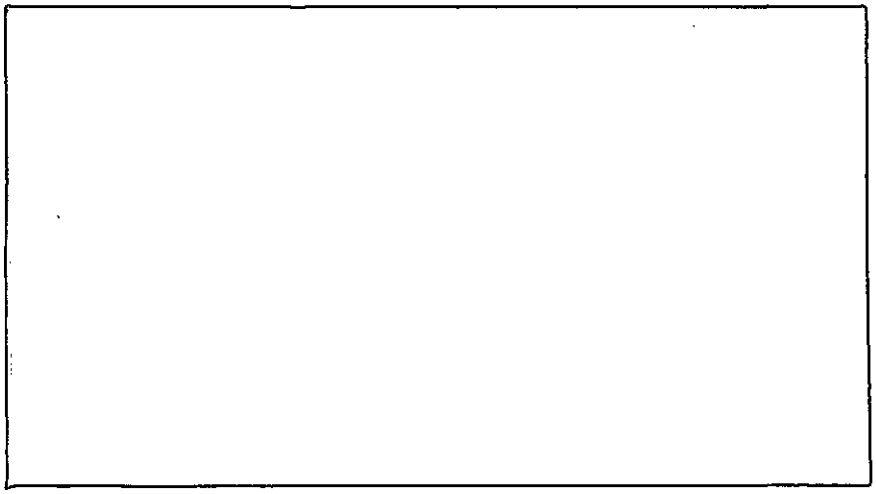
Environmental Engineering



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Acute Toxicity Tests: General Description and Materials and Methods Manual I. Fish

Env.Eng.Report No. 72-83-3

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April 1983

Acute Toxicity Tests: General Description and Materials and Methods Manual

I. Fish

by

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Submitted to the
Massachusetts Department of Environmental Quality Engineering
Division of Water Pollution Control
Anthony D. Cortese, Sc.D., Commissioner
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April, 1983

I. ACKNOWLEDGEMENT

Portions of this report have been excerpted from the following sources:

1. American Public Health Association (1975);
2. Peltier (1978);
3. Medeiros et al. (1981);
4. and Weber (1980).

The authors wish to express their thanks to the UMass graduate and UMass and Smith College undergraduate students, that have participated in the bioassay studies at the Environmental Engineering Program Laboratory, Department of Civil Engineering at the University of Massachusetts. Thanks are also extended to Ms. Dorothy Pascoe and Kevin Sheehan for their help in preparing this report.

The research, which helped in contributing to the information provided in this manual, was supported by the Massachusetts Division of Water Pollution Control, Research and Demonstration Project Number 80-32.

II. EXECUTIVE SUMMARY

Toxicity tests determine the concentration of a chemical or percentage of some complex waste which causes either death, or some altered physiological process reflecting interference with the normal life cycle of a test organism, and may be used to assess the impact of a pollutant on aquatic organisms in a receiving water. There are several types of fish toxicity tests including: 1) acute; 2) chronic; 3) subchronic embryo-larval; and 4) early juvenile toxicity tests; as well as 5) avoidance; 6) respiratory activity; and 7) blood chemistry tests. This report describes the principles, techniques, and calculation procedures used in acute toxicity testing with fish as the test organism. Particular methods are described for test protocol using fathead minnows (Pimephales promelas) as the test species. While acute toxicity tests may be conducted using either static, continuous-flow, or renewal procedures, the report emphasizes protocol for static fish toxicity testing. Such tests are conducted by exposing a population of test organisms to varying concentrations of toxicant over some time period and observing mortality at each toxicant concentration so that the LC50 (lethal concentration causing 50% mortality) may be calculated. An ILC50 value (incipient LC50 value below which 50 percent of the test organisms will not die from the toxicant stress even upon prolonged exposure) can also be calculated from acute toxicity data.

The report provides information about selection of test organisms, cleaning of facilities and equipment for toxicity testing, preparation and choice of appropriate dilution water, fish procurement, acclimation and

and prophylactic treatment, sampling procedures and test procedures for both screening toxicity tests and LC50 determinations. The report also describes general test conditions and protocol including temperature, filtration, lighting, feeding and toxicant concentration, as well as the interaction of dissolved oxygen, hardness, alkalinity, pH and ammonia on toxicity data. Methods for data analysis are presented including: 1) Log-concentration vs. percent survival; 2) Probit; and 3) Litchfield Willcoxon abbreviated methods. A computer program is also described which calculates LC50 values and associated confidence intervals. The applicability of such laboratory derived toxicity values to field conditions is addressed.

A listing of required equipment and supplies for conducting routine static fish toxicity tests for one year is presented along with 1982 costs which totaled about $18,000. While continuous flow toxicity tests may occasionally be preferred over static tests for wastes or chemicals having high biochemical oxygen demands or that are unstable or volatile, static toxicity tests usually provide comparable information about the toxicity of a chemical or sample waste as do flow-through tests. Static tests are therefore usually preferred since they are simple and easily controlled and are less capital and labor intensive than flow-through tests. Additionally, they do not sacrifice sensitivity, reproducibility, and applicability to field extrapolation.

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VI. GENERAL DESCRIPTION OF FISH TOXICITY TESTS

Toxicity tests determine the concentration of a chemical which causes either death, or some altered physiological process reflecting interference with the normal life cycle of the test organism. Toxicity tests have been performed by numerous investigators for well over a hundred years. Unfortunately, most of the data collected before 1951 are virtually useless due to the lack of a standardized technique that would allow comparison. In 1951, however, Doudoroff evolved such a protocol that details the procedure to be followed and emphasizes knowledge of test water quality.

Brown (1973) recognized three classes of pollutant materials that may be examined by toxicity tests:

1. Materials essentially chemical ("classically" associated with poisons): metal salts, hydrogen cyanide, etc.
2. Physical materials: mineral particulates, radionuclides, hot water, hypertonic solutions, etc.
3. Biological materials: virus, bacteria, etc.

Toxicity tests using fish as the test organism focus on the first class of contaminants which constitutes most of the pollution problems encountered in the aquatic environment. This is attributable to the large amount of *synthetic residue introduced into the environment and the inability of* most aquatic organisms to survive or continue their normal life functions in the presence of these substances.

Toxicity tests are conducted by exposing fish, invertebrates, or algal species to varying concentrations of toxicant for some period of time. Upon

termination of the test the percent mortality is observed at each toxicant concentration and the LC50 (lethal concentration causing 50 percent mortality) is calculated. The percent mortality at varying exposure times may be expressed using the LC symbol. For example, a 96-hour LC50 is that concentration causing a 50% mortality in 96 hours. The use of LC followed by a percentage number also allows the designation of percentages of mortality other than 50%. For example, one can determine a LC90 to remove undesirable species from fishing areas, or a LC10 to insure survival of fish exposed to industrial wastes. However, the LC50 is the standard measure of toxicity because the mean is a reliable measure of central tendency. LC50 is basically the same as the term LD50 (lethal dosage causing 50% mortality), which is used in pharmacology and mammalian toxicology. The converse of the LC50 term is the TLm (mean tolerance limit). This is the concentration, after some period of exposure, at which a survival of 50% is observed. An additional expression is the median effective concentration of EC50 used to designate a level that depresses some life function or process (reproduction, growth, etc.) by 50%.

Toxicity tests also permit the calculation of the toxicity threshold or incipient lethal level of the toxicant below which 50 percent of the test organisms will not die from the stress factor even upon prolonged exposure. This incipient lethal level is called the ILC50.

The use of fish bioassays can be of substantial value as part of an integrated approach to assessing the toxicity of pure compounds and complex mixtures that pollute national waters. Fish are natural inhabitants of aquatic systems and provide a direct measure of toxicity which cannot be determined with chemical analyses alone.

VII. TYPES OF FISH TOXICITY TESTS

There are several types of fish toxicity tests (see EPA, 1978 for more detailed information or categorization) including:

1. Acute toxicity tests determine the toxicant concentration causing 50% mortality in the test population within some defined time interval (usually 48 to 96 hours).

2. Chronic tests determine effects, other than death, on test populations. These adverse effects include growth inhibition, interference with reproduction, and other abnormal behavior. Chronic tests are conducted over extended periods of time (several weeks).

3. Subchronic embryo-larval, early juvenile toxicity tests are performed for 30 days and provide a maximum acceptable toxicant concentration (MATC).

4. Respiratory activity tests (stress tests) measure the gill purge (cough) and ventilation rates of fish as they are affected by the presence of a toxicant. This response is determined by an electrical amplification of the test organisms bioelectric signal during respiration.

5. Avoidance tests observe the test organisms ability to evade a concentrated toxicant regime in their aquatic environment.

6. Blood chemistry tests determine the blood levels of toxicants subsequent to the exposure of test organisms to varying toxicant concentrations.

Each of these tests provide different information about the effects of a toxicant. The Environmental Engineering Laboratory at UMass/Amherst has been involved with acute toxicity testing for several years. This type of toxicity test is particularly useful since it is easy to carry out within a reasonable time period and cost.

The purpose of this manual is to provide information about the methods and techniques used for acute toxicity testing, necessary calculations (including a computer program for calculation of LC50 values),

enumeration of personnel hours and equipment needs and costs. In addition, *in situ* application of lethal concentration values (LC) are addressed. A general flow diagram for acute toxicity testing is presented in Figure 1. Each of these steps is discussed separately in the report.

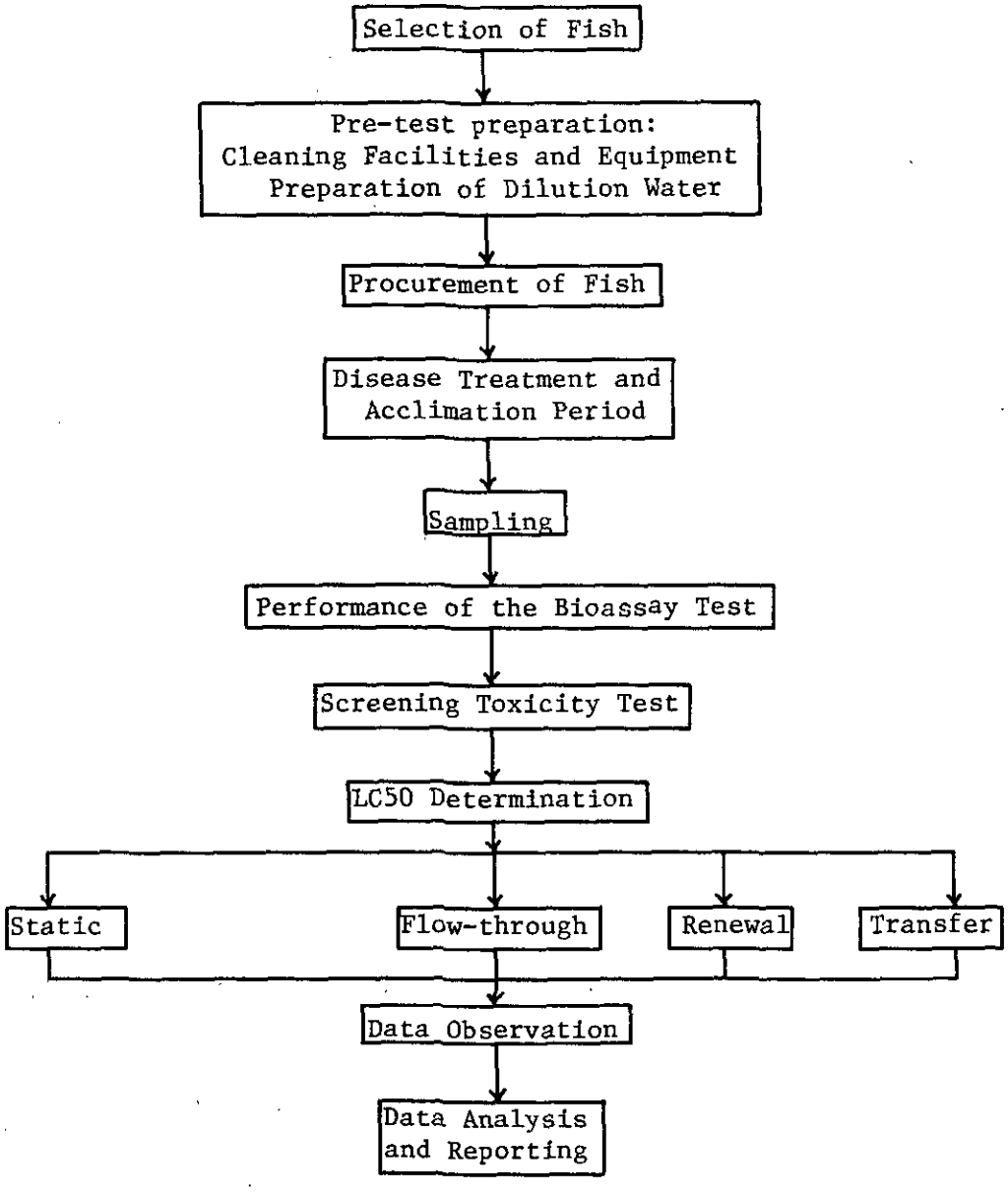

Figure 1. Flow Diagram of the General Acute Toxicity Test Procedure

VIII. ACUTE FISH TOXICITY TEST PROCEDURE

Selection of Test Organisms

Test animals should be adaptable to laboratory conditions and available in adequate numbers of a usable size (less than 2 grams fish/liter test solution). When possible, the test animals should preferably be native to the watershed under investigation, and be a species of recreational or commercial value.

APHA (1975) lists additional factors that should be considered in choosing a test organism that include:

1. their sensitivity to the materials or environmental factors under consideration;
2. their geographical distribution, abundance and availability throughout the year;
3. availability of culture methods and knowledge of their environmental requirements;
4. their general physical condition and freedom from parasites and diseases;
5. limitations of space and time;
6. consideration of the size of the organism and the length of its life cycle (Note that some studies require large organisms with long life cycles.); and
7. their amenability to comparison with other toxicity data obtained in previous research or other toxicity investigations.

In many instances it is necessary to use a species that is sensitive to most toxicants but not found in the receiving water. Non-indigenous species may be used for the following reasons:

1. Data rarely exist as to which species indigenous to a given receiving water is the most sensitive species.

2. Many times, because of the impact of the effluent on a receiving water, all sensitive resident species may have been previously eliminated and only extremely pollution tolerant species remain. Using the remaining species would give an unrealistic estimate of effluent toxicity.

3. A sensitive indigenous species may not be commercially available for use in tests or their culturing requirements may not be known.

4. Test organisms should not be used if they have been exposed to pollutants or other stress. The history of these organisms collected from a receiving water would be unknown. They could have previously been exposed to pollutants and, therefore, tests could provide erroneous results.

The length of the test organisms should not vary by more than 50 percent between the smallest and the largest. For fish, researchers contend that a variation of more than a few millimeters is too much because the weight of a fish increases as the cube of its length. A natural population of fish, however, is varied in size, and the application of toxicity data derived from a very uniform group of fish to problems involving natural populations may be questionable. The test animals must also belong to the same age as well as size class. Immature specimens are commonly used because differences attributable to sex are minimized.

The mean length and weights of the test organism must be determined. Weighing may be accomplished by placing 10 randomly selected fish in a preweighed beaker containing dilution water. Care must be taken not to add additional water to the beaker in the process of transferring the fish from the holding tank into the beaker. The beaker is then reweighed. The difference between the two weight values divided by the number of fish is then equal to the mean fish weight. Alternatively, several randomly selected fish may be sacrificed and then weighed and measured.

A large variety of fish species have been used by investigators in toxicity testing. Recommended species and associated test temperatures (Peltier, 1978) are presented in Table 1.

The fathead minnow (Pimephales promelas) has been tested by many researchers providing a large data base. Fathead minnows are also known to be sensitive to most toxicants.

Table 1 Recommended Fish Species Used in Toxicity Testing[1]

Fish Species	Test Temperature (^{o}C)
Coho salmon, Oncorphynchus kisutch	12
Rainbow trout, Salmo gairdneri	12
Brook trout, Salvelinus fontinalis	12
Goldfish, Carassius auratus	22
Fathead minnow, Pimephales promelas	22
Channel catfish, Icatalurus ponctatus	22
Bluegill, Lepomis macrochirus	22

[1]From Peltier (1978)

Cleaning of Facilities and Equipment

Prior to receipt of test organisms for toxicity testing, it is crucial that all vessels used in the test are sufficiently free of interfering contaminant chemicals. All test containers, glassware, tubing and other equipment used to perform the bioassay, whether new or used, therefore must be cleaned in the following manner:

A. Wash with warm water and synthetic detergent made especially for laboratory glassware (e.g. Alconox or Liquinox).

B. Rinse thoroughly with warm tap water.

C. Rinse with fresh 20 percent hydrochloric acid to remove metals and bases.

D. Rinse with warm tap water.

E. Soak in 10 percent chlorox for about 30 minutes. When pesticide contamination is suspected, test containers must be rinsed with acetone prior to step A above.

F. Rinse thoroughly with tap water.

G. Rinse five times with distilled water.

Glassware used for analytical determinations may require special cleaning procedures appropriate to the particular analysis.

Preparation of Dilution Water

Prior to receiving fish used in toxicity testing, dilution water must be prepared so that there is no delay in transferring the test organisms to the holding aquarium for the required acclimation period. Dilution water is used both during the acclimation period as well as for diluting effluent or toxicant additions when performing bioassay tests. Dilution water can consist of either reconstituted distilled

water (Table 2) or natural water sampled either upstream from the pollutant discharge or from some other relatively uncontaminated source.

Dilution water is acceptable if healthy test organisms survive in it through the acclimation period (holding tank) without showing signs of stress, such as discoloration or unusual behavior (e.g. abnormal swimming). For effluent toxicity testing, the dilution water should be a representative sample of the receiving water, and should be obtained from a point as close as possible to, but upstream or outside of the zone influenced by the effluent. For flow-through bioassays (described in a later section) it is preferable to pump the dilution water continuously to the acclimation tank and dilutor. However, it may be more practical to transport batches of water in tanks to the testing site as the need arises, and then continuously pump water to these systems.

Table 2 Preparation of Reconstituted Waters[a]

2a. Quantities (mg/l) of reagent grade chemicals required to prepare recommended reconstituted fresh waters and the resulting water qualities.

Water Type	Reagent Added					Final Water Quality		
	$NaHCO_3$	$CaSO_4$	$2H_2O$	$MgSO_4$	KCL	pH[b]	Hardness[c]	Alkalinity[c]
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 8.0	7.6-	160-180	110-120
Very hard	384	240.0	240.0		16.0	8.0-8.4	280-320	225-245

2b. Quantities of reagent-grade chemicals to be added to aerated, soft reconstituted freshwater for buffering pH. The solutions should not be aerated after addition of these chemicals.

pH[d]	Volume (ml) of solution added to 15 liters of water		
	1.ON NaOH	1.0 m KH_2PO_4	0.5 m H_3BO_3
6.0	1.3	80.0	--
6.5	5.0	30.0	--
7.0	19.0	30.0	--
7.5	--	--	--
8.0	19.0	20.0	--
8.5	6.5	--	40.0
9.0	8.8	--	30.0
9.5	11.0	--	20.0
10.0	16.0	--	18.0

[a]From Peltier (1978)

[b]Approximate equilibrium pH after aeration and with fish in water.

[c]Expressed in mg/l as $CaCO_3$.

[d]Approximate equilibrium pH with fish in water.

The same water source should be used for holding tank water and test dilution water.

Pretreatment of the dilution water should be limited to filtration through a nylon sieve having 2 mm or larger holes to remove debris and/or break up large floating or suspended solids. The water should be obtained from the receiving water as close as possible to the time the test begins. It should not be obtained more than 96 hours prior to testing. If acceptable dilution water cannot be obtained from the receiving water, some other uncontaminated, well-aerated surface or ground water, or reconstituted water can be used.

Reconstituted water is generally used at the University of Massachusetts Environmental Engineering Laboratory since it contains known concentrations of chemicals and is very accessible. Additionally, toxicity tests are reproducible when using reconstituted water and results are easily compared to results of other investigations since water quality parameters specific to a particular natural water can be avoided. The major disadvantage of using reconstituted water is applying the findings of a test to a particular water body that may contain chelators, metals, or organic compounds that may increase or decrease the toxicity of an effluent.

Reconstituted water should ideally have a total hardness, total alkalinity and specific conductance within 25 percent, and pH within 0.2 units, of the actual receiving water at the time of testing. Holding tank water should be sampled weekly for these parameters which may change due to evaporative loss or reaction with fish waste metabolites. These water parameters should be kept constant by dilution with distilled water or

addition of appropriate chemicals (Table 2). Most inland waters of New England are soft, with associated low alkalinities (Frey, 1971), therefore the Environmental Engineering facility generally uses soft reconstituted water as the dilution water.

The chemicals used for reconstituting water should be dissolved in 8-12 liters of water prior to adding the chemicals to the holding tank. $CaSO_4 \cdot 2H_2O$ and $MgSO_4$ should be dissolved in separate vessels to increase solubilization of these salts. Actual concentrations of chemicals added to reconstitute very soft, soft, hard and very hard water types are presented in Table 2.

Procurement of Fish

Fathead minnows may be cultured under laboratory conditions or can be readily obtained from the Newtown Toxicology Station (EPA, Cincinnati, Ohio), Joe's Bait House located in Windsor Locks, Connecticut, and other biological supply companies (see Appendix D). Since the test organisms were not cultured in this laboratory, this report will not address the methods needed for culturing fish. Methods for fish culturing are provided in Brauhn and Schoettger (1975).

Fish should be shipped in polyethlyene bags in secure containers such as cardboard boxes (About 100 fathead minnows/gallon that are 1-2 grams will survive several hours before oxygen depletion becomes significant.). Transportation time should be minimized as much as feasible since the fish are being stressed by crowding.

Immediately upon arriving at the laboratory, the fish should be transferred to the holding tank. This is accomplished by floating the bag

containing the fish in the holding tank water until temperature equilibrium is achieved. The fish may then be slowly released into the tank. Water contained in the holding tank should be prepared at least 24 hours prior to the receipt of test fish. Fathead minnows which have been raised in hard water may be safely transferred into softer water once temperature equilibrium has been achieved (Pickering, personal communication; Plotkin, personal observation).

Acclimation Period

Fish should be allowed to acclimate at least ten days but preferably 30 days before a test begins. Peterson and Anderson (1969) concluded that the acclimation of metabolism to temperature change takes at least two weeks regardless of direction. Presently, it seems the longer the period the better; however, variations in other conditions associated with temperature changes in the laboratory may make this approach impractical. At any rate temperature fluctuations should not exceed $3^{\circ}C/12$ hours (Peltier, 1978).

Aquaria used to hold the fish during the acclimation period should ideally be flow-through systems with a flow of at least two tank volumes per day (Peltier, 1978). A recirculating system (such as that used by the University of Massachusetts Environmental Engineering Laboratory) may also be employed. The Environmental Engineering tank holds 400 liters and recycles every 1.5 minutes. It was purchased from Frigid Units located in Toledo, Ohio. The system consists of a two chambered filter. One chamber contains polyester fiber and the other chamber is filled with activated carbon plus some ammonia removing pellets. The pellets remove the ammonia, from the water, which the fish release as metabolic waste products. The

filter media should be renewed at least once per month in order to maintain adequate water quality for fish survival. The filter precludes the necessity of changing the water itself. Particulates not removed by filtration should be removed once a week by syphoning with acid washed nylon tubing. The Frigid Units holding tank will accommodate about 1 gram of fish per liter (about 400 fish 2 to 4 cm in length) without causing stressful crowding.

Alkalinity, hardness, pH and conductivity should be kept constant (see dilution water section). A plexiglass shield should be placed over the section of the tank near the water recirculating pump to prevent water splashing out of the tank. Distilled water can be added as needed to replace evaporative loss.

During the acclimation period fish should be fed at least once a day. Feed may consist of either dry constituents such as "Tetra Min Staple Food" (obtained at fish supply stores), or may be supplemented with live food such as Daphnia. It has been observed that fathead minnows remain very healthy over periods of many months feeding upon only the "Tetra Min Staple Food" (Plotkin, personal observation).

Food should be introduced into the holding tank in a quantity such that the entire amount is eaten within five to ten minutes. Food remaining after this time will usually not be eaten and may have a deleterious effect upon the water quality by promoting microbial growth in the tank. Feeding should be terminated, two days prior to the initiation of a test.

Test organisms should not be used for a test if they appear to be stressed, diseased or if there is more than five percent mortality during

the 48 hours prior to the performance of a bioassay test. Diseased or impaired organisms should be removed immediately and discarded.

Disease Treatment

During the two week acclimation period, fish should be chemically treated to cure or prevent disease. A number of products are available for an assortment of problems. If tanks are kept clean and water periodically changed, disease generally will not be a problem. If severe disease occurs the entire lot should be discarded. Antibiotic treatment of fish during the acclimation period is a good procedure to destroy pathogenic bacteria and is therefore a recommended preventative measure. Tetracycline at a concentration of 15 mg/l for 24-48 hours will kill most bacterial pathogens. Other prophylactic and therapeutic treatments are presented in Table 3. A review of icthyological treatments for diseases and parasites is presented in APHA (1975).

Table 3 Recommended Prophylactic and Therapeutic Treatments for Freshwater Fish[a,b]

Disease	Chemical	Concentration (mg/l)	Duration of Treatment[c]
External Bacteria	Oxytetracyclina hydrochloride (water soluble)	25[d]	30-60 min
	Procaine Penicillin G in Dihydrostreptomycin sulfate solution (Franklin Lab, Denver, CO)	(3 ml/100 gal)	48-72 hrs
	Benzalkonium chloride (HYAMINE 1622)	1-2[d]	30-60 min
	Nitrofurzone (water mix)	3-5[d]	30-60 min
	Neomycin sulfate	25	30-60 min
Monogenetic trematodes fungi, and external protozoa[e]	Formalin plus zinc-free malachite green oxalate	25 0.1	1-2 hrs
	Formalin	150-250	30-60 min
	Potassium permanganate	2-6	30-60 min
	Sodium chloride	15000-30000 2000-4000	5-10 min dip (f)
	DEXON^{R} (35% Active Ingredient)	20	30-60 min
Parasitic copepods	Trichlorofon (MASOTEN^{R})	0.25[d]	Continuous[g]

[a]From Peltier (1978)

[b]This table indicates the order of preference of treatments that have been reported to be effective, but their efficacy against diseases and toxicity to fish may be altered by temperature or water quality. Caution: test treatments on small lots of fish before making large-scale applications. Fish should not be treated the first day they are in the facility. Before using a treatment other than those listed in this table, additional information should be obtained from sources such as Davis (1953), Hoffman and Meyer (1974), Reichenbach-Klinke and Elkan (1965), Snieszko (1970), and Van Duijn (1973).

[c]Treatment may be accomplished by (1) transferring the fish to a static treatment tank and back to a holding tank; (2) temporarily stopping the flow in a flow-through system, treating the fish in a static manner, and then resuming the flow to flush out the chemical; or (3) continuously adding a stock solution of the chemical to a flow-through system by means of a metered flow or the technique of Brungs and Mount (1967).

[d]Active ingredient.

[e]One treatment is usually sufficient except for Ichthyopchirius ("Ich"), which must be treated daily or every other day until no sign of the protozoan remains. This may take 4-5 weeks at 5-10°C and 11-13 days at 15-21°C. A temperature of 32°C for one week is lethal to "Ich."

[f]Minimum of 24 hours, but may be continued indefinitely.

[g]Continuous treatment should be employed in static or flow-through systems until no copepods remain, except that treatment should not be continued for more than 4 weeks and should not be used above 27°C.

Sampling

Sampling of an effluent for bioassay is based upon short and long term operation of the dischargers. To assess the maximum amount of damage by a discharge, samples should be taken during a period when the effluent is most concentrated or toxic. If this cannot be easily assessed, then the following guidelines should be followed.

For complex toxicant solutions, such as municipal or industrial wastewaters, bioassay testing is best performed at the site of the wastewater using a continuous sampling technique and a continuous-flow testing procedure. The various types of bioassay, along with the rationale used in choosing a particular testing procedure is discussed in another section of this report. For continuous-flow on-site bioassays, the effluent is pumped directly and continuously from the discharge line to the dilutor system for the duration of the test. However, if the effluent cannot be

pumped directly and continuously to the dilutor system, then either a static or continuous-flow bioassay can be performed in a bioassay laboratory which is not necessarily located at the site of the wastewater discharge. In this case either a static or continuous-flow "off-site" toxicity test can be performed. The following guidelines should be followed for collection of the effluent.

a. When the measured minimum retention time of the effluent is less than 96 hours, as determined from dye studies, a 6-hour composite sample, consisting of equal volumes taken every 30 minutes, may be collected and transported to the dilutor every six hours for the duration of the test.

b. When the measured minimum retention time of the effluent is between four days (96 hours) and 14 days, as determined from dye studies, then a 24-hour composite sample, consisting of equal volumes taken every hour, may be collected daily for the duration of the test.

c. When the measured minimum retention time of the effluent is greater than 14 days, as determined from dye studies, a single grab sample may be collected daily for the duration of the test.

If the industrial or municipal facility discharges intermittently (i.e. where the waste is discharged at the end of the shift, or end of the week), a composite sample, consisting of equal volumes collected every 30-minutes, may be taken for an 8-hour operating shift or for the duration of the plant operating schedule, or a single grab sample may be taken in the case of a batch discharge.

Samples normally should not be altered except by filtering through a

teflon or stainless steel screen with 2 mm or larger openings. The test should begin as soon as possible but no later than 24 hours after collection of the effluent. Refrigeration does not seem to effect the toxicity of a complex effluent stored over several days (K. Macek, personal communication).

For static bioassays about 60 liters of effluent are required. Samples should be collected in pre-acid washed glass containers. Empty acid gallon bottles are a good type of sample container. Samples should be stored on ice in transit and acclimated to the temperature of the dilution water upon return to the laboratory.

Any unusual conditions to which the test group were exposed, e.g. pesticides or chemotherapeutic agents, should be considered and reported (APHA, 1975). Additionally, such standard water quality parameters such as temperature, dissolved oxygen, pH and conductivity should be determined at the time of sampling. Additional tests may be required depending on the nature of the sample and the objective of the assay.

Evaluation of Acute Toxicity

There are two steps required to evaluate the acute toxicity of a sample:

1. determination of the range of toxicant dosage which results in an observable response; and
2. determination of acute toxicity using toxicant dosages applied over a narrower range.

Screening Toxicity Tests

Ranging Oxygen Test:

The first step is a qualitative screening procedure used to assign a range of toxicity. One such procedure is the Ranging Oxygen Test. Several fish are placed into BOD (Biochemical Oxygen Demand) bottles containing fully aerated dilution water (control) or dilution water plus some level of toxicant. 100, 10, 1, .1, .01, and .001 percent of the toxicant or waste effluent is recommended. The bottles are then sealed and left until 100 percent fish mortality is observed. Care should be taken when sealing the BOD bottles to avoid entrainment of air bubbles. The dissolved oxygen concentration in each BOD bottle is determined immediately after 100 percent mortality is obtained. The DO values in BOD bottles in which fish mortality is attributable to oxygen depletion alone will be on the order of tenths of a mg per liter. DO values in BOD bottles in which fish mortality is attributable to the level of toxicant will be higher since mortality will occur prior to oxygen depletion. Thus DO levels in BOD bottles containing higher toxicant concentrations will be greater than those containing lower toxicant concentrations. If final DO concentration is plotted against the log of the toxicant concentration, an "inflection point" is observed where the DO concentration increases with increasing toxicant level. Toxicant levels just below and above this point are then tested over a finer incremental dosage range for the 96-hour LC50 determination. The incremental range is distributed over a logarithmic interval owing to the exponential response of test organisms to toxicant dosage.

Screening Jar Test

Alternatively, a 24-hour screening jar test can be performed over a wide range of toxicant levels. This is usually performed using ten-fold dilutions of the toxicant from 100 to .001 percent and observing 100 percent mortality and 100 percent survival of two test organisms at different toxicant levels after a 24-hour exposure period. The finer incremental toxicant dosage range to be used for the 96-hour LC50 toxicity test is then determined. This range occurs between the highest toxicant concentration resulting in 100 percent survival and the lowest toxicant concentration resulting in 100 percent mortality.

LC50 Determination

The second stage of evaluation is the determination of the 96-hour LC50 or ILC50. This is a test performed with two replicates per concentration and ten fish per aquarium. The range of toxicant concentration required to observe the 96-hour LC50 value is determined using one of the qualitative screening procedures just described.

Acute toxicity determination using the bioassay test employs replicates of a control and at least five concentrations of effluent or toxicant in an exponential series. To determine the acute toxicity of a sample with reasonable accuracy, a definitive test must meet both of the following criteria:

a. Each concentration of the effluent or toxicant must be at least 50 percent of the preceding concentration.

b. One concentration must have killed (or affected) more than 65 percent of the organisms exposed to it, and one concentration must have killed (or affected) less than 35 percent of the organisms.

If 100 percent effluent does not kill (or affect) more than 65 percent of the organisms exposed to it, the percentage of organisms killed (or affected) by various levels of the effluent in the receiving water must be reported.

A control, of diluent water (preferably ground or synthetic water) without any toxicant, must be employed as a point for comparison. There should be no more than ten percent mortality among the control animals during the course of any valid test with the remaining 90 percent appearing vigorous. When an organic solvent or other dispersing agent is used to prepare test solutions, the test control should contain the maximum concentration of the solvent or dispersant to which organisms in the other solutions are exposed.

At least 20 (10 per replicate) fish should be used for each experimental concentration. A limit must be placed, however, on the weight of the fish per liter of test solution to help minimize depletion of oxygen and excessive build-up of metabolic waste products. For flow-through tests, the number of test organisms should not exceed 5 grams/liter at $20^{o}C$ or about 3.5 grams/liter for temperatures above $20^{o}C$. The number of test organisms in static test containers should not exceed 0.8 grams/liter/day at $20^{o}C$ or about 0.4 grams/liter/day at temperatures above $20^{o}C$.

Very immature fish (not actively feeding on exogenous food) or spawning fish should not be used. Fish weighing between 0.5 and 5.0 grams each are preferred. In any single test, all fish should be taken from the same year-class, and the total length (tip of snout to end of tail) of the longest fish should be no more than 1 1/2 times that of the shortest one.

Test animals are normally captured for transfer from acclimation tanks to test chambers by dip-netting. No more than 20 percent of the total number of organisms transferred to each chamber should be added from a given net capture.

Data Observation

Fish mortality or abnormal behavior such as erratic swimming, cannibolism or discoloration should be observed and recorded at frequent time intervals to obtain data for the ILC50 determinations. Table 4 provides a suitable schedule for mortality observations during a 96-hour test. A test fish is considered dead if there is no gill movement and the organism does not respond to gentle proding. Dead organisms should be immediately removed from the test solution. Procedures for determining the toxic level of the material being tested are described in the "Data Analysis" section of this report. The duration of all tests should be at least 48 hours (96-hour tests are preferable). When more than half of the test fish survive at the highest test concentration, the test must be continued for at least a total of 96 hours.

Table 4

Frequency of Mortality
Observations for a
96-Hour Bioassay

Time Interval*	Observation Interval*
0-12	2
12-24	4
24-48	8
48-96	12

*Units in hours

IX. TYPES OF LC50 ACUTE TOXICITY TESTS

The determination of the 96-hour LC50 or ILC50 can be evaluated using several experimental approaches with respect to the flow of the toxicant and diluent solutions. These are classified into the three groups: static, continuous flow, and renewal bioassays. A comparison of continuous flow and static toxicity tests will be the topic of a future report to the Massachusetts Division of Water Pollution Control.

Static toxicity tests are simple and easily controlled. They are suitable for the detection and evaluation of compounds whose toxicity is associated with low oxygen demand, and low concentrations of volatile compounds. The major limitations with static toxicity tests are: (1) the toxic substance being tested may be removed by volatilization, precipitation, or detoxification by the test organisms, and (2) the test must be relatively short term because of the stress and accumulation of metabolic byproducts.

Test containers for the static test should ideally be made of glass. Containers made of nontoxic and nonreactive material are also acceptable. Containers should be at least 20 cm deep to allow for a minimum water depth of 15 cm. Suitable containers such as wide-mouthed five gallon glass jars can be obtained from Empire State Bottle Company, Buffalo, N.Y. (see Appendix B).

For static bioassay tests, the toxicant should be added to the test containers and completely mixed with a clean stirring rod prior to the addition of the fish.

Flow-through toxicity tests are more complicated in design, but new developments in apparatus and techniques have made their usage practical. They

are used mainly to test chronic toxicity of industrial effluents and chemicals that have high biochemical oxygen demands, are unstable or volatile, or are removed appreciably from solution by precipitation on the test organism. The protocol provides for well-oxygenated solutions, non-fluctuating concentrations of the toxicant, and removal of metabolic byproducts. Furthermore, it more closely approximates the natural conditions of receiving streams, and thus permits extended exposures to detect chronic toxicity and to identify "safe" concentrations of the toxicant. The shortcomings inherent in the flow-through approach are: (1) the large amount of space needed, (2) the complicated experimental apparatus, and (3) the large amounts of wastes and diluent water needed.

Unlike static tests, the protocol for flow-through testing permits feeding without danger of fouling or toxicant interaction. This allows for the testing of organisms over their entire life cycle as well as for determination of mortality-abnormality effects.

Test chambers used in flow-through tests may be constructed of 1/4 inch plate glass held together with clear silicone adhesive. Silicone adhesive does adsorb organochlorine and organophosphorus pesticides, which may subsequently desorb into uncontaminated solutions. Therefore, as little of the adhesive as possible should be in contact with water. Extra beads of adhesive inside the containers should be removed.

Alternatively, a stainless steel system can be employed. Joints should be welded rather than soldered. The containers must be designed to permit the desired flow-through characteristics of the test.

Toxicant and dilution water can be stored in 100 gallon polyethylene

containers. The appropriate volumes of toxicant and dilution water can be delivered to test containers via tygon tubing and positive displacement pumps. Alternatively, an inexpensive serial dilution apparatus such as that developed by Mount and Warner (1965) can be employed. In such an apparatus, incoming water is mixed with the toxicant at a specified proportion by a device called a diluter. The modern apparatus is equipped with a fail-safe which shuts off toxicant release when diluent stops or flow rate diminishes appreciably. This method assures a constant concentration of toxicant and O_2 in tested water regardless of volatility or oxygen demand. The UMASS bioassay facility employs stainless steel tanks and positive displacement pumps for toxicant and diluent water delivery.

The continuous flow apparatus for flow-through bioassay testing should be tested for 24 hours prior to adding toxicant or fish to assure flow rates for the toxicant and dilution water are constant. The turnover rate for the flow-through water should be at least six times per day.

Renewal toxicity tests are considered as intermediate between static and continuous flow. They differ from the static test in that the test solution is occasionally renewed. Here, organisms can be fed without accumulation of metabolites and excess food. The test concentration is brought back by replacement of the water, not as gradually as the continuous flow, but by abrupt increases.

Renewal of test solution for the purpose of maintaining more or less uniform concentrations of any volatile or unstable toxic materials, and an adequate DO (dissolved oxygen) can be accomplished by transferring the

test animals quickly to fresh solutions. Test solutions, for such toxicants, must be renewed every 24 hours or less.

Transfer Toxicity Tests are a modification of renewal tests and are used to vary the concentration of toxicant to which the fish are exposed. Fish may be transferred alternately by dip-netting between high and low concentrations of toxicant solutions over designated time intervals. This type of test can also be performed with a flow-through system by varying the proportions of incoming toxicant and dilution water. These tests provide toxicity information that in some instances may be more applicable to actual field conditions where toxic effluents are discharged intermittantly.

The types of toxicty tests discussed above may be modified by the inclusion of a circulatory pump to simulate flowing water and keep the animals active.

X. GENERAL TEST CONDITIONS AND PROTOCOL

Physical and Chemical Data

The number of analytical tests necessary depend upon the nature of the toxicant(s) being tested. Analyses that should be performed in all tests and the methods for their determination are presented in Table 5. Other constituents of the water which markedly influence the toxicity of the material being tested should be additionally monitored. The BOD of waste materials having a high oxygen demand should also be determined. Determination of these water quality constituents are normally made before introducing the fish, and again, at the end of the test (except DO and pH as noted below).

Dissolved Oxygen

Dissolved oxygen levels in the dilution water in the holding tank at the start of a bioassay test should be at or near saturation (aeration may be needed). Care should be taken however not to supersaturate the solution. In most cases aeration should not be employed during a test since this might alter the results. However, DO concentration should not be allowed to fall below 40 percent of saturation for warm-water species or 60 percent of saturation for cold water species. If the DO concentration falls below the appropriate level, the test organisms may be additionally stressed, therefore aeration may be needed, and in the flow-through bioassay test, the turnover rate of the solutions may also be increased in the case of waste materials having a very high BOD.

Table 5

Recommended Water Quality Parameters to be Determined During Bioassay Testing

Parameter	Method	Reference
Dissolved Oxygen	Oxygen Meter (YSI Instrument)	--
Hardness	EDTA Titrimetric Method	EPA (1979)
Alkalinity	HCl Titrimetric Method	EPA (1979)
pH	pH Meter that reads 0-14 pH units 0.1 increments	--
Specific Conductance	YSI Meter and Probe	--
Temperature	Mercury Thermometer ($0^{o}C$-$100^{o}C$)	--
Ammonia	Orion Auto-analyzer and Probe or Scaled Down Indophenol Method	Ram (1979)

Hardness, Alkalinity, pH, and Specific Conductance

When the dilution water used is reconstituted or taken from a source other than the receiving water of the effluent, the experimenter must decide what level of hardness and alkalinity is desirable. For example, if an effluent being tested normally discharges into a soft, poorly buffered stream, then the reconstituted water should have about the same pH, alkalinity, hardness, and specific conductance as the receiving stream. Various formulas for reconstituted water have been previously presented in Table 2.

Ammonia

Ammonia tends to increase in concentration during a static 96-hour test as a result of fish metabolic activity. This should be monitored by the method presented in Table 5. It is the un-ionized portion of total ammonia (NH_3) that is toxic to fish. By measuring the pH and total ammonia concentration, the un-ionized fraction can be determined using Table 6. If the un-ionized ammonia level approaches a harmful level for the species being tested, the water should be changed. For fathead minnows, a guideline for the maximum allowable un-ionized ammonia concentration during acute bioassay testing is 5 percent of the 96-hour LC50 value equal to 1.59 mg/l as reported by DeGraeue et al. (1980). These investigators determined this LC50 value using water containing a hardness of 655 mg $CaCO_3$/l. Since toxicity generally decreases with hardness, the ammonia LC50 may be higher using softer water.

Table 6 Percentage of Ammonia that is Unionized in Distilled Water at Various Temperatures and pH Values

Temperature (°C)	Percent Unionized Ammonia								
	pH								
	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0
7	0.01	0.05	0.15	0.46	1.45	4.44	12.8	31.7	59.5
12	0.02	0.07	0.22	0.68	2.13	6.44	17.9	40.8	68.5
17	0.03	0.10	0.32	1.00	3.08	9.14	24.1	50.1	76.1
22	0.04	0.14	0.45	1.43	4.39	12.7	31.5	59.2	82.1
27	0.06	0.21	0.65	2.03	6.15	17.2	39.6	67.4	86.8

Temperature

Generally the temperature of the water must be kept at 25°C ± 4°C for warm-water fish and 15°C ± 4°C for cold-water species. Fathead minnows have a recommended test temperature of 22°C (Peltier, 1978).

Filtration

Filtration may be necessary when an effluent is very turbid due to the presence of particulate material. The particulate material may interfere with fish respiration. In some instances continued precipitation occurs after initial filtration. Slow settling and mixing induced by the swimming action of the fish may keep the particles suspended. In very turbid solutions these particulates may interfere with observing fish mortality. To help avoid this problem, a one-fourth inch mesh polyethylene screen can be situated several inches above the bottom of the test containers to prevent the fish from contacting the precipitate and resuspending the settled material into the supernatant solution.

Lighting

Ambient light intensities should be 50-100 ft-candles. A day/night cycle is important with a minimum of at least eight hours of illumination per day. The UMass Environmental Engineering lab maintains a cycle of 14 hours illumination and 10 hours darkness.

Toxicant Concentration

Concentrations of diluted municipal or industrial wastes are expressed as percent by volume, while concentrations of non-aqueous wastes or individual

chemicals are expressed in terms of milligrams per liter (mg/l) or micrograms per liter ((µg/l). These concentrations are conveniently expressed on a logarithmic scale (see "calculation" section).

The inclusion of any water of hydration as part of the weight of the solute, i.e., $CaSO_4 \cdot 2H_2O$, should be clearly indicated. When an impure chemical is used, all ingredients should be reported. The range of toxicant concentration to be examined by the full-scale tests should include at least two replicates of five or six concentrations. This range must first be determined by exploratory tests as previously described.

Feeding

Fish are not fed during the static bioassay test since feeding might cause fouling of the test solution. Feeding is additionally terminated in the holding tank two days prior to conducting a static bioassay. Feeding is permitted during the flow-through test although this is not required during short testing intervals such as a four day acute toxicity study.

Feeding is necessary in tests lasting longer than 96 hours such as in chronic flow-through testing.

Table 7 Corrected Values of 0% or 100% Effect

Expected Value	Corrected Value 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	7.0	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	90.0	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	96.9	97.1	97.4	97/8	98.0	98.4	98.7	99.0	99.3	99.7

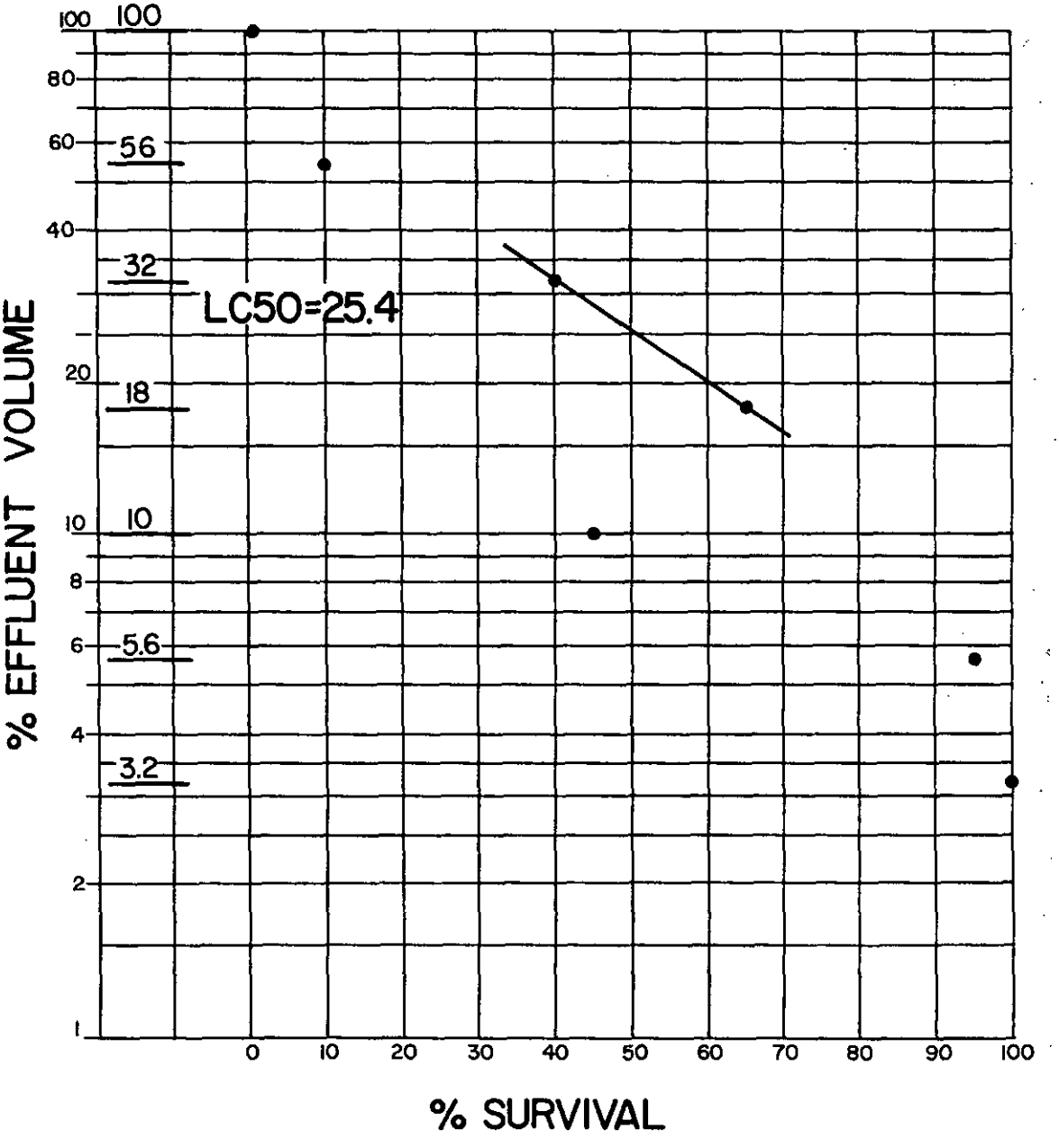

Figure 2. Plotted data and fitted line for log-concentration versus percent-survival method.

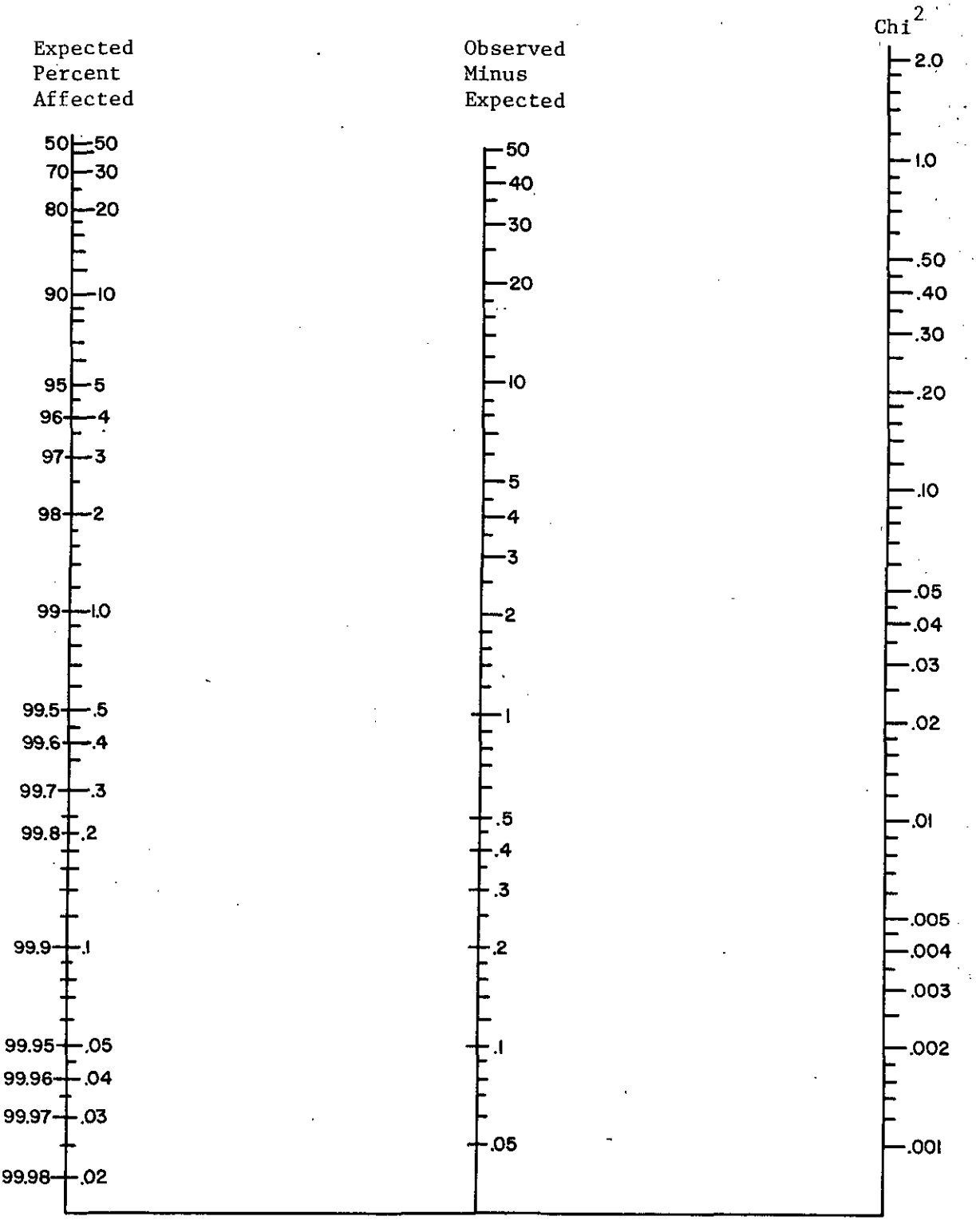

Figure 3. Nomograph for obtaining Chi^2 from expected-percent- and observed-minus-expected values (Step 4).

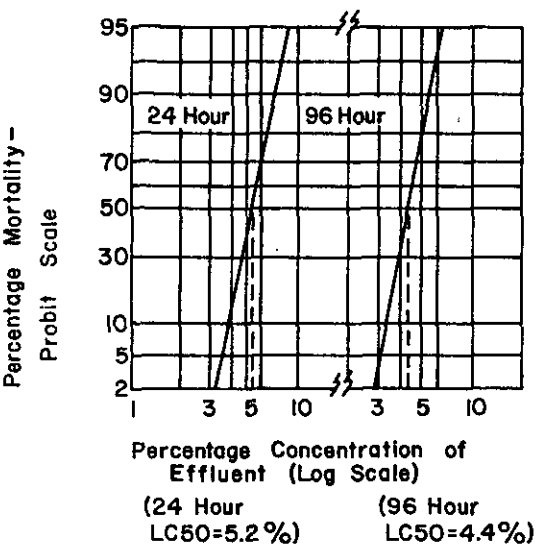

Figure 4. Examples of determining median lethal concentrations at two representative times by probit analysis and the line of best fit.

the Chi^2 scale, the contribution to Chi^2. Sum the contributions to Chi^2 and multiply the total by the average number of organisms per effluent volume (i.e., the number of organisms used in K concentrations divided by K, where K is the number of percent-affected organism values plotted). The product is the "calculated" Chi^2 of the line. The degrees of freedom (N) are two less than the number of points plotted, i.e. N=K-2. If the calculated Chi^2 is less than the Chi^2 given in Table 8 for N degrees of freedom, the data are non-heterogeneous and the line is a good fit. However, if the calculated Chi^2 is greater than the Chi^2 given in Table 8 for N degrees of freedom, the data are heterogeneous and the line is not a good fit. In the event a line cannot be fitted (the calculated Chi^2 is greater the the tabular Chi^2), the data cannot be used to calculate the confidence interval around the LC50. Litchfield and Wilcoxon provided an alternate method for calculating the 95 percent confidence limits under these circumstances. However, the toxicity test should be repeated.

Step 5: Determine the confidence limits of the LC50.

a. Read from the fitted line the percent effluent volumes for the corresponding 16, 50, 84 percent effects (LC16, LC50 and LC84).

b. Calculate the slope function, S, as:

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

c. From the tabulation of the data determine N', which is defined as the total number of test organisms used within the expected-percent-affected organism interval of 16 percent and 84 percent. Calculate the exponent $(2.77 / \sqrt{N'})$ for the slope function and the factor, f_{LC50} used to establish the confidence limits for LC50.

$$f_{LC50} = S^{(2.77 - \sqrt{N'})}$$

The f_{LC50} can be obtained directly from the nomogram in Figure 3 by laying a straight edge across the appropriate base and exponent values and reading the resultant "f" value.

d. Calculate the confidence limits of the LC50 as follows:

(1) Upper limit for 95% probability = $LC50 \times f_{LC50}$

(2) Lower limit for 95% probability = $LC50 / f_{LC50}$

log LC50 vs log time (Figure 7).(3) Alternatively, the incipient lethal level may be made by selecting a time beyond that where acute toxicity ceases, and plotting percent killed (probit scale) for each concentration containing specimens that have survived to that time (Figure 8). The toxicant concentration corresponding to the 50 percent mortality value is identified as the incipient LC50. A good summary of references for computing confidence intervals is included in Sprague (1969, I).

Applications of LC Values

Much work has been done on extrapolation of acceptable field concentrations of test compounds from laboratory derived LC data. However, so many variables are involved that no satisfactory "fudge factor" has yet been evolved. Somewhat of a precedent was set at one location when pulping waste was tested at 0.1 dilution of the LC50 value and the results proved satisfactory to all concerned. Since then a basic rule of thumb used by many industries is to apply a 0.1 dilution factor to the LC50 value. Such general evaluations have justifiably received criticism from many investigators, some claiming that a 0.01 dilution of the LC50 value is a more accurate estimation of the true toxicity threshold. The relationship between the LC50 value and threshold toxicity, if one exists, varies with the specific toxicant. One can generalize to generic types with only the greatest trepidation.

The assessment of a potential toxicant and the subsequent resolution of an application factor (AF) or its reciprocal, the chronicity value, involves the manipulation of two numbers generated by laboratory toxicity tests:

Probit Method

1. General Procedure

Step 1: Draw a best fit line of the test results on logarithmic-probit paper (Figure 4) with concentration on the log scale and mortality on the probit scale. Give the most consideration to points between 16 and 84%.

Step 2: Test results are tabulated for at least one exposure time. The selected time should be the longest one used (usually 96 hours). Use only the less extreme concentrations for more than one 0% or 100% value.

Step 3: The concentration causing 50 percent mortality (LC50) can be read from the fitted line as shown in Figure 4.

Computer Program

A computer program that calculates the LC50 values and confidence intervals using mortality data was developed at EPA and later modified by personnel in the Environmental Engineering Laboratory. A copy of the program is presented in Appendix C of this report.

Calculation of the Incipient LC50 Value (ILC50)

Several procedures have been utilized for determining ILC50 values: (1) The percent survival at each concentration of toxicant is recorded over frequent time intervals (Figure 5). The median period of survival at each toxicant concentration is then estimated and is used in determining the toxicity threshold limit as shown in Figure 6; (2) The percent mortality is observed several times over the entire dilution series. LC50 values after each of these periods of exposure are interpolated from a graph similar to Figure 6 with the percentage concentration of the effluent (log scale) on the x-axis and percentage mortality (probit scale) on the y-axis (Figure 4). The ILC50 value is then evaluated from a plot of

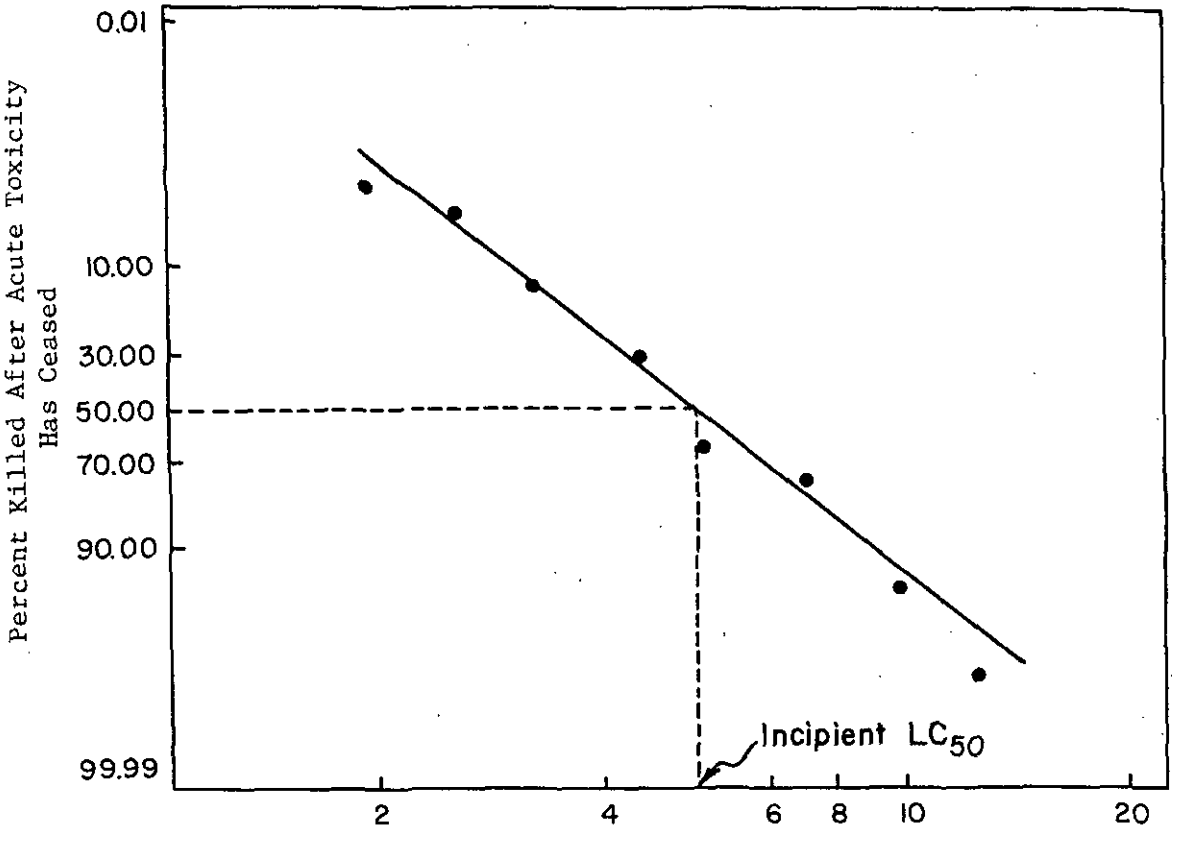

Figure 8. Concentration of toxicant, ppm.

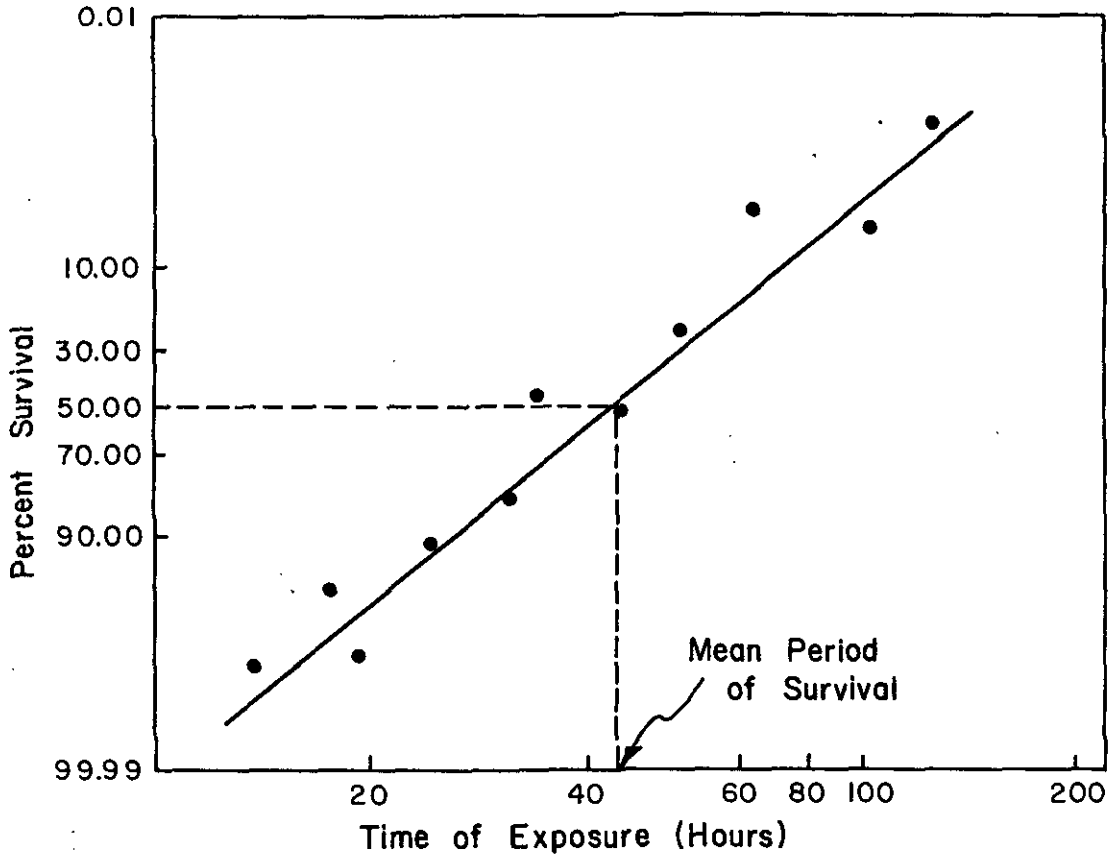

Figure 5. Determination of mean period of survival for a given concentration of toxicant.

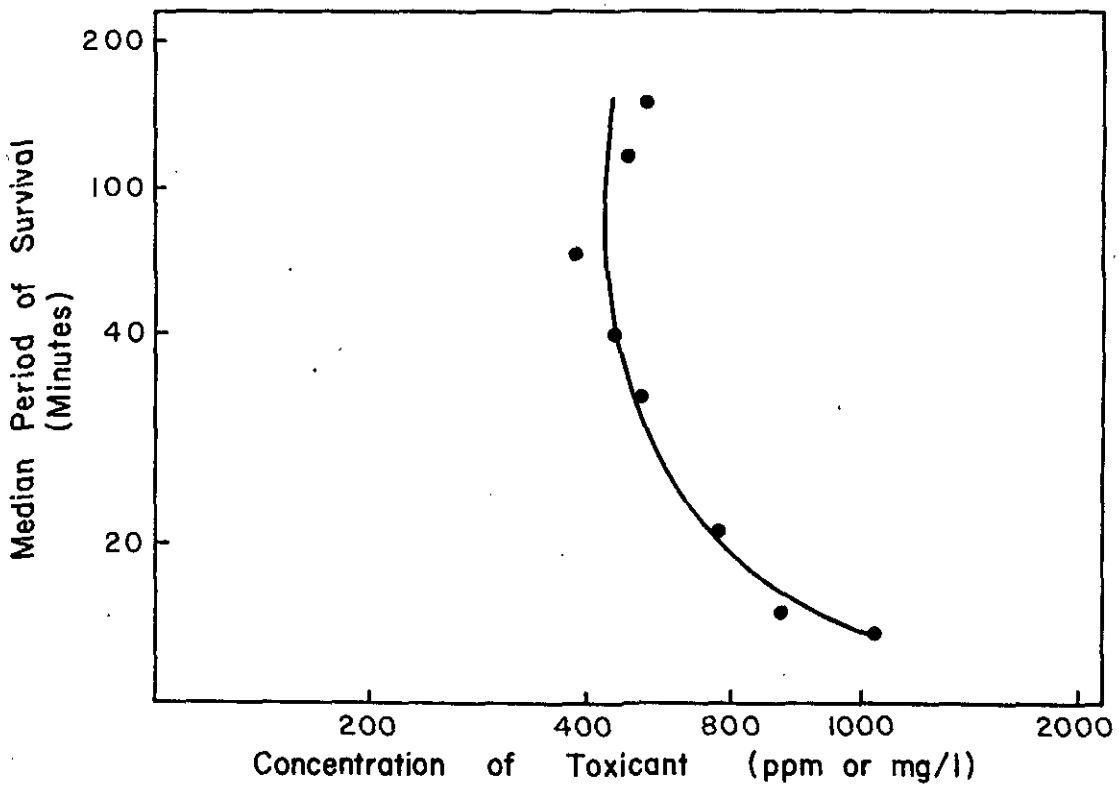

Figure 6. Determination of incipient letal concentration.

1) Long term exposure of test organisms to much lower toxicant concentrations than are lethal to fish may still cause impaired function or performances such as in swimming ability, appetite and growth, resistance to disease, reproductive capacity and the general ability to compete with other species. For example, a certain concentration of a toxicant may have no noticeable chronic toxic effect on a particular species of fish in the lab, but due to subtle impairment of some ability, the species might be outcompeted in the natural environment and be completely eliminated from a waterway after a period of years.

2) Water quality parameters such as alkalinity, hardness, pH, temperature and conductivity can drastically change the LC50 (Moreno, 1981) and MATC under field conditions.

3) Low levels of a toxicant may have no effect on the species of fish tested but could have a toxic effect on other biota that the fish consume. Thus certain species of fish might be selected against due to a change in food availability.

4) It is important to realize that the AF will change according to the species and toxicant being tested. Pickering and Gast (1972) demonstrated the AF for fathead minnows exposed to cadmium in hard water was 0.005-0.008 of the 96-hour LC50 value. Mount (1968) determined the AF for the same species with copper as the toxicant was 0.03-0.08 of the 96-hour LC50. Eaton (1974) showed the AF for the Bluegill (Lepomis machrochirus) with cadmium as the toxicant was 0.0015-0.0039 of the ILC50.

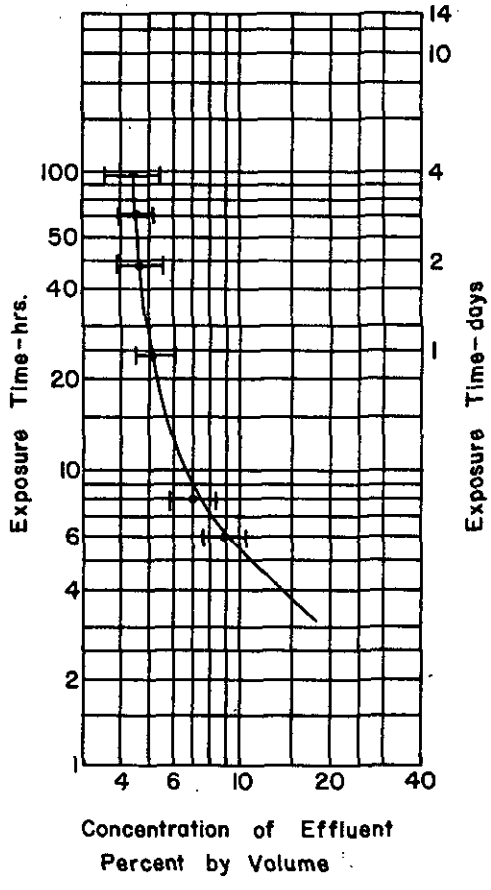

Figure 7. Toxicity curve, drawn as the experiment proceeded, from LC50's determined as shown in Figure 6. Curve has become almost asymptotic with the time axis. The 95 percent confidence limits are shown for each LC50, although in many bioassays they would be calculated only for the selected or final LC50.

1. the 96-hour LC50 estimate of acute toxicity, or the incipient LC50 (ILC50) estimate of toxicity threshold; and
2. the safe concentration (SC) or the maximum allowable toxicant concentration (MATC).

The AF is derived by dividing the MATC by the 96-hour LC50 or the ILC50 (APHA 1975). The AF, once determined, permits estimation of MATC values for a given toxicant from just the 96-hour LC50 value.

While the generation of the latter requires no great competence, estimation of the MATC is a more difficult task. The definition of some threshold of inhibition of a life function (growth, fecundity, etc.) over a sustained period demands a high level of expertise. For this reason a current list of AF values would be extremely useful to laboratories evaluating sample toxicity.

The MATC can be determined directly by chronic toxicity studies. This determination involves placing fish fry in toxicant and observing growth inhibition or spawning interference. Such studies are performed by E.G. and G. Bionomics, Inc., of Wareham, Massachusetts. The MATC is determined from the geometric mean of the greatest concentration that causes the same response as the control, and the lowest concentration where a different response is observed.

An alternative to determining an AF or MATC value based upon long-term chronic exposure is the estimation of safe levels by relatively short-term exposures of embroyos and larvae as suggested by Eaton (1974). Such a test may be completed in 30 days (EPA, 1978). In determining the MATC, several factors should be considered.

Some AF values have been compiled while many are still unknown. The National Technical Advisory Committee on Water Quality Requirements for Aquatic Life (1968) has divided toxicants into three groups and provided the appropriate LC50 and AF values. A general application factor must be applied to toxicants at times when MATC values are unknown in the interest of expediting pollution control. AF values ranging from 0.01 to 0.1 of the 96-hour LC50 have been suggested. Sprague (1969, II) suggested the AF could roughly be approximated by 0.05 based on the 96-hour LC50 or 0.1 using the 20 day LC50. Considering the findings that AF values vary greatly and may be well below 0.05 such as in the studies previously alluded to, a general AF value of 0.005 using the 96-hour LC50 is a more reasonable value.

XII. REFERENCES

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XIII. APPENDICES

Appendix A

Equipment and Supply Costs for Conducting Acute Fish Toxicity Tests

Item	Specifications	Costs (1981 dollars)	Vendor[1]
EQUIPMENT			
Holding tank	400 liter recirculates every 1.5 minutes	$1700	Frigid Units
Static testing jars	20 glass jars (5 gallons $18 each)	$360	Empire State Glass
Flow through system	stainless steel aquaria;	$2000	
	tygon tubing;	$500	Cole Parmer
	7 double head and one 10 channel positive displacement pumps to accommodate 24 inflow-outflows	$3000	Cole Parmer
Water purifying system	Reverse osmosis water purifying system	$2000	Millipore Corp
pH meter	Range 0-14, pH units $\pm$ 0.1	$500	Fisher Scientific
Thermometer	Mercury-0^{o}C-100^{o}C g	$10	Fisher Scientific
Lighting	Must provide about 100 ft-c	$200	
Light timer	Must automatically turn off and on lights at specified intervals	$200	
Dissolved oxygen probe and meter	Reads 0-20 mg0/1	$1000	Yellow Springs Instrument Co.
Conductivity meter	Reads 0-50,000 umhos	$1000	
Analytical balance	Capable of reading to fourth decimal point	$2600	Fisher Scientific
Mixing tanks	3,208 liter polyethylene tanks	$300	Fisher Scientific

APPENDIX A (continued)

Item	Specifications	Costs (1981 dollars)	Vendor[1]
Mixer	3 ft shaft & propeller	$100	Fisher Scientific
Oven	Capable of 120°C	$800	Fisher Scientific
Dessicator	Used for drying chemicals	$100	Fisher Scientific
Vacuum pump	Used with filtering apparatus	$350	Fisher Scientific
Filtering apparatus	For use with 47 mm filter	$200	Millpore Corp.
Atomic Absorption Spectrophotometer	To measure heavy metals	contract out at $8-15 per metal per sample	Water Quality Analyses Lab.
Constant Temp. Room	-	-	-
SUBTOTAL[2]		$16,920	
SUPPLIES			
Polyester fiber	Used for filtering recirculating water in holding tank	$25/4 lbs	Pet store
Activated charcoal	Used to remove organic compounds from holding tank water	$25/10 lbs	Frigid Units
Ammonia removing pellets	Removes NH_3 from holding tank water	$23/gal	Commonwealth Aquarium
Assorted Glasware	2 each of 25 & 10 ml burets; 5, 100 ml volumetric flasks; 3, 1 liter volumetric flasks; 4 each of 0.5,1.0,2.0,3.0, 5.0,20.0, 25.0 & 50.0 ml volumetric pipets; 1 each of 25, 100, 500 ml grad. cylinders	$400	Fisher Scientific

APPENDIX A (continued)

Item	Specifications	Costs (1981 dollars)	Vendor[1]
Filters	0.45 μm membrane filters glass fiber filters	$18/100 $6.00/100	Fisher Scientific
Chemicals	Certified ACS reagent grade, for addition to dilution water and performing chemical tests- $NaHCO_3$, $CaSO_4$ $2H_2O$; $MgSO_4$; KCl; NaOH; KH_4PO_4; methyl orange; potassium acid phthalate, Na_2EDTA; NH_4Cl; NaOH	$500	Fisher Scientific

SUBTOTAL[2] $997

GRAND TOTAL[2] $17,917

[1] See Appendix B for dealer addresses

[2] Does not include costs of heavy metal analyses

[3] Sufficient for one year (about 20 acute toxicity tests)

APPENDIX B

Dealer Addresses

1. Ace Glass Incorporated
   PO Box 688
   1430 NW Boulevard
   Vineland, New Jersey
   (609)692-3333

2. Carolina Biological Supply Co.
   2700 York Road
   Burlington, NC 27215
   (919) 584-0381

3. Cole-Parmer Instrument Company
   7425 North Oak Park Avenue
   Chicago, Illinois 60648
   (800) 323-4340

4. Commonwealth Aquarium
   362 Boylston Street
   Brookline, Massachusetts 02146
   (617) 232-0067

5. Empire State Glass
   Buffalo, N.Y.
   (716) 896-4527

6. Fisher Scientific
   461 Riverside Avenue
   P.O. Box 379
   Medford, Massachusetts 02155
   (617) 391-6110

7. Frigid Units, Inc.
   3214 Sylvania Ave.
   Toledo, Ohio 43613
   (419) 747-6971

8. Hach Company
   PO Box 907
   Ames, Iowa 50010
   (800) 525-5940

8. Millipore Corporation
   Bedford, Massachusetts 01730
   (800) 225-1380

10. Perkin-Elmer
    Norwalk, Connecticut 06856
    (203) 762-1000

11. Yellow Springs Instrument Company
    Box 279
    Yellow Springs, Ohio 45387
    (513) 767-7241

Appendix C

Computer Program Used to Calculate LC50 Values and Confidence Intervals

```
LIST

10 DIM A(10),B(10),C(10),D(10),E(10),F(10),G(18,18),H(10),I(10),J(10),K(10)
20 DIM L(10),M(10),N(10),P(10),T(10),V(10),X(10),Y(10),Z(10)
22 PRINT "DATA SHOULD BE INPUT FROM THE HIGHEST TO THE LOWEST CONCENTRATION."
23 PRINT "THE CONCENTRATIONS AND NUMBER OF FISH ARE REAL NUMBERS, AND"
24 PRINT "SHOULD CONTAIN DECIMAL POINTS."
25 PRINT "THIS PROGRAM WILL ACCEPT A MAXIMUM OF TEN CONCENTRATIONS."
26 PRINT
27 PRINT "NUMBER OF CONCENTRATIONS = "
30 INPUT M
40 FOR J=1 TO M
45 PRINT "CONCENTRATION NUMBER";J;"="
50 INPUT C(J)
60 NEXT J
61 PRINT "DID ALL CONCENTRATIONS HAVE THE SAME NUMBER OF FISH AT THE"
62 PRINT "START OF THE TEST? YES/NO?"
63 INPUT A$
64 IF A$="NO" THEN 71
65 PRINT "NUMBER OF FISH IN EACH CONCENTRATION AT THE START OF THE TEST ="
66 INPUT Q
67 FOR J=1 TO M
68 LET E(J)=Q
69 NEXT J
70 GO TO 95
71 FOR J=1 TO M
75 PRINT "NUMBER OF FISH AT START OF TEST IN CONCENTRATION";C(J);"="
80 INPUT E(J)
90 NEXT J
95 PRINT ">>>NOTE: IF THE MORTALITY IS ZERO, INPUT THE VALUE OF 0.001.  THIS"
97 PRINT "WILL NOT AFFECT YOUR RESULTS AT ALL"
100 FOR J=1 TO M
105 PRINT "NUMBER OF FISH DEAD IN CONCENTRATION";C(J);"="
110 INPUT D(J)
116 IF D(J)=0.001 THEN 118
117 GO TO 120
118 D(J)=0
120 NEXT J
130 PRINT "CONC.","NUMBER","NUMBER","PERCENT","BINOMIAL"
140 PRINT " ","EXPOSED","DEAD","DEAD","PROB.(%)"
150 K0=0
160 FOR J=1 TO M
170 L(J)=LGT(C(J))
180 P(J)=D(J)/E(J)
190 I(J)=1
240 B1=0
250 N1=E(J)
260 IF P(J)>.5 THEN 290
270 N2=D(J)
280 GO TO 300
290 N2=E(J)-D(J)
300 FOR N=0 TO N2
310 LET B2=1
315 LET B3=1
320 FOR I=(N1-N+1) TO N1
330 B2=B2*I
340 NEXT I
350 FOR I=2 TO N
360 B3=B3*I
370 NEXT I
380 B1=B1+(B2/B3)
390 NEXT N
400 B(J)=100*B1*(0.5^N1)
410 PRINT C(J),E(J),D(J),100*P(J),B(J)
450 A1=SQR(D(J)/(E(J)+1))
460 A2=ATN(A1/(SQR(1-A1*A1)))
470 IF D(J)=E(J) THEN 510
480 A3=SQR((D(J)+1)/(E(J)+1))
490 A4=ATN(A3/(SQR(1-A3*A3)))
500 GO TO 520
510 A4=1.57079633
520 A(J)=180*(A2+A4)/(2*3.14159)
530 NEXT J
540 FOR J=1 TO M
550 IF P(J)>0 THEN 610
560 IF J>1 THEN 590
```

```
570 Y(J)=-2.67*(1-P(J+1)/2)
580 GO TO 810
590 Y(J)=-2.67*(1-P(J-1)/2)
600 GO TO 810
610 IF P(J)<1 THEN 665
620 IF J<M THEN 630
630 Y(J)=2.67*(1-(1-P(J-1))/2)
640 GO TO 810
650 Y(J)=2.67*(1-(1-P(J+1))/2)
660 GO TO 810
665 P1=P(J)
670 I1=0
680 IF P1<=0.5 THEN 710
690 P1=1-P1
700 I1=1
710 P2=SQR(LOG(1/P1/P1))
720 P3=((0.010328*P2+0.802853)*P2+2.515517)
730 Y(J)=P3/(((0.001308*P2+0.189269)*P2+1.432788)*P2+1)-P2
740 IF I1=1 THEN 750
745 GO TO 760
750 Y(J)=-Y(J)
760 IF K0>0 THEN 790
770 X3=C(J)
780 GO TO 800
790 IF X3=C(J) THEN 810
800 K0=K0+1
810 NEXT J
820 LET B4=0
830 LET B5=0
840 LET B6=0
850 LET B7=0
1010 FOR J=1 TO M
1020 IF B(J)<1776 THEN 1040
1030 GO TO 1230
1040 IF P(J)<.5 THEN 1090
1050 IF B(J)>2.5 THEN 1090
1060 B4=C(J)
1070 B5=B(J)
1080 NEXT J
1090 FOR J=M TO 1 STEP -1
1100 IF P(J)>.5 THEN 1150
1110 IF B(J)>2.5 THEN 1150
1120 B6=C(J)
1130 B7=B(J)
1140 NEXT J
1150 B8=100-B5-B7
1160 IF B4>0 THEN 1190
1170 PRINT "THE BINOMIAL TEST SHOWS THAT";B6;"AND +INFINITY CAN BE"
1180 GO TO 1200
1190 PRINT "THE BINOMIAL TEST SHOWS THAT";B6;"AND";B4;"CAN BE"
1200 PRINT "USED AS STATISTICALLY SOUND CONSERVATIVE 95 PERCENT"
1210 PRINT "CONFIDENCE LIMITS SINCE THE ACTUAL CONFIDENCE LEVEL"
1220 PRINT "ASSOCIATED WITH THESE LIMITS IS ";B8;"PERCENT."
1230 FOR J=1 TO M
1240 IF P(J)<0.5 THEN 1270
1250 G1=J
1260 NEXT J
1270 FOR J=M TO 1 STEP -1
1280 IF P(J)>0.5 THEN 1310
1290 G2=J
1300 NEXT J
1310 IF P(G2)<P(G1) THEN 1340
1320 M1=((C(G1))*(C(G2)))^.5
1330 GO TO 1370
1340 G3=(45-A(G2))/(A(G1)-A(G2))
1350 G4=L(G2)+(L(G1)-L(G2))*G3
1360 M1=10^G4
1370 PRINT "AN APPROXIMATE LC50 FOR THIS SET OF DATA IS ";M1
1380 PRINT
1390 IF K0>1 THEN 1440
1400 PRINT "WHEN THERE ARE LESS THAN TWO CONCENTRATIONS AT WHICH THE PERCENT"
1410 PRINT "DEAD IS BETWEEN 0 AND 100, NEITHER THE MOVING AVERAGE NOR THE"
1420 PRINT "PROBIT METHOD CAN GIVE ANY STATISTICALLY SOUND RESULTS."
1430 GO TO 3850
1440 I4=0
1450 FOR S=M-1 TO 1 STEP -1
```

```
1460 FOR N=1 TO M-S+1
1470 IF S<3 THEN 1580
1480 FOR J=1 TO S-2
1490 J(J+2)=((L(N+J+1)-L(N+1))/(L(N+1)-L(N)))-J
1500 NEXT J
1510 FOR J=1 TO S
1520 T(J)=-.5*(S-1)+(J-1)+J(J)
1530 NEXT J
1540 LET W1=0
1545 LET W2=0
1550 FOR K=1 TO S
1560 W1=W1+T(K)*(0.5+E(N+K-1))
1565 W2=W2+T(K)*T(K)*(0.5+E(N+K-1))
1570 NEXT K
1580 K1=1
1590 V(N)=0
1600 FOR J=N TO N+S-1
1610 IF S>2 THEN 1640
1620 M(J)=1
1630 GO TO 1650
1640 M(J)=W2-T(K1)*W1
1650 G(J,N)=M(J)*(0.5+E(J))
1660 V(N)=V(N)+G(J,N)
1670 K1=K1+1
1680 NEXT J
1690 K(N)=0
1700 F(N)=0
1710 FOR J=N TO N+S-1
1720 K(N)=K(N)+G(J,N)*L(J)/V(N)
1730 F(N)=F(N)+G(J,N)*A(J)/V(N)
1740 NEXT J
1750 F(N)=(INT((1E6)*F(N)+0.5))/(1E6)
1760 NEXT N
1770 K2=1
1780 FOR J=1 TO M-S
1790 X(K2)=0
1800 K4=0
1810 FOR N=J TO J+S
1820 IF P(N)=0 THEN 1850
1830 IF P(N)=1 THEN 1850
1840 K4=K4+1
1850 NEXT N
1860 IF K4<2 THEN 1920
1870 IF F(J)=F(J+1) THEN 1920
1880 IF F(J)<45 THEN 1920
1890 IF F(J+1)>45 THEN 1920
1900 X(K2)=J
1910 K2=K2+1
1920 NEXT J
1930 IF X(1)=0 THEN 2360
1940 IF I4=1 THEN 1980
1950 PRINT ">>>>RESULTS CALCULATED USING THE MOVING AVERAGE METHOD"
1960 PRINT "SPAN","G","LC50","95 PERCENT CONFIDENCE LIMITS"
1970 I4=1
1980 FOR N=1 TO K2-1
1990 P=X(N)
2000 Q=X(N)+1
2010 Y=F(Q)-F(P)
2020 A=(45-F(P))/Y
2030 M2=K(P)+(K(Q)-K(P))*A
2040 M3=10^M2
2045 LET V1=0
2050 LET V2=0
2055 LET V3=0
2060 FOR J=1 TO S
2070 V1=V1+((3282.81*(G(P+J-1,P)^2))/((4*E(P+J-1)+2)*(V(P))^2))
2080 V2=V2+((3282.81*(G(Q+J-1,Q)^2))/((4*E(Q+J-1)+2)*(V(Q))^2))
2090 IF J=1 THEN 2110
2100 V3=V3+((3282.81*G(P+J-1,P)*G(Q+J-2,Q))/((4*E(P+J-1)+2)*V(P)*V(Q)))
2110 NEXT J
2120 V4=V1+V2-2*V3
2130 V5=V1-V3
2140 Z=1.96
2150 G=Z*Z*V4/(Y^2)
2160 IF G=1 THEN 2330
```

```
2170 R=V1-2*A*V5+(A^2)*V4-G*(V1-((V5^2)/V4))
2180 IF R<0 THEN 2330
2190 V6=Z*SQR(R)/(V4*(1-G))
2200 V7=(A-G*V5/V4)/(1-G)
2210 L2=K(P)+(K(Q)-K(P))*(V7-V6)
2220 U2=K(P)+(K(Q)-K(P))*(V7+V6)
2230 L3=10^L2
2240 U3=10^U2
2250 IF G<1 THEN 2310
2260 IF A<(V5/V4) THEN 2290
2270 PRINT S,G,M3,"0",U3
2280 GO TO 2340
2290 PRINT S,G,M3,L3,"+ INFINITY"
2300 GO TO 2340
2310 PRINT S,G,M3,L3,U3
2320 GO TO 2340
2330 PRINT S,G,M3,"    0","+ INFINITY"
2340 NEXT N
2350 GO TO 2420
2360 NEXT S
2370 IF I4=1 THEN 2420
2380 PRINT "THE MOVING AVERAGE METHOD CANNOT BE USED WITH THIS SET OF DATA"
2390 PRINT "BECAUSE NO SPAN WHICH PRODUCES MOVING AVERAGE ANGLES THAT BRACKET"
2400 PRINT "45 DEGREES ALSO USES TWO PERCENT DEAD BETWEEN 0 AND 100 PERCENT."
2410 PRINT
2420 IF K0>1 THEN 2462
2430 PRINT "WHEN THERE ARE LESS THAN TWO CONCENTRATIONS AT WHICH THE PERCENT"
2440 PRINT "DEAD IS BETWEEN 0 AND 100, THE PROBIT METHOD CANNOT GIVE ANY"
2450 PRINT "STATISTICALLY SOUND RESULTS.PRINT "
2460 GO TO 3850
2462 LET S1=0
2463 LET S2=0
2464 LET S3=0
2465 LET S4=0
2470 LET K3=0
2472 LET I3=0
2480 FOR J=1 TO M
2485 S1=S1+L(J)
2490 S2=S2+Y(J)
2500 S3=S3+L(J)*L(J)
2510 S4=S4+L(J)*Y(J)
2520 NEXT J
2530 B=(S4-S1*S2/M)/(S3-S1*S1/M)
2540 A=(S2-B*S1)/M
2543 LET F1=0
2546 LET F2=0
2549 LET F3=0
2550 LET F4=0
2553 LET F5=0
2556 LET F6=0
2560 FOR J=1 TO M
2570 Z(J)=A+B*L(J)
2580 Z1=ABS(Z(J))
2590 IF Z1>8 THEN 2700
2600 Z2=0.3989422804*EXP(-.5*Z1*Z1)
2610 Z3=1/(1+0.2316419*Z1)
2620 Z4=((1.330274*Z3-1.821256)*Z3+1.781478)*Z3
2630 P=Z2*Z3*((Z4-.3565637812)*Z3+.3193815)
2640 IF Z(J)<0 THEN 2680
2650 Q=P
2660 P=1-P
2670 GO TO 2730
2680 Q=1-P
2690 GO TO 2730
2700 P=1E-16
2710 Z2=1E-15
2720 GO TO 2640
2730 W3=E(J)*(Z2/P)*(Z2/Q)
2740 W4=Z(J)+(P(J)-P)/Z2
2750 F1=F1+L(J)*W3
2760 F2=F2+W3*W4
2770 F3=F3+L(J)*L(J)*W3
2780 F4=F4+W3*W4*W4
2790 F5=F5+L(J)*W3*W4
2800 F6=F6+W3
2810 NEXT J
```

```
2820 F7=F5-F1*F2/F6
2830 F8=F3-F1^2/F6
2840 B=F7/F8
2850 A=(F2-B*F1)/F6
2860 K3=K3+1
2870 D1=0
2880 FOR J=1 TO N
2890 D1=(A+B*L(J)-Z(J))^2+D1
2900 NEXT J
2910 IF D1<1E-11 THEN 2960
2920 IF K3<25 THEN 2543
2930 PRINT "NO CONVERGENCE IN 25 ITERATIONS.  THE PROBIT METHOD PROBABLY"
2940 PRINT "CANNOT BE USED WITH THIS SET OF DATA."
2950 GO TO 3850
2960 F9=F1/F6
2970 F0=F4-F2^2/F6
2980 C1=F0-F7^2/F8
2990 V=M-2
3000 IF V=0 THEN 3430
3010 IF C1/V>20 THEN 3220
3020 J=1
3030 FOR I=V TO 2 STEP -2
3040 J=J*I
3050 NEXT I
3060 C2=C1^(INT((V+1)/2))*EXP(-C1/2)/J
3070 IF INT (V/2)=V/2 THEN 3100
3080 C3=SQR(2/C1/3.14159)
3090 GO TO 3110
3100 C3=1
3110 C4=1
3115 C5=1
3120 V=V+2
3130 C5=C5*C1/V
3140 IF C5<1E-10 THEN 3170
3150 C4=C4+C5
3160 GO TO 3120
3170 C6=1-C2*C3*C4
3180 IF C6>=0.001 THEN 3200
3190 C6=0
3200 V=M-2
3210 GO TO 3250
3220 C6=0
3230 GO TO 3250
3240 C6=1492
3250 IF C6<=0.05 THEN 3290
3260 T5=1.96
3270 H=1
3280 GO TO 3370
3290 IF V=1 THEN 3330
3300 IF V=2 THEN 3350
3310 T5=1.95996+1/(.413*V-.423)
3320 GO TO 3360
3330 T5=12.706
3340 GO TO 3360
3350 T5=4.303
3360 H=C1/V
3370 G=H*T5^2/B^2/F8
3380 E1=SQR(H/F8)
3390 E2=B-T5*E1
3400 E3=B+T5*E1
3410 PRINT ">>>>>RESULTS CALCULATED USING THE PROBIT METHOD"
3415 PRINT
3420 PRINT "ITERATIONS","G","H","GOODNESS OF FIT PROBABILITY"
3430 IF C6=1492 THEN 3460
3440 PRINT K3,G,H,C6
3450 GO TO 3470
3460 PRINT K3,G,H," (CANNOT BE CALCULATED)"
3470 PRINT
3480 IF C6>0 THEN 3510
3490 PRINT "A PROBABILITY OF 0 MEANS THAT IT IS LESS THAN 0.001"
3500 PRINT
3510 IF C6>0.05 THEN 3550
3520 PRINT "SINCE THE PROBABILITY IS LESS THAN 0.05, RESULTS CALCULATED"
3530 PRINT "USING THE PROBIT METHOD PROBABLY SHOULD NOT BE USED."
3540 PRINT
```

```
3550 PRINT "SLOPE =",B
3560 PRINT "95 PERCENT CONFIDENCE LIMITS =";E2;"  AND  ";E3
3570 PRINT
3580 M4=-A/B
3590 M5=10^(M4)
3600 PRINT "LC50 =",M5
3610 IF G=1 THEN 3770
3620 H1=H*((1-G)/F6+(M4-F9)^2/F8)
3630 IF H1<0 THEN 3770
3640 H2=SQR(H1)
3650 L4=M4+G*(M4-F9)/(1-G)-H2*T5/((ABS(B))*(1-G))
3660 U4=M4+G*(M4-F9)/(1-G)+H2*T5/((ABS(B))*(1-G))
3670 L5=10^L4
3680 U5=10^U4
3690 IF G<1 THEN 3750
3700 IF M4>F9 THEN 3730
3710 PRINT "95 PERCENT CONFIDENCE LIMITS = 0 AND ";U5
3720 GO TO 3790
3730 PRINT "95 PERCENT CONFIDENCE LIMITS =";L5;" AND + INFINITY"
3740 GO TO 3790
3750 PRINT "95 PERCENT CONFIDENCE LIMITS ";L5; " AND ";U5
3760 GO TO 3790
3770 PRINT "95 PERCENT CONFIDENCE LIMITS = 0  AND + INFINITY"
3790 IF I3=1 THEN 3850
3800 M4=(-2.32679-A)/B
3810 M5=10^M4
3820 PRINT "LC1   = ",M5
3830 I3=1
3840 GO TO 3610
3850 GO TO 10
3860 END
```

Appendix D

Laboratory Worksheet

BIOASSAY/TOXICITY TEST DATA

DATE: _____

TEST METHOD:

TEST ORGANISM: _____ STARTING TIME: _____

WATER SOURCE

AND HARDNESS: _____ FINAL TIME: _____

TEMPERATURE: _____

DISSOLVED O_2: _____

pH: _____

24, 48 or 96 HOUR TEST

# SURVIVING/% MORTALITY																
TIME INTERVAL		2h	4h		8h		12h		24h		36h	48h		72h	96h	
ACTUAL TIME																
TIME INTERVAL																
TOXICANT CONC.																
CONTROL	A															
	B															
	A															
	B															
	A															
	B															
	A															
	B															
	A															
	B															
	A															
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	A															
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	A															
	B															