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Standard Guide for Ventilatory Behavioral Toxicology Testing of Freshwater Fish[1]

This standard is issued under the fixed designation E1768; 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 information on methods to measure and interpret ventilatory behavioral responses of freshwater fish to contaminants.

1.2 Ventilatory responses are often some of the first prelethal symptoms exhibited by animals to environmental stressors (**1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, **9**, **10**).[2] Continued, abnormal ventilatory behavior (that is, rapid or shallow breathing, erratic breathing) can indicate physiological damage that may be irreversible. Such damage could eventually result in decreased survival, growth, or reproduction of the organism, or all of these.

1.3 Ventilatory responses of some fish species can be measured relatively easily and quickly, providing a useful tool for biomonitoring studies of wastewaters, pure chemicals, surface water, and ground water.

1.4 Appropriate studies of ventilatory responses can yield definitive endpoints such as no observable effect concentration (NOEC) or an EC_{50}, often more rapidly than standard toxicity test methods (**11**, **12**).

1.5 The mode of action of test substances and the type of chemical toxicant can be determined by examining ventilatory behavioral responses in conjunction with other physiological responses (**8**, **9**, **10**, **11**, **12**).

1.6 Fish ventilatory behavior can be assessed in real-time using appropriate computer hardware and software (**12**, **13**, **14**, **15**, **16**, **17**, **18**, **19**). Such systems have proved useful for long-term, on-line monitoring of wastewater effluents, pure chemicals, and surface waters (**12**, **15**, **20**, **21**, **22**, **23**, **24**, **25**). These systems are usually technically complex and will not be discussed in this guide.

1.7 Given the technological constraints of electrical components, it is currently not feasible to monitor bioelectric signals, such as those elicited in ventilatory behavior, in saline (>2 ppt) or high conductivity (>3000 µmhos/cm) water using the procedures discussed in this guide. Therefore, this guide is restricted to the testing of freshwater matrices.

1.8 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this 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.* For specific safety precautions, see Section 6.

1.10 This guide is arranged as follows:

2. Referenced Documents

2.1 *ASTM Standards:*[3]

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

E943 Terminology Relating to Biological Effects and Environmental Fate

E1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians

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

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[2] The boldface numbers given in parentheses refer to a list of references at the end of the text.

[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.

E1241 Guide for Conducting Early Life-Stage Toxicity Tests with Fishes
E1604 Guide for Behavioral Testing in Aquatic Toxicology

3. Terminology

3.1 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 only used 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 a 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 *cough*—gill purge in fish; when a fish reverses or greatly increases the flow of water over the gills and back out to the ambient water. Such activity is used to cleanse the gills by removing particles or other material on the gill plate(s).

3.2.2 *electrode*—device (metallic or chemical based) that receives bioelectric signals from the organism.

3.2.3 *ventilation*—breathing, respiratory process of organism.

3.2.4 *waveform*—representation of analog electrical signal depicting breathing response of organism over time, usually represented on a strip chart recorder or computer monitor.

4. Summary of Guide

4.1 The potential toxicity of water or a pure chemical in water is assessed by measuring changes in fish ventilatory behavior during exposure using a flow-through system. Significant effects are determined by comparing specific ventilatory responses of fish under control conditions with responses of those same fish during exposure conditions. A set of control fish may also be used in the test design in order to evaluate non-toxic changes in ventilatory response over time, particularly when longer-term monitoring is desired.

4.2 Ventilatory responses are observed by using non-invasive metallic or chemically-based electrodes, a signal amplification and filtration system, and strip chart recorder (or other recording device) to display the ventilatory waveform. In short-term tests (<24 h in duration), changes in ventilatory behavior to exposure of a test material can be analyzed after the test by manually analyzing strip chart recordings of the waveform elicited over time by each fish. In experiments >24 h in length or in continuous real-time monitoring applications, ventilatory waveform data are aquisitioned, analyzed, and stored via a microcomputer equipped with an analog to digital processor, disk or magnetic tape storage, and appropriate software. With the aid of a computer and analog to digital board, responses can be monitored and analyzed on a real-time basis. The computer-analyzed response reduces potential subjective biases due to manual analysis of strip-chart recordings.

5. Significance and Use

5.1 Responses that reflect oxygen consumption or utilization have often been targeted as useful indicators of incipient toxic conditions (**26**, **27**, **28**, **29**, **30**). In addition, sustained acute fish ventilatory behavioral responses reflect a physiological change in the organism and therefore might have ecological relevance.

5.2 For some time, the technological means have been available to log and display ventilatory signals over time. As a result, there are a considerable number of studies which examined ventilatory behavior of fish and other aquatic organisms. A large number of substances at lethal levels have been shown to elicit ventilatory responses relatively quickly (**13**, **19**, **20**, **31**, **32**, **33**, **34**). For many pollutants, a significant response was often generated in less than 1 h of exposure to concentrations approaching the 96 h LC50. Studies performed using subacutely toxic samples of effluents or individual pollutants (concentrations well below the reported LC50 concentration), often documented responses within 1 to 10 h of exposure (**11**, **18**, **21**, **30**, **35**, **36**).

5.3 Given the data obtained thus far, it appears that fish ventilatory behavior may be a very sensitive and rapid indicator of acute toxicity if various aspects of this behavior (that is, rate and amplitude) are assessed and analyzed simultaneously. It appears that the more aspects of ventilatory behavior that are assessed, the more sensitive and rapid the system is (**11**, **12**, **21**, **22**).

5.4 Although a variety of organisms have been examined including crayfish (**37**), aquatic insect larvae (**31**), and bivalves (**13**), most research in aquatic ventilatory behavior has used freshwater fish species. This is largely because fish are generally more ecologically "visible" in their importance in aquatic systems and many species (particularly the salmonids and centrarchids) have large opercular flaps that yield relatively clear ventilatory signals for measurement and evaluation. Species eliciting relatively small bioelectric ventilatory signals are more difficult to use given the electrode and amplification systems referenced in this guide.

5.5 Changes in ventilatory behavior have been shown to be a reliable indicator of accidental toxic spills or "slugs" of pollutants in wastewater and drinking water systems (**15**, **20**, **23**, **24**, **33**).

6. Safety Precautions

6.1 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. (**Warning—**Special procedures might be necessary with radiolabeled test materials and with test materials that are, or are suspected of being carcinogenic.)

6.2 Many materials can adversely affect humans 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 manipulating test materials under a ventilated hood or in an enclosed glovebox, enclosing and ventilating the exposure chambers, and use of respirators, aprons, safety glasses and gloves.

7. Responses Measured

7.1 Ventilatory parameters in fish that have been shown to be affected by toxicity include ventilatory rate (opercular movement over time), depth of ventilation (amplitude), coughing or gill purge rate, and erratic episode frequency due to sudden movement of the organism. Most commonly, changes in ventilatory rate (F_v) have been used as a bioindicator of toxic conditions (**11**, **12**, **13**, **19**, **20**, **21**, **30**, **31**, **33**, **34**, **35**, **36**). However, depth of ventilation and cough rate have been reported to be more sensitive indicators of toxicity for some compounds (**11**, **19**, **38**, **39**, **40**).

7.2 Manually, changes in ventilatory rate are often determined by changes in the number of peaks per unit area on a strip-chart recording. Depth of ventilation (tidal volume) or signal amplitude, is measured from top to the bottom of the waveform (see Fig. 1).

7.3 Cough rate has been more difficult to determine because several different types of coughs may be evident, each with its own characteristic wave form pattern (see Fig. 1 and (**11**, **39**, **40**)). Also, without the use of video techniques, (**11**, **41**), the actual occurrence of a cough is not always clear. Researchers who have investigated cough responses have interpreted most abnormal peaks or pattern changes on a strip-chart recording as a cough. Work by Diamond et al. (**11**) however, indicated that many of these changes may in fact be due to general activity, and not coughing responses. Various aspects of monitoring fish coughs have been reviewed by Drummond and Carlson (**40**).

7.4 Erratic episode frequency or activity episode frequency has also proved to be a useful response in some studies (**11**, **12**, **21**). These episodes are represented on a strip chart recording as a multi-peak, high frequency (and often high amplitude) cluster of signals which can be easily distinguished from the normal ventilatory signal (Fig. 1).

8. Test System

8.1 Several techniques have been developed to monitor fish ventilatory behavior. The simplest and most reliable method monitors the bioelectric potentials generated during ventilatory movements by means of noninvasive electrodes (**11**, **12**, **16**, **20**, **21**, **29**) or silver/silver chloride (**15**). These electrodes generally consist of stainless steel wire or screen and are attached to the monitoring chamber such that the fish is not restrained or stressed (see Fig. 2).

8.2 The spatial orientation of the electrodes within the monitoring chamber affects the intensity with which the ventilatory signal is received and recorded. Since reception of the bioelectric signal is dependent on there being a polarity or electrical gradient between the electrodes, electrodes are placed opposite each other in the monitoring chamber to achieve maximum sensitivity. Several different electrode arrangements have been utilized including top and bottom of the chamber (see Fig. 2(*a*) and (**12**, **15**, **21**)), front and back (**29**), and sides of the chamber (see Fig. 2(*b*) and (**11**, **14**, **20**)). Each of these arrangements may have advantages and disadvantages in terms of signal reception and the ability to detect subtle changes in amplitude, body movement, or cough rates. Information at this time suggests that a top and bottom electrode arrangement (see Fig. 2(*a*), will reduce ventilatory signal alteration due to changes in fish position relative to the electrodes in comparison with a side electron orientation (**12**). Test chambers must be clean prior to testing as described in Practice E729, and made of appropriate construction materials such as glass or plexiglass (see Guide E1241 and Practice E729).

8.3 Test organisms and chambers must be isolated so as to reduce external stimuli such as experimenter movement, vibration, and visual cues. This is generally achieved by placing a single fish in each chamber and by placing opaque dividers between test chambers. The entire system (all test chambers) should be isolated within a light-proof box or continuous light compartment.

8.4 The electrical signal (microvolts), generated by ventilatory movements, that is received by the electrodes, must be conditioned prior to use. First, the electrical components of the system must be properly grounded to avoid erratic signal reception. Second, electrical noise, particularly that arising from normal 60 cycle electrical current (such as from lights, strip chart recorder, and amplifier), must be eliminated so that the fish ventilatory signal is received with minimal interference. Third, the bioelectric signal must be amplified from microvolts to millivolts in order to electrically record the signal. A capacitor is also usually required to reduce ventilatory signal offset from the baseline (0 V) level and thereby ensure that the entire waveform is recorded. Instrumentation has been described and documented that accomplish signal conditioning for use in fish ventilatory systems (**14**, **15**, **16**, **18**, **22**). The conditioned signal, once obtained is interfaced to a strip chart recorder or computer, or both, for data collection and analysis (see Fig. 3 and (**13**, **14**, **16**, **17**, **20**, **22**)). The ventilatory signal appears as a distinct pattern on a strip-chart recorder (see Fig. 1). Changes in this pattern in the presence of toxic materials are considered toxic responses if: control organisms exposed simultaneously to control water only do not elicit similar response changes, the same fish previously exposed to control water only (<3 h before receiving the test water), exhibited normal ventilatory patterns/responses (see 9.5 and 10.3), and water quality and external physical environmental factors are relatively constant.

8.5 Dilution water in testing must be acceptable to the fish and in adequate supply (see Guide E729 and Guide E1241). A flow-through system should be used. Dilution water or a pollutant is introduced into the test chamber by a gravity feed system from a head-box or via pumps (see Guide E1604). Flow

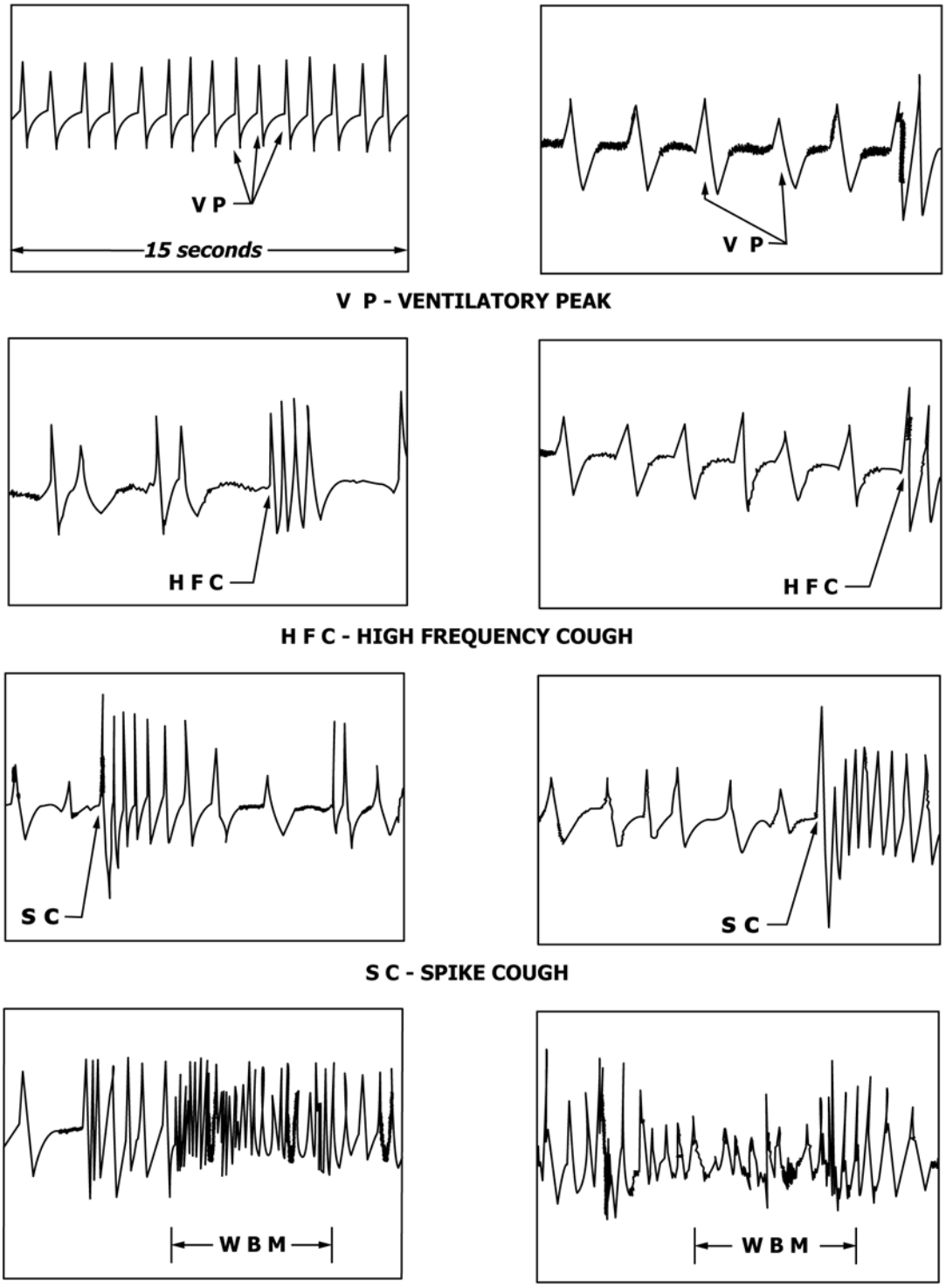


FIG. 1 Typical Ventilatory Signal Recordings

rate will depend on the study objectives and test system design. Flow rate must be adequate in order to maintain dissolved oxygen levels.

8.6 Photoperiod will effect ventilatory behavioral responses in fish (**19**, **20**, **36**) and therefore is a factor that needs to be considered in any fish ventilatory experiments lasting more

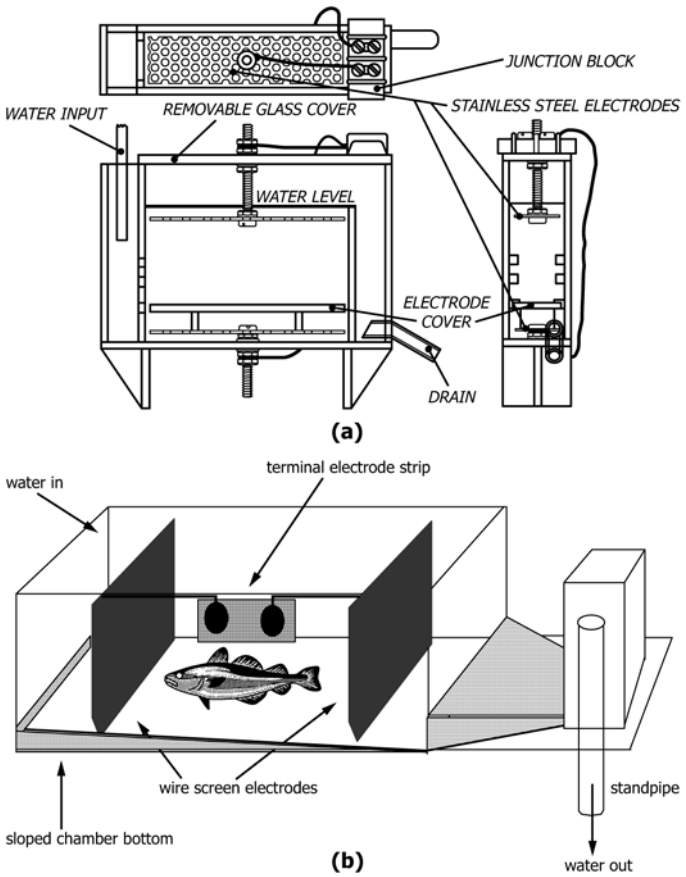
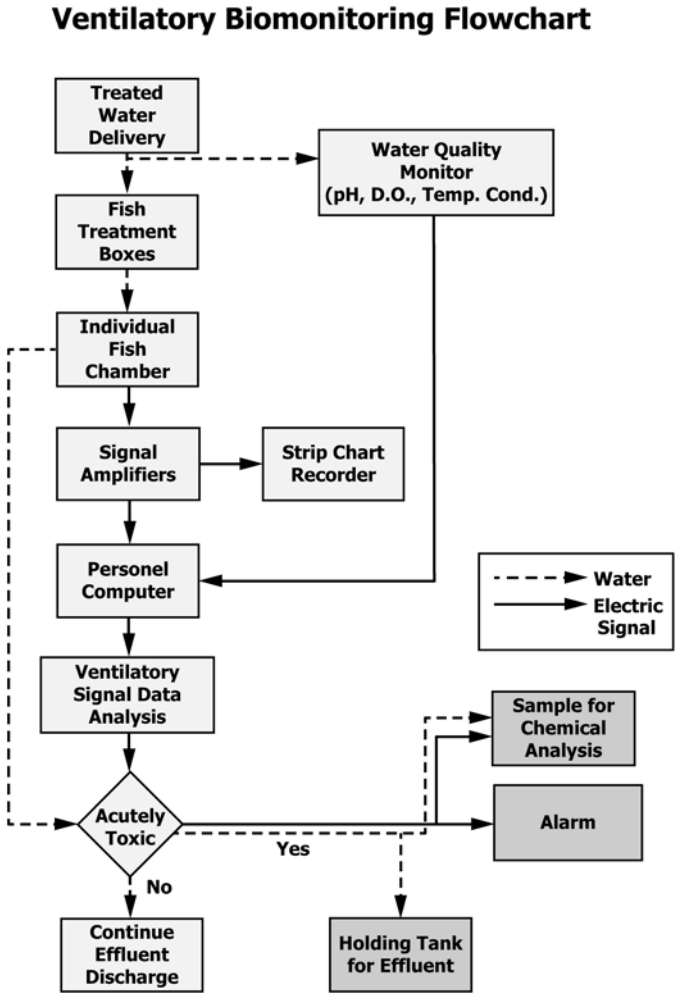


FIG. 2 Diagram of Exposure Chamber Designs Used in Fish Ventilatory Behavior Testing



NOTE 1—Solid lines with white arrows depict water flow through the system. Dotted lines with black arrows depict the signal and data flow.

FIG. 3 The Biomonitoring System

than 1 to 2 h. Most researchers have chosen to dampen the effects of photoperiod by utilizing either a constant light (**12, 18, 21**) or constant dark regime (**11, 20**). In either case, fish should be acclimated to the photoperiod of the test system prior to the experiment (see Practice E729 and Guide E1604).

8.7 Test organisms must not be diseased or injured and must be obtained from uncontaminated field sites or, preferably, from contaminant-free cultures. Organisms must be acclimated to the test water and conditions of the test apparatus (see 9.2). All organisms should be uniform in age and size and obtained from the same source (see Guide E1241 and Practice E729).

8.8 A list of potential test organisms is presented in Table 1. Many of these species are commercially available or easily cultured, or both. Furthermore, juvenile life stages of some of these species have been shown to be sensitive to some pollutants and, therefore, might be appropriate species for examining pollutant effects. There are few studies comparing the relative sensitivity of fish species in ventilatory behavior testing. Most studies have used bluegill sunfish (*Lepomis macrochirus*) as the test species probably because they are widely available, they are easily maintained over a wide range of temperatures and pH, and they have relatively large opercular flaps which elicit a strong ventilatory signal. Furthermore, juvenile bluegill (<6 cm length) have been shown to be relatively sensitive to a number of pollutants (**11, 25, 29**). Fish species with very small opercular flaps (minnows for example) or having naturally high activity rates or erratic breathing patterns, or both, are not recommended as test organisms using this procedure.

TABLE 1 Summary of Freshwater Species That Have Been Utilized in Respiratory Behavioral Toxicity Testing

Taxonomic Name	Common Name	Types of Tested Pollutants[A]
Oncorhynchus *mykiss*	rainbow trout	metals, pesticides, hydrocarbons, effluents (**1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 21, 24, 35, 39, 40**)
Lepomis macrochirus	bluegill	metals, pesticides, effluents, hydrocarbons (**11, 12, 14, 20, 22, 25, 29, 30, 35, 38**)
Oncorhynchus gorbuscha	pink salmon	oil, hydrocarbons (**34**)
Orconectes sp.	crayfish	metals, effluents (**37**)
Hexagenia limbata	hexagenia	metals, effluents, pesticides (**13**)
Hydroperla crosby	stonefly	pesticide (**31**)
Cordalis cornutus	hellgranite	pesticide (**31**)
Notemigonus crysoleucas	golden shiner	pesticide (**31**)
Salvelinus fontinalis	brook trout	suspended solids (**38**)

[A] The references in parentheses are located at the end of the text.

8.9 Fish ventilatory behavior is affected by water quality factors. For example, some data indicate that rainbow trout

may be very sensitive to nontoxic changes in temperature (**32**). Clearly, this type of problem needs to be considered when studying specific toxicant responses. Temperature might affect certain ventilatory behavior responses of bluegill and brook trout (*Salvelinus fontinalis*) as well (**38**). Therefore, it is important to maintain a similar water temperature in all fish monitoring chambers throughout an experiment.

9. Test Procedure

9.1 Organisms should not be fed during testing unless study objectives dictate that feeding is necessary (such as in long-term exposures and on-line monitoring applications) since feeding activity will obscure ventilatory signals.

9.2 Organisms must be acclimated to the experimental conditions prior to testing and data collection. The appropriate length of the acclimation period will vary with the fish species, life stage, and type of monitoring system utilized. Previous work by Gruber et al. (**20**), and Diamond et al. (**11**) using juvenile bluegill, constant darkness, and a completely isolated monitoring system indicated at least a 24 to 48 h acclimation period (under control conditions) prior to any data collection. Work by Shedd et al. (**12**) using juvenile bluegill, constant light and a semi-isolated monitoring system indicated a 72 h acclimation period. Improper acclimation of the test organisms can increase observed variability in control fish responses, masking any pollutant-induced changes in ventilatory response over time. Proper fish acclimation is indicated by a slow, consistent signal over time with very few indications of coughs or movement (see Fig. 1). As a guide, baseline (control) frequencies for rainbow trout and bluegill are approximately 1.5 and 1.0 Hz (breaths/second), respectively (**15**, **19**, **21**, **24**) and will vary with fish age/size, water temperature, and specific features of the monitoring system. Shedd et al. (**12**) suggest that fish acclimation to the test system is indicated when ventilatory depth or amplitude is constant over prolonged time periods (hours). If proper acclimation is not evidenced under control conditions, one of the following procedures should be utilized:

9.2.1 Extend the acclimation period for at least 24 h and continue monitoring organisms. If organisms are still not displaying acclimated ventilatory behavior, investigate one or more of the following:

9.2.2 Re-examine culture history of organisms and determine whether disease or other anomaly is present. If so, discard the batch of organisms and restart the study with a new batch.

9.2.3 Examine the test system to be sure that visual or vibratory influences, or both, are absent or negligible.

9.2.4 Examine the electrical components of the test system to be certain that connections are secure and that electrical signal noise is minimized.

9.2.5 For studies lasting ≥24 h, fish might need to be acclimated to the test system photoperiod prior to use in the ventilatory system. Studies by Shedd et al. (**12**) and van der Schalie (**18**) suggest a two week acclimation period using a constant light photoperiod.

9.3 Care should be taken to control sources of variability other than the pollutant effect desired. Therefore, water flows, lighting, temperature, and so forth must be similar among test chambers (see Guide E1192). Placement of treatments within the study area should be randomized to minimize extraneous effects on the study design.

9.4 The number of fish required per treatment will vary with the study objectives and the experimental design. Two general types of study designs have been commonly employed in fish ventilatory behavioral studies: those monitoring individual fish responses over short periods of time (<24 h) and those monitoring the group response of individual fish exposed over long time periods to either control water or the test water (for example, effluent, drinking water intake). The latter employ automated computer monitoring due to the large quantity of data produced (**12**, **13**, **19**, **21**, **28**). In this design (see Fig. 3), four to eight fish have been used for each treatment, and individual fish responses are analyzed over time (using a moving average or other statistical approach) in order to detect whether a change in behavior has occurred for each fish. Changes recorded for exposed fish are then compared to the changes recorded during the same time interval for control fish. If the exposed fish (or some predetermined percentage of the exposed fish) exhibit a significant change in behavior while control fish (or some predetermined percentage of control fish) do not, it is inferred that the behavioral change observed in exposed fish was due to the test material.

9.4.1 Those studies using a short-term test design monitor individual fish responses before and after introduction of a toxicant (**11**, **22**, **29**, **32**). These studies are typically less than 24 h in length. A minimum of three replicate chambers are recommended per treatment including controls in this test design. Regardless of the type of test design, test organisms should be of similar size, age, and preferably from the same culture batch. Organisms should be randomly assigned to chambers (treatments).

9.5 Control data for all fish used in a given experiment should be collected following acclimation over at least a 1 h period prior to dosing with the test material in short-term tests. Long-term studies typically necessitate a longer control data acquisition period (≥96 h; (**12**)). Longer control periods may be necessary for some test species and monitoring systems.

9.6 The test water should be delivered to test chambers using the same system used for the control water and must be at the same flow rate and temperature. Control organisms continue to receive control water. Monitoring should continue over at least the next hour in all chambers. Longer exposures (24 to 96 h) may be desirable or necessary if low pollutant doses are used or chronic toxicity estimation is being investigated.

9.7 Since the test water gradually replaces the control water in test treatments over time, exposure conditions are characterized by performing periodic chemical evaluations of water leaving the fish chambers. The frequency of chemical monitoring will depend on the flow rate and chamber volume used in the test system. Turnover rates or volume replacement time can be calculated (**11**) for test chambers, which will determine the frequency of chemical monitoring.

9.8 It might be useful to provide a recovery period in which control water is delivered to all chambers, especially when

sublethal conditions are being monitored. The time period for recovery should be at least as long as the exposure period.

9.9 Once used in a ventilatory test, fish must not be reused in any other tests.

10. Data Collection and Analysis

10.1 Data can be collected either manually using a multichannel strip chart recorder or in an automated manner using a microcomputer equipped with a multichannel multiplexer, analogue-to-digital electronic converter, appropriate software, and enough disk space or magnetic tape to store the data from each fish. Manual data collection is satisfactory for short-duration tests (<24 h) but is unwieldy to interpret and may yield biased endpoints for longer tests. Computer monitoring can adequately collect data for any test duration and real-time monitoring but must be validated, using simultaneous manually-collected data, prior to use (**12**, **18**, **20**). If strip chart recordings are obtained, it is useful to divide the data into 5 or 10 min segments. This reduces investigator bias in analyzing the data if subsets of the data are used. If the test is very short (4 h or less), all data should be used in quantifying response variables for each organism. For longer tests, it might be necessary to measure variables in randomly selected segments from the control, exposure (and recovery) phases of the test.

10.2 At a minimum, the following parameters must be measured for each time period or phase of the experiment:

10.2.1 Mean frequency (ventilatory rate or peaks/min),

10.2.2 Mean amplitude (ventilatory depth or volts/peak),

10.2.3 Cough frequency (coughs/min), and

10.2.4 Swimming activity episodes (high frequency bursts/min).

10.2.5 Other parameters which might be useful to analyze include orientation shifts (polarity changes in the waveform (only using the side electrode placement)), changes in the waveform signal not evidenced as coughs or activity episodes (such as waveform flutters), and variability in frequency or amplitude over time.

10.3 Due to inherent variability in ventilatory behavior among individual fish, it is advisable to compare pre-exposure (control) conditions with post-exposure conditions, for each fish individually rather than comparing mean responses of exposed fish with those of control fish at a given time. Averaging responses of several fish in a given treatment (including controls) typically yields large variances which reduce the sensitivity of the test.

10.4 Sophisticated statistical techniques might be necessary in order to evaluate response data if: the ventilatory response variable being measured is not an "all-or-none" response (such as ventilatory frequency, swimming activity, or cough frequency) or a relatively high degree of sensitivity to pollutants is necessary, or both. In test designs comparing individual fish responses before and after short-term exposure to a toxicant, one computes the difference or ratio of response values for each fish in a given treatment (including controls) with its own control values prior to exposure at pre-determined time intervals. A mean difference or ratio is then computed for a given ventilatory response variable for fish in each treatment at a given time. This mean difference or ratio is then compared to the mean difference or ratio observed in control fish using an *a posteriori* means test or linear contrast if select comparisons are to be examined. To examine the response over multiple time intervals, a repeated measures ANOVA statistical design can often be used (**11**) with the above data. If enough time data points are available for each fish, trend analysis or time series evaluation can be effective statistical tools (**19**, **41**).

10.5 Under acutely toxic conditions, fish have been reported to respond with increased ventilatory frequency regardless of the type of test material. Acutely toxic doses of pesticides (**4**, **6**, **9**, **28**, **31**), heavy metals (**1**, **2**, **7**, **11**, **29**), petroleum products (**21**, **33**, **35**), and various wastewater effluents (**14**, **20**) have elicited this response in rainbow trout and bluegill. In many of these instances increased ventilatory frequency is accompanied by increases in cough rate (**11**, **22**, **41**). Diamond et al (**11**) also reported increased erratic activity and body quivers under acutely toxic exposures to certain pesticides.

10.6 Under sub-acutely toxic exposure conditions, greater separation of behavioral responses has been observed with different types of pollutants. Diamond et al. (**11**) and Van der Scalie et al (**18**) observed that increased ventilatory frequency and decreased ventilatory depth were the primary responses of bluegill and rainbow trout exposed to sub-acutely toxic doses of various metals. For chlorinated hydrocarbons such as trichloroethylene or dieldrin, increased cough rate was the primary ventilatory response in bluegill (**11**). McKim et al (**8**, **9**) observed different ventilatory responses of rainbow trout to sub-acutely toxic doses of phenols, pesticides, and various organic pollutants.

11. Interferences

11.1 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 when measuring behavioral responses during toxicity tests:

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

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

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

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

11.1.5 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.

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

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

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

11.1.9 Behavioral responses to toxic substances may subside over time.

11.2 In addition to the potential interferences cited above, other factors can decrease or suppress the ability of the monitoring system to accurately receive and record the bioelectric signals elicited by the fish:

11.2.1 High conductivity or saline test water (>2 ppt. salinity) will reduce the polarity between the electrodes regardless of their spatial orientation in the test chamber, resulting in a dampened or non-existent signal.

11.2.2 Nonshielded or improperly grounded electrical circuits will increase the noise-signal ratio resulting in poorly interpretable signals and lack of test sensitivity.

11.2.3 Excessive physical vibrations, noise, or other stimuli may increase the electrical noise of the system and affect fish responses.

12. Acceptability of Test

12.1 Generally, excessive mortality among controls (Practice E729 and Guide E1241), high variability (>50 % C.V. for any one type of response) in the behavioral response of controls, disease, or variation in water quality or experimental parameters beyond acceptable limits 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.

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

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

12.2.2 The dilution water was not acceptable to the test organisms.

12.2.3 Appropriate negative and solvent controls were not included in the test.

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

12.2.5 Individual test organisms were not impartially or randomly assigned to test chambers or compartments.

12.2.6 Temperature, dissolved oxygen, and concentration of test material were not measured, or within the acceptable range.

12.2.7 Test water was not delivered at the same flow rate or temperature as the negative control water.

12.2.8 Fish were not acclimated to negative control water prior to the test.

12.2.9 Disturbances such as vibrations, slamming doors, casting shadows, abrupt changes in lighting, or water flow were not minimized or eliminated.

12.2.10 Mean frequency, amplitude, cough frequency, and swimming activity episodes were not measured for each test fish.

13. Documentation

13.1 The record of the results of an acceptable behavioral toxicity test should include the following information either directly or by reference to available documents:

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

13.1.2 Source of test material, its lot number, geographical location or transect coordinates, 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.

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

13.1.4 Source, history, and age of test organisms, scientific name (and strain when appropriate), name of person who identified the organisms and the taxonomic key used, history, and age; if a brook 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, report the number of males and females used as well as type of hormone and frequency and timing of injections.

13.1.5 Description of experimental design and exposure chambers (and compartments), the depth and volume of 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.

13.1.6 Description of behavioral procedure and apparatus used in measurement of response. Volume and quality of water used in the apparatus, method of selection of test organisms and stocking density in experimental metering system, and flow rate as volume additions per 24 h.

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

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

13.1.9 Schedule for obtaining samples of test solutions and methods for validation studies and reagent blanks.

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

13.1.11 A table of ventilatory data of the test organisms in each test chamber (and compartment) in each treatment, including the controls, in sufficient detail to allow independent statistical analysis.

13.1.12 Methods used for and results of statistical analysis of data.

13.1.13 Summary of general observations of other effects.

13.1.14 Results of all associated toxicity tests.

13.1.15 Anything unusual about the test, any deviation from these procedures, and any other relevant information.

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

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