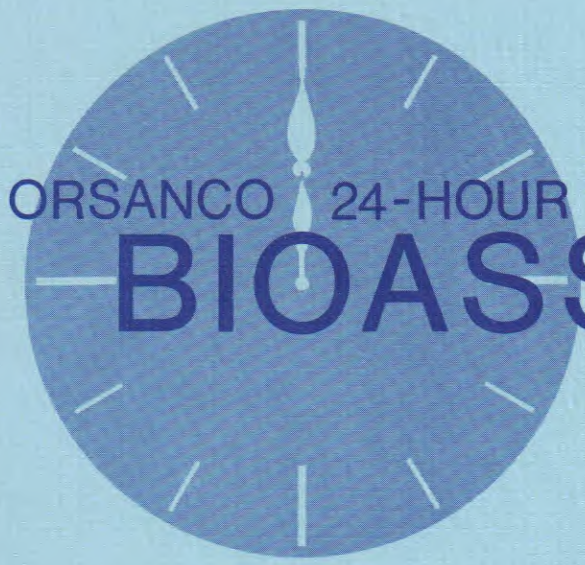

ORSANCO 24-HOUR BIOASSAY

Developed by the

BIOLOGICAL WATER QUALITY COMMITTEE

with the cooperation of the

CHEMICAL INDUSTRY COMMITTEE

of the

OHIO RIVER VALLEY WATER SANITATION COMMISSION

An interstate agency representing: Illinois • Indiana • Kentucky • New York • Ohio • Pennsylvania • Virginia • West Virginia

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developed by the

ORSANCO Biological Water Quality Committee

with the cooperation of the

ORSANCO Chemical Industry Committee

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OHIO RIVER VALLEY WATER SANITATION COMMISSION

414 Walnut Street Cincinnati, Ohio 45202

January 1974

FOREWORD

In 1972 the Ohio River Valley Water Sanitation Commission (ORSANCO) asked a group of eminent scientists to serve as an advisory committee on the biological quality of water and to develop a standardized bioassay for appraising the toxicity of wastewater discharges to aquatic life. Their task was to assist ORSANCO in the implementation of Pollution Control Standard No. 1-70 paragraph (E) which states:

"Reduction of any material or, if necessary, all materials contained in any discharge which singly or in combination are toxic or harmful to aquatic life to such a degree or degrees that the calculated concentration(s) of such material or materials in the river does not exceed one-twentieth of the 96-hour median tolerence limit (96-hour LC50) for aquatic life."

The objective was to develop a standardized procedure, based on available technology, that could be followed easily by technical personnel and that would produce reliable measurements of toxicity. This report describes such a procedure. It has been evaluated by more than twenty laboratories and found to be a suitable screening test to identify effluents that require further analysis by more precise techniques.

This report was approved for publication by ORSANCO on January 9, 1974. The Commission wishes to acknowledge the valuable contributions made by the ORSANCO Chemical Industry Committee in evaluating the bioassay.

WILLIAM L. KLEIN

Acting Executive Director

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INTRODUCTION

The bioassay described in the following protocol was developed as a screening test to assist in implementing ORSANCO's Pollution Control Standard No. 1-70 paragraph (E) relating to toxicity of wastewater discharges to aquatic life. The committee also envisioned that the procedure would have application in connection with the NPDES permit program under PL 92-500 in the event a toxicity standard of effluents was required by the states.

The efforts of the committee were directed toward modifying existing methodology to provide a simple, easily used procedure, and not toward the development of a new method. The committee recognized that some compromises would be necessary to achieve this goal. However, in view of the types and variability of wastewaters, the promise of wide application of the bioassay far outweighed the shortcomings that might result from standardizing the procedure. For example, the bioassay as outlined by the American Public Health Association et al. (1971) in "Standard Methods for the Examination of Water and Wastewater" permits many alternatives that may be used by an investigator and that may, if not properly applied, introduce varying results. By standardization these variations are minimized, and a procedure is set forth that can be followed by technical personnel unaccustomed to performing toxicity measurements with the knowledge that the results will provide useful information on the toxicity of a wastewaster discharge to aquatic life in the receiving water. The fish selected as the test organism, the goldfish (*Carassius auratus* (Linnaeus)), should provide information which is indicative of the needs of the predominant fish in the Ohio Valley. The results obtained from the bioassay can then be used to determine where a toxicity problem exists and whether additional toxicity studies are required.

RATIONALE FOR SCREENING BIOASSAY

Purpose

Living organisms, unlike any other analytical tool available, will respond to every possible substance or mixture of substances at some level, no matter what their chemical or physical characteristics may be. Even water as pure as distilled water is toxic to aquatic organisms such as fish and aquatic insects because some dissolved ions are lacking or others are too abundant. The universality of the detection capability of the living organism is unequaled among instruments devised by man. The purpose of this bioassay is, therefore, to use this capability of living organisms to assist the discharger and the regulatory agency to recognize contaminants in effluent streams that may have biological significance in the receiving water at the concentrations being discharged.

WHAT THIS TEST IS NOT

This test is not a research tool. It has not been designed to give the precision of a highly controlled bioassay test performed in a research laboratory. It will not produce sophisticated results for scientific publication in professional journals. It is not designed to meet a variety of research situations, nor is it flexible. It is not to be altered by personal whims or dislikes.

This test is not intended to determine concentrations of contaminants that will be safe for the most sensitive fish in the Ohio River or its tributaries. It is not intended to locate the most sensitive animals in the river, nor is it intended to protect goldfish in the Ohio River.

This test is not intended to estimate or determine a receiving water standard for the Ohio River and its tributaries. It is not intended to estimate a safe concentration that will not cause significant harm in the Ohio River.

WHAT THIS TEST IS

This test is a simple bioassay using the universal response and the generally great sensitivity of an aquatic organism, a fish, to measure the toxicity of effluents being discharged into the Ohio River and its tributaries. Its purpose is to give the regulatory agency an estimate of the acute toxicity of an effluent at the point of discharge, as that toxicity is affected by all of the variables and contaminants present in the receiving water. It is intended that the test will be affected by what is put into the river upstream from the discharge.

This bioassay is intended to serve as a screening mechanism to indicate whether or not effluents require additional testing to meet the requirements of ORSANCO's Pollution Control Standard No. 1-70. This effluent standard requires that dischargers measure the acute toxicity of their effluent to aquatic organisms and further *calculate* whether there is sufficient dilution water in the river to allow the waste to be diluted to one-twentieth of the LC50 after mixing with the river. It has been generally assumed that, where chronic toxicity data are not available, one-twentieth of the LC50 concentration of the majority of toxicants will be safe for most aquatic organisms.

This bioassay is intended to be rigid, standardized, and reproducible. It is intended to give an estimate of the average acute toxicity of wastes, not extreme estimates. It is intended to be only as precise as needed for the designated purposes.

Test Duration: 24-Hour vs. 96-Hour Bioassay

The test time is short—24 hours. The test is not intended to give a threshold lethal concentration or a chronically safe concentration. The intention is to measure acute (short-term) toxicity of the effluent at the point of discharge. The short test time will minimize the possibility of an unacceptable depletion of dissolved oxygen during the test, which may be caused by the high biological and chemical oxygen demand that is likely to exist in the diluent water and in many of the effluents to be tested. This was a prime consideration in excluding longer static tests. In addition, some tests will have to be performed many miles from the source of the diluent water. Therefore, longer tests requiring water changes or flow-through conditions are not feasible for the purpose of this test. The committee recognizes the values of flow-through tests of longer duration. These values were weighed against the extreme costs to thousands of dischargers, and it was clear that the use of more sophisticated tests would proportionately reduce the number of tests

that could be carried out. The committee recognized that even if the precision of the test was reduced by a factor of five or ten; this lack of precision was dwarfed by the variability of the effluents from hour to hour, day to day, or season to season.

Although the Pollution Control Standard No. 1-70 requires a 96-hour LC50 determination, experience shows that the additional toxicity that can be measured between 24 to 96 hours is often small and is likely to be insignificant when compared to the variability of the effluent tested. At least at the outset the 24-hour test can be a first approximation of the 96-hour LC50 and can be used to identify the more important concerns.

Equipment and Personnel

Procedure for the bioassay test is designed so that individuals untrained in the techniques of toxicity measurement can perform the test in a wide variety of circumstances. Every effort has been made to use commonly available equipment with a minimum of gadgetry to control physical and chemical conditions. The personnel performing the test need have only common sense and a keen eye to provide useful and reproducible results. The design of the test and the reports required are only those needed by the regulatory agency to fulfill the effluent-standard requirements. The test is not designed to provide the highest degree of information from each bioassay, but the design does not prevent the discharger from gaining a great deal of additional information from the test should he so desire.

Disease Treatment

Prophylactic treatments for disease that are recommended in the protocol undoubtedly affect the response of the test organism to the toxicant in the bioassay test. Similarly, most other events during the life of the test organism preceding the bioassay also influence the response. The designers of this test recognize that a lack of mortality during the holding period does not preclude an outbreak of an epidemic of disease during the test period within the same group of fish, since all fish carry bacterial pathogens and a variety of parasites at all times. The impact of this normal condition of all fish is minimized by reducing these bacteria and parasites to a common base level for all tests. No evidence was found in the literature to indicate that the effect of disease treatments changed the response of fish to toxicants as much as disease, change in conditioning water, time of year, sexual development, or many other variables. The committee chose to standardize the treatment of the test fish and accept some alteration of the response to the effluent chemicals as a result of the disease treatment. This alteration is believed to be insignificant in comparison with the variability of effluent toxicity mentioned above.

Selection of Test Species

The selection of the goldfish as the test species was based on many criteria that were either essential or desirable. Most important of all the criteria was the availability of massive numbers of fish of the appropriate size during all weeks of the year. This criterion alone reduced the selection to a few species. The limitation imposed by the conditions under which the tests must be performed (such as temperature, type of water, and geographical location) excluded some of the few species remaining as candidates, such as the guppy. Neither the most sensitive nor the most resistant species was considered desirable, and therefore one of intermediate sensitivity to a wide variety of toxicants was chosen. In addition, several other criteria were fulfilled by the choice of the goldfish as the test species: culture practices for the fish are well established; shipping and handling procedures are well known; the species is in the same family as most of the fish in the Ohio River; and the fish are available economically from a reasonably homogeneous gene pool.

KNOW YOUR GOLDFISH

Shopping for Fish

An inbred strain of goldfish, available from Ozark Fisheries and Grassyfork Fisheries, has been selected for use as the test fish. It is available the year around, has shown a below-average

amount of disease, and has produced favorable results in bioassay tests.

The purchase of goldfish from a neighborhood shop is not a sound investment. The fish are not always of good quality, even though the purchaser is willing to pay the price. Most fish that a dealer has for sale will be relatively mature fish and may have defects or diseases that cannot be seen. For the hobbyist this presents no problem, because they are still suitable for his aquarium if no better specimens are available. In many cases the dealer should not be blamed for offering defective or diseased fish, because they may be the only specimens available to him.

From the standpoint of bioassay, such fish are not worthy of use. Poor quality fish will only produce inaccurate results. The results will indicate much more toxicity than may actually be present. In other words, the results will be conservative and will be to the benefit (i.e., a greater safety factor) of a regulatory agency and to the detriment of a company or municipality. The more healthy the fish, the better the results. Hence, it behooves the person performing the bioassay to buy fish from a reputable source.

Another problem that frequently arises is that the conditions in holding tanks are not like those under which the fish were raised and maintained at the hatchery. The fish at the hatchery are maintained in flowing water and suffer little discomfort when transferred from pond to pond. However, when they are shipped and transferred to holding tanks, the fish are under considerable stress. The loss connected with using poor fish will be even greater under these conditions.

Tank Size, Shape, and Care

The shape of a tank is significant. A shallow, broad-surfaced tank will be able to support more fish than the same volume of water in a tall and narrow tank. Although we have recommended no more than 250 fish in a 100-gallon tank for holding and acclimation purposes, a good general rule to follow is to use the largest possible aquarium that can reasonably be used in a laboratory. In other words—the bigger, the better.

Fiber glass, glass, stainless steel, or porcelain-coated steel are recommended materials for the construction of tanks. Tanks made from polyethylene or polypropylene or glass-reinforced polyesters are probably satisfactory provided they are thoroughly cleaned with a good detergent and rinsed well. Information from plastic manufacturers indicates that residual oils are present after pressure forming of the plastic which may be toxic to fish. Thorough cleaning of the tank should remove these potentially toxic films. Once the tanks have been thoroughly cleaned with detergent and rinsed with water, there should be no additional leaching from the plastic. Remember, thorough cleaning of tanks is necessary between receipt of new shipments of fish from the hatchery.

Goldfish are notorious for gluttonous appetites which result in a great deal of waste in the water; therefore, filters and frequent cleaning of the tanks are necessary. The cleaning can be accomplished with a siphon made from a piece of hose to which a dowel has been fastened to facilitate handling. Place a screen over the end of the hose and dowel to prevent loss of fish. Keep close to the bottom when siphoning off settled material.

One type of filter frequently used for tanks combines the aerator with the filter. Such a filter provides not only filtration but aeration with the same stream of aeration bubbles. Both types of cleaning are necessary for proper maintenance of the tank water. Do not use sand, gravel, or bottom-type filters.

Water and Aeration

Fish should be held in well-aerated, nonchlorinated or dechlorinated water. Aeration should be provided by an aquarium-type pump connected to aerators preferably on the bottom of the tank. A spare air pump should be available in case of failure to prevent any loss of fish from lack of oxygen. Of course, if a continuous supply of well-aerated, nonchlorinated water is available, a continuous flow can be maintained through the tank. Such an arrangement is usually the best way to maintain clean, well-aerated water in a holding tank.

Temperature and Heaters

Goldfish do best in temperatures between 60° and 80° F (16°-27° C). For this and other reasons room temperature was selected for the test; ordinarily a method for cooling water will not be re-

quired. The combination of a broad-surfaced tank, no cover, and vigorous aeration will increase the rate of evaporation and consequently cool the water during summer temperatures. Low-cost heaters are available and are easily installed in a tank and will maintain the temperature within ±2° F (1.1° C). Temperature should not be permitted to go above 85° F (29° C), because at this temperature the metabolism of the fish is increased sufficiently to produce undue stress, which in turn can cause poor results during a bioassay.

Feeding

Three types of food are available for goldfish: dried foods, live foods, and frozen foods. The fish food should have a protein content of between 30% and 40%, and vegetable substances should be included in the mixture. We have recommended a dried food developed to specifications of the U.S. Bureau of Sport Fisheries. This food is of high quality and will serve as an excellent basic nutrient. "Live" foods can be obtained in frozen form, but experience indicates that they are a potential source of infection and should be used with caution.

Feed the fish once a day and feed only as much food as will be consumed in 10 minutes. The amount to be fed is a matter of trial and error until the proper quantity can be determined.

Diseases

Goldfish are subject to a large range of diseases like any other fishes resident in the Ohio Valley. The causative agents of most fish diseases are fungi, bacteria, or parasites. Although a fish may not be infected with all three, the preventive treatment outlined below (section II, C) has been designed to protect the health of the fish from any one of them.

Transferring Fish

Handle fish gently, and work as rapidly as possible when transferring them from tank to tank. Do not keep them out of water any longer than necessary. Use a bobbinet dipnet for transfer. The less handling required, the better.

Hydrogen-Ion Concentration (pH) and Hardness

Goldfish are not fussy, and no particular pH or hardness is required if water conditions are reasonable. If a large amount of water is removed during the siphoning process and replaced with fresh dilution water, the build-up of waste products from the fish will be substantially reduced. Change from 75% to 90% of the water weekly and replace with nonchlorinated or dechlorinated water. Normally, sufficient water will be removed in the siphoning operation during cleaning to accomplish this change.

ORSANCO 24-HOUR STATIC BIOASSAY

In the description of the bioassay protocol given below, all steps of the process are clearly set forth in detail. To facilitate use of the procedural directions the information is presented in four sections:

I. Equipment and Materials;
II. General Requirements of the Test;
III. 24-Hour Static Test Procedure; and
IV. Calculation of Allowable Discharge Rate.

I. Equipment and Materials

The equipment and materials needed to perform the 24-hour static bioassay are tabulated below. Many of the items will be stocked in the average laboratory or can be purchased from a

local laboratory supply house. A source is given for those items that may not be readily available from a supplier. The goldfish *must be obtained* from one of the sources indicated. In the following list a single asterisk indicates availability from laboratory supply houses, and a double asterisk, availability from aquarium supply houses.

QUANTITY	DESCRIPTION	SOURCE
12	Test container--5-gallon glass widemouth bucket with handle and cap--Cat. No. AT 5062 (Type 132-K-450CH)	Owens Illinois Glass Container Division P.O. Box 1035 Toledo, Ohio 43601
1	Thermometer 0^{0} -100^{0}C	*
1	pH meter, electrometric or colorimetric type	*
1	Dissolved oxygen test setup (reagents and apparatus)	Hach Chemical Co. Ames, Iowa 50010
1 lb.	Potassium permanganate (ACS grade)	*
1 lb.	Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) (ACS grade)	*
144	O-TABS	Pemble Laboratories Rt. 3 River Falls, Wisconsin 54022
100	Tetracycline hydrochloride, 250-milligram capsule (when ordering, user must specify that chemical is for laboratory test purposes only; see appendix, p. 16)	American Cyanamid Co. Fine Chemicals Dept. Pearl River, N.Y. 10965
5 (option)	Tetracycline hydrochloride, 10-gram vial (when ordering, user must specify that chemical is for laboratory test purposes only; see appendix, p. 16)	American Cyanamid Co. Fine Chemicals Dept. Pearl River, N.Y. 10965
250	Goldfish (inbred strain) *Carassius auratus* (Linnaeus) (Common comets 1-1/2 to 2-1/2 inches; specify ORSANCO test fish when ordering)	Ozark Fisheries Inc. Stoutland, Missouri 65567 Tel.: 314-765-3322 or Grassyfork Fisheries Co., Inc. Martinsville, Indiana 46151 Tel.: 317-342-4127 (Airmail ship only)
2	Air pump (aquarium-type, heavy duty) *oil-free*, heavy duty (provided *oil-free* compressed air is not available)	**

2 sets	Air disperser (bottom tank type)	**
2	Filters, tanks, outside	**
1	Holding tank (100-gallon) (may be made of glass, porcelain, fiber glass, stainless steel, polyethylene, polypropylene, glass-reinforced polyester, or wood)	**
3	Storage container tank, 30-gallon or larger (may be made of glass, porcelainized steel, fiber glass, stainless steel, polyethylene, polypropylene, glass-reinforced polyesters, or wood)	**
1 (option)	Pump, electric centrifugal, 10 gpm or more for water-transfer purposes--Cat. No. P8492	Scientific Products Evanston, Illinois 60201
10	Jar, glass, wide-mouth (1-gallon)	*
5 lb.	Fish food--dry, #4 granules without antibiotics for minnows and warmwater fish (U.S. Bureau of Sport Fisheries specifications)	Glencoe Mills Glencoe, Minnesota 55336
2	Dipnet--fish-nylon bobbinet or equal	**
1	Filter, activated carbon (housing and cartridge): Cartridge, Cat. No. 40468-01; Housing, Cat. No. 40245-01	Cuno Engineering Corp. Meriden, Connecticut 06450

II. General Requirements of the Test

A. TEST FISH

1. Use as test fish the common goldfish (inbred strain), *Carassius auratus* (Linnaeus), available from Ozark Fisheries Inc. or Grassyfork Fisheries Co., Inc.
2. Fish must be less than 2-1/2 inches total length.

B. HOLDING

1. Clean all tanks with a detergent and rinse thoroughly with water before using. (Caution: Do not use tanks treated by manufacturer to retard growth of mildew, fungi, or bacteria.)
2. Place no more than 250 fish in the holding tank in nonchlorinated or dechlorinated water as soon as possible after receipt from hatchery (section C, day 1). Do not leave fish in extreme temperature (e.g., on a loading dock in sun during summer). To reduce stress avoid abrupt temperature changes of water in which fish are shipped (section C, day 3).

3. Keep dissolved oxygen levels above 5 milligrams/liter. Supply dissolved oxygen (DO) from oil-free compressed air released from dispersers near the bottom of the tank. In many cases the filter and dispersers can be operated as an integral unit. Aerate holding tank continuously from the day it is filled until all fish are removed for testing.

4. Remove debris from bottom of tank daily with a siphon or pump.

5. Feed fish daily starting on day 6 and continuing until 2 days preceding the test. Fish food will normally be consumed in 10 minutes. If fish do not consume all the food added during this period, regulate the amount added until all food is consumed.

6. Fish will be held in holding tanks for 19 days before they are used in the test. This period of time is necessary for disease-prevention treatment (section II, C) and for acclimation to effluent-receiving water (section II, D).

C. DISEASE-PREVENTION TREATMENT

All fish shall be given preventive treatment for disease or parasites upon receipt from the hatchery. Treat the fish in the holding tank in the following day-by-day sequence and *do not stop aeration at any time.*

Day 1: Two to three days before the fish arrive, fill the holding tank with nonchlorinated water to the 5-gallon level and mark the level permanently with waterproof marker. Add 45 more gallons of nonchlorinated water and mark the 50-gallon level. Fill three storage containers with nonchlorinated water and bring to room temperature. Aerate the water continuously. (Caution: Be sure to dechlorinate with a carbon filter if water contains chlorine.)

Day 3: Fish arrival. Without opening, float the plastic shipping bag (water and fish) in the tank and acclimate to tank temperature for 1 - 2 hours before releasing the fish to tank water.

Day 4: (a) Add 5 grams ACS grade potassium permanganate ($KMnO_4$) to 450 milliliters distilled or deionized water. When dissolved dilute up to 500 milliliters. This is a 1% solution of $KMnO_4$. This solution is used on days 4 and 5, and any remaining solution is then discarded.

(b) Add 190 milliliters of the $KMnO_4$ solution to the holding tank containing 50 gallons of water. (Caution: Stir while adding the $KMnO_4$ to achieve a uniform solution.) Hold the fish in the $KMnO_4$ solution for 1/2 hour. Do not exceed the 1/2-hour limit, but continue the treatment to that time even if the fish begin to surface or otherwise seem stressed.

(c) After 25 minutes of treatment, begin to drain the holding tank to the 5-gallon mark. At 30 minutes add water from the storage container to bring the tank volume to 50 gallons.

(d) Add to the holding tank 1 - 2 milliliters of 0.1 *N* sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) solution, or until the water turns yellow-brown. (To prepare 0.1 *N* sodium thiosulfate solution dissolve 25 grams sodium thiosulfate in volumetric flask and dilute to 1 liter with distilled or deionized water.) Color change is slow, so do not be concerned if the water does not become yellow-brown immediately. Refill storage container with nonchlorinated water. *Be sure water is at room temperature before it is used on day 5.*

Day 5: Repeat steps (b), (c), and (d) from day 4. It is not necessary to refill the storage containers on this day.

Day 6: (a) Add 4,750 milligrams of tetracycline (the material from nineteen 250-milligram capsules) to 500 milliliters of warm tap water. Shake until dissolved. Pour this solution into the holding tank and mix gently. Leave the fish in this solution. Fish are fed for the first time on this day.

(b) Fill storage containers with effluent-receiving water for acclimation (section II, D). Aerate water.

Day 7: Feed fish and clean bottom of tank with siphon. Aerate water.

D. ACCLIMATION PERIOD

All fish selected for testing shall be acclimated at room temperature in effluent-receiving water for at least 10 days before the beginning of the bioassay. The 10-day period is *in addition*

to the time required for disease-prevention treatment. Acclimation water shall be representative of the receiving stream and shall be taken as near upstream from the waste discharge as possible. *Do not use distilled or deionized water.* Do not use fish if more than 5% die during the 2 days immediately preceding the test. Do not use disease-prevention treatment during acclimation period. Do not feed during last 2 days.

Day 8: Turn off aeration. Decrease volume in the holding tank to a depth of 3 inches, fill with receiving water from the storage containers immediately. Feed fish. Aerate water. If fish die, notify ORSANCO.

Days 9-17: Acclimate fish in receiving water. Feed daily. Aerate water.

Days 18-19: Acclimate fish in receiving water. Aerate water. Do *not* feed fish. If fish are not scheduled for use on day 20, continue to feed fish daily until 2 days before test is started.

E. TEST CONTAINERS

Test containers shall be made of glass as specified in section I. They will provide a relatively large area at the surface of the water to aid in maintaining oxygen levels. The volume of solution in the test container *must be* 16 liters. *Plastic liners are not to be used.* Test containers must be cleaned thoroughly with a detergent and warm water, followed by a rinse with tap water and distilled water before use and between tests. (Caution: Hot water will crack containers.)

F. DILUTION WATER

Dilution water for the test shall be a representative sample of the receiving stream taken as near upstream from the discharge as possible. It shall be used unfiltered. Use acclimation water, or water from same source. Aerate dilution water before the start of test, but *not in test containers during the test. Experimentally prepared dilution water is not acceptable.*

G. TEMPERATURE

All tests shall be performed at room temperature (i.e., 23° ± 3° C, 73° ± 5° F). A maximum temperature of 25° C (77° F) is recommended, but in no case should the maximum summer temperature of the receiving stream be exceeded.

H. PHYSICAL AND CHEMICAL DETERMINATIONS

The following measurements shall be made on the test solution and reported with bioassay results:

1. Dissolved oxygen (at start and end of test); 125-milliliter bottle or DO electrode is recommended.
2. Temperature (at start and end of test).
3. pH (at start of test only).

I. CONTROL TEST

A control test with dilution water only (no sewage or industrial waste added) shall be performed concurrently and in exactly the same manner and under the same conditions as set forth for the test solutions in the 24-hour bioassay.

If the mortality of the fish in the control test is more than two fish out of 10, this suggests (1) unhealthy fish that are likely to be more sensitive to wastes, (2) residual toxicity from the container, or (3) toxicity from the dilution water itself. If mortality is excessive, call ORSANCO.

J. EXPLORATORY TEST

Exploratory tests may be performed before full-scale testing to determine the range of concentration for wastes of unknown toxicity and to reduce time and work. Such tests should be 6- to 8-hour static tests of 100%, 10%, 1%, and 0.1% concentrations of waste in dilution water. Place two fish in 2 liters or more of test solution for each concentration.

K. WASTEWATER-SAMPLE PREPARATION

The wastewater sample to be tested shall consist of six equal samples collected at 4-hour intervals during any 24-hour period to form a composite sample. All dilutions required for a single test must be prepared from the composite sample. Undissolved materials present in wastewater samples shall be uniformly dispersed by agitation immediately before addition to the test con-

tainer. Unnecessary exposure of the wastewater sample and its dilution to the atmosphere by agitation must be avoided.

L. CONCENTRATIONS TO BE TESTED

The test solutions shall be prepared by mixing composited wastewater (section II, K) and dilution water (section II, F) to achieve the desired concentrations. Test concentrations must include concentrations which kill less than half of the fish and more than half, or else exactly half. At least five consecutive concentrations of waste from the dilution table (below) and a control with dilution water only (section II, I) must be tested. There shall be two test containers for each concentration. Each container shall be filled with 16 liters of test solution. After preparation of the test solution, perform the required chemical and physical determinations (section II, H).

Dilution Table

To prepare test solutions mark a test container at the 16-liter volume, add volume of wastewater specified in right-hand column for appropriate concentration, and fill container to the mark with dilution water.

Test concentration (percentge waste)	Milliliters of wastewater to be added
100	16,000 = 16 liters
50	8,000
25	4,000
10	1,600
5	800
2.5	400
1.0	160
0.5	80
0.25	40
0.1	16
0.05	8
0.025	4
0.01	1.6

M. FEEDING OF FISH DURING THE TEST

Do not feed the fish during the 24-hour test or for 48 hours before the test.

N. TEST DURATION

All tests shall last 24 hours.

O. NUMBER OF FISH

Use 10 fish in each concentration of test solution and divide them between two test containers with a liquid volume of 16 liters per container. Put one fish in first container, the second fish in the second container, and so on alternately until five fish are in each container.

P. OBSERVATION OF TEST RESULTS

Fish are considered dead when all movement has ceased, whether spontaneous or in response to gentle prodding. Remove dead fish as soon as observed. Dead fish left in the container for more than 1 hour may cause others to die because of decomposition and subsequent reduction of oxygen.

Q. REPORTING OF SURVIVAL

Record on the Bioassay Record Sheet (see appendix, p. 17) the number of fish surviving at the end of 24 hours in each concentration of test solution.

R. RENEWAL OF TEST SOLUTION

The test solution *must not be renewed* during the 24-hour period.

S. MAINTENANCE OF OXYGEN LEVELS

If the wastewater causes significant oxygen depletion (to 4 milligrams/liter or less), it is permissible to add O-TABS to reoxygenate the test solution. Add one tablet at a time, using additional tablets only as necessary during the 24-hour exposure to maintain at least 4 milligrams/liter of oxygen.

III. 24-Hour Static Test Procedure

A. Prepare the test solutions and control (sections II, I and II, L).
B. Add fish within 1 hour after preparation of the test solutions and control.
C. Record the number of fish surviving in each test container at the end of the 24-hour period on the Bioassay Record Sheet.
D. Estimate the 24-hour LC50 in accordance with the following instructions on the sheet *Bioassay Paper* (see appendix, p. 18). If lower concentrations are required on the graph, the numbers on the horizontal scale may be divided by 10, 100, etc. as necessary.

Example

An LC50 is a lethal concentration at which 50% of the experimental animals survived, or it is an interpolated value, based on percentages of fish surviving at two or more concentrations, at which less than half and more than half survived. Estimation of the LC50 by interpolation involves merely the plotting of the data on the logarithmic scale, and percentage survival on the probability axis or scale. A straight line is drawn between two points representing survival at the two successive concentrations that were lethal to more than half and to less than half of the fish. The concentration at which this line crosses the 50% survival line is the LC50. Figure 1 (see appendix, p. 19) illustrates this procedure, which is commonly referred to as straight-line graphical interpolation. Hypothetical results used in developing the graph are given in the table. The LC50 for this hypothetical waste is 4.5%. It is permissible to use this method of straight-line interpolation even if the lower concentration gives 100% survival and the next higher concentration in succession gives 0% survival.

IV. Calculation of Allowable Discharge Rate

Calculate allowable discharge rate to river in accordance with the following formula:

$$V = \frac{0.646\,F}{\left(\frac{2000}{C}\right) \cdot 1}$$

where V = volume of waste permitted to be discharged in the stream (in million gallons/day);
F = flow in the stream above the discharge (in cubic feet/second); and
C = percentage concentration of waste to achieve the 96-hour LC50.

If $C = 20\%$, the formula can be simplified with less than 1% error to:

$$V = 3.23 \times 10^{-4}\, C \times F.$$

(See appendix, p. 20, for detailed explanation.)

REFERENCES

American Public Health Association, American Water Works Association, Water Pollution Control Federation. 1971. Standard methods for the examination of water and wastewater. 13th ed. 874 p.

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Davis, H.S. 1953. Culture and diseases of game fishes. Univ. California Press, Berkeley, Calif. 332 p.

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Reichenbach-Klinke, H.H., and E. Elkan. 1965. The principal diseases of lower vertebrates. Academic Press, New York, 600 p.

Snieszko, Stanislas F. (ed.) 1970. A symposium on diseases of fishes and shellfishes. Spec. Publ. 5, American Fisheries Society, Washington, D.C. 526 p.

Van Duijn, C., Jr. 1967. Diseases of fishes. 2nd ed. Iliffe Books, Ltd., London. 309 p.

APPENDIX

NEW DRUGS OR BIOLOGICALS FOR

INVESTIGATIONAL USE FOR IN VITRO TESTING OR IN LABORATORY RESEARCH ANIMALS

UNDER THE U.S. FEDERAL FOOD, DRUG, AND COSMETIC ACT

SECTIONS 505, 507

Name of Investigator .....

Address .....

.....

Date .....

Name of Drug or Biological .....

This is to certify that the undersigned is regularly engaged in conducting in vitro testing or in laboratory research animal tests and that the drug or biological named above will be used only for such laboratory studies or animal testing and will not be used in humans.

(Signed) .....

BIOASSAY RECORD SHEET

Series:_____ Municipality or Company:_____ Date:_____

Technician:_____ Starting Hour:_____

Material being tested:_____

Source:_____

Source of dilution water:_____

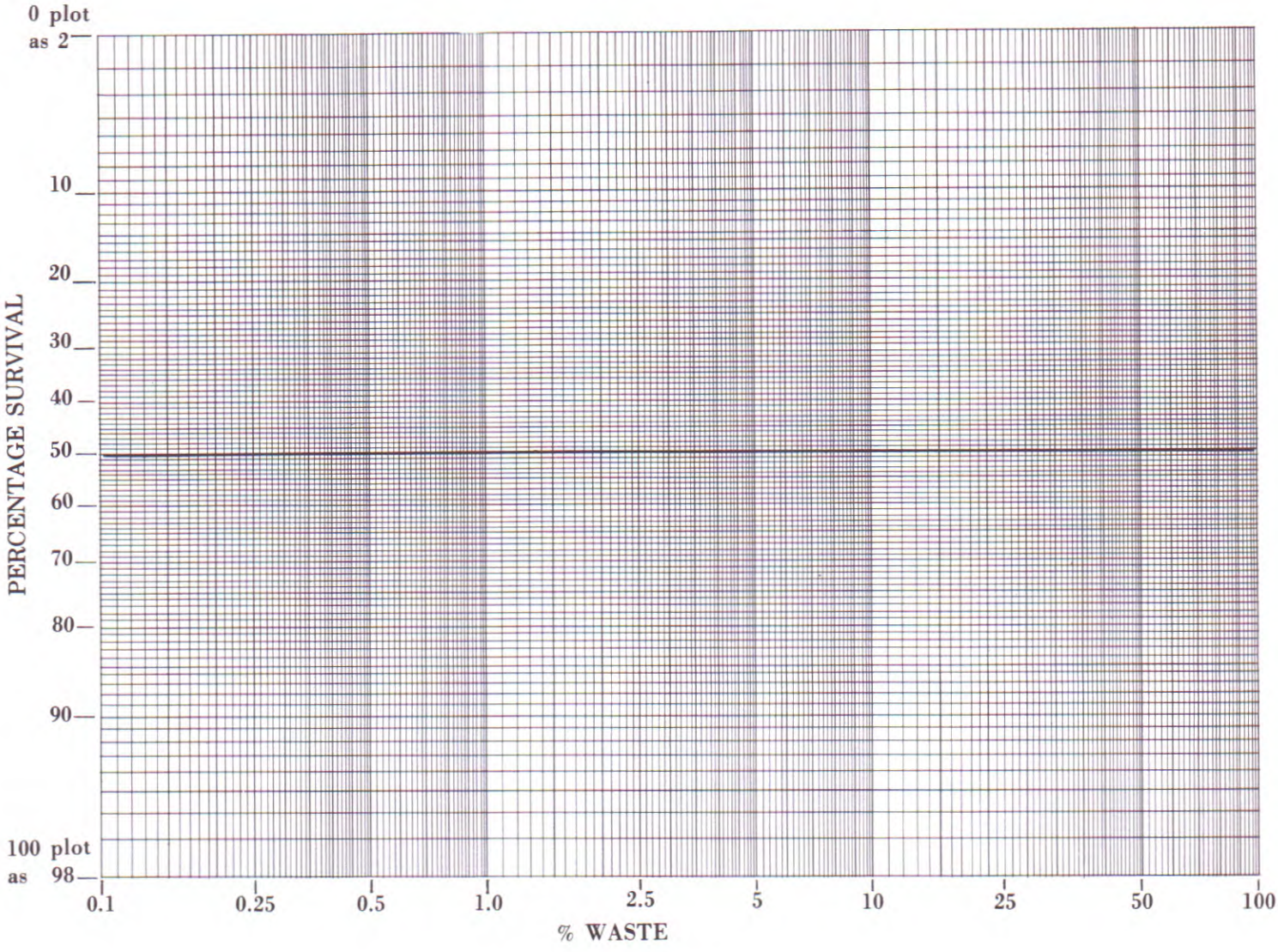
Test species:_____ Temp. range:_____

No. individuals per percent waste:_____ O-TABS Used:_____

Start									
Percent waste									Control
DO									
Temperature									
pH									
24 hours									
Number Surviving									
% Survival									
DO									
Temperature									
pH									
48 hours									
Number Surviving									
% Survival									
DO									
Temperature									
pH									
96 hours									
Number Surviving									
% Survival									
DO									
Temperature									
pH									

BIOASSAY PAPER (Log-probit)

Code:_____



Material Tested:_____

Starting Date: _____ Hour: _____

Concentrations expressed as (circle one): %, mg/liter_____

Test species:_____ Temperature:_____

Dilution water source and characteristics: _____

_____

_____

Other notes: _____

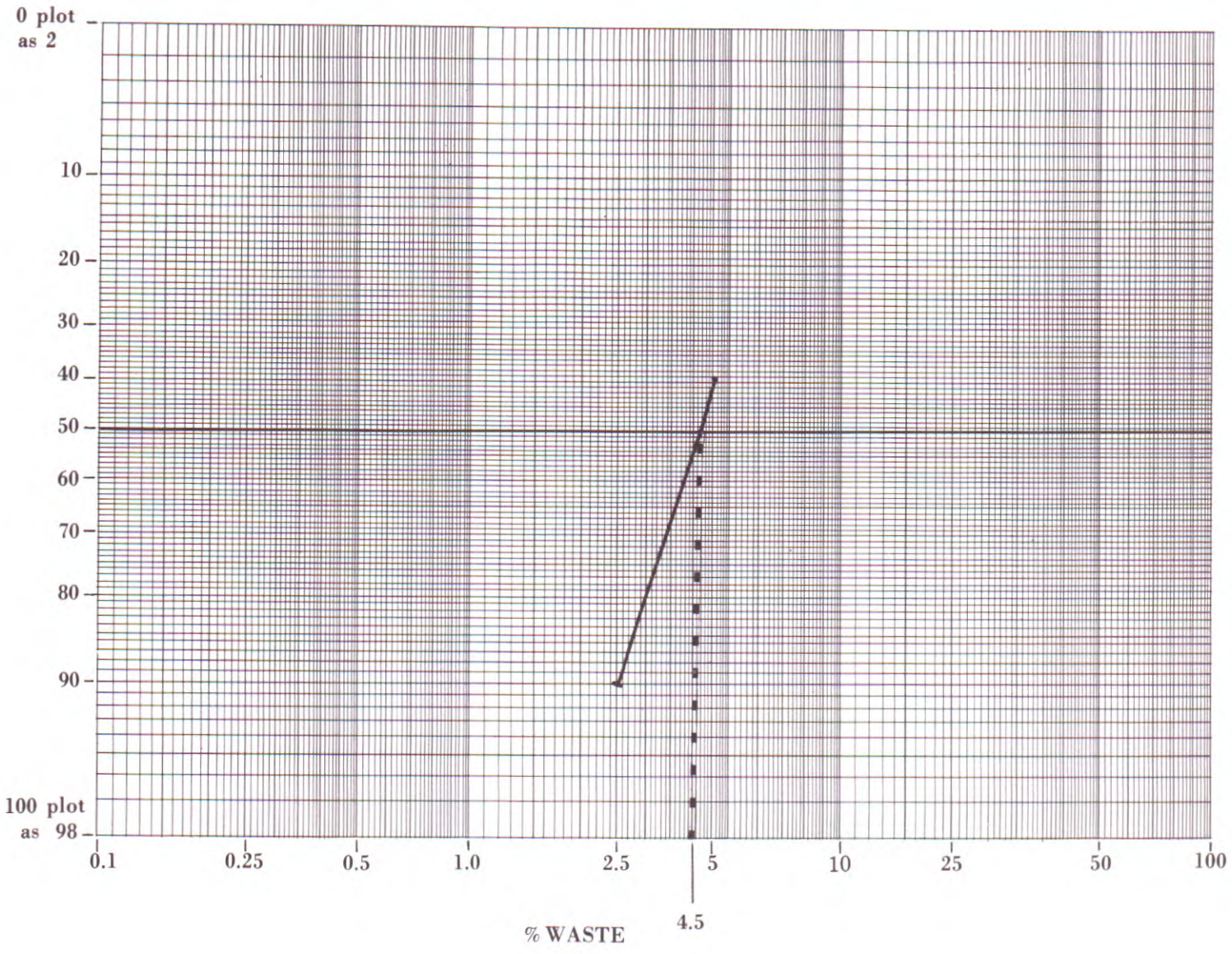
_____

Final Results:

Time interval		24 hr.	48 hr.	96 hr.	
LC50					

Observer:_____

BIOASSAY PAPER (Log-probit) Code:_____



%waste	25	10	5	2.5	1.0			Control
Number surviving	0	0	4	9	10			10
%survival	0	0	40	90	100			100

Figure 1. Estimation of LC_{50} by straight-line graphical interpolation. The waste is hypothetical.

Explanation of Formula for Calculation of Allowable Discharge Rate

I. Paragraph E of ORSANCO Pollution Control Standard No. 1-70 states:
"Reduction of any material or, if necessary, all materials contained in any discharge which singly or in combination are toxic or harmful to aquatic life to such a degree or degrees that the calculated concentration(s) of such material or materials in the river does not exceed one-twentieth of the 96-hour median tolerance limit (96-hr. TL_m) for aquatic life."

II. Standard Methods (American Public Health Association, 1971, p. 565, 3c) defines the percentage concentration of wastewater in the bioassay as

$$\frac{\text{(Volume of wastewater)}}{\text{(Volume of wastewater \& volume of dilution water)}} \times 100$$

and defines V, F, and C in the formula as:

V = volume of wastewater (permitted to flow into the stream);
F = volume of dilution water (or flow in the stream above the discharge);
C = percent of waste to achieve the LC50 (96-hour) without regard to the units of V and F.

Paragraph E states that C must not exceed 1/20 of the above percentage concentration of wastewater so that formula is restated as:

$$\frac{1}{20} C = \frac{V}{V+F} \times 100$$

or

$$C = \frac{2000\ V}{V+F}$$

or

$$V = \frac{F}{\frac{2000}{C} - 1}$$

where V and F are both measured in the same units. However, if V is to be measured in million gallons/day and F in cubic feet/second, F must be multiplied by 0.646 as a unit conversion factor and the formula assumes the form:

$$V = \frac{0.646\ F}{\frac{2000}{C} - 1}$$

where V = volume of waste permitted to be discharged to the stream (in million gallons/day);
F = flow in the stream above the discharge (in cubic feet/second); and
C = percentage concentration of waste to achieve LC50 (96-hour).

EXAMPLE NO. 1

If C = 1% and F = 6500 cubic feet/second, then

$$V = \frac{0.646\ F}{\frac{2000}{C} - 1}$$

$$= \frac{0.646 \text{ x } 6500}{\frac{2000}{1} - 1}$$

$$= 2.10 \text{ million gallons/day}$$

or with the simplified formula:

$$V = 3.23 \text{ x } 10^3 \, C \text{ x } F$$
$$= 3.23 \text{ x } 10^3 \text{ x } 1 \text{ x } 6500$$
$$= 2.10 \text{ million gallons/day}$$

If flow of discharge is greater than 2.10 million gallons/day, toxicity of waste must be decreased to meet Pollution Control Standard No. 1-70, paragraph E.

EXAMPLE NO. 2

If C = 20% and F = 50 cubic feet/second, then

$$V = \frac{0.646 \, F}{\frac{2000}{C} - 1}$$

$$= \frac{0.646 \text{ x } 50}{\frac{2000}{20} - 1}$$

$$= 0.326 \text{ million gallons/day}$$

If flow of discharge is greater than 0.326 million gallons/day, toxicity of waste must be decreased to meet Pollution Control Standard No. 1-70, paragraph E.

REGULATORY AGENCIES of the SIGNATORY STATES

ILLINOIS
Environmental Protection Agency
State of Illinois
Springfield, Illinois 62706
Phone: 217-525-6580

INDIANA
Indiana Stream Pollution Control Board
1330 West Michigan Street
Indianapolis, Indiana 46206
Phone: 317-633-4420

KENTUCKY
Department of Natural Resources
Commonwealth of Kentucky
275 East Main Street
Frankfort, Kentucky 40601
Phone: 502-564-3410

NEW YORK
Environmental Health Services
NYS Dept. of Environmental Conservation
50 Wolf Road
Albany, New York 12201
Phone: 518-457-6533

OHIO
Environmental Protection Agency
State of Ohio
P. O. Box 1049
Columbus, Ohio 43216
Phone: 614-469-6116

PENNSYLVANIA
Department of Environmental Resources
P. O. Box 2063
Harrisburg, Pennsylvania 17120
Phone: 717-787-2666

VIRGINIA
Water Quality Section
State Water Control Board
P. O. Box 11143
Richmond, Virginia 23230
Phone: 703-770-2241

WEST VIRGINIA
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Department of Natural Resources
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