Designation: D 6139 – 00

An American National Standard

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 D 6139; 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 *This standard does not purport to address all 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 to determine the applicability of regulatory limitations prior to use.* Specific hazards are discussed in Section 10.

2. Referenced Documents

2.1 *ASTM Standards:*

D 1129 Terminology Relating to Water[2]

D 1193 Specification for Reagent Water[2]

D 1293 Test Methods for pH of Water[2]

D 4447 Guide for Disposal of Laboratory Chemicals and Samples[3]

D 5291 Test Methods for Instrumental Determination of Carbon, Hydrogen, and Nitrogen in Petroleum Products and Lubricants[4]

D 5864 Test Method for Determining Aerobic Aquatic Biodegradation of Lubricants or Their Components[5]

E 943 Terminology Relating to Biological Effects and Environmental Fate[5]

2.2 *ISO Standard:*[6]

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

2.3 *APHA Standards:*[7]

2540B Total Solids Dried at 103–105°C

9215 Heterotrophic Plate Count

3. Terminology

3.1 *Definitions:*

3.1.1 Definitions of terms applicable to this test method which are not described herein, appear in the Compilation of ASTM Standard Definitions (1990) or Terminology E 943.

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; activated sludge 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 test material caused by organisms or their enzymes.

3.1.4.1 *Discussion*—Biodegradation is only one mechanism by which substances are removed from the environment.

3.1.5 *biomass, n*—any material, excluding fossil fuels, which is or was a living organism or component of a living organism.

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

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

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

[3] *Annual Book of ASTM Standards*, Vol. 11.04.

[4] *Annual Book of ASTM Standards*, Vol. 05.03.

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

[6] Available from American National Standards Institute, 11 West 42nd St., 13th Floor, New York, NY 10036

[7] Methods from *Standard Methods for the Examination of Water and Wastewater*, latest edition. Available from the American Public Health Assoc. (APHA), 1015 18th St., N.W., Washington, D.C. 20036.

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 incubation of an inoculum in the presence of the test material which is done prior to the initiation of the test and 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 is not expected to change the overall extent of biodegradation of the test material.

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 carbon dioxide (ThCO $_2$), n*—the amount of CO_2 which could theoretically be produced from the complete biological oxidation of all of the carbon in a test material.

3.1.16 *ultimate biodegradation, n*—degradation achieved when the test 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 and secretions).

3.1.17 *ultimate biodegradation test, n*—a test which estimates the extent to which the carbon in a product has been converted to CO_2 or methane, either directly by measuring the production of CO_2 or methane, or indirectly by measuring the consumption of O_2.

3.1.17.1 *Discussion*—The measurement of new biomass is not attempted.

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 Method D 5291 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.

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.

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

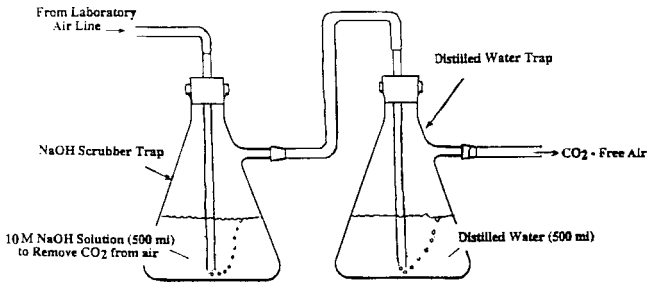

FIG. 1 NaOH Scrubber – Flask Trap Assembly for Providing CO_2-Free Air

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).

6.2 *Incubation/Biodegradation Apparatus – Gledhill-type Shake Flask Units*[8] (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 to 4-L) may be used if 2 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 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.

[8] W.E. Gledhill, "Screening Test for Assessment of Ultimate Biodegradability: Linear Alkyl Benzene Sulfonate", *Applied Microbiology* (1975) 30: 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, pg. 39277, Section 796.3100; 40 CFR 796.3100 (1994).

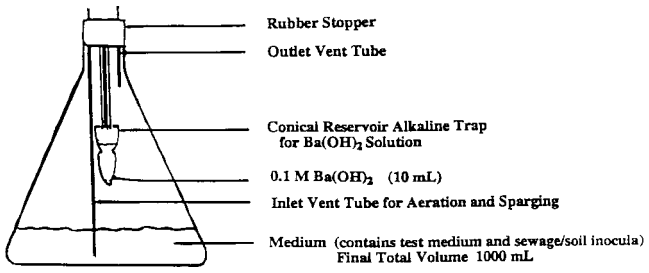

FIG. 2 Gledhill Shake Flask System for CO_2 Evolution

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.[9] 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 D 1193.

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* (0.190–0.210 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 hs 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 blender[10] 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 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 carry-over 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 pre conditioning 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:*

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

[9] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, D.C. 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.

[10] A high sheer/high speed blender has been found suitable for this purpose.

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:[11]

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, 8, 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 ±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 to 200 rpm).

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 D 5291 or an equivalent procedure.

9.3 The test material shall be added to provide 10 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 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 D 4447 or as prescribed by current regulations.

11. Preparation of Apparatus

11.1 *Cleaning*—The following is a suggested method for cleaning glassware and equipment to avoid organic contamination which may affect test results. The glassware and equipment used to prepare and store stock solutions and test solutions should be cleaned before use. Items should be washed with detergent and rinsed with water, a water-miscible organic solvent, water, acid (such as 10 % concentrated hydrochloric acid), and at least twice more with distilled, deionized water. Some organic solvents *may* leave a film that is insoluble in water. The presence of this film is not acceptable and may lead to false positive results. At the end of every test, all items that are to be used again should be immediately (*a*) emptied, (*b*) rinsed with water, and (*c*) cleaned as stated previously.

12. Procedure [12]

12.1 For each blank, test material and reference being tested, prepare a 1 % inoculum by the following dilution:

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

[12] Procedure was adapted from the experimental guidelines of the EPA Chemical Fate Testing Guidelines for Aerobic Aquatic Biodegradation, EPA Publication 560/6-82-003, No. CG-2000 (August 1982) [also described in Federal Register, pg. 39277, Section 796.3100, September 27, 1985 and in 40 CFR 796.3100 (1994)] or those reported by W.E. Gledhill, "Screening Test for Assessment of Ultimate Biodegradability: Linear Alkyl Benzene Sulfonate", *Applied Microbiology* (1975) 30: 992–929.

12.1.1 Add 900 mL of water to each of the 2-L Erlenmeyer flasks.

12.1.2 To each 2-L Erlenmeyer flask, add 10 mL of the phosphate buffer stock solution; 1 mL each of the magnesium sulfate, calcium chloride, ammonium sulfate, and trace elements stock solutions; 4 mL of the ferric chloride stock solution; and 10 mL of the sludge inoculum or a sufficient volume of the inoculum to give 30 mg/L suspended solids. The Erlenmeyer flasks now contain at least 928 mL of solution.

12.1.3 In the case in which a pre-adapted combined inoculum is used and in which the test materials are structurally related, use the same composite inoculum (see 8.3.6) for the blank, reference, and test flasks.

12.2 Aerate the aqueous mixture in the Erlenmeyer flasks with CO_2-free air for at least 1 h to purge the system of CO_2. Carbon dioxide-free air can be bubbled into the aqueous medium with the aid of a long glass tube (or equivalent). A sodium hydroxide scrubbing apparatus used to provide CO_2-free air is shown in Fig. 1.

12.3 Measure the pH in each Erlenmeyer flask by Test Methods D 1293 or an equivalent method. Using dilute HCl or NaOH, adjust the pH to 7 ± 0.5 before adding the test material or reference material.

12.4 The carbon concentration of the test material or reference material in the test medium shall be 10 to 20 mg C/L. Calculate the weight of the test or reference material needed to produce 10 to 20 mg of carbon per litre based upon the carbon content of the material as determined previously (see 9.2).

12.5 *Addition of the Test Material or Reference Material:*

12.5.1 Add the test material or reference material gravimetrically to the replicate Erlenmeyer flasks. If in order to accomplish this, the material is weighed into or onto a small object, then both the material and the object shall be added to the flask.

NOTE 3—An example of a small object might be a glass fiber filter. The test or reference material is added to the respective shake flasks as a measured weight adsorbed onto the surface of the filter. This enables an accurate weight to be dosed into each flask and increases the surface area of the hydrophobic test or reference material. A blank glass fiber filter should also be added to each blank shake flask.

12.5.2 Sonication of the test material or reference material in 5 mL of water while still in/on a small object is allowed as a means of obtaining a better dispersion of insoluble materials in the test medium. If sonication is performed, the object shall also be added to the flask. In addition, if sonication is performed on the test material, the reference material shall also be sonicated in an identical manner prior to its addition to the test medium.

12.6 Along with the flasks containing test materials or reference materials, additional replicate flasks shall contain the test medium and the inoculum with no additional carbon source added. These flasks shall be blanks.

12.7 Add sufficient volume of water to achieve a final volume of 1000 mL in each flask.

12.8 Add 10 mL of the 0.1*M* $Ba(OH)_2$ solution to the conical alkaline trap unit.

12.9 Place the charged $Ba(OH)_2$ trap and stopper securely on top of the Erlenmeyer flask. Connect the tops of the inlet and outlet vent tubes with a single piece of non-permeable tubing and ensure that each assembled Gledhill shake flask unit is a closed system. The stopper should fit tightly on the Erlenmeyer flask and the tubing connecting the inlet and outlet vent tubes should be secured tightly in place to ensure that the test system is closed and to prevent any leaks (for example, the loss of evolved CO_2 or the entry of ambient CO_2 into the flask).

12.10 Start the test by agitating the Erlenmeyer flasks in a shaker table or other form of agitation such as a stir plate set at a moderate speed (for example, 150 to 200 rpm). Ensure that each flask is agitated at about the same speed.

12.11 Run the test at 22 ±2°C, and record the test temperature with an appropriate calibrated temperature measurement device throughout the test period.

12.12 At the time when the temperature is taken, it is also a convenient time to inspect the shake flasks to ensure that the stopper and connecting vent tubing are tightly secured in place.

12.13 Maintain the Erlenmeyer flasks in darkness to prevent photodegradation of the test material and growth of photosynthetic bacteria and algae.

12.14 *Carbon Dioxide Evolution and Analysis:*

12.14.1 The CO_2 produced in each Erlenmeyer flask reacts with $Ba(OH)_2$ in the conical trap and is precipitated as barium carbonate ($BaCO_3$).

12.14.2 When significant $BaCO_3$ precipitate is evident, the $Ba(OH)_2$ solution is removed from the conical trap for analysis. The trap is rinsed with 10 mL of CO_2-free distilled water and this water rinse combined with the collected $Ba(OH)_2$ solution.

12.14.2.1 *Analysis*—The amount of CO_2 produced is determined by titrating the $Ba(OH)_2$ with standard hydrochloric acid. Titrate the combined $Ba(OH)_2$-water rinse mixture immediately after removing and collecting it from each Erlenmeyer flask. *Exercise care to minimize exposure to air to avoid absorbing ambient* CO_2. Titrate with standard hydrochloric acid to a colorless phenolphthalein end point using a graduated burette. If an automatic titrator is used, titrate to a potentiometric end point of pH 8.3 (that is, phenophthalein equivalent end point).

12.14.3 The trap is refilled with 10 mL of fresh 0.1M $Ba(OH)_2$ solution. The inlet and outlet tubes are opened, and the aqueous solution in the flask is then sparged with CO_2-free air through the inlet tube. Flasks should be sparged for approximately the same length of time (for example, a few minutes) for the blank and for the reference and test materials. The outlet and inlet tubes are resealed tightly and the flasks are placed back on the shaker or stirrer, and agitation resumed.

12.14.4 Sampling of the $Ba(OH)_2$ traps and titration may be required every one to three days for the first ten days and then every five to seven days until a plateau of CO_2 evolution is reached. A plateau is reached after evidence of biodegradation has occurred and the production of CO_2 is either no longer detectable or is equal to the CO_2 produced by the blanks for two consecutive sampling times.

12.14.5 Because the results of the titration are added to produce the final test result, the uncertainty in the final result increases as the number of titrations increases. Consideration of this point is advisable when establishing the frequency of

titrations. Titrations of trap samples shall be carried out at the same frequency for the blank, and reference and test flasks, and the method of analysis shall follow the procedure described in 12.14.2.1.

12.14.6 The test shall continue for at least 28 days or, if longer, until the CO_2 evolution has reached a plateau. If on day 28, a plateau has not been achieved, that is, CO_2 production is still being detected in the conical trap (significant $BaCO_3$ precipitation), perform a titration on the $Ba(OH)_2$ solution in the trap and continue to monitor CO_2 evolution until a plateau has been reached.

12.14.7 Once the CO_2 evolution has reached a plateau, measure the final pH of the Erlenmeyer flask contents before adding concentrated acid to terminate the biodegradation study. A minimal volume of the aqueous solution can be removed for pH determination. Then add 1 mL of concentrated hydrochloric acid or concentrated sulfuric acid to each of the Erlenmeyer flasks to decompose inorganic carbonate and to release the trapped CO_2. Restopper the Erlenmeyer flasks and continue agitation overnight to collect the released CO_2 in the barium hydroxide traps. Remove the trap sample on the next day and perform the final titration on the barium hydroxide solution as described in 12.14.2 and 12.14.2.1.

13. Calculation

13.1 The total organic carbon content of the test material (or reference material) determined by elemental analysis is used to calculate the theoretical quantity of CO_2 evolution for each replicate as follows:

13.1.1 Test material (or reference material) contains a specific weight fraction of carbon, therefore:

$$Yi = w\ xTi \tag{1}$$

where:

- Yi = carbon charged to the test medium in the ith replicate, mg,
- w = weight fraction of carbon in test material expressed as a decimal, and
- Ti = test material (or reference material) added to the test medium in the *i*th replicate, mg.

13.1.2 The theoretical amount of CO_2 which could be produced if all of the carbon in the material is converted to CO_2, is then calculated by knowing 12 g carbon yields 44 g CO_2, therefore:

$$Xi = \frac{44}{12} \times Yi \tag{2}$$

where:

- Xi = theoretical amount of CO_2, which could be produced from Yi, mg, and
- Yi = carbon charged to the test medium in the *i*th replicate, mg.

13.2 *Amount of CO_2 Produced:* (For the *i*th replicate flask for the test material (or reference material):

13.2.1 Calculate the amount of CO_2 produced for a specific titration (Cxi) for the test material (or reference material) as follows:

$$Cxi = \frac{M}{2} \times (Zb - Z\,ti)\ x\ 44 \tag{3}$$

where:

- Cxi = CO_2 produced for a specific titration in the ith replicate, mg,
- M = molarity of HCl,
- Zb = HCl needed to titrate $Ba(OH)_2$ solution for blank controls (the average HCl titration volume of the blank control replicates will be used), mL, and
- Zti = mL of HCl needed to titrate $Ba(OH)_2$ solution for test material (or reference material) for the*i*th replicate.

This equation corrects for background CO_2 from the blank control in which no test or reference material (that is, no additional carbon source) was added.

13.2.2 Calculate the total amount of CO_2 *(Cti)* produced for the ith replicate for the test or reference material by summing the results obtained for each titration.

$$C_{ti} = \Sigma\, C_{xli}\ \ldots\ C_{xni} \tag{4}$$

From the *Cti* values for the replicates, determine the mean and standard deviation for the test material (or reference material).

13.2.3 Percent of theoretical CO_2 evolved (Pi) for the ith replicate is calculated as shown below:

$$Pi = \frac{Cti}{Xi} \times 100 \tag{5}$$

where:

- Cti = total amount of CO_2 calculated by summing the CO_2 produced at each titration interval for a given test or reference material for the *i*th replicate, and
- Xi = theoretical CO_2 produced from a given material for the *i*th replicate, mg.

From the replicate values, determine the mean and standard deviation for percent of theoretical CO_2 evolved for the test material (or reference material).

14. Report

14.1 Report the following data and information:

14.1.1 Information on the inoculum, including source, date of collection, storage, handling, and if used, the method for pre-adaptation to the test material.

14.1.2 *Method and Results of Biomass Determination*:

14.1.2.1 Viable microorganisms per millilitre in the inoculum at the beginning of the test, or

14.1.2.2 Sludge dry-weight expressed as milligrams solids per mL mixed liquor.

14.1.3 Identification of the reference material.

14.1.4 Carbon content of the test and reference materials.

14.1.5 Method for determining carbon content if Test Methods D 5291 was not used.

14.1.6 Information on preparation of the test material and reference material, including any procedures for enhancing their dispersion into the test medium.

14.1.7 Percent of theoretical CO_2 accumulated at the plateau and the number of days to reach the plateau for each test and reference material. If the plateau is reached prior to 28 days, the percent of theoretical CO_2 accumulated at the end of 28 days should be reported as well.

14.1.8 Cumulative average percent of theoretical CO_2 over time until the end of the study should be reported in tabular

form for each test and reference material.

14.1.9 The cumulative average percent of theoretical CO_2 over time should be displayed graphically for each test and reference material because the lag-phase, that is, delay in the onset of biodegradation, as well as the rate of biodegradation are important. It is optional to graphically plot the percentage of theoretical CO_2 over time for the individual replicates.

14.1.10 The replicate standard deviation (if applicable) for each test material and reference material evaluated.

14.1.11 The average of all replicates unless one or more replicates may be excluded based on statistical grounds as given in ISO 4259. In that case, report the excluded data and the reason for exclusion.

14.1.12 The temperature range of the test.

14.1.13 Initial and final pH.

15. Precision and Bias

15.1 The precision and bias of the procedure in this test method for measuring the aerobic aquatic biodegradability of lubricants or their components has not been determined.

16. Keywords

16.1 aerobic biodegradation; aquatic biodegradation; degree of biodegradation; lubricant biodegradability; municipal sewage; sewage; sludge; theoretical CO_2 evolution