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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 D5660; 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 E729 and E1192), 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]

D888 Test Methods for Dissolved Oxygen in Water

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https://standards.iteh.ai/catalog/standards/sist/c3f8d19-8eea-41d3-9de2-8625a6349558/astm-d5660-962009

[1] This test method is under the jurisdiction of ASTM Committee D34 on Waste Management and is the direct responsibility of Subcommittee D34.03 on Treatment, Recovery and Reuse.

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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.

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D1125 Test Methods for Electrical Conductivity and Resistivity of Water
D1129 Terminology Relating to Water
D1193 Specification for Reagent Water
D1293 Test Methods for pH of Water
D3370 Practices for Sampling Water from Closed Conduits
E729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians
E943 Terminology Relating to Biological Effects and Environmental Fate
E1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians

3. Terminology

3.1 *Definitions*—The IC20 is defined in terms of a modification of the definition of IC50 as it appears in Terminology E943. The terms turbidity and volatile matter are defined in accordance with Terminology D1129. 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 E729. 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 D1193, 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 E729. 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).

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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

[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.

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 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 D3370. 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.

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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.

[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.