TOXICITY TESTS

T*oxicity tests are experiments designed to evaluate the doses /concentrations of toxicants and the duration of exposure required to produce a criterion effect.* The criterion of effect may be death of the exposed organisms or any other parameter, such as changes in cellular or subcelluar structures, behaviours, physiology, haematological, immunological or biochemical parameters.

In other words, these tests may be defined as *tests, which are performed to measure the degree of response of an organism, or a group of organisms produced by a specific level of stimulus (i.e. level and duration of exposure to test chemical).* These tests provide a database that may be used to assess the risk associated with a situation in which the test chemical, the organism and the exposure conditions are well defined.

The effects produced by a chemical in laboratory animals can be used to predict the possible effects on human beings. Because on the basis of dose per unit body surface, toxic effects in human-beings are usually in the same range as those in experimental animals. On body weight basis, humans are generally more vulnerable than experimental animals, probably by a factor of ten. Keeping in view these quantitative differences, the toxicity tests may be used to evaluate relatively safe doses for human beings.

The aquatic toxicity tests are erroneously and frequently referred to as ***bioassays***. Bioassay may be defined as a *test, which is designed to evaluate the relative potency of a chemical by comparing its effect on a living organism with that of a standard preparation.* Sprague (1973) defines bioassay as a *test in which the quantity or strength of material is determined by the reaction of a living organism to it.* Cairns (1980) defines bioassay as *merely a dose-response evaluation.* The living organisms are used to determine their responses to a series of different chemical concentrations.

A test procedure must be selected keeping in view the following points :

(i) The test should have sound scientific basis;
(ii) It should be widely accepted by the scientific community;
(iii) It should be economical and easy to conduct;
(iv) It should be realistic and as sensitive as possible to measure the effect;
(v) It should be repeatable in various laboratories with similar results;
(vi) It should be able to predict the effects of a wide range of chemicals on different organisms.
(vii) The data should be useful for risk assessment.

Types of toxicity test The toxicity tests may be performed at various levels in accordance with the need.

(A) ***Test types based on number of species*** On the basis of test organisms and conditions, the test may be categorized as below :

(1) Single species test,
(2) Multi species test, and
(3) Ecosystem test.

(1) Single species test Single species test is mostly performed in the laboratory. These tests are conducted with individual species that are considered representative of broad classes of organisms (For example, invertebrates, fish, mammals, *etc*.). The results of these tests provide information on the toxicity of specific chemicals in different types of organisms under defined conditions.

Single species tests are easy to conduct and may be standardized and replicated. These tests under controlled laboratory conditions are helpful in establishing *cause-effect relationships*. But, these tests cannot be used to know the adaptive ability of natural populations.

(2) Multi species test These tests are conducted with several species of organisms and can also be performed in laboratory. They often involve laboratory *microcosms* consisting of small scale enclosures (of glass or plastic) containing samples (of different species) from the natural ecosystem.

In multispecies tests, effects on several species of organisms can be identified which may provide information more directly related to ecological consequences of the chemical. Multispecies laboratory tests are useful in understanding the cause-effect relationship than in natural ecosystems.

(3) *Ecosystem test* Ecosystem tests can also be conducted in laboratory. These studies involve *model ecosystem* consisting of some common producers and consumers of various grades. The laboratory ecosystem tests are more close to the natural conditions. Hence, the data obtained from ecosystem tests can be easily relied upon.

(B) Test types based on exposure of toxicants On the basis of exposure of toxicants, the toxicity tests may be categorized into two groups:

(a) Single dose test, and
(b) Multiple dose test.

(a) Single dose test In single dose test, the toxicant is applied only once and the desired effect is recorded after desired duration of exposure.

(b) Multiple dose test The information obtained from single dose test is used for the identification of multiple dose. The upper dose for multiple exposure should not be more than the dose expected to cause 5-10% mortality. The multiple dose test is usually performed in two species, one being non-rodent with exposure by the route of intended use. A minimum three dose

levels and more commonly five dose levels are employed using 15 animal per sex per dose level. Multiple dose study not only characterizes the dose -response relationship of a test substance, but also provides data for more reasonably predicting the maximum tolerance levels for the species during potential life-time exposures.

(C) ***Types of test based on duration of exposure*** Based on duration of exposures, the toxicity tests can be divided into four groups, of which the important ones are:

(1) Acute toxicity test, and

(2) Chronic toxicity test.

1. ACUTE TOXICITY TEST

Acute toxicity may be defined as *severe effects experienced by the organisms during short-term exposure to toxicants.*

Acute toxicity tests are principally designed to determine the dose/concentration of a test material producing deleterious effects on a group of test organisms during short-term exposure under controlled laboratory conditions.

The easily detectable deleterious response is death of the exposed organisms and lack of movement of gill in fish or lack of response to gentle prodding are generally used criteria for the death of organisms. Therefore, most common acute toxicity tests are *acute lethality tests.* Usually 50% response is the most accepted and reproducible measure of toxicity and 96 hour is the standard exposure time, because it covers the period of acute lethal action.

The results of acute toxicity tests are generally represented in terms of LD_{50} (median lethal dose) in case where the actual amount of poison is known while in case where actual amount of poison responsible for causing a particular *effect* (*e.g.* death or immobility) is not known and only the concentration of poison is known (such as in cases of aquatic toxicity and inhalation toxicity), the term LC_{50} (median lethal concentration) is used. But, in case of some invertebrates (for example, daphnids, midge larvae, *etc*.) death is not easily determined and the criterion of effect is immobilization, which is defined as lack of movement. In this case, the results of toxicity test is determined in terms of EC_{50} (*i. e*. median effective concentration). Acute toxicity tests provide rapid estimates of dose/concentration of test chemicals that cause direct irreversible harm to the organisms. It thus provides a practical means for:

(i) estimating the upper limit of xenobiotics producing toxic effects;

(ii) evaluating relative toxicities of various toxicants;

(iii) determining relative sensitivity of different organisms to toxicants;

(iv) evaluating the effects of environmental variables (*e.g.* water quality and other parameters) on the toxicity of chemicals;

(v) developing an understanding of ***dose-response relationship*** or ***concentration-response relationship***;

(vi) understanding the significance of duration of exposures;

(vii) assessing doses /concentrations for chronic exposures;

(viii)determining most sensitive species of organisms and indicator species.

Acute toxicity test may be performed in various exposure conditions. The toxicants may be exposed :

(i) in aquatic medium (aquatic toxicity)

(ii) in gaseous state (inhalation toxicity)

(iii) mixed in food or culture medium for terrestrial organisms and microbes, respectively,

(iv) directly applied on the body of organisms (topical application or dermal toxicity),

(v) injected in the body of organisms (injection method).

(a) Test organisms Selection of appropriate test organisms is one of the essential steps for the toxicity test. This is not only necessary for the accuracy of results, but also for the extrapolation of meaningful, and ecologically significant results. The organisms selected for toxicity test should have certain basic characteristics, *viz.* (i) The species should be abundant and widely available. (ii) It should be amenable to routine maintenance in the laboratory and techniques for laboratory rearing should be available, (iii) The species should represent broad range of sensitivity, (iv) It should be recreationally, commercially or ecologically important, and (v) There should be adequate background information about the species for the proper interpretation of data.

The test organisms may either be collected from wild populations or taken from cultured laboratory animals. The animals can also be procured from commercial suppliers, but all organisms in a test should be taken from the same source.

(b) Test chemical Any chemical may be selected for the test as per need. It may be either a pure chemical or commercial formulation or mixture of chemicals or a factory effluent. The stock solution may be prepared a day before the commencement of test and all test solutions should be prepared from the same stock. The undissolved chemical should be uniformly dispersed either by shaking or mixing the stock solution. If possible, the test chemical may be directly applied or mixed in the dilution water without use of any solvent or carrier. However, if a solvent/carrier is necessary, the organic chemicals, such as triethylene glycol (TEG), dimethylformamide (DMF), are preferred because they possess: (i) low toxicity, (ii) low volatility, and (iii) ability to dissolve many organic chemicals. In case other solvents (such as, acetone, methanol and ethanol) are used, their amount should not be more than 0.5ml/l of test solution.

For the sake of convenience, the acute toxicity test may be divided into two groups:

(A) Aquatic toxicity test, and

(B)Test on terrestrial organisms.

(A) Acute aquatic toxicity test

Test for the toxicity of chemicals on aquatic organisms in the aquatic medium for short-term exposure may be termed as acute aquatic toxicity test.

(a) Exposure systems Exposure of certain toxicants (such as, volatile chemicals, highly bioaccumulative chemicals and chemicals with low water solubility) to aquatic organisms pose several practical problems. Therefore, a number of exposure systems are normally used in toxicity testing: (i) Static system, (ii) Renewal system, and (iii) Flow through system.

(i) Static system Static exposure systems are much simpler in design and operation than flow through ones. They generally consist of exposure vessels in which the test organisms are subjected to the same test solution for the duration of test. The solution is not changed or renewed. These systems are simple and cheaper, but the main problems of static system are:

(a) decrease in the concentration of test chemical through loss from volatilization, microbial degradation, sorption or organism uptake, and

(b) low dissolved oxygen concentration for test materials having high biochemical oxygen demand (BOD) or as a result of accumulation and microbial degradation of faecal matter.

Owing to these limitations, static systems are used for short-term test with non-volatile or slowly degrading chemical having a low loading of test organisms.

(ii) R*enewal system* Renewal system may be placed in between static and flow through exposure systems. The exposure vessel is essentially the same as in the static system. But, instead of exposing the text organisms to the same solution throughout the test, solutions are

periodically renewed by their respective concentrations. These systems have three major disadvantages: (a) increased handling of test organisms; (b) increased stress and possibility of injury, and (c) fluctuation of concentration between changes of solutions.

This system is mostly used for smaller organisms, such as *Daphnia* and for organisms sensitive to water currents, *e.g.* copepods.

(iii) Flow through system In flow through exposure systems, the test organisms are subjected to relatively fresh solutions of toxicants, which flow into and out of the exposure vessels. The flow of test solution may either be ***continuous*** or ***intermittent***. This system maintains a constant concentration of test material, a constant water temperature and dissolved oxygen concentration near saturation level. This system is preferred for acute toxicity studies. These systems are especially recommended for highly volatile chemicals, as concentration of test material and water temperature can be maintained at fairly constant levels and dissolved oxygen may be kept near saturation for prolonged period.

(b) Diluent water Aquatic toxicity tests are conducted by dissolving the chemicals in water (aquatic medium). The physico-chemical characteristics of water (such as, water temperature, dissolved oxygen concentration, pH, *etc*.) are known to greatly influence the toxicity of various chemicals. Therefore, it is desired to estimate the physico-chemical characteristics of water used as diluent or for the preparation of test solutions by some standard methods as given by **APHA** *et al.* (1998) or other agencies. It is also advisable to report the test results along with some important characteristics of diluent water.

(c) Test procedures Doudoroff *et. al.* (1951) for the first time recognized the need to develop uniform and standardized test procedures to maximize the comparability of toxicity data. Thereafter, a variety of test methods have been developed by American Public Health Association, U.S. Environmental Protection Agency (USEPA), American Society for Testing and Materials (ASTM), World Health Organization (WHO) and Organization for Economic Cooperation and Development (OECD) to evaluate the hazard and potential toxicity of chemicals.

Davis (1977) has summarized the advantages of using standardized test procedures as follows:

(i) It provides uniform and useful tests for selection by a variety of laboratories;

(ii) It increases the accuracy of the data;

(iii) It allows replication of the test,

(iv) It permits initiation and performance of the test by different persons,

(v) It is useful for routine monitoring,

(vi) It facilitates comparison of the data and thus increases the usefulness of the published data.

Thus, a standardized test method with reference to test species and toxicant maximizes the comparability, replicability and reliability of the data. Therefore, such laboratory protocols are useful for studying the relative toxicity of various chemicals and sensitivity of different species.

The tests is conducted in two steps; thus consists of two types of tests: (i) ***Exploratory test*** or ***Range finding test***, and (ii) ***Definitive test***.

(i) Exploratory test In order to evaluate the toxicity of a new chemical or unknown chemical, first of all exploratory tests are performed to find out the toxic range of that particular chemical. The exploratory test requires less effort than definitive test, but the results of exploratory test go a long way toward ensuring the success of definitive test.

Generally, groups of 3-5 animals are exposed to 3-4 concentrations of test chemicals along with control groups and these test concentrations are selected in logarithmic ratio (*e.g.* 0.01, 0.1,1.0,10, 100 mg/l or ppm) In case of effluents, per cent concentrations are selected in logarithmic ratio. For meaningful results, test durations and other conditions are maintained similar or more close to those of definitive test.

(ii) Definitive test On the basis of data obtained from exploratory tests, final definitive tests are designed to evaluate LC_{50} or EC_{50} of desired chemical. Appropriate number of test animals (at least 10) should be exposed to at least five test concentrations within the toxic range. The sequence of concentration of test chemical/ effluent should be such that the ratio of a concentration to its predecessor is always the same (*e.g.* 10, 5, 2.5,1.25 and 0.62 ppm). The range of selected concentration should be such that there is at least one concentration causing less than 35% mortality and at least one causing more than 65% mortality of organisms. The use of more than five test concentrations permits calculation of narrower confidence limits of LC_{50} or EC_{50}.

(d) Beginning of test The important steps for beginning a static test are:

(i)addition of appropriate amount/volume of test chemical to the dilution water in a test vessel,

(ii) shaking the mixture by stirring with a glass rod,

(iii)determination of physico-chemical characteristics of water, and

(iv)transfer of animals to test and control solutions usually after one hour of addition of test chemical.

The flow through tests are performed much in the same way as static tests. The test organisms are transferred to the test solutions and control, when they flow through the exposure system.

(e) FeedingThe feeding of test organisms are usually avoided during test. In case when the test duration covers the period of most of the life-span of organisms and if cannibalistic animals used as test animals cannot be separated, the feeding becomes necessary. In case of feeding during tests, the left over food should be immediately removed in order to avoid the reduction in dissolved oxygen content in the test solutions and the control.

(f) Duration Acute toxicity test is normally conducted upto 96 hours and it starts at the time when the test animals are first placed in test or control solution. But, in certain other cases (for instance, daphnids) it is conducted only upto 48 hours; because the aim is to test only upto a specific stage in the developmental process as also because of problems due to longer exposures.

(g) Biological data Usually death is the criterion of effect considered for acute toxicity test, in such cases the toxicity is conventionally represented in terms of LC_{50}. The mortality of organisms is recoded after every 24±1 hour from the beginning of the test until the termination and the dead animals are immediately removed from the test solution to avoid further depletion of dissolved oxygen on account of contamination.

In addition to mortality data, the behavioural changes in the test organisms following their exposure to test solutions are also recorded. During all tests, general observations of erratic swimming, loss of reflex, discolouration, changes in behaviour, excessive mucus production, hyperventilation, opaque eyes, curved spine, haemorrhaging, moulting and cannibalism should be observed and recorded.