Designation: E1604 – 12

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.

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]

[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.

Current edition approved Dec. 1, 2012. Published February 2013. Originally approved in 1994. Last previous edition approved in 2007 as E1604-04(2007). DOI: 10.1520/E1604-94R07.

[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. A15 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 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 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. A10 % 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 often contain unacceptably high concentrations of copper, lead, zinc, fluoride, chlorine, or chloramines, and quality is often 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 is often 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 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 if precautions are inadequate. 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.

12.5 *Feeding*—Feeding is essential to survival, growth, and reproduction. Feeding inhibitions induced by hazardous substances can result in starvation, impaired growth, decreased fitness, and reproductive failure. Variables of feeding commonly measured during toxicity tests include latency of response to prey and the maximum distance from which the organism responds to prey, prey selectivity, feeding efficiency and prey-handling time, strike and capture frequencies, bioenergetics, and learning (**21**).

12.6 *Predator Avoidance*—Most fish, amphibians, and macroinvertebrates are vulnerable to predation during their life cycle. Hazardous substances may increase a prey organism's vulnerability to predation by disrupting defensive responses or decreasing the organism's ability to escape predators. Variables of prey vulnerability commonly measured during toxicity tests include frequency of capture, schooling, shelter seeking, defensive reactions, and learning (**22**).

12.7 *Reproduction*—Reproduction is essential to the maintenance of a population. Hazardous substances can disrupt reproduction through reduced gametogenesis, egg viability, and behavioral modifications. Behavioral variables of reproduction that can be measured during toxicity tests include reproductive migrations, territoriality, courtship, spawning, nest preparation, maintenance and defense, and parental behavior (**23**).

13. Behavioral Test Method Selection Criteria

13.1 Selection criteria will vary depending on the purpose of the specific toxicity test (**24**). Criteria for the behavioral methods used in biomonitoring might include sensitivity, low cost, biotic and abiotic variation, and standardization. Criteria

for the behavioral methods defining no-observed-effect concentrations may emphasize criteria for sensitivity, standardization, validity, and realism. Behavioral methods for predicting ecological impacts of test materials would emphasize criteria such as realism, validity, and predictive capabilities.

13.1.1 *Documentation*—There should be sufficient investigations or published reports to provide guidance for the conduct of procedure and estimates as to expected outcome. Otherwise, sufficient preliminary study should be conducted to assess the suitability of the response as a measure of toxicity.

13.1.2 *Biotic and Abiotic Influences*—Nonexperimental sources of variation on the behavioral responses should be defined in order to ensure that these variables are addressed in the experimental design and physical setting of the experiment. These precautions will minimize variation of the response of the individuals tested.

13.1.3 *Realism*—The interpretation of the response in terms of the organism's ability to survive and in relation to the viability of the population should be unambiguous.

13.1.4 *Validation*—Responses observed during the toxicity test ought to reflect responses that occur in the field.

13.1.5 *Sensitivity*—The test procedure should produce measurable responses at low, environmentally relevant exposure concentrations.

13.1.6 *Predictive Capabilities*—The test should be predictive of responses of populations and communities to the exposure.

13.1.7 *Costs*—Costs per test should be realistic relative to alternative procedures and reflect the societal value of the resource.

13.1.8 *Standardization*—Conditions and components of the test system should be defined sufficiently to allow different laboratories to obtain similar results. Statistical criteria for detecting and interpreting the responses of a test system should be well-defined, as should the criteria for rejecting test results.

13.2 Precise, objective, operational definitions of behavioral endpoints measured during toxicity tests are required.

14. Experimental Design

14.1 The experimental design for different behavioral toxicity tests will vary depending on the endpoint to be measured, species to be tested, and length of exposure.

14.2 The experimental unit is defined as the smallest physical entity to which treatments can be assigned independently. Because water or air cannot flow from one exposure chamber to another, the exposure chamber is the experimental unit. Behavioral responses measured from organisms from the same chamber are considered to be multiple observations of the same experimental unit. As the number of exposure chambers per treatment increases, the number of degrees of freedom increases, and therefore the power of a significance test increases. Thus, degrees of freedom in behavioral tests increase only when representative organisms from replicate exposure chambers are studied. Several precautions must be taken to ensure that the experimental design does not affect the results of the test: (*1*) all exposure chambers should be treated as similarly as possible, when considering parameters such as temperature and lighting (unless these are the variables tested); (*2*) each exposure chamber, including replicate exposure chambers, must be physically treated as a separate entity; and (*3*) treatments must be assigned randomly to individual exposure chamber locations. The assignment of test organisms to each chamber must be randomized.

14.3 One of the two following experimental designs will be appropriate in most cases:

14.3.1 If it is necessary to determine whether a specific concentration, effluent, or sediment affects behavior, only that concentration, effluent, or sediment and a control are necessary. Controls might include dilution water or solvent control water, or both, to which no test material has been added. Sediments and effluents collected from relatively uncontaminated reference sites may also be used. Preexposure responses may also be compared with those observed during exposure. Reference toxicant may serve as positive control.

14.3.2 When the purpose of the study is to calculate an endpoint, two or more toxicant concentrations should be applied during the exposure. Control treatments include dilution water or solvent water controls, or both, to which no test material has been added, or sediments and effluents collected from reference sites. It is important that the reference sites have been characterized sufficiently to ensure that minimum contamination exists. A geometric series of at least five concentrations is commonly used, with each concentration being at least 50 % of the next higher concentration. Tests using numerous treatments over a broad range of concentrations are valued since they also provide information on dose-response relationships.

14.3.2.1 In tests of single chemical compounds, the range of concentrations selected should include sublethal concentrations that are expected to occur in the environment.

14.3.2.2 In tests of effluents, a 50 % dilution series should be tested using water from an upstream or reference site as a diluent for the effluent to be tested.

14.3.2.3 Toxicity tests of field-collected sediments should include sediments collected in reference areas and areas adjacent to contaminated sites. Sediments "spiked" with the compound may be mixed with reference sediments to create a dilution series of contaminated sediments.

14.3.2.4 When limited information is available on the toxicity of the compound, sediment, or effluent, preliminary exposures should be conducted to establish the relative lethality of the toxicant.

14.4 Organisms should be assigned randomly to treatment groups, and individuals should be sampled randomly for behavioral responses during exposure.

14.5 The species and life stages selected for study should be appropriate for the problem in question. For example, early life stage organisms may display sufficient sensitivity and acclimate readily to the laboratory environment, but they would not provide information on reproductive behavior. The timing of exposure should recognize cyclical responses and circadian rhythmicity of the behavioral response.

14.6 The duration of exposure will depend on the chemical, species, and behavioral endpoint selected for study.

14.7 Behavioral responses may be measured continuously or after selected intervals of exposure, especially during biomonitoring or during avoidance and attractance tests.

14.8 Measurements of responses can be made during exposure as well as during recovery to determine the stability of response over time, as well as the extent to which the behavioral response recovers or that delayed effects occur.

14.9 The measurement of multiple endpoints will enhance the characterization of a substance's toxicity.

15. Acceptability of Test

15.1 A behavioral test generally will be rejected based on excessive mortality among controls, high variability in the behavioral response of controls, disease, or variation in water quality or experimental parameters beyond acceptable limits. The criteria for such limits will vary depending on the substance, species, and response being tested, as well as the objectives of the study.

15.2 A behavioral toxicity test should be considered unacceptable if one or more of the following occurred:

15.2.1 All test chambers (and compartments) were not identical, or were not treated as separate entities.

15.2.2 The exposure water was not acceptable to the test organisms.

15.2.3 The natural geochemical properties of test sediments or effluents collected from the field were not within the tolerance limits of the test species.

15.2.4 Appropriate negative and solvent controls, or reference sediments or effluents, were not included in the test.

15.2.5 The concentration of solvent used affected the survival, growth, or reproduction of the test organisms.

15.2.6 All animals in the test population were not obtained from the same source, were not all of the same species, or were not of acceptable quality.

15.2.7 Treatments were not assigned randomly to individual test chamber locations, and the individual test organisms were not assigned impartially or randomly to test chambers or compartments.

15.2.8 Each test chamber and replicate must contain the same amount of sediment, determined either by volume or weight.

15.2.9 The temperature, dissolved oxygen, and concentration of the test material were not measured or were not within the acceptable range.

15.2.10 Organisms exposed to negative control or reference sediments and effluents did not survive, grow, or reproduce as required for the test organisms.

15.2.11 Behavioral responses measured during the toxicity test were defined ambiguously.

15.2.12 More than 20 % of the control organisms failed to respond or were abnormal in their behavior.

15.2.13 Variability of the behavioral measurement for controls exceeded 50 % of the mean value.

16. Calculation of Test Results

16.1 The primary data to be analyzed from a behavioral toxicity test will vary depending on the response measured and may include (*1*) frequency, proportion, magnitude, or presence and absence of the behavioral response; (*2*) measures of growth, mortality, reproductive, developmental, morphological, histological, and physiological and biochemical variables; and (*3*) concentration of test material in the test solutions.

16.2 The variety of procedures that can be used to calculate the results of behavioral toxicity tests can be divided into two categories: those that test hypotheses and those that provide point estimates. No procedure should be used without careful consideration of (*1*) the advantages and disadvantages of various alternate procedures and (*2*) appropriate preliminary tests, such as those for outliers and for heterogeneity. The calculation procedure(s) and interpretation of results should be appropriate to the experimental design.

17. Report

17.1 Include the following information either directly or by reference to available documents in the record of the results of an acceptable behavioral toxicity test:

17.1.1 Name of the test and investigator(s); name and location of the laboratory; and dates of initiation and termination of the test.

17.1.2 Source of the test material; its lot number, geographical location or transect coordinants, composition (identities and concentrations of major ingredients and major impurities), known chemical and physical properties, and identity and concentration(s) of any solvent used.

17.1.3 Source of the dilution water; its chemical characteristics; description of any pretreatment; and results of any demonstration of the ability of a species to survive, grow, and reproduce in the water.

17.1.4 Source, history, and age of the test organisms; scientific name (and strain, when appropriate); name of the person who identified the organisms and the taxonomic key used; observed diseases, disease treatments, holding, acclimation, and procedures; if the organism is cultured, the number of males and females and number of nests and substrates used. If hormonal injections were used, the number of males and females used as well as the type of hormone, frequency, and timing of injections.

17.1.5 Description of the experimental design and exposure chambers (and compartments); depth and volume of solution in the chambers; number of organisms and test chambers (and compartments) per treatment; and procedure used for thinning, loading, and lighting. Also include a description of the metering system and flow rate as volume additions per 24 h.

17.1.6 Description of the behavioral procedure and apparatus used in the measurement of response; volume and quality of water used in the apparatus; method of selection of test organisms and stocking density in the experimental apparatus; procedure for lighting and temperature control; metering system; and flow rate as volume additions per 24 h.

17.1.7 Source and composition of food; concentrations of test material and other contaminants; and feeding methods, frequency, and ration.

17.1.8 Range and time-weighted average of the measured test temperature and the methods of measuring or monitoring, or both.

17.1.9 Schedule for obtaining samples of the test solutions and methods used to obtain, prepare, and store them.

17.1.10 Methods used for, and results (with standard deviations or confidence limits) of chemical analyses of water quality; and concentration of the test material, impurities, and reaction and degradation products. Include methods for validation studies and reagent blanks.

17.1.11 A table of data on the survival, growth, and behavior of the test organisms in each test chamber (and compartment) in each treatment, including the controls, in sufficient detail to allow independent statistical analysis.

17.1.12 Methods used for and results of statistical analysis of the data.

17.1.13 Summary of general observations of other effects.

17.1.14 Results of all associated toxicity tests.

17.1.15 Anything unusual concerning the test, any deviation from these procedures, and any other relevant information.

17.1.16 Published reports should include enough information to identify the procedures used and quality of the results clearly.

18. Keywords

18.1 aquatic toxicity; behavior; locomotory activities; respiration

REFERENCES

(1) Marcucella, H., and Abramson, C. I., "Behavioral Toxicology and Teleost Fish," *The Behavior of Fish and Other Aquatic Organisms*, Academic Press, New York, NY, 1978, pp. 33-77.

(2) Rand, G. M., "Behavior," *Fundamentals of Aquatic Toxicology: Methods and Applications.* Hemisphere Publishing, New York, NY, 1985, pp. 221-264; Atchison, G. J., Henry, M. G., and Sandheinrich, M. B., "Effects of Metals on Fish Behavior: A Review," *Environmental Biology of Fishes*, Vol 18, 1987, pp. 11-25; Olla, B. L., Pearson, W. H., and Studholme, A. L., "Applicability of Behavioral Measures in Environmental Stress Assessment," *Rapports et Proces-Verbaux des Re'unions Commission Internationale pour l'Exploration Scientifique de la Mer Mediterranee Monaco*, Vol 179, 1980, pp. 162-173.

(3) Little, E. E., "Behavioral Toxicology: Stimulating Challenges for a Growing Discipline," *Environmental Toxicology and Chemistry*, Vol 9, 1990, pp. 1-2; Keenleyside, M. H. A., *Diversity and Adaptation in Fish Behavior, Zoophysiology,* Vol 11, Springer-Verlag Berlin, 1979, 208 pp.

(4) Little, E. E., Flerov, B. A., and Ruzhinskaya, N. N., "Behavioral Approaches in Aquatic Toxicity: A Review," *Toxic Substances in the Aquatic Environment: An International Aspect*, American Fisheries Society, Water Quality Section, Bethesda, MD, 1985, pp. 72-98.

(5) Kane AS, Salierno JD, Gipson GT, Molteno TCA, Hunter C. Video-based movement analysis system to quantify behavioral stress response of fish. Water Research 382004:3993- 4001.

(6) Brewer et al. Brewer, S.K., Little, E.E., DeLonay, A.J., Beauvais, S.L.,Jones, S.B., Ellersieck, M.R., 2001. Behavioral dysfunctions correlate to altered physiology in rainbow trout, Oncorvnchus mykiss exposed to cholinesterase-inhibiting chemicals. *Arch. Environ. Contam. Toxicol*. 40, 2001. 70– 76.

(7) Baatrup E. Measuring complex behavior patterns in fish-Effects of endocrine disruptors on guppy reproductive behavior. *Human and Ecologicl Risk Assessment*15:53-62. 2009

(8) Beitinger, T. L., "Behavioral Reactions for the Assessment of Stress in Fishes," *Great Lakes Research*, Vol 16, 1990, pp. 495-528.

(9) "Natural Resource Damage Assessments: Final Rule," *Federal Register*, Vol 51, 1986, pp. 27674-27753.

(10) Diamond, J. M., Parson, M. J., and Gruber, D., "Rapid Detection of Sublethal Toxicity Using Fish Ventilatory Behavior," *Environmental Toxicology and Chemistry* , Vol 9, 1990, pp. 3-12; Miller, D. C., Lang, W. H., Graeves, J. O. B., and Wilson, R. S., "Investigations in Aquatic Behavioral Toxicology Using a Computerized Video Quantification System," *Aquatic Toxicology and Hazard Assessment: Fifth Conference, ASTM STP 766*, ASTM, Philadelphia, PA, 1982, pp. 206-220; Smith, E. H., and Bailey, H. C., "Development of a System for Continuous Biomonitoring of a Domestic Water Source for Early Warning of Contaminants, *Automated Biomonitoring: Living Sensors as Environmental Monitors*, Ellis Horwood, Chichester, England, 1988, pp. 182-205.

(11) Geckler, J. R., Horning, W. B., Neiheisel, T. M., Pickering, Q. H., Robinson, E. L., and Stephan, C. E., "Validity of Laboratory Tests for Predicting Copper Toxicity in Streams," *EPA-600/3-76-116*, U.S. Environmental Protection Agency, 1976; Giattina, J. D., and Garton, R. R., "A Review of the Preference-Avoidance Responses of Fishes to Aquatic Contaminants," *Residue Reviews*, Vol 87, 1983, pp. 43-90; Cherry, D. S., and Cairns, J., Jr., "Biological Monitoring, V, Preference and Avoidance Studies," *Water Research* , Vol 16, 1982, pp. 263-301.

(12) U.S. Environmental Protection Agency, "Ambient Water Quality Criterion for Chlorine—1984" *EPA 440/5-84-030* , National Technical Information Service, Springifield, VA, 1985.

(13) For information on the toxicities of various substances to aquatic life, see: *Water Quality Criteria*, Nation Academy of Sciences—National Academy of Engineering, 1972; *EPA-R3-73-033*, National Technical Information Service, Springfield, VA, 1973, pp. 172-193; U.S. Environmental Protection Agency, *Federal Register*, Vol 45, Nov. 28, 1980, pp. 79318 -79379; U.S. Environmental Protection Agency, *Federal Register*, Vol 50, July 29, 1985, pp. 30784 -30796; Mayer, F. L., Jr., and Ellersieck, M. R., "Manual of Acute Toxicity; Interpretation and Data Base for 410 Chemicals and 66 Species of Freshwater Animals," *United States Fish and Wildlife Service Resource Publication 160*, 1986.

(14) Bullock, G. L., and Stuckey, H. M., "Ultraviolet Treatment of Water for Destruction of Five Gram-Negative Bacteria Pathogenic to Fishes," *Journal of the Fisheries Research Board of Canada*, Vol 34, 1977, pp. 1244-1249.

(15) Rucker, R. R., and Hodgeboom, K., "Observations on Gas-Bubble Disease of Fish," *Progressive Fish-Culturalist*, Vol 15, 1953, pp. 24-26; Penrose, W. R., and Squires, W. R., "Two Devices for Removing Supersaturating Gases in Aquarium Systems," *Transactions of the American Fisheries Society*, Vol 105, 1976, pp. 116-118; Solderberg, R. W., "Aeration of Water Supplies for Fish Culture in Flowing Water," *Progressive Fish-Culturist*, Vol 44, 1983, pp. 89-91.

(16) Marking, L. L., Dawson, V. K., and Crowther, J. R., "Comparisons of Column Aerators and a Vacuum Degasser for Treating Supersaturated Culture Water," *Progressive Fish-Culturist*, Vol 45, 1983, pp. 81-83.

(17) American Public Health Association, American Water Works Association, and Water Pollution Control Federation, *Standard Methods for the Examination of Water and Wastewater*, 16th ed., Washington, DC, 1985, pp. 413-415; Green, E. J., and Carritt, D. E., "New Tables for Oxygen Saturation of Seawater," *Journal of Marine Research*, Vol 25, 1967, pp. 140-147.

(18) Little, E. E., and Finger, S. E., "Swimming Behavior as an Indicator of Sublethal Toxicity in Fish," *Environmental Toxicology and Chemistry*, Vol 9, 1990, pp. 13-19, Besch, W. K., Kemball, A., Meyer-Waarden, K., and Scharf, B., "A Biological Monitoring System Employing Rheotaxis of Fish," *Biological Monitoring of Water and Effluent Quality, ASTM STP 607*, ASTM, Philadelphia, PA, 1977, pp. 56-74.

(19) Sutterlin, A. M., "Pollutants and the Chemical Sense of Aquatic Organisms: Perspectives and Review," *Chemical Senses and Flavor*, Vol 1, 1972, pp. 167-178; Giattina, J. D., and Garton, R. R., "A Review of the Preference-Avoidance Responses of Fishes to Aquatic Contaminants," *Residue Reviews*, Vol 87, 1983, pp. 43-90; Cherry, D. S., and Cairns, J., Jr., "Biological Monitoring, V, Preference and Avoidance Studies," *Water Research* , Vol 16, 1982, pp. 263-301.

(20) Henry, M. G., and Atchison, G. J., "Influence of Social Rank on the Behavior of Bluegill, Lepomis macrochirus, Exposed to Sublethal Concentrations, of Cadmium and Zinc," *Journal of Fish Biology*, Vol 15, 1979, pp. 309-315.

(21) Sandheinrich, M. B., and Atchison, G. J., "Sublethal Toxicant Effects on Fish Foraging Behavior: Empirical vs Mechanistic Approaches," *Environmental Toxicology and Chemistry*, Vol 9, 1990, pp. 107-120.

(22) Brown, J. A., Johansen, P. H., Colgan, P. W., and Mathers, R. A., "Changes in the Predator-Avoidance Behavior of Juvenile Guppies (Poecilia reticculata) Exposed to Pentachlorophenol, *Canadian Journal of Zoology*, Vol 63, 1985, pp. 2001-2005; Little, E. E., Archeski, R. D., Flerov, B. A., and Kozlovskaya, V. I., "Behavioral Indicators of Sublethal Toxicity in Rainbow Trout," *Archives of Environmental Contamination and Toxicology*, Vol 19, 1990, pp. 380-385.

(23) Stafford, C. L., and Ward, J. A., "Effects of Monochloramine on Courtship and Spawning in the Cichlid Fish Etroplus maculatus," *Predators and Prey in Fishes*, Dr. W. Junk Publishers, The Hague, 1983, pp. 213-220.

(24) Hammons, A. S., *Methods for Ecological Toxicology: A Critical Review of Laboratory Multispecies Tests*, Ann Arbor Science Publishers, Ann Arbor, MI, 1981.