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Industrial Wastes

BIO-ASSAYS FOR CONTROL OF INDUSTRIAL EFFLUENTS *

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Waste effluents from many industries are toxic to fish and other aquatic life. The control or treatment of these wastes for safe release into receiving waters presents a problem of considerable magnitude.

Much effort has been expended by some industries to solve this problem, primarily through chemical analysis for possible toxic components and then generally by a search of the literature to find out how toxic a particular component may be. Unfortunately, this approach has not been successful, nor can it be expected to be productive of satisfactory answers.

Many of the wastes are chemically complex and the necessary techniques for separating and analytical methods for determining all components have not been developed nor are methods sensitive enough to detect all materials at the small concentrations at which they may be toxic. Even when analytical methods are available, toxicity information often is lacking and toxicity cannot always be attributed to a single component. In complex wastes, there may be a number of different toxicants which, when mixed with or under the influence of other chemicals in the effluent or receiving water, may produce an entirely different toxicity from that of the pure components.

The bio-assay solves the problem because it can be used to evaluate directly the toxicity of chemically complex wastes. In many cases, it provides the quickest and most economical approach to identifying and solving waste disposal problems.

While the use of bio-assays by industry is increasing, neither their present value nor the extent of their future use has been fully realized. The tremendous expansion of the chemical industry, increased demand for water by various users, and the need for re-use of water necessitates greater emphasis on toxicity determinations, rather than complete reliance on B.O.D. and chemical determinations for the safe disposal of industrial wastes.

Practical methods of conducting bio-assays have been developed (1) (2) which are relatively easy and economical to use. Some industries have used bio-assays, and in the future many others may find it necessary and desirable to establish bio-assay laboratories. The number of requests received by the Public Health Service from state water-pollution control agencies and from industries for information on the practical use of bio-assays are increasing. Simplified instructions are needed for establishing a bio-assay laboratory and for conducting and using bio-assays. This paper describes bio-assay methods that have been satisfactorily used with industrial effluents. It includes information on the equipment,

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costs, and other requirements necessary for setting up a laboratory, and the many ways in which industry can use bio-assays in the control of industrial effluents.

The Value of Bio-Assays

The literature on the toxicity of various chemicals to fish is voluminous. In addition to numerous articles in American and foreign journals, a number of reviews and summaries of available data on the toxicity of materials to fishes have been published (3)(4)(5)(6). Most of the information available is concerned with the toxicity of a pure chemical to a particular fish under certain experimental conditions which often are not fully described. A review of the literature on almost any chemical will show a wide range of lethal or harmful values obtained by different investigators (*e.g.*, copper, 0.02 to 200 p.p.m.; cyanide, 0.05 to 10 p.p.m.; and phenol, 0.4 to 400 p.p.m.). Such values, of course, reflect the use of different test fish, dilution waters, test exposure times, and other experimental conditions. Some of the values obtained are widely quoted as "safe" or "lethal" concentrations with little or no reference to the experimental conditions. Many of these concentrations are not only of little value but also may be misleading.

Even if the toxic components of a complex waste and their individual toxicities are known, it would be difficult or impossible for an industry to use such data to indicate the toxicity of the total waste in a particular receiving water. A limited amount of toxicity information is available for mixed industrial effluents, but this may vary greatly even in the same type of industry depending on operational conditions.

Toxicity of the same or similar wastes may vary widely in different receiving waters. Many factors may influence the toxicity of a waste to fish. Among these are temperature, dissolved oxygen, carbon dioxide, pH, alkalinity, hardness, synergy, and antagonism.

Hydrogen cyanide, cyanogen chloride, and some of the simple metal cyanides are highly toxic to fish. The toxicity is usually greater at high temperatures and low dissolved oxygen and pH levels. The toxicity of complex cyanides is generally lower than hydrogen cyanide but some of these are greatly influenced by water quality characteristics. It has been reported (7) that ferro- and ferri-cyanides when exposed to sunlight are subject to photo-decomposition with the result that they break down and become toxic at concentrations as low as 2 p.p.m. The toxicity of many of the metal-cyanide complexes is greatly influenced by pH. Doudoroff (8) has reported that fish can withstand more than 1,000 times as much nickel cyanide complex at pH 8 as at pH 6.5. He also found that a reduction in pH from 7.8 to 7.5, can result in more than a 10-fold increase in the toxicity of a $NaCN$-$NiSO_4$ mixture. Thiocyanates may be decomposed by chlorination with the formation of highly toxic cyanogen chloride. It is thus apparent that the concentration of cyanide in an undiluted cyanide-bearing waste is not necessarily a reliable measure of the toxicity of the waste in the receiving stream.

Ammonia becomes rapidly more toxic as the pH increases above 8.0. This is probably because the NH_4OH molecule is much more toxic than the NH_3 ion. Being a weak base, ionization of NH_4OH is depressed as the pH increases, with the result that more and more molecules are present and thus the toxicity increases. Ellis (3) found that the toxicity of ammonium compounds increased 200 per cent or more between pH 7.4 and pH 8.0. Acid salts of ammonia can be expected to be less toxic in soft water with lower buffering capacity because they lower the pH and decrease the concentration

of NH_4OH molecules. The total amount of ammonia present as indicated by chemical analysis is not necessarily a measure of toxicity.

The toxicity of metal salts may be greatly influenced by pH, alkalinity, and hardness. In studies carried on at the Robert A. Taft Sanitary Engineering Center, it has been found that many of the metals are much more toxic in soft water than they are in hard water. For example, beryllium and uranium were 60 to 80 times more toxic to fathead minnows in soft water than they were in hard water. Copper has killed fish at concentrations as low as 0.06 p.p.m. in soft water with a low pH; whereas it is well known that during algicidal operations in some lakes, concentrations well above 0.5 p.p.m. have been applied without lethal effects to fishes. The toxicity of solutions containing titanium, vanadium, zirconium, and other metals may be reduced due to precipitation or complexing with materials present in the water, thus reducing the amount of metal ion in solution. The toxicity is greatly increased at lower pH values.

Moderate concentrations of metal salts such as those of sodium, magnesium, potassium, or calcium, which are harmless to fish in sea water and in highly mineralized fresh water, can be injurious when they occur alone or in physiologically unbalanced solutions. The toxicity of copper, zinc, and other heavy metals is counteracted by calcium and other antagonistic metal cations. On the other hand, copper and zinc, copper and cadmium, and zinc and nickel are strongly synergistic. Doudoroff found that 0.025 p.p.m. of copper with 1 p.p.m. zinc in soft water was more toxic to fathead minnows than 0.2 p.p.m. copper, or 8.0 p.p.m. of zinc alone.

Dissociation, recombination, and oxidization are also of importance in determining the toxicity of a waste in a receiving stream. Recent studies at the Sanitary Engineering Center disclosed that fish lived longer in a relatively high concentration of Na_2S than they did in weak solutions. In the weaker solutions oxidation produced SO_4 which, due to dissociation and recombination, caused a lowering of the pH. This in turn increased the amount of H_2S formed by the dissociation of Na_2S. Since it is the H_2S molecule which is largely, if not entirely, responsible for the toxicity, lowering of the pH depresses the ionization of the H_2S and the toxicity increases.

It is evident from the foregoing that the character of the receiving water can cause wide variations in the toxicity of many materials to fishes. Furthermore, wastes already in a stream or added subsequently may influence the toxicity of another waste through antagonistic or synergistic action. Chemical analyses alone cannot indicate toxicity in most instances; however, a knowledge of the chemical makeup of a waste can be helpful in applying bioassay results. Furthermore, analytical methods are not available for all substances which may be toxic to fishes, nor are they sensitive enough to detect some materials at the low concentrations at which they are toxic. Nor would a knowledge of the main constituents of a waste and their individual toxicities indicate the toxicity of the complex or its variation with changes in water quality.

In view of this situation it is believed that water quality criteria expressed in general numerical terms cannot be set up for all materials to apply over extensive areas or even to all parts of the same stream. If such a cook-book approach is used, permissible levels must be set so low that the allowable concentration is safe, even under those situations in which the material is most toxic. It is apparent, therefore, that rigid standards applied over wide areas without regard to normal stream characteristics, will penalize

those industries on streams in which the toxicity of the wastes is naturally low.

There are three main criteria which can be assigned numerical values applicable over extensive areas. These are criteria for temperature, D. O., and pH. While the toxicity of some substances is not significantly affected by water quality, it is believed desirable from the standpoint of fairness and economy to adopt the tailor-made approach for toxicity criteria and establish safe levels based on the amount of a particular waste that can be added to a particular stream at a specific point without detriment to aquatic life. These safe levels can best be determined by means of bio-assays made with the waste in question by using water from the receiving stream for dilution and by employing local fish as bio-reagents. The bio-assay takes into consideration most of the factors governing toxicity and is a direct approach to the problem. However, plant operational and chemical data are necessary for the proper interpretation and application of bio-assay results.

Bio-Assay Facilities and Equipment

Various bio-assay procedures using fish (1)(2)(9)(10)(11)(12)(13) and other organisms (14) have been described. The procedure recommended by the subcommittee on toxicity of the Federation of Sewage and Industrial Wastes Associations (2) has proved satisfactory with a variety of industrial effluents. Briefly this procedure consists of preparing different concentrations of an effluent or other test material with a selected dilution water, adding the test fish, and observing their reactions over a definite time period. From the observed mortality in the different concentrations, median tolerance limit (TL_m) values are estimated by straight line graphical interpolation. The tests are usually conducted for a 96-hr. period with 24-, 48-, and 96-hr. TL_m values estimated by this method. The TL_m (concentration that kills fifty per cent of the test fish) is a direct comparative measure of toxicity.

This discussion is concerned with some of the practical problems that have been experienced in conducting bio-assays with a variety of industrial effluents. For further details or explanatory material, reference (2) should be consulted. The major requirements for conducting bio-assays are: (a) an adequate supply of test fish and dilution water and (b) laboratory facilities for holding fish and conducting the bio-assays.

Test Fish

The test fish used in the studies should be a species adaptable to the laboratory conditions of temperature, feeding, and handling. They should be of small size (for the experimental conditions described in this paper they should not exceed 3 in. in length nor weigh more than 2 g.) and should be readily available. This discussion is confined primarily to the use of so-called warm water species which will be most commonly used in bio-assay laboratories. However, cold water species such as trout or salmon are good test fish and are easily kept in the laboratory if temperatures of 55° to 65° F. can be maintained and adequate water supplies are available. Where these fish are important in receiving waters, provision should be made for their use.

Availability of an adequate supply of healthy fish of desirable uniform size may be the major factor in the selection of a test species. Almost any species that can be satisfactorily maintained in the bio-assay laboratory can be used. A suitable supply of test fish may be generally available from such sources as bait dealers, fish hatcheries (state, federal, or private), pet shops, and so-called fish farms which specialize in goldfish or other species

normally used in household aquaria. Supplies may also be obtained by seining or trapping in local waters or they may be raised in outdoor or indoor facilities. Local conservation officers or fisheries personnel may be of help in locating an acceptable supply.

Species which have been used successfully in bio-assay laboratories and are commonly available from bait dealers are fathead minnows (*Pimephales promelas*), bluntnose minnows (*Hyborhynchus notatus*), and Baltimore minnows (uncolored goldfish). Other species often handled by bait dealers which are satisfactory, although not generally available in suitable size or numbers, are creek chubs (*Semotilus atromaculatus*), suckers (*Catostomus* sp.), stonerollers (*Campostoma anomalum*), and dace (*Rhinichthys* sp.). Other species which are frequently available, although much more difficult to handle in a bio-assay laboratory, are shiners (*Notropis* sp.) and golden shiners (*Notemigonus crysoleucas*). From the standpoint of size, ease of handling, adaptability to laboratory conditions, and uniformity of test results, the fathead minnow is an excellent choice.

Many state, federal, and private fish hatcheries in various parts of the United States raise warm water game species such as sunfish (*Lepomis* sp.) and bass (*Micropterus* sp.). Juveniles (1½ to 2½ in. long) of most of the centrarchids (sunfish, crappies, black bass, etc.) are satisfactory as test fish for bio-assay laboratories, although somewhat more care is required in acclimating these fish to laboratory conditions, especially feeding. One advantageous use of the centrarchids is that bio-assay results can be applied directly since these fish are generally the most important in receiving waters. Bluegill sunfish (*Lepomis macrochirus*) have been successfully used by many bio-assay laboratories.

Among usable species available from pet stores or fish farms are the goldfish (*Carassius auratus*) and guppies (*Lebistes reticulatus*). While these fish are not normally of importance in receiving waters and bio-assay results may not be applied directly, they are among the most desirable test fish from the standpoint of maintenance in the laboratory and uniformity of available stock. If provision is made for comparison with important local species, these fish may be desirable test fish for routine bio-assay work.

Many of the previously named, as well as additional suitable species, may be obtained by seining or trapping from local ponds, lakes, or streams if adequate numbers of uniform size can be obtained at various seasons of the year. Some species, readily obtainable at some seasons, are almost impossible to obtain at others. Provision of adequate holding facilities such as small outdoor earthen ponds, outdoor or indoor concrete pools, or large glass aquaria may overcome this difficulty. Species such as fathead minnows, bluegill sunfish, or goldfish can be raised in outdoor earthen ponds while guppies may be raised in indoor pools or aquaria.

For uniform test results it has been found desirable to use a single species for the bio-assay work. The sensitivity of different species to different chemicals varies greatly. Fish that are tolerant of low oxygen conditions are not necessarily tolerant of toxic materials. The tolerance also may be dependent on the type of chemical. For example, bluegill sunfish are more tolerant of copper and chlorine than fathead minnows, while the reverse is true for certain insecticides and detergents. Even the supposedly resistant goldfish is fairly sensitive to some chemicals. When investigating a particular effluent, a few comparative tests can relate the sensitivity of the test fish to that of other species which may be of major importance in the receiving water.

This information is essential when using bio-assay results to determine safe dilutions in a receiving stream.

The cost of test fish may vary considerably depending on their source and location. Small bait minnows are usually reasonable, the price generally ranging from $1.00 to $5.00 per hundred, while costs of other species range from $5.00 upward.

When stock supplies of test fish are brought into the laboratory some losses may be expected. The extent of the loss will depend on the care used in their capture and transport. Losses occur more frequently among certain species of wild fish, usually within two or three days after capture with few subsequent losses. The fish should not be used for bio-assays until they have been acclimated to laboratory conditions for at least a week; a longer acclimation period should be used if excessive losses continue.

Provision must be made for feeding the stock fish. A commercial dry dog food, ground and screened to remove dust and to secure a size readily taken by the fish, has proven satisfactory for feeding several test species. Daily feeding is adequate for most species. Centrarchids, which normally feed on living material, do well on this type of food if they are taught to eat it by feeding small quantities several times daily. Care must be taken not to overfeed. Avoidance of extremely fine food material also helps to keep the aquaria clean.

Dilution Water

The recommended diluent for preparing test concentrations is water from the receiving stream or lake, just upstream from the effluent outlet and outside its zone of contamination. If this upstream water is already contaminated to the extent that fish will not survive after suitable aeration, other diluent sources must be used. In such cases the characteristics of the receiving water must be considered in evaluating bio-assay results.

Springs, streams, or lakes in the same locality have also proven satisfactory as sources of dilution water. Such substitute dilution waters should have characteristics similar to those of the receiving stream, particularly with respect to pH, alkalinity, and hardness, and should not be subject to wide fluctuations in these characteristics. Some modification may be made to meet these requirements by the addition of distilled or demineralized water or certain chemicals.

While ordinary city tap water has often been used in conducting bio-assays, this source is not generally satisfactory. Many city water supplies fluctuate widely in major characteristics, especially pH, alkalinity, and hardness, and this fluctuation may cause difficulty in obtaining uniform bio-assay results. Most city supplies also contain 0.2 to 1.0 p.p.m. residual chlorine which may be detrimental or even fatal to the test fish. Chlorine can be removed by passing the water through an activated carbon column, by allowing it to stand in sunlight for a day or two, or by vigorous aeration. Well water supplies are not generally satisfactory.

If the characteristics of the receiving water fluctuate widely, it may be desirable to use two or more diluent waters to cover this range of fluctuation. The characteristics of the diluent water should be known and reported for each series of bio-assays. The use of a good uniform experimental water, especially if obtained from the receiving stream, helps greatly in interpretation of bio-assay results.

Adequate storage facilities must be provided so that the diluent water, when hauled or piped into the laboratory, can be held until it reaches room temperature. It should be aerated to equilibrium with atmospheric gases before being used for bio-assays.

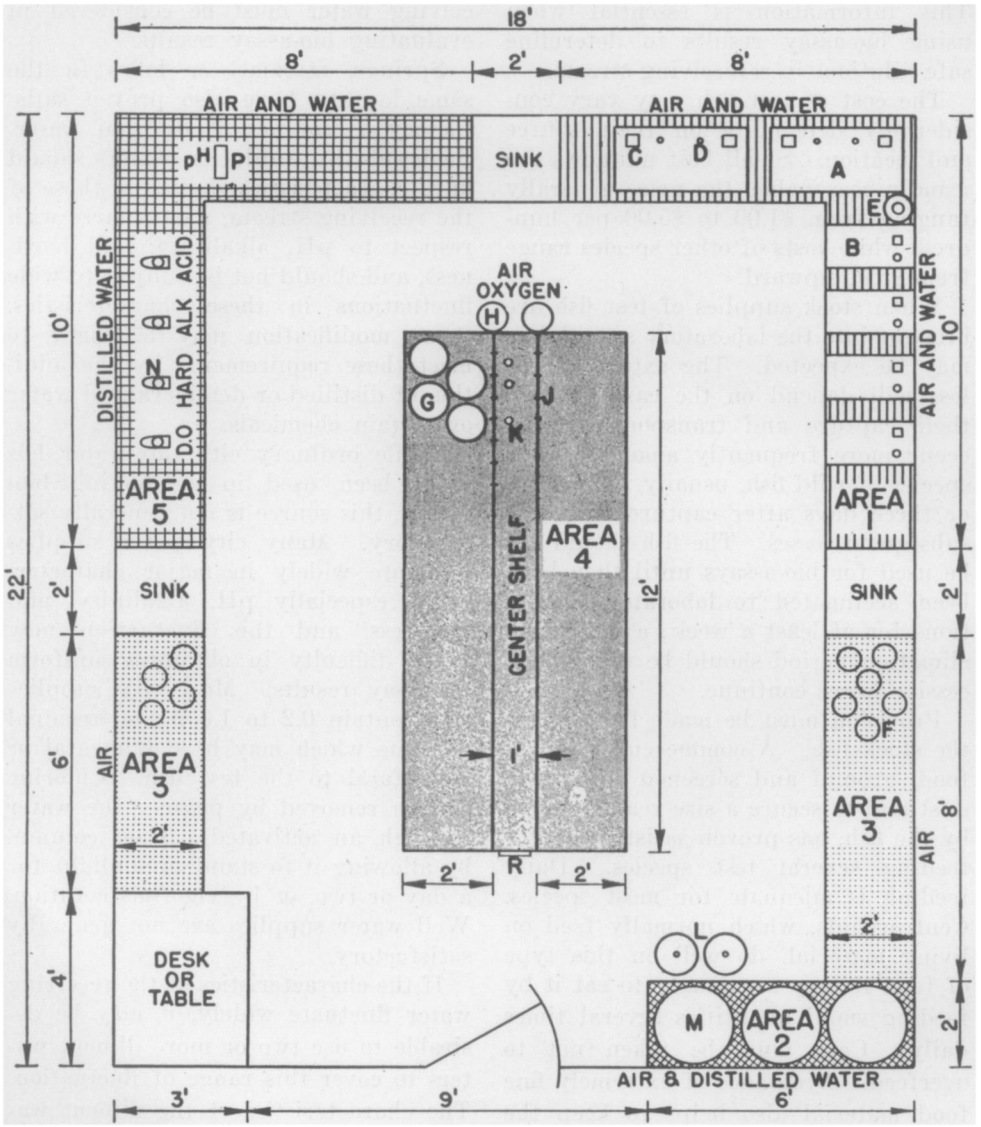

FIGURE 1.—Floor plan of a proposed bio-assay laboratory.

Laboratory Space, Facilities, and Equipment

Experience has indicated that the average industrial analytical laboratory is suitable for conducting bio-assays of industrial effluents. Most laboratories have a satisfactory degree of temperature control (70° to 80° F.) for bio-assays with warm water species of fish. For research or precise comparative work with pure chemicals, a further degree of temperature control such as an air conditioned laboratory, a constant temperature room, or water bath may be necessary. Most laboratories also have suitable bench space, sinks, and utilities, including compressed air, water, and ordinary chemical glassware.

A laboratory arrangement suitable and convenient for conducting bio-assays is shown in Figure 1. The cost

TABLE I.—Description and Cost of Bio-Assay Equipment

Item	Description	Cost Each ($)	Number Needed
A	50-gal. glass aquarium (36 in. × 18 in. × 18 in.)	80.00	2
B	15-gal. glass aquarium (24 in. × 12 in. × 12 in.)	13.00	4
C	Inside aquarium filter	2.00	8
D	Air stone	0.35	30
E	Activated carbon column (3½-in. dia., 3 ft. high)	50.00	1
F	1-gal. widemouth glass bottle (6½-in. dia., 9 in. high)	0.30	60
G	5-gal. widemouth glass bottle with bail handle and screw cap (10-in. dia., 18 in. high)	2.00	60
H	Oxygen cylinder with pressure reducing valve	50.00	2
J	Three-way air valve	0.70	40
K	Petri dish (4-in. dia.)	0.60	60
L	5-gal. polyethylene bottle with handle and screw cap (11-in. dia., 22 in. high)	7.75	20
M	50-gal. polyethylene tank with spigot and cover (22-in. dia., 32 in. high)	60.00	2
N	Squeez-o-matic burette (10 or 25 ml.)	14.00	4
P	pH meter	250.00	1
R	Recording thermometer (5-day)	180.00	1

of some of the items, not generally available in analytical laboratories, are listed in Table I.

Many other arrangements could be used; however, this one gives some idea of the bench space requirements for holding and acclimating test fish, preparing and storing dilution water, preparing test concentrations, conducting exploratory and full-scale bio-assays, and performing the necessary chemical control tests. Adequate space is provided for conducting four or five full-scale bio-assays simultaneously, which is about capacity for one person to handle. With additional shelf space underneath the main benches, and help in washing glassware, obtaining dilution water, and making chemical analyses, a considerably greater number of tests can be conducted. In a similar facility as many as 20 bio-assays of industrial effluents were conducted in a seven-day period.

If a special constant-temperature facility is desired, space requirements can be considerably reduced by using a two- or three-tier shelf arrangement for bio-assays and fish storage, and by removing the chemical work, washing of glassware, and other ancillary activities to an adjoining room. The bench or shelf space needed for each full-scale bio-assay is approximately 6 ft. long by 2 ft. wide.

Area No. 1, Figure 1, provides space for holding and acclimatizing fish. Each of the large aquaria for holding fish (A) is adequate for accommodating about 500 average-size test specimens. An air supply, adequate to provide continuous aeration must be available. Aquarium filters (C) of the inside type have been found most desirable and are helpful in keeping the aquaria clean and in lengthening the time between water changes. Ordinary tap water can be used for holding fish if chlorine has been removed by passing the water through an activated carbon column (E) and if temperature changes are not too abrupt. The smaller aquaria (B) may be used for acclimating the test fish to the experimental water or for holding them without food for periods immediately preceding the tests. They may also be useful for retaining previously used undamaged fish that may be re-used for exploratory tests.

Area No. 2 provides for the storage and preparation of dilution water.

The polyethylene items described (L; M) have been satisfactory for this use, although other inert materials of various sizes and shapes may be used. Additional supplies of distilled or demineralized water are also desirable, particularly if studies are to be made of the effect of a change in water quality characteristics on the toxicity of effluents.

Area No. 3 can be used for preparing experimental test concentrations of the effluent in the dilution water and for exploratory tests (F) to determine the approximate toxic range. In these tests air or oxygen may be needed, depending on the nature of the effluents.

Area No. 4 supplies bench space for holding 20 full-scale test aquaria (G) on each side and permits the simultaneous conduct of at least four full-scale bio-assays. Air and oxygen must be provided. A convenient arrangement for supplying air or oxygen is through a system of small tubing and three-way air valves (J) so that the supply to each test aquarium can be regulated independently. This system may be attached either to the air supply or to an oxygen cylinder (H) equipped with a pressure reducing valve and regulator. Small petri dishes (K), each containing a filter paper soaked in formalin, are convenient for holding and observing dead fish. A recording thermometer (R) is useful for temperature records.

Area No. 5 provides space for the chemical tests necessary for oxygen control and for the information to be used in interpreting bio-assay data. Squeez-o-matic burettes (N) have been found useful for the rapid performance of certain chemical tests.

Additional items needed for fish handling are dip nets, a food grinder, screens, and miscellaneous rubber or plastic tubing for air and oxygen lines. Various items of chemical glassware are needed for preparing test concentrations for bio-assays, for preparing chemical reagents, and for conducting the necessary analytical tests. Other major equipment items generally available in industrial laboratories may include a water still and/or demineralizer, an air compressor, and an analytical balance.

Conducting Bio-Assays

Collection of Effluent Samples

The collection of effluent samples for bio-assay studies and their correlation with plant operations is of extreme importance. The nature of the plant operation and the physical character of the receiving water primarily determine the most effective sampling program. This program must be carefully worked out for each industrial plant. Where operations are variable, and intermittent streams of possibly toxic materials are released, grab samples taken at the proper time would indicate damage to aquatic life that may not be apparent from composite samples. Where effluents are impounded or are released into waters of little or no immediate consequence to aquatic life, composite samples may be adequate. A thorough bio-assay study may include grab and 4- to 24-hr. composite samples of final and process effluents.

Effluents may be collected manually or with automatic samplers. Provision should be made for obtaining information on waste flow, volume, temperature, and other pertinent chemical or operational data. This may be extremely important in evaluating bio-assay results.

The size of the sample needed may vary depending on the toxicity. Five- to ten-gallon samples are normally adequate for final effluents of moderate to low toxicity while 1-gal. samples may be sufficient for highly concentrated process effluents. Metal containers should not be used for collecting or storing effluents. The glass bottles used for the test aquaria are satisfactory as are many other types of glass

jugs, bottles, or carboys. The polyethylene bottles are satisfactory but should be used with caution if the effluents contain a large percentage of oils or solvents.

The effluents should be brought to the laboratory and bio-assays started as quickly as practical, preferably within a few hours. However, the tests may have to be delayed in order for hot or cold effluents to come to room temperature. Some effluents may lose toxicity fairly rapidly due to chemical or biological changes or due to the loss of volatile materials. When samples are stored for more than 24 hr., it is preferable to keep them in full, tightly stoppered bottles, and refrigerated.

Test Concentrations

Test concentrations of effluents are usually prepared on a per cent-by-volume basis in the experimental water. The logarithmic series suggested by Doudoroff *et al.* (2) has been satisfactory and convenient for work with industrial effluents. The series 100, 56, 32, 18, and 10 per cent, etc., which represents quarter points, evenly spaced on semi-logarithmic paper, is normally adequate for obtaining the desired information on the variable effluents from most industries. A higher degree of accuracy can be obtained when necessary, by using intermediates, *e.g.*, 75, 42, 24, 13.5 per cent, etc.

For effluents of completely unknown toxicity, much time and effort may be saved by conducting small-scale or exploratory bio-assays to determine the approximate toxic range. Test solutions are prepared over a wide range of concentrations; for example—100, 10, 1, etc., or 100, 32, 10, 3.2, etc. per cent effluent. Two fish are added to 2 l. of each of the exploratory test solutions in the 1-gal. widemouth glass bottles. Observations for a short period, a few hours or overnight, will indicate test concentrations for the full-scale experiments.

In the full-scale tests, 10 fish are generally adequate for each test concentration. Five fish are added to 10-l. duplicate samples in the large glass bottles. The concentrations to be used in the full-scale tests are determined on the basis of the information obtained from the exploratory tests. For example, if fish are killed in concentrations of 10 per cent and above, and are not affected in concentrations of 1 per cent in the exploratory tests, the full-scale tests are set up in concentrations of 10, 5.6, 3.2, 1.8, and 1.0 per cent. Controls using dilution water only should be set up for each series of experiments. Sufficient information may be obtained in some instances from exploratory or partial full-scale experiments.

Physical and Chemical Determinations

Certain physical and chemical determinations should be made on each concentration in the series during the course of the bio-assay. Determinations that are usually necessary are temperature, dissolved oxygen, pH, alkalinity, acidity, and hardness. Others that may be useful, depending on the type of effluent, are turbidity, color, conductivity or total solids, ammonia, cyanide, phenols, sulfide, various metals, etc.

The determination of dissolved oxygen is necessary to differentiate between fish mortality due to oxygen deficiency and that due to toxicity. It is also necessary to assure control of oxygen conditions during the experiment. High or low pH values (above 9 or below 5) which may cause fish mortality can be readily determined. A change in pH values may have a definite effect on the toxicity of many substances.

The physical and chemical control tests are normally made on test solutions before adding fish, after fish mortality, and at the termination of the bio-assay. Some of these tests, especially the D.O. determination, may be needed more frequently.

It has been found desirable to reduce to a minimum the volume of solution used for routine tests and to use the most rapid methods consistent with desired accuracy (*e.g.*, dissolved oxygen—100 ml. sample, 0.2 p.p.m. accuracy; alkalinity and hardness—20 to 50 ml. sample, 5 p.p.m. accuracy). Small rubber or plastic tubing may be used to siphon the samples directly from the bio-assay aquaria into small D.O. bottles (125 ml. glass-stoppered bottles) and other containers such as small beakers or flasks.

Aeration or Oxygenation of Test Solutions

The bio-assay method described is designed so that surface absorption of oxygen from the atmosphere normally provides an adequate amount of dissolved oxygen for the fish during the test period. Many industrial effluents, however, have a high chemical or biochemical oxygen demand which may cause oxygen depletion. While it is desirable to refrain from the use of air or oxygen when possible, dissolved oxygen levels must be maintained at concentrations adequate for fish survival, usually 3 p.p.m. or greater, through the addition of air or oxygen from an artificial supply.

Aeration with compressed air has been generally unsatisfactory in work with industrial effluents. Small additions of air will not ordinarily maintain the necessary quantities of oxygen whereas vigorous aeration may drive off volatile materials and greatly speed up biological oxidation.

Methods of maintaining adequate dissolved oxygen which have proven satisfactory are: (a) supersaturation of the dilution water with pure oxygen which will take care of immediate chemical demand, (b) renewal of test solutions at fixed periods (12 or 24 hr.) before the initial oxygen is exhausted, (c) maintenance of an interface of pure oxygen at the solution surface, and (d) the addition of pure oxygen at a slow rate (30 to 180 bubbles per minute) by means of a suitable cylinder and valve arrangement. The last method was successfully used in a

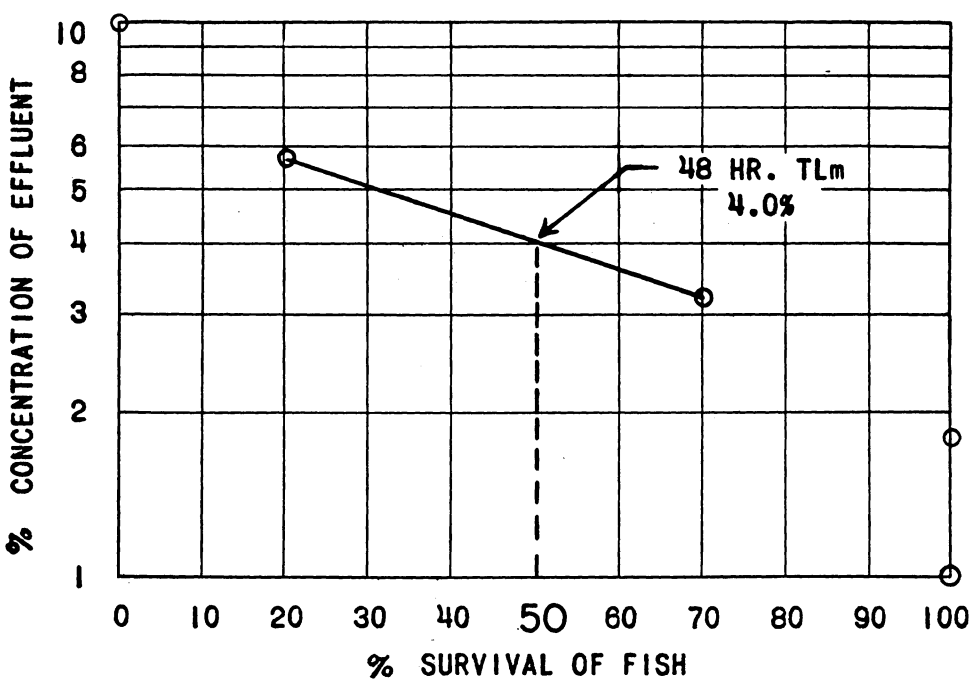

FIGURE 2.—Graphical interpolation for estimating TL_m.

large-scale bio-assay program with a variety of industrial effluents. The toxicity of effluents known to contain volatile materials was not greatly influenced by the addition of oxygen in this manner.

Bio-Assay Results

Computation of Results

Observations on the reactions of test fish are generally made for a 96-hr. period. The 24-, 48-, and 96-hr. TL_m (median tolerance limit) values are estimated through straight-line graphical interpolation as shown in the following example:

Concentrations of 10, 5.6, 3.2, 1.8, and 1.0 per cent effluent in the selected dilution water were prepared and 10 fish added to each. The 48-hr. observations gave the results presented in Table II.

The per cent survival of fish is plotted against log concentrations and the closest points on each side of the 50 per cent survival line are connected. The interpolation for 50 per cent survival (Figure 2) obtains a TL_m of 4.0 per cent.

Experience with a variety of industrial effluents has shown major differences in 24- and 48-hr. TL_m values, although there was little or no difference in the 48- and 96-hr. values, except for an occasional effluent which produced fish mortality over the longer period. This exception, of course, is related to the nature of the chemicals in the effluent. It is believed that 48-hr. values may be sufficient in many situations; however, initial work with an unknown effluent should embrace at least the 96-hr. period. The differences in these values may be of assistance in interpreting and applying laboratory bio-assay results.

The TL_m thus obtained is a direct, comparative measure of toxicity under the test conditions. Obviously a concentration of effluent that causes direct injury to fish on short-time exposure would not be safe for all aquatic life over an extended period. It would be quite definite, however, that this concentration could not be exceeded without detrimental effects. To insure adequate safety to aquatic life, liberal application factors (sometimes erroneously called safety factors) must be used.

Application Factors

An application factor which relates short-term laboratory bio-assay results to actual conditions, is necessary in order to estimate the concentration of effluent which will have no detrimental effect on aquatic life in the receiving water. Need for such a factor and its numerical value are based primarily on the following considerations:

1. As commonly used the bio-assay procedure determines the concentration of a waste at which one-half of the test fish survive for a short period. What is needed, however, is not 50 per cent but 100 per cent survival, and not for short periods, but under conditions of continuous exposure. Thus an application factor is required to compensate for this difference. If continuous flow, long-time experiments are performed with 100 per cent survival as an endpoint, the numerical value of this factor is reduced.

The difficulties in routinely performing experiments of this kind are obvious. Because of the normal biological variation of individuals of the same species, the use of 100 per cent survival as an endpoint will not give satisfactory comparative results. It is be-

TABLE II.—Fish Survival

Effluent Concentration (%)	Condition of Fish	Survival (%)
10	All dead	0
5.6	2 alive	20
3.2	7 alive	70
1.8	All alive	100
1.0	All alive	100

lieved that for most industrial waste work, use of the described short-term procedure and the application factor determined for that type of waste will be more desirable. From present knowledge, this factor may vary, depending on the chemical nature of effluents, but it may fall within fairly narrow limits for most effluents, perhaps in the vicinity of 2 to 3. Work is being conducted at the Sanitary Engineering Center to develop factors for various types of industrial effluents.

2. Test data apply only to the species of fish used. Other fish and fish food organisms may be more sensitive. If very sensitive species are used in bio-assay work, the application factor can be reduced, but such species are more difficult to maintain in the laboratory. Furthermore, the sensitivity of species may vary depending on the nature of the effluent as mentioned in the previous discussions on test fish. The use of a good laboratory fish and the relating of results to other species appear to be the more practical approach. Before this procedure can be effectively carried out research is needed to determine the relative susceptibility of the important fishes and their food organisms at all stages of their life history to the major toxic effluents or waste chemicals.

3. Industrial effluents are often highly variable in volume and toxicity. While measurement of a few samples may indicate the degree of toxicity, the maximum toxicity and the total contribution of toxic materials may be missed. The maximum toxicity of the waste may be the limiting factor from the aquatic life standpoint. Thus, some factor must be provided to allow for this variability; however, if a large number of bio-assays have been conducted and the maximum conditions of toxicity have been ascertained and used in the computation of the toxicity discharged to the stream, this factor may be minimized.

4. Other variables which may have a considerable effect on toxicity, but are generally provided for in the experimental procedure, are temperature, dissolved oxygen, pH, alkalinity, hardness, synergy, and antagonism. Natural purification factors such as biological oxidation and chemical changes such as oxidation or hydrolysis to nontoxic products, precipitation or complexing, loss of volatiles, etc., must also be considered. Any large differences in these characteristics between experimental and actual conditions must be taken into account and appropriate factors used.

After each of the preceding points has been considered, and in the absence of experimental work for the determination of an application factor, a tentative numerical value may be assigned as an application factor. This may be applied as a fractional value of the TL_m to estimate a safe concentration in the receiving water. For example, with an application factor of 10, the indicated "safe" concentration is one-tenth of the TL_m value.

A proposed practical method of applying bio-assays to meet many specific industrial waste problems is as follows:

1. Perform bio-assays to obtain TL_m values.

2. Compute the dilution water required to reduce the toxicity of the total flow or volume of effluent to 50 per cent mortality of the test fish. This value can be computed directly by using the following formula:

$$\text{Dilution volume} = \frac{100 - TL_m}{TL_m} \times \text{Effluent flow}$$

This value can be in any convenient unit (c.f.s., m.g.d., etc.) and gives a direct measure of the toxicity contributed by an effluent.

3. Multiply the dilution volume by the application factor (in the form of a whole number) to give the total dilu-

tion water requirement for safety to aquatic life.

Research and field study are necessary for establishment of reasonably definite application factors for various type effluents under different conditions. A tentative value of 10 is suggested when using the basic bio-assay procedure with a fish of intermediate tolerance, provided there is no great difference in the 24-, 48-, and 96-hr. TL_m values. The individual factors can be reasonably ascertained at present in many industrial situations. Periodic follow-up studies of the receiving water biota may be necessary to determine the adequacy of a program of this nature.

Use of Bio-Assays by Industry

There are many purposes for which industry could effectively use bio-assays. The toxicity of final effluents and the probable effects on the receiving water can be determined. Toxicity may be traced to process effluents, which may be modified, eliminated, or treated. The location of toxicity sources makes possible the treatment of much smaller quantities of waste or it may determine that mixing and lagooning or storage with regulated release is adequate. The effectiveness of treatment processes can be established. Leaks, spills, or other losses of chemicals into waste streams can be detected and located. The toxicity of wastes from new products or processes developed in research laboratories can be evaluated. When locating new plants, the quantity of dilution water necessary or the degree of waste treatment required may be ascertained in advance of construction. Effluents can be continuously monitored by means of continuous flow bio-assays to detect spills or any changes that may affect the aquatic life in the receiving waters.

The examples which follow may serve to illustrate the use of bio-assays in connection with industrial waste problems.

Synthetic Fiber

A large synthetic fiber plant located on a highly valued recreational stream released effluent which effectively destroyed aquatic life. While this could be shown by examining the stream biota, conventional physical and chemical tests (temperature, pH, dissolved oxygen, B.O.D., coliforms, etc.) gave no evidence of serious pollution. Bio-assays showed this effluent to be highly toxic to fish under minimum river flow conditions and also helped to locate the major toxic component (zinc). Attention to the elimination of zinc in the treatment process restored the aquatic life of this river in a short time.

Multiple Industries

A number of effluents from different industries were entering a receiving water which was itself of little or no consequence from the aquatic life standpoint. Toxicity, which built up in this basin, was suspected of causing

TABLE III.—Comparative Toxicity of Industrial Effluents

Effluent	Number of Samples	Flow (c.f.s.)	Median Tolerance Limit (TL_m), Per Cent Concentration		Dilution Volume (c.f.s.)
			Range	Average	
A	3	20.6	37–42	40.0	32
B	7	17.4	4.4–16	9.1	174
C	7	10.5	13.5–28	20.0	42
D	8	2.8	4.0–24	9.6	26
E	14	9.85	2.9–22.5	11.0	80

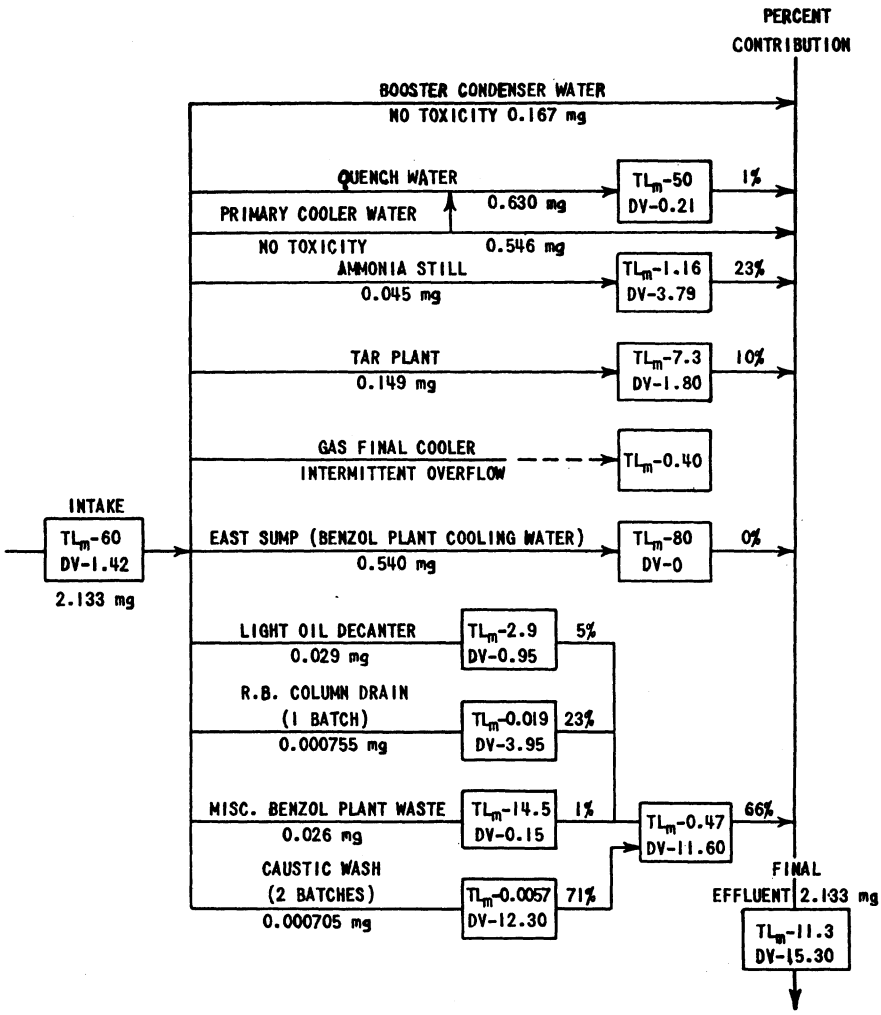

FIGURE 3.—Schematic diagram showing toxicity of major coke plant wastes discharged during an 8-hr. period.

difficulty in waters of recreational importance into which this basin drained. Bio-assays were made on effluents to evaluate contributions of toxicity. The results are presented in Table III and illustrate how total contributions of toxicity can be directly indicated.

By-Product Coke

Samples of an effluent from a by-product coke plant appeared to vary greatly in toxicity. A study was made to evaluate the sources of toxicity in this plant which did not dephenolize its waste. Figure 3 shows the toxicity of the various process effluents and their contribution to the final effluent. A large percentage of the waste toxicity was contained in several small-volume effluents. If other means of disposal could be found for these process effluents, the toxicity entering the receiving water would be greatly reduced.

Petrochemical

Samples of effluent from a petrochemical plant were found to be highly toxic. This discovery led to a careful check of process and sewer systems.

Certain imperfections in the system were found and corrected. Later samples indicated that the toxicity of the waste had been greatly reduced.

Metal Wastes

Bio-assays at a metal separations plant determined that one major effluent was highly toxic and must be eliminated from the receiving stream for protection of aquatic life. Other effluents which made up a larger volume could be released safely, if first mixed (lagooned) and released in a regulated volume according to stream flows.

Other uses undoubtedly could be found for a bio-assay laboratory to make it an increasingly valuable part of an industrial waste laboratory.

While the mechanics of performing bio-assays are such that they may be performed by other types of investigators, an aquatic biologist could make maximum use of bio-assay results both in their interpretation and application.

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