

A Short Methodology Review: for the evaluation of biocides against biofilms in recirculating water systems

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Biofilm formation in recirculating water systems such as cooling towers lead to many undesired conditions in terms of public health concern, operational damage and economic loss. Accumulation of pathogenic bacteria (i.e. *Legionella pneumophila*), increased resistance to antimicrobial compounds, a significant energy loss by increasing heat transfer resistance, equipment damage through microbiologically influenced corrosion (MIC) and premature replacement of equipment constitute some of the important adverse effects of biofilm. To eliminate or reduce the biofouling problem various mechanical and chemical methods are used. The most common approach is chemical treatment primarily using biocides, since mechanical methods have disadvantages due to application difficulties, economical losses because of the system closure during applications and above all the risk of transmission of pathogens. Manufacturers recommend a variety of biocides and concentrations for decontamination recirculating water systems, however, these recommendations are not suited for every system and target microorganisms. To establish a successful biocide program in full scale systems, it is necessary to evaluate the antibacterial activity of correct biocide and/or combinations against both planktonic and sessile bacteria in terms of dosage and contact times in laboratory conditions with taking into consideration of important issues. Thus, the minimum amount of biocide which is necessary for biofouling control will be used and that will not affect either the environment or the performance of the plant itself adversely. In this review, important considerations for accurate evaluation of antibacterial activity will be highlighted.

Keywords Cooling towers; biocides; disinfection; biofilms; *Legionella pneumophila*

1. Introduction

Circulating cooling systems are key enablers of any industrial process. In such systems, microbial clusters or layers are predominant due to reasons such as the presence of nutrients and water, suitable temperatures, high surface-volume ratio. Biofilm layers are potential niches for pathogenic organisms, especially *Legionella pneumophila* [1] and can lead to problems such as decrease in heat transfer rate and significant energy losses due to increased heat transfer resistance, increase in frictional resistance and blockage in pipes, local corrosion of metallic substrata (microbiologically induced corrosion [MIC]) [2-4].

As a result of adverse effects of biofouling, serious economic losses and hygiene problems including those associated with legionellae may occur [5-8]. Serious expenses required to eliminate biofouling in systems, increase the capacity of the equipment and premature replacement of corroded equipment before the schedule. Costs for biofouling prevention is estimated to reach billion dollars for individual countries or industries per year [9]. In this context, to eliminate or reduce the biofouling problem various mechanical and chemical methods are used [6, 10-15].

Usage of mechanical technics for struggle with biofilm in industrial systems often not practical and disrupts the use of equipment [6, 7]. Mechanical techniques have disadvantages such as application difficulties, insufficient reach to the thin pipe system, economical losses because of the system closure during applications and above all the risk of transmission of pathogens. Therefore, antimicrobial biocides are preferred primarily to solve biofouling problem in this type systems. The usage of biocides ensures efficient operation of the system by preventing negative states of the biofilms, the reduction of other water treatment chemicals and control of the potential pathogenic organisms [5, 6, 10-15].

Manufacturers recommend a variety of biocides and concentrations for decontamination industrial systems. However, these recommendations are not suited for every system and target microorganisms [16, 17]. Since, improper use of biocides and/or doses lead to inadequate control of systems and serious economic damage as much as biofouling, to control biofouling effectively organisms to be combated, the correct biocide and/or combinations, dose frequency and contact time should be predetermined against both sessile and planktonic organisms [13]. As a result, the minimum amount of biocide that is necessary for biofouling control treatment will be used and that will not affect either the environment or the performance of the plant itself adversely [7, 8, 10, 13, 18].

Since different biocides would be useful for different systems depending on the natural structure of the biofilm and each biofilm is unique, best biocide selection should be determined in practical conditions [19]. On the other hand, it is difficult to conduct well controlled disinfection studies in real industrial cooling towers because of the large volume; decrease bactericide concentrations after addition due to system blow-down (water discharge) and interaction with organic and inorganic compounds; requirement of the employment of specialized personnel to maintain the necessary equipment. Furthermore, since full scale studies depend on local conditions and open nature of these systems, it is

difficult to reproduce and generalize *in situ* disinfection results [20]. To overcome these obstacles, perform systematic and long-term studies and evaluate recommended “environmentally friendly” new generation biocides, experiments may be conducted in a constructed model system.

Investigation of the effects of antimicrobial agents against biofilm and construction of model systems requires the integration of microbiology, engineering and chemistry. The differences in effectiveness of biocide also depend on mode of action of the biocide, chemical composition and physicochemical properties of biocides as well as on biocide application conditions i.e. microbial growth, reactor dynamics. Depending on the structure of biocide any of these parameters could become a major important factor [7].

In this respect, in the current study it is intended to revise important considerations for accurate evaluation of antibacterial activity of water treatment biocides with taking into account the above-mentioned parameters.

2. System

Since different factors may affect the adherence microorganisms on surfaces and biofilm growth in industrial equipments: (i) temperature, (ii) water flow velocity, (iii) the presence of nutrient and oxygen, (iv) surface material type, (v) microbial species [5, 6]; in order to apply the results obtained from pilot-scale testing to full-scale systems, model system should have design and run to the nearest operating conditions of full-scale system.

2.1. Temperature

The optimum incubation temperature is about 40°C for many bacteria found in cooling water, and this temperature level is the most common in industrial water coolers, especially during the summer months. Microbial activity is very sensitive to temperature, even minor changes in the value of the temperature makes considerable changes in the development of biofilms such as its thickness [5]. Thus, for the evaluation of biocides properly against biofilms in such systems, water temperature should be kept constant at 35°C or 40°C with an immersed electric heater to simulate actual water temperature in the cooling system for maximum microbial growth.

2.2. Water Flow Velocity

While in most bioreactors there is low velocities (of the order of 0.1 to 50 m h⁻¹) and medium/high substrate concentrations, in real recirculating water systems higher velocities (of the order of 0.1 to 3 m h⁻¹) and low organic substrate concentrations is seen [5, 6, 21]. There are some contradictions about the impact of water velocity on biofilm growth. It is generally considered that increasing the water velocities resulted in a greater flux of nutrients, and a greater transport and penetration of biocides due to the greater mass transfer, a greater shearing of biofilms [22]. Higher flow velocities facilitate transport of both planktonic bacteria and the nutrients to the biofilm and promote biofilm formation. Contrarily, increased velocity and consequently increased shear force is also cause specific detaching of biofilms and thus, promote biofilm suppression [5, 6, 23, 24]. To model biofilms formed under the real operating conditions, water flow velocity should be high, regular and at constant speed. Additionally it is necessary to ensure that water is distributed evenly throughout the test coupons, if required by adding apparatus such as nozzle [25].

2.3. The Presence of Nutrient and Oxygen

Recirculating water systems contain oxygen-saturated water due to the aeration provided by continuous circulation of system water and intimate contact of water/air during the cooling process. While oxygen was provided with the aeration, the continuous water flow also provides a continuous supply of nutrients [5].

2.4. Surface Material Type

One of the crucial points in biofilm formation is the surface material type [26]. Since each material does not allow biofilm formation equally, for modeling of real cooling tower system, materials which are used in the construction of cooling towers may be prepared as biofilm surfaces.

2.5. Microbial Species

Hence bacteria communicate each other with production of diffusible bacterial metabolites, bacteriocins, quorum sensing molecules; they are not randomly distributed in a biofilm but rather settled to best meet the needs of each. Acquisition of new genetic characteristics by intercellular communication and transfer of genetic material within populations of cells and between bacterial populations regulate the diversity and distribution of bacteria in biofilm. Owing to transmissible, genetic elements at accelerated rates in biofilms, bacteria especially pathogens, increase survival capabilities by acquisition of multiple antibiotic resistance, virulence factors and environmental survival capabilities [27-30]. Thus, in order to accurate assessment of biocide efficacy against bacterial populations, inoculation of real system water is very important also in this aspect in such simulations.

3. Water Characteristics of System

In recirculating water systems, cooling process is accomplished by a combination of sensible heat transfer and evaporation of water. As a result of continuous evaporation in the system the dissolved and suspended solids will be concentrated, for this reason, a portion of the recirculating water discharged either continuously or intermittently from the tower as blowdown. To balance the water evaporated and blow down from the system sufficient fresh make-up water must be added continuously, thus makeup keeps the water volume constant, blowdown controls the solids content [10, 15]

While common practice is to use lakes or rivers as make-up water sources, public potable water is also used usually. Microorganisms and organic/inorganic load will enter to the system via make-up water, thus for modeling microbial flora of a system, at the beginning of the experiment real cooling tower system water should be inoculated to the model system and the same make-up water supply of real system should be used.

Furthermore, in model system make-up and discharge should be made likewise with actual cooling tower. Since quality of water influence biofouling in system, system water should be analyzed periodically in view of physico-chemical parameters (i.e pH, conductivity, total dissolved solids, dissolved oxygen and different chemical salts).

4. System Cleaning

Before start experiments, system must be cleaned mechanically and followed by chemically with a disinfectant solution (e.g 100 ppm sodium hypochlorite solution) at least for 24 hours. At the end of disinfection process, disinfectant solution should be neutralized with an appropriate chemical solution circulation or rinse with plenty of water at least for 24 hours. After neutralization, system should be completely drained and left to dry by covering with a sterile lid until starting to experiments [14, 25, 26].

5. Coupon Holder

Test coupon holder should be designed as to be placed easily to the system and capable of holding numerous coupons to allow repetitions. Segmentation and cleaning/sterilization of holder should be easy; holder material should be durable, non-toxic and inert chemical characteristics for microorganisms. Coupons in holder should stay without contact each other, not fall out by the water flow from the holder, however in sampling for experiments coupons should be taken and removable easily. Water should contact with each side of coupons and equally be distributed.

In figure 1, plexiglass coupon holder which was designed by us and made in CNC (Computer Numerical Control) machine can be seen.

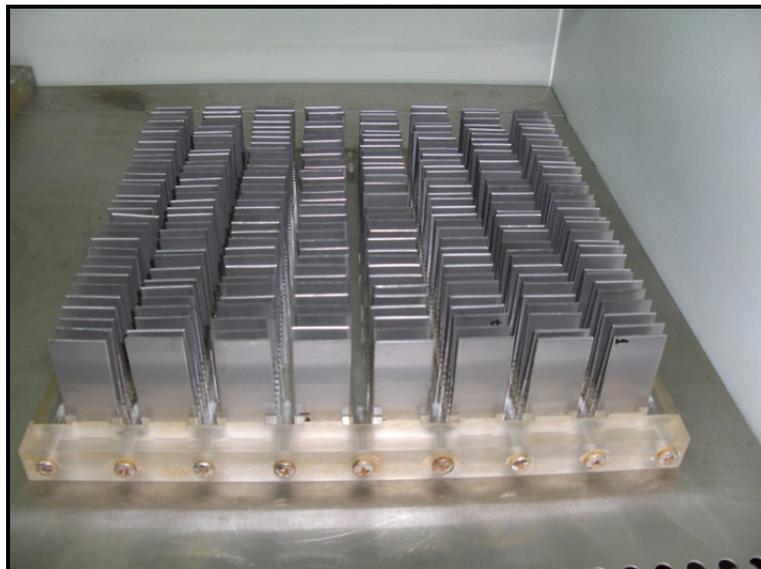


Fig. 1 A designated plexiglass coupon holder

6. Cleaning and Sterilization of Coupons

As mentioned above, since the characteristics of the surface material greatly influence the densities of biofilm formation, coupons should be selected the same material with the construction of cooling towers which will be modeled.

Surface roughness and composition play an important role in the initial stages of biofilm formation. Generally, it has been known that microorganisms accumulate more easily on damaged or irregular rough surfaces; detachment of biofilm due to shear forces from the flow is reduced on the rougher surface because cells were protected from the fluid flow on rough surfaces [5, 6, 22].

In this regard, abrasive cleaning should be avoided to not create roughness or pitting during cleaning the test coupon surfaces. Thus, the difference in the biofilm load between coupons is prevented due to the more than usual accumulation of microorganisms on damaged locations of coupons. On the other hand, to eliminate in differences bacterial density on each coupons in each sampling minimum three coupons should be taken from the system. Owing to these precautions, the reproducibility of results will not be damaged and the variations or deviations in results are decreased.

After cleaning with a non-toxic detergent, coupons should be rinsed with tap water and disinfected by an appropriate disinfection method (i.e. irradiation by using transilluminator or autoclaving) according to material type. After sterilization the coupons should be tested for the presence of any residual detergent by placing coupons onto bacterial lawns on appropriate medium and after incubation plates should be examined for the presence of growth inhibition zones around the coupons [17, 31, 32]. The coupons may be placed into slide-holders situated in the water basins, biofilms were allowed to develop within the aqueous phase of the system.

7. Biofilm Ageing

It has been known that characteristically initiation, exponential growth and steady state phases exist in mature biofilm development. From early colonization to the mature biofilm, physiological and metabolic changes seen in the biofilms due to ageing, for example, differences are expected in the amount and types of produced extracellular compounds in a certain development phase [5, 9]. It is well known that such changes can alter susceptibility to antimicrobial agents. Since sensitivities of old and young biofilms to antimicrobials may differ, test time should be kept long to permit maturation of the biofilm.

In mature [reached a structural equilibrium (steady state)] biofilm layer, sessile and planktonic cells are in continuously exchange and in this phase the net biofilm growth is balanced by the biofilm detachment and growth/detachment rate of biofilm remains constant [5, 9].

As a result of biofilm detachment, resistant bacteria pass into the bulk water and create a risk especially the spread of pathogens to the environment by dissemination of contaminated aerosol from system. Due to the potential harbouring a variety of pathogens, protecting them from biocides and continuous release of microbial colonies from biofilm back to the bulk water, biofilm communities are also considerable. Therefore, for successful evaluation of biocide efficacy, both sessile and planktonic populations of model system containing matured biofilm have to be tested periodically.

8. Biocide

All biocides should be safely handled and applied in a safe manner as instructed on the label or Material Safety Data Sheet (MSDS). MSDS of the compound provide information such as physical data (melting and boiling point, etc.), chemical properties, toxicity, health effects, first aid, reactivity, storage, disposal, protective equipment, and spill-handling procedures. Owing to the MSDS, ecological risk assessment of the biocide will be made by definition of supporting information such as dosage rates and frequencies, aquatic toxicity data, antidote (neutralizing agent) of the biocide to be tested.

The effectiveness of biocides in cooling towers should also be evaluated in terms of criteria such as efficiency, cost, causing corrosion in equipment, creating secondary contamination risk for the environment [13]. Since, in general cooling tower water are discharged back to the natural environment (lake, sea etc), testing and evaluation the activity of efficient and environmentally friendly biocides would provide advantages both for industrial operations and environment [15].

Cooling tower water generally enriched in mineral content and organic materials which are concentrated by cooling tower circuit become significant inactivators of certain oxidizing microbial biocides. Furthermore, pH of the cooling system water influences the activity of biocides. For these reasons, more closely simulate natural field conditions, biocides should be resolved in the model system water and active biocide concentration should be measured at first preparation and end of the incubation period.

Additionally, since application of biocides to the system by using dosage pump will require a separate system for each dose and control group, *in vitro* testing of the efficacy of biocide against both planktonic and biofilm samples received from the model system would be advantageous.

9. Neutralizing Agent

According to the standard tests, for accurate determination of antimicrobials within a specified contact time, complete and immediate inactivation (neutralization) of the antimicrobial agent is needed. Since inefficient or incomplete neutralization and likewise the toxicity of neutralizing agent against bacteria resulting in an overestimation of antimicrobial activity, the accurate neutralization activity against biocide and the toxicity of quenching agent against both biofilm and planktonic bacteria should be predetermined [33].

10. Standard Test Methods for Evaluation of Antibacterial Agents

Before starting to the experiments it should be investigated to determine whether there are standard tests for the evaluation of antimicrobial activity. Biocide assays should meet the criteria of the standard test method and also techniques which will be used to assess the effectiveness of biocide should be defined, required equipment and chemicals should be provided.

11. Methods/ Techniques

For implementation of *in vitro* results to real industrial systems and execute a successful biocide program in such systems, microbial burden monitoring and data evaluation should be done by using different laboratory techniques. Selected techniques for this purpose should be validated each other and provide easier and more accurate assessment. Besides, alternative techniques that are not mentioned in the standard test method should be investigated [7, 8, 10, 13, 18].

Conventional plate count, light microscopy, electron microscopy, epifluorescence microscopy, measurement of respiration rate, components of microorganisms and adenosine triphosphate (ATP) concentrations, also a variety of molecular techniques are the most commonly used techniques to assess the extent of microbial burden/microbiological activity [6, 8, 10].

11.1. Total Viable Cell Count

Conventional plate or colony forming units (cfu) count is commonly used in determination of bacteria number after biocidal treatment. However, as a result of inhibition and injury of metabolism and/or nutrient shock due to the high nutrient content of the medium, bacteria may become viable but non-culturable (VBNC). This method has drawbacks such as allowing growth of a low fraction of the microorganisms present in the sample on a single medium, requirement of long time to obtain results (24-240 hours) and lower determination of cfu numbers than the numbers of actually present cells. Therefore, success of biocide is overestimated and this technique is questionable. [6, 8, 34].

11.2. Microscopic Analyses

In biofilm analyses, while light microscopy provides preliminary information about the bacterial morphology and general biomass appearance, epifluorescence microscopy can provide information about the bacterial activity and total cell counts, as well as 2D distribution of bacteria. Light microscopy is rapid and does not usually require complex sample pre-treatment procedures, however, has a limited capability for biofilm analysis and its resolution is restricted. Therefore, when used on live biofilms, classical light microscopy is suitable for the examination of the early phases of biofilm formation [35, 36].

Fluorochrome staining is commonly applied in connection with epifluorescence microscopy for biofilm monitoring and fluorescent dyes commonly used in this technique are acrydine orange (AO), rhodamine 4,6-diamidino-2-phenylindole (DAPI) and 5-cyano-2, 3-ditoyl tetrazolium chloride (CTC). AO fixes to RNA and DNA and may be used as index of physiological activity since slow growing cells, with low RNA/high DNA content fluoresce green and fast growing cells with high RNA content fluoresce orange. Rhodamine fluoresces in proportion to cellular proton motive force. DAPI stains targets DNA in both dead live all the cells and allow for total cell counts. CTC staining visualizes metabolically active bacteria, as it becomes red fluorescent when dye is reduced via electron transport activity during cellular respiration. Combined application of CTC with DAPI allows distinguishing between active and total bacteria populations within a sample. Therefore, the vitality of microorganisms which can not be detected by traditional plate method will be determined easily and more accurately [12, 36].

Epifluorescence microscopy also allows the identification of bacterial communities in biofilms when used conjunction with labelled oligonucleotide ribosomal RNA probes [36].

As depth examination of thick biofilm is not possible with light and epifluorescence microscopy, biofilm should be removed from its substrata and homogenized with appropriate techniques and then transferred onto microscopic slides for examination [36].

Since homogenization procedures result in the distortion of the natural structure of biofilm, physical and optical examination of inherent structure of thicker biofilms can be achieved through the use of confocal laser microscopy (CSLM) combined with microelectrodes and image analysis. CSLM in combination with fluorescently labelled oligonucleotide probes can be applied to study 3D spatial distributions of certain bacterial groups or strains in biofilms [36].

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) which are early microscopic techniques used in biofilm monitoring, have special importance in clarification of biofilm structure. Although electron microscopy does not allow for *in vivo* and *in situ* studies of intact biofilms and CSLM overcomes some of the inherent drawbacks of electron microscopy, SEM and TEM are still important techniques due to their high magnifications [36]. However, it should be noted that SEM, TEM and CSLM are suitable techniques for biofilm analysis and can not provide information about the vitality.

11.3. Measurement of Respiration Rate

Measurement of oxygen utilization (dissolved oxygen uptake) may provide information about the activity index for entire communities [37]. Since aerobic and facultative anaerobic microorganisms found in industrial systems utilize oxygen during growth, monitoring oxygen consumption provides a simple, rapid method for assessing biocide performance [38].

11.4. Components of Microorganisms

Different components such as organic nitrogen, carbon, chlorophyll, lipopolysaccharide lipid, protein, carbohydrate, fatty acid analysis, glycocalyx can give information about the dynamic activity of the native microbial populations. Although, according to microorganisms to be investigated different components can be measured as biochemical indicators, these methods are not used for assessing biocide performance [37].

11.5. ATP

Adenosine triphosphate (ATP) molecule is universal energy currency and a key regulator of enzyme activity in all alive cells. In the presence of luciferin substrate and luciferase enzyme, ATP dependent oxidation of luciferin produces oxyluciferin, CO₂, AMP, inorganic phosphate and light. The quantity of generated light is proportional to the amount of ATP present, thus, the light units can be measured to estimate the biomass of cells in a sample. While most common microbiological test methods only detect certain categories of organisms in long period of time, ATP tests have several advantages over traditional microbiological methods in that they allow simple and rapid measurement of all kind of the microbiological organisms in the sample with reproducible results.

ATP content of the cells is related to the homeostatic regulation and thus relatively constant in living cells. When a cell died intracellular ATP is reduced by ATPases and ATP levels are decreased quickly. Biocide efficacy is assessed by determination of total ATP and free ATP concentrations in both biofilm and bulk water samples using different ATP bioluminescence swabs. Total ATP tests measure both the ATP that is bound up within living cells as well as ATP that is floating free in the water. Free ATP tests measure only the ATP floating free outside of living cells. The microbial ATP concentration is determined by the difference between total and free ATP. Monitoring of total and free ATP levels during biocidal treatment may indicate the efficiency of the clean-up effort and help determine the appropriate scheduling to maximize the biocidal activity. Thus, this process allows microbiological risk assessment of system in a few minutes *in situ* [1; 25, 39, 40, 41, 42].

11.6. Molecular Techniques

PCR (polymerase chain reaction), fluorescence in situ hybridization using 16S rRNA-targeted oligonucleotides and immunologic probes combined with confocal laser scanning microscopy are the most common molecular techniques in order to assess the effectiveness of biocides against biofilm [27, 43].

Since < 1% of the bacterial population from oligotrophic environments may be cultured, usage of conventional cultivation techniques in conjunction with molecular techniques allows the determination of the spectrum of microorganisms present in biofilms and understanding of the ecological significance of biofilm bacteria [27, 43].

Although molecular techniques are able to characterize the microbial community structure and activity in natural and artificial environments, all these techniques have limitations as well as advantages to their use in natural ecosystems. Some methodological problems include cell permeability, target site accessibility, target site specificity/sensitivity, low signal strength of the fluorescently labelled probes. Especially, in quantification of biocide efficacy the determination of vitality is very crucial and these methods fail to discriminate between live and dead bacteria. With the recent molecular genetic advances, new techniques such as viable real time PCR (vPCR) have been developed. vPCR method allow to

detect accurate and reliable quantification of viable microorganism (especially waterborne pathogens) in environmental samples after biocide application [44].

12. Evaluation of results

Many factors may influence the efficacy of biocides such as structure and application method of biocide, contact time, microflora burden, structure and the surface of the material to be disinfected, target microorganisms, concentration and dissolution of biocide in water, environmental conditions, pH and temperature [45]. Any of these parameters can become important factor depending on the biocide structure [7].

Biofilms quite diverse due to many factors like surface type, the presence of nutrients and oxygen, microbial species, the flow rate of the water. Since each biofilm is unique, knowledge obtained from the experiments made with a specific type of biofilm can not be quoted other types of biofilms. Although there have been several attempts to develop a standard laboratory systems for the production of artificial biofilms, to test the effectiveness of biocide, a standard model which represents all biofilms could not been found until now. Thus, in order to implementation of laboratory results to cooling towers, model system design and operation should be made the nearest operating conditions of full-scale systems as well as parameters influence the efficacy of biocides should be taken into considerations.

For the selection of suitable test methods to accurate evaluation of the efficacy of biocides against biofilms in recirculating water systems ASTM E1427 guide can be taken into consideration. Selection of complementary test methods enables simulation of *in vitro* results to the field conditions. According to the selected methods efficacy endpoints such as % kill rate, % reduction in CFU (Colony Forming Units), reduction in ATP concentration should be researched and determined. After measurements, values should be calculated as per ml and per cm² for planktonic and biofilm samples, respectively and compared with control groups. In antimicrobial researches endpoint values of some of the new methods are not specified, in this case, examination of the results of these studies by institutions and organizations which identify standards will provide the determinations the endpoint limits of the tests and accurate assessment of microbial load of industrial systems. In this sense, *in vitro* and *in situ* antimicrobial studies with taking into account the above-mentioned parameters will be beneficial in terms of determinations of new standards; the best biocide type/dosage selection for a specific biofilm and accurate evaluation of antibacterial activity of water treatment biocides.

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