Evaluation of the antimicrobial activity of colloidal silver-hydrogen peroxide against model cooling tower biofilm

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In this study a model cooling tower system was experimentally infected by *L. pneumophila* ATCC 33152 and has been run to the nearest operating conditions of full-scale system. Effectiveness of colloidal silver-hydrogen peroxide biocide was assessed against both planktonic and biofilm bacteria (on stainless steel, glass, polyvinyl chloride slides) monthly during 6 months experiment period. Each month, samples were analyzed for heterotrophic plate count (HPC), the presence of *Legionella* spp., viable and total counts, the total and free ATP concentration. A considerable variation was found in the response of biofilm to the biocide when biofilm reached the mature phase (from the 4th month). The reduction to zero in HPC values, from the 2nd and 5th month in planktonic and biofilm phase, respectively; has been interpreted as induction of the viable but non-culturable form state in bacteria. The results emphasized that biocide should be applied on a regular basis prior to the settlement of pollution in the system and microbial burden monitoring/data evaluation should be done periodically by using different microbiological techniques for the proper evaluation of the system.

Keywords: Biofilm; cooling tower; heterotrophic plate count; *Legionella pneumophila*; ATP; DAPI-CTC

1. Introduction

Biofouling is a major problem in cooling tower systems which are an integral component of many industrial processes. The chemical and physical conditions of cooling water systems provide an ideal environment for microbial growth and biofilm formation. In addition to the accumulation and dissemination of pathogenic organisms, especially *Legionella pneumophila* [1-3]; biofilms also lead to many undesired conditions such as equipment damage through corrosion, decreased energy efficiency, local blocking of cooling towers and increased heat transfer resistance [2, 4, 5]. As a result of adverse effects of biofouling, serious economic losses and public health risk problems including those associated with legionellae may occur [6]. The most common approach for diminishing economic losses, operational and public health problems in industrial field is chemical treatment primarily using biocides. The usage of biocides in parallel with the microbiological analysis will be beneficial for the efficient system operation and the accumulation/dissemination of pathogenic organisms [1, 4, 7].

Biocides are important for a successful and regular cooling water treatment program. However, recommended biocides/dosages for decontamination of industrial systems by manufacturers may not be suitable for each system and target microorganisms, since the natural structure of the each biofilm is unique. Owing to the fact that, the conduction of well controlled disinfection studies in full scale industrial cooling towers is difficult, the antimicrobial activity of biocides should be pre-evaluated against both planktonic and sessile bacteria under *in vitro* and *in situ* conditions, before field applications [8-10]. For implementation of *in vitro* results to the full scale systems and achievement of a successful biocide program in such systems, microbiological growth and/or activity monitoring should be done by using different laboratory techniques in parallel with the biocide usage, at regular intervals. To assess the extent of microbial burden conventional plate count, different microscopy techniques, fluorescent antibody staining techniques, measurement of adenosine triphosphate (ATP) concentrations and a variety of molecular techniques have been used [4].

Various oxidizing and nonoxidizing biocidal compounds have been examined for their efficacy in both laboratory simulations and field treatment of *Legionella* contaminations [8, 9, 11-18]. In this study, we investigated of colloidal silver-hydrogen peroxide (CSHP) which is a commercial formulation recommended by manufacturers for decontamination of cooling towers. Colloidal silver-hydrogen peroxide is an alternative oxidizing compound as being safe for humans and environment, also known to be effective even in low concentrations, stable in a wide range of temperatures and has no build-up resistance by microorganisms. This compound shows activity based on the fast oxidative-reductive chemical reaction against nucleic acids, functions of protein and enzymes [12, 19].

Although colloidal silver-hydrogen peroxide is recommended for disinfecting cooling towers, there is a lack of published data about the efficacy of this compound against both planktonic and sessile microflora of cooling tower. Therefore, in the current study, inhibitory characteristics of the recommended dosages (100 and 200 mg l^{-1}) of this biocide at different contact times were investigated against both planktonic and biofilm bacteria monthly during the 6 months experiment period.

2. Materials and Methods

In the current study, laboratory scale recirculating cooling tower model system under constant hydraulic conditions was used to simulate cooling tower installations. For modeling microbial flora of cooling tower, real cooling tower system water (at 35ºC) was inoculated to the model system at the beginning of the experiment and has been run to the nearest operating conditions of full-scale system for six months. Additionally, to mimic *Legionella* contaminated cooling systems, model system was experimentally infected with *L. pneumophila* standard strain (ATCC 33152) suspension (1 ml *L. pneumophila* inoculum of 10^5 cell ml^{-1}). Throughout the experiment, no chemicals (disinfectant, pH regulators or anti-scaling agents) were added to the system in order to exclude their possible negative effects on microorganisms and biofilm formation. Public potable water was used to replenish water lost by evaporation and blowdown.

2.1 Biocide preparation

100 and 200 mg l^{-1} concentrations of colloidal silver-hydrogen peroxide were prepared in sterile demineralized water.

2.2 Determination of biocidal activity against microorganisms

Both bulk water and biofilm samples on different surfaces (SS, G, PVC) were collected from the model system monthly and divided into three groups: (a) treated with 200 mg l^{-1} biocide (b) treated with 100 mg l^{-1} biocide (c) untreated controls. At 0, 1 and 24 hours contact times, samples were analyzed in terms of HPC values, the presence of *Legionella* spp., the total and free ATP concentration. Samples were also examined for direct enumeration of viable and total bacteria by CTC/ DAPI staining method using epifluorescence microscopy.

2.2.1 Heterotrophic plate count and *Legionella* analysis

For planktonic samples, model system water was taken into the sterile containers and biocide concentrations were applied separately.

To prepare biofilm samples, SS, G, PVC coupons were removed aseptically from the basin, dip-rinsed in sterile phosphate buffer to remove unattached cells. Coupons were exposed to the 100 and 200 mg l^{-1} biocide, individually. Biocide exposed and unexposed biofilm samples on coupons were scraped by sterile scalpel and homogenated [20].

At 0, 1, 3, 6 and 24 h contact times, biocide treated planktonic and sessile samples were neutralized with 0.4% sodium thiosulfate. The resulting suspensions were serially diluted to 10^{-5}.

For HPC determinations, diluted biofilm homogenates and bulk water samples were spread-plated (0.1 ml) onto R2A agar (Oxoid, UK) plates and incubated at 28°C for 10 days [21]. After incubation, colonies were counted with a Colony Counter Device (åCOLyte Super Colony Counter, Synbiosis) and recorded as cfu ml^{-1} for bulk water and cfu cm^{-2} for biofilm samples. HPC determinations were done in triplicate.

For *Legionella* spp. analysis, biocide exposed and unexposed bulk water and biofilm samples were divided into two parts and one of them was treated with acid solution for 15 min (KCl–HCl solution, pH 2.2), the other part was treated with heat at 50°C for 30 min, to reduce overgrowth of commensal flora. Pretreated and untreated samples were inoculated (0.1 ml) onto alpha-ketoglutarate supplemented buffered charcoal-yeast extract (BCYE, Oxoid, UK) agar containing glycine, vancomycin, polymyxin, natamycin and incubated at 37°C for 14 days. Analyses were carried out in triplicate. Definitive identification was performed by latex agglutination (OXOID) [22, 23].

2.2.2 Enumeration of viable and total bacteria by CTC/ DAPI staining method

The number of total and respiring cells in biocide exposed and unexposed planktonic/biofilm samples were determined by a modification of the procedure reported by Rodriguez et al. (1992). The tetrazolium salt 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Sigma-Aldrich) was used in conjunction with DNA-binding 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) fluorochrome to differentiate metabolically active cells from dead cells [24]. Following the manufacturer's instructions, all red signals were considered live cell while blue signals were considered dead.

All samples were incubated with 50 mM CTC redox dye solution (5 mM final concentration of dye) in the dark at 28°C for 4 hours. Then, samples were counterstained with 1.0 μg.ml^{-1} DAPI for 1 h. After incubation, samples were filtered through a vacuum filtration onto black 0.2 μm pore size polycarbonate filters (Millipore, USA). The air-dried filters were mounted on a glass microscope slide with non-fluorescent immersion oil below the filter and coverslipped, respirometrically active cells were enumerated microscopically [25, 26]. Results were recorded as log cell ml^{-1} for bulk water and log cell cm^{-2} for biofilm samples.

2.2.3 ATP measurement

Biocide treated and untreated samples were taken using total and free ATP swabs, after extraction ATP from cells by cationic agents in swabs, released ATP was quantified after reaction with the luciferase-luciferin reagent. Within a few seconds, swabs were inserted into the luminometer (Uni-Lite, Biotrace) and the intensity of light was returned to the

RLUs [27-30]. Results were recorded as RLU ml^{-1} for bulk water and RLU cm^{-2} for biofilm samples. The microbial ATP concentration was determined by the difference between total and free ATP.

2.2.4 Statistical of analysis

The data were analysed using the Graphpad Prism 6 statistical package. The mean and standard deviation (SD) within samples were calculated in all cases. Biocide exposed and unexposed groups were compared by the Student's t-test. Kruskal-Wallis non-parametric test was used to detect differences of the biocide dosages under the same conditions. The value of $p < 0.05$ was considered statistically significant.

3. Results and Discussion

In this study, two recommended dosages of colloidal silver-hydrogen peroxide biocide were tested for efficacy in reducing planktonic and sessile microbial populations of a model cooling tower system, including *L. pneumophila*, during 6 months experiment period. Bulk water and biofilm samples which grown on different surfaces (SS, G, PVC) were collected from the system monthly and exposed to the biocide dosages for 0, 1 and 24 hours. After contact times, samples were evaluated in terms of heterotrophic plate count (HPC), the presence of *Legionella* spp., epifluorescence microscopy, the total and free ATP concentration and compared material dependence of antibacterial activity of biocide.

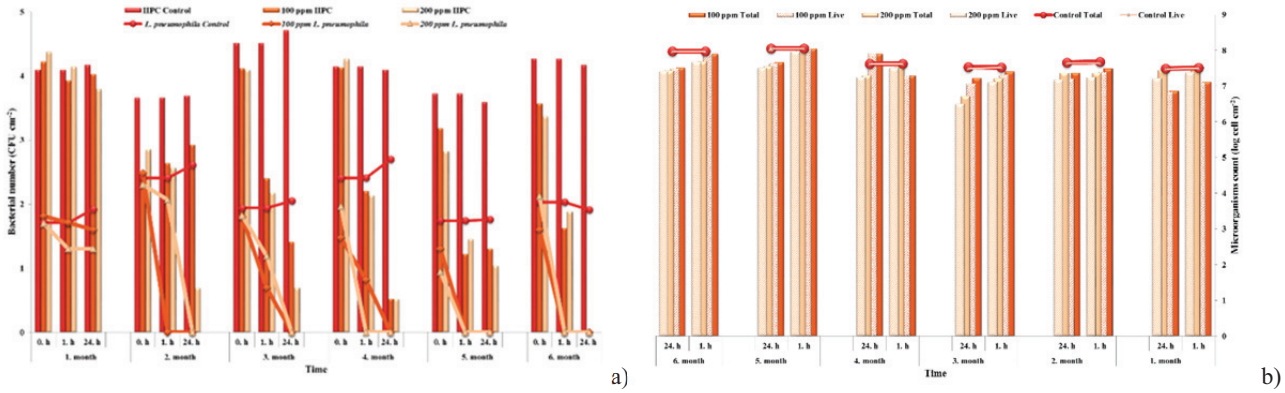
Since biocides currently being used may induce the development of resistance and pose a risk to human health and the environment, alternative biocides have been seeking for cooling tower water treatment programs. In the current study, colloidal silver-hydrogen peroxide compound which is a commercial formulation recommended by manufacturers for decontamination of cooling towers, was studied. Colloidal silver-hydrogen peroxide is an alternative oxidizing compound, safe for humans, stable in solution even in a wide range of temperatures [12, 19]. To our knowledge, no information on the effect of colloidal silver-hydrogen peroxide against found in cooling towers has been reported.

In biocide efficacy tests, ASTM E645−13 standard test method for evaluation of microbicides used in cooling water systems was taken into consideration, for the interpretation of results [31]. According to this standard, microbicides can 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.

90% kill or 1 log reduction would be the minimum level of performance considered to show efficacy of a microbicide according to this guidelines. The reduction in the number of microorganisms at each biocide concentration was calculated relative to the count of the control sample. The control samples (without biocide) showed a stable population with no more than a 1-1.5 log increase or 0.5 log decrease in growth, during the test period for a 24 hours contact time period. In our study, tested biocide dosages have provided minimum ≥1 log (90% kill) decrease in cultivable sessile HPC (Fig. 1a, 2a, 3a) ; and also in cultivable planktonic HPC, except after 1 h contact with the 100 mg l^{-1}, during 6 months at 1 and 24 h contact times (Fig. 4a).

With the both tested biocide dosages, sessile *L. pneumophila* counts were reduced to zero after 24 hours contact from the 2nd month; and the same result was obtained after 1 hour exposure from the 5th month, on all slides (Fig. 1a, 2a, 3a).

Treatment with 200 mg l^{-1} and 100 mg l^{-1} biocide for 24 h resulted in zero planktonic *L. pneumophila* counts from the 1st month and from the 5th month, respectively. The same reduction has been achieved at time point zero with 200 mg l^{-1} from the 3rd month and 100 mg l^{-1} biocide from the 5th month (Fig. 4a).



a) b)

Fig. 1 Log reduction of sessile bacteria counts on stainless steel coupons exposed to colloidal silver-hydrogen peroxide at different contact times and concentrations a) cultivation counts b) Total (live+dead) and live microorganisms counts.

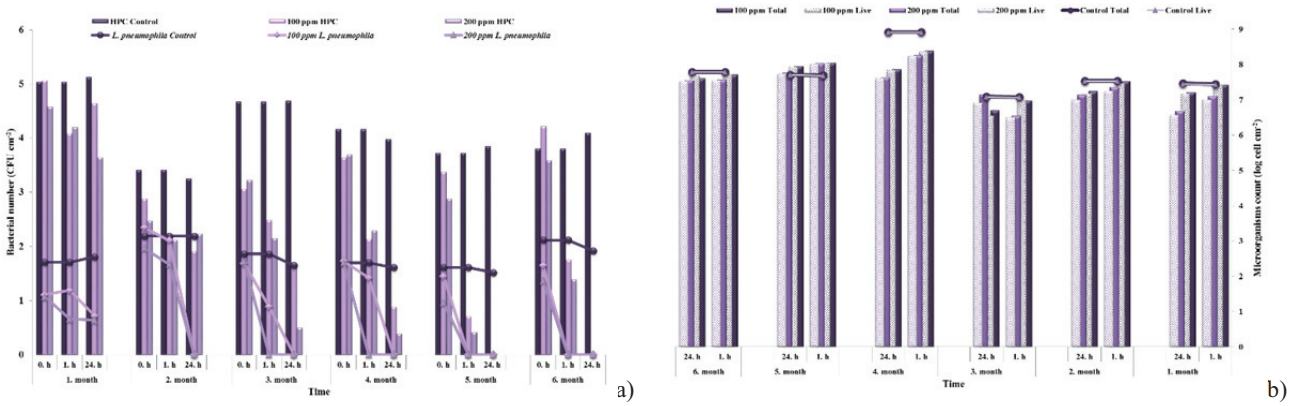


Fig. 2 Log reduction of sessile bacteria counts on glass coupons exposed to colloidal silver-hydrogen peroxide at different contact times and concentrations a) cultivation counts b) Total (live+dead) and live microorganisms counts.

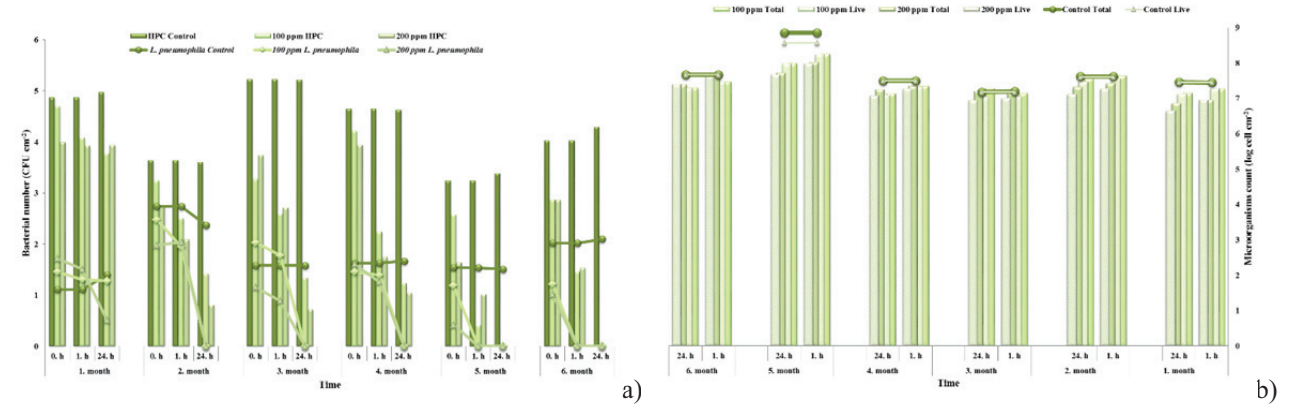


Fig. 3 Log reduction of sessile bacteria counts on PVC coupons exposed to colloidal silver-hydrogen peroxide at different contact times and concentrations a) cultivation counts b) Total (live+dead) and live microorganisms counts.

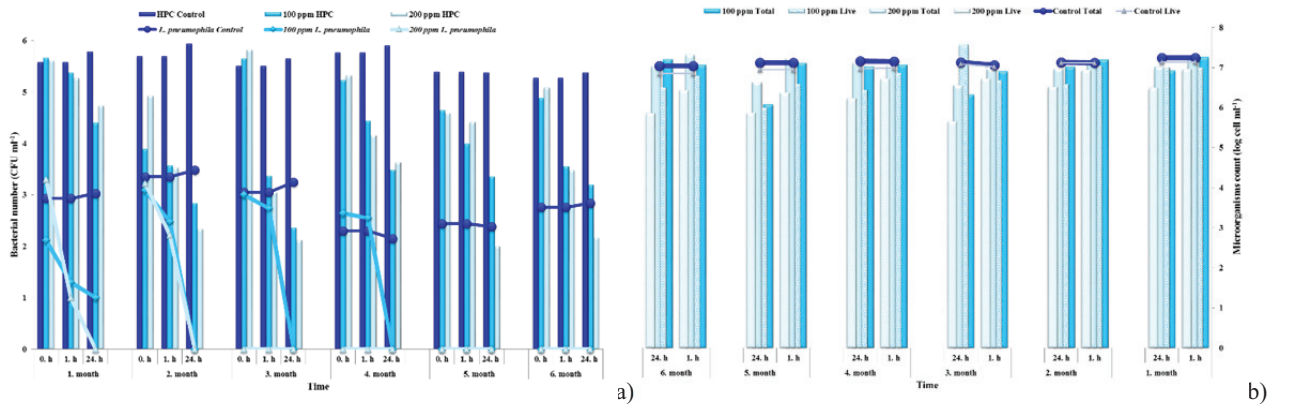


Fig. 4 Log reduction of planktonic bacteria counts exposed to colloidal silver-hydrogen peroxide at different contact times and concentrations a) cultivation counts b) Total (live+dead) and live microorganisms counts.

The authors have pre-evaluated the antibacterial activity of the same biocide against three different *L. pneumophila* strains *in vitro* [12] and the compound was found effective at the recommended concentrations (30-100 mg l^{-1} for cooling towers with continuous treatment, 1.000-3.000 mg l^{-1} for legionellae). It was determined that concentrations of 1.000-5.000 mg l^{-1} colloidal silver-hydrogen peroxide achieved 5 log reduction at 0 h contact time. Furthermore it has been reported that all tested strains were killed by lower doses (100 and 50 mg l^{-1}) in 3 h contact time [12]. On the other hand, in the present study, 100 mg l^{-1} reduced to zero both planktonic and sessile *L. pneumophila* bacteria after 24 h exposure from the 2nd month. With reference to these studies, it can be concluded that the efficacy of biocide has been decreased in the presence of complex and mixed microbial flora which reflects the real system conditions.

Since, heterotrophic plate count does not recover all microorganisms present in a system, especially after biocidal treatment, ASTM E645−13 standard address a guideline for unconventional measurement of microbes which can be found in Guide E1326 (Standard Guide for Evaluating Nonconventional Microbiological Tests Used for Enumerating Bacteria) [32]. One of these unconventional methods is the measurement of adenosine-5′-triphosphate (ATP), which is the primary energy donor for life processes. Since, ATP does not exist in association with non-living detrital material;

the ATP firefly (luciferin-luciferase) method is suitable for the determination of viable microbial biomass. On the other hand, it should be noted that ATP measurement method indirectly estimate the microbial load, since ATP quantity per cell varies with species and physiological state of the organism and because of the instability nature of ATP. Use of the total and free ATP tests together provides a much better indication of the biocide efficacy, since, total tests measure bound up ATP within living cells and floating free ATP in the water; free tests measure only free floating ATP. In assessing the efficacy of biocides, the difference between the two represents is used as microbial ATP. In total and free ATP values, significant reduction ($p < 0.05$) was detected compared to control in both planktonic and biofilm samples and values were varied according to months (Table 1, 2, 3, 4).

Table 1 Total and free ATP levels **(**RLU cm^{-2}**)** of sessile samples on stainless steel coupons exposed to colloidal silver-hydrogen peroxide at different contact times and concentrations.

		Contact Times			
		1 h		24 h	
Months	**Dosages (mg l^{-1})**	**Total ATP**	**Free ATP**	**Total ATP**	**Free ATP**
	Control	1465	49	1712	62
	200	60.53	82.41	14.73	16.06
1	100	57.93	57.83	54.66	49.13
	Control	1105	149	1128	130
	200	40.73	30	7.73	8.13
2	100	30.53	33.26	13.33	10.4
	Control	2075	215	2382	256
	200	33.26	22.6	24.46	44.26
3	100	74.73	41.26	14.6	9.46
	Control	2493	225	2434	213
	200	66.86	44.6	22.66	17.86
4	100	76.86	81.4	24.46	23.13
	Control	2264	200	2370	184
	200	37.73	60.26	47.08	69.73
5	100	31.73	41.86	20	23.33
	Control	2072	203	2418	190
	200	48.26	47.6	32.86	26.46
6	100	37.46	37.4	32.66	41.78

Table 2 Total and free ATP levels **(**RLU cm^{-2}**)** of sessile samples on glass coupons exposed to colloidal silver-hydrogen peroxide at different contact times and concentrations.

		Contact Times			
		1 h		24 h	
Months	**Dosages (mg l^{-1})**	**Total ATP**	**Free ATP**	**Total ATP**	**Free ATP**
	Control	1777	110	1828	130
	200	45.26	45.26	30.05	75.63
1	100	35.36	32.10	22.89	14.47
	Control	1753	176	1847	190
	200	16.15	14.68	11.36	4.05
2	100	17.57	19.73	6.47	7.78
	Control	1572	146	1630	131
	200	30.57	26.78	54.47	29.31
3	100	33.52	34.84	19.26	16.27
	Control	1852	98	184	107
	200	64.15	47.52	22.78	13.63
4	100	37.73	21.78	33.68	23.21
	Control	1804	107	1826	114
	200	17.57	33.05	17.68	51
5	100	25.42	30.78	11.78	10.68
	Control	1779	51	1770	72
	200	17	26.42	37.68	36.94
6	100	17.84	26.15	8.94	14.94

Table 3 Total and free ATP levels **(**RLU cm^{-2}**)** of sessile samples on PVC coupons exposed to colloidal silver-hydrogen peroxide at different contact times and concentrations.

		Contact Times			
		1 h		24 h	
Months	**Dosages (mg l^{-1})**	**Total ATP**	**Free ATP**	**Total ATP**	**Free ATP**
	Control	1829	80	1885	89
	200	102.36	123.61	26.21	24.89
1	100	56.89	53.47	40.26	36.73
	Control	1531	130	1681	91
	200	24.89	24.31	29.26	24.78
2	100	18	18.26	11.68	5.73
	Control	1716	156	1839	198
	200	43.57	39.10	18.73	29.36
3	100	70.94	74.78	35.52	8.78
	Control	1935	70	1920	54
	200	56.68	42.63	24.89	16.36
4	100	62.42	49.47	22.10	17.36
	Control	1684	42	1773	76
	200	25.31	34	17.84	21.73
5	100	27.94	44	11.10	5.52
	Control	1699	75	1747	61
	200	13.42	17.57	10.31	12.31
6	100	13.68	18.31	5.47	16.21

Table 4 Total and free ATP levels **(**RLU ml^{-1}**)** of planktonic samples exposed to colloidal silver-hydrogen peroxide at different contact times and concentrations.

		Contact Times			
		1 h		24 h	
Months	**Dosages (mg l^{-1})**	**Total ATP**	**Free ATP**	**Total ATP**	**Free ATP**
0.day	Control	178	106		
	Control	1780	424	1287	69
	200	505.8	42.4	388	560
1	100	823	1289	454	710
	Control	2950	404	2630	230
	200	170.2	47.4	83.83	42.2
2	100	74.6	32.2	83.6	46.6
	Control	2274	414	2262	338
	200	90.2	40.2	42.4	40.6
3	100	137.2	62.2	82.5	29.4
	Control	1590	654	1648	672
	200	80.4	48	63.2	46.6
4	100	72.8	49.2	47.8	44.8
	Control	1422	592	1264	408
	200	35.6	49.2	34.8	33
5	100	33.4	36.8	29.8	39.2
	Control	1576	904	1749	832
	200	259.8	128	195.8	193.6
6	100	261	183	236.6	392

Although assessment of biocidal activity were interpreted based on ASTM E645−13 standard, this ASTM standard was not specifically developed for legionellae. On the other hand, ASTM D5952-08 [35], which provides information on microbiological analysis of environmental samples for legionellae, has been associated with ASTM E645−13 standard. While, maximum allowable bacterial counts for heterotrophic bacteria and legionellae is not specified in ASTM E645−13 [31] and D5952-08 [33] standards; from other sources it has been known that > 100.000 cfu ml^{-1} HPC, > 1.000 cfu ml^{-1} legionellae counts indicate a potentially hazardous situation for cooling towers. < 10^4 cfu ml^{-1} heterotrophic plate count [2], < 10 cfu ml^{-1} *Legionella* counts [34] and < 300 relative light units (RLU) total ATP, < 150 free and < 300 RLU microbial ATP values are clean conditions criteria [35]. Tested biocide concentrations achieved < 10^4 cfu ml^{-1} HPC values, at 1 and 24 hours contact times except first months. < 10 cfu ml^{-1} cultivable *L. pneumophila*

counts were obtained from the second month in both bulk water and biofilm samples, after treatment with both dosages (Fig. 1a, 2a, 3a, 4a). However, maximum allowable limits have not been specified, in those of standards. for biofilms, yet.

Biofilms quite diverse due to many factors like surface type, the presence of nutrients and oxygen, microbial species, and the flow rate of the water. Knowledge obtained from the experiments made with a specific type of biofilm cannot be quoted and adapted to the other types of biofilms, each biofilm is unique. 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. Similarly, ASTM E1427-00 standard which was prepared for the evaluation of the efficacy of biocides against biofilms, has been withdrawn in 2009 and it has not been replaced [36]. Nevertheless, total viable and nonviable cells with associated biofilm were determined with epifluorescence technique which proposed in ASTM E1427-00 standard.

Statistical analysis revealed that there were not significant differences between the tested biocide dosages according to HPC, DAPI-CTC staining, and ATP measurement of both planktonic and biofilm samples ($p < 0.05$). There was no statistically significant difference in material dependence in terms of antibacterial activity of biocide. With biofilms, cultivable HPC values were reduced to zero after contact 24 h with the both dosages, at the 5th month on G and PVC slides and at the 6th month on all slides' surfaces. On the other hand, the significant increase in the respiration activity (values are > %98) was determined (Fig 2a, 2b, 2c, 2d). This indicates that fluorescent staining is superior to the conventional plate count for the biocide efficacy evaluation. Furthermore, it has been seen that tested compound could induce viable but not culturable (VBNC) state in bacteria. On the other hand, maximum allowable limits for fluorescent stained cells have not been specified, in any of antimicrobial standard.

As a conclusion:

i) The goal of water treatment program is to maintain microbial control of equipment and to avoid conditions which allow legionellae to multiply, since complete eradication of microbial populations may not be possible and without preventive measures, water systems can rapidly be fouled by uncontrolled microbial growth. However, microbial load should be controlled in allowable range and applied eradication procedure should be verified using different microbiological analysis.

ii) There have also been increasing federal and state regulatory restrictions regarding microbial control of water systems and existing standards may not be totally predictive of microbicidal effectiveness in the field. Therefore, standards about microbiological control in industrial water systems should be detailed and updated according to scientific experimental studies, maximum allowable limits and/or minimum level of performance considered to show efficacy of a microbicide should be determined for new microbiological analysis techniques.

iii) The efficacy of biocide has been decreased in the presence of complex microbial flora, while the antibacterial activity of tested biocide seems to be maintained according to valid standards. Therefore, for full scale treatments the compound may be beneficial when applied in different concentrations. Fluorescent microscopy results showed that biocide induced VBNC state. Since it has been known that, bacteria retain their virulence and pose a risk for public health in VBNC state, maximum allowable limits for tests which determine VBNC state should be specified in standards.

iv) A considerable variation was found in the response of biofilm to the biocide when biofilm reached the mature phase (from the 4th month). The reduction to zero in HPC values, from the 2nd month in planktonic phase and from the 5th month in biofilm phase, has been interpreted as induction of the viable but non-culturable form (VBNC) state in bacteria because of the increased resistance mechanisms of biofilm and cell exchange between planktonic and biofilm phases. The results of the study emphasized that biocide should be applied on a regular basis prior to the settlement of pollution in the system and microbial burden monitoring/data evaluation should be done periodically by using different microbiological techniques for the proper evaluation of the system.

Acknowledgements This study was supported by Research Fund of Istanbul University (Project numbers: UDP 47144, BYP 2270).

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