



Designation: **D6731—18** **D6731 – 22**

## Standard Test Method for Determining the Aerobic, Aquatic Biodegradability of Lubricants or Lubricant Components in a Closed Respirometer<sup>1</sup>

This standard is issued under the fixed designation D6731; 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 ( $\epsilon$ ) indicates an editorial change since the last revision or reapproval.

### 1. Scope\*

1.1 This test method covers a procedure for determining the degree of biodegradability of lubricants or their components in an aerobic aqueous medium on exposure to an inoculum under controlled laboratory conditions. This test method is an ultimate biodegradation test that measures oxygen demand in a closed respirometer.

1.2 This test method is suitable for evaluating the biodegradation of volatile as well as nonvolatile lubricants or lubricant components.

1.3 This test method is applicable to lubricants and lubricant components which are not toxic and not inhibitory to the test microorganisms at the test concentration.

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 given 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.*

### 2. Referenced Documents

#### 2.1 ASTM Standards:<sup>2</sup>

[D1129 Terminology Relating to Water](#)

[D1193 Specification for Reagent Water](#)

[D1293 Test Methods for pH of Water](#)

[D4175 Terminology Relating to Petroleum Products, Liquid Fuels, and Lubricants](#)

[D4447 Guide for Disposal of Laboratory Chemicals and Samples](#)

[D6384 Terminology Relating to Biodegradability and Ecotoxicity of Lubricants](#)

<sup>1</sup> 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.

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<sup>2</sup> 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.

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

**E943 Terminology Relating to Biological Effects and Environmental Fate**

2.2 *ISO Standards:*<sup>3</sup>

**ISO 4259-1,2 Petroleum products—Determination and application of precision data in relation to methods of test**

**ISO 6107-2 Water quality—Vocabulary—Part 2**

**ISO 8192 Water quality—Test for inhibition of oxygen consumption by activated sludge for carbonaceous and ammonium oxidation**

**ISO 9408 Water quality—Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium by determination of oxygen demand in a closed respirometer**

2.3 *OECD Standards:*<sup>4</sup>

**OECD 301F Ready Biodegradability-Manometric Respirometry**

2.4 *APHA Standards:*<sup>5</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 appear in the Compilation of ASTM Standard Definitions and the following terminology standards: **D1129**, **D4175**, **D6384**, **E943**, and ISO 6107-2.

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*—(a) taking place in the presence of oxygen; (b) living or active in the presence of oxygen.

3.1.4 *biochemical oxygen demand (BOD), n*—the mass concentration of dissolved oxygen consumed under specified conditions by the biological oxidation of organic or inorganic matter, or both.

##### 3.1.4.1 Discussion—

BOD determination is performed using empirical tests employing standardized laboratory procedures. These tests measure oxygen utilization during a specified incubation period for the biochemical degradation of organic material (carbonaceous demand) in water.

3.1.5 *biodegradation, n*—the process of chemical breakdown or transformation of a test material caused by microorganisms or their enzymes.

##### 3.1.5.1 Discussion—

Biodegradation is only one mechanism by which materials are removed, transformed, or both, in the environment.

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

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

3.1.8 *pre-adaptation, n*—the ~~incubation~~ pre-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.

##### 3.1.8.1 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 more rapid biodegradation of the test material but ~~is not expected to~~ a change in the overall final extent of biodegradation of the test material.

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

<sup>3</sup> Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036, <http://www.ansi.org>.

<sup>4</sup> Available from Organisation for Economic Cooperation and Development (OECD), 2 rue André Pascal, F-75775, Paris Cedex 16, France, <http://www.oecd.org>.

<sup>5</sup> From Standard Methods for the Examination of Water and Wastewater, latest edition. Available from the American Public Health Assoc., 1015 18th St., NW, Washington, DC 20036.

3.1.10 *sludge, n*—a water-formed sedimentary deposit.

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

#### 4. Summary of Test Method

4.1 Biodegradation of a lubricant or the component(s) of a lubricant is determined by measuring the oxygen consumed when the lubricant or component is exposed to microorganisms under controlled aerobic aquatic conditions. This value is then compared to the theoretical amount of oxygen (ThO<sub>2</sub>) which is required to oxidize all of the elements (that is, carbon, hydrogen, nitrogen, and so forth) in the test material. This test method mixes the test material (lubricant or component) with aerobic microorganisms in a closed respirometer containing a defined aquatic medium and measures the biodegradation of the test material by following the decrease in oxygen in the respirometer.

4.2 The test material is the sole source of carbon and energy in the medium. A reference material known to biodegrade, such as low erucic acid rapeseed oil (LEAR or canola oil) is run alongside the test material to confirm that the inoculum is viable and capable of biodegrading suitable materials under the test conditions. The test material or reference material concentration is normally 50 mg/L to 100 mg/L, providing a theoretical oxygen demand of at least 50 mg O<sub>2</sub>/L but no more than 200 mg O<sub>2</sub>/L. The ThO<sub>2</sub> of the test and reference materials will be determined from measured elemental compositional analysis and will be calculated as in 13.1.

4.3 The inoculated medium is stirred in a closed flask and the consumption of oxygen is determined either by measuring the amount of oxygen required to maintain a constant gas volume in the respirometer flask, or by measuring the change in volume or pressure (or a combination of the two) in the apparatus.

4.4 Evolved CO<sub>2</sub> (carbon dioxide) is absorbed in an alkaline trap solution (for example, 10 M NaOH or KOH) or other CO<sub>2</sub>-absorbing system suspended within the test vessel, typically in the headspace of the test vessel.

4.5 Biodegradation is followed over a specified period by determining the consumption of oxygen. The amount of oxygen utilized in oxidation of the test and reference material is corrected for oxygen uptake by the inoculum in the blank controls and is expressed as a percentage of the theoretical oxygen demand (ThO<sub>2</sub>) calculated from the empirical formula of the material. Evaluation of the biodegradability of the test material is made on the basis of these data. Normally the test duration is 28 days; however, the test may be terminated if oxygen consumption has plateaued. The test may be extended as long as the systems' integrity is maintained and the inoculum in the blank systems is viable. The duration of the test will be dependent on the length of time required for the rate of test material biodegradation to achieve a plateau. A graphical illustration of the test results for a biodegradable material is presented in Fig. 1.

#### 5. Significance and Use

5.1 Results from this test method suggest the degree of aerobic, aquatic biodegradation of a lubricant or lubricant component. The rate and extent of oxygen consumption is measured upon exposure of the test material to an inoculum within the confines of a controlled laboratory setting. Test materials which 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 method, low results do not necessarily mean that the test material is not biodegradable under environmental conditions, but indicate that further testing is necessary to establish biodegradability.

5.3 If the pH value at the end of the test is outside the range from 6 to 8 and if the percentage degradation of the test material is less than 50 %, it is advisable to repeat the test with a lower concentration of the test material or a higher concentration of the buffer solution, or both.

5.4 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 must be regarded as invalid and shall be repeated using a fresh inoculum if the reference material does not demonstrate biodegradation to the extent of >60 % of the ThO<sub>2</sub> within 28 days.

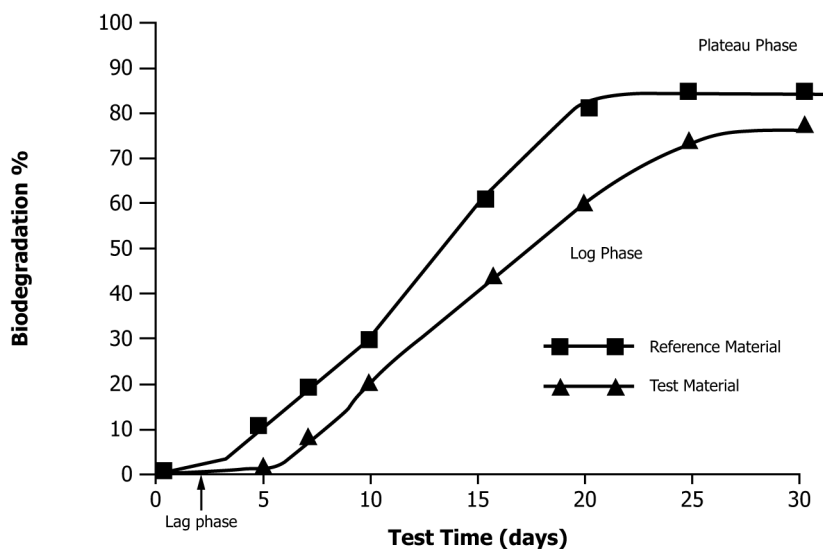


FIG. 1 Respirometric Test—Biodegradation Curve

5.5 Information on the toxicity of the test material to the inoculum may be useful in the interpretation of low biodegradation results. Toxicity of the test material to the inoculum may be evaluated by testing the test material in combination with the reference material in inhibition control systems. If an inhibition control is included, the test material is assumed to be inhibiting if the degradation percentage of the reference material is lower than 40 % (ISO 8192). In this case, it is advisable to repeat the test with lower concentrations of the test material.

5.6 Total oxygen utilization in the blank at the end of the test exceeding 60 mg O<sub>2</sub>/L invalidates the test.

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

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

## 6. Apparatus

### 6.1 Closed Respirometer:

6.1.1 The principle of a closed respirometer is given in Fig. 2. When testing volatile compounds, the apparatus used shall be appropriate or adapted to this particular purpose in accordance with the manufacturer's instructions. Exercise care that the closed respirometer apparatus is well sealed to prevent any loss (for example, leakage) of volatile compounds from the system or of oxygen into the system.

6.1.2 The test mixture is stirred by a magnetic stirrer in the test flask, which is filled with sufficient volume to minimize headspace and prevent delay of O<sub>2</sub> and CO<sub>2</sub> diffusion through the air-water phases. This volume is dependent on the selected flask size, and is normally specified by the manufacturer of the respirometer. If biodegradation takes place, the microorganisms consume oxygen and produce carbon dioxide. Oxygen from the headspace is then dissolved in the liquid to reestablish chemical equilibrium. The carbon dioxide produced by the microorganisms diffuses into the headspace where it is trapped in an absorbent solution or material and the total pressure in the flask then decreases.

6.1.3 This pressure drop is detected by a manometer, which produces a signal that results in the electrolytic generation of oxygen. When the original pressure is re-established, the signal is stopped and the quantity of electricity used is measured. The amount of electricity used is proportional to the amount of consumed oxygen. This is indicated on a plotter or a printer, or the data are collected using an appropriate software program.

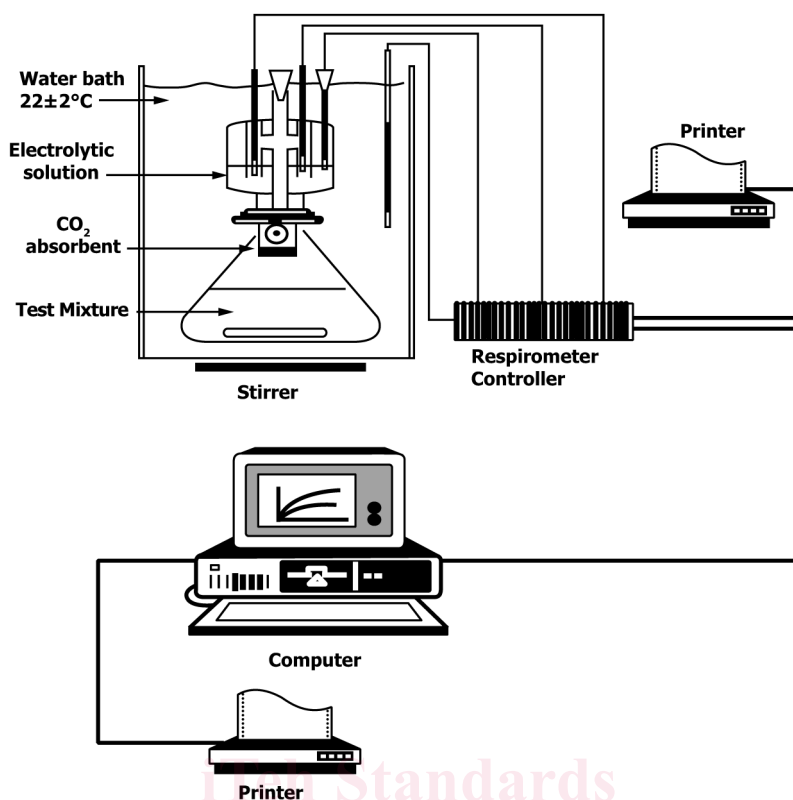


FIG. 2 Principle of a Closed Respirometer

6.2 Water-Bath or Constant Temperature Room, to comply with 11.2.

6.3 Centrifuge.

6.4 pH-meter.

6.5 Analytical Balance, capable of weighing to appropriate precision and accuracy (for example,  $\pm 0.0001$  g).

## 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>6</sup> Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without decreasing 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 *Calcium Chloride Solution*—Dissolve 27.5 g of anhydrous calcium chloride ( $\text{CaCl}_2$ ) or 36.4 g of calcium chloride dihydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) in water and dilute to 1 L.

<sup>6</sup> *Reagent Chemicals, American Chemical Society Specifications, 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.

7.3.2 *Ferric Chloride Solution*—Dissolve 0.25 g of iron (III) chloride hexahydrate ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ) in water and dilute to 1 L. Prepare this solution just before use or add a drop of concentrated hydrochloric acid (HCl) (**Warning**—Corrosive, fumes cause irritation.) or 0.4 g/L of ethylenediamine-tetraacetic acid (EDTA).

7.3.3 *Magnesium Sulfate Solution*—Dissolve 22.5 g of magnesium sulfate heptahydrate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) in water and dilute to 1 L.

7.3.4 *Phosphate Buffer Solution*—Dissolve 8.5 g of anhydrous potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ), 21.75 g anhydrous potassium monohydrogen phosphate ( $\text{K}_2\text{HPO}_4$ ), 33.4 g disodium hydrogen phosphate dihydrate ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ), and 0.5 g ammonium chloride ( $\text{NH}_4\text{Cl}$ ) in water and dilute to 1 L. Alternatively, 50.3 g of disodium hydrogen phosphate, heptahydrate ( $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ ) may be used in place of  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ . The pH of this solution shall be about 7.4.

7.3.5 *Sodium Hydroxide 10 M Solution*—(**Warning**—Causes severe burns.) Cautiously dissolve 400 g NaOH (**Warning**—Causes severe burns.) in distilled water to a final volume of 1 L. Filter the solution to free it of solids.

7.3.6 *Potassium Hydroxide 10 M Solution*—(**Warning**—Causes severe burns.) Cautiously dissolve 561 g KOH (**Warning**—Causes severe burns.) in distilled water to a final volume of 1 L. Filter the solution to free it of solids.

## 8. Inoculum Test Organisms

8.1 *Sources of the Inoculum*—Activated sewage-sludge from a sewage-treatment plant that treats principally domestic waste may be considered 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. 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. 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 minimal or no effluent from industry.

8.1.1.1 Using  $\text{CO}_2$ -free air, aerate sludge in the laboratory for 4 h. Depending on the number of test systems, sufficient volume of the mixed liquor is sampled and homogenized for 2 min at medium speed using a high-shear/high-speed blender. Withdraw a sample for the determination of the dry weight of the suspended solids (8.2.2). Keep the inoculum continuously well mixed until all sample preparation is completed to avoid solids settling.

8.1.1.2 Calculate the volume of homogenized mixed liquor necessary to achieve a final sludge dry-weight concentration in the test medium of 30 mg/L (suspended solids, 8.2.2). The inoculum prepared from the homogenized mixed liquor may be used to prepare a composite inoculum (8.1.5), pre-adapted to the test material (8.3.1), or added directly to the test systems (12.2).

8.1.1.3 Alternatively, settle the homogenized sludge for 30 min or longer (if required) and decant the liquid supernatant for use as inoculum. The inoculum prepared from the supernatant may be used to prepare a composite inoculum (8.1.5), pre-adapted to the test material (8.3.2), or added directly to the test systems (12.2).

8.1.1.4 It is optional to pre-condition the inoculum. Pre-conditioning consists of aerating the activated sludge for up to seven days. Sometimes pre-conditioning improves the precision of the test method by reducing the amount of oxygen consumption in the blank controls.

NOTE 1—Exercise care in pre-conditioning because of the sensitivity of inocula to prolonged aeration and starvation conditions. Pre-conditioning should be applied in situations where it is known that the inoculum source consistently shows a high internal respiration rate.

8.1.2 *Inoculum from Secondary Effluent*—Alternatively, the inoculum can be derived from the secondary effluent of a treatment plant or laboratory-scale unit receiving domestic sewage.

8.1.2.1 Allow the secondary effluent to settle for 1 h and collect the supernatant or filter the effluent through a coarse filter paper. After supernatant collection or effluent filtration, aerate the sample using  $\text{CO}_2$ -free air in the laboratory for 4 h. The inoculum may be used to prepare a composite inoculum (8.1.5), pre-adapted to the test material (8.3.2), or added at this point to the test systems (12.2). Up to 100 mL of this type of inoculum may be used per litre of medium.



8.1.3 *Inoculum from Surface Water*—A further source for the inoculum is surface water. In this case, collect a sample of an appropriate surface water (for example, river or lake) and keep aerobic until required.

8.1.3.1 Filter the surface water through a coarse filter paper or glass wool plug, and discard the first 200 mL. Aerate the remaining filtered sample using CO<sub>2</sub>-free air in the laboratory for 4 h. The inoculum may be used to prepare a composite inoculum (8.1.5), pre-adapted to the test material (8.3.1), or added directly to the test systems (12.2). Up to 100 mL of this type of inoculum may be used per litre of medium.

8.1.4 *Inoculum from Soil*:

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

8.1.4.2 Allow the suspension to settle for 30 min.

8.1.4.3 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. The soil inoculum may be used to prepare a composite inoculum (8.1.5), pre-adapted to the test material (8.3.1), or added directly to the test systems (12.2). Up to 100 mL of this type of inoculum may be used per litre of medium.

8.1.5 *Composite Inoculum*—The four inoculum sources may be combined in any proportion and mixed well.

8.2 *Determination 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<sup>6</sup> to 10<sup>7</sup> colony-forming units 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 for inoculum from activated sludge, 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 any inoculum type to a test material is allowed. A sufficient volume of pre-adapted inoculum in test medium shall be incubated for 14 days to yield a minimum of 100 mL of inoculated medium for each respirometer test system; that is, 100 mL for each blank, test material, and positive control material replicate. When developing pre-adapted inoculum for more than one test material, individual cultures will be prepared separately for each test material. Pre-adaptation can be accomplished as follows:<sup>7</sup>

8.3.1 *Pre-adaptation of Homogenized Mixed Liquor Inoculum*—Supplement the calculated volume of homogenized mixed liquor inoculum necessary to achieve a suspended solids concentration of 30 mg/L (8.1.1.2) with 25 mg/L of vitamin-free casamino acids and 25 mg/L of yeast extract.

8.3.1.1 Add the supplemented inoculum to a 2 L Erlenmeyer flask. Add 10 mL of phosphate buffer solution, 1 mL of magnesium sulfate solution, 1 mL of ferric chloride solution, and 1 mL of calcium chloride solution to the 2 L Erlenmeyer flask. Add sufficient volume of water to the 2 L Erlenmeyer flask to achieve a total volume of 1000 mL. Prepare separate inoculated test medium for each test material requiring pre-adaptation.

8.3.2 Pre-adaptation of inoculum prepared from one of the following sources: activated sludge supernatant, 8.1.1.3; secondary effluent, 8.1.2.1; surface water, 8.1.3.1; soil, 8.1.4.3; or composite, 8.1.5. Supplement inoculum with 25 mg/L of vitamin-free casamino acids and 25 mg/L of yeast extract.

8.3.2.1 Add 100 mL of the supplemented inoculum prepared in 8.3.2 to a 2 L Erlenmeyer flask. Add 10 mL of phosphate buffer solution, 1 mL of magnesium sulfate solution, 1 mL of ferric chloride solution, and 1 mL of calcium chloride solution to the 2 L Erlenmeyer flask. Add sufficient volume of water to the 2 L Erlenmeyer flask to achieve a total volume of 1000 mL. Prepare separate inoculated test medium for each test material requiring pre-adaptation.

<sup>7</sup> Sturm, R. N., "Biodegradability of Nonionic Surfactants: Screening Test for Predicting Rate and Ultimate Biodegradation," *Journal of American Oil Chemists Society*, Vol 50, 1973, pp. 159–167.