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GUIDELINES FOR PERFORMING STATIC ACUTE TOXICITY FISH BIOASSAYS IN MUNICIPAL AND INDUSTRIAL WASTE WATERS

By

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FOREWORD

This document was prepared at the Fish and Wildlife Water Pollution Control Laboratory of the California Department of Fish and Game under State Water Resources Control Board Interagency Agreement No. 3-4-4. The manual is designed primarily for the use of personnel engaged in compliance monitoring, relative to the toxicity of waste effluents discharged to California waters.

The results of bioassays with aquatic organisms are subject to a great degree of variability depending on the test species, water characteristics, and of the testing system itself (Brungs, 1973). All these must be understood and accounted for before bioassays are conducted to ensure that the most accurate and useful data are obtained. In the past, broad interpretations have been made from instructions included in the bioassay section of Standard Methods (APHA, 1971), often to the extent that reliability of the data has been questionable. It is the intent of these guidelines to eliminate the broad interpretations and provide more specific procedures based on current information.

The manual will also serve as the basis for evaluating performance of bioassay laboratories requiring certification as set forth by the California Administrative Code, Subchapter 9, Chapter 3, Sections 2235.13, which states that chemical, bacteriological, and bioassay analyses shall be required to be conducted at a laboratory certified for such analyses by the State Department of Health.

INTRODUCTION

The primary purpose of this manual is to provide guidelines for laboratory personnel performing routine static fish bioassays as they relate to the State National Pollutant Discharge Elimination System (NPDES). This program requires waste dischargers to "self-monitor" and report on the quality of their effluent discharged to surface waters. The manual emphasizes routine waste monitoring procedures; therefore, the methods presented here may not always apply to non-routine bioassay tests such as those used by regulatory agencies to delineate suspected sources of a fish kill.

There are two basic types of assays used to measure the effects of toxicity to fish, acute and chronic. Acute toxicity bioassays measure the lethal action in which death occurs within a short period of time, usually within four days. Chronic assays measure sublethal effects such as abnormal growth or reproduction, which occur over a period of several months.

Two methods of assays are in general use: the continuous flow-through assay in which the test solution is renewed continuously and the acute static bioassay in which test organisms are held in containers of standing test water that may, or may not, be changed during the test period.

The measure of acute toxicity most often used with fish bioassays is either the 96-hour median tolerance limit (TL50 or TLm) or the 96-hour median lethal concentration (LC50). The term "tolerance limit" applies to a level of any measurable lethal agent, including temperature and pH, while the expression "lethal concentration" denotes the concentration of a specific toxicant. To identify a specific toxicant in a waste effluent is often quite difficult if not impossible. Under these circumstances, depite the differences between

"tolerance limit" and "lethal concentration," the terms as used in this manual will be considered synonomous.

In discussing toxicity as a parameter in waste water management, the acute toxicity bioassay test must be placed in its proper perspective. First, the test is limited in its ability to determine long-term safe concentrations because a level that kills 50 percent of the subjects cannot be considered safe. Moreover, the test ignores all of the possible adverse effects other then death. It is important to remember that even where mortalities do not occur in the acute toxicity bioassay, there is no assurance that chronic toxicity is absent. The primary functions of the acute toxicity test as outlined in this manual are to monitor compliance with discharge requirements and to establish an index to the toxicity of a discharged effluent.

To assure a successful and meaningful test, it is essential that the discharger follow closely the methods presented here for static toxicity tests. As new techniques and improvements are made in bioassay procedures, the manual will be updated to reflect these advancements. For the benefit of laboratory personnel desiring additional information concerning bioassay tests, a list of supplemental readings is included.

I. DETAILED TEST PROCEDURES

A. Laboratory Facilities

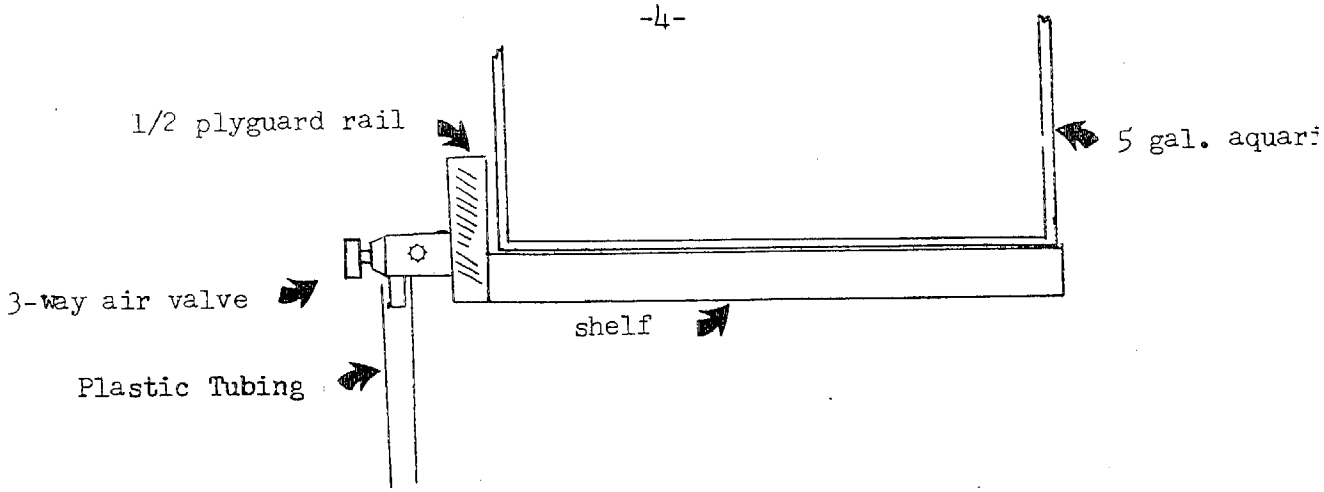
1. To avoid stressing test organisms, tests must be conducted in areas of minimal disturbance from laboratory equipment and personnel. The area should be well ventilated and free of fumes, both to prevent contamination of test solutions and to protect personnel from volatile chemicals and waterborne pathogens that may be dispersed from open bioassay chambers.

2. The specified temperature ranges for bioassays must be maintained for the duration of the test. If a satisfactory degree of temperature control is not possible with existing heating and cooling systems, a constant-temperature room or recirculating water bath must be provided.

3. A photoperiod of 16-hours light and 8-hours dark is desirable in areas where fish are being held and tested. If this cannot be provided, then fish should be held under subdued lighting conditions or an on-off dimmer switch used on existing lighting to avoid sudden changes in light intensity.

4. Suitable bench space should be provided to maintain test aquaria and acclimating tanks. This includes space for six-19 liter (5 gallon) test containers and four-4 liter (1 gallon) test containers per test. Two or more 75-150 liter (20-40 gallon) tanks should be available for acclimating and holding tanks. Shelves can be used for supporting test containers to conserve space (Figure 1).

5. Other items, such as chemicals and glassware required for preparing test concentrations and conducting analytical tests, should be available



AIR REGULATION TO TANK - DETAIL

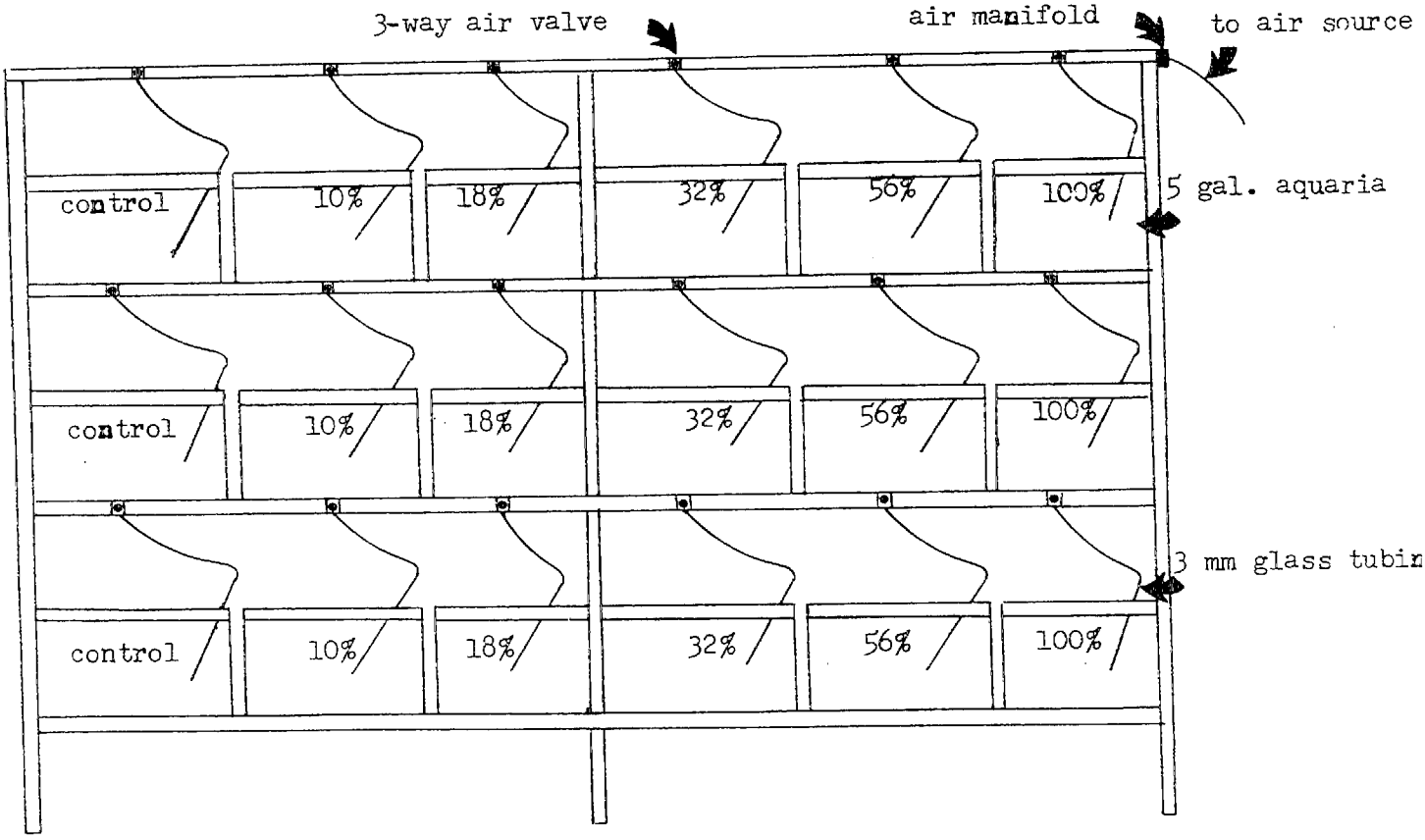


FIGURE 1. Suggested shelving arrangement for static bioassays, including test aquaria, and air regulating systems.

in the laboratory. Table 1 lists the minimal equipment required to conduct bioassay tests.

6. Special waste disposal facilities, such as evaporation ponds or a Class I waste disposal site [1/] must be used for disposal if the material being tested is highly toxic. Municipal sewage systems are unacceptable for disposal of this type of material.

B. Test Containers and Equipment

1. All tanks and materials that may contact any water into which fish are to be placed must not contain toxic substances that can be leached or dissolved by water. Holding and acclimating tanks can be made of any material that is nontoxic to the test fish. Test containers and materials subjected to contact with the wastes being tested should be carefully chosen to minimize leaching and sorption. Standard 19-liter (5 gallon) glass aquaria or widemouthed pickle jars are recommended. Several 4 liter (1 gallon) jars should be available for preliminary tests. Plexiglass® (acrylic plastics), polypropylene, and Teflon® can be used for most wastes; however, sorption of some materials, especially on plastics, may make cleaning of these containers difficult. Known toxic materials such as zinc, copper, lead, rubber, etc. should not come in contact with dilution water or toxicant solutions. See Section I. E.1. for precautions to be taken when tap water is used for dilution purposes.

1/ Locations of Class I waste disposal sites are designated by the California Regional Water Quality Control Boards.

TABLE 1. Description Of The Minimum Test Equipment Required To Conduct Bioassays.

Item	Description	Quantities Needed (one complete test)
A	Temperature control (constant temperature room or waterbath)	1
B	75-150 liter (20-40 gallon) aquaria	2
C	Aquaria filter	2
D	19 liter (5 gallon) aquaria or pickle jars	6
E	4 liter (1 gallon) wide mouth jars	4
F	Fish dip net (small mesh)	1
G	Aerator pump or compressor (oil free)	1
H	Oxygen cylinder with double-stage regulator [2/]	1
I	3 mm I.D. glass tubing	6 ft.
J	4 mm I.D. (1/8") flexible air line Tygon or plastic tubing	25 ft.
K	Adjustable air valves or 6-place manifold for air lines	6
L	Activated carbon column or deionizer [3/]	1
M	1000 ml graduated cylinder	1
N	100 ml graduated cylinder	1
O	10 ml pipet (0.1 ml divisions)	1
P	Meter stick	1
Q	Balance (sensitivity 0.1 gm)	1
R	Thermometer (1°C divisions)	1

2/ Oxygen cylinders required where dissolved oxygen levels cannot be maintained with controlled aeration.

3/ Tap water used for dilution purposes or for holding fish should be passed through a carbon column. If waters are suspected to be high in metals, an ion exchange unit should be used and the water reconstituted with the appropriate salts.

TABLE 1 (CONTINUED)

Item	Description	Quantities Needed (one complete test)
S	Recording Thermograph (5 day, span 0-50°C or less)	1
T	pH meter	1
U	Probability or semi-logarithmic graph paper	1

Laboratories must have the capabilities of performing the following analyses:

Total Alkalinity

Total Hardness

Dissolved Oxygen

Residual Chlorine

Conductivity (saltwater tests)

Salinity or chlorinity (saltwater tests)

pH

C. Cleaning Test Containers

1. Test containers must be thoroughly cleaned before use to remove possible toxic residue. Soap and hot water cleaning followed by thorough rinsing (3-5 times) with hot water is usually adequate. In some cases, depending on the toxicant, an acid or solvent rinsing may be necessary. Acid removes metals and bases; an organic solvent removes organic compounds. This procedure must be followed by 3 to 5 rinses with fresh water. Precautions should be taken not to dissolve the adhesive holding the aquaria together when using acid and solvent rinses.

D. Effluent Samples

1. With the exception of regulating temperature and oxygen, tests will be conducted on the effluent as discharged to the receiving waters.
2. Samples must be collected in thoroughly cleaned containers. Containers should be completely filled with the effluent before capping. Sample degradation by biological action can be minimized by storing samples at 4°C. Tests should begin as soon as possible after collecting the sample. Where samples are known to contain volatiles that may be toxic, or where samples may undergo rapid changes, bioassay tests must be conducted within four hours of sample collection. All other samples must be tested within 24 hours after the samples are collected.
3. A grab sample should be taken, as opposed to a composite sample, if the waste is known to contain toxic volatiles or may undergo rapid chemical and/or biological degradation. If the time of greatest toxicity can be determined for a discharge, a grab sample should be taken at that time. This may be determined by evaluation of chemical data or by testing fish periodically to establish the range of toxicity.

E. Dilution Water

1. Fresh or saltwater waste discharged to a freshwater environment: The suitability of the dilution water to maintain fish must be determined before tests are run. A practical criterion for acceptable water is that fish will survive in it for the duration of acclimation without showing signs of stress or unusual behavior. Where possible the receiving waters should be used for dilution purposes. The water must be of good quality and collected within 48 hours of starting the test. If an acceptable dilution water cannot be obtained from the receiving water, then water of similar quality must be obtained or prepared from some other source. Such water can be prepared by adding appropriate chemicals to available ground or surface water of good quality or the preparation of a reconstituted water (Table 2). The hardness, alkalinity, and pH of the dilution water must be similar to that of the receiving water.[4/] Dechlorinated domestic water should be used only as a last choice and then only if the residual chlorine levels are less than 30 ug/l and copper and zinc less than 10 ug/l.

Reconstituted water is prepared by adding known amounts of specified reagent grade chemicals to glass distilled or deionized water (Table 2). Deionized water may contain phenols and/or formaldehydes in concentrations toxic to fish. This is especially true of infrequently used or recently recharged tanks. When testing the suitability of deionized or distilled water to support fish life, the appropriate salts must be added for the fish to properly osmoregulate.

2. Freshwater wastes discharged to a marine environment: The acute toxicity of a freshwater discharge to a marine or estuarine system should be

4/ The Environmental Protection Agency (EPA) proposes that the hardness and alkalinity of the dilution water be within 25 percent and pH within 0.2 unit of those of the receiving water.

TABLE 2. Quantities Of Reagent-Grade Chemicals Required To Prepare Reconstituted Freshwaters And The Resulting Water Qualities. 5/

Type 6/	Salts Required (mg/l)				pH	Total Hardness mg/l $CaCO_3$	Total Alkalinity mg/l $CaCO_3$
	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCl			
Very soft	12	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Soft	48	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Hard	192	120.0	120.0	8.0	7.6-8.0	160-180	110-120
Very hard	384	240.0	240.0	16.0	8.0-8.4	280-320	225-245

5/ Adapted from Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians (1975).

6/ Reconstituted water of similar pH, hardness and alkalinity as that of the receiving waters should be used. For a freshwater discharge to a marine or estuarine environment, use formulation for very hard water. For hard water preparations each salt should be dissolved separately before combining.

tested using a freshwater species and reconstituted freshwater diluent (use formulation for very hard water (Table 2). This dilution water should be adequate when the test is for compliance monitoring purposes and to establish an index to the toxicity of the waste. The use of freshwater diluent avoids physiological problems associated with testing fish in varying salinities as would be experienced if seawater were used as the diluent. The use of seawater species and saltwater diluent may be determined to be desirable upon development of new techniques and selection of standard marine test species.

3. Saltwater wastes discharged to a marine environment: When possible a good quality marine water should be used for making dilutions. An alternative to this may be either the reconstituted seawater (Table 3) as prescribed by the Environmental Protection Agency (1975), or commercially available aquarium sea salts, from which tetrasodium ethylenediaminetetraacetate (EDTA) has been omitted. Reconstituted seawater of 34 $\pm$ 2 g/l salinity should be used in tests for marine species and 22 $\pm$ 2 g/l salinity for estuarine species. The initial salinity of the reconstituted seawater given in Table 3 is 34 $\pm$ 0.5 g/l. The desired test salinity is attained at time of use by dilution with glass distilled or deionized water (see precautions for using deionized water in Section I. E.1.).

F. Test Organisms (see also Section III, Care, Handling and Diseases of Fishes, Pages 38-45).

1. Species and source: Availability is an important aspect in the selection of test species. In most instances, obtaining test organisms directly from wild populations is not desirable. With the exception of threespine stickleback, Gasterosteus aculeatus, and California killifish, Fundulus parvipinnis, fishes listed in Table 4 can be purchased from

TABLE 3. Recommended Procedure For Preparing Reconstituted Seawater.[7/]

Add to 890 ml deionized water the following reagent-grade chemicals in the amounts and order listed. Each chemical must be dissolved before another is added.

Chemical	Amount
NaF	3 mg
$SrCl_2 \cdot 6H_2O$	20 mg
H_3BO_3	30 mg
KBr	100 mg
KCl	700 mg
$CaCl_2 \cdot 2H_2O$	1.47 g
Na_2SO_4	4.00 g
$MgCl_2 \cdot 6H_2O$	10.78 g
NaCl	23.50 g
$Na_2SiO_3 \cdot 9H_2O$	20 mg
$NaHCO_3$	200 mg

If the resulting solution is diluted to 1 liter, the salinity should be 34 ± 0.5 g/l (ppt). The desired test salinity is attained at time of use by dilution with deionized water. Reconstituted seawater of 22 ± 2 g/l salinity should be used for tests with estuarine fish.

7/ Adapted from Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians (1975).

licensed fish breeders throughout the State.[8/] This list is revised annually and available from the offices of the California Department of Fish and Game (see Appendix A for addresses). Sticklebacks may be captured from wild stocks [9/] or purchased from several sources in the San Francisco Bay Area, while killifish are common to bays of Southern California. Care must be taken not to use spawning fish or fish which have recently spawned. Table 4 is a tentative list of test species and is subject to change as more information becomes available on other test organisms.

2. Holding fish: To avoid unnecessary stress to the test organisms, changes in temperature and water quality should be made gradually. Before transferring fish from transporting tanks to holding tanks, there should be no more than a 2°C temperature difference between tanks. Dissolved oxygen should be at or near saturation and the hardness and alkalinity similar to the water from which the fish are being removed. During prolonged holding periods, it is usually better to maintain fish in waters of similar hardness as those in which they are reared, and at temperatures 2 to 3 degrees lower than the suggested test temperatures. The source of fish, date of collection, and environmental conditions such as type of

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8/ A person desiring to propagate and raise fish in natural or artificial ponds shall file with the Department of Fish and Game each year a written application for a domesticated fish breeder's license (California Fish and Game Code, Chapter 5, Article 3, Sections 6450-6456).

9/ Persons desiring to capture fish for use in scientific research and not for resale must apply to the California Department of Fish and Game for a scientific collector's permit (California Fish and Game Code, Chapter 3, Article 1.5, Sections 1000-1003). A commercial fishing license and collector's permit is required by the Department of Fish and Game for those persons procuring fish for the purpose of reselling them (California Fish and Game Code, Chapter 1, Article 3, Sections 7850-7855). The unarmored threespine stickleback, Gasterosteus aculeatus williamsoni, found in parts of Southern California and the Mojave River, are an endangered species and may not be captured.

TABLE 4. Tentative Listing Of Test Species And Temperatures.

Freshwater	Test Temperature °C 10/
Rainbow Trout, Salmo gairdnerii	14-17
Golden Shiner, Notemigonus crysoleucas	20-23
Marine	
Threespine-stickleback, Gasterosteus aculeatus	14-17 18-21
California killifish, Fundulus parvipinnis	14-17 18-21

10/ Tests must be conducted within the specified temperature range.

water (fresh, salt, bay, stream, etc.), pH, and conductivity should be recorded. In addition, different stocks of fish should be kept separate in holding and acclimation tanks, i.e., do not mix batches of fish collected from different areas or time intervals, even of the same species.

3. Acclimating fish: Ideally, fish used in bioassays should be conditioned gradually over a seven-day period to test temperatures, dilution water and laboratory conditions similar to those under which tests are to be performed. Where laboratories are conducting tests on a variety of samples, it may be impractical to acclimate fish to a number of dilution waters. Under these circumstances, stocks of fish can be divided and acclimated to different hardnesses, i.e., soft, hard, or very hard waters as listed in Table 2. Fish to be used in tests are selected from the acclimation tank having similar water quality characteristics as the required dilution water. In this situation two controls, as discussed in Section I. K.2., will be required: one being the water in which fish were acclimated and the other the dilution water being used.

Overcrowding of fish must be avoided in holding and acclimation tanks. A 76-liter (20 gallon) tank with filter will maintain 60 to 80 test fish having an average weight of 1 to 2 grams (g) each. During the acclimation period, fish should show no symptoms of disease, abnormalities in appearance or behavior. If more than 10% mortality occurs in the fish stock during initial acclimation, disease should be suspected and steps taken to chemically treat the fish. If the disease is severe, it is often best to destroy the entire group of fish and start anew. After treatment, fish must not be used in tests for at least 10 days, and then only if treatment has been successful. If more than 10% of the fish die in the four-day period immediately preceding the bioassay, this batch of fish should not be used in tests.

4. Feeding: Fish should be fed regularly up to 48 hours before starting tests but not after this period or during tests.

5. Size and number: In any single test, all fish must be from the same batch and year class. Small fish weighing between 0.5 and 5 g and not more than 7 centimeters (2.8 inches) standard length (tip of snout to base of tail, Figure 2), are generally the most convenient size for bioassay testing. The length of the largest individual fish should not be more than 1.5 times the length of the smallest. To avoid overloading of test containers it is desirable to have no more than 1 g of fish per liter of solution, with a maximum not to exceed 2 g per liter. If there is some question as to whether fish are within the specified size range, initial weights should be taken on a random sample of fish from the holding or acclimating tank prior to the start of the bioassay. Fish used for this purpose must not be used in the bioassay test.

The minimum number of test animals for establishing the LC50 is ten per concentration. The reliability of tests may be increased by using more fish per concentration, providing the criterion for the weight-volume ratio is maintained, or by testing replicate concentrations. The mean resulting from combining the replicate values is used in the LC50 determination.

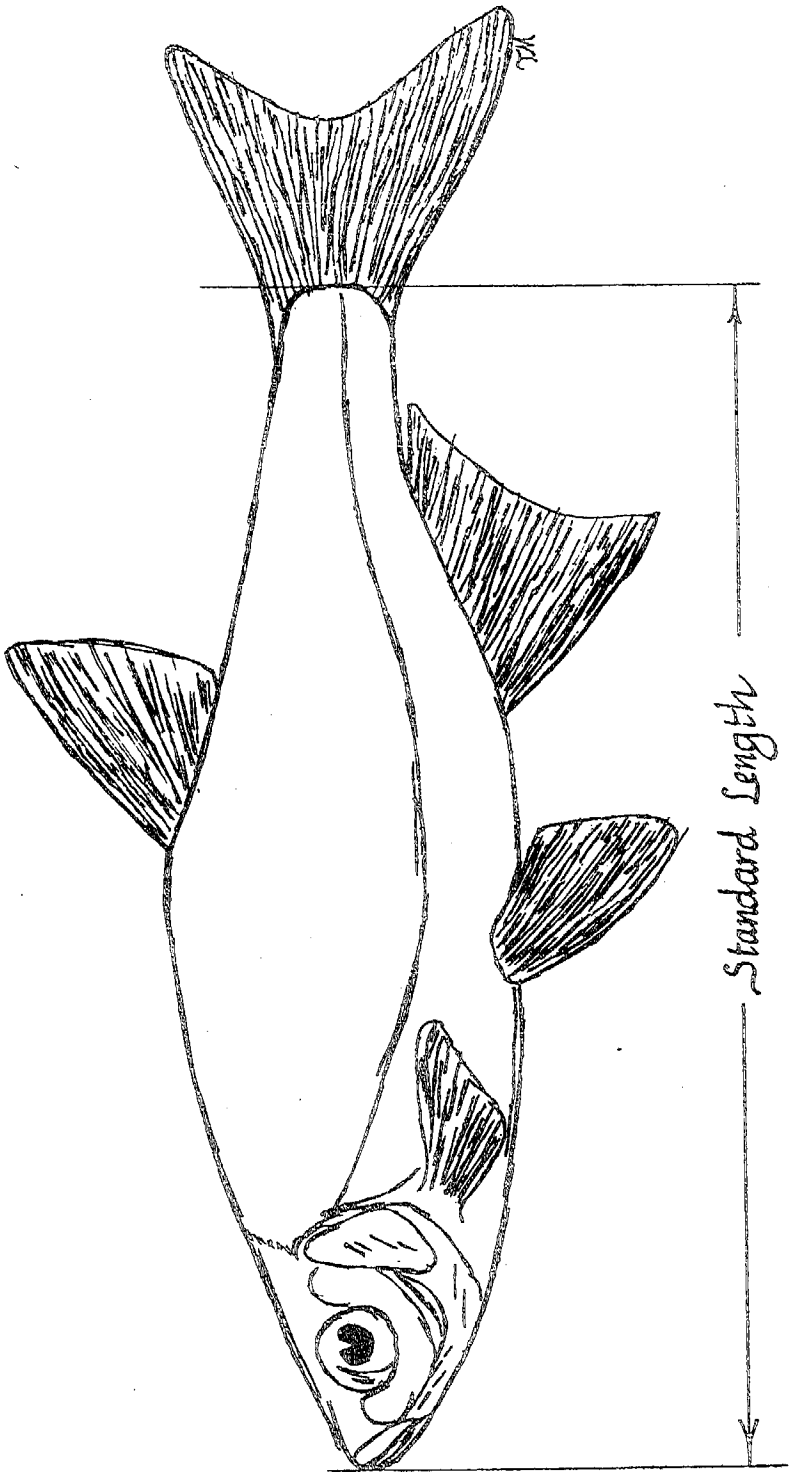

FIGURE 2. Standard length measurement of fish.

G. Test Temperatures

1. Freshwater tests must be performed within a range of 14 to 17°C for coldwater species and 20 to 23°C for warmwater species (Table 4).
2. Saltwater tests must be performed within a range of 14 to 17°C or 18 to 21°C based on receiving water temperatures. Bay and ocean waters of Southern California may warrant the upper temperature range while waters of Northern California the lower range.
3. To assure that tests are being conducted within the prescribed temperature range, at least one concentration, or the control, must be monitored with a continuous recording thermograph. Where tests are conducted in either a temperature controlled room or recirculating water bath air or water temperatures may be monitored. In conjunction with continuous recordings, temperatures will be taken on all concentrations at a minimum of 24-hour intervals.

H. Dissolved Oxygen (D.O.) Concentrations

1. When surface absorption does not maintain D.O. levels above 70% air saturation, oxygen may be supplied by initial oxygenation of the diluent, periodic renewal of test solutions, or by controlled aeration or oxygenation during the test. When oxygen is used, care must be taken not to supersaturate solutions. The solubility of oxygen at varying chloride concentrations is given in Table 5.
2. To avoid undue loss of volatile toxicants, diffuser stones shall not be used in test aquaria. Instead, glass or rigid plastic tubing such as 1 or 2 ml disposable pipettes, can be used to deliver oil-free air or

TABLE 5. Solubility Of Oxygen In Freshwater and Seawater Exposed to Water-Saturated Air. 11/

TEMPERATURE °C	CHLORINITY ppt												APPROXIMATE DIFFERENCE PER 1,000 PARTS CHLORIDE
	0		4		8		12		16		20		
	Percent Saturation												
	100	70	100	70	100	70	100	70	100	70	100	70	
	Dissolved Oxygen-mg/l												
14	10.3	7.2	9.9	6.9	9.5	6.7	9.1	6.4	8.7	6.1	8.3	5.8	0.10
15	10.1	7.1	9.7	6.8	9.3	6.5	8.9	6.2	8.5	6.0	8.1	5.7	0.10
16	9.9	6.9	9.5	6.7	9.1	6.4	8.7	6.1	8.3	5.8	8.0	5.6	0.10
17	9.7	6.8	9.3	6.5	8.9	6.2	8.5	6.0	8.2	5.7	7.8	5.5	0.10
18	9.5	6.6	9.1	6.4	8.7	6.1	8.3	5.8	8.0	5.6	7.7	5.4	0.09
19	9.3	6.5	8.9	6.2	8.5	6.0	8.2	5.7	7.8	5.5	7.5	5.3	0.09
20	9.1	6.4	8.7	6.1	8.4	5.9	8.0	5.6	7.7	5.4	7.4	5.2	0.09
21	8.9	6.2	8.6	6.0	8.2	5.7	7.9	5.5	7.5	5.3	7.2	5.1	0.09
22	8.7	6.1	8.4	5.9	8.0	5.6	7.7	5.4	7.4	5.2	7.1	5.0	0.08
23	8.6	6.0	8.2	5.7	7.9	5.5	7.6	5.3	7.3	5.1	7.0	5.0	0.08
24	8.4	5.9	8.1	5.7	7.7	5.4	7.4	5.2	7.1	5.0	6.8	5.0	0.08

11/ Prepared from data given in Green and Carritt (1967)

oxygen to test chambers. The rate of aeration must not exceed 30 milliliters of air per minute, [12/] which is equivolent to approximately 175 ± 10 bubbles per minute delivered through a 3 mm I.D. tube.

3. Safety precautions must be observed when using compressed oxygen, i.e., prohibition of smoking, open flames, etc. All oxygen cylinders must be properly secured and equipped with a double-stage regulator.

I. Sample Size

1. Prior to collecting samples and setting up tests, consideration must be given to the volume of sample necessary to conduct both the fish bioassay as well as the required chemical determinations. The volume of each test concentration must be large enough to permit the removal of a portion of each sample for chemical analysis during the test period while still remaining consistent with the fish weight to volume ratio discussed in I. F.5.

J. Preliminary Tests

1. Where the toxicity of the waste sample is unknown and materials do not degrade or volatize, time and effort can be saved by conducting preliminary bioassays to determine the range of concentrations that should be used in definitive tests. In preliminary tests, a wide range of concentrations is tested, such as 100, 10, and 1 and 0.1%. Tests are

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12/ Milliliters of air per minute can be determined using an air bubble flow meter. If a meter is not available, an estimate can be made by filling a graduated cylinder with water and inverting in water. Air is then bubbled into the cylinder and regulated so as to displace 30 ml of water in one minute.

run for 24 hours using two to four fish in 2 liters of solution. These tests should be in compliance with the fish weight to test volume ratio stated in Section I. F.5. When the critical concentration range has been adequately defined, definitive tests can be prepared. In cases where preliminary bioassays cannot be conducted due to the nature of the effluent, i.e., due to volatile or rapidly degradable toxicants (Section I. D.2.), and the toxicity of the waste is unknown, then the definitive test will require a greater number of concentrations to be assured of adequately bracketing or determining the LC50. Generally, after running several tests on the same effluent, the approximate LC50 can be established for that waste and the number of concentrations required in future tests reduced to five.

K. Definitive Tests

1. A range of concentrations are used in the definitive which fall between the highest concentration at which all or most fish survive in the preliminary test and the lowest concentration at which all or most fish died.

2. Test concentrations: For the determination of the LC50, at least five concentrations in a geometric series and a control (containing no waste water) must be used. Each concentration in the series must be at least 55 percent of the next higher one. All dilutions for a given test must be prepared from the same sample of waste and dilution water. All undissolved material in the sample must be uniformly dispersed by gentle agitation before being added to test containers. The final volume of test solution to be used can be pre-marked on the outside of each test container and the predetermined volumes of waste water added. The diluent should then be brought up to the final mark. A gentle swirl with a glass rod is usually sufficient for mixing.

The series of concentrations given in Table 6 is widely used in fish bioassays. In this table, column 1 is generally adequate for establishing the LC50 for most effluents. A closer approximation of the LC50 can be obtained by using the intermediate concentrations listed . . . . in columns 2 and 3. Although the table can be used to express concentrations in milligrams per liter (mg/l) or milliliters per liter (ml/l) its use here is primarily to express percent waste by volume. In column 1, one hundred (100) represents one hundred percent waste, fifty-six (56), fifty-six percent waste and so on. The values given may be divided by any power of 10 to determine lower concentrations. For example, in the first column 100 and 10 may be changed to 1 and 0.1, with the values in the other columns changed accordingly.

If in a preliminary test, concentrations of 1%, 10%, 50%, and 100% waste by volume give the following results,

% Concentration	% Mortality
1	0
10	20
50	70
100	100

the geometric series of dilutions recommended for the definitive test would be 5.6%, 10%, 18%, 32%, and 56% concentrations.

The minimum volume for each concentration is 10 liters. The depth of the liquid should be uniform in all containers and should never be less than 12 cm. The purpose of this restriction is to limit the loss of volatiles. The rate of loss varies with the ratio of the exposed surface area to the volume of the liquid (APHA, 1971). At least ten fish must be used per concentration. In some situations, it may be desirable to

'BLE 6. Bioassay Dilution Chart Based On A Logarithmic Scale [13/]

Col. 1	Col. 2	Col. 3
100		
		87
	75	
		65
56		
		49
	42	
		37
32		
		28
	24	
		21
18		
		15.5
	13.5	
		11.5
10		

13/ When expressing concentrations as percent waste by volume, it is convenient to use figures in columns 1, 2, and 3 as percentages.

divide test fish between two or more containers of the same concentration to maintain the acceptable ratio of fish to solution. In other situations, replicate concentrations may be required by the State or Regional Board to increase reliability of results.

3. Controls: With each series of tests, a control test must be performed under the exact same conditions as in the prescribed test. Usually one control, using the diluent as the medium in which the control fish are held, is sufficient. In instances where the test diluent and the acclimating water are different, two controls must be run, i.e., 100% diluent and 100% acclimating water.[14/] In order for the test to be valid, there must be no more than 10% mortality among the control fish during the course of the test.

4. Handling and transferring of fish to test aquaria: Test animals shall be transferred in a random manner, using a small mesh dip net, from the acclimation aquarium to the test containers within one hour after the preparation of the test solutions. Randomization can be accomplished by sequentially adding two fish to each concentration, repeating the procedure until the desired number of fish per concentration has been reached. Any specimen that does not appear healthy or has been dropped or mishandled during transfer must be rejected. Temperatures of prepared test solutions must be within the specified test range and dissolved oxygen not below 70% saturation, (Section I. G. and H.).

14/ If there is greater than a 10% difference in mortality between the diluent water and acclimating water used for controls, further tests must be conducted to determine if the diluent is of suitable quality to support fish life.

5. Duration: A test begins when the organisms are first exposed to the potential toxicant and extends for 96 hours. If the test time is other than the 96-hour period, it must be reported as such. For example, a test termination after 72 hours would be reported as the 72-hour LC50. Fish mortalities and environmental conditions are recorded every 24 hours (Section I. L.1).

6. Renewal of solutions: In instances where effluents may have volatile or unstable toxic constituents, or a high dissolved oxygen demand, periodic renewal of the test solutions will provide more uniform concentrations of these materials. Fresh solutions should be made up every 24 hours or less, and test animals transferred quickly but gently to the new test solution by means of a dip net.

7. Biological data: Dead fish must be removed as soon as they are observed, with the total number of dead in each test chamber counted and recorded every 24 hours. Fish are considered dead in the absence of gill movement and loss of all ability to move or respond to stimuli. When removing dead fish from the test aquarium a different net should be used for each concentration or dead fish removed from the controls first and then those from the lowest concentration to the highest to avoid contamination with solutions from higher concentrations. Nets should be placed in a disinfectant, such as Wescodyne®, when not in use and rinsed thoroughly before reusing.

The minimum, maximum, and average weights and lengths, to the nearest 0.1 g and mm (Section I. F.5), must be determined by measuring a representative sample of fish (15 to 20 individuals) prior to the test or on surviving fish after the test.

8. Disposal of surviving fish: Surviving fish, including controls, must not be used in future tests and must be destroyed before being disposed of. The California Fish and Game Code, Chapter 5, Article 1, Section 6400 states that it is illegal to return fish to State waters without written permission of the Department of Fish and Game. Fish can be killed quickly and humanely by placing them in water to which a few drops of ether, chloroform, or chloretone have been added (Sturges and Nicola, 1975).

L. Physical and Chemical Determination

1. The DO, pH, and temperature must be measured in all controls and dilutions before introducing fish and at 24-hour intervals. Additional checks of these parameters are advisable at any time fish are observed dying. If a freshwater dilution is made, the total hardness and total alkalinity must also be measured in the control, low, medium and high effluent concentrations at the beginning of each test. If a brackish or marine dilution water is used, its salinity, chlorinity, or specific conductance must be measured. A continuous recording thermograph is required for all tests (Section I. G.3.). Specified toxicants as required by the appropriate regulatory agency, must be measured at the beginning and end of the test in the low, medium and high concentrations. Concentrations of other constituents known to be present in the diluent in amounts that may influence the toxicity of the material being tested should be determined.

M. Percent Survival Tests

1. Waste effluent discharge requirements may require reporting percent survival in the undiluted waste in lieu of, or in conjunction with,

the standard 96-hour LC50. When run in conjunction with the LC50, the undiluted waste is treated in the same manner as all the other concentrations being tested. When conducting percent survival tests only, the same basic procedures apply as used in the standard bioassay with the exception that dilutions are not made and 20 fish minimum are used in the undiluted waste and 20 in the control. One or more test containers may be used, providing consistency is maintained in the weight-volume ratio stated in Section I. F.5. Fish are acclimated for seven days to test temperatures and receiving waters, or waters similar in pH, hardness, and alkalinity as that of the receiving waters. Where a freshwater waste is being discharged to marine waters, fish are acclimated to the formulation given in Table 2 for very hard water. A control is to be used for each test using the same type water used for acclimating the fish. Tests are conducted for 96 hours and reported as percent survival in the undiluted waste.

N. Expression of Results. Three of the most commonly used methods of expressing results are:

1. Estimation of the median lethal concentration, LC50: The straight-line graphical interpolation method (Doudoroff et. al., 1951) is the easiest and most often used procedure for estimating the LC50. The data are plotted on either semilogarithmic or log probit paper, with the test concentrations entered on the log scale and the percent mortality on the arithmetic or probit scale.[15/] Data points representing two successive concentrations that were lethal to more than half and to less than half

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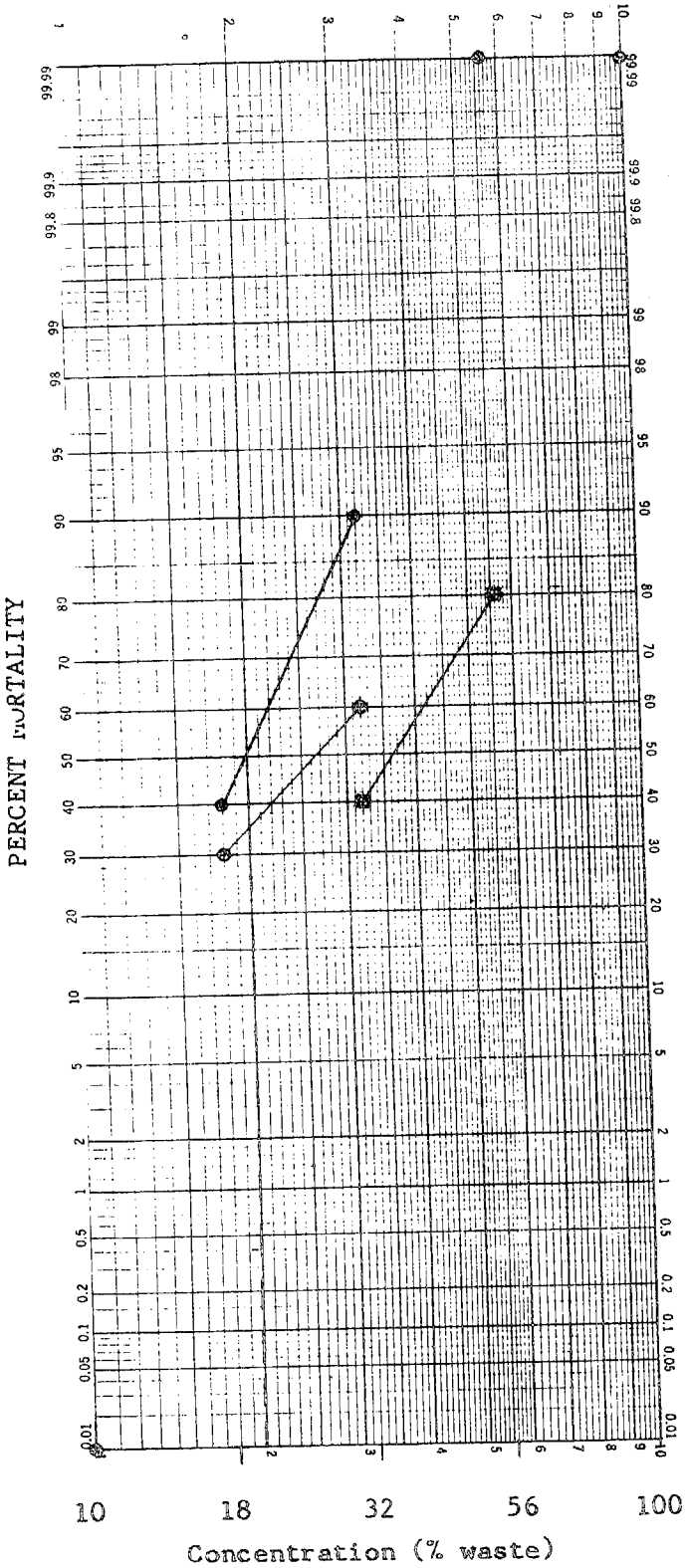
15/ K&E Probability graph paper 46-8080 and 46-8040 or semi-logarithmic 46-5810 can be used in the graphical interpolation method.

the fish, respectively, are connected with a straight line. The point at which the plotted line and the 50% mortality line intersect is the concentration representing the LC50. Hypothetical experimental data are presented in Figure 3 for this determination. When survival is greater than 50% in the highest concentration tested, the percent survival for this concentration must be reported.

When using the straight-line method, a practical decision must be made as to the validity of the test. In the final analysis of the data, only two points are used to determine the LC50; however, consideration should be given to all plotted points. In an acceptable test, the points should lie on a reasonably straight line with an increase in concentration representing an increase in mortalities. There should be less than 10% mortality in the controls, and ideally, one concentration should have killed more than 60 percent of the organisms exposed to it. The next lowest concentration should have killed less than 40 percent of the organisms. Methods are discussed in Section I. O. for the statistical evaluation of data and the construction of confidence limits for bioassay tests.

2. Percent survival: Percent survival is usually based on the number of fish surviving in the undiluted waste for 96 hours. It can, however, be based on survival of fish in any designated concentration of waste for any time period.

3. Toxic units: The use of toxic units to express bioassay test results has been proposed by Sprague (1969) and its use reviewed by Brown and Beck (1972) for the San Francisco Bay-Delta Toxicity Study. When an LC50 can be determined, i.e., when the waste water is sufficiently toxic



Conc. Waste %	Percent Mortality		
	24 hr.	48 hr.	96 hr.
100	100	100	100
56	80	100	100
32	40	60	90
18	0	30	40
10	0	0	0

LC50	24 hr. □	48 hr. ○	96 hr. ●
	37%	27%	20%

% Survival. 100% Waste = 0

$$\text{tc Units} = \frac{100}{\text{96-hr. LC50, \%}}$$

$$\text{tc Units} = \frac{100}{20} = 5$$

FIGURE 3. Sample information worksheet for estimating median lethal concentration (LC50) by straight-line graphical interpolation.

to measure the concentration killing at least 50% of the test animals in the acute bioassay, the toxicity of the waste can be expressed as toxic units where:

$$\text{Toxicity Concentration, Tc (toxic units)} = \frac{100}{\text{96-hr. LC50 \%}}.$$

The determination is the 96-hour LC50 concentration in percent and the units of toxicity concentration are "toxic units." Note there is an inverse relationship between toxic units and LC50, i.e., the greater the toxicity the larger the toxic units and the smaller the LC50 designation. The toxicity concentration would be 1.0 toxic units in waste with a LC50 of 100%.

The matter of expressing toxicity concentrations when fish mortalities are less than 50% in 100% waste is discussed by Esvelt (1971) in the San Francisco Bay-Delta Toxicity Study. In this study the following expression was derived for golden shiner exposed to chlorinated municipal wastes:

$$\text{Tc (toxic units)} = \frac{\text{Log (100-S)}}{1.7}$$

in which S, the percentage survival, is calculated by the expression:

$$S = \left(1 - \frac{x + 1}{n}\right) 100$$

where n is the total number of fish tested in 100 percent waste and x is the number of dead fish at 96 hours. It should be understood that this expression is for golden shiner exposed to chlorinated municipal wastes and are not necessarily applicable to other wastes and test species.

O. Statistical Evaluation

1. The precision of any bioassay is limited by the biological variation of individuals within the species. When there is a large natural variability of response among the test subjects, the analysis of numerical data can only be effected satisfactorily with the aid of statistical techniques. Finney (1964, 1971) reviews a variety of methods available for the statistical evaluation of bioassays. The most widely used methods are the

probit, moving average, Spearman-Karber (1908, 1931), and the Litchfield-Wilcoxon (1949) methods. These and other methods are being evaluated for their applicability in the "self-monitoring" program. For the present time, the Litchfield-Wilcoxon (1949) method is suggested for use because of its ease of computation. This method allows a rapid graphic method for approximating the LC50, slope of the curve, and confidence limits by persons with little or no statistical experience. An example of this method is included in Appendix B.

P. Worksheets and Reports. Figures 4 and 5 illustrate the type of worksheets that can be used to record bioassay and chemical data. These forms can be revised accordingly to meet the specific needs of the discharger. The worksheets and/or reports should include the following information where applicable.

1. Test identification.
2. Name of investigator and laboratory performing test.
3. Date and time samples were collected and received at the laboratory.
4. Description of the material being tested, including its source and the concentration of specified toxicants.
5. Source and chemical characteristics of dilution water. If dilution water is other than receiving water then the total alkalinity, total hardness and pH of the receiving waters should be reported.
6. Information about test organisms, including common and scientific name, source, length and weight (average, minimum, maximum).
7. Information on acclimation, including water source, number of days, temperature, and percent dead in acclimation tank.
8. Time interval for solution renewal (if applicable).
9. Bioassay test concentration, volume and depth of test solution and number of organisms per concentration.

10. Type of aeration (compressed air or oxygen) if used.

11. Chemical and physical measurements, including DO, temperature, pH, hardness, and alkalinity.

12. Number of mortalities after 24, 48, 72, and 96 hours. Determination of 96-hour LC50 and 95% confidence limits. When required the percent survival in the undiluted waste.

13. Full description of any abnormalities of test organisms. If fish appear healthy, note this.

14. Any other relevant information.

Address

Static Bio y Worksheet

Test No. _____ Investigator: _____ Date & Time Sampled _____

Date Started _____ Time Started _____ Renewal of Test Solution at _____ Hour Interval

Test Con'c	Initial			24 Hrs.				48 Hrs.				72 Hrs.				96 Hrs.			
	pH	DO	Temp	No. Dead	pH	DO	Temp	No. Dead	pH	DO	Temp	No. Dead	pH	DO	Temp	No. Dead	pH	DO	Temp
Control																			

Species _____ Avg. Length _____ Max. Length _____ Min. Length _____
(Both common & scientific name & source)

Number of test animals per concentration _____ Avg. Weight _____ Max. Weight _____ Min. Weight _____

Volume of test solution _____ Depth _____ Type Aeration _____

Acclimatization _____ days at _____ °C. Percent dead in acclimatization tank _____ Water Source _____

Notes _____ Dilution Water Source _____
(Describe condition of fish during test especially any abnormalities in behavior and color.)

LC50 _____ Percent Survival (Undiluted Effluent) _____ Confidence Limits _____

Remarks _____

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FIGURE 4. Bioassay worksheet.

Bioassay Chemical Data Sheet

Test No. _____ Date and Time Sample Collected _____

Analysis By _____ Date Sample Received _____

Type Discharge (give full description of material and source) _____

_____

_____

_____

Concentration of specified toxicants at time of collection _____

_____

_____

Test Con'c	TEST START					96 Hr.		
	Hardness $CaCO_3$	Alkalinity $CaCO_3$	Specified Toxicants			Specified Toxicants		
Control								
1								
2								
3								
4								
5								
6								

Salinity or Conductivity (marine and brackish dilution water) _____

Receiving water (if other than dilution water)

Alkalinity _____

Hardness _____

pH _____

FIGURE 5. Bioassay chemical data sheet.

II. TEST PROCEDURES CHECK LIST

The following guide is intended as a check list for laboratory personnel conducting static bioassays. References given in parentheses following each step refer to the detailed explanation of bioassay procedures in Section I of this manual.

A. Preliminary Tests. Where the approximate toxicity of the waste sample is unknown and toxic materials in the sample do not degrade or volatize, preliminary tests should be conducted (Section I. J.1.).

1. Premark final volume of test solution on containers. Minimum volume per test aquarium in preliminary tests is 2 liters.

2. Concentrations of 100, 10, and 1 and 0.1% waste are usually adequate to determine the range of concentrations required in definitive tests. To prepare the above dilutions, add the volume of waste indicated in the table below and bring up to the 2-liter mark with dilution water.

Concentration (% waste)	Volume of Waste (ml)	Volume of Dilution Water (ml)
100%	2,000	0
10%	200	1800
1%	20	1980
0.1%	2	1998
0	0	2000 (control)
Total (ml)	2,222	7778
Gallons Required	1	2½

3. A Control must be run under the same conditions as those prescribed for the tests (Section I. K.3.).

4. DO must be maintained at a minimum of 70% air-saturation (Section I. H.).
5. Temperatures must be within the prescribed range (Section I. G.).
6. Tests are conducted for 24 hours using 2 to 4 fish per aquarium remaining consistent with the weight-volume ratio (Section I. F.5.).

B. Definitive Tests (Section I. K.).

1. Nineteen-liter (5 gallon) wide-mouthed jars or aquaria are the recommended test containers (Section I. B.1.).
2. Premark final volume of test solution on the outside of the containers with a waterproof felt pen. Minimum volume per test aquarium is 10 liters; at least five concentrations and a control must be used per test (Section I. K.2.).
3. Full-scale tests are conducted on a range which falls between the highest wastewater concentration, at which all fish survived in the preliminary tests, and the lowest concentration at which all or most fish died (Section I. K.2.).
4. With each series of tests, a control must be run under the same conditions as those prescribed for the tests. If the dilution water is other than what fish were acclimated to, two controls are required: (1) dilution water; (2) acclimating water (Section I. K.3.).
5. Dissolved oxygen and temperatures must be within the prescribed range before adding fish to test containers. A test begins when the organisms are first exposed to the toxicant and extends for 96 hours (Sections I. G. and H.).
6. A minimum of 10 fish (see size and weight Section I. F.5.) is added randomly to each concentration.

7. Temperature, DO and pH of each dilution and control must be recorded at the start of the test and at 24-hour intervals. A continuous recording thermograph is required to monitor room temperature or water temperature in the control or one of the concentrations. In freshwater tests, total hardness and total alkalinity must be determined in the controls, low, medium and high concentrations at the beginning of the test. If marine or brackish dilution waters are used, salinity, chlorinity or conductivity must be reported. (Sections I. G., H., and L.).

8. Determinations of specified toxicants must be made prior to beginning the test and at the end of the test in the low, medium and high concentrations.

9. Remove dead fish as soon as observed. All or a portion (15-20) of the surviving fish must have weight-length measurements taken to the nearest 0.1 gm and mm (Section I. K.7.).

10. Surviving fish should be destroyed (Section I. K.8.).

11. Calculate the LC50 or percent survival for the 96-hour period (Section I. N. and O.).

III. CARE, HANDLING AND DISEASES OF TEST FISHES

The condition of test organisms is crucial to the performance of a successful and credible bioassay test. Generalizations concerning the care and handling of any fish must be approached with caution due to inherent complexities relative to species, size, behavior, habitat, etc. The following information, however, based largely upon the experiences of the California Department of Fish and Game should be helpful in developing methods suited to individual needs for the maintenance of a good healthy stock of fish.

Unnecessary stress to fish must be avoided. Stress conditions will cause a greater susceptibility of fish to disease, infection and toxicants and, therefore, will bias test results. Some of the more common causes of stress are: the handling and transporting of fish; changes in environmental conditions such as temperature, dissolved oxygen, water, photoperiods, disturbances from outside sources and overcrowding. Wild populations of fish captured for use would lik be under greater stress in captivity than would commercially-reared stock. Fish should be handled as little as possible. When they must be handled, it should be done carefully and as quickly as possible using fine-meshed dip nets.

One of the most convenient methods for transporting small quantities of fish (50-100/container) is the use of inexpensive polystyrene ice chests with lids. These chests have a capacity of 20-30 liters (5-8 gallons) and can be lined with polyethylene bags to insure water tightness. Water temperatures will remain relatively constant for several hours in these containers. To reduce the probability of disease and infection, it is advisable to transport fish in a weak solution of oxytetracycline HCl (Terramycin®), about 0.5 gm/30 liters.

Dissolved oxygen must be maintained near air-saturation levels during transport. Two methods of aeration can be used: (1) battery-operated aerators,

either dry cell or 12 volt automobile lighter plug-in which bubbles air into the fish tank during transport; or (2) top-loading, where fish are placed in a polyethylene bag partially filled with water and the bag inflated with compressed oxygen and sealed by tying. This method allows an adequate supply of oxygen for several hours, depending upon the size and number of fish.

Test organisms arriving at the laboratory must not be subjected to abrupt changes in water quality and temperature. Before fish are transferred from transporting to holding tanks, the pH and hardness of the water should be similar in both tanks to prevent osmotic shock, and differences in water temperature not greater than 2°C. New groups of fish should not be mixed with fish already on hand. Tanks must be located away from any potential source of mechanical disturbances including furnaces, air conditioning units, etc. An ordinary sequence of darkness and light, either from normal daylight or controlled photoperiod, is desirable. If a photoperiod cannot be provided, then tanks should be located in areas of subdued light and a dimmer switch used to control light intensity. When possible, fish should be held in the dilution waters in which they will be tested. However, any good quality water capable of supporting fish life, including dechlorinated tap water, can be used. The severity of disease and infection can usually be reduced by holding fish at lower temperatures rather than at higher temperatures until such time fish are to be acclimated to test conditions. Coldwater fish are best held at 13° to 15°C and warmwater species from 18° to 20°C. Stickleback generally do better at 15° to 18°C in salinities of 15 to 25 g/l (ppt).

The size of the holding and acclimation tank is of individual preference although several 70 liter (20 gallon) aquaria are more convenient to work with than one large tank. Of major importance is not to overcrowd fish. Holding

and acclimation tanks should be sterilized with 200 mg/l hypochlorite or Wescodyne® for one hour followed by several freshwater rinses before introducing new fish. Substrate or side mount filters should be used on all holding and acclimating tanks. Ultraviolet sterilizers or ozone generators will help minimize the chance of diseases. Filtering materials, i.e., glass wool and activated charcoal, should be replaced at a frequency of about once a week or sooner if debris accumulates in the filter. Unionized ammonia concentrations should not exceed 20 ug/l (E.P.A., 1975) in holding and acclimating tanks. Levels less than this can usually be maintained by passing water through activated charcoal and changing 1/3 of the aquarium water every one to two weeks. The concentration of unionized ammonia can be calculated from the concentration of total ammonia, pH, and temperature (Table 7). To maintain appropriate salinity levels in salt water holding tanks, fresh water should be added to make up for loss of water through evaporation. Dissolved oxygen concentrations must be maintained between 70 and 100% saturation.

Fish should be fed daily or at least five times a week on a routine basis. It is best to feed a small amount of food several times a day as compared to a large amount once a day. Do not overfeed. Accumulation of food in tanks and filters usually indicates overfeeding. Fish food may be purchased dry or in frozen moist form. Food for commercially-reared fish can usually be purchased from the dealer selling the fish. Stickleback feed well on fresh or frozen brine shrimp, although from cursory examination it appears they require some roughage in their diets. This can be supplied by maintaining aquatic plants in holding and acclimating tanks or by feeding a blended fish food, such as Tetra-Min® available from most aquarium supply shops.

Prevention is the key word in disease control. Prevention begins with good handling techniques, adequate space and proper water quality and

TABLE 7. Percentage Of Un-Ionized Ammonia (NH_3) In Distilled Water At Different Temperatures And pH Factors. 16/

Temperature °C	pH								
	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0
14	.03	.08	.25	.80	2.48	7.43	20.2	44.5	71.7
16	.03	.09	.29	.90	2.87	8.54	22.8	48.3	74.7
18	.03	.11	.34	1.07	3.31	9.78	25.5	52.0	77.4
20	.04	.13	.40	1.24	3.82	11.2	28.4	55.7	79.9
22	.05	.15	.46	1.43	4.39	12.7	31.5	59.2	82.1
24	.05	.17	.53	1.65	5.03	14.4	34.6	62.6	84.1
26	.06	.19	.61	1.90	5.75	16.2	37.9	65.9	85.9

16/ Prepared from data given in Thurston, Russo and Emerson (1974).

sanitation conditions, i.e., clean aquaria and filters. Treatment is usually of little value unless the disease is diagnosed early and the underlying causes of the disease identified. Some of the obvious changes in behavior of fish suffering from a disease, parasite or other physical affliction may be: (1) loss of appetite; (2) abnormal distribution in aquarium such as swimming close to the surface and/or fish separated from the main school of fish; (3) loss of equilibrium; and (4) darting and twisting movements.

Leitritz (1962) outlines several gross external symptoms to be aware of: (1) discolored areas on the surface of the body, head and fins; (3) swelling of the body and gills; (4) popeye; and (5) hemorrhaging.

In general, if a disease problem does occur, it is best to destroy the fish. All equipment and tanks fish have come in contact with must be sterilized before new fish are introduced into the system. If the disease originated in the hatchery from which the fish were purchased, and the problem has not been corrected, loca a new source of fish.

The following review describes briefly a general classification of the more common disease-causing organisms among fish in California and the recommended treatment of these diseases. Treatment is based primarily on methods used in trout hatcheries; however, many diseases are common to both cold and warmwater species. Persons considering the use of any of the reported techniques are advised that it first be tested on a small lot of fish before making large-scale applications. The efficacy of these treatments against diseases is related to many factors such as pH, temperature, salinity, the presence of interfering substances, etc. In addition, the physiological condition of the fish also influences the success of a treatment. Heavily diseased or stressed fish may not be able to tolerate otherwise safe and effective treatments. The susceptibility of fish to toxicants is also affected by the developmental stage, age, size and often the sex of the fish.

Various methods are employed for the treatment of diseases. The four most commonly used methods are: (1) the "dip" method in which a strong solution is used for a relatively short period. The fish are netted, surplus water allowed to drain off, and the net containing the fish placed in the solution [18/] for a designated time period. Fish are then placed in freshwater; (2) the "bath" method of treatment uses a weaker concentration for a longer period of time than the dip method. The chemical is added to a tank and left for a specified period of time after which the solution is drained from the tank and replaced with freshwater; (3) in the "prolonged" or indefinite bath" chemicals are added to the water and left indefinitely. Degradation or dissipation of the chemical occurs within the tank with no dilution by freshwater. This method is often used in the prevention of diseases; (4) if fish are feeding and will accept artificial feeds, they can possibly be treated by feeding medicated foods. This treatment is often used for internal and bacterial gill diseases.

A. External Bacterial Diseases

1. Columnaris disease: The most commonly encountered of all external bacterial infections.

   a. Appearance: Grayish-white lesion with erosion of skin often exposing underlying musculature.

   b. Treatment: The best success in treating external bacterial diseases is the use of oxytetracycline HCl (Terramycin®) or copper sulfate. Terramycin® is the preferred method, since it is effective and much safer to use than copper sulfate. Expose fish to 0.1 gm water-soluble Terramycin® per 4 liters of water for one-hour bath then transfer to freshwater. When using copper sulfate, dip fish in a 1:2000 part

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18/ When transferring fish from one tank to another, be certain DO levels and temperatures are adequate in the tanks to maintain fish.

solution (1 gm per 2 liters of water) for 1 to 2 minutes. The addition of 0.1% sodium chloride solution (1 gm per liter water) reduces the stress of copper to fish. On occasions when external treatments do not provide adequate control, it is necessary to feed a diet containing Terramycin®.

2. Bacterial gill disease: One of the more common diseases in California. The conditions under which this disease flourishes are water temperatures above 15°C and crowding of the fish.

   a. Appearance: In early stages the gills may be swollen and clubbed with large amounts of mucous present. Fungus is often found as a secondary invader on the gills of infected fish.

   b. Treatment: Terramycin® or copper sulfate as for Columnaris.

B. Fungus: Generally fungus is a secondary invader and is introduced when the protective coating of scales and mucous is lost because of handling injury c bacterial damage.

1. Saprolegnia sp.: Most common of the fungal infections.

   a. Appearance: Cottony appearance in blotchy areas of skin.

   b. Treatment: Fish affected by fungus may be dipped i a 1:15,000 malachite green solution (1 gm per 15 liters of water) for one minute; or 0.20 mg/l malachite green solution can be added as an indefinite bath to the holding tank twice weekly for three consecutive weeks.

C. External Protozoa

1. "Ich" Ichthyophthirius multifilus: Probably the most common protozoan on almost all kinds of freshwater fishes. Warmwater and crowding are conductive to the outbreak of "Ich".

a. Appearance: Whitish globular colonies from slightly smaller than the head of a pin to microscopic.

b. Treatment: Malachite green treatment as for Saprolegnia.

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A P P E N D I X A

California Fish and Game offices where a licensed fish breeder's list may be obtained.

California Department of Fish and Game
Headquarters Office
1416 Ninth Street
Sacramento, California 95814

California Department of Fish and Game
Region I
627 Cypress Avenue
Redding, California 96001

California Department of Fish and Game
Region 2
1001 Jedsmith Drive
Sacramento, California 95819

California Department of Fish and Game
Region 3
Building C, P.O. Box 47
Yountville, California 94599

California Department of Fish and Game
Region 4
1234 East Shaw Avenue
Fresno, California 93726

California Department of Fish and Game
Region 5
350 Golden Shore
Long Beach, California 90802

A P P E N D I X B

LITCHFIELD-WILCOXON METHOD

The Litchfield-Wilcoxon Method can be used to establish confidence limits for fish bioassays. For the complete method including the necessary tables and nomographs for the calculation of significantly heterogeneous data, parallelism of two curves, and confidence limits for the slope of the curve, the reader is referred to the original publication.

The following symbols are used for determining the confidence limits of the LC50.

K = The number of concentrations plotted

n = K-2 = Degrees of freedom for $(Chi)^2$

LC50 = Median Lethal Concentration

s = Slope function

fLC50 = Factors for LC50

N' = Total number of animals used between 16 and 84 percent expected effect

Exponent 2.77 is a constant for any bioassay.

Procedure

A. The data and graph. (an example of this method follows on page 57)

1. List the actual concentration used, the number reacting/number tested, and the percent effects. Do not list more than two consecutive 100 percent effects at the upper end or more than two consecutive 0 percent effects at the lower end of the curve.
2. Plot concentrations against percent effect on logarithmic-probability paper (NO. 46 8040, Keuffel and Esser Co.) leaving space for but omitting any 0 or 100 percent effects.

With a transparent straight-edge fit a temporary straight line through the ...nts, particularly those in the region of 40 to 60 percent effect.

B. Plotting 0 or 100 percent effects

1. Read and list the expected percent effects as indicated by the line drawn, for each concentration. If the expected value for any concentration is less than .01 or greater than 99.99 delete such concentrations and effects from the list.

2. Using the expected effect, record and plot from Table 8 a corrected value for each 0 or 100 percent effect which is listed. Inspect the fit of the line to the completely plotted data. If it is obviously unsatisfactory, refit the line and repeat the preceding two steps to obtain a new set of expected and corrected values. The derivation of this table is beyond the scope of this manual; however, its purpose is to reduce adverse influences caused by extreme mortalities or survival in high and low concentrations.

When the line appears to fit satisfactorily, as is almost always the case with the first line, proceed to the $(Chi)^2$ test.

C. The $(Chi)^2$ test

1. List the difference between each observed (or corrected) effect and the corresponding expected effect.

2. Using each difference and the corresponding expected effect, read and list the contributions to $(Chi)^2$ from Nomograph No. 1. page 60 (A straight edge connecting a value on the expected percent scale with a value on the difference scale, will indicate at the point of intersection of the $(Chi)^2$ scale, the contribution to $(Chi)^2$).

3. Total the contributions to $(Chi)^2$ and multiply by the average number of animals per concentration, i.e., the total number of animals/K. This

is the $(Chi)^2$ of the line. The degrees of freedom are two less than the number of concentrations plotted, i.e., n = K-2.

4. If the $(Chi)^2$ of the line is less than the value of $(Chi)^2$ given in Table 9 for n degrees of freedom, the data are not significantly heterogeneous, i.e., the line is a good fit. If the $(Chi)^2$ curve exceeds the value of $(Chi)^2$ given in Table 9, the data are significantly heterogeneous and the line is not a good fit. Refit the line to see if the $(Chi)^2$ of the line can be reduced below the permissable $(Chi)^2$.

D. The LC50 and fLC50

1. Read from the line on the graph the concentration for 16, 50, and 84 percent effects (LC16, LC50, and LC84).

2. Calculate the slope function, S, as:

$$S = \frac{LC84/LC50 + LC50/LC16}{2}$$

3. Obtain from the data tabulation, N' the total number of animals tested at those concentrations whose expected effects were between 16 and 84 percent.

4. Calculate the exponent in the expression:

$$LC50 = S^{2.77/\sqrt{N'}} = S^{\text{exponent}}.$$

To carry out this step, obtain first the $\sqrt{N'}$, then solve $2.77/\sqrt{N'}$ = exponent. Next, using this exponent and the value of S, read the fLc50 on the center scale of Nomograph No. 2 page 61 by laying a straight edge across the correct scale values.

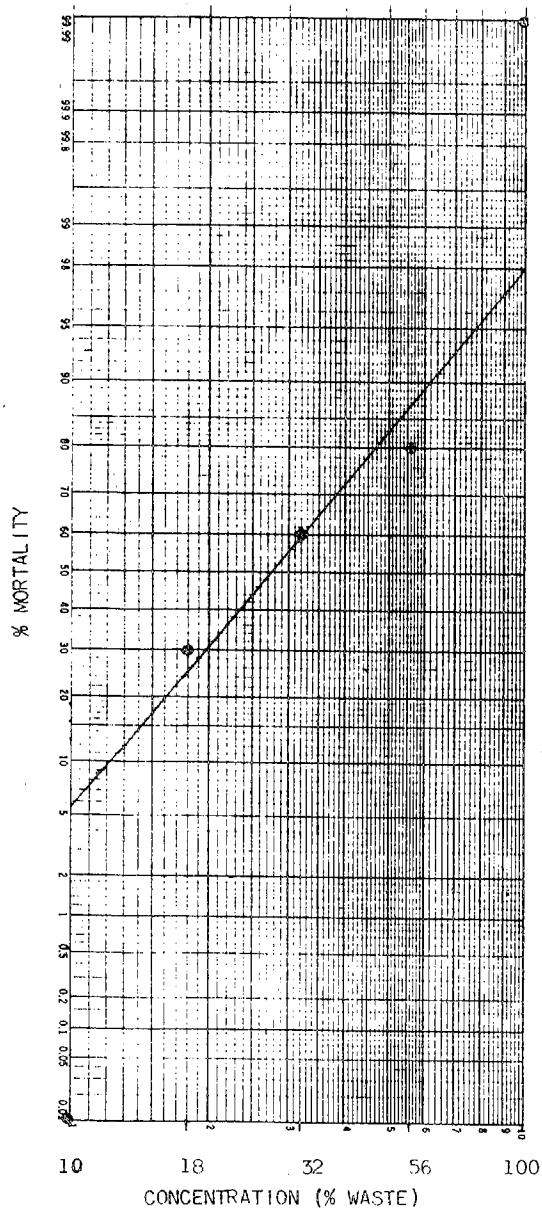
5. Calculate the confidence limits of the LC50 as:

$$\left.\begin{array}{l} LC50 \times fLC50 = \text{upper} \\ LC50/fLC50 = \text{lower} \end{array}\right\} \text{ limit for 19/20 probability}$$

The following example illustrates the use of the Litchfield-Wilcoxon Method for establishing confidence limits for the LC50.

% Concentration	Expected % Mortality [1]	Observed % Mortality [2]	Observed Minus Expected	Contribution To $(Chi)^2$ [3]
10	6	0 (2)	4	.025
18	26	30	4	.007
32	60	60	0	0
56	87	80	7	.04
100	98	100 (99.3)	1.3	.008
			Total	.080

1/ Values read from line plotted in graph below.
2/ Numbers in parentheses are corrected values for 0 and 100 percent effect from Table 8.
3/ Values from nomograph No. 1.



Total test animals = 50
Number of concentration (K) = 5
Animals/Concentration = 50/5 = 10
$(Chi)^2$ = .08 x 10 = .8
Degrees of Freedom, n = K-2 = 5-2 = 3
$(Chi)^2$ from Table 9 for 3 degrees of freedom = 7.82
.08 is less than 7.82; therefore, the line is a good fit.
LC84 = 51.5 % waste (read from adjoining graph)
LC50 = 27.2 % waste (read from adjoining graph)
LC16 = 14.5 % waste (read from adjoining graph)

$$s = \frac{LC84/LC50 + LC50/LC16}{2} = \frac{51.5/27.2 + 27.2/14.5}{2} = 1.88$$

N' = 30 (number of animals tested between 16 and 84% mortality)

$$fLC50 = (s)^{2.77/\sqrt{N'}} = 1.88^{2.77/\sqrt{30}} = 1.88^{0.51} = 1.38$$ (from Nomograph No. 2)

Exponent 2.77 is a constant for any bioassay.

Confidence limits of the LC50:

LC50 x fLC50 = 27.2 x 1.38 = 37.5 upper limit

LC50/fLC50 = 27.2/1.38 = 19.7 lower limit

TABLE 8. Corrected Values [19/] Of 0 or 100 Per Cent Effect (Body of Table) For Observed Mortalities Corresponding To Expected Values (Margins).

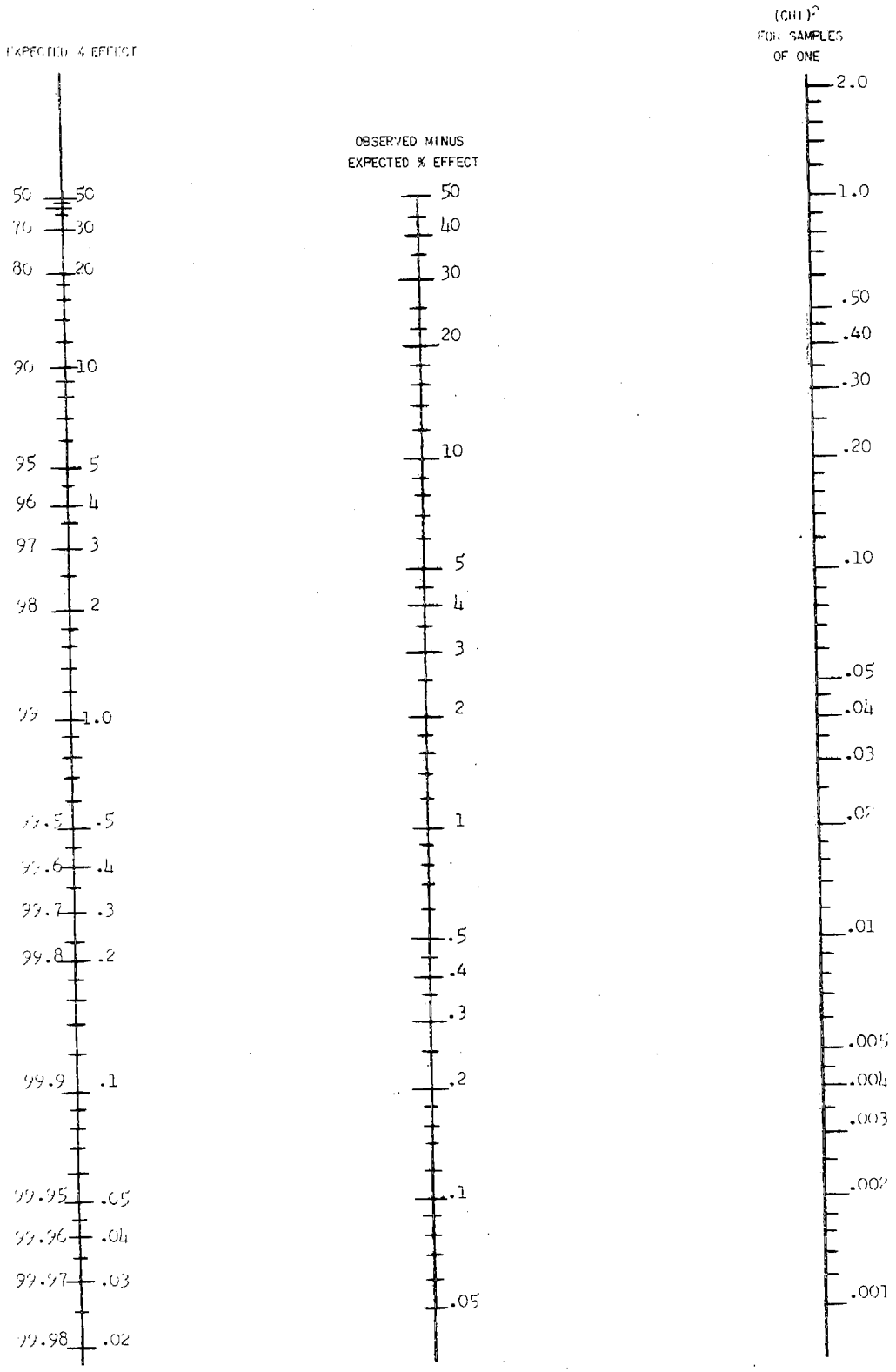
Expected	0	1	2	3	4	5	6	7	8	9
0	---	0.3	0.7	1.0	1.3	1.6	2.0	2.3	2.6	2.9
10	3.2	3.5	3.8	4.1	4.4	4.7	4.9	5.2	5.5	5.7
20	6.0	6.2	6.5	6.7	7.0	7.2	7.4	7.6	7.8	8.1
30	8.3	8.4	8.6	8.8	9.0	9.2	9.3	9.4	9.6	9.8
40	9.9	10.0	10.1	10.2	10.3	10.4	10.4	10.4	10.4	10.5
50	---	89.5	89.6	89.6	89.6	89.7	89.7	89.8	89.9	90.0
60	90.1	90.2	90.4	90.5	90.7	90.8	91.0	91.2	91.4	91.6
70	91.7	91.9	92.2	92.4	92.6	92.8	93.0	93.3	93.5	93.8
80	94.0	94.3	94.5	94.8	95.1	95.3	95.6	95.9	96.2	96.5
90	96.8	97.1	97.4	97.7	98.0	98.4	98.7	99.0	99.3	99.7

19/ These values are derived from the maximal and minimal corrected probits of Bliss (1938). The use of this table is similar to the use of standard logarithm tables. Units are found in the vertical columns and tens in horizontal lines. For example, look at the graph on page 56. The expected values extrapolated for 10 and 100% concentration are 6% and 98% mortality, respectively. To find the "corrected" mortality for 6% expected go to line 0 (0 tens) and column 6 (6 units: 0 + 6 = 6) and read 2.0% mortality. Similarly to find the "corrected" value for 98% expected go to 90 for the horizontal line and column 8 (8 units; 90 + 8 = 98) and read 99.3%. Use the values found in the table to calculate the expected minus observed values.

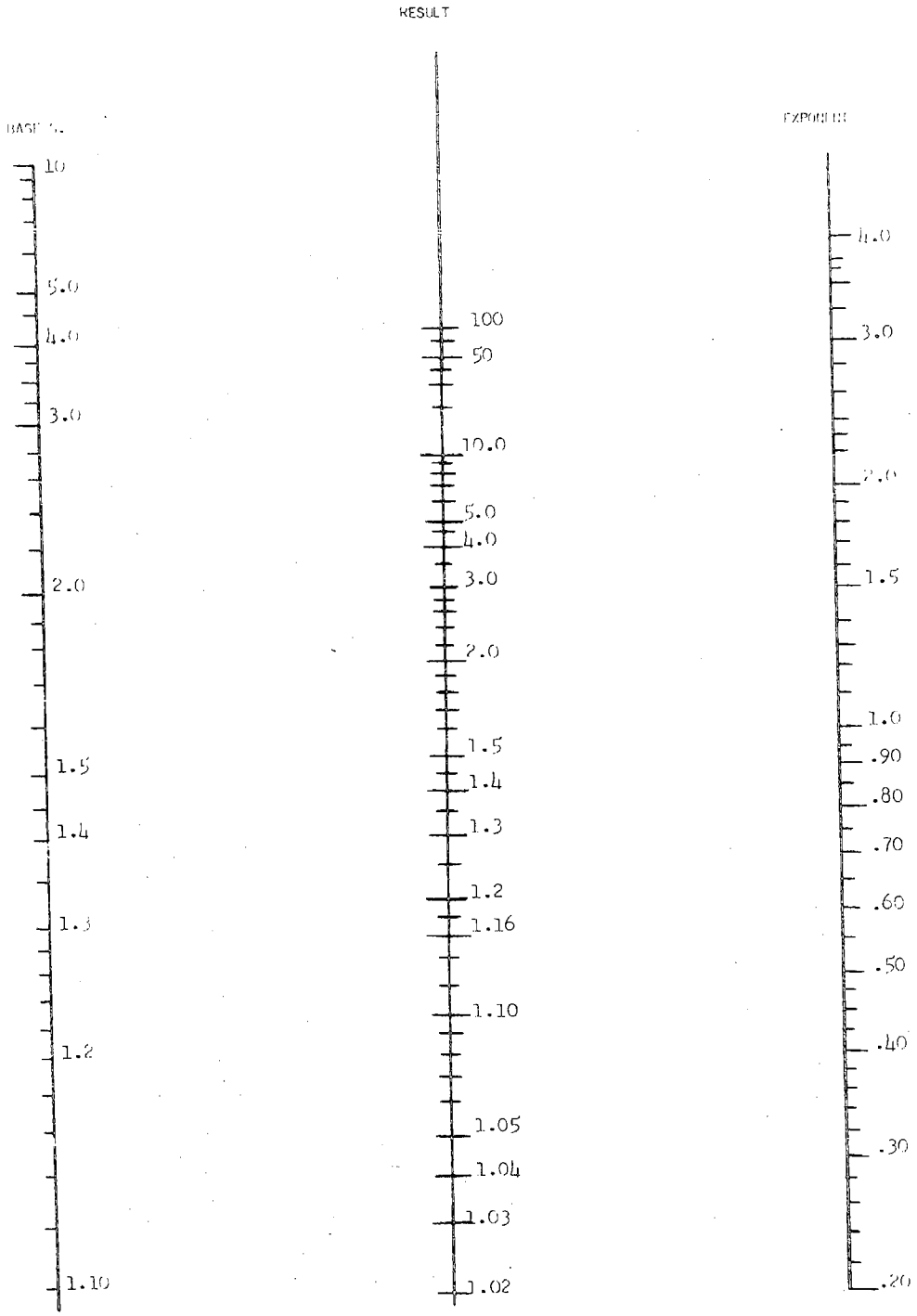
TABLE 9. Values [20/] Of $(Chi)^2$ For P = .05

Degrees of Freedom	$(CHI)^2$
1	3.84
2	5.99
3	7.82
4	9.49
5	11.1
6	12.6
7	14.1
8	15.5
9	16.9
10	18.3

20/ Values of $(Chi)^2$ for p = .05 are the same as may be found in more extensive tables.



No. 1 Nomograph For Obtaining $(Chi)^2$ From Expected % Effect And Observed Minus Expected % Effect.



NO. 2 Nomograph For Raising Base S To A Fractional Exponent.

A P P E N D I X C

GLOSSARY

Acclimate - To accustom test organisms to a different environment, i.e., water, temperature, laboratory conditions, etc.

Acute Toxicity - Any direct lethal action, usually demonstrable within four days for fish.

Bioassay - Test in which the quantity or strength of material is determined by the reaction of a living organism to it. (Sprague, 1973).

Chronic Toxicity - Long-term effects that may be related to changes in appetite, metabolism, reproduction or even possible mutation of genes.

Clubbing - Thickened and fussed gill tissue in fish due to bacterial gill disease.

Composite Sample - A combination of individual samples of water or wastewater taken at selected intervals that may be proportioned to the flow at time of sampling.

Continuous-Flow Bioassay - Test solution is renewed continually or by frequent periodic additions. Can be used for either acute or chronic toxicity tests.

Definitive Test - Full-scale bioassay test consisting of 5 to 6 different concentrations and a control.

Geometric Series - A sequence of concentrations in which a ratio of each concentration to the preceding one is the same throughout the sequence.

Grab Sample - A single sample of wastewater taken at neither set time nor flow.

Lethal - Causing death, or sufficient to cause it, by direct action.

96-Hour Median Lethal Concentration (LC50) - That concentration in which 50 percent mortality occurs. This term cannot be used with grammatical accuracy of lethal "concentrations" of water temperature and pH (Sprague, 1969). Despite the differences between "tolerance limit" and "lethal concentration;" the terms used in this manual will be used interchangeably.

96-Hour Median Tolerance Limit (TLm) - That concentration in which 50 percent of the fish survive for 96 hours. Can designate a level of any measurable lethal agent, including temperatures, pH, and the like.

Osmosis - The diffusion which takes place between two fluids or solutions through a permeable or semipermeable membrane, and tending to equalize their concentrations.

Percent Survival Test - Exposure of test organisms to a sample of undiluted waste for 96 hours and the percent survival reported.

Preliminary Test - Exploratory bioassay usually conducted for 24 hours to determine the range of concentrations that should be tested in the definitive test.

Specified Toxicants - The analysis and reporting of certain constituents in the waste in conjunction with bioassay tests that may be required by the proper regulatory agency.

Static Toxicity Test - Test organisms are held in containers of standing test water that may, or may not, be changed during the test period. Used primarily for the determination of acute toxicity.

Sub-Lethal - Below the level which directly causes death.

Toxic Unit - Is the units of toxicity and is defined as the toxicity concentration of a waste divided by its LC50.

Toxicity Concentration (Tc) - Is the toxicity concentration of a waste divided by its LC50 (100/96-hr. LC50, %) and expressed in toxic units.