

# 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 the proliferation of zebra mussel infestations in their cooling water intake systems under control. 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. 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 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 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 20 years as discussed in the following section.

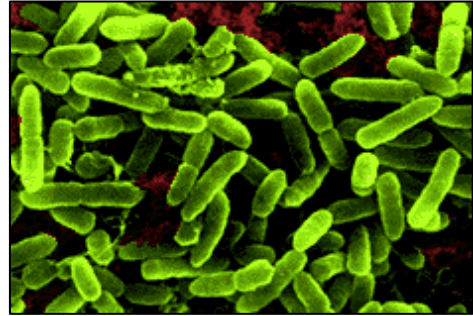
## Prior Participation in Commercial Success

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

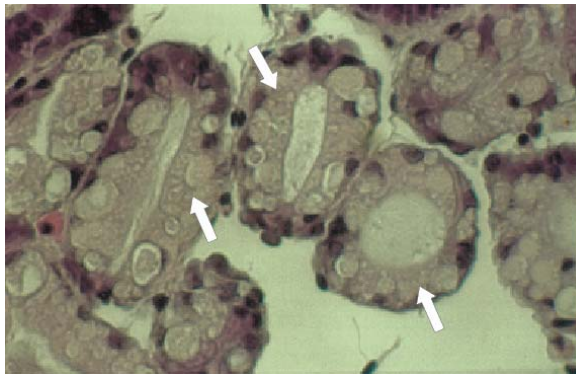
The Empire State Electric Energy Research Corporation (ESEERCO<sup>1</sup>) — faced with the threat of zebra mussels fouling electric power facilities within New York State — contracted with our NYSM Field Research Laboratory in 1991 for the screening of bacteria as potential biological control agents. 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 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 of more than 700 bacterial strains identified a North American isolate, strain CL145A of *Pseudomonas fluorescens*, to be lethal to zebra mussels. *Pseudomonas fluorescens* 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 — the control of zebra mussels (Molloy 2002). A patent for this purpose has been issued in both the United States (Molloy 2001) and Canada (Molloy 2004).



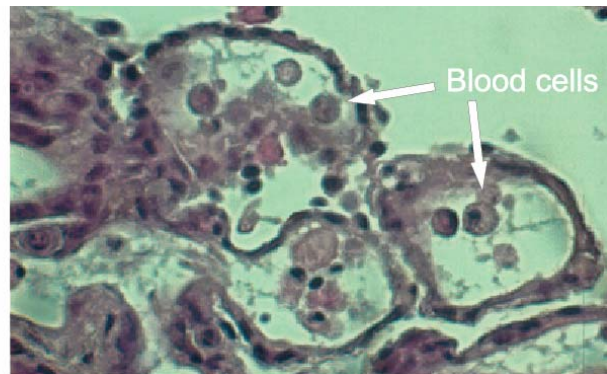
**Individual cells of *P. fluorescens*.**

### 2. Zebra Mussels Die from a Natural Toxin: 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 CL145A, however, a toxin 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 toxin, not from infection. Techniques have already been developed at our laboratory that 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 CL145A 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-

<sup>1</sup> 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 CL145A cells as "normal" bacterial food by zebra mussels 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 CL145A. Thus, the bacteria are capable of killing all zebra mussels, irrespective of mussel size.



#### 5. Mussel Species: Both Species Can Be Killed

The bacterium is lethal to both species of zebra mussels present in North America, *Dreissena polymorpha* and *Dreissena bugensis*.

#### 6. Water Hardness: Mussel Kill is Highest 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 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. 50-100 ppm (dry bacterial mass per unit volume) for 6 hr consistently obtain high mussel mortality.

**11. Mussel Siphoning Behavior: 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**

Trials routinely achieving high 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) and at the Rochester Gas & Electric's Russell Power Station on Lake Ontario (Rochester, New York).



**Very high mussel kill (>95%) was consistently achieved in 6-hr treatments 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.**

### 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 CL145A 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 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 zebra mussels, providing evidence that the toxin 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 toxin. 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 CL145A and confirmed that it did produce trace amounts of HCN. Then our testing focused on determining whether HCN was the toxin that was responsible for causing zebra mussel death. Our experiments demonstrated that treating CL145A cells with an irreversible flavoenzyme inhibitor, diphenyleneiodonium 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 toxin that caused mussel death. Further efforts to identify the toxin 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 toxin.

### 15. Current Research Activities and Plans

Funded by the U.S. Department of Energy National Energy Technology Laboratory (DOE-NETL 2006) and the National Science Foundation, we are currently working on the development of a dead-cell formulation that will have good shelf life and low environmental impact. CL145A cell toxicity is being improved in experiments that manipulate components of a chemically-defined fermentation medium as well as the gene(s) within the bacterium that produce the mussel-killing toxin.

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