



# Standard Test Method for Determining Aerobic Aquatic Biodegradation of Lubricants or Their Components<sup>1</sup>

This standard is issued under the fixed designation D 5864; 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 reappraisal. A superscript epsilon ( $\epsilon$ ) indicates an editorial change since the last revision or reappraisal.

## 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 laboratory conditions.

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 lubricants that are not volatile and are not inhibitory at the test concentration to the organisms present in the inoculum.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* Specific hazards are discussed in Section 10.

## 2. Referenced Documents

### 2.1 ASTM Standards:

- D 1129 Terminology Relating to Water<sup>2</sup>
- D 1193 Specification for Reagent Water<sup>2</sup>
- D 1293 Test Methods for pH of Water<sup>2</sup>
- D 4447 Guide for Disposal of Laboratory Chemicals and Samples<sup>3</sup>
- D 5291 Test Methods for Instrumental Determination of Carbon, Hydrogen, and Nitrogen in Petroleum Products and Lubricants<sup>4</sup>
- E 943 Terminology Relating to Biological Effects and Environmental Fate<sup>3</sup>

### 2.2 ISO Standard:<sup>5</sup>

- 4259:1992(E) Petroleum Products—Determination and Application of Precision Data in Relation to Methods of Test

### 2.3 APHA Standard:<sup>6</sup>

- 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 that are not described herein appear in the *Compilation of ASTM Standard Definitions*, 1990<sup>7</sup> or Terminology E 943.

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

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

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

3.1.5 *blank*, *n*—a flask containing the test medium and the inoculum with no additional carbon source added.

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

3.1.7 *lag phase*, *n*—the period of physiological activity and diminished cell division following the addition of microorganisms to a new culture medium.<sup>8</sup>

3.1.8 *log phase*, *n*—the period of growth of microorganisms during which cells divide at a constant rate.<sup>8</sup>

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

3.1.10 *pre-adaptation*, *n*—the incubation of an inoculum in the presence of the test substance which is done prior to the initiation of the test and under conditions similar to the test conditions.

3.1.10.1 *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

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<sup>2</sup> *Annual Book of ASTM Standards*, Vol 11.01.

<sup>3</sup> *Annual Book of ASTM Standards*, Vol 11.04.

<sup>4</sup> *Annual Book of ASTM Standards*, Vol 05.03.

<sup>5</sup> Available from American National Standards Institute, 11 West 42nd St., New York, NY, 10036.

<sup>6</sup> From *Standard Methods for the Examination of Water and Wastewater*, latest edition. Available from the American Public Health Association, 1015 18th St., N.W., Washington, DC 20036.

<sup>7</sup> Available from ASTM Headquarters.

<sup>8</sup> Adapted from *McGraw-Hill Dictionary of Scientific and Technical Terms*, 4th ed., 1989.

changes in the microbial population of the inoculum leading to more rapid biodegradation of the test substance.

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

3.1.12 *theoretical CO<sub>2</sub>, n*—the amount of CO<sub>2</sub> which could hypothetically be produced from the complete biological oxidation of all of the carbon in a substance.

3.1.13 *ultimate biodegradation, n*—degradation achieved when the test substance is totally utilized by microorganisms resulting in the production of CO<sub>2</sub>, (and possibly methane in the case of anaerobic biodegradation), water, inorganic compounds, and new microbial cellular constituents (biomass or secretions, or both).

3.1.14 *ultimate biodegradation test, n*—a test that estimates the extent to which the carbon in a product has been converted to CO<sub>2</sub> or methane, either directly, by measuring the production of CO<sub>2</sub> or methane, or indirectly, by measuring the consumption of O<sub>2</sub>.

3.1.14.1 *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 measured by collecting and measuring the CO<sub>2</sub> 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<sub>2</sub> which could be generated if all of the carbon in the test material were converted to CO<sub>2</sub>. CO<sub>2</sub> is a product of aerobic microbial metabolism of carbon-containing substances and so is a direct measure of the test substance's ultimate biodegradation. CO<sub>2</sub> production is quantified by trapping it in a Ba(OH)<sub>2</sub> solution and titrating the solution to calculate the amount of CO<sub>2</sub> absorbed.

4.2 The carbon content of the test substance is determined by Test Method D 5291 or an equivalent method and the theoretical CO<sub>2</sub> is calculated from that measurement. It is necessary to directly measure the carbon content of the test substance 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<sub>2</sub> production.

#### 5. Significance and Use

5.1 Results from the test method suggest, within the confines of a controlled laboratory setting, the degree of aerobic aquatic biodegradation of a lubricant or components of a lubricant by measuring the evolved carbon dioxide upon exposure of the test material to an inoculum. The plateau level of CO<sub>2</sub> evolution in this test method will suggest the degree of biodegradability of the lubricant. Test substances that achieve a high degree of biodegradation in this test may be assumed to easily biodegrade in many aerobic aquatic environments.

5.2 Because of the stringency of this test, a low yield of CO<sub>2</sub> does not necessarily mean that the test substance is not biodegradable under environmental conditions, but indicates that further testing is necessary to establish biodegradability.

5.3 Information on toxicity to the inoculum of the test substance 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 is considered an acceptable active aerobic inoculum available over a wide geographical area in which to test a broad range of lubricants. An inoculum derived from soil or natural surface waters, or both, or any combination of the three sources, is also appropriate for 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 substance known to biodegrade is necessary in order to verify the activity of the inoculum. The test must be regarded as invalid and should be repeated using a fresh inoculum if the reference does not demonstrate a biodegradation of > 60 % of the theoretical CO<sub>2</sub> evolution within 28 days.

5.6 A total CO<sub>2</sub> evolution in the blank at the end of the test exceeding 75 mg CO<sub>2</sub> per 3 L of medium shall be considered as invalidating the test.

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

5.8 The ratio of carbon incorporated into cellular material to carbon released as CO<sub>2</sub> 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.

#### 6. Apparatus

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

6.1.1 The following are required to produce a stream of CO<sub>2</sub>-free air of sufficient volume to test up to three materials and the accompanying reference and blank controls in triplicate:

6.1.1.1 *Five 1-L plastic bottles*, containing 700 mL of 10-M sodium hydroxide (NaOH),

6.1.1.2 *Two empty 1-L Erlenmeyer flasks*, to prevent liquid carryover, and

6.1.1.3 *One 1-L Erlenmeyer flask*, containing 700 mL of 0.0125 M barium hydroxide [Ba(OH)<sub>2</sub>] solution.

6.1.2 Connect the bottles 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 at a constant rate.

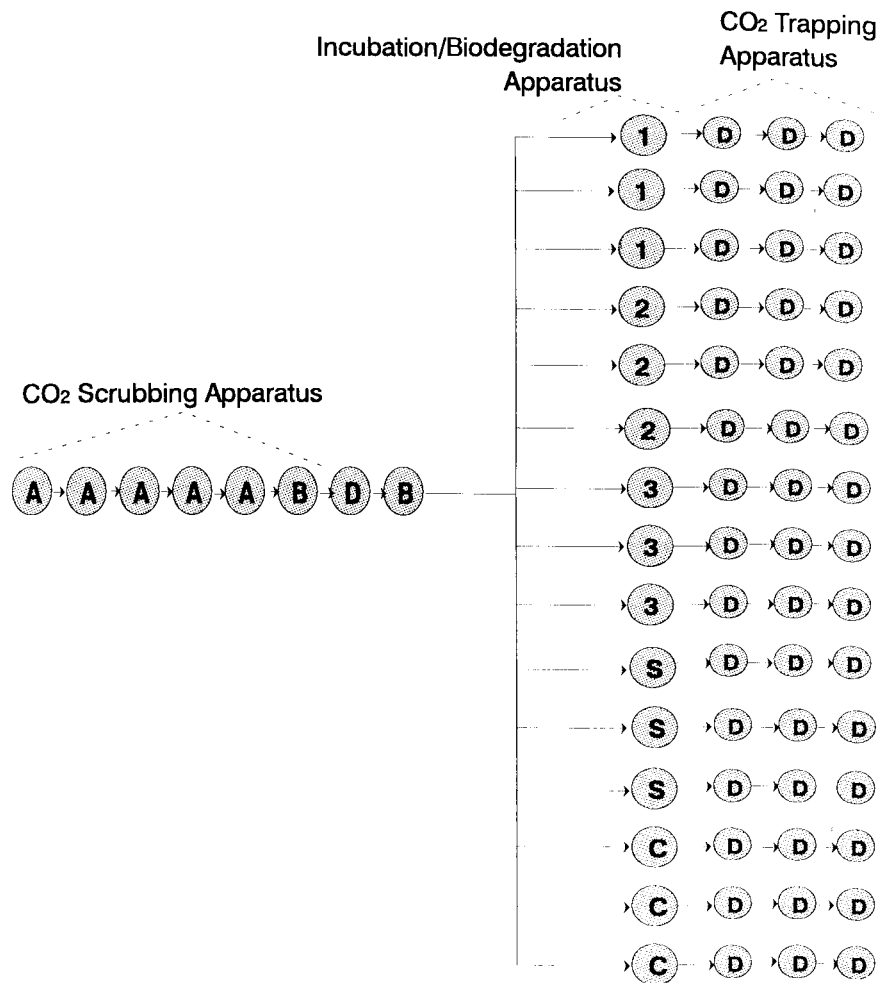
6.1.3 For each additional test substance to be tested, add one additional 1-L plastic bottle filled with 700 mL of 10 M sodium hydroxide.

6.1.4 The CO<sub>2</sub> scrubbing apparatus upstream of the Erlenmeyer flask containing the Ba(OH)<sub>2</sub> solution may be replaced by an alternative system which effectively and consistently produces CO<sub>2</sub> free air (that is, containing less than 1 ppm CO<sub>2</sub>).

6.2 *Incubation/Biodegradation Apparatus*—Each test material, reference, or control requires the following:

6.2.1 *Three 4-L Erlenmeyer flasks*,

6.2.2 *Stoppers*, which are non-permeable to CO<sub>2</sub>.



- A = NaOH
- B = Empty
- C = Blank
- S = Standard
- D = Ba(OH)<sub>2</sub>
- 1 = Test substance 1
- 2 = Test substance 2
- 3 = Test substance 3

FIG. 1 Aerobic Aquatic Biodegradation Testing Schematic

6.2.3 *Flexible Plastic Tubing*, which is non-permeable to CO<sub>2</sub>.

6.2.4 *Agitators or Stirrers*, for each 4-L Erlenmeyer flask.

6.3 *Analytical Balance*, to weigh out test material or reference material before or as adding to the test flask,

6.4 *Trapping Apparatus for Measuring Production of CO<sub>2</sub>*—For each incubation apparatus, the following are required:

6.4.1 *Several 200-mL Bottles*, fitted with gas bubblers and containing 100 mL 0.0125 M Ba(OH)<sub>2</sub> carbon dioxide scrubbing solution.

6.5 *Titration Apparatus for Measuring Production of CO<sub>2</sub>*:

6.5.1 *100-mL burette*.

6.6 *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.<sup>9</sup> 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 g of ammonium sulfate ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) in water and dilute to 1 L.

<sup>9</sup> *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmaceutical Convention, Inc. (USPC), Rockville, MD.

7.3.2 *Calcium Chloride Solution (27.5 g/L)*—Dissolve 27.5 g of calcium chloride (CaCl<sub>2</sub>) in water and dilute to 1 L.

7.3.3 *Ferric Chloride Solution (0.25 g/L)*—Dissolve 0.25 g of ferric chloride hexahydrate (FeCl<sub>3</sub> 6H<sub>2</sub>O) in water and dilute to 1 L.

7.3.4 *Magnesium Sulfate Solution (22.5 g/L)*—Dissolve 22.5 g of magnesium sulfate heptahydrate (MgSO<sub>4</sub> 7H<sub>2</sub>O) in water and dilute to 1 L.

7.3.5 *Phosphate Buffer*—Dissolve 8.5 g potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>), 21.7 g potassium monohydrogen phosphate (K<sub>2</sub>HPO<sub>4</sub>), 33.4 g sodium monohydrogen phosphate dihydrate (Na<sub>2</sub>HPO<sub>4</sub> 2H<sub>2</sub>O), and 1.7 g ammonium chloride (NH<sub>4</sub>Cl) in water and dilute to 1 L.

7.4 The test medium will contain the following reagents diluted to 1 L with water.

7.4.1 *Ammonium Sulfate Solution*, 1 mL,

7.4.2 *Calcium Chloride Solution*, 1 mL,

7.4.3 *Ferric Chloride Solution*, 4 mL,

7.4.4 *Magnesium Sulfate Solution*, 1 mL, and

7.4.5 *Phosphate Buffer Solution*, 10 mL.

7.5 *Barium Hydroxide Solution*, 0.0125 M, is prepared dissolving 4.0 g Ba(OH)<sub>2</sub> 8H<sub>2</sub>O per litre of distilled water. 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<sub>2</sub> from the air. It is recommended that 5 L be prepared at a time when running a series of tests.

7.6 *Difco Vitamin-free Casamino Acids*.

7.7 *Yeast Extract*.

7.8 *Phenolphthalein*.

7.9 *Standardized Hydrochloric Acid (0.0480–0.0520 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 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 minimal or no effluent from industry.

8.1.1.1 Using CO<sub>2</sub>-free air, aerate sludge in the laboratory for 4 h. Remove and homogenize 500 mL of the mixed liquor for 2 min at medium speed in a blender<sup>10</sup> or equivalent high speed mixer.

8.1.1.2 If using sludge supernatant as the inoculum, allow the homogenized sludge to settle for 30 min. If the supernatant still contains high levels of suspended solids at the end of 30 min, allow to settle for a further 30 to 40 min or adapt laboratory conditions to obtain better settling. Once sufficient settling is achieved, decant sufficient volume of the supernatant to provide a 1% (by volume) inoculum. Avoid carry-over of sludge solids which might cause inconsistencies in the measurement of CO<sub>2</sub> production. The supernatant inoculum is aerated immediately and continuously until use.

8.1.1.3 If using mixed liquor as the inoculum at 30 mg/L solids, determine the dry weight of sludge solids per unit volume in samples of well-mixed homogenized sludge using APHA Test Method 2540B. Calculate the volume of mixed liquor needed to achieve a final sludge dry-weight concentration in the test medium of 30 mg/L. Continue to aerate the mixed liquor until use.

NOTE 2—The use of mixed liquor at 30 mg/L solids without pre-adaptation (8.3) may result in exceedance of the CO<sub>2</sub>-production criterion in the blanks (75 mg CO<sub>2</sub> total). It is recommended that this inoculum type be used only if the inoculum will be pre-adapted.

8.1.2 *Inoculum from Soil*:

8.1.2.1 Suspend 100 g of soil in 1000 mL of water.

NOTE 3—Soils with an extremely large content of clay, sand or organic carbon are unsuitable.

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 use.

8.1.3 *Inoculum from Surface Water*:

8.1.3.1 Filter surface water through a coarse paper or glass wool plug, discarding the first 200 mL.

8.1.3.2 Aerate the filtrate until used.

8.1.4 *Composite Inoculum*:

8.1.4.1 The three inoculum sources may be combined in any proportion and mixed well.

8.2 *Determination of Biomass*:

8.2.1 APHA Test Method 9215, or equivalent, shall be used to enumerate the microorganisms in the inoculum. The inoculum shall contain 10<sup>6</sup> to 20 × 10<sup>6</sup> colony-forming units per millilitre.

8.3 Pre-adaptation of the inoculum is allowed and can be accomplished as follows:<sup>11</sup>

8.3.1 Supplement inoculum with 25 mg/L Difco vitamin-free casamino acids and 25 mg/L yeast extract.

8.3.2 Add 100 mL of supplemented inoculum and 900 mL test medium to a 2-L Erlenmeyer flask.

8.3.3 Add test compounds incrementally during the acclimation period at concentrations equivalent to 4, 8, and 8 mg carbon/L on days 0, 7, and 11, respectively.

8.3.4 On day 14, homogenize the culture in a blender for 15 s and refilter the medium through glass wool prior to use as the inoculum for the test. If preadaptation is conducted for a series of functionally or structurally related chemicals, media from the separately prepared inoculum flasks may be combined before final filtration.

## 9. Test Substance and Reference Substance

9.1 The carbon content of a test substance shall be measured by Test Method D 5291 or equivalent procedure.

9.2 The test substance shall be added to provide 10–20 mg carbon per litre (mg C/L) in the test medium. This will ensure that sufficient carbon is present to yield CO<sub>2</sub> which can be

<sup>10</sup> A Waring blender has been found suitable for this purpose.

<sup>11</sup> R. N. Sturm, "Biodegradability of Nonionic Surfactants: Screening Test for Predicting Rate and Ultimate Biodegradation," *Journal of American Oil Chemists Society* (1973), Vol 50, 159–167.



adequately measured by the trapping procedure described in this test method should the test substance be biodegradable.

9.3 *Reference*—A substance known to be biodegradable shall be tested simultaneously with the test substance.

9.3.1 For water soluble test substances, suggested reference substances are sodium benzoate or aniline.

9.3.2 For water insoluble test substances, the suggested reference substance is 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 % by weight erucic acid.

9.3.3 The reference will be added in the same manner as the test substance to provide a carbon concentration of 10 to 20 mg C/L in the flask.

9.3.4 The results from flasks containing the reference verify the viability of the inoculum.

9.4 The test method in this section will be performed in triplicate on all test and reference substances.

9.5 Care should be taken to obtain representative samples from test and reference substances.

## 10. Hazards

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

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 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 Erlenmeyer flasks 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 lots of some organic solvents might 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 emptied, rinsed with water, and cleaned as stated above.

## 12. Procedure

12.1 For each blank, test substance, and reference being tested, prepare an inoculated culture medium by the following dilution:

12.1.1 Add 2470 mL of water to each of the 4-L Erlenmeyer flasks.

12.1.2 To each 4-L Erlenmeyer flasks, add 3 mL each of ammonium sulfate, magnesium sulfate, and calcium chloride stock solutions, 30 mL of the phosphate buffer stock solution, 12 mL of the ferric chloride stock solution, and 30 mL of the activated sludge supernatant (or soil suspension filtrate, or composite inoculum) or a sufficient volume of activated sludge mixed liquor to give 30 mg/L suspended solids in the final volume of 3 L.

12.2 Aerate the Erlenmeyer flasks with CO<sub>2</sub>-free air for 24 h to purge the system of CO<sub>2</sub>.

12.3 After the aeration procedure, fill three CO<sub>2</sub>-absorber bottles with 100 mL 0.0125 M Ba(OH)<sub>2</sub> and connect in series to the outlet of each Erlenmeyer flask (see Fig. 1).

12.4 Measure the pH in each Erlenmeyer flask by Test Method D 1293. Using dilute HCl or NaOH, adjust the pH to 7 ± 0.5 before adding the test or reference substances.

12.5 The carbon concentration of the test substance or reference substance in the test medium shall be 10 to 20 mg/L. Calculate the weight of the test or reference substance needed to produce 10 to 20 mg of carbon per litre based upon the carbon content of the substance as determined above (see 9.1).

12.6 *Addition of the Test Substance or Reference Substance:*

12.6.1 Add the test substance or reference substance gravimetrically to three of the Erlenmeyer flasks to begin the testing period. If in order to accomplish this, the substance is weighed into or onto a small object, then both the substance and the object shall be added to the flask.

12.6.2 Sonication of the test substance or reference substance in 5 mL water while still in or on a small object is allowed as a means of obtaining a better dispersion of insoluble substances 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 substance, the reference substance shall also be sonicated in an identical manner prior to its addition to the test medium.

12.7 Along with the flasks containing test substances or reference substances, three flasks shall contain the test medium and the inoculum with no additional carbon source added. These flasks shall be blanks.

12.8 Add a sufficient volume of water to achieve a final volume of 3000 mL in each flask.

12.9 Run the test at 20 to 25°C, and record the test temperature throughout the test period.

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

12.11 *Carbon Dioxide Evolution and Analysis:*

12.11.1 Start the test by bubbling CO<sub>2</sub>-free air through the test solutions at a rate of 50 to 100 mL/min per Erlenmeyer flask.

12.11.2 Agitate each flask throughout the test with a magnetic stirrer or shaker table set at a moderate speed (~200 rpm). Ensure that each flask is agitated at the same speed.

12.11.3 The O<sub>2</sub> produced in each Erlenmeyer flask reacts with Ba(OH)<sub>2</sub> and is precipitated as barium carbonate (BaCO<sub>3</sub>). Periodically remove the CO<sub>2</sub> absorber nearest the

Erlenmeyer flask for titration. This should be done before BaCO<sub>3</sub> precipitate is evident in the second flask.

12.11.3.1 Titrations may be required every two to three days for the first ten days and then every fifth day until a plateau of CO<sub>2</sub> evolution is reached. A plateau is reached after evidence of biodegradation has occurred and the production of CO<sub>2</sub> is either no longer detectable or is equal to the CO<sub>2</sub> produced by the blanks for two consecutive sampling times.

12.11.3.2 Because the results of the titrations 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.

12.11.4 Move the remaining two absorbers one place closer to the Erlenmeyer flask and place a new absorber filled with 100 mL of fresh 0.0125 M Ba(OH)<sub>2</sub> at the far end of the series.

12.11.5 The test shall continue for at least 28 days or until the CO<sub>2</sub> evolution has reached a plateau. If on day 28 a plateau has not been achieved, that is, CO<sub>2</sub> production is still being detected in the absorber nearest the Erlenmeyer flask, perform a titration on the CO<sub>2</sub> absorber nearest the Erlenmeyer flask and continue to monitor CO<sub>2</sub> evolution until a plateau has been reached.

12.11.6 Once the CO<sub>2</sub> evolution has reached a plateau, again measure the pH of the Erlenmeyer flask contents, add 1 mL of concentrated hydrochloric acid to each of the test Erlenmeyer flasks to decompose inorganic carbonate and release trapped CO<sub>2</sub>. Continue aerating the Erlenmeyer flasks overnight to collect the released CO<sub>2</sub>.

12.11.7 The amount of CO<sub>2</sub> produced is determined by titrating the remaining Ba(OH)<sub>2</sub> with standard hydrochloric acid. Titrate the 100-mL Ba(OH)<sub>2</sub> solutions immediately after removing the bottles closest to the Erlenmeyer flasks. At the completion of the procedure, titrate all remaining bottles. Exercise care to minimize exposure to air to avoid absorbing ambient CO<sub>2</sub>.

12.11.7.1 Titrate with standard hydrochloric acid to a phenolphthalein end-point.

12.11.7.2 Alternatively, titrate by automatic titration to an end-point of pH 7.

### 13. Calculation

13.1 The total organic carbon content of the test substance determined by elemental analysis is used to calculate the theoretical quantity of CO<sub>2</sub> evolution as follows:

13.1.1 Test substance contains a specific weight fraction of carbon, therefore:

$$Y = w \times T \quad (1)$$

where:

$Y$  = mg of carbon charged to the test medium,

$w$  = weight fraction of carbon in test substance expressed as a decimal, and

$T$  = mg of test substance added to the test medium.

13.1.2 The theoretical amount of CO<sub>2</sub> which could be produced if all of the carbon in the substance is converted to CO<sub>2</sub>, is then calculated by knowing 12 g carbon yields 44 g CO<sub>2</sub>, therefore:

$$X = \left( \frac{44}{12} \times Y \right) \quad (2)$$

where:

$X$  = the theoretical amount (mg) of CO<sub>2</sub> which could be produced from  $Y$ , and

$Y$  = mg of carbon charged to the test medium.

13.2 Amount of CO<sub>2</sub> Produced:

13.2.1 Calculate the amount of CO<sub>2</sub> produced for a specific titration ( $C_x$ ) as follows:

$$C_x = \frac{M}{2} \times (Z_b - Z_t) \times 44 \quad (3)$$

where:

$C_x$  = mg CO<sub>2</sub> produced,

$M$  = molarity of HCl,

$Z_b$  = mL of HCl needed to titrate blank barium hydroxide solution, and

$Z_t$  = mL of HCl needed to titrate test barium hydroxide solution.

13.2.2 Calculate the amount of CO<sub>2</sub> produced for a specific blank titrate ( $C_a$ ) as follows:

$$C_a = \frac{M}{2} \times (Z_c - Z_b) \times 44 \quad (4)$$

where:

$C_a$  = mg CO<sub>2</sub> produced,

$M$  = molarity of HCl,

$Z_c$  = mL of HCl needed to titrate unused barium hydroxide solution, and

$Z_b$  = mL of HCl needed to titrate blank barium hydroxide solution.

13.2.3 Calculate the total amount of CO<sub>2</sub> produced in the test or reference flasks ( $C_t$ ) by summing the results obtained for each titration.

$$C_t = \sum C_{x_1} \dots C_{x_n} \quad (5)$$

13.2.4 Calculate the total amount of CO<sub>2</sub> produced in the blank flasks ( $C_b$ ) by summing the results obtained for each blank titration.

$$C_b = \sum C_{a_1} \dots C_{a_n} \quad (6)$$

13.2.5 Percent of theoretical CO<sub>2</sub> evolved ( $P$ ) is calculated as shown below:

$$P = \frac{C_t}{X} \times 100 \quad (7)$$

where:

$C_t$  = the sum of all titrations for a given test substance or reference substance, and

$X$  = the theoretical mg of CO<sub>2</sub> produced from a given substance.

### 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-adaption to the test substance.

14.1.2 Method and results of biomass determination:

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

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

14.1.3 Identification of the reference substance.

14.1.4 Carbon content of the test and reference substances.

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

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

14.1.7 Percent of theoretical CO<sub>2</sub> accumulated at the plateau and the number of days to reach the plateau for each test and reference substance.

14.1.8 Cumulative average CO<sub>2</sub> evolution over time until plateau should be reported and displayed graphically for each test and reference substance because lag-phase, that is, delay in the onset of biodegradation, as well as the rate of biodegradation are important.

14.1.9 The replicate standard deviation for each test substance and reference substance evaluated.

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

14.1.11 The average total CO<sub>2</sub> accumulated in the replicate blank flasks.

14.1.12 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 is being determined.

## 16. Keywords

16.1 aerobic biodegradation; aquatic biodegradation; degree of biodegradation; lubricant biodegradability; municipal sewage; sewage; sludge; theoretical CO<sub>2</sub> evolution

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