Designation: E 645 – 97

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 *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.

3.1.5 *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).

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

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

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

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 E-35 on Pesticides and is the direct responsibility of Subcommittee E35.15 on Antibacterial and Antiviral Agents.

Current edition approved April 10, 1997. Published June 1997. Originally published as E 645 – 78. Last previous edition E 645 – 91.

[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 calibrations, or other suitable delivery systems, such as 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 reagents used are the water or other solvent used in preparing the microbicide stock solutions. Reagent grade organic solvents should be used if water is not a suitable diluent for a particular microbicide. If a solvent other than water is used, however, an additional control that has solvent without any of the microbicide added to the cooling water sample should be used to demonstrate that the chosen 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 or procedures, such as ATP measurement, may 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 $MgCl_2$ 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 Tower Water Sample*:

7.5.1 The cooling tower 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 of the samples should be obtained or analysis of the additives should be conducted. The samples

[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.

shall not be exposed to temperature extremes during transit. If a variation of 1.0 pH unit exists between the time of sampling and testing, the sample should be discarded. The test procedure should be initiated within 24 h after collection.

7.5.2 Deposits of microbial composition should be collected in sterile containers from any affected areas of the cooling tower, such as the distribution deck, slats, or sump area. The deposit samples should be transported with the water sample following the same precautions. Upon receipt at the laboratory, microscopic examination of the deposits should be conducted to confirm that it is microbiological in nature. If testing for algicide or fungicide activity, or both, the sample must contain algae or fungi, or both.

8. Preparation of the Test Samples

8.1 The cooling water sample may be used as received or inoculated with known microorganisms. If only the water is used as a substrate and known microorganisms[5] will be added as inoculum, then the water should be filter-sterilized prior to the addition of microorganisms. If a biofilm sample or microbiological deposit is available, it may be used as the inoculum in filter-sterilized cooling water or synthetic cooling water. A homogenate should be prepared with the biofilm deposit not more than 10 % of the total weight of the samples.

8.2 The cooling water sample should be placed on a stirrer and mixed continuously. Transfer 100 ± 2 mL (or 100 ± 2 g) to sterile flasks or bottles. Prepare at least duplicate flasks or bottles for each microbicide concentration to be tested. In addition, prepare duplicate controls to which no microbicide will be added. If a solvent other than water is used to make the microbicide stock solutions, also include solvent control bottles that contain as much of the solvent as is added to the microbicide test containers (see 8.1).

8.3 After the test aliquots have been transferred to flasks, determine the viable count of microorganisms in the control flask in accordance with standard microbiological method. Suggested dilutions for this are 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. Bacterial numbers should be at least 10^5 bacteria/mL. Algae and fungal numbers are determined by pour plate, spread plate, or MPN. A minimum of 100 algae CFU/20 mL of cooling water or 20 fungi CFU/20 mL of cooling water is necessary to conduct the efficacy against algae or fungi. Other nonconventional microbial detection methods may be used (see Guide E 1326).

9. Preparation of Microbicide Stock Solutions

9.1 The appropriate test concentrations for a particular microbicide must be determined by the investigator. Usually, active-ingredient concentrations are in the range from 1 to 50 mg/L. For this range, concentrations of 1.0, 2.0, 5.0, 10.0, 25, and 50 mg/L would be tested. It is convenient, therefore, to make two microbicide stock solutions. The first should be 5.0 mg/mL and the second should be a 1 to 10 dilution of the first stock solution. The first stock solution is used to make the 10.0, 25.0, and 50.0-mg/L test concentrations, by addition of 0.2, 0.5, and 1.0 mL, respectively, per 100 mL of sample. The second stock is used to make the more dilute test concentrations. Using this method, the volume of microbicide stock solution added will not exceed 1 % of the total volume of the cooling water aliquots. This relative volume is an important consideration, particularly when solvents other than water are used to make the microbicide stock solutions. If an acceptable reduction in microbial numbers will not be achieved with these concentrations, the investigator must make the appropriate adjustments in the microbicide stock solutions and in the selected test concentrations in order to achieve this end. Microbicide test stock solutions should be prepared no more than 3 h before the test.

9.1.1 The investigator will determine the minimal concentration for efficacy based upon customer/laboratory registration needs.

9.2 The initial microbicide stock solution is prepared by weighing the microbicide on an analytical balance, transferring it to a volumetric flask, and bringing it to correct volume. Aluminum weighing dishes are not recommended because of the reactive nature of aluminum.

10. Addition of Microbicide to Test Samples

10.1 It is necessary to stagger the starting times by adding the microbicide stock solution to the test aliquots at timed intervals. Intervals of 1.5 to 2 min are convenient, but the time selected will depend on the speed of the investigator. The test flasks should be plated in the same order in which the microbicide was added.

10.2 Since different microbicides vary in their mode of action, the exposure time should be consistent with the microbicide's effect on microorganisms. The exposure time(s) is left to the discretion of the investigator, but a 3-h (±5 min) contact time must be included in each test. A 24-h time sampling period is also suggested. Alternative time intervals may be necessary to establish speed of kill, persistence of effect, or effect of system cycle times, or combination thereof.

10.3 An appropriate test temperature for the samples must be chosen by the investigator. A suggested temperature is within ±5°C of the temperature of the water in the sump at the time the water was collected. Before the microbicide is added to begin the 3-h exposure time, or any other time interval, the test samples should be equilibrated at the temperature chosen.

10.4 The sample is mixed in a shaking incubator at 100 rpm/min.

11. Plating the Samples

11.1 Enumerate the bacteria in the cooling water homogenate using standard microbiological methods, such as pour-plate, spread-plate, dry film, or MPN.[6,7] If nonconventional methods for microbial determinations, that is, ATP detection, are to be used refer to Guide E 1326. Do not use these methods

[5] Pesticide Assessment Guidelines, Subdivision G, Product Performance, U.S. Environmental Protection Agency, November 1982, Section 92.4, or most current edition.

[6] Standard Methods for Examination of Water and Wastewater, American Public Health Association, 19th ed., 1995.

[7] Methods for General and Molecular Bacteriology, American Society for Microbiology, Washington, DC, 1994.

interchangeably, since variation in results may occur. Inactivation of microbicides must be achieved by appropriate neutralizers or dilutions if cidal claims are to be made (see Practices E 1054). The dilutions chosen for bacterial enumeration are dependent upon the concentration of organisms in the test samples and the efficacy of the microbicide being tested. Dilutions between 10^{-1} to 10^{-5} will usually be appropriate. Incubate the plates or tubes at the selected temperature for 48 h, or at the recommended time period for a given medium, and determine the viable count of bacteria.

11.2 Enumerate fungi by spread-plate, dry film, or filtering and placing filter on an appropriate medium. Do not use methods interchangeably since variation in results may occur. Inactivation of microbicides must be achieved by appropriate neutralizer or dilutions if cidal claims are to be made. Dilutions chosen for fungal enumeration depend upon the method of enumeration, spread plate or filtering. Incubate the plate or tubes at the selected temperature, and record the counts after 48 h and up to a period of 7 days.

11.3 Algae may be estimated by using the MPN, filtering and placing the filter on an appropriate medium, spread plate procedure, or chlorophyll content. Media should inhibit bacterial and fungal growth. Inactivation of microbicides must be achieved by appropriate neutralizer or dilution if cidal claims are to be made. Incubate the cultures at the selected temperature under constant illumination using standard fluorescent lights (at a distance of 6 to 12 in.). Record the viable counts after 5 days and up to a period of 14 days. Viable counts of algae also may be made by filtering a portion of the test sample or dilution, transferring the membrane filter (0.22 µm) to the surface of the agar medium, and counting the colonies that develop. Algal biomass also may be estimated from the chlorophyll content of the samples.[6]

12. Interpretation of Results

12.1 The results of the test should be discarded if any of the following occurs:

12.1.1 The initial counts in the cooling water homogenate control samples (without microbicide) are less than 100 000 bacteria/mL.

12.1.2 The viable count of the control sample decreases by more than 1 log during the specified test period.

12.1.3 Initial counts of algae and fungi are less than 100 algae CFU/20 mL of cooling water or 20 fungi CFU/20 mL of cooling water, as determined by pour plate, spread plate, or MPN. If chlorophyll content is used, the investigator must establish baseline values.

12.1.4 The viable counts of fungi or algae must be at least equivalent to control samples or higher.

12.1.5 The viable count of the solvent control sample, if microbicide diluent is other than water, decreases by more than 1 log during the specified time period.

12.2 The untreated control should show a stable population or a slight increase in growth during the test period. The reduction in the number of microorganisms at each biocide concentration (*B*) tested relative to the initial count of the control sample (*A*) prior to exposure (see 8.2) may be expressed in terms of percent kill as follows:

$$\text{percent kill} = \frac{(A - B)}{(A)} \times 100$$

where:

A = initial count of microorganisms of the control sample, and

B = number of microorganisms detected after biocide concentration.

The efficacy of the test microbicide also may be determined on a logarithmic basis, whichever the investigator prefers.

12.3 The investigator will determine the minimal microbicide threshold concentration for efficacy based upon customer/laboratory registration needs.

12.4 The presence of fungi and algae in cooling waters is unpredictable and dependent upon many variables. It is desirable, however, to determine the effectiveness of microbicides on these organisms in cooling systems. This may be achieved with this procedure on a growth-no growth response basis. By this approach, the effective concentration is that level where no growth is observed. If no organisms are detected within the ranges selected for effective bacterial control, it can be assumed that fungi and algae will be controlled within the same range. If growth is present at concentrations that provide effective control of bacteria, however, additional studies should be made to establish an effective range of microbicide concentrations.

12.5 Water from several sites of cooling towers or from several cooling towers, or both, should be tested by the investigator to gain a better understanding of the efficacy of a given microbicide.

13. Precision and Bias

13.1 A precision and bias statement cannot be made for this test method at this time.

14. Keywords

14.1 algae; bacteria; biofilm; cooling tower; cooling water; efficacy; fungi; microbicide deposits/slime