Designation: D7978 – 14

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

2. Referenced Documents

2.1 *ASTM Standards:*[3]

D975 Specification for Diesel Fuel Oils
D1129 Terminology Relating to Water
D1655 Specification for Aviation Turbine Fuels
D4175 Terminology Relating to Petroleum, Petroleum Products, 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 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

[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 and Cleanliness 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.

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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.

5.4 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

[5] Available from International Air Transport Assocation (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.

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.

8.6 *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.

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

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).

12.3.6 If using the loop dispenser (8.3), for testing heavy and residual fuels only, open the peel pack at the handle end and remove the dispenser, taking care not to touch the loop and lower part of the shaft.

12.3.7 *Aviation Kerosine Fuels*—The volume to test is 0.5 mL. Dispense this volume directly into the gel culture medium bottle.

12.3.8 *Other Middle Distillate Fuels and Gasoline*—The volume to test is 0.25 mL. Dispense this volume directly into the gel culture medium bottle.

12.3.9 *Heavy and Residual Fuels*—The volume to test is 0.01 mL. Use the syringe (8.2) to dispense a drop of fuel onto the loop dispenser (8.3). Allow surplus fuel to drain away, but ensure that the circle of the loop is filled, and then stab it into the gel culture medium in the bottle and agitate briefly to transfer the fuel into the gel. Alternatively, use a pipette (8.5) to dispense 0.01 mL fuel directly into the gel culture medium bottle.

12.3.10 The volume of specimen tested can be changed in order to increase or decrease the detection level of the test. However, the repeatability and reproducibility of the test has not been validated for testing fuel volumes greater than those stated above. Testing specimen volumes greater than those specified above is outside the scope of this test method.

NOTE 6—Although accuracy and minimum detection levels can be improved by conducting replicate tests, the test method is designed for both field and laboratory use and, in the field, any increase in accuracy obtained by replication will usually be sacrificed for operational expediency.

12.3.11 The syringe and loop dispenser and other disposable measuring devices shall be used only once and discarded after use. If reuseable measuring devices are used, they shall be freshly cleaned and resterilized between each use.

12.3.12 Once the fuel has been dispensed into the gel culture medium bottle, replace and tighten the cap and proceed in accordance with 12.4.

12.4 *Dispersing the Test Portion in the Gel Culture Medium:*

12.4.1 Loosen and break up the gel culture medium in the bottle containing the dispensed test portion by tapping the bottle firmly on the palm of the hand or on a large rubber bung or thick rubber mat. Take care not to break the glass bottle.

12.4.2 Shake the bottle vigorously for approximately 30 s to liquefy the gel and disperse the test specimen.

NOTE 7—After shaking the gel, it should be slightly viscous but free of lumps and have uniform clarity. The presence of air bubbles in the gel will not affect the test.

12.4.3 Stop shaking abruptly so that the gel collects in the bottom of the bottle. Proceed immediately to 12.4.4 before the gel resets.

12.4.4 Tap the bottle in the palm of the hand until the gel forms a flat layer on one of the larger flat bottle sides. Ensure a uniform layer of gel reaching all corners of the bottle is obtained.

12.5 *Incubating and Examining the Gel Culture Medium:*

12.5.1 Transfer the gel culture medium bottle to a warm, dark location or incubator (7.1) to maintain a temperature of 25 °C ± 3 °C. Keep the gel on the lowest surface of the bottle. The gel will set firm after a few hours. It is important not to tilt the bottle or disturb the gel during this time. In normal circumstances, the culture medium test shall be incubated for four days.

12.5.1.1 When conducting the test in the field, it will not always be possible to maintain incubation temperature within the specified range. Occasional temperature fluctuations (for example, overnight) below the specified temperature range should not affect the number of colonies that develop and will not critically affect the test result, but microbial colonies can take longer to become visible and thus an extension of the specified incubation time should be applied. If temperature falls below the specified range during incubation, the incubation time should be extended by a time equivalent to the total time the temperature is estimated to have fallen below the specified range. If the incubation temperature falls below the specified range for a period or periods totaling four days or more, microbial contamination will be underestimated and the result should be considered invalid. The temperature of the culture medium should not be allowed to drop below 4 °C. Incubation of the culture medium above the specified temperature range can prejudice the ability to detect some types of microorganism. However, where microbial contamination occurs in tanks or systems where the contents have a higher temperature than 25 °C, the contaminating microorganisms will often have a preference for growth at higher temperatures, in such cases it can be considered valid to incubate the test at the temperature of the system sampled.

12.5.2 If possible, examine the culture medium daily during the incubation period. As a minimum, examine the test on at least one occasion in the first three days of incubation and again on a final day of incubation. During each observation, count visible red or purple colonies as they develop. All red or purple colonies in all parts of the test bottle should be counted, including any that are in gel that is not part of the flat layer. Once a colony is counted, do not count it again even if it grows larger; it is the number of colonies that are required, irrespective of their size. Avoid agitating or shaking the bottle during incubation and examination.

12.5.2.1 It is recommended to mark colonies with a permanent marker pen (8.6) on the glass of the gel culture medium bottle to ensure that they are not counted twice. It is usually possible to count up to about 250 colonies.

NOTE 8—Colonies are usually circular but can have irregular edges. Different types of microorganisms can grow at different rates in the gel culture medium, and therefore, the colonies can be of different sizes. At the recommended incubation temperature, colonies of bacteria and yeasts usually develop within one to two days and remain quite small. Mold colonies develop more slowly but eventually produce large colonies that can have a powdery or "fuzzy" appearance. Generally, the more colonies there are in the test bottle, the smaller the colonies will be. Colonies will tend to become visible more quickly in samples with a higher viable microbial content.

12.5.2.2 It can be useful to photograph tests at each time of examination for subsequent reference when determining final colony counts.

12.5.2.3 If color change suspected to be due to fuel antioxidant additives interferes with ability to count or estimate microbial colonies (see 6.1), the fuel can be retested using a smaller test volume (for example, 0.01 mL) so that the interference effected is diluted out; if the original result was genuinely due to heavy microbial contamination, it would normally be expected that the retest will show a discernible number of red/purple colonies and the background color will be less intense.

NOTE 9—Some bacteria are motile and can, on prolonged incubation, spread through the gel, producing a large irregularly-shaped colony, streak, or patch of red or purple color in the gel. These bacteria usually grow quickly, and thus, if tests are examined while the colonies are still small (for example, after one or two days incubation), they are more easily counted.

12.5.2.4 If irregularly-shaped streaks or patches are observed, the center of each streak or patch should be counted as a single colony. Continue incubation and count any new colonies that develop.

NOTE 10—If the number of colonies is greater than 100, although a reasonable colony count can usually be obtained, the colony count will exceed the range currently defined for quantitative assessment of microbial content and consequently accuracy and precision can be low.

12.5.2.5 If colonies cannot be counted, estimate the approximate colony count as per 12.5.3.

12.5.3 If the number of colonies is too numerous to count, visually compare the test to the chart provided in the Annex A1, holding the test bottle against a white background.

NOTE 11—The chart provides an estimated colony count to the nearest factor of ten. The result can only be considered semi-quantitative as the number of colonies present exceeds the range considered acceptable for fully quantitative assessment of microbial content. If samples have a very high viable microbial content, individual colonies will not be distinguishable and the whole gel becomes red or purple (see picture in chart showing a test with ≥10 000 colonies). In such cases, the microbial content is equal to or exceeds the maximum detection level of the test.

12.6 *Testing Water Phase of Samples (optional):*

12.6.1 If an assessment of microbial content of free water phase is required, after completion of fuel phase analysis as described in 12.3 to 12.4, allow the sample to stand until water phase has settled to the bottom of the sample. Use the syringe (8.2) or a pipette (8.4) to remove water from the bottom of the sample and transfer to a separate small, sterile container (8.7). Avoid transferring any of the fuel with the water.

12.6.1.1 To enable ready access to water phase, it might be necessary to first decant off some fuel phase from the sample.

12.6.2 Once water has been removed from any fuel, invert the container into which the water has been transferred three times to homogenize prior to withdrawing the aliquot for test. Remove the cap of a gel culture medium bottle; do not touch the inside of the cap or bottle neck. Place the cap on a clean surface.

12.6.3 As appropriate for the volume of water to be tested (see 12.6.3.1), use the loop dispenser (8.3), syringe (8.2), or pipette (8.4 or 8.5) to measure the required volume of water to test.

12.6.3.1 The usual volume of water to test is 0.01 mL. The volume of water specimen tested can be changed in order to increase or decrease the detection level of the test. The test can be used to test water volumes up to 1 mL. For example, for water present in samples from aircraft fuel tank drains, it can be appropriate to test 0.1 mL of water to improve the detection level of the test because under normal circumstances, water in aircraft tanks is clean condensate water and will have a lower microbial count than water in other fuel facilities.

12.6.4 If using the loop dispenser (8.3), open the peel pack at the handle end and remove the dispenser, taking care not to touch the loop and lower part of the shaft. Immerse the loop in the water and withdraw. Alternatively, use a syringe (8.2) to withdraw some of the water and dispense a drop of water onto the loop dispenser. Allow surplus water to drain away, but ensure that the circle of the loop is filled. Immediately stab the loop into the gel culture medium and agitate to transfer the test portion into the gel.

12.6.5 If using the syringe (8.2) to take larger volumes of water, 0.1 mL to 1 mL, open the sterile syringe pack at the handle end and remove the syringe, taking care not to touch the lower barrel and nozzle. Draw just over the required volume of water into the syringe and then, with the syringe nozzle uppermost, expel air and water surplus to the volume needed for the test. Dispense the required measured volume directly into the gel culture medium bottle.

12.6.6 Once water has been dispensed into the gel culture medium bottle, replace and tighten the cap, and proceed in accordance with 12.4 and 12.5.

12.6.6.1 The syringe and loop dispenser (8.2 and 8.3) and other disposable measuring devices shall be used once only and discarded after use. If reuseable measuring devices are used, they shall be freshly cleaned and resterilized between each use.

13. Calculation or Interpretation of Results

13.1 Calculate the number of microbial CFU/L of fuel as shown in 13.1.2 and/or the number of microbial CFU/mL of water, as shown in 13.1.3.

13.1.1 If the number of colonies is estimated by visually comparing to the chart provided in Annex A1, only a semi-quantitative estimate of the colony count is obtained. For the recommended test volumes of 0.5 mL, 0.25 mL, and 0.01 mL, the chart provides the corresponding approximate number of microbial CFU/L of fuel or microbial CFU/mL of water based on the equations shown below.

13.1.2 *Fuel Phase Test Portion*—Calculate the number of microbial CFU/L of fuel using the following equation:

$$\text{Number of microbial CFU/L} = \frac{CC \times 1000}{V} \tag{1}$$

where:

CC = number of colonies counted or estimated, and
V = volume of specimen tested in mL.

13.1.3 *Water Phase Test Portion*—Calculate the number of microbial CFU/mL of water using the following equation:

$$\text{Number of microbial CFU/mL} = \frac{CC}{V} \tag{2}$$

where:

CC = number of colonies counted or estimated, and
V = volume of specimen tested in mL.

13.2 *Expression of Results:*

13.2.1 *Results Derived from a Test Where the Number of Colonies was Counted:*

13.2.1.1 Report the result as the viable aerobic microbial content in CFU/L of fuel or CFU/mL of water to the nearest whole number. If the recommended test volumes are used, the calculation will provide results to the nearest number of colony forming units shown in Table 1 and shall be reported as such. If the number of colonies counted was greater than 100, it shall

TABLE 1 Limits of Resolution for Recommended Sample Types and Specimen Volume Tested

Sample Type	Volume Tested (mL)	Report Result to the Nearest
Aviation kerosene fuels	0.5	2000 CFU/L
Other middle distillate fuels and gasolines	0.25	4000 CFU/L
Heavy and residual fuels	0.01	100 000 CFU/L
Water associated with fuel	0.01	100 CFU/mL

be stated on the test report that the colony count exceeded the range defined for quantitative assessment of microbial content by the test method and that accuracy and precision could be low.

13.2.1.2 Report the volume of specimen tested and the conditions of test incubation, including total incubation time and, as far as is known, the nominal incubation temperature and any fluctuation outside the range specified.

13.2.1.3 Report the visual appearance of the original sample prior to any separation.

13.2.1.4 If the time and conditions of storage or transit of samples prior to testing do not comply with the recommendations of Practice D7464, this shall be stated on the test report. If the test is carried out more than 24 h after sampling, it is possible that the results will not reflect the viable microbial content present at the time of sampling.

13.2.2 *Results Derived from a Test Where the Number of Colonies was Estimated:*

13.2.2.1 If the result was derived from a test where the number of colonies was estimated (see 12.5.3), using the chart provided in Annex A1, report the result as the viable aerobic microbial content in approximate number of microbial CFU/L of fuel or CFU/ mL of water to one significant figure in scientific notation to the nearest power of ten, as shown in the right hand column of the chart (for example, approximately 10^6 CFU/L of fuel).

13.2.2.2 It shall be stated in the test report that the result is a semi-quantitative estimate as colonies were too numerous to count.

13.2.2.3 Report the volume of specimen tested and the conditions of test incubation, including total incubation time and, as far as is known, the nominal incubation temperature and any fluctuation outside the range specified.

13.2.2.4 Report the visual appearance of the original sample prior to any separation.

14. Report

14.1 The test report shall contain at least the following information:

14.1.1 A reference to this test method.

14.1.2 The type and complete identification of the product tested.

14.1.3 The result of the test (see Section 13).

14.1.4 Any deviation, by agreement or otherwise, from the procedure specified.

14.1.5 The date the test was commenced (that is, test aliquot of specimen added to test bottle) and completed (that is, final examination after incubation).

14.1.6 The time and date the sample was taken.

14.1.7 The type of facility or equipment tested.

14.1.8 The location of the sampling point and the sampling method.

15. Precision and Bias

15.1 There are inherent difficulties in assessing precision of microbiological procedures for fuels on account of the inherent variability of the determinant and various determinable and indeterminable sources of inaccuracy (see Guide D7847).

15.2 The precision of any microbiological analytical method will generally be considerably less than that of methods widely used in the petroleum industry for analysis of physical and chemical properties of fuels.

15.3 An interlaboratory study has been conducted for this test method to investigate precision and further information is provided in research report RR IP 613-2013.[9]

15.3.1 Because the numbers and viability of microorganisms in fuel samples can change during transport between different operators or different laboratories, it is not possible to determine the reproducibility of the test between laboratories. The laboratory study therefore established intermediate precision for different operators, testing nominally replicate samples, using different test kits (8.1 and 8.2) and the same incubator (7.1) at the same test location.

15.3.2 Nine samples of distillate fuel were blind-coded and tested in duplicate in random order by seven participants simultaneously at a single site. The samples included Jet A-1 (DEFSTAN 91-91), automotive diesel (EN590), marine diesel (ISO 8217 DMA), F-76 (DEFSTAN 91-4)[2] and gas oil (BS2869 Class D).[10] The samples were either microbiologically contaminated field samples or samples in which microbiological growth was allowed to develop naturally in the laboratory.

15.3.3 The repeatability was determined to be 1.423 (x + 5000).

15.3.4 The intermediate precision, established as qualified in 15.3.1, was determined to be 1.548 (x + 5000).

15.3.5 The established precision values are consistent with what would reasonably be expected for a microbiological test method. Precision is appropriate for the intended application, as described in Section 5, where it is usually sufficient to establish orders of magnitude differences in contamination level in order to determine whether contamination is absent, light, moderate or heavy.

15.4 *Bias*—Since there is no accepted reference material suitable for determining the bias of this test method, bias cannot be determined.

[9] A copy of research report RR IP 613-2013 may be obtained upon request from Energy Institute, 61 New Cavendish St., London, UK, http://www.energyinst.org.

[10] BS2869, Fuel Oils for Agricultural, Domestic and Industrial Engines and Boilers, Specification, Sept 2010, BSI London, ISBN 978 0 580 76376 2.

16. Keywords

16.1 bacteria; biocontamination; biodeterioration; colony forming units (CFU); culturable aerobic microorganisms; fuel; fungi; microbial contamination; microbial growth; microbe; microbiology; MicrobMonitor2; thixotropic gel culture; viable aerobic microorganisms

ANNEX

(Mandatory Information)

A1. CHART FOR THE VISUAL ESTIMATION OF THE NUMBERS OF COLONIES

A1.1 See Table A1.1.

TABLE A1.1 Chart for the Visual Estimation of the Numbers of Colonies

<table>
<tr><th colspan="3">Fully Quantitative Range of Detection
Count exact number of colonies</th></tr>
<tr><th>No. of Colonies Counted (CC)</th><th colspan="2">Calculation of Microbial Content</th></tr>
<tr><td>No colonies</td><td colspan="2" rowspan="3">Calculate the microbial content using the appropriate equation for fuel or water sample:
$\text{Number of microbial CFU/L} = \frac{CC \times 1000}{V}$
OR
$\text{Number of microbial CFU/mL} = \frac{CC}{V}$
where:
CC = Number of colonies counted or estimated, and
V = Volume of sample tested in millilitres</td></tr>
<tr><td>Count exact number of colonies (10 shown)</td></tr>
<tr><td>If possible, count exact number of colonies (100 shown)</td></tr>
<tr><th colspan="3">Semi-Quantitative Range of Detection
Estimate number of colonies by comparison to pictures</th></tr>
<tr><th>No. of Colonies Estimated</th><th>Volume Tested</th><th>Approx. Microbial Content</th></tr>
<tr><td rowspan="3">c. 100 colonies</td><td>0.25 mL or 0.5 mL fuel</td><td>c. 10^5 CFU/L</td></tr>
<tr><td>0.01 mL of fuel*</td><td>c. 10^7 CFU/L</td></tr>
<tr><td>0.01 mL of water</td><td>c. 10^4 CFU/mL</td></tr>
<tr><td rowspan="3">c. 1000 colonies</td><td>0.25 mL or 0.5 mL fuel</td><td>c. 10^6 CFU/L</td></tr>
<tr><td>0.01 mL of fuel*</td><td>c. 10^8 CFU/L</td></tr>
<tr><td>0.01 mL of water</td><td>c. 10^5 CFU/mL</td></tr>
<tr><td rowspan="3">≥ c. 10,000 colonies</td><td>0.25 mL or 0.5 mL fuel</td><td>≥ c. 10^7 CFU/L</td></tr>
<tr><td>0.01 mL of fuel*</td><td>≥ c. 10^9 CFU/L</td></tr>
<tr><td>0.01 mL of water</td><td>≥ c. 10^6 CFU/mL</td></tr>
</table>

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