Evaluation of bacterial resistance to Chloramine T Biocide and effects on Rifampicin Antibiotic Susceptibility as a consequence of biocide usage in Cooling System Biofilm including *Legionella pneumophila*

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The aim of the study was to investigate the development of bacterial resistance to Chloramine T and subsequent effects on rifampicin susceptibility. Both the efficacy of biocide and antibiotic on the mature biofilm and the co-evaluation of biocide and antibiotic resistance in *Legionella pneumophila* were investigated. 4 month-old mature biofilm samples which grown in an open recirculating model system to simulate the environment of an industrial cooling water system. After treatment with antimicrobials, the samples were analyzed for heterotrophic plate count, the presence of *Legionella* spp., total and direct viable count, the total and free ATP concentration. As an oxidizing biocide preferred in the study, Chloramine T trihydrate is purported not to induce resistance build-up; according to our results, it correspondingly did not build up a cross resistance against the rifampicin preferred in the treatment of Legionnaires' Disease.

Keywords: Chloramine T trihydrate; Rifampicin; Cooling tower; Cross resistance

1. Introduction

Cooling tower is a unit which removes heat. The cooling water flows to the operating units in order to pick up the heat and cool or condense process streams, and the hot water emerged as a result of this operation is returned to the cooling water [1].

Cooling towers provide an ideal environment for microbial growth and have been attributed as a source for accumulation and dissemination of pathogenic organisms, especially *Legionella pneumophila* [2, 3]. Microorganisms are carried to cooling tower water system via make-up water and air, and adhere to the solid surfaces in order to reach nutrients more easily. Using nutrients, they reproduce and release extracellular polysaccharide substances (EPS), and thus form biofilm. The formation of biofilm brings about serious problems such as energy loss, pipe blockage and corrosion of metal surfaces in cooling tower [4-6].

Cooling systems potentially carry a risk for public health due to the fact that *Legionella* infections have mostly been detected in contaminated aerosols which are produced even at such distances as over 6 km [7]. Inhalation of *Legionella*-contaminated aerosols may cause Legionnaires' disease or Pontiac fever in humans [8].

The most common approach to eliminate or reduce the biofouling problem in contaminated systems is chemical treatment primarily by using biocides [9]. Owing to the fact that biofilm bacteria are more resistant than their planktonic counterparts to the biocides [10], increasing biocide concentrations can lead to microbial resistance build-up first to the biocide and then consequently to the antibiotics [11]. Manufacturers recommend Chloramine T trihydrate (N-chloro-p-toluene sulfonamide) as a commercial formulation since it inhibits biological growth in cooling towers. It is widely known that Chloramine T is stable in solutions at high temperatures and has only minor corrosive effect on materials. According to our knowledge, there is very little information at hand on the effect of Chloramine T acting against bacteria *in vitro* conditions but no information about cooling towers [12, 13].Consequently, in this study, the effect of Chloramine T biocide and the co-evaluation of Chloramine T biocide and rifampicin antibiotic on 4-month old mature biofilm which included *L. pneumophila* were investigated in a model cooling water system under laboratory conditions. For this purpose, after the application of antibacterial compounds, the samples were analyzed in terms of heterotrophic plate count (HPC), the number of *L. pneumophila*, the epifluorescence microscopy, the total and free ATP concentration.

2. Materials and Methods

2.1 Model system

The experimental study was performed by using a 100-liter polypropylene laboratory scale in an open recirculating model system to simulate the environment of an industrial cooling water system (Fig. 1). The municipal water was recirculated with a pump (550 W, 40 l.min-1, Pedrollo, Italy) and heated to keep temperature constant at 37°C by a heater (AT–100, 100 W, Atman, Germany). The cover lid had openings to ensure the fresh air and daylight entry. The microbial flora was developed spontaneously but *L. pneumophila* was inoculated to the model system. The municipal water was used to replenish water lost by evaporation and blowdown. The glass coupons were inserted vertically into

the coupon–holders situated in the water basins. The chemicals such as disinfectants, pH regulators and anti-scaling agents were not added to the system in order to protect it from negative effects that may come about on microorganisms and biofilm formation.

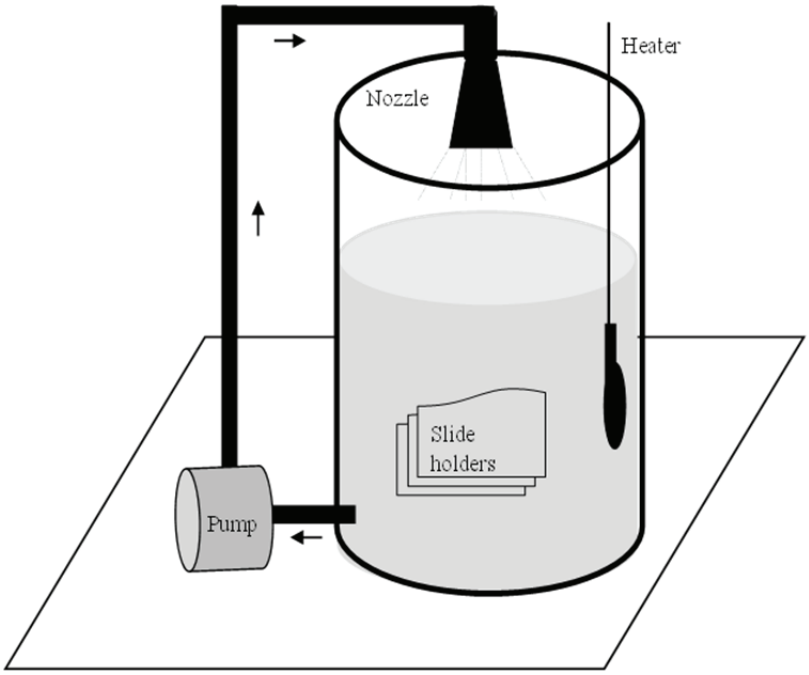

Fig. 1 The schematic diagram of model recirculating water system, with arrows indicating the flow direction.

2.2 Physico-chemical quality of the water in the model system

The system water was investigated for some physico-chemical parameters at the 4th month, such as pH, dissolved oxygen, conductivity, total dissolved solids (TDS).

2.3 Biocide and Antibiotic Preparation

2000 mg l^{-1} dosages of Chloramine T trihydrate and recommended rifampicin antibiotic concentration for patients with Legionnaires' disease (4.16 mg ml^{-1}) were prepared in sterile demineralized water. Free chlorine doses were measured by the N, N-diethyl-p-phenylene diamine (DPD) procedure [Hach, Model CN–66]. The difference between total and free chlorine value was multiplied by 3.97 and calculated as active residual biocide concentration [12].

2.4 Determination of Biocidal and Antibiotic Activity against Heterotrophic Bacteria and *L. pneumophila*

4 month-old mature biofilm samples which had been grown on glass surfaces were divided into 4 groups: 1st group was exposed to the recommended dosage of biocide for cooling water systems (2000 mg l^{-1}) for 24 hours, 2nd group to 4.16 mg ml^{-1} rifampicin antibiotic for 24 hours, 3rd group slides to the same antibiotic concentration for 24 hours after the same biocide concentration and contact time exposure, and 4th group was used as control. After all contact times, samples were analyzed for heterotrophic plate count (HPC), the presence of *Legionella* spp., total (DAPI) and direct viable count (CTC), total and free ATP concentration.

The biofilm on the surfaces was scraped by a sterile cotton swab and suspended in 10 ml sterile tap water by vortexing for 60 sec and stomaching 60 sec [14]. The resulting suspensions were serially diluted from 10^{-1} to 10^{-5}. To estimate the number of aerobic heterotrophic bacteria, triplicate 100 µl volumes of each dilution (10^{-1} to 10^{-5}) were spread onto R2A agar (OXOID, UK) plates and aerobically incubated at 27 °C for 7 days [15]. After incubation, for each sample, the dilution that was counted contained between 30 and 300 colonies. The number of colonies was enumerated by a colony counter (åCOLyte Super Colony Counter, Synbiosis), and recorded as CFU.cm^{-2} for biofilm samples.

For the detection of *L. pneumophila*, 5 ml of biofilm suspensions was acid-treated with KCl–HCl for 5 min (pH 2.2). Additionally, 2 ml of water suspension was heat-treated at 50 °C for 30 min; 100 µl pretreated samples and untreated samples with acid/heat were inoculated in a buffered charcoal yeast extract (BCYE) (Oxoid, UK) agar. All plates were incubated at 37 °C for 10–14 days. Analyses were carried out in triplicate. The suspected colonies were subcultured to tryptone soy agar. A latex agglutination kit was used for the identification of *L. pneumophila* [16, 17].

2.5 CTC/DAPI Staining

The redox dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and the DNA-binding fluorochrome, 4′,6-diamidino-2-phenylindole (DAPI) have been used to detect direct viable counts of actively respiring bacteria and the enumeration of the total bacteria cells, respectively [18, 19].

The biofilm suspensions (900 µl) were incubated with aliquots of a 50 mM CTC redox dye solution to a final concentration of 5 mM in the dark at 28°C for 4 h. Following the CTC incubation, 1.0 $\mu g.ml^{-1}$ DAPI solution was added to samples and were incubated for 1 h, in the dark, at 28°C. After incubation, the samples were filtered by vacuum filtration onto black 0.2 µm pore size polycarbonate filters (Millipore, USA) [20, 21]. The membrane was air-dried and mounted on a glass slide with non-fluorescent immersion oil below the filter and coverslipped. Microscope slides were examined under oil immersion in a Nikon 80i microscope equipped with a 100 W mercury lamp and appropriate filters for CTC, and DAPI. The number of bacteria was estimated from the counts of 20 randomly chosen microscopic field (at X 1000). An eyepiece with a graticule calibrated was used for all bacterial counting. Estimation of number of cells in each sample was calculated as follows:

$$N = \frac{S \times n}{C \times V} \times D$$

where N, number of microorganisms per milliliter; S, real area of filtration; n, average number of microorganisms per field of vision; C, real area of microscopic range; V, volume of filtered sample; D, sample dilution.

2.6 ATP measurement

The total and free ATP swabs were used to take the homogenized biofilm samples. The swab systems extract ATP from microbial cells. The released ATP reacts with luciferin and luciferase which are in a specialized container in the swab; subsequently, the whole contraption is inserted into the luminometer (Uni-Lite Biotrace) in a few seconds and the relative light units (RLUs) were returned [22-25]. The difference between total and free ATP determines the microbial ATP concentration.

2.7 Statistical Analysis of Data

Bacterial counts were transformed to log10 and standard deviations of the means were calculated. Results were analyzed statistically by Student's t-test. All the analyses were done using SPSS for Windows Version 11.5 and $p<0.05$ was accepted as statistically significant.

3. Results and Discussion

In the current study, the effectiveness of Chloramine T trihydrate biocide which is recommended to use in cooling towers to inhibit bacterial growth and of rifampicin antibiotic which is used to treat in Legionnaires' Disease were tested individually against 4-month old mature biofilms which included *L. pneumophila*. Additionally, the development of bacterial resistance to Chloramine T and the subsequent effect on rifampicin susceptibility in *L. pneumophila* were investigated. Although chlorine is widely used in many water systems and cooling towers, since all forms of chlorine are highly corrosive and toxic to humans and environment and free chlorine produces carcinogenic trihalomethanes after a reaction with organic matter, the usage of alternative biocides has been suggested in recent years. In this study, it has been argued that Chloramine T is a potential alternative to chlorine, as it is safe for humans, non-cytotoxic, stable in solutions at high temperatures, environmentally friendly and has no risk of inducing bacterial resistance [13]. Chloramine T [$C_7H_7SO_2NNaCl(3H_2O)$] should be considered different from monochloramine. Pure monochloramine (NH_2Cl) is an unstable liquid and it is produced by adding chlorine to a solution containing ammonia, whereas Chloramine T is an organic compound produced by chlorinating benzene sulfonamide [26]. Chloramine T is capable of binding to enzymes and altering their characteristics [27]. To our knowledge, little information on the effect of Chloramine T against bacteria that may be found in cooling towers [28] and no information about antibiotic susceptibility subsequent to biocide exposure in cooling towers has been reported.

In general, the data of biocidal treatment tests are based on laboratory experiments; however, these studies do not reflect the conditions of real systems and the fate and the resistance of the microorganisms are unknown after biocide applications. Therefore, in this study, the biofilm in a constructed model system was formed to mimic the natural microbial flora of a full-scale cooling system. Since it has been known that different factors such as pyhsico-chemical characteristics of water may influence the activity of biocide, in this study, pH, dissolved oxygen, conductivity, total dissolved solids (TDS) were measured (Table 1).

Table 1 The Physico-chemical parameters of the model system at 4th month

	pH	DO ($mg\ L^{-1}$)	TDS ($mg\ L^{-1}$)	Conductivity ($\mu S\ cm^{-1}$)
Model system	8.19	6.07	644	880
Make-up water	7.00	8.02	210	430

The model system water was drained in order to maintain a steady concentration of dissolved solids intermittently from the model system. The tap water was used as make-up water to balance the water losses by evaporation and blow down (drainage) from the system. Nevertheless, it was determined that all the physico-chemical parameters of the model system were higher than the physico-chemical parameters of the make-up water. These organic/inorganic deposits facilitate microorganisms' proliferation, which will increase the system's fouling.

Traditionally, the effectiveness of biocides against microorganisms is determined in agar media by using the colony counting method. However, because of injury of metabolism or nutrient shock, this method is not effective to determine actual bacterial counts in the biofilm of water systems. Alternatively, in order to determine metabolically active biomass, different methods are suggested such as measurement of ATP, determination of total and live microbial populations by staining with specific fluorescent dyes [4, 29, 30].

The heterotrophic bacteria and *L. pneumophila* counts in the biofilm samples treated with biocide and antibiotic and in the untreated control samples were presented in Table 2.

Table 2 Heterotrophic bacteria and *L. pneumophila* counts of biofilm samples exposed to Chloramine T, Antibiotic (Rifampicin), Chloramine T+ Antibiotic (Rifampicin)

($CFU\ cm^{-2}$)	Chloramine T (24 Hour)	Antibiotic (Rifampicin) (24 Hour)	Chloramine T+ Antibiotic (Rifampicin)	Control (24 Hour)
HPC	log 0.47	0	0	log 4.89
L. pneumophila	0	0	0	log 3.27

CFU: Colony Forming Unit

After the application of Chloramine T, the heterotrophic bacterial counts on the surfaces were decreased in comparison to the control. In addition, the heterotrophic bacterial counts decreased to zero as a result of the application of antibiotic, and Chloramine T+antibiotic. *L. pneumophila* counts were decreased to zero at the end of the contact time by all tested antimicrobial agents (Table 2). Chloramine T, rifampicin, and Chloramine T+rifampicin achieved > 4 log reduction in the cultivable heterotrophic bacteria, and > 3 log in the *L. pneumophila* counts.

It has also been detected that apart from biocide that was exclusively applied in the plate count, the application of antibiotic, and biocide + antibiotic reduced the count of the cultivable bacteria in the biofilm to zero, but the rate of viability did not decrease in the samples stained with fluorescent dyes (Table 3), (Fig. 2).

Table 3 Total (live + dead) and live microorganisms count (log $cell.ml^{-1}$) of biofilm samples exposed to Chloramine T, Antibiotic (Rifampicin), Chloramine T+ Antibiotic (Rifampicin)

	Chloramine T (24 Hour)	Antibiotic (Rifampicin) (24 Hour)	Chloramine T+ Antibiotic (Rifampicin)	Control (24 Hour)
Total microorganisms count[a]	6.44	7.82	7.66	5.86
Live microorganisms count[b]	6.41	7.81	7.65	5.84
Respiration activity (%)	93.75	97.19	98.00	97.72

[a]Total microorganisms' counts determined by DAPI + CTC staining
[b]Live microorganisms' counts determined by CTC staining

When biofilm was exposed to antibiotic for 24 hours following the treatment with biocide it has been observed that the biocide has not affected the biofilm and the bacteria have recovered and grown stronger after the 24-hour antibiotic application.

Antimicrobials have negative impacts on the cultivability of bacteria and could induce viable but not culturable (VBNC) state in bacteria [31]. Therefore, to measure the total cell count and respiratory active cells, cultivation-independent DAPI/CTC staining technique was used. In our study, the bacteria count obtained from culture results was much lower than that in the result of DAPI-CTC analysis.

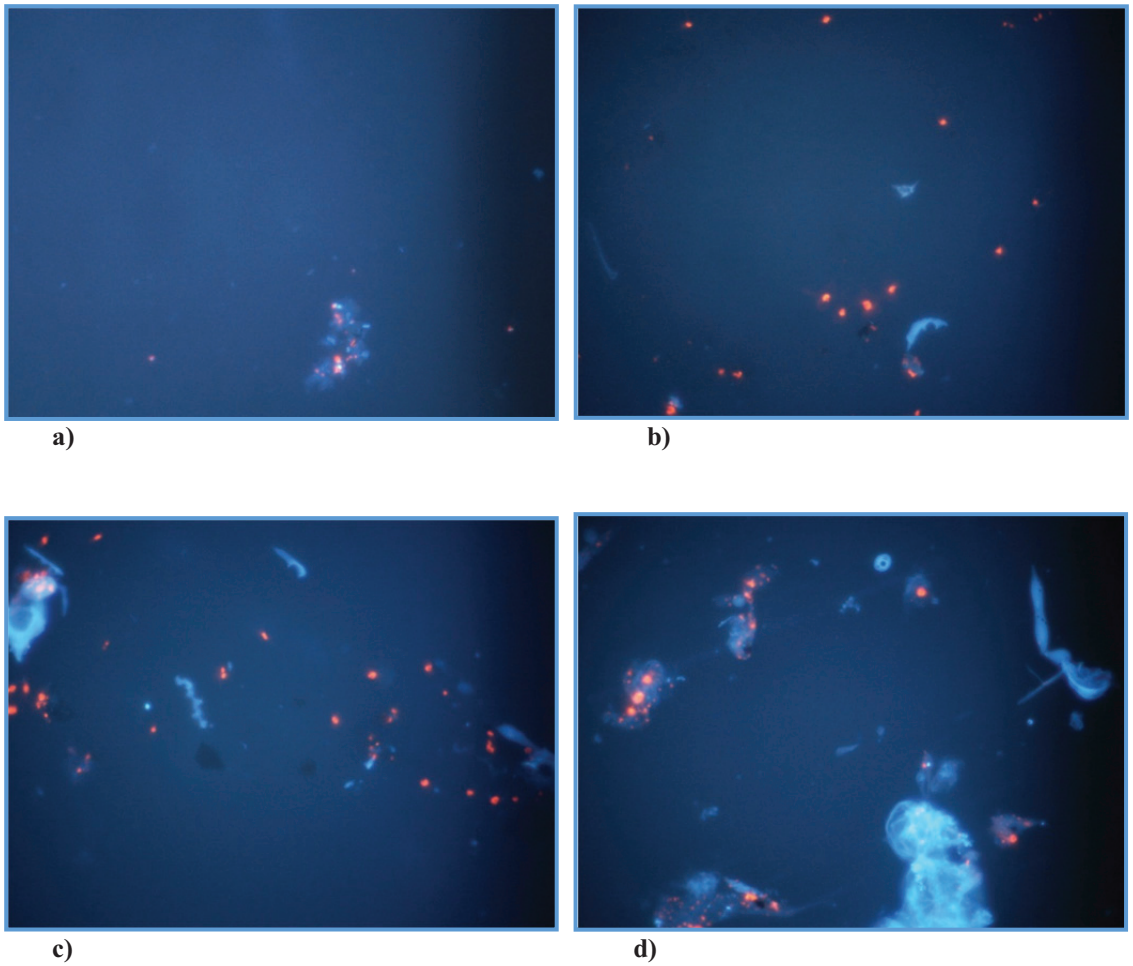

Fig. 2 Representative epifluorescence microscopy images of biofilm samples exposed to a) Chloramine T trihydrate, b) Rifampicin, c) Chloramine T trihydrate+ rifampicin and d) Control

The total and free ATP values were shown in Table 4. It has been detected that all the antimicrobial applications ensured a significant rate of reduction in the total and free ATP values compared to the control ($P < 0.05$). In addition, ATP measurements support DAPI-CTC results. Therefore, DAPI-CTC and ATP measurements must be absolutely conducted in addition to the colony counting method.

Table 4 Total and free ATP levels (RLU.ml^{-1}) of biofilm samples exposed to Chloramine T, Antibiotic (Rifampicin), Chloramine T+ Antibiotic (Rifampicin)

Chloramine T (24 Hour)		Rifampicin (24 Hour)		Chloramine T+ Rifampicin		Control (24 Hour)	
Total ATP	**Free ATP**	**Total ATP**	**Free ATP**	**Total ATP**	**Free ATP**	**Total ATP**	**Free ATP**
6.4	14.1	7.1	37	4.4	6.6	81	29.5

RLU: Relative Light Unit

According to the standard test method for evaluating the efficacy of microbicides used in cooling water systems [32], the minimum level of biocidal performance has been defined only for the culture method as 90% kill or 1 log reduction. On the other hand, while the usage of new and different techniques has been suggested, in recent years, the minimum level of performance has been considered to show the efficacy of a microbicide according to these new test techniques has not yet been specified in any of the standard. Since, these new techniques are essential for the evaluation of both water systems and biocidal activity accurately, standards should be revised.

It has been known that VBNC bacteria are alive and lose their ability to grow on microbiological culture media [31, 33]. Especially, it has been shown that *L. pneumophila* can enter into a VBNC state as a consequence of antimicrobial treatments, but retain its virulence and multiply in its host. As a result, the dissemination of legionellae (both culturable and VBNC state) from cooling towers continue to pose serious risk for the public health. Therefore, biocide which will not cause co-resistance to antibiotics should be selected. In this context, in the current study, the tested biocide does not

generate resistance to the rifampicin antibiotic which is widely used in the treatment of Legionnaires' disease. Although Chloramine T biocide induces VBNC in bacteria, this biocide can be recommended since it does not cause subsequent resistance to the antibiotic in bacteria.

As can be seen in our experimental results, if the colony counting method is used as the only base for the evaluation of biocide, biocide will be evaluated as effective; however, if DAPI-CTC results are taken into consideration, it will be able to be seen that biocide is weakly effective.

As a conclusion,

i) The standard methods for evaluating the efficacy of microbicides used in cooling water systems should be supplemented by different, special growth-independent assays.

ii) Chloramine T biocide can be recommended since it does not cause subsequent resistance to rifampicin antibiotic which is widely used in the treatment of Legionnaires ' disease.

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