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Research Article

Non-target trials with *Pseudomonas fluorescens* strain CL145A, a lethal control agent of dreissenid mussels (Bivalvia: Dreissenidae)

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Abstract

In an effort to develop an efficacious and environmentally safe method for managing zebra mussels (*Dreissena polymorpha*) and quagga mussels (*Dreissena rostriformis bugensis*), we initiated a research project investigating the potential use of bacteria and their natural metabolic products as biocontrol agents. This project resulted in the discovery of an environmental isolate lethal to dreissenid mussels, *Pseudomonas fluorescens* strain CL145A (*Pf*-CL145A). In previous published reports we have demonstrated that: 1) *Pf*-CL145A's mode of action is intoxication (not infection); 2) natural product within ingested bacterial cells lyse digestive tract epithelial cells leading to dreissenid death; and 3) high dreissenid kill rates (>90%) are achievable following treatment with *Pf*-CL145A cells, irrespective of whether the bacterial cells are dead or alive. Investigating the environmental safety of *Pf*-CL145A was also a key element in our research efforts, and herein, we report the results of non-target trials demonstrating *Pf*-CL145A's high specificity to dreissenids. These acute toxicity trials were typically single-dose, short-term (24-72 h) exposures to *Pf*-CL145A cells under aerated conditions at concentrations highly lethal to dreissenids (100 or 200 mg/L). These trials produced no evidence of mortality among the ciliate *Colpidium colpoda*, the cladoceran *Daphnia magna,* 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*). Low mortality (3-27%) was recorded in the amphipod *Hyalella azteca,* but additional trials suggested that most, if not all, of the mortality could be attributed to some other unidentified factor (e.g., possibly particle load or a water quality issue) rather than *Pf*-CL145A's dreissenid-killing natural product. In terms of potential environmental safety, the results of these invertebrate and vertebrate non-target trials are encouraging, but it would be unrealistic to think that dreissenids are the only aquatic organisms sensitive to *Pf*-CL145A's dreissenid-killing natural product. Additional testing is needed to better define *Pf*-CL145A's margin of safety by identifying the sensitivity of other susceptible organisms. The results of these non-target safety trials – in combination with equally promising mussel control efficacy data – have now led to *Pf*-CL145A's commercialization under the product name Zequanox®, with dead cells as the product's active ingredient. The commercial availability of only dead-cell Zequanox formulations will eliminate the risk of any possible non-target infection by *Pf*-CL145A, further reducing environmental concerns. During the non-target project reported herein, the limited quantities of *Pf*-CL145A cells that we were able to culture severely restricted the number and size of our trials. In contrast, the availability of Zequanox will now greatly expand the opportunities for non-target testing. The trials reported herein – exposing non-target organisms under aerated conditions to unformulated, laboratory-cultured cells – clearly point to *Pf*-CL145A's potential for high host specificity, but non-target trials with Zequanox – using *Pf*-CL145A cells cultured, killed, and formulated using industrial-scale protocols – will be even more important as they will define the non-target safety limits of the actual commercial products under a wide range of environmental conditions.

Key words: zebra mussel; quagga mussel; *Dreissena polymorpha*; *Dreissena rostriformis bugensis*; biocontrol; Zequanox®

Introduction

Due to their severe economic (O'Neill 2008) and environmental (Karatayev et al. 2002) impacts, zebra mussels (*Dreissena polymorpha* (Pallas, 1771)) and quagga mussels (*Dreissena rostriformis bugensis* (Andrusov, 1897)) are widely regarded as the "poster children" of freshwater invasive species. Unfortunately there is still no practical method available for their control in open waters, such as lakes and rivers. Within infested infrastructures, chlorination is currently the most commonly used chemical control method, but due to environmental concerns, it is widely regarded as an unacceptable long-term solution. Chlorine's potential to cause lethal and sublethal impacts on non-target organisms and its formation of carcinogenic substances, such as trihalomethanes, are some of the primary environmental issues of concern (Mackie and Claudi 2009).

In an effort to develop an effective and environmentally safe alternative for managing dreissenid infestations, we initiated an investigation of the potential use of bacteria and their natural metabolic products as biocontrol agents (Molloy 1991). Screening trials evaluating the activity of environmental bacterial isolates resulted in the discovery of a strain of *Pseudomonas fluorescens* (Trevisan) Migula, 1895 that was lethal to dreissenids (Molloy 1998, 2001). Designated as *P. fluorescens* strain CL145A (*Pf*-CL145A), our research (Molloy et al. 2013a,b) has demonstrated that: (1) the mode of action in killing dreissenids is intoxication (not infection), with dead bacterial cells equally as lethal as live cells; (2) mussel intoxication is the result of a natural metabolic product associated with the bacterium's cell wall; (3) death from intoxication is accompanied by the selective destruction of the mussel's digestive tract epithelium; (4) the strain's intoxicating natural product is a heat-labile secondary metabolite whose toxicity significantly degrades within 24 h when applied to water; (5) *Pf*-CL145A appears to be an unusual strain since it was the only one among ten *P. fluorescens* strains evaluated to cause high dreissenid mortality; and (6) pipe trials conducted under once-through conditions indicated *Pf*-CL145A cells are efficacious against both zebra and quagga mussels, with high mortalities achievable against both species. Investigating the environmental safety of *Pf*-CL145A was also a key element in our research efforts, and herein, we report the results of non-target trials with aquatic organisms indicating that *Pf*-CL145A cells appear to have a very high level of specificity to dreissenids.

Methods

Unless otherwise indicated, all trials were conducted at the New York State Museum Field Research Laboratory (NYSMFRL) in Cambridge, New York – a former state fish hatchery that receives a continuous flow of water directly from a stream-fed pond on the property. Trials included a wide variety of non-target aquatic organisms, including ciliates, daphnids, amphipods, fish, and bivalves (Table 1). Species were selected in response to requests by funding agencies or in anticipation of requirements for United States Environmental Protection Agency product registration. As outlined below, these trials were conducted using a variety of methodologies, many of which evolved as a result of improvements and refinements during this decade-long research and development effort. Initial trials used live *Pf*-CL145A cells, but following the discovery in the late 1990s that dead cells killed equally as well as live cells (Molloy 2001; Molloy et al. 2013b), we switched to exclusively using dead cells in subsequent trials.

Production of Pf-CL145A cells

Pf-CL145A was cultured at the NYSMFRL in static flasks following Molloy (2001). Cell pellets were harvested from final whole cultures by centrifugation and resuspended in dilution water (80 ppm KH_2PO_4, 405 ppm $MgCl_2 \bullet 6H_2O$ in deionized water that was pH-adjusted to 7.2 with NaOH). To produce larger quantities of *Pf*-CL145A, cells were cultured 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. To produce dead *Pf*-CL145A cells, ionizing radiation (gamma or electron beam) was used to kill the cells (Molloy et al. 2013b).

Confirmation of the activity of the Pf-CL145A cells used to treat non-targets

As a quality control to confirm the high dreissenid-killing activity of the *Pf*-CL145A cells used in these non-target trials, assays against zebra and/or quagga mussels were conducted concurrently in separate testing chambers. Mussels (length, 5-15 mm) were field-collected from local water bodies, including the Mohawk River (Crescent, New York), Lake Ontario (Rochester, New York), and Hedges Lake (Cambridge, New York), transported to the laboratory in coolers, and maintained in aerated, unchlorinated tap water in aquaria with filtration (Tetra Whisper® filters) at 20-23ºC. One day prior to each assay, a defined number of mussels, typically 25-100, were placed in 1-L glass testing jars containing oxygen – saturated , hard water (192 ppm $NaHCO_3$, 120 ppm $CaSO_4{\bullet}2H_2O$, 120 ppm $MgSO_4$, 8 ppm KCl; United States Environmental Protection Agency 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 gentle aeration initiated. Typically there were 2-3 jars treated and one untreated control. *Pf*-CL145A cells were applied for either 24 or 48 h at 100-200 ppm (dry weight cells per unit water volume, e.g., 1 ppm = 1 mg/L). At the end of the exposure period, treated water was poured off, and mussels were rinsed and placed in clean plastic dishes containing fresh, oxygen-saturated, hard water. Dead mussels were identified as those having gaping shells that did not respond to gentle touch. Assays were conducted at 20-23ºC, with mortality assessed and water changed daily for an additional 8-9 days (to achieve a total 10-day test period).

Ciliate – *<u>Colpidium colpoda</u>* (Ehrenberg, 1838)

Colpidium colpoda, a species commonly used in toxicity tests (Modoni 2000; Rogerson et al. 1983), was collected from a stream seepage area on the NYSMFRL property and cultured on rice seeds in stream seepage water at 10-17°C in glass Petri dishes. Trials were conducted in six glass embryo dishes (30 × 30 mm) each containing one ciliate in 0.5 ml stream water at 23(±1)°C. Immediately prior to treatment, almost all water was removed and replaced with stream water containing 100 ppm of live *Pf*-CL145A cells. Ciliates were exposed for three days, with mortality checked daily. An identical set of six embryo dishes served as untreated controls.

Cladoceran – <u>Daphnia magna</u> Straus, 1820

Daphnia magna were obtained from the New York State Department of Environmental Conservation (NYSDEC) Bureau of Environmental Protection (Avon, New York). Colony maintenance and testing followed United States Environmental Protection Agency (2002) guidelines for aquatic effluent acute toxicity testing. Daphnids were cultured in polystyrene cups containing 20 ml of oxygen-saturated, moderately-hard water (96 ppm $NaHCO_3$, 63 ppm $CaSO_4{\bullet}2H_2O$, 66 ppm $CaCl_2{\bullet}2H_2O$, 30 ppm $MgSO_4$, 4 ppm KCl; United States Environmental Protection Agency 2002). The colony was maintained at 22(±1)°C on a 16/8-h light/dark cycle at a light intensity of 525-1075 lux. Daphnids were transferred daily to cups containing fresh, moderately-hard water and fed the alga *Selenastrum capricornutum* Printz, 1914 and YCT (yeast, Cerophyll®, and digested trout starter food). 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 h old.

A series of four identical trials were conducted. In each trial, five neonates (<24 h old) were transferred to eight glass Petri dishes each containing 25 ml of oxygen-saturated, moderately-hard water. Four Petri dishes were treated at 200 ppm with *Pf*-CL145A dead cells, and four served as untreated controls. Neonates were fed at the start of the test and again at 24 h. The Petri dishes were placed in an incubator at 23(±1)°C, and dissolved oxygen levels remained ≥ 5.0 ppm throughout the exposure. After 48 h, the daphnids were transferred to new Petri dishes containing 25 ml of fresh, oxygen-saturated, moderately-hard water, and fed YCT and algal concentrate. Daily water renewals and mortality assessments were performed for an additional eight days.

Amphipod – *<u>Hyalella azteca</u>* (Saussure, 1858)

United States Environmental Protection Agency (2000) protocols for measuring the toxicity of sediment-associated contaminants served as a

guideline for these trials. Juvenile *Hyalella azteca* were obtained from Chesapeake Cultures (Hayes, Virginia). The amphipods were held and tested in oxygen-saturated, hard water at 22(±2)°C under a 16/8-h light/dark cycle. Amphipods (7-14 days old) were treated at 0 (untreated control), 25, 50, 100, and 200 ppm with dead *Pf*-CL145A cells in glass jars holding 200 ml of hard water, with exposures lasting 24 or 48 h. Each glass jar held ten amphipods and there were three replicates per treatment. Following exposure, amphipods were transferred to clean glass containers holding fresh, oxygen-saturated, hard water. Daily posttreatment observations and survival assessment continued for 12-13 days, with amphipods fed YCT and water renewed approximately every other day. As an additional control to assess whether any observed amphipod mortality was due to the dreissenid-killing cell toxin, trials were conducted with dead "toxin-inactivated" *Pf*-CL145A cells, i.e., prior to treating the amphipods the dead bacterial cells were exposed to heat (50-70°C) in a water bath – a process that inactivates the dreissenid-killing toxin (Molloy et al. 2013a).

Fathead minnow – Pimephales promelas Rafinesque, 1820

A trial was conducted with three-month-old fathead minnows obtained from Cosper Environmental Services, Inc. (Bohemia, New York). Upon receipt, fish were placed in aquaria containing aerated, unchlorinated tap water with filtration to begin acclimation to the testing temperature of 23°C. One day prior to treatment, 25 fish were transferred to four 20-L aquaria containing 12 L of aerated, unchlorinated tap water. Live *Pf*-CL145A cells were applied at 100 ppm to three of these aquaria. After 72 h of exposure, fish were transferred by hand net through a water rinse and into clean aquaria containing fresh, aerated, unchlorinated, tap water with filtration. Fish had been fed every other day, alternating between flake fish food (Tetris®) and frozen brine shrimp, until 24 h before treatment, and this feeding protocol was resumed after the three-day exposure. Mortality assessments were performed immediately prior to a water change every other day for an additional 17 days.

In addition to the above laboratory trial, two once-through trials were conducted within Rochester Gas and Electric's Russell Station – a coal-fired power plant in Rochester, New York receiving Lake Ontario water. Fathead minnows (length, 35-55 mm) were supplied by the NYSDEC Bureau of Environmental Protection (Avon, New York). Two aluminum baskets, each containing 50 fish, were placed within two 80-L troughs that received water directly from a service-water pipe at 5 L/min. Thirty meters upstream of the troughs a metering pump injected an aqueous suspension of dead *Pf*-CL145A cells for 24 h directly into the service water pipe to create a continuous 50 ppm concentration. Two other 80-L troughs (each also similarly containing two aluminum baskets with 50 fish) served as the untreated controls and received water from the service water pipe at a location upstream of the bacterial injection point. During the pretreatment and treatment periods, immersion heaters maintained the service water entering the troughs at 10(±1)°C in the first trial and 21(±1)°C in the second trial. During the 41-day posttreatment period, fish continued to be held under once-through conditions, but without immersion heaters, with temperatures ranging from 9-20°C and 15-23°C in the first and second trials, respectfully. Fish were fed fish chow pellets and checked for mortality daily during posttreatment periods.

Bluegill – Lepomis macrochirus Rafinesque, 1819

Juvenile bluegills were obtained from Hopper-Stephens Hatcheries, Inc. (Lonoke, Arkansas) and upon receipt were transferred to 75-L aquaria containing aerated, unchlorinated tap water at 12°C with filtration that was allowed to warm slowly to the ambient laboratory temperature of 20(±1)°C. Fish chow pellets were provided approximately every other day up until the day before treatment, at which time 15 bluegills (mean (±SD) length, 50.5 (±2.9) mm) were transferred to each of six 19-L glass aquaria containing 12 L of stream water under aeration. Three aquaria were treated with dead *Pf*-CL145A cells at 100 ppm, and three remained as untreated controls. After 72 h of exposure, fish were transferred by hand net through a water rinse and into clean 19-L glass aquaria containing aerated stream water with filtration. Fish were fed, water was changed, and mortality recorded daily for an additional 11 days.

Brown trout – Salmo trutta Linnaeus, 1758

Young-of-the-year brown trout (standard length, 2.5-3.5 cm) were supplied by the NYSDEC Bureau of Habitat (Rome, New York). Fish were maintained in stream water under once-through conditions at 6(±1)°C and were fed trout starter food daily up until the day before treatment, at which time 25 fish were placed in each of six plastic containers (32 × 20 × 18 cm), each containing two liters of aerated stream water at 6(±1)°C. Three containers were treated with dead *Pf*-CL145A cells at 100 ppm, and three remained as untreated controls. After 72 h of exposure, trout were transferred to clean plastic containers with screened lids and again held under once-through conditions in stream water. Fish were fed starter food pellets and checked for mortality daily during the 39 days posttreatment period at 6(±1)°C.

Blue mussel – Mytilus edulis Linnaeus, 1758

Blue mussels were obtained from the Long Island Power Authority (Melville, New York) and were maintained in 19-L aquaria in a solution of 2.8% (w/v) Instant Ocean® in aerated, unchlorinated tap water with filtration at 20(±1)°C. Twenty-five blue mussels (length, 12-19 mm) were placed in each of four 1-L glass testing jars containing 500 ml of aerated Instant Ocean water and held overnight. The following morning, three jars were treated with live *Pf*-CL145A cells at 100 ppm, with the remaining jar serving as the untreated control. After five days of exposure, water was poured off, and mussels were transferred to clean jars containing fresh, aerated Instant Ocean water. Mortality was checked and water changed daily for an additional 14 days. Dead mussels were identified as those having gaping shells that did not respond to gentle touch.

Unionid mussels – Laboratory trials with Pyganodon grandis (Say, 1829), *Pyganodon cataracta* (Say, 1817), *Lasmigona compressa* (Lea, 1829), *Strophitus undulatus* (Say, 1817), *Elliptio complanata* (Lightfoot, 1786), *and Lampsilis radiata* (Gmelin, 1791)

A series of four laboratory trials were conducted using adult unionids collected from four water bodies: Moseskill River (Fort Edward, New York), Webatuck Creek (Wassaic, New York), Lake Champlain (Ausable Point State Park, Peru, New York), and Lake Cossayuna (Cossayuna, New York). Species identifications were made following Strayer and Jirka (1997), with selected additional confirmations by D. Strayer (Cary Institute for Ecosystem Studies) and W. Harman (State University of New York at Oneonta).

Trial #1: Twenty unionids (mean (±SD) length, 48.8 (±11.5) mm) were collected from the Moseskill River. Five were randomly chosen and placed in each of four 38-L aquaria containing 12 L of unchlorinated tap water with aeration. Live *Pf*-CL145A cells were applied to three aquaria at 100 ppm, and exposure continued for three days at 16(±1)°C. The remaining aquarium served as the untreated control. Following exposure, the unionids were rinsed by hand in tap water and transferred to clean 115-L aquaria filled with aerated, unchlorinated tap water with filtration. 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 28 days posttreatment. This trial represented our initial unionid trial, and our taxonomic inexperience prevented us from keying these 20 individuals to species prior to initiating the experiment. At the end of the trial, however, individuals were sacrificed and identified (with expert confirmation) as being 2 *Pyganodon grandis*, 3 *L. compressa*, and 10 *S. undulatus* in the treated group and 1 *P. grandis* and 4 *S. undulatus* in the untreated control.

Trial #2: Fifteen *Elliptio complanata* from Webatuck Creek were placed in each of four 38-L aquaria containing 28 L of unchlorinated tap water with aeration. Live *Pf*-CL145A cells were applied to three aquaria at 100 ppm, and the unionids were exposed for three days at 16(±1)°C. The remaining aquarium served as the untreated control. Following exposure, the unionids were rinsed by hand in tap water and transferred to clean 115-L aquaria filled with aerated, unchlorinated tap water with filtration. 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 27 days posttreatment.

Trial #3: Fifteen *Lampsilis radiata* from Lake Champlain were placed in each of four 38-L aquaria containing 28 L of aerated, unchlorinated tap water. Live *Pf*-CL145A cells were applied to three aquaria at 100 ppm, and the unionids were exposed for three days at 16(±1)°C. The remaining aquarium served as the untreated control. Following exposure, the unionids were rinsed by hand in tap water and transferred to

clean 115-L aquaria filled with aerated, unchlorinated tap water with filtration. Water was changed in the aquaria each week. Unionids were fed *Chlorella* three times per week and were checked daily for mortality for 27 days posttreatment. Mean (±SD) length of the *L. radiata* was 76.3 (±7.8) mm.

Trial #4: *Elliptio complanata* and *Pyganodon cataracta* were collected from Lake Cossayuna and held in aquaria containing aerated stream water with filtration 23(±1)°C. One day prior to treatment, 4-L glass testing bottles were set up each containing one unionid in two liters of aerated, oxygen-saturated, hard water at 23(±1)°C. For each of the two species, live *Pf*-CL145A cells were applied at 100 and 200 ppm for 24 and 48 h, with each of the four dosages having ten treated replicates and one untreated control. At the end of the exposures, unionids were removed from the treated water, rinsed by hand in stream water, and placed in 38-L aquaria containing aerated stream water with filtration. Stream water (containing naturally suspended plankton) was refreshed every two days, without any additional food added. Mortality was monitored daily for 33 days following the 24-h exposures and 32 days following 48-h exposures. Mean (±SD) lengths of *E. complanata* and *P. cataracta* were 80.6 (±7.3) mm and 73.0 (±7.0) mm, respectively.

Unionid mussels – Mesocosm trial with Pyganodon cataracta

Mesocosms enclosures were constructed of open-ended, transparent fiberglass cylinders (diameter × height, 30 cm × 30 cm). They were pushed approximately 5 cm into the sand on the bottom of a cement-walled pond (length × width, 6.8 m × 1.2 m) through which stream water flowed on the NYSMFRL property. The water in each cylindrical mesocosm enclosure had a depth of ca. 18 cm and a volume of ca. 15 L. *Pyganodon cataracta* (mean (±SD) length, 82.7 (±9.7) mm) were collected from Lake Cossayuna, and ten were placed directly into each of four mesocosm enclosures, and aeration was supplied to each. The next day, live *Pf*-CL145A cells were applied at 200 ppm to the 21°C water within three of the mesocosms. The remaining mesocosm enclosure served as the untreated control. At the end of a 48-h treatment period, the cylinders were removed and replaced with mesh cages having 1.5 cm openings through which stream water currents could readily flow. Unionids remained in these mesh-sided cages until final mortality was scored 28 days later. The entire test was conducted at 21(±3)°C.

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. Where appropriate, i.e., for tests where mean mortality among treated organisms was higher than the mean in the untreated controls, *p*-values are included in Table 1.

Results

Treatment with *Pf*-CL145A cells (Table 1) resulted in little to no mortality among non-target organisms and high mortality against dreissenids – the latter confirming the high activity of the bacterial cells used in this study.

Ciliates – *Colpidium colpoda*: Not only was no mortality observed in any of the six treated or six control dishes (Table 1), but there was no statistical difference in the reproductive rate among treated and untreated ciliates. Each dish started off with one ciliate, but at the end of the 72-h test there was a mean (±SD) of 7.0 (±3.6) ciliates in treated dishes and 4.3 (±3.5) in the controls (*t*-test, $p=0.221$).

Cladocerans – *Daphnia magna*: There was no evidence that *Pf*-CL145A cells were lethal to daphnids at 200 ppm for 48 h (Table 1). Overall mean (±SD) mortality in the four trials was 5.0(±5.8)% in treated dishes and 6.3(±7.5)% in untreated controls.

Amphipods – *Hyalella azteca*: Treatment with dead *Pf*-CL145A cells resulted in mean mortalities (range, 3.3-26.7%) almost always higher than in the untreated controls (range, 0.0-3.3%; Table 1), with one of the treatments (200 ppm for 48 h) statistically significant (*t*-test, $p=0.039$; mean (±SD) mortalities of 26.7 (±15.3)% and 0.0(±0.0)%, respectively, in treated and control). To evaluate whether the observed amphipod mortality was the result of the dreissenid-killing toxin, additional trials were conducted using

Table 1. Safety trials of *Pseudomonas fluorescens* strain CL145A with aquatic non-target organisms.[a]

Aquatic non-target organism	Concentration of bacteria (Duration of exposure)	Treatment temperature (°C)	Posttreatment observation period (days)	Mean control mortality (±SD)	Mean treatment mortality [h] (±SD)	Mean dreissenid mortality in parallel treatment [i] (±SD)
Ciliate *Colpidium colpoda*	100 ppm (72 h) [b]	23(±1)	0	0.0 ±0.0%	0.0 ±0.0%	— [j]
Cladoceran *Daphnia magna*	200 ppm (48 h) [c]	23(±1)	8	0.0 ±0.0%	0.0 ±0.0%	No data
	200 ppm (48 h) [c]	23(±1)	8	15.5 ±10.0%	10.0 ±11.6% $^{p=0.537}$	95.3 ±4.6%
	200 ppm (48 h) [c]	23(±1)	8	0.0 ±0.0%	0.0 ±0.0%	90.7 ±3.1%
	200 ppm (48 h) [c]	23(±1)	8	10.0 ±11.5%	10.0 ±20.0% $^{p=0.785}$	92.0 ±3.5%
Amphipod *Hyalella azteca*	25 ppm (24 h) [c]	22(±2)	13	0.0 ±0.0%	6.7 ±11.5% $^{p=0.863}$	61.3 ±12.9%
	50 ppm (24 h) [c]	22(±2)	13	0.0 ±0.0%	10.0 ±17.3% $^{p=0.745}$	As above
	100 ppm (24 h) [c]	22(±2)	13	0.0 ±0.0%	16.7 ±5.8% $^{p=0.141}$	As above
	100 ppm (24 h) [c]	22(±2)	13	3.3 ±5.8%	16.7 ±11.5% $^{p=0.73}$	As above
	100 ppm (48 h) [c]	22(±2)	12	3.3 ±5.8%	26.7 ±30.6% $^{p=0.62}$	As above
	200 ppm (24 h) [c]	22(±2)	13	3.3 ±5.8%	3.3 ±5.8% $^{p=1.0}$	As above
	200 ppm (48 h) [c]	22(±2)	12	3.3 ±5.8%	17.4 ±15.5% $^{p=0.848}$	98.7 ±2.3%
	200 ppm (48 h) [c]	22(±2)	12	3.3 ±5.8%	16.7 ±11.5% $^{p=0.094}$	96.0 ±4.0%
	200 ppm (48 h) [c]	22(±2)	12	0.0 ±0.0%	26.7 ±15.3% $^{p=0.039}$	96.0 ±6.9%
	Toxin-inactivated trials – Cells were heat-treated to inactivate the dreissenid-killing toxin:					
	100 ppm (24 h) [d]	22(±2)	13	0.0 ±0.0%	10.0 ±0.0% $^{p=0.000}$	0.0 ±0.0%
	200 ppm (48 h) [d]	22(±2)	12	3.3 ±5.8%	23.3 ±15.3% $^{p=0.514}$	20.0 ±6.9%
Fathead minnow *Pimephales promelas*	100 ppm (72 h) [b]	23(±1)	17	4.0%	2.7 ±2.3%	46.7 ±18.0%
	50 ppm (24 h) [c,e]	10(±1)	41	2.5 ±3.5%	0.0 ±0.0%	70.8 ±1.4%
	50 ppm (24 h) [c,e]	21(±1)	41	3.0 ±1.4%	1.0 ±1.4%	78.3 ±1.4%
Bluegill *Lepomis macrochirus*	100 ppm (72 h) [c]	20(±1)	11	4.4 ±3.9%	6.7 ±6.7% $^{p=0.823}$	96.0 ±4.0%
Brown trout *Salmo trutta*	100 ppm (72 h) [c]	6(±1)	39	1.3 ±2.3%	2.7 ±2.3% $^{p=0.519}$	71.3 ±2.4% [k]
Blue mussel *Mytilus edulis*	100 ppm (120 h) [b]	20(±1)	14	4.0%	1.3 ±2.3%	100.0 ±0.0%
Unionids *Elliptio complanata*	100 ppm (72 h) [b]	16(±1)	27	0.0%	0.0 ±0.0%	96.0 ±0.0%
	100 ppm (24 h) [b]	23(±1)	36	0.0%	0.0 ±0.0%	97.8 ±1.1%
	100 ppm (48 h) [b]	23(±1)	35	0.0%	0.0 ±0.0%	97.7 ±1.2%
	200 ppm (24 h) [b]	23(±1)	36	0.0%	0.0 ±0.0%	97.7 ±1.6%
	200 ppm (48 h) [b]	23(±1)	35	0.0%	0.0 ±0.0%	98.2 ±1.6%
Lampsilis radiata	100 ppm (72 h) [b]	16(±1)	27	0.0%	0.0 ±0.0%	100.0 ±0.0%
Lasmigona compressa	100 ppm (72 h) [b]	16(±1)	28	— [g]	0.0 ±0.0%	98.7 ±2.3%
Pyganodon grandis	100 ppm (72 h) [b]	16(±1)	28	0.0%	0.0 ±0.0%	As above
Strophitus undulatus	100 ppm (72 h) [b]	16(±1)	28	0.0%	0.0 ±0.0%	As above
Pyganodon cataracta	100 ppm (24 h) [b]	23(±1)	33	0.0%	0.0 ±0.0%	97.8 ±1.1%
	100 ppm (48 h) [b]	23(±1)	32	0.0%	0.0 ±0.0%	97.7 ±1.2%
	200 ppm (24 h) [b]	23(±1)	33	0.0%	0.0 ±0.0%	97.7 ±1.6%
	200 ppm (48 h) [b]	23(±1)	32	0.0%	0.0 ±0.0%	98.2 ±1.6%
	200 ppm (48 h) [b,f]	21(±3)	28	0.0%	0.0 ±0.0%	97.8 ±1.3%

a Unless otherwise indicated, all tests were single-dose acute toxicity treatments in closed containers followed by a posttreatment observation period after which mean mortalities were determined.
b Organisms were treated with live *Pf*-CL145A cells.
c Organisms were treated with dead *Pf*-CL145A cells.
d The dreissenid-killing toxin within the dead *Pf*-CL145A cells was inactivated with heat (50-70°C for 30-60 min).
e These fish trials were in once-through service water at a coal-fired power station (Rochester Gas and Electric's Russell Station).
f This unionid trial was an outdoor mesocosm trial.
g Too few specimens of this unionid species to have an untreated control.
h *p*-values are indicated only in trials where mortality among treated is higher than in untreated controls and mean (±SD) values are present.
i Mortality in untreated controls <10%.
j No dreissenid efficacy test was conducted at the time of the ciliate *C. colpoda* test; the same culturing methods, however, were used to produce *Pf*-CL145A cells in numerous tests before and after the *C. colpoda* test, with 75-100% dreissenid mortality routinely achieved.
k The 71.3% mean mortality recorded against zebra mussels at 6°C indicated bacterial cells used against the brown trout were very efficacious; in a concurrent trial at 20ºC with the same bacterial inoculum 97.3 ±4.6% was achieved against zebra mussels.

dead *Pf*-CL145A cells that had been heated to inactivate the toxin. In these "toxin-inactivated trials" (Table 1), concurrent tests against zebra mussels, as expected, resulted in low mussel kill (range, 0.0-20.0%), but the same level of mortality (range, 10.0-23.3%) was observed among the amphipods. Thus, although the amphipods did experience some mortality following *Pf*-CL145A treatment, these additional toxin-inactivated trials provided evidence that their deaths may not have been due to the dreissenid-killing natural product.

Fish – *Pimephales promelas*, *Salmo trutta*, and *Lepomis macrochirus*: There was no evidence of mortality among fathead minnows, brown trout, or bluegills following exposure to *Pf*-CL145A cells (Table 1). This included trials with live cells against fathead minnows and dead cells against all three species.

Bivalves – *Mytilus edulis, Elliptio complanata, Lampsilis radiata*, *Lasmigona compressa*, *Pyganodon grandis*, *Strophitus undulatus*, and *Pyganodon cataracta*: No mortality was recorded among any of the non-target bivalves treated with live cells (Table 1). This included a total of 75 blue mussels treated in the laboratory trial, 30 *P. cataracta* treated in the mesocosm experiment, and 185 unionids treated in the four laboratory trials (2 *P. grandis*, 3 *L. compressa*, and 10 *S. undulatus* in the Moseskill River Trial #1; 45 *E. complanata* in the Webatuck Creek Trial #2; 45 *L. radiata* in the Lake Champlain Trial #3; and 40 *E. complanata* and 40 *P. cataracta* in the Lake Cossayuna Trial #4).

Discussion

These non-target trials indicated a high level of specificity of *Pf*-CL145A cells to dreissenid mussels. Low mortality (range, 3-27%) was recorded in trials with the freshwater amphipod *H. azteca,* but additional trials using toxin-inactivated *Pf*-CL145A cells suggested that most, if not all the mortality, could be attributed to some other unidentified factor (possibly increased particle load or a water quality issue) rather than the dreissenid-killing natural product.

The results of these non-target trials – in combination with equally promising dreissenid control trials (Molloy et al. 2013a) – have recently led to *Pf*-CL145A's commercialization under the product name Zequanox®, with dead cells as the active ingredient (Marrone Bio Innovations 2012a). The commercial availability of only dead-cell Zequanox formulations will eliminate the risk of any possible non-target infection by *Pf*-CL145A, further reducing environmental concerns.

In terms of potential environmental safety, therefore, the results of these non-target trials are encouraging, but it would be unrealistic to think that dreissenids are the only aquatic organisms susceptible to *Pf*-CL145A's dreissenid-killing natural product. Additional trials are needed to better define *Pf*-CL145A's margin of safety by identifying the sensitivity of other susceptible organisms. The limited quantities of *Pf*-CL145A cells that we were able to culture severely restricted the number and size of our non-target trials. In contrast, the present availability of Zequanox will now allow expanded and far more comprehensive non-target trials by multiple investigators, and such studies are already occurring (Marrone Bio Innovations 2012b). The trials reported herein – exposing non-target organisms under aerated conditions to unformulated, laboratory-cultured cells – clearly point to high host specificity, but trials with Zequanox – using *Pf*-CL145A cells cultured, killed, and formulated following industrial-scale protocols – will be even more important as they will define the non-target safety limits of the actual commercial products under a wide range of environmental conditions.

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