DRAFT TANZANIA STANDARD

Industrial effluents – Bio-Assay method for evaluating acute toxicity

TANZANIA STANDARD

Industrial effluents – Bio-Assay method for evaluating acute toxicity

0 Foreword

0.1 The waste water and industrial effluents discharged into the Tanzania surface waters have adverse effects on aquatic life owing to the toxic nature of the effluents. However, the degree of toxicity of these effluents is still unknown.

0.2 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, the 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 acute toxicity.

0.3 The bio-assay is very useful in connection with the control or effluent disposal. Chemical examination of complex industrial effluents alone may not yield sufficient information. May 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 is not known. The toxicity of effluents can be greatly influenced by interaction between their present in widely varying amounts in the receiving waters. Therefore, the toxicity of an industrial effluent to local fish and other aquatic organisms in their natural habitat has to be evaluated directly through biological test under appropriate experimental conditions.

0.4 In the preparation of this standard, assistance has been derived from the following standard:

IS: 6582-2:2001 Bio-assay method for evaluating acute toxicity of industrial effluents and wastewaters-Part 2-using toxicity factor to zebra fish (brachydanio rerio)

1. SCOPE:

This draft Tanzanian standard prescribes an alternative method for determination of acute lethal toxicity of waste water and industrial effluents to zebra fish (*Brachydanio rerio*) under specified conditions.

2 NORMATIVE REFERENCES

There are no normative references.

3 TERMS AND DEFINITIONS

For the purpose of this standard, the following definitions shall apply.

3.1 dilution Water

The dilution water shall have a pH of 7.8 ± 0.2 and a calcium hardness of approximately 200 mg/1 expressed as CaCO3. It shall be prepared in accordance with 7.1.

3.2 Test Water

the mixture of waste water and dilution water, which is used for testing.

3.3 Dilution Factor (T)

the numerical expression of volumetric proportion of the waste water in test water. 'T' denotes the toxicity unit.

3.4 Toxicity Factor, TF

the lowest Dilution Factor T of the Test Water at which all fish survive. TF denotes the toxicity for fish. This is a dimensionless toxicity unit, which is used as standard parameter for compliance monitoring of waste water discharges. Higher the TF, the greater is the toxicity of the tested waste water.

3.5 Death of Fish

A fish is declared dead if it does not move when touched.

3.6 Range Finding Test

a preliminary test for samples with unknown toxicity to determine the range of dilution within which the TF value is expected.

3.7 Standard Test

is the confirmatory test performed to determine the TF value, under strict compliance with the procedure prescribed in this method?

4 GENERAL PRINCIPLE

4.1 Fish are affected by toxic substances chiefly in following two ways:

a) Epithelia absorb toxic substances, getting damaged in this process, for instance, the gills stick together or get congested with mucus and get destroyed.

b) Besides the above immediate effects, harmful substances are adsorbed through the gills, skin or intestine, thereby impairing physio logical functions. These effects may ultimately lead to the death of fish.

4.2 These toxic effects are determined in terms of the death of the test organism in standard dilution series of waste water samples with standard dilution water.

5 SAMPLING

5.1 Point of Sampling

In those cases, where the effluent at a specific point is to be tested, the question of choosing the point of sampling does not arise. However, where the composition of an effluent as finally discharged by the sewage treatment plant is to be ascertained, the point of sampling shall be the final outlet of the treatment plant.

5.2 Frequency of Sampling

When it is required to find out variations in the composition of the effluent during a specified period, such as that of peak discharge, the samples shall be taken at short and appropriate intervals, say, every 5, 10, 15 or 30 min and analyzed.

To study the average conditions over a cycle of operations or a period (usually 24 hours) or during the daily working period of the treatment plant, the collection of composite sample shall be adopted. The composite sample shall be made by collecting at appropriate intervals samples from the common channel or drain at a point where the flow of the effluent is likely to be most representative of the entire volume, and mixing.

The volume of the individual samples shall be a fixed proportion of the volume of the effluent flowing at that time. The interval should depend upon the frequency of variation in the nature of the effluent and the volume of flow. Care shall be taken to take the samples in such a way as to maintain the true proportion of suspended solids. Samples shall not be taken by skimming the top or scraping the bottom. A point about one-third of the way from the bottom shall normally be selected. The samples shall be drawn gently without unnecessary aeration. In most cases, collection of samples every hour would be sufficient.

5.3 Sampling Instrument

Porcelain-lined or enameled pails, in which the lining is unbroken, or glass vessels shall be used for taking samples. The vessels used for taking the sample shall be wide-mouthed and small enough for the contents to be transferred quickly to the sample container without leaving behind any deposit or scum. Automatic sampling devices, if available, may be used.

5.3.1 Each individual sample shall be deposited in a receptacle of sufficient size to hold the entire composite sample. Clean and dry carboys, other large glass containers or enameled buckets with lids without chipping may be used for pooling the sample.

5.4 Sample Containers

5.4.1 The quantity of sample required for analysis shall be taken from the composite sample after thorough mixing in order to keep the solids in suspension.

5.4.2 The sample for analysis shall be drawn in clean glass-stoppered bottles, which shall be rinsed with a portion of the sample. New bottles shall be washed with acid and thoroughly rinsed with distilled water before being brought into use. About 2 to 3 litres of the sample will be required for analysis. The bottle containing the final sample shall be filled so that a small air bubble is present after closure to prevent leakage or even breakage arising from any subsequent changes in temperature. The stopper shall be firmly inserted and, if the sample is to be transported some distance, tied down to keep in position.

5.4.3 The label on the bottle shall bear the name of the sampling authority, details of the type of sample, place, date and time of sampling.

5.5 Preservation of Samples

5.5.1 The samples shall be kept at a low temperature (about 4°C) during collection and thereafter.

5.5.2 No single method of preservation is applicable for the sample for all the tests. The analysis shall be carried out, preferably, immediately after collection. Storage at 3° to 4°C in a well-insulated ice box or refrigerator is the best way to preserve most samples till the next day. Where chemical preservatives are used as specified for individual tests, these shall be added to each portion of sample taken for the particular test and not to the entire sample.

6 MATERIAL

6.1 Test-Fish Species

Brachydanio of 30 ± 5 mm, corresponding to approximately 0.2 to 0.3 g of mass.

6.2 Stock of Fish

6.2.1 The fish shall be kept at temperature of approximately 25°C in aerated chlorine free potable water of roughly similar characteristics as the dilution water. The population density of fish shall not exceed 1 g per litre.

6.2.2 The daily illumination shall be in the range of 12 to 16 h. The stock shall be kept on a normal diet. The fish shall be free of manifest diseases or visible malformations. The minimum acclimatization period shall be 10 days prior to test under conditions of water quality and illumination similar to those used in the test. Mortality shall not exceed 1 percent per week.

6.2.3 For each test, fish shall be selected from the same stock tank, the population of which is under conditions of water quality and illumination similar to those applied in the test.

6.3 Equipment

All equipment shall be of inert material, preferably of stainless steel or borosilicate glass.

6.3.1 Glass Beaker — 3 l capacities.

6.3.2 Measuring Pipettes — 5 and 10 ml.

6.3.3 Volumetric Pipettes — 10, 20 and 50 ml.

6.3.4 Volumetric Flask — 100 ml and 1 litre.

6.3.5 Measuring Cylinders — 100 ml and 1 litre.

6.3.6 Beakers — 100, 200, 300 ml and 1 litre.

6.3.7 Thermometer

6.3.8 Oxygen Meter

6.3.9 pH Meter

6.3.10 Conductivity Meter 6.3.11 Tanks for Fish Stock and Dilution Water

6.3.12 Water Baths

6.3.13 Aquaria

6.3.14 Aerators

6.3.15 Thermostats

6.3.16 Air and Water Filter with Activated Charcoal

6.3.17 Hand nets

6.4 Reagents

6.4.1 Calcium Chloride ($CaCl_2, 2 H_2O$)

6.4.2 Magnesium Sulphate ($MgSO_4, 7 H_2O$)

6.4.3 Sodium Bicarbonate ($NaHCO_3$)

6.4.4 Potassium Chloride (KCl)

6.5 Quality of Reagents

Unless specified otherwise, pure chemicals and distilled water shall be employed in the tests. NOTE – 'Pure chemicals' shall mean chemicals that do not contain impurities which affect the results of analysis.

7 PROCEDURE

7.1 Preparation of Dilution Water

7.1.1 Dilution water is prepared by mixing 25 ml each of the following four stock solutions and diluting to 1 litre with water. Thereafter the pH is adjusted using sodium hydroxide or hydrochloric acid solution.

7.1.1.1 Calcium chloride solution — Dissolve 11.76 g of calcium chloride dihydrate (CaCl2, 2H2O) in water and dilute to 1 litre.

7.1.1.2 Magnesium sulphate solution — Dissolve 4.93 g of magnesium sulphate heptahydrate (MgSO4,7H2O) in water and dilute to 1 litre.

7.1.1.3 Sodium bicarbonate solution — Dissolve 2.59 g of sodium bicarbonate (NaHCO3) in water and dilute to 1 litre.

7.1.1.4 Potassium chloride — Dissolve 0.23 g of potassium chloride (KC1) in water and dilute to 1 litre.

7.1.2 Aeration of the Dilution Water

The dilution water shall be aerated for 24 h prior to the test. 7.2 Preparation of Test Solutions

7.2.1 Range Finding Test

In case the range of toxicity of an effluent is unknown, a range-finding test should be performed prior to the standard test to determine the concentration range within which 0 percent and 100 percent mortality is observed within 24 h. For example, in case of an effluent with unknown toxicity, the following dilution series with a dilution factor of 10 may be used;

Dilution factor(T)	**Ratio of Waste Water to Test Water (by Volume)**	**Parts of Dilution Water to be Added to one Part of Waste Water (by Volume)**
1	1:1	0
10	1:10	9
100	1:100	99
1,000	1:1,000	999

7.2.2 Standard Test

7.2.2.1 The test water is prepared by adding the effluent to the dilution water in a rounded logarithmic (geometric) progression scale as given below:

Dilution factor(T)	Ratio of Waste Water to Test Water (by Volume)	Parts of Dilution Water to be Added to one Part of Waste Water (by Volume)
1	1:1	0
2	1:2	1

4	1:4	3
8	1:8	7
16	1:16	15
32	1:32	31
64	1:64	63
125	1:125	124
250	1:250	249
500	1:500	499
1 000	1:1 000	999

7.2.2.2 Up to five consecutive concentrations have to be selected based on previous experience with regard to potential toxicity of the effluent. Each test vessel is filled with 2 litres of test water. One control vessel containing only dilution water is provided for each test. The test may be performed with one replicate.

7.3 Test Conditions

Following test conditions shall be maintained:

a) 24 h prior to the test, feeding shall be stopped.

b) Fish for a single test, shall be selected from a tank with population of the same stock.

c) After obtaining the correct temperature, 5 fishes shall be placed in each of the vessels. The fishes shall be selected randomly from the stock population and distributed at random in the various vessels with the help of a small fine-mesh dip net of soft inert material.

d) Any fish dropped or otherwise harmed during the transfer shall be discarded. All the fish, for a single test shall be introduced to test vessels within a period of 30 min.

e) The samples and test solutions shall not be aerated or treated; else extreme BOD content or extreme pH values may influence the result.

f) The duration of the test shall be 96 h.

g) Count of the dead fish in each vessel shall be taken each after 2 h, 6 h, 24 h and 48 h; the dead fish shall be removed from the vessels.

h) In case of fish dying in the control vessel, the test shall be discarded and fresh testing shall be repeated.

i) The dissolved oxygen concentration, the pH and temperature in each vessel shall be measured at the beginning of the test and each after 24 h and 48 h.

j) The handling of fish, solutions and all test procedures shall be carried out in premises free of harmful concentrations of vapors. Any disturbances that may change the behavior of fish shall be avoided.

k) All tests shall be carried out under normal laboratory illumination with natural photo period.

l) The temperature shall be maintained at 25 ± 1.0°C.

8 CALCULATIONS

8.1 The dilution factor of the test solution with the highest concentration of effluent in which all fish survive shall be recorded in rounded numbers as TF. Example: If TF = 8, it shows that all or some of the fish die in the test solution for a T value, less than 8 (1-part waste water +7 parts of dilution water) and all are alive in test solutions for a T value of 8 and above, after 48 h.

9 VALIDITY

9.1 The results shall be considered valid if the following requirements are met

9.1.1 The TF value of the reference chemical for each stock of fish should be in agreement with results obtained previously in the same laboratory, as a reference material potassium dichromate is used. In dilution water containing 100 mg/l potassium dichromate, all fish should survive (that is TF = 1).

9.1.2 All the conditions defined in the test method are fulfilled.

10 REPORT

10.1 The test report shall include the following information.

10.1.1 The specification of the test effluent and full information for identification of test samples.

10.1.2 Any deviation from the procedure specified in this standard and the reason for this, including a description of the circumstances which could have influenced the results

11 Quality Assurance and Quality Control (QA/QC)

To ensure validity and reliability of bio-assay results, the following procedures shall be implemented.

11.1Reference Toxicant Tests

i). **Purpose:** To monitor the sensitivity of test organisms and laboratory performance over time.

ii). **Frequency:** Conduct with each new batch of organisms, or at least monthly when tests are performed regularly.

iii). **Reference Toxicant:** Suitable chemicals include sodium chloride (NaCl), potassium chloride (KCl), or sodium dodecyl sulfate (SDS).

iv). **Control Chart:** Plot results (e.g., 48-h EC50 for Daphnia, 96-h LC50 for fish) on a control chart. Calculate mean and warning limits (±2 standard deviations). If results fall outside control limits, investigate cause and reject concurrent effluent tests until resolved.

11.2 Test Organism Acceptability (Controls)

i). **Control Survival:** Survival in the control (diluent water only) shall be ≥ 90% at test end. For fish tests (Clause 4), this means no more than 10% mortality. For Daphnia tests (Clause 6), this means no more than 10% immobilization. If control survival is <90%, the test is invalid and shall be repeated.

ii). **Health:** Organisms shall be active, show no abnormal behavior, and meet the fitness criteria specified in Clause 4.2.6 (for fish) and Clause 6.2 (for Daphnia).

11.3 Physicochemical Parameters

Measure and record the following at the beginning and end of the test (and at 24-hour intervals for tests lasting beyond 48 hours) in all test concentrations and the control:

i). **Dissolved Oxygen (DO):** Must remain ≥ 4.0 mg/L throughout the test. If DO falls below this level, the test results for that concentration may be invalidated. Any aeration or oxygenation (as per Clause 5) shall be noted.

ii). **Temperature:** Must be maintained within ± 2°C of the selected test temperature (e.g., 20°C, 25°C) and recorded.

iii). **pH:** Shall be measured; a drift of > 1.5 pH units in any test concentration should be noted in the report.

11.4 Test Validity Criteria

A bio-assay is valid for calculating an LC50/EC50 only if **ALL** the following are met:

a) Control survival ≥ 90% (2.7.2.1).

b) DO remained ≥ 4.0 mg/L in the control and in the test concentrations used for calculation (2.7.3.1).

c) Reference toxicant test (conducted within the last 30 days) confirms organism sensitivity is within established control chart limits (2.7.1.4).

11.5 Replication and Statistics

i). **Replication:** Where possible, use replicates to improve statistical power. For Daphnia tests, it is recommended to use at least 4 groups of 5 animals per concentration (total n=20) rather than a single group of 50.

ii). **Data Analysis:** Calculate LC50/EC50 using appropriate statistical methods (e.g., Probit Analysis, Spearman-Karber Method). Report the 95% confidence limits alongside the point

estimate. Graphical interpolation (as described in Clause 4.7) may be used for estimation, but statistical software is preferred for definitive values.