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Standard Guide for Conducting Toxicity Tests with Bioluminescent Dinoflagellates[1,2]

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1. Scope

1.1 This guide covers two distinct procedures, based on similar principles, for obtaining data concerning the adverse effects of a test material (added to dilution water) on oceanic bioluminescent dinoflagellates.

1.1.1 The endpoint for both procedures is based on a measurable reduction or inhibition in light output from the dinoflagellates. Both procedures are similar in that when bioluminescent dinoflagellates are exposed to toxicants, a measurable reduction in bioluminescence is observed from their cells following mechanical stimulation when compared to control cells. In the first procedure, cells of the bioluminescent dinoflagellate *Gonyaulax polyedra* can be tested over a range of up to seven days of exposure (or longer) to a toxicant. The second procedure uses another species, *Pyrocystis lunula*, for a 4 h test.

1.2 Both procedures can measure the toxic effects of many chemicals, various marine and freshwater effluents, antifouling coatings, leachates, and sediments to bioluminescent dinoflagellates (**1-5**).[3] Compounds with low water solubility such as large organic molecules may be solubilized with methanol, ethanol, and acetone solvents for testing (**4**) (see Guide E729).

1.3 An IC50 in light output (bioluminescence) is the recommended endpoint (**1**). However, percent inhibition of bioluminescence is an appropriate endpoint in some cases (**5**).

1.4 Other modifications of these procedures might be justified by special needs or circumstances. Although using appropriate procedures is more important than following prescribed procedures, results of tests conducted using unusual procedures are not likely to be comparable to results of other tests. Comparison of results obtained using modified and unmodified versions of these procedures might provide useful information concerning new concepts and procedures for conducting acute and chronic tests.

1.5 The values stated in SI units are to be regarded as the standard.

1.6 *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.*

2. Referenced Documents

2.1 *ASTM Standards:*[4]

D1141 Practice for the Preparation of Substitute Ocean Water
D5196 Guide for Bio-Applications Grade Water
E178 Practice for Dealing With Outlying Observations
E729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians
E1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians
E1218 Guide for Conducting Static Toxicity Tests with Microalgae
E1241 Guide for Conducting Early Life-Stage Toxicity Tests with Fishes
E1733 Guide for Use of Lighting in Laboratory Testing

3. Terminology

3.1 *Definitions:*The words "must,""' should," "may," "can," and "might" have very specific meanings in this guide.

3.1.1 *can*—is used to mean is (are) able to.

3.1.2 *may*—is used to mean is (are) allowed to.

3.1.3 *might*—is used to mean could possibly.

[1] This guide is under the jurisdiction of ASTM Committee E47 on Biological Effects and Environmental Fateand is the direct responsibility of Subcommittee E47.01 on Aquatic Assessment and Toxicology.

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[2] This standard Guide is a document developed using the consensus mechanisms of ASTM, that provides guidance for the selection of procedures to accomplish a specific test but which does not stipulate specific procedures.

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

[4] 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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3.1.4 *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.

3.1.5 *should*—is used to state that the specified condition is recommended and ought to be met if possible.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *bioluminescence*—production of light by living organisms due to an enzyme-catalyzed chemical reaction.

3.2.2 *dark phase*—that part of the daily cycle (night) when dinoflagellates are not being exposed to ambient light and produce the greatest levels of bioluminescence when stimulated.

3.2.3 *dinoflagellate*—unicellular, eukaryotic, flagellated fresh or marine organisms that have photosynthetic and non-photosynthetic species. Dinoflagellates have brownish plastids containing chlorophyll *a*, chlorophyll *c*, and a mixture of carotenoid pigments, including peridinin that is unique to this phylum.

3.3 *IC50*—a statistically or graphically estimated concentration of test material that, under specified conditions, is expected to cause a 50 % inhibition of a biological process (such as growth, reproduction, or bioluminescence) for which the data are not dichotomous.

3.4 *lux*—a unit of illumination equal to the direct illumination that is everywhere 1 m from a uniform point source of one candle intensity or equal to 1 $^{1m}/_{m2}$.

3.5 *Pyrocystis lunula mutant*—a mutant that produces 30 % greater light than its progenitor.

4. Summary of Guide

4.1 *Experimental Design*—A dinoflagellate test intended to allow calculation of an IC50 usually consists of one or more control treatments and a geometric series of at least five concentrations of test material. In the medium or solvent control(s), or both, dinoflagellates are exposed to medium to which no test material has been added. Samples are usually diluted from the highest tested concentration through a series of dilutions to 6.25 % of the highest tested concentration. Except for the controls and the highest concentration, each concentration should be at least 50 % of the next higher one unless information concerning the concentration-effect curve indicates that a different dilution factor is more appropriate. At a dilution factor of 0.5, five properly chosen concentrations are a reasonable comprise between cost and the risk of all concentrations being either too high or too low.

4.1.1 The primary focus of the physical and experimental design and the statistical analysis of the data is the experimental unit, which is defined as the smallest physical entity to which treatments can be independently assigned (**6**). As the number of test cuvettes (experimental units) increases, the number of degrees of freedom increases, and, therefore, the width of the confidence interval on a point estimate decreases and the power of an hypothesis test increases.

4.1.2 With respect to factors that might affect results within the test chamber and the results of the test, all cuvettes in the test should be treated as similarly as possible. For example, within the test chamber, the temperature affecting each test cuvette should be as similar as possible unless the purpose of the test is to study the effect of light or temperature. Prior to the test replicates are usually arranged into rows. Placement of the cuvettes must be randomized.

4.1.3 The minimum desirable number of test chambers and cell density per treatment should be calculated from the expected variance among test cuvettes and either the maximum acceptable width of the confidence interval on a point estimate or the minimum difference that is desired to be detectable using hypothesis testing (**7**). A coefficient of variation of 10 % or less in light production between cuvettes is desirable, however, reliable toxicity trends can be observed with a coefficient of variation as high as 30 %.

4.2 *Summary*—If the sample has a salinity of less than 33 ± 2 g/Kg (parts-per-thousand), commercial grade aquarium sea salt may be added directly to the water sample to bring it into this range. Testing of the dinoflagellates is accomplished by placing individual cuvettes containing the test material, medium, and cells into a darkened test chamber which is attached to a photomultiplier tube (PMT). The top of the test chamber must be removable and house a small motor that drives a steel shaft terminating in a propeller. The propeller is seated into each cuvette and, as the contents are stirred at a constant voltage, bioluminescence is generated and measured by the PMT. At the end of each stir period, the accumulated "PMT counts" are shown on an LED display. Each test period is completed at preset intervals thereafter until completion of the toxicity test. *Pyrocystis lunula* mutant can be used to conduct a 4 h acute test. *Gonyaulax polyedra* can be used in tests conducted for four to seven days or longer depending on the purpose of the test. Mean light output (stimulated bioluminescence expressed as PMT counts) is calculated for each treatment and control. Light output means (as percent of controls) are plotted against time. An IC50 can be estimated for each days of the test (**1**).

5. Significance and Use

5.1 Protection of aquatic species requires prevention of unacceptable effects on populations in natural habitats. Toxicity tests are conducted to provide data to predict what changes in viable numbers of individual species might result from similar exposure in the natural habit. Information might also be obtained on the effects of the material on the health of other species. Bioluminescent dinoflagellates represent an important eucaryotic group which are widely distributed in the oceanic environment.

6. Hazards

6.1 Many materials can affect humans adversely if precautions are inadequate. Therefore, skin contact with all test materials, solutions, and leachates should be minimized by such means as wearing appropriate protective gloves (especially when washing equipment or putting hands in test solutions), laboratory coats, aprons, and safety glasses. Information on toxicity to humans (**8**), recommended handling procedures (**9**), and the chemical and physical properties of the test material should be studied before a test is begun.

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6.2 Disposal of stock solutions and test solutions might pose special problems in some cases. Therefore, health and safety precautions and applicable regulations should be considered before beginning a test.

6.3 To prepare dilute acid solutions, concentrated acid should be added to water, not vice versa. Opening a bottle of concentrated acid and mixing with water should be performed only in a well-ventilated area or under a fume hood.

7. Laboratory Equipment

7.1 *Facilities*—The culture trays for the dinoflagellates, a microscope for estimating the cell stock density, the preparation of test materials, leachate preparation, pipetting of the cells into cuvettes, and the testing of the dinoflagellates with an appropriate test chamber-PMT combination. Sufficient laboratory counter space should accomodate "wet" preparation of all stock solutions, cell counting, and culture of the dinoflagellates. The glassware should be clean rinsed with a high quality water such as deionized or distilled. The dinoflagellates must be maintained in a temperature incubator of 18 to 20°C as abrupt changes in their temperature could effect the viability of the cells and their light output. The incubator must be fitted with cool white fluorescent bulbs (40 watts each) to provide illumination of approximately 1075 lux to *Pyrocystis lunula* and 4000 lux to *Gonyaulax polyedra* (4000 lux is the approximate equivalent of 6 to 10 w/m^2; see Guide E1733) for the growth of the photosynthetic dinoflagellates. The light fluence should be monitored adjacent to various test chambers at the height of the surface of the test solutions. Light fluence must not deviate by more than 10 % from the desired level. Layers of cheese cloth or screen material may be used to attenuate light incident upon flasks and test chambers, if necessary. A timer should be provided to turn the lights on and off in a prescribed 12:12 h (light:dark) cycle. For convenience of testing in the laboratory, the cells can be exposed to light during the hours from 2200 to 1000. The cells would then be in their dark phase from 1000 to 2200. The cells are most stimulable 3 to 5 h into their dark phase and consequently produce maximum levels of light (bioluminescence) during this period. Cells must be shielded from ambient room lights during their dark phase and during testing. A black cloth may be used for this purpose. The operator may conduct tests in a darkened laboratory with a red light for ease of operation. This practice can prevent unnecessary exposure of the test organisms to light, which could cause unpredictable bioluminescence.

7.2 *Culture and Test Chambers*—Optical grade disposable spectrophotometric cuvettes or clear, borosilicate sample vials should be used as test chambers. Cultures should be maintained in borosilicate Erlenmeyer flasks. All vials and flasks should be seawater-aged for several days prior to first time use. Disposable cuvettes should be soaked in deionized water for several hours prior to use in a test. Disposable cuvettes should be discarded following the test.

7.3 *Bioluminescence Measurement System*—One possible configuration for a toxicity test system uses a 2-in. diameter RCA 8575 photomultiplier tube (PMT) with an S-20 response (300 to 820 nm; peak sensitivity 428 nm) (**2**). Another system uses a 931B or H957-06 miniature PMT (**5**). The top of the test chamber must be removable and house a small adjustable motor which drives a stainless steel shaft terminating in a plastic propeller. The propeller is seated into the cuvette and, as the contents are stirred, bioluminescence is generated and measured by the PMT. At the end of each stir period, the accumulated PMT counts are shown in an LED display. Each test period is conducted at preset intervals (either at 4 or 24 h) until completion of the toxicity test.

8. Medium

8.1 Either synthetic seawater or seawater that is enriched are appropriate media for culture and dilution purposes. Natural seawater by itself is not adequate to maintain high densities of dinoflagellates in culture. Seawater with added nutrients (Enriched Seawater Medium-ESM; see Guide E1218) or Synthetic Dinoflagellate Medium (SDM) (**10**) are used to ensure growth in control replicates and cultures. Either ESM or SDM is recommended for use in these tests.

8.1.1 *Purity of Reagents*—Reagent grade chemicals should be used for the preparation of enriched seawater medium (ESM) and synthetic dinoflagellate medium (SDM). Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available (see Guide E1218).[5] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

8.2 *Preparation of Enriched Seawater Medium (ESM) and Synthetic Dinoflagellate Medium (SDM)*—ESM is recommended both for the culture of the stock cells and all test dilutions prepared for a toxicity test using *G. polyedra* (**2**) while SDM is recommended for preparing the culture and all test dilutions of *P. lunula* (**4**) (refer to Annex A1 and Annex A2 for details). Comparable seawater media may be substituted so long as the following requirements are met.

8.3 *Requirements:*

8.3.1 The medium must allow satisfactory growth of the cells. A culture of *G. polyedra* or *P. lunula* should reach cell densities of at least 2000 cells/mL. Starter cultures of *G. polyedra* may take several weeks before adequate cell densities are attained. *P. lunula* requires a substantially longer period of time because cell division occurs approximately every four days in contrast to daily division of *G. polyedra*. A culture of no less than 2000 cells/mL is recommended in both tests for use in inoculating test replicates.

8.3.2 The medium should have a salinity of 33 ± 2 g/Kg and have a pH in the range of 7.8 to 8.2 (*G. polyedra*) or 7.6 to 8.0 (*P. lunula*).

[5] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmaceutical Convention, Inc. (USPC), Rockville, MD.

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8.4 *Stock Solutions of ESM*—Stock solutions to enrich the seawater are prepared by dissolving reagents into 1 L of deionized or distilled water. A specific volume of stock solution is then added to natural seawater. The pH of the enriched seawater may have to be adjusted by adding 0.1 *N* NaOH or HCl. Sterilize (see A1.3.3) the medium and let cool to 19°C before adding the dinoflagellate cells. Sodium silicate may be omitted from the complete seawater medium as dinoflagellates do not require silicates for growth.

9. Procedure

9.1 *Salinity Adjustment of Sample*—If an effluent is being tested, the salinity may need to be adjusted to 33 ± 2 g/Kg with a commercially available aquarium sea salt to provide the proper environment for the cells. The salinity of the sample should match the seawater control salinity prior to testing. Using a temperature-compensated hand-held refractometer, the salinity of the sample and that of the ambient seawater source should be tested. The difference between the two measurements is used to determine the amount of sea salt to be added to the sample. Multiply the salinity difference by 1.2 to calculate the grams of sea salt to be added to each litre of sample for the desired salinity (33± 2 g/Kg). Test the salinity of the adjusted sample to ascertain that the proper salinity was achieved. Filter the adjusted sample with a 0.45 µm membrane filter or centrifuge the sample to remove particulates that might interfere with the bioluminescence measurements. If a commercial sea salt is added to an effluent sample, a sea salt control must be tested to detect any potential bioluminescence inhibition. Because ESM and SDM contain added nutrients and the effluent may not, an equivalent concentration must be added to the effluent sample. This would eliminate any bias in the 100 % effluent sample.

9.2 *Stock Culture Cells*—Each test cuvette or chamber is prepared by addition of a known volume from a stock culture of the test species, sample, and medium. The assumption is then made that the stock is well mixed so that the number of organisms added to each test chamber is the same.

9.3 *Estimating the Stock Culture Cell Concentration*—Swirl the flask containing the stock culture of algal cells. Redistribute the cells within the culture flask by moving the flask side-to-side a few times, then forward and back a few times. Pipet a 1.0 mL aliquot of the stock cells into a small beaker or volumetric flask. Add 25 mL of filtered (0.45 µm) seawater to the beaker and mix. From this diluted cell stock, pipet a 1.0 mL aliquot into a counting cell (that is, Sedgewick-Rafter or other settling chamber) for cell enumeration. Repeat 2 to 4 times and record results. For motile cells of *G. polyedra* add one to two drops of full strength formalin (37 %) to the counting cell to fix the cells during the counting procedure. Allow time for cells to settle then count while viewing at approximately 40X magnification. Average the cell counts and multiply by 26 to get the stock cell culture density (cells/mL). An electronic particle counter may be used.

9.4 *Initial Treatment Solution Calculations*—A large enough batch of medium with cells should be obtained so that the desired volume of each control test solution can be prepared, the necessary volume of each test solution can be prepared, and the desired analysis can be performed on the medium. Treatments are the different test concentrations of a chemical or dilutions of a sample. To determine the total volume of each test solution (V_f) needed, use the formula V_f = (number of test chambers)× (volume of each cuvette or sample vial) × (number of tests or days that are to be conducted). Next, using this calculated volume, determine how much of the original stock culture (V_s) will be added to each treatment solution to get the desired cell concentration in each treatment (C_f) using the following formula:

$$C_f V_f = C_s V_s \quad (1)$$

where:

C_s = concentration of original cell stock culture,
V_s = volume of original cell stock culture needed to be added to test solutions,
C_f = final cell concentration of test solutions (recommended 200 cells/mL), and
V_f = final volume of solution needed for each test solution (mL).

9.5 *Test Solutions*—Dilutions for a sample toxicity test are usually 100, 50, 25, 12.5, and 6.25 %, with no sample added to the control. Use pipets and graduated cylinders to deliver volumes. ESM or SDM may be used as the dilution medium in combination with the test sample. For example, if 100 mL of medium, sample, and cell stock volume is prepared for each of the five test solutions and a control, the control would contain 96 mL of medium and 4 mL of cell stock. The 6.25 % test solution would contain 6.25 mL of sample, 4 mL of cell stock, and 89.75 mL of medium. Once these test solutions are prepared, they are well mixed by swirling the flasks (as in 9.2) and subsamples are pipetted to the appropriate cuvettes or vials for subsequent testing in the detector system.

9.6 *Test Duration*—Decisions to be made concerning experimental design are the duration of the test, dilution factor, number of treatments, number of test cuvettes, and cell density per cuvette. Selection of these parameters should be based on the purpose of the test and the type of procedure used to calculate the results. Depending on the particular requirement for the test, testing can be conducted from an initial 4 h exposure period to as long as seven days. For example, after cells are pipetted into cuvettes, they could be tested in 4 h to screen for potential toxicity. If a longer duration is required, the light production can be measured every day for up to seven days, providing an adequate number of cuvettes are prepared during the initial setup. All testing should be conducted 3 to 5 h into the cells' dark phase.

9.7 *Biological Data*—A reduction in light produced by bioluminescent dinoflagellates in response to exposure to a toxicant present in the sample is the adverse effect. These effects may be expressed after as little as 4 h exposure. Some toxicants may require a longer period of exposure to be detected and measured. The common endpoint used to measure this light reduction is the IC50.

9.8 *Other Measurements—Bioluminescence Measurement System Noise*—Before each session of data collection, a blank

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