



Standard Guide for Behavioral Testing in Aquatic Toxicology¹

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1. Scope

1.1 This guide covers some general information on the selection and application of behavioral methods useful for determining the sublethal effects of chemicals to fish, amphibians, and macroinvertebrates.

1.2 Behavioral toxicity occurs when chemical or other stressful conditions, such as changes in water quality or temperature, induce a behavioral change that exceeds the normal range of variability (1).² Behavior includes all observable, recordable, or measurable activities of a living organism and reflects genetic, neurobiological, physiological, and environmental determinants (2).

1.3 Behavioral methods can be used in biomonitoring, the determination of no-observed-effect and lowest-observed-effect concentrations, and the prediction of hazardous chemical impacts on natural populations (3).

1.4 Behavioral methods can be applied to fish, amphibians, and macroinvertebrates in standard laboratory toxicity tests, tests of effluents, and sediment toxicity tests.

1.5 The various behavioral methods included in this guide are categorized with respect to seven interdependent, functional responses that fish, amphibians, and macroinvertebrates must perform in order to survive. These functional responses include respiration, locomotion, habitat selection, feeding, predator avoidance, competition, and reproduction (4). These responses can be documented visually or through video or acoustic imagery. Electronically recorded information can be derived through manual techniques or through the use of digital image analysis software. (5, 6, 7)

1.5.1 The functional responses are not necessarily mutually exclusive categories. For instance, locomotion, of some form of movement, is important to all behavioral functions.

1.6 Additional behavioral methods for any category may be added when new tests are developed as well as when methods are adapted to different species or different life stages of an organism.

1.7 This guide is arranged as follows:

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1.8 The values stated in SI units are to be regarded as the standard.

1.9 *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.* Specific precautionary statements are given in Section 9.

2. Referenced Documents

2.1 ASTM Standards:³

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

E1241 Guide for Conducting Early Life-Stage Toxicity Tests with Fishes

E1383 Guide for Conducting Sediment Toxicity Tests with Freshwater Invertebrates (Withdrawn 1995)⁴

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² The boldface numbers in parentheses refer to the list of references at the end of this standard.

³ 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.

⁴ The last approved version of this historical standard is referenced on www.astm.org.

3. Terminology

3.1 *Definitions*—The words “must,” “should,” “may,” “can,” and “might” have very specific meanings. “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 the factors that directly relate to the acceptability of the test. “Should” is used to state that the specified condition is recommended and ought to be met if possible. Although the violation of one “should” 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.”

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *behavior*—the complex of observable, recordable, or measurable activities of a living organism.

3.2.2 *behavioral toxicity*—the phenomenon observed when a behavioral response varies beyond the range of normal as a result of exposure to chemical or other stressors.

4. Summary of Guide

4.1 The potential toxicity of chemical substances in water, food, or sediments is assessed by measuring the behavior of fish, amphibians, and macroinvertebrates during exposure, using static, flow-through, or food exposure systems. The behavioral response of organisms exposed to chemical substances in water, effluents, food, or sediments is compared with the behavioral responses of control organisms. The behavioral responses measured during toxicity tests are highly sensitive to sublethal exposure. The behavioral measures are relevant to essential life functions that fish, amphibians, and macroinvertebrates often must perform in order to survive and include respiration, locomotion, habitat selection, feeding, predator avoidance, competition, and reproduction. Data are obtained to determine the effects of toxic substances on behavior from short (for example, 1 h) or long-term (partial to full life cycle) exposures.

5. Significance and Use

5.1 Protection of a species requires the prevention of detrimental effects of chemicals on the survival, growth, reproduction, health, and uses of individuals of that species. Behavioral toxicity provides information concerning sublethal effects of chemicals and signals the presence of toxic test substances.

5.1.1 The behavioral responses of all organisms are adaptive and essential to survival. Major changes in the behavioral responses of fish, amphibians, and macroinvertebrates may result in a diminished ability to survive, grow, or reproduce and cause significant changes in the natural population (8).

5.2 The results from behavioral toxicity tests may be useful for measuring injury in the assessment of damages resulting from the release of hazardous materials (9).

5.3 Behavioral toxicity test methods may be useful for long-term monitoring of effluents (10).

5.4 The results from behavioral toxicity data can be used to predict the effects of exposure on fish, amphibians, and aquatic invertebrates likely to occur in field situations as a result of exposure under similar conditions, including the avoidance of exposure by motile organisms (11).

5.5 The results from behavioral toxicity tests might be an important consideration for assessing the hazard of materials to aquatic organisms. Such results might also be used when deriving water quality criteria for fish and aquatic invertebrates organisms.

5.6 The results from behavioral toxicity tests can be used to compare the sensitivities of different species, relative toxicity of different chemical substances on the same organism, or effect of various environmental variables on the toxicity of a chemical substance.

5.7 The results from behavioral toxicity tests can be used to predict the effects of long-term exposure.

5.8 The results of behavioral toxicity tests can be useful for guiding decisions regarding the extent of remedial action needed for contaminated aquatic and terrestrial sites.

5.9 The behavioral characteristics of a particular organism must be understood and defined before a response can be used as a measure of toxicity. The range of variability of any behavioral response of unexposed organisms is influenced by genetic, experiential, physiological, and environmental factors. Thus it is important to avoid selecting test organisms from populations that may vary significantly in these factors.

5.10 The results of behavioral toxicity tests will depend on the behavioral response measured, testing conditions, water quality, species, genetic strain, life stage, health, and general condition of test organisms. Therefore, the behavioral response may be affected by the test environment.

6. Interferences

6.1 A number of factors can suppress, elicit, or alter behavioral responses and thus influence behavioral test results and complicate data interpretation. The following factors should be considered in the experimental protocol or in the discussion of results when measuring behavioral responses during toxicity tests:

6.1.1 The pretest handling of test organisms resulting from collection, transfer, and maintenance of the culture environment can affect the response observed during exposure to toxic substances.

6.1.2 The health, nutritional state, and physical condition of the organism can influence the test.

6.1.3 Behavioral responsiveness may vary by species, genetic strain, population, gender, and developmental stage of the organism.

6.1.4 Prior exposure to hazardous materials, environmental stresses, and pathogens can affect the behavioral responses.

6.1.5 Social status, such as dominance or sex of the individuals tested, and experiential factors, such as prior experience with predator or prey species, can influence the behavioral

response. Individuals tested in isolation may respond differently than when tested in groups.

6.1.6 Cyclical changes (circadian, seasonal, annual, and reproductive) in behavioral responses can occur.

6.1.7 The behavioral response can be affected by apparatus design and by the procedural sequence of the measurement method.

6.1.8 Behavioral responses will vary according to the extent to which test organisms acclimate to the physical variables of the testing environment, including water quality, temperature, water flow, light, cover, and substrate, as well as their recovery from handling, acceptance of diet, and adjustment to novel testing chambers.

6.1.9 Behavioral responses to toxic substances may subside over time.

7. Test Facility

7.1 *Facilities*—The facility should include a constant temperature area for culturing and testing. Test and culture chambers may be placed in a temperature-controlled recirculating water bath or in a constant-temperature area. Air used for aeration should be free of fumes, oil, and water and can require filters to remove oil, water, and bacteria. The test facility should be well-ventilated and free of fumes. Enclosures may be necessary to ventilate test chambers.

7.1.1 Culture and animal care facilities should not be in a room in which toxicity tests are conducted, stock solutions or test solutions are prepared, or equipment is cleaned.

7.1.2 A timing device should be used to provide a light:darkness cycle. A 15 to 30-min transition period, allowing for a gradual change in light intensity when the lights are turned on or off, may be desirable for reducing stress caused by instantaneous illumination or darkness.

7.2 *Construction Materials*—Consistent with specifications delineated, for example, in Guide E1241, equipment and facilities that come into contact with stock solutions, test solutions, food, sediment, air, or water, into which the test organisms are placed, should not contain substances that can be leached or dissolved in amounts that affect the test organisms adversely. The materials should be chosen to minimize sorption of test materials.

7.3 *Water and Air Delivery Systems*—The water delivery system used in flow-through testing can be one of several designs. The system should be capable of delivering equal volumes of water at an equal rate of flow to each replicate treatment container. Various metering systems, using different combinations of siphons, pumps, solenoids, valves, etc., have been used successfully to control the flow rates of water and toxic substances (see Guide E1241).

7.3.1 The metering system should be calibrated before the test by determining the flow rate of water and air through each test chamber. The general operation of the metering system should be visually checked daily throughout the test. The water delivery system should be adjusted during the test if necessary. At any particular time during the test, flow rates through any two test chambers should not differ by more than 10 %.

7.4 *Test Chambers*—In a behavioral toxicity test with fish, amphibians, and macroinvertebrates, the measurement of be-

havioral response may take place directly in the exposure vessel, or the organisms may be transferred to a specific apparatus or observation chamber for the purpose of measuring a behavioral response (see section 8.1.8). The independent experimental unit for such tests is based on the smallest physical exposure unit between which there are no water, air connections, or common access to sediment or food. All test chambers must be identical, and the test compartments within each chamber must be identical and placed in analogous locations within each test chamber.

7.4.1 Test chambers may be constructed in several ways and of various materials, depending on the experimental design and contaminants of interest. Clear silicone adhesives, suitable for aquaria, should be used sparingly since they sorb some organic compounds that may be difficult to remove. New test chambers sealed with silicone adhesives should be weathered for at least 48 h in water of the same quality as that used in the toxicity test to leach potentially toxic compounds from the adhesive.

7.4.2 Apparatus will vary according to the response being measured and species and life stage being tested. Organisms may be observed directly in the exposure chamber, or they may be transferred to specialized apparatus for measurement of the response. Recording of response may require (1) direct visual observation, (2) video-recorded observation, or (3) electronically recorded observation.

7.5 *Cleaning*—Test chambers, water delivery systems, equipment used for preparing and storing exposure water, and stock solutions should be cleaned before use. Consistent with Guide E729, new items should be washed in the following manner: (1) detergent wash, (2) tap water rinse, (3) water-miscible organic solvent wash, (4) tap water rinse, (5) acid wash (such as 10 % concentrated hydrochloric acid), and (6) rinse at least twice with distilled, deionized, or test water. Test chambers should be rinsed with test water just before use.

7.5.1 Many organic solvents leave a film that is insoluble in water. A 10 % nitric acid solution, for example, may cause deterioration of silicone adhesive. A rinse with 10 % concentrated hydrochloric acid may be preferable. A dichromate-sulfuric acid cleaning solution can generally be used in place of both the organic solvent and the acid (see Guide E729), but the solution might attack silicone adhesive and leave potentially mutagenic residues of chromium on glass. Non-chromium cleaning solutions are also available.

7.5.2 Upon completion of a test, all items that are to be used again should be immediately (1) emptied of water, sediment, or effluent (which should be disposed of properly; (2) rinsed with water; (3) cleaned by a procedure appropriate for removing the test material (for example, acid to remove metals and bases and detergent, organic solvent, or aqueous slurry of activated carbon to remove organic chemicals); and (4) rinsed at least twice with distilled, deionized, or overlying water.

7.6 *Acceptability*—Before a toxicity test is conducted in new test facilities, it is desirable to conduct a non-toxicant test, in which all test chambers contain uncontaminated water or sediment. The behavior of the test species will demonstrate whether facilities, water, control sediment, and handling techniques are adequate to result in acceptable species-specific