

Biological Control of Zebra Mussels with *Pseudomonas fluorescens*: An Overview

Improvements Over Existing Chemical Control Methods

Power generation facilities require annual maintenance and preventive programs to keep in check the proliferation of zebra mussel infestations in their cooling water intake systems. Currently it is necessary at many of these stations to administer controlled dosages of chlorine or other types of chemicals for this purpose. Although such applications meet all existing water pollutant discharge regulatory limits, evidence exists to suggest that natural resource interest groups and regulatory agencies are reexamining the negative long-term use of chemicals for this purpose. Both groups have made it clear that safe, non-chemical alternatives for controlling mussel fouling would be environmentally beneficial. Chlorine, for example, can combine with organic compounds in water resulting in the formation of trihalomethanes, dioxins, and other potentially carcinogenic substances (United States Environmental Protection Agency 1999; Thornton 2000). Should future regulatory actions result in the loss of chemical biocides, without an available control option, electric generation organizations and many other industries that rely on 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, zebra mussel control method to replace chlorine and other biocides is critically needed by coal-burning plants.

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 zebra mussel management in power generation facilities? Sounds illogical? Well, it is widely accepted that the screening of the 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 "biotoxins" which have potential as highly selective biopesticides. In fact, the use of microbial biotoxins 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 laboratory at the New York State Museum (NYSM) has been involved in such research for over 20 years as discussed in the following section.

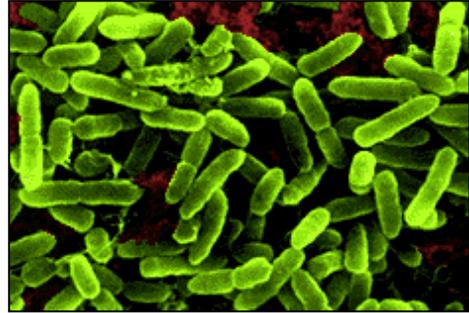
Prior Participation in Commercial Success

In the interest of eliminating polluting pesticides and thereby protecting biodiversity in New York State, the NYSM Field Research Laboratory assisted two decades ago in the commercial development of a 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.

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 the 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 biotoxins could be used as lethal agents for this new aquatic pest, the zebra mussel. The research efforts funded by ESEERCO proved this hypothesis to be true (Molloy 1998). Extensive laboratory screening trials with more than 700 bacterial strains identified a North American isolate, strain CL0145A of *Pseudomonas fluorescens*, to be lethal to zebra mussels. This bacterial species is worldwide in distribution and is present in all North American waterbodies. Normally it is a harmless bacterial species that is found protecting the roots of plants from rot and mildew. Our research, however, has shown that this same species can be fortuitously used for another purpose — control of zebra mussels (Molloy 2002). A patent for this purpose has been issued in the United States (Molloy 2001) and is pending in Canada.



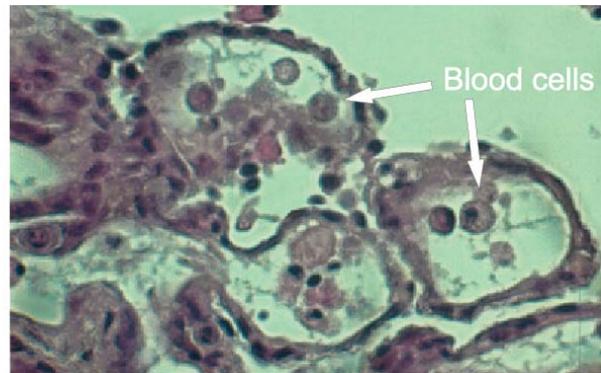
Individual cells of *P. fluorescens*.

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

Although phytoplankton is their preferred food, zebra mussels can filter out and consume bacteria as a food source (Mikheev and Sorokin 1966; Frischer et al. 2000). When a zebra mussel ingests artificially high densities of strain CL0145A, however, a biotoxin within these bacterial cells destroys the mussel's digestive system. Dead cells are equally as effective against zebra mussels as live cells, providing clear evidence that the mussels die from a biotoxin, not from infection. Techniques have already been developed at our laboratory which kill the bacteria without any reduction in their lethality to zebra mussels. Future commercial products based on this microbe will contain dead cells, thus further reducing environmental concerns.



Normal epithelial cells (arrows) lining digestive gland tubules.



Epithelial cells destroyed and digestive gland hemorrhaging following bacterial treatment.

3. Mussel Feeding: Bacteria Are Readily Ingested

Although ingestion of CL0145A cells is clearly a suicidal behavior for zebra mussels, they appear to have no adverse reaction to feeding on the cells and filter normally throughout a typical 6-hr, once-

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

through pipe treatment. In contrast, biocides, like chlorine, that are currently being used for zebra 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 CL0145A cells as "normal" bacterial food by zebra mussels is thus fortuitous and facilitates the use of this microbe as a biocontrol agent.

4. Mussel Length: All Mussel Sizes Can Be Killed

All zebra mussel sizes tested to date (length, ca. 1-25 mm) appear to be equally susceptible to kill by CL0145A. Thus, the bacteria are capable of killing all zebra mussels, irrespective of their size.



5. Mussel Species: Both Species Can Be Killed

The bacterium is lethal to both species of zebra mussels present in North America, *D. polymorpha* and *D. bugensis*. *D. polymorpha*, the most widely distributed of these two species, appears to be the most susceptible.

6. Water Hardness: Kill Best in Hard Water, the Preferred Zebra Mussel Habitat

Tests to date suggest that bacterial treatments may have reduced efficacy in soft waters with pH values less than ca. 7.4. Zebra mussels, however, rarely reach high population densities in such soft (near neutral) 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

Tests to date 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.

8. Water Temperature: Higher Kill at Warmer Temperatures

Mussel kill increases with water temperature, with >95% mortality consistently achieved in routine testing at 23°C. High mortality is still achievable even in very cold waters, e.g., 75% kill at 7°C, indicating that the bacteria are actually more effective at lower temperatures than currently commercialized chemical molluscicides used for zebra mussel control. The latter commercial biocides, e.g., chlorine, can not achieve such high mussel kill below about 15°C, thus limiting their application to warm water periods.

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

Tests to date indicate that unusually high levels of naturally-occurring particles in water (e.g., suspended mud at greater than 100 ppm) can sometimes 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. This should be easily achievable.

10. Treatment Concentration and Duration: For Maximum Kill Treat at ca. 100 ppm for 6 hr

Tests to date indicate that treatments of ca. 100 ppm (dry bacterial mass per unit volume) for 6 hr consistently obtain >95% mussel mortality at 23°C.

11. Mussel Siphoning Behavior: To Ensure Highest Kill Do Not Disturb Normal Mussel Feeding

In nature, a zebra 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, food particles are egested through the exhalant siphon. Testing to date has 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.



12. Trials at Power Plant: High Kill Can Be Achieved in Service Water

Several trials in artificial pipes have been conducted within a New York Power Authority electric power station on the Mohawk River (Crescent, New York). All tests have been carried out using power plant service water flowing through clear, ca. 6-cm diameter pipes to allow observation of mussels throughout the experiments. Tests have consistently achieved >95% mussel kill throughout the entire pipe length following treatments of ca. 100 ppm (dry bacterial mass per unit volume) for 6 hr at 23-25°C.



High mussel kill (>95%) has been consistently achieved in trials inside a power 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.

13. Nontarget Trials: Outstanding Species 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 tested to date, including ciliates, bivalves, and fish:

- **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.
- **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*).
- **Fish:** No bacteria-induced mortality to 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*). Fish can not tolerate exposure to high levels of live bacteria, possibly due to low dissolved oxygen. Because of this sensitivity, our fish trials were conducted with dead bacteria, and the results indicated that applications of these dead bacteria, while harmless to the fish, were highly lethal to the zebra mussels. Future commercial products based on this microbe will contain almost exclusively dead cells.

Although the above-mentioned ciliate, bivalve, and fish laboratory trials have suggested that bacterial strain CL0145A may truly have promise as an environmentally-safe biocontrol agent, it is naive to think that this strain will prove so selective as to only affect zebra mussels. For this reason, further nontarget tests are planned as part of the proposed research.



Brown trout

Fathead
minnows

Sunfish

Ciliates

Blue
mussel

Unionids

There has been no mortality to nontarget species tested to date.

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

Research was undertaken to characterize, isolate, and identify the specific mussel-killing natural product that is associated with *P. fluorescens* strain CL0145A 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 zebra mussels, providing evidence that the biotoxin was heat-labile and protein in nature. Even though we were able to separate the toxin from the cells by chemical treatment (i.e., make the cells nontoxic), we were unable to develop an effective method to deliver the solubilized toxin molecules to the zebra mussels on particles that they would ingest. As a result, we altered our biochemical experimental approach and decided to search the literature for documented products from *P. fluorescens* that matched characteristics of our biotoxin. A candidate molecule investigated was glycine dehydrogenase, an enzyme that catalyzes the conversion of the amino acid glycine to hydrogen cyanide (HCN). First we analyzed strain CL0145A and confirmed that it did produce trace amounts of HCN. Then our testing focused on determining whether HCN was the biotoxin that was responsible for causing zebra mussel death. Our experiments demonstrated that treating CL0145A cells with an irreversible flavoenzyme inhibitor, diphenyliodonium chloride (DPI), successfully blocked the cell's ability to produce HCN. Even though DPI-treated cells no longer produced trace amounts of HCN, the cells still remained equally lethal to zebra mussels, demonstrating that HCN was not the biotoxin that caused mussel death. Further efforts to identify the biotoxin are on hold due to lack of resources. Plans,

however, are being drawn up for seeking funds to investigate genetic approaches for determining the identity of the biotoxin.

15. Current Research Activities and Plans

Funded by the U.S. Department of Energy National Energy Technology Laboratory, we are currently developing a dead-cell formulation that is economical to produce, has good shelf life, and demonstrates low environmental impact, yet maintains toxicity against zebra mussels in power plant service water pipes. CL0145A cell toxicity is being improved in experiments that manipulate components of a chemically-defined fermentation medium. Economic medium components will then be evaluated for use in commercial-scale production runs. Although non-target safety data have been encouraging (see Section 13 above), further safety testing is planned with both cells and final formulation as required by the U.S. Environmental Protection Agency. Treatment experiments in service water pipes are planned at Rochester Gas & Electric's Russell Power Station.

References

- Frischer, M., Nierzwicki-Bauer, S., Parsons, R., Vathanodorn, K., and Waitkus, K. 2000. Interactions between zebra mussels (*Dreissena polymorpha*) and microbial communities. *Canadian Journal of Fisheries and Aquatic Sciences* 57:591-599.
- Mikheev, V. P. and Sorokin, Y. L. 1966. Quantitative studies of *Dreissena polymorpha* habits using the radiocarbon method. *Zhurnal Obshchei Biologii* 27:463-472. (In Russian.)
- Molloy, D. (ed.) 1982. Biological Control of Black Flies (Diptera: Simuliidae) with *Bacillus thuringiensis* var. *israelensis* (Serotype 14): A Review with Recommendations for Laboratory and Field Protocol. *Miscellaneous Publications of the Entomological Society of America* 12(4):30 pp.
- Molloy, D. P. 1990. Progress in the biological control of black flies with *Bacillus thuringiensis* var. *israelensis*, with emphasis on temperate climates. Pages 161-186 in *Bacterial Control of Mosquitoes and Black Flies: Biochemistry, Genetics & Applications of Bacillus thuringiensis israelensis and Bacillus sphaericus* (H. de Barjac and D. Sutherland, eds.) Rutgers University Press, New Brunswick, New Jersey.
- Molloy, D. P. 1991. Biological control of zebra mussels: Use of parasites and toxic microorganisms. *Journal of Shellfish Research* 10:260.
- Molloy, D. P. 1992. Impact of the black fly (Diptera: Simuliidae) control agent *Bacillus thuringiensis* var. *israelensis* on chironomids (Diptera: Chironomidae) and other nontarget insects: Results of ten field trials. *Journal of the American Mosquito Control Association* 8:24-31.
- Molloy, D. P. 1998. The potential for using biological control technologies in the management of *Dreissena* spp. *Journal of Shellfish Research* 17:177-183.
- Molloy, D. P. 2001. A Method for Controlling *Dreissena* Species. U. S. Patent and Trademark Office, U. S. Department of Commerce. Patent No. 6,194,194 February 27th. 4 pp.
- Molloy, D. P. 2002. Biological control of zebra mussels. Pages 86-94 in *Proceedings of the Third California Conference on Biological Control*. University of California, Davis.
- Molloy, D. and Jamnback, H. 1981. Field evaluation of *Bacillus thuringiensis* var. *israelensis* as a black fly (Diptera: Simuliidae) biocontrol agent and its effect on nontarget stream insects. *Journal of Economic Entomology* 74:314-318.
- Molloy, D. P. and Struble, R. H. 1989. Investigation of the microbial control of black flies (Diptera: Simuliidae) with *Bacillus thuringiensis* var. *israelensis* in the Adirondack Mountains of New York. *Bulletin of the Society of Vector Ecology* 14:266-276.
- Rodgers, P. B. 1993. Potential of biopesticides in agriculture. *Pesticide Science* 39:117-129.
- Thornton, J. 2000. *Pandora's Poison: Chlorine, Health, and a New Environmental Strategy*. MIT Press, Cambridge, Massachusetts. 599 pp.
- United States Environmental Protection Agency. 1999. Wastewater technology fact sheet: Chlorine disinfection. U. S. Environmental Protection Agency, Washington, DC. EPA/832-F99-062. 7 pp.

Acknowledgments

Prior funding for this zebra mussel biocontrol project from the following agencies is gratefully acknowledged:

- ESEERCO New York State Utilities
- New York Sea Grant
- New York State Department of Environmental Conservation
- New York State Energy Research and Development Authority
- U.S. Army Corps of Engineers
- U.S. Department of Energy National Energy Technology Laboratory
- U.S. Fish and Wildlife Service