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

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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:*[2]

D1193 Specification for Reagent Water

D4129 Test Method for Total and Organic Carbon in Water by High Temperature Oxidation and by Coulometric Detection

E895 Practice for Determination of Hydrolysis Rate Constants of Organic Chemicals in Aqueous Solutions

E896 Test Method for Conducting Aqueous Direct Photolysis Tests

E1194 Test Method for Vapor Pressure

E1195 Test Method for Determining a Sorption Constant (K_{oc}) for an Organic Chemical in Soil and Sediments

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**),[3] 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

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[2] 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.

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

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.

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.