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Standard Guide for
Measurement of Behavior During Fish Toxicity Tests[1]

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1. Scope

1.1 This guide covers some general information on methods for qualitative and quantitative assessment of the behavioral responses of fish during standard laboratory toxicity tests to measure the sublethal effects of exposure to chemical substances. This guide is meant to be an adjunct to toxicity tests and should not interfere with those test procedures.

1.2 Behavioral toxicosis 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**). Behavior includes all of the 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, in the determination of no-observed-effect and lowest-observed-effect concentrations, and in the prediction of hazardous chemical impacts on natural populations (**3**).

1.4 The behavioral methods described in this guide include locomotory activity, feeding, and social responses, which are critical to the survival of fish (**4**).

1.5 This guide is arranged as follows:

1.6 The values stated in inch-pound units are to be regarded as standard. The values given in parentheses are mathematical conversions to SI units that are provided for information only and are not considered standard.

1.7 *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.* While some safety considerations are included in this guide, it is beyond the scope of this guide to encompass all safety requirements necessary to conduct behavioral toxicity tests. Specific hazards statements are given in Section 7.

2. Referenced Documents

2.1 *ASTM Standards:*[2]

E140 Hardness Conversion Tables for Metals Relationship Among Brinell Hardness, Vickers Hardness, Rockwell Hardness, Superficial Hardness, Knoop Hardness, Scleroscope Hardness, and Leeb Hardness
E729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians
E1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses
E1241 Guide for Conducting Early Life-Stage Toxicity Tests with Fishes
E1383 Guide for Conducting Sediment Toxicity Tests with Freshwater Invertebrates[3]
E1604 Guide for Behavioral Testing in Aquatic Toxicology

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 relate directly to the acceptability of the test. "Should" is used to state that the specified condition is recommended and ought to be

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

[3] Withdrawn.

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 *aggressive behavior*—behavioral reactions made in response to a conspecific resulting in the repulsion of individuals. Aggressive social behaviors include reactions of approaches; displays of coloration, posture, or body movements; bodily contact; or biting that results in the retreat of the responding conspecific or in the initiation of similar responses.

3.2.2 *feeding behavior*—a response resulting in the consumption of material, including orientation and movement toward the material, sucking or striking motions directed at the material, capture by mouth, spiting or holding, and swallowing of the material.

3.2.3 *locomotory behavior*—neuromuscular responses that result in movement of the fish's body or a portion of the body in space to cause a change in position or orientation in space, as well as reflexive movements of body parts.

3.2.4 *schooling or shoaling behavior*—responses of social attraction that reflect a tendency to remain in the vicinity of a conspecific.

4. Summary of Guide

4.1 This guide is intended to describe behavioral methods that can be applied during routine bioassays. Qualitative behavioral assessment procedures are intended to provide limited behavioral characterizations that require minimal facility modifications, equipment, or training and are inexpensive to conduct. Quantitative behavioral assessments are more rigorous measurements of behavior and are intended for laboratories that have an interest in behavioral testing and can provide limited modifications of facilities and conventional video recording equipment and limited staff training.

4.1.1 Qualitative behavioral screening of spontaneous behavioral activity provides a broad view of toxicant effects during exposure to contaminants. Abnormal behavioral responses observed among fish are documented on a daily basis using a behavioral checklist that includes categories of responses such as lack of feeding, lethargic or frenzied activity, abnormal swimming movements or postures, and lack of response or hyperreactivity to external stimuli (**5**). The behavioral aberrations are based on the absence of response and on obvious differences from the response of untreated fish. Although no attempt is made to quantify the magnitude of response, the consistent observation of response over time provides a quantitative measurement of the response. Early detection of behavioral abnormalities may warrant quantitative measures of specific behavioral patterns.

4.2 Quantitative measurements of locomotory, feeding, and social behaviors of fish can be conducted during standard laboratory exposures, including static, flow-through, sediment, and food exposures from direct observation or overhead video recordings to determine the effects of sublethal exposure (**6**). These behavioral responses are highly sensitive to sublethal exposure and are relevant to survival (**7**). Data are obtained to determine the effects of toxic substances on behavior from short (for example, 96 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 tests provide information concerning the sublethal effects of chemicals and signal the presence of toxic test substances.

5.1.1 The locomotory, feeding, and social responses of fish are adaptive and essential to survival. Major changes in these responses may result in a diminished ability to survive, grow, avoid predation, or reproduce and cause significant changes in the natural population (**8**). Fish behavioral responses are known to be highly sensitive to environmental variables as well as toxic substances.

5.2 Results from behavioral toxicity tests may be useful for measuring injury resulting from the release of hazardous materials (**9**).

5.3 Behavioral responses can also be qualitatively assessed in a systematic manner during toxicity tests to discern trends in sublethal contaminant effects (**5**).

5.4 The assessment of locomotory, feeding, and social behaviors is useful for monitoring effluents and sediments from contaminated field sites as well as for defining no-effect concentrations in the laboratory or under controlled field conditions. Such behavioral modifications provide an index of sublethal toxicity and also indicate the potential for subsequent mortality.

5.5 Behavioral toxicity data can be used to predict the effects of exposure likely to occur in the natural environment (**10**).

5.6 Results from behavioral toxicity tests might be an important consideration when 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.

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

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

5.9 The behavioral characteristics of a particular organism need to be understood and defined before a response can be used as a measure of toxicity (**11**). Swimming, feeding, and social behavior varies among species as well as among life stages within a species; the most effective test methods are therefore those tailored to a particular life stage of a single species. The range of variability of any behavioral response of unexposed organisms is influenced by genetic, experiential, physiological, and environmental factors. It is thus important to avoid selecting test organisms from populations that may vary in these factors.

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

5.11 No numerical value or range of values has been defined as the norm for swimming, feeding, or social behavior for any fish; the detection of abnormal activity is therefore based on comparisons of the responses of exposed fish, either with activity measured during a baseline or pre-exposure period or observations of fish under a control treatment (**10**).

5.12 These measures are incorporated readily into standard toxicity test protocols, with minimal stress to the test organism.

6. Interferences

6.1 A number of factors can suppress, elicit, or alter locomotory, feeding, and social responses and thus influence behavioral test results and complicate data interpretation. The following factors should be considered 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 behavioral responses.

6.1.5 Social status, such as the dominance or sex of the individuals tested, and experiential factors, such as prior experience with the predator or prey species, can influence the behavioral response. Individuals tested in isolation may respond differently from when tested in groups.

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

6.1.7 The behavioral response can be affected by the apparatus design and 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 It is very important to eliminate disturbances to the test system, such as vibrations, slamming doors, casting shadows, abrupt changes in lighting, or water flow, that may frighten the fish or disrupt ongoing activity.

6.1.10 Behavioral responses to toxic substances may subside over time.

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

6.1.12 Generally, excessive mortality among controls (see Guides E729 and E1241), high variability in the behavioral response of controls, disease, or variation in water quality or experimental parameters beyond acceptable limits, and inconsistent visualization of the organism are the basis for rejecting a behavioral test. The criteria for such limits will vary depending on the substance, species, and response being tested, as well as the objectives of the study. Guide E1604 should be consulted regarding the acceptability of behavioral test results.

7. Safety Precautions

7.1 Many substances may pose health risks to humans if adequate precautions are not taken. Information on toxicity to humans, recommended handling procedures, and the chemical and physical properties of the test material should be studied and all personnel informed before an exposure is initiated. (**Warning—** Special procedures might be necessary with radiolabeled test materials and with test materials that are, or are suspected of being, carcinogenic.)

7.2 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 the following: (*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.

8. Responses Measured

8.1 Qualitative changes in behavior can be assessed during the course of toxicant exposure by observing changes in responses such as feeding inhibition, lethargic or frenzied activity, abnormal swimming movements or postures, lack of response or hyperreactivity to external stimuli, abnormal coloration, heightened or inhibited aggression, or aberrant respiratory patterns and coughs (**5**).

8.2 *Locomotion*—Locomotory responses are essential to survival in most organisms and are often very sensitive to hazardous substances (**10**). Disruption of locomotory behavior can impair the ability of fish to perform essential life functions that might rely on agile, efficient, and vigorous swimming. Variables of locomotory behavior commonly measured during standard toxicity tests include the frequency and duration of activity, form and posture of locomotion, and larval development of locomotion. In addition, movements of the organism unrelated to locomotion, including postures and grooming movements, as well as tremors and spasms, may be observed during toxicity tests.

8.3 *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. Feeding behavior includes variables such as orientation to the food material; movement toward, striking, or sucking movements used to capture the material; oral contact with, and acceptance of, the material as indicated by consumption or rejection (spitting) of the material, as well as latency of response to prey or food material; and the maximum distance from which the organism responds to prey, prey selectivity, feeding efficiency, and prey-handling time, strike, and capture frequencies (**12**).

8.4 *Social*—Aggression and social attraction (shoaling) are observed commonly in captive fishes.

8.4.1 Aggressive responses play an important role in the dispersion of individuals and distribution of habitat resources. Aggressive responses of an individual result in the displacement of a conspecific. Variables involved in aggressive responses include changes in posture, coloration, or body movements and movements toward, or contact between, conspecifics, which results in the displacement of one individual, but most commonly measure the frequency and magnitude of aggressive interactions. Bodily contacts include bites as well as nudging or pushing of one individual against another. Displacement can include rapid retreat from an area, change in position within the water column, or reduced individual distance, that is, the characteristic three-dimensional volume of space surrounding an individual (**13**).

8.4.1.1 Stress arising from aggressive interactions may potentiate the toxicity of a chemical substance during toxicity tests.

8.4.2 Shoaling (schooling) plays an important role in the formation of aggregations to minimize predation and to facilitate feeding or reproduction (**14**). Shoaling responses are measured as nearest neighbor distances, or volume of space occupied by the aggregation. Other variables measured during laboratory toxicity tests include the rapidity and density of aggregation in response to an external stimulus (for example, tap on aquaria wall) and the duration of aggregation following the stimulus (**15**).

9. Test Organisms

9.1 The 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.

9.1.1 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 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.

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

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

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

9.1.5 All organisms in a test should 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.

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

9.1.7 In the event that the fish have been disturbed, there should be a reasonable period of time after the disturbance has occurred before the behavioral observations are made. A resumption of ongoing activity, unrestricted movement within the chamber, resumption of feeding, decrease in schooling, recovery of coloration, or posture or return to the behavioral condition that existed before the disturbance can be used to judge recovery from the disturbance.

10. Facility

10.1 *Facilities*—The test facility is that used for standard toxicity tests that are conducted routinely in the laboratory. Descriptions of such facilities appear in ASTM documents, including the following: Guides E729, E1023, E1241, E1383, and E1604. These provide guidance on construction materials, water and air delivery systems, test chambers and cleaning, and water supply.

10.2 *Water Supply*—The requirements for dilution water used in behavioral toxicity tests, and water used to hold the organisms before testing, should be acceptable to the test species and uniform in quality, and they must allow satisfactory survival, without inducing signs of disease or apparent stress, such as discoloration or unusual behavior. These requirements must follow those established for toxicity tests delineated in Guides E729, E1023, E1241, and E1383, and Tables E140.

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

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

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

10.4 *Test Chambers*—Behavioral observations are made directly in the exposure vessel during standard toxicity tests (**16**). ASTM standards such as Guides E729, E1023, E1241, E1383, and E1604 should be consulted regarding the construction and cleaning of exposure chambers.

10.4.1 The behavioral observations will normally be conducted from an overhead view of the organisms within each test chamber. Such observations require a clear, unobstructed, and continuous view of the organism. Some modifications of the exposure chamber may therefore be necessary to facilitate the behavioral observations.

10.4.2 Modifications to the standard toxicity test facilities may be required to ensure a clear, unobstructed, continuous observation of the fish for qualitative measurements. Such modifications may include the mounting of overhead mirrors or the addition of an overhead track or cable to which a video camera can be mounted to provide an unobstructed image of the fish. Water, air, or effluent supply lines and distribution boxes may need to be removed from the field of view temporarily for an unobstructed view of the fish. Exposure jars may need to be replaced with containers having openings of the same dimensions as the sides. If the fish can be tracked consistently at different depths within the chamber, a shallower exposure chamber or isolation to a standard depth may be required. Partitioned areas may also be added temporarily to facilitate the observation.

10.4.3 When such modifications are not possible, a sample of fish may be moved to an observation chamber to conduct the behavioral observation. The observation chamber should be of a size that does not limit the movements of the fish but is viewed readily by the video camera. The fish will require a period of recovery from handling (see 9.1.7).

10.4.4 For quantitative measurements, a video camera should be mounted over the exposure chambers to provide the overhead view of the fish. The most useful mounting would be an overhead track that would allow movement of the camera over each test chamber.

10.4.5 The fish should contrast sufficiently with the exposure chamber to be observed readily and continuously. Clear chambers should have a bottom covering to provide contrast. The covering should be a neutral pastel, such as grey or beige. This will eliminate unnecessary background images, which is particularly important if computer-assisted assessment procedures are used.

10.4.5.1 Contrast within the exposure chambers could be achieved by constructing the chambers of opaque material, painting the external surface of the chamber bottom, or covering the bottom with a self-adhesive vinyl plastic. It is important that such applications be uniform and prevent air bubbles, and so forth, which may obscure the image of the fish. The exposure chambers could also be placed directly over a solid background material. These materials should not be in contact with the exposure water. Consideration should be made as to the durability of these materials to withstand customary cleaning as well as the expense and ease of their replacement.

10.4.5.2 It is also important that the field of view observed through the video camera provide a continuous view of the fish. Fish moving out of the field of view during the observation would invalidate the measurement. This can be accomplished by appropriate vertical positioning of the camera above the exposure chamber, by the selection of camera lenses, including macro, wide-angle, or telephoto lenses, and by selecting exposure chamber dimensions to facilitate a continuous view of the fish.

10.4.5.3 It may be necessary to partition a portion of the exposure chamber for the purpose of observing the behavioral response. Temporary partitions could be added prior to the observation period. The partitions should be constructed of materials that do not contain substances that can be leached or dissolved in amounts that affect the test organisms adversely. The materials should be chosen to minimize the sorption of test materials. Partitions 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 (see Guide E729).

10.5 *Video Equipment*—Any video recording equipment commonly available for consumer use is sufficient for recording behavior during aquatic toxicity tests (**7**). Features most important for recording behavior depend on the lens and lighting combinations that will produce a clear picture in sufficient detail. The following equipment and materials may be required:

10.5.1 *Standard ½-in. (12.5-mm) VHS Video Recorder*, with camera or camcorder.

10.5.2 *Telephoto Zoom Lens* (12.5 to 75 mm, 1:1.4)—A standard lens for most TV cameras is sufficient for recording juvenile fish ranging from 2 to 5 cm in length. An 8.5-mm wide-angle camera lens (with C-mount adaptor) may be necessary when recording broad areas. A macro lens (50 mm, 1:3.5) is useful for recording the response of larval fish but may require a restriction of space.

10.5.3 *VHS ½-in. (12.5-mm) Video Tape*, by any manufacturer.

10.5.4 *Fish-Holding Chamber,* or diluter aquaria, with contrasting background.

10.5.5 *Overhead Camera Track,* or tripod-mounted camera, positioned for overhead view.

10.5.6 *Glass Partitions,* for isolating fish within the holding chamber.

10.5.7 *Stopwatch.*

10.5.8 *Material Such as Cardboard,* for shading in the event of glare.

10.6 A video camera or camcorder is mounted on a track above the exposure chambers, or a tripod-mounted camera is positioned above the exposure chambers. The camera is moved overhead from one chamber to another, and a video recording of each chamber is made for an interval of time. Information on fish swimming, feeding, and social behaviors is obtained during playback of the video tape.

11. Qualitative Behavioral Assessment Method

11.1 Behavioral screening methods provide a qualitative assessment of the spontaneous behavioral activity of fish during exposure and involve the daily use of a behavioral checklist to document responses such as lack of feeding, lethargic or frenzied activity, abnormal swimming movements or postures, and lack of response or hyperreactivity to external stimuli (**5**). The behavioral aberrations are based on the absence of response and on obvious difference from the response of untreated fish. There are no controls for the abnormal response of untreated fish other than the absence of grossly aberrant responses. Although no attempt is made to quantify the magnitude of response, the consistent observation of response over time provides a quantitative measurement of the response. The early detection of behavioral abnormalities by this screening method may warrant subsequent quantitative measures of specific behavioral patterns.

11.2 Direct observation of fish in the exposure chamber is conducted during this screening procedure, and no additional equipment is thus needed. However, video recording with an overhead video camera can be used to create a permanent record of the response.

11.3 The checklist (Fig. 1) indexes categories of response, including the following (**5**):

Location in water column	confined to bottom, mid water column, confined to surface
Swimming posture	swims on side, head-up swimming
Mode of swimming	swims on side, frequent sinking or rising, swims in circles or spirals, serpentine body movement, loss of equilibrium, tremors, convulsions
Swimming activity	hyperactivity, fast swimming, lethargy, or stationary
Excitability	unresponsive, hyperresponsive (jumps or swims into aquarium walls) to external stimuli
Feeding	no response, or limited feeding
Social	frequent bites or chases (for example, bluegill), loss of schooling (for example, fathead minnow)
Respiration	exaggerated gill movement, rapid gill movement, frequent coughs
Morphological	coloration very light/very dark, partial body coloration, bent spine, lesions, fin erosion, excess mucus

11.4 Observations are conducted daily or several times per week during the exposures of fish to a dilution series of effluent or toxicants (**16**). Controls are unexposed fish held under similar conditions. The observer evaluates each treatment group for several minutes by each response indexed on the checklist. Abnormal responses are noted by a checkmark on the survey form if more than four fish or 10 % of the test population in the replicate treatment group exhibits the response.

11.5 Observations are conducted at the same time each day and should be made prior to or 1 h after daily activities that might stress the fish. The observer must avoid startling the fish; if ongoing activity is interrupted, the observer should wait until the fish resume movement in the exposure chamber or are calm, if agitated. Overhead video recordings can also be made at this time. A count of the number of fish responding in an abnormal manner should be made if the sample size is small. Rough estimates of feeding (for example, 25, 50, and 75 %) from the amount of food remaining 30 to 60 min after feeding can also be made if uniform rations are provided to each treatment group at feeding.

11.6 Repeated observations of control groups may facilitate the recognition of abnormal responses among exposed fish. If in doubt concerning the nature of the response, note on the checklist, and make a second observation of the control group.

11.7 The all or none nature of the data, and the lack of quantification of the number of fish responding, limit statistical testing to Probit or Logit procedures or categorical data analyses (**17, 18**). Because these methods are used to determine trends and to characterize gross behavioral changes, consistent observation of the response over daily observations is critical in defining the abnormality. Daily survey sheets should be reviewed for the occurrence of abnormal responses by three to four fish per replicate treatment (or 10 % of the exposure group). Only obvious abnormalities should be considered; subtle responses for which the observer was uncertain should not be considered further. Spurious responses of several individuals are not considered further. Responses that persist over time and that show dose response in terms of when they are initiated during the exposure are most likely to be detected by this method (**19**). The data are plotted as the date or duration of exposure when the abnormality first appears consistently during the exposure relative to the exposure concentration. For example, if lethargic activity among the fish exposed to Concentration *X* was observed on Day 4 of exposure, on Day 8 for Concentration *Y*, and on Day 10 for Concentration *Z*, then the date of the first occurrence for lethargic activity would be plotted as Day 4 for Concentration *X*, Day 8 for Concentration *Y*, and Day 10 for Concentration *Z*. Intermittant responses lasting 1 to 2 days may also be plotted, depending on the investigator's confidence in the observation.

12. Quantitative Behavioral Measurements

12.1 Aberrant behavioral responses can be assessed quantitatively through measures of specific behavioral responses, including swimming activity, feeding, and social responses (**16**). These measures are incorporated into standard toxicity test protocols readily, with minimal stress to the test organism. The use of video tape recordings is strongly recommended to minimize the handling of test organisms and the interference of behavior by the presence of the observer. Modifications to the facility may be required to facilitate such observations (see 10.4.2). Alternatively, advanced digital image analysis software, available from several commercial sources, provides an effective means of analyzing behavioral data from video images, particularly for the analysis of swimming paths. (**20, 21, 22**)

12.2 Exposures are conducted on a replicated series of dilutions of an effluent or of a single toxicant. Responses can be measured several times during the exposure to provide information on how the response changes with the duration of exposure, occurrence of delayed toxic effects, and extent to which abnormal behaviors recover. Fish that do not demonstrate strong social tendencies such as schooling or aggressive interactions can be tested in groups. The responses of aggressive fish and those that school will be influenced by social