Designation: E 1259 – 01

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

This standard is issued under the fixed designation E 1259; 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 is designed to evaluate antimicrobial agents for the prevention of microbially influenced deterioration of liquid fuels (as defined by Specification D 396, D 910, D 975, D 1655, D 2069, D 2880, D 3699, D 4818 and D 6227), 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 *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:*

D 396 Specification for Fuel Oils[2]
D 910 Specification for Aviation Gasolines[2]
D 975 Specification for Diesel Fuel Oils[2]
D 1655 Specification for Aviation Turbine Fuels[2]
D 2069 Specification for Marine Fuels[2]
D 2880 Specification for Gas Turbine Fuels[3]
D 3699 Specification for Kerosine[3]
D 4814 Specification for Automotive Spark-Ignition Engine Fuel[4]
D 6227 Specification for Grade 82 Unleaded Aviation Gasoline[4]
D 6469 Guide to Microbial Contamination in Fuels and Fuel Systems[4]

2.2 *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 *antimicrobials*, *n*—chemical agents that are cidal or static to microorganisms.

3.1.2 *microbial-induced deterioration*, *n*—decomposition/degradation of material (fuel) or making unsuitable for use, as a result of metabolic activity or the presence of microbes

3.1.3 *microbicide*, *n*—an agent that kills microbes: bacterial vegetative cells, fungal vegetative cells and spores, algae and protozoa. This term is applied to chemical agents that kill microbes.

4. Summary of Test Method

4.1 This test method is conducted on a reference fuel, and determinines the antimicrobial efficacy under well-defined conditions that include specific inocula *Pseudomonas aeruginosa*, American Type Culture Collection, (ATCC) No. 33988, *Hormoconis resinae*, ATCC No. 20495, and *Yarrowia tropicalis (*formerly *Candida tropicalis*, ATCC No. 18138; as well as water/fuel ratios, and time of containment. It is designed for destructive sampling at regular intervals during bottom water buildup. This test method allows for impact of fuel/water partitioning and time, on the antimicrobial agent, as well as the effect of continual rechallenge. Every 2 weeks, water phase is increased by 0.25 % while concomitantly, a paired system is destructively tested. Thus, at 4 weeks, there is an increase in the water phase to 0.5 %, at 6 weeks 0.75 %, and at 8 weeks 1.0 %. At each sampling time interval, treated and untreated aliquots are checked for the three types of organisms in the initial inoculum. These counts are coupled with gross observations of each system for biofilm formation and interfacial growth.

5. Significance and Use

5.1 Guide D 6469 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 distillate fuels. The effect of environmental conditions, such as a variety of fuel additives,

[1] This test method is under the jurisdiction of ASTM Committee E35 on Pesticides and is the direct responsibility of Subcommittee E35.15 on Antimicrobial Agents.

Current edition approved April 10, 2001. Published June 2001. Originally published as E 1259 – 88. Last previous edition E 1259 – 94.

[2] *Annual Book of ASTM Standards*, Vol 05.01.

[3] *Annual Book of ASTM Standards*, Vol 05.02.

[4] *Annual Book of ASTM Standards*, Vol 05.03.

[5] Available from the Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402.

metal surfaces, and climatology, are variables that can be included in specific tests using this protocol.

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

6.3 *Sterilizer*—Any suitable steam sterilizer capable of producing the conditions of sterility is acceptable.

6.4 *Separatory Funnels*—Sixteen 1-L funnels.

6.5 *Ring Stand*, suitable for supporting separatory funnel.

6.6 *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 150–200 mL capacity and tight closure may be used.

7.4 *Fuel.*[6]

7.5 *Synthetic Bottom Water.*[7]

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*.

8. Inoculum

8.1 *Inoculum Preparation and Maintenance*:

8.1.1 *Inoculum Revitalization*—Cultures are *Pseudomonas aeruginosa*, ATCC No. 33988, *Hormoconis resinae*, ATCC No. 20495, and *Yarrowia tropicalis* (formerly *Candida tropicalis*), ATCC No. 18138. Obtain lyophilized preparations from ATCC. Before initiating fuel antimicrobial tests, revitalize each of the three cultures in accordance with the instructions contained with each culture.

8.1.2 *Maintenance and Preparation of Inocula*—All three cultures are transferred from slants of a specified agar, (*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 (French square), and then overlaid with 10 times the volume of fuel. This two-phase system is kept at room temperature (18-24°C) for seven days, and the interface with half the bottom water is transferred weekly to a similar system weekly until used. The bacterial levels expected are about 10^7 CFU/mL, the yeast levels 10^6 CFU/mL, and mold levels 10^4 spores/mL. For the test inoculum, the bacteria are diluted 1:100 while yeast and molds are diluted 1:10. The counting of the inoculum is done directly from the prepared synthetic bottom water mixture at time zero, just prior to adding inoculum to each setup, and at each subsequent time point.

NOTE 1—**Caution:** In the distillate fuel industry, additives, including biocides, are calculated on a weight per weight basis so that the specific gravity of both the fuel and the biocide (if a liquid formulation) must be taken into account.

9. Procedure

9.1 *The Setup*—For each biocide, four setups are needed plus four for the control. A typical evaluation could include three levels of the biocide in addition to the control system with no biocide.

$$4 \times 3l + 4c = 16ts \quad (1)$$

where:

l = level,
c = control, and
ts = total setups.

9.1.1 To each l-L funnel, add 800 mL of test fuel and the appropriate level of stock biocide so that the desired concentration of biocide is achieved.

9.1.2 Stock biocide is made by adding it to a solvent that will dissolve the biocide.

9.1.3 Next, add 2 mL of synthetic bottom water medium (0.25 % of fuel volume) containing the appropriate levels of each organism. Close the funnel. Shake vigorously for 10 s. Place in ring stand. Open stopper slightly to allow volatile gas to escape. Close and leave for two weeks.

9.2 *Sampling*—At 2, 4, 6, and 8 weeks, the following protocol is observed. The bottom water fraction including the fuel/water interface and a minimal amount of fuel is bled from the separatory funnel and mixed vigorously in a vortex for 10 s. Before settling, aliquots are removed for bacteria, yeast and mold plate counts. Dilutions, 1:100, 1:1000, and 1:10000, are prepared in sterile synthetic bottom water. These will be used for pour platings for bacteria and yeast, respectively. In addition, three 0.1 mL portions will be used for bacteria and yeast pour plates, and for spread plates for mold counts. For estimating *Pseudomonas aeruginosa*, use soy casein digest agar; for *Yarrowia tropicalis*, use Sabouraud's Dextrose Agar with gentamicin 0.5 µg/mL; and for *Hormoconis resinae*, use 0.01 % potassium tellurite in 1.5 % bacteriological agar.

9.3 *Reinoculation*—Immediately after the sacrificing (destructive sampling) of one-system biocide level, 2 mL of synthetic bottom water solution containing additional inoculum is added to each of the three remaining setups/biocide level. Again, the procedure described in 8.1.2 and 9.1.3 is repeated. After two more weeks, 9.2 is again repeated and the process is repeated at weeks 6 and 8.

[6] Representative fuel samples from each product grade are available from all petroleum refiners.

[7] Items 7.5-7.12 are available from a variety of media manufacturers and chemical supply companies.

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 observation of the condition of each system will be made with the intent of using these data as part of the evaluation.

NOTE 2—*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.

11. Precision and Bias

11.1 It is not practical to specify the precision of the procedure in Test Method E 1259 because detection and enumeration of microorganisms is subjective and not absolute. Since there is no accepted reference material suitable for the procedure in Test Method E 1259, 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

APPENDIX

(Nonmandatory Information)

X1. ALTERNATIVE PROCEDURES

X1.1 Setups using 1-L French squares or other suitable container, can substitute for separatory funnels. Although the interface is not so readily discerned, the bottom water can be removed by careful pipetting.

X1.2 *Fuel*—Potentially, the inclusion of additives may not only increase the growth rate of the inoculum but may also affect the efficacy of the biocide. In any specific testing for fuel suppliers, it would be unrealistic to exclude these additives from the study (see also Practice D 4054).

X1.3 *Bottom Water Level and Time of Storage*—The test described here does not consider either long term storage with minimal bottom water (for example, 8 weeks at 0.25 % water) or shock dosing a heavily contaminated system with a water soluble biocide, or bleed off of bottom water with loss of biocide.

X1.4 *Corrosion*—There is provision for use of metal coupons for both evaluation of biocide corrosivity and microbial-induced corrosion.

X1.5 *Inoculum*—For specific testing, it may be advisable to use contaminated fuel as an inoculum after determination of the identity of the contaminant.