

U.S. HOUSE OF REPRESENTATIVES
COMMITTEE ON NATURAL RESOURCES
SUBCOMMITTEE ON WATER AND POWER

Hearing on:

*THE SILENT INVASION: FINDING SOLUTIONS TO MINIMIZE THE IMPACTS
OF INVASIVE QUAGGA MUSSELS ON WATER RATES,
WATER INFRASTRUCTURE AND THE ENVIRONMENT*

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June 24, 2008

Chairwoman Napolitano and members of the Subcommittee, I am Denise Mayer, Senior Research Scientist at the New York State Museum's Field Research Laboratory. Originally from the shores of Lake Superior in northern Wisconsin, I have a long history in the formal and informal study of sensitive aquatic ecosystems and their susceptibility to invasive species, and I have been involved in microbial and ecological research for almost 20 years. I am honored to be here today to provide information about a green technology for the management of quagga and zebra mussels that shows promise to help protect sensitive industries and ecosystems from these fouling invasive mussels.

One of the goals of the NYSM is to make information about the ecology of New York State available to the public and to protect biodiversity through applied scientific research. Industries with raw-water dependent infrastructures are at risk to fouling by mussels and are interested in ecologically friendly alternatives to the broad spectrum biocides currently required to manage mussel infestations in their pipes. Due to its history in biopesticide development, the NYSM's Field Research Laboratory, under the direction of Dr. Daniel Molloy, was approached by the power industries in New York (a research consortium called the Empire State Electric Energy Corporation (ESEERCO)) in the early 1990's hoping that he might discover a highly effective biological control agent that could reduce the use of the polluting broad-spectrum biocides they were forced to use for mussel control due to a lack of safer alternatives (http://sgnis.org/publicat/papers/jsr17_1.pdf). His NYSM laboratory examined the biocontrol potential of the natural enemies of these mussels (parasites and predators) and also evaluated bacteria that contained novel byproducts with pesticidal properties. His laboratory's screening trials of more than 700 bacterial strains did successfully identify a North American bacterium, strain CL145A of *Pseudomonas fluorescens*, that was highly lethal to zebra and quagga mussels.

Since its discovery, his research team at the NYSM, of which I am the lead scientist, has been developing *Pf*-CL145A into an effective and environmentally benign alternative to chemical control for the management of zebra and quagga mussels for use in industrial water systems. Over 3 million dollars in research funds have been awarded to our NYSM laboratory by agencies in NY State (ESEERCO, NY Power Authority, NY Sea Grant, NYS Energy Research and Development Authority, NYS Department of Environmental Conservation) and federal agencies (U.S. Department of Energy's National Energy Technology Laboratory, U.S. Army Corps of Engineers, U.S. Fish and Wildlife Service, National Science Foundation, U.S. Bureau of Reclamation) for the development of this product. Without the foresight of these funding agencies to find alternatives to polluting biocides, the development of this product would not have been possible. Among the key findings of our research to date are:

- The mode of action of *Pf*-CL145A is toxin related as opposed to infection. Applications are as effective whether the bacterial cells are alive or dead. Thus, to reduce the risk of proliferation and ecological impact, killed cell formulations are applied.
- Because quagga and zebra mussels naturally filter feed on bacteria in water, they readily ingest *Pf*-CL145A cells, and the toxin kills by destruction of their digestive gland. This is in contrast to most chemical treatments which are sensed by the mussels causing them to stop feeding and thereby requiring extended treatment periods.
- The bacterial product is effective across a wide range of biotic and abiotic factors. We have characterized the following variables: water temperature, oxygenation, velocity, turbidity and chemistry (pH and hardness), mussel density, species (quagga and zebra), size, life stage (larval, juvenile and adult).
- The toxicity of the bacterial product is highly specific to quagga and zebra mussels. At dosages that produce high mussel mortality, no bacteria-induced mortality has been observed among any of the nontarget species tested, including fish, ciliates, daphnids and native bivalves.
- Mammalian toxicological studies predict human safety when working with this product.

More detailed information about *Pf*-CL145A is available in the document that I have also submitted as part of my written testimony to the Subcommittee.

Until recently our research focused entirely on industrial applications, i.e., controlling mussels in pipes. After the expansion of the quagga mussel's range to the West, however, there has been considerable interest among state and federal agencies as to its potential usefulness in the control of mussels in open waters, like rivers and lakes. With this interest, we began assessing the effectiveness of *Pf*-CL145A against mussel larvae (i.e., veligers) and have demonstrated their sensitivity to the toxic bacterium. Just over one year ago, when quagga mussels were first discovered in Lake Mead, near the Hoover Dam, the management of the State of Nevada's Lake Mead Fish Hatchery allowed us to conduct a closed system laboratory trial to demonstrate the effectiveness of *Pf*-CL145A at killing quagga mussels from within their facility. Recently the U.S. Bureau of Reclamation has allied with us in a partnership that includes Marrone Organic Innovations (MOI), our commercial partner, to assess the effectiveness of this bacterial product to manage populations of quagga mussels in lakes and reservoirs as well as within closed pipe systems. The U.S. Environmental Protection Agency, as well as regulatory agencies in Arizona, California and Nevada, have been working closely with us and MOI to obtain the permits necessary for experimental field trials and eventual product registration. At the same time, individual states, including Nevada, Arizona and California, have expressed interest in teaming with us to expedite field trials at scales not requiring an experimental use permit (i.e., less than 2 acres) this summer. We would be pleased to form additional partnerships this year with state, federal and private agencies interested in assessing *Pf*-CL145A's effectiveness in controlling quagga and zebra mussels under field conditions. I welcome interested parties to contact me directly if you're interested in participating in field trials out West.

Pf-CL145A is unique in its effectiveness and environmental safety for mussel control and it stands as the only non-chemical control method being developed that could potentially be used in both raw-water infrastructures and open waters with minimal ecological impact. Once formulated to maximize its effectiveness in open-water conditions, the use of this management tool could help slow the spread and impact of quagga and zebra mussels particularly in the Western States where the infestation is still in its early stages. The timing of the availability of this management tool could prove to be critical to preservation of sensitive ecosystems if the mussel invasions are detected early, when we believe *Pf*-CL145A could be most effective. It is in this regard that we feel an urgency to expedite the final phases of R&D on this bacterial control method. When mussels invaded the Central and Eastern U.S., no such environmentally safe method existed to slow their spread, but now we believe that such a tool potentially does exist and field trials are urgently needed to assess the impact of *Pf*-CL145A treatments in open waters.

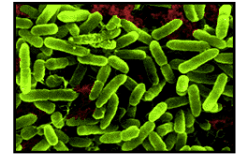
In conclusion, I'd like to thank the Subcommittee and staff for inviting me here today and express my appreciation to many supporters of this project, including the U.S. Department of Energy, Marrone Organic Innovations, the National Science Foundation, the U.S. Fish and Wildlife Service, the U.S. Bureau of Reclamation and well as the US Environmental Protection Agency. We look forward to continuing to work with them and others as we make further improvements in developing bacterial formulations for effective mitigation of quagga and zebra mussels. I'll be happy to answer any questions that you may have.



Adult mussels

**Overview of a Novel Green Technology:
Biological Control of Zebra and Quagga Mussels
with *Pseudomonas fluorescens***
(Version 8: Updated June 20, 2008)

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*P. fluorescens* cells

Clogged water pipe

Summary

Responding to a request from the North American electric power generation industry for the development of a more environmentally benign method for controlling infestations of zebra and quagga mussels in their pipes, researchers at New York State Museum launched a program in the 1990s to find a highly selective biological control agent. Funded by 3 million dollars in private, state and federal support, their efforts were successful in identifying a naturally occurring bacterial strain with a cellular byproduct that is lethal to these fouling mussels, yet safe for other aquatic organisms. This bacterial strain has proven itself effective in experiments to control mussel infestations in power plant pipes, and the U.S. Bureau of Reclamation is now taking the lead in research to evaluate its potential for controlling these mussels in open waters, such as rivers and lakes. Funding to bring this biocontrol agent to commercialization was recently awarded to the biopesticide company Marrone Organic Innovations by the U.S. National Science Foundation. Product availability is targeted for 2010.

Improvements Over Existing Chemical Control Methods

Power generation facilities require annual maintenance and preventive programs to keep infestations of fouling zebra and quagga mussels (*Dreissena* spp.) in their cooling water intake systems under control. Currently it is necessary at many of these and other infested raw-water dependent infrastructures to administer controlled dosages of chlorine or other types of biocides for this purpose. Although such applications meet all existing water pollutant discharge regulatory limits, evidence exists to suggest that



Coal-fired power plant on Lake Ontario.
(Photo credit: Rochester Gas & Electric)

natural resource interest groups and regulatory agencies are reexamining the negative long-term use of biocides for this purpose. Both groups have made it clear that safe, non-chemical alternatives for controlling mussel fouling would be environmentally beneficial. Chlorination, for example, is a common control method, and when chlorine combines with organic compounds in water, potentially carcinogenic substances such as trihalomethanes and dioxins are formed (United States Environmental Protection Agency 1999; Thornton 2000). Should future regulatory actions result in the loss of

chemical biocides, without an alternative control option, electric generation organizations and many other industries that rely on the withdrawal of surface waters for operational reasons are certain to experience economic penalties. These losses would be the result of decreased production brought on by increased facility maintenance and downtime. Thus, the availability of an equally effective, yet far more environmentally benign, mussel control method to replace chlorine and other biocides is critically needed by power plants and other infested facilities.

Research Paradigm

Why would one look to use a naturally-occurring, non-parasitic, non-infectious microbe, such as the ubiquitous soil-water bacterial species, *Pseudomonas fluorescens*, to serve as an innovative, novel strategy for mussel management in power generation facilities? Sounds illogical? Well, it is widely accepted that the screening of diverse biochemicals found in tropical plant species is a worthwhile activity due to the discovery of drugs that can prevent or cure animal diseases, particularly cancers. Production of these biochemicals, however, did not evolve in these plants for this purpose, and the effect of these plant substances on animal diseases, although fortuitous, is purely coincidental. Using the same logic, we can also look to microorganisms for unique biochemicals or toxins which have potential as highly selective biopesticides. In fact, the use of microbial toxins already has a clear record of commercial success and environmental safety in the control of invertebrate pests in North America, as well as globally (Rodgers 1993), and our New York State Museum (NYSM) laboratory has been involved in such research for over two decades as discussed in the following section.

Prior Participation in Commercial Success

In the interest of eliminating polluting pesticides and thereby protecting biodiversity in New York State, our NYSM Field Research Laboratory assisted in the commercial development of *Bti* — the selectively toxic bacterium, *Bacillus thuringiensis* subsp. *israelensis* — as the first biological control agent for black flies (Simuliidae). This bacterium, because of its extraordinary nontarget safety (Molloy 1982, 1990, 1992; Molloy and Jamnback 1981; Molloy and Struble 1989), has now completely replaced broad-spectrum, chemical pesticides throughout New York State and elsewhere in North America for the control of these biting flies. The commercial use of this microbial agent is not small scale; large waterbodies, such as the Susquehanna River in Pennsylvania and the New River in West Virginia, are routinely treated with this bacterial species to control larval black fly populations.



Treatment of the Susquehanna River with *Bti* bacteria for black fly larval control.
(Photo credit: Pennsylvania Department of Environmental Protection)

Research Progress To Date

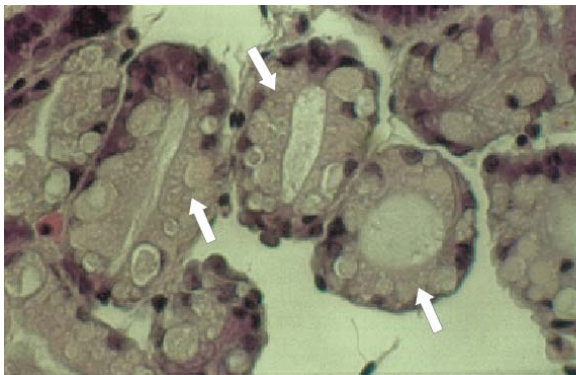
1. Inception of Project: Research Funded by Private Electric Power Industry

The Empire State Electric Energy Research Corporation (ESEERCO¹) — faced with the threat of zebra mussels fouling electric power facilities within New York State — contracted with our NYSM Field Research Laboratory in 1991 for the screening of bacteria as potential biological control agents. Based on the successful development of the environmentally safe, biological control agent for aquatic black fly larvae (see above), it was hypothesized (Molloy 1991) that bacteria also existed in nature whose toxins could be used as lethal agents for these new aquatic pests, zebra and quagga mussels. The research efforts funded by ESEERCO proved this hypothesis to be true (Molloy 1998). Extensive laboratory screening trials of more than 700 bacterial strains identified a North American isolate, strain CL145A of *Pseudomonas fluorescens*, to be lethal to these mussels. Of all 10 strains of *P. fluorescens* that have been laboratory tested to date, only *Pf*-CL145A has been found to be highly lethal, i.e., at dosages that produce >90% zebra mussel kill with *Pf*-CL145A, the other 9 strains of *P. fluorescens* caused only 0-11% mortality.

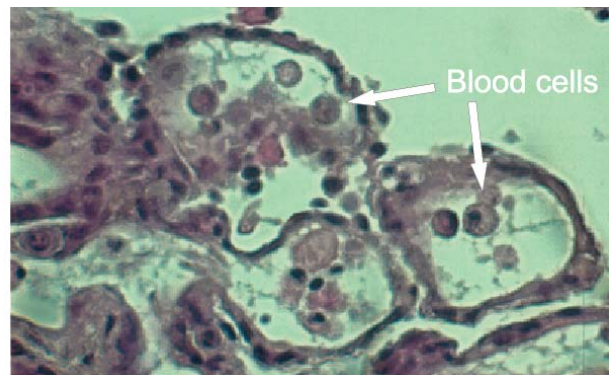
Pseudomonas fluorescens is worldwide in distribution and is present in all North American waterbodies. In nature it is a harmless bacterial species that is found protecting the roots of plants from rot and mildew. It is so ubiquitous that it is a common food spoilage organism in the average household refrigerator. Our research, however, has shown that the *Pf*-CL145A strain of this species may be fortuitously used for another purpose — the control of *Dreissena* spp. (Molloy 2002). A patent for this purpose has been issued in both the United States (Molloy 2001) and Canada (Molloy 2004).

2. Mussels Die from a Natural Toxin: Dead Bacteria Kill Equally As Well

Although phytoplankton is their preferred food, *Dreissena* mussels can filter out and consume bacteria as a food source (Mikheev and Sorokin 1966; Frischer et al. 2000). When a zebra or quagga mussel ingests artificially high densities of strain CL145A, however, a toxin within these bacterial cells destroys the mussel's digestive system. Dead cells are equally as lethal as live cells, providing clear evidence that the mussels die from a toxin, not from infection. Techniques have already been developed at our laboratory that kill the bacteria without any reduction in their lethality to the mussels. Future commercial products based on this microbe will contain dead cells, thus further reducing environmental concerns.



In healthy mussels, epithelial cells (arrows) appear as a thick layer lining the tubules of the digestive gland.



Following bacterial treatment, epithelial cells are destroyed. Blood cells are abundant as the digestive gland hemorrhages.

¹ A research consortium of New York State's electric power generation companies.

3. Mussel Feeding: Bacteria Are Readily Ingested

Although ingestion of CL145A cells is clearly a suicidal behavior for *Dreissena* mussels, they appear to have no adverse reaction to feeding on the cells and filter normally throughout a typical 6-hr, once-through pipe treatment. In contrast, biocides, like chlorine, that are currently being used for mussel control cause them to quickly shut their valves since the mussels apparently sense an adverse effect. This necessitates more prolonged chlorination periods, such as continuous treatments of three weeks or more. The apparent acceptance of CL145A cells as "normal" bacterial food by these mussels facilitates the use of this microbe as a biocontrol agent.

4. Mussel Length: All Mussel Sizes Can Be Killed

All *Dreissena* mussel sizes tested to date (length, ca. 1-25 mm) appear to be equally susceptible to kill by CL145A. Thus, the bacteria are capable of killing, irrespective of mussel size.



5. Mussel Species: Both Species Can Be Killed

The bacterium can kill zebra mussels (*Dreissena polymorpha*) and quagga mussels (*Dreissena rostriformis bugensis*) — the 2 species that invaded North America in the 1980s. Tests to date consistently achieve higher kill against zebra mussels.



6. Water Hardness: Mussel Kill is Highest in Hard Water – the Preferred *Dreissena* Mussel Habitat

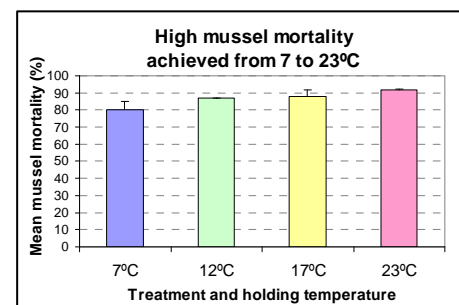
Tests to date suggest that bacterial treatments may have reduced efficacy in soft waters with pH values less than ca. 7.4. *Dreissena* mussels, however, rarely reach high population densities in such (near neutral or acidic) waters, and thus, infested pipes in power plants typically will have more alkaline waters where bacterial efficacy will not be impaired.

7. Dissolved Oxygen: Keep Oxygen Levels High to Ensure Highest Kill

Laboratory tests indicate that very low oxygen levels (<2 ppm) can sometimes result in a 20% decline (e.g., 75% vs. 95%) in mussel kill. This is possibly due to lower feeding by the mussels on suspended bacteria under such low oxygen conditions. Thus, wherever possible, bacterial treatments should occur in waters of high dissolved oxygen – the preferred environment of *Dreissena* mussels.

8. Water Temperature: Higher Kill at Warmer Temperatures

Susceptibility increases with water temperature, with >90% mortality consistently achieved in routine lab testing at 23°C against zebra mussels. High mortality is still achievable even in very cold waters, e.g., near 80% kill at



Mortality increases with temperature.

7°C, indicating that the bacteria are actually more effective at lower temperatures than currently commercialized chemical molluscicides used for *Dreissena* control. The latter commercial biocides, e.g., chlorine, can not achieve such high mussel kill below about 10°C, thus limiting their application to warm water periods. Mussels die more quickly at higher water temperatures, with all kill typically achieved within two weeks at 23°C and two months at 7°C.

9. Suspended Particles: Avoid Treating in Periods of Very High Particle Loads To Ensure Highest Kill

Tests to date indicate that unusually high levels of naturally-occurring particles in water (e.g., suspended mud at greater than 100 ppm) can result in a 20% decline (e.g., 75% vs. 95%) in mussel kill. This is possibly due to competitive displacement, i.e., lower feeding by the mussels on the suspended bacteria vs. mud particles. Thus, wherever possible, bacterial treatments should not occur in waters of very high particle loads.

10. Mussel Siphoning Behavior: Do Not Disturb Normal Mussel Feeding

In nature, a dreissenid mussel typically has its two shells spread apart and extends an inhalant siphon tube from between its shells to take food particles into its mantle cavity. After passing through the digestive system, fecal material is egested through the exhalant siphon. Testing has generally indicated that the more active this siphoning behavior is, the higher the mortality that will be achieved by a bacterial treatment. Thus, any stress factors (e.g., vibrations, shadows) that cause the mussels to close their shells during treatment will likely reduce mortality.



11. Treatment Concentration and Duration: Treat for about 6 hr for Maximum Kill

Laboratory and facility trials indicate that ca. 6-hr treatments of ca. 50-100 ppm (dry bacterial mass per unit volume) consistently obtain the highest mussel mortality. Exposing mussels to longer treatment durations or higher bacterial concentrations is (particularly >12 hr) achieves very limited further benefit.

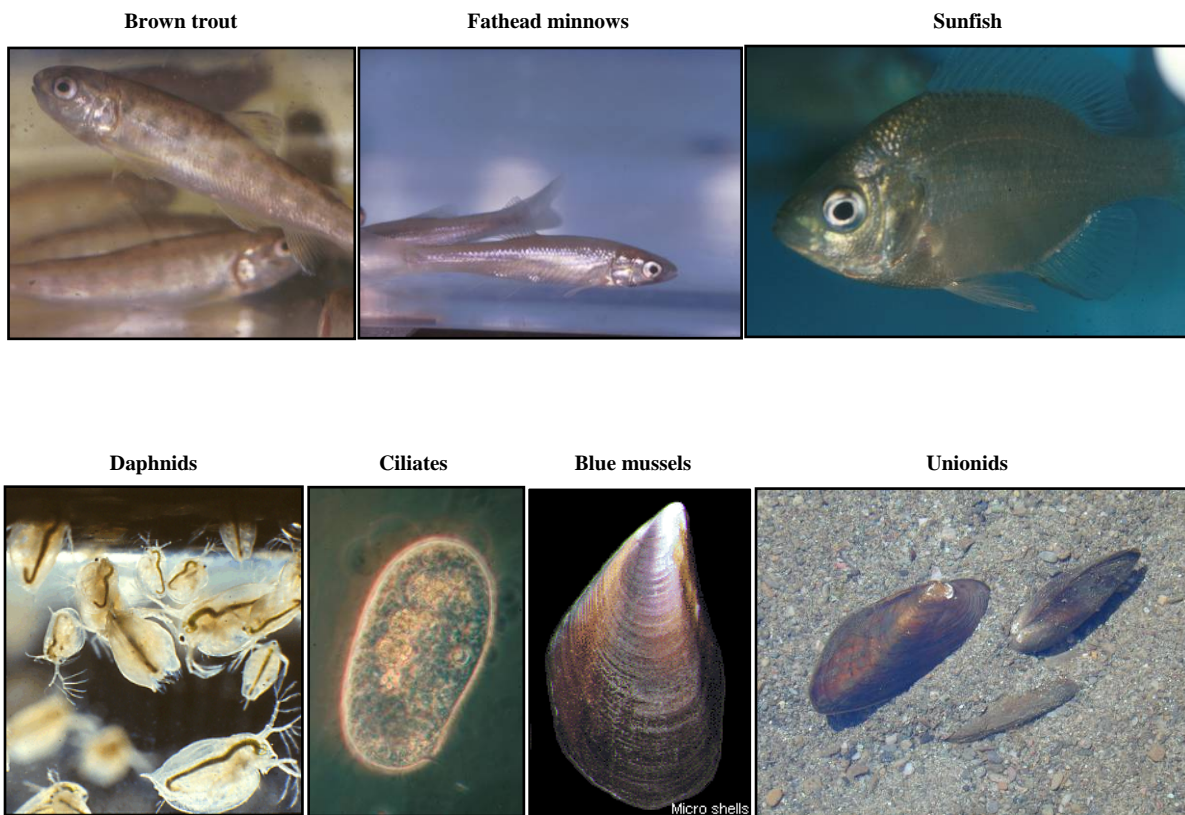
12. Nontarget Trials: Outstanding Specificity

Laboratory trials to date have been very encouraging regarding nontarget safety. At dosages which produced high zebra mussel mortality (76–100%), no bacteria-induced mortality was recorded among any of the nontargets, including fish, ciliates, daphnids, and bivalves:

- **Fish:** No biotoxin-induced mortality has been observed in the three fish species thus far tested: fathead minnows (*Pimephales promelas*), young-of-the-year brown trout (*Salmo trutta*), and juvenile bluegill sunfish (*Lepomis macrochirus*). Laboratory and facility trials have indicated that fish can not tolerate exposure to high levels of live bacteria. Fish trials conducted with dead bacteria, however, have indicated that applications of killed cells were

harmless to the fish, but yet were still highly lethal to the *Dreissena* mussels. To protect fish, as well as to reduce overall environmental concerns, future commercial products based on this microbe will contain almost exclusively dead cells.

- **Ciliates:** Trials with the common freshwater ciliate *Colpidium colpoda* indicated that the bacteria were not only nonlethal, but served as a food source permitting higher rates of ciliate reproduction than ciliates held in untreated streamwater.
- **Daphnids:** The microcrustacean *Daphnia magna* is an aquatic filter feeder that ingests small suspended particles including bacteria, making it an appropriate organism for non-target tests. Laboratory assays indicate that the bacteria are not lethal to this species.
- **Bivalves:** Bacterial exposures caused no mortality to blue mussels (*Mytilus edulis*) or any of 6 native North American unionid clam species (*Pyganodon grandis*, *Lasmigona compressa*, *Strophirus undalatus*, *Lampsilis radiata*, *Pyganodon cataracta*, and *Elliptio complanata*).



There has been no non-target mortality from the bacterial toxin yet observed.

13. Trials at Power Plants: High Kill Can Be Achieved in Service Water

Trials routinely achieving high zebra mussel mortality (ca. 70–97%) in pipes have been conducted at the New York Power Authority (NYPA) electric power station on the Mohawk River (Crescent, New York). Trials at the Rochester Gas & Electric's Russell Power Station on Lake Ontario (Rochester, New York) have been primarily against quagga mussels (the less susceptible of the 2 North American *Dreissena* spp.) and routinely achieved 50-70% mortality.



Very high zebra mussel kill (>95%) was consistently achieved in 6-hr treatments at 100 ppm inside a NYPA hydropower plant under flow-through conditions (3 replicate pipes 17 m in length were used in this trial). Experiments to date indicate that there should be no limit on the length of pipe that can be successfully treated.



Pouring suspension of bacterial cells in preparation for pipe treatments within power plants. Advances in fermentation have allowed increasingly larger volumes of bacteria to be produced, thus allowing larger volumes of water to be treated in pipes.

14. Identity of the Natural Product that is Lethal to *Dreissena* Mussels

Research was undertaken to characterize, isolate, and identify the specific *Dreissena*-killing natural product that is associated with *P. fluorescens* strain CL145A cells. Treatment of toxic cells with lysozyme or deoxycholate appeared to separate the toxin molecules from the bacterial cells, suggesting that the toxin was associated with the outer membrane of the cells. Protease treatments also decreased toxicity, suggesting that the membrane-associated toxin was likely a protein. Cells that were mildly heated lost their ability to kill mussels, providing evidence that the toxin was heat-labile and protein in nature.

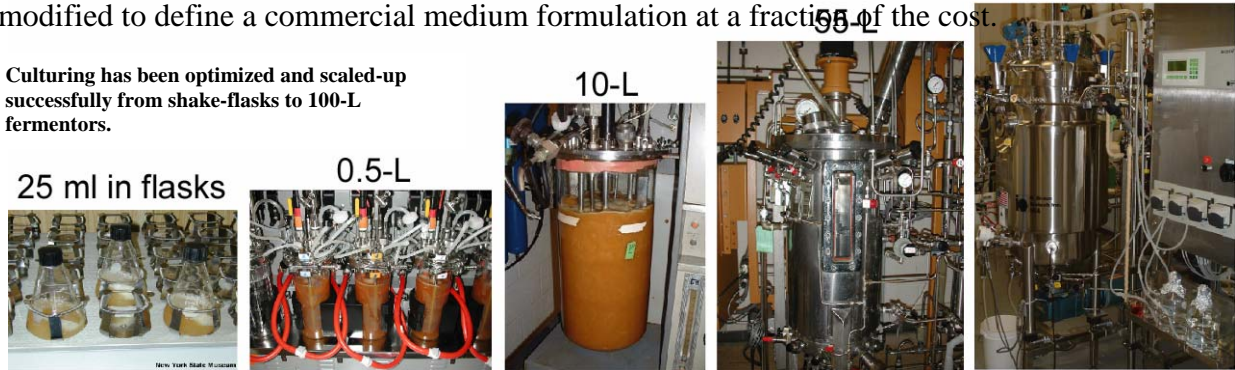
A biochemical approach was undertaken in an attempt to get the toxin to adhere to particles which could hopefully be used to identify the toxin. Even though the toxin was able to be separated from the cells by chemical treatment (i.e., make the cells nontoxic), efforts to develop an effective method to deliver the solubilized toxin molecules to the mussels on particles that they would ingest were unsuccessful. As a result, we altered their biochemical experimental approach and decided to search the literature for documented products from *P. fluorescens* that matched characteristics of the mussel-killing toxin. A candidate molecule investigated was glycine dehydrogenase, an enzyme that catalyzes the conversion of the amino acid glycine to hydrogen cyanide (HCN). Analysis of strain CL145A confirmed that it did produce trace amounts of HCN. Testing then focused on determining whether HCN was the toxin that was responsible for causing mussel death. These experiments demonstrated that treating CL145A cells with an irreversible flavoenzyme inhibitor, diphenylethidium chloride (DPI), successfully blocked the ability of strain CL145A to produce HCN. Even though DPI-treated cells no longer produced trace amounts of HCN, the cells still

remained equally lethal to the mussels, demonstrating that HCN was not the toxin that caused mussel death. Efforts are now underway at the NYSM to use genetic approaches to determine the identity of the toxin (see next section).

15. Culturing Optimization and Fermentation Scale-up:

Toxic *P. fluorescens*-CL145A cells are harvested during late linear to stationary growth phase under defined culture conditions. Toxic cells are routinely harvested from shake-flasks and 0.5-L fermentors at the bench-scale and fermentation has been scaled to 100-L. Media components and fermentations parameters have been optimized to result in a proprietary medium and protocol that consistently produce toxic cells. The optimized medium has been further modified to define a commercial medium formulation at a fraction of the cost.

Culturing has been optimized and scaled-up successfully from shake-flasks to 100-L fermentors.



16. Commercialization Partnership

Following a nation-wide search, the NYSM chose to partner with Marrone Organic Innovations (<http://www.marroneorganicinnovations.com/>), a biopesticide company whose staff have unparalleled experience in the discovery, development, and marketing of natural products for pest management. These joint research efforts are funded by the National Science Foundation and are being directed toward increasing bacterial cell toxicity via additional fermentation work and further understanding the chemistry of the toxic moiety so that cells can achieve even higher mussel kill and thus be more competitive with current polluting chemical control methods. Other critical steps toward commercialization include analytical method development, identification of the mussel-killing toxin, formulation improvement, and additional nontarget toxicology studies that will be mandated by the USEPA for product registration. Commercial product availability is targeted for 2010.

17. Recent and Current Project Activities

Research on this NYSM project was recently supported for eight years by the U.S. Department of Energy's National Energy Technology Laboratory (DOE-NETL 2008). Funding to bring this biological control agent to commercialization through a partnership with the NYSM was recently awarded to the biopesticide company Marrone Organic Innovations by the U.S. National Science Foundation (<http://www.nsf.gov/awardsearch/showAward.do?AwardNumber=0750549>). Thus far the research focus of this green technology project has been controlling these mussels within power plant pipes, so that the use of the polluting chemical control agents can be reduced. The lack of non-target impact when treating with dead bacterial cells, however, may allow this novel green technology to also be used for *Dreissena* mussel control in lakes and rivers. This has drawn the

attention of the U.S. Bureau of Reclamation, and this federal agency is now taking the lead in coordinating research to evaluate the bacterium's potential for controlling zebra and quagga mussels in such open waters. Recent preliminary trials against zebra and quagga veligers have suggested that this planktonic larval stage may actually be even more susceptible to the bacterial treatment than attached mussel stages. This suggests that bacterial treatments in open waters could significantly reduce veliger densities, thereby preventing high mussel density buildup and slowing the spread of mussel populations.

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- U.S. Department of Energy National Energy Technology Laboratory
- U.S. Fish and Wildlife Service