Efficacy of Colloidal Silver-Hydrogen Peroxide and 2-Bromo-2-nitroporopane-1,3-diol Compounds Against Different Serogroups of *Legionella pneumophila* Strains

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Abstract Cooling towers are considered as amplifier and disseminator sources for *Legionella* spp. despite preventive treatments. Information which was obtained from biocidal tests could improve the effectiveness of treatments. Therefore, the choice of appropriate biocides and the applying of biocides in correct dosages and contact times are important. Various oxidizing and non-oxidizing biocides have been investigated in vitro for their effectiveness against legionellae. Colloidal silver-hydrogen peroxide (CSHP) and 2-bromo-2-nitroporopane-1,3-diol (BNPD) biocides were selected as an example for oxidizing and non-oxidizing agents, respectively, in view of bactericidal action against different serogroups of *L. pneumophila* strains [serogroup 1 (S1) and serogroup 2–14 (S2)] which are isolated different cooling towers in the vicinity of Istanbul, Turkey and reference strain. In the current study, oxidizing biocide was found more effective than non-oxidizing biocide in terms of contact times, log reductions and recommended dosages. At the recommended concentrations for cooling towers (100 ppm), while CSHP compound killed all strains in 3 h contact time, BNPD compound killed S2 and reference strain in the same contact time, S1 strain after 6 h contact time. The results of the present study showed that effective biocide applications can be achieved by pre-determining minimum inhibitory concentration (MIC) and minimum contact time of different biocides to kill target bacteria.

Keywords *Legionella pneumophila* · Biocide · Cooling tower

Introduction

Legionella pneumophila is the causative agent of Legionnaires' disease and Pontiac fever. The primary route of dispersion of this aetiological agent is the inhalation of water aerosols containing the bacteria. Well-known sources of the legionellosis are frequently contaminated cooling tower waters or potable waters. Irrefutable studies demonstrate that the overall prevention strategy against these bacteria in cooling towers depends on regular maintenance and cleaning of the towers in conjunction with chemical biocide control [1–5]. A number of different oxidizing and non-oxidizing agents biocides have been examined to determine their effectiveness against these bacteria, in vitro [1, 6–8]. In this sense, an oxidizing and a non-oxidizing agent were selected as representative samples for two different groups.

In the current study, a mixture of colloidal silver-hydrogen peroxide (CSHP) and 2-bromo-2-nitroporopane-1,3-diol (BNPD) which are commercial formulations recommended by manufacturers for decontamination and inhibiting biological growth in cooling towers and air-conditions were investigated. Although these biocides recommended for decontamination of water systems, there is no published data about the efficacy of this compound against different legionellae which are isolated from Turkey. CSHP is an alternative oxidizing compound for its being safe for humans and environment, effective even in low concentrations, stable in a wide range of temperatures up to the boiling disinfection, has no build-up resistance by microorganisms. CSHP compound shows activity based on the fast oxidative–reductive chemical reaction against

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nucleic acids, functions of protein and enzymes [9]. On the other hand, BNPD has a broad spectrum of antibacterial activity which is a non-oxidizing compound, stable under varying conditions of temperature, relative humidity and exposure to natural light, slightly toxic to avian species, freshwater fish and invertebrates [10]. The antibacterial activity of BNPD relates to its interaction with essential thiols within the cell for the growth inhibition and generation of free radicals causing cell death [11–13].

The purpose of the present study is to determine the lethal activity of various concentrations of a mixture of CSHP and BNPD compounds on *L. pneumophila*. The susceptibility of environmental isolates [SG1 (S1) and SG 2–14 (S2)] and reference strain of *L. pneumophila* (SG1 ATCC 33152) to biocides was also assessed at different contact times.

Materials and Methods

Test Organisms

All tests were performed with three different *L. pneumophila* strains confirmed by monoclonal antibody tests. S1 and S2 strains were isolated from different cooling towers in the vicinity of Istanbul. Reference strain of *L. pneumophila* was obtained from Hertfordshire University Biodeterioration Center. In a previous study strains were investigated by Randomly Amplified Polymorphic DNA (RAPD) method and they were genotypically different [14]. Cultures were grown on Buffered Charcoal Yeast Extract Agar (BCYEA, Oxoid) to late log phase, then cells were harvested and a suspension was prepared in Cl₂-free sterile tap water to a concentration of Mc Farland 1 standard. The suspension was diluted in 1/10 ratio and used in the experiments.

Biocide

Different concentrations of CSHP (Roam Chemie, Belgium) and BNPD (Merck, Germany) compounds were prepared in sterile demineralised water. Concentrations of 5,000, 4,000, 3,000, 2,000, 1,000, 500, 400, 300, 200, 100, 50 ppm were used for CSHP and concentrations of 5,000, 1,000, 900, 800, 700, 600, 500, 400, 300, 200, 150, 100, 50 and 25 ppm were used for BNPD. Concentrations used in the present study were determined by minimum inhibitory concentration (MIC) tests.

Determination of MIC

Macrodilution method was followed for the determinations the minimum inhibitory concentrations of biocides for *L. pneumophila* strains.

Different concentrations of biocides were prepared in test tubes and each tube was then inoculated with *L. pneumophila* inocula (final concentration 10⁵ CFU ml⁻¹). The tubes were incubated at 37°C for 4 days and then examined for visual turbidity. The lowest concentration of the biocide, at which growth was inhibited (indicated by lack of turbidity), was taken as MIC. Samples of 10 µl were drawn from each tube without turbidity and were subcultured on BCYE plates to determine bactericidal concentration.

Neutralization of Biocides

CSHP were neutralized using 0.4% sodium thiosulphate solution, whereas BNPD was neutralized using sodium thioglycolate at an equimolar concentration to the highest BNPD concentration.

Determination of Biocidal Activity

In the current study, suspension test method which was described by Skaliy et al. [6] was modified based on ASTM (1991) E 645-91 [15] standard test method which is used for determination of the efficacy of microbicides used in cooling systems.

The bactericidal activity of sub-MIC biocide concentrations was assessed by adding bacterial suspension into sterile demineralised water containing different concentrations of biocide. After shaking this mixture gently, aliquots were drawn at 0th, 3rd, 6th, 24th and 168th hours of incubation, and transferred into appropriate neutralizer in sterile tap water.

An aliquot of the neutralized sample was serially diluted with sterile tap water. 0.1 ml from each of suitable dilutions was inoculated on BCYEA. After inoculation, plates were

Table 1 MIC values of CSHP against different serogroups of *L. pneumophila*

Concentration (ppm)	S1	S2	Reference strain
5,000	–	–	–
4,000	–	–	–
3,000	–	–	–
2,000	–	–	–
1,000	–	–	–
500	+	–	–
400	+	–	–
300	+	–	–
200	+	+	+
100	+	+	+
50	+	+	+
Control	+	+	+

– growth negative, + growth positive

Table 2 MIC values of BNPD against different serogroups of *Legionella pneumophila*

Concentration (ppm)	S1	S2	Reference strain
5,000	–	–	–
1,000	–	–	–
900	–	–	–
800	–	–	–
700	–	–	–
600	–	–	–
500	–	–	–
400	–	–	–
300	+	+	–
200	+	+	+
150	+	+	+
100	+	+	+
50	+	+	+
25	+	+	+
Control	+	+	+

– growth negative, + growth positive

sealed in polyethylene bags to prevent dehydration. After 7-days incubation at 37°C, the number of survivors was determined by counting the colonies (CFU/mL) using a

colony counter device (aCOLyte Super Colony Counter, Synbiosis). Plates on which colonies did not develop were incubated for an additional 7 days before discarding. At the time points given above, biocide–bacteria suspensions were incubated at 37°C and aliquots were processed as described above. The controls consisted of *Legionella* bacteria in Cl₂-free sterile tap water containing no biocide [16, 17].

All of the experiments were done in triplicates. The activity of biocides was evaluated according to log CFU reductions in bacteria. Concentrations providing 4-log reduction in CFU value was accepted as effective [18].

Statistical Analysis

Results were analyzed statistically by student's *t*-test. Differences were considered significant when $P < 0.05$.

Results and Discussion

Contaminated water systems with legionellae must be eradicated by alternative biocides without delay [5, 6]. Various oxidizing and non-oxidizing agents have been investigated for legionellae decontamination of water

Table 3 Survival of different serogroups of *L. pneumophila* (log CFU ml⁻¹) exposed to CSHP at different contact times and concentrations (the initial numbers of (N_0) strains log 6.29, log 5.09 and log 5.96 for S1, S2 and reference strain, respectively)

Time (h)	Concentration (ppm)						
	Control	500	400	300	200	100	50
S1							
0	5.75	5.02 ± 0.02	4.99	5.22 ± 0.01	3.47	4.82 ± 0.01	4.41
3	5.47 ± 0.02	0	0	0	0	0	0
6	5.36 ± 0.01	0	0	0	0	0	0
24	5.53 ± 0.02	0	0	0	0	0	0
168	5.58 ± 0.02	0	0	0	0	0	0
Time (h)	Concentration (ppm)						
	Control	200			100		50
S2							
0	6.98 ± 0.02		4.74 ± 0.05		1.47		4.78
3	4.94 ± 0.01		0		0		0
6	4.88 ± 0.02		0		0		0
24	5.55		0		0		0
168	5.29 ± 0.01		0		0		0
Time (h)	Concentration (ppm)						
	Control	200			100		50
Reference strain							
0	4.78 ± 0.02		4.07 ± 0.05		3.84 ± 0.02		4.27 ± 0.09
3	4.46 ± 0.01		0		0		0
6	4.88 ± 0.02		0		0		0
24	5.55		0		0		0
168	5.27 ± 0.03		0		0		0

systems [2–6, 19–22]. In this sense, CSHP and BNPD were examined as an example for oxidizing and non-oxidizing agents, respectively, in view of bactericidal action against different serogroups of *L. pneumophila* strains.

MIC values of CSHP biocide were found as 1,000 ppm concentration for S1 and 300 ppm concentration for S2 and reference strains. According to determined MIC values, it was found that S1 is the most resistant strain and the susceptibility of reference and S2 strains was found to be similar against CSHP (Table 1). On the other hand, it was observed that the susceptibility of environmental strains is similar and reference strain is the most susceptible strain against BNPD (Table 2). 400 ppm BNPD concentration was found as MIC for S1 and S2 strains, 300 ppm for reference strain.

CSHP compound was found effective at the recommended concentrations (30–100 ppm for cooling towers with continuous treatment, 1,000–3,000 ppm for legionellae). It was determined that concentrations of 1,000–5,000 ppm of CSHP achieved >5 log reduction at 0 h contact time. Furthermore all strains were killed by lower doses (100 and 50 ppm) in 3 h contact time (Table 3).

Domingue et al. [7] was stated that >99% of *L. pneumophila* population was inactivated with 1,000 µg/ml H₂O₂

within 30 min contact time. However, in our study we have found that the 1,000 µg/ml CSHP achieved ≥5 log reduction at 0 h contact time. The difference in the results may be originated from chemical structures of tested compounds, since CSHP contains silver nitrate as a stabiliser and activator for hydrogen peroxide [9]. Our results are in agreement with those of García and Pelaz [8] in terms of log reductions of the *L. pneumophila* and the effectiveness of the CSHP at the recommended concentrations.

Recommended BNPD concentration for cooling towers (100 ppm) killed S2 and reference strains at 3 h contact time, however S1 strain was killed at 6 h contact time. The antibacterial activity of lower doses BNPD (50 and 25 ppm) against S1 and reference strains required longer contact times than recommended concentrations (Table 4).

The results presented here agreed with those of Wright et al. [3] who found BNPD to be effective against environmental serogroup 1 strain with the 100 ppm dose which reduce the number of viable bacteria by more than 4 logs after contact for 9 h. In our study, it was also observed that for killing environmental S1 strain required longer contact times (Table 4).

Elsmore [1] assigned that BNPD was effective against legionellae both in laboratory experiments (50 ppm) and

Table 4 Survival of different serogroups of *L. pneumophila* (log CFU ml⁻¹) Exposed to BNPD at different contact times and concentrations (the initial numbers of (*N*₀) strains log 5.72, log 5.51 and log 5.30 for S1, S2 and reference strain, respectively)

Time (h)	Concentration (ppm)						
	Control	300	200	150	100	50	25
S1							
0	4.84 ± 0.01	4.51	5.30 ± 0.07	5.61 ± 0.01	6.08 ± 0.01	5.1 ± 0.01	4.86 ± 0.02
3	4.92 ± 0.01	0	4.24 ± 0.02	5.14 ± 0.1	3.5 ± 0.01	4.0 ± 0.01	5.53 ± 0.05
6	5.73 ± 0.03	0	0	0	0	4.3 ± 0.01	0
24	5.86 ± 0.04	0	0	0	0	0	0
168	5.87 ± 0.03	0	0	0	0	0	0
Time (h)	Concentration (ppm)						
	Control	300	200	150	100	50	25
S2							
0	5.17 ± 0.03	5.0 ± 0.03	5.34 ± 0.02	5.39 ± 0.01	5.21 ± 0.03	5.08	5.13 ± 0.04
3	5.09 ± 0.01	0	0	0	0	0	0
6	4.95 ± 0.01	0	0	0	0	0	0
24	4.95 ± 0.02	0	0	0	0	0	0
168	4.87 ± 0.02	0	0	0	0	0	0
Time (h)	Concentration (ppm)						
	Control	200	150	100	50	25	
Reference strain							
0	5.28 ± 0.02	4.50 ± 0.03	4.98 ± 0.04	4.78 ± 0.01	4.45 ± 0.01	4.96 ± 0.05	
3	4.93 ± 0.03	0	0	0	0	4.2	
6	5.01 ± 0.01	0	0	0	0	0	
24	4.78 ± 0.02	0	0	0	0	0	
168	4.58 ± 0.01	0	0	0	0	0	

cooling systems (70 ppm). On the other hand, the others [21, 23] indicated that BNPD was ineffective against legionellae. In our study, the efficacy of BNPD showed variability according to strains and contact times. These differences can be related to the following reasons: (i) strains were isolated from different sources, (ii) strains may be improved resistance, if they have been exposed to biocides before isolation, (iii) strains belong to different serogroups of *L. pneumophila*.

The differences biocide treated (both CSHP and BNPD) and untreated samples were statistically significant ($P < 0.05$).

The susceptibility of the strains against both tested biocides was in the order of: reference strain $> S2 > S1$ in terms of log reduction from initial counts. Environmental strains might be exposed antibacterial compounds and thus, may exhibit improved resistance [24].

In general, oxidizing biocides were found to be more effective than non-oxidizing ones against legionellae [5, 16, 17]. In the present study oxidizing biocide (CSHP) was found more effective than non-oxidizing biocide (BNPD) in terms of contact times, log reductions and recommended concentrations. Although BNPD is accepted as effective a biocide for decontamination of legionellae according to Elsmore's criteria [18] it should be considered that BNPD was found effective against legionellae above recommended concentrations. Since several factors such as bacterial strains and the background conditions in the water system (e.g. pH, temperature, and organic and inorganic constituents' levels) affect the efficiency of a biocide against bacteria, results which were found as effective should be tested under field trial conditions [5, 18, 25].

Each manufacturer recommends various concentrations for decontamination of water systems by biocides; however these concentrations may not be proper for each system and each target microorganism. The results of the present study showed that effective biocide applications can be achieved by pre-determining MIC and minimum contact time of different biocides to kill target bacteria. If water treatment biocides will not be applied to systems in correct dosages and contact times, it would be detrimental rather than beneficial. Also, the current study is important for extending previous data on *L. pneumophila* isolates from different geographic areas.

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References

1. Elsmore R (1986) Biocidal control of legionellae. *Isr J Med Sci* 22:647–654
2. Swango LJ, Wilt GR, Killen AD, Williams DE, Worley SD (1987) Inactivation of *Legionella pneumophila* by hypochlorite and an organic chloramines. *Appl Environ Microbiol* 53(12): 2983–2986
3. Wright JB, Ruseska I, Costerton JW (1991) Decreased biocide susceptibility of adherent *Legionella pneumophila*. *J Appl Bacteriol* 71:531–538
4. Yamamoto H, Ezaki T, Ikeda M, Yabuuchi E (1991) Effects of biocidal treatments to inhibit the growth of legionellae and other microorganisms in cooling towers. *Microbiol Immunol* 35(9): 795–802
5. Kim BR, Anderson JE, Mueller SA, Gaines WA, Kendall AM (2002) Literature review-efficacy of various disinfectants against *Legionella* in water systems. *Water Res* 36(18):4433–4444
6. Skaliy P, Thompson TA, Gorman WG, Morris GK, McEachern HV, Mackel DC (1980) Laboratory studies of disinfectants against *Legionella pneumophila*. *Appl Environ Microbiol* 40(4): 697–700
7. Domingue EL, Tyndall RL, Mayberry W, Pancorbo OC (1988) Effects of three oxidizing biocides on *Legionella pneumophila* serogroup 1. *Appl Environ Microbiol* 54(3):741–747
8. García MT, Pelaz C (2008) Effectiveness of disinfectants used in cooling towers against *Legionella pneumophila*. *Chemotherapy* 54:107–116
9. Silver Peroxide (Undated) Available on the Internet at: www.silverperoxide.com/Brochure%20silver-%20peroxide.pdf
10. Bronopol (Undated) Available on the Internet at: <http://www.vce.org/bronopol.html>
11. Shepperd JA, Waigh RD, Gilbert P (1988) Antibacterial action of 2-bromo-2-nitropropane-1, 3-diol (bronopol). *Antimicrobial Agents Ch* 32(11):1693–1698
12. Denyer SP, Stewart GSAB (1998) Mechanisms of action of disinfectants. *Int Biodeter Biodegr* 41:261–268
13. Karsa DR (2007) Biocides. In: Johansson I, Somasundaran P (eds) *Handbook for cleaning/decontamination of surfaces*, vol 1, F2. Elsevier, Amsterdam, pp 593–624
14. Zeybek Z, Türetgen I, Kimiran-Erdem A, Filoglu G, Cotuk A (2009) Profiling of environmental *Legionella pneumophila* strains by randomly amplified polymorphic DNA method isolated from geographically nearby buildings. *Environ Monit Assess* 149: 323–327
15. ASTM Committee on Standards (1991) E 645-91 standard test method for efficacy of microbicides used in cooling systems, pp 325–332
16. Kimiran-Erdem A, Sanli-Yurudu NO, Cotuk A (2007) Efficacy of a quaternary ammonium compound against planktonic and sessile populations of different *Legionella pneumophila* strains. *Ann Microbiol* 57(1):121–126
17. Sanli-Yurudu NO, Kimiran-Erdem A, Cotuk A (2007) Studies on efficacy of chloramine T trihydrate (*N*-chloro-*p*-toluene sulfonamide) against planktonic and sessile populations of different *Legionella pneumophila* strains. *Int J Hyg Envir Heal* 210: 147–153
18. Elsmore R (1993) Microbiocides and the control of *Legionella*. In: Barbaree JM, Breiman RF, Dufour AP (eds) *Legionella current status and emerging perspectives*. ASM 1325 Massachusetts Ave, N. W. Washington, DC, pp 250–253
19. Grace RD, Dewar NE, Barnes WG, Hodges, GR (1981) Susceptibility of *Legionella pneumophila* to three cooling tower microbicides. *41(1)*: 233–236
20. Soracco RJ, Gill HK, Fliermans CB, Pope DH (1983) Susceptibilities of algae and *Legionella pneumophila* to cooling tower biocides. *Appl Environ Microbiol* 45(4):1254–1260
21. Green PN, Pirrie RS (1993) A laboratory apparatus for the generation and biocide efficacy testing of *Legionella* biofilms. *J Appl Bacteriol* 74(4):388–393

22. Lin YS, Stout JE, Yu VL, Vidic RD (1998) Disinfection of water distribution systems for *Legionella*. *Semin Respir Infect* 13(2): 147–159
23. Bentham RH, Broadbent CR (1995) Field trial of biocides for control of *Legionella* in cooling towers. *Curr Microbiol* 30: 167–172
24. Fux CA, Shirtliff M, Stoodley P, Costerton JW (2005) Can laboratory reference strains mirror ‘real-world’ pathogenesis? *Trends Microbiol* 13(2):58–63
25. Kusnetsov JM, Tulkki AI, Ahonen HE, Martikainen PJ (1997) Efficacy of three prevention strategies against *Legionella* in cooling water systems. *J Appl Microbiol* 82:763–768