Designation: E979 – 20

Standard Practice for Evaluation of Antimicrobial Agents as Preservatives for Invert Emulsion and Other Water Containing Hydraulic Fluids[1]

This standard is issued under the fixed designation E979; 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.

INTRODUCTION

Invert emulsion hydraulic fluids typically contain 60 % mineral oil and 40 % water (by volume). These fluids routinely are prepared using proprietary, oil-soluble, emulsifying agents, as well as other emulsifiable constituents. They are recommended for use where conditions indicate a low-cost, fire retardant product, compatible with water-based metal working fluids.

The high water content of these hydraulic fluids makes them susceptible to microbial attack. Uncontrolled microbial growth in these fluids can cause cartridge filter unit plugging, maladorous conditions, or general biodeterioration. Problem microorganisms associated with these fluids include bacteria and fungi.

The hydraulic system is essentially a closed one in which water of evaporation is added to maintain a fixed volume. The inclusion of an efficacious preservative in the water containing hydraulic fluids can prevent microbial growth and the resulting problems that follow.

1. Scope*

1.1 This laboratory practice is designed to evaluate the utility and effectiveness of antimicrobial agents intended to control microbial growth in invert emulsions and other water containing hydraulic fluids.

NOTE 1—Procedures for preparation of water soluble hydraulic fluids and recovery of organisms appear in Practice E2169.

1.2 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

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

1.4 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1129 Terminology Relating to Water

D4454 Test Method for Simultaneous Enumeration of Total and Respiring Bacteria in Aquatic Systems by Microscopy (Withdrawn 2015)[3]

D5465 Practices for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods

E1326 Guide for Evaluating Non-culture Microbiological Tests

E2169 Practice for Selecting Antimicrobial Pesticides for Use in Water-Miscible Metalworking Fluids

E2523 Terminology for Metalworking Fluids and Operations

E2694 Test Method for Measurement of Adenosine Triphosphate in Water-Miscible Metalworking Fluids

[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.

***A Summary of Changes section appears at the end of this standard**

3. Terminology

3.1 Terms used in this practice are defined in Terminologies D1129 and E2523.

4. Summary of Test Method

4.1 The antimicrobial agent to be evaluated is incorporated into an emulsion system by (*a*) addition to the aqueous phase employed in the preparation of the emulsion, (*b*) in doses to the formulated system, or (*c*) by other methods suitable for the test compound.

4.2 A heavy bacterial or fungal inoculum, or both, is then added.

4.3 The resulting mixture is aerated and passed over the surface of a simulated filter system for a minimum period of eight weeks either continuously or with shutdowns to simulate actual operations conditions.

4.4 The degree of microbial control is determined by periodically quantifying the bioburden in the emulsion by direct microscopic count (Test Method D4454), plate count (Practice D5465), or other appropriate method (Guide E1326) and visual observations for microbial fouling of the simulated filter surface.

NOTE 2—A knowledge of standard microbiological techniques is required for this procedure. It is also required that good laboratory practices be followed throughout these tests. This means appropriate containment for the microbiological systems being evaluated. The systems should be maintained in an enclosure so that during the aeration process the mists and aerosols generated do not contaminate the laboratory environment.

5. Significance and Use

5.1 This procedure is designed to determine the effectiveness of antimicrobial agents intended for microbial control in invert emulsions and other water containing hydraulic fluids.

6. Apparatus

6.1 *Air Supply*—Any air source which is free from organic vapors, organic matter, or other objectionable material may be used.

NOTE 3—If desired, air may be sterilized as follows:

Pack two 150-mm long drying tubes (bulb type) loosely with glass wool in a series with neoprene stoppers, glass tubing, and neoprene tubing. Wrap loosely in aluminum foil and steam sterilize at 15 to 20 psi for 30 minutes. Cool to room temperature while still wrapped. In-line pre-sterilization air filters are available from most local laboratory supply houses.

Insert into air line with bulbs on upstream side. Average lifetime in continuous use is two weeks. Discard sooner if upstream filter becomes wet or contaminated with oil.

6.2 *Colony Counter*—Any one of several types may be used.

6.3 *Incubator*—Any cabinet capable of maintaining a temperature of 35 ± 1 °C may be used.

6.4 *Test Cabinet*—A large cabinet capable of maintaining a temperature of 35 ± 1 °C, able to house several two litre beakers, and into which an air line can be introduced.

6.5 *Sterilizer*—Any suitable steam sterilizer capable of producing the conditions of sterilization is acceptable.

6.6 *Simulated Filters:*

6.6.1 *Strainer,* 3-in. epoxy coated, ¼-in. mesh gutter strainer.[4]

6.6.2 *Screen,* 16 by 18 in. fiberglass screening material.

NOTE 4—Fiberglass mesh screening material (16 by 18 in.) is available from any local hardware dealer.

6.6.3 *Wire,* 20-gauge, galvanized or stainless steel.

6.7 *Tubing,* ¼-in. ID Tygon.

NOTE 5—Tygon is available from most local laboratory supply houses.

6.8 *T-Connectors,* ¼-in. polypropylene.

6.9 *Laboratory Blender*—Any standard adjustable speed laboratory blender having a 2-L capacity glass or metal container is satisfactory.

6.10 *Hypodermic Needle,* 16-gauge needle.

6.11 *Microscope,* Brightfield microscope equipped with 40× and 100× objectives.

6.12 *Labware:*

6.12.1 *Culture Dishes*—100 by 15 mm sterile culture dishes made of glass or plastic are required for making standard plate counts.

NOTE 6—Presterilized and disposable plastic petri dishes are available from most local laboratory supply houses.

6.12.2 *Bacteriological Pipettes of 1.1 or 2.2-mL capacity.*

NOTE 7—Presterilized and disposable 1.1-mL bacteriological pipettes are available from most local laboratory supply houses.

6.12.3 *Water Dilution Bottles*—Any sterilizable glass containers having a 150 to 200-mL capacity and tight closures may be used.

NOTE 8—Milk dilution bottles of 160-mL capacity having screw-cap closures are available from most local laboratory supply houses.

6.12.4 *Two-Liter Borosilicate Glass Beakers.*

6.12.5 *Bent Glass Rod.*

6.12.6 *Screw Cap Culture Tubes,* autoclavable, 15 by 150 mm.

6.13 *Water Bath*—Maintain at 46 ± 2 °C to anneal agar based microbiological media.

6.14 *Aluminum Foil.*

7. Reagents and Materials

7.1 *Invert Emulsion Emulsifier.*[5]

7.2 *Paraffinic Mineral Oil.*

7.3 *Deionized or Distilled Water* (>2 MOHM quality)

[4] The sole source of supply of the apparatus known to the committee at this time is Billy Penn Corp., Philadelphia, PA 19122. 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.

[5] The sole source of supply of a satisfactory emulsifier for the preparation of invert emulsion hydraulic fluids (Compound #5162) known to the committee at this time is the Lubrizol Co., Wickliffe, OH. 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.

7.4 *Gentamicin Sulfate.*[6]

7.5 *Arlacel 80.*[7]

7.6 *Tween 60.*[7]

7.7 *Phosphate Buffer— For serial dilutions.*

7.8 *Mineral oil, sterile.*

7.9 *Microbiological Media*—General retrieval media consistent with good microbiological practices are acceptable. Examples are as follows:

7.9.1 *Soybean-Casein Digest Agar,* U.S.P. XIX, Medium II.

NOTE 9—Soybean-casein digest agar is available in dehydrated form from most laboratory media supply houses.

7.9.2 *Fluid Soybean-Casein Digest Medium,* U.S.P. XIX, Medium III.[8]

7.9.3 *Sabouraud Dextrose Agar,* U.S.P. XIX, Medium 20.[8]

7.9.4 *Sabouraud Dextrose Broth,* U.S.P. XIX, Medium 21.[8]

7.9.5 *Sulfate American Petroleum Institute (API) Agar,*[7]for enumeration of sulfate reducing bacteria.

7.10 *Inoculum:*

7.10.1 The inoculum may vary according to the users' requirements. It may be either undefined or defined.

7.10.1.1 An undefined inoculum may consist of microorganisms isolated from a "spoiled" invert emulsion hydraulic fluid which exhibits microbiologically induced phase generation, or which is known to have caused plugging of a hydraulic system filter due to microbial slime, and grown in a nutrient medium.

7.10.1.2 An undefined inoculum may consist of the following: (*1*) equal volumes of fluid soybean-casein digest and "spoiled" (see 7.10.1.1) hydraulic fluid aerated at 35 °C for 24 h (typically) until the bacterial count reaches 10^9 CFU/mL, (*2*) equal volumes of sabouraud dextrose broth and "spoiled" (see 7.10.1.1) hydraulic fluid aerated at 35 °C for 24 h (typically) or until fungal count reaches 10^6 to 10^7 CFU/mL, or (*3*) equal volumes of (*1*) and (*2*) if both bacteria and fungi are the desired test organisms.

7.10.2 A defined inoculum consisting of a mixed culture of specific microorganisms may also be used.

7.10.2.1 The defined inoculum may be prepared by isolating and identifying specific microorganisms from a "spoiled" (see 7.10.1.1) hydraulic fluid emulsion and culturing the bacterial isolates in soybean-casein digest medium and the fungal isolates in sabouraud dextrose broth until there are 10^9 CFU bacteria or 10^6 to 10^7 CFU fungi, or both, per mL, respectively.

7.10.2.2 Other microorganisms of particular interest (Rossmoore and Szlatky)[9] may be used such as: *Pseudomonas fluorescens*, *Pseudomonas cepacia*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Desulfovibrio desulfuricans*, *Aspergillus niger*, *Cephalosporium* sp., *Fusarium* sp., *Candida* sp.

7.10.2.3 Equal mixtures of any two of the above bacterial species or two of the above mold species, or both, plus the *Candida* species to provide a final titer of 10^9 CFU bacteria, or 10^6 to 10^7 CFU fungi, or both, per mL, should be used as an inoculum for the emulsion system.

7.11 *Antimicrobial Agents*—The chemical agents to be evaluated as preservatives.

8. Preparation of Simulated Filters

8.1 Cut the epoxy-coated, ¼-in. mesh gutter strainers 16 by 18 in. mesh fiberglass screening material into 3 by 5 in. sections. Secure the screening to the strainers with 20-gauge wire or with staples.

8.2 *Preparation of Aerators*—Cut tubing (see 6.7) into 13-in. sections. Bend tubing in a circle and connect both ends using a T connector (see 6.8). Connect third arm of T connector to a 20-in. length of tygon tubing. This tubing will be connected to the main air supply line. Using a hot 16-gauge needle, carefully punch a series of holes, ½ in. apart, along the outer circumference of the tubing which forms the ring. Also punch similar holes ½ in. apart on the upper and lower surface of the tubing, at right angles to the holes previously punched. These holes allow the air from the air source to bubble up through the hydraulic fluid producing a cascading effect over the surface of the simulated filter.

9. Preparation of Microbiological Medium

9.1 Microbiological media should be prepared in accordance with manufacturer's instructions. Media to be augmented with antibiotics should be annealed in a 46 ± 2 °C water bath before antibiotics are added. Antibiotics should be added just before pouring. Use 100 g gentamicin sulfate per mL to suppress bacterial growth on fungal recovery media.

10. Microbiological Methods

10.1 Solubilize the invert emulsion aliquot (see 7.1) according to the procedure of McConville, et al.,[10,11] as follows:

10.1.1 Disperse 1 mL of the invert emulsion in 1 mL of Arlacel 80 and bring the volume up to 10 mL with 10 % Tween 60 solution.

[6] The sole source of supply of gentamicin sulfate known to the committee at this time is as Garamycin Reagent Solution, available in two concentrations of 10 and 50 mg/mL, from the Schering Corp., Kenilworth, NJ 07033.. 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] The sole source of supply of the reagents (Arlacel 80, Tween 60, and Sulfate API Agar) known to the committee at this time is Sigma Aldrich Co., St. Louis, MO 63178, http://www.sigmaaldrich.com. 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.

[8] The sole source of supply of the media, available in dehydrated form, known to the committee at this time is Baltimore Biological Laboratories, Cockeysville, MD or Difco Laboratories, Detroit, MI. 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.

[9] Rossmore, H. W., and Szlatky, K. ,"Characterization of the Microbial Flora of Invert Emulsion Hydraulic Fluids," *Int. Biodetn. Bulletin*, Vol 13, No. 4), 1977, pp. 96–100.

[10] McConville, J. F., et al., "Method for Performing Aerobic Plate Counts of Anhydrous Cosmetics Utilizing Tween 60 and Arlacel 80 as Dispersing Agents," *Applied Microbiology* , Vol 27, 1974, pp. 5–7.

[11] Hoffman, N. M., "Hydraulic Fluid of 95-Percent Water," *Lubrication Engineering*, Vol 35, No. 2, 1979, pp. 65–71.

10.2 Quantify the bacterial bioburden in the solubilized invert emulsion samples (see Test Methods D4454 and E2694, Practice D5465, or Guide E1326).

NOTE 10—Do not use enumeration procedures interchangably since each bioburden parameter measures a different aspect of the microbial population and different culture methods are likely to select for different microorganisms, thereby contributing to test variability. Incubate all plates for three days at 35 °C.

10.3 Enumerate the mold and yeast populations in the solubilized invert emulsion samples by using the same procedures as in 10.2, but use sabouraud dextrose agar containing 100 µg of gentamicin sulfate per mL as the plating medium. Incubate all plates for five days at 35 °C.

10.4 Enumerate sulfate reducing bacteria populations in the solubilized invert emulsion samples by serially diluting 1.0 mL aliquots in 9.0 mL molten, API agar in 15 by 150 mm screwcap culture tubes. Prepare a series of 10^{-1} and 10^{-4} dilutions. Gently tip tube back and forth several times to mix inoculum with API agar while minimizing aeration. Warm pipet gently over bunsen burner flame before transferring a sample from one dilution tube to the next in a series. Once inoculated and the API agar has gelled, fill each culture tube with sterile mineral oil. Incubate at 35 ± 1 °C. Observe for the formation of black colonies weekly for four weeks. Record final titer.

11. Procedure

11.1 Add 67.5 mL of emulsifier (see 7.1) to 832.5 mL of paraffinic mineral oil (see 7.2).

11.2 Transfer mixture into a 2 L blender cup and add 450 mL of deionized water and 150 mL of a broth inoculum prepared as described in 7.8.

NOTE 11—Add the water and inoculum very gradually with the blender running at slow speed to avoid raising the temperature of the mixture above 24 to 38 °C.

11.3 Continue mixing until stable water-in-oil emulsion is produced. This emulsion will serve as the untreated control sample.

11.4 Prepare the treated emulsion samples as described in 11.1 – 11.3, but add the antimicrobial to be tested to the deionized water in a concentration which will provide the desired antimicrobial test dose in the completed emulsion or in a manner consistent with industrial practice and proposed recommendations. When preparing the treated emulsions, make sure to add the inoculum to the blender after all of the deionized water containing the antimicrobial has been incorporated.

11.5 Add each test emulsion sample to a separate 2 L beaker (see 6.12.4). Mark height of emulsion in beaker on outside of container.

11.6 Place a simulated filter assembly (see 8.1) into each beaker.

11.7 Surround the simulated filter cone with the circular aerator tube (see 8.2) in such a way that a cascading effect is produced by the air issuing from the holes causing the emulsion to bubble over the top of the simulated filter. See Fig. 1 for assembled apparatus.

11.8 Place beakers in 35 ± 1 °C chamber.

11.9 Connect individual beaker air supply lines to the main air supply tube using T connectors.

11.10 Aerate the systems continually, for four days, discontinue aeration for 64 h to simulate a weekend shutdown, and then reinitiate aeration. Continue this on/off schedule for a minimum of eight weeks or until the simulated filter becomes completely plugged or the emulsion "splits."

11.11 *Sampling and Maintenance Schedule:*

11.11.1 Check systems daily to determine the need for make-up water. Add deionized water to each system as needed to maintain the system at its original volume.

11.11.2 Observe the simulated filters daily for evidence of fouling (slime build-up), and record findings. When fouling is observed, sample fouling deposit by subculturing onto an appropriate microbiological medium (see 7.7). Use standard plating and microscopic techniques to confirm the microbiological nature of the deposit. This will help distinguish between microbiological and non-microbiological deposits.

11.11.3 Remove a sample of the test emulsion from each beaker, after each simulated "weekend shutdown", for microbiological plate count analysis (see Section 10). If claims relating to the control of anaerobes will be made for the antimicrobial agents employed, sampling from the bottom of the system should be done consistently with standard microbiological techniques for the retrieval of anaerobes.

12. Interpretation of Results

12.1 For the test(s) to be valid, microbiological fouling of the simulated filter assembly in the untreated control beaker must be observed. Total contaminant titers in the untreated controls should be at least 10^8 CFU bacteria/mL and 10^6 CFU fungi/mL at test termination.

12.2 Visible slime production in the hydraulic fluid formulation employed in this procedure, accompanied by plugging of the simulated filters, is indicative of failure in the field because of plugging of the cartridge filters employed in most industrial hydraulic systems. Thus, visible slime production and plugging of the simulated filter indicates inadequate protection of the hydraulic fluid by the antimicrobial concentration under test as confirmed by microbiological subculturing techniques. High bacterial titers (>10^7 CFU/mL) in treated invert emulsion hydraulic fluids indicate a low degree of bioresistance. High bacterial titers will lead to fluid failure. The appearance of fungal involvement, (>10^2 CFU/mL) with or without subsequent plugging of cartridge filters, is also an indication of poor bioresistance. The detection of both microbial populations are warnings of ultimate system failure and should be regarded as a signal for either a change, or an addition of antimicrobial agents. When several preservatives are being tested, they should be compared by their inhibitory effect relative to the untreated control. Duration of efficacy, in weeks, should also be compared.

13. Precision and Bias

13.1 A precision and bias statement cannot be made for this practice. Selection of test fluid, microbicide treatment, challenge inoculum, bioburden quantification protocol, or any