TECHNICAL REPORT
(40751R011)

Report Title:	**Factors Affecting Zebra Mussel Kill by the Bacterium *Pseudomonas fluorescens***
Type of Report:	**Final**
Reporting Period Start Date:	**October 1, 2000**
Reporting Period End Date:	**December 31, 2003**
Principal Author:	**Daniel P. Molloy**
Date Report Was Issued:	**February 24, 2004**
DOE Award No.:	**DE-FC26-00NT40751**
Name and Address of Submitting Organization:	**New York State Education Department State Education Building - Room 125 Albany, NY 12234**

DISCLAIMER

ABSTRACT

The specific purpose of this research project was to identify factors that affect zebra mussel kill by the bacterium *Pseudomonas fluorescens*. Test results obtained during this three-year project identified the following key variables as affecting mussel kill: treatment concentration, treatment duration, mussel siphoning activity, dissolved oxygen concentration, water temperature, and naturally suspended particle load. Using this latter information, the project culminated in a series of pipe tests which achieved high mussel kill inside power plants under once-through conditions using service water in artificial pipes.

TABLE OF CONTENTS

EXECUTIVE SUMMARY

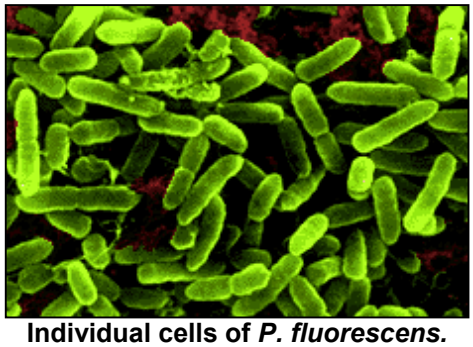
Under this USDOE-NETL contract, the bacterium *Pseudomonas fluorescens* strain CL0145A was being evaluated as a biocontrol agent for zebra mussels. The specific purpose of the contract was to identify factors that affect mussel kill. Test results obtained during this three-year project indicated that key experimental factors are: treatment concentration, treatment duration, mussel siphoning activity, dissolved oxygen concentration, water temperature, and concentration of naturally suspended particles. Variables of lesser importance are: mussel size, zebra mussel species, water pH, whether bacterial concentration was very constant or pulsating during a treatment, presence of air bubbles passing through a pipe, pipe orientation, mussel density, and water velocity. Using this latter information on the relative importance of abiotic and biotic factors, high zebra mussel mortality was achieved in a series of pipe tests inside power plants under once-through conditions using service water in artificial pipes.

REPORT

Background Leading Up to This USDOE Project

Power generation facilities require annual maintenance and preventive programs to keep in check the proliferation of zebra mussel infestations in their service water and 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. 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.

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 the New York State Museum (D. P. Molloy, Principal Investigator) 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, it was hypothesized 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. 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. A patent for this purpose has been issued in the United States and is pending in Canada.



Individual cells of *P. fluorescens.*

USDOE Project DE-FC26-00NT40751

In October 2000 this project was initiated to investigate the importance of selected biotic and abiotic factors in achieving zebra mussel kill with *P. fluorescens* strain CL0145A. The following information was acquired during the three-year research period.

1. Mussel Siphoning

Is mussel siphoning critically important to achieving kill? 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 indicated that the more active this siphoning behavior is, the higher the mortality

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

that will be achieved by a bacterial treatment (Quarterly Technical Report R40751R09). Thus, any stress factors (e.g., pipe vibrations) that cause the mussels to close their shells during treatment will likely reduce mortality. Although ingestion of CL0145A cells through the mussel's inhalant siphon is clearly a suicidal behavior for zebra mussels, they appear to have no adverse reaction to feeding on the cells and siphon normally throughout a typical 6-hr, once-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.

Siphons of a zebra mussel.

2. Mussel Length

Are small and large mussels equally susceptible to kill? Tests provided evidence that the bacteria are capable of killing all sizes of zebra mussels equally, e.g., mussels averaging 9.4 mm and 15.0 mm had the same mean mortality (Quarterly Technical Report R40751R01).

3. Mussel Species

Is bacterial treatment equally lethal to the two zebra mussel species present in North America, *Dreissena polymorpha* and *Dreissena bugensis*? To address this question, laboratory experiments were conducted with sympatric populations of these two species occurring both in Lake Erie and Lake Ontario. Test results indicated that both species are of near equal susceptibility (Quarterly Technical Report R40751R02). Thus, the bacterium should be equally effective in controlling zebra mussels in pipes, irrespective of which species is present.

4. pH of Water

What is the effect of the pH of water on the efficacy of treatment? To address this question, tests were conducted in a variety of synthetic fresh water types with pH ranges of 7.2 to 7.8 (very soft water), 7.5 to 7.9 (soft water), 7.8 to 8.1 (moderately hard water), 8.1 to 8.4 (hard water), and 8.4 to 8.6 (very hard

water). Tests were encouraging since the bacteria were effective at killing zebra mussels at all pH values ranging from 7.2 to 8.6 (Quarterly Technical Report R40751R06).

5. Dissolved Oxygen Concentration

What is the effect of oxygen concentration on the efficacy of treatment? To address this question, tests were conducted in conditions having dissolved oxygen (DO) concentrations ranging from very low (0 to 1 ppm) to very high (6 to 8 ppm). Results indicated that strain CL0145A was effective at killing zebra mussels at all DO concentrations, but that the highest mussel mortality can be achieved in conditions of moderate to high DO (Quarterly Technical Report R40751R07).

6. Water Temperature

What is the effect of water temperature on the efficacy of treatment?. To address this question, tests were conducted at water temperatures of 23, 17, 12, and 7ºC (Quarterly Technical Report R40751R05). Results indicated that strain CL0145A was effective at killing zebra mussels at all four temperatures. Mussel kill increased with water temperature, with >95% mortality consistently achieved in tests at 23ºC. High mortality, however, was still achievable even in very cold waters, e.g., 75% kill at 7°C.

An additional series of warm and cold water tests indicated that the total percentage mussel mortality achieved should be the same at 13ºC and 23ºC (Quarterly Technical Report R40751R10). This is significant because the development of a zebra mussel control method that is equally efficacious in such a wide range of temperatures broadens its usefulness as a potential commercial product. Moreover, the cold water tests indicated that this bacterial control agent is actually more effective at lower temperatures than currently commercialized chemical molluscicides that are used for zebra mussel control. The latter commercialized products, such as chlorine, are not able to achieve high mussel kill below ca. 18ºC.

7. Suspended Particles

Could mussel kill be reduced by the presence of other suspended particles? Since bacterial cells are lethal only if ingested by mussels, waters containing very high levels of naturally suspended particles might reduce the mortality that can be achieved by a bacterial treatment. If true, this inhibition might occur as a result of particle exclusion, i.e., there could be reduced ingestion of bacterial cells since they represent a reduced percentage of all particles ingested. Our tests indicated that a range of particle concentrations that might naturally exist in a turbid river did not inhibit mussel kill by the bacterial cells, but that an artificially high load of natural particles was capable of causing a reduction in kill (Quarterly Technical Report R40751R09). To be conservative, therefore, future pipe treatments should be timed to occur when intake waters have relatively low quantities of naturally suspended particulate matter.

8. Treatment Concentration & Duration

What dosage should be used to achieve high mussel kill? As expected, we learned that kill was dose dependent, i.e., the concentration of bacteria suspended in water was directly correlated with mussel kill (Quarterly Technical Report R40751R04). Laboratory tests were also conducted to determine the most effective method of treating zebra mussels with a defined mass of inoculum under warm (ca. 23°C) and cold (ca. 13°C) water conditions (Quarterly Technical Report R40751R10). The specific question addressed at each temperature was whether higher mortality could be achieved by exposing mussels to lower concentrations of inoculum over a longer period of time *versus* higher concentrations over a shorter time period. The results of the warm water tests suggested that as long as the total quantity of bacteria applied during the entire treatment period was the same, similar mussel mortality would be achieved in treatments lasting 1.5 hr to 12.0 hr. It was noteworthy that 1.5-hr treatments in the warm water tests consistently achieved >90% mussel kill as no other zebra mussel control method has been reported that can achieve such high kill following such a single, short treatment period. The results of the cold water tests suggested that when treating with a defined mass of bacteria, higher mortality will most likely be achieved by treating for 3 to 12 hr (*versus* 1.5 hr).

9. Constant *versus* Pulsating Bacterial Concentration

Is it critically important to maintain a very constant bacterial concentration during a treatment? Test results were encouraging since they suggested that it was not important whether the bacterial concentration was constant or pulsating during a treatment, as long as the total mass of bacteria applied was the same to all pipes over the same time period (Quarterly Technical Report R40751R10).

10. Air Bubble Disturbance with a Pipe

Could air bubbles passing through a pipe effect mussel kill? Test results were encouraging since they suggested that if air bubbles were being carried by currents through power plant pipes, mussel kill should not be adversely effected during a treatment (Quarterly Technical Report R40751R10).

11. Pipe Orientation

Might mussel kill be different inside a vertical *versus* horizontal pipe? Test results were encouraging since they suggested that the orientation of a pipe should not effect mussel kill (Quarterly Technical Report R40751R10).

12. Mussel Density

What is the effect of mussel density on the efficacy of treatment? Test results were encouraging since pipes with mussels in low density (6.3 mussels/cm) had the same kill as pipes with high mussel density (32.1 mussels/cm). This suggests that high mussel kill will be achievable irrespective of the density of mussels in a pipe.

13. Water Velocity

What is the effect of water velocity on the efficacy of treatment? Test results were encouraging since mussel kill was the same irrespective of whether mean water velocity was 4.4 or 8.8 cm/sec. This suggests that high mussel kill will be achievable in sections of pipes where mussels attach under these flow rates.

14. Trials at Power Plant

Laboratory trials can be effective, but how does the bacteria perform in actual power plant service water? Small-scale, service-water tests in artificial pipes within a hydropower station demonstrated that exposure to bacteria can result in high zebra mussel mortality (Quarterly Technical Report R40751R10). Tests were conducted using service water (mean 23°C) within a New York Power Authority hydrostation on the Mohawk River (Crescent, NY). Four acrylic pipes (L x D - 25 x 5 cm) were each seeded with 100 mussels (9-mm mean length) on the day prior to treatment and then were treated at ca. 80 ppm with CL0145A cells throughout a 6-hr period while maintaining a constant water flow rate (4L/hr) within each pipe. Identical untreated control pipes with 100 mussels (3 replicates) were also maintained. Following the 6-hr treatment period, mussels continued to be held in pipes and received fresh service water (23°C) for 14-days. The entire test was conducted twice over a one-month period. Mean (±SD) mussel mortalities in these two tests were 97.2 (±1.5)% and 99.7 (±0.6)%. In contrast, mean (±SD)

Examples of the acrylic minipipes used in the tests. The pipe with clear water on left is an untreated control, whereas water in other pipes has a grayish color due to presence of suspended bacteria during treatment. Mussels are visible attached to walls of pipe.

mussel mortalities in untreated control pipes were, respectively, 2.0 (±1.7)% and 3.7 (±1.5)%.

Once-through, 6-hr treatments conducted within a power plant in larger diameter artificial acrylic pipes (5.7 cm diameter) demonstrated that high mussel kill could be achieved along the entire pipe length. Two tests (each with 3 replicate pipes) were carried out during the summer of 2003. The initial test was conducted at ca. 141 ppm using 26°C service water in pipes of 8.6 m length and achieved a mean (±SD) kill of 96.0 (±1.7)% and 95.7 (±2.5)%, respectively, at the beginning and end of the pipes (upstream control mortality = 6.7 (±3.2)%). The second test was conducted at ca. 116 ppm using 23°C service water in pipes of 17.1 m length and achieved a mean (±SD) kill of 97.0 (±1.0)% and 97.0 (±2.7)%, respectively, at the beginning and end of the pipes (upstream control mortality = 3.7 (±0.6)%). These results were encouraging since they demonstrated that, based on the knowledge we had gained from laboratory tests to date, we were able to design treatment protocols which successfully achieved very high mussel kill in service water within a power plant.

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.

PLANS FOR FUTURE RESEARCH

Based on the success of the above-mentioned research, this project will be continued under a new three-year USDOE Cooperative Agreement (i.e., DE-FC26-03NT41909). A primary objective of this new agreement is to learn how to economically produce and formulate *Pseudomonas fluorescens* cells in order that they will prove to have good product shelf-life, be environmentally safe, and be highly effective in killing zebra mussels in power plant service water pipes. Accomplishment of this latter objective and a favorable evaluation of the environmental safety data regarding large-scale bacterial treatment of power plant water systems by the U.S. Environmental Protection Agency would make commercialization at the end of the three-year agreement a reality. The third year of this new agreement includes treatment of the entire house service water system of a coal-fired power plant in Rochester as a demonstration of the feasibility of this zebra mussel control method.

TECHNOLOGY AND INFORMATION TRANSFER

Presentations

Molloy, D. P. Black flies and zebra mussels: Can we really control them with biological agents? July 16, 2001. Rensselaer Polytechnic Institute Darrin Fresh Water Institute, Lake George.

Molloy, D. P., Mayer, D. A., Karatayev, A. Y., Burlakova, L. E., and Gaylo, M. J. Challenges in the scale-up of *Pseudomonas fluorescens:* A promising biopesticide for zebra mussel control. Annual Meeting of the Society for Industrial Microbiology. July 31, 2001. St. Louis, Missouri.

Molloy, D. P., Mayer, D. A., Gaylo, M. J., Presti, K. T., Karatayev, A. Y., and Burlakova, L. E. Progress in the biological control of zebra mussels with microbial toxin. Annual Meeting of the National Shellfisheries Association. April 17, 2002. Mystic, Connecticut. (Poster.)

Molloy, D. P., Mayer, D. A., Gaylo, M. J., Presti, K. T., Karatayev, A. Y., and Burlakova, L. E. Biological control of zebra mussels with microbial toxin: An overview of research progress. Annual Meeting of the American Society for Microbiology. May 20, 2002. Salt Lake City, Utah. (Submitted poster.)

Mayer, D. A., and Molloy, D. P. 2002. Progress in developing a microbial agent for the biological control of zebra mussels. July 18, 2002. New York State Museum Seminar Series. Albany, New York. (Invited lecturer.)

Mayer, D. A., Molloy, D. P., and Presti, K. T. Progress in the culturing scale-up of *Pseudomonas fluorescens* strain CL0145A for use as a biopesticide against zebra mussels (*Dreissena polymorpha*). Annual Meeting of the Society for Industrial Microbiology. August 13, 2002. Philadelphia, Pennsylvania. (Submitted poster.)

Molloy, D. P. Biological control of zebra mussels. Third California Conference on Biological Control. August 16, 2002. University of California, Davis, California. (Invited speaker.)

Molloy, D. P. Les epèces nonindigènes – un problem international. September 18, 2002. Université de Metz, France. (Invited lecturer.)

Molloy, D. P. Potential for the biological control of zebra mussels. November 7, 2002. Department of Biology, Stephen F. Austin State University, Nacogdoches, Texas. (Invited lecturer.)

Molloy, D. P. David versus Goliath: Controlling zebra mussels with a tiny microbe. March 6, 2003. Great Lakes Conference, Michigan State University, East Lansing, Michigan. (Invited speaker.)

Molloy, D. P., Mayer, D. A., and Presti, K. T. Biological control of zebra mussels with microbial toxin: Small-scale once-through pipe tests. Twelfth International Conference on Aquatic Invasive Species. June 10, 2003. Windsor, Ontario. (Submitted paper.)

Mayer, D. A., Molloy, D. P., Gaylo, M. J., and Presti, K. T. Small-scale flow-through application of *Pseudomonas fluorescens* strain CL0145A in the service water of a hydropower facility for the biological control of zebra mussels. Annual Meeting of the Society for Industrial Microbiology. August 11, 2003. Minneapolis, Minnesota. (Submitted poster.)

Molloy, D. P. David versus Goliath: Controlling zebra mussels with a tiny microbe. November 7, 2003. NYSDEC Pesticide Workshop, SUNY Forest Ranger School, Wanakena, New York. (Invited speaker.)

Mayer, D. A. *Pseudomonas fluorescens* Strain CL0145A as a Biological Control Agent against Zebra Mussels. December 4, 2003. Drew University, Madison, New Jersey. (Invited speaker.)

Media Coverage

Poughkeepsie Journal. May 7, 2002. Fight zebra mussel invasion of Hudson. Page 6A.

WPTZ (NBC Affiliate in Plattsburgh, NY). May 13, 2002. Natural born killer? (2 minute story)

Science News. June 1, 2002. Mussel muzzled: Bacterial toxin may control pest. 161, pages 339-340.

Chicago Tribune. June 16, 2002. Menacing zebra mussels may run aground: Scientist digs up soil bacteria that can kill mollusks. Section 4, page 3.

Environment News Service - Electronic International Daily Newswire. June 19, 2002. Bacteria toxin kills zebra mussels. http://ens-news.com/ens/jun2002/2002-06-12-09.asp#anchor7

International Water and Dam Construction. 2002. World News: Zebra mussel control. July Issue: page 5.

Fulton, E. 2003. Common bacterium shows promise in controlling zebra mussels. *Hydro Review* 22(1):74-75.