FINAL REPORT

CHRONIC TOXICITY OF SACRAMENTO RIVER WATERSHED SAMPLES TO LARVAL FATHEAD MINNOWS

RESULTS OF COMPARATIVE QUALITY ASSURANCE TOXICITY TESTS 1998-1999

Volume 1 of 3
Report and Attachments

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CHRONIC TOXICITY OF SACRAMENTO RIVER WATERSHED SAMPLES TO LARVAL FATHEAD MINNOWS

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1.0 EXECUTIVE SUMMARY

Comparative fathead minnow 7-day chronic toxicity tests were conducted at the University of California, Davis, Aquatic Toxicology Laboratory (UCDATL) and AQUA-*Science* (A-S) on samples from the Sacramento River Watershed Program (SRWP). The purpose of the study was to provide QA/QC support for the SRWP program and to elucidate the role of ambient pathogens in any observed toxicity. A total of 76 samples were tested in thirteen monthly test events from February, 1998 through March, 1999. Water samples were collected from six sites; five sites on the Sacramento River (Alamar, Bend, Colusa, Freeport and Keswick) and one site on the Feather River. Endpoints monitored were fathead minnow survival and growth. Effects on fathead minnow survival and/or growth were observed in 38% (29 of 76) of samples tested in both laboratories. The number of samples that exhibited toxicity was similar in both laboratories: 23% at UCDATL and 20% at A-S. However, only 5% (4 of 76) of the samples tested produced toxicity in both laboratories. Antibiotic addition prevented the toxicity of each of these samples. Characteristics of the toxicity included high replicate variability, delayed onset and prevention by antibiotics. These characteristics, along with the results of histopathology evaluations on fathead minnows exposed to SRWP samples, strongly suggest that the observed toxicity was due to ambient water-borne pathogens (bacteria and fungi), and was probably not the direct result of chemical contaminants. This type of anomalous toxicity has recently been reported in fathead minnow chronic toxicity tests conducted with ambient water from around the U.S. and is currently under study by several research groups and a panel of experts. At this time, the recommendations from these groups are to utilize procedures, including antibiotic addition, filtration or U.V. sterilization to eliminate effects of pathogens in ambient samples. However, it is our view that these procedures should not be undertaken until pathogen-contaminant interactions in this test protocol are more fully understood. Recommendations for future studies include implementing rigorous test container cleaning protocols, investigating cold-shock and bacterial contamination of fathead minnow food as possible causes of the anomalous toxicity, and determining the feasibility of using alternative testing procedures for evaluating ambient toxicity.

2.0 BACKGROUND

In conjunction with the monitoring component of the Sacramento River Watershed Program (SRWP), three species chronic toxicity tests (larval fathead minnows, *Ceriodaphnia*, and algae) were conducted by the University of California, Davis, Aquatic Toxicology Laboratory (UCDATL) on water samples collected from 24 sites in the Sacramento River Watershed beginning in 1996. Fathead minnow mortality was observed in several toxicity tests conducted on SRWP water samples collected in 1997 and 1998 (Larson, 1998). The cause(s) of this toxicity was unknown, but may have been related to the presence of water-borne pathogen(s) (Marty, 1998). To enhance the Quality Assurance/Quality Control aspects of the SRWP chronic toxicity testing program, and to further elucidate the role of pathogens on the toxicity of SRWP samples, the California Urban Water Agency (CUWA) contracted with AQUA-*Science* (A-S) to conduct the chronic fathead minnow bioassays on approximately 25 percent of the SRWP samples tested by the UCDATL.

During the period from February, 1998 through March, 1999, comparative toxicity tests were conducted on a total of 76 samples in 13 monthly test events. The fathead minnow toxicity tests conducted at A-S employed antibiotic additions and special cleaning procedures to characterize the role of pathogens in the toxicity of the SRWP samples. The results of these comparative bioassays are reported herein.

3.0 MATERIALS AND METHODS

3.1 Test Samples

Fathead minnow toxicity tests were conducted at A-S on selected test water samples that were collected monthly from the study sites by UCDATL personnel (Appendix I). The GPS coordinates, description of the sample location and rationale for site selection are shown in Appendix II. The sites used in the comparative studies were selected because they had produced toxicity in one or more testing events during previous studies (Larson, 1998).

The samples were collected in chemically clean 1-gallon glass amber bottles as single, sub-surface grabs. The samples were stored on wet ice until delivery to A-S within 24

hours of collection. At A-S, the samples were stored in the dark at 4 °C until testing was initiated within 24 hours of sample delivery.

3.2 Fathead Minnow Toxicity Tests

3.2.1 Test Procedures

The 7-day chronic larval fathead minnow bioassays were conducted according to EPA protocol (USEPA, 1994. see Appendix III). Larval fathead minnows (< 24 hours old) were obtained from Aquatox, Inc. (Hot Springs, AK) via overnight carrier. Parallel bioassays for each test sample were conducted in 500 mL polyethylene Tripour™ beakers containing 250 mL of test solution with antibiotic addition, and in 250 mL Teflon™ beakers containing 200 mL of test solution without antibiotic addition. Each sample was tested with four replicates of ten fish. Fish were fed newly hatched brine shrimp (*Artemia*) nauplii three times daily. Each day during the test period, mortality was recorded, test containers were cleaned using the procedures described in Section 3.2.4, and test solutions were renewed (80% replacement). A single sample was used for the daily change-outs during the 7-day tests. Tests were conducted in a temperature-controlled room at 25 ± 1 °C with a photoperiod of 16 hours light:8 hours dark. At test termination, fish were killed with MS-222, rinsed with distilled water, dried in an oven at 100 °C for 20-24 hours, and weighed using an analytical balance with 0.1 mg sensitivity (Denver Instrument Co., Model A22DS). Mortality and weight data were analyzed using a computer program (ToxCalc™ 5.0, Tidepool Scientific Software).

3.2.2 Water Quality Measurements

The following water quality measurements were taken daily in the freshly prepared test solutions, using the indicated equipment: temperature (Tracable™ electronic thermometer); pH (Beckman™ Model 210 pH meter); dissolved oxygen (Orion™ Model 835 dissolved oxygen meter); conductivity (Orion Model 135 conductivity meter); alkalinity (Hach™ digital titrator, Method 8203); and hardness (Hach digital titrator, Method 8213). In addition, temperature, pH and dissolved oxygen were measured daily in all 24-hour solutions at sample renewal.

3.2.3 Antibiotic Additions

Two antibiotics, Maracyn™ (0.26 mg/L Minocyclin) and Maracyn II™ (erthromycin, 5.2 mg/L), were added to one set of the samples, as described in 3.2.1, to ascertain the role of ambient pathogens in toxicity of the test samples to the fathead minnows. The antibiotic concentrations used were recommended by the manufacturer of the two products (Mardel Laboratories, Glendale Heights, IL). Antibiotic stock solutions were freshly prepared at the beginning of each test period.

3.2.4 Test Container Cleaning

As described in Section 3.2.1, two types of test containers were used for the fathead minnow toxicity tests: plastic 500-mL Tripour beakers and 250-mL Teflon beakers. The plastic beakers are the test containers that are typically used in fathead minnow toxicity tests conducted at A-S. The 250-mL Teflon beakers were added to the test protocol because trial tests indicated they were easier to clean, e.g., the film on the bottom of the test containers could be more easily removed. In addition, it was thought that adsorption of the potential toxicants onto the container surfaces would be less in the Teflon containers than in the plastic containers.

Both types of test containers were rigorously cleaned daily during sample renewal using a special procedure developed at A-S. A glass turkey baster fitted with a 2.5-cm section of Tygon™ tubing (1.5 cm o.d. x 1.2 cm i.d., Cole-Parmer, Vernon Hills, IL) was used to remove feces and excess food, and to scrape off the film that formed on the bottom and sides of the test containers between sample change-outs.

4.0 RESULTS AND DISCUSSION

Fathead minnow chronic bioassays were conducted on 76 split samples from the SRWP in thirteen test events from February, 1998 through March, 1999. A summary of the results of the first nine test events has been previously submitted (Miller, 1998).

The results of the comparative chronic larval fathead minnow toxicity tests on split samples, conducted at A-S and at UCDATL, are shown in Attachment 1, summarized in Table 1 and discussed on an event-by-event basis below.

Table 1 Summary of Results of Comparative Chronic Fathead Minnow Toxicity Conducted at AQUA-*Science* and the UCDATL

Test Event (Date)	*Test Lab*	*Test Site*					
		Alamar	*Bend*	*Colusa*	*Freeport*	*Feather River*	*Keswick*
98-01 (2/29/98)	A-S	n/t		n/t	n/t	n/t	n/t
	UCDATL	●*▲*	●*▲*		●[a]▲[a]		
98-03 (3/18/98)	A-S		△*				
	UCDATL						
98-04 (4/22/98)	A-S						
	UCDATL					●	
98-05 (6/23/98)	A-S						
	UCDATL						
98-06 (7/21/98)	A-S		△*○*		△*	△*	
	UCDATL		●				
98-07 (8/20/98)	A-S			△*			△*
	UCDATL	▲	▲	▲	▲	▲	▲
98-08 (9/17/98)	A-S					△*	
	UCDATL						
98-09 (10/22/98)	A-S	△*	△*	△*	△*		△*
	UCDATL						
98-10 (11/18/98)	A-S						△*
	UCDATL		●				
98-11 (12/15/98)	A-S		△*			△*	
	UCDATL					▲	
99-01 (1/21/99)	A-S						
	UCDATL		●▲				
99-02 (2/18/99)	A-S						
	UCDATL			●		●▲	
99-03 (3/19/99)	A-S						
	UCDATL			▲			▲

n/t = sample not tested
a = not tested with antibiotic
* = Effect not present in test with antibiotic addition
○ = Significant effect of survival in test conducted at A-S
△ = Significant effect on growth in test conducted at A-S
● = Significant effect on survival in test conducted at UCDATL
▲ = Significant effect on growth in test conducted at UCDATL

Note: In addition to the samples shown above, toxicity tests were conducted on one sample from Sacramento Slough and two samples from the American River (see Attachment 1 for test results)

Event 98-01 (2/29/98): A total of five of eight samples tested by UCDATL (Alamar, Bend, Freeport, Sacramento Slough, and American River) detected significant effects on mortality and/or growth. However, these effects were absent in the two toxic samples that contained antibiotic. None of the 5 samples tested at A-S detected toxicity.

Event 98-02 (3/2/98): This test was an evaluation of the antibiotic addition procedure at A-S and is not shown in Table 1.

Event 98-03 (3/28/98): One sample produced significant growth effects at A-S (Bend), which were not present in the antibiotic-treated sample. None of the samples tested at UCDATL produced significant toxicity.

Event 98-04 (4/22/98): One sample (Feather River) produced significant growth effects at UCDATL. None of the samples tested at A-S produced significant toxicity.

Event 98-05 (6/23/98): No toxicity was detected in any of the samples tested by either laboratory.

Event 98-06 (7/21/98): Toxicity was detected in A-S tests on Bend (survival and growth), Freeport (growth) and Feather River (growth). However, no toxicity was observed in the antibiotic-treated samples from these sites. The UCDATL tests detected effects on growth in the Bend sample.

Event 98-07 (8/20/98): Effects on growth were detected in all of the samples tested at UCDATL. Two of the samples tested at A-S (Colusa and Keswick) produced effects on growth, which was not present in the antibiotic-treated samples.

Event 98-08 (9/17/98): One sample tested at A-S (Feather River) produced effects on growth, which was not present in the antibiotic-treated aliquot. None of the samples tested at UCDATL produced toxicity.

Event 98-09 (10/22/98): Growth effects were observed in five samples tested at A-S (Alamar, Bend, Colusa, Freeport and Keswick). However, the effects were absent in the antibiotic-treated replicates. No toxicity was detected in any of the samples tested at UCDATL.

Event 98-10 (11/18/98): Survival effects were detected in one sample tested at UCDATL (Bend), which were not detected at A-S. The growth effects seen in one sample tested at A-S (Keswick) were not present in the sample with antibiotic present.

Event 98-11 (12/15/98): Growth effects seen in two samples at A-S (Bend and Feather River) and one sample at UCDATL (Feather River) were not present in the antibiotic-treated sample.

Event 99-01 (1/21/99): Effects on growth and survival were detected in one sample (Bend) at UCDATL. None of the samples tested at A-S detected toxicity.

Event 99-02 (1/21/99): Toxicity was detected in two samples tested at UCDATL (Colusa and Feather River), but was not present in any of the samples tested at A-S.

Event 99-03 (3/19/99): Two samples tested at UCDATL (Colusa and Keswick) produced effects on growth and/or survival. None of the samples tested at A-S produced detectable effects.

Summary of Results

- 29 of the 76 samples (38%) tested in both laboratories produced significant effects on growth and/or survival.
- The number of samples which produced toxicity was similar in both laboratories: 15 of 76 (20%) at A-S and 18 of 76 (23%) at UCDATL. No toxicity was detected in the samples with antibiotic additions.
- Only 4 of the 76 samples (5%) produced significant toxicity in both laboratories and antibiotic addition prevented the toxicity of each of these samples.

5.0 QUALITY ASSURANCE

The 7-day fathead minnow toxicity test protocol requires that control survival be ≥80% and that growth be ≥0.250 mg/fish. The control performance for the fathead minnow toxicity tests with the SRWP samples are shown in Table 2.

Table 2 Control Performance for SRWP Fathead Minnow Chronic Toxicity Tests

Test Event[a]	*Survival (%)*	*Growth (mg/fish)*[b]
98-01	95	0.251
98-03	98	0.254
98-04	99	0.286
98-05	99	0.270
98-06	100	0.252
98-07	99	0.257
98-08	100	0.309
98-09	100	0.317
98-10	100	0.196
98-11	99	0.250
99-01	98	0.320
99-02	86	0.333
99-03	100	0.317
Mean ± SD	***98 ± 4***	***0.275 ± 0.03***

a Test event 98-02 was a trial test to calibrate antibiotic additions
b Mean of 8 replicates with 10 fish/replicate

As shown above, minimum requirements for survival were achieved in all 13 test events. Mean control survival was 98 ± 4%. Minimum requirements for growth were achieved in 12 of the 13 test events. In event 98-10, the fish were dried for an excessively long period due to technician error. Therefore, these control weights are considered invalid.

6.0 DISCUSSION

The vast majority of the SRWP samples that produced toxicity in this study had characteristics that suggested that ambient pathogens had a role in the toxicity. These characteristics are discussed below.

High Replicate Variability: Samples that exhibited toxicity had unusually high variability among replicates. In extreme cases, survival among the four replicates ranged from 0-100%. This anomaly has been observed by others in ambient water fathead minnow toxicity tests conducted nationally (Kszos, et. al, 1997; Grothe, et. al., 1976; Norberg-King and Mount, 1986; and Stewart, et. al., 1990).

Antibiotic Addition Prevents Toxicity: In this study, none of the antibiotic-treated samples produced significant toxicity. This suggests that ambient bacteria have a causal role in the toxicity. Histopathological analysis of fathead minnows exposed to SRWP samples also suggested that ambient water-borne pathogens (bacterial and fungi) had a causal role in the anomalous mortality (Marty, 1998). However, the report also notes that other non-toxicant variables may have contributed to the anomalous mortality, including shipping stress, age of test organisms, water hardness, and test container hygiene (cleanliness).

Delayed Mortality: The mortality typically occurred after day 4 of the study. This pattern has been reported by others (Kszos, et. al., 1997).

Seasonal Pattern of Mortality: Most of the anomalous toxicity was observed in the fall and winter months. This characteristic has been observed in other studies (Grothe, et. al., 1996; Kszos, et. al., 1997). The role of pathogens in this characteristic of the toxicity has not been established, although compromised immune system function caused by cold-shock during transport of the test organisms is one possibility.

Other Evidence for Role of Pathogens: In tests conducted by others, treating the sample with bactericidal levels of UV light increased mean survival time and lowered among-replicate variability. Also, testing of fathead minnows singly (1 fish/beaker) demonstrated that the conventional protocol system (four replicates of 10 fish/beaker) facilitates the spread of pathogens from fish to fish (Kszos, et. al., 1997).

The results of this study and other recently published reports suggest that water-borne pathogens, including bacteria (*Flexibactor* sp., *Aeromonas* sp.) and fungi (*Saprolegnia*), can produce anomalous results in chronic fathead minnow tests with ambient waters. Characteristics of this anomalous toxicity include:

- Prevention of toxicity by antibiotic treatment or UV sterilization and filtration of the samples
- High replicate variability
- Delayed onset of toxicity
- Presence of bacteria and fungi in moribund minnows

Some experts have suggested that procedures such as sterilization by filtration, UV or heat treatment, or antibiotic addition should be used in conjunction with the standard testing protocol to eliminate or control the pathogens in the test samples to avoid the anomalous mortality problem (SETAC, 1999). We think that each of these procedures has unknown consequences which may complicate assessing the "true" toxicity of the ambient samples. For example, UV treatment can destroy organic compounds and generate singlet oxygen, which is extremely toxic and reactive (Stewart, 1990). Autoclaving can destroy heat-liable organic toxicants. Filtration can remove dissolved and suspended hydrophobic or metallic toxicants. Moreover, the use of antibiotic addition, while very effective in eliminating anomalous toxicity, poses serious questions about the interaction of toxicants with naturally occurring pathogens in ambient waters.

The difficult question of how to control this anomalous fathead minnow mortality is currently under study by a special committee of the Society of Environmental Toxicology and Chemistry (SETAC). We have provided comments to this committee and a draft report has been published (Appendix IV). A final report on this issue is due from the committee in February, 2000.

Currently, we are focussing on two possible causes of the pathogen-related mortality: cold stress, which may be occurring during transport of the test organisms, especially during the winter months; and the role of bacteria associated with the brine shrimp, which are fed to the fathead minnows during the toxicity test.

7.0 RECOMMENDATIONS

Until the anomalous toxicity issue is fully characterized and a consensus is reached on how to deal with the problem, we recommend the following.

1. Discontinue the antibiotic addition to split samples. Although this procedure is effective in eliminating the vast majority of the anomalous toxicity, the interaction of ambient pathogens with chemical and metallic contaminants is poorly understood.
2. Adopt a rigorous test chamber cleaning protocol in all ambient fathead minnow toxicity tests. The use of Teflon test chambers facilitates the cleaning procedure because the film that forms on the bottom of the test containers is more easily removed. Although the rigorous cleaning protocol is somewhat less effective in preventing anomalous toxicity than the addition of antibiotic, this procedure does not have unknown consequences on assessing the "true"

toxicity of the ambient samples associated with other possible solutions, including antibiotic treatment, UV treatment and filtration sterilization.

3. If toxicity is detected in ambient samples, retest the sample to confirm the presence of toxicity before Toxicity Identification Evaluation studies are conducted on the sample.
4. Investigate the role of cold-shock and brine shrimp-borne pathogens in the anomalous toxicity. Communicate these findings, as appropriate, to the SETAC expert committee.
5. Investigate the feasibility of using alternative test protocols, such as the rainbow trout development test to supplement or replace the fathead minnow tests for the SRWP samples. This development test has several attractive features including relatively low cost, applicability to the SRWP samples since salmonids are resident and threatened in the system, and the amenability of the test to computerized scoring.

8.0 REFERENCES

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APPENDIX I

Location of Test Sites for Comparative Fathead Minnow Toxicity Tests

Test Event (Date)	*Test Site*							
	Alamar	*Bend*	*Colusa*	*Freeport*	*Sac. Slough*	*Feather River*	*American River*	*Keswick*
98-01 (2/29/98)		√	√	√			√	
98-03 (2/28/98)	√	√	√	√		√	√	√
98-04 (4/22/98)	√	√	√	√	√	√		
98-05 (6/23/98)	√	√	√	√		√		√
98-06 (7/21/98)	√	√	√	√		√		√
98-07 (8/20/98)	√	√	√	√		√		√
98-08 (9/17/98)	√	√	√	√		√		√
98-09 (10/22/98)	√	√	√	√		√		√
98-10 (11/18/98)	√	√	√	√		√		√
98-11 (12/15/98)	√	√	√	√		√		√
99-01 (1/21/99)	√	√	√	√		√		√
99-02 (2/18/99)	√	√	√	√		√		√
99-03 (3/19/99)	√	√	√	√		√		√

APPENDIX II

Description of Access Directions of the Samples Sites Monitored in the 1997-98 Sacramento River Watershed Toxicity Survey

Map ID[a]	*Site*	*Total No. of Samples Collected*	*GPS*[b] *Coordinates*	*Access Directions*	*Rationale for Site Selection*[c]				
					1	*2*	*3*	*4*	*5*
21	Sacramento River at Alamar (this site is considered mainstem Sacramento River)	12	LN 38° 31.780 W 121° 37.650	The sample was collected from the upper end of the Alamar Marina dock. The Alamar Marina is located about 50 feet upstream from the I-5 Veterans bridge.				x	x
5	Sacramento River at Bend Bridge (this site is considered mainstem Sacramento River)	13	LN 40° 09.242 W 121° 11.955	Bank samples were collected from the south shore of the Sacramento River at the Bend Road Bridge			x	x	x
6	Sacramento River at Colusa (this site is considered mainstem Sacramento River)	13	LN 39° 12.827 W 122° 00.015	Bridge samples were collected from the middle of the river, from the Hwy. 45 Bridge near the City of Colusa. This site is upstream of all major agricultural drains.					x
23	Sacramento River at Freeport Blvd. (this site is considered mainstem Sacramento River)	13	LN 38° 27.426 W 121° 30.123	Shore samples were collected from the upstream end of the fuel dock at the Freeport Marina.				x	x
18	Feather River (this site is considered a major tributary of the Sacramento River)	12	LN 38° 53.017 W 121° 36.824	Bank samples were collected from shore approximately 2 miles downstream of the Hwy. 99 bridge accessed off of Garden Hwy,	x		x	x	x
4	Sacramento River d/s Keswick Dam (this site is considered mainstem Sacramento River)	11	LN 40° 35.627 W 122° 23.919	Bank samples were collected from the east side of the Sacramento River approximately 200 meters upstream of Lake Redding Weir. This sample represents the discharge from Shasta Dam, Spring Creek Debris Dam and Spring Creek Power Plant	x	x	x	x	x

a Numbers refer to site locations on the map of the study area

b GPS = Global Positioning System

c Numbers refer to the following rationale for selection:

1. The site was a significant source of flow or loads into the watershed
2. The site was a representative type of drainage (i.e., agricultural, urban, mining)
3. The site was a critical or sensitive habitat area (i.e., spawning and nursery area for anadromous fishes)
4. The site had existing indications of water quality degradation (i.e., previous toxicity or water quality objective exceedances)
5. The site afforded opportunities to collaborate with other monitoring programs (BPTCP, AMP, NAWQA, RMP, DWR, DPR, etc.)

d d/s = downstream

APPENDIX III

Summary of Test Protocol

SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE FATHEAD MINNOW, *PIMEPHALES PROMELAS,* LARVAL SURVIVAL AND GROWTH TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS

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1. Test type:	Static-renewal
2. Temperature:	25 ± 1 °C
3. Light quality:	Ambient laboratory illumination
4. Light intensity:	10-20 μE/m^2/s (50-100 ft-c, ambient laboratory levels)
5. Photoperiod:	16 h light, 8 h darkness
6. Test chamber size:	500 mL (minimum)
7. Test solution volume:	250 mL (minimum)
8. Renewal of test solutions:	Daily
9. Age of test organisms:	Newly hatched larvae less than 24 hours old. If shipped, not more than 48 hours old, 24 hours range in age
10. No. larvae per test chamber:	15 (minimum of 10)
11. No. replicate test chambers per concentration:	4 (minimum of 3)
12. No. larvae per concentration:	60 (minimum of 30)
13. Source of food:	Newly hatched *Artemia* nauplii (less than 24 hours old)
14. Feeding regime:	Feed 0.1 g newly hatched (less than 24-hour old) brine shrimp nauplii three times daily at 4-hour intervals, or as a minimum, 0.15 g twice daily, 6 hours between feedings (at the beginning of the work day prior to renewal, and at the end of the work day following renewal). Sufficient nauplii are added to provide an excess. Larvae fish are not fed during the final 12 hours of the test.
15. Cleaning:	Siphon daily, immediately before test solution renewal
16. Aeration:	None, unless D.O. concentration falls below 4.0 mg/L. Rate should not exceed 100 bubbles/min
17. Dilution water:	Uncontaminated source of receiving or other natural water, synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals, or DMW (see Section 7, Dilution Water)

SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR THE FATHEAD MINNOW, *PIMEPHALES PROMELAS,* LARVAL SURVIVAL AND GROWTH TOXICITY TESTS WITH EFFLUENTS AND RECEIVING WATERS (continued)

18. Test concentrations:	Effluents: Receiving water:	Minimum of 5 and a control 100% receiving water or minimum of 5 and a control
19. Dilution factor:	Effluents: Receiving waters:	≥ 0.5 series None or ≥ 0.5
20. Test duration:	7 days	
21. Endpoints:	Survival and growth (weight)	
22. Test acceptability criteria:	80% or greater survival in controls; average dry weight per surviving organism in control chambers equals or exceeds 0.25 mg	
23. Sampling requirements:	For on-site tests, samples collected daily, and used within 24 h of the time they are removed from the sampling device. For off-site tests, a minimum of three samples collected on days one, three, and five with a maximum holding time of 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4)	
24. Sample volume required:	2.5 L per day	

APPENDIX IV

Draft Report of SETAC Expert Panel

POTENTIAL PATHOGENIC INTERFERENCE IN SHORT-TERM CHRONIC WET TESTS USING FATHEAD MINNOWS

BACKGROUND

Some facilities have observed unusual patterns of receiving water control survival in short-term chronic WET tests with fathead minnows(*Pimephales promelas)* when samples from the receiving water are used as the test dilution and control water (diluent). The problem takes the form of apparently random mortality across replicates of receiving water controls and effluent exposures, often resulting in invalid tests despite adequate lab water control performance. This phenomenon may produce test results which are difficult to interpret and may complicate decisions regarding the toxicity of the surface water and of the effluent being tested.

The discussion that follows is based on the experiences of the authors and their colleagues, professional contacts, and clients. Except when otherwise noted, the experimental results described herein are taken from unpublished technical reports submitted for compliance with NPDES permits. Because of the variety of data sources, it is not practical to provide detailed data summaries or to secure permission to cite those reports in this document. Although technical reports submitted in conjunction with NPDES compliance are part of the public record it is typically a point of professional courtesy to not cite data and technical reports without the permission of the pemittees. Readers who require further information on particular results and data sets are encouraged to contact members of the WET Advisory Panel on Performance Evaluation and Interpretation of WET Data.

The phenomenon appears to be due to a biological agent. It can appear not only in WET tests using the receiving water as diluent but also in effluent from Aonce through@ non-contact cooling water operations and in tests on ambient waters. (By Atests on ambient waters@, we mean those tests on surface waters where the toxicity of the surface water is itself of interest). This discussion will focus on WET tests using receiving water as diluent. In these tests, the receiving water is being used as diluent because it provides a test matrix that more closely approximates in-stream conditions than reconstituted laboratory water. In these cases, the toxicity of the effluent in the receiving water matrix is of interest. Mortality in the test, which is due to pathogens in the diluent, can be viewed as interference, which prevents conclusions regarding the toxicity of the effluent. When this problem is common it may be appropriate to treat the diluent in a way that removes the interference. Various methods of sample treatment will be discussed below. While we recognize that manipulating the diluent will alter it in unknown ways, we suggest that the treated diluent or synthetic dilution water may still represent a suitable matrix and their use may be preferable to repeating invalid tests. The decision to use laboratory water or treated receiving steam as diluent should be the result of a dialogue between the permittee and the regulator.

Biological interference in tests using receiving waters may be contrasted with the presence of such interference in tests using effluents and ambient waters. With effluents and ambient waters the issue may be whether the pathogen is masking the presence of a chemical that is, by itself, toxic. This issue is relatively easy to resolve. More difficult is the possibility that

the pathogen infection is facilitated by some predisposing factor and would not occur without that factor. This problem becomes one of describing a toxin/pathogen interaction where the pathogenesis itself is of interest.

Such is not the case in WET tests using receiving waters as diluent. In these WET tests we are interested in the toxicity of the effluent in the receiving water matrix and biological infections originating from the matrix constitute interference. Accordingly, this discussion will focus on problems encountered in WET tests using receiving water as diluent and defer the obvious (and interesting) toxicity identification issues to another discussion.

Q: What are some common characteristics of this phenomenon?

A: The phenomenon typically shows the following characteristics:

1. Effects are seen in *Pimephales promelas* 7-day chronic tests but not in 96-h acute tests with fathead minnows.

2. Effects are not seen in chronic tests using invertebrates (e.g. *Ceriodaphnia dubia*) conducted concurrently with the fathead minnow tests.

3. In some areas the effects appear to occur seasonally, occurring anytime during the late fall to early spring. In other areas, effects have been noted in all months of the year.

4. There is typically high variability in survival among replicates. It is not uncommon for mortality in a particular concentration to range from 0% to 100% among replicates.

5. The dose response is often non-monotonic (i.e, atypical). That is, mortality is not always highest in the highest sample concentrations. In tests where receiving waters are used for dilution, the receiving-water controls and the lower effluent concentrations may show lowered survival and high replicate variability while higher effluent concentrations do not show effects.

6. Mortality is often first noted in receiving-water controls and lower effluent concentrations on day 4 of the chronic test, but not before (and not in the 4 day acute test). 7. There is sometimes fungal growth on fish, especially in the gill area. This fungal growth has been attributed to *Saprolegnia* sp

While many of these characteristics may not be observed in any single test, items 1, 4, and 5 together will be the most common.

Q: What evidence is there to suggest that the problem is pathogenic and not due to poor laboratory performance?

A: Toxicity tests in which receiving water is used as diluent should include a performance control using laboratory water. Consistent differences between adequately performing lab water controls and receiving water controls indicate that the mortality in the receiving controls is a characteristic of the diluent and not the procedure.

The phenomenon does not appear to be due to lax QA/QC as it is observed in states which maintain an active QA/QC certification program for biomonitoring laboratories. In Wisconsin, for example, these results have been shown in tests from all ten laboratories that have performed tests for compliance in the state of Wisconsin during the last ten years (including the state=s lab). Each of these laboratories is certified by the state of Wisconsin and has shown no QA, procedural, or other discrepancies which could be expected to cause these problems to occur. A variety of culturing conditions (e.g., water supply sources, in-house vs. outside sources of organisms, etc.) exists at each of these laboratories.

It seems unlikely that the phenomenon is due to inadequate cleaning of test vessels. As described above, the phenomenon is observed in laboratories where independent QA scrutiny is present. If the problem is due to poor Abeaker hygiene@ it should be common with all types of samples, not just receiving waters and once through effluents. This explanation is also not consistent with many results in which the phenomenon is seen in the receiving water controls and low effluent concentrations (which contain mostly receiving water) but not in the high effluent concentrations and undiluted effluent. The effects of inadequate beaker cleaning might be expected to affect all concentrations.

Toxicity Identification Evaluation (TIE) of samples has shown that any form of sterilization is effective in eliminating or reducing mortality. For some samples, one or more of the following techniques [autoclaving, pasteurization, addition of antibiotics, filtration (0.2 u pore size), and irradiation with ultraviolet light] have been shown to effectively improve survival and reduce variability among replicates. Attempts to associate the lowered survival with chemical toxicity have not been successful.

One set of experiments showed that when living fish carrying fungus (recall that the fungus is probably a secondary effect; in this experiment the presence of fungus was simply a means of identifying affected fish) are removed from the test beakers on days two and three, the remaining fish were much more likely to survive than when fish were removed only if they died.

These observations are compelling evidence of the presence of a pathogen, but to date, a specific pathogenic cause of the phenomenon has not been isolated and identified. A number of bacteria have been identified in tests showing the phenomenon, including *Flexibacter auranticus, Flexibacter columnaris, Flavobacterium spp.* (Wisconsin Department of Natural Resources, unpublished data.) Histopathological examination of dead and dying fish from toxicity tests exhibiting the phenomenon has shown the presence of what is most likely a bacteria belonging to the *Aeromonas hydrophila* complex. A secondary infection of the fungus *Saprolegnia*, sp. is sometimes also present. Attempts to correlate *Aeromonas* abundance with mortality have not been successful. While *Aeromonas* is usually considered to be an opportunistic pathogen it is likely that virulent strains may exist.

Q: How common is the phenomenon?

A: Several facilities in various regions of the country have observed the phenomenon. However, in many cases the data are anecdotal and not well documented. The phenomenon may occur whenever surface waters are used in testing throughout the year. It is seen in a wide variety of receiving waters, including headwaters, outstanding resource waters, and other locations where no known point or non-point impacts were expected. It can be seen in tests in which samples from the receiving water are used as diluent as well as in effluents from facilities that use surface waters for non-contact cooling (e.g. power plants) and in tests on ambient waters.

In Texas, eight power plants have observed similar effects as noted above in their effluents (once-through cooling waters) and receiving-water controls. These seem to occur most often during late fall to early spring.

Data from New England indicate that shallow, slow running, highly urbanized streams/rivers are more likely to experience this phenomenon and a seasonally effect has also been noted. Data from Massachusetts and Rhode Island show rivers, which consistently produce this phenomenon, others which show it periodically, and some which do not show it at all.

In Wisconsin, the effects described above have occurred during all months of the year. Of a total of 1,308 chronic *P. promelas* tests performed in the last ten years, 346 (26%) have shown unacceptable survival (<80%) in receiving-water controls. Of a total of 124 receiving waters used in these tests, 91 (73.3%) have shown these unacceptable receiving water control survival during one or more tests. These receiving waters range in size from large rivers (Mississippi & Wisconsin Rivers - 7Q10 > 1,200 cfs) to shallow, intermittent streams (7Q10 = 0 cfs). These results have also occurred with waters taken from lakes, pools, and impoundments (Wisconsin Department of Natural Resources, unpublished data).

In Tennessee, Kszos et al (1997) reported this phenomenon in numerous streams, including headwater streams. During a 10-year period, 4 reference sites (headwater streams having diverse benthic invertebrate communities and lacking known anthropogenic contaminants) near Oak Ridge, TN, were tested using the *P. promelas* chronic test system. Out of 94 tests, 16 (16.8%) had mean survival < 60% and only 31.6% of the tests had survival >90% (vs. 98.5% of laboratory-water controls with survival >90%).

Variability was examined by comparing coefficients of variation (CV=s) in survival in effluents with water from ambient sites for cases where mean survival was 40% to 70%. CV=s of Alow survival@ ambient water tests were twice as high (48.2%) as those from Alow survival@ effluent tests (23.6%, p = 0.0007). Treatment of ambient water samples with ultraviolet (UV) light greatly reduced the frequency of low survival.

Q: Is this pathogen-induced mortality observed in effluents?

A: Effluents made up of Aonce through@ cooling water derived from surface water may show the characteristics outlined above. The phenomenon has also been observed in at least one municipal effluent. However, for reasons discussed above, this discussion will focus on pathogen induced mortality in receiving water diluent.

Q: What can be done about it?:

A: The main objective of conducting toxicity tests using receiving steam as diluent should be to produce valid tests which reflect characteristics of the effluent in a test matrix which approximates in-stream conditions as closely as possible. Towards this end a simple solution would be to use reconstituted lab water as diluent. If it is desired that the diluent more closely resemble the receiving water then some sort of sterilization of the receiving water diluent is necessary.

There are a number of effective means of removing the pathogen and each method has advantages and disadvantages. Manipulating a sample changes it in unknown ways and this could conceivably change toxicity test results. Our purpose here is not to recommend one method for all situations. The method chosen should be the result of a dialogue between the permittee and regulator. A pilot study comparing sterilized and unsterilized samples should be conducted to evaluate the effectiveness of any chosen method. Any toxicity tests using sterilized sample must also include a Ablank@ preparation consisting of similarly sterilized laboratory water. All methods will add to the cost of testing but should be more cost effective than repeating invalid tests.

1) Heating: Heating the sample will reduce or remove the effect of the pathogen. The more aggressive the heating the more complete the removal but the more the sample itself is likely to be affected. Heating will remove oxygen and the sample must be aerated before use.

A form of pasteurization in which the sample is heated to near 100°C will usually remove the pathogen effect. Advantages of this approach are that it is simple to perform and requires little in the way of specialized equipment. Sufficient volumes can be treated relatively quickly with minimal effort. Disadvantages may include concentration of the sample through evaporation, loss of volatile compounds and unknown effects of heating on the sample

Autoclaving has been found to be effective and should always remove pathogenic effects. Containers used in autoclaving should be Apreconditioned@ by autoclaving laboratory water in them to remove toxins that could leach out of the container walls. The advantage of autoclaving is that one is assured of complete pathogen removal. The disadvantages are that a large autoclave is needed and a relatively long heating and cooling time is required for the large volume of sample. Autoclaving would appear, intuitively at

least, to be among the more intrusive means of sterilization.

2) Irradiation with UV light: In-line UV sterilizers are readily available at modest prices and are part of the equipment of many laboratories. Sample can be pumped through the apparatus at a rate specified by the manufacturer of the apparatus. There should be no need to sterilize tubing used in the procedure as bacterial contamination routinely encountered in a toxicity testing laboratory is not of concern. What is important is that the bacteria originally present in the receiving water sample are killed. It is very important that the UV light source be changed according to the manufacturer=s recommendations. These light sources have limited lifetimes and their effectiveness will decrease with age. The delivery pump and the light source should be on the same electrical circuit so that if the power to one is interrupted, both pieces of equipment will cease operating. Common sense QA/QC procedures should be put into place to assure that the light source is on at the beginning and at the end of the procedure.

Advantages of UV sterilization include availability of equipment and ease of use. Sufficient sample can be prepared upon sample arrival to begin test preparation early in the day and the remaining sample required for testing can be sterilized unattended during the remainder of the day. Disadvantages may include unwanted effects of UV light on the sample (e.g. photo activation of some organic compounds) and decreased effectiveness with turbid or stained samples.

3) Filtration using a 0.2 υm filter has been found to consistently remove the effect of the pathogen. Filtration through larger pore sizes is not consistently effective. The advantage of filtration is that it appears to be every bit as effective in pathogen removal as autoclaving. A significant disadvantage is the effort involved in sample treatment. This difficulty can be reduced if the sample is prefiltered through glass fiber filters and if an apparatus is available which allows the use of large diameter (e.g. 137 mm) filters. This type of equipment is readily available (e.g. for TCLP sample prep) but expensive. Another disadvantage is that removal of suspended solids may influence the bioavailability of chemical pollutants. The seriousness of this problem would have to be evaluated on a site-specific basis. However, for many potential pollutants (e.g. metals, pesticides) concerns about potential changes in toxicity to fish may be overshadowed by actual toxicity to invertebrate test organisms.

4) Addition of antibiotics. The addition of wide spectrum antibiotics has been found to be effective in removing the pathogen effect. Any antibacterial treatment such as those commonly used in aquaculture or home aquarium maintenance (e.g. oxytetracycline, chloramphenicol, and actinomycin) should be effective in removing

the effect. A pilot study to determine the effective dosage is recommended.

Advantages of antibiotic use include effectiveness and ease of use. The chemicals themselves are inexpensive and readily available. Large volumes of sample can be easily and quickly treated. As with other treatment procedures, antibiotic treatment may alter the sample in unknown or undesirable ways. Addition of some antibiotics may cause the sample to become cloudy.

SUMMARY

Interpretation of short-term chronic WET tests using fathead minnows may sometimes be complicated by the presence of what appears to be a bacterial pathogen. This phenomenon may be observed whenever surface waters (receiving waters, Aonce through@ effluents, ambient waters) are used in testing. When present in samples used as diluent, this pathogen can be viewed as a source of interference and measures to remove it may be appropriate. If a permittee suspects that pathogenic interferences are influencing their WET tests, they should begin a dialogue with the appropriate regulatory authority to determine the best course of action. The interference may be removed by using reconstituted lab water as diluent or by sterilizing the receiving water diluent in any number of ways. It is acknowledged that laboratory water will differ from the receiving water and that manipulating the receiving water sample will alter it in unknown ways. We suggest that the treated diluent or synthetic dilution represents a suitable matrix and their use may be preferable to repeating invalid tests.

LITERATURE CITED

Kszos, L.A., A.J. Stewart and J.R. Sumner. 1997. Evidence that variability in ambient fathead minnow short-term chronic test is due to pathogenic infection. Environ. Toxicol. and Chem. 16:351-356.

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