Designation: D 5660 – 96 (Reapproved 2004)

Standard Test Method for Assessing the Microbial Detoxification of Chemically Contaminated Water and Soil Using a Toxicity Test with a Luminescent Marine Bacterium[1]

This standard is issued under the fixed designation D 5660; 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 test method **(1)**[2] covers a procedure for the rapid evaluation of the toxicity[3] of wastewaters and aqueous extracts from contaminated soils and sediments, to the luminescent marine bacterium *Photobacterium phosphoreum*,[4] prior to and following biological treatment. This test method is meant for use as a means to assess samples resulting from biotreatability studies. Sensitivity data for *P. phosphoreum* to over 1300 chemicals have been reported in the literature **(2).** Some of the publications are very relevant to this test method **(3)**. The data obtained from this test method, when combined with respirometry, total organic carbon (TOC), biochemical oxygen demand (BOD), chemical oxygen demand (COD), or spectrophotometric data, can assist in the determination of the degree of biodegradability of a contaminant in water, soil, or sediment **(3).** The percentage difference between the IC20 of treated and untreated sample is used to assess the progress of detoxification.

1.2 This test method is applicable to the evaluation of the toxicity (to a specific microbe) and its implication on the biodegradation of aqueous samples from laboratory research bio-reactors (liquid or soil), pilot-plant biological treatment systems, full-scale biological treatment systems, and land application processes (see Notes 1 and 2).

NOTE 1—If the biologically treated material is to be discharged in such a manner as to potentially impact surface waters and ground water, or both, then the user must consult appropriate regulatory guidance documents to determine the proper test species for evaluating potential environmental impact **(4).** Correlations between data concerning reduction in toxicity produced by this test method and by procedures for acute or short-term chronic toxicity tests, or both, utilizing invertebrates and fish (see Guides E 729 and E 1192), should be established, wherever possible.

NOTE 2—Color (especially red and brown), turbidity, and suspended solids interfere with this test method by absorbing or reflecting light. In these situations data are corrected for these effects by use of an absorbance correction procedure included in this test method (see 5.3, 6.1, and 6.2).[5]

1.3 The results of this test method are reported in terms of an inhibitory concentration (IC), which is the calculated concentration of sample required to produce a specific quantitative and qualitative inhibition. The inhibition measured is the quantitative reduction in light output of luminescent marine bacteria (that is, IC20 represents the calculated concentration of sample that would produce a 20 % reduction in the light output of exposed bacteria over a specified time).

1.4 The values stated in SI units are to be regarded as the standard. The values given in parentheses are for information only.

1.5 *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.* Specific hazard statements are given in Section 9.

2. Referenced Documents

2.1 *ASTM Standards:* [6]

D 888 Test Methods for Dissolved Oxygen in Water

[1] This test method is under the jurisdiction of ASTM Committee D34 on Waste Management and is the direct responsibility of Subcommittee D34.07 on Municipal Solid Waste.

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[2] The boldface numbers in parentheses refer to the list of references at the end of this standard.

[3] Toxicity measured as toxic inhibition of bacterial light output.

[4] Microbics Corp. is currently the only known supplier of the reagents (test organism *Photobacterium phosphoreum* strain NRRL B-11177) specific to this test method. There are two known manufacturers of analyzers that can be used to measure bioluminescence under temperature control: Microbics Corp., 2232 Rutherford Road, Carlsbad, CA 92008 (Microtox Model 500 and Model 2055 Analyzers), and Pharmacia LKB, 9319 Gaither Road, Gaithersburg, MD 20877 (LKB Wallac Model 1250 and Model 1251 Luminometers). Other instruments would be considered when they become available. Please notify ASTM Subcommittee D34.09 if you are aware of any additional systems or instruments capable of performing this testing.

[5] At present (1993) use of the color correction scheme described in this procedure is known to be effective only with the Microbics Corporation's toxicity analyzers, due to the fact that the correction mathematics involve the detailed geometry of both the ACC and the light meter. Please notify ASTM Subcommittee D34.09 if you are aware of any other source of equipment capable of providing color or turbidity correction, or both, for the *P. phosphoreum test*. Data validating the absorbance correction procedure are available from Microbics Corp.

[6] 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.

D 1125 Test Methods for Electrical Conductivity and Resistivity of Water
D 1129 Terminology Relating to Water
D 1193 Specification for Reagent Water
D 1293 Test Method for pH of Water
D 3370 Practices for Sampling Water
E 729 Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians
E 943 Terminology Relating to Biological Effects and Environmental Fate
E 1192 Guide for Conducting Acute Toxicity Tests on Aqueous Effluents with Fishes, Macroinvertebrates, and Amphibians

3. Terminology

3.1 *Definitions*—The IC20 is defined in terms of a modification of the definition of IC50 as it appears in Terminology E 943. The terms turbidity and volatile matter are defined in accordance with Terminology D 1129. These terms are as follows:

3.1.1 *color*—that is, the presence of dissolved matter that absorbs the light emitted by *P. phosphoreum* (that is, wavelength of 490 ± 100 nm).

3.1.2 *IC20*—a statistically or graphically estimated concentration of test material that, under specified conditions, is expected to cause a 20 % inhibition of a biological process (such as growth, reproduction, or bioluminescence) for which the data are not dichotomous.

3.1.3 *turbidity*—reduction of transparency of a sample due to the presence of particulate matter.

3.1.4 *volatile matter*—that matter that is changed under conditions of the test to the gaseous state.

4. Summary of Test Method

4.1 This test method covers the determination of acute toxicity of aqueous samples to luminescent marine bacteria, *P. phosphoreum*.

4.2 Wastewater samples are osmotically adjusted to the appropriate salinity for the test species *P. phosphoreum*. A sodium chloride (NaCl) concentration of 2 % has been found optimal for this test organism for freshwater tests, or about 3.4 % NaCl for seawater samples. This provides the necessary osmotic protection for the bacteria, which are of marine origin.

4.3 Samples should not be pH adjusted unless the user is not concerned about toxic effects related directly to pH. Altering the sample pH will usually alter the solubility of both organic and inorganic constituents of the sample. Altering the pH can also cause chemical reactions that will change the integrity of the sample, and greatly alter the exhibited toxicity of the sample. If sample pH is considered secondary to organism response, then the optimal pH for the bacterium *Photobacterium phosphoreum* is 6.7.

4.4 Comparison of inhibitory concentrations (IC20s) for untreated wastewater (or extracts of untreated soils) versus those for biologically treated wastewater (or extracts of treated soils), calculated from measured decreases in light output of exposed bacteria, allows for an assessment of the reduction in toxicity to the marine bacterium *P. phosphoreum* (see 1.1, 1.2, and Note 1).

4.5 Samples that are highly colored, or contain solids that cannot be removed without seriously compromising sample integrity, can be analyzed using an absorbance correction procedure. This procedure determines the amount of light absorbed by the wastewater at a concentration near the nominal IC20 versus the baseline light output established by measuring the light absorbed by the clear diluent.

5. Significance and Use

5.1 This test method provides a rapid means of determining the acute toxicity of an aqueous waste, or waste extract, prior to and following biological treatment, and contributes to assessing the potential biodegradability of the waste (see 1.1, 1.2, and Note 1). The change in toxicity to the marine bacterium *P. phosphoreum* with respect to time may serve as an indication of the biodegradation potential. Sample analyses are usually obtained in 45 to 60 min, with as little as 5 mL of sample required **(5).**

5.2 Samples with high suspended solids concentrations may test nontoxic to the bacteria, while still exhibiting significant toxicity to freshwater organisms, due to those suspended solids.

5.3 The absorbance correction procedure included in this test method allows for the analysis of highly colored lightabsorbing samples, by providing a means for mathematically adjusting the light output readings to account for light lost due to absorption.[5]

6. Interferences

6.1 Some test samples that are highly colored (especially red and brown) interfere with this test method, but the absorbance correction procedure can be used to correct for this interference.[5]

6.2 Turbidity due to suspended solids interferes with this test method. The absorbance correction procedure can be used to correct for this interference and is preferable to other alternatives. Pressure filtration, or centrifuging and decanting, will also remove this interference. Some toxics may be lost through adsorption and volatilization during filtration or centrifugation, thus impacting the exhibited toxicity.[5]

7. Apparatus

7.1 *Fixed or Adjustable Volume Pipetter*, 10 µL, with disposable tips.

7.2 *Variable Volume Pipetter*, 10 to 1000 µL, with disposable tips.

7.3 *Variable Volume Pipetter*, 1 to 5 mL, with disposable tips.

7.4 *Timer or Stopwatch.*

7.5 *Glass Cuvettes*, 11.75 mm OD, 10.5 mm ID by 50 mm height, 4-mL volume.

7.6 *Absorbance Correction Cuvettes (ACC)*—Optional item, but required to analyze highly colored samples or those containing suspended particulates.[5]

7.7 *Variable Voltage Chart Recorder (optional)*—Useful when using some types of light meters.

7.8 *Computer (optional)*—Useful with some light meters, for which software is also available, to facilitate data capture and reduction.

7.9 *Light Meter*, for cuvettes listed in 7.5.[4,5]

7.10 *Temperature Control Devices* (temperature-controlled room, water bath, refrigerators, or other device)—One capable of maintaining 5.5 ± 1°C and one capable of maintaining 15 ± 0.5°C.

8. Reagents and Materials

8.1 *Test Reagents*:

8.1.1 For purposes of this test method, test reagents are defined as the reagents actually used in performance of the test method. The necessary requirement with regard to qualification of test reagents is that this test method provide acceptable results when reference toxicants are tested using the test reagents. They are then considered to be non-toxic for purposes of this test method.

8.1.2 *Microbial Reagent*—Freeze-dried *Photobacterium phosphoreum*. This is the only test reagent that is currently (1993) available from only one source.[4] While other acceptable means of preservation may become available in the future, freeze-dried *P. phosphoreum* is specified in this test method because a large number of users concur in the opinion that the strain is well standardized by this method of preservation, and that the same strain does not provide comparable response to reference toxicants when preserved by other methods, or when freshly cultured and harvested at the user's laboratory, as described by Anthony A. Bulich, et al **(1).** Another consideration is that a large body of published results, for which freeze-dried *P. phosphoreum* was used, has accumulated since about 1980 **(1,2,3,5,6)**.

8.1.3 *Reconstitution Solution*—Nontoxic water.

8.1.4 *Diluent*—Nontoxic 2 % sodium chloride (NaCl), or 3.4 % NaCl, reconstituted seawater or sea water (depending upon the type of sample and purpose of the test). The *P. phosphoreum* test has been performed at osmotic pressures equivalent to 1 to 6 % NaCl, but has long been standardized at 2 % for freshwater samples. The major requirement is that the osmotic pressure be held constant within each test, to minimize transient variations in luminescence due to variations in osmotic pressure. The higher salinity (and osmotic pressure) of marine samples dictate the use of a diluent other than 2 % NaCl. Both reconstituted seawater and clean seawater have been used as diluent. A procedure for preparing reconstituted salt water, and formula, are given in Table 3 of Guide E 729. Actual seawater has also been collected at remote sites and used as diluent for testing aqueous samples of marine origin. The most important requirement is that the diluent must be qualified for use with this test method (see 8.1.1).

8.2 *Reagent Chemicals*—Reagent grade chemicals are recommended for use in preparation of test reagents and reference toxicants. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society.[7] Other grades may be used, but there will be more risk that the resulting test reagents will fail to qualify (see 8.1.1).

8.2.1 *Sodium Chloride (NaCl)*—Used in preparation of diluent, and for adjusting the osmotic pressure of samples to that of the chosen diluent.

8.2.2 *Phenol, or Other Common Organic Toxicant*—Used as a reference toxicant.

8.2.3 *Zinc Sulfate Heptahydrate, or Other Common Inorganic Toxicant*—Used as reference toxicant.

8.3 *Purity of Water*— Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D 1193, Reagent Water, Type I or II, Subtype A. Test reagents prepared from reagent water are to be qualified for use with this test method (see 8.1.1).

8.4 When this test method is used in conjunction with other tests employing higher organisms, appropriate dilution water for bulk samples should meet the acceptability criteria established in Section 8 of Guide E 729. In addition, all such dilution water used for comparative testing with this test method and invertebrates and fish is to be assayed on *P. phosphoreum* (minimally once per month).

9. Hazards

9.1 The handling of wastewaters entails potential hazards due to exposure to chemical and biological contaminants. Appropriate safety measures, such as the wearing of protective clothing (gloves, apron, face shield, respirator, etc.) and maintaining proper hygiene, are utilized to minimize the chance of exposure. This test method is to be performed in a well-ventilated area.

9.2 Appropriate, environmentally safe procedures prescribed by regulatory agencies are utilized in the disposal of used waste samples.

9.3 Due to the presence of aqueous samples and electrical instrumentation in close proximity, care must be taken to prevent electrical shock.

10. Technical Precautions

10.1 Osmotic adjustment of freshwater test samples, to 2 % sodium chloride concentration, is required due to the use of a marine bacterium as a test organism. Osmotic adjustment may make some components of a wastewater less soluble, reducing concentrations in solution and altering exhibited toxic inhibition.

10.2 Samples containing highly volatile components are to be handled as little as possible to reduce losses due to stripping. Mixing procedures (see 13.8.4) are modified by performing only one pipet mixing per sample dilution versus the usual five pipet mixings. Volatile samples, which can be analyzed by UV spectrophotometry, allow the investigator to measure the average sample concentration of volatiles over the actual test period.

10.3 The addition of any preservative or other chemical agent, including acid or base to alter pH, will in all likelihood impact the exhibited toxicity of the sample. These practices should be avoided in most cases, unless the user is specifically testing to determine the effects of these sample modifications.

10.4 The use of a reference toxicant, such as phenol or zinc sulphate, is recommended for validation of data produced with

[7] *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. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

different lots of test reagents (that is, bacteria, reconstitution solution, and diluent) or for individual lots used over an extended period of time. A good practice is to perform a reference toxicant analysis with each new lot of bacterial reagent received and new lots of test reagents prepared (or purchased). Under normal conditions, with reagent in good condition, tests on phenol produce an IC50 (5 min) between 13 and 26 mg phenol/L, and tests of zinc sulfate heptahydrate produce an IC50 (15 min) between 5 and 12 mg $ZnSO_4 \cdot 7H_2O$/L (or, 1.1 to 2.7 mg Zn/L). The corresponding nominal ranges are IC20 (5 min) = 3 to 6 mg phenol/L and IC20 (15 min) about 1.5 to 4.5 mg $ZnSO_4 \cdot 7H_2O$/L (or, 0.34 to 1.02 mg Zn/L).

10.5 In order to verify that changes in observed toxicity are due to treatment, it is essential to have control samples for biodegradation test systems. Typical controls would be sterilized (autoclaved) waste samples. These samples undergo toxicity assessment for comparison with the treated samples; that is, they undergo the same physical manipulations and testing as the inoculated or nutrient-enhanced treatment systems, but all microbial action has been terminated by sterilization at the outset of the test series. It is necessary to compare the toxicity (IC20s) of autoclaved and untreated samples immediately after autoclaving in order to determine changes due to autoclaving **(3)**. Autoclaving of samples for use as control samples requires special consideration and sample handling techniques. The following procedure is recommended:

10.5.1 Completely fill new borosilicate jars with sample, and seal them with autoclavable caps having TFE-fluorocarbon liners, to minimize loss of volatile toxicants during autoclaving.

10.5.2 Soil and sediment samples are to be moist, for optimal effectiveness of autoclaving.

10.5.3 Bring the autoclave to 121°C and hold the sample jars there for one to two hours, then turn off the heat and allow the autoclave to cool very slowly, to avoid large transient positive pressure inside the jars, which might cause them to fracture.

10.5.4 It is recommended that the autoclaving be repeated 24 h later as a precaution against survival of spores. In addition, or alternatively, commercially available spore strips or preparations may be added to a jar of soil and included in the autoclave load as a direct means of validating the effectiveness of the autoclave cycle.

11. Sampling

11.1 Collect aqueous samples in accordance with Practices D 3370. Soil and other solid material samples, for aqueous extraction, should be sampled in such a way as to reduce the risk of loss of volatile components.

11.2 All sample containers (vials or bottles) should be made of borosilicate glass that has been thoroughly cleaned using a nontoxic soap wash, HCl wash, and water rinse (twice). All sample containers should be sealed with TFE-fluorocarbon-lined caps.

11.3 Prepare all dilutions required for a single toxicity evaluation from the same treated or untreated wastewater sample. Portions of the sample shall be stored, until needed, at a temperature of 2 to 4°C in completely filled, tightly stoppered borosilicate-type glass containers. TFE-fluorocarbon-lined caps are used to seal collection bottles to minimize adsorption or sample contamination.

11.4 Uniformly disperse (by mild agitation), any undissolved material present in a wastewater sample, before withdrawing a measured portion for osmotic adjustment and subsequent analysis. Undissolved material, which will interfere with light transmission during analysis, should be adjusted for or removed from the osmotic pressure-adjusted sample as described in Section 6. Avoid violent agitation and unnecessary exposure of the sample to the atmosphere.

12. Calibration and Standardization[8]

12.1 Use the procedure specified by the manufacturer of whatever light-measuring instrument is being utilized. The procedure should include a mechanism for zeroing the instrument for no light production and a procedure for setting the output range.

12.2 If a chart recorder is being used, it should be calibrated against either the digital reading of the photometer or the voltage output of the photometer to the recorder.

13. Procedure [9]

13.1 Samples taken from a treatment process are collected using an ASTM acceptable sampling procedure (see Section 11).

13.2 For aqueous samples, visually evaluate the sample for suspended particulates and color. Both of these factors can interfere with measured light output readings. If either of these conditions is present use one of the methods described in 6.2 to remove or account for the interference.

13.3 For solid phase samples prepare the test sample as follows:

13.3.1 Wet sediment should be centrifuged to separate the pore water. Centrifuge 50 to 100 g of sediment at 2000 to 4000 g, for 10 to 20 min at 4°C. Decant the pore water and use the resulting pellet of solids as if it were a soil sample.

13.3.2 Homogenize 10 to 50 g of representative soil sample by hand mixing with a spatula for 10 min.

13.3.3 Weigh a representative 3 to 5-g portion of the homogenized sample to the nearest 0.01 g, then air dry at 20 to 25°C for 16 h. After drying, reweigh the dried sample.

13.3.4 Take a 2-g sample from the homogenized soil or sediment and add 20 mL of the appropriate diluent.

13.3.5 Mix the soil/diluent mixture for 16 h using an orbital shaker set at 200 r/min.

13.3.6 Centrifuge the sample at 2000 to 4000 g, for 10 to 20 min at 4°C.

13.3.7 Decant 10 to 15 mL of the aqueous phase for use in the analysis of toxic inhibition.

13.4 Positive pressure filtration (through a prerinsed, glass-fiber filter) can be used to remove suspended solids, while

[8] Calibration and standardization procedures will vary depending on the instrument being used to measure the bacterial light output.

[9] This is a generic procedure that will require modification depending on the particular instrument being used to measure microbial light output.

minimizing loss of volatile organics. Rinsing the filter with nontoxic water, prior to sample filtration, reduces organic leaching from the filter. Note the potential sample alterations mentioned in 6.2.

13.5 Take 5 mL of the aqueous sample from 13.2 to 13.3 and measure the pH (Test Methods D 1293), dissolved oxygen (DO) (Test Methods D 888), conductivity (Test Methods D 1125), and salinity.

13.6 Adjust the sample salinity to either 2 % NaCl or 3.4 % NaCl (for samples of marine origin) by adding sodium chloride to 10 mL of sample. Adjust the pH and DO only if those factors are not concerns in the process under investigation. Be aware of the potential changes in overall sample chemistry as noted in 6.2.

13.7 If the user is adjusting the sample pH to determine the effect thereof, the acid or base, or both, used for the adjustment should be noted, and the quantity required in the adjustment should be recorded. Sample dilution and chemical species changes must be taken into account if pH adjustment is necessary.

13.8 Samples of unknown toxicity are screened, prior to definitive testing, using the following range finding procedure:

13.8.1 Prepare a cuvette of bacterial reagent (*Photobacterium phosphoreum*) by adding 1 mL of nontoxic water at 5.5 ± 0.5°C to a bottle of lyophilized luminescent bacteria and transferring the reconstituted bacteria to a cuvette maintained at 5.5 ± 0.5°C.

13.8.2 Prepare 10 test cuvettes, by adding 0.5 mL of diluent and 10µ L of reconstituted bacteria. Maintain the test cuvettes at 15 ± 0.5°C.

13.8.3 Without waiting the normal 15-min temperature acclimation period, place one of the test cuvettes of bacteria into the photometer, and measure the light output for 10 to 20 s. If the instrument used allows the output value to be adjusted, adjust the output to read 90 units. Otherwise record the output value as it is.

13.8.4 Add l0 µL of the unknown sample to the cuvette being measured. Mix the contents with a 250-µL pipet by aspirating and dispensing its full volume five times, or as an alternative, mix the contents by briskly flicking the cuvette with a finger (cuvette flicking method).

13.8.5 Measure the light output of the exposed bacteria for 10 to 20 s.

13.8.6 If the loss of light output is greater than 20 % within several minutes, dilute the sample ten-fold, and repeat 13.8.3-13.8.5 with one of the unused cuvettes prepared in 13.8.2 using the diluted sample. Repeat this procedure until a sample dilution produces a loss of light of less than 20 % during the first few minutes after sample addition. Observe the bacterial response for 5 min, and then estimate graphically the 5-min toxic response. This information gives the tester a good indication of the sample concentration range which will produce a statistically sound IC20, if the sample is toxic to that extent.

13.9 The procedure for running a toxicity test using *Photobacterium phosphoreum* is as follows:

13.9.1 Place 20 clean cuvettes in a temperature-controlled area at 15 ± 0.5°C, and one additional clean cuvette at 5.5 ± 1°C. Set the cuvettes in two rows of ten, and use a labeled test tube rack or other device to identify the cuvettes as A1–A10 and B1–B10.

13.9.2 Add 1 mL of nontoxic water to the cuvette being held at 5.5°C.

13.9.3 Add the appropriate amount of diluent to Cuvettes A1–A9 (being maintained at 15°C) to obtain the desired concentrations after serial dilution (for example, for a 2:1 serial dilution, 1.5 mL of diluent is added to A1–A9). Cuvette A10 is left empty for the primary sample concentration.

13.9.4 Add 0.5 mL of diluent to Cuvettes B1–B10 (which serve as the test cuvettes).

13.9.5 Add 1.5 mL of the osmotically adjusted primary sample concentration (diluted or not) to Cuvette A10, and an appropriate amount to A9. Mix the diluted contents of A9 by aspirating and dispensing, by pipette, 500 µL of sample five times; or by briskly flicking the cuvette with a finger. Complete the serial dilution of the test sample by transferring an appropriate volume of A9 to A8 and A8 to A7 . . . A3 to A2, using one of the mixing methods previously described. In the example of a 2:1 serial dilution scheme, the dilution would be performed as follows: 1.5 mL of 100 % sample (note that the actual concentration is 91 to 100 % depending on the need for and method of salinity adjustment) added to Cuvettes A10 and A9 and mix A9, 1.5 mL of A9 to A8 and mix, 1.5 mL of A8 to A7 and mix, 1.5 mL of A7 to A6 and mix, 1.5 mL of A6 to A5 and mix, 1.5 mL of A5 to A4 and mix, 1.5 mL of A4 to A3 and mix, 1.5 mL of A3 to A2 and mix, and remove and discard 1.5 mL of A2.

13.9.6 Allow 5 to 10 min for samples to reach thermal equilibrium, then check to verify that the temperature of the reconstitution solution is 5.5 ± 1°C and that the test cuvettes have reached 15 ± 0.5°C.

13.9.7 While the prepared test cuvettes are temperature equilibrating, remove a vial of lyophilized bacteria from refrigeration and rapidly add the precooled 1-mL volume of reconstitution solution into the vial, swirl the vial to mix, and return the reconstituted bacteria to the cuvette which is replaced at a temperature of 5.5 ± 1°C. Mix the reconstituted bacteria by aspirating and dispensing 0.5 mL of solution, by pipet, 20 times. The reagent dilution is started within 5 min of bacterial reconstitution, in order to maintain maximum sensitivity.

13.9.8 Transfer 10 µL of reconstituted bacterial reagent to each Cuvette B1 through B10. Wipe the pipet tip of excess reagent before each transfer. Mix the contents of each cuvette using a 250-µL pipet to aspirate and dispense the solution five times, or by the cuvette flicking method.

13.9.9 Allow the bacteria in the test cuvettes to achieve a stable light output level by remaining undisturbed at 15°C for 15 min. This allows the bacteria to recover from the shocks of reconstitution, shift in temperature, and dilution of nutrients.

13.9.10 Cycle the cuvettes through the photometer, and adjust the light output levels to read between 80 and 100 units if possible (some units will automatically perform this task with the initial I_0 light readings). Cuvette output reading is performed in the order of B1, B2, B3 ... B10.

13.9.11 Take the initial (I_0) readings by cycling the cuvettes, one cuvette every 25 s, and recording the light output of each cuvette (B1 through B10) for 5 s. Record the time with each reading so that the 5, 15, and 30-min exposure periods are accurately timed.

13.9.12 Start the addition of the test samples (Cuvettes A1–A10) to the test cuvettes (Cuvettes B1–B10) immediately following the reading of the light output of Cuvette B10, the last cuvette in the cycle. The addition starts with 0.5 mL of A1 (the nontoxic blank) added to Cuvette B1, mixing the sample by the pipet technique or flicking technique. The sample additions proceed from low concentration to high concentration, adding 0.5 mL of A2 to B2 and continuing up to A10 to B10, allowing 25 s between each sample addition. The time of each addition is recorded so that the light output of each challenged test cuvette can be measured 5, 15, and 30 min after the sample addition.

13.9.13 The test cuvettes (B1 through B10) are cycled through the photometer 5 min after the sample additions and the light output of the bacteria is recorded for each cuvette. This procedure is repeated at 15 and 30 min to observe any time-dependent increases in toxic inhibition (that is, toxicity due to metals).

13.9.14 The recorded light outputs are used to calculate IC values by plotting or mathematical determination.

13.10 The procedure used to correct for absorbance in highly colored aqueous samples, as described in 6.1, is as follows:

13.10.1 Pipet 1.5 mL of diluent into the outer chamber of a clean absorbance correction cuvette (ACC) and place it in the photometer.

13.10.2 Pipet 1.0 mL of diluent into a standard cuvette (A1) and place it at 15°C.

13.10.3 Pipet 2.0 mL of sample of chosen concentration C_c (the concentration closest to the nominal ICxx) into each of two standard cuvettes (A2 and A3), and place them at 15°C.

13.10.4 Allow 10 min for the solutions to reach thermal equilibrium.

13.10.5 Pipet 50 µL of reconstituted bacterial reagent into Cuvette A1. Mix five times with a 500-µL pipet or flick the cuvette briskly.

13.10.6 Remove the ACC from the photometer long enough to transfer a sufficient amount of bacterial solution from cuvette A1 into the inner chamber of the ACC to get a volume level equal to that of the diluent level in the outer chamber.

13.10.7 Return the ACC to the photometer. Adjust the light output reading of the ACC to 90 units (if possible), then record the light output for 10 to 20 min until a stable baseline or steady drift baseline is established.

13.10.8 Using a clean aspirator, remove the diluent from the outer chamber of the ACC while the ACC remains in the photometer.

13.10.9 Remove as much of the diluent as possible with an aspirator. Transfer 1.5 mL of test sample from Cuvette A3 into the outer chamber of the ACC.

13.10.10 Record the light output for 10 min or more. The light levels recorded for the sixth through tenth minute will be used in data reduction.

14. Calculation

14.1 The following equations are used to determine 20 % inhibitory concentrations (IC20s) from light output readings produced using the methods described in Section 13:

14.1.1 Calculate the blank ratios (which will be used to normalize the Γ responses calculated in 14.1.2) for 5, 15, and 30 min, using the following equations:

$$R(t) = I(t)b/I(0)b \quad (1)$$

where:

$R(t)$ = blank ratio for time t,
$I(0)b$ = initial light reading for the blank cuvette (zero time, just before transferring toxicants), and
$I(t)b$ = final light reading for the blank cuvette (t min after transferring toxicants).

14.1.2 Calculate the 5, 15, and 30-min gamma responses, Γ(t), for each of the eight test cuvettes, normalized for reagent pipetting errors and normal drift of luminescence with time, using the following equation:

$$\begin{aligned}\Gamma(t) &= \text{Light Lost/Light Remaining} \\ &= [R(t)I(0) - I(t)]/I(t) \\ &= [R(t)I(0)/I(t)] - 1 \end{aligned} \quad (2)$$

where:

$I(0)$ = initial light reading for any given test cuvette at zero time, just before challenging the organisms,
$I(t)$ = light reading for the corresponding test cuvette at time (t),
$R(t)$ = blank ratio for time (t) as defined in 14.1.1, and
$\Gamma(t)$ = Γ effect calculated for each exposure time (t); that is, at 5, 15, and 30 min.

It should be noted that $1n\,\Gamma(t) = 1n\,(D/(1 - D))$ (see 14.1.4) is identical to Berkson's logit P/Q = logit $P/(1 - P)$ **(7).** The method described in this test method is, therefore, a logit analysis.

14.1.3 Use linear regression[10] of $1n\,\Gamma(t)$ on $1\,n\,C$, with $1n\,\Gamma(t)$ as the dependent variable, to obtain the 1 n-1n regresssion equation,

$$1n\,\Gamma(t) = b(1n\,C) + 1n\,a \quad (3)$$

then solve this equation for $1n\,C$ to obtain the estimating equation,

$$1n\,C = (1/b)[1n\,\Gamma(t)] - [1n\,a] \quad (4)$$

where:

C = concentration of sample,
$1n\,a$ = intercept of the 1 n-1n regression line with the ordinate $1n\,C = 0$, which will be a constant number, but different for each exposure time (5, 15, and 30 min),

[10] Standard regression analysis should be used, with care given to make certain that the quality of the data warrants the conclusions drawn. The estimating equation reserves the variables compared to the conventional dose response curve to facilitate solution of the equation for C for a specified Γ. This estimating equation is simply the regression equation rearranged to make $1n\,C$ a function of Γ(t).

b = slope of the 1n-1 n regression line, which will also be a constant number, but different for each exposure time (5, 15, and 30 min), and

$\Gamma(t)$ = toxic responses for corresponding concentrations, for each exposure time (5, 15, and 30 min).

14.1.4 In order to find IC20s, solve the above estimating equation for C when $\Gamma(t) = 0.25$, corresponding to 20 % reduction of light output (see 1.3), for 5, 15, and 30-min data. These concentrations (Cs) are the IC20s for 5, 15, and 30 min, respectively. The relationship between Γ and percent reduction of light output (% D) is:

$$\Gamma = \%\,D/(100\,\% - \%\,D) \quad \text{or} \quad \%\,D = 100\,\% \times \Gamma/(1 + \Gamma) \tag{5}$$

It may be easily seen that IC20 (that is, % D = 20 %) corresponds to Γ = 20 %/(100 % − 20 %) = 20 %/80 % = 0.25. The estimating equation must be satisfied by these corresponding values of C and Γ. Substituting these specific values into the estimating equation results in the following:

$$1n(IC20) = 1/b1n(0.25) + 1/b1n\,a = 1/b(-1.3863) + 1/b1n\,a \tag{6}$$

Once the right side of the equation is reduced to a single number, say N, IC20 is the antiln of N. The antiln (N) is simply e^N, where e = 2.7182818...; that is, the base of the natural logarithms.

14.2 The following equations use data obtained in 13.9 and 13.10 to determine corrected light loss when a sample is highly colored and light absorbing or highly turbid, or both.[11]

14.2.1 Considerable labor can be saved when it is possible to calculate the values of A_c for all sample concentrations (C) based upon measurement of only one concentration (C_c) in the ACC, using the equation given in 14.2.2. When the sample is such that this approach is not applicable, [13] determine A_c for each concentration that yielded a significant Γ (that is Γ between 0.02 and 100) by direct measurement with each such concentration in the ACC. The equation in 14.2.2 must then be solved for each set of ACC data, I_0/I_F, with $C/C_C = 1$ in each case. It should be noted that A_C is considered to be zero for concentrations having Γ responses of 0.02 or less.

14.2.2 When applicable (see 14.2.1),[11] calculate absorbance due to color (A_C) for the ACC for all concentrations (C) of sample tested in the toxicity cuvettes which gave significant Γ responses, using the following equation:

$$A_C = (C/C_C)\,[3.1\;1n(I_0/I_F)] \tag{7}$$

where:

A_C = calculated absorbance expected if concentration C were to be measured in the ACC, for each concentration tested in the toxicity test which gave a significant Γ. [12] (Alternatively, each A_C is calculated using I_0 and I_F results from direction measurements in the ACC.)

I_0 = initial light level, measured in the ACC (for diluent),

I_F = final light level, measured in the ACC (for C_C),

C_C = chosen concentration measured in the ACC (in 13.10),

C = each sample concentration tested in the toxicity cuvette, which gave a significant Γ (that is, 0.02 or larger), and

3.1 = composite factor for the ACC which corrects for geometrical differences between it and the standard test cuvette.[5]

14.2.3 Calculate the transmittance (T_C) of the toxicity cuvette for each sample concentration tested that gave a significant Γ, using the following formula:

$$T_C = (1 - e^{-Ac})/A_C \tag{8}$$

where:

T_C = unity (that is, 1.00) for concentrations having insignificant Γ responses, corresponding to A_C = zero.

14.2.4 Calculate the corrected gamma responses ($\Gamma_C(t)$) for 5, 15, and 30-min data for each concentration tested, using the following equation:

$$\Gamma_C(t) = T_C(1 + \Gamma(t)) - 1 \tag{9}$$

where:

$\Gamma(t)$ = Γ response observed for each concentration (C) in the test, at each test time (5, 15, and 30 min), and

$\Gamma_C(t)$ = color-corrected toxic response for each test time (5, 15, and 30 min).

14.2.5 Determine the color-corrected IC20 ($IC20_C$) for 5, 15, and 30-min data as described in 14.1.3, using the $\Gamma_C(t)$ values determined in 14.2.3 for each exposure time.

14.3 The following equation is used to correct the IC20s determined for soil and sediment samples in either 14.1.3 or 14.2.4 (if color/turbidity corrected) to dry-weight basis. The wet and dry weights of a representative soil/sediment sample were determined in 13.3.3.

$$IC20(t)_{DRY} = IC20(t)_{WET} \times (\text{dry weight})/(\text{wet weight}) \tag{10}$$

15. Data Interpretation

15.1 *Choice of Exposure Time*—the exposure time of choice is, in general, that which provides the greatest sensitivity. However, the IC20 having the smallest 95 % confidence interval may be preferred in cases in which the confidence interval varies appreciably with time of exposure. Consistency of choice between control samples and treated samples is of major importance for comparative studies. Finally, it should be noted that organics generally cause fast (5 to 10 min) response, while some metals continue to affect the luminescence of *P. phosphoreum* beyond 30 min. The changes in relative IC20 for the various exposure times as treatment progresses may, therefore, provide some additional information with regard to progress of treatment or further treatability, or both.

[11] In samples where absorbance due to concentration does not behave in accordance with Beer's Law or the samples causing significant Γ responses (0.02 or larger) are turbid, or both, it is necessary to directly measure the absorbance in the ACC for each sample concentration toxicity tested that gave a significant Γ response, by this test method. If desired, verify conformance to Beer's Law by performing the test in 13.10 with a second concentration in the ACC, for example, the highest concentration of interest. Using the equation in 14.2.1, calculate two values of A_c for the lower concentration using both ACC results. If the ratio of the two A_c values is between 0.98 and 1.02, the deviation from Beer's Law is within acceptable limits.

15.2 Compare the IC20 values (calculated concentration at $\Gamma = 0.25$) for the treated and untreated sample. Any toxicity reduction of 20 % or more, compared to the untreated system control sample or the raw starting material, is considered to be significant and a potential indication of biodegradability (see 1.1, 1.2, and Note 1).

15.3 Care must be taken to account for toxicity reduction that is not due to biodegradation (that is, adsorption, volatilization, and sample preparation errors). Control samples not exposed to biodegradation are essential as part of the data validation process (see 10.5).

16. Report

16.1 The record of the test and published reports of the results of the test should contain the following information:

16.1.1 Name of test, investigator, and laboratory; and the date the test was conducted;

16.1.2 Detailed description of the test sample including its source (detail biodegradation system used), composition (identity and concentration of major ingredients and major impurities), known physical and chemical properties, and identity and concentration of any solvents or other additives used;

16.1.3 The source of the dilution water, its chemical characteristics, and a description of any pretreatment;

16.1.4 Detailed information about the reagents used, including lot number, date received, reference toxicant data for the reagent lot, and any noted abnormalities;

16.1.5 A detailed description of the toxic inhibition analysis performed on the sample, including the test date, exposure times, test temperature, pH of sample before and after testing, all parametric data about sample, observations during test, and data reduction results (see 1.1, 1.2, and Note 1).

17. Precision and Bias

17.1 Quality data are produced when test procedures are followed as stated. The greatest source of error will be due to operator error. Errors are most likely to occur during sample preparation, salinity adjustment, filtration (if required), sample dilution, reagent dilutions, sample transfer and mixing steps, and data interpretation and resulting calculations. Use of the proper equipment and development of the appropriate skills required for using the test equipment are necessities in producing quality data.

17.2 Precision of the data may be improved by running a split sample duplicate analysis, repeating the procedures listed in 13.9 with the duplicate sample. Duplicate analyses can be performed simultaneously, or the duplicate sample can be analyzed separately. The duplicate sample must be protected from incurring further biodegradation or other physical/chemical changes. The results of the duplicate analyses are compared for any irregularities (obvious differences) in response versus exposure concentration. If such irregularities are noted, the sample should be retested if at all possible.

17.3 The raw data generated by the test procedures will determine whether an IC20 can be calculated with reasonable accuracy.

17.4 The determination of 95 % confidence intervals, using an acceptable procedure, will assist the investigator in determining the quality of generated IC20s (computer programs are available to perform these calculations).

17.5 An interlaboratory comparison study **(5)** was conducted on the toxic inhibition procedure described in this test method. The study involved 18 laboratories in four round robins, during which a total of six blind samples (five toxic and one nontoxic) were analyzed. The coefficient of variation (CV) ranged from 14.29 to 18.57 for the pooled data set, while the overall CV (regardless of sample) was calculated to be approximately 17.8 %.

17.6 The lack of an internal standard for this test method makes it impossible to determine the bias.

18. Keywords

18.1 bioluminescence; bioremediation; contaminated soil; contaminated water; detoxification; marine bacterium; toxicity

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