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DRAFT ZANZIBAR NATIONAL STANDARD

Bio-assay methods for evaluating acute toxicity of industrial effluents and waste waters

ZANZIBAR BUREAU OF STANDARDS

First Edition

Foreword

This draft Zanzibar National Standard has been developed by Water Quality Standards Technical Committee (TCE1). In accordance with ZBS general procedures, this draft standard is presented to the public in order to receive any technical and editorial comment concerns.

Technical Committee Representatives

This Draft Zanzibar National Standard was prepared by Water Quality Standards Technical committee which consist of representatives from the following organizations:

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Introduction

The bio-assay procedures given in the standard are intended for use in laboratories for evaluating the toxicity of industrial effluents and waste waters to fish. The test can be used to determine whether or not an effluent or effluent component is acutely toxic and, if toxic, its degree of toxicity. It also serves as a basis for judging whether or not an effluent can be discharged at a given rate without causing direct injury to fish and other aquatic organisms in the receiving water due to its toxicity.

The bio-assay is very useful procedure in connection with the control of effluent disposal. Chemical examination of complex industrial effluents alone does not usually yield sufficient information because many of their various toxic components cannot be readily detected, separated and measured by chemical means. Moreover, the degree of toxicity of each of the numerous substances and mixtures of chemicals could be unknown. The toxicity of effluents can be greatly influenced by interaction between their individual components and the dissolved minerals present in widely varying amounts in the receiving waters. Therefore, the toxicity of an industrial effluent to local fish in their natural habitat has to be evaluated directly through biological test under appropriate experimental conditions.

In the preparation of this standard, the reference was made to the following sources:

IS:6582-1971 (Reaffirmed 2019), *Bio-assay methods for evaluating acute toxicity of industrial effluents and waste waters.*

Bio-assay methods for evaluating acute toxicity of industrial effluents and waste waters

1 Scope

This standard lays down the bio-assay methods for evaluating the acute toxicity of industrial effluents and waste waters to fish.

2 General principles and directions

2.1 Measure of acute toxicity

The measure of acute toxicity is the concentration of the test sample in suitable diluent (referred to as diluent water) at which just 50 percent of the test animals are able to survive for a specified period of exposure. This is known as the median tolerance limit (TL_m). The concentration of the sample in the diluent water is expressed as percent by volume. For example, a 10 percent concentration or a TL_m of 10 percent equals 1 part of the test sample in 9 parts of the diluent water. To evaluate toxicity, different concentrations of the test sample are tested so that the concentration lethal to 50 percent of the test animals (within the prescribed period) may be found or estimated by interpolation.

2.2 Provision of dissolved oxygen during test

Since death of the fish may be caused also by deficiency of dissolved oxygen (D.O) in waste water, it is necessary that adequate dissolved oxygen is maintained in the sample under test.

2.3 Uniformity of Experimental Procedure

Reasonable uniformity of experimental procedure and of the manner of presentation of results is essential; widespread adoption of uniform methods will promote the accumulation of comparable data. However, rigid standardization of experimental material and conditions is not desirable, for it would tend to defeat the purpose of practical test. Strict comparability of test results could be achieved only by sacrificing much of their relevance and applicability to practical problems in specific localities. Experimental water (water used as diluent) and test animals best suited for the purpose of each bio- assay should be selected. With respect to other features, the test can be more or less uniform.

2.4 Sampling

Samples of effluents or waste waters which are not constant in their composition shall be collected at different times and shall not be unnecessarily combined to make composite samples, because knowledge of the maximal toxicity of a variable effluent is often required in connection with the control of effluent disposal in flowing water rather than knowledge of the average toxicity. Extensive damage to the aquatic life of a receiving stream can result from brief, intermittent discharges of a highly toxic waste, even though the toxicity of the effluent at other times and its average toxicity are negligible. Therefore, if the composition and toxicity of an effluent vary considerably, it is necessary to test a number of individual (grab) samples taken at times when the effluent is likely to be highly toxic, hence maximal toxicity can be determined. A composite sample of an effluent consisting of portions collected at regular time intervals can be useful only when average toxicity is to be evaluated.

2.5 Storage of Samples

Samples of industrial effluents or waste waters should be stored in completely filled, stoppered bottles at a fairly uniform temperature which should not greatly exceeding the initial temperature. Samples containing photosensitive material should be kept in brown bottles or bottles covered with dark paper. If the effluent contains organic matter subject to bacterial decomposition, the samples shall be

refrigerated (without freezing) and held at a temperature between 0°C and 4°C. Duration of storage prior to testing should be kept to a minimum.

2.6 Interference

Extreme volatility, instability and rapid detoxification of effluent components and excessive oxygen demand can render the Routine Bio-assay method inapplicable, or can cause serious bias in the results. Extreme volatility, instability, or detoxification of important constituents is indicated when the recorded average survival time of the test animals in a fresh medium is much less than survival time in the corresponding older, used medium, if adequate D.O is present throughout both tests.

3 Selection test method

3.1 The basic routine bio-assay method given under clause 4 constitutes the simplest procedure. It is widely applicable, being suitable for the detection and evaluation of acute toxicity which is not associated with excessive oxygen demand and which is due to substances that are relatively stable and are not extremely volatile. The routine bio-assay method is designed so that the surface absorption of oxygen from the atmosphere plus some oxygen from the diluent generally provides an adequate amount of D.O. for the fish during the test period. Many industrial effluents have a high chemical or biochemical oxygen demand, which may cause oxygen depletion in test solutions. Although it is usually necessary to use artificial supplies of air or oxygen, D.O. shall nevertheless be maintained at levels adequate for the test fish.

3.2 Uncontorlled aeration with compressed air has been generally unsatisfactory in bio-assay of industrial effluents. Small additions of air will not ordinarily maintain the necessary quantities of D.O., whereas vigorous aeration may drive off volatile toxicants and may greatly speed up biological oxidation.

3.3 The following three methods of maintaining adequate D.O. are given under modifications of routine bio-assay method in clause 5:

a) controlled artificial oxygenation of test solutions;
b) initial oxygenation of diluent water and
c) renewal of test solutions

3.4 Bio-assay method using Daphnia

The microcrustacean *Daphnia magna* as an experimental animal in bio-assay may be used as an alternative procedure and has many advantages. These organisms are small in size (reaching a maximum size of 5 mm) and so a great number of them can be reared in a small space. They can be grown in small bottles or cultured in mass in large aquaria. They are very sensitive to even low concentration of toxic substances. They are easy to culture and require only water containing bacteria or other equivalent for food. The organisms mature early giving birth to young ones within the first week of their life. After the first brood they give rise to new brood every 2 or 3 days. An average of 20 to 30 young ones may be produced in each brood. Their life span is short (about two months).

3.5 When the experimental data are reported, any deviation from the basic routine procedure should be described in order to avoid misinterpretation of test results, and their significance.

4 Routine bio-assay method

4.1 Apparatus

4.1.1 Test containers

Six to twelve containers are usually required. These shall be chemically clean wide-mouth glass bottles or cylindrical glass jars 25 to 30 cm in diameter, capable of holding 15 to 20 litres of water. These dimensions would be suitable for tests with fish which are 5 to 7 cm long. Smaller or larger glass jars or rectangular glass aquarium tanks may be suitable for smaller or larger fish.

4.1.2 Acclimatizing tanks

Large rectangular glass aquarium tanks of 60 to 200 litres capacity, provided with arrangement for aeration with compressed or pumped air released near the bottom of the tank, shall be used. These shall be placed well in advance in the testing laboratory so that the temperature of the water may approximate the room temperature.

4.2 Test animals

4.2.1 Types of Fish

Fresh-water fishes commonly inhabiting unpolluted waters in the locality are preferable. They should be adaptable to laboratory conditions of temperature, feeding and handling. The following fresh-water species are recommended:

a) Cyprinodontidae(Top minnows); and
   i)Aplocheilus panchax
b) Cyprinidae(True minnows)
   i)Nuria darinca
   ii)Rasbora daniconius
   iii)Danio Malabaricus
   iv)Barbus ticto
   v)Barbus dorsalis
   vi)Amblypharyagodon melettinus
   vii)Cyprinus carpio
c) *Clarias gariepinus and Clarias batrachus (catfish)*
d) *Chaetodon miliaris (millets)*
e) *Chanos chanos (Milk fish)*
f) *Caridea (Shrimps)*
g) *Oreochromis niloticus (Tilapias)*

Fish belonging to the same species shall be used for evaluation of each individual toxicity. They shall be identified as to genus and species.

4.2.2 Source

The fishes may be obtained from any single common source (stream, lake, hatchery, etc) but preferably from the body of water receiving the tested pollutant. They should all be collected and brought to the laboratory for acclimatization instantly.

4.2.3 Size

Specimens of more or less uniform size, averaging 5 to 7.5 cm in length are preferable. The largest shall be not more than 15 times as long as the smallest.

4.2.4 Stocks

Stocks of fish for test shall be kept initially in any suitable enclosures or containers (small ponds, concrete or wooden tanks, glass aquaria etc) and in any water of suitable quality and temperature in which they will remain in good condition foranalysis.

4.2.5 Acclimatization and feeding

The test fish shall be acclimatized for at least 10 days to laboratory conditions similar to those under which the tests are to be performed, with regard especially to the temperature and chemical characteristics of the dilution water. They shall be fed fairly regularly during the acclimatization period. They shall not be fed for about 48 hours before they used in a test. They shall also not be fed during the test.

4.2.6 Fitness

The incidence of specimens dying or becoming seriously diseased in the acclimatizing aquarium during a period of 4 days immediately preceding a test shall be less than 10 percent. Otherwise the lot of test fish shall be deemed unfit for use. The specimens used for test shall not show any symptoms of disease or abnormalities of appearance or behaviour when transferred for the sample investigation.

4.3 Diluent Water

4.3.1 The water to be used as a diluent and acclimatizing medium shall be obtained from the same body of water receiving the sample under investigation. When the toxicity of an effluent alone is to be determined the diluent water shall be obtained at a point where there is no pollution from any source.

4.3.2 When the water receiving an effluent to be tested is subject to previous contamination with other effluents, the toxicity of the effluent under test in conjuction with the previous contamination shall be considered in judging rates of discharge which will not be acutely lethal. For this purpose, the diluent water shall be taken immediately upstream of the point of discharge of the effluent to be tested but outside the zone of its influence. Such an evaluation is possible only when the test animals can live in the diluent water.

4.3.3 If uncontaminated diluent water cannot be obtained from the body of water under consideration, water of similar quality, with respect to its dissolved mineral content, should be obtained from another source, or else prepared artificially from distilled or demineralised water to which appropriate amounts of minerals are added to simulate natural water conditions. The pH, temperature, alkalinity, and hardness of this artificially prepared water shall be adjusted to match as closely as practicable those of the natural water.

4.3.4 The diluent water shall be settled or filtered if excessive amount of suspended matter is percent. The dissolved oxygen content of the diluent water shall be not below 4 mg/L. The diluent water shall be well aerated with compressed air. It shall not contain residual chlorine.

4.4 Number of test Fish

At least 10 fish shall be used for each experimental concentration of the sample under test. They may be placed in one container with the test dilution, or preferably divided equally among two or more jars containing solutions of the same strength. It is desirable to maintain a ratio of about 1 g of weight of fish per litre of liquid.

4.5 Dissolved Oxygen Content of Test Samples

The D.O. content of test solutions, or dilutions, shall not fall below 4 mg/L throughout the period of the experiment.

4.6 Procedure

4.6.1 Preliminary Test

When testing effluents of completely unknown toxicity, much time and effort can be saved by conducting preliminary bio- assay to determine approximate range of concentration of the effluent which should be covered in the full scale test. Prepare wide ranges of concentrations of sample under test, for example, 100,10,1,0.1 percent with the diluent water. Place two or more test fishes in an appropriate volume of each concentration contained in wide-mouth glass bottles. Observe after 24 hours. Select for full scale test the dilution ranges between the lowest concentration at which all fish survive for 24 hours and the highest concentration at which all or most fish die in 24 hours. The preliminary assay will also show whether excessive oxygen depletion occurs during the test period.

4.6.2 Full scale Bio-assay

Test at least 4 to 6 concentrations arranged at equal logarithmic intervals within (and including) the limits of dilution ascertained from the preliminary test. A concurrent control test shall also be run under exactly similar conditions using the diluent water alone. The control shall be deemed satisfactory if not more than 10 percent of the fish placed in it die within the test period.

4.6.3 Preparation of test dilutions of the sample

Shake the sample to disperse suspended matter uniformly. Withdraw measured portions of the sample and add to measured quantity of the diluent water and mix by gentle stirring. Avoid violent agitation.

4.6.4 Transfer of test fish

Transfer from the acclimatizing aquarium not less than 10 fish into each dilution. This shall be done within 30 minutes after preparation of the dilution. In transferring test fish special care shall be taken to handle them without causing injury.

4.6.5 Duration of test and observations

4.6.5.1 The test fish shall not be fed during the course of the test. Observe the behaviour of the fish under test at regular intervals for at least 48 hours. The test period shall be extended to 96 hours if more than half of the test fish survive for 48 hours at the lowest concentration tested. Note especially the symptoms exhibited by the test fish such as abnormal behaviour and loss of equilibrium. The test fish shall be deemed dead if they show no movement even when gently prodded with a glass rod. The dead fish shall be removed as soon as observed.

4.6.5.2 At the end of the test period transfer the surviving fish from each jar with as little agitation as possible to separate jars containing adequate amounts of the diluent water and observe for a further period of 24 hours. The number of fish which die in excess of those dying in the control within this period shall also be reported separately for the particular dilution.

4.6.5.3 When required, minimum quantities of test liquids may be removed for such determinations as dissolved oxygen. The volume of liquid in the test container shall then be made up with previously prepared dilution of similar strength*.*

4.7 Estimation of TL_m

Plot the percentages of surviving fish in each concentration of semi- logarithmic co-ordinate paper with the sample concentrations laid off on the logarithmic scale and survival percentages on the arithmetic scale. Fit a straight line to the points, particularly those between 20 and 80 percent survival. A line drawn from the point where this line crosses the 50 percent survival line to meet the concentration ordinate at right angles gives the TL_m concentration for the particular period of exposure involved.

4.8 Reporting of results

4.8.1 If all the test fish survive in the test sample (without any dilution) even after 96 hours the sample shall be reported as free from acute lethal toxicity.

4.8.2 While reporting the TL_m value,information should be given about the species of fish used and their origin, weight and length; the temperature at which the test was carried out; pH and D.O. content of the test solution; the duration of the test; and the source of the diluent water and its mineral composition.

5 Modifications of routine bio-assay method

5.1 Controlled artificial oxygenation of test solutions

If the sample under test has an excessive biochemical or other oxygen demand, it would be necessary to artificially aerate the test solution to maintain the minimum dissolved oxygen concentration. This shall be done by bubbling (30 to 180 bubbles per minute) pure oxygen from an oxygen cylinder provided with pressure reduction valve and arrangements for proper disbursement of the gas without violent agitation of the test animals. Supersaturation with dissolved oxygen shall be avoided.

5.2 Initial Oxygenation of Diluent water

Another modification is the addition of oxygen to the diluent water. An industrial effluent can be free from oxygen and it can also have a considerable immediate (chemical) oxygen demand which shall be satisfied. The D.O. content of each test dilution shall be adequate at the beginning of the test. In order to introduce the required amount of oxygen without recourse to artificial aeration or excessive dilution of the test effluent, additional oxygen may be dissolved in the diluent water before it is mixed with the effluent. The oxygen content or the immediate oxygen demand of the effluent sample shall be determined. The required oxygen content of the diluent to be added, or the minimum degree of dilution with well- oxygenated (supersaturated) water of known oxygen content that will ensure an adequate initial D.O. level, can then be estimated. Oxygenation of the diluent water shall be done by bubbling compressed oxygen gas through it in a tall container.

5.3 Renewal of test solution

A third modification involves renewal of the liquids tested for the purpose of maintaining more or less uniform concentrations of any volatile and unstable toxic components and adequate D.O. content. This modification is recommended especially when there are reasons for believing that the toxicity of the liquids declines rapidly during the course of a test. Periodic renewal of the liquid tested, at daily or other convenient intervals necessary to maintain the test conditions, presents fewer difficulties. This can be accomplished by transferring the test animals quickly, by means of dip net, to a test container with liquid. Renewal of the test medium at intervals of 24 hours is often both convenient and sufficient, but renewal at shorter intervals, such as 12 or 8 hours, is sometimes necessary or advisable. Constant- flow systems, in which the diluted wastes or test soltions are renewed continually (that is, replaced with fresh mixtures flowing constantly into the experimental containers), may also be used:

6 Method using daphnia

6.1 Apparatus

6.1.1 Culture and Test Containers

These shall be made of glass and be chemically clean. Wide-mouth bottles of capacity 150 mL and of medium height may be used.

6.2 Test animals

6.2.1 Type of Daphnia

The Daphnia shall be of species adaptable to the laboratory conditions of test and temperature, easily handled, available at all times of the year, present in normal streams and of value to fish either directly or indirectly. Daphnia magma meets all these requirements and hence is usually adopted as the test animal. In order to obtain reliable results the use of organisms 8 hours old or less is recommended.

6.2.2 Number of test animals

Fifty organisms shall be used for testing each concentration of the material under investigation.

6.2.3 Rearing of experimental animals.

The experimental animals shall be reared in the laboratory in the culture medium. The medium shall be prepared as follows:

Mix 5 g of air-dried horse manure with 20 g of dried sandy muck in 1 litre of water. Allow the infusion to stand for two days and strain through bolting silk cloth. Allow the filtrate to stand for 4 to 6 days and use the clear supernatant.

6.2.4 Culture of test animals

Use 150- ml wide- mouth bottles. Place one mature female in each of a series of bottles filled with the culture medium. After 4 or 5 days add to each bottle, every alternate day, 1 mg of a suspension prepared by mixing 1 mg of dried yeast with 1 mL of water. Transfer the young ones from the bottles every day to a stock tank to prevent depletion of food materials to the mother. Occassionally add some yeast to the medium in the stock tank. Use a translucent viewing screen for making observations. A dark field illumination helps . Rotating the bottles while observing will ensure visibility of all animals in it.

6.2.5 Preparation of animals for test

Remove all young Daphnias from the culture bottles with a pipette 8 hours prior to the beginning of the test. Wash the Daphnia three times in diluent water (see 7.3) by transferring to a bottle containing the diluent water and allowing them to remain in the water for 5 minutes.

6.3 Diluent water

Unpolluted water which is low in dissolved salts and hardness, with pH between 6.5 to 8.5 shall be used.

NOTE 1: Daphnia are very sensitive to chlorine and copper. These shall be absent in the diluent water.

6.4 Procedure

6.4.1 Preliminary test

Prepare dilutions of the sample under test covering a wide range of concentrations, for example, 100,10,1,0.1 and 0.01 percent with diluent water. Place 10 or more of the Daphnia in each concentration sand observe for a period of 24 hours. From this determine the approximate concentration range required as in 5,6,1 and carry out the full scale test.

6.4.2 Full scale bio-assay

Set up six 150- mL wide-mouth bottles. Prepare the various concentrations of the sample under investigation using the diluent water. Concentrations in a series of dilutions using the logarithmic values are preferable. Keep the sixth bottle with the diluent water only as control. Transfer 50 Daphnia prepared for test as in 6.2.5 in each bottle. Observe and record the number of Daphnia which are immobilised in each test container at the end of 12 hours and 24 hours after their introduction and also at the end of 48 hours if the tests are continued beyond 24 hours.

6.5 Estimation of TL_m

From the observations obtained, obtain the value of TL_m concentration as prescribed in 5.7.

6.6 Reporting of Results

6.6.1 If most of the test animals survive in the test sample (without any dilution) even after 48 hours, the sample shall be reported as free from acute toxicity.

6.6.2 While reporting the TL_m value, information shouldbe givenabout the test animals used, the temperature at which the test was carried out, the duration of the test, and the source of the diluent water and its mineral composition.

