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A MANUAL ON BIOASSAYS IN THE LABORATORY AND THEIR TECHNIQUES

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CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
INDIAN COUNCIL OF AGRICULTURAL RESEARCH
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ERNAKULAM, COCHIN - 682 014

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Front cover : The sewage canal from the Ernakulam Main Market opens into the Ernakulam Channel of the Cochin Backwater.

Back cover : Heaps of sulphur on the sides of the Mattanchery Channel.

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PREFACE

The world is advancing towards modernisation and industrialisation, but it should not be at the cost of our precious environment and natural wealth. In the process of urbanisation and industrialisation, the wastes find their way into the sea which serves as the world's largest 'sink'. All these wastes and effluents if not controlled, may adversely affect the biota and the effects ultimately reach the human beings in the form of contaminated food or lead to the scarcity of aquatic wealth. It is our utmost duty to protect the aquatic environment from such hazardous effects by suitable means. These may include scientific, legal and managerial approaches for controlling pollution. It is essential to assess the levels of pollution to find out the safer limits through bioassays.

Though many reports are available on the techniques of bioassays, interpretation of results, etc. in most of the cases it is doubtful whether the bioassays have been conducted with standard procedures. The authors of this Special Publication have carried out bioassays following standard techniques and the results are included in the Ph. D. thesis of the first author and approved for the award of the Ph. D. degree by the Cochin University of Science and Technology. These results have been given as examples in this manual. This manual with its comprehensive expressions, definitions and techniques, will be of great use as a practical guide to the researchers, students and planners for conducting bioassays systematically, and interpreting the results. It also reviews most of the available reports on this important subject.

I appreciate the efforts of the authors and record my thanks to Dr. K. Rengarajan for editing this manual.

M. Devaraj
Director

Central Marine Fisheries
Research Institute

Cochin - 14,
October 1995.

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1

INTRODUCTION

Toxicity tests, otherwise called "Bioassay tests" are performed widely from the 18th century itself, in order to evaluate the impact of chemicals both on aquatic and terrestrial organisms. However, there has been enormous growth of activity in this field after the second world war, owing to the production of a large number of pesticides and the resulting pesticide regulatory schemes led to a boom in toxicity testing using both invertebrates and fishes. Further, during the 1960s and 1970s, the hazards posed by chemicals in the environment were brought out by several well publicised events, which included methyl mercury poisoning to human beings in Minimata Bay, Japan, reproductive failure in seals and minks in several parts of the world caused by PCBs and other organochlorines, kills of terns and eiders and decline in certain predatory bird population, etc. Under these circumstances, the international regulatory agencies such as the Organisation for Economic Co-operation and Development (OECD) and the European Economic Community (EEC) emphasised the need for the conduct of toxicity tests for the following reasons :

1. To approve the manufacture of pesticides, oil spill dispersants, other chemicals, etc.
2. To determine the tax payable for industrial discharges.
3. To monitor the effluent discharges and to develop control measures.
4. To establish water quality criteria.
5. To formulate directives relating to packing, labelling and classification of dangerous substances.

Therefore, OECD framed a package of test procedures for achieving the above objective, from a minimum requirement of simple toxicity tests involving fish and algal species to more extensive tests covering bio-degradation and bio-accumulation.

Thus, since 1950, the acute toxicity testing has become the "Work house" for the detection, evaluation and abatement of water pollution (Buikema *et al.*, 1982). Information generated from various toxicity tests can be of use in the management of pollution for the purpose of (i) prediction of environmental effects of a waste, (ii) comparison of toxicants or animals or test conditions and (iii) regulation of discharge (Buikema *et al.*, 1982).

A single toxicity test does not serve all purposes equally well and toxicity tests are only one of many techniques available. Through common usage "bioassay" is used to indicate studies that determine (i) the suitability of environmental conditions for aquatic life, (ii) favourable and unfavourable concentrations or levels of environmental factors, (iii) the effects of various combinations of the environmental factors on the toxicity of wastes, (iv) the relative toxicity of different wastes to biomonitors, (v) the relative sensitivity of the biomonitoring agent to the effluent or toxicant, (vi) the amount of waste treatment needed to meet water pollution control requirements, (vii) the effectiveness of different waste treatment methods and (viii) the permissible discharge rates of effluent and other wastes. The objective of a toxicity test is to define the concentrations at which a test material is capable of producing some selected response, usually deleterious, in a population under controlled conditions of exposure. The appropriate way to do this is by use of the "quantal response" (*i.e.* by having only two experimental alternatives dead or alive, all or none) from which the relation between concentration and percentage effect can be defined (Ward and Parrish, 1982). In their simplest application, acute toxicity tests are time-dependent. That is, the length of exposure is predetermined, usually 48 to 96 hours (Ward and Parrish, 1982). According to Reish and Oshida (1987) the 96-hour test is the most common. In many cases the effects of test material occur rapidly and are well defined in these time periods. However, because some test materials will not reach a "threshold" (the point in time where no significant increase in mortality or effect occur) within a 96 hour period, the "toxicity curve" (explained in a later section) will not be completed. In that case a time-independent test is a better one for determination of

the acute toxicity of a test material than a time-dependent test. In general, the bioassay upto 96 hour is described in this manual. The toxicity tests with aquatic organism can be conducted by applying the test material directly to the test organisms (such as by injection or in food), but acute tests are conducted by exposing the test organisms to test solutions which contain various concentrations of the test material. One or more controls are concurrently carried out in which the organisms are exposed to similar conditions, but without toxicant to provide a measure of experimental acceptability. The control experiments indicate the suitability of the dilution water, test conditions, handling procedures, etc. Death is generally used as a criteria of a change in the 96-hour test while the extension of duration can be adopted for investigation of the various other related physiological, biochemical or behavioural changes. The concentration which causes a 50% live-death response is defined as the lethal concentration or LC50.

2

TYPES OF ACUTE TOXICITY TESTS

An acute test is one involving a stimulus severe enough to bring about a response, usually within a few hours or in 4-7 days (APHA-AWWA-WPCF, 1976). There are generally five types of acute toxicity tests (Ward and Parrish, 1982; Reish and Oshida, 1987).

2.1. *Static test*

In the static acute test, the test solutions and the test organisms are placed in test chambers and kept there for the duration of the test. The 96-hour bioassay is almost always of the static type.

2.2. *Renewal test*

The renewal acute test is similar to the static acute test except that the test organisms are periodically exposed to fresh test solutions of the same composition, usually once in every 24 hours, either by transferring the test organisms from one test chamber to another or by replacing the test solution.

2.3. *Flow-through test*

In a flow-through acute test, the test solutions flow into and out of the test chambers on a once-through basis for the duration of the test. This type of test is perhaps more realistic of field conditions, but there are many inherent problems connected with this type of test, such as large space requirements, the large quantity of water used and a complete water delivery system. There are basically two types of flow-through acute tests.

(a) Time-dependent test

In this the time of termination is predetermined. Commonly, the 96-hour period has been used. The

shape of the toxicity curve may not be clearly established during the 96-hour period, in this test.

(b) Time-independent test

It has no predetermined temporal end-point. This test is allowed to continue until mortality has ceased and the toxicity curve reveals a threshold. Usually this is for 7-10 days, although it may be longer.

2.4. Short-term test

This type of bioassay is conducted for a short period of time, usually 48 or 96 hours. The 96-hour test period is the most frequently used. Organisms except phytoplankton, echinoderm larvae and possibly zooplankton are not fed during the test and the solution is not changed.

2.5. Long-term test

This type of bioassay is conducted from 7 days to one or more months depending upon the species used and the type of data desired. Some long-term tests are simply extensions of the 96-hour tests which generally involve feeding the organisms and may involve renewing the test solution. More time and experience is required in order to conduct this type of experiment.

3

TERMINOLOGIES IN TOXICITY TESTS

The term "bioassay" used in this manual is according to the definition by FAO (1977) which states :

3.1. Bioassay definition

"Bioassay signifies a test in which a living tissue, organism or a group of organisms is used as test material for the determination of the potency of any physiologically active substance of unknown activity".

"Bioassay" has different meanings in different countries. Some workers prefer the term "Toxicity test" rather than bioassay.

3.2. Lethal Concentration (LC50)

"LC" is used to express the results of bioassay having lethality as the criterion of toxicity. It is the concentration of a substance that is lethal to 50% of the test organisms in 96 hours. The prefix indicates the period of exposure. The suffix indicates the percentage of death in the experiment.

3.3. Effective Concentration (EC50)

It is used when some effect other than lethality is being studied. The median effective concentration (EC50) is the concentration producing a specific effect or response in 50% of the test organisms. This effect, as measured, can involve any other percentage, such as 20 or 90% *i.e.* EC20 or EC90. The time is also used as a prefix to it, *e.g.* 96 hr EC50.

3.4. Inhibiting Concentration (CI50)

CI50 is a technique which measures the concentration of a potential pollutant, which reduces the capacity of a marine bacteria to bioluminescence by 50%. The Backman Microtox system was developed to measure CI50. This technique is simple, quick and relatively inexpensive. This technique received attention as a

replacement for the relatively slow, complex and expensive LC50 and EC50 tests for environmental monitoring.

3.5. Inhibiting Concentration (General Inhibition) (IC50)

Inhibiting concentration of a toxic product is that which results in the 50% reduction of a physiological parameter. There is a growing demand in environmental research for tests measuring sublethal toxicity levels. Therefore, IC50 as distinct from LC50 is becoming a popular parameter.

3.6. Incipient lethal level

It is the concentration at which the acute toxicity ceases, that is, the concentration at which 50% of the population can live for an indefinite time. The synonyms are incipient lethal level (Fry, 1947), ultimate median tolerance limit (Doudoroff,1951), lethal threshold concentration (Lloyd and Jordan, 1963) and asymptotic LC50 (Ball, 1967).

3.7. Safe Concentration (SC)

It is the maximum concentration of a toxicant that has no observable harmful effects after long-term exposure over one or more generations.

3.8. Maximum Allowable Toxicant Concentration (MATC)

This is the level of toxic waste that may be present in the receiving water without causing significant harm to its productivity and all its usefulness. The MATC is determined by a long-term bioassay of a partial life cycle with the sensitive life stages or a full life cycle of the test organism in which a range of concentrations of the toxicant under test that do not demonstrate significant harm to the test organism is determined.

3.9. Application Factor

"The application Factor" (AF) is obtained from the following:

$$AF = \frac{\text{MATC}}{\text{Incipient LC50}}$$

3.10. Safe Application Factor Equation (SAFE)

It is estimated by dividing LC0 (the maximum concentration at which all the test animals survived for 96 or 168 hr) by LC100 (the minimum concentration at which all the test animals died in 96 or 168 hr).

4

BASIC REQUIREMENTS FOR BIOASSAYS

The major requirements for bioassays are

- supply of good quality water,
- adequate space and wet laboratory,
- availability of experimental organisms,
- availability of equipments and facilities,

  1. aquaria ranging in size from 4 - 100 litres or larger (holding pond) for fish,
  2. nets for covering the tanks and for transferring fish from holding tank to the test container,
  3. a mobile bioassay laboratory for field studies which consists of wooden frames and disposable polyethylene bags,
  4. compressed air system equipped with plastic tubing, air stones, and glass tubing,
  5. filter papers,
  6. 125 and 250 ml Erlenmeyer flasks,
  7. pipettes of various sizes,
  8. glass or plastic petridishes,
  9. analytical balance,
  10. compound microscope,
  11. pH meter, oxygen cylinder with regulator,
  12. probability graph papers, nomograph papers and calculator,
  13. haemocytometer,
  14. fluorometer to measure chlorophyll,
  15. gas chromatograph,
  16. atomic absorption spectrophotometer.

5

CONDUCT OF BIOASSAY TESTS

5.1. *Test organisms*

5.1.1. *Selection of test organisms*

The selection of test organisms for the 96 hour bioassay is dependent upon many criteria. Based on the previous experience of the laboratory personnel conducting the bioassays and the laboratory facilities available, the test organisms are to be selected. Some bias in selection of the test organism is unavoidable.

Because of their economic, recreational and ecological importance, the fish is commonly used as a test organism. Many species of fish are sensitive to pollutants and are good candidates for bioassays. For most of them the biology is known. The selected species should be indigenous to the area of impact. Since many fresh water species are reared in government or private farms one can obtain the test specimens from them rather than collect them from natural waters. However, in some cases it may be necessary to collect them from natural waters. Since very few marine fish are cultured, it will be necessary to collect these fish from the natural environment. If no species are available from the area, then representative species from nearby localities offer an alternative source. Only of a single species should be used in a test. The length of the largest fish in an bioassay should not be more than 1.5 times the length of the smallest individual used (APHA-AWWA-WPCF, 1976). If great precision is desired, extreme care should be taken to select fish varying in length by only a few millimetres. It must be remembered that the weight of a fish increases as the cube of its length, and a fish half again as long as another fish may weigh several times as much as the smaller fish. A natural population of fish is varied in size and the application of toxicity data derived from studies on uniform-sized fish to problems involving natural populations may be questionable.

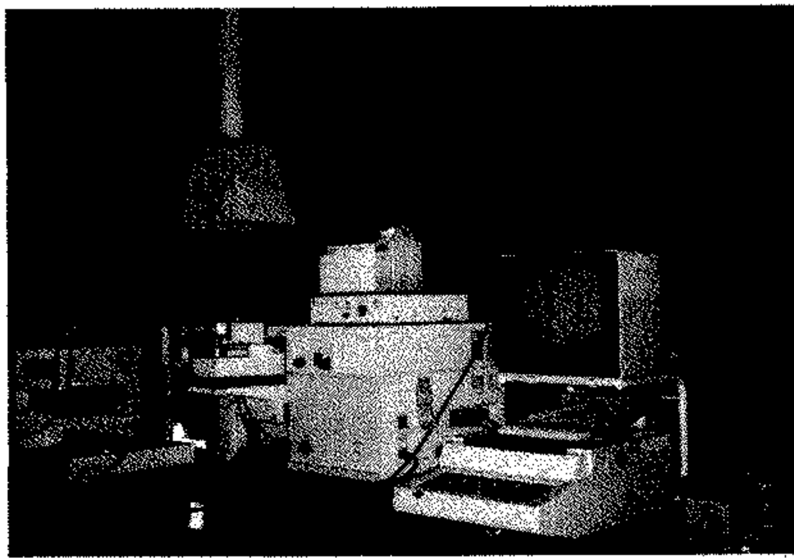


Plate I. Hitachi Polarized Zeeman Atomic Absorption Spectrophotometer.

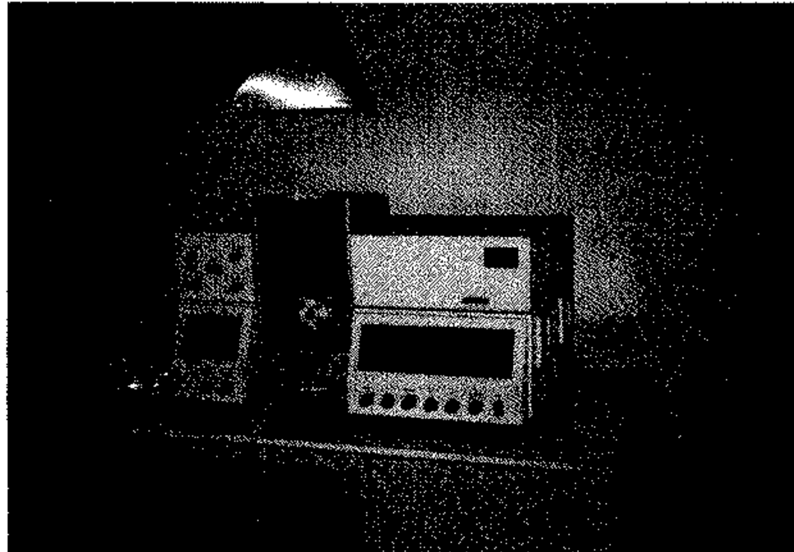


Plate II. Perkin Elmer - 2380 model Atomic Absorption Spectrophotometer.

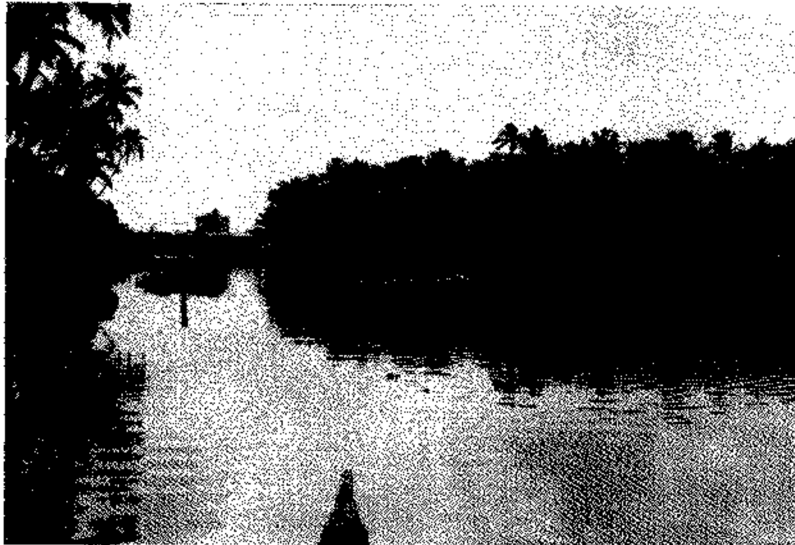


Plate III. A rivulet near Cochin with an industrial establishment on the bank.

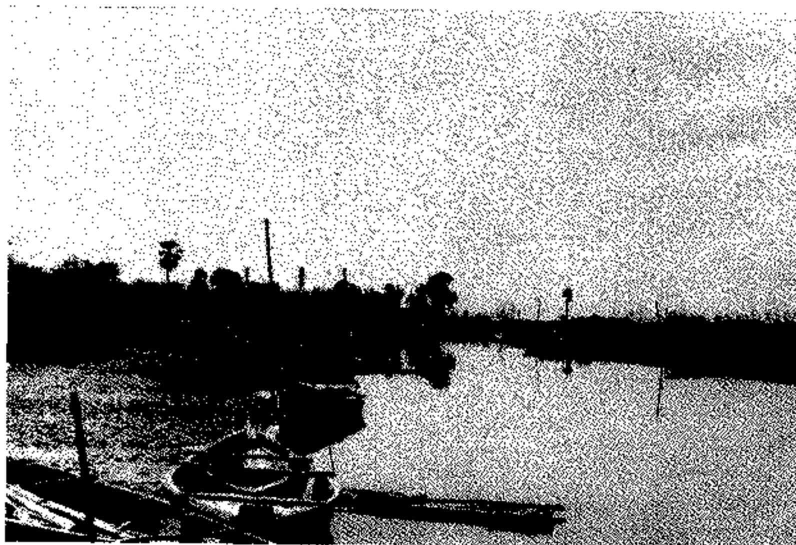


Plate IV. Ennore Creek near Madras with thermal and industrial plants.

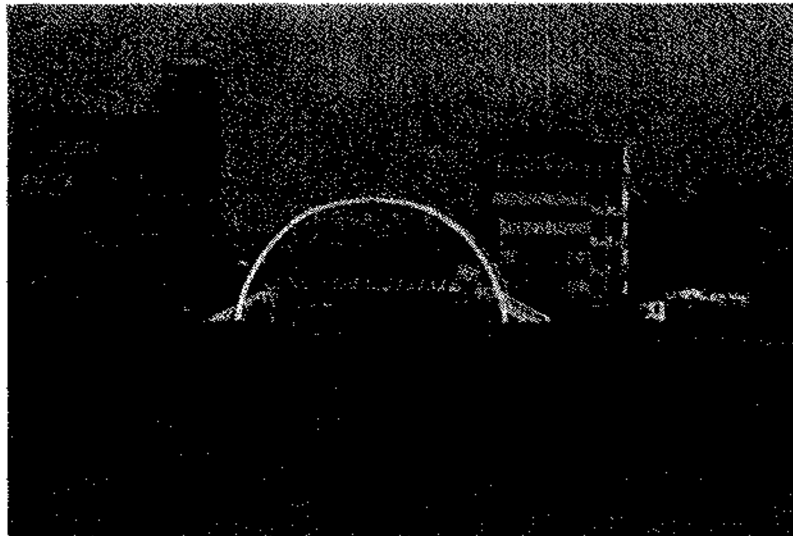


Plate V. The canal from the Ernakulam Main Market opens into the Ernakulam Channel of the Cochin Backwater with slimy and stinking sewage.

Plate VI. A close up view of the canal.

Plate VII. Karuvelipady Canal adjacent to the Cochin Fishery Harbour, discharges slimy sewage.

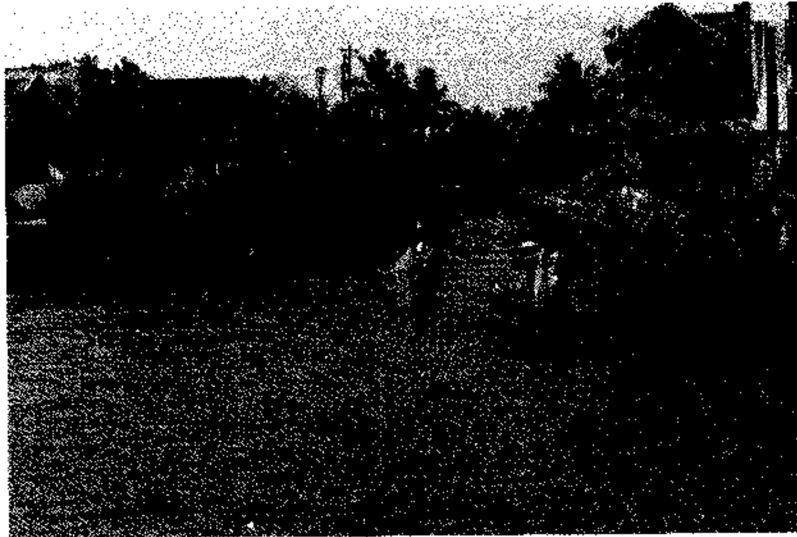


Plate VIII. A canal near Mattanchery with sewage joins into the Mattanchery Channel of the Cochin Backwater.

The other considerations in the selection of test organisms for bioassay are :

a. their sensitivity to the material or environmental factors under consideration,

b. their geographical distribution, abundance and availability throughout the year,

c. the availability of culture methods for rearing them in the laboratory,

d. their healthiness and

e. their suitability for bioassay tests.

As per the APHA-AWWA-WPCF (1976) guideline, smaller organisms not over 5 to 8 cm long and having a short life cycle are desirable for bioassay studies. The test organisms are not selected from polluted areas where the organisms are in poor health condition or where they have an unusually high burden of potential toxicants, especially those under test. Taking test animals, particularly fish from areas where disease and parasites are prevalent are to be avoided. Similarly deformed individuals are to be eliminated. Knowledge of the environmental requirements and food habits are important in the selection of test organisms.

Fish have been the most popular test organism, because they are presumed to be the best understood organisms in the aquatic environment and perceived as most valuable by the majority of laymen (Buikema *et al.*, 1982).

According to United States Environmental Protection Agency (USEPA, 1979) the selection of test organisms is to be based on four criteria :

1. the organism must be a representative of an ecologically important group,

2. the organism must occupy a position within a food chain leading to man,

3. the organism must be widely available, amenable to laboratory tests, easily maintained and genetically uniform,

4. there must be adequate background data on the organism (*i.e.* on physiology, genetics, taxonomy, etc.).

Rosenberger *et al.* (1978) added other criteria which included economic importance, type of test, sensitivity to the toxicant and consistency in response to the toxicant. The "Red Book" (USEPA, 1976) and others have suggested the use of indigenous species for establishing a toxicological data base.

Selection of test animal should depend on the objective of the study. For relative comparison of effluent or wastewaters at a point of time, any organism may be appropriate. For relative comparisons of toxicity over time, the species easily held or cultured in the laboratory are more useful.

5.1.2. Collection and transportation of test organisms

The collection, transfer and transport of test animals for bioassay should be done in a manner which minimizes injury and physiological trauma. Fishes can be collected with the use of different types of nets. Temperature, salinity (in the case of marine organisms), dissolved oxygen, pH and total hardness at the collection site have to be monitored to understand the quality of the water into which the organisms are transferred on arrival at the laboratory. Before collection, sufficient clean water (from the site) should be kept ready in transporting tanks. If the organisms are to be transported any distance by boat, oxygenated boxes/ bags are to be provided where they can be held. If they are transported by truck, the animals are kept in large tanks supplied with water from the area in which they were collected. To avoid stress, the aeration facility has to be provided for the holding tanks. The spawn, fry and fingerlings are transported in polyethylene bags with oxygen (Jhingran, 1983). Ideally, the transport containers, especially for highly active species should be circular or elliptical to prevent animals from crowding in corners or damaging themselves by striking the walls (Marine Technology Society, 1974). According to Mohapatra (1994) the fishes can be transported with great care to the laboratory in plastic bins of 100 litre capacity mounted on the jeep's trailer. If the transport is for a longer distance lasting more than 30 minutes, additional

safeguards must be taken to shield the animals from the sunlight and from extreme temperature and dissolved oxygen depletion.

5.1.3. Acclimatization of the test animal

Whether fish is collected by the investigator in the field or purchased from a vendor, a substantial loss by death occurs due to the stress of capture and the rigorous transportation. After the third or fourth day in the laboratory, mortalities will decline if the fishes are healthy. Many species of fish adapt quite readily to laboratory conditions and will often begin to feed within a week. While conducting bioassays, in acclimatization tanks, Mohapatra (1989, 1994) noticed no deaths in the grey mullet *Liza parsia*. According to him 2-tonne capacity plastic pools are convenient for use as acclimatization containers. It is also suggested to avoid fungal attack on test animals, the water may be treated with 11 mg of malachite green/1000 lt. The organisms can be fed once in a day. The faecal matter and other waste materials are to be siphoned off daily to reduce the ammonia content in water. The use of biological filter is recommended. The acclimatization period varies according to the convenience of the research worker and the species to be experimented. Bennett and Dooley (1982) and Mohapatra (1989, 1994) suggested one week acclimatization for fishes. At the end of the acclimatization the test organisms must be in excellent condition to withstand the experiments on them. There should be less than 2% mortality during acclimatization (APHA-AWWA-WPCF, 1976). No disease should occur and deaths should be less than 1% in the 4 days before the tests. There should be no evidence of abnormalities or unusual behaviour during acclimatization. In general, organisms should not be subjected to more than a 5°C gradual change in water temperature in any 24-hour period and for salt-water organisms no more than a 5‰ salinity change in 24 hours (Ward and Parrish, 1982).

Organisms should be handled to the minimum extent possible. When handling is necessary, it should be accomplished as gently and quickly as possible. Small scoop dip nets are best for handling larger organisms.

To reduce mortality and diseases in stock tanks, a variety of antibiotics is used immediately after collection or during transport or on arrival at the laboratory. Holding in tetracycline 15 mg/lt for 24 to 48 hour can be very helpful (APHA-AWWA-WPCF, 1976). The organisms treated with antibiotics are used for bioassay only after 10 days. The use of any type of chemotherapeutic agents in bioassay tanks should be avoided and if used these may cause synergistic effects on the test animals.

Many books are available on the care and feeding of fish. For freshwater fish consult such reference as Hunn *et al.* (1968). Papers by May (1970), Houdo and Palko (1970), Boyd and Simmons (1974) and Mohapatra (1994) are useful for maintaining marine species. Fishes can generally be maintained on dried commercial fish food; however, an occasional feeding with live food may increase their vitality. Aeration to the tanks may be done from an oil-free compressor. The aerators available in the market can also be used. Careful attention to maintain the dissolved oxygen level at 5 mg/l in the culture tanks is important. An oil trap filtration system may be necessary if oil droplets appear in the plastic tubing.

5.2. *Test solutions*

The test material can be one or more pure chemicals or a complex mixture such as a formulation or an effluent. Sometimes the test solutions are not true solutions, because they contain undissolved test material. Test solutions are often prepared by dissolving a test material in a solvent, preferably water, to form a suitable stock solution and then by adding a portion of the stock solution to dilution water. The test solutions are prepared immediately prior to initiation of the experiment. If the chemicals are in precipitate forms, the chemicals are either discarded or filtered to remove the precipitate. If filtered, the concentration of the chemical is verified by analytical methods.

5.2.1. *Carrier solvents*

Solvents are not to be used to dissolve the chemicals unless it is absolutely essential and, if one is used, not more than 0.5mg/lt is added (Reish and Oshida, 1987). In such cases, a second control

series is conducted with the solvent alone at the highest concentration employed (APHA-AWWA-WPCF, 1976; Reish and Oshida, 1987). The APHA standard methods recommend that the following solvents could be used as carrier solvents, especially for hydrophobic substances.

1. Acetone
2. Dimethly formamide (DMF)
3. Ethanol
4. Methanol
5. Isopropanol
6. Acetonitrile
7. Tri-ethylene Glycol (TEG)

Note: Concentration should not exceed 0.5 ml/lt for static and 0.1 ml/lt for flow-through test.

If the toxicant to be used is in solid form such as a metallic salt, a stock solution should be made. This solution should be approximately two orders of magnitude greater than the highest test solution (*i.e.* if the highest test concentration is 10 mg/lt then the stock solution should be 1000 mg/lt). This minimizes errors due to weighing and making dilutions (Reish and Oshida, 1987). When 1 mg of salt in 1 litre of solvent is dissolved, it gives 1 ppm test solution and 10 mg in 1 litre gives 10 ppm salt solution, etc. For example, 1 gm copper sulphate ($CuSO_4 5H_2O$) is used for the preparation of the test solution, the bioassay results should be expressed for copper sulphate only. To find out the results in terms of copper, the following formula should be used :

$$\text{Grams of } CuSO_4 5H_2O \text{ containing 1.0 g of Cu} = \frac{\text{Molecular wt. of } CuSO_4 5H_2O}{\text{Molecular wt. of Cu}}$$

i.e. 1 g of $CuSO_4 5H_2O$ contains 0.2545 g of Cu.

In general the formula is :

$$\text{Grams of compound containing 1 g of element} = \frac{\text{Molecular weight of compound}}{\text{Molecular weight of element}}$$

5.2.2. Petrochemicals

Crude oil is less toxic than refined petroleum. The highly volatile components evaporate largely within a 24 hour period (Reish and Oshida, 1987.) In order to standardize the method of preparation of test solutions, a procedure was developed by the American Petroleum Institute to eliminate the lower carbon chain gases (the volatile component) from the oil and utilize the water-soluble fraction of a particular petroleum. The use of the water soluble fraction in toxicity testing approximates what is actually occurring in nature. The procedure of getting the test solution of oil is given by Reish and Oshida (1987) in detail.

5.2.3. Pesticides

There are so many pesticides developed and used in the past few decades. They may possess long half-lives and therefore will persist in the environment for a long time. Now-a-days the effort is being made to develop more specific target pesticides which will have very short half-life in the environment. Most of the pesticides are soluble in water. Along with solubility in water, the organophosphates are biodegradable. Mohapatra (1989) worked on "Nuvan", an organophosphate whose main component is Dichlorvos and is soluble in water. In due course the media treated with "Nuvan" turned green and it is ássumed that the phosphate group might have been used for plankton production. The pesticides with low solubility in water require an organic solvent, such as acetone. The procedure of mixing the DDT in water through acetone is given by Reish and Oshida (1987). The other solvents used are dimethyl formamide (DMF), ethanol, methanol and triethylene glycol. If these are not satisfactory, isopropanol, acetonitrile, dimethyl acetamide or ethylene glycol (APHA-AWWA-WPCF, 1976) are used. Only minimal amount of solvent is necessary to disperse the toxicant, not exceeding 0.5 mg/l in static and 0.1 mg/l in flow-through test solutions.

5.2.4. Dredge spoils and sediments

The contaminated sediments generally adversely affect the organism present at the disposal site. Techniques are available for testing the effects of dredge spoils and sediments on the organisms inhabiting the area. The toxicity of any toxic substance in the water column is temporary, but can be long-term in the benthos. The toxic effect of the sediments which settle on the benthos is described below.

After collection the sediment is sieved through 0.5 mm mesh and if necessary, a little water can be used. The water and sediment are allowed to settle for 6 hours. The water is siphoned off and the sediment can be stored upto two weeks at 2 - 4°C. The sediment should neither be dried nor frozen. While conducting the bioassay, 3 cm of sediment is used in the aquaria. In a 55 litre aquarium, 20 litres of water should be added. A minimum of 3 hours is needed for the settlement of the sediment. Now the aquaria with the medium and the sediment are ready for conducting the toxicity tests. It will not be necessary to change the water in each aquarium if the test is conducted for only 96 hours. If extended upto 10 to 20 days, 50-75% of the water is removed at 48-hour intervals. The aquaria are observed each day for dead organisms. At the end of the experiment the water is removed, sediments are sieved through 0.5 mm mesh sieve for collecting the test organisms. The control test is conducted either with uncontaminated sediments or in aquaria without any sediment. The data are analysed for percent survival, mean and standard deviation for both contaminated and clean test series. If the standard deviations do not overlap, then the differences are significant. This test is conducted in replicate for better results. Reish and Oshida (1987) have given the procedures for collecting contaminated sediments and conducting sediment bioassays in detail.

5.2.5 Sewage

Sewages are liquid effluents in which the composition and concentration of the different chemicals are unknown. Here the bioassay is useful in determining the water quality of a body of water which receives the waste discharges from many sources. The procedure employed for conducting the toxicity test here is

similar to that of general bioassay discussed in this manual. The dilutions of the sewage are made between 0 and 100% for conducting the tests.

5.2.6. Selection of dilution water

"Dilution water" is the water used for control tests and for making concentrations of the test substance. It may be a receiving system water, dechlorinated tap water or an artificial reconstituted water (Marking and Dawson, 1973; ASTM, 1980). If the purpose of the test is to estimate the effluent effects on the receiving system, then the receiving water should be used. If the receiving system water is toxic, then dechlorinated tap water or artificial water is more appropriate. Generally in a receiving system, the dilution water should be collected from a point above the entrance of the effluent into the waterway. The dilution water should contain undetectable levels of priority pollutants and pesticides (ASTM, 1980). During acclimatization and test period the test organisms in it, should not show any signs of stress *e.g.* discolouration or unusual behaviour (Peltier, 1978).

To conduct bioassay for euryhaline (estuarine and brackish-water) fish in water of various salinities, Mohapatra (1989, 1994) suggested that the water from marine environment is to be diluted with freshwater to the required salinities. It is necessary to filter the water to remove microorganisms and suspended sediment. Depending upon the species of test organism used, the salinity should not vary more than 1.0‰ during the holding period.

If the municipal tap water is used as dilution water or for preparation of stock solution and dilution water, it should be aerated for atleast 24 hours before use to eliminate the possible chlorine residues. It is advised to collect water from the collection sites in which the test animals are collected, and transported to the laboratory for use as the dilution water.

The dilution water should be clean, uncontaminated and of constant quality and should meet the following specifications (Ward and Parrish, 1982) :

Hardness (fresh water)	40-200 mg/lt as $CaCO_3$
Suspended solids	< 20 mg/lt
Total organic carbon	< 10 mg/lt
Un-ionized ammonia	< 20 μg/lt
Residual chlorine	< 3 μg/lt
Total organophosphorus pesticides	< 50 ng/lt
Total organochlorine pesticides including PCBs	< 50 ng/lt

5.3. *Test procedures*

5.3.1. *Laboratory conditions*

In general the animals are maintained in similar environmental conditions as in the field. The toxicity of a given test material may be modified by the physical and chemical properties of the dilution water such as temperature, pH, hardness, bicarbonate alkalinity, total dissolved solids, salinity and dissolved oxygen.

5.3.2. *Experimental containers, test solutions and loading rates*

The test chamber should be atleast 1.5 times the average height and average dimensions of the test organisms. It should be either made of stainless steel or glass or fibreglass.

The test solution in the chamber should be 150 mm deep for organisms over 0.5 g and atleast 50 mm deep for smaller organisms to limit the escape of volatile components in the tested solution (Katz, 1971). All the test containers should have uniform depths of test solutions. For test solutions please refer section 5.2.

The number of organisms should be optimal and it should not be high.

The loading should not exceed 0.8 g/lt at 17° C or below and 0.5 g/l at high temperature in static tests.

In flow-through tests, 1 g/lt of test media has to be passed through the tanks in 24 hours or 10 g/lt at any given time at 17°C or below. At higher temperatures the recommended loading is 0.5 g/lt/day or 5 g/lt at any given time.

5.3.3. Standard toxicants

For comparing the results from different laboratories, the choice of standard toxicant as described by Kalverkamp *et al.* (1979) could be followed. The choice of the toxicants depends on the following :

1. The lethality should be low *i.e.* in mg/l range.
2. It should be easy to measure the concentration of the toxicant in the test media.
3. The toxicant should be available in the "purest form".
4. It should be highly soluble in water.
5. The ionizable compounds pH should be atleast away by one unit.
6. It should have a known mode and site of action.

Fogels and Sprague (1977) added two more criteria :

1. It should have definite threshold of toxicity for commonly tested species and fish, and
2. there should be minimum change in the toxicity at different levels of hardness and pH.

Examples

1. Sodium pentachlorophenate - difficult to analyse.
2. Dodecyl sodium sulphate - loses its toxicity upon storage.
3. Phenol - most suitable.
4. Sodium azide - tried.
5. Copper sulphate - tried.

Alexandor and Clark (1978) contented that phenol is of limited use and also concluded that a series of physiological and behavioural tests may be more useful than reference toxicants.

5.3.4. General environmental parameters

Dissolved oxygen

The dissolved oxygen content of the solution tested should not fall below 5 ppm when using cold water fish or below 4 ppm when warm water fish is used (Katz, 1971). Before beginning the test, the dilution water is vigorously aerated. If dissolved oxygen falls to the level at which the fish compensate by increasing their respiratory rates, then the results will be erroneous. It is well documented that the lethality of many compounds is increased at low dissolved oxygen contents. This is explained by the fact that the fish will compensate for a low dissolved oxygen content by increasing their respiratory rates. Hence, more toxicant is passed over their gills and is absorbed. Physiologically this phenomenon is explained by the fact that under an oxygen deficiency, the level of haemoglobin in the blood of the fish increases and the rate of blood circulation through the gills is enhanced. It is necessary to supply air to the animals during the acclimation period and to some during the bioassay. A single large compressed air pump is more satisfactory than many aquarium pumps.

Temperature

It is recommended that tests should be performed at uniform temperatures (Doudoroff *et al.*, 1951). For warm water fish, temperature between 20 and 25° C and for cold water species temperature between 12 and 18° C are recommended. In Indian condition in some laboratories the temperature often recorded more than 25° C. Temperature should not fluctuate more than 2° C during the test period. From the temperature coefficient it can be seen that with a reduction in water temperature by 10° C, the time of manifestation of poisoning symptoms is accelerated by 1.9 to 3.4 times (Metelev *et al.*, 1983).

Ammonia

In an aqueous solution, ammonia is present in ionized (NH_4+) and more toxic, easily diffusable and highly lipid-soluble un-ionized (NH_3) forms. The concentration of un-ionized ammonia must not exceed 20 µg/lt (APHA-AWWA-WPCF, 1976).

Ammonia serves as a substrate for the production of nitrite that is also highly toxic (Boyd, 1982). According to Inhaber (1974), ammonia for fish and aquatic life should not exceed 1.0 ppm.

Nitrite

Nitrite, an intermediate product in the nitrification of ammonia to nitrate and bacterial denitrification of nitrate to nitrogen, is highly toxic to cultured animals. Denitrification takes place best under anaerobic conditions, although some investigators (Smith *et al.,* 1972) have found that denitrification can proceed under oxygen concentrations of upto 1 mg/lt. The toxicity of nitrite increases with a decrease of pH. According to King and Spottee (1974), in closed seawater systems, the NO_2-N level should not exceed 0.1 mg/lt.

pH

The toxicity of ammonia, ammonium salts, cyanides and certain compounds of chromium, iron (chloride and sulphate), manganese, copper, lead is influenced by the pH of the dilution water (Metelev *et al.,* 1983). The effect of the pH on toxicities of different metals is also well documented by Cusimano *et al.* (1986) and Stripp *et al.* (1990). A weak relationship exists between the resistance of fish to phenol and pH level. Ammonia toxicity increases in an alkaline medium. The toxicity of cyanides decreases as the pH increases. Most fishes can tolerate a pH range of 5.0 to 9.0. It is advisable to conduct the bioassay in the dilution water with pH in or around the neutral.

Carbon dioxide

In an aquatic medium, the carbon dioxide, pH and dissolved oxygen have a close relationship among themselves. The unutilized

feed and other organic solid wastes in the medium are converted to metabolites. In the presence of oxygen, the microorganisms convert these metabolites to less harmful/harmless forms *i.e.* nitrate, sulphate and carbon dioxide. The carbon dioxide in higher concentration interferes with the utilization of dissolved oxygen (Boyd, 1982). The carbon dioxide in water produces carbonic acid and thus reduces the pH.

Hardness

It has long been known that the toxic effect of ammonia, salts of alkalies, alkaline earth metals and heavy metals decreases in hard and sea water. Physiologically this phenomenon is explained by the fact that highly mineralized water containing calcium, potassium, sodium, magnesium and barium salts decreases the solubility of toxic substances, forming insoluble sediments with them and hence reducing their toxicity many times over. A close relationship exists between the resistance of fish to the toxic effect of salts of heavy metals and the degree of hardness of water (Metelev *et al.*, 1983; Bradly and Sprague, 1985; Hutchinson and Sprague, 1989; Moni and Dhas, 1989; Sayer *et al.*, 1989).

5.3.5. Design of experiment

Normally each bioassay involves a series of five test concentrations and a control with an additional control if solvents or emulsifiers are used. The test organisms are exposed in duplicate containers for each concentration and control (Fig. 1) (Mohapatra, 1989). The use of more organisms and replicate test containers for each toxicant concentration is often desirable to reduce variability. There should not be water connections between the test containers. If each concentration is run in duplicate, 12 containers are required for each test. The containers required may be made of glass, fibreglass, etc. The 40 - 100 litre capacity fibreglass tanks are quite economical and useful for conducting the test (Mohapatra, 1989, 1994). Precautions should be taken to avoid contamination of the controls.

In short-term static or renewal tests with fishes, it has been the usual practice to use 10 or more test organisms in each toxicant concentration. When 10 numbers are used, the death of one is

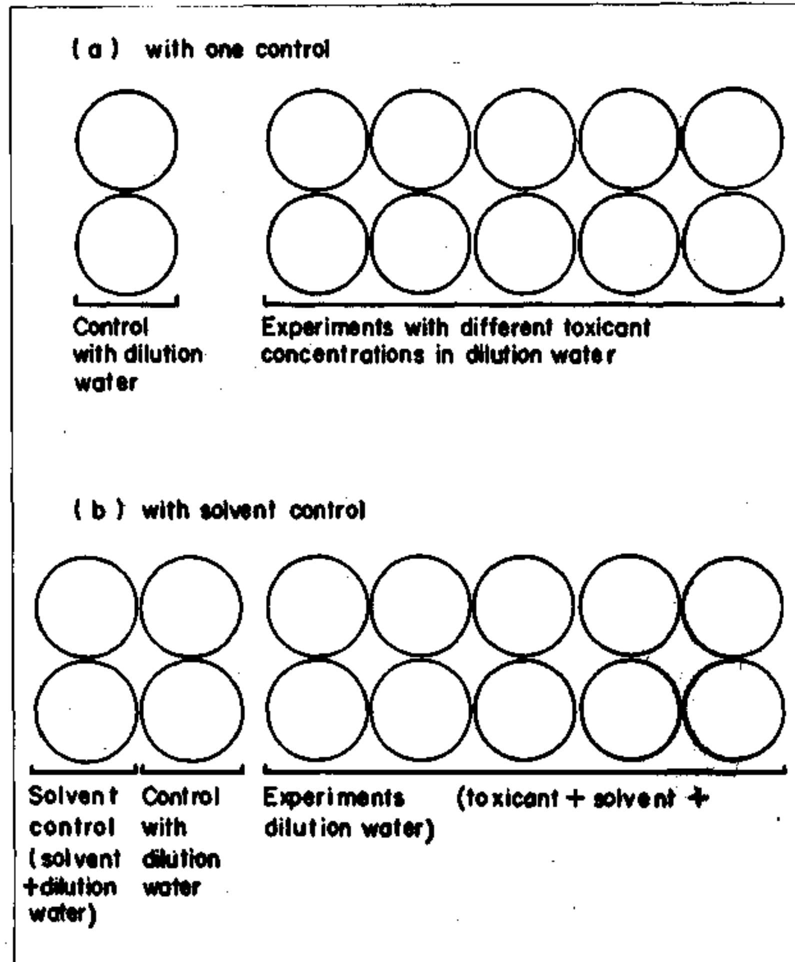


Fig. 1. Design of bioassay experiment.

counted as 10% death. According to Reish and Oshida (1987) a minimum of 10 animals per concentration for larger species and 20 for smaller species are to be used.

The number of organisms to be exposed in each test concentration is governed by a number of factors : (i) the size of the organisms, (ii) the extent of cannibalism and (iii) the availability of dilution water, toxicant and test organisms.

5.3.5.1. *Control test*

The control series is an essential part of any experiment. Control tests are typically conducted by placing animals in dilution water with no toxicant. As a rule of thumb a toxicity test is valid if control mortality is less than 10%. Where the solvent is used, a second test should be conducted and the test is valid, if the mortality of test animals is less than 10%. For example, a control contains 20 test animals. If 1 or 2 organisms die in the control, the test is valid. If 3 or more die, it is 15% and more, and the test should be repeated. The control mortality is usually indicative of a problem. The main causative factors for such problems are (i) handling , (ii) stress, (iii) diseases, (iv) poor experimental condition, (v) dirty test chambers, (vi) toxic dilution water, etc.

5.3.5.2. *Range-finding bioassay*

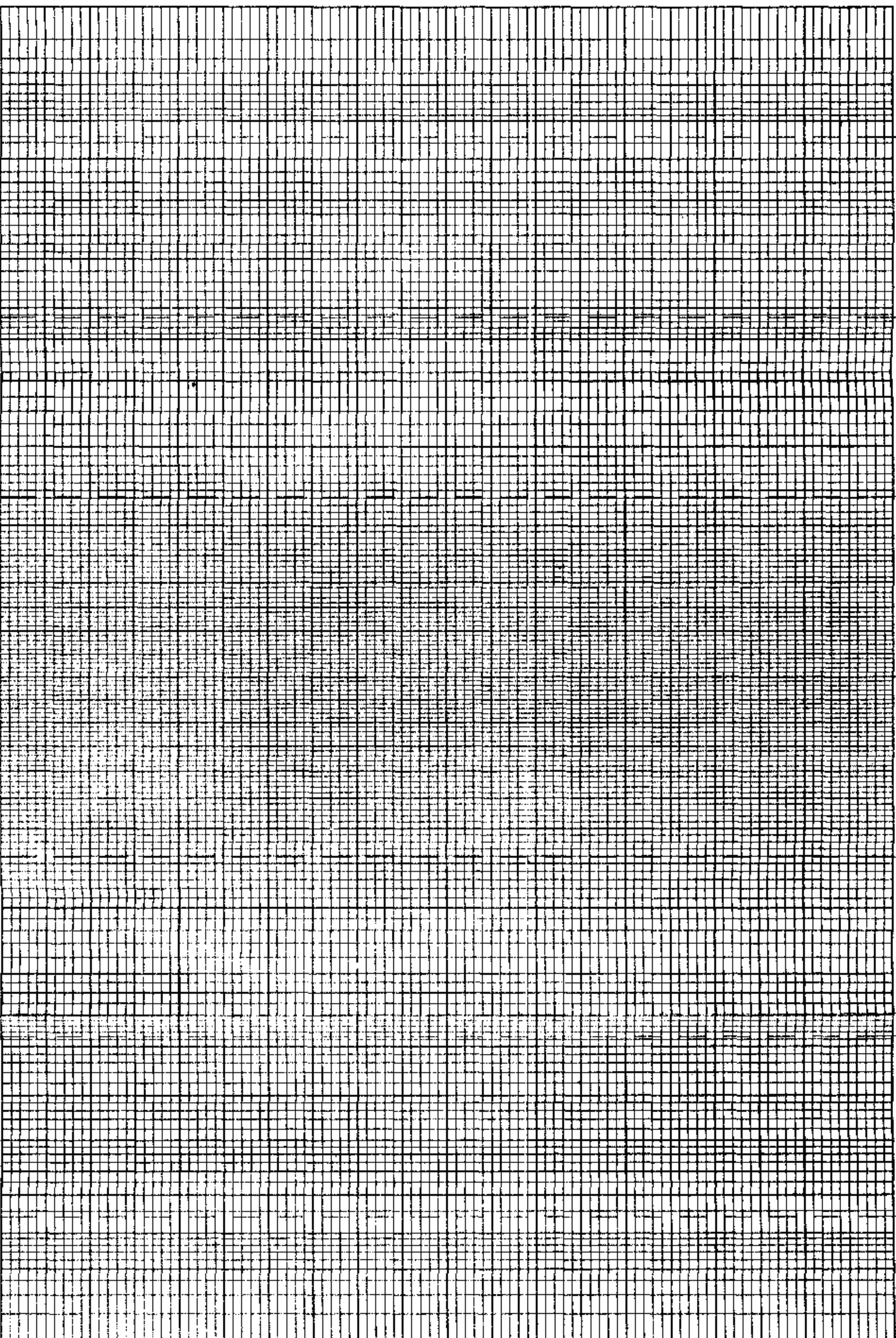
While working with an unfamiliar waste or effluent or material of unknown toxicity the investigator should select a series of concentrations in logarithmic scale for conducting small scale range finding or exploratory bioassays (APHA-AWWA-WPCF, 1976; Buikema *et al.*, 1982; Ward and Parrish, 1982). The authors also proved that the selection of concentrations can be done in geometric scale and dealt subsequently. Generally the scale is 0.1, 1.0, 10 and 100% for effluent and 0.01, 0.1, 1.0, 10, 100, 1000, etc. (in ppm) for solid toxicants such as heavy metals, their salts, gammaxine, etc. and for liquids such as pesticides, insecticides, fungicides, etc. The above scale is convenient. Suppose 90% animals died at 10% waste and non in 1% waste, the investigator should select the concentrations between 1 and 10% for conducting bioassay. Suppose 70% animals died at 10% waste and 20% at 1% waste, the investigator may select one concentration below 1%, one as 1%,

one in between 1 and 10%, one as 10% and another above 10%. It is very important to understand the expected LC50 value from the bioassay after conducting the range-finding bioassay for selection of test concentrations. Mohapatra (1994) conducted range-finding bioassays exposing the grey mullet *Liza parsia* to copper sulphate, zinc sulphate, lead nitrate and their combination in 1:1:1 ratio. The concentrations were in logarithmic scale such as 1.0, 10, 100 and 1000 ppm. Ten animals were released to each concentration and mortality after 12 hours in each tank was recorded. In copper sulphate and zinc sulphate 0, 20 and 100% mortality was recorded in 10, 100 and 1000 ppm concentrations respectively. In lead nitrate the mortality of 0% in 100 ppm and 100% in 1000 ppm was recorded. In 1:1:1 combination of all these metallic salts 0, 10 and 100% mortalities were recorded in 10, 100 and 1000 ppm respectively. Based on the Table (page 715) of APHA-AWWA-WPCF (1976) the concentrations between 56 and 180 ppm were selected for copper sulphate and zinc sulphate and between 75 and 210 ppm for lead nitrate and their combinations. While selecting concentrations it is imperative to decide the duration of exposure. Range-finding tests are usually short-term static bioassays of 12 hour duration (Mohapatra, 1994), 24 hour duration (Buikema *et al.*, 1982), 24 to 96 hour duration (APHA-AWWA-WPCF, 1976; Ward and Parrish, 1982).

Generally groups of 2 to 5 animals (Buikema *et al.*, 1982), 5 animals (Ward and Parrish, 1982) are exposed to three to five widely spaced toxicant concentrations and a control. Mohapatra (1994) suggested that 10 animals in each toxicant concentration are ideal. The authors here also suggest 10 animals in each concentration, as the death of one is easy to calculate as 10% and approaches the accuracy. If 5 animals in each concentration are dealt with, the death of one will be calculated as 20%. The number of animals depends on their availability also.

The greater the similarity between the range-finding test and the definitive test (bioassay), the more useful the result of the range-finding test will be.

% P



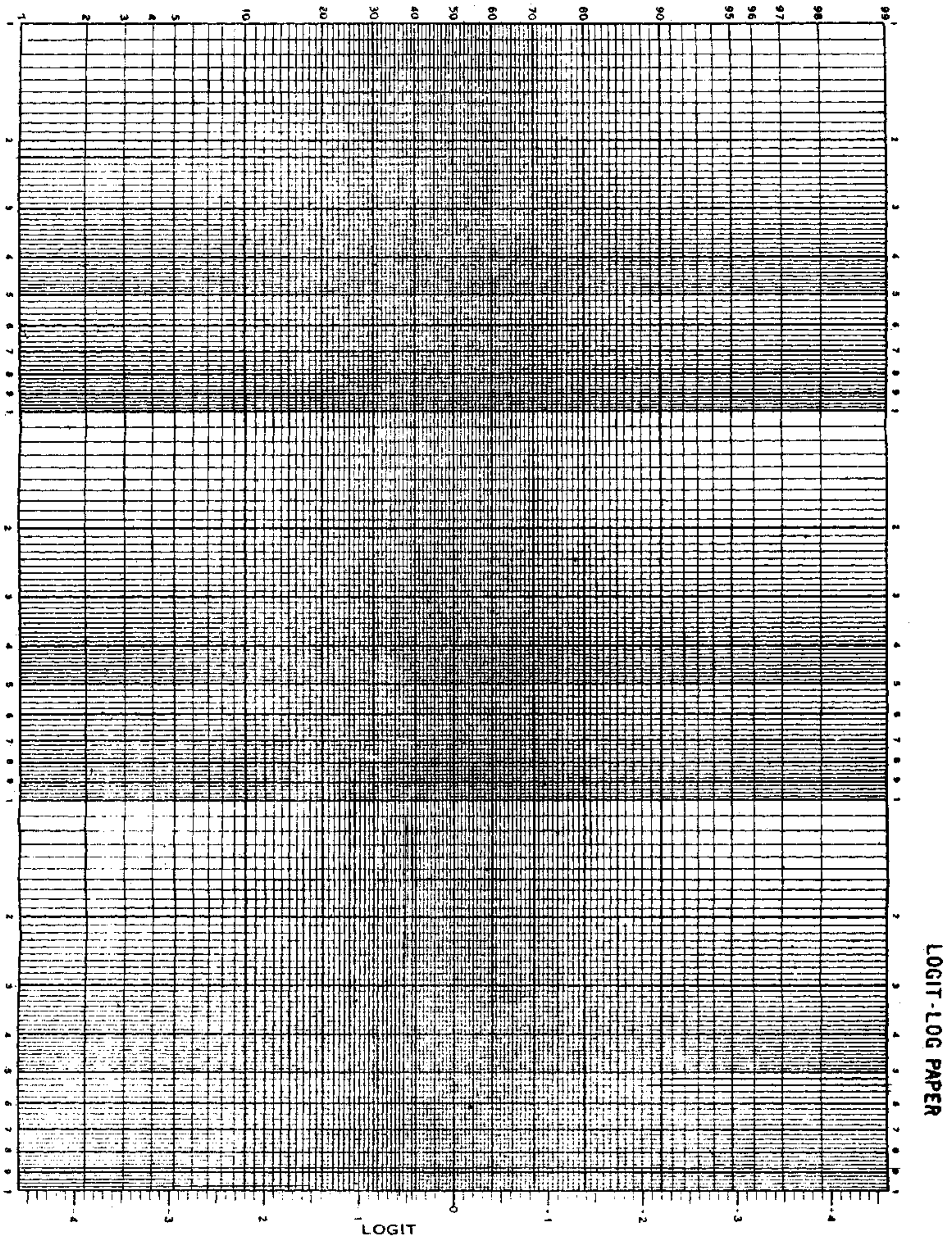


Fig. 2 b. A specimen Logit - log graph paper.

It is desirable to have the concentrations tested, as they include the one that killed all the organisms and others that killed very few or none of the organisms. If the lowest concentration in the series killed all the organisms, another series in logarithmic scale below the lowest concentration is arranged.

5.3.5.3. Selection of test concentrations

Before going to the selection of test concentrations for bioassay, it is explained here the logic behind it. For graphical analysis of data the "Probability graph paper" is used (Reish and Oshida, 1987; Mohapatra and Noble, 1991; Mohapatra, 1994; Mohapatra and Rengarajan, MS). This type of graph paper is shown in Fig. 2a. It has the lines in arithmetic scale on the X-axis. The concentrations for exposure in arithmetic scale are selected at the start (Mohapatra, 1989). It is because of equal spacings on probability graph paper while plotting the concentrations on the X-axis. The data were analysed applying the "probit analysis" to it on a computer and the same compared with that of graphical analysis. It was found that both the methods (graphical as well as computer) gave similar results upto three decimals (Mohapatra and Noble, 1991). Concentrations can also be selected based on the Table (page 715) given in APHA-AWWA-WPCF (1976). The concentrations after converting to logarithms (either to base 'e' or '10') will be equally spaced on X-axis of the "Probability graph paper". For example : in between 1 and 10 ppm the selected concentrations are

i. from Table : 1.0. 1.8, 3.2, 5.6 and 10 ppm, and after log conversion (to base 10), will be 0, 0.2553, 0.5051, 0.7482 and 1.0 ppm respectively,

ii. from Table : 1.15, 1.8, 2.8, 4.2 and 6.5 ppm after log conversion will be 0.0607, 0.2553, 0.4472, 0.6232 and 0.8129 ppm respectively,

iii. from Table : 1.35, 2.4, 4.2 and 7.5 ppm after log conversion will be 0.1303, 0.3802, 0.6232 and 0.8751 ppm respectively,

iv. from Table : 3.2, 4.2, 5.6, 7.5 and 10.0 after log conversion will be 0.5051, 0.6232, 0.7482, 0.8751 and 1.0 respectively.

The log concentration of the toxicant on the X-axis and the percentage death of animals on the Y-axis can also be plotted on "Logit-log graph paper" (Fig. 2 b) for obtaining the lethal concentrations.

The values given in the Table of APHA-AWWA-WPCF (1976) can be multiplied or divided by a factor of 10 to get the higher or lower concentrations respectively.

As stated earlier the geometric scale can also be used for selection of concentrations for bioassay. The logic put-forth here by the authors is clearly evident from the following examples.

Example 1

Suppose the concentrations are in multiples of 2 *i.e.* 1, 2, 4, 8, 16 ppm; log (base to 10) conversion will give 0, 0.3010, 0.6021, 0.9031 and 1.204 ppm respectively.

Example 2

For multiples of 3 *i.e.* 1, 3, 9, 27, 81 ppm and after log conversion will be 0, 0.4771, 0.9542, 1.431, 1.908 ppm respectively.

Example 3

For multiples 0.5, *i.e.* 10, 5, 2.5, 1.25, 0.625 ppm; log conversion will be 1, 0.699, 0.398, 0.097 and - 0.204 ppm respectively.

The geometric scale converted logarithmically will be equally spaced on probability graph paper. Again the geometric scale values can be multiplied or divided by a factor of 10 respectively to get the higher or lower concentrations for bioassay .

While graphical analysis is done with logarithmic values on the X-axis of the "probability graph paper", the "LC50" value or other "LC" values are obtained in log also. The antilog of the obtained value will give the real value. It is explained in a following section with examples.

5.3.5.4. Definitive test

For definitive test (bioassay) the use of a control and at least five concentrations of test material in a geometric progression *i.e.* a sequence in which the ratio of a concentration to its predecessor is always the same as explained by Ward and Parrish (1982). To calculate the LC50 or EC50 the following points should be taken into consideration.

i. Except for the controls, the concentration of the test material in each treatment should be at least 50% of the next higher test concentration.

ii. One treatment other than the control should have killed or affected less than 35% of the organisms exposed to it, and one treatment should have killed or affected more than 65% of the organisms (Ward and Parrish, 1982).

The authors suggest the extreme limits to be 16% and 84%. In the graphical method of analysis, the values in between 16% and 84% of mortality is used for calculation.

5.3.6. Experimental conditions

5.3.6.1. Number of test animals

In general, the bioassay is conducted by placing 10 or more individuals of a single species in each replicate of 4-6 different concentrations of a test substance and a control. A minimum of 10 species should be used per experimental tanks. For a given test under similar conditions, increasing the number of test organisms increases the precision. According to APHA-AWWA-WPCF (1976) in a series of tests with sewage effluent, it was found that with 10 fish, the 95% confidence interval was within ± 20% of the mean, while 20 fishes were exposed in each test concentration, it was within ± 14% of the mean value. The use of at least 10 animals for bioassay is suggested by various authors. The total number of animals required to conduct a bioassay is (5 concentrations and 1 control x 3 replicates) 180. If the animals are costly or have limited availability, the bioassay can be conducted (in duplication) with 120 animals (Mohapatra, 1994). If a solvent control is needed

another 20 animals will be required. A desirable size of the animal is 8 cm long weighing 5 grams (Sprague, 1973).

5.3.6.2. Volume of test solution

According to Reish and Oshida (1987), 10 litres of test solution are required per gram of fish. For 10 test specimens, weighing 5 grams each, a test container of 500 litre capacity is required. According to Buikema *et al.* (1982) 10 fathead minnows, 50 - 75 mm in length, can be tested in 15 litres of exposure media. Brown *et al.* (1974) conducted acute toxicity tests on rainbow trout of 130 - 150 mm fork length in perspex aquarium tanks of 40 lt capacity. They exposed 10 of fishes to each concentration. Mohapatra (1989, 1994) conducted bioassay with *Liza parsia* in 40 lt capacity fibreglass tanks. He observed the healthiness of these fishes in control tanks and came to the conclusion that, being a sturdy species, 10 fishes could be accommodated in each tank of 40 l capacity.

5.3.6.3. Release of organisms to test solutions

Static tests are begun by introducing the test organisms to the test chambers within 1 hour after the toxicant is added to the dilution water. The addition of the test material into the dilution water should be followed by swirling the test solution with a glass or teflon rod to disperse the test material immediately. Flow-through tests are begun by placing the test organism in the test chambers after the test solution have been flowing through the test chambers long enough, so that the test material concentrations are constant. The placement of the test animals in the test containers should be done randomly. A representative sub-sample of the test (organism) population should be impartially distributed to the test chambers, either by adding 2 (if there are to be 10 of fewer organisms per chamber) or 4 (if there are to be more than 10 organisms per chamber) test organisms to each chamber; and then adding 2 or 4 more and repeating the process until each test chamber has the desired number of test organisms in it (Ward and Parrish, 1982). The selection of the test organisms for a particular concentration can be made by following a random number Table (Zar, 1974) or by drawing the numbers from a hat.

5.3.6.4. Feeding the test animals

Test organisms should not be fed while in the test chambers (Ward and Parrish, 1982; Reish and Oshida, 1987; Mohapatra, 1989, 1994). Minimal feeding may be necessary, however, if (i) cannibalistic animals cannot be separated or restrained or (ii) the test duration constitutes a relatively large portion of the test organisms' life span.

5.3.6.5. Recording of quantal response

The number of fish living or dead in the various concentrations is recorded at the end of 24, 48, 72 and 96 hours or at whatever time interval, as deemed necessary (Katz, 1971). According to Mohapatra (1994) it is more useful if one can start counting the dead animals at 6 hr, 12 hr and then continued as suggested by Katz (1971). "Death" is the criterion for effect, most often used to study acute toxicity with aquatic organisms. Common effects generally used for determining an EC50 are immobilization and loss of equilibrium (Ward and Parrish, 1982). Lack of opercular movement in fish is an indication of pending death (APHA-AWWA-WPCF, 1976; Reish and Oshida, 1987). Dead animals are removed as soon as they are observed. The suggestion of APHA-AWWA-WPCF (1976) includes the counting of the number of the dead or affected organisms in each test container at 1.5, 3, 6, 12 and 24 hrs after beginning the test and once or twice a day thereafter.

5.3.6.6. Other information

Other effects can be used for determining an EC50, but the effect and its definition must always be reported. Indications of erratic swimming, discolouration, changes in behaviour, excessive mucus production, hyperventilation, opaque eyes, curved spine, haemorrhage, moulting, cannibalism, etc. are to be noted. All data should be recorded carefully.

A report on an acute toxicity test should include information on the test species, environmental parameters such as temperature,

light intensity, photoperiod, water chemistry such as hardness, alkalinity, conductivity, pH, dissolved oxygen, and measured toxicant concentration; biological factors such as food type and quantity, age, sex, stage, etc.

5.3.6.7. Cleaning of test containers

After the experiment the test solution should be disposed of in a safe manner. The test containers must be scrubbed and washed thoroughly with a chemical cleaner to remove all traces of the toxicant. The scrubber is the detergent. The chemicals generally used are 10% hydrochloric acid (HCl) or nitric acid (HNO_3).

6

DATA ANALYSIS

6.1. Determination of LC50

The LC50 value can be determined graphically from a line drawn through the data points or by a variety of computational methods described by Litchfield and Wilcoxon (1949); Finney (1971, 1978); Hamilton *et al.* (1977) and Koejman (1981). The method chosen will depend on the distribution and number of partial effects between 0 and 100% mortality. "Probit analysis" (*e.g.* Finney, 1971) is a commonly used parametric technique for analyzing toxicity data. Most investigators emphasize the LC50 in the evaluation of toxicity data. However, the LC50 does not provide all the information about the dose-response line. The graphical method explained here provides the maximum information about the "Response curve". This is easily performed without a computer and the investigator can avoid the lengthy computations for obtaining results.

The results of a bioassay (Mohapatra, 1994) is presented in Table 1. The average cumulative percentage mortality for each concentration is tabulated against toxicant concentration. The total number of test animals in each concentration is taken for calculation. For example, in 1 ppm, in one series 40% death is recorded and in other series (duplication) 50% death is recorded. The number of animals exposed in a concentration in a series is 10. So the total number of animals is 20, concentration is 1 ppm, and the percentage of mortality is 45 for graphical analysis. The addition of number of test animals for each concentration gives the maximum accuracy in calculation (Mohapatra, 1989, 1994).

TABLE 1. *Mortality rate of* Liza parsia *in different concentrations of copper sulphate : zinc sulphate : lead nitrate :: 1 : 1 : 1 : during acute toxicity studies (Each concentration tested with 20 animals)*

Exp. period (hr)	Concentration (ppm) 75		100		115		135		210	
	Nos	%	Nos	%	Nos	%	Nos	%	Nos	%
6	-	-	-	-	-	-	4	20	6	50
12	-	-	-	-	1	5	6	30	16	80
24	1	5	2	10	4	20	10	50	20	100
48	1	5	2	10	5	25	12	60	20	100
72	1	5	4	20	7	35	16	80	20	100
96	4	20	6	30	11	55	17	85	20	100

6.2. Arithmetic graphic method for LC50

In the case of "arithmetic graphic method" on graph paper (Coordinated paper), the percent death is plotted on the ordinate against the concentration on the abscissa. Each data point for 96 hr (Table 1) is plotted and the points connected to form a graph (Fig. 3). A horizontal line is drawn from the 50% death point (on ordinate) to intersect the plot. A vertical line is drawn from the intersection point to the abscissa. The intersect point on the abscissa corresponds to the 96 hr LC50 *i.e.* 112.5 ppm. In this type of graphical analysis the result obtained may not be correct. Only two points *i.e.* one immediately before and other immediately after 50% mortality on the graph governs the LC50 result. If any one of them does not represent the correct result of the experiment, the LC50 will be erroneous. In Fig. 3, the points against 100 and 115 ppm on graph paper give the LC50 value while other 3 points for 75, 135 and 210 ppm have no role for obtaining LC50 value except for drawing the graph. The authors do not advise this method for LC50 calculation.

6.3. *Logarithmic method for LC50*

In "logarithmic method" the LC50 calculation is similar to the arithmetic method except that "semi-log paper" is used for plotting the results (Fig. 4). The concentration is plotted logarithmically along the X-axis as the concentrations used are

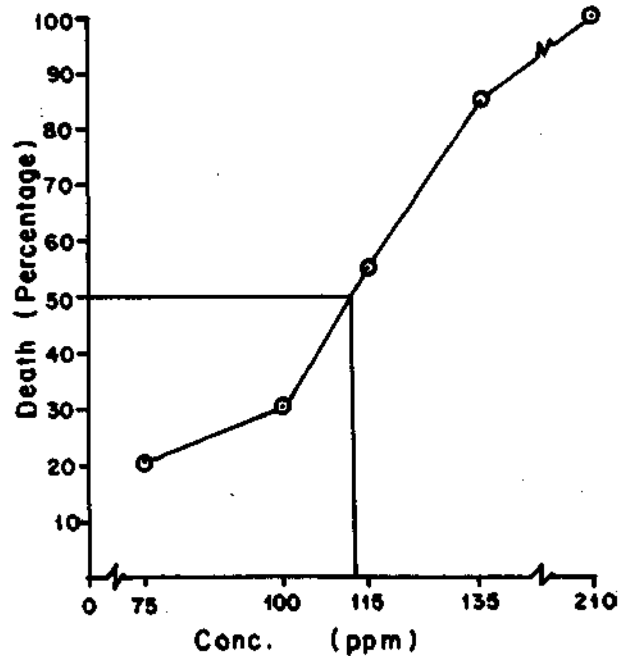


Fig. 3. Response curve for obtaining 96 hrs LC50 of Copper sulphate : Zinc sulphate : Lead nitrate :: 1 : 1 : 1 on arithmetic graph paper (Data taken from Table 1).

based on a logarithmic scale and percentage of death on the arithmetic scale. A straight line is drawn between the two points representing the percentage dead at the two successive concentrations that were lethal to more than half and to less than half of the test organisms. The concentration at which this line

crosses the 50% lethality line, is the estimated LC50 value *i.e.* 114 ppm. To all the points on graph paper a best-fit can be drawn after suitable statistical analysis. In a dose-response curve (on arithmetic scale), it is typically shown that percent mortality rapidly increases and then levels out as the concentration increases. Buikema *et al.* (1982) have explained from their experience that log increase in toxicant concentration yields equivalent increase in response, but this function is toxicant-dependent. Often, when the concentration axis is plotted on a log scale, the idealised dose-response curve is a cumulative normal distribution describing the tolerance levels of individuals in the population. Thus, at a low concentration of toxicant, only a few very intolerant individuals of the exposed group are killed. At intermediate concentration, most of the exposed group is affected, and at high concentrations, only very few tolerant members survive. By transforming the response data to a probit scale, the dose-response curve becomes a straight line (Buikema *et al.*, 1982).

Goodness of fit test (x^2)

Occasionally toxicity data are not normally distributed and do not fit a log-probit model. A chi-square (x^2) goodness of fit test will indicate whether the model sufficiently describes the relationship between dose and response. If the chi-square test does not indicate significant differences between observed and expected responses, the model is appropriate and the LC50 can be calculated from the data. A significant chi-square may indicate large random or systematic deviations of the observed data from the log-probit model. Random deviations from the log-probit line have the greatest effect on goodness of fit when they occur in the middle rather than at the ends of the line, because the centre portion of the line is more heavily weighed in estimating the dose-response curve. The probit method of calculation of the LC50 minimises the extremes in the results and maximises the middle percentage survivals (or dead).

6.4. *Probability / Probit method for LC50*

In probit method of analysis the data are plotted on logarithmic probit paper, otherwise known as "Logit - log paper", (Fig. 2 b) with the concentrations on logarithmic and the percentages of the dead on the probit scale. The probability graph paper is shown in Fig. 2 a in which the X-axis is in arithmetic scale. In that case the log (to the base '10' or 'e') concentrations (converting the

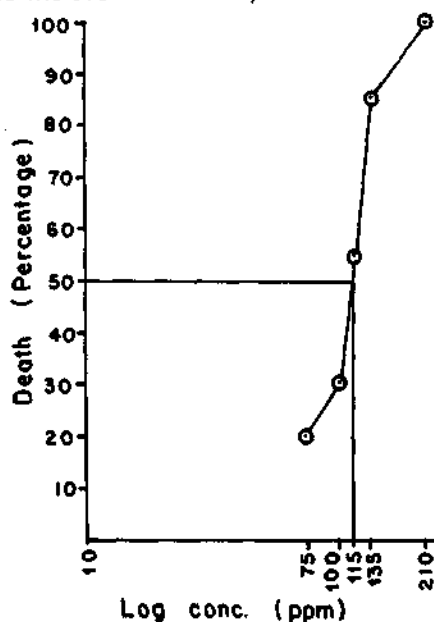


Fig. 4. Response curve for obtaining 96 hrs LC50 of Copper sulphate : Zinc sulphate : Lead nitrate : : 1 : 1 : 1 on semi log paper (Data taken from Table 1).

tested concentrations to log) should be taken for plotting in X-axis. The probability / probit graph papers neither have 0 nor 100 point. Pal (1982) explained the plotting of data (cumulative percentage *vs* toxicant concentration) on the arithmetic probability paper. According to him when the data approximates a normal distribution the plotting will give a linear line. He suggested that for positively-skewed distributions, logarithmic transformations are effective for converting the skewed character of the data into a normal one.

The model analysis of data (given in Table 1) by "Probability graphical method" is explained step by step in Example 1 in this chapter. Efforts have been put into make the analysis as simple as possible for getting better results of LC50 and other supporting information.

To construct the graph (response curve) the percentage mortality on the vertical axis (Y-axis) against the concentration (logarithmic) on the horizontal axis (X-axis) (Mohapatra, 1989, 1994) is plotted. Death in Y-axis of probability / probit paper is on a probit or probability scale. As the probit scale never reaches 0 or 100%, any such point should be plotted with an arrow indicating their true position. These points can also be avoided in plotting on graph papers. The utmost consideration is given to points between 16 and 84% mortality (Reish and Oshida, 1987). APHA-AWWA-WPCF (1976) suggested that a line tò be fitted to the points by eye. But it is felt by the authors that this type of eye fitting line may not be the true best-fit. The best-fit to the points should be drawn based on the regression equation (y=a+bx) as shown in Fig. 5 a - f. It is also demonstrated in the reports of Reish and Oshida (1987). Regression is a functional relationship between one dependent variable 'x' (the concentration) and one independent variable 'y' (the percentage mortality). In the above-said equation the 'a' and 'b' are constants where 'a' is the intercept of the straight line on the Y-axis and 'b' its slope indicating the rate at which 'y' changes with a change in 'x' (Pal, 1982). The formulae for 'a' and 'b' are as follows :

$$b = \frac{\sum xy - \frac{\sum y \ \sum x}{n}}{\sum x^2 - \frac{(\sum x)^2}{n}}$$

where n = no. of tests

$$a = \frac{\sum y - b\sum x}{n}$$

After finding out 'a' and 'b', 'y' is taken as 30 and 70 or 40 and 60 (approximately) and the corresponding 'x' for these 'y' values are found out. Any pair (x, y) the investigator can use for drawing the best-fit to the points on graph paper. The correlation coefficient (r) for the variables in the regression equation (y = a + bx) as given below is found out :

$$r = \frac{\sum xy - \frac{\sum x \sum y}{n}}{\sqrt{\left[\sum x^2 - \frac{(\sum x)^2}{n}\right]\left[\sum y^2 - \frac{(\sum y)^2}{n}\right]}}$$

Generally the 'r' value lies between -1 and +1. A correlation coefficient measures the degree of association between the sets of paired variates. A significance test of it can show whether that degree of association is likely to be more than a matter of chance.

The concentration causing 50% mortality from the fitted line is read out : this is the estimated log LC50 for the selected exposure time. The antilog of it is taken to get the actual concentration. Similarly the LC16 and LC84 from the fitted line are also found out. On probability paper the 16% and 84% lines are darker (thicker) ones. In research studies, it is desirable to have at least one of the responses in the range of 16 to 84% mortality because they represent ± 1.0 probit about the median response. At least two partial mortality values are required in order to estimate the LC50 (APHA-AWWA-WPCF, 1976). In more exacting tests, it is desirable to have several partial mortalities between 16 and 84%. The LC50 estimated by graphical procedures is almost always satisfactorily accurate (APHA-AWWA-WPCF, 1976; Mohapatra and Noble, 1991).

6.5. Binomial test for LC50

According to Stephan (1977), almost all concentration-mortality data produced by acute mortality test can be analysed to produce a statistical "best estimate" of LC50 and its 95% confidence limits. The exception is the case in which none of the

test concentrations contain test organism mortality >0% <100%. The binomial test can then be used to obtain an estimate of the LC50 as LC50 = $(AB)^{1/2}$, where A = highest test material concentration in which none of the test organisms died and B = lowest concentration in which all the organisms died. The confidence level associated with these limits depends on the number of organisms exposed and can be calculated from the formula :

$$\text{confidence level} = 100\ [\ 1-2(1/2)^{N}]$$

where N = number of exposed organisms.

If six or more organisms are used for treatment, the confidence level represented by A and B is always above 95%.

The LC50 may be estimated by probit analysis. In routine tests this requires, as a minimum, that a line be drawn by eye to fit the results plotted on logarithmic-probability paper (APHA-AWWA-WPCF, 1976) or on arithmetic probability paper. The best-fit (response curve) can be drawn even with three data points on the graph paper.

6.6. *LC50 related statistical analysis*

The other formulae given by Reish and Oshida (1987) are given below :

i. The slope function :

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

ii. The confidence limit :

$$f_{LC50} = S^{\sqrt{\frac{2.77}{n}}}$$

(where f = confidence limit, 2.77 = constant, n = total number of organisms tested at those exposure concentrations whose expected results are between 16 and 84%)

iii. The upper and lower 95% confidence limits (called as fiducial limits):

$$\text{Upper limit} = LC50 \times f_{LC50}$$

$$\text{Lower limit} = \frac{LC50}{f_{LC50}}$$

The 95% confidence limits are an additional statistical treatment of the data; it defines the limits of the calculated LC50 taking into account the variability of the system. If the 95% confidence limits are narrow, then the results of the experiment are considered good. It is useful in comparing the results of one replicate to another. If it falls within a narrow limit, then the results of these two replicates are the same and are statistically insignificant. If the confidence limits do not overlap then the differences are statistically significant. The level of confidence, say 68% or 95%, the range within which a value predicted will lie very much. It is estimated the confidence limits for the LC50 of the longest exposure time (APHA-AWWA-WPCF, 1976). According to Mohapatra (1989, 1994) it is essential to estimate the fiducial limits of LC50 for 12, 24, 48, 72 and 96 hour exposures for better construction of toxicity curves. Fiducial limits are a statement that there is a 19 of 20 chance that the LC50 value falls within the specified limits (P = 0.05).

In the case of replicates or in different test conditions the significant differences between two LC50s may be tested more exactly by the formula developed by Litchfield and Wilcoxon (1949):

$$1.96\ SE_{Diff} = \text{Antilog} \sqrt{(\text{Log } f_1)^2 + (\log f_2)^2}$$

If the ratio $\frac{\text{Greater LC50}}{\text{Smaller LC50}}$ exceeds the value for 1.96 SE_{Diff} then the LC50's are significantly different.

All other things being equal as the slope of the line increases (becomes steeper) the fiducial limits decrease (Buikema *et al.*, 1982).

Fiducial limits are also affected by the number of partial kills *i.e.* responses between 0 and 100% mortality, that occur and the number of organisms per container. The relationship between the LC50 value and the mean test concentration also affects the width of the fiducial limits. As the distance between the LC50 value and the mean test concentration increases, the width of the fiducial limit increases (Buikema *et al.*, 1982). When LC50 values are extrapolated from the log-probit line beyond the last data point of the series (*i.e.* if LC50 is 70 ppm and the last highest concentration tested is 45 ppm) the calculation of fiducial limits is meaningless. Even if fiducial limits could be calculated, the width of the interval will be large and may approach infinity. The estimate of the slope with fiducial limits provides information about the standard deviation of the log tolerance distribution.

When a formal probit analysis is carried out with a computer it is always done with a graph such as Fig. 5 to check the reasonableness of the computed LC50. The percentage of the test organisms that die or show the effect in the control treatment must not be used in the calculation of the results (Ward and Parrish, 1982). No correction should be made for control mortality.

Example 1 : Calculation of LC50 values from the data given in Table 1.

(a) 6 hr LC50

Concentration (ppm)	(x) log conc. (in ppm)	(y) % death
135	4.905	20
210	5.347	50

Best-fit (response curve) is given in Fig. 5 a.

LC16 = Antilog 4.840 = 126.5 ppm
LC50 = Antilog 5.347 = 210.0 ppm
LC84 = Antilog 5.870 = 354.2 ppm
S = 1.699
N = 40
$f_{LC\,50}$ = 1.251
Upper limit = 262.8 ppm
Lower limit = 167.8 ppm

(b) 12 hr LC50

Concentration (ppm)	(x) log conc. (in ppm)	(y) % death
115	4.745	5
135	4.905	30
210	5.347	80

b = 125.0
a = -586.7
r = 0.908
y = -586.7+125.0 x

Best-fit (response curve) is given in Fig. 5 b.

LC16 = Antilog 4.810 = 122.7 ppm
LC50 = Antilog 5.078 = 160.5 ppm
LC84 = Antilog 5.350 = 210.6 ppm
S = 1.310
N = 40
f_{LC50} = 1.126
Upper limit = 180.6 ppm
Lower limit = 142.6 ppm

(c) 24 hr LC50

Concentration (ppm)	(x) log conc. (in ppm)	(y) % death
75	4.317	5
100	4.605	10
115	4.745	20
135	4.905	50
210	5.347	100

b = 77.23
a = -337.33
r = 0.913
y = -337.33+77.23 x

Best-fit (response curve) is given in Fig. 5 c.

LC16 = Antilog 4.535 = 93.22 ppm
LC50 = Antilog 5.025 = 152.2 ppm
LC84 = Antilog 5.510 = 247.2 ppm
S = 1.628
N = 40
f_{LC50} = 1.238
Upper limit = 188.4 ppm
Lower limit = 122.9 ppm

(d) 48 hr LC50

Concentration (ppm)	(x) log conc. (in ppm)	(y) % death
75	4.317	5
100	4.605	10
115	4.745	25
135	4.905	60
210	5.347	100

b = 95.65
a = -419.1
r = 0.917
y = -419.1+95.65 x

Best-fit (response curve) is given in Fig. 5 d.

LC16 = Antilog 4.530 = 92.8 ppm
LC50 = Antilog 4.910 = 135.6 ppm
LC84 = Antilog 5.285 = 197.4 ppm
S = 1.458
N = 40
f_{LC50} = 1.18
Upper limit = 160.0 ppm
Lower limit = 114.9 ppm

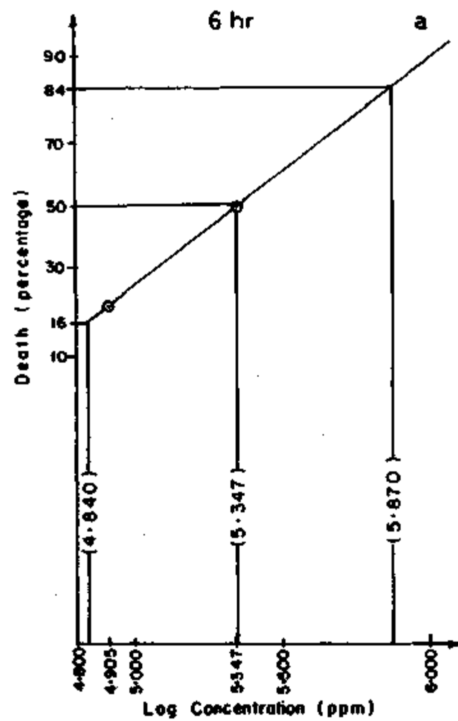


Fig. 5 a. Response curve of Copper sulphate : Zinc sulphate : Lead nitrate : : 1 : 1 : 1 at 6 hrs.

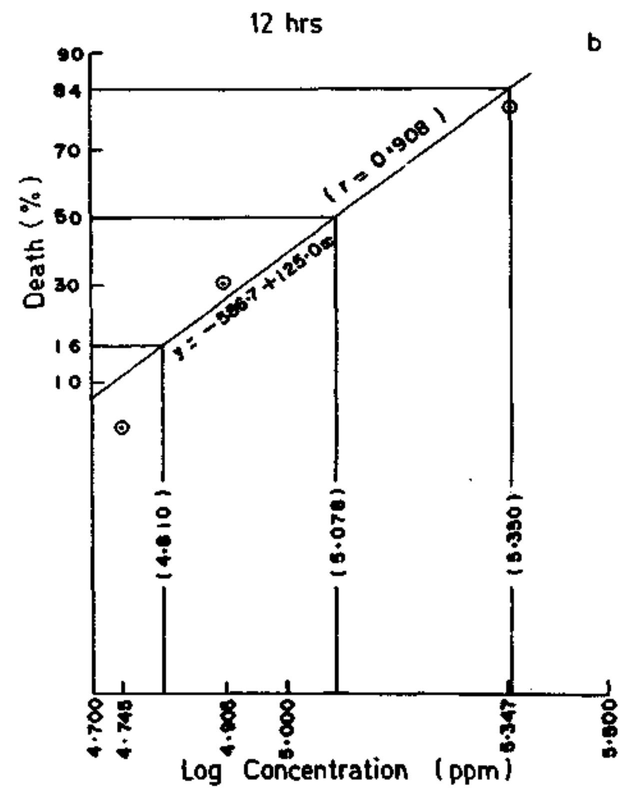


Fig. 5 b. Response curve of Copper sulphate: Zinc sulphate : Lead nitrate : : 1 : 1 : 1 at 12 hrs.

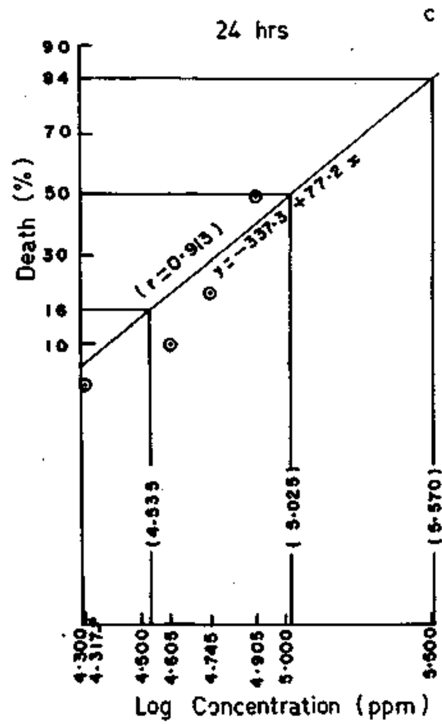


Fig. 5 c. Response curve of Copper sulphate : Zinc sulphate : Lead nitrate :: 1 : 1 : 1 at 24 hrs.

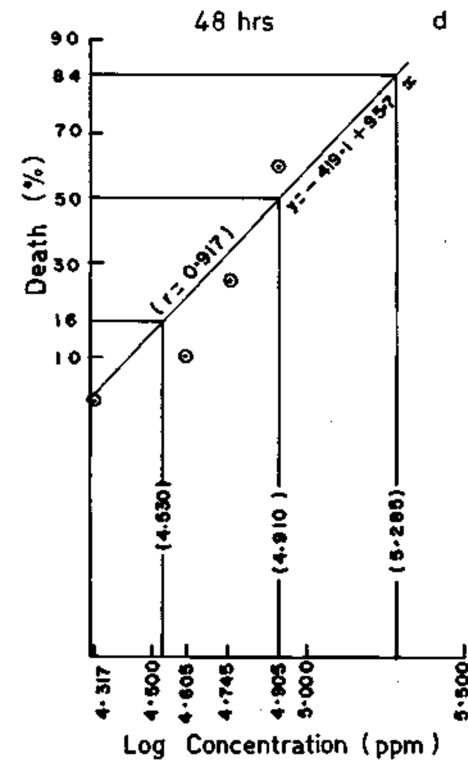


Fig. 5 d. Response curve of Copper sulphate : Zinc sulphate : Lead nitrate :: 1 : 1 : 1 at 48 hrs.

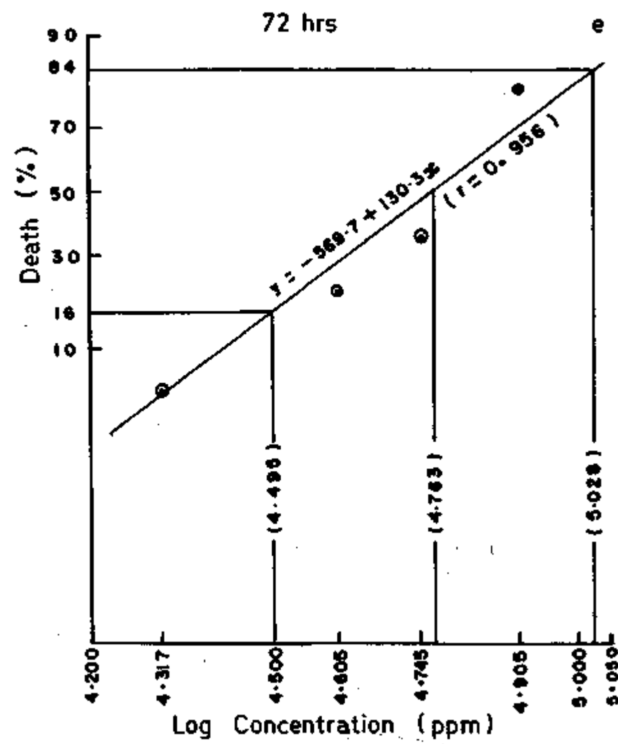


Fig. 5e. Response curve of Copper sulphate : Zinc sulphate : Lead nitrate : : 1 : 1 : 1 at 72 hrs.

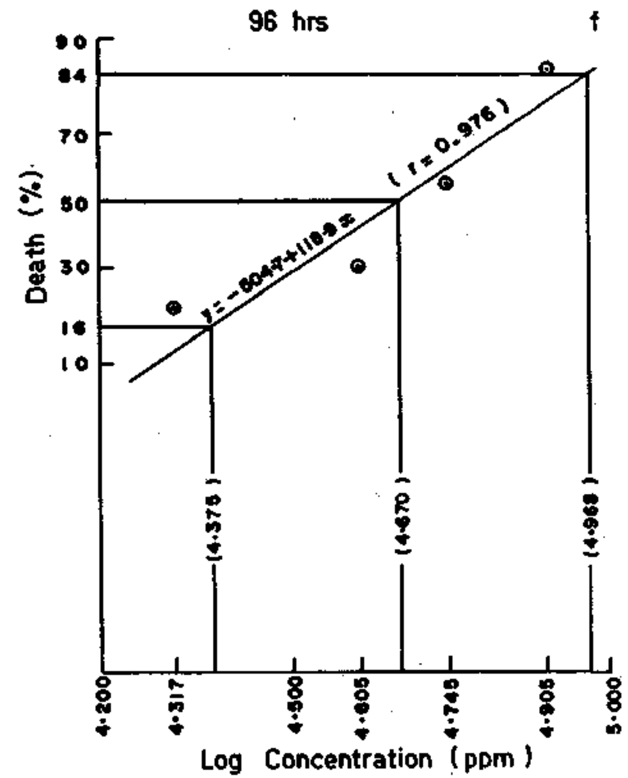


Fig. 5 f. Response curve of Copper sulphate : Zinc sulphate : Lead nitrate : : 1 : 1 : 1 at 96 hrs.

(e) **72 hr LC50**

Concentration (ppm)	(x) log conc. (in ppm)	(y) % death
75	4.317	5
100	4.605	20
115	4.745	35
135	4.905	80
210	5.347	10

b= 130.25
a= -569.7
r= 0.956
y= -569.7+130.25 x

Best-fit (response curve) is given in Fig. 5 e.

LC16 = Antilog 4.495 = 89.6 ppm
LC50 = Antilog 4.763 = 117.1 ppm
LC84 = Antilog 5.028 = 152.6 ppm
S = 1.305
N = 60
f_{LC50} = 1.10
Upper limit = 128.8 ppm
Lower limit = 106.5 ppm

(f) **96 hr LC50**

Concentration (ppm)	(x) log conc. (in ppm)	(y) % death
75	4.317	20
100	4.605	30
115	4.745	55
135	4.905	85
210	5.347	100

b = 118.9
a = -504.7
r = 0.976
y = -504.7+118.9 x

Best-fit (response curve) is given in Fig. 5 f.

LC16	=	Antilog 4.375	=	79.4 ppm
LC50	=	Antilog 4.670	=	106.7 ppm
LC84	=	Antilog 4.968	=	143.7 ppm
S	=	1.345		
N	=	60		
f_{LC50}	=	1.112		
Upper limit	=	118.6 ppm		
Lower limit	=	95.9 ppm		

The results obtained from Example 1 are summarised below in Table 2.

TABLE 2. *Acute toxicity results*

Exposure period (in hr)	LC50 (in ppm)	CL	95% Fiducial limits: Upper limit (in ppm)	95% Fiducial limits: Lower limit (in ppm)
6	210.0	1.251	262.8	167.8
12	160.5	1.126	180.6	142.6
24	152.2	1.238	188.4	122.9
48	135.6	1.180	160.0	114.9
72	117.1	1.100	128.8	106.5
96	106.7	1.112	118.6	95.9

Toxicity curve

The series of LC50s should be used along with their fiducial limits (upper limit and lower limit) to construct a "toxicity curve" as the experiment proceeds, ending with something similar to Fig. 6. It gives the investigator an overall picture of the progress of the test and also indicates when acute lethality has stopped. This will be indicated by the curve becoming asymptotic to the time axis (Sprague, 1970). The LC50 for an exposure time that is in the asymptotic part of the curve is termed as the "threshold" or "incipient LC50".

To construct a "toxicity curve" the LC50 values are plotted on two cycle "Log-log (nomograph)" paper with the LC50s on the X-axis and time in hours along the Y-axis (Fig. 6). The toxicity curve may not pass through all the LC50 points on the log graph

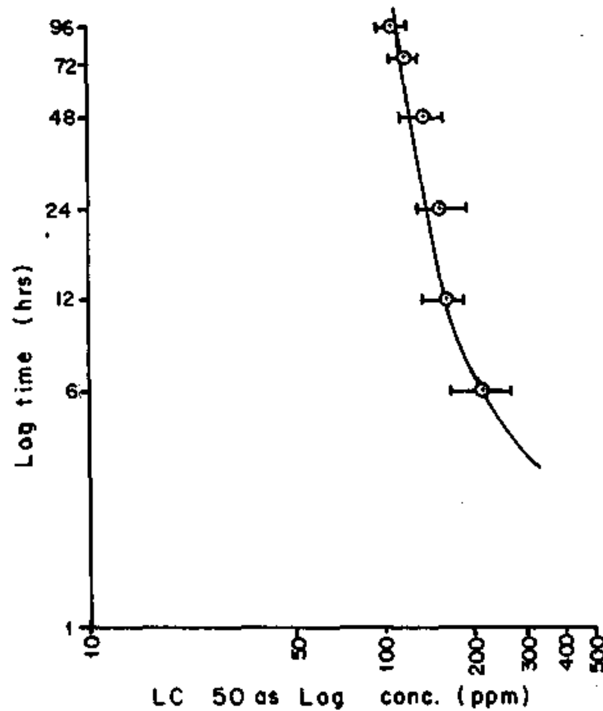


Fig. 6. Toxicity curve of Copper sulphate : Zinc sulphate : Lead nitrate : : 1 : 1 : 1.

paper at all times. It should pass if not LC50, in between fiducial limits of LC50. If the test curve becomes asymptotic, then the 96 hr LC50 is a reasonably accurate figure and the test considered valid. On the other hand if the curve does not approach a vertical asymptote, the test cannot be considered valid. In that case the test concentrations may be too high or the test organisms may not be in good health.

7

JOINT TOXICITY

It is a simple approach for determining the interaction of two or more test materials to test organisms. Suppose there are two toxicants namely 'A' and 'B'. For *e.g.* 10 ppm of 'A' produces the response on test animals. Similarly 10 ppm of 'B' will produce some results. The response in 10 ppm of A and B together to the test animals is observed. If this combination just causes the response, the actions of A and B are exactly "additive". If it causes more than the given response of A or B alone, then it is "more-than additive". If it does not cause the response, then the test materials are either "less-than additive", show no interaction or are antagonistic (Ward and Parrish, 1982). It is better explained in "Gaddum's diagram" (Gaddum, 1948; Sprague, 1970).

Another example is explaining the antagonistic effect of one toxicant over the other. If 1.0 unit of 'A' is required to just produce the response, irrespective of the concentration of 'B' present (as long as it is 1.0 unit), then 'A' is causing the response and 'B' is neither helping nor hindering. If more than 1.0 unit of 'A' is required to produce the effect because of the presence of 'B', this indicates antagonism with 'B' antagonizing the effect of 'A'.

7.1. Calculation of incipient LC50

The system for predicting joint toxicity is simple and explained with example in this chapter. The calculation is based on incipient or time-independent LC50s. According to Sprague (1970) the 48 or 96 hr LC50 can be used for calculations. It has often been found that the incipient lethal levels are little different from TLm (LC50) values corresponding to exposure periods of 96 hr or more (Laws, 1981). In the vicinity of a median survival time of 96 hour, the toxicity curves are almost vertical. In practice, it is frequently found that acute toxicity studies involve measurements of 96 hr TLm value rather than the more laborious testing required to estimate incipient lethal levels, the rationale

being that 96 hr TLm (96 hr LC50) values are likely to be little different from the true incipient lethal levels. In other words, if an organism can survive exposure to a stress of 96 hour, it can probably survive (ignoring chronic stress complications) for an almost indefinite period of time at a stress level little different from the 96 hr TLm (96 hr LC50).

7.2. Toxic unit

The strength of a given test material (measured in any suitable chemical units) is expressed as a fraction or proportion of its lethal threshold concentration (measured in the same units) when 1.0 toxic unit equals the incipient LC50. Hence, if this number is greater than 1.0 toxic unit, more than half of a group of test organisms will be killed by exposure to this test material. If the number is less than 1.0 toxic unit, then half of the test organisms will not be killed. Thus the strength of any test material may be calculated as follows :

$$\text{Toxic units} = \frac{\text{actual concentration in solution}}{\text{lethal threshold concentration (LC50)}}$$

For a mixture, the number of toxic units may be calculated for each of the component pollutants. Since the strength of all are expressed in the same units, they may be added together (EIFAC, 1980). Once again, if the total of toxic units is 1.0 or larger the mixture is predicted to be lethal. Solutions rated at 5 to 10 toxic units might be expected to be lethal to fish in a few hours. The actual value above 1.0 represents the solution required to bring the toxicant or mixture of toxicants down to the just-lethal level (Sprague, 1970).

Example

The 96 hr LC50 of 1:1:1 combination of copper sulphate, zinc sulphate and lead nitrate = 106.7 ppm (Table 2).

The individual contribution $= \frac{106.7 \text{ ppm}}{3} = 35.57$ ppm

The 96 hr LC50 of Copper sulphate = 85.6 ppm (Mohapatra, 1994)

The 96 hr LC50 of Zinc sulphate = 60.3 ppm (Mohapatra, 1994)

The 96 hr LC50 of Lead nitrate = 103.5 ppm (Mohapatra,1994)

The toxic unit of Copper sulphate = $\frac{35.57}{85.6}$ = 0.4155

The toxic unit of Zinc sulphate = $\frac{35.57}{60.3}$ = 0.5898

The toxic unit of Lead nitrate = $\frac{35.57}{103.5}$ = 0.3436

The toxic units are 0.4155 + 0.5898 + 0.3436 = 1.3489 and is found greater than unit (*i.e.* 1). Thus the chemicals are indeed strictly "additive".

7.3. Combined toxic effects

Most sewer water form commercial and domestic installations have a complex chemical composition; consequently it is important to know not only the toxicity of individual components, but also their combined effect. The combined effect of the components is manifested in the form of synergism, antagonism or an independent combined effect.

Synergism is the phenomenon of interaction between two or several components at which the toxic effect is greater than the total effect of each component individually. According to Metelev *et al.* (1983) combination of heavy metals (copper and zinc, copper and cadmium, nickel and zinc), ammonia and phenol, ammonia and cyanides, ammonia and chlorine, formic acid and sulphates, etc. are synergistic. Salts of potassium, calcium and sodium are antagonistic in nature.

Calcium salts neutralize the effects of magnesium salts. Other antagonistic combinations are sodium chloride and calcium chloride, prussic acid and ferric oxide, potassium permanganate and rotenone, methylene blue and rotenone, lime water and salts of heavy metals (copper, zinc, tin, iron, etc.), lime and fluorides, lime water and silicofluorides, etc.

The toxicity of salts of heavy metals and fluorides in soft and distilled water is higher than in hard and sea water. This property of lime and other alkaline elements is used in some purification installations for rendering sewer water non-poisonous.

8

APPLICATION OF LC50

Acute toxicity tests can provide meaningful comparison of toxicant lethality between animals or toxicants or test conditions. It can reliably monitor changes in the lethality of complex mixtures to an organism. But the acute toxicity test (bioassay), by itself, can not adequately predict a concentration of toxicant unlikely to harm a population or ecosystem. When the question of interest in the prediction of a concentration unlikely to cause dreadful effects to a complex ecosystem and its population, additional information for that purpose is essential.

The toxicity test or bioassay has been of great value to the industry for the solution of many toxicity problems. It gives the waste producer some idea of the toxicity of the waste. If it is determined that the waste is not very toxic and is discharged into an extremely large body of water with almost immediate and thorough mixing, then the industry would have good reason to believe that its chances of seriously affecting biological organisms are small. But, if the waste is of great toxicity and is discharged into a relatively small stream on which other water users make legitimate demands or even into a large stream that is accepting other waste discharges, then the waste disposal problem is serious. In many cases, the derived toxicity information indicates that the plant management must construct some effective treatment facilities involving large and continuing expenses. Then it estimates what dilution or degree of treatment is necessary to meet the demands of the regulatory agency. At this point the 96 hr LC 50 (96 hr TLm) becomes painfully apparent. With this value the conservation agency and the fisheries interests could not be satisfied with a level of toxicity that would only allow 50% of a fish population to survive - 50% would not be strong and they would be affected to some undesirable degree by the waste. What is necessary is the dilution or treatment that will ensure a healthy, vigorous, normally growing population of organisms that can carry on all of their life activities.

8.1. Dilution factor

A dilution factor of 10 (1/10th of the LC50) was suggested at one time for a kraft pulp waste. The dilution factor of 10 has been unofficially and unfortunately adopted as adequate by many agencies and has become rather firmly entrenched in practice, for no good reason (Katz, 1971). There were attempts by some biologists who were dealing with a few specific toxicants to develop formulae which would utilize the data obtained by short-term toxicity tests (bioassays) to determine the dilution required to protect aquatic populations. In most of the predictions, the application factor (AF) is actually used along with LC50 values. According to National Academy of Sciences and National Academy of Engineering (NAS/NAE, 1973) the application factors vary from 0.1 to 0.0001. The more conservative numbers are for bioaccumulative substances. But the estimates of "safe" concentrations in environments cannot be guaranteed as valid (Buikema *et al.*, 1982) and often no significantly justifiable basis exists for using a particular AF. The most commonly used reference value, the LC50, often is not a reliable estimate of the incipient lethal level, especially for bioaccumulative materials.

8.2. Application Factor

Mount and Stephan (1967) suggested that an AF could be empirically derived by determining the ratio of the maximum concentration of a material not having an effect on growth, reproduction (including spawning behaviour, hatching and lack of teratogenic effects) to the LC50. The application factor is :

$$AF = \frac{MATC}{LC\ 50}$$

where MATC is the "maximum allowable toxicant concentration" and the LC50 is usually for 48 or 96 hours depending on the test species in question.

Mount and Stephan (1967) and Mount (1968) have derived experimentally some tentative application factors and these studies have demonstrated much variation, depending on the toxicant. Available data indicate that these factors must vary according to

the toxicant if they are to be reasonable and equitable as well as effective. No single application factor can be equally appropriate to all toxic materials. According to Laws (1981) typically the application factor is a number on the order of 0.01 - 0.1. The choice of the appropriate AF is based on experiments in which the chronic effect of a pollutant has been specifically studied. Since in many cases appropriate chronic stress studies have not been performed with the particular organism or pollutant concerned, it is often necessary to estimate the appropriate AF based on the chronic stress studies involving similar organisms and/or similar pollutants.

An application factor ranging from 0.030 to 0.099 was calculated for zinc conducting bioassay on the Guppy *Poecilia reticulata* (Pierson, 1981). The Environmental Protection Agency (EPA) guidelines indicate that lead concentration in both marine and freshwater systems should not exceed 1% of the 96 hr LC50 (96 hr TLm) for sensitive species (Laws, 1981). Mohapatra (1994) conducted chronic stress studies (based on bioaccumulation of heavy metals) on *Liza parsia* exposing it to copper, zinc and lead and found that 0.01 cannot be used as application factor for these metals. The higher dilutions are needed in the case of bioaccumulative substances. The application factor can be obtained from chronic stress studies, where no significant differences exist between the control tests and the experiments with lower concentrations of test material (Mohapatra, 1994).

8.3. Non-lethal concentration

Mohapatra and Noble (1991) have tried graphically to obtain the non-lethal concentration of "Nuvan" for application of it in fish farming as a medicine against ectoparasites such as *Lernea* and *Argulus*. They used the LC16, LC50 and LC84 values obtained from response curves for 24, 48, 72 and 96 hour for plotting on coordinate paper. For a definite exposure duration the corresponding LC16, LC50 and LC84 were joined for obtaining curves and their approach on X-axis was studied carefully and 0.2 ppm for "Nuvan" was found

non-lethal to *Liza parsia*. For short applications this concentration can be used. The LC16, LC50 and LC84 values obtained from Example 1 are plotted for non-lethal concentration of Copper sulphate, Zinc sulphate and Lead nitrate and shown in Fig. 7. All the curves are seen approaching 72 ppm on X-axis which indicates 0% mortality at that concentration of toxicants.

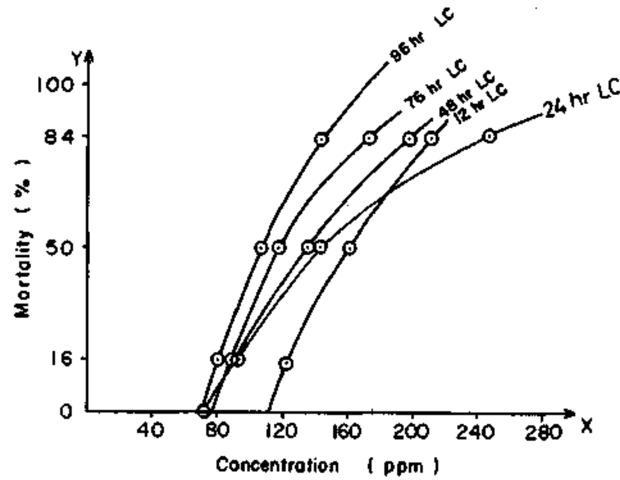


Fig. 7. Finding of non-lethal concentration of Copper sulphate : Zinc sulphate : Lead nitrate :: 1 : 1 : 1 to *Liza parsia*.

8.4. *Safe Application Rate (SAR)*

It is stated in the study of Basak and Konar (1977) that the SAR of an insecticide is determined by multiplying SAFE (Safe Application Factor Equation) by LC50 (48 or 96 hr).

9

CONCLUSION

The ideal toxicity bioassay should attempt to duplicate natural conditions, but the results obtained in the laboratory experiments have certain limitations. Data obtained by the use of the standard bioassay procedure in which the fish is immersed in a jar are questionable, when applied to natural systems. In a stream into which an effluent is being discharged continuously, for example the organisms are being subjected to a constant renewed toxicant. To duplicate this situation, it is desirable to conduct a bioassay in a constant flow-through system. A constant flow system is not practicable in most laboratories, because of the need of a relatively large extent of space, the complicated experimental apparatus, the large amount of wastes and dilution waters. In such cases the static bioassay is of quite useful. It may sometimes be called upon to work with a volatile waste or one in which the toxic substance is destroyed or removed from the solution either by volatilization, precipitation or detoxification by the test animal. This problem is sometimes solved by the periodic transfer of the test animal to freshly prepared solutions of the toxicant at regular intervals dictated by experience and many workers make this change at 12 or 24 hr intervals.

For a comparison of two effluents or two process streams entering a waste water treatment facility, a static test may be sufficient. If the interest lies in more precisely determining the toxicity of a volatile compound or the toxicity of a waste water to riverine organisms, then a continuous flow test would be more appropriate.

Ani and Mohapatra (1993) have demonstrated the use of Probit analysis in the starvation experiments on the prawn *Penaeus indicus*. For getting the response curve, they took the percentage mortality on the Y-axis and the log duration (days) on the X-axis. In this way they found the 50% response (death) value at 10 days 14 hours and 10 minutes.

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GLOSSARY

(Brief definition / explanation / expansion of some important terms used in this Manual)

Abscissa : The intercept between a fixed point and foot of an ordinate.

Acclimatize : Become or make accustomed to a new climate or a new environment, new condition, etc.

Accumulation : To go on increasing; the action or process of accumulating.

Acute toxicity : A relatively short-term lethal or other effect, usually defined as occurring within 4 days for fish and macroinvertebrates and shorter time for smaller organisms (APHA-AWAA-WPCF, 1985).

Alkalinity : Alkalinity of a water is its acid-neutralizing capacity. It is the sum of all the titratable bases.

Analytical : Using analysis.

Antagonism : The ability of one toxic substance to reduce or eliminate the effect of another, the inhibitory action of one species on the other (Holmes, 1979).

Antibiotics : Substances that can destroy or prevent the growth of bacteria.

Application Factor (AF) : A factor applied to acute toxicity tests to estimate toxicant concentration that is safe for chronic or life-time exposure of test organism (NAS/NAE, 1972).

$$AF = \frac{MATC}{96\ hr\ LC50}$$

Where MATC is "Maximum Allowable Toxicant Concentration".

Aquarium : Artificial pond or glass tank where live fish and other water creatures and plants are kept.

Asphyxia : Lack of oxygen or an excess of carbon dioxide in the system resulting in suspended animation or loss of consciousness, caused by interrupted respiration, particularly from suffocation or drowning, or the inhalation of irrespirable gasses (New Webster's Dictionary, 1989).

Asymptotic LC50 : Toxicant concentration at which LC50 approaches a constant for a prolonged experimental time.

Bioassay : Bioassay signifies a test in which a living tissue, organism or group of organisms is used as a reagent for the determination of the potency of any physiologically active substance of unknown activity (FAO, 1977).

Best-fit : For any bivariate distribution there are atleast two lines of "best-fit" depending upon the method by which the "best-fit" is defined. But because of the convention of assigning the independent variable (or that for which values are given) to the X-axis and the dependent variable (or that for which values are to be interpolated) to the Y-axis, the regression line of Y on X *i.e.* Y = a + bx is more commonly used. The equation of this line is given by

$$(Y' - \bar{Y}) = r. \frac{Sy}{Sx} (x - \bar{x})$$

where $(Y' - \bar{Y})$ are the residuals which are perpendicular to the X-axis, 'r' is the product moment correlation, $\bar{x}$ and $\bar{y}$ are the mean and Sx and Sy are the standard deviations of the given values of X and Y respectively. Thus, the unknown value Y^1 differs from the average of its set of data $\bar{Y}$ by the same amount as the unknown value $\bar{x}$ differsfrom its average $\bar{x}$ (Pal, 1982).

Biodegradable : Substances that can be broken down by micro-organisms (Holmes, 1979).

Biomonitors : Biological agents used for continuous observation or record or test of a system or environment.

Cannibalism : Practice of eating one's own kind.

Chi-square (x^2) : Let 'O' be the total number of items or frequencies in a class and 'E' the corresponding expected frequencies. Then the chi-square statistic is given by

$$X^2 = \Sigma \frac{(O - E)^2}{E}$$

where the symbol χ is the greek letter chi' (pronounced 'kie'). Large values of chi-square occur when actual and expected results differ considerably, making the chi-square large. Therefore, more the sample results deviate from what would be expected. If any value of chi-square comes out to be greater than the values given in the chi-square table (*i.e.*, calculated $\chi^2 \geq \chi^2_{\alpha}$) we will be in position to reject the hypothesis (Pal, 1982).

Chemotherapy : Treatment of disease by drugs and other chemical substances.

Chronic toxicity : Long-term effects that may be related to changes in appetite, growth, metabolism, reproduction, and even death and mutations (Kopperdahl, 1976).

Conductivity : Conductivity is a numerical expression of the ability of an aqueous solution to carry an electric current.

Confidence limits : It gives a measure of accuracy with which a population mean is known in relation to the sample mean (Pal, 1982).

100 (1-α)% Confidence Interval : A 100 (1-α)% confidence interval for a parameter θ is an interval of the form $[L_1, L_2]$, in which L_1 and L_2 are statistics such that $P [L_1 \leq \Theta \leq L_2] 1-\alpha$ regardless of the actual value of θ (Milton *et al.*, 1986).

Coordinate : Either of two numbers of letters used to fix the position of a point on a graph or map : X and Y coordinates on a graph.

Correlation coefficient (r) : The existence of association between pairs of characters where the probability of a individual having a given value of one variate depends on the value it bears of the other variate where the frequency arrays suffer by more than such differences as could be caused by random sampling variation.

The correlation coefficient measures the degree of association between the sets of paired variates.

Cumulative : Gradually increasing in amount, force, etc. by one addition after another.

Detoxification : Action of removing poison or harmful substances *e.g.* additive drugs.

Dilution water : The water used in the control test and experimental tanks, and also for making dilution of the test solutions.

Ecosystem : Ecological unit consisting of a group of plants and living creatures interacting with each other and with their surroundings (Cowie, 1989).

Effective Concentration (EC) : Toxicant concentration affecting a specific response *e.g.* respiration rate, loss of equilibrium in a given time for *e.g.* 96 hr EC50 (Committee on Methods for Toxicity Tests with Aquatic Organisms, 1975).

Effluent : Discharge of liquid waste matter, sewage, etc. *e.g.* from factory into a river or water body.

Epithelium : Any tissue which covers an external or internal surface, or lines a cavity or the like, and which performs protective, secreting, or other functions, as the epidermis or the lining of blood vessels (New Webster's Dictionary, 1989).

Erratic : Irregular or uneven in movement, quality or behaviour.

Euryhaline marine organisms : This group is formed by those forms which extend in their distribution from the sea to the upper reaches of the estuary and can tolerate salinity as low as 15‰. A few however can tolerate salinities of even 5‰ (Jhingran, 1991).

Exposure time : Time of experiment of test organism to test solution.

Fiducial : A line or point established accurately as a basis of reference.

Food chain : The trophic levels (producers, consumers and decomposers) are linked together in a sequence of bonds known as the food chain.

Fungicide : They are active against fungi, parasitic plants comprising molds, mildews, mushrooms and rusts that destroy higher plants, fabrics, glass, finished products like adhesives, leather, paints, etc. A fungus destroying substance.

Haemoglobin : Substance carrying oxygen in the red blood cells of vertebrates.

Haemorrhage : Heavy bleeding.

Half-life : Time taken for the radioactivity of a substance to fall to half its original value.

Heavy metal : The metals having specific gravity of 5.0 or above.

Hyper ventilation : Ventilation to an excessive degree : above; over.

Incipient : In its early stages; beginning to happen.

Insecticide : Substances used for killing insects.

intercept : It gives the place at which the regression line intersects the Y-axis. In the regression context, it gives the predicted value of Y when x = 0 (Milton *et al.*, 1986).

LC50 : Concentration killing 50% of exposed organisms at a specific time of observation *e.g.* 96 hr LC50 (Committee on Methods for Toxicity Tests with Aquatic Organisms, 1975).

Lethal Concentration (LC) : Toxicant concentration producing death of test organisms.

Liza : The genus *Liza* belongs to family Mugilidae. The important identifying characters include : scales without a membraneous digitated hind margin; pectoral axillary scale rudimentary or absent (Anonymous, 1974).

Liza parsia : It is the estuarine mullet. The important diagnostic characters include : first dorsal fin origin nearer to snout tip than to caudal fin base; 11 transverse scales (Anonymous, 1974).

Linear correlation : If the amount of change in one variable tends to bear constant ratio to the amount of change in the other variable, then the relation is linear.

MATC (Maximum Allowable Toxicant Concentration) : Toxicant concentration that may be present in a receiving water without causing significant harm to productivity or other uses.

Mean : It is defined as the sum of the values in a series divided by the number of the series.

$$\text{Mean} = \frac{X_1 + X_2 + X_3 + \text{-----------} + X_N}{N}$$

or

$$\text{Mean} = \frac{\Sigma X}{N}$$

Where Σ = sum of the values of the items and N = number of items.

Molecular weight : It is the relative average weight of a molecule of the substance as compared to the weight of the carbon atom taken as 12 on the atomic mass unit scale (Nanda *et al.*, 1983).

Moulting : Casting of the exoskeleton or chitinous covering on the body of crustaceans, or the loose of the outer covering before a new growth.

Mucus : Sticky slimy substance produced by the mucous membrane; any similar slimy substance.

Nomograph paper : It is also known as log-log paper. Paper ruled with two sets of mutually perpendicular, parallel lines spaced according to the logarithms of consecutive numbers rather than the numbers themselves.

Normal Distribution : A random variable X is said to be distributed normally with mean μ and variance ...if its probability function is given by

$$f(x) = \frac{1}{\sigma\sqrt{2\pi}} \exp\left[-\frac{1}{2}\left(\frac{x-\mu}{\sigma}\right)^2\right]$$

$-\infty < x < \infty$

$-\infty < \mu < \infty$

$\sigma > 0$

$\pi \approx 3.1416$

exp ≈ 2. 7183 (Milton *et al.*, 1986).

Ordinate : A straight line parallel to an axis cutting off an abscissa.

Pesticide : Chemical substance used to kill pests especially insects.

Petroleum : Mineral oil that found underground and is obtained from well sunk into the ground, from which petrol, paraffin, diesel oil, etc. are obtained by processing.

pH : The negative of the logarithm of hydrogen ion concentration in aqueous solution; low pH is acid, high pH is alkaline, pH 7 is neutral (New Webster's Dictionary, 1989).

Photoperiod : The period of light exposure in a 24-hour cycle that controls growth and development of certain plants and animals (New Webster's Dictionary, 1989).

Plankton : It is defined as free floating animal and plant organisms, whose intrinsic power of locomotion, if present is so feeble that they remain almost at the mercy of currents and waves.

ppm : Parts per million *i.e.* 10^{-6}.

Precipitation : Separation of a solid substance from the liquid in which it held.

Probability graph paper : The graphical representation of the frequency distribution or frequency table is known as the "frequency curve".

By joining the cumulative class frequencies plotted at the upper or lower class boundaries, one gets the cumulative frequency curve better known as the "ogive curve". Ogive curves resemble the letter "S".

As mentioned above, the cumulative frequency curve is nearly S-shaped, with the relative size of two tails of the S determined by the symmetry or skewedness of the distribution. If a distribution closely follows the pattern of a normal curve, it is possible to straighten out the S by using a special vertical scale known as a probability scale. This scale is so designed that any normal cumulative distribution will graph as a straight line. This graph paper with one arithmatic (ordinary) and one probability scale is known as the arithmatic probability graph paper (Pal, 1982). In the test it is also used as the probit paper.

Pulp : Soft mass of wood fibre used for making paper; wood pulp.

Quantal response : That is by having only two experimental alternatives - dead or alive, all or none (Ward and Parrish, 1982).

Regression of Y on x : Let x be a mathematical variable and let Y be a random variable. The curve of regression of Y on x is the graph of the mean value of Y for various values of x. That is, it is the graph of μ y/x (Milton *et al.*, 1986).

Regression (Linear) : A curve of regression of Y on x is said to be linear if and only if

$$\mu\ y/x = \alpha + \beta x$$

for α and β real numbers (Milton *et al.*, 1986).

Response : The measured biological effect of the material tested. In acute toxicity tests this usually is death.

Safe Concentration (SC) : It is the maximum concentration of a toxicant that has no observable harmful effects after long-term exposure over one or more generations (APHA-AWWA-WPCF, 1976).

Sewage : Waste matter from human bodies, factories, towns, etc. that flow away in sewers.

Sewer : Underground pipe or passage that carries sewage away to be treated or purified.

Significant levels : Significant levels nearer 100% gives less scope for the result to occur by chance. At 99% probability a result can be considered to be highly significant, but below 95% the result is only suggestive. Thus the probability of our being in error is called the significance level of the test and it is usually taken at 1% and 5%. So the level of significance can be defined as the probability of rejecting a hypothesis.

Slope : In a linear regression equation ($\mu\, y/x = \alpha + \beta\, x$) the Beta, the coefficient of x is called the slope of the line. It tells us the direction and steepness of the line. Positive values of β indicate that the line rises from left to right; negative values indicate a line that falls from left to right (Milton *et al.*, 1986).

Solvent : Substance (especially liquid) that can dissolve another substance.

Standard Deviation (SD) : The root of the average of the squares of the differences from their mean, $\bar{x}$, of a number, n, of observations, x.

$$SD = \frac{1}{n} \Sigma \ (x - x\ )^2$$

Static test : Test in which solutions and test organisms are placed in test chambers and kept there for the duration of the test (Committee on Methods for Toxicity Tests with Aquatic Organisms, 1975).

Stress : A constraining, urging or impelling physical force; strain.

Swirl : It cause water, air, etc. to move or flow with twists and turns and with varying speed.

Synergism : The phenomenon where two substances such as hormones, interact to produce an effect greater than the sum of their individual effects (Holmes, 1979).

Threshold : The minimum input that produces a corrective action in an automatic control system.

TLm (Median Tolerance Limit) : The test material concentration at which 50% of test organisms survive for a specified experiment time, synonym to LC50 (NAS/NAE, 1972).

Total hardness : The concentration of calcium (Ca) plus magnesium (Mg) expressed as equivalent calcium carbonate, is the total hardness.

Toxicity : Adverse effect to a test organism caused by pollutants, generally a poison or mixture of poisons (APHA-AWWA-WPCF, 1985).

Volatile : Having the quality off quickly by evaporation; able to vaporize freely in the air (New Webster's Dictionary, 1989).

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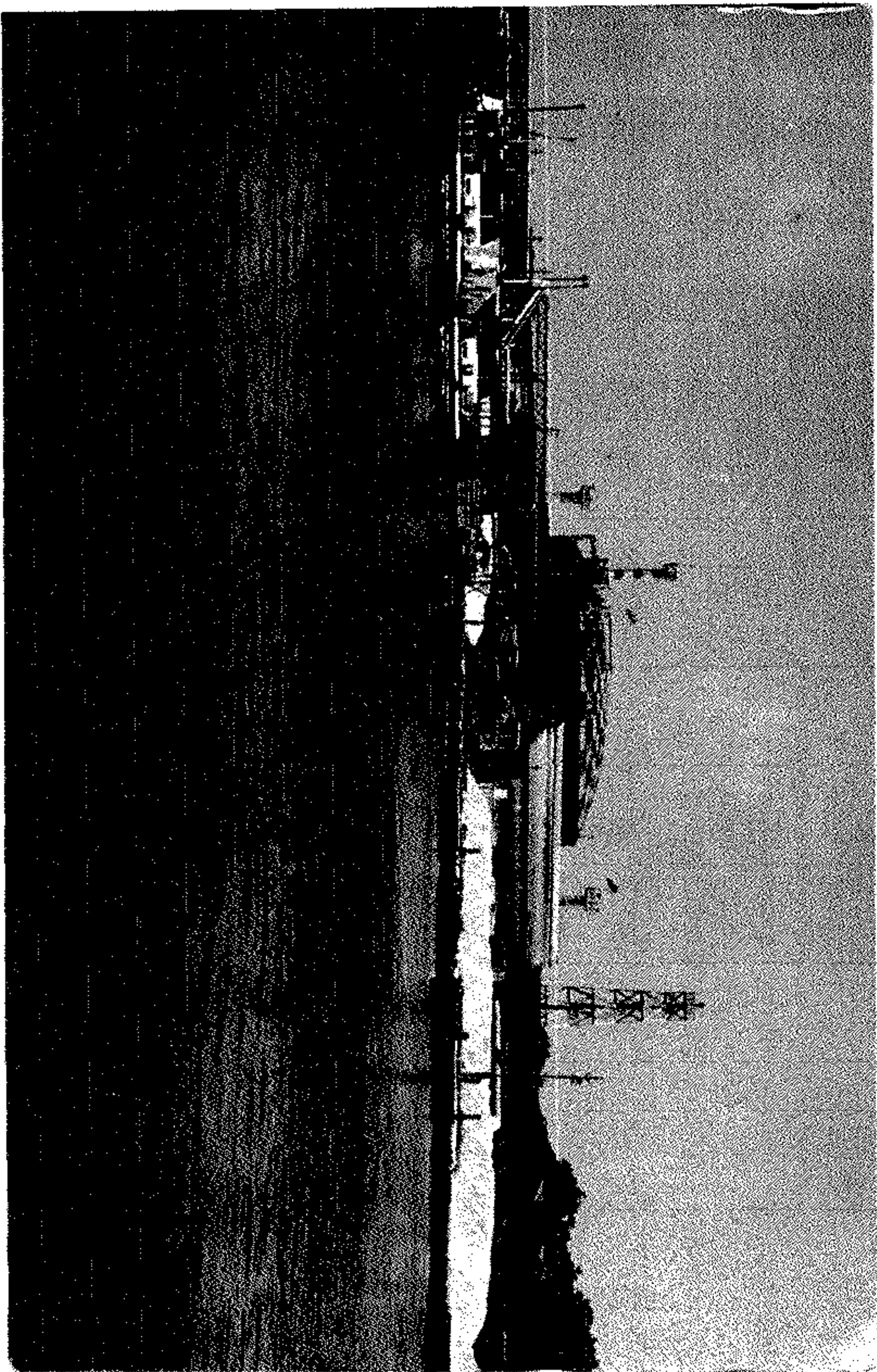

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