TECHNICAL REPORT
(40751R05)

Report Title:	**Impact of Water Temperature on Zebra Mussel Mortality**
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DISCLAIMER:

ABSTRACT:

These tests conducted this past quarter have indicated that the bacterium *Pseudomonas fluorescens* strain CL0145A is effective at killing zebra mussels at water temperatures ranging from 7 to 23ºC. Percent kill will likely be somewhat lower at very low temperatures, e.g., 7ºC, but even at such low temperatures high mussel kill can still be achieved (>70% kill). This is significant because the development of a zebra mussel control method that is efficacious in such a wide range of temperatures broadens its usefulness as a potential commercial product.

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EXECUTIVE SUMMARY:

Subtask 2.2 of the Statement of Work requires that the Contractor examine the effect of water temperature on the efficacy of treatment with *Pseudomonas fluorescens* strain CL0145A. To address this subtask, tests were conducted at water temperatures of 23, 17, 12, and 7ºC. Results indicated that strain CL0145A was effective at killing zebra mussels at all four temperatures. This is significant because the development of a zebra mussel control method that is efficacious in such a wide range of temperatures broadens its usefulness as a potential commercial product. These tests indicated that this bacterium is 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.

EXPERIMENTAL MATERIALS AND METHODS:

Culturing *Pseudomonas fluorescens* strain CL0145A:

Under shaken conditions, 250-ml Erlenmeyer flasks containing 25 ml buffered tryptic soy broth (bTSB) were each inoculated with 0.4 ml stock frozen bacterial culture. Flasks were then incubated at 26(±1)?C on orbital shakers at 200 rpm for 24 hr. These shake-flask seed cultures were subsequently used to inoculate 250-ml Erlenmeyer flasks (1 ml/flask) containing 100 ml bTSB and incubated at 26(±1)?C under static conditions for ca. 72 hr.

Obtaining the Cell Fraction Inoculum:

Final whole cultures were centrifuged (30 min at ca. 1,450 x g) in 50-ml batches, supernatants were discarded, and cell pellets were resuspended in dilution water (80 ppm KH_2PO_4, 405.5 ppm $MgCl_2?6H_2O$ in deionized water, pH adjusted to 7.2 with NaOH). The optical density of the cell fraction (CF) inoculum was determined by taking 1 absorbance reading (spectrophotometer, ? = 660 nm) from each of 3 separate CF samples. Based on an

absorbance equation developed during previous trials (author, unpublished data), the optical density of the CF was then used to calculate the volume of CF inoculum required to treat the mussels at the desired target concentration of 200 ppm (dry bacterial cell mass/unit volume). Two 1-ml samples of the CF inoculum were air dried in a dessicator and then weighed on a Denver Instruments balance to determine actual treatment concentration.

Maintaining Constant Water Temperatures:

Water baths were set-up prior to the test to maintain constant water temperatures of 7, 12, 17, and 23ºC. To maintain 7ºC, an 88-L aquarium containing unchlorinated tap water was set up inside a 7ºC walk-in cooler. To maintain 12ºC and 17ºC, 50-L insulated coolers within the walk-in cooler were filled with unchlorinated tap water and fitted with an aquarium heater set to 12 or 17ºC. To maintain 23ºC, a 44-L aquarium was set up in a 23ºC room, filled with tap water, and fitted with an aquarium heater set to 23ºC. In each water bath, the water was circulated using a submersible pump. Water temperature was monitored and recorded throughout the experiment using a digital thermometer.

Treatment of Zebra Mussels:

Two separate tests were conducted using zebra mussels collected from the Mohawk River (Crescent, NY) having mean lengths (±SD) of 8.34 ± 1.81 mm (test #1) and 8.77 ± 1.70 mm (test #2). The water used in all testing jars was hard, synthetic freshwater (Peltier and Weber, 1985).

Prior to treatment, mussels were either transferred directly from 7?C in the walk-in cooler to the 7ºC water bath or placed in an aquarium containing 7°C unchlorinated tap water and allowed to warm slowly while closely monitoring the temperature. When the temperature reached 12ºC and 17?C mussels were transferred to the 12ºC and 17?C water baths, respectively. Likewise, when the water temperature reached the ambient laboratory temperature of 23?C, mussels were transferred to the 23?C water bath. One day before treatment, 100 mussels were placed in 500-ml testing jars containing ca. 100 ml of synthetic hard water to a depth of 3 cm. Mussels were maintained at the target temperature before, during, and following treatment. The morning of treatment unattached mussels were removed and replaced with attached mussels, the testing jars were filled with 495 ml of aerated hard water, and fit with airstones set at gentle aeration. The aerating testing jars were placed in the appropriate water bath to maintain temperature during treatment.

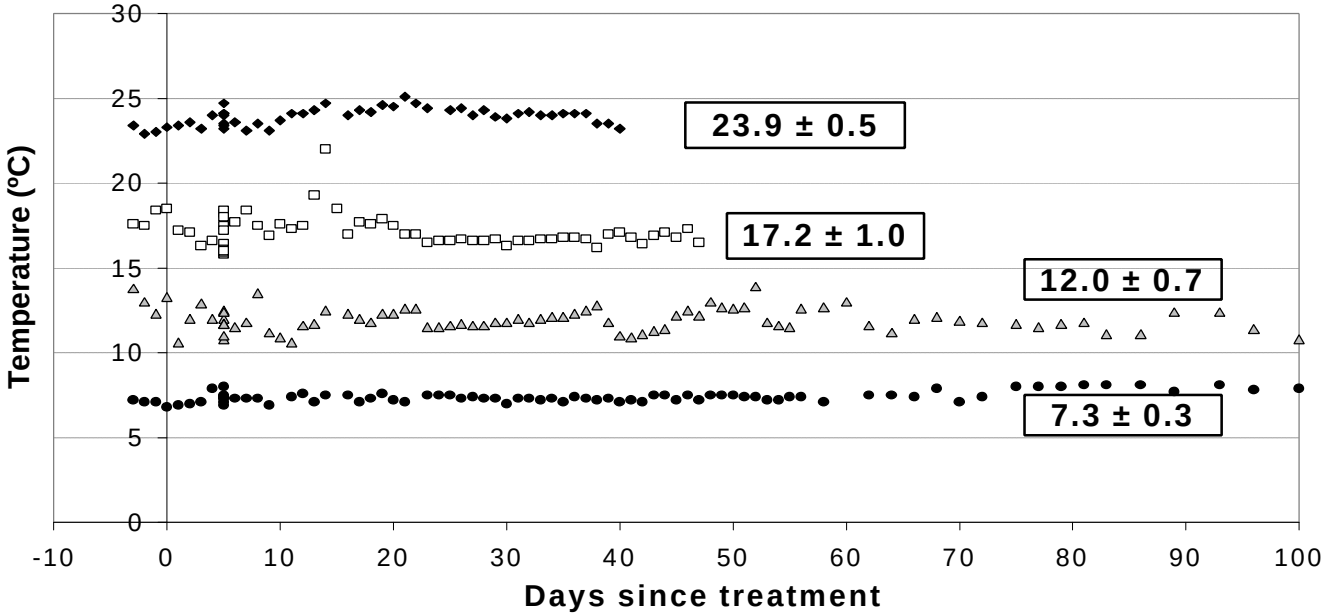
For each test, 3 replicate testing jars were treated with bacteria and one untreated control testing jar, each containing 100 mussels, were exposed for a period of 48 hr at an initial target concentration of 200 ppm of CL0145A cells. Following treatment in the testing jars, mussels were transferred to 7.6 x 7.6 cm plastic dishes in ca. 100 ml oxygenated hard water that had been adjusted to the appropriate temperature. During the post-treatment observation period, these dishes were floated in water baths set to the treatment temperature. Mussel mortality was scored and water changed with temperature-adjusted oxygenated hard water for periods which varied by temperature depending upon when the accumulation of mortality ceased (i.e., in test #1 and test #2, respectively, 30 and 21 days at 23?C, 40 and 37 days at 17?C, 91 and 90 days at 12?C, and 91 and 90 days at 7?C).

All binomial mortality data were analyzed following angular transformation (Sokal and Rohlf, 1995).

RESULTS AND DISCUSSION:

The water bath protocol that was used to maintain constant temperatures worked well. All mean temperatures stayed within the desired range of ±1ºC (actual mean temperatures ± SD were: 23.9 ± 0.5, 17.2 ± 1.0, 12.0 ± 0.7, and 7.3 ± 0.3ºC) (Fig. 1).

Fig. 1: Temperature recorded in each water bath before, during, and following treatment. The values in boxes represent mean ± SD over the entire period monitored.



Both water temperature tests achieved very similar results. Cells of strain CL0145A effectively killed zebra mussels at all four temperatures tested. Although higher mean mortality was achieved at warmer temperatures overall (i.e., 92.3 and 88.9% at 23ºC, 85.3 and 91.0% at 17ºC, 86.6 and 88.1% at 12ºC, and 83.7 and 76.4% at 7ºC), there was no significant difference between these mean values ($p>0.05$)[1] (Table 1). Mortalities at 23, 17, 12, and 7ºC occurred, respectively, within the following periods and then leveled off: ca. 2-15, 2-20, 4-40 and 8-50 days (Fig. 2). Thus, the lower the temperature of the water, the longer it took for mortality to commence and accumulate (Fig. 2). Total mortality of mussels treated in very cold temperature conditions may take up to three times as long as in warmer temperatures (three months at 7ºC vs. one month at 23ºC).

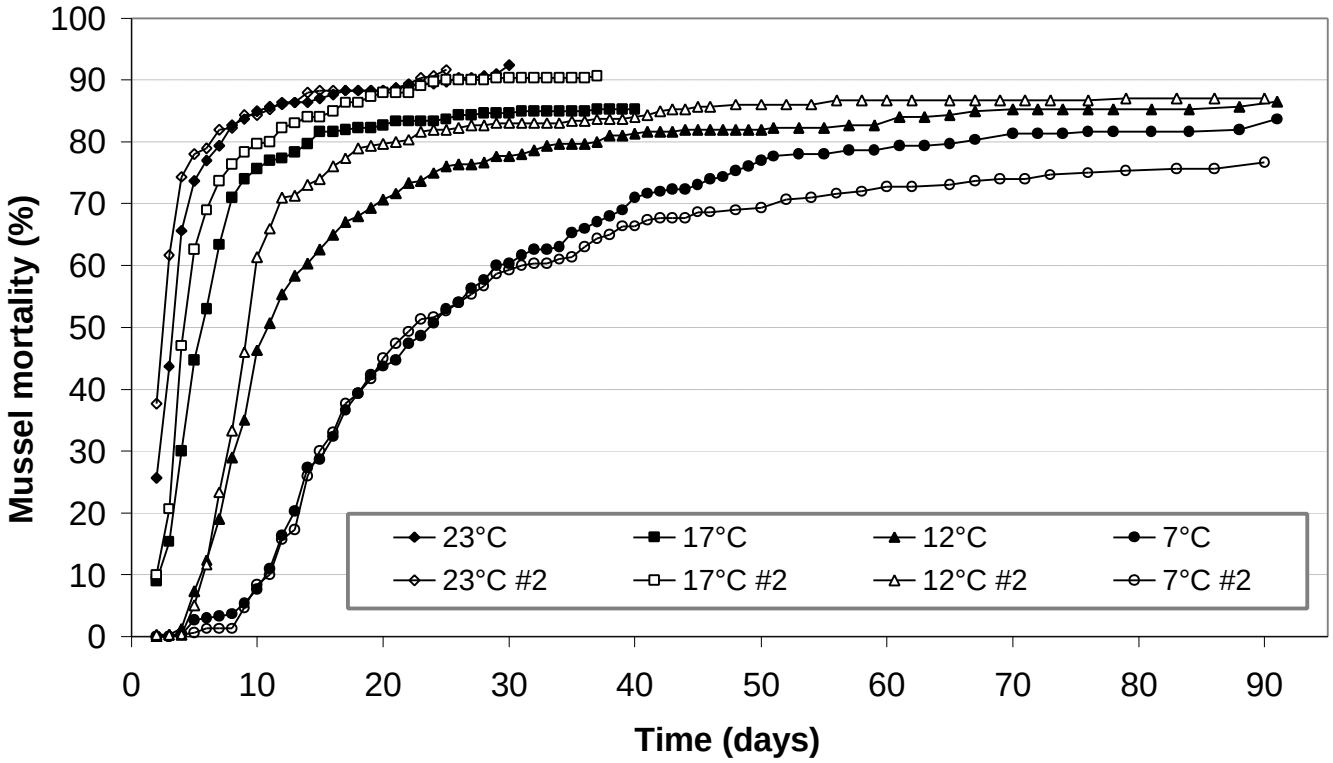
The results obtained in this test are very similar to those from an earlier test (author, unpublished data) in which mussels were treated at 6ºC and 20ºC, obtaining 73.0 ± 1.2% and 95.0 ± 1.3% mortality, respectively. In this earlier test the difference between mortality achieved at 20 and 6ºC was significant ($p=0.05$). Although the mortality data from different temperatures described in this report are not statistically significant by the Scheffe test, this is the third test to suggest that somewhat lower mortality will be achieved at very cold temperatures (e.g., 7ºC). The protocol for treatment concentration (ca. 200 ppm) and length of exposure (ca. 48 hr) used to treat mussels at colder temperatures (i.e., 7ºC) was the same as at warmer temperatures (i.e., 23ºC) in this test. However, since metabolic processes are slower at cold temperatures, under such treatment conditions (i.e., 7ºC) mussels may require longer exposure periods to ingest enough toxic bacterial mass to result in high mussel kill. Treating mussels at lower temperatures for a longer exposure time may result in mortality more similar to that achieved at warmer temperatures.

[1] Analysis of angular transformed mortality values using Scheffe test.

Table 1: Zebra mussel mortality at 23, 17, 12 and 7ºC after a 48 hr exposure to 144 and 183 ppm (test #1 and #2, respectively) treating 100 mussels/testing jar containing 500 ml of hard water.

Actual Water Temperature (Mean ± SD)	Test #	Period of mortality accumulation (days)	Mean zebra mussel mortality ± SD% (% mortality in untreated control)
23.9 ± 0.5ºC	1	30	92.3 ± 3.5% (1%)
	2	21	88.9 ± 11.5% (9%)
17.2 ± 1.0ºC	1	40	85.3 ± 4.6% (2%)
	2	37	90.8 ± 2.0% (0%)
12.0 ± 0.7ºC	1	91	86.55 ± 3.9% (1%)
	2	90	88.12 ± 13.6% (0%)
7.3 ± 0.3ºC	1	91	83.7 ± 4.6% (1%)
	2	90	76.4 ± 8.1% (0%)

Fig. 2: Zebra mussel mortality accumulation at 23, 17, 12 and 7ºC (data from two tests).



The results of these tests at 23, 17, 12 and 7ºC represent good news for the development of *P. fluorescens* strain CL0145A and its use for the biological control of zebra mussels since the chemical control methods currently used are virtually non-effective at low temperatures. Chlorine and other general molluscicides are only efficacious at temperatures above 18ºC. Our temperature experiments (combining these two tests with previous trials) indicate that *P. fluorescens* strain CL0145A applications can kill mussels at temperatures ranging from 6 to 27ºC. Chemical control measures are often hindered by natural occurrences that lower water temperature such as seasonal changes or weather patterns that stir up cooler water (i.e., lake winds off the Great Lakes). Thus, CL0145A's effectiveness at these lower temperatures will expand its potential use beyond the temperature limitations currently involved with the use of chemicals to kill zebra mussels.

CONCLUSIONS:

Our experiments (combining the two tests conducted this past quarter with previous trials) indicate that the use of *P. fluorescens* strain CL0145A is not temperature limited. This bacterial strain has effectively killed zebra mussels at all temperatures tested to date, i.e., 6-27ºC. Percent mussel kill will likely be somewhat lower at 6ºC than =12ºC, but high mussel kill (>70%) is possible even at such low temperatures (e.g., 6ºC). This indicates that this bacterium is more effective than currently commercialized chemical molluscicides that are used for zebra mussel control. The effectiveness of these latter commercialized products, like chlorine, is significantly diminished below ca. 18ºC.

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TECHNOLOGY AND INFORMATION TRANSFER:

Presentations

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.)

Media Coverage

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