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# Standard Guide for Acute Toxicity Test with the Rotifer Brachionus[1]

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## 1. Scope

1.1 This guide describes procedures for obtaining laboratory data concerning the acute toxicity of chemicals and aqueous effluents released into fresh, estuarine, or marine waters. Acute toxicity is measured by exposing *Brachionus* newly hatched from cysts to a series of toxicant concentrations under controlled conditions. This guide describes a test for using *B. calyciflorus*, a fresh water rotifer, and the Appendix describes modifications of this test for estuarine and marine waters using *B. plicatilis*. These procedures lead to an estimation of acute toxicity, including the concentration expected to kill 50 % of the test rotifers (LC50) in 24 h. Procedures not specifically stated in this guide should be conducted in accordance with Guide E729 and Guide E1192.

1.2 Modifications of these procedures might be justified by special needs or circumstances. Although using appropriate procedures is more important than following prescribed procedures, the results of tests conducted using modified procedures might not be comparable to rotifer acute tests that follow the protocol described here. Comparison of the results using modified procedures might provide useful information concerning new concepts and procedures for conducting acute toxicity tests on chemicals and aqueous effluents.

1.3 This guide is organized as follows:



1.4 These procedures are applicable to most chemicals, either individually or in formulations, commercial products, or mixtures. This guide can also be used to conduct investigations of the effects on rotifer survival of pH, hardness, and salinity and on materials such as aqueous effluents, leachates, oils, particulate matter, sediments, and surface waters. This guide might not be appropriate for materials with high oxygen demand, with high volatility, subject to rapid biological or chemical transformation, or that readily sorb to test chambers.

1.5 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* For specific hazards statements, see Section 8.

## 2. Referenced Documents

2.1 *ASTM Standards:*[2]

E380 Practice for Use of the International System of Units (SI) (the Modernized Metric System)

E729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians

E943 Terminology Relating to Biological Effects and Environmental Fate

E1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians

## 3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *rotifer cyst*—a rotifer embryo arrested at an early stage in development, enclosed in an envelope and resistant to desiccation and temperature extremes. Rotifer cysts are often

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

incorrectly referred to as resting eggs. Upon hydration, embryonic development resumes until a neonate female emerges from the cyst.

3.1.2 *rotifer neonate*—a newly hatched, freely swimming rotifer. All neonates hatched from cysts are females.

3.1.3 *strain*—a geographically identified population of a single species. Strains are usually separated by considerable distances and can be characterized genetically through isozyme analysis or physiologically by their population dynamics and sensitivity to toxicants.

3.1.4 The words "must," "should," "may," "can," and "might" have very specific meanings in this guide. "Must" is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specified condition, unless the purpose of the test requires a different design. "Must" is used only in connection with factors directly relating to the acceptability of the test (see 13.1). "Should" is used to state that the specified condition is recommended and ought to be met if possible. Although violation of one "should" statement is rarely a serious matter, violation of several will often render the results questionable. Terms such as "is desirable," "is often desirable," and "might be desirable" are used in connection with less important factors. "May" is used to mean "is (are) allowed to," "can" is used to mean "is (are) able to," and "might" is used to mean "could possibly." Thus, the classic distinction between "may" and "can" is preserved, and "might" is never used as a synonym for either "may" or "can."

## 4. Summary of Guide

4.1 Rotifer cysts are induced to hatch in 16 to 22 h by incubating them at 25°C in standard dilution water. These neonates are then exposed immediately to two or more concentrations of test material plus a control in covered dishes. After 24 h, the percent of dead animals in each dish is recorded. An appropriate statistical method is used to calculate an LC50 or some other appropriate endpoint.

## 5. Significance and Use

5.1 An important goal of aquatic toxicology is to determine the effects of toxic compounds on species that play a central role in aquatic communities. Rotifers have a major impact on several important ecological processes in freshwater and coastal marine environments. As filter-feeders on phytoplankton and bacteria, rotifers exert substantial grazing pressure that at times exceeds that of the larger crustacean zooplankton (**1, 2**).[3] Rotifer grazing on phytoplankton is highly selective (**2-4**) and can influence phytoplankton composition, the coexistence of competitors, and overall water quality (**5**). The contribution of rotifers to the secondary production of many aquatic communities is substantial (**6-9**). In fresh water, rotifers often account for the major fraction of zooplankton biomass at certain times of the year (**10, 11**). Rotifers and other zooplankton are a significant food source for many larval fish, planktivorous adult fish (**12, 13**), and several invertebrate predators (**14-16**). The high metabolic rates of rotifers contribute to their role in nutrient cycling, which might make rotifers more important than crustaceans in certain communities (**17, 18**).

5.2 In addition to their important ecological role in aquatic communities, rotifers are attractive organisms for toxicological studies because an extensive database exists on the basic biology of this group. Techniques have been published for the culture of many rotifer species (**3, 19**). The rotifer life cycle is well defined (**20, 21**), and the factors regulating it are reasonably well understood (**22-25**). Several aspects of rotifer behavior have been examined closely (**26-29**). The biogeography of many rotifer species has been characterized (**30, 31**), and the systematics of the group are well described (**32, 33**).

5.3 Toxicity tests with rotifers of the genus *Brachionus* are more easily performed than with many other aquatic animals because of their rapid reproduction, short generation times, sensitivity (**34**), and the commercial availability of rotifer cysts. *Brachionus spp.* have a cosmopolitan distribution that spans six continents (**31**), and they are ecologically important members of many aquatic communities impacted by pollution. The use of *B. plicatilis* in an acute toxicity test for estuarine and marine environments and *B. rubens* in fresh water has been described, as well as their sensitivity to several toxicants (**35, 36**).

5.4 The test described here is fast, easy to execute, sensitive, and cost-effective. Obtaining test animals from cysts greatly reduces some of the major problems in routine aquatic toxicological testing such as the limited availability of test animals and the inconsistency of sensitivity over time. Rotifers hatched from cysts are of similar age and are physiologically uniform, thus eliminating pre-test conditions as a source of variability in the toxicity test. Cysts can be shipped inexpensively worldwide, allowing all laboratories to use standard, genetically defined strains that have been calibrated with reference toxicants. The convenience of an off-the-shelf source of test animals that require no pre-conditioning is likely to permit new applications of aquatic toxicity tests.

5.5 Sensitivity to toxicants is compound and species specific, but the sensitivity of *B. calyciflorus* is generally comparable to that of *Daphnia* (**37**).

5.6 Rotifer cysts are commercially available, but they can also be obtained from natural populations and from laboratory cultures. Techniques for rotifer cyst production in laboratory populations have been described (**24, 25, 38, 39**). However, using a well-characterized rotifer strain is best since strains are known to have differing toxicant sensitivities.

## 6. Apparatus

6.1 *Laboratory Facilities*—Preparation of the test, storage of the dilution water, and all stages of the test procedure should take place in an atmosphere free from dust and toxic vapors.

6.2 *Equipment*—The equipment required for this test includes: a constant temperature bath or environmental chamber capable of maintaining 25°C, petri dishes with covers or multiwell tissue culture plates, micropipets with smoothed openings, test tubes or petri dishes for hatching cysts, a

[3] The boldface numbers in parentheses refer to the list of references at the end of this standard.

stereomicroscope capable of 10 to 15× magnification, and a 20 to 40 W fluorescent light.

## 7. Dilution Water

7.1 Reconstituted fresh water is prepared with high-quality deionized or distilled water to which 96 mg of $NaHCO_3$, 60 mg $CaSO_4 \cdot 2H_2O$, 60 mg $MgSO_4 \cdot 7H_2O$, and 4 mg KCl are added per litre (**40**). This moderately hard dilution water (with a hardness of 80 to 100 mg $CaCO_3$ per litre and alkalinity of 60 to 70 mg per litre) is stirred for 24 h and adjusted to pH 7.5 using concentrated hydrochloric acid or sodium hydroxide. This dilution water may be used for up to seven days, but then it should be discarded. The dissolved oxygen content should be at least 90 % of saturation at the beginning of the test. Unexpected and inconsistent results can often be traced to problems with the dilution water, so it should be prepared and stored very carefully.

7.2 Other reconstituted dilution waters may be used as described in Guide E729. In addition, natural dilution water sometimes might be desirable (Guide E729). Cyst hatching and LC50s in these dilution waters might differ from those previously reported (**37**).

## 8. Hazards

8.1 Many materials can affect humans adversely if precautions are inadequate. Therefore, skin contact with all test materials and solutions should be minimized by wearing appropriate protective gloves, especially when washing equipment or putting hands in test solutions. Laboratory coats, aprons, and protective glasses should always be worn, and pipets should be used to remove organisms from test solutions. Special precautions, such as covering test chambers and ventilating the area surrounding the chambers, should be taken when conducting tests on volatile materials. Information on toxicity to humans (**41-45**), recommended handling procedures (**46-49**), and chemical and physical properties of the test material should be studied before a test is begun. Special procedures might be necessary with radiolabeled test materials (**50, 51**) and with test materials that are, or are suspected of being, carcinogenic (**52**).

8.2 Although the disposal of stock solutions, test solutions, and test organisms poses no special problems in most cases, health and safety precautions and applicable regulations should be considered before beginning a test. Removal or degradation of the test material might be desirable before disposal of the stock and test solutions.

8.3 Cleaning of equipment with a volatile solvent such as acetone should be performed only in a well-ventilated area in which no smoking is allowed and no open flame, such as a pilot light, is present.

8.4 An acidic solution should not be mixed with a hypochlorite solution because hazardous fumes might be produced.

8.5 To prepare dilute acid solutions, concentrated acid should be added to water, not vice versa. Opening a bottle of concentrated acid and adding concentrated acid to water should be performed only in a well-ventilated area.

8.6 Becuase water is such a good conductor of electricity, ground fault systems and leak detectors should be used to help avoid electrical shocks.

## 9. Test Material

9.1 *Single Chemical*—Guide E729, sections on stock solutions, solvents, solvent controls, and test concentrations apply to this test.

9.2 *Effluents*—Guide E1192, sections on collection, preservation, treatment, and test concentrations of effluents, apply to this test.

## 10. Test Organisms

10.1 Test animals are obtained by hatching cysts. Rotifer cyst hatching should be initiated approximately 16 h before the start of the toxicity test. Hatching is initiated by placing *B. calyciflorus* cysts in the dilution water (see 7.1) and incubating at 25°C at an illumination level of 1000 to 3000 lux. Hatching should begin after approximately 15 h, and by 20 h approximately 50 % of the cysts should have hatched. A hatching percent of 50 % is common. Cooler temperatures, low or high pH, low light, elevated hardness, and alkalinity can all delay hatching. If hatching is delayed, the cysts should be checked hourly to ensure collection of the test animals within 0 to 2 h of hatching. It is important to obtain 0 to 2-h-old animals for the test because there is no feeding during the toxicity test. Consequently, food deprivation begins to cause mortality after about 32 h at 25°C. If rotifers are older than 32 h at the end of the test, excessive control mortality might result.

## 11. Test Procedure

11.1 *Experimental Design:*

11.1.1 Decisions concerning aspects of the experimental design, such as the dilution factor, number of treatments, and number of test chambers per treatment, should be based on the purpose of the test and the type of procedure that is used to calculate the results. One of the following types of experimental designs will probably be appropriate in most cases.

11.1.2 If it is necessary to determine only whether a specific concentration affects survival, then a pass/fail type of test consisting of a single concentration and controls is useful. An example of this design would be a test in which a control is compared to a 100 % effluent concentration (**40**).

11.1.3 To determine the LC50 for a test material, a concentration series including a control should be prepared according to Guide E1192. Tissue culture plates containing 24 wells are convenient for LC50 determination because they permit a control and five test material concentrations on a single plate. However, other containers may be used. Tests are conducted in 1 mL of test solution with ten animals per well. This design might be modified to fit the question being asked more appropriately.

11.2 *Brachionus calyciflorus,* is a small animal approximately 250 µm in length, which is one-fourth the size of newborn *Daphnia*. Although they are small and require magnification for transferring, they swim slowly and are easy to catch with a micropipet. Newly hatched rotifers are white and

are most visible against a dark background. A stereomicroscope with 10 to 15× magnification and dark field, substage illumination is ideal. Since they are moderately phototactic, rotifers tend to congregate around the edges of a dish.

11.3 Rotifers should be transferred using a micropipet with a bore large enough to allow animals to enter and exit without injury. The volume of medium carried over with the rotifers should be minimized.

11.4 Several rotifers should be collected with a micropipet and transferred to a rinsing well containing the appropriate concentration of toxicant. Rotifers can then be transferred to the test wells, observing under the microscope their exit from the micropipet and entry into the test solutions. Rotifers must be randomly assigned to the test chambers. This procedure permits counting exactly ten animals per well and confirms their arrival into the test well in good condition. This procedure should be repeated until all control and treatment wells are loaded. A piece of parafilm should be stretched across the top of the plate and the cover put on tightly. The temperature, pH, and hardness of the test solutions must be recorded at the beginning and end of a test. Dissolved oxygen must be measured at the beginning of a test. Because test chambers contain only one mL, it is technically difficult to measure dissolved oxygen at the end of a test. However, brachionids are not sensitive to low oxygen levels (**53**).

11.5 Plates containing rotifers should be incubated at 25 ± 1°C for 24 h. Incubation should be conducted in darkness unless the investigator believes that light is necessary to activate toxicity of the test material. Containers of water should be placed in the incubator to maintain high humidity and prevent desiccation of the test wells. A summary of recommended test conditions is given in Table 1.

11.6 After 24 h, the live and dead rotifers in each well should be counted at 10 to 15× magnification and recorded. With experience, it is easy to determine rotifer death based on lack of movement, clearing of internal tissues, and retraction of the corona. If it is questionable whether an animal is dead, observe the rotifer for 5 s. Lack of movement, including mastax and foot movement, indicates death. Mortality in the controls must be 10 % or less; otherwise the test is considered invalid.

**TABLE 1 Recommended Test Conditions for the Definitive Acute Toxicity Test with the Rotifer *B. calyciflorus***

| Test Type | Static Acute |
|---|---|
| Duration | 24 h |
| Endpoint | LC50 |
| Temperature | 25°C |
| Dilution water | Reconstituted, moderately hard freshwater (see 7.1) |
| Photoperiod | OL:24D (continuous darkness) |
| Test chamber size | 2.5 mL |
| Test solution volume | 1.0 mL |
| Test concentrations | 5 plus a control |
| Total volume required for test | about 125 mL |
| Age of test animals | 0–2 h |
| Number neonates per concentration | 3 |
| Number of neonates per concentration | 30 |
| Feeding | none |
| Aeration | none |
| Test acceptability | <10 % control mortality |

11.7 *Range Finding Test*—This test is to determine the "critical range" within which mortality changes from 0 % at the low concentration to 100 % at the high concentration. A series of logarithmically spaced concentrations or dilutions of the test material is prepared with reconstituted fresh water. For example, the following concentration series might be used for a chemical: 0.01, 0.1, 1, 10, 100, 1000 mg/L. For effluents, the following concentrations might be used: 0.01, 0.1, 1, 10, 100 %. If effluent characteristics (that is, $NH_3$, $NO_2$, and so forth) are altered by aeration, the stabilization times for the effluents and controls must be decreased. The range-finding test is conducted with only one test well per concentration. An additional well with ten rotifers in the dilution water is included as a control. It should be noted that a range-finding test with an effluent will require at least 24 h storage of the effluent before a definitive test. This could be a significant factor with an effluent containing easily degraded compounds.

11.8 *Definitive Test*—This test is conducted to determine the 24 h LC50 for *B. calyciflorus*. From the critical concentration range obtained in the range-finding test, concentrations or dilutions of the test material should be chosen from a geometric scale. A control and five or more concentrations are usually examined. Refer to Guide E729, for further guidance on the experimental design of acute toxicity tests.

11.9 *Reference Chemical Test*—It is desirable to determine a 24-h LC50 using a reference chemical at least once every 10 to 15 tests with *B. calyciflorus* in order to demonstrate test animal sensitivity and conformity of the experimental procedure with that of other laboratories.

11.9.1 Many chemicals have been used as reference toxicants (**54**). A reference toxicant is more likely to be useful when used in conjunction with tests on materials that have the same mode of action as the reference toxicant.

11.9.2 Extensive data exist on copper toxicity to *B. calyciflorus*, including an international intercalibration involving more than 100 laboratories (**55**). If copper is used as a reference toxicant, the following concentrations of copper could be tested: control, 14.0, 18.4, 24.3, 31.9, and 42.0 µg/L. To prepare a 1000 µg/L copper stock solution, dissolve 39.4 mg of copper sulfate (copper comprises 25.4 % of $CuSO_4 \cdot 5H_2O$ by weight) in 100 mL of reconstituted fresh water (see 7.1) at 25°C with the aid of a magnetic stirrer. Add 1 mL of this solution to reconstituted fresh water in a 100 mL volumetric flask and bring to volume. This produces a final copper concentration of 1000 µg/L. It is desirable to prepare the stock solution immediately before use and to adjust its pH to 7.5 with KOH. The *B. calyciflorus* 24 h LC50 for copper is 30µ g/L with 95 % confidence limits of 10 to 50 µg/L based on interlaboratory comparisons (**55**). If a reference test with copper produces an LC50 that falls outside these limits, it might indicate that proper experimental procedures are not being followed. It is especially important to perform a reference test each time a new batch of cysts or a new water source is used. A round-robin involving 172 laboratories in Europe, the United States,

and Canada demonstrated that copper LC50s with reference *B. calyciflorus* cysts had intralaboratory and interlaboratory coefficients of variation of 11 % and 67 %, respectively (**55**). Copper LC50s for three *B. plicatilis* geographic strains (a marine congener of *B. calyciflorus*) ranged from 35 to 166 µg/L, indicating that cysts from different strains can produce results that differ markedly. New *B. calyciflorus* strains should be calibrated with reference chemicals before they are used for toxicity testing.

## 12. Calculation of Results

12.1 For a design using multiple concentrations of the test material, an LC50 and and its 95 % confidence limits might be calculated on the basis of the measured initial concentrations, if available, or the calculated initial concentrations.

12.2 The acute toxicity test produces quantal data, that is, counts of the number of animals in two mutually exclusive categories, that is, alive or dead. A variety of methods can be used to calculate an LC50 and its 95 % confidence limits from a set of quantal data that is binomially distributed (**56-59**).

12.2.1 When two or more concentrations of toxicant cause partial mortality of the test animals, the probit method will usually produce statistically sound information on the LC50. In some situations, it may be necessary to use a non-parametric procedure such as the Spearman-Karber method to obtain statistically sound information. In any case, the method used should appropriately take into account the number of test chambers per treatment and the number of test animals per chamber.

12.2.2 When partial mortality is observed in fewer than two concentrations of toxicant, the binomial test can usually be used to obtain statistically sound information concerning the LC50. The binomial test does not provide confidence limits. If desired, an interpolation procedure may be used to obtain an approximately LC50.

12.3 If used as a pass/fail acute toxicity test, it is not necessary to calculate an LC50. In this case, the comparison of a control with a 100 % effluent concentration using a t-test or a non-parametric Wilcoxon Rank Sum test might be appropriate (**40**).

## 13. Acceptability of the Test

13.1 A definitive rotifer acute test is usually considered unacceptable if one or more of the following has occurred:

13.1.1 All test chambers were not identical.

13.1.2 A required dilution water or solvent control was not included in the test.

13.1.3 Individual test animals were not randomly assigned to test chambers.

13.1.4 Greater than 10 % mortality occurred in the controls.

13.1.5 Temperature, pH, hardness, and dissolved oxygen were not measured.

13.2 Calculation of an LC50 should be considered unacceptable or inappropriate if either or both of the following has occurred:

13.2.1 No treatment other than a control treatment killed or affected less than 37 % of the test animals exposed to it.

13.2.2 No treatment killed or affected more than 63 % of the animals exposed to it.

## 14. Report

14.1 Report the following:

14.1.1 The origin of the rotifer strain and, if applicable, the batch number of the cysts used.

14.1.2 Information on the test chemical or effluent, including its origin, purity, CAS number, and the manufacturer's lot number. For effluents, the origin, collection method, storage time, and conditions should be described.

14.1.3 The test temperature, pH, hardness, and dissolved oxygen at the beginning of the test, the temperature, pH, and hardness at the end of the test, and the laboratory performing the test. Because the test chambers contain only 1 mL, it is technically difficult to measure dissolved oxygen at the end of the test. It is also known that brachionid rotifers are not sensitive to low oxygen levels (**53**).

14.1.4 The type of dilution water and the type and concentration of any solvents used.

14.1.5 The calculated 24 h LC50 with 95 % confidence limits.

14.1.6 The method used to calculate the LC50.

14.1.7 The concentrations tested and percent mortality in each for both range finding and definitive tests.

14.1.8 The data confirming the validity of the results:

14.1.8.1 The percent mortality in the controls,and

14.1.8.2 The date, reference chemical, and LC50 for the most recent reference test performed on the batch of cysts currently in use.

14.1.9 Any deviation from the standard procedure and any problem encountered during the test.

## 15. Keywords

15.1 acute toxicity; cysts; freshwater; invertebrate; LC50; marine; rotifers; toxicity test

# APPENDIX

**(Nonmandatory Information)**

## X1. AN ACUTE TOXICITY TEST FOR ESTUARINE AND MARINE WATERS USING BRACHIONUS

X1.1 Toxicity in estuarine and marine waters can be examined with the rotifers *B. calyciflorus* or *B. plicatilis*. It is possible to use *B. calyciflorus* as test animals at salinities of 5 g/kg or less. *B. calyciflorus* is preferred over *B. plicatilis* at these salinities because the former is considerably more sensitive to most chemicals tested (**37**). *B. plicatilis* can be used at salinities above 5 g/kg with the appropriate modifications to the freshwater protocol.

X1.2 The sensitivity of *B. plicatilis* is more variable than that of *B. calyciflorus*. For some toxicants, such as copper and sodium dodecyl sulfate (SDS), *B. plicatilis* is more sensitive than the mysid *Mysidopsis bahia* (**35**). For others, such as free ammonia and cadmium, *B. plicatilis* is much less sensitive than *M. bahia*.

X1.3 For *B. plicatilis*, the dilution water is a reconstituted seawater at 15 g/kg called ASPM (**60**). It is prepared with high-quality deionized water, to which is added 11.31 g NaCl, 0.36 g KCl, 0.54 g $CaCl_2$, 1.97 g $MgCl_2 \cdot 6H_2O$, 2.39 g $MgSO_4 \cdot 7H_2O$, and 0.17 g $NaHCO_3$ per litre. This reconstituted seawater is stirred for 24 h, and the pH is adjusted to 8.0 with hydrochloric acid or sodium hydroxide. Full salinity seawater (34 g/kg) can also be prepared by adding 2.3 times more of each salt above to 1 L of deionized or distilled water. Reconstituted seawater can be diluted to a salinity of 1 to 5 g/kg with deionized water and used for low-salinity *B. calyciflorus* tests. Reconstituted seawater may be used for up to seven days, but then it should be discarded. Unexpected and inconsistent results can often be traced to problems with the dilution water, so it should be prepared and stored very carefully. Other reconstituted dilution waters can be used as described in Guide E729. In addition, the use of natural seawater might sometimes be desirable. Cyst hatching and LC50 in these dilution waters might differ from those previously reported (**35, 61**).

X1.4 *B. plicatilis* cyst hatching should be initiated approximately 24 h before the start of the toxicity test. Hatching is initiated by placing cysts in standard seawater at 15 g/kg and incubating at 25°C and 1000 to 3000 lux illumination. Hatching should begin after approximately 22 h, and approximately 50 % of cysts should have hatched by 24 h. A hatching percent of 50 % is common. Low temperatures, high salinity, high pH, and low light can all delay hatching. If hatching is delayed, check the cysts hourly to ensure collecting the test animals within 0 to 2 h of hatching. It is important to obtain 0- to 2-h-old animals for the test because there is no feeding during the test. The effects of food deprivation begin to cause mortality after about 80 h at 25°C.

X1.5 *Reference Chemical Test*—A24 h LC50 using a reference chemical should be determined at least once every 10 to 15 assays with *B. plicatilis* in order to demonstrate conformity of the experimental procedure of that laboratory with other laboratories.

X1.5.1 A large amount of data on copper toxicity exists for *B. plicatilis* (**55**). If copper is used as a reference toxicant, the following concentrations of copper should be tested in three replicates: control, 20.0, 33.6, 56.6, 95.1, and 160.0 µg/L. This dilution series is prepared from a 1000 µg/L copper stock solution and its pH adjusted to 8.0 with NaOH. The *B. plicatilis* 24 h LC50 for copper is 80 µg/L with 95 % confidence limits of 30 to 130 µg/L based on interlaboratory comparisons. If a laboratory's reference test with *B. plicatilis* produces an LC50 falling outside these 95 % confidence limits, it might indicate that proper experimental procedures are not being followed. It is especially important to conduct a reference test each time a new batch of cysts or a new water source is used.

X1.5.2 A round-robin conducted in Europe, the United States, and Canada involving 122 labs demonstrated that copper LC50s with reference *B. plicatilis* cysts had intralaboratory and interlaboratory coefficients of variation of 20 % and 67 %, respectively (**55**).

X1.6 Since food deprivation does not begin to cause mortality in *B. plicatilis* until 80 h, the duration of this test can be increased from 24 h to 48 or 72 h with little modification of protocol. Longer toxicant exposures significantly lower LC50 estimates. For example, the cadmium 24 h LC50 is 39.1 mg/L, as compared to LC50s of 9.2 and 5.2 mg/L for 48 and 72 h exposures, respectively (**61**). Similarly, the 24 h LC50 of 1.9 mg/L for sodium pentachlorophenate is significantly lower by 53 % and 86 % with 48 and 72 h exposures, respectively.

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