This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.

Designation: D6139 – 22

Standard Test Method for Determining the Aerobic Aquatic Biodegradation of Lubricants or Their Components Using the Gledhill Shake Flask[1]

This standard is issued under the fixed designation D6139; 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 covers the determination of the degree of aerobic aquatic biodegradation of fully formulated lubricants or their components on exposure to an inoculum under controlled laboratory conditions. This test method is an ultimate biodegradation test that measures carbon dioxide (CO_2) evolution.

1.2 This test method is intended to specifically address the difficulties associated with testing water insoluble materials and complex mixtures such as are found in many lubricants.

1.3 This test method is designed to be applicable to all non-volatile lubricants or lubricant components that are not toxic and not inhibitory at the test concentration to the organisms present in the inoculum.

1.4 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.* Specific hazards are discussed in Section 10.

1.6 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

[1] This test method is under the jurisdiction of ASTM Committee D02 on Petroleum Products, Liquid Fuels, and Lubricants and is the direct responsibility of Subcommittee D02.12 on Environmental Standards for Lubricants.

Current edition approved April 1, 2022. Published April 2022. Originally approved in 1997. Last previous edition approved in 2018 as D6139 – 18. DOI: 10.1520/D6139-22.

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1193 Specification for Reagent Water

D1293 Test Methods for pH of Water

D4447 Guide for Disposal of Laboratory Chemicals and Samples

D5291 Test Methods for Instrumental Determination of Carbon, Hydrogen, and Nitrogen in Petroleum Products and Lubricants

D5864 Test Method for Determining Aerobic Aquatic Biodegradation of Lubricants or Their Components

E943 Terminology Relating to Biological Effects and Environmental Fate

2.2 *ISO Standard:*[3]

4259:1992(E) Petroleum Products—Determination and application of precision data in relation to methods of test

2.3 *APHA Standards:*[4]

2540B Total Solids Dried at 103–105°C

9215 Heterotrophic Plate Count

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3. Terminology

3.1 *Definitions:*

3.1.1 Definitions of terms applicable to this test method that are not described herein appear in the ASTM Online Dictionary of Engineering Science and Technology[5] or Terminology E943.

[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] Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036, http://www.ansi.org.

[4] Methods from *Standard Methods for the Examination of Water and Wastewater*, latest edition. Available from the American Public Health Association (APHA), 800 I Street, NW, Washington, DC 20001.

[5] *ASTM Online Dictionary of Engineering Science and Technology* (Stock#DEFONLINE) is available on the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org.

*A Summary of Changes section appears at the end of this standard

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3.1.2 *activated sludge, n*—the precipitated solid matter, consisting mainly of bacteria and other aquatic microorganisms, that is produced at a domestic wastewater treatment plant and is used primarily in secondary sewage treatment to microbially oxidize dissolved organic matter in the effluent.

3.1.3 *aerobic, adj*—(*1*) taking place in the presence of oxygen; (*2*) living or active in the presence of oxygen.

3.1.4 *biodegradation, n*—the process of chemical breakdown or transformation of a material caused by organisms or their enzymes.

3.1.4.1 *Discussion*—Biodegradation is only one mechanism by which materials are transformed in the environment.

3.1.5 *biomass, n*—biological material including any material other than fossil fuels which is or was a living organism or component or product of a living organism.

3.1.5.1 *Discussion*—In biology and environmental science, biomass is typically expressed as density of biological material per unit sample volume, area, or mass (g biomass / g (or / mL or / cm^2) sample); when used for products derived from organisms biomass is typically expressed in terms of mass (kg, MT, etc.) or volume (L, m^3, bbl, etc.).

3.1.5.2 *Discussion*—Products of living organisms include those materials produced directly by living organisms as metabolites (for example, ethanol, various carbohydrates and fatty acids), materials manufactured by processing living organisms (for example: pellets manufactured by shredding and pelletizing plant material) and materials produced by processing living organisms, their components or metabolites (for example, transesterified oil; also called biodiesel).

3.1.6 *blank, n—in biodegradability testing*, a test system containing all system components with the exception of the test material.

3.1.7 *inoculum, n*—spores, bacteria, single celled organisms, or other live materials, that are introduced into a test medium.

3.1.8 *lag phase, n*—the period of diminished physiological activity and cell division following the addition of microorganisms to a new culture medium.

3.1.9 *log phase, n*—the period of growth of microorganisms during which cells divide at a positive constant rate.

3.1.10 *mixed liquor, n*—in sewage treatment, the contents of an aeration tank including the activated sludge mixed with primary effluent or the raw wastewater and return sludge.

3.1.11 *pre-adaptation, n*—the pre-incubation of an inoculum in the presence of the test material under conditions similar to the test conditions.

3.1.11.1 *Discussion*—The aim of pre-adaptation is to improve the precision of the test method by decreasing variability in the rate of biodegradation produced by the inoculum. Pre-adaptation may mimic the natural processes which cause changes in the microbial population of the inoculum leading to a more rapid rate of biodegradation of the test material but not to a change in the final degree of biodegradation.

3.1.12 *pre-condition, n*—the pre-incubation of an inoculum under the conditions of the test in the absence of the test material.

3.1.13 *supernatant, n*—the liquid above settled solids.

3.1.14 *suspended solids (of activated sludge or other inoculum samples), n*—solids present in activated sludge or inoculum samples that are not removed by settling under specified conditions.

3.1.15 *theoretical CO_2, n*—the amount of CO_2 which could in theory be produced from the complete oxidation of all of the carbon in a material.

3.1.16 *ultimate biodegradation, n*—degradation achieved when a material is totally utilized by microorganisms resulting in the production of CO_2 (and possibly methane in the case of anaerobic biodegradation), water, inorganic compounds, and new microbial cellular constituents (biomass or secretions, or both).

4. Summary of Test Method

4.1 Biodegradation of a lubricant or the component(s) of a lubricant is estimated by collecting and measuring the CO_2 produced when the lubricant or component is exposed to microorganisms under controlled aerobic aquatic conditions. This value is then compared to the theoretical amount of CO_2 which could be generated if all of the carbon in the test material were converted to CO_2. Carbon dioxide is a product of aerobic microbial metabolism of carbon-containing materials and so is a direct measure of the test material's ultimate biodegradation. The evolved CO_2 is trapped in a $Ba(OH)_2$ or other alkaline solution and the amount of CO_2 absorbed is determined by titrating the remaining hydroxide in solution.

4.2 The carbon content of the test material is determined by Test Methods D5291 or another appropriate method and the theoretical CO_2 is calculated from that measurement. It is necessary to directly measure the carbon content of the test material instead of calculating this number, because of the complexity of the mixture of compounds present in lubricants.

4.3 Biodegradability is expressed as a percentage of theoretical CO_2 production.

5. Significance and Use

5.1 Results from this CO_2 evolution test method suggest, *within the confines of a controlled laboratory setting*, the degree of ultimate aerobic aquatic biodegradability of a lubricant or components of a lubricant. Test materials which achieve a high degree of biodegradation in this test method may be assumed to easily biodegrade in many aerobic aquatic environments. (See also Test Method D5864.)

5.2 Because of the stringency of this test method, a low yield of CO_2 does not necessarily mean that the test material is not biodegradable under environmental conditions, but indicates that further testing needs to be carried out in order to establish biodegradability.

5.3 Information on the toxicity of the test material to the inoculum may be useful in the interpretation of low biodegradation results.

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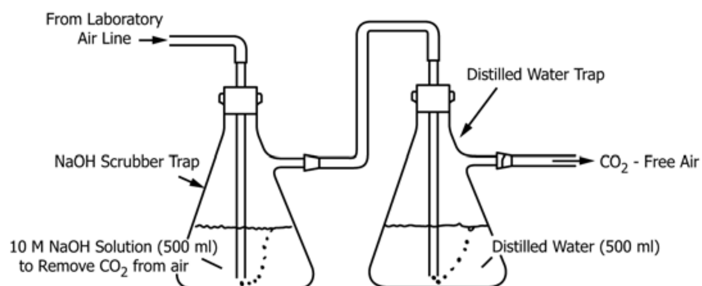

FIG. 1 NaOH Scrubber – Flask Trap Assembly for Providing CO_2-Free Air

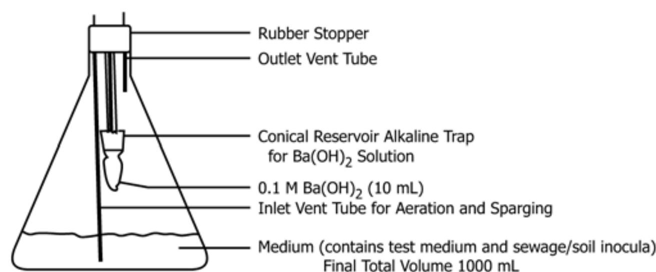

FIG. 2 Gledhill Shake Flask System for CO_2 Evolution

5.4 Activated sewage-sludge from a sewage treatment plant that principally treats domestic waste may be used as an aerobic inoculum. An inoculum derived from soil or natural surface waters, or any combination of the three sources, may also be used in this test method.

NOTE 1—Allowance for various and multiple inoculum sources provides access to a greater diversity of biochemical competency and potentially represents more accurately the capacity for biodegradation.

5.5 A reference or control material known to biodegrade under the conditions of this test method is necessary in order to verify the activity of the inoculum. The test method must be regarded as invalid and should be repeated using a fresh inoculum if the reference does not demonstrate biodegradation to the extent of >60 % of the theoretical CO_2 within 28 days.

5.6 The water solubility or dispersibility of the lubricant or components may influence the results obtained and hence the procedure may be limited to comparing lubricants or components with similar solubilities.

5.7 The ratio of carbon incorporated into cellular material to carbon metabolized to CO_2 will vary depending on the organic substrate, on the particular microorganisms carrying out the conversion, and on the environmental conditions under which the conversion takes place. In principle, this variability complicates the interpretation of the results from this test method.

5.8 The behavior of complex mixtures may not always be consistent with the individual properties of the components. The biodegradability of the components may be suggestive of whether a mixture containing these components (that is, a fully formulated lubricant) is biodegradable but such information should be used judiciously.

6. Apparatus

6.1 *Carbon Dioxide Scrubbing Apparatus* (see Fig. 1):

6.1.1 The following are required to produce a stream of CO_2-free air for aeration and for sparging aqueous solutions and mixtures (for example, test medium, sewage inoculum):

6.1.1.1 *Erlenmeyer flask*, one 1 L with side arm containing 500 mL of 10 *M* sodium hydroxide (NaOH), and fitted with a rubber stopper and an inlet tube that extends below the level of the NaOH solution or an equivalent apparatus or system.

6.1.1.2 *Erlenmeyer flask*, one 1 L with side arm containing 500 mL of distilled water and fitted with a stopper and inlet tube, or an equivalent apparatus or system.

6.1.1.3 It is optional to add an empty 1 L Erlenmeyer flask in series with the flasks to prevent liquid carryover.

6.1.1.4 It is optional to add a 1 L Erlenmeyer flask containing 500 mL of 0.1 *M* barium hydroxide [$Ba(OH)_2$] solution to monitor for possible breakthrough CO_2.

6.1.2 Connect the flasks in series as shown in Fig. 1, using vinyl or other suitable non-gas-permeable tubing, to a pressurized air system and purge air through the scrubbing solution.

6.1.3 The CO_2 scrubbing apparatus upstream of the Erlenmeyer flask containing the $Ba(OH)_2$ may be substituted with an alternative system which effectively and consistently produces CO_2-free air (that is, containing <1 ppm CO_2).

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6.2 *Incubation/Biodegradation Apparatus—Gledhill-type Shake Flask Units*[6] (see Fig. 2)—Each test material, reference, or blank control requires the following:

6.2.1 *Erlenmeyer Flasks, 2 L*—2 L Erlenmeyer flasks are used to hold the 1 L of total final aqueous volume but larger volume Erlenmeyer flasks (as large as 3 L to 4 L) may be used if 2 L to 3 L final aqueous volumes are required. The amounts described here are for 1 L final aqueous volumes carried out in 2 L Erlenmeyer flasks; scale procedure accordingly if larger final aqueous volumes and larger Erlenmeyer flasks are necessary.

6.2.2 *Stoppers*—Each stopper is fitted with a conical alkaline trap, an outlet and an inlet vent tube (see Fig. 2). Ensure that the stopper fits tightly in the Erlenmeyer flask to prevent any leaks.

6.2.3 *Conical Alkaline Trap Tube or Unit*—Glass, 40 mL conical tube (borosilicate glass, No. 8120 centrifuge tube or equivalent) welded to a glass support rod, or an equivalent apparatus, will be used to hold the $Ba(OH)_2$ solution for trapping the evolved CO_2 from aerobic biodegradation. The opening in the alkaline trap tube is large enough to permit CO_2 diffusion into the barium hydroxide solution. The support rod of the conical trap shall fit tightly in the stopper.

6.2.4 *Inlet and Outlet Vent Tubes*—The inlet vent tube attached to the stopper extends down into the flask so that it will be immersed below the surface of the aqueous medium and will be used for sparging. The outlet vent tube will be

[6] Gledhill, W. E., “Screening Test for Assessment of Ultimate Biodegradability: Linear Alkyl Benzene Sulfonate,” *Applied Microbiology* Vol 30, 1975, pp. 992–929. Also see description of Gledhill shake flask unit in EPA Chemical Fate Testing Guidelines for Aerobic Aquatic Biodegradation, EPA Publication 560/6-82-003, No. CG-2000 (August 1982); Federal Register, September 27, 1985, p. 39277, Section 796.3100; 40 CFR 796.3100, 1994.

situated significantly above the level of the aqueous medium and will be used for venting. The two vent tubes shall fit tightly in the stopper.

6.2.5 Flexible tubing which is non-permeable to CO_2 will be used to connect the tops of inlet and outlet vent tubes to form a closed system.

6.2.6 *Agitators*—Incubator-shaker table unit or equivalent, or stirrers may be used to agitate the aqueous mixture in the Erlenmeyer flasks.

6.3 *Analytical Balance*, to weigh out test material or reference material to be added to the test flask (capable of weighing to appropriate precision and accuracy, for example, ±0.0001 g).

6.4 *Titration Apparatus for Measuring the Production of CO_2:*

6.4.1 Appropriate graduated burette filled with standard HCl solution.

6.4.2 Alternatively, an automatic titration apparatus in which the burette dispenser is filled with standard HCl solution. Automatic titrations are carried out to a potentiometric end point of pH 8.3 (that is, phenolphthalein end point equivalent)

6.5 *Glass Wool*, for filtering the inoculum.

7. Reagents and Materials

7.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where such specifications are available.[7] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

7.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water as defined by Type II of Specification D1193.

7.3 Prepare the following stock solutions:

7.3.1 *Ammonium Sulfate Solution* (40 g/L)—Dissolve 40.0 g ammonium sulfate [$(NH_4)_2SO_4$] in water and dilute to 1 L.

7.3.2 *Calcium Chloride Solution* (27.5 g/L)—Dissolve 27.5 g anhydrous calcium chloride ($CaCl_2$) in water and dilute to 1 L.

7.3.3 *Ferric Chloride Solution* (0.25 g/L)—Dissolve 0.25 g ferric chloride hexahydrate ($FeCl_3 \cdot 6\ H_2O$) in water and dilute to 1 L.

7.3.4 *Magnesium Sulfate Solution* (22.5 g/L)—Dissolve 22.5 g magnesium sulfate heptahydrate ($MgSO_4 \cdot 7\ H_2O$) in water and dilute to 1 L.

7.3.5 *Phosphate Buffer*—Dissolve 8.5 g potassium dihydrogen phosphate (KH_2PO_4), 21.7 g potassium monohydrogen phosphate (K_2HPO_4), 50.3 g sodium monohydrogen phosphate heptahydrate ($Na_2HPO_4 \cdot 7\ H_2O$) [or alternatively, 33.4 g of sodium monohydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2\ H_2O$), the dihydrate equivalent form], and 1.7 g ammonium chloride (NH_4Cl) in water and dilute to 1 L.

7.3.6 *Trace Elements Solution*—Dissolve 0.035 g manganous chloride tetrahydrate ($MnCl_2 \cdot 4\ H_2O$), 0.057 g boric acid (H_3BO_3), 0.043 g zinc sulfate heptahydrate ($ZnSO_4 \cdot 7\ H_2O$), and 0.037 g ammonium molybdate tetrahydrate [$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$] in water and dilute to 1 L.

7.4 *Barium Hydroxide Solution*, 0.1 *M*, is prepared by dissolving 32.0 g barium hydroxide octahydrate [$Ba(OH)_2 \cdot 8H_2O$] in distilled water and diluting to 1 L. Filter free of solid material, confirm molarity by titration with standard acid, and store under nitrogen sealed as a clear solution to prevent absorption of CO_2 from the air. It is recommended that 2 L be prepared at a time when running a series of tests.

7.5 *Vitamin-free Casamino Acids.*

7.6 *Yeast Extract.*

7.7 *Phenolphthalein.*

7.8 *Standardized Hydrochloric Acid* (0.190 M to 0.210 M).

8. Inoculum Test Organisms

8.1 *Sources of the Inoculum*—The following provides several options for where and how to obtain an appropriate inoculum:

8.1.1 *Inoculum from Activated Sludge*—Activated sludge freshly sampled (that is, less than 24 h old) from a well-operated predominantly domestic sewage treatment plant (that is, one with no recent upsets and operating within its design parameters) may be used. This sewage treatment plant should receive no more than 25 % of its influent from industrial source(s).

8.1.1.1 Using CO_2-free air, aerate sludge in the laboratory for 4 h. Five hundred millilitres of the mixed liquor is sampled and homogenized for 2 min at medium speed in a high sheer/high speed blender or equivalent high-speed mixer. Allow to settle for 30 min.

8.1.1.2 If the supernatant still contains high levels of suspended solids at the end of 30 min, allow to settle for another 30 min to 40 min.

8.1.1.3 Decant sufficient volume of the supernatant to provide either a 1 % (by volume) inoculum or 30 mg/L of suspended solids for each test Erlenmeyer flask. Avoid carryover of sludge solids which might interfere with the measurement of CO_2 production.

8.1.1.4 It is optional to pre-condition the inoculum. Preconditioning consists of aerating the activated sludge in mineral medium solution for up to seven days. Sometimes preconditioning improves the precision of the test method by reducing blank values.

NOTE 2—Exercise care in pre-conditioning because of the sensitivity of inocula to prolonged aeration and starvation conditions. Pre-conditioning should be applied mainly in situations where it is known that the inoculum source consistently shows a high internal respiration rate.

8.1.2 *Inoculum From Soil:*

[7] *ACS Reagent Chemicals, Specifications and Procedures for Reagents and Standard-Grade Reference Materials*, 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.

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8.1.2.1 Suspend 100 g of soil in 1000 mL of water.
8.1.2.2 Allow the suspension to settle for 30 min.
8.1.2.3 Filter the supernatant through a coarse filter paper or glass wool plug, and discard the first 200 mL. The filtrate is aerated immediately and continuously until used.
8.1.3 *Inoculum from Surface Water:*
8.1.3.1 Filter surface water through a coarse filter paper or glass wool plug, discarding the first 200 mL.
8.1.3.2 Aerate the filtrate until used.
8.1.4 *Composite Inoculum*—The three inoculum sources may be combined in any proportion and mixed well.

8.2 *Enumeration of Microorganisms:*
8.2.1 APHA Test Method 9215, or equivalent, shall be used to enumerate the microorganisms in the inoculum. The inoculum shall contain 10^6 to 10^8 colony-forming units (CFU) per millilitre. It is optional to measure the total bacterial count of the inoculum using the dip slide technique with a commercially available diagnostic kit.
8.2.2 Alternatively, APHA Test Method 2540B shall be used to determine the sludge dry-weight per unit volume. Calculate the volume of mixed liquor necessary to achieve a final sludge dry-weight concentration in the test medium of 30 mg/L (suspended solids).

8.3 Pre-adaptation of the inoculum is allowed and can be accomplished as follows:[8]
8.3.1 Supplement inoculum with 25 mg/L vitamin-free casamino acids and 25 mg/L of yeast extract.
8.3.2 The test medium solution shall be prepared as follows: each litre of the test medium is prepared by measuring out the following volumes of the six stock solutions listed below, combining them, mixing, and diluting to 1 L with water. Multiples of this test medium solution can be prepared at one time (scale volumes proportionally).
8.3.2.1 *Ammonium Sulfate Solution*, 1 mL,
8.3.2.2 *Calcium Chloride Solution*, 1 mL,
8.3.2.3 *Ferric Chloride Solution*, 4 mL,
8.3.2.4 *Magnesium Sulfate Solution*, 1 mL,
8.3.2.5 *Phosphate Buffer Solution*, 10 mL,
8.3.2.6 *Trace Elements Solution*, 1 mL.
8.3.3 Add 100 mL of supplemented inoculum and 900 mL test medium to a 2 L Erlenmeyer flask
8.3.4 Add test materials incrementally during the acclimation period at concentrations equivalent to 4 mg, 8 mg, and 8 mg carbon/L on days 0, 7, and 11, respectively.
8.3.5 The inoculum flask(s) will be maintained at a temperature of 22 °C ± 2 °C in the dark and will be agitated on a shaker table or with a magnetic stirrer at a moderate speed (for example, 150 r/min to 200 r/min).
8.3.6 On day 14, homogenize the culture in a blender for at least 1 min and refilter the medium through glass wool prior to use as the inoculum for the test. If pre-adaptation is conducted for a series of functionally or structurally related test materials (may include reference material), media from the separately prepared inoculum may be combined before final filtration. The enumeration of microorganisms in the final pre-adapted inoculum shall be carried out using the method described in 8.2.

9. Test Material and Reference Material

9.1 This section addresses specific requirements pertaining to the carbon concentrations of the test material and reference material as well as the appropriate choice of reference materials.

9.2 The carbon content of a test material shall be measured by Test Methods D5291 or an equivalent procedure.

9.3 The test material shall be added to provide 10 mg to 20 mg carbon per litre (mg C/L) in the test medium. This will ensure that sufficient carbon is present to yield CO_2 which can be adequately measured by the trapping procedure described in this test method should the test material be biodegradable.

9.4 *Reference*—A material known to be biodegradable shall be tested simultaneously with the test material.
9.4.1 For water-insoluble test materials, the suggested reference is a low erucic acid rapeseed oil, also called LEAR, such as canola oil. The fatty acid profile of low erucic acid rapeseed oil shall contain a maximum of 2 % erucic acid by weight.
9.4.2 Sodium benzoate or aniline is suggested as a reference material if the test material is water-soluble.
9.4.3 The reference will be added in the same manner as the test material to provide a carbon concentration of 10 mg to 20 mg C/L in the flask.
9.4.4 The results from flasks containing the reference verify the viability of the inoculum.

9.5 The test method will be performed in a minimum of two replicates on all test and reference materials although triplicates are recommended.

9.6 Exercise care to obtain representative samples from test and reference materials.

10. Hazards

10.1 This test method includes the use of hazardous chemicals. Avoid contact with chemicals and follow the manufacturers' instructions and Material Safety Data Sheets (MSDS).

10.2 This test method includes the use of potentially harmful microorganisms. As such, execution of this test method must be carried out under the guidance of qualified personnel who understand the safety and health aspects of working with microorganisms. Minimally, review the test method with an industrial hygienist before initiating any activity. Avoid contact with the microorganisms by using gloves and other appropriate protective equipment and sterile procedures. Use good personal hygiene.

10.3 Sterilize materials and supplies contaminated with biologically active cultures before discarding or reusing them.

10.4 Chemicals should be disposed of as described in Guide D4447 or as prescribed by current regulations.

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[8] Sturm, R. N., "Biodegradability of Non-ionic Surfactants: Screening Test for Predicting Rate and Ultimate Biodegradation," J. Am. Oil Chemists Soc, Vol 50, 1973, pp. 159–167.