Designation: E 645 – 02

Standard Test Method for Efficacy of Microbicides Used in Cooling Systems[1]

This standard is issued under the fixed designation E 645; 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 outlines a procedure for evaluating the efficacy of microbicides (algicides, bactericides, and fungicides) that will be used for controlling microbial growth in cooling water systems. The microbicides will be evaluated using simulated or real cooling tower water against microbes from cooling water, microbiological deposits (biofilms) from operating cooling systems, or microorganisms known to contaminate cooling water systems, or a combination thereof. This test method should be performed by individuals familiar with microbiological techniques.

1.2 *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 3731 Practices for Measurement of Chlorophyll Content of Algae in Surface Waters[2]

D 4412 Test Methods for Sulfate-Reducing Bacteria in Water and Water-Formed Deposits[3]

E 1054 Practices for Evaluating Inactivators of Antimicrobial Agents Used in Disinfectants, Sanitizer, Antiseptic, or Preserved Products[2]

E 1326 Guide for Evaluating Nonconventional Microbiological Tests Used for Enumerating Bacteria[2]

E 1427 Guides for Selecting Test Methods to Determine the Effectiveness of Antimicrobial Agents and Other Chemicals for the Prevention, Inactivation, and Removal of Biofilm[2]

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *algicide, n*—a substance that kills algae; unicellular chlorophyll-containing plants.

3.1.2 *bactericides, n*—an agent that kills bacteria. This term is applied to chemical agents that kill all bacteria, but not necessarily bacterial spores.

3.1.3 *biofilm, n*—an accumulation of cells immobilized on a substratum and frequently embedded in an organic polymer matrix of microbial origin.

3.1.4 *cooling system, n*—an assemblage of equipment for the removal of heat from processes or equipment, or both. The most common medium used for removal or transfer of heat is water. The heated water then can be discharged into a receiving body (once through cooling system) or it can be cooled and reused (recirculating cooling system).

3.1.5 *cooling tower, n*—a structure used to dissipate heat in open recirculating cooling systems.

3.1.6 *cooling water, n*—medium used to transfer heat in cooling systems.

3.1.7 *fungicides, n*—an agent that kills fungi (molds and yeasts), both fungal vegetative cells and spores. This term is applied mostly to chemical agents.

3.1.8 *microbial biofouling, n*—the unwanted accumulation of cells and their products on surfaces. Many times this accumulation is accompanied by deposition of organic and inorganic material.

3.1.9 *microbicides, 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 Microbicides are evaluated against microbes under conditions simulating a cooling water system. Microbicides at concentrations that are expected to control the microbes are added to cooling water. At selected time periods, the amount of microbes in the water are determined and compared to values at the start of the experiment. Bacteria (aerobic and anaerobic), fungi or algae, or both, may be detected by a number of methods, such as plate counting, Most Probable Number (MPN), Adenosine-5′-Triphosphate (ATP). The investigator will determine the minimal microbicide concentration for efficacy based upon laboratory registration needs.

5. Significance and Use

5.1 This test method determines potentially effective microbicides for use in cooling water systems using cooling water

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

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[2] *Annual Book of ASTM Standards*, Vol 11.05.

[3] *Annual Book of ASTM Standards*, Vol 11.02.

and deposits/biofilm obtained from the field. The addition of deposits/biofilms addresses the need to include the major source of microorganisms in cooling water systems. Even with this addition, however, laboratory results may not be predictive of microbicide effectiveness in the field. This, in part, is due to conditions in the field that effect microbicide efficacy and are hard to mimic in the laboratory, that is, blow down rate, addition of makeup water, water hardness, hydrocarbon leaks, pH, sediment loading, dissolved solids, microbes in slime, and deposits (biofilms) on surfaces. Another factor is the difficulty in enumerating microbes in the water due to the lack of adequate recoverable medium. Guidelines that address formation of and testing for surface-attached microbes (biofilms) may be found in Guides E 1427, while a guideline for unconventional measurement of microbes is found in Guide E 1326.

6. Apparatus

6.1 *Balance*—An analytical balance sensitive to 0.1 mg should be employed to weigh the candidate microbicide to be used in the preparation of stock solutions.

6.2 *Containers*—Flasks, bottles, or test tubes suitable for shaking shall be sterilized prior to use.

6.3 *Colony Counters*—Manual, such as Quebec, Buck, or Wolffhuegel, or a proven colony image analyzer (electronic/scanner type) are suitable for counting plates after incubation. A hand tally or automatic recording device on the manual counter is desirable.

6.4 *Spiral Plater*.

6.5 *Constant Temperature Shaker*—A reliable constant-temperature shaker ±2°C (water bath or incubator shaker) shall be used to provide mixing and aeration and to maintain temperature during the contact period at a setting within the temperature range selected in 10.2.

6.6 *Petri Dishes*, sterile, 100 by 15-mm plastic or borosilicate glass.

6.7 *Pipettes*—Standard pipettes, sterile, with appropriate NaCl to a volumetric flask, fill with reagent water to the calibrations, or other suitable delivery systems, such micropipetters, can be employed.

6.8 *Sterilizers*—Pressurized steam sterilizer (for media, containers, and so forth), hot air oven (180 ± 2°C for 2 h) for containers, and filter apparatus for filter sterilization (disposable filter units, 250 mL, 0.22-µm pore size).

6.9 *Stirrer*—A stirrer is required to mix the cooling water sample while it is being dispensed into test containers. This can be a magnetic stirrer, a propeller-type stirrer, or any other suitable device.

6.10 *Volumetric Flasks*, 100 mL, are convenient for preparing microbicide stock solutions. Smaller volume flasks may be used where appropriate.

6.11 *Blender*—A blender, stomacher, sonic bath, or vortex mixer, may be necessary to homogenize the microbial deposit before mixing it with the cooling water.

6.12 *Microscope*, provides a magnification of 400 to 1000× and is complete with a suitable light source. Phase contrast or dark-field capability is desirable.

7. Reagents and Materials

7.1 *Purity of Reagents*—The principal reagent used is water, but other solvents may be necessary in preparing the microbicide stock solutions. Reagent grade organic solvents are normally used if water is not a suitable diluent for dissolving a microbicide. If a solvent is used, an additional control must be performed that has solvent without any microbicide added to the cooling water sample. This is conducted to demonstrate that the solvent has no appreciable effect on the test results.

7.2 *Purity of Water*—All reference to water as a diluent or reagent shall mean distilled water or water of equal purity, unless otherwise noted.

7.3 *Culture Media*:

7.3.1 A general bacterial agar medium, such as Glucose Extract Agar, Tryptic Soy Agar, R2A Agar, and so forth, is used for conducting bacterial counts on test samples. Other media, such as selective or differential types may be used. ATP measurement may also be used to monitor the bacteria.

7.3.2 A general fungal medium, such as inhibitory mold agar, Sabouraud dextrose agar, and so forth, is used to for conducting fungal counts on the samples. This medium must be able to inhibit the growth of bacteria.

7.3.3 Bristol's medium,[4] or a suitable equivalent, is the recommended medium for the growth of algae.

7.4 *Dilution Water Blanks*—Sterile, 99 or 9-mL phosphate buffered saline or magnesium chloride dilution blanks are convenient for diluting test samples for viable counts. Buffer strength and salinity can be adjusted to mimic experimental or field conditions.

7.4.1 *Phosphate Buffered Dilution Water Blanks*.

7.4.1.1 *Phosphate Buffer Solution, Stock*—Dissolve 34.0 g of potassium dihydrogen phosphate (KH_2PO_4) in 500 mL of water. Adjust pH to 7.2 ± 0.2 with NaOH solution (40 g/L) and bring to 1000 mL with water. Sterilize by filtration or autoclave.

7.4.1.2 *Phosphate Buffered Saline Dilution Water*—Add 1.25 mL of stock phosphate buffer solution and 8.75 g of NaCl to a volumetric flask, fill with reagent water to the 1000-mL mark, and mix. Final pH should be 7.2 ± 0.2. Dispense in amount that will provide 99 ± 2 mL or 9 ± 1 mL after sterilization into screw-cap dilution bottles or tubes. Sterilize immediately.

7.4.2 *Phosphate Buffered Magnesium Chloride Dilution Water*—Add 1.25 mL of stock phosphate buffer solution and 5.0 mL of magnesium chloride solution (81.1 g $MgCl_2 \cdot 6 H_2O$/L, reagent grade water) to 1000 mL of water. Adjust pH to 7.2 ± 0.2. Dispense in amount that will provide 99 ± 2 mL or 9 ± 1 mL after sterilization into screw-cap dilution bottles or tubes. Sterilize immediately.

7.5 *Cooling Water Sample*:

7.5.1 The cooling water sample shall be collected in a sterile container (1-gal or 2.2-L plastic bottles are convenient). The temperature and pH should be determined at the time of sample collection. The presence of additives in the cooling tower water may affect the efficacy of the microbicides, therefore, a history

[4] Starr, R. C., and Zeikus, J. A., "The Culture Collection of Algae at the University of Texas at Austin," *Journal of Psychology*, Vol 23(5): pp. 1–47, 1987.