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Standard Test Method for Determination of the Viable Aerobic Microbial Content of Fuels and Associated Water—Thixotropic Gel Culture Method[1]

This standard is issued under the fixed designation D7978; 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 describes a procedure that can be used in the field or in a laboratory to quantify culturable, viable aerobic microorganisms present as contaminants in liquid fuels, including those blended with synthesized hydrocarbons or biofuels, with kinematic viscosities (at 40 °C) of ≤24 mm^2 s^{-1} and heavy and residual fuels with kinematic viscosities (at 40 °C) of ≤700 mm^2 s^{-1} and in fuel-associated water.

1.1.1 This test method has been validated by an ILS for a range of middle distillate fuels meeting Specifications D975, D1655, ISO 8217 DMA, and NATO F-76.[2]

1.2 This test method quantitatively assesses culturable, viable aerobic microbial content present in the form of bacteria, fungi, and fungal spores. Results are expressed as the total number of microbial colony forming units (CFU)/L of fuel or total number of CFU/mL of associated water. The number of CFU should not be interpreted as absolute values but should be used as part of a diagnostic or condition monitoring effort; for example, these values can be used to assess contamination as absent, light, moderate, or heavy.

NOTE 1—This test method is technically equivalent to IP 613, although the two methods are not currently jointed.

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

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

1.5 *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:*[3]

D975 Specification for Diesel Fuel
D1129 Terminology Relating to Water
D1655 Specification for Aviation Turbine Fuels
D4175 Terminology Relating to Petroleum Products, Liquid Fuels, and Lubricants
D4176 Test Method for Free Water and Particulate Contamination in Distillate Fuels (Visual Inspection Procedures)
D6469 Guide for Microbial Contamination in Fuels and Fuel Systems
D6974 Practice for Enumeration of Viable Bacteria and Fungi in Liquid Fuels—Filtration and Culture Procedures
D7464 Practice for Manual Sampling of Liquid Fuels, Associated Materials and Fuel System Components for Microbiological Testing
D7847 Guide for Interlaboratory Studies for Microbiological Test Methods
E2756 Terminology Relating to Antimicrobial and Antiviral Agents

2.2 *Energy Institute Standards:*[4]

IP 385 Determination of the Viable Aerobic Microbial Content of Fuels and Fuel Components Boiling Below 390 °C—Filtration and Culture Method
IP 613 Determination of the Viable Aerobic Microbial Content of Fuels and Associated Water—Thixotropic Gel Culture Method
Guidelines for the Investigation of the Microbial Content of Petroleum Fuels and for the Implementation of Avoidance

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

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[2] Defense Standard 91-4, Fuel, Naval, Distillate (NATO Code: F-76, Joint Service designation DIESO F-76), Issue 9, 3 May 2013, UK Defense Standardization

[3] 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.

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

and Remedial Strategies, 2nd Edition, 2008, Energy Institute, London, ISBN 978 0 85293 524 8

2.3 *Other Standards:*

ISO 8217 DMA Petroleum Products, Fuels (Class F), Specifications of Marine Fuels, 4th Edition 2010, ISO Switzerland

IATA Guidance Material on Microbiological Contamination in Aircraft Fuel Tanks, 4th Edition, December 2011[5]

3. Terminology

3.1 *Definitions*—For definitions of terms used in this test method, refer to Terminologies D1129, D4175, and E2756.

3.2 *Definitions:*

3.2.1 *colony, n*—a discreet visible aggregate of microorganisms that develops when a viable microorganism, or particle containing viable microorganisms, is introduced into a gel-based nutritive culture medium and reproduces there.

3.2.1.1 *Discussion*—A period of incubation is necessary to allow sufficient reproduction. This test method utilizes a reactive compound that shortens the time for colonies to become visible and stains them so that they appear as red or purple spots.

3.2.1.2 *Discussion*—Typically, bacterial colonies become visible to the naked eye only after the colony contains $\geq 10^9$ individual cells. Consequently, the time required for a colony to become visible is dependent on the organism's generation (doubling), which can range from <30 min to >1 week.

3.2.2 *culture medium, n*—solid, semi-solid, or liquid preparations that contain nutrients that support microbial growth, and usually other chemical agents that can inhibit or stimulate growth by specific microorganisms or that can indicate the presence of all culturable or specific microorganisms.

3.3 *Definitions of Terms Specific to This Standard:*

3.3.1 *thixotropic gel, n*—a sheer thinning gel that is semi-solid while static and becomes a liquid when a sheer force is applied.

3.4 *Acronyms and Abbreviations:*

3.4.1 *CC*—number of colonies (colony count).

3.4.2 *CFU*—colony forming unit.

3.4.3 *N*—number of CFU/mL (in water) or CFU/L (in fuel).

3.4.4 *TNTC*—too numerous to count.

3.4.5 *V*—volume tested, mL

4. Summary of Test Method

4.1 A known volume of fuel or water is added to the test kit[6], which consists of a rectangular, transparent glass bottle containing a patented sterile, thixotropic gel-based culture medium capable of sustaining the growth of a wide range of microorganisms encountered in liquid fuels and petroleum products (8.1).

4.2 The gel liquefies when the bottle is shaken, dispersing the fuel or water specimen containing any microorganisms.

4.3 The gel is allowed to reset into a flat layer on one of the larger sides of the bottle.

4.4 The bottle is incubated in the dark in this position for four days. The gel contains components that sustain the growth of viable, culturable microorganisms and the fuel specimen itself contributes additional nutrients.

4.5 Viable, culturable microorganisms in the volume of fuel or water tested grow into visible colonies, and a reactive compound changes the color of these colonies to red or purple such that they can be easily counted or their number estimated.

4.6 The number of colonies formed is considered in relation to the volume of specimen added to the test, and expressed as CFU/L of fuel, or CFU/mL if the result relates to a test of water in a fuel system sample.

5. Significance and Use

5.1 This test method is intended to provide a tool for assessing whether fuel storage and distribution facilities or end user fuel tanks are subject to microbial growth and alert fuel suppliers or users to the potential for fuel quality or operational problems and/or the requirement for preventative or remedial measures.

5.2 This test method detects numbers of microbial colony forming units (CFU), the same detection parameter used in the laboratory standard procedures Practice D6974 and IP 385. However, whereas Practice D6974 and IP 385 provide separate assessment of numbers of viable aerobic bacteria CFU and numbers of viable fungal CFU, this test method provides a combined total count of viable aerobic bacteria and fungal CFU.

5.3 This test method is designed to detect a recognized group of microorganisms of significance in relation to contamination of distillate fuels, but it is recognized that microbiological culture techniques do not detect all microorganisms that can be present in a sample. Culturability is affected primarily by the ability of captured microbes to proliferate on the growth medium provided, under specific growth conditions. Consequently, a proportion of the active or inactive microbial population present in a sample can be viable but not detected by any one culture test.[7] In this respect, the test is indicative of the extent of microbial contamination in a sample ,and it is assumed that when a fuel sample is significantly contaminated, some of the dominant microbial species present will be quantifiably detected, even if not all species present are culturable.

[5] Available from International Air Transport Association (IATA), 800 Place Victoria, PO Box 113, Montreal H4Z 1M1, Quebec, Canada or 33, Route de l'Aeroport, PO Box 416, 1215 Geneva 15 Airport, Switzerland, http://www.iata.org.

[6] The sole source of supply of the test kit known to the committee at this time is ECHA Microbiology Ltd., Cardiff, CF3 0EF, UK. 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.

[7] White, J. et al., "Culture-Independent Analysis of Bacterial Fuel Contamination Provides Insight into the Level of Concordance with the Standard Industry Practice of Aerobic Cultivation," *Applied and Environmental Microbiology*, Vol. 77, No. 13, July 2011, pp. 4527-4538.

5.4 Many samples from fuel systems can be expected to contain a low level of "background" microbial contamination, which is not necessarily of operational significance. The minimum detection level of this test method is determined by the volume of specimen tested and is set such that microbial contamination will generally only be detected when it is at levels indicative of active proliferation.

5.5 The test will detect culturable bacteria and fungi that are metabolically active and dormant fungal spores. Presence of fungal spores in a fuel sample can be indicative of active microbial proliferation within a fuel tank or system, but at a point distant from the location sampled. Active microbial growth only occurs in free water, and this can be present only as isolated pockets at tank or system low points. Because fungal spores are more hydrophobic than active cells and fungal material (mycelium), they disperse more readily in fuel phase and are thus more readily detected when low points cannot be directly sampled and only fuel phase is present in samples.

5.6 This test method can determine whether microbial contamination in samples drawn from fuel tanks and systems is absent or present at light, moderate, and heavy levels.

5.7 The categorization of light, moderate, and heavy levels of contamination will depend on the fuel type, the sampling location, the facility sampled, and its specific operating circumstances.

5.8 Further guidance or interpretation of test results can be found in Guide D6469, in the Energy Institute Guidelines for the investigation of the microbial content of petroleum fuels, and for the implementation of avoidance and remedial strategies and in the IATA Guidance Material on Microbiological Contamination in Aircraft Fuel Tanks.

5.8.1 Further guidance on sampling can be found in Practice D7464.

5.9 Testing can be conducted on a routine basis or to investigate incidents.

5.10 Microbiological tests are not intended to be used to determine compliance with absolute fuel specifications or limits. The implementation of specification limits for microbiological contamination in fuels is generally not appropriate, and microbial contamination levels cannot be used alone or directly to make inferences about fuel quality or fitness for use.

5.11 When interpreting results, it must be appreciated that the test result applies only to the specific sample and specimen tested and not necessarily to the bulk fuel. Microbiological contamination usually shows a highly heterogeneous distribution in fuel systems, and therefore, analysis of a single sample will rarely provide a complete assessment of the overall levels of contamination present.

5.12 Water phase will usually contain substantially higher numbers of microbial CFU than fuel phase and, consequently, a different interpretation of results is required.

6. Interferences

6.1 Some antioxidant additives that can be present in the fuel being tested can cause a uniform light peach or orange color in the gel culture medium (usually within 12 h). This color change will not interfere with the growth of any microorganisms, and in most cases, microbial colonies can be counted or estimated ignoring the background color. If antioxidants are present at very high concentration, the color change in the gel culture medium can be so strong that users can find it difficult to distinguish from the appearance of a test in which more than 10 000 microbial colonies have grown. The effect can be compensated for by testing a smaller volume of specimen, as described in 12.5.2.3 in the test procedure.

6.2 Some bacteria are motile and can, on prolonged incubation, spread through the gel culture medium, producing large irregularly-shaped colonies, streaks, or patches of red or purple color that are difficult to count. Procedures for compensating for this effect are described in 12.5.2.4 in the test procedure.

6.3 If microorganisms other than those in the specimen are introduced into the culture medium, they can give rise to spurious colonies. To avoid this, the test should be conducted in as clean an environment as practicable, and care should be taken to avoid touching surfaces of dispensing apparatus, pipettes, and sample containers that come into direct contact with the sample or culture medium.

7. Apparatus

7.1 *Incubator,* capable of operating at 25 °C ± 3 °C. The design of the incubator shall ensure the culture medium is not exposed to light during incubation. The use of an incubator is not essential (see 12.5.1.1).

7.2 *Temperature Measuring Device,* accurate to ±2 °C, to measure incubation temperature.

8. Reagents and Materials

8.1 *MicrobMonitor2,*[8] commercially available test kit comprised of a rectangular, sterile transparent glass bottle fitted with a cap and seal and containing a sterile, thixotropic gel-based culture medium capable of sustaining the growth of a wide range of microorganisms encountered in liquid fuels and petroleum products and associated water.

8.2 *Disposable Syringe,* sterile and fuel compatible, capacity 1 mL graduated in 0.1 mL units used for dispensing the recommended volume of middle distillate fuels described in 12.3.4.

8.3 *Disposable Loop Dispenser,* sterile and fuel compatible, capacity 0.01 mL, used for dispensing 0.01 mL of water or heavy and residual fuel.

NOTE 2—The disposable syringes (8.2) and loop dispensers (8.3) are provided with the test kit.

8.4 *Pipettes,* with or without disposable tips, sterile and fuel compatible, capacity 0.1 mL to 1 mL (optional), and can be purchased pre-sterilized or can be sterilized.

8.5 *Pipettes,* with or without disposable tips, sterile and fuel compatible, capacity 0.01 mL (optional), and can be purchased pre-sterilized or can be sterilized.

[8] MicrobMonitor2 is a registered trademark of ECHA Microbiology Ltd., Cardiff, CF3 0EF, UK.

8.6 *Marker Pen,* permanent with fine or medium tip and capable of writing on glass (optional).

8.7 *Container,* transparent, and fuel compatible, capacity 10 mL to 150 mL (optional). Borosilicate glass has been found to be suitable. Containers shall meet the cleanliness requirements for sample containers stipulated in Practice D7464. Suitable disposable, sterile containers are commercially available. Some incorporate a mechanism for separation of fuel and water phases.

8.8 *Disinfectant Solution or Chlorine-release Pills,* see 9.1.

9. Hazards

9.1 *Warning Statement*—When examining the culture medium during incubation, do not open the bottles. Wash hands after handling the bottles. Once the final examination of the culture medium has been conducted, the test bottle shall be decontaminated, for example, by adding chlorine-release pills to the test bottle and shaking vigorously for 30 s or by immersing the open bottle and cap in a strong disinfectant solution. The bottle shall then be left for 24 h before discarding in accordance with local regulations.

10. Sampling, Test Specimens, and Test Units

10.1 Samples shall be collected and handled in accordance with Practice D7464.

10.2 It is preferable to take a sample of about 1 L in volume to enable easier visual observation of the sample for water, dirt, particulates, and suspected microbial growth. However, less than 1 mL of specimen is actually required for the test.

NOTE 3—The numbers of viable microorganisms in a fuel sample will be highly dependent on the sampling location and also whether free water is present in the sample; test results should be interpreted accordingly. Usually, microbial contamination will be present in greater abundance near the tank bottom or system low point, particularly at any fuel-water interface and in free water settled or suspended in the fuel. For routine monitoring, testing low point (for example, dead bottom or drain) samples is advisable as these will provide the earliest and most consistent indication of tank or system contamination. Testing representative fuel samples (for example, upper, middle, and lower layer samples) can provide some indication of the extent of contamination in bulk fuel, but results will be applicable only to the specific sample at the time of sampling; microbial contamination in bulk fuel is generally unevenly distributed and levels of contamination detected at the same sampling location will vary with time. Contamination levels in bulk fuel in storage tanks will generally decrease with increasing product settling time and will increase if tank bottoms are disturbed, for example, during fuel receipts.

10.3 If fuel phase is to be tested (see 12.3), the container to which sample is transferred shall be filled sufficiently to enable the disposable syringe (8.2) or pipette (8.4) to be inserted to a depth of approximately 3 cm below the surface of the fuel. If water phase is also to be tested (see 12.6), the container to which sample is transferred shall enable ready access to settled water in the bottom of the sample.

11. Preparation of Apparatus

11.1 If an incubator (7.1) is to be used, use a temperature measuring device (7.2) to ensure it is operating at 25 °C ± 3 °C.

11.2 If an incubator is not used, use a temperature measuring device (7.2) to determine the temperature of the location used to incubate the test. Measurements should be taken at sufficient frequency to establish temperature fluctuations outside the nominal specified range, in order to establish compliance with the incubation requirements described in 12.5.1.1.

12. Procedure

12.1 Visually inspect the sample to identify if water is present in accordance with the procedures described in Test Method D4176.

NOTE 4—Because free water phase cannot always be recovered in samples, for consistency, when conducting routine microbiological monitoring, it is recommended that the fuel phase from above any free water phase present is always tested. In some circumstances, for example when investigating contamination sources or assessing the extent of microbial growth in a tank or system, it can be also informative to assess the microbial content in any free water present in the sample. Presence of discolored water (brown or black), a lacy interface between the fuel and water layers, or soft, organic debris in the fuel or water layer are all indications of likely microbiological activity.

12.2 If the sample is not in a container suitable for visual inspection or does not enable use of the measuring devices (8.2, 8.3, 8.4, or 8.5), to remove an aliquot for test in accordance with the procedures described below in 12.3 for fuel phase test or 12.6 for water phase test, transfer the sample first to a suitable, container (8.7).

12.3 *Testing the Fuel Phase of Sample:*

12.3.1 Immediately prior to analysis, shake the sample vigorously by hand for approximately 30 s and then allow to stand for 12 min ± 1 min. If the depth of the fuel phase in the sample is less than 6 cm, then allow a settling time of 2 min/cm.

NOTE 5—The prescribed settling time will typically enable any suspended free water to settle out from the top 6 cm of sample. In some cases, for example where fuels are wet and visibly hazy, water phase will not separate readily from the fuel phase, and higher microbiological counts can be expected. Depending on the test objectives, an additional settling time can be applied to the sample prior to testing.

12.3.2 If the bottles of gel culture medium have been stored refrigerated, allow them to equilibrate to ambient temperature before they are used. Avoid prolonged exposure of the culture medium to direct sunlight or other bright light at all times.

12.3.3 Immediately before dispensing the fuel specimen, remove the cap seal and cap of a gel culture medium bottle; do not touch the inside of the cap or bottle neck. Discard the seal and place the cap on a clean surface.

12.3.4 Using a syringe (8.2) or an alternative measuring device (8.4), draw fuel from approximately 3 cm below the surface of the fuel phase of the sample. If there is less than 6 cm depth of fuel, draw the aliquot for test from approximately halfway down the fuel phase. The transfer of visible interfacial particulate, water droplets, or emulsion in the aliquot to be tested shall be avoided.

12.3.5 If using the syringe (8.2), open the sterile syringe pack at the handle end and remove the syringe, taking care not to touch the lower barrel and nozzle. Draw fuel into the syringe until it is more than half full and then, with the syringe nozzle pointing upwards, expel air. Expel fuel surplus to the volume needed for the test (see 12.3.7 to 12.3.9 below).