TECHNICAL REPORT
(40751R07)

Report Title:	**Impact of Oxygen Concentration on Zebra Mussel Mortality**
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DISCLAIMER:

ABSTRACT:

These tests have indicated that the bacterium *Pseudomonas fluorescens* strain CL0145A is effective at killing zebra mussels in environments having dissolved oxygen (DO) concentrations ranging from very low to very high. The results suggest that the highest mussel kill can be achieved in moderately to highly aerated environments, while kill may be 0-20% lower under conditions of very low oxygen. For example, under highly oxygenated conditions 97% kill was achieved while conditions having low DO produced 79% mussel kill. Service water measured in a local power plant indicated that DO concentrations were in the range of 8-9 ppm (e.g., highly aerated) within their pipes. Therefore, we will not expect to see decreases in the efficacy of CL0145A treatments due to oxygen levels within such power plant pipes.

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EXECUTIVE SUMMARY:

Subtask 2.2 of the Statement of Work requires that the Contractor examine the effect of oxygen concentration on the efficacy of treatment with *Pseudomonas fluorescens* strain CL0145A. To address this subtask, tests were conducted in conditions having dissolved oxygen (DO) concentrations ranging from very low (0-1 ppm) to very high (ca. 6-8 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.

EXPERIMENTAL MATERIALS AND METHODS:

Culturing *Pseudomonas fluorescens* strain CL0145A:

Under shaken conditions, 250-ml Erlenmeyer flasks containing 25 ml of buffered tryptic soy broth (bTSB) were each inoculated with 0.4 ml of 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.

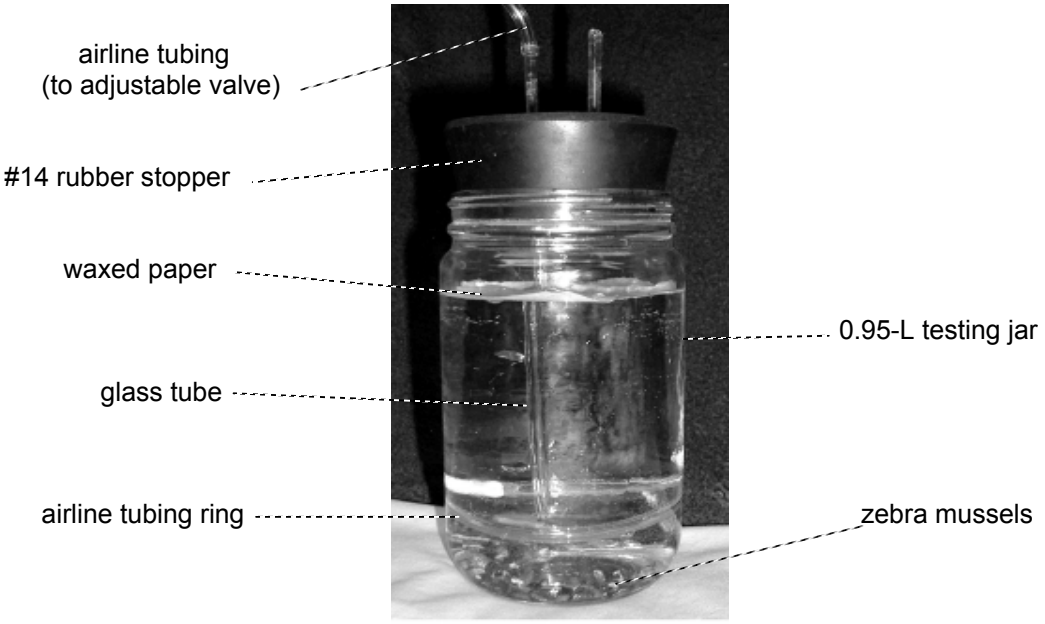
Obtaining the Cell Fraction Inoculum:

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 $MgCl_2 \bullet 6H_2O$ 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, λ = 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.

Producing Testing Conditions with a Range of Oxygen Levels:

Treatment vessels were 0.95-L glass jars each containing 750 ml of aerated hard synthetic fresh water (Peltier and Weber, 1985) and capped with a #14 rubber stopper fit with a glass tube (200 mm x 5 mm (length x outside diameter)) extending into the jar to 25 mm from the bottom (Fig. 1). A range of dissolved oxygen (DO) levels during the treatment period was achieved by decreasing the water-air surface area, delivering various levels of aeration, and by keeping the mussels near the bottom of the treatment vessel. A circle of waxed paper was cut to 85 mm in diameter with a 10 mm diameter hole near the center and placed on the surface of the water to reduce the water-to-air surface area. Aeration was provided and finely controlled through a pressurized air line with an air valve connected to the glass tube. Jars receiving no air were tightly sealed with screw cap lids. Mussels in the testing jars were kept near the bottom of the jar by friction-fitting an 85-mm diameter ring of vinyl airline tubing into the inside of the jar approximately 3 cm from the bottom to inhibit mussel migration during the treatment period.

Figure 1: Testing jar set-up used in oxygen tests.



Using the set-up described above, ranges of DO levels were achieved in two ways:

1) constant aeration tests: maintained constant incremental levels of aeration, ranging from no aeration to very high aeration, allowing DO levels to change, and
2) constant DO tests: levels of aeration were manually adjusted to maintain three distinct ranges of DO.

DO was monitored over time in each testing jar by submersing a DO probe to within 2.5 cm from the bottom of the testing jar (Orion model 97-08-99 probe connected to an Orion model 720A meter) and recording the measurement in mg/L (ppm).

Treatment of Zebra Mussels:

Five separate tests were conducted using zebra mussels collected from the Mohawk River (Crescent, NY) having mean lengths (±SD) of 9.01 ± 1.98 mm (tests #1 and #2), 8.77 ± 1.68 mm (tests #3 and #4), and 9.08 ± 1.47 mm (test #5). The water used in all testing jars was hard, synthetic freshwater (Peltier and Weber, 1985).

Prior to treatment, mussels were transferred from 7°C in a walk-in cooler to an aquarium containing 7°C unchlorinated tap water and allowed to warm slowly to ambient laboratory temperature (23°C). One day before treatment, 100 mussels were placed in 0.95-L testing jars containing ca. 100 ml of aerated hard synthetic water to a depth of 3 cm. The morning of treatment, unattached mussels were removed and replaced with attached mussels, the testing jars were filled with 750 ml of aerated hard synthetic water, fit with rubber stoppers and aerated as described above.

Constant aeration tests: Twenty testing jars, each containing 100 zebra mussels, were set up as described above (Fig. 1) with incremental levels of aeration ranging from no aeration to very high aeration. Mussels were exposed for 24 hr at an initial target concentration of 200 ppm of CL0145A cells. In addition, 1 highly aerated and 3 non-aerated jars were set up without bacterial treatment as untreated controls. An additional test was conducted comparing mussel mortality in testing jars with no aeration and high aeration.

Constant DO tests: Nine testing jars, each containing 100 zebra mussels, were set up as described above (Fig. 1) and treated at an initial target concentration of 200 ppm of CL0145A cells. DO was monitored in the testing jars and the level of aeration was manually adjusted to maintain three distinct DO concentrations of 1-2 ppm, 4-5 ppm and 7-8 ppm. Thus, mussels in 3 replicate testing jars at each DO level were exposed for 12 hr.

Following treatment, mussels were transferred to 7.6 x 7.6 cm plastic dishes containing ca. 100 ml of oxygenated hard water, and dead removed. The mussels were held in these dishes for an additional 9 days at 23ºC, and the water was changed, the dead were removed, and the mortality was scored each day.

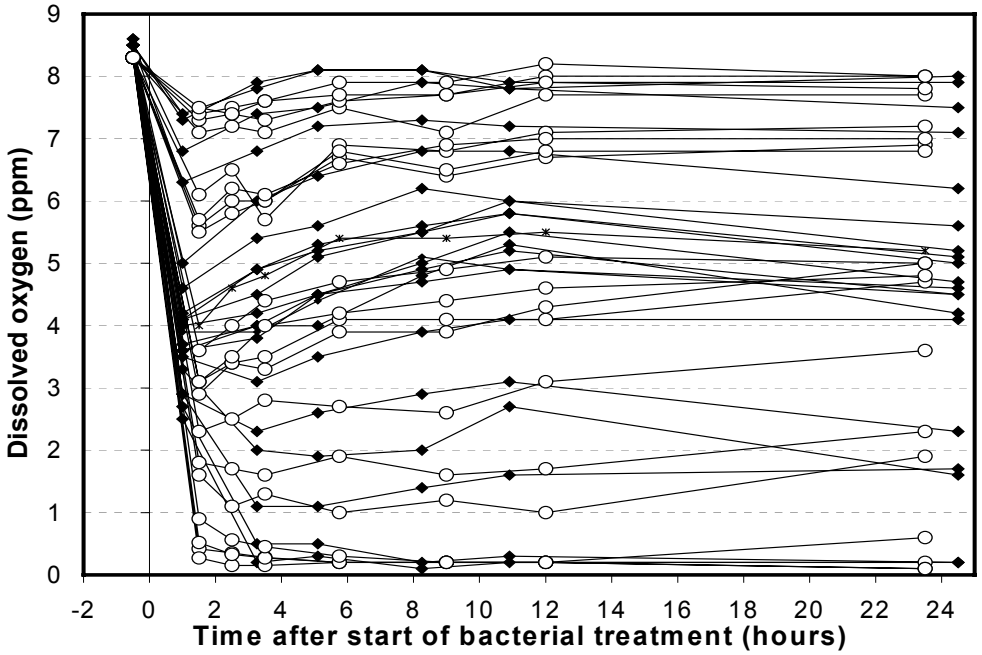
All binomial mortality data were analyzed following angular transformation (Sokal and Rohlf, 1995).

RESULTS AND DISCUSSION:

1. Oxygen Levels Obtained

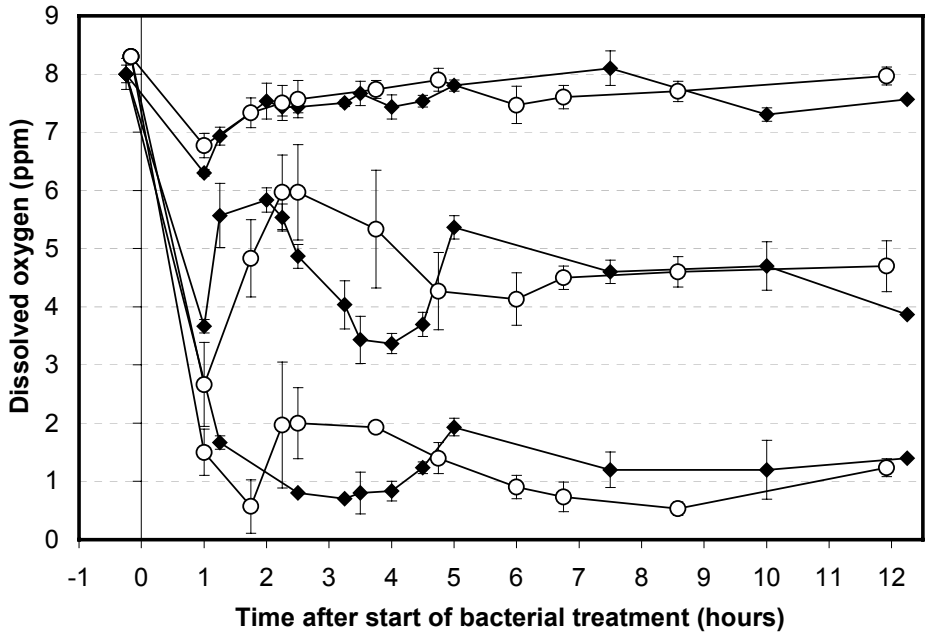
Constant aeration tests:
As desired, the testing jar protocols that were used provided testing conditions having a variety of DO levels. In jars provided with constant aeration during the 24-hr treatment period, DO levels typically dropped during the initial 2 hr of treatment and then remained relatively constant throughout the remaining 22 hr, ranging from 0.15 to 8.2 ppm (Fig. 2).

Figure 2: DO in testing jars provided with incremental levels of constant aeration (first test (♦) and second test (o)).



Constant DO tests:
As desired, we were able to maintain relatively constant levels of DO by adjusting the level of aeration in testing jars (Fig. 3). Targeted DO concentrations of 1-2, 4-5, and 7-8 ppm produced actual mean DO levels of 1.3, 4.6, and 7.5, respectively, in the 2 tests combined. Therefore, these conditions provided a series of incremental levels of DO during treatment in which to analyze the effects of DO concentration during application of CL0145A cells on zebra mussel mortality.

Figure 3: DO in testing jars at "constant" DO levels of 1-2 ppm, 4-5 ppm, and 7-8 ppm (first test (♦) and second test (o)). Bars represent standard deviation between data from three replicate testing jars.



2. Mussel Mortalities Obtained

Mussel mortality results indicate that DO concentration in the treatment water may impact the efficacy of CL0145A treatments. Treatment conditions consisting of constant aeration and 24-hr exposure (Table 1) resulted in similar mussel mortalities as in conditions in which a constant DO level was maintained for a 12-hr exposure (Table 2). In test #2, high mussel mortalities were achieved with both treatment protocols irrespective of DO concentration (range = 97.3 to 99.5% kill (Tables 1 and 2)). These results contrasted with results from tests #1 and #3 in which lower mortalities were achieved under conditions of lower DO concentration. For example, in conditions of constant aeration, we achieved 78.7 and 79.0% mean mussel mortality (tests 1 and 3, respectively) when treating in the 0-1 ppm DO range (i.e., very low aeration) and achieved 96.4 and 97.0% mean mussel mortality (tests 1 and 3, respectively) when treating in the 6-8 ppm DO range (i.e., very high aeration) (Table 1). When the DO in testing jars was maintained at 1-2, 4-5, and 7-8 ppm, respective mean mortalities were 83.5, 85.0, and 94.7% (Table 2, test #1). These data suggest that in low oxygen conditions where no or very little air is added to the treated jar, we will likely achieve 0-20% less mortality than in an aerated jar.

Table 1: Constant aeration tests: Mean mussel mortality after 24 hr exposure to 230, 214, and 193 ppm CL0145A cells (test #1, #2, and #3, respectively) treating 100 mussels/testing jar under constant levels of aeration. Represents total mortality accumulated 9 days following exposure.

Range of DO concentration*	Test #	Mean zebra mussel mortality ± SD% (% mortality in untreated control(s))
0-1 ppm	1	78.7 ± 7.5% (4.3 ± 3.5%)
	2	99.5 ± 0.6% (1.3 ± 1.5%)
	3	79.0 ± 4.2% (1.3 ± 0.6%)
2-3 ppm	1	78.7 ± 8.1%
	2	97.9 ± 1.5%
4-5 ppm	1	92.9 ± 5.5%
	2	98.0 ± 1.7%
6-8 ppm	1	96.4 ± 4.3% (1%)
	2	98.7 ± 1.3% (0%)
	3	97.0 ± 2.7% (0%)

* Approximate DO range during 2 to 24 hr after initial exposure.

Table 2: Constant DO tests: Mean mussel mortality after 12 hr exposure to 230 and 214 ppm CL0145A cells (test #1 and #2, respectively) treating 100 mussels/testing jar in ranges of constant DO. Represents total mortality accumulated 9 days following exposure.

Attempted constant DO range	Actual mean DO achieved over 12 hr treatment period	Test #	Mean* zebra mussel mortality ± SD%
1-2 ppm	1.3 ppm	1	83.5 ± 7.4%
		2	98.3 ± 1.5%
4-5 ppm	4.6 ppm	1	85.0 ± 7.5%
		2	97.3 ± 2.1%
7-8 ppm	7.5 ppm	1	94.7 ± 4.9%
		2	98.0 ± 1.0%

*n=3

CONCLUSIONS:

Results from these tests indicate that very high mussel mortality can be achieved in environments having DO concentrations ranging from very low to high, but occasionally (for unknown reasons) low DO readings result in lower mortality. The tests suggest that high mussel kill will be consistently achieved in moderate to high DO waters. To predict results in future power plant trials (i.e., service water), the DO concentration in the service water should be assessed. Recent DO measurements of flowing service water at the Crescent power plant (New York Power Authority) on two separate occasions revealed that the water was very highly oxygenated (i.e., 8-9 ppm DO). Therefore, according to these tests, CL0145A treatment applications in pipes under such high DO conditions should produce optimal mussel mortality.

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:

Presentations[1]

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.)

Media Coverage

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

[1] Some of these presentations were given prior to the current reporting period of October - December 2002, but were inadvertently omitted in prior reports.