Overview of a Novel Green Technology: Biological Control of Zebra and Quagga Mussels with *Pseudomonas fluorescens*

(Version 6: Updated August 24, 2007)

Daniel P. Molloy and Denise A. Mayer
New York State Museum

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 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.

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

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

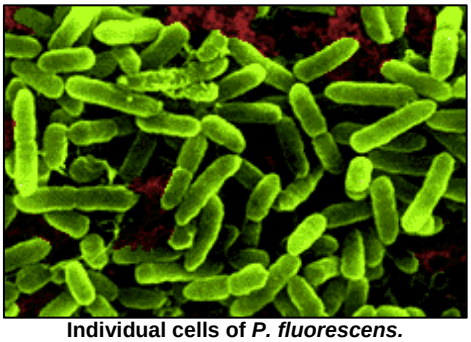
In the interest of eliminating polluting pesticides and thereby protecting biodiversity in New York State, our NYSM Field Research Laboratory assisted in the commercial development of *Bti* — the selectively toxic bacterium, *Bacillus thuringiensis* subsp*. israelensis* — as the first biological control 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 Resources)

Research Progress To Date

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

The Empire State Electric Energy Research Corporation (ESEERCO[1]) — 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.



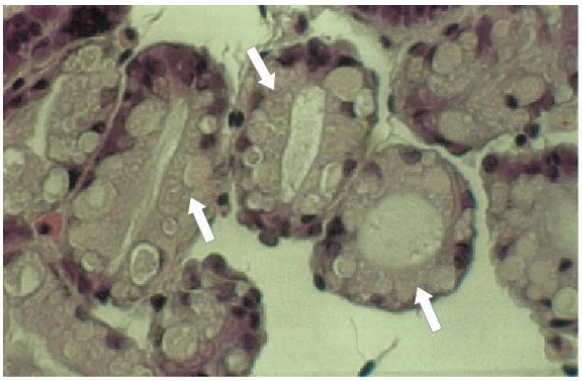
Individual cells of *P. fluorescens.*

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).

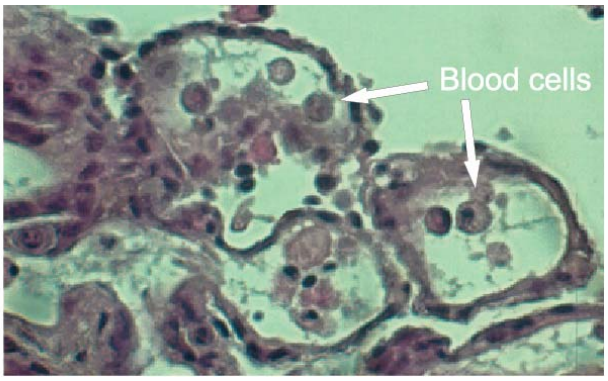
[1] A research consortium of New York State's electric power generation companies.

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.

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. Susceptibility of the planktonic mussel stage has not yet been tested.

All mussel sizes show the same susceptibility.

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

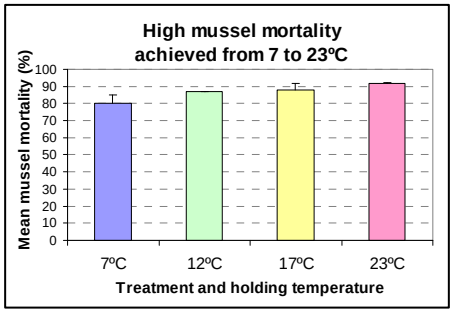
Tests to date suggest that bacterial treatments may have reduced efficacy in soft waters with pH values less than ca. 7.4. *Dreissena* mussels, however, rarely reach high population densities in such (near neutral or acidic) waters, and thus, infested pipes in power plants typically will have more alkaline 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 23ºC against zebra mussels. High mortality is still achievable even in very cold waters, e.g., near 80% kill at 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 10ºC, 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 23ºC and two months at 7ºC.



Mortality increases with temperature.

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*).

Brown trout | **Fathead minnows** | **Sunfish**

Daphnids | **Ciliates** | **Blue mussels** | **Unionids**

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.

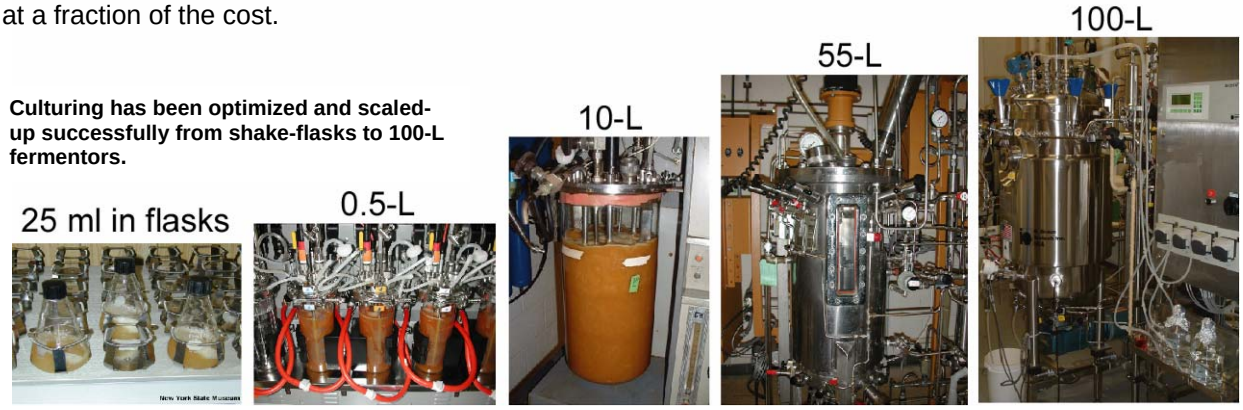
A biochemical approach was undertaken in an attempt to get the toxin to adhere to particles which could hopefully be used to identify the toxin. Even though the toxin was able to be separated from the cells by chemical treatment (i.e., make the cells nontoxic), efforts to develop an effective method 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, diphenyleneiodonium 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. fluoresens*-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. Current and Recent Project Activities

Research on this NYSM project is currently supported by the U.S. Department of Energy National Energy Technology Laboratory (DOE-NETL 2007). With this funding, the complete genome of strain CL145A has been sequenced, and research is currently underway to identify the gene(s) that produce(s) the mussel killing biotoxin. Successful identification of the gene(s) could lead to identification of the biotoxin molecule and ultimately to the production of cells of higher toxicity.

Recently a grant from the National Science Foundation (NSF 2007) supported experimentation which developed a protocol to produce a dead-cell, powdered commercial formulation that would have good shelf life due to biotoxin stability.

The lack of non-target impact when treating with dead cells may allow this novel green technology to also be used for *Dreissena* mussel control in open waters, such as lakes and rivers. Thus far, however, the research focus of the project has been controlling these mussels within power plant pipes. 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 that 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.

17. Commercialization Partnership

Following a nation-wide search, the NYSM has chosen 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 will be 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.

References

DOE-NETL (Department of Energy National Energy Technology Laboratory). 2007. Power Systems Advanced Research – Environmentally-Safe Control of Zebra Mussel Fouling Fact Sheet. Accessed March 14, 2007 @ http://www.netl.doe.gov/publications/factsheets/project/Proj291.pdf

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. United States Patent and Trademark Office, U. S. Department of Commerce. Patent No. 6,194,194. (Filed December 17, 1997 & issued February 27, 2001.) 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. P. 2004. A Method for Controlling *Dreissena* Species. Canadian Intellectual Property Office, Industry Canada. Patent No. 2,225,436. (Filed December 27, 1997 & issued December 21, 2004.) 11 pp.

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.

NSF (National Science Foundation) 2007. STTR Phase I: Stabilization of a Protein Toxin - An Essential Step in the Commercialization of an Innovative Method for Controlling Zebra Mussels. Accessed March 14, 2007 @ http://www.nsf.gov/awardsearch/showAward.do?AwardNumber=0610252

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

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

Private

- ESEERCO New York State Power Generation Utilities

State

- New York Sea Grant
- New York State Department of Environmental Conservation
- New York State Energy Research and Development Authority

Federal

- National Science Foundation
- U.S. Army Corps of Engineers
- U.S. Department of Energy National Energy Technology Laboratory
- U.S. Fish and Wildlife Service