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Standard Practice for Evaluating Water-Miscible Metalworking Fluid Bioresistance and Antimicrobial Pesticide Performance[1]

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1. Scope

1.1 This practice addresses the evaluation of the relative inherent bioresistance of water-miscible metalworking fluids, the bioresistance attributable to augmentation with antimicrobial pesticides or both. It replaces Methods D3946 and E686.

1.2 In this practice relative bioresistance is determined by challenging metalworking fluids with a biological inoculum that may either be characterized (comprised of one or more known biological cultures) or uncharacterized (comprised of biologically contaminated metalworking fluid or one or more unidentified isolates from deteriorated metalworking fluid). Challenged fluid bioresistance is defined in terms of resistance to biomass increase, viable cell recovery increase, chemical property change, physical property change or some combination thereof.

1.3 This practice is applicable to antimicrobial agents that are incorporated into either the metalworking fluid concentrate or end-use dilution. It is also applicable to metalworking fluids that are formulated using non-microbicidal, inherently bioresistant components.

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]

D1129 Terminology Relating to Water
D888 Test Methods for Dissolved Oxygen in Water
D1067 Test Methods for Acidity or Alkalinity of Water
D1193 Specification for Reagent Water
D3342 Test Method for Dispersion Stability of New (Unused) Rolling Oil Dispersions in Water
D3519 Test Method for Foam in Aqueous Media (Blender Test) (Withdrawn 2013)[3]
D3601 Test Method for Foam In Aqueous Media (Bottle Test) (Withdrawn 2013)[3]
D4627 Test Method for Iron Chip Corrosion for Water–Miscible Metalworking Fluids
D5465 Practice for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods
E70 Test Method for pH of Aqueous Solutions With the Glass Electrode
E1326 Guide for Evaluating Non-culture Microbiological Tests Used for Enumerating Bacteria
E2169 Practice for Selecting Antimicrobial Pesticides for Use in Water-Miscible Metalworking Fluids
E2523 Terminology for Metalworking Fluids and Operations
E2563 Practice for Enumeration of Non-Tuberculosis *Mycobacteria* in Aqueous Metalworking Fluids by Plate Count Method
E2564 Practice for Enumeration of *Mycobacteria* in Metalworking Fluids by Direct Microscopic Counting (DMC) Method
E2657 Test Method for Determination of Endotoxin Concentrations in Water-Miscible Metalworking Fluids
E2694 Test Method for Measurement of Adenosine Triphosphate in Water-Miscible Metalworking Fluids
E2756 Terminology Relating to Antimicrobial and Antiviral Agents
E2889 Practice for Control of Respiratory Hazards in the Metal Removal Fluid Environment

2.2 *Other Standards:*

AOAC 960.9 Germicidal and Detergent Sanitizing Action Disinfectants[4]

[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.

[4] *AOAC International Methods of Analysis*, AOAC International, Gaithersburg, MD.

9215A.6a Heterotrophic Plate Count Media, Plate Count Agar[5]
9216 Direct Total Microbial Count[5]
Microbiological Test <71>[6]

2.3 *Government Standard:*
40 CFR 156 Labeling Requirements for Pesticides and Devices

3. Terminology

3.1 For definitions of terms used in this guide refer to Terminologies D1129, E2523, and E2756.

3.2 *Definitions:*

3.2.1 *active ingredient, n*—the chemical component or components of an antimicrobial pesticide that provides its microbicidal performance.

3.2.2 *antimicrobial pesticide, n*—chemical additive registered under 40 CFR 152, for use to inhibit growth, proliferation or both of microorganisms.

3.2.3 *as supplied, adj*—antimicrobial pesticide finished product including the active ingredient(s), solvent and any additional inactive ingredients.

3.2.4 *biocide, n*—any chemical intended for use to kill organisms.

3.2.5 *bioresistant, adj*—ability to withstand biological attack.

3.2.5.1 *Discussion*—Bioresistant, or recalcitrant, chemicals are not readily metabolized by microorganisms.

3.2.6 *biostatic, adj*—able to prevent existing microbial contaminants from growing or proliferating, but unable to kill them.

3.2.6.1 *Discussion*—Biostatic additives may be registered antimicrobial pesticides or unregistered chemicals with other performance properties. The difference between biocidal and biostatic performance may be attributed to dose, chemistry or both.

3.2.7 *dose, n*—concentration of antimicrobial pesticide added to treated solution.

3.2.7.1 *Discussion*—Dose is generally expressed as either ppm active ingredient (a.i.) or ppm as supplied (a.s.).

3.2.8 *inactive ingredient, n*—component of antimicrobial pesticide that is not directly responsible for the pesticide's antimicrobial performance.

3.2.8.1 *Discussion*—Inactive ingredients may include, but are not limited to solvents and chemicals that improve the pesticide's non-biocidal performance properties, such as miscibility and reactivity with non-target molecules in the treated material.

3.2.9 *minimum inhibitory concentration (MIC), n*—lowest treatment-dose that will prevent test population from growing, proliferating or otherwise contributing to biodeterioration.

3.3 *Abbreviations:*
3.3.1 *a.i.*—active ingredient
3.3.2 *a.s.*—as supplied
3.3.3 *ATCC*—american type culture collection
3.3.4 *CFU*—colony forming unit

4. Summary of Practice

4.1 End-use dilutions of one or more water-miscible metalworking fluids are dispensed into microcosms. The fluids may be fresh or aged, dosed with one or more antimicrobial pesticides or undosed. Microcosms are challenged with either uncharacterized or characterized biological inocula. After inoculation, microcosms are aerated either continuously or periodically to simulate recirculation conditions in coolant systems. Chips may also be added to microcosms to simulate chip accumulation in coolant systems.

4.2 After inoculation, fluid samples are drawn from each microcosm periodically and tested for the parameters of interest, including but not limited to microbial viable counts. Depending on the test objectives, the test duration may range from 24 h to three months.

4.2.1 Shorter test periods are used to evaluate microbicide speed of kill and metalworking formulation initial bioresistance.

4.2.2 Longer test periods are used to evaluate metalworking fluid formulation resistance to repeated challenges. For tests lasting longer than one-week, 10 to 80 % of the fluid is exchanged weekly with fresh fluid before the additional challenge. The percentage of fluid exchange should reflect anticipated fluid turnover rates in fluid's end-use application.

4.3 Bioresistance is determined as the test fluid's relative ability to prevent the proliferation of challenge microbes, retain its original chemical or physical properties of some combination of the above. The bioresistance of test formulations is defined relative to that of a benchmark or control formulation.

5. Significance and Use

5.1 This practice provides laboratory procedures for rating the relative bioresistance of metalworking fluid formulations, for determining the need for microbicide addition prior to or during fluid use in metalworking systems and for evaluating microbicide performance. General considerations for microbicide selection are provided in Practice E2169.

5.2 The factors affecting challenge population numbers, taxonomic diversity, physiological state, inoculation frequency and biodeterioration effects in recirculating metalworking fluid systems are varied and only partially understood. Consequently, the results of tests completed in accordance with this practice should be used only to compare the relative performance of products or microbicide treatments included in a test series. Results should not be construed as predicting actual field performance.

6. Apparatus

6.1 *Air Supply,* air provided at no more than 110 kPa.

NOTE 1—Any air source that is free of organic vapors, organic matter or other objectionable material may be used. Sterile air need not be used

[5] Available from American Public Health Association (APHA) *Standard Methods for the Examination of Water and Wastewater* 800 I Street, NW Washington, DC 20001.

[6] Available from U.S. Pharmacopeia (USP), 12601 Twinbrook Pkwy., Rockville, MD 20852-1790, http://www.usp.org.

for the uncharacterized inoculum, but shall be used for the characterized inoculum. If necessary, air may be sterilized either by inserting, in series, two commercially available in-line sterile filters designed for this purpose. Alternatively an in-line filter may be prepared as follows: Pack two 150 mm long drying tubes (bulb-type) loosely with borosilicate glass wool in series with neoprene stoppers, glass tubing and neoprene tubing. Wrap loosely in aluminum foil and steam sterilize at 103 to 138 kPa for 30 min or dry heat sterilize at 160°C for 2 h. Cool to room temperature while wrapped. Insert into air line with bulbs on upstream side. Whether using a commercial or fabricated filter, average lifetime in continuous use is two weeks. Discard sooner if upstream filter becomes wet or contaminated with oil.

6.2 *Aquarium Tubing,* 6.35 mm diameter, silicone or vinyl.

6.3 *Autoclave,* with both steam cycle (80 to 100°C) and sterilization cycle (15 min at ≥ 121°C) capability.

6.4 *Adjustable Volume Pipetters,* with sterile disposable tips. Pipetters will be used to deliver 1.0 µL to 2 mL volumes.

6.5 *Glassware:*

NOTE 2—Sterile laboratory ware or sterile disposable laboratory ware should be used according to standard microbiological practice.

6.5.1 *Glass Tubing,* 6.35 mm i.d., cut into 15 cm lengths with ends fire-polished.

6.5.2 *French Square Bottles,* 960 mL, with metal cap.

NOTE 3—Alternatively, 1 L capacity canning jars may be used.

6.5.3 *Pipetes, Bacteriological,* 10 and 2.2 mL.

6.6 *Incubator,* capable of maintaining a temperature of 25 ± 2°C.

NOTE 4—Although an incubator is preferred, incubation may be performed at ambient room temperature.

6.7 *Manifold,* aquarium style, multi-valve.

NOTE 5—The number of manifolds and valves per manifold will depend on the number of microcosms in the test array. Air for each microcosm shall be supplied through a single air valve. Where used, air sterilization filters shall be placed between the air valve and microcosm aeration tube.

6.8 *Metal Punch,* 1 cm diameter.

7. Reagents and Materials

7.1 *Reagents:*

7.1.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where such specifications are available.[7]

7.1.2 *Water Purity*—Unless otherwise indicated, references to water shall be understood to mean reagent water as defined by Type III of Specification D1193.

7.1.3 *Antimicrobial Pesticide(s):*

NOTE 6—The measurement of antimicrobial pesticide (microbicide) efficacy in a medium as complex as metalworking fluid is relative, not absolute. Consequently, when this method is used to evaluate microbicide performance (8.3 or 8.4), it is prudent to always evaluate at least two antimicrobial treatments. Preferably one treatment should serve as a positive control; its efficacy in the test system having been established previously.

7.1.4 *Metalworking Fluid(s):*

NOTE 7—The number of metalworking fluids available is almost limitless. Recommendations for the use of any particular fluid cannot be made. If the primary intent is to evaluate the general efficacy of the microbicide(s) being tested, then it/they should be tested in various types of formulations. If the primary intent is to protect a particular formulation, then a microbicide-free version of that formulation should be used as the control and base-fluid to which the treatments are added.

7.1.4.1 *End-use Dilution Metalworking Fluid*—Dilute metalworking fluid concentrate in synthetic hard water (AOAC 960.9) to achieve the concentration at which it is used typically in recirculating metalworking fluid systems.

NOTE 8—Depending on the metalworking process, metal alloy being worked and formulation chemistry, metalworking fluid end-use dilution may range from 2 % (v/v) to > 15 % (v/v). If the formulation(s) being tested is (are) likely to be used at a variety of end-use strengths, they should be tested minimally at the high and low ends of the anticipated end-use concentration range. If the test objective is to evaluate microbicide performance in multiple metalworking fluid formulations, a 5 % (v/v) end-use dilution is appropriate.

7.2 *Materials:*

7.2.1 *Inoculum*—The microbial inoculum may vary according to the user's requirements. It may be either characterized or uncharacterized. The challenge population should be acclimated to the metalworking fluid before being used in this method. Acclimatization shall be achieved by growing the challenge in the end-use dilution, negative-control metalworking fluid formulation.

7.2.1.1 Prepare an uncharacterized inoculum by adding 50 mL of spoiled metalworking fluid to 850 mL of freshly prepared end-use dilution, negative-control metalworking fluid. Aerate at 25 ± 2°C or at ambient room temperature for 24 h or until the microbial viable count reaches 10^9 CFU · mL^{-1}. Replace 800 mL of this fluid with freshly prepared portion of the negative-control fluid. Repeat the aeration and metalworking fluid replacement procedure for a minimum of three cycles before using the preparation as an inoculum.

7.2.1.2 Prepare a characterized inoculum by using standard microbiological techniques to isolate, maintain and identify specific microbes from spoiled metalworking fluid. Alternatively, cultures of specific interest may be obtained from a commercial type culture collection. Examples of commercial cultures that may be used are: *Aeromonas hydrophila* (ATCC 13444), *Candida albicans* (ATCC 752), *Desulfovibrio desulfuricans* (ATCC 7757), *Escherichia coli* (ATCC 8739), *Flavobacterium ferrugineum* (ATCC 13524), *Fusarium oxysporum* (ATCC 7601), *Klebsiella pneumonia* (ATCC 13883), *Mycobacterium immunogenum* (ATCC 700505), *Proteus mirabilis* (ATCC 4675), *Pseudomonas aeruginosa* (ATCC 8689), *Pseudomonas oleovorans* (ATCC 8062) and *Saccharomyces cerevisiae* (ATCC 2338). Before using a characterized inoculum for metalworking fluid bioresistance testing, acclimate the inoculum following the procedure described for an uncharacterized inoculum (7.2.1.1). **Warning—**Microbes recovered from metalworking fluids may be pathogenic. Do not pipet by mouth.

[7] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For Suggestions on the testing of reagents not listed by the American Chemical Society, see *Annual Standards for Laboratory Chemicals,* BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary,* U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

NOTE 9—As more bioresistant metalworking fluid formulations are developed, microbicide-free control fluid may not support microbial growth at normal end-use dilutions. If microbial viable counts do not increase by at least three logs within 48 h (for example, 10^4 CFU · mL^{-1} at time 0; 10^7 CFU · mL^{-1} at time 48), then the coolant should be augmented with 1 part in 10 of soybean-casein digest (7.1.3).

7.2.2 *Metal Chips:*

NOTE 10—Although ferrous chips are suitable for most tests, alternative materials may be substituted if the fluid is to be used with specific materials such as non-ferrous metals or ceramics. Chips should be prewashed with toluene (or similar non-polar solvent), then methanol (or similar polar solvent) and dried before use.

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

7.2.3.1 Plate count agar (APHA Standard Methods 9215A.6a)

7.2.3.2 Soybean-casein digest medium (USP/NF Microbiological Test <71>).

7.2.3.3 Yeast extract-malt extract-glucose agar (APHA Standard Methods 9610B.2c)

7.2.3.4 Commercially available dip-slides prepared with bacterial recovery medium on one side and fungal recovery medium on the other side.

8. Procedures

8.1 Completed microcosm is shown in Fig. 1. To prepare jar lids, use 1 cm diameter metal punch to create two holes. Aeration tube will be placed into one of the holes. The second hole is used as a vent and as a sampling port. The microcosm is assembled after fluids (and chips, see 7.2.2) are placed in the jar.

NOTE 11—If the microcosms are not to be placed in a fume hood, the two holes in the lid may be fitted with silicone rubber stoppers. If stoppers are used, the aeration tube (6.5.1) should be inserted into the stopper so that once the microcosm is assembled, the submerged end of the tube will be 1.0 to 1.5 cm above the bottom of the jar. A 5 cm vent tube may be prepared from the glass tubing described in 6.5.1. The vent tube should be inserted into the second stopper so that once the microcosm is assembled, the tube extends 1 to 2 cm into the jar. An in-line filter (6.1) should be connected to the vent using a short (2 cm) section of aquarium tubing.

8.2 *Assessing Bioresistance*—This test is designed to compare the bioresistance of two or more metalworking fluid formulations.

8.2.1 *System Preparation*—Prepare one jar for each test formulation, plus one jar for the control formulation against which the test formulation(s) will be compared. If chips are to be used, add 10 g chips to the bottom of each jar, then add 85 mL of inoculum (7.2.1). Add 715 mL test fluid to maintain a ratio of 1 part inoculum to 9 parts fresh test fluid. Place an intact lid onto the jar and invert the container at least 10 times to allow for complete mixing.

8.2.2 *Aeration*—Replace the intact lid with a two-hole lid (8.1). Insert the aeration tube into one 1 cm diameter hole, and insert the vent tube into the other hole. Start aeration and adjust air flow so that each aeration tube releases 1 to 3 bubbles/s. Continue aeration for five days. Add water (7.1.2) to return total volume to 800 mL. Suspend aeration for 2.5 days (the

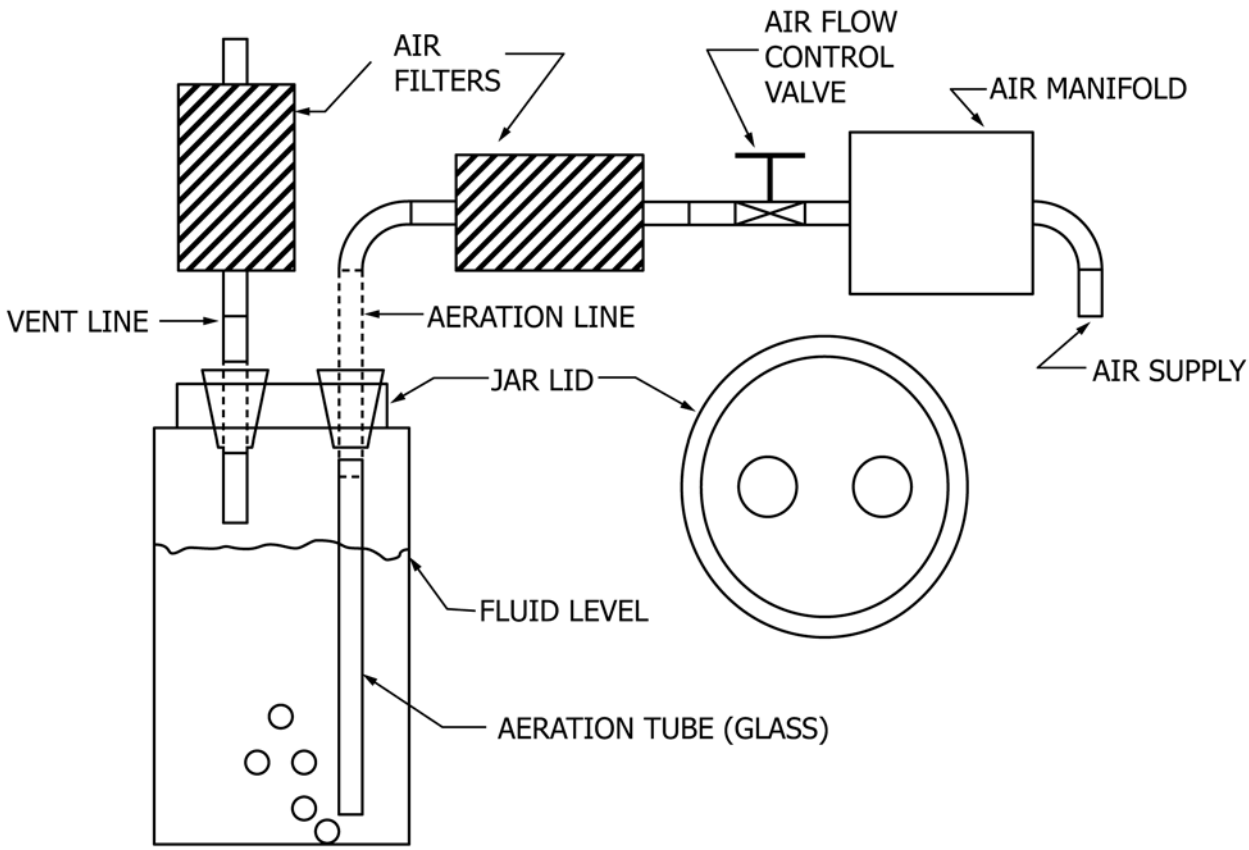

FIG. 1 Microcosm Setup

equivalent of a weekend shutdown) and then resume aeration for 5 more days. Add water as before to make up for evaporation losses.

NOTE 12—Inadequate aeration is likely to permit microcosms to become anoxic. Anoxic condition will inhibit the proliferation of obligately aerobic bacteria and fungi in the inoculum, thereby creating a bias in the test results. Excessive aeration may cause foaming.

8.2.3 *Sample Regimen:*

8.2.3.1 Remove samples for testing five times during the evaluation run: (*1*) T_1 before starting aeration and within 30 min of having mixed fresh metalworking fluid with the inoculum; (*2*) T_2 after the first 2 days of aeration; (*3*) T_3 after five days of aeration; T_4 after the weekend non-aeration period; and (*4*) T_5 after the second 5 day aeration period.

8.3 *Microbicide Performance; Assessing Speed of Action*—This test is designed to determine how quickly a microbicide treatment will reduce microbial viable count recoveries. Microbicide is added to metalworking fluid that is pre-challenged.

8.3.1 *System Preparation*—Prepare one jar for each microbicide treatment to be evaluated, plus one jar as the untreated control. Prepare sufficient inoculum (7.2.1) so that 800 mL can be added to each jar (for example: if three microbicide treatments are to be tested, there will be a total of four jars - control plus one per treatment; at 800 mL per jar, 2,400 mL inoculum is needed). Add 800 mL of inoculum to each microcosm jar. Put the screw cap cover onto each jar and insert the aeration tube. Start aeration and adjust air flow so that each aeration tube releases 1 to 3 bubbles/s.

8.3.2 *Treatment*—Add a volume of microbicide sufficient to achieve the required treatment dose to each test microcosm. Use Eq 1 to determine the appropriate treatment volume.

$$V_m = V_f \times D \quad (1)$$

where:

D = is the treatment dose in µL microbicide / L metalworking fluid,
V_m = is the microbicide treatment volume in µL, and
V_f = is the total metalworking fluid volume in L (per 8.2.1, V_m = 0.8 L).

NOTE 13—In accordance with 40 CFR 152, microbicide manufacturers list recommended use levels on their product labels. When screening a variety of microbicides, the investigator may choose to work with the maximum allowable dose of each product tested. Subsequent or alternative evaluations may include the minimum and maximum recommended doses, or minimum, medium (half-way between minimum and maximum allowed) and maximum allowed doses. Suppliers typically provide recommendations for product use as supplied (a.s.). When comparing alternative microbicides, it may be useful to compare performance on an active ingredient (a.i.) basis.

8.3.3 *Aeration*—aerate (8.2.2) continuously for the duration of the test.

8.3.4 *Sampling Regimen:*

8.3.4.1 Remove samples for biological evaluation six times during the course of this test: (*1*) no more that 10 min before adding treatment (T_1), (*2*) T_2 after 4 h, (*3*) 8h T_3 , (*4*) 24h T_4, (*5*) 72h (T_5), and (*6*) 5 days (T_6).

8.4 *Microbicide Performance; Assessing Preservation Effectiveness*—This method is used to determine the effectiveness of a single microbicide or combination of microbicides to protect a metalworking fluid against biodeterioration. It can be used to determine product and dose effectiveness (see Note 12).

8.4.1 *System Preparation*—Prepare one jar for each microbicide treatment to be evaluated, plus one jar as the untreated control. If chips are to be used, add 10 g chips to the bottom of each jar, then add 715 mL test fluid. Add 85 mL of inoculum (7.2.1) and cover the jar with an intact lid. Invert the jar at least 10 times to allow for complete mixing.

NOTE 14—If this test is being run to evaluate the effectiveness of tankside microbicide treatment, add the appropriate volume of microbicide (8.3.2) and mix the fluid (8.4.1) before adding the inoculum. Mix the fluid a second time after adding the inoculum (8.4.1).

8.4.2 *Aeration*—Replace the intact lid with a two-hole lid (8.1). Insert the aeration and vent tubes (8.2.2) and aerate for five days. After five days, suspend aeration for 2.5 days, and then resume aeration for an additional five days. Repeat this cycle for a minimum of six weeks or until microbicide failure occurs (9.2).

8.4.3 *Sample Regimen:*

8.4.3.1 After aeration is suspended (8.2.2), observe the physical condition of the fluid.

8.4.3.2 Add water (7.1.2) to replace lost volume. Mix the fluid with a glass rod, pipet or similar device, and then remove 640 mL of the fluid. Perform chemical, microbiological and physical tests on the 640 mL portion removed (9). Add 630 mL of freshly prepared end-use dilution metalworking fluid (7.1.4) and then add 10 mL inoculum (7.2.1). If test is intended to evaluate tankside treatment, re-treat the fluid with the same microbicide(s) and dose(s) used in accordance with Note 13.

NOTE 15—Fluid loss from recirculating metalworking fluid systems results from a combination of evaporation, drag-out and splash. Evaporation tends to increase metalworking fluid concentration. Drag-out tends to decrease metalworking fluid concentration. Splash (misting, etc.) tends to reduce the overall fluid volume. The net effect of these three processes and their relative impacts on fluid volume and concentration varies widely amongst metalworking operations. Removing 640 mL of fluid weekly simulates approximately 11 % daily fluid loss. If the actual loss rates are known, the replacement volumes used in this test may be adjusted accordingly to reflect field operating conditions more accurately.

8.4.3.3 Sample weekly for at least 6 weeks, or until microbicide fails (9.2).

8.5 *Bioresistance and Microbicide Performance Evaluation:*

NOTE 16—As discussed in Practice E2169, both bioresistance and microbicide performance are defined operationally in terms of specific objectives. Whether using a bioresistant formulation or an antimicrobial pesticide treatment, the objectives center around preventing microbes from either proliferating in the fluid, changing one or more properties of the fluid, or both.

NOTE 17—The methods listed in this section are meant to be merely representative of the types of tests that may be performed to evaluate the effectiveness of a treatment against the adverse effects of uncontrolled microbiological metabolic activity, growth and proliferation on the test fluids. Additional or alternative tests may be used to evaluate particular performance properties of interest to the investigator.

8.5.1 *Gross Observations*—Visual examination is a useful means of assessing MWF failure qualitatively or semi-quantitatively.

8.5.1.1 Visible flocs of biomass within the fluid or slime accumulation on test vessel walls indicated that the MWF is supporting microbial community proliferation.

8.5.1.2 Fluid discoloration in test vessels indicates biodeterioration.

8.5.1.3 Phase separation in treated test vessels may be compared with that in the control vessel(s).

NOTE 18—In the test systems described in this practice, factors that may contribute to MWF gross property changes in actual metalworking operations are absent. Consequently, changes in MWF gross properties may be attributed to biodeterioration.

8.5.2 *Chemical Tests:*

8.5.2.1 Alkalinity (Method D1067) and pH (Method E70) may be used to determine the inoculum's effect on the formulation's chemistry.

8.5.2.2 Dissolved oxygen may be measured to determine whether the inoculum is metabolically active in the test fluid. Use Method D888 to measure dissolved oxygen in the fluid immediately after sampling and after 1 h quiescence. Use freshly prepared, uninoculated end-used dilution metalworking fluid as a negative control. Use fluid from the inoculated untreated microcosm as a positive control.

8.5.2.3 Additive manufacturers may have specific tests for their products. Assays for specific metalworking fluid components, including microbicides, may be used to determine whether and to what extent these components are removed or altered due to the metabolic activity of the inoculum.

8.5.3 *Microbiological Tests:*

NOTE 19—In addition to the microbiological tests listed below, it may be desirable to evaluate microbicide performance to, or to evaluate the relative ability of metalworking fluid formulations to support the growth (or select for) of one or more specific target cultures. Since additional health and safety risks may be attendant to such testing, these tests should be performed only by qualified laboratories equipped with the appropriate containment facilities.

8.5.3.1 Culturable microbiological population densities may be determined by Method D5465.

NOTE 20—Alternatively, instructions provided by dip-slide manufacturers (7.2.3.3) will provide acceptable viable count data.

NOTE 21—When testing MWF bioresistance to or antimicrobial pesticide performance against *M. immunogenum* use Practice E2563 or Practice E2564, or both.

8.5.3.2 Direct counting (APHA 9216) provides data on the total number of microbial cells present, but normally does not permit differentiation between viable and non-viable cells.

NOTE 22—There are a number of modifications of the direct count method that theoretically differentiate between viable (metabolically active) and non-viable (metabolically inactive) cells. None of these methods has been standardized by a consensus organization such as ASTM. Investigators considering the use of non-standardized methods are referred to Guide E1326.

8.5.3.3 Adenosine Triphosphate (ATP) may be assayed (Test Method E2694) to determine total biomass.

NOTE 23—Alternative, non-conventional biomass assays may be used, consistent with the guidance provided in Guide E1326. Non-conventional microbiological test data may not covary with tradition test method data (8.5.3.1 and 8.5.3.2). Microbial constituent molecules may persist after microbial cells are killed. The concentration of a molecule, such as ATP, endotoxin or other biomarker may vary with microbial species, physiological state of the test microbes or both. Consequently, it is important to ensure that the measured parameter covaries with other parameters indicative of the fluid's biostability (for example: pH, emulsion stability, lubricity, etc.).

8.5.3.4 **Warning—**Endotoxins are known to cause respiratory disease in the metalworking environment (Practice E2889). Endotoxin concentration can be determined by Method E2657.

8.5.4 *Physical Tests:*

8.5.4.1 Dispersion stability (Method D3342) may be used to determine the inoculum's effect on emulsifiable oil and semi-synthetic formulation stability.

8.5.4.2 Corrosivity (Method D4627) testing may be used to determine the inoculum's impact on the metalworking fluid's anticorrosive properties.

8.5.4.3 Foaming tendency (Test Methods D3519 and D3601) may be used to determine whether the inoculum has affected the metalworking fluid's foaming characteristics.

9. Evaluation of Results

9.1 This practice is designed to give relative values rather than absolute values for fluid bioresistance or microbicide performance. Different fluids may have different stabilities to large numbers of microorganisms and, therefore, there is greater variation in microbial levels that cause physical and chemical changes in fluids. The net change and rate of change for each parameter monitored are both indicative of fluid biodeterioration. Parameter changes in test formulations should be compared to changes in both negative (uninoculated) and positive (inoculated; untreated or benchmark formulation) controls.

9.2 As discussed in Notes 15 and 16, the assessment of treatment effectiveness should be based on predetermined criteria for each parameter monitored. At the investigator's discretion, failure should be defined as the point at which any single monitored criterion exceeds control limits in two successive samples or when any predetermined combination of multiple criteria exceed control limits in two successive samples from a test microcosm.

10. Report

10.1 Report the following information:

10.1.1 The metalworking fluid formulation used, identifying the composition of the control and test fluids,

10.1.2 The antimicrobial pesticide(s) and dose(s) used,

10.1.3 The inoculum used for each test, including viable count or biomass data or both and taxonomic makeup or source.

10.1.4 Makeup water volumes used at the end of each aeration interval (8.2.2 and 8.4.3.2).

10.1.5 Data for each parameter monitored for each test and control microcosm at each time interval, T,

10.1.5.1 For Bioresistance (8.2), T_1 = at start up, T_2 = 5 days, T_3 = 7.5 days, and T_4 = 12.5 days.

10.1.5.2 For Microbicide Performance; Speed of Kill (8.3), T_1 = no more than 10 min before microbicide addition, T_2 = 4 h, T_3 = 8 h, T_4 = 24 h T_5 = 72 h, and T_6 = 5 days.

10.1.5.3 For Microbicide Performance; Preservation Effectiveness (8.4) T_1 = at start up, T_2 = 5 days, T_3 = 14 days (2 weeks), T_4 = 3 weeks, T_5 = 4 weeks, T_6 = 5 weeks, and T_7 = 6 weeks.

NOTE 24—Testing may be extended beyond 6 weeks if one or more test formulations have not failed by that time.

10.1.6 Failure criterion for each parameter monitored,

10.1.7 Computed differences between test and control data for each system and at each time interval,

10.1.8 Time interval at which each test formulation failed (9.2).

11. Precision and Bias

11.1 *Precision*—Since precision will depend on the fluids, challenge microbes and microbicide treatments used to perform individual investigations, no statement on precision is made.

11.2 *Bias*—Since there is no accepted reference material suitable for the bias in this practice, no statement on bias is made.

12. Keywords

12.1 antimicrobial; bacteria; bactericide; biocide; bioresistant; coolant; fungi; fungicide; metalworking fluid; microbicide; mold; pesticide; yeast