TECHNICAL REPORT
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Report Title:	**Impact of the Duration of Bacterial Exposure on Zebra Mussel Mortality**
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DISCLAIMER:

ABSTRACT:

These tests indicated that: 1) duration of exposure to bacterial strain CL0145A of *Pseudomonas fluorescens* is a key variable in obtaining zebra mussel mortality; 2) that given a choice of exposure periods up to 96 hr, the longer the exposure period, the higher the mean mortality that will be achieved; 3) that the first few hours that the mussels are exposed to the bacteria are the most important in achieving kill; 4) that the mortality achieved by exposure periods ≥ 72 hr may be somewhat amplified by the degraded water quality conditions which can develop in recirculating water systems over such extended time periods.

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EXECUTIVE SUMMARY:

Subtask 2.3 of the Statement of Work requires that the Contractor examine how the duration of time that zebra mussels are exposed to *Pseudomonas fluorescens* strain CL0145A affects mortality rates. To address this subtask, two series of tests were conducted at a treatment concentration of ca. 300 ppm (mg of bacterial mass/L). In both tests, 100 mussels (mean length = 6.6 mm) were held in glass jars containing 500 ml of recirculating water at 23(±1)ºC. Mean mortality increased with exposure period in both tests. In test #1, bacterial exposures of 12, 24, 48, and 72 hr resulted, respectively, in 74, 78, 81, and 93% mean mortality. In test #2, bacterial exposures of 3, 6, 12, 24, 48, 72, and 96 hr resulted, respectively, in 49, 58, 70, 72, 78, 83, and 97% mean mortality. Thus, these tests indicated that the duration of exposure period can be critically important to achieving high mussel mortality, with the initial few hours being the most effective in producing kill. Evidence is presented that the mortality achieved by exposure periods ≥ 72 hr may be somewhat amplified by the degraded water quality conditions which can develop in recirculating water systems over such extended time periods. This is fortuitous, however, since in future trials in power plant pipes mussels that do not die solely from the toxin associated with strain CL0145A may finally succumb due to the additional stress of the degraded water quality conditions inherent in such recirculating water systems.

EXPERIMENTAL MATERIALS AND METHODS:

Culturing *Pseudomonas fluorescens*:

Under static conditions, 10-ml tubes containing 6 ml of tryptic soy broth (TSB) were each inoculated with 0.1 ml stock frozen bacterial culture. The tubes were then incubated at 26(±1)ºC under static conditions for ca. 41 hr. Tubes were subsequently used to inoculate 250-ml flasks (1 tube per flask) containing 100-ml TSB and incubated at 26(±1)ºC 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 300 ppm (mg dry bacterial mass/L). Three 0.5 ml samples of the CF inoculum were air dried in a dessicator then weighed on a Cahn C-30 microbalance to determine actual treatment concentration.

Treatment of Zebra Mussels:

Two tests were conducted at 23(±1)ºC using zebra mussels collected from the Mohawk River having a mean length (±SD) of 6.6 ±1.4 mm. Both tests used hard, synthetic, freshwater (Peltier and Weber (1985) in all test containers and were 10 days in duration (i.e., period of mussel exposure to the bacteria + post-treatment observation period).

In Tests #1 and #2, mussels were exposed to bacteria, respectively, for 12, 24, and 48 hr and for 3, 6, 12, 24, 48, 72, and 96 hr. In each test, 100 mussels were placed in a 1-L glass jar containing ca. 250 ml of aerated water and allowed to acclimate for ca. 24 hr pretreatment. Immediately before bacterial treatment, the volume of water in each jar was brought up to ca. 500 ml. Jars were then treated with bacterial CF and maintained under aerated conditions throughout the exposure period.

For each exposure duration, 5 jars of mussels were treated with toxic strain CL0145A of *P. fluorescens*. An additional 5 jars were treated in each test at the same exposure durations with strain CL0239 of *P. fluorescens*, a strain demonstrated in previous tests (author, unpublished data) to be nontoxic to zebra mussels. These additional treatments with strain CL0239 served as a "quality control" to indicate what mussel mortality, if any, might occur over time among zebra mussels simply due to the declining water quality conditions resulting from having mussels in testing jars containing high concentrations (i.e., 300 ppm) of a nontoxic bacterium. In each test, 3 jars of 100 mussels were also maintained as untreated controls.

In past jar tests in which mussels were exposed to bacteria using recirculating water (i.e., the bacterial exposure was not on flow-through conditions), the question had arisen as to whether mussels found dead at the end of an exposure period might have indirectly inflated mortality in the jar by contributing to poor water quality conditions during the exposures. To address this issue, 10 additional test jars were treated as outlined above with toxic strain CL0145A as part of Test #2. Five of these jars were treated for 72 hr, but were also inspected and dead mussels removed at 48 hr. The remaining 5 jars were treated for 96 hr and had their dead mussels removed at 48 and 72 hr.

Following bacterial exposure, mussels in all jar tests were transferred to petri dishes containing ca. 70 ml of previously aerated water and held under static conditions. Water was changed and dead mussels removed each day. All binomial mortality data were analyzed following angular transformation (Sokal and Rohlf (1995).

RESULTS AND DISCUSSION

Test #1

Mean mortality resulting from treatment with the toxic strain increased from 74% to 93% as the duration of bacterial exposure was extended from 12 to 72 hr (Table 1 and Fig. 1). In contrast and as expected, mussels in jars treated with the nontoxic strain had virtually no mortality at any of the exposure periods (Table 1). The slight mortality (ca. 1%) recorded following the 72-hr exposure to the nontoxic strain, however, did suggest that the water quality in jars treated with the nontoxic stain may have declined by 72 hr to a point where mussels were sufficiently stressed to incur some mortality. If true, then the 93% mean mortality achieved by the 72 hr exposure to the toxic strain may have been due not only to the toxin associated with strain CL0145A, but also in part from stress resulting from degraded water quality conditions. Statistical analysis supported this theory since mean mortality among mussels treated with the toxic strain for 12, 24 or 48 hr did not differ significantly from each other ($p=0.113$), but did differ significantly from the mortality achieved by the 72 hr exposure ($p<0.001$) (Fig. 1).

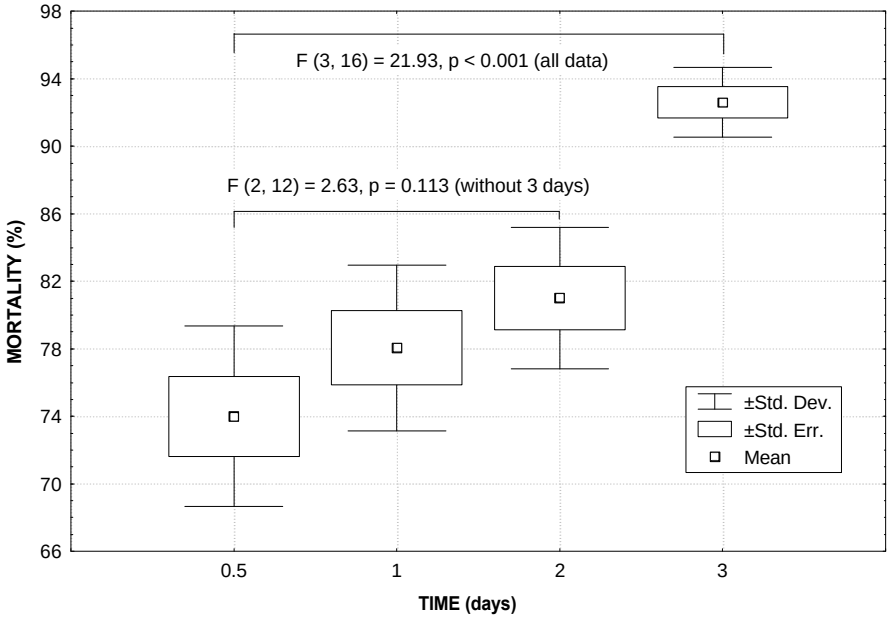
Table 1: Zebra mussel mortality resulting from exposure to toxic and nontoxic strains of *P. fluorescens* in Test #1.*

Exposure period (hr)	Mean mortality ±SD %**	
	Toxic strain CL0145A	Non-toxic strain CL0239
12	74.0 ±5.3%	0.0 ±0.0
24	78.1 ±4.9%	0.0 ±0.0
48	81.0 ±4.2%	0.0 ±0.0
72	92.6 ±2.1%	0.4 ±0.6

* Target concentration for both the toxic and nontoxic strains was 300 ppm; actual treatment concentrations were, respectively, 300 and 269 ppm.

** Mean mortality among 5 treated jars of 100 mussels; mean mortality ±SD among 3 "control" jars of 100 untreated mussels = 0 ±0.0%.

Fig. 1. Graphic expression of zebra mussel mortality resulting from exposure to toxic strain CL0145A of *P. fluorescens* in Test #1. Results of 1-way ANOVA.



Test #2

Mean mortality increased from 49% to 97% as the duration of bacterial exposure was extended from 3 to 96 hr (Table 2 and Fig. 2). Mortality resulting from exposure periods ranging from 12 hr to 72 hr, i.e., 70% to 83%, were similar to that recorded in Test #1, i.e., 74% to 93% (Table 1, Fig. 1). In Test #2, mortality increased gradually with increasing exposure period, such that each successive exposure period resulted in mortality that was not significantly different from the shorter exposure preceding it. The only exception to this was that mean mortality resulting from the 96 hr exposure (i.e., 97%) was significantly different ($p<0.01$) from that resulting from the 72 hr exposure (i.e., 83%). The reason for this relatively rapid increase in mortality from 72 hr to 96 hr exposures may have been due to a decline in water quality. The mortality from the nontoxic treatments support this hypothesis: mussel mortality resulting from nontoxic treatments was close to zero following exposures of 3, 6, 12, 24, and 48 hr, but increased to 3% following the 72-hr exposures. Similar results were recorded in Test #1 in which mean mortality in jars treated with the nontoxic strain was slightly elevated following the 72 hr exposure. In Test #2, however, the poor water quality conditions that can result from holding mussels in a container of bacteria-treated water for > 72 hr became more obvious with a mean mortality of 44% resulting from the 96 hr exposure to the nontoxic strain. These results indicated that the 97% mean mortality achieved by the toxic strain following the 96 hr exposures was likely amplified from the degraded water quality conditions resulting from having mussels confined in water treated with bacteria for ≥ 72 hr. Levels of ammonia, for example, increase in such recirculating test jars due in part to mussels feeding on bacteria, irrespective of whether the bacteria are toxic or not (author, unpublished data).

To what extent did the presence of dead mussels in test jars treated with the toxic strain amplify mortality? The test data indicated that the presence of dead mussels made no difference in the final mean mortality rates achieved. There was no statistical difference in mortality at the end of the 10-day test in jars whether dead mussels were allowed to remain until the end of the 72 hr exposure (mean mortality ±SD = 82.6 ±6.7%, Table 2) or whether they were also removed at 48 hr (mean mortality ±SD = 86.8 ±5.1%). Likewise, there was no statistical difference in mortality at the end of the 10-day test whether dead mussels were allowed to remain in the jars until the end of the 96 hr exposure (mean mortality ±SD = 97.2 ±1.7%, Table 2) or whether they were also removed at 48 hr and 72 hr (mean mortality ±SD = 96.2 ±1.5%). Thus, even though mussels are known to starting dying from the toxic stain within 24 hr of exposure, their decomposing bodies did not apparently degrade water quality conditions sufficiently to inflate mortality rates under our testing conditions.

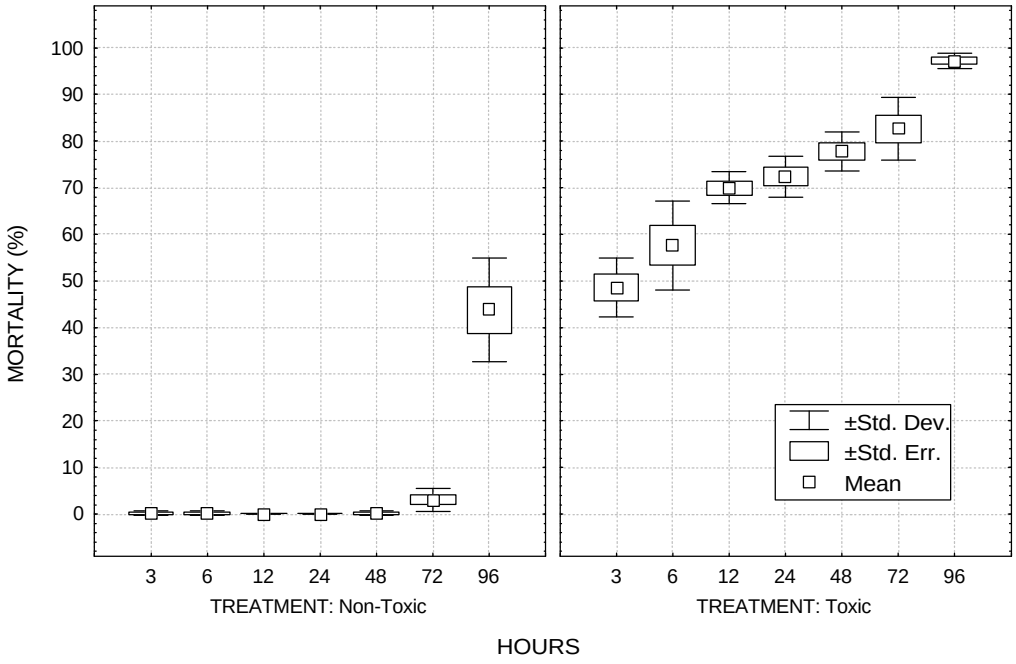
Most of the mussel death from exposure to the toxic strain resulted from the initial few hours of treatment, i.e., mean mortality resulting from just 3 hr of exposures was 49%, whereas an additional 3 hr exposure raised mortality by only 9% (i.e., 6 hr mortality = 58%, Table 2). This is not surprising since strain CL0145A kills because of its biotoxin, and the latter data fit a typical toxicological model whereby the higher the mortality, the harder it is to achieve.

Table 2: Zebra mussel mortality resulting from exposure to toxic and nontoxic strains of *P. fluorescens* in Test #2.*

Exposure period (hr)	Mean mortality ±SD %**	
	Toxic strain CL0145A	Nontoxic strain CL0239
3	48.6 ±6.4 a	0.2 ±0.5 f
6	57.6 ±9.6 a,b	0.2 ±0.5 f
12	70.0 ±3.5 b,c	0.0 ±0.0 f
24	72.4 ±4.4 c	0.0 ±0.0 f
48	77.8 ±4.2 c,d	0.2 ±0.5 f
72	82.6 ±6.7 d	3.0 ±2.5 f
96	97.2 ±1.7 e	43.8 ±11.2 a

* Target concentration for both the toxic and nontoxic strains was 300 ppm; actual treatment concentrations were, respectively, 306 ppm for and 290 ppm; mean mortality ±SD among 3 untreated control jars of 100 mussels = 1.0 ±1.0%.
** Mean mortality among 5 treated jars of 100 mussels; values followed by same letter are not statistically different ($p>0.05$).

Fig. 2. Graphic expression of Test #2 data. Zebra mussel mortality resulting from exposure to *P. fluorescens* nontoxic strain CL0239 and toxic strain of *CL0145A.*



These tests in recirculating water with strain CL0145A of *P. fluorescens* indicated that:

- exposure period can be critically important to achieving high mussel mortality rates;
- the longer the exposure period, the higher the mean mortality that will be achieved;
- the initial few hours that mussels are exposed to treatments are the most effective in achieving kill.

By including parallel trials with a nontoxic strain of *P. fluorescens*, these tests have also provided evidence that the mortality achieved by exposures ≥ 72 hr are not due solely to the toxin associated with strain CL0145A, but rather are amplified by the degraded water quality conditions which can develop in recirculating water systems over such extended time periods. This is fortuitous, however, since in future trials in power plant pipes, mussels that do not die solely from the toxin associated with strain CL0145A may finally succumb due to the additional stress of the degraded water quality conditions which tend to develop in such recirculating water systems.

CONCLUSIONS:

Subtask 2.3 of the Statement of Work requires that the Contractor examine the impact of the duration of bacterial exposure on zebra mussel mortality. These recirculating water tests used exposure periods from 3 to 96 hr and found that the longer the exposure period, the higher the mean mortality achieved. The results of these tests also indicated that the mortality resulting from exposures ≥ 72 hr may have been due in part to the additional stress of degraded water quality conditions – something that can be used to our advantage to bolster the mortality rate in future pipe tests using recirculating water in power plants.

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