DEVELOPMENT OF A NONDESTRUCTIVE METHOD FOR MEASURING LENGTH OF FATHEAD MINNOW LARVAE AS AN INDICATOR OF GROWTH DURING A SEVEN DAY TOXICITY TEST

By

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CHAPTER I

INTRODUCTION

The Clean Water Act of 1972 established as a national objective the protection of the physical, chemical, and biological integrity of the waters of the United States (USEPA, 1987). In order to meet these objectives, each wastewater discharger in the U.S. was issued a discharge permit under the National Pollution Discharge Elimination System (NPDES) that specified the water quality parameters required to comply with these objectives. The NPDES permits are a mechanism to phase in water quality standards in sequential 5-year periods, or rounds. The first round permits, issued from about 1975 to 1980, included restrictions on the discharge of biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), and other primary pollutants (USEPA, 1987). Second round permits, issued from about 1980 to 1985, were more restrictive, including limits on priority pollutants (a compilation of total toxic organics (TTO's)and metals), nutrients such as nitrate-nitrogen and ammonia-nitrogen, and fecal coliform (USEPA, 1991). These first two rounds of NPDES permits were designed to address the protection of the chemical integrity of our nation's waters. In 1984, the

United States Environmental Protection Agency (USEPA) implemented a program to use bioassays to prohibit discharge of toxic contaminants (USEPA, 1991). Third round permits, issued from 1985 through 1993, required for the first time that wastewater dischargers use biomonitoring to protect the biological integrity of our waters. The biomonitoring tests developed by the USEPA for whole effluent toxicity (WET) testing of fresh water have been most broadly applied by regional EPA water quality divisions (USEPA, 1991). The USEPA and the states may also impose permit limits on effluent toxicity where violations of water quality standards exist or are projected, and may require an NPDES permittee to conduct a toxicity reduction evaluation (TRE) (Fed. Reg., 1989). TRE's are systematic investigations which combine whole effluent and/or chemical specific testing for toxicity identification and characterization. TRE's are intended to locate the source of effluent toxicity and evaluate what pollution control actions or waste water treatment plant modifications should be implemented in order to achieve compliance with a permit limit (USEPA, 1991).

Static-renewal WET tests using growth and survival of newly hatched fathead minnow (*Pimephales promelas*) larvae have been used for several years to assess the lethal and sublethal effects of aquatic contaminants. These tests are currently required by many NPDES permits to assess and control the discharge of toxic substances (Norberg-King, 1989). Since introduction of these tests by Norberg and Mount (1985) in the early 1980's, there was debate regarding

the practicality, reproducibility, and sensitivity of the test (Mayes, Shafer, and Barron, 1988 and Norberg-King, 1989). Some of the criticisms included the intensive labor requirements involved with obtaining fathead minnow weight measurements, the variation in growth of control fish, and the low sample size resulting from the necessary pooling of data since individual fish weights are impractical to obtain (Mayes, Shafer, and Barron, 1988).

Growth of young fathead minnows is considered an important endpoint of these static-renewal tests because of the ecological impact of growth rates of organisms and the increased sensitivity of growing organisms to toxicants. Growth may be expressed as a change in weight or length over time, and may be measured by drying and weighing the fish or by measuring fish length. Weight has been the more accepted determination of growth because early length measurement techniques involved anesthetization and confinement of fish for direct measurement. These techniques were typically stressful to the fish.

Photographic methods allow routine length measurements without handling or removing fish from the water. Further benefits of photographic methods include reduced handling stress to the test organisms, a permanent photographic record of test animals, and the ability to postpone actual length measurements (Sauter and Harrison, 1985).

With the development and availability of new technology, photographic length measurement techniques can be further improved, and could possibly enhance current

aquatic toxicity testing procedures. The primary objectives of this study were to:

1. Determine if fish length measuring techniques could be modified to develop a nondestructive method using a commercially available video camera, computer, and software (image analysis techniques).

2. Determine if length measurements are acceptable indicators of sublethal toxicity.

3. Determine if sublethal toxicity can be detected earlier than the end of the 7-day subchronic test.

The null hypotheses tested were:

Ho: There is no linear correlation between length and weight of fathead minnow larvae.

Ho: Length measurements are not as sensitive as weight measurements for indicating sublethal toxicity.

Ho: Sublethal toxicity cannot be detected earlier than the end of a 7-day subchronic test.

CHAPTER II

REVIEW OF LITERATURE

Growth as a Toxicological Endpoint

Growth is considered an important toxicological endpoint of chronic and subchronic testing because of its ecological impact and its sensitivity. Reduced growth may affect competition for food and habitat, time to maturation, and susceptibility to predation and disease (Woltering, 1984). Several studies have shown growth to be a sensitive measure of toxic stress, and indicate 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).

Methods for determining growth

Growth may be measured by drying and weighing fish or by measuring fish length. Dawson, *et al.* (1988) utilized head-tail length measurements of *Xenopus sp.* and fathead minnow embryos as an index of growth in a study to determine the effects of metal-contaminated sediment extracts on the development, growth and survival of these organisms.

Meteyer *et al.* (1988) measured total larval length and dry weight of sheepshead minnow (*Cyprinodon variegatus*) to determine developmental effects of cadmium exposure during a 7-8 day test. The resulting cadmium-exposed larvae were shorter than the control larvae. The study indicated that dry weight was a less sensitive growth indicator than total length. Simple linear regression analyses from preliminary studies by Stebler indicated that growth measured as change in weight was highly correlated with growth measured as change in length (Stebler, 1988). The slope coefficient of the regression equation varied depending on the age of the fish and the type of effluent sample studied, yet 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. 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).

Photographic methods allow routine length measurements without handling or removing fish from the water. Further benefits of photographic methods include reduced handling stress to the test organisms, a permanent photographic record of test animals, and the ability to postpone actual length measurements (Sauter and Harrison, 1985). Sauter and Harrison (1985) proposed a modification of photographic

techniques using instant photography, a digitizing tablet, and a computer. Previous methods required expensive equipment, and often produced images difficult to measure resulting from fish in a curved position or several fish crowded together. The method proposed by Sauter and Harrison (1985) improved the efficiency and eliminated some of the previous disadvantages of photographic measurements.

Fish length may be a more desirable measurement of growth because current USEPA protocol fish weight methods (Weber *et al.*, 1989) rely on obtaining average weights of all fish fry per replicate, reducing the number of treatment replicates to four. Statistical power and test sensitivity may be maximized by increasing the sample size. Sample size may be increased by analyzing data for individual fish (Mayes *et al.*, 1988). Although weighing individual fish is time consuming and requires delicate and expensive equipment, measuring the length of individual fish using image analysis methods can be accomplished relatively inexpensively.

CHAPTER III

MATERIALS AND METHODS

Bioassay Procedure

Fathead minnow (*Pimephales promelas*) larval survival and growth tests were conducted on ten sources of wastewater according to USEPA method 1000.0 (Weber *et al.*, 1989): six municipal wastewater effluents (identified as M1 through M6), one industrial wastewater effluent (identified as I1), and two Copper Sulfate ($CuSO_4$) reference toxicant samples (identified as RT1 and RT2). Head to tail lengths of fathead minnow larvae were measured for each day of the seven-day tests. Fish weights were measured on day seven of the tests. Extra replicates were set up in the M3 test so that weights could be measured on day two, day four and day seven of the test. Tests were conducted at Stover Biometric Laboratories (SBL) in Stillwater, Oklahoma in a test exposure room under constant temperature and photoperiod conditions. The exposure room temperature was maintained at 25 C ± 1 C, with a photoperiod of 16 hours of light and eight hours of dark during each 24 hour period. Test organisms were fed 0.1 ml of concentrated *Artemia sp.* nauplii twice daily, with a minimum of six hours between

feeding. Fathead minnow larvae were supplied from cultures maintained by SBL.

Sample Collection and Preparation

Twenty-four hour composites of wastewater effluents were received from seven municipal and one industrial wastewater treatment plants. Three samples were collected by each treatment plant's personnel for each test. Samples were received on the first, third, and fifth days of the test, or the first, third and sixth days of the test, depending upon sampling and shipping schedules. Tests were set up within 36 hours after the first sample was removed from the sampler. Each sample was diluted with receiving stream water or synthetic dilution water to produce 1000 ml of each of the appropriate test concentrations, according to the treatment plant's NPDES permit. Dissolved oxygen and pH were measured for each dilution daily, before renewal of test vessels. Dissolved oxygen, pH, conductivity, hardness, and alkalinity were measured for each dilution the day a new sample was received. Ammonia and chlorine levels were measured on the 100% effluent sample and the control the day a new sample was received. Copper sulfate ($CuSO_4$) reference toxicant samples were prepared using a $CuSO_4$ reference toxicant received from the Environmental Protection Agency's Environmental Monitoring Systems Laboratory, Cincinnati, Ohio.

Test Set Up

Each test was set up with fathead minnow fry 24-hours old or younger. Each dilution was divided into four 150 ml glass evaporating dishes for all tests except M3. Fathead minnow fry were then randomly added to the dishes until each vessel contained 15 fry. Any obviously unhealthy or malformed fry were replaced with randomly selected healthy fry. The M3 test was set up with eight replicates, each containing fifteen fish.

Test renewal and photography of fish

Each day of the test, the test solution was siphoned from each vessel until the fish were unable to swim freely, but were still submerged. Fish were allowed a minimum of two hours after the first daily feeding before the test vessels were siphoned. Any dead fish and detritus were removed in the siphoning process. The low final siphoning volume ensured that all fish in the test vessel remained within the same plane for photographing, and prevented surface distortion caused by swimming movements. Once siphoned, each vessel was filmed for approximately 60 seconds using a Sony® 8mm 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. The height of the camera was set so that the bottom area of the test vessels almost completely

filled the camera's field of view. Two clear plastic 70 mm rulers were placed on the plane of focus 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. Fish images were collected and stored on 8mm video cassette tapes. The test solution was renewed after filming on days one through six of the test. Fresh test solution was carefully poured into the vessel, which was then returned to the test shelf. Immediately after filming on day seven of the test, the fish were placed on filters for weighing.

Length measurements of fish

A 386/16MHZ micro-computer with ZIP IMAGE® processing software and High Resolution Technologies (HRT)® video frame grabber were used to digitize images of the fish. Head to tail fish lengths were measured using a program developed in Turbo C® with HRTlib, an image processing/support library for the HRT512-8 video frame grabber. The measuring program created a data file of fish lengths for each vessel. With minimal editing, this data file was imported directly into the statistical software package Toxstat®, version 3.0 (Gulley *et al.*, 1987).

Weight Measurements of Fish

After the fish were filmed on the final day of the test, they were weighed according to the following protocol. One Whatman #1 filter was placed in an aluminum weighing pan for each vessel within the test. The filters were rinsed with deionized water and placed in an oven set at 100 C to bake for a minimum of six hours. After this drying period, the filters were removed from the oven and placed in a glass desiccator. After cooling in the desiccator for a minimum of one hour, the filters were weighed to the nearest ten micrograms. The fish in each vessel were rinsed with deionized water to remove sediment and food debris, and were then placed onto the appropriate preweighed filter. The filters and fish were dried for two hours in the oven set at 100 C. After drying, the filters and fish were placed in the desiccator for one hour. After this cooling period, the fish and filters were weighed to the nearest ten micrograms to obtain net weights per filter. The original weight of each filter was subtracted from the weight of the filter with fish to provide a dry weight for all the fish in the vessel. Dry weight was converted to mean dry weight per fish by dividing by the number of fish on the filter. The M3 test was set up with eight replicates of fifteen fish per replicate. Weights for the M3 fish were measured on day two and day four by randomly removing forty fish from the replicates and weighing them according to the above protocol. The remaining M3 fish were weighed on day seven.

Statistical Analysis

Day seven length and weight values were compared for all tests, and day four length and weight data were compared for the M3 test. The correlation coefficient (r) and Student's t value for n-2 degrees of freedom were determined for each data set. The data were analyzed independently as separate tests and as a combined data set. Student's t values were used to test the null hypothesis Ho: $\rho = 0$.

All other statistics were determined using Toxstat, version 3.0 (Gulley *et al.*, 1987). Data for each test were analyzed independently. Shapiro-Wilk's test was used to determine normality of the data, and Bartlett's test was used to determine homogeneity of variance. The No Observed Effect Level (NOEL) and Lowest Observed Effect Level(LOEL) were determined for both weight and length for each test using the most appropriate of the following tests: Bonferroni T-test, Dunnett's test, Steele's Many-One Rank test, or the Wilcoxon Rank Sum test with Bonferroni Adjustment. Weight NOEL values were determined for day seven data for all the tests, and day four and day seven data for the M3 test. Length NOEL values were determined for days four and seven of all tests, and days two, four, and seven of the M3 test.

A summary of this information was submitted to the Bulletin of Environmental Contamination and Toxicology for publication, and is presented in appendix A.

CHAPTER IV

RESULTS

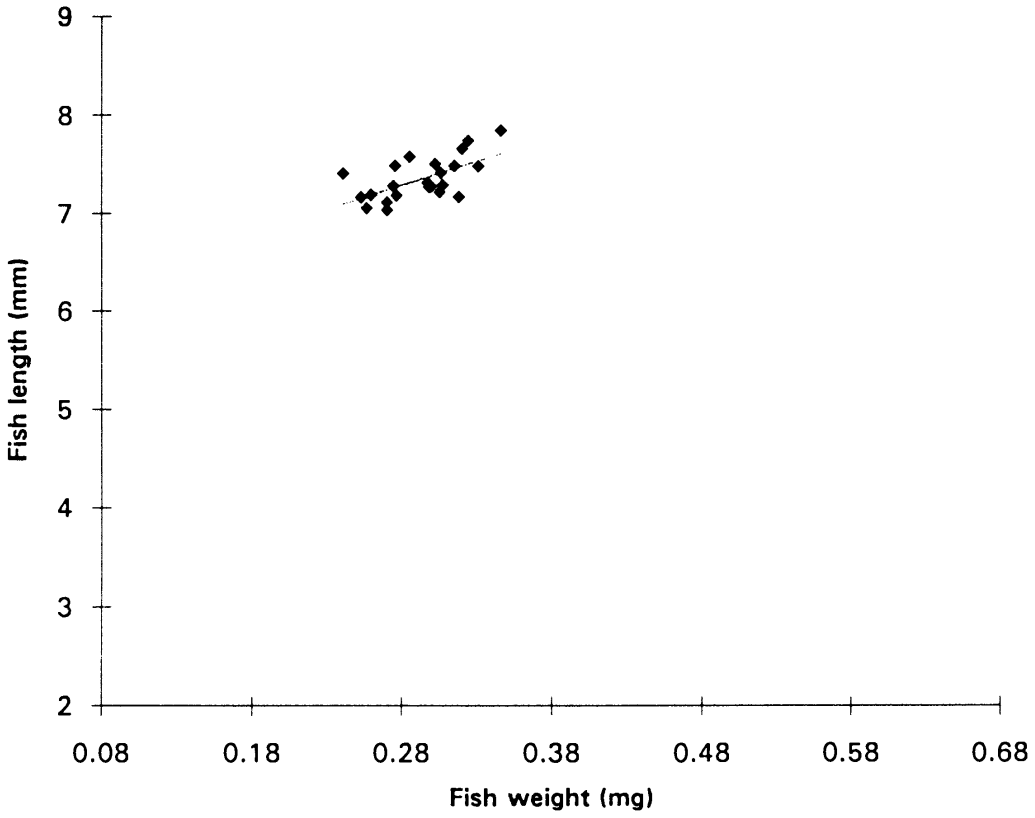
Length-Weight Correlation

Length and weight data were compared for day seven measurements of each test. Length and weight data were also compared for day four of the M3 test (Appendix B). Linear regression analysis of length and weight measurements using the least squared method produced correlation coefficients ranging from 0.053 to 0.653, indicating poor linear correlation (Figures 1 through 11). Student's t values were used to test the null hypothesis of no linear correlation between length and weight (Ho: $\rho = 0$ using $\alpha = 0.05$). The data were analyzed in eleven sets. Data from seven sets failed to reject the null hypothesis. Data from the remaining four data sets rejected this hypothesis. The combined data for all ten tests resulted in a failure to reject Ho (Table 1).

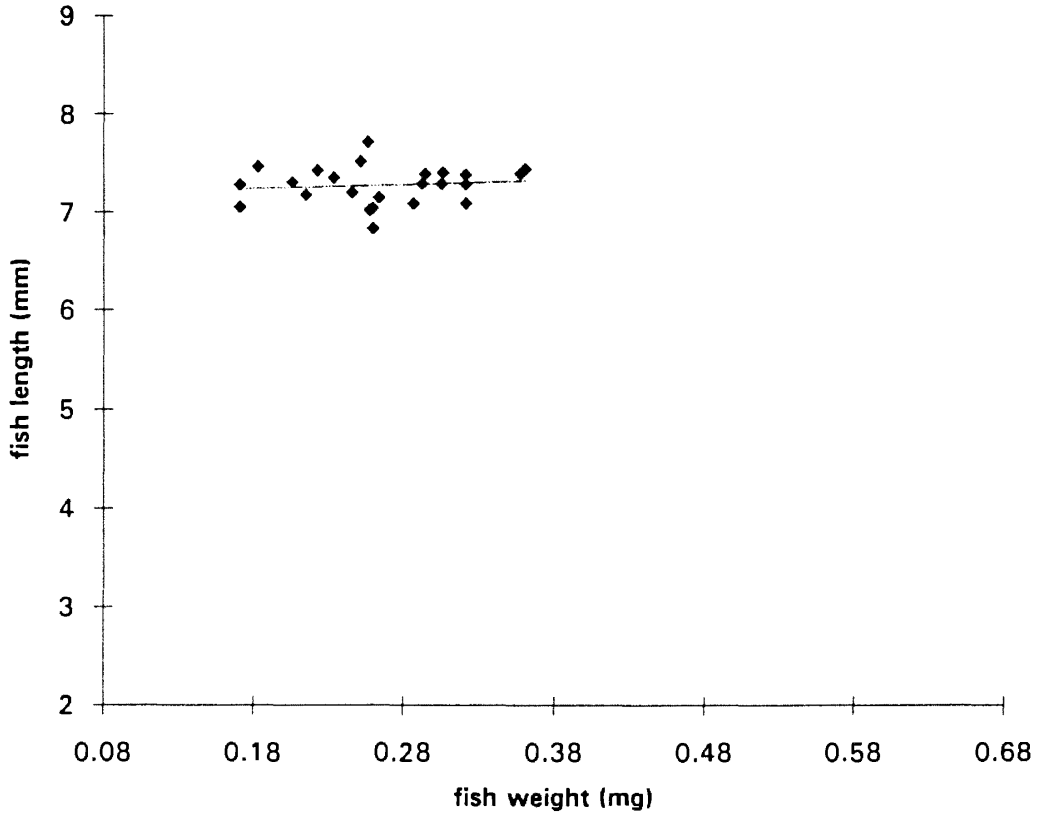
NOEL Comparison

Comparison of the NOEL values for length and weight were identical for the industrial effluent and for five of the municipal effluent tests (Table 2). Length NOEL values

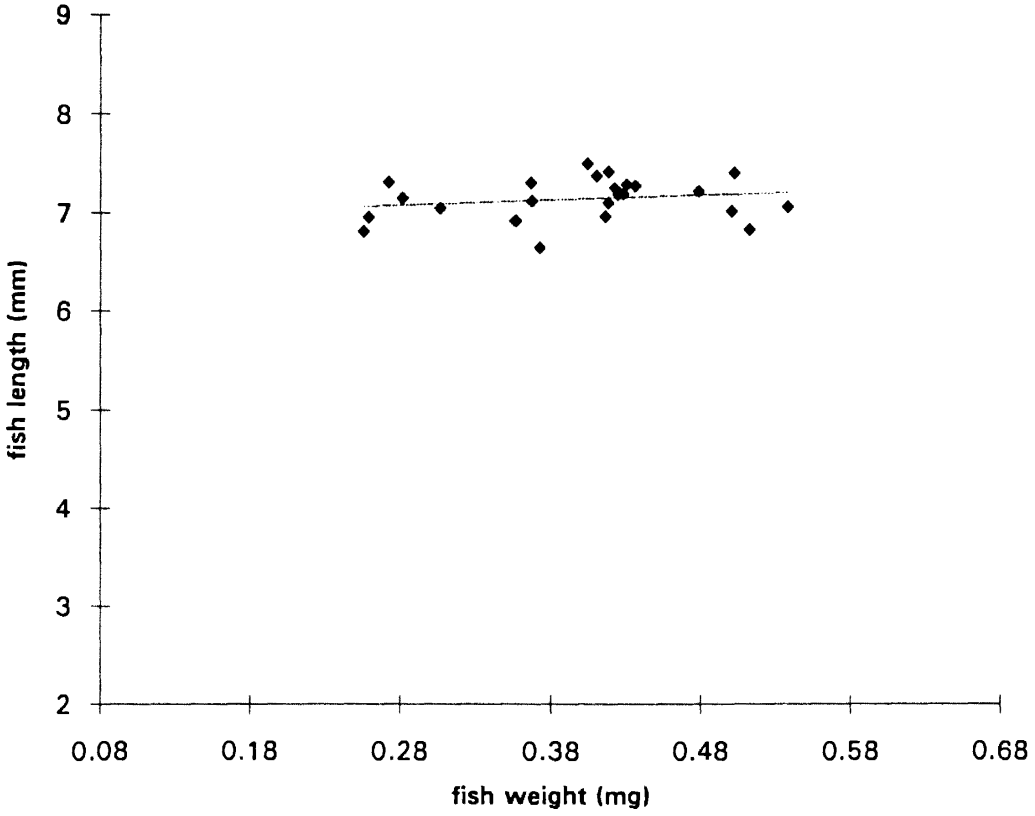
Linear Regression of Length (mm) Verses Weight (mg)
for Seven Day Data of Test I1



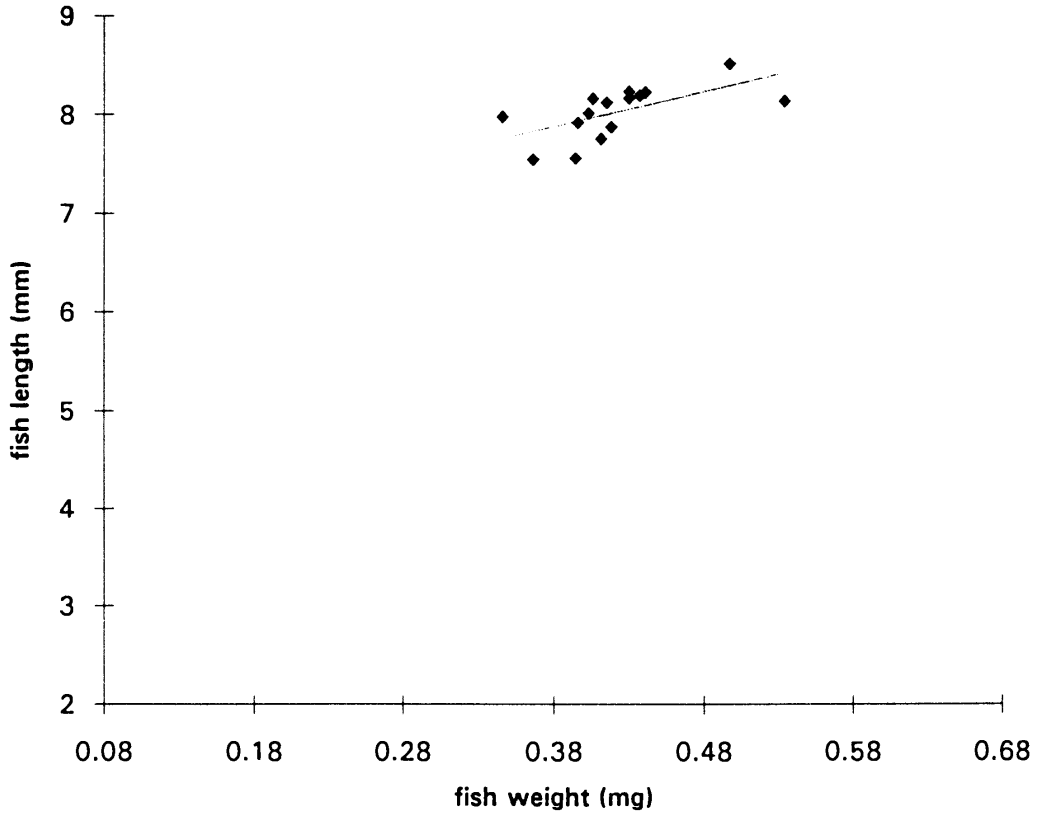
Linear Regression of Length (mm) Verses Weight (mg)
for Seven Day Data of Test M1



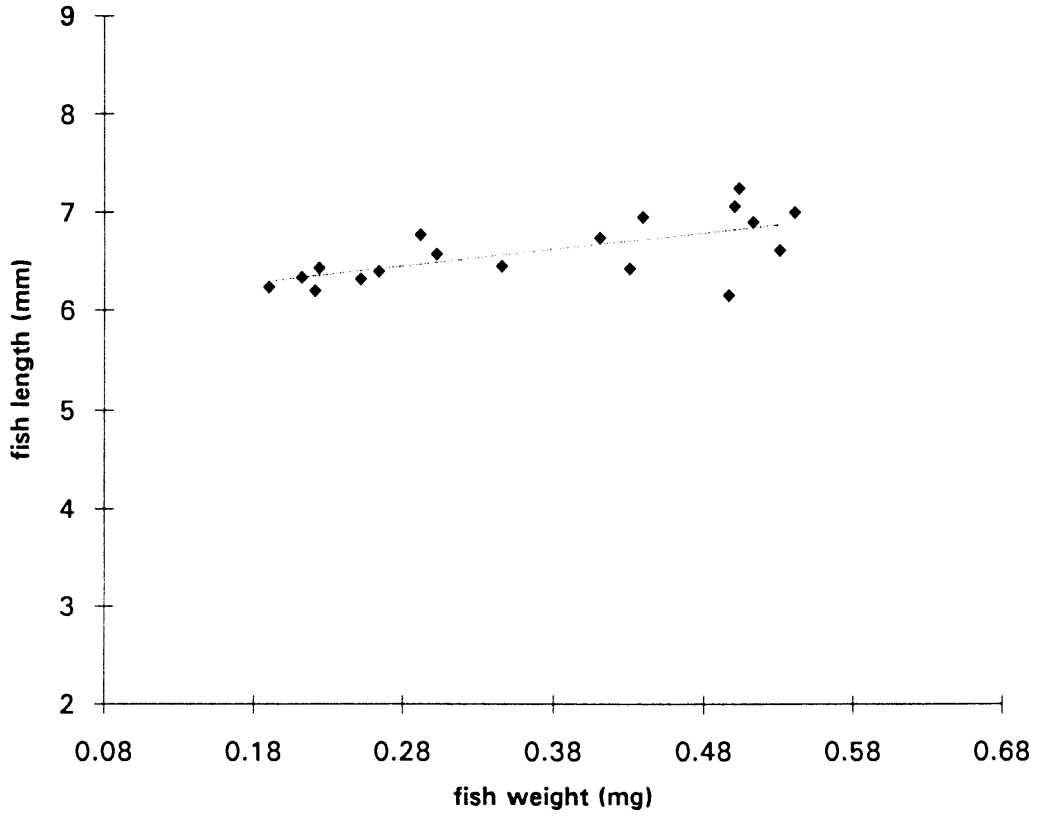
Linear Regression of Length (mm) Verses Weight (mg)
for Seven Day Data of Test M2



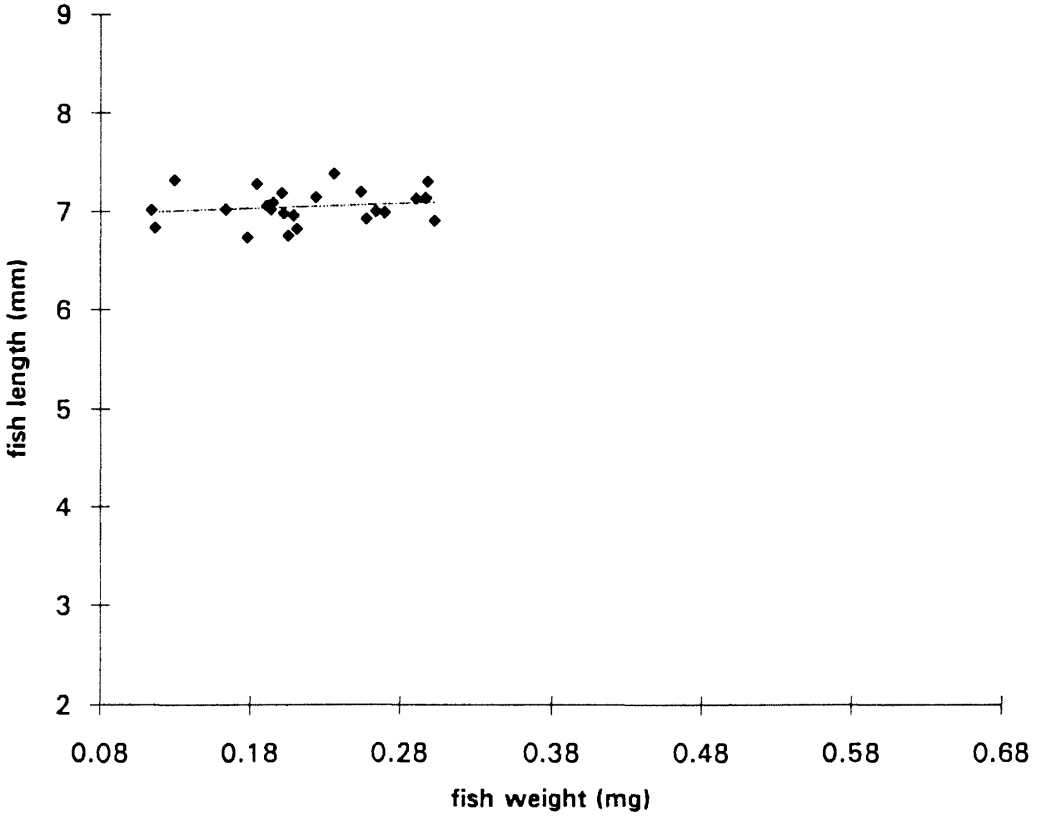
Linear Regression of Length (mm) Verses Weight (mg)
for Seven Day Data of Test M3



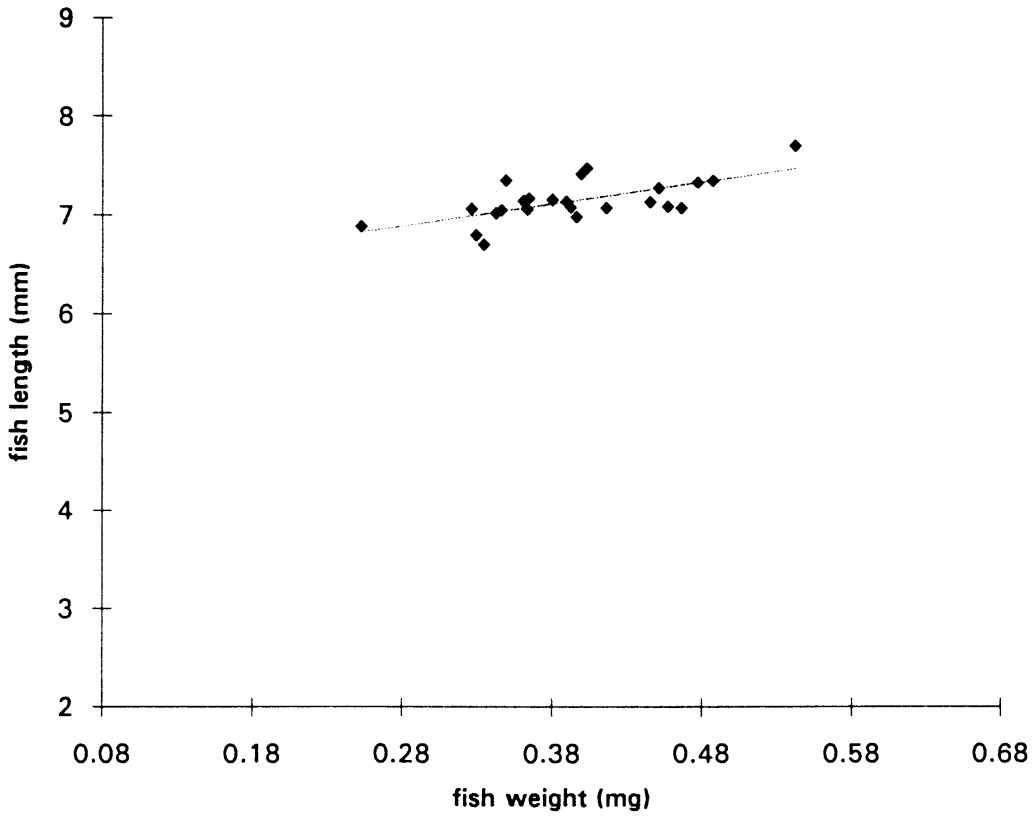
Linear Regression of Length (mm) Verses Weight (mg)
for Four Day Data of Test M3



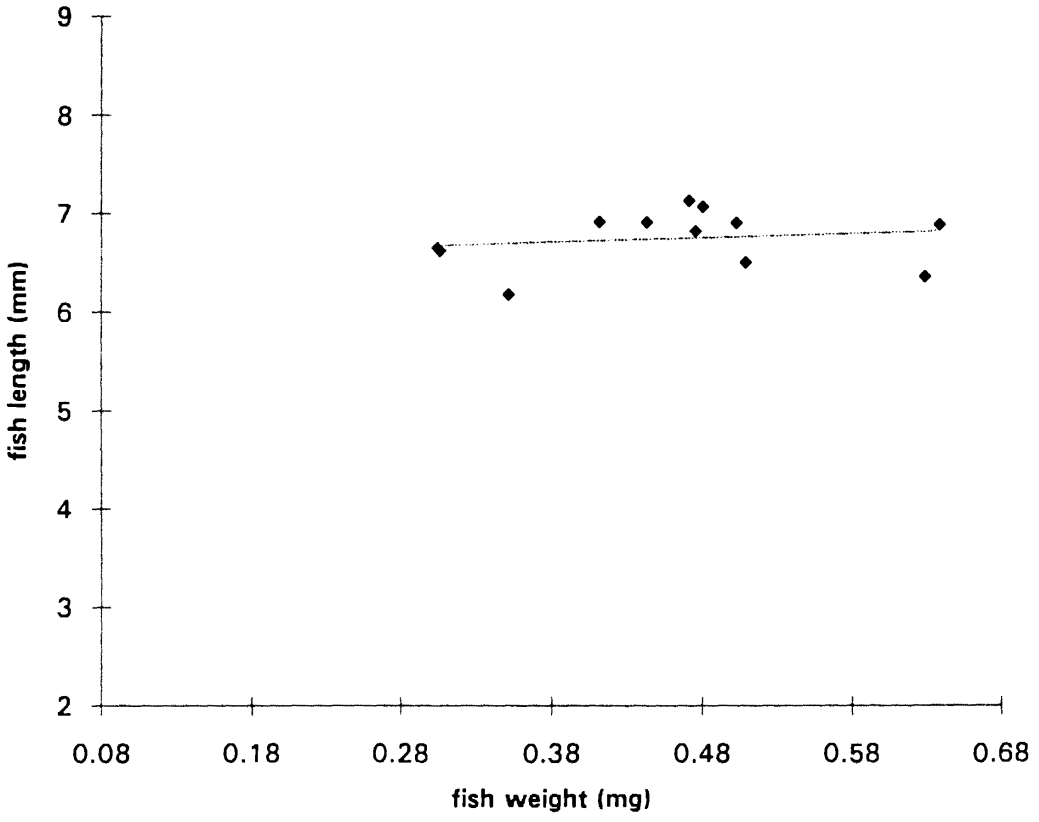
Linear Regression of Length (mm) Verses Weight (mg)
for Seven Day Data of Test M4



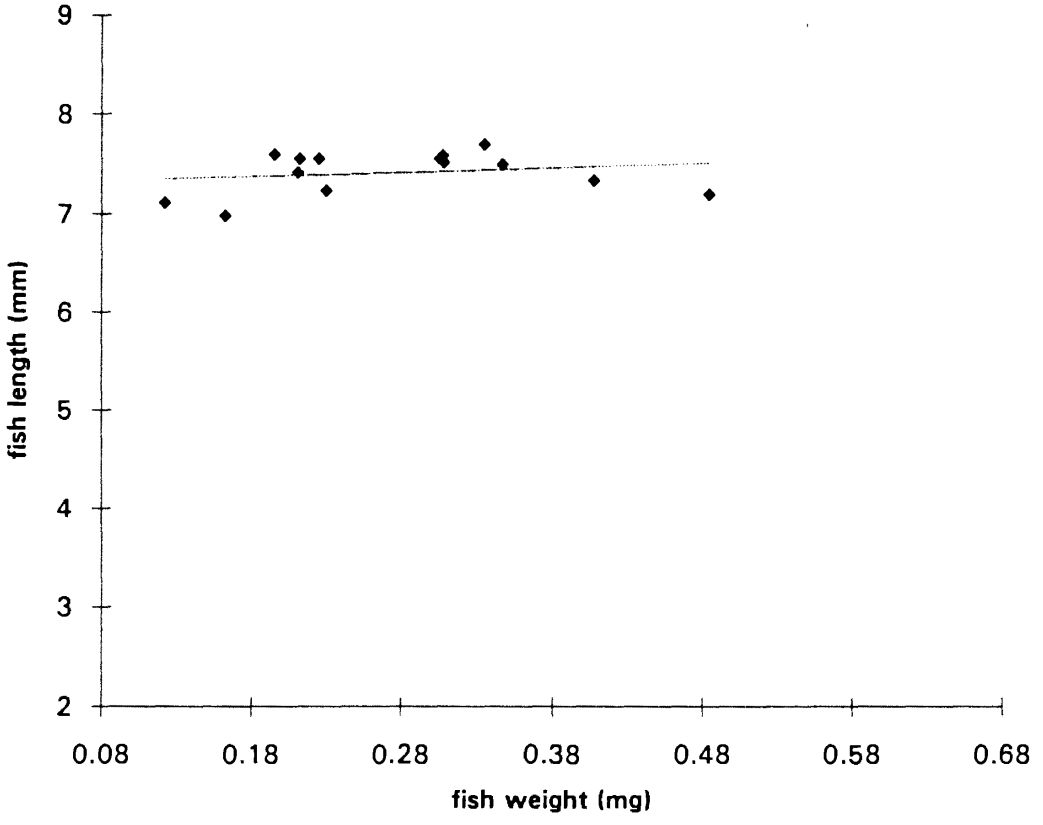
Linear Regression of Length (mm) Verses Weight (mg)
for Seven Day Data of Test M5



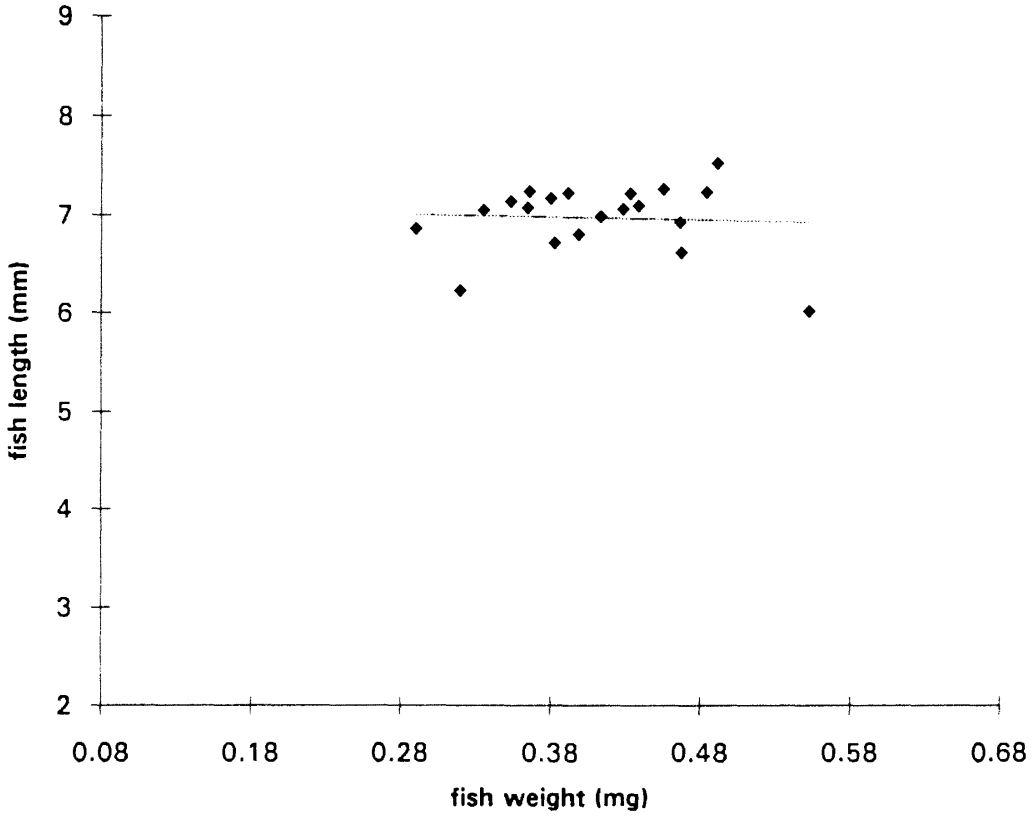
Linear Regression of Length (mm) Verses Weight (mg)
for Seven Day Data of Test M6



Linear Regression of Length (mm) Verses Weight (mg)
for Seven Day Data of Test RT1



**Linear Regression of Length (mm) Verses Weight (mg)
for Seven Day Data of Test RT2**



Linear Regression of Length (mm) Verses Weight (mg)
for Seven Day Data of All Data Sets

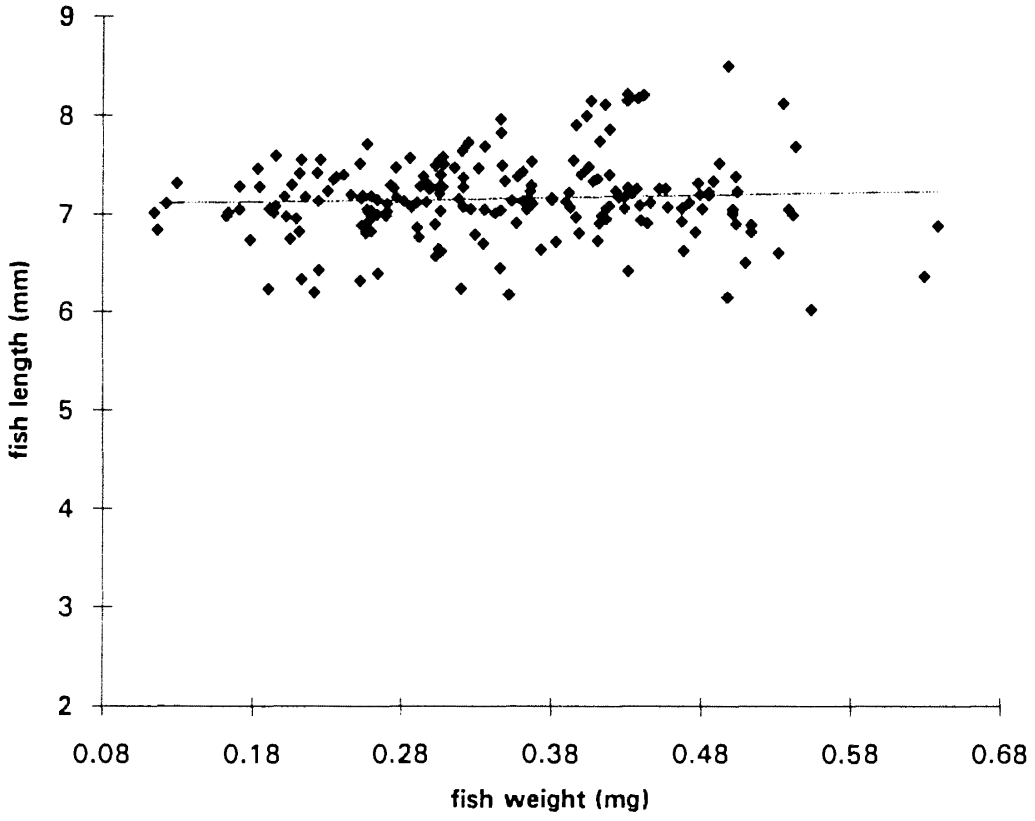


TABLE I

SUMMARY OF REGRESSION COEFFICIENTS AND STUDENT'S T VALUES FOR LENGTH VERSUS WEIGHT COMPARISONS OF FATHEAD MINNOW LARVAE DURING SEVEN-DAY TOXICITY TESTS

Test	r	r squared	t statistic	df	Ho: r = 0
I1	0.616	0.379	3.665	23	Reject
M1	0.116	0.014	0.550	23	FTR *
M2	0.182	0.033	0.868	23	FTR
M3 (day 7)	0.618	0.382	2.837	14	Reject
M3 (day 4)	0.653	0.427	3.560	18	Reject
M4	0.171	0.029	0.813	23	FTR
M5	0.649	0.421	3.998	23	Reject
M6	0.159	0.025	0.511	11	FTR
RT1	0.210	0.044	0.743	13	FTR
RT2	0.053	0.003	0.223	19	FTR
All data Combined	0.066	0.004	0.933	199	FTR

*FTR: Failed to reject

TABLE II

COMPARISON OF NOEL VALUES FOR LENGTH AND WEIGHT MEASUREMENTS OF FATHEAD MINNOW LARVAE DURING SEVEN-DAY TOXICITY TESTS

	NOEL values (% effluent)		LOEL values (% effluent)	
Test	Length	Weight	Length	Weight
I1	100	100	>100	>100
M1	100	100	>100	>100
M2	100	100	>100	>100
M3 (7-day)	100	100	>100	>100
M3 (4-day)	12.5	12.5	25	25
M4	96	96	>96	>96
M5	0	6.25	6.25	12.5
M6	0	25	25	100

	NOEL values (ug/L)		LOEL values (ug/L)	
Test	Length	Survival	Length	Survival
RT1	50	50	75	75
RT2	60	60	75	75

for M5 and M6 were lower than the corresponding weight NOEL values. Weight NOEL values could not be determined for the two $CuSO_4$ reference toxicant tests due to significant mortality. However, survival NOEL values were determined to be identical to the length NOEL values for both reference toxicant tests (Table 2).

Early Measurement of NOEL

NOEL values were calculated for day four length measurements for each test, and day two and four measurements for the M3 test (Table 3). Four day length NOEL values were equivalent to seven day values for five of the nine toxicity tests. Of the four unequal NOEL values, three underestimated toxicity (the four-day NOEL value was greater than the seven-day value), and one overestimated toxicity (the four-day NOEL value was less than the seven-day value).

TABLE III

COMPARISON OF FATHEAD MINNOW LENGTH NOEL VALUES
FOR FOUR AND SEVEN DAY EXPOSURE PERIODS

	NOEL values (% effluent)		
Test	Day 2	Day 4	Day 7
I1	--	100	100
M1	--	100	100
M2	--	100	100
M3	12.5	12.5	100
M4	--	96	96
M5	--	100	0
M6	--	25	0

	NOEL values (Ug/L)		
Test	Day 2	Day 4	Day 7
RT1	--	75	50
RT2	--	85	60

CHAPTER V

CONCLUSIONS

Length-Weight Correlation

Seven of the eleven data sets analyzed during this study indicated that there is little or no linear correlation between length and weight of fathead minnow larvae. The four data sets that indicated some linear correlation had r-values of less than 0.7. This does not support earlier analyses by Stebler (1988). Discussions with Dr. S. L. Burks in 1993 indicated that feeding protocols have changed since the study conducted by Stebler. If earlier tests were fed lower amounts, the length-weight correlation might have been affected because weight would not increase as rapidly. Stebler's data indicated that fish older than seven days did not consistently maintain a linear length-weight correlation.

Sensitivity of Length as a Growth Endpoint

Comparison of NOEL values derived from length and weight data indicated that length is as sensitive a growth endpoint as weight, and in some cases may be more sensitive. Length NOEL values for six of the eight data sets were equal

to the corresponding weight NOEL values. Length NOEL values for two of the data sets were greater than the corresponding weight NOEL values.

Early Detection of Sublethal Toxicity

NOEL values calculated from four day length data did not consistently match those calculated from seven-day length data. Of the nine data sets compared, the NOEL values for four- and seven-day data were equal for five data sets. Of the four NOEL values that did not match, three four-day NOEL values were greater than the seven-day values. These results indicate that four-day NOEL values can be less sensitive than seven-day values. Although a four-day test would be less expensive and less labor intensive, it could potentially underestimate the toxicity of a sample to fathead minnow larvae.

Evaluation of Methodology

The length measurement technique was successfully utilized to measure fish lengths during nine seven-day chronic tests. The technique allowed daily measurements of test fish without any adverse effects resulting from stress during handling.

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APPENDIXES

APPENDIX A

COMPARISON OF LENGTH AND WEIGHT AS INDICATORS OF SUBLETHAL TOXICITY IN SEVEN-DAY SUBCHRONIC TOXICITY TESTS

Comparison of Length and Weight as Indicators of Sublethal Toxicity in Seven-Day Subchronic Toxicity Tests

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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 disease (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. 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).

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Fish length may be a more desirable measurement of growth because current USEPA protocol fish weight methods (Weber et al., 1989) rely on obtaining average weights of all fish fry per replicate, reducing the number of treatment replicates to four. Test sensitivity may be maximized by increasing the sample size. Sample size may be increased by analyzing data for individual fish (Mayes et al., 1988). Although weighing individual fish is time consuming and requires delicate and expensive equipment, measuring the length of individual fish using image analysis methods can be accomplished relatively inexpensively.

Image analysis measurements provide a nonintrusive 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 nonintrusive method for measuring the length of fish fry during routine 7-day subchronic toxicity tests are described in this report. Image analysis techniques were developed using video recording equipment, image capture software, and computer digitizing equipment. These methods allowed nonintrusive length measurements of test fish for each day of a 7-day subchronic test.

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 sample 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. The filming and data processing procedures are outlined in Figure 1. Fish larvae in each vessel were filmed using a Sony 8 mm Handycam^R 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. 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 a 100° C oven. After 24 hours, the filters were removed from the oven and placed in a desiccator. After cooling in the desiccator for one hour, the filters

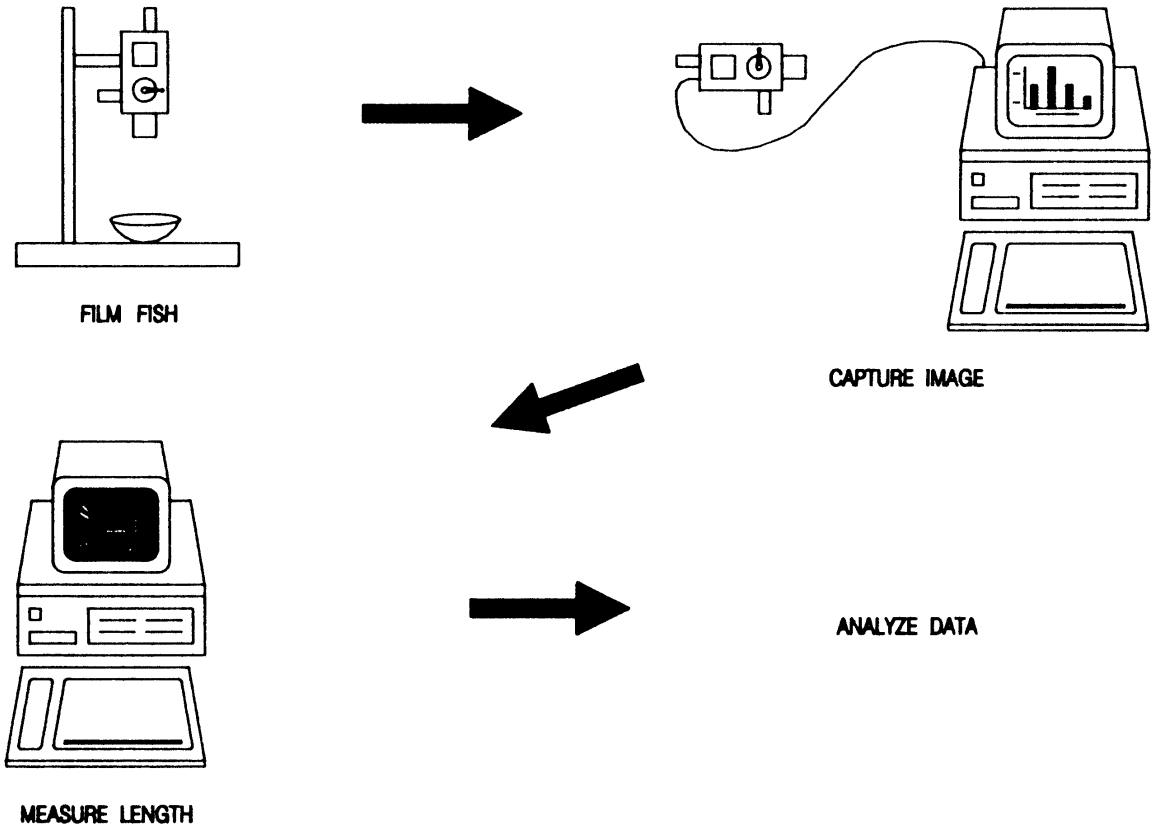

FIGURE 1 - FLOWCHART OF FILMING AND DATA PROCESSING PROCEEDURES FOR NONINTRUSIVE IMAGE ANALYSIS LENGTH MEASUREMENTS OF *P. promelas* LARVAE DURING A 7-DAY SUBCHRONIC TEST

were weighed. After length measurements were taken, 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 could not be determined for filters containing fewer than seven fish because resulting net weights were beyond 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 regression 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. Reference toxicant data are presented in Table 2. Length and weight NOEL values for the industrial wastewater effluent test, the four day municipal wastewater effluent test, and four of the seven-day 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 affects in length were determined as early as day six for the fifth municipal effluent (M5) and day four for the sixth municipal effluent (M6). Due to significant mortality in the two $CuSO_4$ reference toxicant tests, weight NOEL values could not be determined. However, survival NOEL values were determined to be identical to the length NOEL values for both reference toxicant tests (Table 4).

Preliminary data by Stebler (1988) 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 regression 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 indicated that length may successfully be used to predict growth inhibition. All tests conducted indicated length to be as sensitive or more sensitive than weight as an indicator of growth. Preliminary results also indicated that growth effects may be determined before the end of a seven-day test when length measurements are used.

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 minutes to film the test vessels, 60 minutes to digitize the images, and 30 minutes to measure the lengths of the fish. Approximate time requirements for weight measurements for the same test are as follows: 30 minutes to weigh the empty filters, 90 minutes to place fish on the filters, 120 minutes to oven dry the fish, 30 minutes to cool the fish to room temperature, and 30 minutes to weigh the filters with fish. Thus, measuring fish length

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)			Length (mm/fish)		
		n	mean	SE	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
	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

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

Table 3. Comparison of No Observed Effect Level (NOEL) values for length measurements 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 days of exposure.

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

	NOEL values (µg/L)	
Test	**length**	**survival**
RT1	50	50
RT2	60	60

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 seven day 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- or cost- effective.

Benefits of length measurements include increased sample size, nonintrusive techniques, and the capability of maintaining permanent visual records of the test organisms and results. Fish length may also be a more sensitive indicator of growth than weight. Image analysis measuring methods may be especially beneficial for use in chronic Toxicity Identification Evaluations, particularly with samples prone to toxicity degradation.

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APPENDIX B

LENGTH AND WEIGHT MEASUREMENTS FOR FATHEAD MINNOW LARVAE DURING A SEVEN-DAY TOXICITY TEST

Appendix B: Length and Weight Measurements for Fathead Minnow Larvae During a 7-day Toxicity Test

I1		M1		M2		M3 (day 7)		M3 (day 4)	
Mean per replicate		Mean per replicate		Mean per replicate		Mean per replicate		Mean per replicate	
weight (mg)	length (mm)	weight (mg)	length (mm)	weight (mg)	length (mm)	weight (mg)	length (mm)	weight (mg)	length (mm)
0.271	7.116	0.287	7.090	0.501	7.018	0.438	8.201	0.190	6.232
0.271	7.042	0.260	7.050	0.357	6.918	0.416	8.131	0.212	6.336
0.300	7.288	0.264	7.160	0.429	7.189	0.397	7.925	0.221	6.201
0.260	7.197	0.322	7.090	0.307	7.046	0.535	8.146	0.224	6.433
0.308	7.292	0.322	7.289	0.437	7.269	0.498	8.520	0.252	6.326
0.241	7.415	0.306	7.300	0.538	7.061	0.404	8.023	0.264	6.402
0.303	7.508	0.358	7.398	0.513	6.832	0.442	8.233	0.292	6.782
0.299	7.273	0.246	7.208	0.373	6.649	0.431	8.173	0.303	6.578
0.347	7.846	0.307	7.414	0.417	6.966	0.431	8.239	0.346	6.450
0.253	7.167	0.361	7.443	0.256	6.820	0.407	8.169	0.411	6.739
0.307	7.419	0.171	7.053	0.479	7.212	0.419	7.882	0.411	6.741
0.276	7.492	0.322	7.391	0.368	7.120	0.412	7.759	0.431	6.430
0.321	7.664	0.293	7.296	0.273	7.308	0.367	7.545	0.440	6.952
0.275	7.282	0.257	7.729	0.259	6.958	0.347	7.986	0.497	6.150
0.306	7.222	0.234	7.364	0.425	7.183	0.395	7.563	0.501	7.059
0.298	7.314	0.295	7.400	0.367	7.304			0.504	7.245
0.316	7.490	0.223	7.431	0.419	7.414			0.513	6.902
0.277	7.192	0.252	7.529	0.503	7.401			0.531	6.615
0.325	7.748	0.215	7.185	0.405	7.497			0.541	7.002
0.286	7.587	0.206	7.309	0.282	7.147				
0.257	7.060	0.258	7.026	0.431	7.286				
0.319	7.172	0.260	6.836	0.423	7.243				
0.300	7.279	0.183	7.470	0.411	7.375				
0.332	7.482	0.171	7.283	0.419	7.098				

Appendix B: Length and Weight Measurements for Fathead Minnow Larvae During a 7-day Toxicity Test (continued)

M4		M5		M6		RT1		RT2	
Mean per replicate		Mean per replicate		Mean per replicate		Mean per replicate		Mean per replicate	
weight (mg)	length (mm)	weight (mg)	length (mm)	weight (mg)	length (mm)	weight (mg)	length (mm)	weight (mg)	length (mm)
0.202	6.985	0.543	7.704	0.472	7.131	0.336	7.705	0.492	7.534
0.201	7.189	0.404	7.479	0.476	6.830	0.195	7.606	0.485	7.238
0.254	7.200	0.488	7.351	0.481	7.067	0.308	7.601	0.414	6.995
0.303	6.911	0.478	7.328	0.444	6.919	0.309	7.525	0.354	7.148
0.299	7.308	0.452	7.275	0.412	6.917	0.348	7.512	0.365	7.088
0.129	7.317	0.467	7.071	0.509	6.513	0.225	7.562	0.439	7.105
0.184	7.288	0.446	7.129	0.307	6.629	0.211	7.426	0.381	7.175
0.191	7.058	0.458	7.084	0.305	6.656	0.408	7.351	0.456	7.275
0.236	7.385	0.365	7.169	0.628	6.370	0.306	7.567	0.336	7.058
0.297	7.144	0.397	6.982	0.352	6.180	0.162	6.984	0.366	7.243
0.116	6.835	0.364	7.061	0.638	6.896	0.122	7.109	0.429	7.072
0.264	7.010	0.393	7.079	0.503	6.914	0.212	7.561	0.434	7.228
0.195	7.091	0.390	7.137			0.485	7.209	0.399	6.819
0.209	6.963	0.347	7.054			0.230	7.244	0.392	7.236
0.205	6.757	0.327	7.070					0.383	6.728
0.258	6.933	0.343	7.022					0.291	6.878
0.163	7.023	0.253	6.891					0.467	6.937
0.114	7.010	0.361	7.142					0.320	6.239
0.178	6.742	0.400	7.422					0.553	6.031
0.211	6.828	0.350	7.356					0.468	6.631
0.224	7.142	0.330	6.802						
0.270	6.995	0.335	6.710						
0.291	7.140	0.381	7.154						
0.194	7.023	0.417	7.076						

VITA

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Master of Science

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