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

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ε[1] NOTE—Warning notes were editorially moved into the text in August 2003.

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 either inch-pound or SI units are to be regarded as the standard. The values given in parentheses are for information only.

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:*

E 140 Standard Hardness Conversion Tables for Metals Realtionship Among Brinell Hardness, Vickers Hardness, Rockwell Hardness, Superficial Hardness, Knoop Hardness, and Scleroscope Hardness[2]

E 729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians[3]

E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses[3]

E 1241 Guide for Conducting Early Life-Stage Toxicity Tests with Fishes[3]

E 1383 Guide for Conducting Sediment Toxicity Tests with Freshwater Invertebrates[4]

E 1604 Guide for Behavioral Testing in Aquatic Toxicology[3]

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,

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[2] *Annual Book of ASTM Standards*, Vol 03.01.

[3] *Annual Book of ASTM Standards*, Vol 11.04.

[4] Discontinued, replaced by E 1706. See 1994 *Annual Book of ASTM Standards*, Vol 11.05.

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 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 E 729 and E 1241), 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 E 1604 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 E 729.

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 E 729, E 1023, E 1241, E 1383, and E 1604. 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 E 729, E 1023, E 1241, and E 1383, and Tables E 140.

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 E 1241.

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 E 1383.

10.4 *Test Chambers*—Behavioral observations are made directly in the exposure vessel during standard toxicity tests **(16)**. ASTM standards such as Guides E 729, E 1023, E 1241, E 1383, and E 1604 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 E 729).

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). The video recording equipment is readily available for purchase or rental, easy to operate, and permits analyses not possible through direct observation.

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 interactions; responses measured among individuals within a group should therefore be assessed as multiple observations of a group.

12.3 *Preparation for Video Observation for Swimming Activity, Feeding, and Social Behavior*—Successful, error-free analysis of the video tape requires a high-contrast image of the fish against its background, with a minimum of structure or clutter in the background that may obscure or hide the image of the fish. The image should be in good focus and free of surface glare or distortions from moving water. The fish need to be present within the field of view continuously. Food should be withheld for 4 to 6 h prior to the observation period. Food, feces, and other debris should be removed from the chamber 2 h prior to recording.

12.3.1 Toxicant delivery lines and other materials obscuring the full field of view should be removed at least 30 min before

Date: Time of Observation: Observer:

	EXPOSURE CHAMBER											
	1	2	3	4	5	6	7	8	9	10	11	12
Location in Aquaria												
confined to bottom												
mid to upper water column												
water surface												
Mode of Swimming												
stationary												
swims on side												
serpentine swimming												
head-up swimming												
frequent sinking and rising												
Feeding												
Slow response to food												
no response												
Activity/Excitability												
hyperactive												
lethargic												
unresponsive												
Morphological												
bent spine												
hypermucosity												
light/dark coloration												
Other (aggression, schooling,												
respiration)												

NOTE 1—Redrawn from Drummond, et al **(5)**.

FIG. 1 Daily Behavioral Checklist

observation. Air and water flow should be adjusted to ensure that water surface distortions do not obscure the image of the fish. The fish should not be chased, netted, or stressed unduly at this time. The fish should be allowed to recover for at least 2 h before conducting the observation in the event of such disturbances.

12.3.2 Video tape is loaded in the video recorder in a fully rewound position, and the tape footage indicator is reset to zero. The video tape cartridge should be labeled clearly with the date, study number, and description, with corresponding information added to the data sheet. The overhead camera is brought into position and focused such that the perimeter of the chamber is framed by the video image. The image is checked to ensure a high-quality, glare- and shadow-free image. Water flow to the chamber should be stopped during the recording.

12.3.3 If the size of the chamber is too large to be included completely within the field of view, or if the organisms are too small to be viewed easily within the field of view, the fish should be confined within a smaller area by adding glass partitions to the chamber and framing this partitioned area within the field of view, as described in Step 5. The camera lens may also need to be changed to create a suitable image.

12.3.4 Several chambers can be videotaped within the same field of view if the resulting video image of the individual fish is large enough to provide consistent observation.

12.3.5 From five to ten fish will be isolated within the partitioned area. The fish are allowed to recover from handling for 2 h prior to observation. Resumption of activity, or movement throughout the area, is a good indication of recovery from handling.

12.3.6 The video recording begins with a 10-s recording of a title card showing the date and treatment code of the fish to be observed. The starting footage is recorded on the data sheet, along with the study code, date, and treatment.

12.3.7 A 2 to 4-min timer is started, and the chamber is video taped for that period of time. At this time, notes on the behavioral activity of the organisms can be made for reference when data are collected during playback of the tape.

12.3.8 The tape footage is noted on the data sheet at the conclusion of this interval. Food may then be added to the chamber. The food material is spread evenly over the surface of the partitioned area. The timer is reset and the recording continued for 5 min. This recording will be used to quantify the feeding activity.

12.3.9 Food items may include commercial food or brine shrimp that the fish are fed routinely. Two important considerations for food selection are that definite strikes (mouth closure and lateral or forward head movements) are observed and that fish readily eat the food item with a minimum latency. The feeding movements of larger fish may be too subtle to quantify feeding, and it may be necessary in these cases to use a larger food item, such as an adult *Daphnia magna*, to evoke a more definite strike. Preconditioning may be necessary for novel food to be eaten readily. The novel food item is available to each group of fish during preconditioning over a period of several days to allow the fish an opportunity to capture and consume the food.

12.3.10 While feeding is being recorded, one fish within the field of view should be monitored by counting the number of strikes the fish makes at the food over a 3-min period. The strike frequency should be recorded on the data sheet, along with the tape footage reading observed at the end of the 5-min recording.

12.3.11 At the conclusion of this interval, partitions are removed and rinsed thoroughly, airstones and toxicant supply lines to the chamber are replaced, and the water flow is restored. The camera can then be moved into position over the next exposure chamber for video observation of another treatment group.

12.4 *Measures of Swimming Activity from Video Recording*:

12.4.1 A fully rewound videotape is placed in the video recorder, and the footage indicator on the video recorder is reset to 0000.

12.4.2 Playback is started and advanced to the picture of the treatment identification card displaying the experiment, data, and treatment.

12.4.3 At the conclusion of the identification card recording, a 1-min timer is activated and the videotape is played for 1 min. At the end of the 1-min playback, the PAUSE button on the recorder is activated for a still-frame image, and the tape footage is noted on the data sheet. It is necessary to replay the video tape to quantify the activity of each fish when two or more fish are recorded in the field of view. This requires identification of each fish as well as an accurate starting point from which to begin the playback and the behavioral measurement. To accomplish this, the PAUSE button on the record is activated to create a still-frame image on the screen, and a felt-tipped marker is used to mark the position of each fish in view with a number. The mark should be made directly on the monitor screen and should be positioned at the anterior-most position of the fish.

12.4.4 One fish is selected, and the 2-min timer is started simultaneously along with the video recorder.

12.4.5 Several methods can be used to quantify swimming activity, depending on how active the fish are.

12.4.5.1 *Cumulative Frequency of Moves*—The swimming behavior of intermittently active fish such as juvenile bluegill or larval fathead minnows can be measured by counting each time the fish starts movement during the 2-min period **(20)**.

12.4.5.2 *Duration of Activity/Inactivity*—The swimming activity of species that swim with long bouts of movement, such as rainbow trout, can be measured by timing the duration of continuous activity or inactivity **(4)**. A stopwatch is to be activated each time the fish initiates a bout of activity to record the cumulative duration of movement during the 2-min interval.

12.4.5.3 *Frequency of Grid Crossing*—Rapidly moving organisms may be especially difficult to measure, particularly when several individuals are in the field of view, and spatial aspects of the movement can be measured in such cases. A grid is drawn on the monitor screen to bisect the image of the exposure chamber into four equal quadrants. During replay of the videotape, a crossing is recorded each time the fish crosses a grid line by one body length. A cumulative total for the

chamber is recorded when the activity is too frequent to track individuals accurately.

12.4.6 The cumulative frequency or duration of response is recorded for each individual at the conclusion of the observation period.

12.4.7 To assess the next individual, the videotape is rewound to the initial start point, and the images of the fish are aligned with the head position numbers marked earlier on the monitor screen. The next fish is selected, and swimming activity is measured.

12.4.8 Contaminant impacts to other swimming variables such as distance traveled or speed of swimming may be indicated but are somewhat more difficult to quantify. Distance can be measured by tracing the path of a fish's movement directly on the video monitor during the slow advance of the video playback, and then the length of the path of travel is measured. Speed is calculated by dividing the distance traveled by the total time of the playback. Computer-assisted methods can also be used to assess these parameters of locomotory behavior **(21, 22)**.

12.4.9 The frequency of other body movements such as tremors **(23)**, as well as stretching or fin-cleaning movements **(13)**, can also be recorded from the overhead video. Tremors include a rapid, jerky movement of the tail and caudal fin, which may involve the entire body and occur mainly when the fish initiates movement. Tremors should not be confused with the S-shaped body postures observed occasionally during aggressive interactions. Tremors do not include coughs (gill purge reactions), feeding movements, or movements of dorsal or pectoral fins.

12.5 *Measures of Feeding Behavior from Video Playback*—Feeding behavior is highly sensitive to sublethal contaminant exposure, and reduced feeding activity can be quantified from overhead video recordings, along with swimming activity.

12.5.1 Fully rewound videotape is placed in the video recorder, and the footage counter is reset to 00000.

12.5.2 The playback is advanced to the image of the treatment identification card for the first treatment group, and information on the playback card is confirmed on the data sheet.

12.5.3 The videotape is advanced to the point at which the food was added to the chamber, the video recorder is then paused to produce a still image (press PAUSE), and that footage is indicated on the data sheet.

12.5.4 Each fish in the field of view is identified by marking a number on the video screen at the head of each fish.

12.5.5 A fish is selected for analysis, and the 5-min stopwatch and video recorder are started simultaneously.

12.5.6 Each feeding movement made by the fish is counted during the 5-min period until the stopwatch alarm sounds, the recorder is then stopped, and the cumulative frequency of feeding movements and the videotape footage is entered on the data sheet.

12.5.7 The feeding sequence includes orientation toward the food item, the approach, a lunge or similarly distinctive attack movement, and mouth closure **(24)**. Although the most sensitive assessment would include measurements of each variable of the feeding sequence, the lunge movements are detected most readily in early life-stage fish.

12.5.8 At the conclusion of the feeding measurement, the videotape is rewound to the initial start point, and the images of the fish are aligned with the head position numbers on the monitor screen. Another fish is selected, and the feeding movements are counted as before.

12.5.9 When all fish of a treatment group have been analyzed for feeding activity, the videotape is advanced to the data card of the next treatment group, and Steps 5 through 8 are repeated.

12.6 *Measures of Aggressive Behavior*—Swimming behavior and other responses may be disrupted by aggressive interactions among the individuals of an exposure group. The aggressive behavior can be used as a measure of toxicity in such cases **(13, 23)**.

12.6.1 Observations of aggression can be made of current "live" responses viewed directly from the TV monitor or retrieved from videotape.

12.6.2 The monitoring of "live" activity begins at the activation of the timer for the 3 to 4-min video sample interval.

12.6.3 To measure aggression from the video playback, the fully rewound videotape is placed in the VCR, and the footage indicator is reset to zero.

12.6.4 The videotape is advanced to the tape footage position of the selected treatment group to the beginning of the sample interval where the treatment identification card is shown.

12.6.5 Monitoring of activity begins when the data card is removed from the field of view, at which time the alarm timer is activated for the 3 to 4-min observation period.

12.6.6 The number of aggressive interactions is counted during the 3 to 4-min interval. Aggressive interactions include all interactions between two fish, where the movement of one fish results in the movement or reorientation of another away from the first. An approach is the lowest level of aggression and is the movement of one fish toward another resulting in the retreat of the second fish. A chase is the rapid advance of one fish and concomitant rapid retreat of the second fish. A nudge is the intentional physical snout contact of an advancing fish against the body of another fish. A bite is open jawed contact of an advancing fish with the body of another fish. A bite is the highest level of aggression.

12.6.7 At the end of the sample period, the number of aggressive encounters is entered on the data sheet, along with a qualitative score from 1 to 4 of the average intensity of interaction observed for that group, with 1 representing a low intensity of aggression, with a predominance of approach-avoidance interactions, and 4 representing a high intensity of aggression with a predominance of bites.

12.6.8 If significant trends of aggressive intensity with treatment are revealed from this analysis, the footage should be reanalyzed by recording the score for the intensity of each interaction.

12.6.9 Aggressive encounters for each individual fish should be conducted when aggressive activity is too frequent to assess the entire group of fish.

13. Experimental Design

13.1 The experimental design for the behavioral study will follow that of the toxicity test for which the exposure is being conducted.

13.2 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 the power of a significance test therefore 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 test results: (*1*) all exposure chambers should be treated as similarly as possible, such as temperature and lighting (unless these are the variables tested); (*2*) each exposure chamber, including replicate exposure chambers, must be treated physically 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.

13.3 *Statistical Analysis*—Each treatment will generally be replicated at least once in the toxicity test, and behavioral data will be obtained from five to ten fish of each replicate treatment group. Data on the duration of swimming activity should be arsine transformed since the response reflects the proportion of time the fish are active during a defined observation period. The frequency of activity, strikes at prey, and aggressive interactions should be square root transformed to normalize the distribution of data, particularly when the data include zero values. Analysis of variance and Fisher's Least Significant Difference or Duncans Multiple Range tests are performed using SAS General Linear Models procedures (Statistical Analysis Systems, 1982), with one degree of freedom used for each replicate treatment group. A split-plot in time design is preferable when behavioral observations are made at selected exposure intervals. Measures over time will provide a more accurate measure of effective dose and will increase the likelihood of detecting behavioral aberrations as well as determine potential acclimation to the toxicant. Measures during exposure, as well as during recovery, will determine the stability of response over time, as well as the extent to which the behavioral response recovers, or that delayed effects occur.

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

13.5 The measurement of multiple endpoints will enhance the characterization of a substance toxicity.

13.6 Guide E 1604 should be consulted regarding other considerations of the experimental design.

14. Calculation of Test Results

14.1 The primary data to be analyzed from behavioral observations made during a laboratory toxicity test will include frequency, proportion, magnitude, or presence and absence of the behavioral response.

14.2 Commonly, statistical tests are used to determine which of the tested concentrations of a test material caused a statistically significant difference in behavior from the control treatment. The data should be tested for heterogeneity, and appropriate transformations of proportional or percent data should be conducted. A pair-wise comparison technique, analysis of variance, or multiple comparison procedure appropriate to the experimental design should then be used. Presentation of the results of such tests should include the test statistic and its corresponding significance level, as well as an indication of variance. Refer to Guides E 729 and E 1241 for additional guidance on statistical analyses.

14.3 Point estimates such as the EC50 and corresponding confidence interval are occasionally reported for behavioral data, but these may not always be applicable, given the fact that a bimodal response can occur with varying concentrations and durations of exposure.

15. Report

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

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

15.1.2 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 the identity and concentration(s) of any solvent used.

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

15.1.4 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, history, and age; if a brood stock was used, observed diseases, disease treatments, holding, acclimation, and culture procedures (if appropriate), number of males and females or number of nests and substrates used, if natural spawning was used. If hormonal injections were used, the number of males and females used as well as the type of hormone and frequency and timing of injections.

15.1.5 Description of the experimental design and exposure chambers (and compartments), depth and volume of the solution in the chambers, number of organisms and test chambers (and compartments) per treatment, procedure used

for thinning, loading, and lighting, a description of the metering system, and the flow rate as volume additions per 24 h.

15.1.6 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 the test organisms, and stocking density in the experimental apparatus, procedure for lighting, temperature control, description of the metering system, and flow rate as volume additions per 24 h.

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

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

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

15.1.10 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.

15.1.11 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 permit independent statistical analysis.

15.1.12 Methods used for, and results of, statistical analysis of the data.

15.1.13 Summary of general observations of other effects.

15.1.14 Results of all associated toxicity tests.

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

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

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