Designation: E 1259 – 94

Standard Test Method for Evaluation of Antimicrobials in Distillate Fuels (Based on Preliminary Screening and Compatibility)[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 microbial-induced deterioration of distillate fuels (as defined by Specification D 396 as fuel) or system deterioration, or both.

NOTE 1—A knowledge of microbiological techniques is required for these procedures.

1.2 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.

1.3 *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.* See caution statement, Note 2, in Section 8.

2. Referenced Documents

2.1 *ASTM Standards:*

D 396 Specification for Fuel Oils[2]

D 4054 Practice for Evaluating the Compatibility of Additives with Aviation-Turbine Fuels and Aircraft Fuel System Materials[3]

2.2 *Federal Standard:*

40 CFR, Part 160, Good Laboratory Practice Standards[4]

3. Summary of Test Method

3.1 This test method is conducted on a reference fuel for determining 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, 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 equivalent 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.

4. Significance and Use

4.1 The procedure should be used to evaluate the relative efficacy of microbicides in distillate fuels. The effect of environmental conditions including a variety of fuel additives, metal surfaces, and climatology are variables that can be included in specific tests using this protocol.

5. Apparatus

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

5.2 *Incubator*—Any incubator capable of maintaining temperature of 30 to 35 ± 2°C may be used.

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

5.4 *Separatory Funnels*—Eight 1-L funnels.

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

5.6 *Vortex*—Mixer.

6. Reagents and Materials

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

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

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

6.4 *Distillate Fuel.*[5]

6.5 *Synthetic Bottom Water.*[6]

6.6 *Soy Peptone Casein Digest Agar*.

6.7 *Sabouraud Dextrose Agar*.

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[1] This test method is under the jurisdiction of ASTM Committee E-35 on Pesticides and is the direct responsibility of Subcommittee E35.15 on Antimicrobial Agents.

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

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

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

[5] I-H CAT diesel fuel is available from Howell Hydrocarbons Inc., San Antonio, TX 78223-3531.

[6] Items 6.5-6.12 are available from a variety of media manufacturers and chemical supply companies.

6.8 *Agar, Bacteriological Grade*.
6.9 *Potassium Tellurite Solution*—sterile 1 %.
6.10 *Gentamicin Sulfate*—50 µg/mL.
6.11 *Plate Count Agar*.
6.12 *Potato Dextrose Agar*.

7. Inoculum

7.1 *Maintenance and Preparation of Inocula*—All three cultures are transferred from specific agar slants (*a*) *Pseudomonas aeruginosa*, plate count agar; (*b*) *Hormoconis resinae*, and (*c*) *Yarrowia tropicalis*, potato dextrose agar to synthetic bottom water medium in a suitable size screw-cap glass bottle (French square) and overlaid with 10 times the volume of fuel. This two-phase system is kept at room temperature, and the interface with half the bottom water is transferred to a similar system weekly until used. The bacterial levels expected are about 10^7 CFU/mL, Colony Forming Units, 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 the latter two are diluted 1:10. The counting of the inoculum (zero time) is done directly from the prepared synthetic bottom water mixture just prior to adding to each setup at zero time and each subsequent time point.

8. Procedure

8.1 *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.

8.1.1 To each 1-L tunnel, add 800 mL of test fuel with appropriate level of biocide.

NOTE 2—**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.

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

8.2 *Sampling*—At each time point after 0 weeks, 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 plate counts for the yeast, bacteria, and molds with dilutions into sterile synthetic bottom water solution as follows: 1.0 mL into 99 mL for 1:100 and from this dilution subsequent dilutions for 1:1 000 and 1:10 000 are made. These will be used for pour platings for bacteria and yeast, respectively. In addition, three by two 0.1 mL portions will be used for bacteria and yeast pour plates and for spread plates for mold counts; for estimating *Pseudomonas aeruginosa*, soy casein digest agar; for *Yarrowia tropicalis*, sabourauds dextrose agar with gentamicin 0.5 µg/mL; for *Hormoconis resinae*, 0.01 % potassium tellurite in 1.5 % bacteriological agar.

NOTE 3—*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.

8.3 *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 of shaking, etc. described in 8.1 is repeated. After 2 more weeks, 8.2 is repeated and the process described here is repeated at Weeks 6 and 8.

9. Results

9.1 *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.

10. Precision and Bias

10.1 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.

11. Keywords

11.1 antimicrobials; distillate 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 biocide negatively as well as positively. 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 after determination of the identity of the inoculum.