Designation: E1259 – 16

Standard Practice for Evaluation of Antimicrobials in Liquid Fuels Boiling Below 390°C[1]

This standard is issued under the fixed designation E1259; 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 is designed to evaluate antimicrobial agents for the prevention of microbially influenced deterioration of liquid fuels (as defined by Specification D396, D910, D975, D1655, D2069, D2880, D3699, D4814, D6227, D6751, and D7467), system deterioration, or both.

1.2 Knowledge of microbiological techniques is required for these procedures.

1.3 It is the responsibility of the investigator to determine whether Good Laboratory Practice (GLP) is required and to follow them where appropriate (40 CFR, 160), or as revised.

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

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D396 Specification for Fuel Oils
D910 Specification for Leaded Aviation Gasolines
D975 Specification for Diesel Fuel Oils
D1655 Specification for Aviation Turbine Fuels
D2069 Specification for Marine Fuels (Withdrawn 2003)[3]
D2880 Specification for Gas Turbine Fuel Oils
D3699 Specification for Kerosine
D4814 Specification for Automotive Spark-Ignition Engine Fuel
D5465 Practice for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods
D6227 Specification for Unleaded Aviation Gasoline Containing a Non-hydrocarbon Component
D6293 Test Method for Oxygenates and Paraffin, Olefin, Naphthene, Aromatic(O-PONA) Hydrocarbon Types in Low-Olefin Spark Ignition Engine Fuels by Gas Chromatography (Withdrawn 2009)[3]
D6469 Guide for Microbial Contamination in Fuels and Fuel Systems
D6729 Test Method for Determination of Individual Components in Spark Ignition Engine Fuels by 100 Metre Capillary High Resolution Gas Chromatography
D6733 Test Method for Determination of Individual Components in Spark Ignition Engine Fuels by 50-Metre Capillary High Resolution Gas Chromatography
D6751 Specification for Biodiesel Fuel Blend Stock (B100) for Middle Distillate Fuels
D6974 Practice for Enumeration of Viable Bacteria and Fungi in Liquid Fuels—Filtration and Culture Procedures
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
D7467 Specification for Diesel Fuel Oil, Biodiesel Blend (B6 to B20)
D7687 Test Method for Measurement of Cellular Adenosine Triphosphate in Fuel, Fuel/Water Mixtures, and Fuel-Associated Water with Sample Concentration by Filtration
D7978 Test Method for Determination of the Viable Aerobic Microbial Content of Fuels and Associated Water—Thixotropic Gel Culture Method
E1259 Practice for Evaluation of Antimicrobials in Liquid Fuels Boiling Below 390°C
E1326 Guide for Evaluating Non-culture Microbiological Tests

[1] This practice is under the jurisdiction of ASTM Committee E35 on Pesticides, Antimicrobials, and Alternative Control Agents and is the direct responsibility of Subcommittee E35.15 on Antimicrobial Agents.

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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.

[3] The last approved version of this historical standard is referenced on www.astm.org.

2.2 *NACE Standard:*

TM0172 Determining Corrosive Properties of Cargoes in Petroleum Product Pipelines[4]

2.3 *Federal Standards:*

40 CFR Part 79 Fuels and Fuel Additives Registration Regulations[5]

40 CFR Part 152 Pesticide Registration and Classification Procedures[5]

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *antimicrobial, n*—see *biocide*.

3.1.2 *biocide, n*—a physical or chemical agent that kills living organisms.

3.1.2.1 *Discussion*—Biocides are further classified as bactericides (kill bacteria), fungicides (kill fungi), and microbicides (kill both bacterial and fungi). They are also referred to as *antimicrobials*.

3.1.3 *microbially-influenced deterioration, n*—decomposition /degradation of material (fuel) or making unsuitable for use, as a result of metabolic activity or the presence of microbes.

3.1.4 *microbicide, n*—see *biocide*.

3.1.5 *microcosm, n*—a miniature system used to model larger systems.

3.1.5.1 *Discussion*—It is generally impractical to evaluate microbicide performance in large fuel storage system capacities (> 24 000 m^3), consequently small volume (1.0 to 208 L capacity) microcosms are used as model systems.

4. Summary of Practice

4.1 This practice is conducted on a fuel representative of the grade to be treated, and determines the antimicrobial efficacy under well-defined conditions that may include specific inocula or an uncharacterized inoculum from a microbially contaminated fuel system.

4.1.1 Water/fuel ratios and containment time are also defined. This practice allows for impact of fuel/water partitioning and time, on the antimicrobial agent, as well as the effect of continual rechallenge.

4.1.2 At each sampling time interval, treated and untreated aliquots are checked for the treated population survival. Microbiological testing is coupled with gross observations of each system for biofilm formation and interfacial growth.

4.1.3 The size of the test system, total volume of fluid, fuel to bottom-water ratio and test duration may vary depending on the specific objectives of the test.

4.1.4 Before beginning any test plan intended to meet performance testing compliance requirements, confirm that the cognizant authority accepts the test protocol.

[4] Item No. 21204, available from NACE International (NACE), 1440 South Creek Dr., Houston, TX 77084-4906, http://www.nace.org.

[5] Available from U.S. Government Printing Office Superintendent of Documents, 732 N. Capitol St., NW, Mail Stop: SDE, Washington, DC 20401.

5. Significance and Use

5.1 Guide D6469 details the types of problems associated with uncontrolled microbial growth in fuels and fuel systems. Treatment with effective antimicrobial agents is one element of contamination control strategy.

5.2 The procedure should be used to evaluate the relative efficacy of microbicides in liquid fuels boiling below 390°C. The effect of environmental conditions, such as a variety of fuel additives, metal surfaces, and climatology, are variables that can be included in specific tests using this protocol.

5.3 This practice addresses product performance issues only. Regulatory Agencies restrict and control the use of both pesticides (in the U.S.: 40 CFR 152) and fuel additives (40 CFR 79). Regardless of performance in this method, antimicrobials must only be used in compliance with applicable regulations. Specific industries, for example, the aviation industry, may place further restrictions on chemicals used for fuel treatment.

6. Apparatus

6.1 *Colony Counter*—Any of several types, for example, a Quebec Colony Counter may be used.

6.2 *Drums; Steel*—208 L (55 gal) 16 ga. steel, open-head drum with removable 16 ga. lid fitted with 2.05 cm and 1.90 cm threaded ports for venting and sampling.

6.3 *Incubator*—Any incubator capable of maintaining temperature of 30 to 35°C may be used.

6.4 *Glass Jars*—French square or similar configuration.

NOTE 1—Jar capacity should be determined based on the test plan designed fuel to water ratio and the expected sample volume size needed for weekly testing (9.5 and 9.9).

6.5 *Pails; Steel*—18.9 L (5 gal) steel, open-head pail with removable 16 ga. lid fitted with 2.05 cm and 1.90 cm threaded ports for venting and sampling.

6.6 *Sterilizer*—Any suitable steam sterilizer capable of producing the conditions of sterility is acceptable. A pressurized filter sterilization apparatus of appropriate capacity to filter sterilize the test fuels and bottom-water used in the negative control microcosms. A 0.2 µm pore-size methyl cellulose or cellulose acetate membrane should be used as the filtration medium.

6.7 *Vortex*—Mixer.

7. Reagents and Materials

7.1 *Petri Dishes*—100 by 15 mm required for performing standard plate count.

7.2 *Bacteriological Pipets*—10.0 mL and 1.1, or 2.2 mL capacity.

7.3 *Water Dilution Bottles*—Any sterilizable glass container having a 150 to 200 mL capacity and tight closure may be used.

7.4 *Fuel.*

NOTE 2—Representative fuel samples from each product grade are available from all petroleum refiners.

7.5 *Synthetic Bottom Water.*

NOTE 3—In order to promote microbial growth of the inoculum when using the fuel as the sole source of organic nutrients, synthetic bottom water may contain various inorganic nutrients. An example, of a commonly used synthetic bottom water is Bushnell-Haas Mineral Salts medium (BHMSS).[6] with the concentration adjusted to simulate a particular type of bottoms-water (marine, brackish, fresh, etc.).

7.6 *Soy Peptone Casein Digest Agar.*

7.7 *Sabouraud Dextrose Agar.*

7.8 *Agar, Bacteriological Grade.*

7.9 *Potassium Tellurite Solution*—sterile 1 %.

7.10 *Gentamicin Sulfate*—50 µg/mL.

7.11 *Plate Count Agar.*

7.12 *Potato Dextrose Agar.*

NOTE 4—Items 7.5 – 7.12 are available from a variety of media manufacturers and chemical supply companies.

8. Inoculum

8.1 *Inoculum Selection:*

8.1.1 Depending on the objectives of a test plan, one or more characterized cultures (for example: bacterium, yeast and mold) can be selected or microbially contaminated bottoms-water collected from a fuel system can be used.

8.1.2 Contaminated fuel system microbial communities can be quite diverse and contain >50 different taxa. Consequently, when Practice E1259 is to be used in order to assess a product's general antimicrobial performance properties in fuel systems, multi-taxa inocula provide a more realistic challenge population than either single or commonly used, three taxa inocula.

8.1.3 The use of standardized cultures to prepare microcosm inocula facilitates corroborative testing.

8.1.4 Inoculum taxa should be selected from cultures known to grow using fuel as their sole carbon source.

8.1.5 Depending on microcosm design, it can be appropriate to include aerobic and anaerobic taxa. If inhibition of microbiologically influence corrosion is to be assessed, the challenge population should include iron related bacteria, acid producing bacteria and sulfate reducing bacteria as part of the inoculum mixture.

8.1.6 Uncharacterized, bottoms-water, contaminant populations are most appropriate when Practice E1259 is to be used to evaluate microbicide performance efficacy in a single system or family of systems (for example, bulk storage tanks for a specific fuel grade at a specific facility).

8.2 *Inoculum Preparation and Maintenance:*

8.2.1 *Inoculum Revitalization*—Commonly used cultures are *Pseudomonas aeruginosa*, ATCC No. 33988, *Hormoconis resinae*, ATCC No. 20495, and *Yarrowia tropicalis* (formerly *Candida tropicalis*), ATCC No. 48138. However, in accordance with 8.1, additional cultures can be used.

8.2.1.1 Obtain cultures from ATCC. Before initiating fuel antimicrobial tests, revitalize each of the three cultures in accordance with the instructions contained with each culture.

8.2.2 *Maintenance and Preparation of Pre-Inocula*—All cultures are transferred from slants of a specified agar, (for example, *a*) *Pseudomonas aeruginosa* (Plate Count Agar), (*b*) *Hormoconis resinae* Potato Dextrose Agar), and (*c*) *Yarrowia tropicali* (Potato Dextrose Agar)) to synthetic bottom water medium in a suitable size screw-cap glass bottle (6.4).

8.2.2.1 Overlay inoculated bottom water with fuel to give a final fuel to water ratio of 10.

8.2.2.2 Keep this two-phase system at room temperature (20 to 30°C) for seven days.

8.2.2.3 Weekly, transfer the interface, along with half the bottom water to a similar system until the inoculum used.

8.2.2.4 During this inoculum preparation period the bacterial levels should be maintained at approximately 10^7 CFU/mL or non-culture test bioburden equivalent, the yeast levels at approximately 10^6 CFU/mL, and mold levels at approximately 10^4 spores/mL.

8.2.2.5 Freshly collected, microbially contaminated bottoms-water can be maintained per 8.2.2.1 – 8.2.2.4

8.2.3 *Preparation of Challenge (Test) Inoculum:*

8.2.3.1 To prepare the test inoculum, dilute bacterial pre-inocula 1:100 to achieve a population equivalent to approximately 10^5 CFU/mL. Dilute yeast and molds 1:10 to achieve a population equivalent to approximately 10^3 CFU/mL.

8.2.3.2 At time zero, just prior to adding inoculum to each setup, and at each subsequent time point, determine the microbial population density (9.9).

8.2.3.3 If test systems larger than 1.0 L will be used, the challenge inoculum should first be acclimated to growth in systems that contain the same volume and fuel to bottom-water ratio as the test systems.

9. Procedure

9.1 *Test Array Determination*—The test plan determines the number and capacities of microcosms needed for the test plan. Preferably, duplicate microcosms will be set up for each control and test treatment.

9.1.1 Controls may include any combination of:

9.1.1.1 Filter sterilized fuel over filter sterilized water.

9.1.1.2 Challenged, microbicide-free fuel over water.

NOTE 5—Some commercially available fuels contain additives with antimicrobial properties. It may be necessary to filter such fuels through activated carbon filters before using them for microbicide performance testing.

9.1.1.3 *Reference Control*—Microbicide treated fuel over bottom-water.

9.1.2 *Microbicide Treatment Dose*—Testing may be performed using a single dose or a range of doses. Typically the minimum and maximum doses permitted under the microbicide's FIFRA registration are used. One or intermediate concentrations may also be used. For cost-effectiveness comparisons, dose selection may be based on the treatment costs of the microbicide against which the test product is being evaluated.

9.1.3 To determine the number of microcosms needed for the test array, add the total number of control and test treatments and multiply by the number of replicate microcosms required.

9.2 *Determine Microcosm Volume*—Microcosm volume will depend on test objectives.

[6] Bushnell, L.D. and Haas, H.F. 1941. The utilization of certain hydrocarbons by microorganisms. *J. Bacteriol.* 41: 653- 673.

9.2.1 Preliminary microbicidal product screening can be performed in 1 L or 2L microcosms.

9.2.2 Microbicide partitioning between fuel and water phases, in test microcosms and under field conditions, is likely to be affected by fuel to water ratios.

9.2.2.1 Use of a fuel to water ratio of 1000 to 1 is recommended, although fuel to water ratios between 50:1 and 500:1 may also be used, depending on factors such as sample availability.

NOTE 6—All fuel-grades covered by this practice have sufficiently high vapor pressures to permit off-gassing of noxious, potentially toxic volatile organic carbon (VOC) molecules. Small microcosms should be set up inside a fume hood. Microcosms too large to be stored inside a fume hood should be equipped with a vapor trapping system. A simple system can be designed from polyvinylchloride (PVC) piping and buckets filled with activated carbon (see Fig. 1).

9.3 *Determine Bottom-Water Composition*—Depending on the anticipated end-use application, bottom-water composition may range from distilled water (simulating condensate-water accumulation) to sea-water. Recognizing that bottom-water chemistry varies substantially amongst fuel tanks, site-specific testing should be performed using filter-sterilized water from fuel tanks.

9.4 *Determine Challenge Frequency*—The test plan may include a single challenge or repeated challenges. Typically, when repeated challenges are used, they are scheduled for immediately after each sample collection time.

9.5 *Determine Sampling Schedule:*

9.5.1 *Kill-Rate Testing*—For speed of kill or kill-rate testing, collect samples after 30 min; 4, 8, 16, 24, 48, and 72 h.

9.5.2 *Persistence of Effect Testing*—Sample at monthly intervals until microcosm with highest microbicide dose fails (see 10.2.3).

NOTE 7—To simulate long-term storage, replace fuel and bottom-water volumes removed after sampling, but do not re-challenge. To simulate high turnover systems, replace fuel and bottom-water volumes and re-challenge after each sampling.

9.6 *Set Up Microcosms:*

9.6.1 If test will include corrosion testing (NACE TMO172), prepare corrosion coupons and place them in microcosms.

9.6.2 Dispense bottom-water then fuel into each microcosm.

9.6.3 Draw pre-test samples and enumerate fuel and bottom-water viable counts (see Practice D6469 and section 9.9).

9.7 *Add Challenge Inoculum*—Inoculate test and positive control microcosms with challenge population. Draw time zero (T_0) fuel and bottom-water samples (see Practice D6469 and section 9.9).

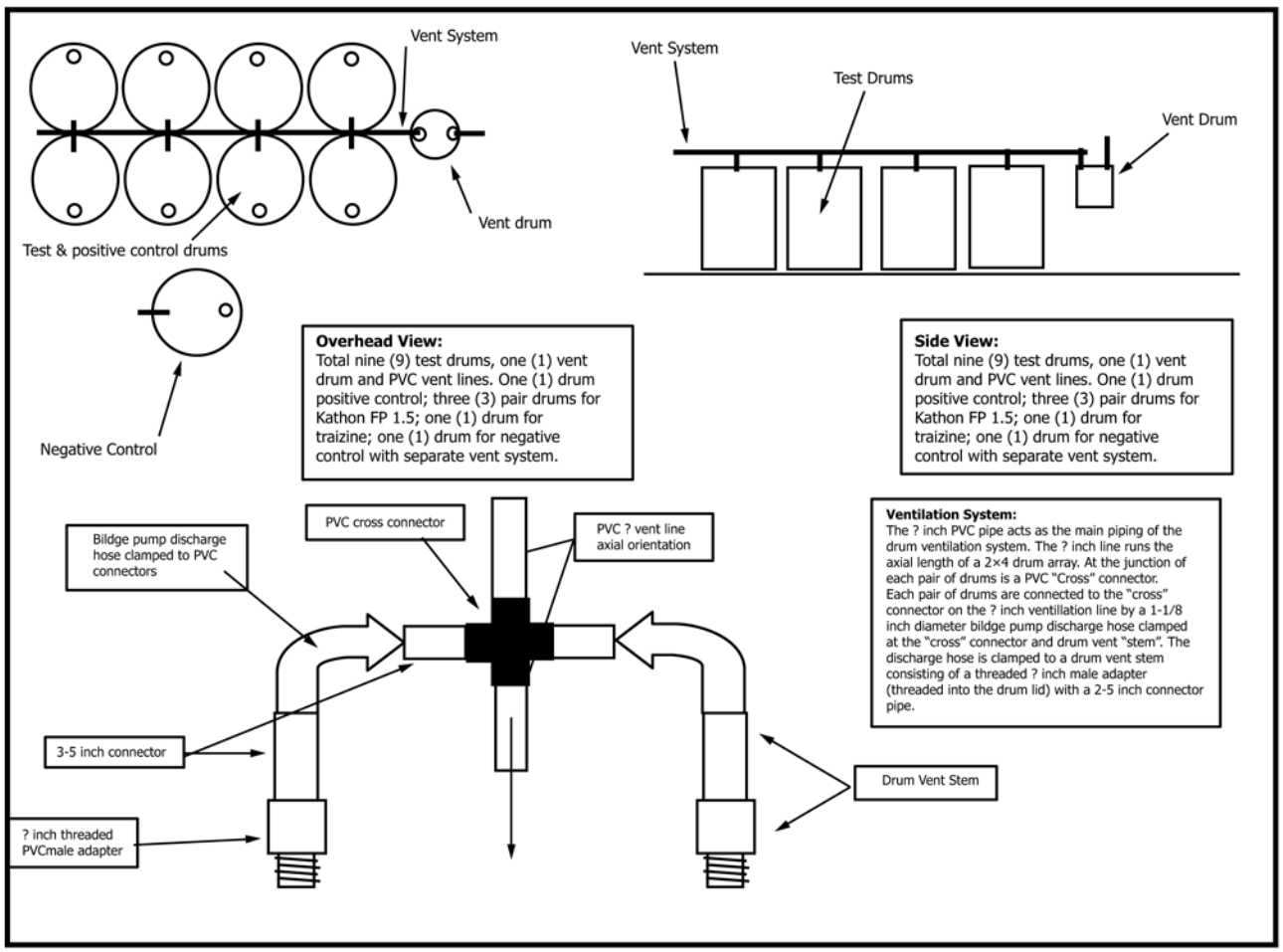

FIG. 1 Schematic Drawing for an Eight-Drum Microcosm Array Ventilation System

NOTE 8—Viable count data may be replaced by or augmented with non-conventional data (see Guide E1326 and Test Method D7463).

9.8 *Sampling*—Predetermined intervals, the following protocol is observed.

9.8.1 *Small (<5.0 L) Microcosms:*

9.8.1.1 Use a 10.0 mL sterile glass pipet to recover 1.0 mL of bottom-water. Transfer the sample to a sterile sample vial (screw capped test tube or bottle).

9.8.1.2 Use a sterile syringe to draw a fuel-phase sample per Practice D6974.

9.8.2 *Large (≥5.0 L) Microcosms:*

9.8.2.1 Draw a fuel-phase sample per Practice D7464.

9.8.2.2 Use the same procedure to draw a bottom-water sample.

9.9 *Microbiological Testing:*

9.9.1 *Bottom-Water*—Enumerate bottom-water bacteria and fungi using either Practice D5465 or an alternative, Guide E1326 validated nonconventional method.

9.9.1.1 Use soy casein digest agar for enumerating *Pseudomonas aeruginosa*; Sabouraud Dextrose Agar with gentamycin 0.5 µg/mL for enumerating *Yarrowia tropicalis*, and 0.01 % potassium tellurite in 1.5 % bacteriological agar *Hormoconis resinae*.

9.9.1.2 For uncharacterized populations, use trypticase soy agar for enumerating bacteria and Sabouraud Dextrose Agar with gentamycin 0.5 µg/mL for enumerating fungi.

9.9.2 *Fuel*—Enumerate fuel-phase bacteria and fungi per Practice D6974, Test Method D7687, or Test Method D7978 or an alternative, Guide E1326 validated, nonculture method.

9.10 *Corrosivity Testing*—Perform corrosion testing per TMO172.

9.11 *Fuel Biodeterioration*—Test for fuel specification property changes as appropriate for fuel grade being used for microbicide performance evaluation (see Specifications D396, D910, D975, D1655, D2069, D2880, D3699, and D6751 for fuel oils, aviation gasolines, diesel fuel oils, aviation turbine fuels, marine fuels, gas turbine fuels, kerosene and biodiesel blend stocks, respectively. Additionally or alternatively, fuel may be tested for changes in the chemical distribution of its constituent molecules (see Test Methods D6293, D6729, or D6733).

9.11.1 For fuel biodeterioration determination, specification tests, fuel chemistry tests or both must be performed at time zero and at the time of test termination. These tests may also be performed at intermediate times in order to evaluate biodeterioration kinetics.

9.11.2 If fuel specification, chemistry or both types of tests are to be included in the experimental design, ensure that the volumes of fuel and bottom-water are sufficient to permit the planned sampling without affecting the volume of the sampled phase by more than 5 %.

10. Results

10.1 *Comparison of Test and Control*—At each interval, the microbiological counts for treated systems will be compared with those of the untreated systems. In addition, gross observations, specification tests (see 9.11), fuel chemistry tests (see 9.11) or any combination of these non-microbiological tests of the condition of each system will be made with the intent of using these data as part of the evaluation.

NOTE 9—*Yarrowia* readily outgrows *Hormoconis* in Sabouraud making distinction of both groups difficult, if not impossible. *Hormoconis resinae* is able to grow in a simple, unsupplemented agar, albeit slowly (about 5 days incubation with a tellurite reduction as an indicator of growth). Under these minimal nutritional conditions, the potassium tellurite may also be inhibitory to the yeast.

10.2 *Test Scoring:*

10.2.1 *Kill-Rate Testing*—Determine the time to achieve 99.9 % reduction; or 4-log reduction if T_0 viable counts are $<10^6$ CFU/mL.

NOTE 10—Before substituting a non-culture test method for culture testing, determine the relationship between changes in the parameter and changes in CFU/mL. Depending on the microbicide being tested non-culture data can be either more conservative than (underestimate actual kill), more optimistic than (overestimate actual kill), or comparable to culture data. See Practice E1326 for additional guidance.

NOTE 11—Metabolically active microbes are found predominantly in the aqueous phase of fuel-water microcosms. Consequently, microbicide efficacy is based primarily on its impact on bottoms-water populations. However, fuel-phase microbiological data can serve as a check to determine if the change in fuel-phase population density is affected by test treatments.

10.2.2 *Persistence Testing*—Determine maximum difference between untreated controls and treated microcosm populations. Score treated microcosms as *failed* when the culturable population density (or its non-culture analogue) in a microcosm has either:

10.2.2.1 Increased by $\geq 2\text{Log}_{10}$ X, relative to the minimum bioburden in that microcosm where X CFU/mL or other quantitative microbiological test parameter,

10.2.2.2 The bioburden in the treated microcosm is ≥ 50% of the bioburden in the challenged, untreated control microcosm (negative control), or

10.2.2.3 Both 10.2.2.1 and 10.2.2.2 have occurred.

10.2.3 *Sources of Variability*—Uncontrollable sources of variability affect test outcomes.

10.2.3.1 *Microbial Population*—Innumerable uncontrollable variables affect the ecology of the mixed challenge population used for microbicide performance evaluations. Consequently, biofilm formation, relative abundances of different test species and the net physiological state of the inoculum will vary amongst microcosms.

10.2.3.2 *Climate*—Climate control facilities are likely to be insufficient to house larger (>100 L) microcosm arrays. Consequently, for test arrays not set up in an a climate controlled environment, climatic conditions (in particular: temperature, relative humidity, and dew point) will effect microcosm ecology.

10.2.3.3 *Fuel Chemistry*—Fuels are manufactured to specifications. Fuels with substantially different chemical profiles (for example, as determined by Test Method D6293) may yield identical specification test results. Moreover, fuels are chemically unstable, consequently, their chemistry changes with time. Production lot variability and aging contribute to variability in a fuels tendency to support microbial growth in fuel associated water.

10.2.3.4 *Sampling*—For larger microcosm systems, 100 % system capture for sampling is impractical. However, microbial population distribution within microcosms >10 L is heterogeneous. Population density is likely to be variable both vertically and horizontally. Populations typically are most dense with biofilms concentrated at the three-way intersection of fuel, water and microcosm wall.

(1) Vertical Population Density Distribution—Microbes tend to be most abundant at interfaces. Consequently (after the three-way interface described in 10.2.3.4), the greatest population densities and levels of metabolic activity are at the fuel-water interface. Population densities and microbial activity decrease logarithmically with distance form the fuel-water interface.

(2) Horizontal Population Density Distribution—Population densities are heterogeneous within zones of nominally high population densities (for example at the fuel-water interface). Viewed vertically, interface growth often appears as islands of biomass. Samples collected between these islands will underestimate population densities and microbial activity. Samples taken within these islands may overestimate these parameters. This spatial variability typically yields a series of peaks and valleys for test results from samples drawn during the course of a microbicide performance evaluation.

11. Precision and Bias

11.1 It is not practical to specify the precision of the procedure in Practice E1259 because detection and enumeration of microorganisms is subjective and not absolute. Since there is no accepted reference material suitable for the procedure in Practice E1259, bias has not been determined.

12. Keywords

12.1 antimicrobials; aviation fuels; biodeterioration; diesel; distillate fuels; gasoline; gas-turbine fuels; marine fuels; microbially-induced deterioration