INTERNATIONAL

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

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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~~, and~~ , 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.

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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 Aviation Gasolines
D975 Specification for Diesel Fuel Oils
D1655 Specification for Aviation Turbine Fuels
D2069 Specification for Marine Fuels
D2880 Specification for Gas Turbine Fuel Oils
D3699 Specification for Kerosine ~~D4057 Practice for Manual Sampling of Petroleum and Petroleum Products~~
D4814 Specification for Automotive Spark-Ignition Engine Fuel
D5465 Practice for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods
D6227 Specification for Grade 82 Unleaded Aviation Gasoline
D6293 Test Method for Oxygenates and Paraffin, Olefin, Naphthene, Aromatic(O-PONA) Hydrocarbon Types in Low-Olefin Spark Ignition Engine Fuels by Gas Chromatography
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 FuelsFiltration 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

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[1] This practice is under the jurisdiction of ASTM Committee E35 on Pesticides 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.

D7467 Specification for Diesel Fuel Oil, Biodiesel Blend (B6 to B20)
E1326 Guide for Evaluating Nonconventional Microbiological Tests Used for Enumerating Bacteria
2.2 *NACE Standard:*
TM0172 Determining Corrosive Properties of Cargoes in Petroleum Product Pipelines[3]
2.3 *Federal Standards:*
~~40 CFR, Part 79,~~40 CFR Part 79 Fuels and Fuel Additives Registration Regulations[4]
~~40 CFR, Part 152,~~40 CFR Part 152 Pesticide Registration and Classification Procedures[4]

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: *Pseudomonas aeruginosa*, American Type Culture Collection, (ATCC) No. 33988, *Hormoconis resinae*, ATCC No. 20495, and *Yarrowia tropicalis* (formerly *Candida tropicalis*, ATCC No. 18138; or an uncharacterized inoculum from a microbially contaminated fuel system. Additionally, 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. 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. 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. Before beginning any test plan intended to meet performance testing compliance requirements, confirm that the cognizant authority accepts the test protocol.

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*—1 L capacity, French square or similar configuration.
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.

[3] ~~Item No. 21204, available from NACE International, Houston TX.~~
[3] Item No. 21204, available from NACE International (NACE), 1440 South Creek Dr., Houston, TX 77084-4906, http://www.nace.org.
[4] Available from U.S. Government Printing Office Superintendent of Documents, 732 N. Capitol St., NW, Mail Stop: SDE, Washington, DC 20401.

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.*~~ Fuel.

NOTE 1—Representative fuel samples from each product grade are available from all petroleum refiners.

7.5 *Synthetic Bottom Water.*
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 2—Items 7.5-7.12 are available from a variety of media manufacturers and chemical supply companies.

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 cultures 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 (20 to 30°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. This procedure may also be followed to maintain and prepare uncharacterized inocula. If test systems lager 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.

NOTE ~~1—**Caution:**In~~ 3—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 *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 ~~2—Some~~ 4—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 Microcosms~~*~~ Volume*—Microcosm volume will depend on test objectives. Preliminary microbicidal product screening may be performed in 1 L microcosms. Achieving the desired fuel to water ratio, to simulate tank storage conditions, may require drum-size (208 L) microcosms. Typical fuel to water ~~rations~~ratios range ~~form~~from 50:1 to 500:1.

NOTE ~~3—All~~ 5—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).