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Development and Evaluation of a Nondestructive Measure of Fish Growth for Sublethal Toxicity Assessment

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Sublethal toxicity is the most frequently observed ecotoxicological response in the study of freshwater pollution (Woltering 1984). Sublethal toxicity can manifest itself in several behavioral and physiological manners. Among the most detrimental sublethal effects are reproductive inhibition and growth retardation. Growth is an important factor because reduced growth may affect competition for food and habitat, time to maturation, and susceptibility to predation and diseases (Woltering 1984). Many tests indicate that larval growth is a sensitive measure of toxic stress and that many fish subjected to sublethal levels of toxicants show a general decrease in total body length (Norberg and Mount 1985; Johnson et al. 1979; Wilson 1976; and Norberg-King 1989). Growth of fish is currently used as an endpoint for many chronic and subchronic bioassays. Fish fry or hatchlings are generally used for these tests because the first few days after hatching are the most sensitive life stage of the organism (Norberg and Mount 1985). Growth may be measured by drying and weighing the fish or by measuring fish length. Simple linear regression analyses from preliminary studies by Stebler (*Stebler EF (1988) [Larval Length Measurement as an alternative to Weight as an Indicator of Growth in the Seven-Day Fathead Minnow Larval Survival and Growth Test], unpublished*) indicated that growth measured as change in weight was highly correlated with growth measured as change in length. Although the slope coefficient of the regression equation varied depending on the age of the fish and the type of effluent sample studied, the correlation coefficients remained high. Although other tests have been conducted to correlate the growth parameters of weight gain and length (Rowe et al. 1982), weight has been the more accepted determination of growth because early length measurement techniques involved anesthetization and confinement of fish for direct measurement. However, these techniques were typically stressful to the fish. Photographic methods have been proposed in order to minimize stress to the fish (Sauter and Harrison 1985).

Image analysis measurements provide a nondestructive technique for analyzing growth data. Measurements can be recorded several times throughout the test rather than only at the end of the test. Image records can be maintained as a permanent record of the test organisms, and fish may be measured any time after test completion.

The development and preliminary validation of a nondestructive method for measuring the length of fish fry during subchronic toxicity assessment are

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described in this report. Image analysis techniques were developed using video recording equipment, image capture software, and computer digitizing equipment. Comparisons were made between fish dry weight (destructive) and fish length (nondestructive) using video image analyses to determine the efficacy of length analysis in assessing deleterious effects on growth.

MATERIALS AND METHODS

Fathead minnow (Pimephales promelas) larval survival and growth tests were conducted on seven municipal wastewater effluents, one industrial wastewater effluent, and two $CuSO_4$ reference toxicant samples according to USEPA method 1000.0 (Weber et al. 1989) with the following modifications. Pyrex brand 150-ml glass evaporating dishes were used as test vessels. Prior to daily filming, the solution level in each vessel was lowered until the fish were unable to swim freely, but still submerged. After filming, the vessel was refilled with the appropriate test solution. Fish larvae in each vessel were filmed using a Sony 8 mm Handycam® video camera set on macro, backlight, and portrait settings. A small fluorescent light table was used for backlighting and a copy stand was used to hold the camera at a fixed focal length during filming. Two clear plastic 70-mm rulers were placed on the plane of focus with the fish within the field of view, and were filmed with each test vessel. One ruler was placed along the horizontal axis, and the other along the vertical axis. The ruler images were used to correct for image distortion and to calibrate the length-measuring program to ensure accurate measurements of the fish. A 386/16MHZ computer with ZIP IMAGE processing software and High Resolution Technologies (HRT) video frame grabber were used to digitize images of the fish. Total head to tail fish lengths were measured using a program developed with HRTlib, an image processing/support library for the HRT512-8 video frame grabber. Treatment mean lengths were compared to control mean lengths to determine No Observed Effects Levels (NOEL) ($P = 0.05$) using the statistical software package Toxstat, version 3.0 (Gulley et al. 1987).

Fish weights were measured at seven days for each test and at four days for one municipal effluent test. Weights were measured according to the following protocol. Whatman #1 filters were placed in aluminum weighing pans and dried in an oven at 100° C. After 24 hr, the filters were removed from the oven and placed in a desiccator. After cooling in the desiccator for one hour, the filters were weighed. Following length measurements, the fish were rinsed with deionized water and placed on the appropriate filter according to treatment replicate. Filters with fish were then dried in the 100° C oven for two hours. The same cooling and weighing process was followed to obtain net weights per filter. These values were then converted to mean dry weight per fish (mg). Mean dry weights for filters containing fewer than seven fish were difficult to evaluate as the resulting net weights were approximated the sensitivity of the balance used. Treatment mean weights were compared to control mean weights to determine NOEL values ($P = 0.05$) using Toxstat, version 3.0 (Gulley et al. 1987). NOEL values were calculated for length and weight for each test. The least squares method of linear regression analysis was used to determine correlation coefficients for the correlation between fathead minnow larval length and weight for each test.

RESULTS AND DISCUSSION

Treatment mean lengths, weights and respective standard error values for the municipal wastewater effluents and industrial wastewater effluent are presented in Table 1. A comparison of weight and length measurements from *P.*

Table 1. Comparison of mean and standard error values for weight and length measurements of *P. promelas* larvae exposed to municipal wastewater effluents.

Test	Conc (%)	Weight (mg/fish) n	mean	SE	Length (mm/fish) n	mean	SE
I1	0	4	0.276	0.322	60	7.161	0.109
	11	4	0.288	0.210	58	7.373	0.111
4	27	4	0.296	0.224	59	7.481	0.120
	42	4	0.300	0.187	60	7.370	0.110
	67	4	0.301	0.195	59	7.503	0.115
	100	4	0.302	0.212	58	7.244	0.113
M1	0	4	0.283	0.205	60	7.098	0.108
	0.7	4	0.308	0.233	57	7.300	0.108
	1.5	4	0.290	0.268	59	7.323	0.117
	3	4	0.270	0.206	56	7.779	0.169
	30	4	0.224	0.188	58	7.392	0.113
	100	4	0.218	0.234	59	7.151	0.111
M2	0	4	0.399	0.270	53	7.043	0.115
	6	4	0.465	0.262	54	6.950	0.117
	13	4	0.380	0.277	53	7.029	0.121
	25	4	0.331	0.265	50	7.188	0.115
	50	4	0.402	0.275	53	7.369	0.116
	100	4	0.421	0.154	49	7.247	0.123
M3	0	3	0.417	0.218	39	8.086	0.136
	12.5	3	0.479	0.294	36	8.237	0.146
	25	3	0.434	0.163	33	8.215	0.151
	75	3	0.413	0.160	32	7.926	0.148
	100	3	0.370	0.228	23	7.742	0.195
M4	0	4	0.240	0.235	58	7.070	0.113
	3	4	0.201	0.258	58	7.240	0.113
	10	4	0.228	0.265	57	7.094	0.115
	30	4	0.217	0.205	58	6.933	0.113
	60	4	0.166	0.224	57	6.904	0.122
	96	4	0.245	0.229	55	7.076	0.121
M5	0	4	0.478	0.244	55	7.455	0.116
	6.25	4	0.456	0.154	52	7.14	0.120
	12.5	4	0.380	0.183	56	7.074	0.115
	25	4	0.352	0.203	60	7.071	0.106
	50	4	0.341	0.250	59	7.199	0.115
	100	4	0.366	0.225	56	6.943	0.120
M6	0	4	0.468	0.181	53	6.990	0.125
	25	4	0.383	0.280	49	6.515	0.136
	100	4	0.530	0.303	29	6.465	0.165
M7	0	4	0.519	0.188	79	6.960	0.100
	12.5	4	0.465	0.234	69	6.755	0.108
	25	4	0.363	0.262	60	6.588	0.113
	75	4	0.265	0.226	68	6.483	0.105
	100	3	0.212	0.214	55	6.226	0.115

promelas larvae exposed to reference toxicant copper sulfate is provided in Table 2. Length and weight NOEL values for the industrial wastewater effluent test, the 4-d municipal wastewater effluent test, and four of the 7-d municipal wastewater effluent tests were determined to be identical (Table 3). The NOEL values for length were less than the NOEL values for weight in the fifth and sixth municipal effluent tests (Table 3). Significant growth effects in length were determined as early as day six for the fifth municipal effluent (M5) and day four for the sixth municipal effluent (M6). Although the limited number of larvae available for weight measurement made evaluation concentration response data difficult, significant length inhibition was noted at copper sulfate concentrations at least two-fold below those concentrations adversely affecting fish weight (Table 4).

Table 2. Comparison of mean and standard error values for weight and length measurements of *P. promelas* larvae exposed to a $CuSO_4$ reference toxicant.

Test	Conc (μg/l)	Weight (mg/fish)			Length (mm/fish)		
		n	mean	SE	n	mean	SE
RT1	0	4	0.287	0.250	59	7.609	0.117
	50	4	0.298	0.278	53	7.466	0.123
	75	4	0.200	0.265	47	7.288	0.129
	100	4	0.437	0.381	19	7.281	0.186
RT2	0	4	0.436	0.253	59	7.233	0.104
	50	4	0.410	0.229	55	7.166	0.117
	60	4	0.391	0.234	51	7.152	0.116
	75	2	0.396	0.187	20	7.007	0.196
	85	3	0.380	0.314	21	6.837	0.188
	150	3	0.447	0.338	11	6.306	0.253

Preliminary data by Stebler (nonpublished) produced length-weight regression coefficients of 0.82 through 0.98 for seven wastewater effluents tested. The tests conducted in this study, however, were unable to duplicate those results. Linear regression analysis of the six municipal wastewater effluent tests, one industrial wastewater effluent test, and two reference toxicant tests produced length-weight correlation coefficients of less than 0.4, indicating somewhat poor linear correlation.

Because fish weight is a factor of both length and width of the fish, it is not surprising that length and weight are not always linearly correlated. However, preliminary data from Stebler (unpublished) indicated that length may successfully be used to predict growth inhibition. All tests conducted indicated length to be as sensitive, if not more sensitive than weight as an indicator of growth. Preliminary results also indicated that growth effects may be determined prior to the end of a 7-d test when length measurements are used. Additional studies need to be conducted to compare the sensitivity of the growth endpoints, including length, to survival. However, both lethal and sublethal responses should be evaluated in subchronic toxicity assessment, at least initially. Specific toxicants and complex mixtures may adversely affect growth, but not survival. Conversely, specific toxicants and mixtures may produce lethal effects without inducing a sublethal response. Concentration thresholds

and toxicokinetics also determine which endpoint is affected. Thus, in aquatic subchronic toxicity studies, an initial evaluation of the most sensitive endpoint may be warranted to determine which parameter (growth or survival) should be used for more definitive testing.

Table 3. Comparison of No Observed Effect Level (NOEL) values for length and weight measurements of *P. promelas* larvae exposed to municipal wastewater effluents.

	NOEL values (% effluent)	
Test	**Length**	**Weight**
I1	100[1]	100[1]
M1	100[1]	100[1]
M2	100[1]	100[1]
M3	100[1]	100[1]
M4	96	96
M5	0	6.25
M6	0	25
M7	12.5[2]	12.5[2]

[1] Highest concentration tested, no toxic effects.
[2] NOEL values determined after 4 d of exposure.

Table 4. Comparison of No Observed Effect Level (NOEL) values for length and weight measurements of *P. promelas* larvae exposed to $CuSO_4$.

	NOEL values (mg/L)	
Test	**Length**	**Weight**
RT1	50	100
RT2	60	150

Approximate time requirements for each day of image analysis methods for a six-dilution test with four test vessels per dilution and 15 fish per test vessel are as follows: 60 min to film the test vessels, 60 min to digitize the images, and 30 min to measure the lengths of the fish. Approximate time requirements for weight measurements for the same test are as follows: 30 min to weigh the empty filters, 90 min to place fish on the filters, 120 min to oven dry the fish, 30 min to cool the fish to room temperature, and 30 min to weigh the filters with fish. Thus, measuring fish length through image analysis methods actually requires less time than measuring fish weight. Image analysis methods could be conducted on one or two days of the 7-d test in addition to weight measurements with very little time increase to the test. However, image analyses for each day of the test may not be time-effective, cost- effective, or informative.

Additional benefits offered by length measurement include nondestructive analysis, and the capability of maintaining permanent computerized visual records of the test organisms and results, minimizing the need for extensive manual record keeping and filing. Fish length may also be a more sensitive indicator of growth than weight. Image-analysis measuring methods may be especially beneficial for use in studies designed to rapidly identify sublethal toxicants. Results obtained in this study, warrant future study to further evaluate the practicability and utility of this assay.

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