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Standard Practice for Evaluation of Microbicides Used in Cooling Water Systems[1]

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1. Scope

1.1 This practice 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 (*1*) microbes from cooling water, (*2*) microbes in microbiological deposits (biofilms) from operating cooling systems, or (*3*) microorganisms known to contaminate cooling water systems, or a combination thereof. This practice should be performed by individuals familiar with microbiological techniques.

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

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D3731 Practices for Measurement of Chlorophyll Content of Algae in Surface Waters (Withdrawn 0)[3]

D4012 Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water

D4412 Test Methods for Sulfate-Reducing Bacteria in Water and Water-Formed Deposits

E1054 Test Methods for Evaluation of Inactivators of Antimicrobial Agents

E1326 Guide for Evaluating Nonconventional Microbiological Tests Used for Enumerating Bacteria

E1427 Guide for Selecting Test Methods to Determine the Effectiveness of Antimicrobial Agents and Other Chemicals for the Prevention, Inactivation and Removal of Biofilm (Withdrawn 2009)[3]

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

3. Terminology

3.1 For definitions of terms used in this practice, see Terminology E2756.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *algicide, n*—a chemical agent that kills algae; unicellular or filamentous chlorophyll-containing plants.

3.2.2 *bactericide, n*—a physical or chemical agent that kills bacteria, but not necessarily bacterial spores.

3.2.3 *biofilm, n*—a dynamic, self-organized accumulation of microorganisms and environmental by-products immobilized on a substrate and embedded in an organic polymer matrix.

3.2.4 *cooling system, n*—equipment and coolant used for the removal of heat from processes, equipment, or both.

3.2.4.1 *Discussion*—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).

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

3.2.6 *cooling water, n*—any water-based solution that absorbs and transfers heat in a heat exchange system.

3.2.7 *fungicides, n*—a physical or chemical agent that kills fungi; that is, vegetative mycelia and/or budding yeasts including spores and/or conidia.

3.2.8 *microbial biofouling, n*—the unwanted accumulation of bacterial, fungal, or algal cells, or any combination thereof and their products on surfaces.

3.2.8.1 *Discussion*—Often this accumulation is accompanied by deposition of organic and inorganic material.

3.2.9 *microbicides, n*—a physical or chemical agent that kills microorganisms.

[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.

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4. Summary of Practice

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 number of microbes or measurable component of the microbes are determined and compared to values at the start of the experiment. Bacteria (aerobic and anaerobic), fungi, and algae may be detected by a number of methods, such as plate counting, Most Probable Number (MPN), chlorophyll content, adenosine-5′-triphosphate (ATP). The investigator will determine the range of microbicide concentration for acceptable efficacy based upon laboratory testing that may be used to satisfy registration or customer needs.

5. Significance and Use

5.1 This practice determines potentially effective microbicides for use in cooling water systems using cooling water 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, laboratory results may not be totally predictive of microbicidal effectiveness in the field. This is because conditions in the field affecting microbicide effectiveness are difficult to mimic in the laboratory. These conditions that affect microbicide efficacy include blow-down rate, addition of makeup water, water hardness, hydrocarbon leaks, pH, sediment loading, dissolved solids, microbes in slime (biofilms), and deposits (salts, iron minerals, organics, and so forth) on surfaces. An additional factor is the difficulty in enumerating all microbes in the water due to the lack of adequate recovery media. Guidelines that address formation of and testing for surface-attached microbes (biofilms) may be found in Guide E1427, while a guideline for unconventional measurement of microbes is found in Guide E1326.

6. Apparatus

6.1 *Balance*—a calibrated analytical balance sensitive to 0.1 mg to weigh the candidate microbicide for preparation of stock solutions.

6.2 *Containers*—flasks, bottles, or test tubes suitable for shaking shall be sterile for 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.

6.4 *Spiral Plater (alternative).*

6.5 *Constant Temperature Shaker*—a reliable constant-temperature shaker ±2°C (water bath or incubator shaker) 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 micropipetters.

6.8 *Sterilizers*—pressurized steam sterilizer (for media, containers, and so forth), hot air oven for containers, and filter apparatus for filter sterilization (disposable filter units, 250 mL, 0.22-µm pore size).

6.9 *Stirrer*—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 to homogenize the microbial deposit before mixing it with the cooling water.

6.12 *Microscope,* providing a magnification range of 400 to 1000× with a suitable light source. Phase contrast or dark-field capability may be necessary.

6.13 *Filter apparatus,* with 0.2 µm filter.

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 used 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, or dry film is used for conducting bacterial counts on test samples. Other media, such as selective or differential types (that is, for the quantification of sulfate-reducing bacteria, Test Methods D4412) may be used for detecting desired bacteria. MPN or ATP measurement may also be used to quantify the bacteria (Guide E1326). Once a specific agar medium or other method of measurement is chosen, it must be used throughout this procedure.

7.3.2 A general fungal medium, such as an inhibitory mold agar or Sabouraud dextrose agar, is used 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 phosphate buffered 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.

[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, No. 5, 1987, pp. 1–47.

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 amounts 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 will 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 effectiveness of the microbicides, therefore, a history of the samples should be obtained or analysis of the water for additives should be conducted. Stop biocide addition at least 4 h before collection of samples, or an appropriate biocide inactivator must be added to the sample. Do not expose samples 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. Samples received from the field must be refrigerated (4 ± 2ºC).

7.5.2 Collect deposits of microbial composition in sterile containers from any affected areas of the cooling tower, such as the distribution deck, slats, or sump area. Transport the deposit samples with the water sample following the same precautions. Upon receipt at the laboratory, conduct microscopic examination of the deposits to confirm that they are microbiological in nature. If testing for algicidal or fungicidal 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 the water is used only as a substrate and known microorganisms[5] will be added as inoculum, the water should be filter-sterilized (using a 0.2 μm filter system) prior to the addition of microorganisms. If a biofilm sample or microbiological deposit is available, it may be used as the inoculum in either filtered or non-filtered sterilized cooling water. The biolfilm or slime must be homogenized/disaggregated so that no clumps are present. This can be accomplished by vortexing, sonicating, or any other method that disperses the clumps. No more than 10 % of the total weight (w/v) of the samples should be biofilm or deposit. A synthetic cooling water may also be used as the sample water.

8.2 Place the cooling water sample on a stirrer and mix 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). The 100-mL water volume is a standard volume used in all previously published evaluation of cooling water microbicides. However, other volumes of cooling water may be used in this test.

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 methods. 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 10^3 algal CFU/mL and/or fungal CFU/mL is necessary to conduct the effectiveness test against algae or fungi in cooling water. Obtaining these numbers of algae and fungi may require addition of algae, fungi or biofilm. Other microbial detection methods may be used (see Guide E1326). Initial or baseline values must be established for these methods.

9. Preparation of Microbicide Stock Solutions

9.1 The appropriate test concentrations for a particular microbicide must be determined by the investigator. Usually, microbicide concentrations depend upon the biocide, its application, and its registration range. For oxidizing biocides, it is important to determine the oxidant demand of the water. The concentration of the oxidizing biocide for testing can depend upon the oxidant demand of the water. In general, non-oxidizing microbicides are used at higher concentrations than oxidizing biocides. The volume of microbicide stock solution added should 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 is not achieved with these concentrations, the investigator must choose another microbicide or make the appropriate adjustments in the microbicide stock solutions and in the selected test concentrations. Microbicide stock solutions should be prepared no more than 3 h before the test.

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. Alternatively, biocide amounts may be added volumetrically for water-based

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

products, which are not viscous, based upon the specific gravity of the samples tested.

9.3 The investigator will determine the minimal acceptable concentration for effectiveness based upon customer requirements or specific registration needs.

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 2 ± 0.5 min. The test flasks should be plated in the same order and staggered time frame 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 effect of the microbicide on microorganisms. The exposure time(s) is left to the discretion of the investigator, but a 3 ± 1 h and a 24 ± 0.25 h contact time must be included in each test. Additional time intervals may be necessary to establish speed of kill, persistence of effect, effect of system cycle times, or combination thereof.

10.3 An appropriate sample test temperature 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 or 150 r/min. All tests must be at the same shaking rate.

11. Plating the Samples

11.1 Enumerate the bacteria in the cooling water homogenate using standard microbiological methods, such as pour-plate, spread-plate, or MPN techniques.[6,7] If non-conventional methods for microbial determinations are used, for example, ATP detection (Test Method D4012), refer to Guide E1326. Do not use these methods interchangeably, since variation in results may occur. Inactivation of microbicides must be achieved with appropriate neutralizers or dilutions if cidal claims are made (see Test Methods E1054). The dilutions chosen for bacterial enumeration are dependent on the numbers 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-plating, or membrane filtration with placement of filter on appropriate medium. Do not use methods interchangeably since variation in results may occur. Inactivation of microbicides must be achieved with appropriate neutralizer or dilutions if cidal claims are made (see Test Methods E1054). Dilutions chosen for fungal enumeration depend upon the method of enumeration. 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, membrane filtration and placement of filter on appropriate medium, spread-plate procedure, or chlorophyll content (Practices D3731). Media should inhibit bacterial and fungal growth. Inactivation of microbicides must be achieved with an appropriate neutralizer or dilution if cidal claims are made (Test Methods E1054. Incubate the cultures at the selected temperature under the appropriate illumination cycle (such as 18 h light and 6 h of darkness) using standard fluorescent lights (at a distance of 15 to 31 cm). Record the viable counts after 5 days and up to 14 days. Viable counts of algae may be made by filtering a portion of the test sample or dilution, and transferring the membrane filter (0.22 µm) to the surface of an agar medium. Algal biomass 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 10^5 CFU/mL for aerobic bacteria, as determined by pour-plate, spread-plate, or MPN. If ATP measurement is used, the investigator must establish baseline values.

12.1.2 The viable aerobic bacterial count of the control sample decreases by more than 0.5 log during the specified test period.

12.1.3 Initial counts of algae and/or fungi are less than 10^3 CFU/mL in cooling water, as determined by spread plate, membrane filtration, or MPN. If chlorophyll content is used, the investigator must establish baseline values. If the investigator has no interest in testing for fungi or algae, then the need for minimum numbers in the test water can be ignored.

12.1.4 The viable counts of fungi or algae in the control samples must be at least equivalent to the numbers at time 0 h.

12.1.5 The viable aerobic baterial count of the solvent control sample, if microbicide diluent is other than water, decreases by more than 0.5 log during the specified time period.

12.2 All duplicate counts are averaged and used for calculations. If a duplicate count differs by more than 1 log, that part of the experiment should be rerun.

12.3 The untreated control should show a stable population with no more than a 1 to 1.5 log increase or 0.5 log decrease in growth for a 24-h test period.

12.4 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 8.3) may be expressed in terms of log reduction or percent kill as follows:

$$\text{log reduction} = \log(A) - \log(B)$$

[6] Microbiological Examination Part 9000, *Standard Methods for Examination of Water and Wastewater*, American Public Health Association, 20th ed., 1998.

[7] Koch, A. L., Growth Measurements, *Methods for General and Molecular Bacteriology*, American Society for Microbiology, Washington DC, 1994, pp. 248–277.

where:

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

B = number of microorganisms detected at a given biocide concentration after a specific contact time.

and where:

1-log reduction = 90 % kill
2-log reduction = 99 % kill
3-log reduction = 99.9 % kill
4-log reduction = 99.99 % kill

12.5 The investigator will determine the minimal microbicide threshold concentration for effectiveness based upon customer requirement or specific registration needs. In most cases, a 90 % kill or 1-log reduction would be the minimum level of performance considered to show efficacy of a microbicide.

12.6 The presence of fungi and algae in cooling waters is unpredictable and dependent on many variables. It is desirable 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 for fungi or algae.

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

13. Keywords

13.1 algae; algicide; bacteria; bactericide; biofilms; cooling towers; cooling tower waters; deposits/slime; efficacy; fungi; fungicide; microbial biofouling; microbicide

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