TECHNICAL REPORT
(40751R04)

Report Title:	**Evaluation of Biotic and Treatment Factors Relating to Bacterial Control of Zebra Mussels**
Type of Report:	**Quarterly**
Reporting Period Start Date:	**January 1, 2002**
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DISCLAIMER:

ABSTRACT:

Testing over the last quarter has indicated the following regarding control of zebra mussels with bacterium *Pseudomonas fluorescens* strain CL0145A:

- the concentration of bacteria suspended in water is directly correlated with mussel kill;
- the ratio of bacterial mass per mussel, if too low, could limit mussel kill; a treatment must be done at a high enough ratio so that mussels do not deplete all the suspended bacteria before the end of the desired exposure period;
- bacteria appear to lose almost all their toxicity after suspension for 24 hr in highly oxygenated water;
- In a recirculating pipe system, the same percentage mussel kill will be achieved irrespective of whether all the bacteria are applied at once or divided up and applied intermittently in smaller quantities over a 10-hr period.

Since this is the fourth quarterly report, a summation of all test results over the last twelve months is provided as a table in this report. The table includes the above-mentioned fourth-quarter results.

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EXECUTIVE SUMMARY:

Testing over the last twelve months has indicated the following regarding control of zebra mussels with bacterium *Pseudomonas fluorescens* strain CL0145A:

1. Factors that are not important:
   - mussel size: bacteria are equally lethal to all zebra mussel sizes;
   - mussel species: bacteria are equally lethal to both species of zebra mussel;
   - bacterial cell viability: bacteria are equally lethal whether they are dead or alive;
   - treatment frequency: the same percentage mussel kill will be achieved irrespective of whether all the bacteria are applied at once or divided up and applied intermittently in smaller quantities over time (e.g., 10 hr).
2. Factors which could be important:
   - ratio of bacterial mass per mussel; this ratio should not be allowed to be so low that mussels can filter out all the suspended bacteria before the end of the desired exposure period.
3. Factors that are very important:
   - treatment duration: highest mussel kill requires exposure to bacteria for about 12-24 hr;
   - bacterial concentration: highest mussel kill (>95%) requires exposure to about 200-300 ppm (1 ppm = 1 mg of bacterial mass per liter of water);
   - water temperature: highest mussel kills are achieved at the highest water temperatures.

Fourth Quarter Results: January 1, 2002 through March 31, 2002

Subtask 2.1: What quantity of bacteria is needed to kill zebra mussels?

1. Bacterial Concentration in Water

The percentage mortality resulting from a pesticide application typically has a direct correlation with the concentration of a pesticide being applied, i.e., higher concentrations tend to produce higher mortalities. Subtask 2.1 of the contract requires examination of this relationship in reference to the use of the bacterium *Pseudomonas fluorescens* strain CL0145A for zebra mussel control. Laboratory trials were conducted which confirmed that this typical direct correlation did exist, i.e., the percentage mussel kill increased with bacterial concentration. For example, concentrations required to kill 50% and 95% of the mussels were, respectively, ca. 4 and 178 ppm.

2. Ratio of Bacterial Mass per Mussel

It was also noted in the above-mentioned tests that chambers holding the same number of 10-mm long mussels per chamber (i.e., 100 mussels) and treated at the same bacterial concentration resulted in lower mortalities in chambers holding lower water volumes. In water volumes of 500 ml and 2,000 ml, a concentration of 100 ppm caused, respectively, ca. 92% and 95% kill. (It required ca. 256 ppm in the 500 ml tests to achieve 95% kill.) This suggested that in addition to bacterial concentration in the water, the ratio of bacterial cell mass per mussel in a testing chamber was also a factor influencing mortality rates. A possible explanation for this was that for any given concentration, the lower the water volume, then the less bacterial mass and the fewer toxic bacterial cells that were available for ingestion per mussel. At 100 ppm in water volumes of 500 ml and 2000 ml, this ratio was, respectively, 0.5 and 2.0 mg/mussel.

Subtask 2.3: Under recirculating Conditions How Long Are Bacterial Suspensions Lethal?

One approach to controlling zebra mussels within power plant pipes is to recirculate bacterial suspensions in pipes. The previous quarterly report (#40751R01) had indicated that under recirculating conditions, the first few hours zebra mussels are exposed to bacteria are the most important in achieving kill. Recent tests have shed some light on why this true. It appears that the bacteria lose their toxicity when recirculated in water over time. When 500 ml of an aqueous bacterial suspension was aerated for 12 hr in glass jars without mussels and then used to treat mussels, the toxicity of the bacterial cells was noted to decline by roughly half. Bacterial cells after 24 hr of aeration in jars had virtually no toxicity when exposed to mussels. The reason for this decline in cell toxicity is unknown, but may be in part a consequence of exposure to oxygen, movement or high temperature since bacteria suspended in cold water without oxygen or agitation do not similarly lose their toxicity in a 24-hr period. These results actually could be of great environmental benefit since once water is released from a power plant, its bacterial cells are all the less likely to cause any nontarget problems in open waters due to their rapid tendency to lose toxicity when suspended in moving, oxygenated water. The loss of toxicity observed, however, does highlight the critical importance of all mussels feeding during the initial few hours during a recirculated treatment.

Subtask 2.3: Given a set quantity of bacterial cells, is it better to treat once with all of them or intermittently with equal portions over time?

Tests were conducted in artificial pipes under recirculating conditions to address this question. Mussel mortality was the same whether all the bacteria was applied at once or divided up into 6 equal portions and applied at 2-hr intervals over a 10-hr period. Thus, there was no benefit to staggering treatments.

Entire Project Period Summary: Four Quarterly Reports Combined

This is the fourth quarterly report for this research project. Although the project's contract was signed in October 2000, research only began in March 2001. As required by the contract, the following project summary is provided for research performed during the last four quarters.

Current Status:

- We are roughly halfway through the project period with DOE funding scheduled to end in February 2003.
- We have had steady success in meeting research objectives, and the project has solid forward momentum toward commercialization of the patented bacterial strain CL0145A of the common, soil bacterium *P. fluorescens* for the biological control of zebra mussels.

Results and Their Significance:

In addition to DOE funding (ca. $193K), this project is being co-funded (ca. $200K) by the New York State Energy Research and Development Authority (NYSERDA).

- DOE funds support laboratory experiments to determine the biotic, abiotic, and treatment factors important in achieving high mussel mortality.
- NYSERDA funds support: 1) tests in pipes within power plants to verify the results obtained from the DOE laboratory experiments, and 2) bacterial culturing research to establish how to mass-produce bacterial cells of the highest possible toxicity in fermentation units.

Table 1. Overview of test results from experimentation over the last twelve months.

Factor Examined	Additional Information
Mussel Size: All zebra mussel sizes are susceptible to bacterial kill, with almost equal mortality.	• There will be no zebra mussel size in pipes that will be immune to the bacterial treatments.
Mussel Species: There are two species of zebra mussels present in North America, *Dreissena polymorpha* and *D. bugensis*, and both are equally susceptible to kill by the bacterium.	• There will be no zebra mussel species in pipes that will be immune to bacterial treatments.
Bacterial Cell Viability: Dead bacteria are equally as effective as live bacteria in killing zebra mussels.	• This is the strongest evidence that the bacteria kill by a toxin, not by infection. • Future commercial products will contain dead bacterial cells without any loss in their lethality to zebra mussels; this should further lessen any environmental concerns.
Treatment Duration: For highest kill due solely to the bacterial toxin, limit treatments to a minimum of 12 hr and a maximum of 24 hr.	• Little additional mortality can be achieved if bacterial treatments are extended beyond 24 hr by continuing to recirculate cells. This is due in part to the fact that at 23ºC, bacteria appear to lose almost all their toxicity after suspension for 24 hr in highly oxygenated water (i.e., either recirculated or aerated). • Even retreating using fresh bacterial cells after 24 hr does not increase mussel mortality.
Continuous or Intermittent Treatment: In a recirculating pipe system, the same percentage mussel kill will be achieved irrespective of whether a given quantity of bacteria is applied at once or divided up and applied intermittently in smaller quantities.	• If pipes will be treated in a closed loop system, there is no need to treat intermittently (e.g., every 2 hr). For example, treat once at 300 ppm rather 6 times at a 50 ppm over a 10-hr period.

Table 1 (Continued)

Treatment Concentration: Bacterial concentration in the water and mussel kill are directly correlated.	• In waters at 23ºC, >95% mussel mortality requires exposure to about 200-300 ppm
Ratio of Bacterial Mass per Mussel: If this ratio is too low, it could limit mussel kill.	• Treatments must be done at a high enough ratio so that mussels do not deplete all the suspended bacteria before the end of the desired exposure period. • Lower than expected mortality might result if a pipe containing a high density of mussels and relatively low water volume was treated and then either sealed off (i.e., flow stopped) or recirculated. In this case, mussels might quickly filter out all the bacteria within a few hours and thereby not have a long enough feeding period (e.g., 24 hr) for a lethal exposure. • Tests indicate that such treatments should include ≥2 mg per 10-mm long mussel. • Since mussels have a maximum rate of ingestion, there is an upper limit on this ratio, above which mussels will simply produce pseudofeces when offered increased quantities of bacteria.
Water Temperature: Although there is some loss of efficacy with declining water temperature, the bacteria can still inflict high mussel mortality at cold water temperatures.	• This indicates that this bacterium is more effective than currently commercialized chemical molluscicides that are used for zebra mussel control. • The current commercialized products are ineffective at water temperatures ca. <18ºC, whereas the bacteria can cause significant mortality even at ca. 7ºC.
Pipe tests: 95% kill of mussels has been achieved in small-scale trials in artificial pipes in a power plant.	• This indicates that we can use the knowledge we gained in our lab tests and apply it to achieving high kill in pipes.
Fermentation Units: Bacteria which were previously only produced in 100-ml batches in flasks under static conditions can now be grown in 1,600-ml batches in fermentation units under agitated conditions.	• This is a very important and major accomplishment for the project. • Bacteria capable of killing zebra mussels must produce a toxin during their growth; it took over a year to find the right agitated culturing conditions in fermentation units to produce such toxic bacterial cells. • Higher volume productions (e.g., 500 L) are now being planned.

Publications:

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. 4 pp.

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.

DOE Project Research Plans:

- **Next 6 months:** Continue experiments to determine if the following factors make a difference in the lethality of the bacteria to zebra mussels: the pH, velocity, oxygen concentration, and turbidity of water.
- **Next 12 months:** Continue experiments to determine if the following factors make a difference in the lethality of the bacteria to zebra mussels: mussel colony thickness on a surface; bacterial strain; presence of algae.

Other Noteworthy Developments Related To This Project

- In addition to cost sharing ca. $200K on this DOE project, New York State Energy Research and Development Authority also awarded a $135K grant to chemically identify the bacterial toxin that kills zebra mussels and a New York State company has expressed interest in its commercialization.
- In contrast to its lethality to zebra mussels, this bacterium has caused no nontarget mortality yet in laboratory and mesocosm tests involving six unionid bivalve species, blue mussels, one ciliate species, brown trout, sunfish, and fathead minnows. As a result of these trials, USFWS awarded $75K to evaluate the use of this bacterium for controlling zebra mussels in open waters; there are no other zebra mussel control agents being considered for use in such natural aquatic habitats.