LARVAL LENGTH MEASUREMENT AS AN ALTERNATIVE TO WEIGHT AS AN INDICATOR OF GROWTH IN THE SEVEN-DAY FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST

By

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PREFACE

I wish to express my appreciation to my major adviser, Dr. L. Herbert Bruneau, for his patience and his faith that I would one day complete my degree requirements. I express my gratitude to Dr. S. L. Burks for the freedom to pursue this work while I was employed with the Water Quality Research Laboratory and for all the guidance he has given me over the years. I thank Dr. Margaret S. Ewing for her encouragement and invaluable assistance in the preparation of this report.

I am especially grateful to Dr. John A. Bantle for allowing me to use his equipment and for suggesting the idea for this project originally. I also extend my thanks to the students and technicians who were helpful in performance of this research, including: Doug Dawson, Debbie Newell, James Rau and Pat Shaw Allen.

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CHAPTER I

ABSTRACT

Linear regression analyses were performed on weights and lengths of larval fathead minnows in an attempt to evaluate these variables as comparable measures of the growth endpoint in short-term chronic assays. Mean dry weight and mean individual total lengths were highly and positively correlated (R values of 0.82 - 0.98) for data generated from seven-day fathead minnow larval survival and growth tests performed on effluents from a variety of treatment facilities (industrial, municipal, and refinery). Length measurements were also found to be accurate, precise, and capable of detecting the same level of significant differences between control and treatment means. In comparison to weight measurements, substantial time can be saved using larval lengths obtained with a very simple setup and cost of equipment can be reduced as well. Length measurements should be considered as an alternative to the mean dry weights presently required by EPA Method 1000.0.

CHAPTER II

INTRODUCTION

Fathead minnows (Pimephales promelas) have been used for many years to evaluate the effects of aqueous contaminants both in short-term acute tests and long-term chronic tests. Recently two methods have been adopted by the U. S. Environmental Protection Agency (USEPA) [1] to use fathead minnows in "short-term chronic" tests which measure contaminant effects upon the especially sensitive life stages of the embryo and the newly hatched larva and estimate chronic toxicity.

Birge et al. [2] developed an eight-day, embryo-larval survival and teratogenicity test for fish and amphibians with mortality and teratogenicity of the larvae as the effects measured. In 1985 Norberg and Mount [3] published a method designed to be a simple, rapid, and cost-effective assessment of subchronic effects of effluents and chemicals to the fathead minnow. Survival and growth are the variables measured. These are two of the four methods incorporated in the USEPA manual [1] and they have been used extensively under the National Pollutant Discharge Elimination System (NPDES) permit program of biological monitoring of wastewaters.

Growth has often been found to be a more sensitive measure of deleterious effects than survival [4, 5, 6] and it has typically been measured using dry weight as an indicator of growth. However, the Frog Embryo Teratogenesis Assay: Xenopus (FETAX) which was standardized by Dumont et al. [7] and has been used by others [8-9] to detect teratogenic or growth inhibiting substances in complex aqueous mixtures, uses length of the frog larva as the indicator of growth. Dawson and coworkers [10] evaluated the effects of metal-contaminated sediment extracts on the development, growth and survival of Xenopus and fathead minnow (Pimephales promelas) embryos and used head-tail length of the organisms as the index of growth. Meteyer et al. [11] studied the effects of cadmium on the development of sheepshead minnow (Cyprinodon variegatus) embryos in a 7 - 8 day test. The effects upon growth were analyzed by measuring the total larval length as well as dry weight. They found that all cadmium treated larvae were shorter than controls and that dry weight was less sensitive than total length as an indicator of growth. Mean dry weight was also not dose-related with mid-range concentrations not significantly different than the controls.

Previous work with the eight-day fathead minnow embryo-larval survival and teratogenicity test [1] in this lab [10 & unpublished work] used total length of larvae to evaluate effects upon growth and found significant differences in larval development based on length. Because the

embryo-larval test does not include growth inhibition as an effect to be measured, the present study focused on larval length compared to larval weight as the measure of growth in the seven-day fathead minnow larval survival and growth test, USEPA Method 1000.0 [1].

Growth in the USEPA Method 1000.0 is defined as an increase in weight and an analytical balance capable of accurately weighing larvae to the nearest 0.1 mg is required. Because the weight of an individual fish may be as little as 0.1 mg, the method calls for preserving, drying and weighing the larvae from each test chamber as a group. The method also allows use of as few as two test chambers per concentration with 10 larvae/chamber. If the fish are weighed as a group, as few as two mean dry weight values may be used to estimate the growth of the fish at each concentration. The statistical tests are few that can handle this situation and in some cases the powers of the tests are severely limited by only duplicate values. Many times the fish exposed to a test solution do not grow uniformly and neither the range of fish weights nor the standard deviation of the fish weights is estimated.

One approach to this problem would be to require individual fish weights. This would require an expensive microbalance capable of accurately reading the weight of the smallest larva. These balances are commercially available but they do not eliminate the tedium necessary to obtain accurate dry weights. In fact these balances require con-

siderably more time and care than an analytical balance to obtain a single reading.

Another approach might be to allow a different measure of the growth of the larvae. In this paper, larval length measurement is considered as an alternative comparable indicator of growth in the seven-day fathead minnow larval survival and growth test. Effluents from municipalities, refineries and chemical industries were used in the tests to determine if length was linearly correlated with weight and to assess the sensitivity of the treatment comparisons. Controlled growth experiments were also used to show that length measurements are accurate, precise and offer the advantages of being less time-consuming, less costly, and less susceptible to data entry error.

CHAPTER III

MATERIALS AND METHODS

Fathead minnow (Pimephales promelas) larval survival and growth tests were performed according to USEPA method 1000.0 [1]. At the end of the seven-day exposure period individual larval length measurements were taken and the fish were subsequently dried and weighed in groups. Both sets of data were analyzed for significant differences in growth. Linear regression analyses were performed on 142 length/weight sets of points. Sigma-Plot [12] software package was used to calculate a regression (r) value and to plot the data. The data were analyzed by individual effluent as well as combined into a single group. Seven effluents representing two chemical industries, three municipalities, and two refineries were included in the comparisons.

A growth experiment using a control creek water (Stebler Creek, Payne County, Oklahoma) was performed using the same USEPA methodology [1] as above to obtain measurements from hatching to approximately 3 weeks of age. Larval fish sampled day-of-hatch, one, two, four, six, and seven days post hatch, and every few days thereafter, were measured.

Precision of measuring techniques was explored by repeating ten individual fish length measurements six times and by taking six tare weights and six gross weights for 10 pans of fish. Standard deviations were calculated and compared. Accuracy of the length measurements was obtained by measuring a one-cm mark on a clear plastic ruler 10 times using the digitizer. The one-cm mark used to calibrate the system was also measured 10 times with the digitizer. Bantle [personal communication] had previously concluded that no distortion occurred across the measuring surface.

Length Measurements

At the end of the test, surviving fish larvae were fixed with four-percent formalin and placed in a 60 mm diameter clear plastic petri dish. The dish was then placed on a photographic enlarger and the fish images magnified about five times their actual size. The image was projected onto white paper, the head-tail length of each fish was measured using a Radio Shack digitizer, and the data entered and stored in a Radio Shack model 16 microcomputer. After all the fish were measured and the data saved in files, treatment means were compared for significant differences ($P = 0.05$) using ANOVA and Dunnett's tests. The statistical tests were part of a software package [13] adapted by Bantle for the analysis of FETAX (Frog Embryo Teratogenicity Assay with Xenopus) experiments [8, 9, 10].

No Observed Effects Levels (NOEL) were calculated for each effluent tested.

Dry Weight Measurements

Aluminum weighing pans were placed in a 100° C drying oven for 24 h before being weighed. Tare weights were taken 2 to 3 times, except during the precision weighing experiment when the pans were each weighed six times. After the length measurements were obtained, the preserved larvae were rinsed in distilled water to remove any formalin residue. The larvae were grouped in the pans according to treatment replicate and placed in the drying oven. After 24 h the pans were removed from the oven and placed in a desiccator to prevent moisture absorption, and gross weights were taken. This procedure was repeated 2 to 3 times except for the precision experiment when pans were weighed six times. Net weights per pan were calculated and converted to mean dry weight per fish expressed in milligrams. The mean dry weights were entered using a TOXSTAT [14] software package and analyzed using ANOVA and Dunnett's test for significant differences in treatment means ($P = 0.05$). NOELs were calculated for each effluent tested.

CHAPTER IV

RESULTS

For each of the seven effluents tested, the NOEL was determined to be the same percent effluent by each method of growth determination (Table I). Mean dry weight and mean individual total lengths were highly and positively correlated for each effluent. Fish exposed to the two industrial effluents both had correlation values of 0.93 between length and weight. The length-weight correlation of fish exposed to the three municipalities had r values of 0.82, 0.92, and 0.96 and the two refineries both had r values of 0.98. Data for one refinery effluent (Fig. 1) is representative of tested effluents, which were similar except that the slope of the line varied. When all sets of data were pooled the r value dropped to 0.79 as a result of different slope functions for each effluent.

Fish grown for seven days under control conditions using creek water exhibited the highest r value (0.99). Even when fish were allowed to grow much older (2 - 3 weeks old) than in a seven-day test, the correlation was still high (0.97) for the larger fish. The plot of the lengths versus weights of the entire range of fish sizes in the control group resulted in a third-order curve (Fig. 2).

However all fish in the seven effluent tests had length measurements of less than 1.0 cm which fall within the flat part of the curve.

When growth as length and as weight were plotted against the percent effluent concentration the curves for the two were parallel (Fig. 3). Similarly when growth was plotted against age in the controlled experiment, the length and weight lines also paralleled each other.

Standard deviations of the six repeated individual fish length measurements averaged 0.005 for fish that were from 0.513 to 0.541 cm in total length. The standard deviation for weights of 10 aluminum weighing pans with dried fish in them averaged 0.127. The pans with fish weighed approximately 1400 mg but when the tare weights were subtracted and the net weights divided by the number of fish per pan, the mean weight per fish was sometimes as low as 0.097 mg.

Length measurements of a 1-cm division on a clear plastic ruler gave a mean of 0.997 cm with a standard deviation of 0.003. Length measurements of the 1-cm mark used to calibrate the system gave a mean of 1.003 cm with a standard deviation of 0.002.

CHAPTER V

DISCUSSION

Fathead minnow larval length was correlated with larval weight in a positive linear fashion (r values > 0.79 in all cases) at least within the size range of fish normally encountered in the seven-day larval survival and growth test. R values of 0.82 - 0.99 indicate that growth is reflected as an increase in both length and weight. For tests of individual effluents the slope of the regression line varied but the r value always remained high. Because each of the tests was run as a complete unit with its own controls, the slope need not be the same in all cases.

For the seven effluents tested, the NOELs were calculated to be the same percent effluent whether using weight measurements or length measurements (Table I). Analysis of mean lengths detected the same level of significant differences as mean weights. However, the use of individual lengths offered greater statistical sensitivity than either mean weight or mean length due to the substantial increase in degrees of freedom.

Meteyer et al. [11] used a Cahn model 4400 Electrobalance for dry weight measurements and a dissecting microscope equipped with an ocular micrometer for length mea-

surements. Even with a very expensive balance and a relatively inexpensive setup for length measurements, they found that total length was more sensitive to cadmium effects on growth than was weight.

The apparatus that was used in this study is no longer available. However, there are now numerous digitizer pads on the market and the prices have dropped. They can be purchased for about $500 and software packages are also available for about $500. A photographic enlarger can also be purchased for the same amount. With this setup, analysis time can be reduced considerably from the time required to take dry weights. In fact, all larvae from a standard effluent biomonitoring test with 5 - 6 dilutions, 4 replicates per treatment and 10 fish per replicate can be measured individually in two hours and the full statistical analysis of the length measurements completed. Two hours is the minimum drying time allowed for the dry weight measurements. Drying, weighing, redrying and reweighing as well as data entry for the statistical analysis may require as long as three days. If larvae are to be weighed individually, an electrobalance (approximately $3000 - $5000) is necessary.

Larval length measurements were found to be accurate and precise. Larval lengths and weights were linearly correlated and detected the same level of significant differences between control and treatment means. In comparison to weight measurements, substantial time can be saved

using larval lengths even with a very simple setup and cost of equipment can be reduced considerably as well. Results obtained demonstrate the usefulness of this method for rapid and cost-effective evaluation of effects of contaminants on growth of fathead minnow larvae in the seven-day survival and growth test.

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Table I. Comparison of the NOEL for growth of fathead minnows exposed to seven effluents using length measurements and weight measurements as the indicators of growth.

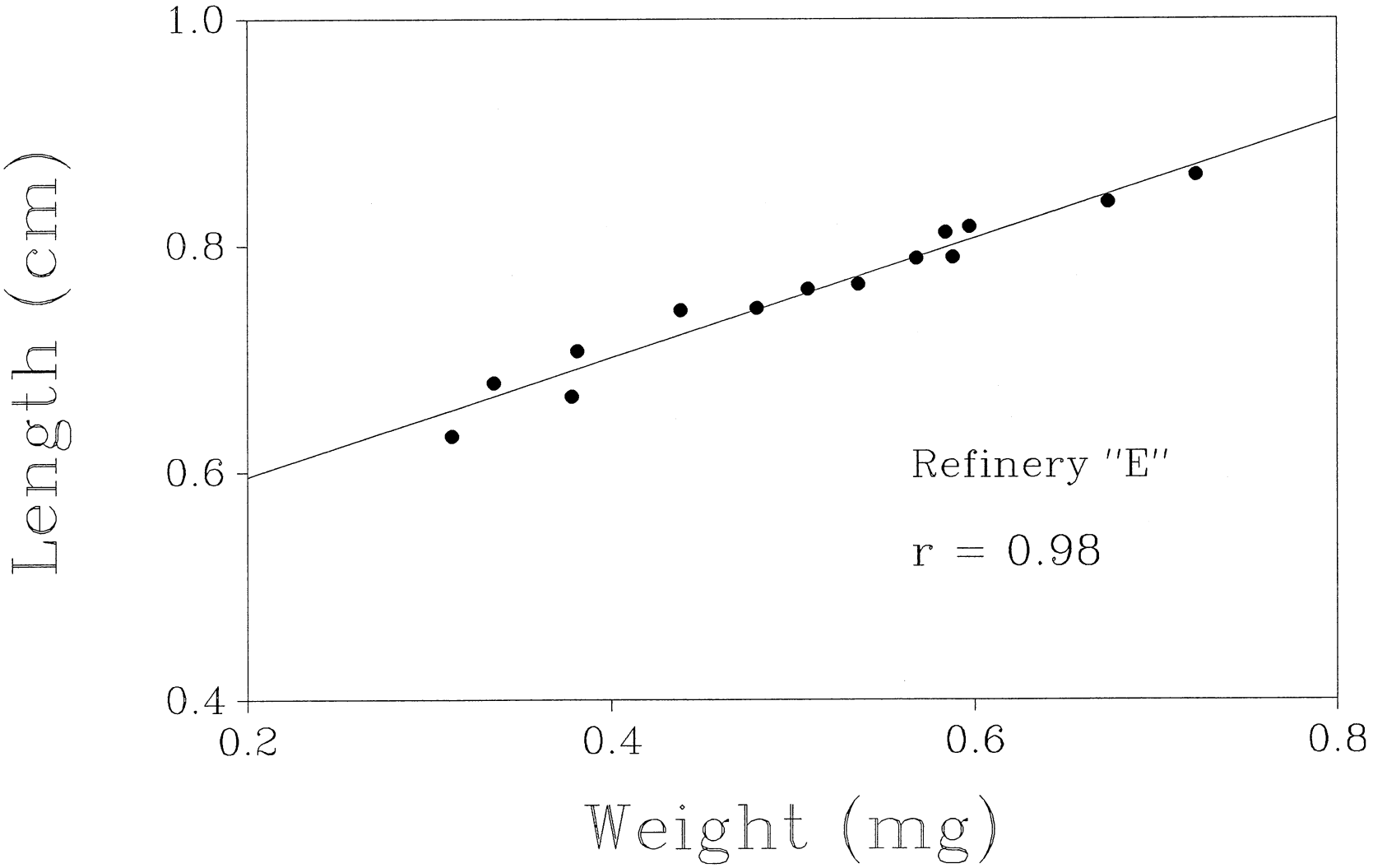
Effluent	Length NOEL (% effluent concentration v/v)	Weight NOEL (% effluent concentration v/v)
Industry F	100	100
Industry U	100	100
Municipal T	100	100
Municipal D	50	50
Municipal M	10	10
Refinery T	1	1
Refinery E	1	1

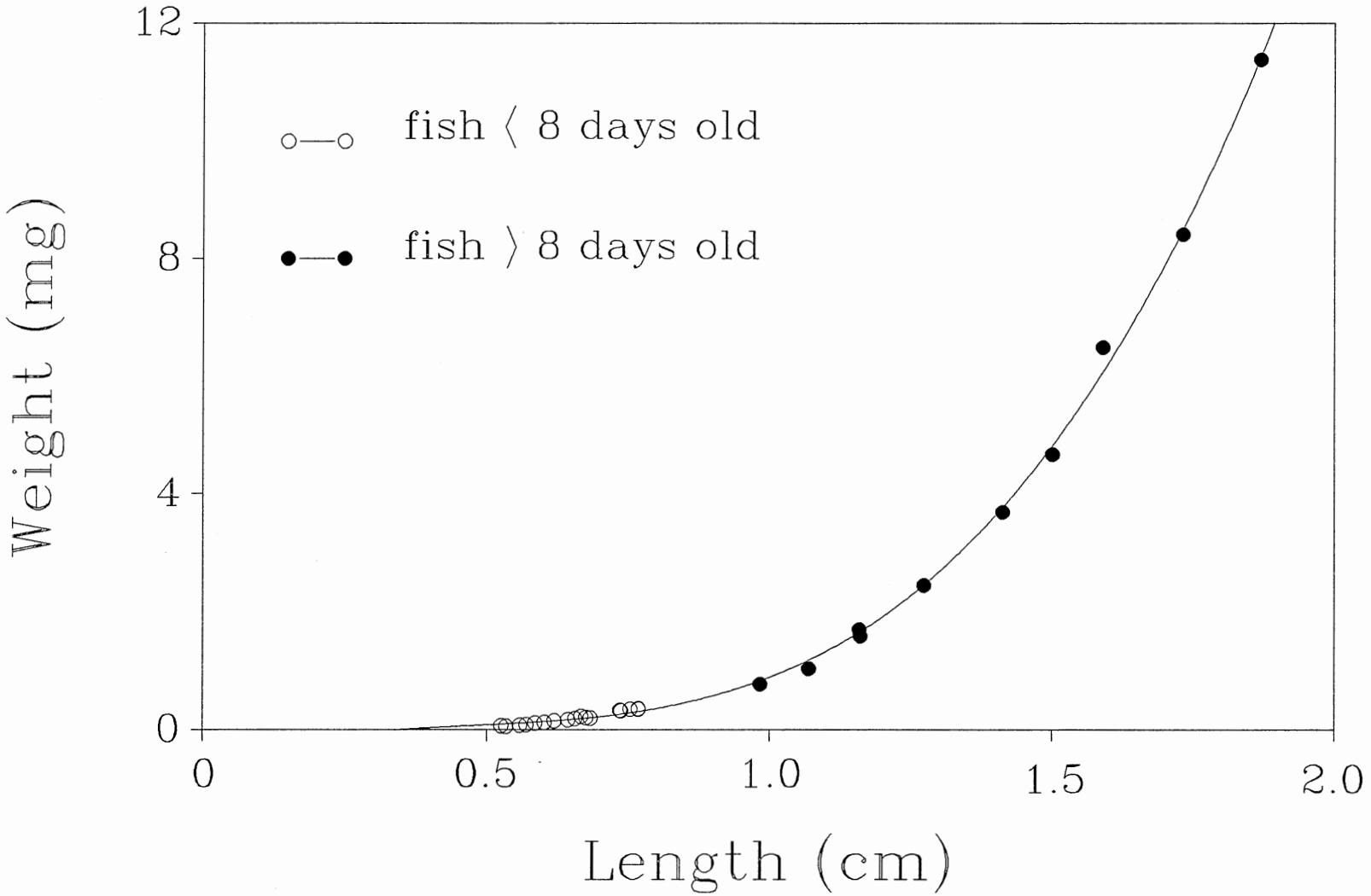
FIGURE LEGENDS

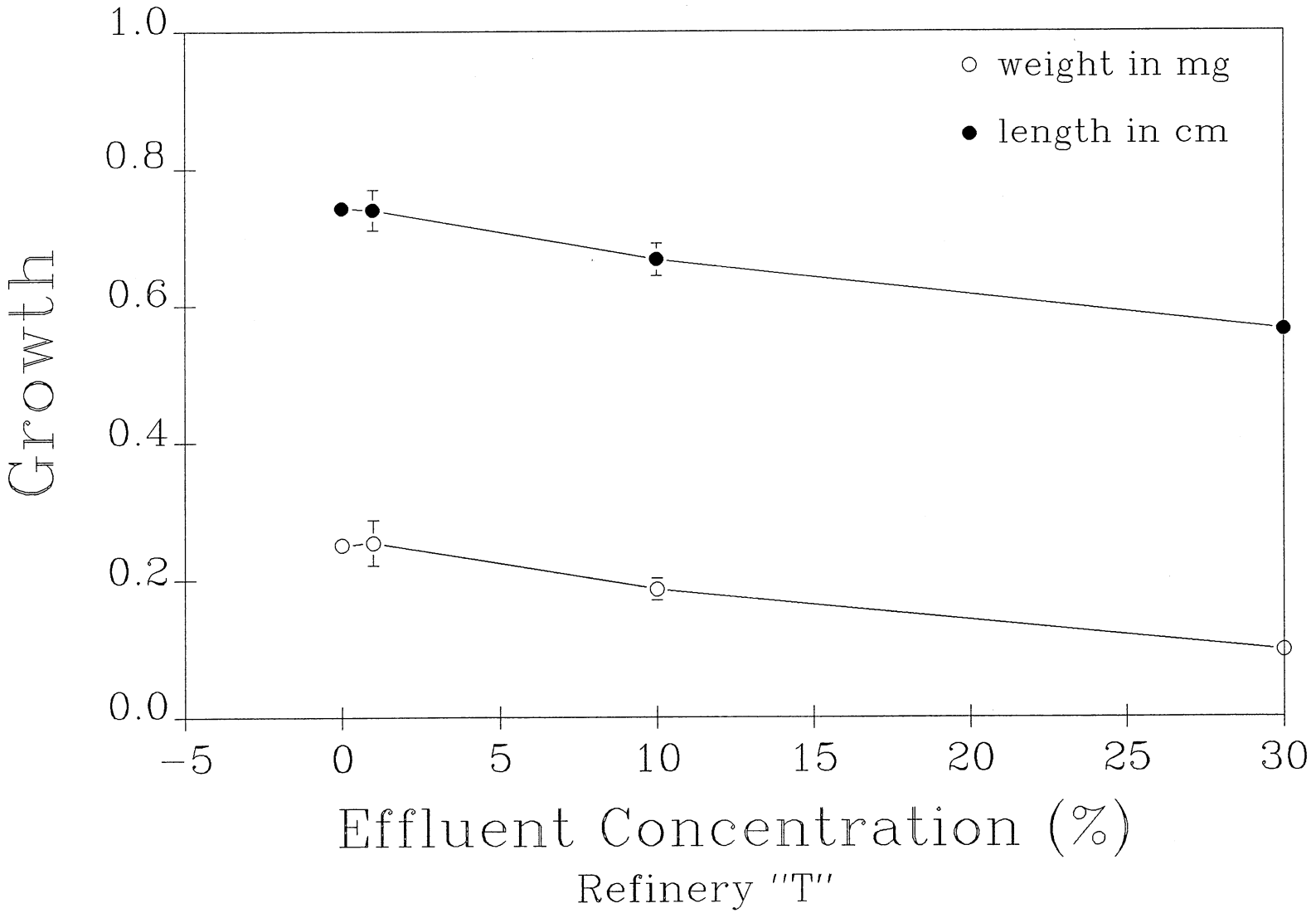
Figure 1. Length versus weight correlation for growth of fathead minnow larvae exposed to Refinery E effluent in the seven-day larval survival and growth test.

Figure 2. Length and weight of fathead minnow larval fish grown in control creek water. Fish range from 24-h to three weeks old.

Figure 3. Percent effluent concentration versus growth response as length and as weight for fathead minnow larvae exposed to Refinery T effluent for seven days in the survival and growth test.


1.0
0.8
0.6
0.4
Length (cm)
0.2
0.4
0.6
0.8
Weight (mg)
Refinery "E"
r = 0.98


12
8
4
0
Weight (mg)
fish ⟨ 8 days old
fish ⟩ 8 days old
0
0.5
1.0
1.5
2.0
Length (cm)


1.0
0.8
0.6
0.4
0.2
0.0
Growth
weight in mg
length in cm
−5
0
5
10
15
20
25
30
Effluent Concentration (%)
Refinery "T"

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