

**TECHNICAL REPORT
(41909R01)**

Report Title: Environmentally Safe Control of Zebra Mussel Fouling

Type of Report: Semi-Annual

Reporting Period Start Date: October 1, 2003

Reporting Period End Date: March 30, 2004

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Date Report was Issued: May 2004

DOE Award No.: DE-FC26-03NT41909

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ABSTRACT

Under this USDOE-NETL contract, the bacterium *Pseudomonas fluorescens* is being developed as a biocontrol agent for zebra mussels. In the first six months of the project, significant progress has been made in tests designed to develop a culturing protocol to increase the toxicity of each bacterial cell and an ionizing radiation protocol for creating a dead-cell formulation. Efforts have also been successful in establishing a research trailer on the grounds of Rochester Gas & Electric's Russell Power Station. Tests will soon be conducted in this trailer to develop treatment protocols designed to achieve high mussel kill under the service water conditions existing at this power plant. Contacts have been established with both the Bureau of Pesticides of the New York State Department of Environmental Conservation and the U.S. Environmental Protection Agency regarding the procedures that will be required to obtain proper permitting for future field release of *P. fluorescens* cells into open waters surrounding the power plant. An initial step in this permitting process will be laboratory trials against the aquatic microcrustacean *Daphnia magna*, and preparations are underway for these tests. Planning is also on track for small-scale treatment of actual service water piping within the Russell Power Station this summer.

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EXECUTIVE SUMMARY

Use of the bacterium *Pseudomonas fluorescens* strain CL0145A represents a potential alternative to the current use of polluting biocides for control of zebra mussel infestations in water pipes. The purpose of this DOE-NETL project is to accelerate research toward commercialization of this biocontrol agent. This is to be achieved by developing economical methods to produce and formulate the bacterial 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 water pipes. During the six month period reported herein, research efforts focused primarily on the following topics:

- Cell toxicity: The more toxic each *P. fluorescens* cell is, the fewer cells that would be required in an actual zebra mussel treatment. The need to use fewer cells should consequently result in lower treatment costs. Laboratory experiments to define key nutrients (e.g., carbohydrate and nitrogen sources) required to produce more toxin per bacterial cell were commenced during the reporting period and progress was achieved.
- Dead-cell formulation: Since cells of *P. fluorescens* strain CL0145A are equally lethal to zebra mussels whether applied dead or alive, we wish to develop a commercial formulation that contains dead cells, as such a formulation will further reduce environmental concerns. Progress was made in these experiments as test results indicated that there is potential for development of an ionizing radiation treatment dosage (e.g., 0.9 Mrad) that will kill almost all *P. fluorescens* cells, yet cause virtually no loss in their lethality to zebra mussels.
- State and federal agencies: In order to treat power plant water pipes and have the effluent discharged into surrounding open waters, an Experimental Use Permit (EUP) must be obtained from the U.S. Environmental Protection Agency (USEPA). Contacts with both the Bureau of Pesticides of the New York State Department of Environmental Conservation and the USEPA have been made and have led to open lines of communication regarding the procedures that will be required to obtain proper permitting for future field release of the bacterium into open waters (as will occur in 2005). An initial step in this permitting process will be laboratory trials scheduled to commence soon against the aquatic microcrustacean *Daphnia magna*.
- Service water pipe treatments: Planning is also on track for the treatment of actual service water piping within RG&E's Russell Power Station this summer. The use of a research trailer on the grounds of the Russell Power Station will allow small-scale laboratory experiments to be conducted using service water under once-through conditions. These trailer experiments will assist in the refinement of protocols to be used in the treatment of the station's service water piping this summer.

INTRODUCTION

Coal-fired power plants within North America need an effective, economical, and non-polluting technique for managing infestations of zebra mussels within their facilities. Due to a lack of options, many facilities have relied on the use of broad-spectrum, chemical biocides for control of these freshwater mussels. However, biocide treatments, such as continuous chlorination for three weeks, are widely regarded as environmentally unacceptable because they can result in the formation of potentially carcinogenic substances. Use of the bacterium *Pseudomonas fluorescens* strain CL0145A represents a potential alternative to the use of polluting biocide treatments and is the leading candidate in the world for the biological control of these macrofouling mussels. The purpose of this DOE-NETL project is to accelerate research toward commercialization of this biocontrol agent. This is to be achieved by developing economical methods to produce and formulate the bacterial 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 water pipes. During the six month period reported herein, research efforts focused on the following 6 project activities:

1. DEVELOPING A CULTURING PROTOCOL TO INCREASE THE TOXICITY OF EACH BACTERIAL CELL.

The more toxic each *P. fluorescens* cell is, the fewer cells that would be required in an actual zebra mussel treatment. The need to use fewer cells should consequently result in lower treatment costs. Laboratory experiments to define the key nutrients and culture conditions required to produce more toxin per bacterial cell were commenced during the reporting period and the progress achieved is reported herein.

2. DEVELOPING A DEAD-CELL FORMULATION.

Since cells of *P. fluorescens* strain CL0145A are equally lethal to zebra mussels whether they are applied dead or alive, we wish to develop a commercial formulation that contains dead cells, as such a

formulation will further reduce environmental concerns. Progress in experiments to kill *P. fluorescens* cells without causing loss of their toxicity are reported herein.

3. OBTAINING A PERMIT FROM THE USEPA FOR TREATING POWER PLANT SERVICE WATER SYSTEMS.

In order to treat power plant water pipes and have the effluent discharged into surrounding open waters, an Experimental Use Permit (EUP) must be obtained from the U.S. Environmental Protection Agency (USEPA). Progress in applying to USEPA for this permit is reported herein.

4. DEVELOPING TREATMENT PROTOCOLS THAT ACHIEVE HIGH MUSSEL KILL.

Throughout this three-year project, an annual summertime treatment is planned in service water pipes within the Russell Power Station, a coal-fired plant owned by Rochester Gas & Electric (RG&E). Treatment of actual power plant pipes will start in Year 1 with a small-scale treatment, then progress to a medium-scale treatment in Year 2, and finally culminate in a full-scale treatment of the entire service water system in Year 3. To guarantee success in achieving high mussel kill in these annual power plant treatments, laboratory experiments will be conducted to refine treatment protocols. Efforts made in planning these tests within a research trailer located at Russell Power Station are reported herein.

5. EVALUATING ENVIRONMENTAL SAFETY.

Nontarget safety trials conducted to date are encouraging, but further experiments to evaluate the environmental impact of bacterial treatment are required to meet USEPA product registration requirements. Plans for commencing these tests with trials against daphnid crustaceans are outlined herein.

6. DEMONSTRATING EFFICACY IN KILLING ZEBRA MUSSELS WITHIN ACTUAL POWER PLANT PIPES.

Progress in planning the annual summertime treatment in service water pipes at RG&E's Russell Power Station is outlined.

EXPERIMENTAL

The following is an overview of the materials and methods used in tests completed in activities #1 and #2. The other 4 activities listed above involved preparation and planning for experiments, not actual testing and data generation, and information about these other 4 activities is included in the RESULTS AND DISCUSSION section.

1. DEVELOPING A CULTURING PROTOCOL TO INCREASE THE TOXICITY OF EACH BACTERIAL CELL.

The following is a general outline of the methodology employed in these culturing tests.

- **Shaken seed cultures:** One 250-ml Erlenmeyer flask containing 25 ml of buffered tryptic soy broth (bTSB) was inoculated with 0.4 ml of stock culture and shaken at 200 rpm at 26°C for 24 hr.
- **Shaken flask cultures:** 0.25 ml from the bTSB 24-hr shaken seed culture was used to inoculate each of 3 replicate flasks containing each experimental medium type. Flasks were shaken at 200 rpm at 26°C for 24 hr.
- **Evaluation of growth of cultures:** Growth of cultures was assessed by measuring optical density of FWC (A_{660} with a Genesys 20 spectrophotometer) in each flask after 24 hr of growth, using the uncultured media of each type as blanks.
- **Evaluation of pH of cultures:** The pH of media during their preparation and at 24-hr FWC was measured using a pH probe (Corning #476346). The initial pH was determined by measuring the pH of uncultured media of each type (i.e., the blank).
- **Production of cell fraction:** The final whole culture (FWC) from each flask was centrifuged separately to produce individual pellets from each flask as true replicates. FWC were centrifuged

(30 min at 1449 x g) in 25-ml batches in 50 ml centrifuge tubes, and cell pellets were resuspended in dilution water (80 ppm KH_2PO_4 , 405.5 ppm $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in deionized water).

- Preparation of cell fraction: The optical density of the cell fraction (CF) inoculum was determined by taking two absorbance readings from the CF at $\lambda = 660 \text{ nm}$ at a dilution that resulted in a reading less than 0.5. The optical density of the CF was then used to calculate the volume of CF required to treat each of 3 replicate micro-chambers (3 replicate micro-chambers treated per each replicate flask) at a target concentration of 25 or 50 ppm. Mean dry bacterial cell mass/ml for CF were calculated from 2-1.0 ml desiccated subsamples using a Denver Instruments balance. The targeted treatment concentration was 25 or 50 ppm (dry bacterial mass/water volume).
- Preparation of zebra mussels: *Dreissena polymorpha* were collected from the Mohawk River near Crescent, NY, brought back to the lab, sieved, and kept at 7°C in unchlorinated tap water with filtration and aeration. Approximately 1 week before the test, mussels were moved from 7°C and acclimated to 23°C in a 5-gal aquarium containing unchlorinated tap water with aeration (aquarium wrapped in towels to slow warming over several days). The day before the test, 20 <6 mm mussels were picked into microchambers containing ca. 5 ml of aerated hard water (Peltier and Weber 1985) and allowed to attach overnight. The morning of the test, unattached mussels were removed and replaced with attached mussels from an extra dish and the water was replaced with 10 ml (micro-chambers) aerated hard water.
- Treatment of zebra mussels with CF: At least one hour before treatment, the micro-chambers were set up with aeration, labeled, and treated (Figs. 1 and 2). Mussels were exposed for the treatment period (24 hr), then the fluid was poured off and mussels were collected in clean plastic dishes with oxygenated hard water to be examined for mortality. Mortality was scored and the mussels were held in the dishes for an additional 6 days, changing the water and scoring mortality each day. All binomial mortality data were analyzed following angular transformation (Sokal and Rohlf 1995).



Fig 1. Three micro-chambers each with 10 ml of water and 20 mussels within a plastic container. Each micro-chamber had a single air line with a cotton-filter to maintain aerated conditions throughout the test.



Fig 2. Row of color-coded, plastic containers each holding three micro-chambers. These testing chambers have proven to produce reliable, accurate data, and their small size allows relatively quick set-up and break-down during an experiment.

2. DEVELOPING A DEAD-CELL FORMULATION.

The following is a general outline of the methodology employed in these tests.

- Source of cells: *P. fluorescens* CL0145A cell fraction (CF) produced in 55-L fermentation runs was suspended in sterile dilution water to make a thick liquid formulation. One ml of this CF was dispensed into each of ca. 50 small square plastic dishes with tight fitting lids (ca. 1.5 x 1.5 x 1 cm (L x W x D)). All dishes containing CF were stored at -80°C.
- Irradiation treatment: Duplicate CF samples were passed by an electron accelerator beam in a tray surrounded by dry ice so that the samples were not allowed to thaw during the exposure period. Actual exposure was quantified with a piece of radiachromic film for each treatment exposure.
- Estimating cell death: The percentage of cells that were killed from the irradiation treatments was determined with standard plate counts of various treatments. The difference in numbers of live cells in the untreated CF and each irradiated CF were represented as percentage kill.
- Preparation of CF: Same methods as in above-mentioned activity #1.
- Preparation of zebra mussels: Same methods as in above-mentioned activity #1.
- Treatment of zebra mussels with CF: Same methods as in above-mentioned activity #1.

RESULTS AND DISCUSSION

This section gives an overview of the progress made during the six-month reporting period in both conducting and planning experiments.

1. DEVELOPING A CULTURING PROTOCOL TO INCREASE THE TOXICITY OF EACH BACTERIAL CELL.

The first step in the experimental approach for increasing the toxin production of strain CL0145A by culture improvement involved the development of a strictly chemically-defined culture medium to replace our current complex chemically-undefined medium. Within the chemically-defined medium, we can manipulate individual components of the medium to progressively build a new medium that enhances toxin production. Experimentation focused on selecting chemically-defined carbohydrate and nitrogen sources for the culture medium. Each component was tested in triplicate and the growth of the cultures and the toxicity of harvested cells was compared to production in our current culture medium as "relative growth" and "relative toxicity," respectively.

Carbohydrate Sources

Eighteen carbohydrate sources were tested in single-substitution tests within our current complex culture medium. Carbohydrates that produced cells having similar or increased levels of relative toxicity were repeated. Of the 18 carbohydrate sources tested, at least 15 resulted in culture growth similar to growth in the standard medium (i.e., relative growth ≥ 0.70), while relatively low growth was achieved with only three carbohydrate sources (Fig. 3). However, the highest growth was consistently achieved in the standard medium. Therefore, these tests suggest that for growth of CL0145A cultures the best carbohydrate tested to date currently is in our standard medium. Four of the 18 carbohydrate sources tested produced cells that were significantly less toxic than cells from our standard medium ($p < 0.05$), while the other 14 carbohydrates achieved various levels of mean relative toxicity ranging from 0.45 to 1.06 (Fig. 4). None of the carbohydrate sources tested appeared to enhance CL0145A cell toxicity; therefore, the carbohydrate source in our current standard medium formulation was selected to be used in future nutrient optimization experiments.

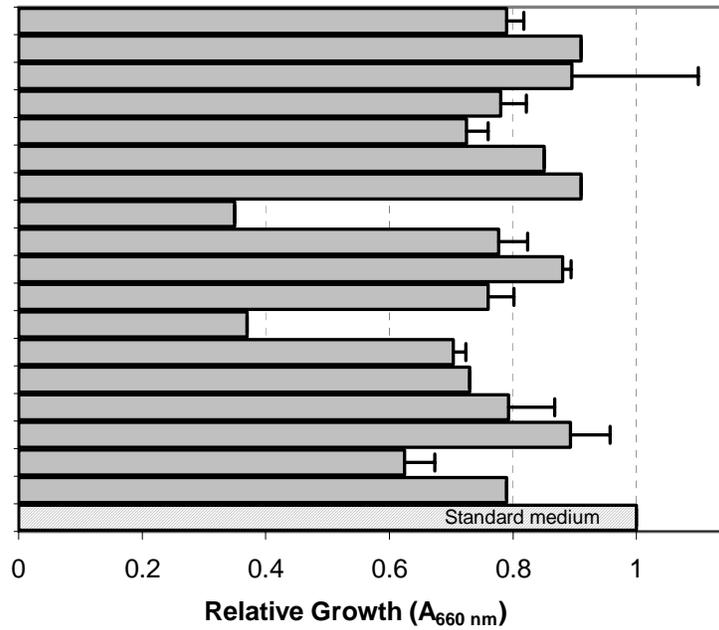


Fig. 3: Relative growth of 24 hr CL0145A cultures in media containing various carbohydrate sources compared to growth in our standard medium. Optical densities ($A_{660 \text{ nm}}$) from cultures with carbohydrates that were selected for to be tested in multiple runs are represented in the graph by their mean \pm SD relative values.

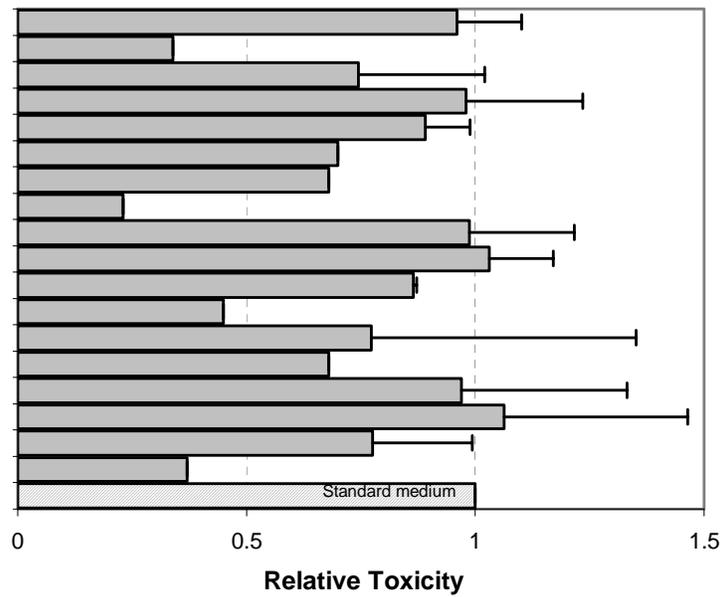


Fig. 4: Relative toxicity of cells harvested from media containing various carbohydrate sources compared to toxicity from cells harvested from our standard medium. Relative toxicities from cultures with carbohydrates that were selected for testing in multiple runs are represented in the graph by their mean \pm SD relative toxicity values.

Nitrogen Sources

Relative growth: The various complex peptone sources comprising our current standard medium were replaced with individual defined nitrogen sources to produce a strictly chemically-defined medium. Eleven nitrogen sources were tested to select a source primarily on the basis of whether the nitrogen source produced culture growth. The selected source(s) will comprise the formulation of the base medium in future amino acid optimization testing. Initially, relative toxicity of cells was a secondary consideration. However, without the presence of an amino acid source, relative growth in the cultures was low, ranging from 0.05 to 0.14 compared to our standard medium. Therefore, to more adequately assess the defined nitrogen sources they were tested again, this time with casamino acids present in the medium. Casamino acids are the acid hydrolyzed peptone formulation that is the basis for individual amino acid concentrations planned in future tests. Overall, the relative growth of the cultures increased in the presence of casamino acids, with maximum relative growth reaching approximately 0.2 (Fig. 5). The addition of casamino acids to the media containing the nitrogen sources increased the relative growth of the cultures in most cases compared to relative growth values without casamino acids (Fig. 5). Thus, casamino acids appeared to enhance primary metabolism in these cultures.

Relative toxicity: Cells harvested from cultures in media containing nitrogen sources with casamino acids ranged in relative toxicity from 1.23 to 4.65, and mean (\pm SD) relative toxicity values from nitrogen sources selected for multiple runs ranged from 1.65 (\pm 0.42) to 3.17 (\pm 1.39). Comparing relative toxicity values without and with casamino acids (Fig. 6) suggests a correlation between the presence of amino acids and the toxicity of CL0145A cells in the presence of the nitrogen sources. The presence of amino acids in the media was apparently, in some cases, essential for toxin production with these nitrogen sources. Future tests will be designed to examine interactions between nitrogen sources over a range of concentrations. In this way, we can select possible combinations of nitrogen sources and their most effective concentrations to achieve the highest toxicity.

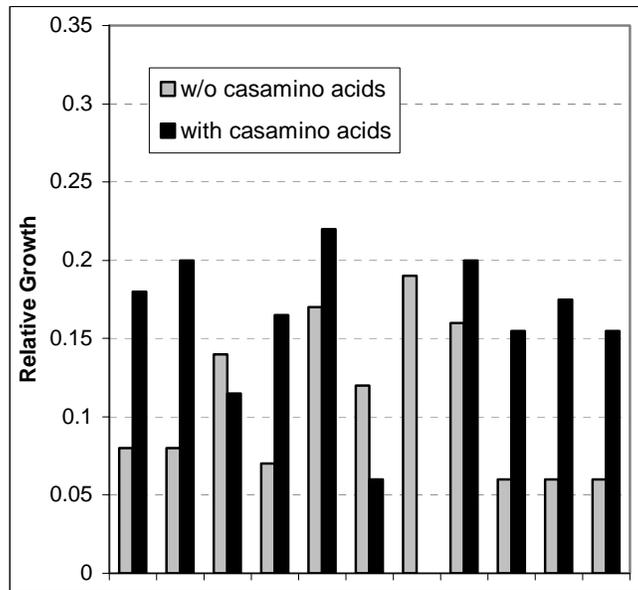


Fig. 5: Comparison of relative growth amongst N source media without and with casamino acids. The standard medium would have a relative growth value of 1.0.

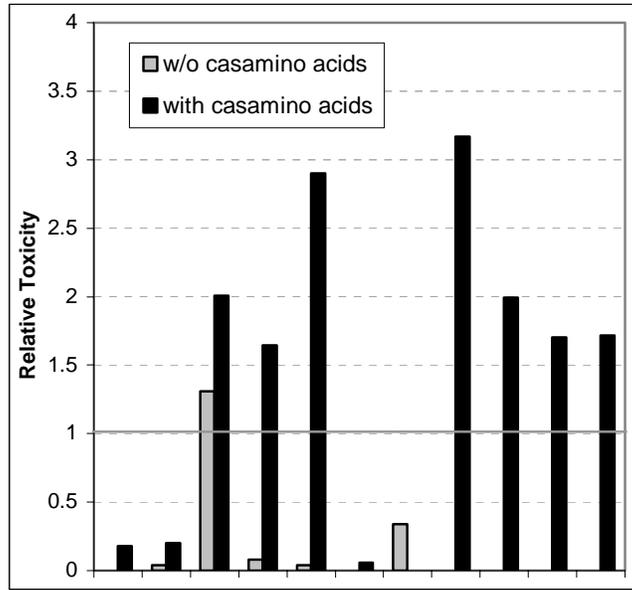


Fig. 6: Comparison of relative toxicity amongst N source media without and with casamino acids. The standard medium would have a relative growth value of 1.0 (solid grid-line).

2. DEVELOPING A DEAD-CELL FORMULATION.

Radiation exposures reduced the number of live cells by >99.99999% in this test without loss in mussel mortality (Table 1, Fig. 7). Maximum cell kill was achieved at an exposure of 0.9 Mrad, while higher exposures did not increase percent cell kill. There was no significant loss in the toxicity of cells exposed up to 1.4 Mrad ($p > 0.05$), but there appeared to be a decline in toxicity with exposures higher than 1.4 Mrad. Results from this test were in agreement with a preliminary test that indicated that a 0.9 Mrad dosage would achieve similarly high cell kill (99.999998%) without significantly reducing cell toxicity. These results were very encouraging since they indicated that there is potential for development of an ionizing radiation treatment dosage (e.g., 0.9 Mrad) that will kill almost all *P. fluorescens* cells, yet cause virtually no loss in their lethality to zebra mussels.

Table 1: Percentage kill and toxicity of cells exposed to ionizing radiation. Zebra mussel mortality after 24-hr exposure to treatment. Untreated control mortality was $3.3 \pm 5.8\%$ (mean of 3 micro-chambers).*

Irradiation exposure	Reduction in live bacterial cells (mean \pm SD)	Actual treatment concentration (mean \pm SD)(ppm)	% Mortality (7-day)	Mean % mortality (\pm SD)	Mean angular transformed mortality (\pm SD)
0 Mrad	0 \pm 0%	24.1 \pm 0.6	65.0, 60.0, 41.7	55.56 \pm 12.29	0.846 \pm 0.129 _a
0.4 Mrad	99.999995 \pm 0.000001%	29.0 \pm 1.3	58.3, 60.0, 33.3	50.56 \pm 14.94	0.791 \pm 0.157 _a
0.9 Mrad	100.000000 \pm 0.000000%	36.8 \pm 1.4	28.3, 30.0, 53.3	37.22 \pm 13.98	0.649 \pm 0.148 _a
1.4 Mrad	100.000000 \pm 0.000000%	37.1 \pm 0.8	30.0, 18.3, 30.0	26.11 \pm 6.74	0.530 \pm 0.076 _{a,b,c}
2.0 Mrad	100.000000 \pm 0.000000%	41.0 \pm 4.0	11.7, 5.0, 16.7	11.11 \pm 5.85	0.296 \pm 0.147 _{b,c}

*Statistical differences in mean angular mortality are indicated with subscripted letters ($p < 0.05$).

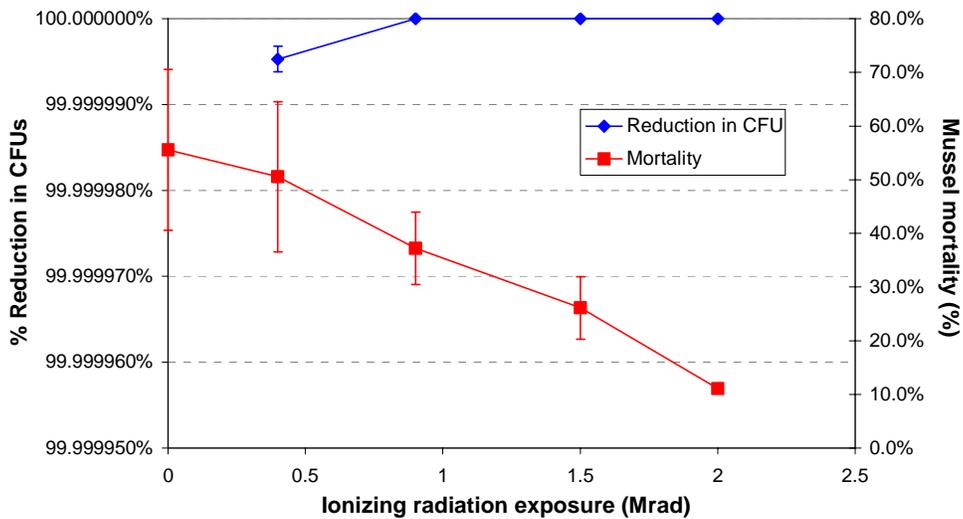


Fig. 7: Effects of ionizing radiation exposure: Live cell reduction and toxicity.

3. OBTAINING A PERMIT FROM THE U.S. EPA FOR TREATING POWER PLANT SERVICE WATER SYSTEMS.

In order to treat power plant water pipes and have the effluent discharged into surrounding open waters, an Experimental Use Permit (EUP) must be obtained from the U.S. Environmental Protection Agency (USEPA). Such discharges will only occur during experiments conducted in 2005, i.e., during the second year of this project. Tests conducted in 2004 on the property of the RG&E Russel Station will discharge all bacteria treated service water into the sewage lines of the facility, thus avoiding the need for an EUP. In anticipation of the need for the EUP in 2005, however, the following steps were taken:

- a presentation was given on our project in Albany (NY) on December 19, 2003 to staff of the Bureau of Pesticides of the New York State Department of Environmental Conservation – the state agency responsible for issuing the local permit to allow discharge of biopesticides;
- a presentation was given on our project on April 6, 2004 to staff of the USEPA’s Biopesticides and Pollution Prevention Division – the federal agency responsible for issuing an EUP;
- USEPA databases were comprehensively reviewed to obtain an understanding of the steps required to obtain an EUP;
- planning began for the kinds of nontarget tests that are typically required for obtaining an EUP; such tests will start with assays against the aquatic microcrustacean, *Daphnia magna* – one of the key nontarget species that USEPA requires safety data on.

4. DEVELOPING TREATMENT PROTOCOLS THAT ACHIEVE HIGH MUSSEL KILL.

To increase the chances for success in achieving high mussel kill in the annual treatment of service water piping at the RG&E Russell Power Station, laboratory experiments will commence soon using service water diverted into pipes within a research trailer on the grounds of the power station (Figs. 8 and 9). Over the last 6 month, this trailer was modified to accommodate these testing plans.



Fig. 8. The Rochester Gas & Electric Company has generously provided use of a research trailer on the grounds of the their Russell Power Station. The trailer contains areas for small-scale pipe tests as well as holding areas for maintaining mussels in fresh service water prior to conducting experiments.



Fig 9. Within the trailer, small-scale tests can be conducted using service water diverted from the power plant. Acrylic pipes, such as the single pipe with green end caps in foreground, will hold zebra mussels during the experiments.

5. EVALUATING ENVIRONMENTAL SAFETY.

As indicated in activity #3, we have begun planning nontarget tests required for obtaining an EUP. Tests will start with assays against the aquatic microcrustacean, *Daphnia magna* – one of the key nontarget species that USEPA will require safety data on (Figs. 10 and 11). Gary Neuderfer, an expert in this type of nontarget testing at the Bureau of Pesticides of the New York State Department of Environmental Conservation, has offered to serve *gratis* as a consultant in these tests.



Fig. 10. *Daphnia magna* is a key nontarget species which the USEPA requires safety data on prior to granting permits (e.g., an EUP) for the field testing of a biopesticide in aquatic environments.



Fig 11. An individual specimen of *Daphnia magna*. These microcrustaceans are filter-feeders and are likely to readily ingest *P. fluorescens* cells since they normally feed on suspended particles such as bacteria.

6. DEMONSTRATING EFFICACY IN KILLING ZEBRA MUSSELS WITHIN ACTUAL POWER PLANT PIPES.

In each year of this three-year project, an experiment involving treatment of service water pipes within RG&E's Russell Power Station will occur. Talks are now underway with RG&E staff to pick a small section of service water piping to be treated this summer. Commercial fermentation companies have also been contacted to obtain pricing for the large fermentation productions (e.g., 100 liters) that will be required for this summer's treatment.

CONCLUSIONS

In the first six months of this project, significant progress has been made in tests designed to identify a culturing protocol to increase the toxicity of each bacterial cell and also in a protocol for using ionizing radiation to create a dead-cell formulation. Efforts have also been successful in establishing a research trailer on the grounds of RG&E's Russell Power Station. Tests will soon be conducted in this trailer to develop treatment protocols that achieve high mussel kill. Contact with both the Bureau of Pesticides of the New York State Department of Environmental Conservation and the USEPA has been established and has led to open lines of communication regarding the procedures that will be required to obtain proper permitting for future field release of the bacterium into open waters (as will occur in 2005). An initial step in this permitting process will be laboratory trials against the aquatic microcrustacean *Daphnia magna*. Planning is also on track for the treatment of actual service water piping within RG&E's Russell Power Station this summer.

REFERENCES

- Peltier, W. H., and Weber, C. I. 1985. Methods For Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms. Third edition. U. S. EPA Office of Research and Development, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio. 216 pp.
- Sokal, R. R., and Rohlf, F. J. 1995. Biometry: The Principles and Practice of Statistics in Biological Research. Third edition. W. H. Reeman and Company, New York. 887 pp.

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.)