Designation: ~~D6974 – 16~~ D6974 – 20

Standard Practice for Enumeration of Viable Bacteria and Fungi in Liquid Fuels—Filtration and Culture Procedures[1]

This standard is issued under the fixed designation D6974; 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 practice covers a membrane filter (MF) procedure for the detection and enumeration of Heterotrophic bacteria (HPC) and fungi in liquid fuels with kinematic viscosities ≤24 $mm^2 \cdot s^{-1}$ at ambient temperature.

1.2 This quantitative practice is drawn largely from IP Method 385 and Test Method D5259.

1.3 This test may be performed either in the field or in the laboratory.

1.4 The ability of individual microbes to form colonies on specific growth media depends on the taxonomy and physiological state of the microbes to be enumerated, the chemistry of the growth medium, and incubation conditions. Consequently, test results should not be interpreted as absolute values. Rather they should be used as part of a diagnostic or condition monitoring effort that includes other test parameters, in accordance with Guide D6469.

1.5 This practice offers alternative options for delivering fuel sample microbes to the filter membrane, volumes or dilutions filtered, growth media used to cultivate fuel-borne microbes, and incubation temperatures. This flexibility is offered to facilitate diagnostic efforts. When this practice is used as part of a condition monitoring program, a single procedure should be used consistently.

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

1.7 *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~~ safety, health, and ~~health~~environmental practices and determine the applicability of regulatory limitations prior to use.*

1.8 *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:*[2]

D1129 Terminology Relating to Water
D1193 Specification for Reagent Water
D4175 Terminology Relating to Petroleum Products, Liquid Fuels, and Lubricants
D5259 Test Method for Isolation and Enumeration of Enterococci from Water by the Membrane Filter Procedure
D6426 Test Method for Determining Filterability of Middle Distillate Fuel Oils
D6469 Guide for Microbial Contamination in Fuels and Fuel Systems
D7463 Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Fuel, Fuel/Water Mixtures, and Fuel Associated Water
D7464 Practice for Manual Sampling of Liquid Fuels, Associated Materials and Fuel System Components for Microbiological Testing
E1326 Guide for Evaluating Non-culture Microbiological Tests

[1] This practice is under the jurisdiction of ASTM Committee D02 on Petroleum Products, Liquid Fuels, and Lubricantsand is the direct responsibility of Subcommittee D02.14 on ~~Stability~~ Stability, Cleanliness and ~~Cleanliness~~Compatibility of Liquid Fuels.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For Annual Book of ASTM Standards volume information, refer to the standard's Document Summary page on the ASTM website.

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

F1094 Test Methods for Microbiological Monitoring of Water Used for Processing Electron and Microelectronic Devices by Direct Pressure Tap Sampling Valve and by the Presterilized Plastic Bag Method

2.2 *Energy Institute Standards:*[3]

IP 385 Determination of the Viable Aerobic Microbial Content of Fuels and Fuel Components Boiling Below 390 °C—Filtration and Culture Method

3. Terminology

3.1 *Definitions:*

3.1.1 For definition of terms used in this method refer to Terminologies D1129 and D4175, and Guide D6469.

3.1.2 *aseptic, adj*—sterile, free from viable microbiological contamination.

3.2 *Acronyms:*

3.2.1 *CFU*—colony forming unit

3.2.2 *HPC*—heterotrophic plate count

3.2.3 *MF*—membrane filter

3.2.4 *MEA*—malt extract agar

3.2.5 *TNTC*—too numerous to count

3.2.6 *TSA*—tryptone soy agar

3.3 *Symbols:*

3.3.1 *N*—number of CFU · L^{-1}

3.3.2 *CC*—number of colonies on membrane filter

3.3.3 *V*—sample volume filtered, mL

4. Summary of Practice

4.1 Any free water present in a fuel sample is removed by settling in a separatory funnel. After the water has been removed, a known volume of the remaining fuel is filtered through a membrane filter aseptically by one of three methods.

4.2 The filter membrane retains microbes present in the fuel. Filter replicate fuel samples through fresh membranes to permit replicate testing, growth on alternative nutrient media, or both.

4.3 After filtration, place each membrane on one of two types of agar growth media, incubate at a designated temperature for three days, and examine for the presence of CFU.

4.4 Incubate the filter media on agar for two more days, then reexamine.

4.5 Count the colonies manually or by electronic counter.

4.5.1 If practical, identify colonies on each agar medium, based on colony color, morphology, and microscopic examination.

4.5.2 Convert bacterial and fungal colony counts to CFU per litre of fuel.

5. Significance and Use

5.1 Biodeteriogenic microbes infecting fuel systems typically are most abundant within slime accumulations on system surfaces or at the fuel-water interface (Guide D6469). However, it is often impractical to obtain samples from these locations within fuel systems. Although the numbers of viable bacteria and fungi recovered from fuel-phase samples are likely to be several orders of magnitude smaller than those found in water-phase samples, fuel-phase organisms are often the most readily available indicators of fuel and fuel system microbial contamination.

5.2 *Growth Medium Selectivity*—Guide E1326 discusses the limitations of growth medium selection. Any medium selected will favor colony formation by some species and suppress colony formation by others. As noted in 6.3, physical, chemical and physiological variables can affect viable cell enumeration test results. Test Method D7463 provides a non-culture means of quantifying microbial biomass in fuels and fuel associated water.

5.3 Since a wide range of sample sizes, or dilutions thereof, can be analyzed by the membrane filter technique (Test Methods D5259 and F1094), the test sensitivity can be adjusted for the population density expected in the sample.

5.4 Enumeration data should be used as part of diagnostic efforts or routine condition monitoring programs. Enumeration data should not be used as fuel quality criteria.

6. Interferences

6.1 High non-biological particulate loads (sediment) can clog the membrane and prevent filtration.

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[3] Available from Energy Institute, 61 New Cavendish St., London, WIG 7AR, U.K., http://www.energyinst.org.uk.

6.2 Each CFU is assumed to originate from a single microbial cell. In reality, microbes often form aggregates which appear as a single colony. Consequently, viable count data are likely to underestimate the total number of viable organisms in the original sample.

6.3 The metabolic state of individual microbes may be affected by numerous physical-chemical variables in the fuel. Injured cells or cells that have relatively long generation times may not form colonies within the time allotted for test observations. This results in an underestimation of the numbers of viable microbes in the original fuel sample.

7. Apparatus

7.1 *Separatory Funnels,* glass, nominal capacity 500 mL.

7.2 *Measuring Cylinders,* glass, nominal capacity 100 mL and 1 L.

7.3 *Pipettes,* glass or sterile disposable plastic, nominal capacity 10 mL, or adjustable volume pipette and sterile disposable plastic tips.

7.4 *Membrane Filter,* ~~mixed esters of cellulose,~~ polyethersulfone (PES) or cellulose acetate (CA), presterilized, preferably gridded, 47 mm diameter, nominal pore size 0.45 µm.

NOTE 1—~~While the recommended filter material is mixed esters of cellulose, the selection of membrane material will depend on individual preference and fuel type.~~The choice of filter material will depend on local availability or preference for use of a disposable filtration device, or both.

NOTE 2—CA filters will become translucent on wetting with fuel, but this is not detrimental to the final recovery of microorganisms.

7.5 *Filtration Unit,* one of:

7.5.1 *Unit,* as described in Test Method D6426, with pre-sterilized in-line filter housing, or

7.5.2 *Hypodermic Syringe,* sterile, 100 mL, with pre-sterilized in-line filter housing, or

7.5.3 *Filter Holder Assembly,* single or manifold, glass, stainless steel, or polypropylene, pre-sterilized.

NOTE 3—If the vacuum filtration option (7.5.3) is chosen, a vacuum source, not more than –66 kPa will also be needed.

7.6 *Forceps,* blunt tipped.

7.7 *Filter Flask,* of sufficient capacity to receive the entire sample being filtered plus washings.

7.8 *Petri Dishes,* disposable plastic or glass, nominal diameter ≥50 mm.

NOTE 4—Pre-poured Petri dishes, containing the growth media described below are available commercially and may be substituted for the dishes listed here.

7.9 *Incubator,* capable of maintaining a temperature of 25 °C ± 2 °C or any other temperature (within the range–ambient to 60 °C), as appropriate.

7.10 *Water Bath,* capable of maintaining a temperature of 47 °C ± 2 °C and receiving 500 mL bottles. Water bath capacity should be sufficient to accommodate at least one bottle of each type of agar growth medium used.

7.11 *Glass Bottles,* screw cap with gas-tight closures, 500 mL nominal capacity.

7.12 *Culture Tubes,* glass, 16 mm by 125 mm, screw cap.

7.13 *Autoclave,* with capacity to hold 500 mL glass bottles upright.

NOTE 5—Items 7.10 – 7.13 are not needed if using commercially prepared Petri dishes, as indicated in Note ~~3~~4.

8. Reagents and Materials

8.1 *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.[4]

8.2 The agar used in preparation of culture media shall be of microbiological grade. Whenever possible, use commercial culture media.

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

8.4 *Chlorotetracycline,* 0.1 % (w/v) aqueous. Dissolve 0.1 g chlorotetracycline in water and dilute to 100 mL. Sterilize by passing through a 0.2 µm filter.

[4] ~~*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~~suggestions on the testing of reagents not listed by the American Chemical Society, see ~~*Annual*~~*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.

8.5 *Detergent Solution* 0.1 % by volume—Dissolve 1.0 mL of polyoxyethylene (20) sorbitan monooleate[5] in 999 mL water. Sterilize, either by passing through a 0.2 µm membrane filter into a sterile vessel, or autoclaving at 121 °C for 15 min.

8.6 *Hydrochloric Acid,* 1 mol HCl · L^{-1}.

8.7 *Lactic Acid,* 10 % (w/v) aqueous. Dissolve 10 g of lactic acid in water and dilute to 100 mL. Sterilize by passing through a 0.2 µm filter.

8.8 *Malt Extract Agar (MEA):*

8.8.1 *Composition/Litre:*

Malt Extract	30 g
Mycological Peptone	5 g
Agar	15 g
Water	1 L

8.8.2 *Preparation*—Suspend the malt extract, mycological peptone and agar in 1 L of water and boil to dissolve. Adjust the pH to 5.4 ± 0.2 using either 1 mL · L^{-1} hydrochloric acid (8.6) or sodium hydroxide 10 % w/v (8.10). Dispense 250 mL portions into 500 mL glass screw-cap bottles (7.11). Sterilize by autoclaving at 121 °C ± 2 °C for 10 min. Cool and maintain the sterilized agar in a water bath (7.10) at 47 °C ± 2 °C. Optionally, after the agar has cooled to 47 °C ± 2 °C, add 1 mL of a 0.1 % aqueous solution of chlorotetracycline (filter sterilized by passing through a 0.2 µm filter, see 8.4) per 100 mL MEA and mix by shaking. If the medium is required at pH 3.5, add 10 % lactic acid (filter sterilized by passing through a 0.2 µm filter, see 8.7) to adjust pH. Once acidified, the MEA shall not be reheated. Make agar plates of the medium by pouring sufficient MEA into sterile petri dishes to give a layer approximately 4 mm thick. Allow to cool and set.

NOTE 6—MEA is available from various manufacturers in dehydrated form and in pre-poured plates with and without added antibiotic, either of which may be used. When sterilizing MEA prepared from commercial dehydrated media, follow the manufacturer's instructions for sterilization. Avoid overheating.

NOTE 7—Alternative media to MEA may be used, providing the ability of any alternative medium to support comparable growth of yeast and molds that are likely to be encountered in test samples can be demonstrated.

NOTE 8—Alternative antibiotics may be used providing their ability to inhibit growth of bacteria but not yeast and molds has been validated.

8.9 *Ringer's Solution, One-Quarter Strength:*

8.9.1 *Composition/Litre:*

Sodium chloride	2.25 g
Potassium chloride	0.105 g
Calcium chloride	0.12 g
Sodium bicarbonate	0.05 g
Water	1 L

8.9.2 *Preparation*—Dissolve salts in 1 L of water and dispense 10 mL portions into screw capped culture tubes (7.12). Sterilize by autoclaving at 121 °C for 15 min.

NOTE 9—One-quarter strength Ringer's salts are available in tablet form from various manufacturers.

8.10 *Sodium Hydroxide,* 10 % (w/v) aqueous. Dissolve 10 g NaOH in water and dilute to 100 mL.

8.11 *Tryptone Soy Agar (TSA):*

8.11.1 *Composition/Litre:*

Tryptone	15 g
Soy protein	5 g
Sodium chloride	5 g
Agar	15 g
Water	1 L

8.11.2 *Preparation*—Suspend the dry ingredients in 1 L of water and boil to dissolve. Dispense 250 mL portions into 500 mL glass screw-cap bottles (7.11). Sterilize by autoclaving at 121 °C ± 2 °C for 10 min. Cool and maintain the sterilized agar in a water bath (7.10) at 47 °C ± 2 °C. Draw a sample and test the pH. If the pH ≠ 7.3 ± 0.3, reject the batch and make a fresh mixture. Make agar plates of the medium by pouring sufficient TSA into sterile petri dishes to give a layer approximately 4 mm thick. Allow to cool and set.

NOTE 10—TSA is available from various manufacturers in dehydrated form and in pre-poured plates.

NOTE 11—Alternative media to TSA may be used, providing the ability of any alternative medium to support comparable growth of bacteria that are likely to be encountered in test samples can be demonstrated.

9. Procedure

9.1 *Sampling:*

9.1.1 Samples shall be drawn in accordance with Practice D7464.

[5] The sole source of supply of Tween 80 known to the committee at this time is Sigma Aldrich Co., St. Louis, MO 63178, http://www.sigmaaldrich.com. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.