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

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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:

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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.

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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.)

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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 E729). Flow

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