# TECHNICAL REPORT
# (R41909R09)

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| **Report Title:** | **Environmentally Safe Control of Zebra Mussel Fouling** |
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| **Principal Author:** | **Daniel P. Molloy** |
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## DISCLAIMER



## ABSTRACT

The two primary objectives of this USDOE-NETL contract were successfully achieved during the project:

#1) to accelerate research on the development of the bacterium *Pseudomonas fluorescens* strain CL145A (*Pf*-CL145A) as a biocontrol agent for zebra mussels (*Dreissena polymorpha*) and quagga mussels (*Dreissena rostriformis bugensis*) ─ two invasive freshwater bivalve species that are infesting water pipes in power plants;

#2) to identify a private-sector company that would move forward to commercialize *Pf*-CL145A as a substitute for the current polluting use of biocide chemicals for control of these dreissenid mussels in power plant pipes.

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## EXECUTIVE SUMMARY

The two primary objectives of this USDOE-NETL contract were successfully achieved during the project.

Objective #1: The project made significant progress during the contract period in the following research areas:

1A. Unique Toxicity of *Pseudomonas fluorescens* Strain CL145A
Of the seven strains of *P. fluorescens* that had been previously laboratory tested, only *Pf*-CL145A had been found to be highly lethal, i.e., at dosages that produce >90% zebra mussel kill with *Pf*-CL145A, the other 6 strains of *P. fluorescens* caused only 0-11% mortality. The unique nature of *Pf*-CL145A as an effective control agent was further verified in tests during this project with three additional strains of *P. fluorescens*. These tests indicated that strain CL045A was again the only strain capable of inflicting high levels of mortality on zebra mussels.

1B. Effective Treatment Dosages
Determining the ideal treatment dosage for large-scale plant treatments will be an important cost-cutting tool and will help ensure maximum efficacy. A majority of the bacteria used to treat mussels in once-through, flowing water passes through the system and is discharged without having ever been ingested, especially at high flow rates. Discovering the treatment concentration and duration that minimizes bacterial waste and maximizes efficacy is essential. A comprehensive series of dosage trials (varying treatment duration and bacterial concentration) indicated that a 6-hr treatment at 50 ppm (i.e., 50 mg dry weight of bacterial cells per liter of power plant intake water) is optimal to achieve mussel kill.

1C. Nontarget Safety
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. Experiments conducted during this project indicated that *Pf*-CL145A was not lethal to this species. This further advanced the potential for commercialization of *Pf*-CL145A as *D. magna* is one of the primary species that the U.S. Environmental Protection Agency uses to assess biopesticide safety.

1D. Economic Commercial Production
In experiments conducted during this project, the cost of the fermentation medium needed to produce high yields of toxic *Pf*-CL145A cells was reduced by ca. 88%. This bacterial approach to zebra mussel control has now become more economically competitive with the cost of biocides currently used by power plants.

1E. Identification of Gene(s) That Produce the Mussel-Killing Biotoxin
*Pf*-CL145A cells are currently not toxic enough to consistently achieve high kill of zebra mussels (i.e., >90%) in infested pipes, and this must be overcome for this environmentally safe biopesticide to compete in the market place with current polluting (yet inexpensive) chemical biocides. Identifying the *Pf*-CL145A gene(s) responsible for

toxin production is viewed as critical in this process. The complete draft genome sequence of *Pf*-CL145A was received and annotation completed. Comparative analyses of the *Pf*-CL145A genome to genome sequences of non-toxic *P. fluorescens* strains have identified regions of the *Pf*-CL145A genome that are unique and thus represent regions that contain candidate toxin-producing genes. Progress was also made toward isolating the genomic regions that are uniquely expressed in populations of *Pf*-CL145A that are toxic to zebra mussels. The sequences of these isolated genomic regions will help us identify target genes that are responsible for toxin production by mapping to the annotated complete genome sequence of *Pf*-CL145A that has previously been achieved.

Objective #2: The project successfully attracted a commercial partner.

Following a nation-wide search, the Principal Investigator's institution, the New York State Museum (NYSM) chose Marrone Organic Innovations (MOI) as its commercialization partner. MOI is a biopesticide company whose staff have unparalleled experience in the discovery, development, and marketing of natural products for pest management. The commercialization partnership between MOI and NYSM was subsequently selected by the National Science Foundation (NSF) for a $500,000 award to further accelerate the path to commercialization of this environmentally safe control agent.

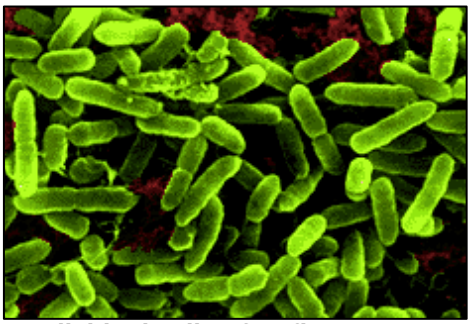
## REPORT

### 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 New York State electric utility industry, faced with the threat of zebra mussels fouling their power generation facilities — 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 CL145A 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. Patents for this purpose were issued prior to the initiation of this DE-FC26-03NT41909 project in the United States and Canada.



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

In 2000-2003, USDOE-NETL funded the PI's laboratory (Project DE-FC26-00NT40751) to identify factors that affect zebra mussel kill by the *Pseudomonas fluorescens* strain CL145A. Test results obtained during that 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.

## USDOE Project DE-FC26-03NT41909

In October 2003 this current project was initiated with two primary objectives:

- Objective #1) to accelerate research on the development of the bacterium *Pseudomonas fluorescens* strain CL145A (*Pf*-CL145A) as a biocontrol agent for zebra mussels (*Dreissena polymorpha*) and quagga mussels (*Dreissena rostriformis bugensis*) ─ two invasive freshwater bivalve species that are infesting water pipes in power plants;

Objective #2) to identify a private-sector company that would move forward to commercialize *Pf*-CL145A as a substitute for the current polluting use of biocide chemicals for control of these mussels in power plant pipes.

## Objective #1

The following research progress was achieved during the project period.

1A. Unique Toxicity of *Pseudomonas fluorescens* Strain CL145A

Of the seven strains of *P. fluorescens* that had been previously laboratory tested, only *Pf*-CL145A had been found to be highly lethal, i.e., at dosages that produce >90% zebra mussel kill with *Pf*-CL145A, the other 6 strains of *P. fluorescens* caused only 0-11% mortality. The unique nature of *Pf*-CL145A as an effective control agent was further verified in tests during this project with three additional strains of *P. fluorescen*s. These tests indicated that strain CL045A was again the only strain capable of inflicting high levels of mortality on zebra mussels.

1B. Effective Treatment Dosages

Determining the ideal treatment dosage for large-scale plant treatments will be an important cost-cutting tool and will help ensure maximum efficacy. A majority of the bacteria used to treat mussels in once-through, flowing water passes through the system and is discharged without having ever been ingested, especially at high flow rates. Discovering the treatment concentration and duration that minimizes bacterial waste and maximizes efficacy is essential. A comprehensive series of dosage trials (varying treatment duration and bacterial concentration) indicated that a 6-hr treatment at 50 ppm (i.e., 50 mg dry weight of bacterial cells per liter of power plant intake water) is optimal to achieve mussel kill. This is a much shorter treatment duration than any commercial mussel control method currently in use in power plants, e.g., chlorination is typically several weeks in duration (~100X longer). Therefore, in addition to reducing power plant concerns/liabilities about the handling of hazardous materials (e.g., materials for chlorination), the use of this environmentally safe, bacterial approach should also lower plant personnel costs required for treatment.

1C. Nontarget Safety

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. Experiments conducted during this project indicated that *Pf*-CL145A was not lethal to this species at a treatment concentration as high as ~200 ppm for 48 hr. This further advanced the potential for commercialization of *Pf*-CL145A as *D. magna* is one of the primary species that the U.S. Environmental Protection Agency uses to assess biopesticide safety. As research continues ion improving toxin production per cell and thus lowering the quantity of bacteria required to achieve high zebra mussel mortality (e.g. >95%), it is expected that future non-target trials with *D. magna* will result in little or no *Daphnia* mortality.

1D. Economic Commercial Production

In experiments conducted during this project, the cost of the fermentation medium needed to produce high yields of toxic *Pf*-CL145A cells was reduced by ca. 88%. This new fermentation medium, in conjunction with a newly revised fermentation protocol, will serve as the basis for future commercial production of large quantities of *Pf*-CL145A at relatively low cost. Thus, this bacterial approach to zebra mussel control has now become more economically competitive with the cost of biocides currently used by power plants.

1E. Identification of Gene(s) That Produce the Mussel-Killing Biotoxin

Identifying the gene(s) responsible for producing the toxin will lead to increased toxin production and a more highly efficacious commercial biopesticide for use against *Dreissena* mussels. Toward this end, the complete draft genome sequence of *Pf*-CL145A was received and annotation completed. Comparative analyses of the *Pf*-CL145A genome to genome sequences of non-toxic *P. fluorescens* strains identified regions of the *Pf*-CL145A genome that are unique and thus represent regions that contain candidate toxin-producing genes. Progress was also made toward identifying the genomic regions that are most highly expressed by *Pf*-CL145A when the cells are highly toxic to zebra mussels. We completed much of the subtractive hybridization procedure that will eventually result in the isolation of genomic fragments that correspond to genes that are uniquely highly expressed when *Pf*-CL145A cells are most toxic to zebra mussels.

## Objective #2

Following a nation-wide search, the Principal Investigator's institution, the New York State Museum (NYSM) chose Marrone Organic Innovations (MOI) as its commercialization partner (http://www.marroneorganicinnovations.com). MOI is a biopesticide company whose staff have unparalleled experience in the discovery, development, and marketing of natural products for pest management. The commercialization partnership between MOI and NYSM was selected by the National Science Foundation (NSF) for a $500,000 award to further accelerate the path to commercialization of this environmentally safe control agent (http://www.nysm.nysed.gov/press/releases/mdan.cfm). MOI's NSF award, combined with its own resources, will be used to develop commercial formulations, optimize the manufacturing process, identify mussel-killing natural compounds produced by the bacterium, conduct field trials, and complete all other tasks required to submit the product to the US Environmental Protection Agency for approval. The NYSM will use its NSF funding to further define environmental safety testing and will also work closely with MOI on other scientific aspects of product development.

## PLANS FOR FUTURE RESEARCH

The recent $500,000 award to MOI and NYSM by NSF to form a commercialization partnership is a direct result of the achievements made during this 2003-2008 USDOE-NETL project. The joint research efforts by MOI and NYSM are now currently directed toward increasing optimizing and 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. Other critical steps toward commercialization include analytical method development, formulation improvement, and any additional toxicology studies that may be mandated by the USEPA for product registration (expected in 2010).

## TECHNOLOGY AND INFORMATION TRANSFER

This project was highlighted in the following presentations:

Mayer, D. A. *Pseudomonas fluorescens* strain CL0145A as a biological control agent against zebra mussels. December 4, 2003. Drew University, Madison , NJ. (Invited speaker.)

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

Molloy, D. P. Project overview: Bacterial control of zebra mussels. December 19, 2003. New York State Department of Environmental Conservation, Albany, NY. (Seminar speaker.)

Molloy, D. P. and Morse, J. T. Control of zebra mussels with the biopesticide *Pseudomonas fluorescens*. April 6, 2004. USEPA Microbial Pesticides Branch, Biopesticides and Pollution Prevention Division, Office of Pesticide Programs. (Seminar speaker.)

Mayer, D. A. Development of *Pseudomonas fluorescens* strain CL0145A as a biological control against zebra mussels (*Dreissena polymorpha*). Department of Biological Sciences of the University at Albany. September 10, 2004. Albany, NY (Invited speaker.)

Mayer, D. A., Molloy, D. P., Morse, J. T., Presti, K. T., Sawyko, P. M., and Sprague, P. A. Biocontrol of zebra mussels: The path to commercialization of *Pseudomonas fluorescens* strain CL0145A. Annual Meeting of the Society for Industrial Microbiology. July 26, 2004. Anaheim, CA (Submitted poster.)

Morse, J. Control of zebra mussels with the biopesticide *Pseudomonas fluorescens*. New York State Department of Environmental Conservation, Avon, NY. May 6, 2004. (Invited speaker.)

Molloy, D. P. Bacteria and zebra mussels: What's known about their interactions? March 17, 2005. Annual Meeting of the New England Association of Environmental Biologists, Lake George, NY. (Invited speaker.)

Mayer, D. A. and Molloy, D. P.. Increased efficacy of *Pseudomonas fluorescens* biocontrol strain CL0145A through the modification of culture media. Annual Meeting of the Society for Industrial Microbiology. August 21-25, 2005. Chicago, IL. (Submitted poster.)

Molloy, D. P., Gaylo, M. J., Morse, J. T., and Mayer, D. A. Further evidence of the environmental safety of the zebra mussel control agent *Pseudomonas fluorescens* strain CL145A: Lack of acute toxicity to *Daphnia magna.* Fourteenth International Conference on Aquatic Invasive Species. May 17, 2006. Key Biscayne, FL. (Submitted poster.)

Molloy, D. P., Gaylo, M. J., Morse, J. T., and Mayer, D. A. Further evidence of the environmental safety of the zebra mussel control agent *Pseudomonas fluorescens* strain CL145A: Lack of acute toxicity to *Daphnia magna.* Annual Meeting of the Society for Industrial Microbiology. July 31, 2006. Baltimore, MD. (Submitted poster.)

Mayer, D. A. Development of an environmental isolate, *Pseudomonas fluorescens* CL145A, for biological control against zebra mussels. April 5, 2007. Skidmore College, Saratoga Springs, NY.

Molloy, D. P. Searching for environmentally-safe biological control agents as alternatives to polluting chemical pesticides. Bio-Chem Mentor Network of New York State. April 27, 2007. Albany, NY. (Invited speaker.)

Mayer, D. A. and Molloy, D. P. Fermentative development of *Pseudomonas fluorescens* CL145A as a biocontrol agent against zebra mussels. Annual Meeting of the Society for Industrial Microbiology. August 1, 2007. Denver, CO (Invited speaker.)

Molloy, D. P. and Mayer, D. A. 2007. Update on *Pseudomonas fluorescens* strain CL145A as a zebra mussel control agent Fifteenth International Conference on Aquatic Invasive Species. September 26, 2007. Nijmegen, Netherlands. (Submitted paper.)

**INVENTIONS**

There were no patentable inventions during the project.