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Comparison of *Artemia* Feeding Regimens on Larval Growth in a Short-Term Fathead Minnow Toxicity Test

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The Fathead Minnow Larval Survival and Growth Test, as described by the U.S. Environmental Protection Agency (U.S. EPA Method 1000.0), uses newly hatched larvae of *Pimephales promelas* to estimate the chronic toxicity of effluents and receiving waters (Weber *et al.* 1989). This short-term test is one of several used widely throughout the United States to monitor effluent toxicity and compliance with National Pollutant Discharge Elimination System permit requirements. Test endpoints include mortality and growth (dry weight). Test protocol stipulates that larvae be fed 0.1 mL (700 to 1,000 individuals) of newly hatched brine shrimp (*Artemia*) nauplii three times per day or 0.15 mL of shrimp twice daily. Tests are acceptable if the average dry weight of controls at test termination is $\geq$ 0.25 mg per fish and control mortality does not exceed 20%.

This investigation compared the effect of different *Artemia* feeding regimens on control fish growth and mortality under general conditions of the fathead minnow larval growth test. Feeding regimens varied in the number of brine shrimp fed per feeding and the daily feeding frequency. These factors may affect growth because *Artemia* survive only a few hours in freshwater, and larval fish feed predominantly on living *Artemia*. Effects of two basic sets of feeding levels (high and low amounts) were compared. In Study I larval fish were provided quantities of brine shrimp at or above the test recommendations. In Study II fish were given *Artemia* amounts generally in the range of and below that of standard test conditions. Feeding frequencies ranged from one to four times daily. The research objective was to determine which *Artemia* feeding regimens optimize larval fish growth without excess feeding. This information could indicate procedural changes which would lead to further test standardization and optimization of results, while reducing maintenance requirements. Increased understanding of the effects of feeding on the test endpoints also will allow for better interpretation of toxicant induced changes.

MATERIALS AND METHODS

Test methods and conditions were those recommended for the Fathead Minnow Larval Survival and Growth Test (U.S. EPA Method 1000.0 in Horning and Weber

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1985) except that all larvae were maintained in control water (no toxicant exposure). Minor changes in the test procedures have recently been published (Weber *et al.* 1989) but feeding conditions are basically as before. Test organisms were *Pimephales promelas* larvae < 24-h old. Larvae were hatched from eggs obtained either from the U.S. EPA Environmental Monitoring and Support Laboratory - Newtown Facility (Study I) or from in-house cultures (Study II). Tests were conducted at 25 ± 1.5°C in an environmental room maintained with a 16-h light/8-h dark photoperiod. Test chambers consisted of covered 1000-mL glass beakers with a solution volume of 500 mL. Tests were conducted with moderately hard (80-100 mg $CaCO_3$/L) reconstituted water (Horning and Weber 1985) aerated before use.

Tests were initiated by placing 10 randomly selected larvae into each test chamber (two per feeding regimen). In addition, at onset of the test, four groups of 10 larvae each were dried and weighed for determination of initial weights. Each day the numbers of live and dead larvae were recorded and dead larvae removed. Water samples were then collected from test chambers for water quality analyses. Afterward, unconsumed brine shrimp were removed and test solutions renewed. Dissolved oxygen and temperature were measured daily. Alkalinity, hardness, and pH were measured a minimum of three times during the test period, according to Standard Methods (APHA-AWWA-WPCF 1985). Growth tests were terminated either 8 d (Study I) or 7 d (Study II) ± 2 hours after initiation. For both studies, larvae were fed on 7 consecutive days, but during Study I fish were not fed on the day of test initiation. Fish were not fed during the last 12 h of any test. Upon termination, the larvae in each test chamber were counted, rinsed, transferred to a pre-weighed aluminum pan and dried for at least four hours at 100°C. After cooling in a desiccator, pans were reweighed to the nearest 0.01 mg.

Two separate studies with different feeding regimens were conducted. During Study I fish were fed quantities of *Artemia* at or above that normally recommended in the standard U.S. EPA protocol (0.1 mL or 700 - 1,000 shrimp per feeding). In this study, fish larvae were fed either once per day (10:00 a.m.), or two, three, or four times per day at 4-h intervals (first feeding at 10:00 a.m.). Amounts fed were 0.1, 0.3, 0.6 and 0.9 mL of concentrated shrimp suspension per feeding. Each feeding test of 16 regimens (four different amounts and four different frequencies) was repeated five times (five trials).

Based on results of Study I, a second study was conducted to investigate larval growth when feeding frequency and amounts were generally at or below that of the standard recommendation. During Study II, larvae were fed approximately 310, 620, 930, or 1,240 shrimp per feeding at a frequency of one, two, or three times per day (4 h between feedings; first feeding at 10:00 a.m.). A standard aliquot of 0.1 mL (≈310 *Artemia*) and multiples thereof were used for feeding. An additional treatment of unfed (control) larvae was also maintained. Each test of 12 feeding regimens plus controls (2 chambers each) was repeated four times (four trials).

Artemia nauplii were cultured according to general methods described in the U.S. EPA Acute Methods document (Peltier and Weber 1985). Brine shrimp eggs (2.0

g for Study I; 1.0 g for Study II) were incubated in glass separatory funnels containing 450 mL of salt solution (27.8 g NaCl/L). For Study I, San Francisco Bay Brand (Newark, CA) eggs were used. The egg source was changed to Aquarium Products (Glen Burnie, MD) for Study II because cysts from this source exhibited a higher and more consistent hatching frequency. During Study I, the number of brine shrimp fed per feeding was estimated based on the range given in the U.S. EPA Short-Term Chronic Methods Manual (700 to 1,000 brine shrimp per 0.1 mL concentrated suspension). For Study II, the numbers of *Artemia* fed were determined based on several counts per test made using aliquots drawn from the shrimp suspensions used for feeding. Aliquots of 0.1 to 0.4 mL were taken daily from one of the three feedings and were selected randomly for quantitation. Counts of these aliquots (n=33) gave an average of 310 ± 36 *Artemia* per 0.1 mL.

Growth effects were tested for significant differences using analysis of variance (ANOVA). When statistical significance was found at the $p<0.05$ level, individual treatment comparisons were made using Tukey's honestly significant difference (HSD) method with a significance level of $p=0.05$ (Sokal and Rohlf 1981).

RESULTS AND DISCUSSION

Mortality for Study I (five trials) averaged 6.9% overall with a range of 3.0 to 12.0% for individual feeding regimens. For any trial, mortality for different feeding regimens never exceeded 20%. In Study II (four trials) mean mortality for all feeding regimens was 2.9%. Average mortality for unfed control larvae was similar (2.5%). Mortality for individual feeding regimens ranged from 0 to 6.3% overall and from 0 to 15% for any given test. For both studies, mortality did not appear to be related to the number of *Artemia* fed per chamber or to water quality parameters. Alkalinity, hardness and pH were not appreciably affected by different feeding conditions. In Study II a decrease in dissolved oxygen, from 7.8 to 6.1 mg/L (mean values), was observed in test chambers as daily brine shrimp concentration increased from 0 to its highest level. Still, percent oxygen saturation always exceeded 70%, well above the 40% minimum specified in the method.

For Study I when feeding ranged from 2,000 to 36,000 *Artemia* per test chamber per day, larval fish growth was independent of the total number of shrimp fed (Fig. 1). Average weight per fish varied from 0.56 mg (larvae given 700 to 1,000 shrimp/d) to 0.73 mg (larvae fed 2,000 to 9,000 shrimp/d). Average initial larval weight was 0.11 mg. Analysis of variance indicated no significant effect of amount fed per day on mean growth. Over the range of feeding in Study I (≈700 to 9,000 shrimp per feeding), larval fish growth also was independent of feeding frequency (Table 1). ANOVA indicated no overall effect of feeding frequency on mean larval growth. However, at a feeding level of 700 to 1,000 *Artemia* per feeding, fish fed once daily had reduced growth (0.56 ± 0.05 mg) compared to larval fish given this amount two (0.73 ± 0.08 mg), three (0.70 ± 0.08 mg), or four (0.71 ± 0.08 mg) times per day.

In Study II over the range of feeding levels studied (0 to about 1,240 shrimp per feeding), fish growth was related to both the total amount fed per day (Fig. 2) and

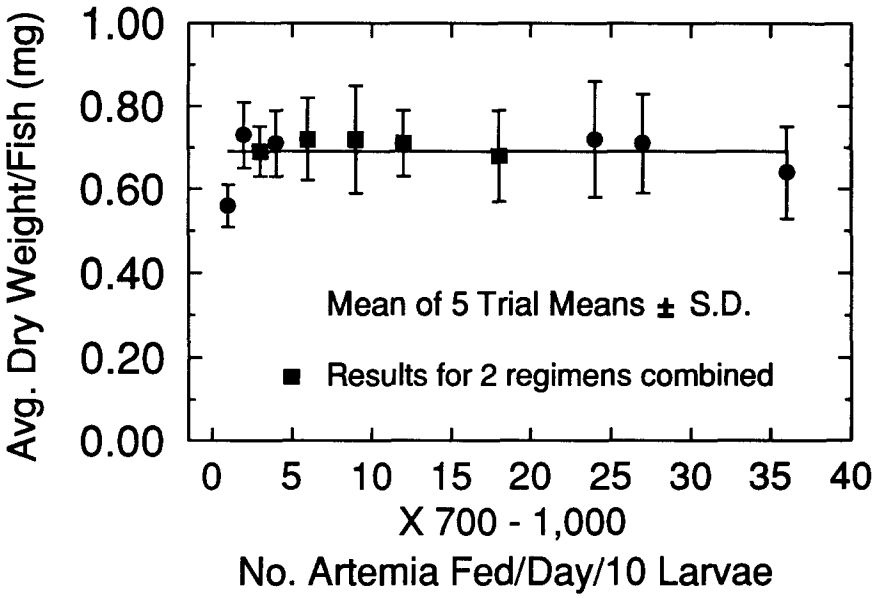

Figure 1. Study I: Effect of total daily feeding amount (≈ 1,000 to 36,000 shrimp/d) on larval fish growth.

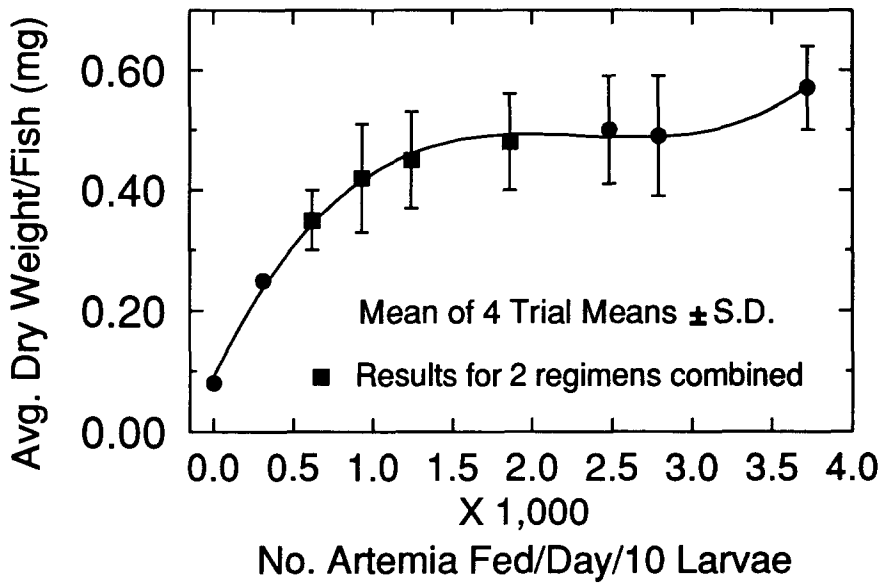

Figure 2. Study II: Effect of total daily feeding amount (0 to 3,720 shrimp/d) on larval fish growth.

the feeding frequency (Table 2). Above a feeding level of about 1,500 to 2,000 shrimp per test chamber per day, growth reached a plateau (Fig. 2). A two-way ANOVA indicated that the effect of amount fed (number of *Artemia* fed per feeding) on growth was significant at the $p<0.001$ level, while the effect of feeding frequency on growth was significant at the $p=0.0001$ level (interaction was not significant; $p=0.87$). Mean growth (average weight per fish) varied from a low of 0.25 mg (fish provided ≈310 shrimp/d) to a high of 0.57 mg (fish given ≈3,700 shrimp/d). Average initial weight of larvae was 0.12 mg. In contrast, unfed control fish averaged only 0.08 mg at test termination. For Study II at each of the feeding levels, larvae fed only once per day exhibited reduced growth compared to those fed either two or three times per day (Table 2). However, this reduced growth was only statistically different at the lower feeding levels of about 310 and 620 *Artemia* per feeding. Over the entire feeding range (310 to 1,240 nauplii per feeding), there were no statistically significant differences in growth between fish larvae which were fed twice or three times daily (Table 2).

Studies I and II indicate that a minimum daily feeding requirement for adequate growth (≥0.45 mg/fish), independent of feeding frequency, is approximately 1,500 to 2,000 shrimp per day for 10 larvae. In this case adequate growth is defined as the beginning of the growth plateau shown in Figures 1 and 2. The current protocol recommended for the Fathead Minnow Larval Survival and Growth Test stipulates feeding of 0.1 mL (≈700 to 1,000 individuals) of newly hatched brine shrimp nauplii three times daily at 4-h intervals or, as a minimum, 0.15 mL twice daily with 6 h between feedings (Weber *et al.* 1989). Study results indicate that both of these feeding schedules should ensure adequate and approximately equal growth of fathead minnow larvae under test conditions of 10 fish per 500 mL solution volume. Either feeding regimen should produce control fish with an average dry weight of at least 0.25 mg, the minimum weight required for test acceptability. In our studies, all feeding regimens tested except unfed controls, produced larvae which averaged ≥0.25 mg. This suggests that the minimum weight acceptability criterion may be too low. Furthermore, most regimens produced larvae which averaged over 0.40 mg. Based on these results, a more realistic minimum criterion might be 0.30 to 0.35 mg per fish, or an increase of 3.0 times from initial weight to final weight. Since initial weight may have some influence on final weight, a criterion based on percent increase in weight may be the most appropriate.

Absolute growth, even at comparable feeding levels, varied somewhat between the two studies. This difference in growth may have been due to the fact that fish in Study I were slightly older at the time of first feeding than those in Study II. Another likely explanation is a difference in the source, size and food quality of *Artemia* nauplii used in the two studies. The biometrics (e.g., length, weight) of newly hatched nauplii have been shown to differ considerably between various *Artemia* strains (Vanhaecke and Sorgeloos 1980). Investigators also have found that larval fish survival and growth varies depending on the geographical strain of brine shrimp fed in the diet (Beck and Bengtson 1982; Klein-MacPhee *et al.* 1980; Usher and Bengtson 1981). Effects are sometimes related to the size of the nauplii. For instance, newly hatched Atlantic silversides (*Menidia menidia*) had a higher

Table 1. Study I: Effect of feeding regimen (high) on larval fish growth[a]

No. *Artemia* fed per feeding per chamber[b]	Feeding Frequency (times per day)			
	1	2	3	4
700 - 1000	0.56 ± 0.05	0.73 ± 0.08	0.70 ± 0.08	0.71 ± 0.08
2100 - 3000	0.67 ± 0.03	0.73 ± 0.13	0.73 ± 0.14	0.70 ± 0.09
4200 - 6000	0.70 ± 0.09	0.72 ± 0.08	0.69 ± 0.15	0.72 ± 0.14
6300 - 9000	0.70 ± 0.14	0.66 ± 0.08	0.71 ± 0.12	0.64 ± 0.11

[a] Growth is expressed as average dry weight (mg) per fish at test termination. All data are means of means ± SD for five trials. Average initial weight per fish was 0.11 ± 0.01 mg. ANOVA indicated no significant effects.
[b] Range indicates approximate number of *Artemia* fed per feeding based on an estimated 700 to 1000 shrimp per 0.1 mL of feeding suspension.

Table 2. Study II: Effect of feeding regimen (low) on larval fish growth[a]

No. *Artemia* fed per feeding per chamber	Feeding Frequency (times per day)		
	1	2	3
310	0.25 ± 0.01	0.38 ± 0.06	0.43 ± 0.10
620	0.33 ± 0.05	0.47 ± 0.07	0.48 ± 0.08
930	0.41 ± 0.09	0.49 ± 0.10	0.49 ± 0.10
1240	0.43 ± 0.10	0.50 ± 0.09	0.57 ± 0.07

[a] Growth is expressed as average dry weight (mg) per fish at test termination. All data are means of means ± SD for four trials. Average initial weight per fish was 0.12 ± 0.01 mg. Control fish (not fed) averaged 0.08 ± 0.01 mg at test termination. Horizontal lines join weights that are not significantly different (Tukey's HSD method; p=0.05).

mortality when fed large Italy strain nauplii in contrast to those fed smaller strains of *Artemia* (Beck and Bengtson 1982). However, growth was greater when larvae were fed the larger nauplii. Results presented above were consistent with these findings. At comparable feeding levels (1,000 - 4,000 shrimp/d), *Artemia* nauplii in Study I (San Francisco Bay Brand) produced greater growth, but slightly higher larval mortality (5.4 ± 1.7 % vs. 2.5 ± 1.9 %; statistically different by one-tailed t-test, p=0.01) than brine shrimp fed in Study II (Aquarium Products Brand). Because the *Artemia* strain used for feeding likely influences larval survival and growth, and potentially test results, we recommend that further research be carried out to determine strain effects. In the future, it may be advantageous for the U.S. EPA to recommend particular brine shrimp strains for uniformity of testing. Indeed, use of a standard geographical strain of *Artemia* for toxicological studies has been previously proposed and discussed by Beck and Bengtson (1982).

Our studies considered the effects of different feeding treatments on fathead minnow larval growth under control conditions only. The influence of *Artemia* feeding on the toxicity of the insecticide chlorpyrifos in flow-through early life-stage tests with sheepshead minnows (*Cyprinodon variegatus*) has been investigated by Cripe *et al.* (1986). They found that inadequate quantities of food may enhance fish sensitivity to a toxicant and increase the variability of survival and growth data. These authors recommended feeding test fish to excess to improve the reliability and statistical power of results from early life-stage tests. While feeding to excess may be advantageous for flow-through tests the same may not be true under static-renewal conditions as found in the Fathead Minnow Larval Survival and Growth Test. Our research points out the necessity of defining what feeding is adequate under appropriate test conditions.

Both studies indicated that feeding three times per day is excessive since, regardless of the feeding amount, growth was never appreciably increased by the extra food offering. There are several other important reasons why feeding only once or twice per day would be preferable. From a practical standpoint, feeding fewer times per day will reduce the time, labor and cost of performing the test. Reduced feeding frequency may also reduce interactions of food with test toxicants or effluents thus minimizing associated effects on bioavailability and/or bioaccumulation. These often overlooked interactions are inherent with toxicity tests that utilize feeding.

Study results indicate that larvae fed sufficient amounts may be fed only once per day. A single daily feeding of 1,500 to 2,000 *Artemia* would appear sufficient to produce an average larval weight of about 0.45 mg per fish. Although a single feeding per day may suffice for attainment of adequate larval growth, a conservative recommendation would be to use this regimen only on weekends, holidays or under extenuating circumstances. Since brine shrimp survive only a few hours in fresh-water, and since larvae principally consume living shrimp, two small daily feedings will provide fish with a preferred food source for a greater portion of the day than one large feeding. Because of this, for routine testing we recommend standardization on a feeding protocol which provides 10 larvae with 700 to 1,000 *Artemia* twice daily.

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