presented in Table 1. In applications of Pf-CL145A achieving high kill to D. polymorpha, no significant mortality was observed among any of the following non-
target organisms: the freshwater ciliate *C. colpoda*, the freshwater zooplankton *D. magna*, three fish species (*P. promelas, S. trutta*, and *L. macrochirus*), and seven bivalve species (*M. edulis, P. grandis, L. compressa, S. undulatus, L. radiata, P. cataracta*, and *E. complanata*). Observed impacts from acute treatments with *Pf*-CL145A cells (killed or live) to non-target aquatic organisms ranged from undetectable to low in the tests described in this series of studies (Table 1). For all species tested, except *H. azteca*, if mortalities were observed during the observation period, the mean mortalities were not higher than those observed in the corresponding untreated controls (p-values displayed in Table 1). Dreissenid mortality in parallel exposures demonstrated efficacy of the *Pf*-CL145A material used in the non-target tests (Table 1). No parallel dreissenid efficacy test, however, was conducted at the time of the ciliate, *C. colpoda*, test. The same culturing methods, however, were used to produce *Pf*-CL145A test material in numerous tests before and following the *C. colpoda* test, and 75-100% lethality was achieved against zebra mussels. Therefore, we are confident that the material used in the ciliate trial was efficacious.

Sensitivity was observed in the freshwater amphipod *H. azteca*, following *Pf*-CL145A applications. After observing mortality at high dose treatments, 200 ppm for 48 hr and 100 ppm for 24 hr, *H. azteca* were exposed to the same dose of toxin-inactivated killed cells. The mean mortality observed following treatment with active killed *Pf*-CL145A cells at 100 ppm and 200 ppm was not significantly different than mean mortalities observed from the inactivated killed *Pf*-CL145A cell controls, suggesting that mortality was not caused by the active dreissenid-killing toxin. The toxin-inactivated material demonstrated low toxicity to zebra mussels, but *H. azteca* were sensitive to the
presence of the material at concentrations of 100 and 200 ppm. The amphipod *H. azteca*
appears to be mildly sensitive to treatments with *Pf-CL145A*, but the data suggest that
the sensitivity can be attributed to the presence of the particulate bacterial cell matter
itself rather than the dreissenid-killing cellular by-product.

**Discussion**

The acute toxicity trials reported in this paper were single-dose, short term
(typically 24 to 72 hour) exposures to *Pf-CL145A* cell suspension at concentrations that
were highly lethal to zebra mussels (100 or 200 ppm). In static, closed-container
treatments at 20°C, the activity of *Pf-CL145A* degrades within 30 hours following
application (Figure 3). Therefore, observed impacts from exposures in application
periods exceeding 24 hours may not represent impacts directly related to the dreissenid-
killing toxin activity, but to indirect impacts related to water quality, such as increased
ammonia. Every non-target species reported in this paper was exposed to *Pf-CL145A* for
a minimum of 48 hours in at least one trial and mortality was not observed during or
immediately following the exposure period, suggesting that both the direct impacts from
the dreissenid-killing toxin and indirect impacts from the degradation of the bacterial
cells mass following *Pf-CL145A* applications should not lead to acute toxic impacts.

In contrast to chemical biocides, which typically kill dreissenids quickly
following exposure, the observed impacts from the activity of *Pf-CL145A* occur over an
extended period of time, depending on the temperature (Figure 1). At 23°C dreissenid
mortality may occur up to 14 days following treatment, while at 7°C mortality may occur
over a span of 60 days. Unlike chemicals that can cause general tissue damage upon
contact, the activity of Pf-CL145A specifically degrades the epithelial cells of the
dreissenid digestive gland tubules (Figure 2) (author’s unpublished data; Molloy et al., in
prep.). The epithelium of the digestive gland tubules is the main center of detoxification
and elimination of xenobiotics (Marigómez et al., 2002) and cellular uptake of particles, i.e.,
pinocytosis (Bayne et al., 1976a). Bivalve hemocytes play important roles in digestion,
nutrient transport, excretion, regeneration and repair, and internal defense (Bayne et al.,
1976b; Giamberini et al., 1996). They migrate from the mussel’s open vascular system to
injured tissues for wound repair as well as to phagocytize foreign particles, such as
bacterial cells. In observations of histological slides produced from dreissenids that had
been exposed to Pf-CL145A at the NYSM, the presence of hemocytes in the tubules was
thus not surprising (Figure 2). These histological observations provided strong evidence
that dreissenid mortality from Pf-CL145A was the result of intoxication rather than
infection and that the toxic action of strain Pf-CL145A is highly selective in that it
destroys only the epithelial lining of the digestive gland tubules. The degradation of the
digestive gland tubule epithelium and subsequent mussel death, i.e., unresponsive gaping
of shells, is not immediate. Therefore, an extended observation period for recording
mussel mortality is required following treatment. For trials with non-target organisms,
we likewise extended the observation period for recording impacts.

The specificity of Pf-CL145A’s activity to dreissenids may be due to the lack of
organ structures homologous to the dreissenid digestive gland in many of the non-target
organisms tested. Unionids have digestive glands that are anatomically similar to those
of dreissenids and might, therefore, be predicted to be sensitive to Pf-CL145A.

Histological examinations of unionid digestive gland tissue were not performed in these
trials, but current and future testing will include digestive gland tissue examination, as well as multiple life stages of unionids (i.e., glochidia, juveniles, and sub-adults) which may be more sensitive than adults.

*Pseudomonas fluorescens* is a known pathogen to fish causing generalized septicemia in most species (Austin and Austin, 2007). Since *Pf*-CL145A can be similarly efficacious to dreissenids whether the cells are alive or dead (Molloy et al., in prep.), applications of the *Pf*-CL145A-based product for dreissenid management should consist entirely of dead *Pf*-CL145A cells. Applications of dead cells of *Pf*-CL145A were not toxic to fish in these trials. The commercial development and use of killed cell formulations will reduce environmental concerns of pathogenicity to fish or proliferation of the bacterial strain.

Due to limited control options, many infested facilities have had to rely on broad-spectrum, chemical biocides to manage pipe infestations. Chlorination, currently among the most common chemical control methods, is tightly regulated. Regulatory agencies limit the levels of total residual chlorine in discharge water due to concerns over environmental impacts (Mackie and Claudi, 2009). This includes its negative impacts on non-targets and the reaction of elemental chlorine with organic compounds in water resulting in the formation of potentially carcinogenic substances, such as trihalomethanes (Mackie and Claudi, 2009). If the chlorinated biocides become more tightly regulated, they may not be available for dreissenid control in the future. Waller et al. (1994) evaluated the efficacy and selectivity of candidate molluscicides, and determined that potassium chloride, Bayluscide, and Clamtrol CT-1 were the most effective and selective among those tested, providing possible substitutions for the use of chlorine for dreissenid
control. The results of our aquatic non-target toxicity trials indicate Pf-CL145A to be a safe alternative to chemical control, and suggest that its development could provide an effective option to include in dreissenid management strategies within industrial systems.

Non-target safety of Pf-CL145A looks promising for a selective control agent for dreissenids; however, some of the limitations of the data presented in this chapter should be acknowledged. These studies were conducted as part of the internal research and development of this strain for dreissenid control, and were not meant to supplant studies by external laboratories using regulated Good Laboratory Practices for USEPA product registration. Rather, these tests provided an initial indication of the level of sensitivity that might be expected and allowed us to predict which groups of organisms should be evaluated in more detail. For industrial treatments, where non-target organisms would be exposed to diluted Pf-CL145A product as the industrial effluent mixes with the source water, environmental exposure concentrations will likely be low due to dilution. Therefore, concerns over exposure of non-target organisms in ecosystems following industrial applications may be relatively low and the types of organisms presented in this chapter may suffice. However, open-water treatments with Pf-CL145A could involve the product’s direct application to areas inhabited by multiple species of organisms. It is not economically feasible that the Pf-CL145A would be applied to the entire water column of a lake or river. Rather, treatments systems designed to deliver Pf-CL145A formulations to the benthic areas of small localized areas of less than 1,000 m² may be considered for dreissenid management. For example, it may be feasible to design applications to unionid mussel beds to selectively reduce the impacts from dreissenids when an open-water formulation is approved for use. For such open-water treatments, additional
species of fish, and more life stages of unionid species should be tested for potential sensitivity. Impacts of Pf-CL145A to juveniles of unionid mussels should be considered since juveniles feed on particles in the benthos by pedal feeding rather than filter feeding as in adults (Yeager et al., 1994; Gatenby et al., 1996), which could impact the juvenile unionids differently. Additional groups that might require further evaluation include other benthic macroinvertebrates such as snails, crayfish, and insects (i.e., larvae of dragonflies, mayflies, stoneflies, etc.). As the sensitivity of more aquatic species of various life stages is evaluated in greater detail, the potential impacts of the product will be evaluated so the pest management strategies can be designed to minimize impacts to other organisms.

References


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molluscicides to zebra mussels (Dreissena polymorpha) and selected nontarget

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juvenile rainbow mussels Villosa iris (Bivalvia: Unionidae). Journal of the North
Table 1: Safety trials with aquatic nontarget organisms. Unless otherwise indicated, all tests were single-dose acute toxicity treatments in closed container bioassays followed by a post-treatment observation period after which mean mortalities were determined.

<table>
<thead>
<tr>
<th>Aquatic non-target organism</th>
<th>Concentration of bacteria (Duration of exposure)</th>
<th>Treatment Temperature</th>
<th>Post-treatment observation period</th>
<th>Mean control mortality (±SD)</th>
<th>Mean treatment mortality (±SD)</th>
<th>Mean dreissenid mortality in parallel treatment (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciliate Colpidium colpoda</td>
<td>100 ppm* (72 hr)</td>
<td>23°C</td>
<td>0 days</td>
<td>0.0 ± 0.0%</td>
<td>0.0 ± 0.0% p=1.0</td>
<td>No data</td>
</tr>
<tr>
<td></td>
<td>200 ppm** (48 hr)</td>
<td>23°C</td>
<td>8 days</td>
<td>0.0 ± 0.0%</td>
<td>0.0 ± 0.0% p=1.0</td>
<td>No data</td>
</tr>
<tr>
<td></td>
<td>200 ppm** (48 hr)</td>
<td>23°C</td>
<td>8 days</td>
<td>15.5 ± 10.0%</td>
<td>10.0 ± 11.6% p=0.537</td>
<td>95.3 ± 4.6%</td>
</tr>
<tr>
<td></td>
<td>200 ppm** (48 hr)</td>
<td>23°C</td>
<td>8 days</td>
<td>0.0 ± 0.0%</td>
<td>0.0 ± 0.0% p=1.0</td>
<td>90.7 ± 3.1%</td>
</tr>
<tr>
<td></td>
<td>200 ppm** (48 hr)</td>
<td>23°C</td>
<td>8 days</td>
<td>10.0 ± 11.5%</td>
<td>10.0 ± 20.0% p=0.785</td>
<td>92.0 ± 3.5%</td>
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<tr>
<td>Cladoceran Daphnia magna</td>
<td>25 ppm** (24 hr)</td>
<td>22°C</td>
<td>13 days</td>
<td>0.0 ± 0.0%</td>
<td>6.7 ± 11.5% p=0.863</td>
<td>98.7 ± 2.3%</td>
</tr>
<tr>
<td></td>
<td>50 ppm** (24 hr)</td>
<td>22°C</td>
<td>13 days</td>
<td>0.0 ± 0.0%</td>
<td>10.0 ± 17.3% p=0.745</td>
<td>61.3 ± 12.9%</td>
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<td></td>
<td>100 ppm** (24 hr)</td>
<td>22°C</td>
<td>13 days</td>
<td>0.0 ± 0.0%</td>
<td>16.7 ± 5.8% p=0.141</td>
<td>61.3 ± 12.9%</td>
</tr>
<tr>
<td></td>
<td>100 ppm** (24 hr)</td>
<td>22°C</td>
<td>13 days</td>
<td>3.3 ± 5.8%</td>
<td>16.7 ± 11.5% p=0.73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 ppm** (48 hr)</td>
<td>22°C</td>
<td>12 days</td>
<td>3.3 ± 5.8%</td>
<td>26.7 ± 30.6% p=0.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 ppm** (24 hr)</td>
<td>22°C</td>
<td>13 days</td>
<td>3.3 ± 5.8%</td>
<td>3.3 ± 5.8% p=1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 ppm** (48 hr)</td>
<td>22°C</td>
<td>12 days</td>
<td>3.3 ± 5.8%</td>
<td>17.4 ± 15.5% p=0.848</td>
<td>98.7 ± 2.3%</td>
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</tbody>
</table>
### Table 1 continued:

<table>
<thead>
<tr>
<th>Aquatic non-target organism</th>
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<th>Mean dreissenid mortality in parallel treatment (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amphipod Hyalella azteca (continued)</strong></td>
<td>200 ppm** (48 hr)</td>
<td>22°C</td>
<td>12 days</td>
<td>3.3 ± 5.8%</td>
<td>16.7 ± 11.5% p=0.094</td>
<td>96.0 ± 4.0%</td>
</tr>
<tr>
<td></td>
<td>200 ppm** (48 hr)</td>
<td>22°C</td>
<td>12 days</td>
<td>0.0 ± 0.0%</td>
<td>26.7 ± 15.3% p=0.039</td>
<td>96.0 ± 6.9%</td>
</tr>
<tr>
<td><strong>Toxin-inactivated treatments:</strong></td>
<td>100 ppm inactivated Δ killed CF (24 hr)</td>
<td>22°C</td>
<td>13 days</td>
<td>0.0 ± 0.0%</td>
<td>10.0 ± 0.0% p=0.323</td>
<td>0.0 ± 0.0%</td>
</tr>
<tr>
<td></td>
<td>200 ppm inactivated Δ killed CF (48 hr)</td>
<td>22°C</td>
<td>12 days</td>
<td>3.3 ± 5.8%</td>
<td>23.3 ± 15.3% p=0.514</td>
<td>20.0 ± 6.9%</td>
</tr>
<tr>
<td><strong>Fathead minnow Pimephales promelas</strong></td>
<td>100 ppm* (72 hr)</td>
<td>23°C</td>
<td>17 days</td>
<td>4.0%</td>
<td>2.7 ± 2.3%</td>
<td>46.7 ± 18.0%</td>
</tr>
<tr>
<td></td>
<td>50 ppm** (24 hr) †</td>
<td>10°C</td>
<td>41 days</td>
<td>2.5 ± 3.5%</td>
<td>0.0 ± 0.0%</td>
<td>70.8 ± 1.4%</td>
</tr>
<tr>
<td></td>
<td>50 ppm** (24 hr) †</td>
<td>21°C</td>
<td>41 days</td>
<td>3.0 ± 1.4%</td>
<td>1.0 ± 1.4%</td>
<td>78.3 ± 1.4%</td>
</tr>
<tr>
<td><strong>Sunfish Lepomis macrochirus</strong></td>
<td>100 ppm** (72 hr)</td>
<td>20°C</td>
<td>11 days</td>
<td>4.4 ± 3.9%</td>
<td>6.7 ± 6.7% p=0.823</td>
<td>96.0 ± 4.0%</td>
</tr>
<tr>
<td><strong>Brown trout Salmo trutta</strong></td>
<td>100 ppm** (72 hr)</td>
<td>6°C</td>
<td>39 days</td>
<td>1.3 ± 2.3%</td>
<td>2.7 ± 2.3% p=0.519</td>
<td>97.3 ± 4.6% (20°C) 71.3 ± 2.4% (6°C)</td>
</tr>
<tr>
<td><strong>Blue mussel (marine) Mytilus edulis</strong></td>
<td>100 ppm* (120 hr)</td>
<td>22°C</td>
<td>14 days</td>
<td>4.0%</td>
<td>1.3 ± 2.3%</td>
<td>100.0 ± 0.0%</td>
</tr>
<tr>
<td><strong>Unionid clams:</strong></td>
<td><strong>Elliptio complanata</strong></td>
<td>100 ppm* (72 hr)</td>
<td>17°C</td>
<td>27 days</td>
<td>0.0%</td>
<td>0.0 ± 0.0%</td>
</tr>
</tbody>
</table>
### Table 1 continued:

<table>
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<th>Mean treatment mortality (±SD)</th>
<th>Mean dreissenid mortality in parallel treatment (±SD)</th>
</tr>
</thead>
</table>
| Lampsilis radiata 100 ppm* (72 hr) 17°C 27 days 0.0% 0.0 ± 0.0% 100.0 ± 0.0% | Pyganodon compressa 100 ppm* (72 hr) 16°C 28 days 0.0% 0.0 ± 0.0% 98.7 ± 2.3% | Pyganodon grandis 100 ppm* (72 hr) 16°C 28 days 0.0% 0.0 ± 0.0% | Lasmigona undulatus 100 ppm* (72 hr) 16°C 28 days 0.0% 0.0 ± 0.0% | Pyganodon cataracta 100 ppm* (72 hr) 23°C 33 days 0.0% 0.0 ± 0.0% 97.8 ± 1.1% | **Organisms were treated with Dead cells of Pf-CL145A.** | *Organisms were treated with Live cells of Pf-CL145A.**

* These fish trials were under flow-through conditions in service water at a coal-fired power station (Rochester Gas and Electric Russell Power Station). **Toxin-inactivated with heat (50-70°C for 30-60 min) Δ This unionid trial was an outdoor mesocosm trial.
Figure 1: Mean zebra mussel mortality at 23, 17, 12, and 7°C over the post-treatment observation period (data from two tests).
Figure 2: Mode of action. Zebra mussels were exposed to 300 ppm live *Pf*-CL145A cells for 24 hours. Mussels (treated and untreated controls) were fixed in 10% buffered formalin, dehydrated in alcohols and toluene, and embedded in paraffin at 24 hours (immediately following treatment exposure). 5-µm serial sections were stained with hematoxylin and eosin. A. Photographs show digestive gland tubules of a mussel after 24 hr of exposure to live *Pf*-CL145A cells showing hemocyte infiltration into lumen (long arrows) and atrophy of epithelium (short arrows). B. Tubules of a control (untreated) mussel showing presence of normal epithelium (short arrows) and clear lumen. Scale bars = 50 µm (Molloy et al., in prep.).
Figure 3: Degradation of Pf-CL145A activity over time. Hard water was treated at a concentration of 200 ppm with Pf-CL145A freeze-dried formulation at 20°C with aeration over a period of 48 hours in triplicate 18-L aquaria. Triplicate 500-ml aliquots of treated water was periodically removed from the aquaria over a period of 24 (Test 1) or 48 hours (Test 2) and applied to zebra mussels in bioassay jars where they were exposed for 24 hours, and then rinsed, placed in fresh hard water and observed and scored for mortality for 7 days (total) with water changes each day.
CHAPTER 2

Impact of Environmental Factors on the Efficacy of Pseudomonas fluorescens Strain CL145A to Zebra Mussels (Dreissena polymorpha) in Laboratory Trials

Abstract

Zebra (Dreissena polymorpha) and quagga mussels (D. rostriformis bugensis) are two of the most detrimental invasive species in fresh waters of North America. Since their initial invasion in the early 1980s, they quickly infested many North American waterbodies. One factor contributing to their success is their ability to thrive across a breadth of environmental conditions characteristic of many of North America’s lakes and streams. Four environmental factors that are key parameters for the establishment of mass infestations of dreissenids are dissolved oxygen, temperature, hardness (i.e., calcium availability), and turbidity (i.e., particle load).

Scientists at the New York State Museum discovered a strain of the common soil bacterium, Pseudomonas fluorescens (strain Pf-CL145A) that has the capacity to cause lethality to zebra and quagga mussels and is being developed into a commercial product for dreissenid control. The efficacy of Pf-CL145A to zebra mussels when applied in laboratory trials across a range of dissolved oxygen (1-2 ppm, 4-5 ppm, and 7-8 ppm), temperature (7, 12, 17, 23°C), hardness (ranging from ca. 20 mg/ml to 240 mg/ml CaCO₃), and particle load (up to 133 ppm) is presented. Significant impacts on efficacy were observed in waters having low pH (<7.8) or very high turbidity (113 ppm), but the levels of both of the factors exceeded that which typically support dreissenid invasions.
Pf-CL145A was highly effective under all conditions tested, indicating that the utility of this dreissenid-control product will likely not be limited by the environmental parameters of dissolved oxygen, temperature, pH or turbidity.

**Introduction**

Zebra mussels (*Dreissena polymorpha*) were first reported in the Great Lakes of North America in mid-1980s (reviewed in Carlton, 2008) and then quickly spread throughout the waterbodies of the central and eastern United States, and recently to the West (San Justo Reservoir, CA) (Figure 4, current distribution). Quagga mussels (*D. rostriformis bugensis*) were found in Lake Michigan in 1997 and have been displacing zebra mussel populations in many areas of the Great Lakes (Nalepa et al., 2009). Quagga mussels were reported in Lake Mead in the Colorado River basin and are now continuing their spread in the southwestern United States. One factor in the success of these invasive species is their ability to thrive across a breadth of water chemistry conditions (Table 2). Clearly, zebra and quagga mussels thrive in the environmental conditions of many of North America’s lakes and streams.

Their ecological (Karatayev et al., 2002) and economic impacts (O’Neill, 2008) characterize these dreissenids as two of the most detrimental invasive species to freshwater ecosystems (Pimentel et al., 2005). With the goal of preserving and protecting aquatic ecosystems, scientists at the New York State Museum Field Research Laboratory (NYSM) launched a project in the 1990s to discover a natural product for dreissenid control that could possibly replace some of the uses of broad-spectrum biocides, such as
chlorine, for dreissenid management. They discovered a strain of the common soil bacterium *Pseudomonas fluorescens* (*Pf*-CL145A), originally isolated from river sediments in North America, that is both efficacious (Molloy, 2001) and selective to dreissenids (Chapter 1). The commercial development of the *Pf*-CL145A cell-based product, Zequanox™, is underway by Marrone Bio Innovations (MBI; Davis, CA), and registration of the killed-cell formulation was issued in July 2011. Since killed cells of *Pf*-CL145A are as lethal to dreissenids as live cells, the toxic effect is from intoxication rather than infection, and commercial product formulations will contain only dead *Pf*-CL145A cells to minimize environmental concerns of proliferation of the strain. *Pf*-CL145A produces a cellular byproduct that, when ingested by filter-feeding zebra and quagga mussels, causes the mussels to die due to degradation of the epithelial lining of the digestive gland tubules after exposure and ingestion (Figure 2; Molloy et al., in prep.). For optimal utility, a management tool for dreissenids, such as *Pf*-CL145A, should be effective across a wide range of the conditions in which dreissenids occur. The tests described in this chapter test the effectiveness of *Pf*-CL145A across a range of environmental conditions where *Pf*-CL145A has potential for dreissenid management. Environmental conditions during *Pf*-CL145A applications may at times fall below or exceed the tolerance limits of dreissenid populations for a limited amount of time or cause fluctuations in certain conditions. These laboratory trials were designed to examine whether the toxic impact of the *Pf*-CL145A might be affected and to predict the degree to which the impact might occur. The objective of these studies was to evaluate the ability of *Pf*-CL145A to maintain activity and be effectively consumed by the treated dreissenid mussels across ranges of dissolved oxygen, temperature, water hardness, and
suspended particle load as evidenced by the level of zebra mussel mortality achieved in laboratory bioassays.

Materials and Methods

Test material - Pf-CL145A cell suspension

Pf-CL145A cell suspension was produced at the New York State Museum Field Research Laboratory (Cambridge, New York) in static flasks following Molloy (2001). Pf-CL145A cell pellets were harvested from liquid culture by centrifugation (30 min at 1449xg) and resuspended in buffered dilution water (80 ppm KH₂PO₄, 405 ppm MgCl₂·6H₂O in deionized water). To achieve the desired treatment concentration, the optical density (A₆₆₀nm) of the cell suspension was determined with duplicate measurements and the treatment volume calculated from a previously determined equation of the line from a graph of dry weight cell suspension and optical density. The actual treatment concentration was confirmed with dry weights of the cell suspension measured as duplicate desiccated subsamples and expressed as mean bacterial cell mass per unit volume.

Statistical analyses

All binomial mortality data were prepared for parametric analysis by angular transformation (Sokal and Rohlf, 1995). Statistical differences (p<0.05) among means in each test were determined by ANOVA and then differences between cases were assessed by Tukey's HSD multiple comparisons test.
Dissolved oxygen concentration during treatment:

Testing chambers with controlled oxygen levels

Glass testing jars (1-L) containing 750 ml of oxygen-saturated, synthetic, hard fresh water (192 ppm NaHCO₃, 120 ppm CaSO₄·2H₂O, 120 ppm MgSO₄, 8 ppm KCl) (USEPA, 2002), were covered with #14 rubber stoppers each fit with a glass tube (200 mm x 5 mm) extending to 25 mm from the bottom of the jar. To achieve and maintain a range of dissolved oxygen levels during the treatment, a piece of waxed paper 8.5 cm in diameter with a 10 mm diameter hole near the center was placed on the water surface to reduce the water-to-air surface area and aeration provided through a pressurized air line connected to the glass tube. Mussels were prevented from migrating vertically to the water surface with a ring of air line tubing inserted to fit at 3 cm from the bottom of the testing jar. Levels of aeration in each testing jar were manually adjusted with an air valve to achieve three ranges of dissolved oxygen through the treatment period. Dissolved oxygen was monitored in each testing jar by submersing a probe inserted to within 2.5 cm from the bottom of the testing jar (Orion model 97-08-99 probe; Orion model 720A meter) and recorded as mg oxygen per liter.

Treatment of zebra mussels

Zebra mussels (mean length 9.0 ±2.0 mm (±SD)) were collected from the Mohawk River (Halfmoon, NY) and held in aerated, unchlorinated tap water until the test. One day prior to treatment, 100 zebra mussels were placed in testing chambers containing 100 ml of oxygen-saturated, hard fresh water at ambient laboratory temperature (22°C). To ensure that only live individuals were present in each container at the beginning of an experiment, only zebra mussels that had attached to the jar...
overnight were used and unattached mussels were removed and replaced with attached mussels from an extra jar. At least one hour before treatment, 750 ml of oxygen-saturated hard water was added to each testing jar and supplied with varying levels of aeration to achieve desired range of dissolved oxygen. *Pf*-CL145A cell suspension was applied at 200 ppm (dry weight cells per unit volume) and exposure continued for 12 hours. During treatment, dissolved oxygen was monitored in the testing jars and the level of aeration was manually adjusted to maintain three distinct concentrations: 1-2 ppm, 4-5 ppm, and 7-8 ppm, in triplicate.

Experimental controls consisted of a single testing jar of 100 mussels under aerated conditions where dissolved oxygen ranged from 7.8 to 8.3 ppm and triplicate jars with 100 mussels each that were supplied with no aeration where mean dissolved oxygen ranged from 6.3 to 1.0 ppm throughout the 24 hour testing period. All experimental controls received no application of *Pf*-CL145A and were held for 24 hours.

*Assessment of mussel mortality*

At the end of the exposure period, treated water was poured off and mussels were rinsed and placed in clean plastic dishes (7.6 x 7.6 cm) with oxygen-saturated hard water. Dead mussels were identified as those having gaping shells that did not respond to a gentle touch with forceps. Mortality was assessed and water changed daily for an additional 9 days (to achieve a total ten-day test period) at which time final percent mortality was calculated. The experiment was repeated and is reported as two separate tests.
Temperature:

Testing chambers with controlled temperature

Water baths, set to four temperatures (7, 12, 17, and 23°C) served to maintain temperatures before, during, and after treatment. Water was monitored and recorded throughout the experiment using a digital thermometer.

Treatment of zebra mussels

Zebra mussels (mean length 8.5±1.8 mm (±SD)) were collected from the Mohawk River (Halfmoon, NY) and held in aerated, unchlorinated tap water at 7°C until the test. One week prior to treatment, mussels were either transferred directly from 7°C in the walk-in cooler to the 7°C water bath or placed in an aquarium containing 7°C unchlorinated tap water and allowed to warm slowly while closely monitoring the temperature. When the temperature reached 12°C, 17°C or 23°C mussels were transferred to the appropriate water baths. One day prior to treatment, 100 zebra mussels were placed in 1-L testing jars containing 100 ml oxygen-saturated, synthetic, hard fresh water. To ensure that only live individuals were present in each container at the beginning of an experiment, only zebra mussels that had attached to the side of the jar overnight were used. Unattached mussels were removed and replaced with attached mussels from an extra jar. Mussels were maintained at the target testing temperature before, during, and after treatment by placing the testing jars in the appropriate water bath. At least one hour before treatment, 500 ml of temperature-adjusted oxygen-saturated hard water was added to each testing jar and supplied with gentle aeration. Pf-CL145A cell suspension was applied at 200 ppm (dry weight cells per unit volume) and exposure continued for 48 hours.
Experimental controls consisted of a single testing jar of 100 mussels under aerated conditions at each testing temperature. All experimental controls received no application of Pf-CL145A and were held for 48 hours.

Assessment of mussel mortality

At the end of the exposure period, treated water was poured off and mussels were rinsed and placed in clean plastic dishes (7.6 x 7.6 cm) with oxygen-saturated hard water that had been adjusted to the appropriate temperature. Dead mussels were identified as those having gaping shells that did not respond to a gentle touch with forceps. Mussel mortality was scored and water changed with temperature-adjusted oxygenated hard water for periods which varied by temperature depending upon when the accumulation of mortality ceased (i.e., in test 1 and test 2, respectively, 30 and 21 days at 23°C, 40 and 37 days at 17°C, 91 and 90 days at 12°C, and 91 and 90 days at 7°C).

Water Hardness:

Testing chambers with different water hardness

Five types of synthetic fresh waters were produced following USEPA (2002) which varied in chemical composition defined by their relative hardness (i.e., very soft to very hard) and exhibited a range of pH. Chemicals were added to deionized water to produce very soft, soft, moderately hard, hard, and very hard waters as indicated in Table 3. The pH of each synthetic fresh water was monitored using a pH probe (Accumet #13-620-287) and meter (Corning model 420).
Treatment of zebra mussels

Zebra mussels (mean length 9.5±1.5 mm (±SD)) were collected from the Mohawk River (Halfmoon, NY) and held in aerated, unchlorinated tap water at 7°C until the test. Two days prior to treatment, mussels were transferred from 7°C unchlorinated tap water to an aquarium in the laboratory containing 7°C unchlorinated tap water (pH 8.0, hardness 170-200 mg/ml CaCO₃), and allowed to warm slowly over a period of two days to the ambient laboratory temperature of 23°C. One week before treatment, mussels were transferred from the aquarium containing unchlorinated tap water to aquaria containing the appropriate synthetic water type. The mussels remained in these aquaria until one day before treatment, when 100 mussels were placed in 1-L testing jars containing 100 ml of the respective water type to a depth of 3 cm. To ensure that only live individuals were present in each testing jar at the beginning of an experiment, only zebra mussels that had attached to the side of the jar overnight were used. Unattached mussels were removed and replaced with attached mussels from an extra jar. At least one hour before treatment, 500 ml of the appropriate water type was added to respective testing jars and supplied with gentle aeration. Pf-CL145A cell suspension was applied, in triplicate, at 100 ppm (Test 1) or 200 ppm (Test 2) (dry weight cells per unit volume) and exposure continued for 24 hours.

Experimental controls consisted of a single testing jar for each water type with 100 mussels under aerated conditions. All experimental controls received no application of Pf-CL145A and were held for 24 hours.
Assessment of mussel mortality

At the end of the exposure period, treated water was poured off and mussels were rinsed and placed in clean plastic dishes (7.6 x 7.6 cm) with 100 ml of the appropriate oxygen-saturated water type. The dishes were covered with mesh to prevent the mussels from escaping and then submersed in 40-L aquaria containing the appropriate water type. Submerged dishes were removed to assess mortality daily. Dead mussels were identified as those having gaping shells that did not respond to a gentle touch with forceps. Mortality was assessed for an additional 9 days (to achieve a total ten-day test period) at which time final percent mortality was calculated. The experiment was repeated and is reported as two separate tests.

Suspended particles:

Preparation of water having a range of particle loads

Water was collected from the Mohawk River (Halfmoon, NY) into 20-L carboys at multiple times when the water appeared to have low turbidity and periods of high turbidity (i.e., suspended particles). For each test, raw (unfiltered) water was compared to water that had been filtered (Whatman filters, numbers 4 and 1) to remove particles greater than 11 μm. The particle load of each water type was measured as the dry weight of particles collected on triplicate 0.45-μm glass fiber filters and expressed as mg/L or ppm. Water having a higher level of suspended particles was prepared by allowing raw water to settle for one hour and then removing approximately 65% of the water from the top of the carboy.
Treatment of zebra mussels

Zebra mussels (mean length 9.0±1.7 mm (±SD)) were collected from the Mohawk River (Halfmoon, NY) and held in aerated, unchlorinated tap water until the test. Two days prior to treatment, approximately 600 mussels were transferred from the aquarium containing tap water to an aquarium containing 17-L of water having the appropriate level of suspended particles. One day before treatment, 100 mussels were placed into 3-L testing bottles each containing ca. 300 ml of the appropriate treatment water (hard water, raw Mohawk River water, or filtered Mohawk River water) to a depth of 3 cm. To ensure that only live individuals were present in each container at the beginning of an experiment, only zebra mussels that had attached to the side of the jar overnight were used. Unattached mussels were removed and replaced with attached mussels from an extra jar. Twenty minutes prior to treatment, the water was poured off and replaced with 2000 ml of the appropriate treatment water in the testing bottles and supplied with gentle aeration. *Pf*-CL145A cell suspension was applied, in triplicate, at 100 ppm (dry weight cells per unit volume) and exposure continued for 24 hours.

Experimental controls consisted of a single testing bottle of water having each level of suspended particles with 100 mussels under aerated conditions which received no application of *Pf*-CL145A and were held for 24 hours. Positive controls consisted of testing bottles containing hard water of 0 ppm suspended particles with 100 zebra mussels and treated at 100 ppm in triplicate.

Assessment of mussel mortality

At the end of the exposure period, treated water was poured off and mussels were rinsed and placed in clean plastic dishes (7.6 x 7.6 cm) with 100 ml of the appropriate
water turbidity. The dishes were covered with mesh to prevent the mussels from escaping and then submersed in 20-L aquaria containing the appropriate water type. Submerged dishes were removed to assess mortality daily. Dead mussels were identified as those having gaping shells that did not respond to a gentle touch with forceps. Mortality was assessed for an additional 9 days (to achieve a total ten-day test period) at which time final percent mortality was calculated. The experiment was repeated and is reported as three separate tests.

Results

Dissolved oxygen concentration during treatment:

Dissolved oxygen levels were maintained within the targeted concentrations of 1-2, 4-5, and 7-8 ppm with manual control of the level of aeration provided to the testing chamber. The measured mean dissolved oxygen concentrations were 1.3, 4.6, and 7.5, respectively, in the 2 tests combined (Figure 5) from 1 hour after Pf-CL145A application to the end of treatment after 12 hours. Starting the test with oxygenated water at 8 ppm dissolved oxygen, the oxygen was readily consumed within one hour following treatment and then was maintained at targeted levels throughout the remaining 12 hour treatment period.

There was no significant statistical difference (p>0.05) observed between mortality to zebra mussels when the dissolved oxygen was allowed to drop from approximately 8 ppm to an average of 1.3, 4.6 or 7.5 ppm in either of two tests (Table 4). At the lowest levels of dissolved oxygen, mussel mortality appeared to trend slightly lower in the first test, 83% mortality at 1.3 ppm dissolved oxygen compared to 85% and
95% mortality at 4.6 and 7.5 ppm dissolved oxygen, respectively, but the difference was not significant (p=0.966 and 0.173, respectively; Table 5). This trend was not observed when the test was repeated (Table 6). These results suggest that if dissolved oxygen levels are allowed to drop to levels as low as 1 to 2 ppm during treatment, the efficacy of Pf-CL145A applications should not be significantly reduced.

Temperature:

Water temperatures during periods before, during and following treatment of zebra mussels with Pf-CL145A remained close to targeted levels (Figure 6). Mean water temperatures in the water baths were 23.9±0.5°C, 17.2±1.0°C, 12.0±0.7°C, and 7.3±0.3°C. There was no significant statistical difference (p>0.05) observed between final mean mortalities achieved to zebra mussels held and treated with Pf-CL145A at any of the temperatures in either of the two tests (Tables 7-9). In each test, however, the lowest mortality was achieved at the lowest temperature (7°C); although not statistically significant, there appears to be a trend of lower kill at lower temperatures. Mortality took longer to commence and accumulated more slowly at lower temperatures (Figure 7). The period of time when 90% of the final mortality was recorded at each temperature occurred between 2 and 9 days, 2 and 12 days, 4 and 30 days, and 5 and 48 days at 23, 17, 12, and 7°C treatments, respectively (Figure 7). The average rate of mortality accumulation described by the linear portion of the curve for each temperature described in Figure 4 was 17, 12, 9, and 3% mortality per day at 23, 17, 12, and 7°C, respectively (Figure 8). Points on each curve were selected for inclusion in the linear regression both visually and for R values (R>0.95; expect for 17°C in test 2 which had a weaker fit (R =
0.94) for fit to the line. Thus, while high mortality can be achieved with Pf-CL145A at lower temperatures, it will take longer to accumulate.

**Water Hardness:**

Different types of synthetic fresh water were used to maintain relatively constant and distinct pH ranges. The synthetic fresh water types that were produced for these tests had measured pH ranging from 7.2 to 8.6 (Table 10). For each test, the mean pH values measured throughout the test period for each water type was statistically distinct (Tables 11-12, p<0.05). Thus, zebra mussels exposed to Pf-CL145A in each of the five types of synthetic fresh water were held and treated within distinct and different conditions of pH. Mean pH was 7.58, 7.78, 7.96, 8.18, and 8.33 in very soft, soft, moderately hard, hard, and very hard synthetic fresh waters, respectively.

Zebra mussel mortality was significantly lower in very soft water in the first test, and very soft water and soft water in the second test (Tables 13-14), suggesting that Pf-CL145A may have lower efficacy in waters of relatively low hardness and that Pf-CL145A may be the most efficacious in waters with calcium levels that tend to have the highest potential of infestation by dreissenids.

**Suspended Particles:**

The resulting dry weight of suspended particles in raw, unfiltered Mohawk River water was 12, 28, and 67 ppm, filtered river water was 2, 3, and 9 ppm, and concentrated river water was 113 ppm (Table 15). Following treatment with 100 ppm of Pf-CL145A cells, the particles contributing to water turbidity and collected on 0.45-µm filters,
represented 11%, 22%, and 40% of the dry weight in unfiltered Mohawk River water, 2%, 3%, and 8% of the dry weight in filtered Mohawk River water, and 53% of the dry weight in concentrated Mohawk River water (Table 15).

Zebra mussel mortality was significantly different in two treatments: when treated in filtered Mohawk River water with a dry weight of 2 ppm in test 2 and in concentrated Mohawk River water with a particle dry weight of 113 ppm in test 3 (Tables 16-18). While it may be that the high particle load in the highly turbid water at 113 ppm reduced the efficacy of the Pf-CL145A application, it is difficult to explain the results from the low turbidity treatment in filtered Mohawk River water at 2 ppm. These results suggest that Pf-CL145A treatments could result in lower efficacy in waters of very high turbidity (>113 ppm).

Discussion

The ability of zebra and quagga mussels to thrive in a wide range of environmental conditions is one factor that contributes to their invasive proliferation in North American waters. For optimal utility, a management tool for dreissenids, such as a Pf-CL145A-based product, should be effective in a wide range of conditions in which dreissenids occur. We evaluated the ability of Pf-CL145A cells to maintain activity, indicated by zebra mussel mortalities achieved after bioassay under controlled conditions in laboratory trials. Environmental conditions during Pf-CL145A applications may at times exceed the tolerance limits of dreissenid populations for a limited amount of time or cause fluctuations in the certain parameters. These tests were designed to examine
whether the toxic impact of the *Pf*-CL145A might be affected and to predict the degree to which the impact might occur.

Dreissenid populations are not sustained in environments with persistent dissolved oxygen levels of less than 3 to 4 ppm (Claudi and Mackie, 1994; Cohen, 2007). We tested the efficacy of *Pf*-CL145A treatments when mean dissolved oxygen levels were allowed to fall to 1.3, 4.6, and 7.3 ppm during the 12 hour treatment and observed no impact on the efficacy of *Pf*-CL145A. Thus, while sustained low dissolved oxygen concentrations may inhibit dreissenid populations, it did not appear that any level of dissolved oxygen tested affected either the toxicity of the natural product associated with the bacterial cells or the ability of the mussels to ingest the particles and pass them to their digestive system for the toxic action to occur. This was a short term (12 hour) treatment and the dissolved oxygen declined mainly due to the respiration of the bacterial cells applied as the treatment. Therefore, other than the treatment period, the mussels were held in conditions of high dissolved oxygen, before and after the treatment period. While these tests did not examine the efficacy of the *Pf*-CL145A product in natural ambient environmental conditions having various levels of dissolved oxygen over an extended period of time, as controlled laboratory experiments, they suggest that the toxic component of *Pf*-CL145A will not be significantly degraded or inactivated over a 12 hour period in conditions having dissolved oxygen levels ranging from roughly 1 to 8 ppm.

Water temperature can limit zebra mussel infestations if sustained mean temperatures are below 10°C or above 32°C, and quagga mussel infestations below 2°C or above 30°C (Table 2). We tested the efficacy of *Pf*-CL145A treatments at four temperatures, 7°C, 12°C, 17°C, and 24°C, to zebra mussels that had been acclimated to
each temperature before treatment, treated and then held under the same temperature conditions until mortality culminated. Mortality data were used to assess both the relative efficacy of *Pf*-CL145A to zebra mussels at various temperatures and the rate that observed mortality occurred at each temperature. While lower mortality was achieved in both tests at 7°C, the difference was not statistically significant, suggesting that *Pf*-CL145A can be highly effective in temperature conditions ranging from 7 to 24°C, but the rate at which the mortality occurs will be slower at lower temperatures (Figure 8, statistically significant at 12 and 7°C, p < 0.05). Interestingly, the rates of mortality accumulation at 12°C and 23°C correspond to predicted rates according to the Van’t Hoff principle, often applied to decomposition (Kirschbaum, 1995; Kätterer et al., 1998), where for every 10 degree increase in temperature the rate of biochemical reactions doubled. Thus, the slower rate of observed mortality at lower temperatures is likely due to the reduced rates of degradation of both the epithelial cells in the digestive gland and the mussel biomass required allowing the bivalve to gape.

The predictable variations in water temperature across seasons may be useful in designing and planning treatment regimes with *Pf*-CL145A to reduce the indirect risks following treatments such as increased biological oxygen demand and nutrient levels from decaying mussels. Waterbodies have been shown to support populations of dreissenids over 100,000 individuals/m² (Griffiths et al., 1991; Ricciardi et al., 1998). The rapid degradation of the biomass associated with mortality of extremely dense local populations could have negative impacts to the ecosystem, but if treatments are planned to occur during periods of lower temperatures, e.g., during the late autumn or spring, the impacts could be reduced.
The pH of water is correlated with calcium, alkalinity, and hardness levels in the water. Our first attempts to examine the impact of varying pH levels on the efficacy of *Pf*-CL145A to zebra mussels failed to produce stable pH levels due to the buffering capacity of the hard water we were attempting to use. Therefore, we modified the experimental design to use different types of synthetic water to sustain different levels of pH throughout the testing period. When significantly lower mussel mortality was observed from *Pf*-CL145A treatments, it was in waters having mean pH below 7.8, very soft and soft water types. Mackie and Claudi (2009) suggest that nuisance infestations of zebra or quagga mussels have the greatest risk potential at pH levels greater than 8.0. Therefore, our results suggest that *Pf*-CL145A will be the most effective in water types having pH levels in which dreissenids are most likely to occur at nuisance levels.

Dreissenid populations tend to reach nuisance levels in waters having concentrations of suspended particles of less than 96 ppm (Table 2). We tested the efficacy of *Pf*-CL145A in water conditions having natural levels of turbidity of 12, 28, and 67 ppm and observed no impact on the efficacy to zebra mussels. A higher level of turbidity was created for these laboratory tests, 113 ppm, and significantly lower mortality was observed. While it may be that the high particle load in the highly turbid water at 113 ppm reduced the efficacy of the *Pf*-CL145A application, it is difficult to explain the results from the low turbidity treatment in filtered Mohawk River water at 2 ppm. Since other treatments were conducted with filtered Mohawk River water at similarly low levels of turbidity, 3 ppm and 9 ppm, the significantly lower mortality at 2 ppm is not likely due to turbidity or the act of filtering. Some other unknown factor that was not detected in the study may have been involved in the lower kill to mussels. The
statistically significant lower mortality achieved from water containing 113 ppm of particles suggests that treatments with Pf-CL145A in highly turbid water may be less efficacious. These conditions would likely only be achieved immediately after very high water weather events and would likely be short-lived in open-water systems.

It was difficult to adequately assess the impact of turbidity on Pf-CL145A in laboratory tests under static conditions due to the filtering of particles by the mussels and settling of the suspended particles. A more realistic assessment of the impact of natural levels of turbidity on Pf-CL145A efficacy will be achieved in flow-through or large scale treatments in industrial or open-water systems.

Four environmental variables that are key parameters for the establishment of mass infestations of dreissenids are dissolved oxygen, temperature, pH, and turbidity. The studies reported herein evaluated the efficacy of an emerging tool for dreissenid control, Pf-CL145A, throughout ranges of each of these environmental variables. The environmental factors that correlated with significant negative impacts on efficacy were applications to water types (i.e., level of hardness) having low pH (<7.8) or very high levels of suspended particles (113 ppm). These factors should not preclude the use of Pf-CL145A in either industrial (i.e., pipes) or open-water systems having conditions that are the most likely to support nuisance infestations of dreissenids. Further assessment of the impact of environmental factors on the effectiveness of Pf-CL145A treatments will be possible during actual field applications when the treatment will be exposed to the interactions of multiple variables, including those not previously examined in laboratory trials. However, variability in these environmental parameters did not appear to present a limitation on the utility of a Pf-CL145A product for dreissenid control.
References


Table 2: Conditions of sustained temperature, dissolved oxygen, hardness, pH, and turbidity supporting risk potential for zebra mussel (*D. polymorpha*) or quagga mussel (*D. rostriformis bugensis*) nuisance infestations (modified from Mackie and Claudi, 2009).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Zebra mussel</th>
<th>Quagga mussel</th>
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<td>Dissolved oxygen (mg/L)</td>
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<td>≥4</td>
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<tr>
<td>Temperature (°C)</td>
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<td>2 - 30</td>
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<td>Hardness, total (mg CaCO₃/L)</td>
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<td>≥36</td>
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<td>pH</td>
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<td>7.0 - 9.5</td>
</tr>
<tr>
<td>Total suspended solids (mg/L)</td>
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<td>8 - 96</td>
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</table>
Table 3: Five types of synthetic fresh waters and their theoretical and actual pH ranges (USEPA, 2002).

<table>
<thead>
<tr>
<th>Water type</th>
<th>Amount of each chemical added to deionized water (mg/L)</th>
<th>Theoretical pH range</th>
<th>Measured initial pH range</th>
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</thead>
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<tr>
<td></td>
<td>NaHCO₃</td>
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<td>MgSO₄</td>
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<td>Very Soft Water</td>
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<td>Soft Water</td>
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<td>Moderately Hard Water</td>
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</tr>
<tr>
<td>Very Hard Water</td>
<td>384.0</td>
<td>240.0</td>
<td>240.0</td>
</tr>
</tbody>
</table>
Table 4: Mean mussel mortality (n=3) after 12 hr exposure to 200 ppm Pf-CL145A cells in testing jars with manually adjusted dissolved oxygen levels during treatment. Data represent total mean mortality accumulated to 9 days following exposure. Mortality in the experimental controls was <5%. Superscripted letters indicate statistical differences (p<0.05) between means for each test (lower case-Test 1, uppercase-Test 2).

<table>
<thead>
<tr>
<th>Targeted dissolved oxygen range</th>
<th>Mean dissolved oxygen between 1 and 12 hr after treatment</th>
<th>Test #</th>
<th>Mean zebra mussel mortality ± SD%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2 ppm</td>
<td>1.3 ppm</td>
<td>1</td>
<td>83.5 ± 7.4% a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>98.3 ± 1.5% A</td>
</tr>
<tr>
<td>4-5 ppm</td>
<td>4.6 ppm</td>
<td>1</td>
<td>85.0 ± 7.5% a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>97.3 ± 2.1% A</td>
</tr>
<tr>
<td>7-8 ppm</td>
<td>7.5 ppm</td>
<td>1</td>
<td>94.7 ± 4.9% a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>98.0 ± 1.0% A</td>
</tr>
</tbody>
</table>
Table 5: ANOVA and Tukey HSD results for Dissolved Oxygen Test 1.

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality</td>
<td>0.05901</td>
<td>2</td>
<td>0.029505</td>
<td>0.068225</td>
<td>6</td>
<td>0.011371</td>
<td>2.594766</td>
<td>0.154176</td>
</tr>
</tbody>
</table>

Tukey HSD test; Variable: Mortality. Marked differences are significant at p < .05000

<table>
<thead>
<tr>
<th>DO</th>
<th>Mortality = 83.5%</th>
<th>Mortality = 85.0%</th>
<th>Mortality = 94.7%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2 ppm</td>
<td>0.965753</td>
<td></td>
<td>0.172972</td>
</tr>
<tr>
<td>4-5 ppm</td>
<td>0.965753</td>
<td></td>
<td>0.237534</td>
</tr>
<tr>
<td>7-8 ppm</td>
<td>0.172972</td>
<td>0.237534</td>
<td></td>
</tr>
</tbody>
</table>

Table 6: ANOVA and Tukey HSD results for Dissolved Oxygen Test 2.

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality</td>
<td>0.003917</td>
<td>2</td>
<td>0.001958</td>
<td>0.028145</td>
<td>6</td>
<td>0.00469</td>
<td>0.41749</td>
<td>0.67645</td>
</tr>
</tbody>
</table>

Tukey HSD test; Variable: Mortality. Marked differences are significant at p < .05000

<table>
<thead>
<tr>
<th>DO</th>
<th>Mortality = 98.3%</th>
<th>Mortality = 97.3%</th>
<th>Mortality = 98.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2 ppm</td>
<td>0.660448</td>
<td></td>
<td>0.830580</td>
</tr>
<tr>
<td>4-5 ppm</td>
<td>0.660448</td>
<td></td>
<td>0.949091</td>
</tr>
<tr>
<td>7-8 ppm</td>
<td>0.830580</td>
<td>0.949091</td>
<td></td>
</tr>
</tbody>
</table>
Table 7: Mean zebra mussel mortality (n=3) at 23, 17, 12, and 7°C after a 48 hour exposure to 200 ppm *Pf*-CL145A cell suspension. Superscript letters indicate significant differences (p<0.05) between means for each test (lower case-Test 1, upper case-Test 2).

<table>
<thead>
<tr>
<th>Temperature (Mean ± SD)</th>
<th>Test #</th>
<th>Period of mortality accumulation (days)</th>
<th>Mean zebra mussel mortality ± SD% (% mortality in untreated control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23.9 ± 0.5°C</td>
<td>1</td>
<td>30</td>
<td>92.3 ± 3.5% (1%)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>21</td>
<td>88.9 ± 11.5% (9%)</td>
</tr>
<tr>
<td>17.2 ± 1.0°C</td>
<td>1</td>
<td>40</td>
<td>85.3 ± 4.6% (2%)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>37</td>
<td>90.8 ± 2.0% (0%)</td>
</tr>
<tr>
<td>12.0 ± 0.7°C</td>
<td>1</td>
<td>91</td>
<td>86.55 ± 3.9% (1%)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>90</td>
<td>88.12 ± 13.6% (0%)</td>
</tr>
<tr>
<td>7.3 ± 0.3°C</td>
<td>1</td>
<td>91</td>
<td>83.7 ± 4.6% (1%)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>90</td>
<td>76.4 ± 8.1% (0%)</td>
</tr>
</tbody>
</table>
Table 8: ANOVA and Tukey HSD results for Temperature Test 1.

<table>
<thead>
<tr>
<th>Variable</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality</td>
<td>0.033180</td>
<td>3</td>
<td>0.011060</td>
<td>0.033335</td>
<td>8</td>
<td>0.004167</td>
<td>2.654283</td>
<td>0.119894</td>
</tr>
</tbody>
</table>

Tukey HSD test: Variable: Mortality. Marked differences are significant at p < .05000

<table>
<thead>
<tr>
<th>Temperature</th>
<th>M</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>7°C</td>
<td>0.866193</td>
<td>0.970474</td>
</tr>
<tr>
<td>12°C</td>
<td>0.970474</td>
<td>0.986430</td>
</tr>
<tr>
<td>17°C</td>
<td>0.986430</td>
<td>0.319930</td>
</tr>
<tr>
<td>23°C</td>
<td>0.319930</td>
<td>0.207938</td>
</tr>
</tbody>
</table>

Table 9: ANOVA and Tukey HSD results for Temperature Test 2.

<table>
<thead>
<tr>
<th>Variable</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality</td>
<td>0.083050</td>
<td>3</td>
<td>0.027683</td>
<td>0.168456</td>
<td>8</td>
<td>0.021057</td>
<td>1.314679</td>
<td>0.335273</td>
</tr>
</tbody>
</table>

Tukey HSD test: Variable: Mortality. Marked differences are significant at p < .05000

<table>
<thead>
<tr>
<th>Temperature</th>
<th>M</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>7°C</td>
<td>0.448714</td>
<td>0.391675</td>
</tr>
<tr>
<td>12°C</td>
<td>0.391675</td>
<td>0.999386</td>
</tr>
<tr>
<td>17°C</td>
<td>0.999386</td>
<td>0.999881</td>
</tr>
<tr>
<td>23°C</td>
<td>0.999881</td>
<td>0.999881</td>
</tr>
</tbody>
</table>
Table 10: Mean zebra mussel mortality in different synthetic fresh water types after 24-hr exposure to 100 and 200 ppm *Pf*-CL145A cell suspension (tests 1 and 2, respectively). Superscript letters indicate significant differences (p<0.05) between means for each test (lower case-Test 1, upper case-Test 2).

<table>
<thead>
<tr>
<th>Synthetic water type</th>
<th>Measured pH range (Mean)</th>
<th>Test #</th>
<th>Mean zebra mussel mortality ± SD (% mortality in untreated control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very Soft Water</td>
<td>7.2 – 7.8^a,A (7.58)</td>
<td>1</td>
<td>83.0 ± 4.6% (3%)^a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>88.7 ± 2.3% (1%)^A</td>
</tr>
<tr>
<td>Soft Water</td>
<td>7.5 – 7.9^b,B (7.78)</td>
<td>1</td>
<td>96.7 ± 0.6% (1%)^b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>81.0 ± 3.5% (0%)^A</td>
</tr>
<tr>
<td>Moderately Hard Water</td>
<td>7.8 – 8.1^c,C (7.96)</td>
<td>1</td>
<td>97.7 ± 1.2% (2%)^b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>97.0 ± 1.0% (0%)^B</td>
</tr>
<tr>
<td>Hard Water</td>
<td>8.1 – 8.4^d,D (8.18)</td>
<td>1</td>
<td>98.3 ± 1.2% (0%)^b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>97.0 ± 2.6% (2%)^B</td>
</tr>
<tr>
<td>Very Hard Water</td>
<td>8.4 – 8.6^e,E (8.33)</td>
<td>1</td>
<td>98.0 ± 1.7% (1%)^b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>99.0 ± 1.0% (2%)^B</td>
</tr>
</tbody>
</table>
Table 11: ANOVA and Tukey HSD results for pH in different water types Test 1.

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>10.49070</td>
<td>4</td>
<td>2.622674</td>
<td>2.243995</td>
<td>95</td>
<td>0.023621</td>
<td>111.0315</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Tukey HSD test: Variable: pH. Marked differences are significant at p < .05000

<table>
<thead>
<tr>
<th>Variable</th>
<th>M</th>
<th>M</th>
<th>M</th>
<th>M</th>
<th>M</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very Soft</td>
<td>7.56</td>
<td>7.74</td>
<td>7.95</td>
<td>8.21</td>
<td>8.47</td>
<td>0.00</td>
</tr>
<tr>
<td>Soft</td>
<td>0.002637</td>
<td>0.000117</td>
<td>0.000117</td>
<td>0.000117</td>
<td>0.000117</td>
<td>0.000117</td>
</tr>
<tr>
<td>Moderately Hard</td>
<td>0.000117</td>
<td>0.000721</td>
<td>0.000120</td>
<td>0.000117</td>
<td>0.000117</td>
<td></td>
</tr>
<tr>
<td>Hard</td>
<td>0.000117</td>
<td>0.000117</td>
<td>0.000120</td>
<td>0.000124</td>
<td>0.000124</td>
<td></td>
</tr>
<tr>
<td>Very Hard</td>
<td>0.000117</td>
<td>0.000117</td>
<td>0.000117</td>
<td>0.000124</td>
<td>0.000124</td>
<td></td>
</tr>
</tbody>
</table>

Table 12: ANOVA and Tukey HSD results for pH in different water types Test 2.

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.967301</td>
<td>4</td>
<td>1.741825</td>
<td>1.982642</td>
<td>90</td>
<td>0.022029</td>
<td>79.06837</td>
<td>0.000000</td>
</tr>
</tbody>
</table>

Tukey HSD test; Variable: pH. Marked differences are significant at p < .05000

<table>
<thead>
<tr>
<th>Variable</th>
<th>M</th>
<th>M</th>
<th>M</th>
<th>M</th>
<th>M</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very Soft</td>
<td>7.58</td>
<td>7.78</td>
<td>7.96</td>
<td>8.18</td>
<td>8.33</td>
<td>0.00</td>
</tr>
<tr>
<td>Soft</td>
<td>0.000701</td>
<td>0.000118</td>
<td>0.000118</td>
<td>0.000118</td>
<td>0.000118</td>
<td></td>
</tr>
<tr>
<td>Moderately Hard</td>
<td>0.000118</td>
<td>0.004026</td>
<td>0.000214</td>
<td>0.000118</td>
<td>0.000118</td>
<td></td>
</tr>
<tr>
<td>Hard</td>
<td>0.000118</td>
<td>0.000118</td>
<td>0.000214</td>
<td>0.015872</td>
<td>0.015872</td>
<td></td>
</tr>
<tr>
<td>Very Hard</td>
<td>0.000118</td>
<td>0.000118</td>
<td>0.000118</td>
<td>0.015872</td>
<td>0.015872</td>
<td></td>
</tr>
</tbody>
</table>
Table 13: ANOVA and Tukey HSD results for mortality in different water types

Test 1.

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality</td>
<td>0.186738</td>
<td>4</td>
<td>0.046685</td>
<td>0.022172</td>
<td>10</td>
<td>0.002217</td>
<td>21.05564</td>
<td>0.000074</td>
</tr>
</tbody>
</table>

Tukey HSD test: Variable: Mortality. Marked differences are significant at p < .05000

<table>
<thead>
<tr>
<th>Water Type</th>
<th>M = 83.0%</th>
<th>M = 96.7%</th>
<th>M = 97.7%</th>
<th>M = 98.3%</th>
<th>M = 90.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very Soft</td>
<td>0.000840</td>
<td>0.000376</td>
<td>0.000259</td>
<td>0.000291</td>
<td></td>
</tr>
<tr>
<td>Soft</td>
<td></td>
<td>0.901666</td>
<td>0.576270</td>
<td>0.711657</td>
<td></td>
</tr>
<tr>
<td>Moderately Hard</td>
<td>0.000376</td>
<td>0.901666</td>
<td>0.963754</td>
<td>0.993511</td>
<td></td>
</tr>
<tr>
<td>Hard</td>
<td>0.000259</td>
<td>0.576270</td>
<td>0.963754</td>
<td>0.999162</td>
<td></td>
</tr>
<tr>
<td>Very Hard</td>
<td>0.000291</td>
<td>0.711657</td>
<td>0.993511</td>
<td>0.999162</td>
<td></td>
</tr>
</tbody>
</table>

Table 14: ANOVA and Tukey HSD results for mortality in different water types

Test 2.

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality</td>
<td>0.271111</td>
<td>4</td>
<td>0.067778</td>
<td>0.030569</td>
<td>10</td>
<td>0.003057</td>
<td>22.17216</td>
<td>0.000059</td>
</tr>
</tbody>
</table>

Tukey HSD test: Variable: Mortality. Marked differences are significant at p < .05000

<table>
<thead>
<tr>
<th>Water Type</th>
<th>M = 88.7%</th>
<th>M = 81.0%</th>
<th>M = 97.0%</th>
<th>M = 97.0%</th>
<th>M = 99.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very Soft</td>
<td>0.197056</td>
<td>0.024140</td>
<td>0.017638</td>
<td>0.001392</td>
<td></td>
</tr>
<tr>
<td>Soft</td>
<td></td>
<td>0.000920</td>
<td>0.000736</td>
<td>0.000221</td>
<td></td>
</tr>
<tr>
<td>Moderately Hard</td>
<td>0.024140</td>
<td>0.000920</td>
<td>0.999552</td>
<td>0.318137</td>
<td></td>
</tr>
<tr>
<td>Hard</td>
<td>0.017638</td>
<td>0.000736</td>
<td>0.999552</td>
<td>0.410544</td>
<td></td>
</tr>
<tr>
<td>Very Hard</td>
<td>0.001392</td>
<td>0.000221</td>
<td>0.318137</td>
<td>0.410544</td>
<td></td>
</tr>
</tbody>
</table>
Table 15: Experiments testing impact of particle load in water on efficacy of single-dose treatment with 100 ppm *Pf*-CL145A suspended cells on zebra mussel mortality. Water was collected from the Mohawk River. Superscript symbols indicate significant differences (p<0.05) between means for each test (lower case letters -Test 1, upper case letters -Test 2, symbols - Test 3).

<table>
<thead>
<tr>
<th>Test</th>
<th>Water type</th>
<th>Particle load in water prior to <em>Pf</em>-CL145A application (percentage of total dry weight from suspended particles after 100 ppm treatment)</th>
<th>Mean % mortality (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hard water</td>
<td>0 ppm</td>
<td>88.7 ± 4.0% ^a</td>
</tr>
<tr>
<td></td>
<td>Filtered River Water</td>
<td>3 ppm (3%)</td>
<td>84.0 ± 1.7% ^a</td>
</tr>
<tr>
<td></td>
<td>Unfiltered River Water</td>
<td>12 ppm (11%)</td>
<td>87.7 ± 2.3% ^a</td>
</tr>
<tr>
<td>2</td>
<td>Hard water</td>
<td>0 ppm</td>
<td>99.3 ± 0.6% ^A</td>
</tr>
<tr>
<td></td>
<td>Filtered River Water</td>
<td>2 ppm (2%)</td>
<td>69.4 ± 1.3% ^B</td>
</tr>
<tr>
<td></td>
<td>Unfiltered River Water</td>
<td>28 ppm (22%)</td>
<td>98.0 ± 1.0% ^A</td>
</tr>
<tr>
<td>3</td>
<td>Hard water</td>
<td>0 ppm</td>
<td>95.7 ± 1.2% †</td>
</tr>
<tr>
<td></td>
<td>Filtered River Water</td>
<td>9 ppm (8%)</td>
<td>96.0 ± 3.0% †</td>
</tr>
<tr>
<td></td>
<td>Unfiltered River Water</td>
<td>67 ppm (40%)</td>
<td>92.7 ± 2.5% †</td>
</tr>
<tr>
<td></td>
<td>Concentrated River Water</td>
<td>113 ppm (53%)</td>
<td>72.7 ± 7.8% *</td>
</tr>
</tbody>
</table>
Table 16: ANOVA and Tukey HSD results for mortality when treated in water containing different amounts of suspended particles Test 1.

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality</td>
<td>0.008265</td>
<td>2</td>
<td>0.004132</td>
<td>0.012255</td>
<td>6</td>
<td>0.002042</td>
<td>2.023257</td>
<td>0.213014</td>
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</tbody>
</table>

Tukey HSD test: Variable: Mortality. Marked differences are significant at p < .05000

<table>
<thead>
<tr>
<th></th>
<th>M = 88.7%</th>
<th>M = 84.0%</th>
<th>M = 87.7%</th>
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<tbody>
<tr>
<td>Hard Water (0 ppm)</td>
<td>0.209356</td>
<td>0.380087</td>
<td>0.877082</td>
</tr>
<tr>
<td>Filtered River Water (3 ppm)</td>
<td>0.209356</td>
<td>0.380087</td>
<td>0.877082</td>
</tr>
<tr>
<td>Unfiltered River Water (12 ppm)</td>
<td>0.877082</td>
<td>0.380087</td>
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</table>

Table 17: ANOVA and Tukey HSD results for mortality when treated in water containing different amounts of suspended particles Test 2.

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
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<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality</td>
<td>0.474251</td>
<td>2</td>
<td>0.237125</td>
<td>0.009763</td>
<td>6</td>
<td>0.001627</td>
<td>145.7344</td>
<td>0.000008</td>
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Tukey HSD test: Variable: Mortality. Marked differences are significant at p < .05000

<table>
<thead>
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<th>M = 69.4%</th>
<th>M = 98.0%</th>
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<tbody>
<tr>
<td>Hard Water (0 ppm)</td>
<td>0.000228</td>
<td>0.000234</td>
<td>0.151871</td>
</tr>
<tr>
<td>Filtered River Water (2 ppm)</td>
<td>0.000228</td>
<td>0.000234</td>
<td>0.151871</td>
</tr>
<tr>
<td>Unfiltered River Water (28 ppm)</td>
<td>0.151871</td>
<td>0.000234</td>
<td>0.151871</td>
</tr>
</tbody>
</table>

Table 18: ANOVA and Tukey HSD results for mortality when treated in water containing different amounts of suspended particles Test 3.

<table>
<thead>
<tr>
<th></th>
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<th>df</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Mortality</td>
<td>0.247904</td>
<td>3</td>
<td>0.082635</td>
<td>0.035558</td>
<td>8</td>
<td>0.004445</td>
<td>18.59164</td>
<td>0.000578</td>
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</table>

Tukey HSD test: Variable: Mortality. Marked differences are significant at p < .05000

<table>
<thead>
<tr>
<th></th>
<th>M = 95.7%</th>
<th>M = 96.0%</th>
<th>M = 92.7%</th>
<th>M = 72.7%</th>
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<tbody>
<tr>
<td>Hard Water (0 ppm)</td>
<td>0.985321</td>
<td>0.664479</td>
<td>0.001289</td>
<td></td>
</tr>
<tr>
<td>Filtered River Water (9 ppm)</td>
<td>0.985321</td>
<td>0.477743</td>
<td>0.000954</td>
<td></td>
</tr>
<tr>
<td>Unfiltered River Water (67 ppm)</td>
<td>0.664479</td>
<td>0.477743</td>
<td>0.004375</td>
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<tr>
<td>Concentrated River Water (113 ppm)</td>
<td>0.001289</td>
<td>0.000954</td>
<td>0.004375</td>
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</table>
Figure 4: Zebra and quagga mussel distribution as of 07/08/2011. These data are updated by the USGS and the map was taken directly from their website:
Figure 5: Dissolved oxygen measured in testing jars over time (first test (♦) and second test (○)). Bars represent standard deviation from the mean of three replicates.
Figure 6: Temperature recorded in each water bath before, during, and following treatment. The values in boxes represent mean ± SD over the entire period monitored.
Figure 7: Mean zebra mussel mortality at 23, 17, 12, and 7°C over the post-treatment observation period (data from two tests).
Figure 8: Trend lines and equations for the linear portion of cumulative mortality to evaluate the rate of morality accumulated over time for test 1 (top) and test 2 (bottom). Each equation is in the form of a linear regression ($y = mx + b$, where $m =$ slope (rate), and $b =$ the $y$ intercept).
CHAPTER 3

Dreissenid Management as an Aid to Native Freshwater Mussel Restoration

Abstract

Native freshwater mussels (unionids) are among the most imperiled of all animals in North America. Recovery efforts are underway for many of the endangered species, but too often these efforts continue to be thwarted by the presence of invasive species, particularly zebra and quagga mussels, *Dreissena polymorpha* and *D. rostriformis bugensis*, respectively (dreissenids). Originally developed to mitigate dreissenid fouling in industrial pipe systems, *Pseudomonas fluorescens* strain CL145A (*Pf*-CL145A) has potential for use as a safe and effective dreissenid management tool to aid in unionid recovery efforts. Applications to open-water systems, however, introduce additional concerns of product safety to non-target species not previously tested and indirect impacts from inputs of organic matter. These concerns can be mitigated, in part, by working with regional native mussel recovery teams to identify appropriate non-target test organisms to further evaluate potential impacts and to define application methods that reduce potential indirect impacts. Potential uses for *Pf*-CL145A in limited-scale open-water applications are discussed as an integrated dreissenid management tool for unionid mussel restoration.
Introduction

Freshwater ecosystems and their associated organisms are in desperate need of conservation and preservation. Fresh water covers only 0.8% of the surface of Earth, yet freshwater ecosystems support approximately 10% of all known species (Strayer and Dudgeon, 2010). With 27% of the described freshwater fauna in North America in threat of extinction (Ricciardi and Rasmussen, 1999), their decline and extinction rates rival that of the rain forests (Ricciardi and Rasmussen, 1999; Wilcove and Master, 2005; Strayer and Dudgeon, 2010). Pressures are imposed on freshwater systems through anthropogenic interactions such as increased siltation, temperature and fragmentation from dam construction, and pollution and eutrophication from industrial, agricultural, and residential runoff.

The diversity and abundance of freshwater fauna provide critical functions and services in these ecosystems. An important group of organisms for the function of freshwater systems are the native pearly mussels (Bivalvia: Unionacea), hereafter unionids. Unionids can be the most abundant benthic organisms in terms of biomass in some systems, often occurring in high-density beds of multiple species (Dame, 1996; Strayer et al., 1999; Raikow and Hamilton, 2001). Unionid mussels serve as couplers of nutrient and energy flows from pelagic to and from benthic communities (Welker and Walz, 1998; Raikow and Hamilton, 2001; Nalepa et al., 1991; Vaughn and Hakenkamp, 2001), particularly in lotic systems where materials would otherwise be transported downstream. Filtering up to 10-100% of the water column per day (Strayer et al. 1999), unionids feed on suspended bacteria, phytoplankton, and fine detritus (McMahon, 1991). They also deposit feces and pseudofeces in the benthos and excrete wastes and nutrients.
into the water (Vaughn and Hakenkamp, 2001). Various life stages of unionids appear to feed differently; studies have suggested that juvenile and sub-adult (i.e., less than 5 years old) mussels may deposit feed or “pedal feed” (Yeager et al., 1994; Gatenby et al., 1996). Others have suggested that up to 80% of the food assimilated by adult unionids is obtained through deposit feeding within the sediment (Raikow and Hamilton, 2001), where they also serve to mix the sediment through their burrowing activities (McCall et al., 1979; 1995). In addition, unionid shells provide habitat for other benthic organisms (Sephton et al., 1980; Beckett et al., 1996) and epiphyton (Vaughn and Hakenkamp; 2001 Gutiérrez et al., 2003). These functions not only demonstrate the flow of nutrients between unionids, the surrounding water, and the sediment surface, but also the penetration of this interaction throughout a surface layer of the sediment within the benthic community. Changes to the diversity and abundance of the unionid community may alter the function and services that these communities perform.

**Status of Unionids in North America**

Among the most imperiled groups of animals in North America are the freshwater pearly mussels (Strayer et al., 2004). Almost 300 species of freshwater mussels have been described as endemic to North America, representing the greatest diversity in the world. Of these, 13% are listed as extinct and 66% of the remaining species are ranked as vulnerable, imperiled or critically imperiled (Master et al., 2000). Freshwater mussels can be among the longest living invertebrates, with life spans ranging from 4 to 190 years depending on the species and population (Haag and Rypel, 2011). Therefore, unionid species that are present, or even abundant, may have population age structures that reveal
that the impediments of the past are having deleterious impacts that have not yet been
detected. More information regarding population age structures from current surveys will
be required to continue to assess and update the status of unionids in North America and
for their effective conservation (Haag and Rypel, 2011).

**Reasons for Unionid Imperilment**

Unionids have been harvested in North America for centuries. Historically, they
were used by humans for producing tools and as a supplemental food source, evidenced
by mounds of shells found near villages in archeological digs (Parmalee and Bogan,
1998; Anthony and Downing, 2001). However, it was when their economic benefits
were realized that mass commercial exploitation of mussels began in North America.
Between the freshwater pearl industry from the mid- to late- 1800s and the pearl button
industry, from 1890 to 1960, freshwater mussels were the source of substantial
economies in the Midwest (Anthony and Downing, 2001). The industrial drive to harvest
large quantities of shells led to substantial declines in mussel populations in many of the
rivers and streams of the United States (Anthony and Downing, 2001). Huge mussel
harvests were recorded. For example, over 9,000 tons of mussel shells were harvested
from an area less than 0.75 km² between 1894 and 1897, representing a catch of over 100
million mussels (Smith, 1898). However, it was the advent of the plastic button (more
than decimated mussel beds) that led to the end of the pearl button industry (Claassen,
1994; Neves, 1999). Currently, and since the 1960s, native mussels are harvested
through a regulated fishery for the culture of freshwater pearls, where each cultured
freshwater pearl is started with a piece of a unionid mussel shell.
Human mediated land and water use continue to impact unionid populations. Siltation from dam construction, high-water weather events, and agriculture lead to alterations in unionid species; from those typically associated with gravel and stone areas to species that can tolerate soft, silt sediments, such as the anodontines (subfamily Anodontinae) (Parmalee and Hughes, 1993; Blalock and Sickel, 1996; Metcalfe-Smith et al., 1998; Box and Mossa, 1999). Many of the extinct and imperiled unionid species were from the genus *Epioblasma*, which require large river and riffle habitat (Watters and Flaute, 2010). The decline of water quality due to pollutants from urban and agricultural sources has been correlated with declining unionid populations (Williams et al. 1993; Neves et al. 1997; Metcalfe-Smith et al., 1998). The construction of dams and impoundments impact unionids by altering the flow dynamics of rivers and streams, the formation of artificial lakes and pools, and altered temperature profiles (Watters and Flaute, 2010). The dams also create physical barriers that can further isolate unionid populations by inhibiting migration of their fish hosts that are critical for propagation and dispersal (Branson, 1974; Collier et al., 1996).

Another group of bivalves is threatening native mussel populations - the invasive exotic species, zebra and quagga mussels (*Dreissena polymorpha* and *D. rostriformis bugensis*) (Ricciardi et al., 1996; 1998; Strayer et al., 1999). In areas infested by zebra and quagga mussels, hereafter dreissenids, significant declines in unionid abundance and diversity have been observed (Gillis and Mackie, 1994; Nalepa et al., 1996; Ricciardi et al., 1996; Schloesser et al., 1996; Schloesser and Masteller, 1999). Dreissenid populations at densities of 4,000-20,000/m² were associated with 90-100% unionid mortality in the St. Lawrence River (Ricciardi et al., 1996) and fouling as low as ten.
dreissenids per unionid can result in unionid population declines (Ricciardi et al., 1996). Strayer and Smith (1996) observed declines in unionid populations in the Hudson River, NY, but the unionids had only low levels of dreissenids attached (less than ten), suggesting that the unionids succumbed to indirect impacts of dreissenids rather than a direct result of fouling. Direct and indirect impacts from dreissenid mussels can result in significant reductions in unionids. Therefore, watershed-wide efforts will be required to sustain ecosystems and unionid populations while unionid population restoration and conservation efforts continue.

**Unionid Recovery Efforts**

Mussel recovery efforts have been employed in many states to restore the quantities and diversity of unionids in North America. In an output received from the Nature Serve database on 09/20/2011 (www.natureserve.org), 333 unionid species and subspecies were listed. Among these listed species, 21% of the descriptions indicated that Recovery Plans had been drafted. Recovery Plans, usually drafted by scientists with the United States Fish and Wildlife Service (USFWS) in association with other agencies and institutions, describe the species’ conservation status, historic and current threats, historic and current distribution, and actions that may be required for the protection, conservation, and/or recovery of the species. Of the Recovery Plans drafted for unionid species, 43.5% mentioned that dreissenids either are or could be a concern for the species’ conservation. Of the Recovery Plans drafted after 1986, when zebra mussels were first reported in North America, 58% mentioned dreissenids as a current or potential threat. Dreissenids have spread across North America and they threaten to colonize water
bodies throughout the continent (Figure 4). Preventing the spread of dreissenids to new areas is the most effective approach to protect unionid populations from their devastating impacts, but for areas where dreissenids have already colonized, control strategies could help sustain unionid populations.

Propagation methods for unionid mussels were first developed in response to desires from the pearl button industry for a sustainable mussel fishery (Pritchard, 2005). The U.S. Bureau of Fisheries Biological Station (Fairport, IA) was created around 1910 to rear mussels to reintroduce into the rivers in order to sustain higher mussel harvests (Pritchard, 2005). The success of reintroductions was limited, however, by degraded habitat from high levels of pollution and siltation (Pritchard, 2005). The Endangered Species Act (1973) introduced the first substantial means to protect and recover unionids and their ecosystems in the United States (Neves, 2004). The National Native Mussel Conservation Committee (1998) published a conservation strategy to conserve and maintain a sustainable level of unionid populations through management and conservation of populations, public awareness, governmental support, and protection and rehabilitation of habitat.

The complexity of the unionid life cycle introduces many challenges to their conservation and propagation. During reproduction, fertilization of the eggs occurs in the female mussel’s marsupia (modified gills) by sperm released by males. The eggs develop into larvae called glochidia. The glochidia typically require attachment to a vertebrate host, usually a fish, for development. Thus, unionid species are closely connected and dependent on the presence of populations of fish species. This parasitic interaction offers an evolutionary advantage of dispersion for a relatively sessile animal that typically lives
in flowing rivers and streams where free-swimming larvae would otherwise be washed downstream. In contrast, dreissenids, having evolved in lentic systems, have a free-swimming planktonic larval stage, called veligers. Dreissenid populations typically do not sustain reproducing populations in small flowing streams unless there is a source reservoir population, such as a pond or lake, which serves as a source of larvae and juveniles for settlement (Kern et al., 1994; Stoeckel et al., 1997; Lucy et al., 2008). The coexistence of unionids in beds comprised of multiple species depends on the availability of a host fish resource of multiple fish species (Rashleigh and DeAngelis, 2007). This adds another level of complexity to the management of unionids since fish species diversity needs to be incorporated in habitat conservation management plans.

Some unionid species are extremely host specific and can use only one or two species of fish successfully; other unionid species are generalists and can use many species of fish as hosts (Strayer, 2008). Various species have evolved elaborate means to attract fish to help ensure that the glochidia are released in close proximity to the gills or fins of the fish for successful attachment (Zanatta and Murphy, 2006). Studies have shown that the most imperiled unionid species rely on fish hosts that have the shortest movement distances (Schwalb et al., 2011). The conservation and management of unionid populations depends on abundant and diverse populations of fish species to accommodate the diverse requirements of multiple species of unionids in our lakes and rivers. Unionid conservation also depends on the free movement of fish within the waterbody. If accessibility to their fish hosts is limited by dams, for example, unionid reproduction may be hindered. More research is needed to identify which species of fish are the most successful host for each species (Barnhart et al., 2008).
Development of a Biopesticide against Dreissenid Mussels

As described in the previous two chapters, a *Pseudomonas fluorescens* CL145A-based biopesticide (*Pf*-CL145A) has been developed that appears to hold promise for the safe and effective control of dreissenids. While most of the research and development of *Pf*-CL145A occurred as treatments within industrial systems such as power plant pipes (i.e., hydropower and/or coal-fired) and little research has been conducted to evaluate its potential for controlling dreissenids in open waters, there has been increasing interest among federal, state, and private entities to develop uses for *Pf*-CL145A in open-water systems.

A concern in applying *Pf*-CL145A directly to freshwater ecosystems is the product’s safety to other freshwater organisms. Since applications of the product to industrial systems typically involve treatment of only a fraction of the total water passing through the plant, the product is diluted before it enters the effluent and subsequently into the surrounding water body from the end of a pipe. Applications directly to dreissenids in lake or river beds, conceivably has more potential to come into direct contact at higher concentrations with non-target freshwater organisms. As discussed in detail in Chapter 1, during development of *Pf*-CL145A a series of non-target laboratory and mesocosm trials were conducted to assess the specificity of *Pf*-CL145A to dreissenids. These acute toxicity trials included a variety of aquatic organisms, including a freshwater ciliate (*Colpidium colpoda*), zooplankton (*Daphnia magna*), freshwater amphipod (*Hyalella azteca*), bluegill sunfish (*Lepomis macrochirus*), fathead minnows (*Pimephales promelas*), brown trout (*Salmo trutta*), blue mussels (*Mytilus edulis*), and adult unionids (*Elliptio complanata, Lampsilis radiata, Lasmigona compressa, Pyganodon grandis,*
*Pyganodon cataracta, and Strophitus undulatus*). The only organism that demonstrated sensitivity to *Pf-CL145A* in these trials was the freshwater amphipod, *H. azteca*, and significant sensitivity was only observed at the highest concentration (200 ppm for 48 hr). All other organisms demonstrated no significant mortality at concentrations that demonstrated efficacy to dreissenids (i.e., mortality observed in treated replicates was not significantly different than mortality observed in untreated controls) (see Chapter 1). Additional acute toxicology studies were contracted by MBI to assess potential human health risks as required by the USEPA for product registration. Tests with living cells (Technical Grade Active Ingredient) on skin and in feeding (oral) applications, and inhalation, indicated no effect on treated rats (MBI, personal communication). The oral LD50 was >5,000 mg/Kg, which is the lowest risk category for pesticide products. Mammalian studies also demonstrate that the bacteria are non-pathogenic to rats. These mammalian toxicity results earned *Pf-CL145A* an Exemption from the Requirement of a Tolerance in a rule published by the USEPA in August 2011, the highest level of mammalian safety by the USEPA.

Another concern in applying a cell-based product, such as *Pf-CL145A*, is the addition of large amounts of organic matter to open-water systems. The degradation of large amounts of organic matter could cause declines in dissolved oxygen and increased levels of ammonia, which can be highly toxic to aquatic organisms, and to unionids in particular (Augspurger et al., 2003; Newton et al., 2003; Newton and Bartsch, 2007). Application methods for *Pf-CL145A* should be designed to help reduce these indirect impacts and are discussed in the following sections.
**Pf-CL145A as an Aid in Unionid Recovery Efforts**

**Collaboration with regional recovery teams**

A dreissenid control strategy could aid unionid conservation efforts in the preservation of extant unionid populations in their existing habitat and in propagation efforts for the reestablishment and recovery of imperiled populations. *Pf-CL145A* has potential to be used in these unionid management and conservation efforts through dreissenid control, but coordination and collaboration with local agencies will be essential for its success. Local unionid recovery teams, including state and federal resource managers, are in the field at the local level and generally have the most current information on the status of individual species and the locations of current populations. They can also offer valuable guidance pertaining to where dreissenid management would be most advantageous to their unionid recovery efforts. Representing the stakeholders that will be assessing any potential product or method, their advisement and concerns should be addressed and incorporated into methods proposed for dreissenid management.

**Susceptibility of regional non-target organisms**

An area of concern expressed by members of unionid recovery teams when considering any management tool is product safety. While initial acute toxicity trials demonstrate specificity of *Pf-CL145A* to dreissenids, the tests are not sufficiently representative to allow prospective users to assess the risks of applying the product to open waters for unionid restoration efforts. Since fish and unionid species can vary between regions, each regional conservation strategy would likely include recommendations for additional acute toxicity trials against certain species prior to the
implementation of a program that integrates the formulated Pf-CL145A product into management practices. Additional taxa will likely be recommended for evaluation of potential sensitivity; these could include other benthic macroinvertebrates such as snails, crayfish, and insects (i.e., larvae of dragonflies, mayflies, stoneflies, etc.). While planning a project in the Midwest to evaluate the Pf-CL145A product for use in the recovery of unionid species in the Upper Mississippi River, mussel recovery teams were consulted and a list of species were compiled that were of interest for further aquatic non-target testing. The recommended species include additional fish and unionid species, including three life stages of each species of unionid (Tables 19 and 21). Freshwater mussel and fisheries scientists at the U.S. Fish and Wildlife Service Genoa National Fish Hatchery (Genoa, WI) and the U.S. Geological Survey Upper Midwest Environmental Science Center (La Crosse, WI), are involved in unionid recovery efforts in the Mississippi River, and in the planning and execution of the Pf-CL145A project. Collaborating with regional scientific institutions is important since they already have established contacts, relationships, and credibility with local authorities and the public that could take years to develop as an incoming institution or commercial company. The non-target tests are currently in progress and the results will not be reported here, but the approach of gaining feedback from ecologists and recovery teams in the region was essential for building a useful data set to accommodate their conservation concerns.

The species and life stages of organisms that were requested for further non-target testing with the Pf-CL145A product were consistent with recovery and restoration efforts in the Upper Mississippi River. It was important to select species across various families or subfamilies to obtain a breadth of susceptibility information; the recommended fish
and unionid species are listed in Tables 19 and 21, respectively. The unionid species that had previously been screened by the NYSM were collected from areas within upstate New York (Table 20), and represent species that are common in the Northeast. Recovery teams recommended a list of representative unionid species from the Midwest regions, particularly the Upper Mississippi River, for susceptibility to the *Pf*-CL145A product (Table 21). In this instance, the recovery teams advised that it would be valuable to test the susceptibility of the product to three life stages - glochidia, juveniles (less than 48 hr since dropping from the gills of host fish), and sub-adults (survived one over-wintering period). Since previous tests were conducted on adult unionids only, the proposed tests significantly expand the breadth of information available to unionid conservation teams as the first trials against early life stages of unionid mussels. The information gained will be used by regional unionid recovery teams to guide decisions on whether, when, and how to apply the *Pf*-CL145A product for dreissenid management.

*Prevent the spread of dreissenids into new waterbodies*

The transfer of water from dreissenid-infested waterbodies can be the source of dreissenid introductions to uninfested waters. Water containing planktonic dreissenid larvae can inadvertently be transferred to new water bodies by recreational users in live wells and bait buckets (Johnson et al., 2001). Although many fish hatchery managers treat transfer water with salt solutions to kill dreissenid larvae (Edwards et al., 2002), it would be beneficial to have an additional method, such as *Pf*-CL145A, to eliminate these larvae from the water without harming the organisms being transferred (e.g. fish or unionids). The prevention of new introductions is the only approach that can prevent the
ecosystem-level alterations caused by dreissenids that impact unionid populations indirectly through habitat alteration and nutrient limitation. Therefore, prevention of the spread of dreissenids to new waterbodies is a high priority.

Testing at the NYSM has provided evidence that Pf-CL145A is highly lethal to dreissenid larvae. Trials were designed with the objective to develop methods to achieve 100% lethality to larvae in transport water systems. Field-collected dreissenid larvae were exposed to 200 ppm Pf-CL145A freeze-dried formulation for 6 hours, transferred to fresh, untreated water, and observed for mortality over a span of two to three days. Larval death was evaluated by observing specimens under magnification with dissecting microscopes. Dead larvae were identified as those displaying no ciliary movement and gaping of shells. While larvae were not dead immediately following the 6 hour treatment, they were dead after an additional ca. 42 hours, and 100% lethality was observed in each test (Table 22). The same treatment concentration, 200 ppm, rarely kills 100% of adult mussels (Table 1). The ability to consistently achieve 100% lethality to large numbers of dreissenid larvae (ca. 4,000 treated larvae observed in each test) provides some evidence that the adult dreissenids that survive following exposure are not likely genetically resistant, but are not susceptible for some other reason(s), possibly from simply not feeding at the time of exposure. Thus, the progeny of the surviving adult dreissenids will likely be susceptible to the toxic effects of a Pf-CL145A product.

Application methods for use of Pf-CL145A to help prevent the spread of dreissenid larvae in transport water will need to be modified to reduce the time until 100% mortality is observed to the larvae (currently two days). These transport water treatment methods will be developed, using the commercial product formulations of Pf-
CL145A, and these protocols will be shared with hatchery personnel and recreational users, where appropriate. For effective control, veliger treatment methods would occur during the full spawning season for dreissenids when veligers can be present. For zebra mussels, spawning begins when temperatures reach 12°C (Neumann et al., 1993), and quagga mussels begin at much cooler temperatures, as low as 4.8°C (Roe and MacIsaac, 1997). Spawning can continue throughout the summer and is dictated by the local climate conditions. Recommendations could be posted at boat launches for recreational users to know which months support the highest risk for veliger transport.

*Dreissenid management in unionid propagation and recovery*

The feasibility for controlling dreissenids in natural aquatic ecosystems is extremely limited, even in a relatively small lake or river. Given the rates of dreissenid population growth, leaving just a few dreissenids in the water body is theoretically sufficient for re-establishment of a population. A successful method for sustainable, waterbody-wide control of dreissenids would require levels of dreissenid mortality or removal approaching 100%. The expense and logistics of treating an entire waterbody to manage dreissenids with Pf-CL145A is economically and ecologically prohibitive. The actual commercial cost of the Pf-CL145A product is not currently available, but since treatments of adult mussels do not eradicate the populations, and would therefore need to be recurring, at least every year or two, extensive waterbody-wide Pf-CL145A applications are not a likely investment. Therefore, cost savings will need to be realized by identifying small-scale treatments of high value, such as industrial pipe systems and small, localized, open-water areas.
From an ecological standpoint, the addition of large amounts of organic matter associated with Pf-CL145A applications to an entire waterbody could also be detrimental. Current effective treatment concentrations are approximately 50 ppm Pf-CL145A for six hours to remove 70-90% of adult dreissenids (author’s unpublished data; Molloy et al., in prep.). If applied to an entire waterbody, without the opportunity for dilution, the degradation of the protein-rich organic matter could lead to indirect impacts such as declines in dissolved oxygen and increases in levels of total nitrogen as ammonia, both of which could severely impact aquatic organisms. These impacts could be limited by applying Pf-CL145A to small, localized areas within a given waterbody, avoiding high concentration treatments, and employing treatment methods to place the treatment near the benthos, rather than throughout the water column, to target adult dreissenids. Defining effective treatment concentrations and durations to achieve the desired level of dreissenid management for each situation will be essential. These precautionary measures are realistic in terms of designing applications to aid in native mussel restoration.

We are developing methods for applying Pf-CL145A to defined areas within a lake or river to achieve maximum benefit in the ecosystem to aid in the conservation and recovery of unionid mussels. There are essentially two areas in unionid recovery where a Pf-CL145A product has potential to make a significant contribution: during unionid propagation and for the preservation of existing populations in mussel beds. It may not be feasible to treat an entire unionid bed with Pf-CL145A if the area is large. For example, the Whiskey Rock Essential Habitat Area in the Upper Mississippi River is 742,500 m² in area and supports an estimated 74,000 to 97,000 Lampsilis higginsii (U.S.
Unionid recovery teams may determine that treatment of a portion of the area could help achieve their unionid management goals. Either situation, treating propagation cages or unionid beds, could be designed to require \textit{Pf}-CL145A applications to a relatively small area within a given waterbody, perhaps 100 to 10,000 m\textsuperscript{2}, respectively.

Restoration efforts have focused on removing attached dreissenids from unionid shells, relocation, and propagation. Unionid species that have recovery plans in place will typically have regional support from state and federal agencies as well as more biological data available from which to evaluate ways to incorporate dreissenid management practices. In some cases, manual removal of dreissenids from cages may be adequate, particularly if the cage is scheduled for removal or maintenance, while in other areas, use of \textit{Pf}-CL145A to clean cages and sub-adult mussels could be beneficial.

The conservation of the Higgins Eye mussel (\textit{Lampsilis higginsii}) in the Mississippi River provides a good example of the impact of dreissenids on recovery efforts (U.S. Fish and Wildlife Service, 2004). Ten Essential Habitat Areas (EHAs), areas identified to be critical for the conservation of the species, have been identified for Higgins Eye: six in the Mississippi River, one in the Wisconsin River, and three in the St. Croix River. Of the ten EHAs, only one is completely free of dreissenids, Interstate Park-St. Croix River (U.S. Fish and Wildlife Service, 2004). The EHA that supported the strongest population of Higgins Eye prior to invasion by dreissenids, Prairie du Chien-Mississippi River, was reported to have nearly 10,000 zebra mussels/m\textsuperscript{2} in 2000 (Miller and Payne, 2001). Recovery teams have focused on relocating Higgins Eye from infested areas to areas where zebra mussels are not an imminent threat. Adult unionids are
manually collected and cleaned of dreissenids in EHAs and programs of propagation and release have been engaged.

A dreissenid management program could aid recovery efforts for endangered unionids such as the Higgins Eye mussel. Applications of the Pf-CL145A product to existing beds or portions of beds in EHAs, depending on the desired area for dreissenid management, or other areas where the species are known to occur could reduce fouling to a level that is not an imminent direct threat. These efforts could help protect the populations that are the source of adult mussels for propagation, thereby helping to protect the genetic diversity of the species. Applications of the Pf-CL145A product could be employed in an as-needed or seasonal basis, when zebra mussel populations, assessed through existing monitoring programs, are identified as being at levels that could harm existing native mussels. Timing for treating mussel beds could be once per year, after dreissenid settlement has occurred, to reduce impacts from fouling over the winter months when unionids may be utilizing their energy reserve and be the most susceptible impact from fouling by dreissenids. Information from acute toxicity trials to unionid life stages will allow managers to make assessments on the seasonal timing of Pf-CL145A product treatments to present the lowest risk to sensitive life stages, should life stages demonstrate different levels of susceptibility. Programs might use the product annually, or less often, based on the current need.

In the case of Higgins Eye, there appears to be sufficient genetic diversity within populations so that there is not a significant genetic difference between populations (Bowen, 2004). Protecting existing populations will help ensure genetic diversity in propagated mussels as long as sufficient numbers of females are used for the source of
glochidia for each new population. Bowen (2004) recommended that a minimum of 100 females be used as the source for each new Higgins Eye population. Propagation cages holding thousands of juvenile unionids can become encrusted with dreissenids and reduce water circulation (and food for unionids) within the structure (Figure 9) and dreissenid mussels can attach to the juvenile unionids themselves (Figure 10). A control product, such as *Pf*-CL145A, could reduce fouling by dreissenids on propagation cages and mussels within the cages. To maximize the value of a dreissenid control program using a product such as *Pf*-CL145A, the treatments would be coordinated and integrated with current and continued efforts of manual cleaning and propagation to help ensure conservation success.

**Conclusions**

The imperilment of North America’s unionoid species should be an indicator that something is vastly wrong with our freshwater systems. While restoration and conservation efforts are critical, the need for continued and improved rehabilitation and prevention of further ecosystem destruction are the most sustainable paths to recovery. Without propagation, restoration, and conservation efforts directed toward unionids, more species will likely see their populations decline by an order of magnitude (Strayer and Malcolm, 2007) or be another in the list of those lost to extinction (Wilcove and Master, 2005). As a safe and effective dreissenid management tool, a *Pf*-CL145A-based product could aid in the recovery and reestablishment of unionid populations in limited-use situations that do not involve whole waterbody applications. Any possible aid to the
recovery of unionids is worth consideration, as their presence and diversity in freshwater ecosystems are critical to the processing of North America’s freshwater resources.

References


Gatenby, C.M., Neves, R.J., and Parker, B.C. 1996. Influence of sediment and algal food


Table 19. Fish species selected for acute toxicity testing.

<table>
<thead>
<tr>
<th>Family</th>
<th>Common name (species)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percidae</td>
<td>Walleye (<em>Sander vitreus</em>)</td>
</tr>
<tr>
<td>Percidae</td>
<td>Yellow perch (<em>Perca flavescens</em>)</td>
</tr>
<tr>
<td>Centrarchidae</td>
<td>Largemouth bass (<em>Micropterus salmoides</em>)</td>
</tr>
<tr>
<td>Centrarchidae</td>
<td>Smallmouth bass (<em>Micropterus dolomieu</em>)</td>
</tr>
<tr>
<td>Centrarchidae</td>
<td>Bluegill sunfish (<em>Lepomis macrochirus</em>)</td>
</tr>
<tr>
<td>Salmonidae</td>
<td>Rainbow trout (<em>Oncorhynchus mykiss</em>)</td>
</tr>
<tr>
<td>Salmonidae</td>
<td>Coaster brook trout (<em>Salvelinus fontinalis</em>)</td>
</tr>
<tr>
<td>Acipenseridae</td>
<td>Lake sturgeon (<em>Acipenser fulvescens</em>)</td>
</tr>
<tr>
<td>Ictaluridae</td>
<td>Channel catfish (<em>Ictalurus punctatus</em>)</td>
</tr>
<tr>
<td>Cyprinidae</td>
<td>Fathead minnow (<em>Pimephales promelas</em>)</td>
</tr>
</tbody>
</table>

Table 20: Fish and unionid species previously tested in acute toxicity trials of *Pf*-CL145A.

<table>
<thead>
<tr>
<th>Fish Family</th>
<th>Common name (species)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrarchidae</td>
<td>Bluegill sunfish (<em>Lepomis macrochirus</em>)</td>
</tr>
<tr>
<td>Cyprinidae</td>
<td>Fathead minnow (<em>Pimephales promelas</em>)</td>
</tr>
<tr>
<td>Salmonidae</td>
<td>Brown trout (<em>Salmo trutta</em>)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Unionid Subfamily</th>
<th>Common name (species)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambleminae</td>
<td>Eastern elliptio (<em>Elliptio complanata</em>)</td>
</tr>
<tr>
<td>Lampsilinae</td>
<td>Eastern lampmussel (<em>Lampsilis radiata</em>)</td>
</tr>
<tr>
<td></td>
<td>Creek heelsplitter (<em>Lasmigona compressa</em>)</td>
</tr>
<tr>
<td></td>
<td>Giant floater (<em>Pyganodon grandis</em>)</td>
</tr>
<tr>
<td>Anodontinae</td>
<td>Creeper (<em>Strophitus undulatus</em>)</td>
</tr>
<tr>
<td></td>
<td>Eastern floater (<em>Pyganodon cataracta</em>)</td>
</tr>
</tbody>
</table>
Table 21: Unionid species selected for acute toxicity testing and current protection status.

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Common name (species)</th>
<th>Federal and/or State protection status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambleminae</td>
<td>Washboard (<em>Megalonaias nervosa</em>)</td>
<td>Threatened: MN; Special Concern: WI</td>
</tr>
<tr>
<td>Lampsilinae</td>
<td>Higgins Eye (<em>Lampsilis higginsii</em>)</td>
<td>Endangered: Federal, IL, IA, MN, MO, WI</td>
</tr>
<tr>
<td>Lampsilinae</td>
<td>Plain pocketbook (<em>Lampsilis cardium</em>)</td>
<td>None</td>
</tr>
<tr>
<td>Lampsilinae</td>
<td>Fatmucket (<em>Lampsilis siliquoidea</em>)</td>
<td>None</td>
</tr>
<tr>
<td>Lampsilinae</td>
<td>Hickorynut (<em>Obovaria olivaria</em>)</td>
<td>Special Concern: MN, MO</td>
</tr>
<tr>
<td>Lampsilinae</td>
<td>Mucket (<em>Actinonaias ligamentina</em>)</td>
<td>Threatened: MN</td>
</tr>
<tr>
<td>Lampsilinae</td>
<td>Black sandshell (<em>Ligumia recta</em>)</td>
<td>Threatened: IL; Special Concern: MN, MO</td>
</tr>
</tbody>
</table>
Table 22: Mortality of dreissenid larvae exposed for 6 hr to 200 ppm Pf-CL145A freeze-dried powder formulation (FDP) in 3-L and 15-L testing volumes (two tests in each volume).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean ± SD% 48 hr mortality (total number of veligers observed)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3-L Testing Volume:</strong></td>
<td></td>
</tr>
<tr>
<td>Untreated Controls</td>
<td>4.3±1.5% (n = 193)</td>
</tr>
<tr>
<td></td>
<td>9.6±2.7% (n = 245)</td>
</tr>
<tr>
<td>Pf-CL145A FDP</td>
<td>100.0±0.0% (n = 4165)</td>
</tr>
<tr>
<td></td>
<td>100.0±0.0% (n = 4364)</td>
</tr>
<tr>
<td><strong>15-L Testing Volume:</strong></td>
<td></td>
</tr>
<tr>
<td>Untreated Controls</td>
<td>0.5±0.9% (n = 184)</td>
</tr>
<tr>
<td></td>
<td>4.7±2.8% (n = 229)</td>
</tr>
<tr>
<td>Pf-CL145A FDP</td>
<td>100.0±0.0% (n = 3978)</td>
</tr>
<tr>
<td></td>
<td>100.0±0.0% (n = 3671)</td>
</tr>
</tbody>
</table>
Figure 9: Propagation cage encrusted with zebra mussels. Photo courtesy of USFWS-Genoa National Fish Hatchery.

Figure 10: Native unionid mussel encrusted with zebra mussels. Photo courtesy of USFWS-Genoa National Fish Hatchery.