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EVIDENCE THAT VARIABILITY IN AMBIENT FATHEAD MINNOW SHORT-TERM CHRONIC TESTS IS DUE TO PATHOGENIC INFECTION

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Abstract—An examination of results from ambient fathead minnow subchronic toxicity tests identified a common characteristic of the tests, which manifests itself as a large among-replicate and between-test variance in survival. The unusual replicate-specific mortality in tests with ambient water appears to be due to pathogenic bacteria or fungi. This finding is based on the following facts: a comparison of survival among replicates in effluents and ambient waters showed that when mean survival was low (40–70%), among-replicate variation for ambient tests was greater than it was for the effluent tests; in 63 tests conducted at three locations over nearly 3 years, treating the water with ultraviolet (UV) light improved survival; a seasonal pattern to survival was present at ambient sites; survival was higher when minnows were separated by using 1 minnow/beaker rather than the standard system of 10 minnows/beaker; and survival tended to be higher in ambient sites contaminated with low levels of chlorine. The existence of the pathogen(s) does not mean that the test cannot be used effectively to assess toxicity of ambient waters; instead, it indicates that factors other than toxicity may need to be taken into consideration when interpreting the results.

Keywords—Ambient toxicity testing Pathogen Fathead minnow

INTRODUCTION

Tests with fathead minnow (*Pimephales promelas*) larvae and *Ceriodaphnia dubia* can be used to estimate the acute or chronic toxicity of effluents or receiving waters [1–5]. In ambient applications, though, the results of these tests may need to be interpreted differently from the results of effluent tests. Here, we provide an example of test results that may lead to inaccurate interpretation when the fathead minnow larval test is used in ambient assessments. Our examples are drawn from diverse effluent and ambient water tests conducted at the Oak Ridge National Laboratory (ORNL) over a 10-year period. Specifically, the anomaly observed manifested itself as high replicate-specific variation in minnow survival, which we attribute to naturally occurring pathogenic bacteria or fungi. Large variation in survival of fathead minnow larvae among replicates has been observed by others (T. Norberg-King, U.S. Environment Protection Agency [EPA] Environmental Research Laboratory, Duluth, MN, USA, and P. Downey, TRAC Laboratories, Inc., Denton, TX, USA, personal communication) and bacterial interferences in whole-effluent acute toxicity tests with fathead minnows have been previously observed in samples of cooling water [6]. An anomaly that confounds interpretation of the *C. dubia* test in ambient applications has also been documented; *C. dubia* frequently have higher fecundity in ambient waters than they do in control water (reconstituted or laboratory water) [7]. These anomalies in laboratory tests of ambient water need to be considered when: predicting the effects of effluent on aquatic communities [8]; determining the no-observed-effect concentration (NOEC) or lowest-observed-effect concentration (LOEC) for ambient permit points [9]; conducting ecological risk assessments [10]; and evaluating the effects of specific toxicants (i.e., chlorine) [11,12].

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MATERIALS AND METHODS

Description of water samples

High variation in minnow survival among replicates was observed while conducting ambient tests for three Department of Energy (DOE) facilities: ORNL, the Oak Ridge Y-12 Plant (Y-12), and the Oak Ridge K-25 Site, all located near Oak Ridge, Tennessee, USA. Each facility has a National Pollutant Discharge Elimination System (NPDES) permit that requires biological monitoring (including ambient toxicity monitoring) of receiving streams [13–15]. The ambient samples were collected from settling basins near Y-12 (New Hope Pond and Lake Reality), Melton Branch (MEK; three sites), White Oak Creek (WCK; six sites), First Creek (FCK; two sites), Fifth Creek (FFK; three sites), Northwest Tributary (NWT; one site), the outfall of White Oak Lake (WOL; one site), Bear Creek (BCK; six sites), and McCoy Branch (MCK; one site). The locations of these sites are shown in Figure 1. The ambient water samples were always collected as daily grab samples. Segments of several of these streams are occasionally toxic due to the presence of chlorine [11–13] and suffer occasional fish kills [16]. Upper BCK is toxic to *C. dubia* in part due to the presence of nickel [5].

The effluent tests, conducted to fulfill NPDES permit requirements, were used to evaluate the toxicity of wastewaters from cooling towers at ORNL and the Y-12 Plant, metal-removing treatment plants at the Y-12 Plant, a sewage treatment plant at ORNL, a coal yard runoff treatment facility at ORNL, an incinerator at the K-25 Site, and photographic wastes from the Y-12 Plant. Most of the effluent samples were collected as daily 24-h composites. Several chemicals have been identified as contributing to toxicity in these effluents by

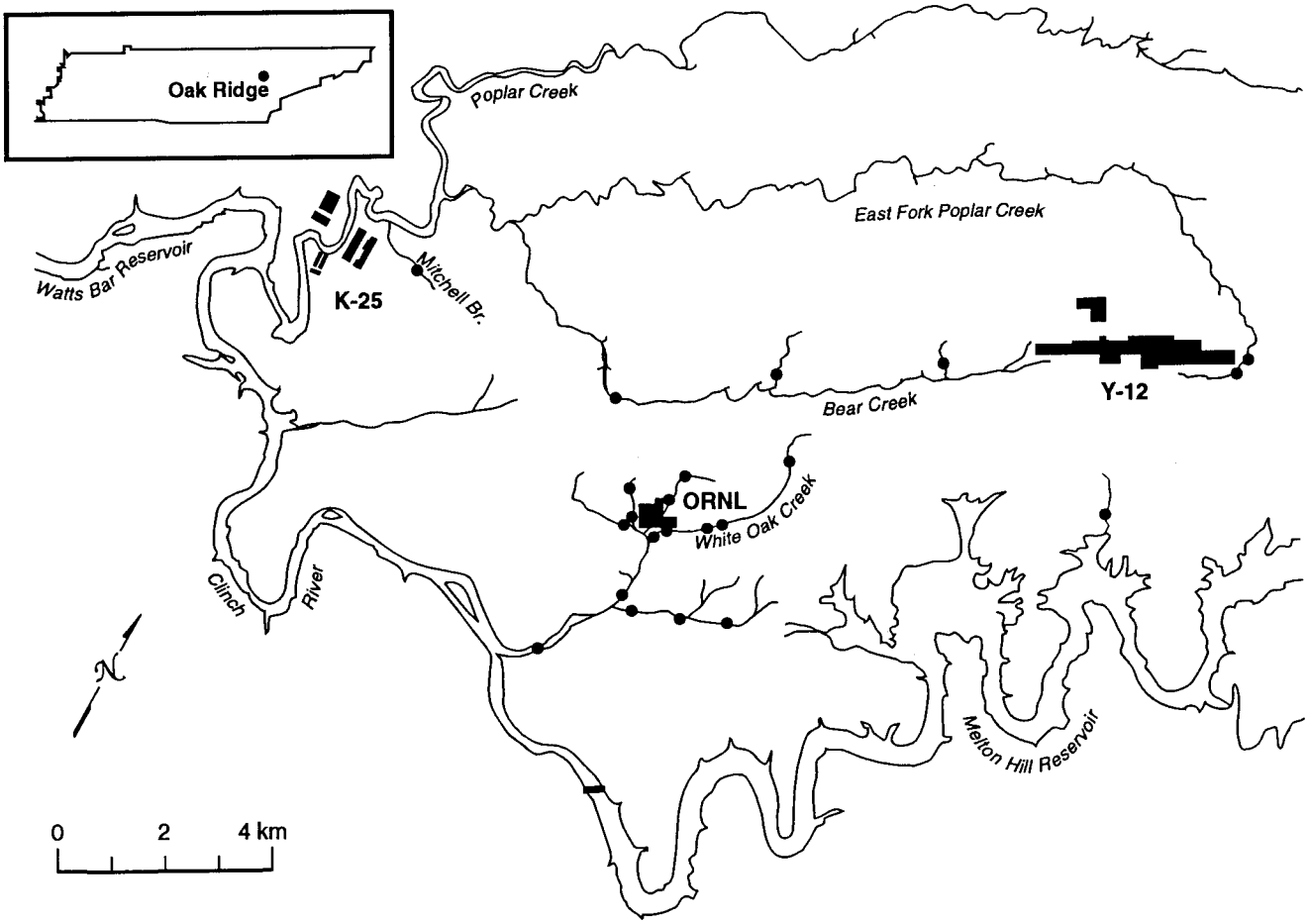

Fig. 1. Location of sampling sites used for ambient toxicity testing on the Oak Ridge Reservation.

conducting pure chemical toxicity tests and by using various toxicity reduction evaluation procedures. Examples include chlorine (sewage treatment plant), ammonia (photographic wastes), nickel [5], hydrogen sulfide, uranium and sodium sulfate (metal-removal treatment plants), and calcium sulfate (coal yard runoff treatment facility) [17].

Toxicity test procedures

The results reported here are from short-term chronic static-renewal tests based on the survival and growth of fathead minnow larvae [cf.2,3,18,19]. Each test used four replicate beakers, each containing 10 larvae and 250 ml or 500 ml of water (the larger volume was used prior to March 1988). The control treatment was dechlorinated tap water (prior to March 1988) or 20% dilute mineral water (moderately hard water) thereafter.

Effluent and ambient water samples were analyzed for pH, alkalinity, hardness, conductivity, and dissolved oxygen. Most ambient samples and several effluent samples were also analyzed for total residual chlorine. The temperature of the water samples was recorded as the sample was collected.

Fathead minnow test anomaly

An anomaly encountered during ambient tests manifested itself as a large among-replicate variation in minnow survival. We determined whether this large variability was a common characteristic of ambient toxicity tests in four ways. First, we compared minnow survival in tests of four sites in headwater streams, upstream of ORNL (reference sites), to survival in control water and in water from sites farther downstream, where chlorine was frequently detected. The headwater stream sites lack anthropogenic contaminants and have diverse benthic invertebrate communities indicative of excellent water quality [13]. Second, we compared the variation in survival in effluents and water from various ambient sites, for cases where mean survival, at the end of the 7-d test, was between 40 and 70%. The 40 to 70% range in mean survival used in this analysis was essentially arbitrary, but includes mean survival values that are low enough to indicate possible toxicity. Fifteen examples of ''low survival'' ambient tests and 15 examples of ''low survival'' effluent tests were selected randomly for this analysis from valid tests where control survival was $\geq$80%. Analysis of variance (ANOVA) [18,19] was used to determine if mean survival or among-beaker variance in survival was different for tests of ambient versus effluent samples. The percentage survival values were transformed (arcsine square root) prior to statistical analysis and prior to the computation of coefficient of variation.

The third approach was experimental. We compared minnow survival in nontreated and ultraviolet (UV) light-treated water from three ambient sites. Two of these sites (Melton Branch at MEK 0.16 and White Oak Creek at WCK 2.65) are downstream of ORNL discharges. The third site (Mitchell Branch at MIK 1.43) is a reference site located upstream of the K-25 Site. A total of 30 tests of water from MEK 0.16, 24 tests of water from WCK 2.65, and 9 tests of water from

MIK 1.43 were conducted from January 1992 to September 1995. Each test used four replicates of each treatment (nontreated versus UV-treated water). Each day, 1-L batches of freshly collected water were exposed to UV light for 20 min in a Lifegard® model QL-25TH (Rainbow Plastics, El Monte, CA USA) water treatment device containing a 25-watt UV light source (254-nm wavelength). Survival was analyzed using a one-tailed binomial test to determine if the number of tests where survival or growth was greater in the UV-treated sample than the nontreated sample was significantly greater than might be expected by chance alone.

Finally, we evaluated whether the test system provided an opportunity for the pathogen to spread from fish to fish within a replicate, thus causing the high variability among replicates. To test this hypothesis, nontreated, UV-treated, or samples filtered through 0.1-μm filters were evaluated for toxicity in three side-by-side tests using either 10 minnows/beaker, or 1 minnow/beaker, with water from MIK 1.43. The one minnow/beaker method used four replicates with 10 beakers/replicate, each containing one larva and 25 ml of water. Larvae were fed brine shrimp (*Artemia*) nauplii (approximately 60 nauplii/beaker) twice daily. The 10 minnows/beaker method used four replicate beakers, each containing 10 larvae and 250 ml of water. Larvae were fed brine shrimp (600 ± 75 nauplii/beaker) twice daily. In both methods, larvae were pooled within a replicate, dried, and weighed to provide estimates of growth. Weight of the larvae was measured to the nearest 0.01 mg with a Cahn (Cahn Instruments, Inc., Cerritos, CA, USA) electrobalance. The survival and growth data were analyzed by ANOVA [20,21]. Percentage survival values were transformed (arcsine square root) prior to statistical analysis.

RESULTS

Survival of fathead minnows in reference sites versus controls

The survival of minnows in water from 15 ambient monitoring sites on five streams near ORNL (including four upstream reference sites on headwater streams) and in control water is summarized in Figure 2. The four reference sites were tested a total of 94 times. In 16 of these 94 tests (16.8%) mean survival was ≤60%. Only 31.6% of the tests at the four reference sites had survival values ≥ 90%. In contrast, 95.8% of the control tests had survival values ≥ 90% (Fig. 2).

Variation in fathead minnow survival in effluent and ambient tests

Results of the effluent and ambient tests used in this analysis are summarized in Table 1. Analysis of variance showed that mean survival values (transformed) for the ''low survival'' effluent and ambient tests did not differ significantly (p = 0.106). However, the coefficients of variation (CVs) for the two groups were not similar (p = 0.0007). The among-beaker CV in survival for the ''low-survival'' ambient tests was, on average, twice as high as that for ''low survival'' effluent tests (23.6% versus 48.2%).

Effects of UV light pretreatment on fathead minnow survival

The frequency distribution of survival in water from the three ambient sites, with and without UV treatment prior to testing, is summarized in Table 2. For the nontreated samples from MEK 0.16 and WCK 2.65, the greatest number of tests had mean survival in the range of 70 to 89% (12 of 30 tests and 11 of 24 tests, respectively). When the samples were treated with UV light, the greatest number of tests had mean survival in the 90 to 100% range (23 of 30 tests and 21 of 24 tests, respectively). There was no distinguishable difference in the frequency distribution of growth in the nontreated and UV-treated samples. In both nontreated and UV-treated samples, growth was normally distributed with the greatest number of tests having mean growth within the 0.48 to 0.59 mg/larvae range.

When survival in the UV-treated and nontreated samples was compared on a test-by-test basis, survival was higher in the UV-treated sample in 73% of the tests of MEK 0.16, 71% of the tests of WCK 2.65, and 100% of the tests of MIK 1.43. The number of tests where UV treatment improved survival was greater than expected by chance alone for each of the sites (binomial test; $\alpha = 0.05$). In 43% of the tests of MEK 0.16 water, UV treatment improved survival by >20%. Likewise, in 25% of the tests of WCK 2.65 and 89% of the tests of MIK 1.43, UV treatment improved survival by >20%. The number of tests where growth was higher in the UV-treated sample than in the nontreated sample was not significantly different for any of the sites.

A seasonal pattern was evident in the frequency of tests where minnow survival was improved by UV treatment. The percentage of tests where UV treatment improved survival by >20% from the nontreated sample was highest during the winter and fall (67% and 64%). During the summer, only 17% of the tests had survival improved by >20% through UV treatment; whereas during the spring, 44% of the tests had survival improved by >20% through UV treatment.

Effects of the test system on fathead minnow survival

Results of the three 10 minnows/beaker versus 1 minnow/beaker tests showed that the standard test method (10 minnows/beaker) contributed to the spread of the pathogen from fish to fish, resulting in low survival. Survival was significantly higher in nontreated water from MIK 1.43 using the 1 minnow/beaker method than it was in nontreated water using 10 minnows/beaker (p = 0.0015). In each of the three tests, mean percent survival (±SD) was 75 ± 27%, 22 ± 33%, and 65 ± 6% in nontreated water using the standard method, whereas mean survival (±SD) was 100 ± 0%, 80 ± 8%, and 93 ± 10% in nontreated water using the 1 minnow/beaker method. Survival was also significantly improved in the standard method when water was treated with UV light or filtered (p = 0.0009). Growth was not significantly different in nontreated, UV-treated, or filtered water using either method (p = 0.09 and p = 0.24). Mean growth was lower in the 1 minnow/beaker test method (ranging from 0.50 to 0.66 mg/larvae) than it was in the standard method (ranging from 0.63 to 0.96 mg/larvae). However, growth was well above the minimum acceptance level designated by the EPA for a valid test (0.25 mg/larvae) [2].

DISCUSSION

This study evaluates low survival outcomes that can be encountered when using the subchronic 7-d fathead minnow larvae survival and growth test to estimate toxicity of ambient waters. One common characteristic that is specific to ambient tests conducted in this study manifests itself as an unusually large variance in survival among replicates. An appreciation for the magnitude of this occurrence and an understanding of

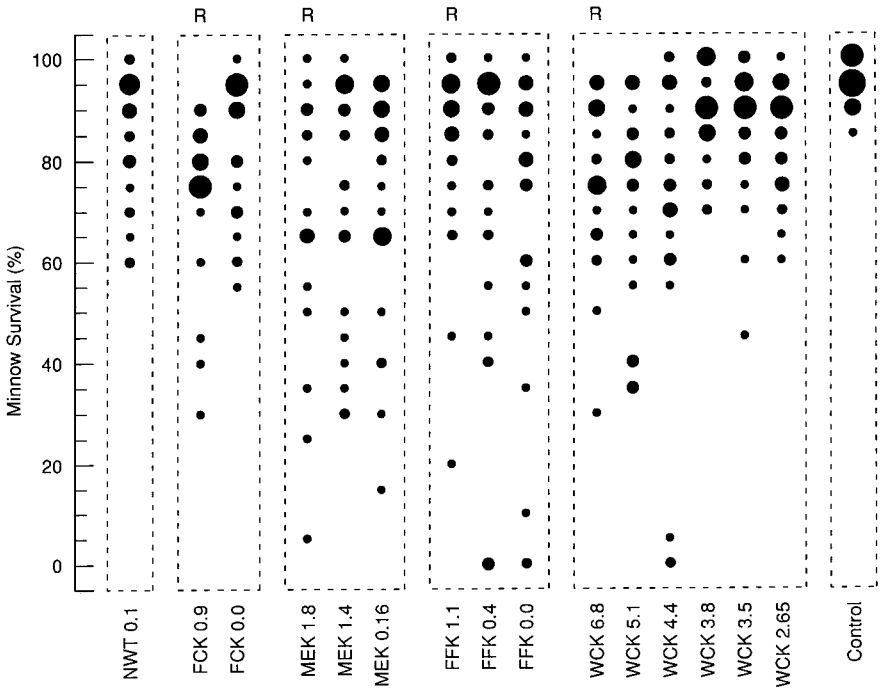

Fig. 2. Survival pattern of fathead minnow larvae in toxicity tests at 15 sites on five streams near the Oak Ridge Reservation. Separate streams (NWT = Northwest Tributary; FCK = First Creek; MEK = Melton Branch; FFK = Fifth Creek; and WCK = White Oak Creek) are indicated by dashed lines. The area of each circle is proportional to the number of tests conducted for each site (n = 37 for MEK 0.16 and control, n = 36 for WCK 2.65, and n = 19–26 for remaining sites). Upstream reference sites (R) are designated at the top of the figure.

its source(s) should permit more accurate interpretations of the results of ambient toxicity tests.

In effluent testing, low mean survival of fathead minnows provides evidence for toxicity, and the amount of intra- and interlaboratory variation in survival is similar to that found for other toxicity tests [22]. In this study, especially of water from noncontaminated headwater streams, it was common for fathead minnow larvae to have low mean survival, and this low mean survival to be accompanied by a large among-replicate variation. In extreme cases, survival values among four replicates ranged from 0 to 100% (e.g., BCK 5.15, Table 2). Our findings indicate that a low mean survival may not necessarily be accepted as evidence for toxicity, if it is accompanied by an unusually large variation (cf. Table 2). These findings must be considered when comparing the survival of fish in a laboratory-derived control water to the survival of the fish in an ambient water. In four noncontaminated stream reference sites at ORNL, 16.8% of the fathead minnow tests had mean survival values $\leq$60%, and 58% of the tests had mean survival values lower than the lowest mean survival in any control. Mean survival in controls was almost always $>$90% (Fig. 2).

Four considerations suggest that pathogenic fungi or bacteria provide the most parsimonious explanation for the large variance in survival of the minnow larvae among replicates in ambient tests. First, treating the ambient water samples briefly with UV light prior to testing increased mean survival and lowered among-replicate variance in survival. In a total of 63 tests conducted at three locations over nearly a 3-year period, survival was consistently improved by UV treatment more times than would be expected by chance.

Second, a seasonal pattern in minnow survival was evident. The number of tests where survival in water from the ambient sites was improved by UV treatment was higher during the fall and winter, and lower during the summer. Seasonal variation in survival patterns provides further evidence of pathogenic infection and has been noted by others (P. Downey, personal communication).

Third, results of our experiment with 1 minnow/beaker demonstrated that the conventional test system (four replicate beakers, each containing 10 larvae) facilitates the spread of the pathogen from fish to fish. Survival was significantly higher in nontreated water from MIK 1.43 using the 1 minnow/beaker method than it was in nontreated water using the 10 minnows/beaker method.

Finally, variation in minnow survival among replicates tended to be greater for upstream reference sites in White Oak Creek and First Creek than for sites farther downstream (cf. WCK 6.8 versus sites WCK 3.8, WCK 3.5, and WCK 2.65, and FCK 0.9 versus FCK 0.0; note Fig. 2). Downstream segments of these two streams receive inputs of chlorinated drinking water [11], and chlorine is more toxic to bacteria (and probably fungi) than it is to fish [23]. Thus, it may not be surprising that fish survival tended to be higher in ambient sites that are contaminated with low levels of chlorine, if pathogenic microorganisms caused the mortality. Unusually large variation in survival of minnow larvae among replicates has been observed by others (T. Norberg-King and P. Downey, personal communication). The mortality observed by TRAC Laboratories has been attributed to *Aeromonas* (bacteria) and *Saprolignia* (aquatic fungi), which infect the gills of the larvae, and was a secondary result due to handling the larvae and the stress of the test system (P. Downey, personal communication). Collectively, these factors provide strong circumstantial evidence for the idea that pathogens may reduce minnow survival in ambient applications of the standard toxicity test method [2]. However, the possibility of toxicity due to naturally oc-

Table 1. Among-beaker variation in fathead minnow survival for 15 effluent tests and 15 ambient toxicity tests in which mean survival was between 40 and 70%

Sample type	Facility	Site[a]	Date	Replicate 1	2	3	4	Mean survival (%)[b]	CV (%)[c]
Ambient water	ORNL	MEK 0.16	April 1989	60	60	10	90	55	45.8
	ORNL	WCK 2.65	April 1989	80	50	50	90	67.5	23.8
	ORNL	MEK 0.16	May 1989	50	70	70	80	67.5	13.8
	ORNL	MEK 0.16	Nov. 1989	40	60	90	80	67.5	25.3
	ORNL	MEK 0.16	Dec. 1989	60	20	10	80	65	52.5
	ORNL	WCK 6.8	Sept. 1986	100	30	30	80	60	49.7
	ORNL	WCK 6.8	Dec. 1989	60	80	90	30	65	30.5
	ORNL	FCK 0.9	Feb. 1988	70	60	40	100	67.5	36.8
	ORNL	FCK 0.9	Dec. 1989	50	10	50	50	40	34.6
	Y-12	BCK NT4	April 1985	0	20	100	100	55	88.3
	Y-12	BCK NT14	April 1985	10	100	100	10	55	76.2
	Y-12	BCK 5.15	Oct. 1985	50	0	30	100	45	88.4
	Y-12	MCK 1.92	Jan. 1990	40	40	60	100	60	43.9
	K-25	MIK 1.43	Jan. 1987	50	70	90	0	52.5	71.2
	K-25	MIK 1.43	March 1988	50	80	100	30	65	42.7
Effluent sample	ORNL	CY 80%	July 1986	60	60	50	90	65	21.4
	ORNL	CY 60%	Jan. 1987	50	60	80	80	67.5	16.6
	ORNL	CY 80%	July 1988	60	40	50	80	57.5	20.9
	ORNL	CY 100%	July 1989	20	30	50	60	40	28.2
	ORNL	CY 100%	Jan. 1990	30	50	60	70	52.5	21.6
	ORNL	CY 100%	July 1990	50	60	90	50	62.5	23.7
	ORNL	STP 50%	Feb. 1991	60	70	50	10	47.5	39.5
	Y-12	PR 10%	April 1987	20	70	50	50	47.5	28.8
	Y-12	PR 20%	July 1988	60	50	60	50	55	6.9
	Y-12	CPCF 5%	Oct. 1986	40	60	40	80	55	23.9
	Y-12	WTF 30%	April 1988	90	50	30	70	60	31.7
	K-25	TI-1 0.5%	Feb. 1989	70	60	50	50	57.5	11.4
	K-25	TI-2 0.5%	Feb. 1989	60	50	10	50	42.5	36.4
	K-25	TI-3 0.5%	Feb. 1989	20	70	50	70	52.5	30.8
	K-25	TI-4 0.5%	Feb. 1989	60	40	50	40	47.5	12.6

[a] Ambient site code numbers designate kilometer positions in the stream; effluent code numbers indicate the tested concentration where mean survival was 40 to 70%.
[b] Mean survival calculated using nontransformed percentage survival values.
[c] CV calculated using arcsine-transformed survival values.

curring organic compounds, which also could be destroyed or modified by exposure to UV light, is not completely excluded.

If the mortality of the minnow larvae in noncontaminated ambient water samples is in fact due to a pathogen, as we suspect, it might be appropriate to pretreat the water samples by filtration, by adding a fungicidal or bacteriocidal chemical, or by exposing the water to UV light to lower the rate of background mortality. Each of these types of treatments, though, can generate additional interferences. Ultraviolet light, for example, can destroy some organic compounds and generate singlet oxygen, which is extremely toxic and reactive [24]. Filtration, too, can remove hydrophobic or metallic contaminants that are sorbed to the surfaces of naturally occurring detrital, sediment, or algal particles. Thus, virtually any treatment to control background mortality in the *P. promelas* test must be evaluated carefully on a case-by-case basis when the tests are being used for regulatory or environmental compliance purposes.

Table 2. Frequency distribution of fathead minnow survival in three ambient sites with and without treatment with ultraviolet (UV) light

Site	Treatment	Survival range (%) <30	30–49	50–69	70–89	90–100
Melton Branch (30 tests)	Nontreated	2	3	4	12	9
	UV treated	0	0	0	7	23
White Oak Creek (24 tests)	Nontreated	0	0	4	11	9
	UV treated	0	0	0	3	21
Mitchell Branch (9 tests)	Nontreated	3	1	2	3	0
	UV treated	0	0	0	4	5

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