Designation: E 1924 – 97

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

This standard is issued under the fixed designation E 1924; 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 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 E 729).

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:*

D 1141 Specification for Substitute Ocean Water[4]

D 5196 Guide for Biomedical Grade Water[5]

E 178 Practice for Dealing with Outlying Observations[6]

E 729 Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians[7]

E 943 Terminology Relating to Biological Effects and Environmental Fate[7]

E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and their Uses[7]

E 1192 Guide for Conducting Acute Toxicity Tests on Aqueous Effluents with Fishes, Macro-Invertebrates, and Amphibians[7]

E 1193 Guide for Conducting Renewal Life-Cycle Toxicity Tests with *Daphnia magna*[7]

E 1218 Guide for Conducting Static 96-h Toxicity Tests with Microalgae[7]

E 1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediment for Toxicological Testing[7]

E 1525 Guide for Designing Biological Tests with Sediments[7]

E 1733 Guide for Use of Lighting in Laboratory Testing[7]

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.

3.1.4 *must*—is used to express an absolute requirement, that

[1] This guide is under the jurisdiction of ASTM Committee E47 on Biological Effects and Environmental Fate and is the direct responsibility of Subcommittee E47.01on 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] *Annual Book of ASTM Standards*, Vol 11.02.

[5] *Annual Book of ASTM Standards*, Vol 11.01.

[6] *Annual Book of ASTM Standards*, Vol 14.02.

[7] *Annual Book of ASTM Standards*, Vol 11.05.

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.

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 E 1733) 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 E 1218) 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 E 1218).[8] 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*).

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

[8] *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 Stated Pharmacopeia and National Formulary*, U.S. Pharmaccutical Convention, Inc. (USPC), Rockville, MD.

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 "dark count" should be recorded to document any ambient light or "system noise" to the phototube. The dark count of the system is obtained without a test cuvette in place. The duration of the dark count should be the same as that of the actual test runs. An average of three dark counts should be sufficient to establish the background noise of the system. The dark count should be insignificant (< 0.5 %) when compared to the amount of light detected from the control test cuvettes. A significantly large dark count (> 2 % of the amount of light detected from the control test cuvettes) may be an indication of a light-leak in the test chamber.

9.8.1 The pH in the control and the high, medium, and low test concentrations should be measured once the test solutions have been prepared and adjusted to the appropriate pH range of the dinoflagellate species used for the test. To minimize potential toxic effects from changes in pH, the test solution pH should be adjusted by adding either 1 *N* NaOH (4 g NaOH in 100 mL distilled water) or 2 *N* HCl (17 mL concentrated HCl to 100 mL distilled water) for the particular dinoflagellate species.

9.8.2 Measurements of the concentration of the test material in the test solution at the beginning and end of the test are desirable. Measurements before and after centrifugation or filtration are desirable to determine what percentage of the test material is not in solution and is not associated with the dinoflagellates.

9.8.3 The use of acceptable solvents to extract test materials and their potential effect on bioluminescent dinoflagellates has not been investigated, although it should be. If a solvent is used, solvent controls must be tested to measure any potential bioluminescence inhibition.

9.8.4 Reference toxicants may be useful to assess the responsiveness of bioluminescent dinoflagellates (see Guide E 729). Sodium dodecyl sulfate, copper sulfate, and other metals have been used to monitor the light output from *G. polyedra* (**1, 3, 11**).

9.9 *Interferences*—Turbidity, suspended solids, or reduced transmission in any concentration or control may interfere with light detection. It is recommended that the controls and highest concentration exhibit similar optical quality. Turbidity can be reduced by centrifugation or filtering the dilutions through a 0.45 µm filter.

9.10 *Analytical Methodology*:

9.10.1 If samples of dilution water, stock solution, or test solutions cannot be analyzed immediately, they should be handled and stored appropriately (**12**) to minimize loss of test material by microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, and volatilization.

9.10.2 Chemical and physical data should be obtained using appropriate ASTM standards whenever possible. For those measurements for which ASTM standards do not exist, methods should be obtained from other reliable sources (**13**).

9.10.3 The precision and bias of each analytical method used should be determined in the dilution water used. When appropriate, reagent blanks, recoveries, and standards should be included whenever specimens are analyzed.

10. Acceptability of Test

10.1 An acceptable dinoflagellate test must have met the following criteria:

10.1.1 The culture medium must have allowed satisfactory growth of the cells (that is, a minimum of 2000 cells/mL in stock cultures).

10.1.2 The medium should have had a salinity of 33 ± 2 g/Kg with a pH in the range of 7.8 to 8.2 (*G. polyedra*) or 7.6 to 8.0 (*P. lunula*). Specific purposes of the test may require a range outside of that advised.

10.1.3 A medium or required solvent control was included in the test.

10.1.4 Temperature and light intensity during the exposure period were monitored for appropriate settings, dependent upon the organism used.

10.1.5 The temperature and light intensity over the exposure area did not vary by more than 10 %.

11. Calculation

11.1 In this acute toxicity test, the dependent variable (for example, bioluminescence expressed as PMT counts) is recorded at intervals throughout the test or at the end of exposure. Each test chamber is discarded immediately after measuring the endpoint. Data generated at any interval should be analyzed to calculate an IC50 for that specific exposure time. The mean light output per treatment, the standard deviation, and the coefficient of variation (standard deviation / mean × 100 %) should be calculated. The mean for each test concentration can be compared with that for the control using the following equation:

$$\%\ of\ control = mean\ of\ test\ concentration\ /\ mean\ of\ control \times 100\ \% \quad (2)$$

After completing these calculations, the values for percent of control for all test concentrations can be plotted against the corresponding concentrations of test material. The IC50 can be determined graphically or by statistical interpolation to find the concentration of test material at which there is a 50 % reduction in light output from the control.

11.2 *Analysis of Percent Inhibition Data*—For each test chamber in each treatment, the percent inhibition should be calculated as

$$I = 100 \cdot [1 - (X/M)] \quad (3)$$

where:

I = percent inhibition of the dependent variable at an exposure concentration,

M = average value of the dependent variable (for example, bioluminescence) across the test chambers for the control treatment(s), and

X = value of the dependent variable for a test chamber for any and all treatments (including the control treatment(s), for which $X = M$ and $I = 0$ %).

On occasion it is convenient to express the bioluminescence detected for the various treatments as a fraction of the control, that is (X/M) in the above equation. This is the fraction of light remaining for treatment compared to the control.

11.2.1 The I for each test chamber should be regressed against the corresponding concentration of test material after transformation of I, concentration, or both, if appropriate (**14**). The IC50 can then be determined by graphical or statistical interpolation as the concentration corresponding to $I = 50$ %. Statistical modeling is not required, however, some transformations may be appropriate (that is, ANOVA) ((**14**), for a discussion of when transformation is appropriate and some transformations that are available).

11.2.2 If possible, the 95 % confidence limits on IC50 should be calculated, appropriately taking into account the number of test chambers per treatment, the number of test organisms exposed in each chamber, the range of concentrations tested, and the variance within each treatment, especially in the control treatment(s).

11.3 *Analysis of Data When Not Expressed as a Percent of*

Control—An appropriate linear or nonlinear inverse regression analysis can be used to calculate the IC50 and its 95 % confidence limits (**15**). A variety of regression models will usually give nearly the same IC50 for a set of data. However, only the correct model, which is not known to be available at this time, will appropriately take into account the variance between the test chambers in the control treatment(s) and give the correct confidence limits.

11.3.1 The values of X may be plotted against the corresponding concentrations of test material, after transformation of X, concentration, or both, if appropriate (**14**). The IC50 can then be determined by graphical or statistical interpolation as the concentration of test material corresponding to $X = M/2$.

11.3.2 An IC near an extreme of toxicity, such as an IC5 or IC95, should not be calculated unless at least one concentration of test material killed or affected a percentage of test organisms, other than 0 or 100 %, near the percentage for which the IC is to be calculated. Other ways of providing information concerning the extremes of toxicity are to report the highest concentration of test material that actually killed or affected no greater a percentage of the test organisms than did the control treatment(s) or to report the lowest concentration of test material that actually killed or affected all test organisms exposed to it. These alternatives are usually more reliable than reporting a calculated result such as an IC5 or IC95 unless at least two treatments produced a percent killed or affected that were close to 5 or 95 %.

11.3.3 It might be desirable to perform an hypothesis test to determine which of the test materials produced an adverse effect. If an hypothesis test is to be performed, the data should first be examined using appropriate outlier detection procedures (see for example, Guides E 178, E 1192, and E 1241) and a test of heterogeneity. Then a pair-wise comparison technique, contingency table test, analysis of variance (ANOVA), or multiple comparison procedure appropriate to the experimental design should be used. Presentation of results of each hypothesis test should include a statement of the hypothesis being tested, the test statistic and its corresponding significant level, the minimum detectable difference, and the power of the test (**16**).

12. Report

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

12.1.1 Names of the test and investigator(s), name and location of laboratory, and dates of initiation and termination of test,

12.1.2 Source of the test material, its lot number, 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,

12.1.3 Procedure used to prepare the medium,

12.1.4 Source of test species, scientific name, name of person who identified the species and the taxonomic key used, and culture procedure used,

12.1.5 Description of the experimental design, test cuvettes, number of test cuvettes per treatment, temperature regulation, and the light regime,

12.1.6 Average and range of the measured air or water temperature and lighting,

12.1.7 Methods used for, and results (with standard deviations or confidence limits) of, chemical analysis of concentration(s) of test material, impurities, and reaction and degradation products, including validation studies and reagent blanks,

12.1.8 Method used for measuring bioluminescence and light measuring system,

12.1.9 A table of bioluminescence data for dinoflagellates in each cuvette in all treatment(s) in sufficient detail to allow independent statistical analysis,

12.1.10 Calculated endpoints, their 95 % confidence limits and calculation method(s) used; specify whether results are based on measured concentrations; for commercial products and formulations; specify whether results are based on active ingredient,

12.1.11 Any stimulation found in any treatment (hormesis), and

12.1.12 Anything unusual about the test, any deviation from these procedures, and other

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

13. Keywords

13.1 bioluminescence; dark phase; dinoflagellate; *Gonyaulax ployedra*; inhibition; light output; *Pyrocystis lunula*; toxicity test

ANNEXES

(Mandatory Information)

A1. METHODS FOR CONDUCTING AN ACUTE (FOUR DAY) AND CHRONIC (SEVEN DAY) BIOLUMINESCENCE TOXICITY TEST USING *Gonyaulax polyedra*

A1.1 *Specifications:*

A1.1.1 *Applications*—The ecological role these minute organisms play as primary producers in the ocean makes them ideal subjects and biological tools in many laboratory situations. This test procedure may be applied to almost any solution to investigate its effects on a single-celled organism common to all oceans.

A1.2 *Laboratory Parameters*—Cultures are maintained in 500 mL Erlenmeyer borosilicate flasks under a light regime of 12:12 h (light:dark). The normal day-night cycle is reversed to accommodate daytime testing. The cells are then in their dark phase and most stimulable for light production after 3 to 5 h. Cultures of *G. polyedra* should be maintained at 19 ± 1°C at a cell density of at least 2000 cells/mL. It is recommended that cultures of *G. polyedra* be in their log phase of growth at the beginning of each test. Media is normally changed at monthly intervals, but higher cell densities may be maintained by changing the media more frequently (**4**). The pH of the media may range from 7.8 to 8.2. For purposes of a test, a pH range of 7.7 to 8.3 is acceptable. Any test dilutions to exceed this range may interfere with test results, as pH may be the cause of an adverse effect. A culture 12 to 20 days old is recommended, as it is likely to contain the required cell density of 2000 cells/mL.

A1.2.1 *Temperature*—Tests with *G. polyedra* must be conducted at 19 ± 1°C.

A1.3 *Culture*—This species can be maintained in either natural or synthetic seawater. Natural seawater or "enriched seawater medium" (ESM) (see Guide E 1218) is considered optimal. ESM is prepared both for the culture of the stock cells (*G. polyedra*) and all medium prepared for a toxicity test. The micronutrient stock solution (A), the macronutrient salt stock solution (B), and the vitamin stock solution (C) should be added to filtered seawater as directed by Guide E 1218.

A1.3.1 *Source*—Fresh seawater should not be collected from areas with an obvious sheen at the surface. Seawater that may appear suspect of contamination, for example, as sheen may be caused by oil or organic contamination. Seawater from these areas should be avoided for culture use or the test.

A1.3.2 All seawater used for the culture of dinoflagellates, should be filtered through membrane filters (0.2 µm) and prepared using enriched seawater medium (ESM) (see Guide E 1218). The micronutrient stock solution (A), the macronutrient salt stock solution (B), and the vitamin stock solution (C) should be added to the filtered seawater as directed in Guide E 1218. Deviations in the enrichment of seawater are acceptable. Precautions to ensure that nutrient levels are adequate in seawater are advised.

A1.3.3 The ESM must be sterilized by microwaving (1500 watts) 1 L for 10 min (**17**). Elimination of bacterial, algal, and fungal contaminants in seawater has been demonstrated with microwaving (**17**). The sterilization of plastic tissue culture vessels and agar media has also been demonstrated with microwave (**18, 19**) and was effective against a wide range of bacterial and viral contaminants (**20**). The salinity of the seawater should be checked and adjusted to 33 ± 2 g/Kg salinity following microwaving and evaporation of the water. To dilute hypersaline seawater deionized water may be added to the sterilized seawater to a final salinity of 33 ± 2 g/Kg. For the purpose of a test, ESM is an appropriate medium for test control dilutions.

A1.3.4 Seawater can be microwaved in 1500 mL Pyrex beakers fitted with a watch glass. Only 1 L of seawater should be microwaved each time. Sterilization of ESM seawater is not necessary to conduct the bioluminescent test because of the short test period with respect to potential contamination problems. ESM must be sterilized for the maintenance of the cell stocks. Extreme heat may break down vitamins during sterilization in the microwave. The vitamin stock solution (C) as well as the micronutrient stock solution (A) and the macronutrient salt stock solution (B) may be added to the sterile seawater following microwaving with a syringe filter.

A1.3.5 Buffering of fresh seawater is not necessary for the culture of dinoflagellates or for the bioluminescent test when using ESM.

A1.3.6 Sodium silicate may be omitted from the macronutrient stock solution B of ESM since it is not required for growth or maintenance of dinoflagellates.

A1.4 *Bioluminescence Test Equipment*—A test chamber to house the cuvettes should be constructed out of non-reactive material impervious to seawater spattering from the stirred cuvettes. The top of the darkened chamber must be removable and house a small motor which drives a stainless steel shaft terminating in a plastic propeller.

A1.4.1 The control box should have face displays for PMT and stirring motor voltages, PMT count, and preset count time settings. At a minimum, the readout of the PMT system is needed to record light output and there must be switches to turn on the PMT and turn off the power before removing the top of the darkened chamber containing the cuvette.

A1.4.2 Neutral density optical filters (ND-1, ND-2) should be arranged in front of the PMT, but between the darkened test chamber housing the cuvette to prevent PMT saturation from the generated bioluminescence.

A1.4.3 *Bioluminescence Measurement System Noise*—Before each session of data collection, a "dark count" should be recorded to document any ambient light or "system noise" to the phototube. The dark count of the system is obtained without a test cuvette in place. The duration of the dark count should be the same as that of the actual test runs. An average of three dark counts should be sufficent to establish the

background noise of the system. The dark count should be insignificant (< 0.5 %) when compared to the amount of light detected from the control test cuvettes. A significantly large dark count (> 2 % of the amount of light detected from the control test cuvettes) may be an indication of a light-leak in the test chamber.

A1.4.4 Mean light output (PMT counts) is calculated for each experimental concentration (usually five) and each control for each measurement period. Light output means are graphed as light output (percent of control) over time. An IC50 is estimated for each day.

A1.5 *Organism—G. polyedra* is a photosynthetic dinoflagellate that is commonly encountered in marine coastal waters of most continents of the world (**3, 4, 21**). This species can be obtained from commercial culture collection agencies.

A1.6 *Test Replicates*—A minimum of five replicates per concentration, with approximately 200 cells per mL in each cuvette, is recommended. More replicates may provide increased certainty in the test, depending on specific purposes of the test. Each test chamber should be inoculated with about 200 cells/mL. This provides an adequate amount of bioluminescence that is needed to observe an adverse effect, if any. An excessive number of cells/mL may result in saturation of the phototube (**2, 3**) with light. To adjust the sensitivity of the phototube there are two different filters (ND-1 and ND-2) that attenuate light levels by approximately a factor of 10 and 100, respectively. For example, a reading taken in the absence of a filter, or with a clear quartz lens, may measure 1×10^6 PMT counts. The same sample, if it had been measured with an ND-1 filter, would measure approximately 1×10^5 PMT counts and with an ND-2 filter, approximately 1×10^4 PMT counts. It is important to note that, because testing takes place while the organisms are in their dark phase, room light should be minimized from reaching the darkened stirring chamber which will result in a false signal. A darkened room is also necessary during testing to prevent photoinhibition of the cells.

A1.7 *Duration*—Measuring light output at 24–h increments until 96 h of exposure (or termination of the test determined by specific requirements) is recommended as a means of observing and checking for consistent inoculation of cells into the replicates of controls and test concentrations and observing the effects of a toxicant on the population of test species. Depending on the toxicity of the material, inhibition of bioluminescence can be observed as soon as all of the experimental units containing the dinoflagellates have been kept in the dark phase long enough so that all cells are equally acclimated to the dark phase. A four day (96–h) test is preferred for consistency with standard aquatic toxicity tests, though effects may be observed at the 24–h measurement period. Some materials may not have adverse effects until a longer exposure is achieved. To estimate these effects, a seven day test is advised.

A1.8 *Biological Data*—Results of dinoflagellate tests should be calculated based on one measurement of light intensity in each cuvette. Light measurements are used to calculate the mean, standard deviation, coefficient of variance, percent of control, and an IC50 should be calculated as an endpoint.

A1.9 *Acceptability of Test*—An acceptable *G. polyedra* test must have met the following criteria:

A1.9.1 The culture and test temperature was not greater than 20°C nor less than 18°C,

A1.9.2 Incident light on the cultures for maintenance and testing was approximately 4000 lux (6 to 10 w/m^2).

A1.9.3 Mean control bioluminescence was not less than 1×10^6 PMT counts (using an ND-2 optical filter) accumulated while stirring each cuvette for 30 s after 24 h.

A1.9.4 The pH of dilution replicates was within the range: 7.7 to 8.3.

A1.10 *Summary Table*—See Table A1.1.

TABLE A1.1 Summary Table

Specifications	
Organism	*Gonyaulax polyedra*
Test Duration	96 or 168 h
Media	Enriched Seawater Media (ESM)
Temperature Range	19 ± 1°C
Salinity Range	31 to 35 g/Kg
pH Range	7.7 to 8.3
Required Growth	2000 cells/mL in culture
Illumination	4000 lux (6 to 10 w/m^2)
Light Regime	12 h light : 12 h dark

A2. METHOD FOR CONDUCTING AN ACUTE (4 H) BIOLUMINESCENCE TOXICITY TEST USING A MARINE DINOFLAGELLATE MUTANT (*Pyrocystis lunula*)

A2.1 *Specifications*:

A2.1.1 *Applications*—This toxicity test has been developed to detect toxic substances in oil-well drilling fluids (**22-24**), marine and terrestrial waters and sediments (**25**), and brines produced from oil and gas wells (**26**). This test is based on the detected inhibition of bioluminescence in dinoflagellates by exposure to a toxic substance.

A2.1.2 The test is applicable to rapid screening of a wide variety of toxic environmental samples. Due to the buffering capacity of the artificial seawater, samples with wide pH extremes (2.0 to 10) do not significantly change the pH of test dilutions. Unless the effluent or solution being tested causes the pH or salinity to exceed the ranges 7.5 to 8.0 and 31 to 35 ppt respectively, in any of the test dilutions, no pH or salinity adjustments are required. A severe test of turbidity using 1 % amino black in the test medium did not significantly reduce bioluminescence measurements compared to untreated controls.

A2.2 *Field Equipment and Equipment*—To perform the toxicity test at a remote site, the test instrument should be portable and have adequate power (internal battery or car

battery hookup). Pre-counted organisms, an adjustable Eppendorf pipette with disposable tips, and a pocket calculator are also required to conduct the toxicity test. Samples may be tested at their collection site, for example, streams, lakes, construction sites, chemical plants, and waste-disposal facilities.

A2.3 *Laboratory Parameters*—Because the organism is sensitive to many metal compounds, water prepared in accordance with Guide D 5196, or equivalent, to three megohms resistivity must be used. Incubation of *P. lunula* requires an environmental chamber that is capable of maintaining a temperature at 20 ± 2°C. It must be equipped with fluorescent lighting and an adjustable timer to vary light and dark cycles. The lights should be shaded to limit the light incident on the cultures to approximately 1075 lux. Fernbach flasks are used for the cultivation of *P. lunula* to provide an appropriate cell volume/surface ratio. A Sedgwick Rafter cell-counting chamber is recommended to facilitate cell counting. An electronic cell counter, if available, may also be used. An automatic pipette is necessary to provide accurate repetitive pipetting (1 % precision) of the culture medium. A photometer capable of detecting and quantifying low-level emissions at 480 nm is required. The photometer must be modified to include an integrating circuit (**24**), a stirrer for providing stress to cells in the test vials, and a timer to ensure equal stress to the test vials. Numerical values of the integrated bioluminescence emission are presented on the LED display. The data can also be saved on a strip chart recorder. Eppendorf pipettes capable of delivering 10 to 500 µL are necessary. Toxicity computations and quality-control statistics may be performed on a pocket calculator that includes statistical functions.

A2.4 *Culture*—Synthetic Dinoflagellate Medium (SDM). The culture medium consists of f/2 medium (one-half strength f-medium) (**27**), modified by the omission of silicate and adjustment to a final pH of 7.6 with dilute HCl. It is prepared with reagent grade (see 8.1.1) salts. The artificial seawater can be prepared as in Guide D 1141. It is prepared with the following: add 1 mL each sodium nitrate-sodium phosphate solution, vitamin solution, trace metal solution, and iron chloride-sodium EDTA solution to approximately 900 mL of artificial seawater. Add 5 g of tris buffer and adjust the final medium to pH 7.6 ± 0.1 with 0.1 normal sodium hydroxide or hydrochloric acid as appropriate. Bring volume to 1 L with artificial seawater. Natural seawater is not used for the culture and testing of *P. lunula*.

A2.4.1 *Sodium Nitrate-Sodium Phosphate Solution*—Dissolve 150 g of $NaNO_3$ and 11.3 g of $NaH_2PO_4 \cdot 2H_2O$ in 1 L of distilled or deionized water.

A2.4.2 *Vitamin Solution*—Dissolve 20 g of thiamine hydrochloride, 0.1 g of Biotin, 0.1 g of B_{12} to 1 L of distilled or deionized water; then dilute 1 mL of this solution to 100 mL with distilled or deionized water.

A2.4.3 *Trace Metals Solution*—Dilute the following in 500 mL of distilled or deionized water:

$CuSO_4 \cdot 5H_2O$	0.0196 g
$ZnSO_4 \cdot 7H_2O$	0.044 g
$CaCl_2 \cdot 6H_2O$	0.02 g
$MnCl_2 \cdot 4H_2O$	0.36 g

A2.4.4 *$FeCl_3 \cdot 6H_2O$-NaEDTA Solution*—Dissolve 3.1448 g of $FeCl_3 \cdot 6H_2O$ and 4.3601 g of NaEDTA ($C_{10}H_{14}O_8Na_2 \cdot 2H_20$) in 1 L of distilled or deionized water.

A2.4.5 *Artificial Seawater*—Dissolve 41.953 g of "sea salt" in sufficient distilled or deioized water to make a 1 L solution (see Specification 1141, and Guide D 5196). Density is 1.025. The resulting salinity and pH are approximately 33 g/Kg (ppt) and 7.6 respectively. If the pH of the culture media is not within the range of 7.5 to 8.0, use 2*N* HCl or 1 *N* NaOH to bring the media within the acceptable range. Composition is:

NaCl	58.49 %
$MgCl_2 \cdot 6H_2O$	26.46 %
Na_2SO_4	9.75 %
$CaCl_2$	2.765 %
KCl	1.645 %
$NaHCO_3$	0.477 %
KBr	0.238 %
H_3BO_3	0.071 %
$SrCl_2 \cdot 6H_2O$	0.095 %
NaF	0.007 %

A2.5 *Organism*—The dinoflagellate *P. lunula* mutant ATCC 40752 may be obtained from the American Type Culture Collection.[9] Its natural ancestor was isolated from tropical Atlantic waters (**28**). On receipt of this axenic culture, sterile techniques must be used for transferring and culture requirements. Transfer aseptically to stoppered 3 L Fernbach flask containing 1 L of sterile culture medium. Illumination is timed to a light cycle of 12 h light (1800 to 0600 h) and 12 h dark (0600 to 1800 h) to adapt the assay procedure to the working day. During the stationary growth phase, culture densities should be expected to contain 2000 to 3000 cells/mL, otherwise an unknown factor may be inhibiting the growth of the cultures.

A2.6 *Test Replicates*—Stock cultures of P. *lunula* must be maintained in SDM at pH 7.6 (± 0.1) at a temperature of 20 ± 2°C. Before measurement, a 10 to 20 mL aliquot is taken from the cell stock culture and diluted 1:6 with SDM. The suspension is gently stirred for 10 min by a magnetic stirrer. A 1–mL aliquot is then removed and counted in the Sedgwick-Rafter counting chamber. The cells are counted and averaged. To obtain the number of cells/mL in the stock culture, this average must be multiplied by six to compensate for the dilution.

A2.6.1 This stock concentration is used to calculate the volume of stock culture combined with SDM and test sample required to reduce the culture concentration to approximately 100 cells/mL in every test solution. SDM and test sample volumes are dependent on the desired range/dilution being assessed. The reduced suspension of cells is allowed to circulate through an automatic pipetter for 30 min. A total of 3 mL of the reduced suspension containing a specific volume of test sample are dispensed into sample vials. This results in test dilutions of 300 cells/ vial or 100 cells/mL of dilution. To develop concentration-effect curves, test sample volumes of 50, 200, and 500 µL : 3 mL are suggested as an example of an

[9] Available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

appropriate range to be combined with SDM and reduced culture, thus creating a percent dilution of 1.6, 6, and 16 %. For quick toxicity screening, samples are dispensed by increments of 50 µL : 3 mL dilutions into each vial. The positive control is sodium lauryl dodecyl sulphate (SDS). Test design is 10 vials control, seven vials experimental (five for screening), and five vials SDS positive control. All vials are stored in a darkened chamber for as long as 4 h before bioluminescence is measured. Once inside the measuring chamber, an integrated circuit measures light for one minute. Light emission values for control and environmental samples are measured by the photometer and displayed by the LED. The light values generated by each vial and displayed by the LED are recorded by hand.

A2.7 *Biological Data*—The data displayed on the LED were used to calculate the mean, standard deviation, and coefficient of variation for control and test vials. The measured adverse effect is percent inhibition of bioluminescence. An IC50 should have been calculated as the endpoint.

A2.8 *Acceptability of Test*—An acceptable *P. lunula* test must have met the following criteria:

A2.8.1 Water used in the preparation of SDM must have had three megohms resistivity.

A2.8.2 *P. lunula* must be have been cultured in buffered SDM at pH 7.6 ± 0.1, at a temperature of 20 ± 2°C, and maintained on a 12:12 h (dark:light) schedule.

A2.8.3 Incident light on the cultures for maintenance and testing must have been approximately 1075 lux.

A2.8.4 The photometer was modified to include an integrating circuit.

A2.8.5 The pH of the dilutions must not exceed 7.5 to 8.0.

A2.9 *Summary Table*—See Table A2.1.

TABLE A2.1 Summary Table

Specifications	
Organisms	*Pyrocystis lunula* (mutant)
Test Duration	4 h
Media	Synthetic Dinoflagellate Medium (SDM)
Temperature Range	20 ± 2°C
Salinity Range	33 ± 2 g/Kg
pH Range	7.5 to 8.0
Required Growth	2000 cells/mL in culture
Illumination	approximately 1075 lux
Light Regime	12 h light : 12 h dark

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