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Standard Guide for Behavioral Testing in Aquatic Toxicology[1]

This standard is issued under the fixed designation E1604; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

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:

1.8 The values stated in SI units are to be regarded as the standard. For an explanation of units and symbols, refer to IEEE/ASTM SI 10.

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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.* Specific precautionary statements are given in Section 9.

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2. Referenced Documents

2.1 *ASTM Standards:*[3]

E729 Guide for Conducting Acute Toxicity Tests on Test

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[1] This guide is under the jurisdiction of ASTM Committee E50 on Environmental Assessment, Risk Management and Corrective Actionand is the direct responsibility of Subcommittee E50.47 on Biological Effects and Environmental Fate.

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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.

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Materials with Fishes, Macroinvertebrates, and Amphibians
E943 Terminology Relating to Biological Effects and Environmental Fate
E1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses
E1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents 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]
E1711 Guide for Measurement of Behavior During Fish Toxicity Tests
E1733 Guide for Use of Lighting in Laboratory Testing
E1847 Practice for Statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines
IEEE/ASTM SI 10 American National Standard for Metric Practice

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.

3.3 For definitions of other terms used in this guide, refer to Guides E729, E1192, E1241, E1383, E1711, E1847, and Terminology definitions in Guide E943.

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, and health 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 invertebrate 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.

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

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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 change 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. Descriptions of toxicity testing equipment appear Guides E729, E1023, E1192, E1241, E1383, and E1711. These provide guidance on construction materials, water and air delivery systems, test chambers and cleaning, and water supply

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:dark 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 (See Guide E1733).

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 Guides E729, E1023, E1241, E1383, and E1847).

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 behavioral 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 12). 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.

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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 control numbers. The magnitude of the within-chamber and between-chamber variance should also be determined.

8. Water Supply

8.1 *Requirements*—In addition to being available in adequate supply, dilution water used in behavioral toxicity tests, and water used to hold organisms before testing, should be acceptable to test species and uniform in quality. To be acceptable to the test species, the water must permit satisfactory survival and growth, without inducing signs of disease or apparent stress, such as discoloration, or unusual behavior.

8.2 *Source*—Natural overlying water should be uncontaminated and of constant quality and should meet the following specifications as established in Guide E729. The values stated help to ensure that the test organisms are not apparently stressed during holding, acclimation, and testing and that the test results are not affected unnecessarily by water characteristics: particulate matter, <5 mg/L; total organic carbon (TOC), <5 mg/L; chemical oxygen demand (COD), <5 mg/L; and residual chlorine, <11 µg/L.

8.2.1 A natural water source is considered to be of uniform quality if the monthly ranges of the hardness, alkalinity, and specific conductance are less than 10 % of their respective averages and if the monthly range of pH is less than 0.4 unit. Natural waters should be obtained from an uncontaminated well or spring, if possible, or from a surface water source. If surface water is used, the intake should be positioned to minimize fluctuations in quality and the possibility of contamination; to maximize the concentration of dissolved oxygen; and to help ensure low concentrations of sulfide and iron. Municipal water supplies can contain unacceptably high concentrations of copper, lead, zinc, fluoride, chlorine, or chloramines, and quality can be variable. Chlorinated water should not be used for, or in the preparation of, exposure water because residual chlorine and chlorine-produced oxidants are toxic to many aquatic animals (**12**). Dechlorinated water should be used only as a last resort because dechlorination can be incomplete.

8.2.2 For certain applications, the experimental design might require the use of water from the test effluent or sediment collection site.

8.2.3 Reconstituted water is prepared by adding specified amounts of reagent grade chemicals to high-quality distilled or deionized water (see Guide E729).

8.3 *Characterization*—The following items should be measured at least twice each year, and more often if (*1*) such measurements have not been determined semiannually for at least two years or (*2*) if surface water is used: pH, particulate matter, TOC, organophosphorus pesticides, organic halides, organochlorine pesticides, polychlorinated biphenols (PCBs), chlorinated phenoxy herbicides, ammonia, cyanide, sulfide, bromide, chloride, fluoride, iodide, nitrate, phosphate, sulfate, calcium, magnesium, sodium, potassium, aluminum, arsenic, beryllium, boron, cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury, molybdenum, nickel, selenium, silver, and zinc, hardness, alkalinity, and conductivity (see Guide E729).

8.3.1 For each method used, the detection limit should be below (*1*) the concentration in the dilution water or (*2*) the lowest concentration that has been shown to affect the test species adversely (**13**).

8.3.2 Water that might be contaminated with facultative pathogens may be passed through a properly maintained ultraviolet sterilizer (**14**) equipped with an intensity meter and flow controls or passed through a filter with a pore size of 0.45 µm or less. Carbon filtration may be required to remove the pathogenic toxins.

8.3.3 Water may require aeration using air stones, surface aerators, or column aerators (**15-17**). Adequate aeration will stabilize the pH, bring the concentrations of dissolved oxygen and other gases into equilibrium with air, and minimize the oxygen demand and concentrations of volatiles. The concentration of dissolved oxygen in water should be between 90 and 100 % saturation (**17**) to help ensure that the dissolved oxygen concentrations are acceptable in the test chambers. Precautions should be taken, however, to ensure that glass air stones are not breaking down with use and that plastic air stones are not absorbing organic chemicals.

9. Safety Precautions

9.1 Many substances may pose health risks to humans if adequate precautions are not taken. Information on toxicity to humans, recommended handling procedures, and chemical and

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physical properties of the test material should be studied and all personnel informed before an exposure is initiated.

NOTE 1—**Warning:** Special procedures might be necessary with radiolabeled test materials and with test materials that are, or are suspected of being, carcinogenic.

9.2 Many materials can affect humans adversely. Contact with test material, sediments, and water should be minimized. Where appropriate, protective gloves, laboratory coats, aprons, protective clothing, and safety glasses should be worn, and dip nets, sieves, or tubes should be used to remove test organisms. When handling potentially hazardous materials, proper handling procedures may include (*1*) manipulating test materials under a ventilated hood or in an enclosed glovebox; (*2*) enclosing and ventilating the exposure chambers; and (*3*) using respirators, aprons, safety glasses, and gloves.

10. Test Material

10.1 Test materials may include pure compounds or commercial formulations of compounds that are added to water or sediment. Test materials collected from field locations may also include complex mixtures of chemical compounds in effluents and sediments.

10.2 Considerations for technical test materials for use in aqueous tests, preparations of stock solutions, use of solvents, and selection of test concentrations of aqueous solutions should follow those outlined in Guide E1241.

10.3 Tests using sediments as the exposure media should follow Guide E1383 for the characterization, collection, storage, preparation of spiked sediment samples, and test concentrations of spiked sediment samples.

11. Test Organisms

11.1 Species and life stages selected for study will depend on the focus of the study and may include standard bioassay organisms when the relative toxicity of a compound is to be determined.

11.2 The species and life stage selected for study should be appropriate for the experimental setting, tolerant of handling and confinement within a reasonable acclimation time, and be willing to accept food in the setting in which the behavioral responses will be observed. The species used should be selected based on (*1*) availability; (*2*) sensitivity to a test material(s); (*3*) ecological relevance to the habitat under study (for example, saltwater or freshwater); and (*4*) tolerance to ecological conditions such as temperature, grain size, and ease of handling in the laboratory. The species of test organism used should be determined using an appropriate taxonomic key.

11.3 Test organisms must not be diseased or injured and must be obtained from relatively uncontaminated field sites or contaminant-free cultures. The organisms must be acclimated to the water quality and testing conditions following the procedures outlined in Guide E729.

11.4 The relative health and quality of test organisms can be verified through an assessment of their behavioral repertoire and bioassays in response to reference toxicants.

11.5 All organisms should be as uniform as possible in age and size class.

11.6 All organisms in a test must be from the same source. Organisms may be obtained from (*1*) laboratory cultures; (*2*) commercial, state, or federal institutions; or (*3*) natural populations from clean areas. Laboratory cultures of test species can provide organisms whose history, age, and quality are known. Local and state agencies may require collecting permits.

11.7 To maintain organisms in good condition and avoid unnecessary stress, they should not be crowded and should not be subjected to rapid changes in temperature or water quality characteristics.

11.8 The addition of shelter or refuge may be required for certain species.

12. Responses Measured

12.1 *Respiration*—Respiratory tissue is frequently in immediate contact with injurious substances. Disruptions in respiratory behavior arise when the substance reduces respiratory efficiency, affects neurological control of respiration, or irritates respiratory membranes (**10**). Respiratory variables commonly measured include respiratory frequency, respiratory volume, and the analog waveform characteristics of the respiratory cycle.

12.2 *Locomotion*—Locomotory responses are essential to survival in most organisms and are often very sensitive to hazardous substances (**18**). Disruption of locomotory behavior can impair the ability of fish, amphibians, and macroinvertebrates to perform essential life functions that might rely on agile, efficient, and vigorous swimming. Variables of locomotory behavior commonly measured include the frequency and duration of activity, form and posture of locomotion, larval development of locomotion, physical capacity for swimming, and bioenergetics. Locomotion may also include the respiratory and feeding movements of sessile organisms.

12.3 *Habitat Selection*—Fish, amphibians, and macroinvertebrates must be capable of detecting and responding appropriately to environmental stimuli in order to seek conditions beneficial to survival and to avoid hazardous conditions. Some chemical substances are detected by fish, amphibians, and macroinvertebrates and elicit avoidance or attractance responses. Chemical substances may alter the ability to detect and respond to environmental stimuli (**19**). Variables of habitat selection that are commonly measured include orientation or preference to temperature, water quality, light, and natural chemical stimuli such as food odors, predator and prey scents, and pheromones.

12.4 *Competition*—Most organisms must compete for available resources. Exposure to hazardous substances may interfere with competitive responses by increasing or decreasing the aggressive interactions between conspecifics and between species (**20**). Stress arising from aggressive interactions may potentiate the toxicity of a chemical substance during toxicity tests. Variables of competition most commonly measured during toxicity tests include the frequency and magnitude of aggressive interactions.

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