Designation: E 1279 – 89 (Reapproved 2001)

Standard Test Method for Biodegradation By a Shake-Flask Die-Away Method[1]

This standard is issued under the fixed designation E 1279; 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 describes procedures for assessing the biodegradation of chemicals in natural surface water samples.

1.2 This test method provides an opportunity to evaluate rates of biodegradation in the presence and absence of natural sediment materials. It also may provide limited information on the abiotic degradation rate, and sorption to sediment and vessel walls.

1.3 This test method allows for the development of a first-order rate constant, based on the disappearance of the test compound with time, and a second-order rate constant, normalized for changes in microbial biomass.

1.4 This test method requires a chemical specific analytical method and the concentrations of test substance employed are dependent on the sensitivity of the analytical method.

1.5 This test method is designed to be applicable to compounds that are not inhibitory to bacteria at the concentrations used in the test method, which do not rapidly volatilize from water, that are soluble at the initial test concentration and that do not degrade rapidly by abiotic processes, such as hydrolysis.

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 1193 Specification for Reagent Water[2]

D 4129 Test Method for Total and Organic Carbon in Water by Oxidation and Coulometric Detection[3]

E 895 Practice for Determination of Hydrolysis Rate Constants of Organic Chemicals in Aqueous Solutions[4]

E 896 Test Method for Conducting Aqueous Direct Photolysis Tests[4]

E 1194 Test Method for Vapor Pressure[4]

E 1195 Test Method for Determining a Sorption Constant [4]

3. Summary of Test Method

3.1 The shake-flask die-away biodegradation method is similar to river water die-away tests described by many authors, including Degens et al **(1)**,[5] Eichelberger and Lichtenberg **(2)**, Saeger and Tucker **(3)**, Paris et al **(4)**, and Cripe et al **(5)**. It differs from most die-away methods by providing for an evaluation of the effects of natural sediments on the transformation of the test compound and by the use of shaking to ensure a dissolved oxygen supply. Each test compound (substrate) is dissolved in water collected from a field site, with and without added natural sediment and with and without sterilization. Initial substrate concentrations typically are relatively low (µg/L), analytical capabilities permitting. Loss of test compound with time is followed by an appropriate, chemical-specific analytical technique. Changes in microbial biomass also may be followed by the use of an appropriate technique such as bacterial plate counts. Data obtained during use of the test method are used to provide the following information: (*a*) the abiotic degradation rate in the presence and absence of sediment and (*b*) the combined biotic and abiotic degradation rate in the presence and absence of sediment.

4. Significance and Use

4.1 Most of the simpler methods used to screen chemicals for biodegradation potential employ measurements that are not specific to the test substance, such as loss of dissolved organic carbon, evolution of respiratory carbon dioxide, or uptake of dissolved oxygen. Such methods generally are used to evaluate the transformation of the test substance to carbon dioxide, water, oxides or mineral salts of other elements, or products associated with the normal metabolic processes of microorganisms (ultimate biodegradability), or both. These methods require the use of relatively high initial concentrations of the test substance, generally 10 mg/L or higher, unless the tests are conducted using ^{14}C-radiolabeled test compounds. Biodegradation tests measuring ^{14}C-CO_2 evolution, for example, can be conducted using initial concentration of test compound at parts per billion. These tests, however, require specialized equipment and the custom preparation of appropriately labeled compound is often very expensive.

[1] This specification is under the jurisdiction of Committee E47 on Biological Effects and Environmental Fate and is the direct responsibility of Subcommittee E47.06 on Environmental Fate of Chemical Substances.

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

[3] *Annual Book of ASTM Standards*, Vol 11.02.

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

[5] The boldface numbers in parentheses refer to the list of references at the end of this test method.

4.2 Die-away biodegradation methods are simple simulation methods that employ water collected from natural water sources and follow the disappearance of an added amount of the test substance resulting from the activity of microorganisms in the water sample. The chemical-specific analytical techniques used to follow the disappearance of the test substance, typically are employed using relatively low initial concentrations of the test substance. Most environmental pollutants are present in the environment at relatively low concentrations (less than 1 mg/L) and it has been observed that biodegradation rates obtained using high test compound concentrations may be quite different from those observed at lower concentrations **(6)**.

4.3 The transformation of the test substance to an extent sufficient to remove some characteristic property of the molecule, resulting in the loss of detection by the chemicalspecific analytical technique, is referred to as primary biodegradation. For many purposes, evidence of primary biodegradation is sufficient, especially when it is known or can be shown that toxicity, or some other undesirable feature, associated with the test compound is removed or significantly reduced as a result of the primary biodegradation. A determination of ultimate biodegradation, on the other hand, is usually required only when treatability or organic loading are issues of concern. Furthermore, many of the simpler methods, such as those measuring CO_2 evolution (see 4.1), may not detect primary biodegradation.

4.4 The use of low substrate concentration enhances the probability of observing first-order, or pseudo first-order, kinetics. Thus, a rate constant for the primary biodegradation reaction and a half-life can be derived from the test compound under defined incubation conditions. Rate constants are required in many environmental fate mathematical models.

5. Apparatus

5.1 *Carefully Cleaned Glass or Plastic Carboys*, required for the collection and transport of field water samples.

5.2 *Field Sediment Samples*, obtained using scoop, beaker, or box sampler, as appropriate.

5.3 *A Rotary Shaker*, capable of holding 2-L Erlenmeyer flasks and shaking at 140 to 150 r/min is required for the incubation of test flasks. Temperature control (±2°C) may be incorporated in an incubator/shaker unit or may be obtained by placing the shaker in a temperature controlled space. The flasks should be constructed of material that minimizes sorption of test or reference compound to the walls of the flasks. In general, glass is the best choice.

5.4 *A Gas Chromatograph*, or other suitable instrument equipped with a detector sensitive to the test compound(s) and reference compound is required for the chemical-specific analysis of the test and reference compounds.

6. Reagents and Materials

6.1 Reference compounds are desirable to evaluate the biodegradation potential of the microbial population. A suitable reference compound will be biodegradable under the test conditions but not so readily biodegradable that it is completely degraded within a small fraction of the normal test period.

7. Sampling

7.1 Take samples from each flask according to a schedule appropriate to the rate of biodegradation of the test and reference substances. Sampling should be sufficiently frequent to establish plots of degradation versus time and to permit the determination of rate constants. Take a minimum of six samples from time zero until completion of the test. A nominal test time of 28 days allows a reasonable period for observations with slowly degraded substances. The test period may be extended beyond 28 days if necessary to calculate a half-life. Tests may be terminated prior to 28 days when more than 50 % of the starting material has disappeared from solution, due to biodegradation.

7.2 Remove duplicate samples of a sufficient size from each flask at appropriate intervals from day 1 ($t = 24$ h) until completion of the test. Centrifuge each sample to remove suspended particulates. Analyze the supernatant (or a suitable extract of the supernatant) to determine the concentration of test or reference compound. A record is maintained of compound concentration versus time for each flask. If adsorption to sediment solids is a significant factor, extract the sediment plug and analyze the extract to more fully account for untransformed test compound.

7.3 If microbial adaptation (a lag phase with little or no loss of test compound followed by relatively rapid loss) is suspected, add additional test compound to that flask and the corresponding control flask, at or near the normal end of the test period. Adaptation is indicated if the microorganisms in the test flask degrade the added compound without a lag period and the control flask, to which test compound has been added, exhibits a lag prior to degradation. Do not use the lag period in the calculation of the biodegradation rate. If there is a lag period due to adaptation, use the end of the lag period as time zero when calculating the first-order constant (see section 8.2.1). For an example, see Cripe et al **(5)**.

7.4 If desired, samples also may be taken for biomass determinations. Sampling times should coincide with the times of sampling for chemical concentrations.

8. Procedure

8.1 *Field Sampling*:

8.1.1 Collect water and sediment from a selected field site (for example, river, lake, or estuary), the day before test initiation. Measure the salinity (when appropriate), water temperature, and pH at the time of sampling. Collect water, from approximately 60 mm below the air/water surface, in clean glass or plastic carboys. Remove floating or suspended particulates, preferably by filtering the water through a 3-µm membrane filter. Collect the upper 5 to 10 mm of underlying sediment by skimming with a beaker, scoop, or box sampler. Screen the sediment through a sieve with 2 mm-openings to remove larger particles and biota. Omit sand by resuspending detritus and fine particles and decanting. This is necessary because sand cannot be quantitatively transferred from a slurry with a pipet. Add field water to or decant it from the sieved sediment until there is approximately a 1:1 ratio between sediment and water volumes. Return the water sample and the sediment slurry to the laboratory in closed containers.

8.1.2 If there is no sediment layer at the field site (for example, the stream or lake bed is all rock), omit the sediment collection and use procedures.

8.2 *Handing of Field Samples*:

8.2.1 Stir the sediment slurry and the site water continuously at room temperature until use in the test method.

8.2.2 Measure the concentration of sediment in the slurry by filtering 5-mL samples of well-mixed slurry through predried (105°, 1 h) 0.45-µm membrane filters. The slurry must be stirred vigorously during sampling to ensure homogeneity. Rinse the slurry sampling pipet, sediment, and filter with 2 to 3 mL of deionized water. The filter and sediment are then dried at 105° for 1 h. Determine the weight of the sediment after the dried filter and sediment have cooled to room temperature in a dessicator. Use the weight of sediment per mL of slurry to calculate the volume of slurry to be used in test flasks.

8.3 *Preparation of Flasks*:

8.3.1 Initial test compound concentration in the method typically is 200 µg/L. This concentration is generally high enough for analytical sensitivity and low enough to be environmentally realistic. Choose other concentrations as appropriate.

8.3.2 *Control Water Flasks*—Add 1 L of site water to each of two 2-L Erlenmeyer flasks.

8.3.3 *Control Sediment Flasks*—Add 900 to 950 mL of site water to each of two 2-L Erlenmeyer flasks. Sufficient sediment slurry is added to each flask to achieve a final (following a second addition of site water) suspended sediment concentration of 500 mg/L (on a dry weight of sediment basis). Add additional site water to achieve a final volume of site water plus sediment equal to 1 L.

8.3.4 *Amended Site Water*—Add sufficient test compound (or reference compound) to 9 to 10 L of site water to produce the desired initial concentration. Generally, analytical sensitivity permitting, the desired initial concentration is 200 µg/L and 2.0 mg of test compound are added to 10 L of site water. Addition of test compound may be accomplished through the addition of a solution of the test compound in a volatile solvent (for example, acetone) to a clean, empty vessel, removal of the volatile solvent by flushing with a clean air or nitrogen stream, and addition of 10 L of site water. Analyze the final solution to determine the concentration of test compound. To compensate for the volume of sediment slurry and formalin added later (8.3.6-8.3.8) an excess of test compound may be added to yield a concentration greater than 200 µg/L. The amount of amended site water added to the active water, active sediment, sterile water, and sterile sediment flasks is then adjusted to yield a final concentration of 200 µg/L test substance. Unamended site water is used, as necessary, to produce a final volume of 1 L in each flask.

8.3.5 *Active Water Flasks*—Add 1 L of amended water to each of two 2-L Erlenmeyer flasks.

8.3.6 *Active Sediment Flasks*—Add 900 to 950 mL of amended water to each of two 2-L Erlenmeyer flasks. Add sufficient sediment slurry to each flask to achieve a final (following a second addition of amended site water) suspended sediment concentration of 500 mg/L. Add additional amended site water to achieve a final volume of water plus sediment equal to 1 L.

8.3.7 *Sterile Water Flasks*—Add 900 to 950 mL of amended water to each of two 2-L Erlenmeyer flasks. Add 20 mL of 37 % formaldehyde solution (formalin) to each flask to act as a sterilant. Add additional amended site water to each flask to achieve a final volume of 1 L. If an interaction between formalin and the test or reference compound is likely or suspected, another sterilization procedure (for example, use of phenylmercuric acetate or autoclaving) may be required.

8.3.8 *Sterile Sediment Flasks*—Add 900 to 950 mL of amended water to each of two 2-L Erlenmeyer flasks. Add sufficient sediment slurry to each flask to achieve a final (following a second addition of amended water) suspended sediment concentration of 500 mg/L. Add 20 mL of formalin to each flask to act as a sterilant. Add additional amended site water to each flask to achieve a final volume of site water, sediment, and formalin equal to 1 L.

8.3.9 *Flask Incubation*—Close the flasks with polyurethane foam plugs and place them on a rotary shaker at 140 to 150 r/min at 25 ± 2°C. If a closer simulation of site conditions is desired, the incubation may be at a site representative temperature ± 2°C. Determine the pH of the water in each flask on day zero and at least every other day for the remainder of the test. Maintain the pH at the value observed at the time of collection, ±0.2 pH units, throughout the test by adding a few drops of 1 N HCl or 1 N NaOH, as required.

8.4 *Preliminary Check*—Compounds which are rapidly lost (50 % or greater decrease in 24 h) from solution due to chemical instability, volatility, or photolysis are not suitable for biodegradation rate determinations using this test method. To determine suitability, a preliminary test may be set up using a 2-L flask containing 1.0 L of reagent water, with a purity equal to or better than Type II of Specification D 1193,[6] to which 20 mL of formalin is added. Amend the flask with test compound to a concentration of about 200 µg/L. Close the amended flask with a stopper and provide with laboratory lighting of the same type and intensity provided to the test shaker flasks. This flask serves as a check on abiotic losses (for example, photolysis, hydrolysis, or evaporation). Sample the flask for test chemical concentration at zero time and after 24 h. If one-half or less of the test chemical is present at 24 h, no further work is done and the method is considered unsuitable for biodegradation testing of the chemical. If abiotic processes are significant, it is recommended that the investigator consult ASTM Test Method E 1194 for the evaluation of vapor pressure, Practice E 895 for hydrolysis, Test Method E 896 for aqueous photolysis, and Test Method E 1195 for sorption.

8.5 *Total Organic Carbon Analysis*—Analyze well-mixed samples from the control sediment and control water flasks for total organic carbon, (TOC) content using a suitable method such as that described in Test Method D 4129. This is used in calculating the equilibrium adsorption coefficient.

8.6 *Equilibrium Adsorption Coefficient*—Sample the sterile sediment flasks at half-hour intervals until the test chemical concentrations are relatively constant at each of two sequential sampling times, indicating no more adsorption to sediment and vessel walls. This generally occurs within the first 6 h. Place

[6] "Reagent Chemicals, American Chemical Society Specifications." Am. Chemical Soc., Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Analar Standards for Laboratory U.K. Chemicals," BDH Ltd., Poole, Dorset, and the "United States Pharmacopeia."

duplicate 25-mL samples from each flask in centrifuge tubes and centrifuge to remove suspended particulates before analysis for test compound (or reference compound) concentrations. Time zero concentration (C_0) is the concentration observed in samples obtained immediately following the preparation of the sterile sediment flasks.

9. Calculation

9.1 *Equilibrium Adsorption Coefficient*:

9.1.1 Calculate the equilibrium adsorption coefficient (K_{OC}) using the following equation:

$$K_{OC} = \frac{\text{µg adsorbed/g organic carbon in sediment}}{\text{µg/mL in solution}} = \frac{1000\ (C_o - C_e)}{C_e(\text{SO})} \quad (1)$$

where:

C_o = test compound concentration at time zero, µg/mL,
C_e = test compound concentration at equilibrium, time, µg/mL,
SO = sediment organic carbon = *CS* − *CW*,
CS = TOC in control sediment sample, g/L, and
CW = TOC in control water sample, g/L.

9.2 *Biodegradation Rates and Half-Lives*:

9.2.1 *First-Order Constants*—Determine first-order rate constants (K_1) by a regression equation of the type in $C = a + K_1 t$, as follows:

where:

C = µg/L test compound,
a = the Y-axis intercept,
K_1 = the slope (first-order rate constant), and
t = time.

See 7.7.3 for information on calculating K_1 if there is microbial adaptation resulting in a lag period.

9.2.2 *Half-Life*—The half-life of the test compound, based on the first-order rate constant, is given by $t_{1/2} = 0.693/K_1$. Calculate the half-life for the test compound in each flask in the test and then calculate an average value for replicate flasks.

9.2.3 *Second-Order Rate Constants*:

9.2.3.1 Second-order rate constants are of interest because some mathematical fate models use a second-order rate expression to describe the biotransformation of chemical compounds in environmental waters. In such models the disappearance rates for compounds are calculated from the concentration of indigenous bacteria, the concentration of the compound and the second-order rate constant.

9.2.3.2 Second-order rate constants (K_2) can be obtained, if desired, by dividing K_1 by the average bacterial concentrations (B). If plate count methods are used, (B) is expressed in colony forming units per mL. Bacterial concentrations normally do not change significantly during these tests, due to low substrate concentrations, and measured bacterial concentrations are averaged to obtain (B). Then calculate K_2 by:

$$K_2 = K_1/(B) \quad (2)$$

10. Report

10.1 Report the following data and information:

10.1.1 Test and reference compound identities.

10.1.2 Site, date, and time of field water and sediment collection.

10.1.3 Temperature, pH, and salinity (when appropriate) of site water at the time of collection.

10.1.4 Concentration of sediment (dry weight) per mL of slurry.

10.1.5 Total organic carbon (g/L) in the control sediment (*CS*) and control water (*CW*) samples.

10.1.6 Measured concentrations of test compound(s) and reference compound at each sampling time during (*a*) the preliminary check, (*b*) adsorption coefficient, and (*c*) test sampling steps.

10.1.7 Equilibrium adsorption coefficient (K_{OC}) calculations and results.

10.1.8 The average first-order rate constants for each replicate pair of flasks. If microbial adaptation was observed (with a lag period following test startup), describe the lag period and how it was evaluated.

10.1.9 The average half-life for the compound in each replicate pair of flasks.

10.1.10 The plate count or other biomass data, if determined.

10.1.11 The average second-order rate constants for each replicate pair of flasks, if determined.

11. Precision and Bias

11.1 The precision and bias for this test method have not been determined.

REFERENCES

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(**2**) Eichelberger, J. W., and Lichtenberg, J. J., "Persistence of Pesticides in River Water," *Environmental Science and Technology*, Vol 5, 1971, pp. 541–544.

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(**6**) Boethling, R. S., and Alexander, M., "Effect of Concentration of Organic Chemicals on Their Biodegradation by Natural Microbial Communities," *Applied and Environmental Microbiology*, Vol 37, 1979, pp. 1211–1216.