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INDIVIDUAL AND COMBINED EFFECTS OF COPPER AND SILVER IONS ON INACTIVATION OF *LEGIONELLA PNEUMOPHILA*

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Abstract—Copper/silver ionization is a new disinfection method that is being used to eradicate *Legionella pneumophila* from hospital hot water recirculating systems. The objective of this study was to determine the susceptibility of *L. pneumophila* serogroup 1 to copper and silver ions alone and in combination. *L. pneumophila* serogroup 1 (*L. p.* sg-1) was completely inactivated (6-log reduction) at copper concentration of 0.1 mg/l within 2.5 h, whereas more than 24 h was required to achieve a similar reduction at the highest silver ion concentration tested (0.08 mg/l). Checkerboard method and *Gard* additive model prediction demonstrated that copper and silver ions in combination could result in additive or synergistic effect depending on the concentration of copper and silver ions. Under the experimental conditions used in this study, synergism of copper/silver ions in eradicating *L. p.* sg-1 was observed at higher concentration combinations of copper/silver ions (e.g. 0.04/0.04 mg/l) while only an additive effect was observed at lower concentration combinations (e.g. 0.02/0.02 mg/l). This study suggested that both copper and silver ions are effective in inactivating *L. pneumophila* and the combined effect is greater than that seen with either ion alone. Copyright © 1996 Elsevier Science Ltd

Key words—copper, disinfection, kinetics, *legionella*, silver, synergism

INTRODUCTION

Legionnaires' disease is a type of pneumonia caused by the bacterium, *Legionella pneumophila*, and has been linked to the presence of *Legionella pneumophila* in water distribution systems (Tobin *et al.*, 1981; Stout *et al.*, 1985). Control of hospital-acquired Legionnaires' disease has been accomplished by disinfecting the hospital water distribution system. The techniques used to eradicate *Legionella* include thermal eradication (superheat and flush), hyperchlorination, ozone, and UV light. However, each of these disinfection modalities has notable disadvantages (Skaliy *et al.*, 1980; Muraca *et al.*, 1987; Muraca *et al.*, 1990). Elevated water temperatures pose a risk of scalding and recolonization often occurs within months. Hyperchlorination requires a chlorine concentration greater than 3 ppm which accelerates corrosion of pipes and increases the concentration of trihalomethanes and other carcinogenic by-products (Jolley *et al.*, 1985).

Heavy metals such as copper and silver ions are known bactericidal agents (Clarke, 1983; Wood, 1984). Copper/silver (Cu^{2+}/Ag^{+}) ionization has been shown to be an effective method for controlling *Legionella* in hospital hot water systems using 0.2–0.4 mg/l of copper and 0.02–0.04 mg/l of silver ions (Liu *et al.*, 1994). The copper and silver ions were introduced by recirculating hot water through a flow cell containing electrodes made of 90:10 copper/silver metal alloy. The advantages of copper/silver ionization compared to other disinfection techniques include relatively low cost, straightforward installation, easy maintenance, and the presence of the residual disinfectant throughout the system. The concentrations used are well below the maximum contaminant levels (MCLs) established by the EPA which are 1.3 mg/l for copper and 0.05 mg/l for silver as a secondary standard. In addition, copper/silver ions are added only into the hospital hot water recirculating lines which facilitates very limited direct contact with human beings.

Eradication of microorganisms with copper/silver ions is attributed to positively charged ions which are both surface-active and microbiocidal. These ions attach onto the negatively charged bacterial cell wall and destroy cell wall permeability. This action coupled with protein denaturation induces cell lysis and death (Friedman and Dugan, 1968; Bitton and Freihofer, 1978; Slawson *et al.*, 1990). However, the effect of individual copper and silver ions as well as the nature of the interaction of these ions in combination against *Legionella* have not yet been investigated. Therefore, the individual and combined

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effects of copper and silver ions on inactivation of *L. p.* sg-1 were evaluated in this study.

MATERIALS AND METHODS

Test organism

An environmental isolate (VAMC No. 1269) of *L. pneumophila* serogroup 1 (*L. p.* sg-1) was selected as the test organism. *L. p.* sg-1 was transferred from −20°C stock broth and inoculated onto buffered charcoal-yeast extract (BCYE) agar medium. Inoculation was repeated after 48 h of incubation at 37°C to achieve logarithmic growth. After 24 h, the culture was removed and suspended in about 30 ml of sterile deionized water. The cells were washed twice by centrifugation at $1000 \times g$ (2500 rpm) for 10 min. Two milliliters of suspension was removed and standardized by comparison with the turbidity of McFarland No. 1 standard (approximate density of 3×10^8 CFU/ml) using a colorimeter (BioMerieux Vitek Inc., Hazelwood, MO). One milliliter of the standardized suspension was transferred to the test solution to achieve the initial concentration of 3×10^6 CFU/ml for each experiment.

Copper and silver test solutions

Copper and silver ion solutions were prepared by dissolving $CuCl_{2(s)}$ and $AgCl_{(s)}$ (Aldrich Chemical Co., Milwaukee, WI) in deionized water. Stock solutions of Cu^{2+} and Ag^+ at 10 mg/l and 1 mg/l, respectively, were prepared in advance and transferred to test solutions using a proper dilution scheme. Actual ion concentrations were confirmed at the beginning of each experiment by atomic adsorption spectrophotometry (AAS). Flame AAS and HGA graphite furnace AAS (AA Model 4000 & 5100, Perkin-Elmer Co., Norwalk, CT) were used to measure high and low concentrations of metallic ions, respectively. Teflon® flasks (250 ml) were used for all batch experiments to prevent loss of Cu^{2+} and Ag^+ caused by the adsorption onto the walls of the container. All test solutions were sterilized by steam sterilization and/or membrane filtration.

Measurement of free active ions in the solution was performed by filtering the liquid sample through 0.2 μm nylon filter (Cole Parmer Co., Chicago, IL) to separate dissolved (free active) copper ions from those attached to the cell wall. Dissolved ion concentration was directly measured by AAS. Concentration of attached ions was determined by acid washing the filter in 3.6 N $H_2SO_{4(aq)}$ followed by AAS analyses.

Checkerboard method

The interaction between copper and silver ions in inactivating *L. pneumophila* was first evaluated by the checkerboard method (Lorian, 1991) in order to quickly determine the inhibitory range of copper and silver concentrations which would later be tested in batch disinfection studies. A microtiter plate (Flow Laboratories, Inc., McLean, VA) with 96 U-bottom wells was used to evaluate the effectiveness of copper/silver ions at multiple dilutions. Each U-bottom well contained the same initial concentration of *L. p.* sg-1 (3×10^6 CFU/ml) and copper and silver ions at different ratios and concentrations which ranged from 0.01–0.16 mg/l. A 0.01 ml sample was taken from each well after 12 h and directly plated onto BCYE culture media (Detection limit = 100 CFU/ml). The lowest ion concentration that resulted in no growth represented the minimum inhibitory concentration (MIC).

The combined effect of copper/silver ions was evaluated for synergy using the checkerboard method. Fractional Inhibitory Concentration Index (FIC Index) (Lorian, 1991) is a quantitative measure of the efficiency of the combination of two antimicrobial agents A and B that can be calculated according to the following equation:

$$\frac{[A]}{MIC_A} + \frac{[B]}{MIC_B} = FIC_A + FIC_B = \text{FIC Index}$$

For the case of Cu^{2+}/Ag^+ ions, this equation can be modified as follows

$$\frac{[Cu]}{MIC_{Cu}} + \frac{[Ag]}{MIC_{Ag}} = FIC_{Cu} + FIC_{Ag} = \text{FIC Index}$$

where, $[Cu]$ (or $[Ag]$) = concentration of copper ions (or silver ions) in a well containing both copper and silver ions which exhibited the lowest inhibitory concentration.

MIC_{Cu} (or MIC_{Ag}) = minimal concentration required to achieve complete eradication by copper ions (or silver ions) alone.

FIC_{Cu} (or FIC_{Ag}) = fractional inhibitory concentration of copper ions (or silver ions).

According to this method, the combined effect of antimicrobial agents can be classified as synergism if the FIC Index is below 1. If the FIC Index is above 2, the interaction of the antimicrobial agents is classified as antagonism. FIC index between 1 and 2 indicates simple additivity of efficiencies for individual antimicrobial agents.

Batch disinfection study

Batch disinfection experiments were used to evaluate the effectiveness of individual copper and silver ions. Approximately 3×10^6 CFU/ml of *L. p.* sg-1 were introduced to a total of 100 ml of deionized water buffered at pH 7.0. Carbonate buffer prepared from 0.05 M sodium bicarbonate solution adjusted to 7.0 with 12 N $HCl_{(aq)}$ was chosen to maintain the constant pH in all experiments. Actual bacterial concentration was determined using the plate count of the sample withdrawn at time

zero. Teflon® flasks were placed on a shaker and temperature was controlled at 37°C. Upon the addition of disinfectant solution, *L. p.* sg-1 concentration was monitored for a predetermined period of time. Sample (1 ml) withdrawn from the batch reactor was mixed with 10 μl neutralizer solution immediately, serially diluted, and plated in duplicate with 0.1 mL of sample solution onto BCYE agar culture media. A neutralizer solution of 14.6% sodium thiosulfate and 10% sodium thioglycolate was used to prevent any further disinfection of *L. pneumophila* during incubation and enumeration (Landeen *et al.*, 1989). The culture plates were incubated for 72 h at 37°C and enumerated for the CFU (detection limit = 10 CFU/ml).

Batch disinfection experiments were also used to evaluate the effectiveness of the combination of copper and silver ions at different concentrations and proportions. The copper/silver ion concentration combination which exhibited the minimum inhibitory concentration in the checkerboard method was chosen as a starting point for batch studies.

Inactivation of a particular organism by a disinfectant is most commonly modeled using the Chick's Law. However, the experimental data obtained in our study did not follow this simple model (correlation coefficient below 0.8) and a *Gard* model was used instead (Montgomery, 1985). According to this model, the inactivation of organisms follows a declining rate as expressed by the following equation:

$$-\frac{dN}{dt} = \frac{kN}{1 + a(Ct)}$$

where, N = Concentration of viable organisms at time t ($N = N_0$ when $t = t_0$)
C = Disinfectant concentration held constant over time
k = First-order rate of deactivation effected at time zero
a = Rate coefficient

When two antimicrobial agents (copper and silver ions) were used in combination, the above equation can be modified as the *Gard* additive model. The differential equation describing the additive behaviour of the two disinfectants can be derived from the original *Gard* model as follows:

$$-\frac{dN}{dt} = \underbrace{\frac{k_1 N}{1 + a_1(C_1 t)}}_{\text{effect of copper}} + \underbrace{\frac{k_2 N}{1 + a_2(C_2 t)}}_{\text{effect of silver}}$$

The integral form of the *Gard* additive model is then:

$$\frac{N}{N_0} = \underbrace{[1 + a_1 \times (C_1 t)]^{-k_1 a_1}}_{\text{effect of copper}} \times \underbrace{[1 + a_2 \times (C_2 t)]^{-k_2 a_2}}_{\text{effect of silver}}$$

Thus, the synergism of two disinfectants is present if the inactivation rate observed in the experimental investigation with combined disinfectants is faster than the rate predicted by the *Gard* additive model using the parameters obtained from rate studies with individual disinfectants.

RESULTS

Checkerboard method

The checkerboard method was used as a preliminary screening test to determine the minimal concentration of copper/silver ions required to achieve 6-log reduction in *L. p.* sg-1 viability. These results were later used as the starting concentrations for the batch disinfection experiments. One checkerboard experiment was performed in duplicate using the concentrations of copper and silver ions in the range 0.01–0.16 mg/l. The minimum concentration of copper/silver ions in combination required to accomplish complete inactivation of *L. pneumophila* within 12 h was 0.04/0.04 mg/l. In contrast, 0.08 mg/l of copper ions alone and 0.16 mg/l of silver ions alone was necessary to achieve the same effect (i.e. $MIC_{Cu} = 0.08$ mg/l, $MIC_{Ag} = 0.16$ mg/l). The FIC index for 0.04 mg/l of copper and silver ions was calculated as follows:

$$\frac{[Cu]}{MIC_{Cu}} + \frac{[Ag]}{MIC_{Ag}} = \frac{0.04}{0.08} + \frac{0.04}{0.16} = 0.75$$

Since the FIC index for this concentration combination was below 1, the interaction of copper and silver ions can be described as synergistic. The batch disinfection studies were then conducted using Cu^{2+}/Ag^{+} concentrations equal to and below 0.04/0.04 mg/l to test the possibility that the synergism would exist even at lower concentrations.

Batch disinfection study I—effectiveness of copper ions

The rate of *L. pneumophila* inactivation observed in the presence of 0.05, 0.1, 0.2, 0.4, and 0.8 mg/l of copper ions is shown in Fig. 1. Each disinfection experiment was performed in triplicate to improve statistical precision of the results. Each data point on time–kill curves represents an average from three experiments performed at different days while each sample was analyzed in duplicate. Vertical bars

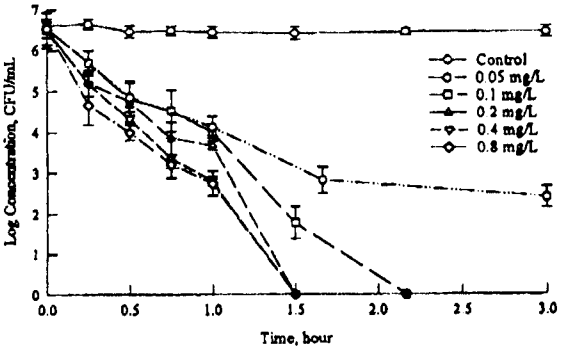

Fig. 1. Inactivation of *L. pneumophila* with copper ions (solid symbols indicate "below detection limit").

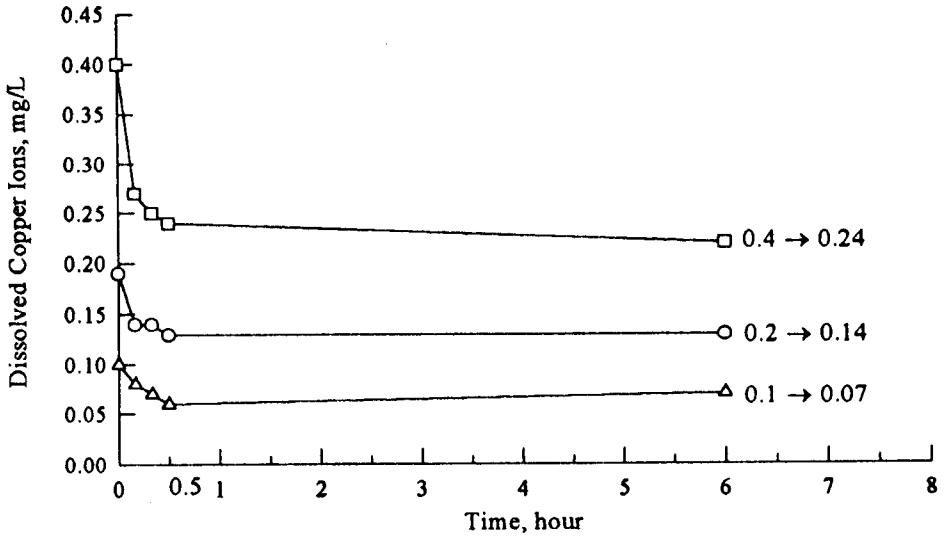

Fig. 2. Variation of dissolved copper ion concentration in test solution during batch experiments.

provided with each data point represent the range of values obtained from these six measurements. As is apparent from Fig. 1, copper ion concentrations in the range from 0.2 to 0.8 mg/l required only 1.5 h to achieve a 6-log reduction, with no significant difference in the rate of *L. pneumophila* inactivation. 0.1 mg/l and 0.05 mg/l of copper ions required 2.5 h and 24 h to achieve the same reduction.

The fate of copper ions in the solution during the 6 h of contact in the batch system is depicted in Fig. 2. Each test was performed in duplicate with initial copper ion concentrations of 0.1, 0.2, and 0.4 mg/l, while the initial concentration of *L. pneumophila* was maintained at 3×10^6 CFU/ml in all cases. There was a rapid decrease of dissolved copper ions in the solution within the first 30 min followed by quick equilibration. The results in Table 1 demonstrated a very good mass balance closure (a close match between dissolved and bound copper ions) indicating that the copper ions lost in the solution were attached to the cells. Equilibrium values achieved in these experiments were used to calculate the amount of copper ions adsorbed by each organism as a function of free dissolved equilibrium copper concentration. These data are presented in Fig. 3, where the uptake capacity in mg Cu^{2+} per CFU (qe) is plotted against free dissolved copper concentration at equilibrium (Ce). The uptake capacity of *L. pneumophila* for Cu^{2+} increased significantly (from 6×10^{-11} to 4×10^{-7} mg Cu^{2+}/CFU) with the increase in equilibrium free Cu^{2+} concentration once the free dissolved copper exceeded 0.2 mg/l. However, the uptake capacity does not change so drastically for the equilibrium free Cu^{2+} concentration in the range of 0.06 to 0.2 mg/l.

Table 1. Fate of copper ions in batch disinfection studies

Initial Cu^{2+} conc. (mg/l)	Final Cu^{2+} conc. (mg/l)		
	Dissolved[a]	Bound[b]	Total
0.1	0.07	0.05	0.12
0.2	0.14	0.05	0.19
0.4	0.24	0.14	0.38

[a]Dissolved Cu^{2+} concentration represents AAS measurement of the filtrate from the test solution.
[b]Bound Cu^{2+} concentration represents AAS measurement of copper ions attached to the cells which was performed by acid washing the filter with 3.6 N $H_2SO_{4(aq)}$.

Batch disinfection study II—effectiveness of silver ions

The results of *L. pneumophila* disinfection experiments with silver ions are shown in Fig. 4. Concentration of silver ions tested in this study were 0.01, 0.02, 0.04, and 0.08 mg/l. More than 24 h was required to completely eradicate *L. pneumophila* even at the highest silver ion concentration tested (0.08 mg/l). The silver ion concentrations of 0.01 and 0.02 mg/l were not sufficient to achieve complete eradication of the organism, and *L. pneumophila* persisted in the system for more than 5 days in both cases (data not shown). The uptake capacity of *L. pneumophila* for silver ions was not investigated because the change in silver ion concentration was below the detection limit of atomic adsorption spectrophotometry.

Batch disinfection study III—effectiveness of combined copper and silver ions

Combination of copper/silver ions at concentrations of 0.02/0.02, 0.02/0.04, 0.04/0.02, and 0.04/0.04 mg/l required 8.2 h, 5.6 h, 3.6 h, and 1.6 h, respectively, to achieve complete inactivation of *L. p.* sg-1 while copper and silver ions alone at these concentration required more than 24 h for complete inactivation. In order to evaluate which concentration combinations of copper/silver ions resulted in synergism, the results from all batch disinfection

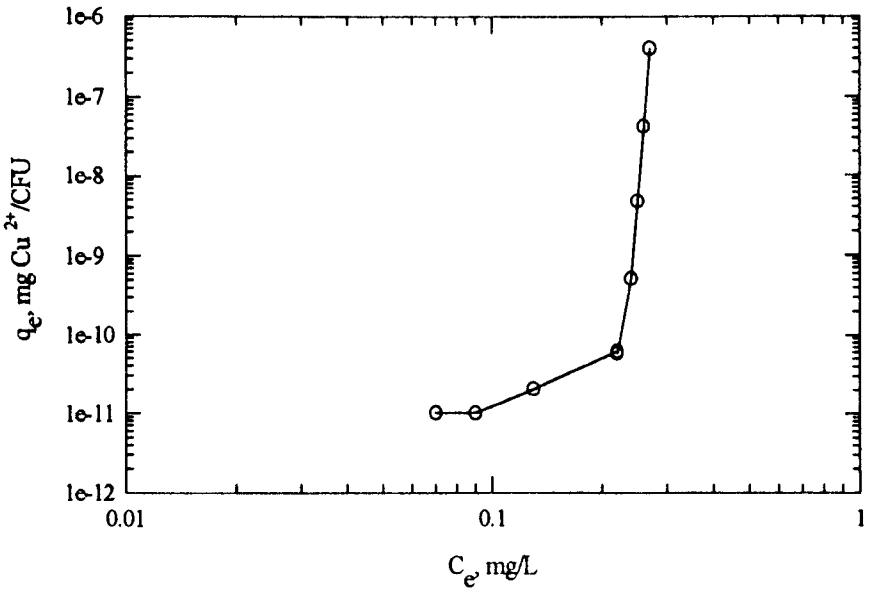

Fig. 3. Adsorptive capacity of *L. pneumophila* for Cu^{2+}.

experiments with copper and silver ions alone were first combined to determine the parameters of the *Gard* model for these disinfectants (Fig. 5). Coefficients a and k for copper and silver ions alone given in Fig. 5 were calculated using a non-linear regression analysis. The Ct value calculated for each data point represented a product of disinfectant concentration and the contact time. Figures 6–9 show the results of batch disinfection studies at four different concentrations of copper and silver ions in combination. Each figure also includes the rate of *L. pneumophila* inactivation predicted by the *Gard* additive model using the parameters given in Fig. 5.

The results showed that there was an additive effect of copper and silver at the copper ion concentration of 0.02 mg/l with silver at 0.02 and 0.04 mg/l since the kill curves observed experimentally are similar to those predicted by the *Gard* additive model (Figs 6 and 7). However, faster disinfection rates were observed at a copper ion concentration of 0.04 mg/l in combination with silver ion concentrations at 0.02 and 0.04 mg/l when compared to the predictions of *Gard* additive model (Figs 8 and 9). Therefore, synergism was observed between copper and silver ions at Cu^{2+}/Ag^{+} = 0.04/0.02 and 0.04/0.04 mg/l, while only an additive effect was observed at lower Cu^{2+}/Ag^{+} concentrations tested in this study.

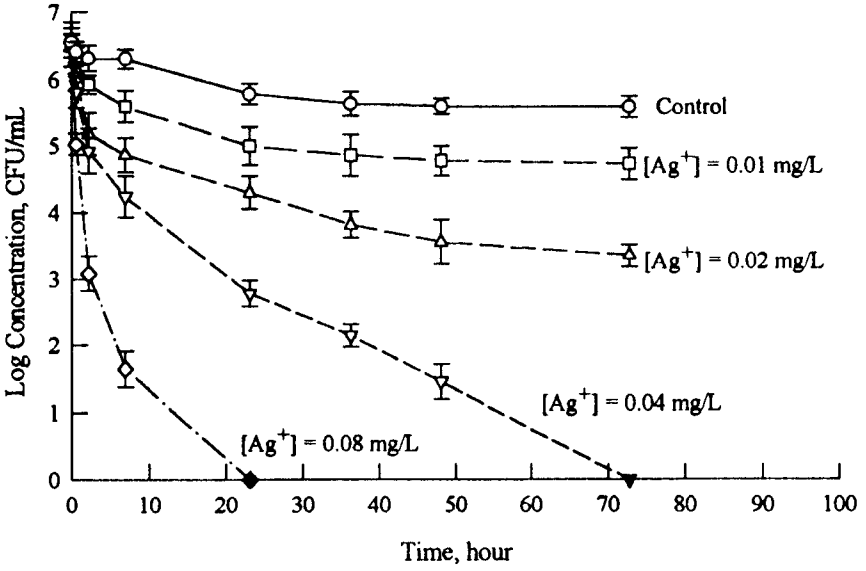

Fig. 4. Inactivation of *L. pneumophila* with silver ions (solid symbols indicate "below detection limit").

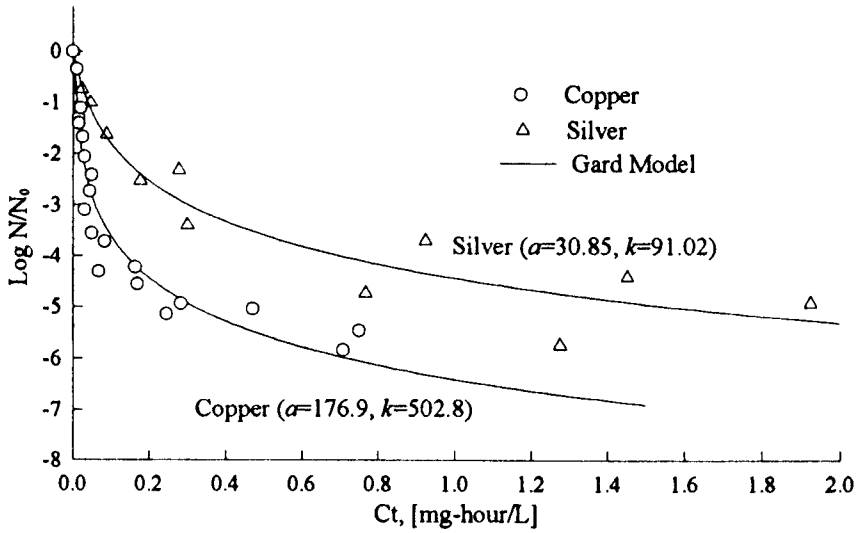

Fig. 5. Inactivation of *L. pneumophila* with individual copper and silver ions.

DISCUSSION

The use of copper and silver ions for inactivating *Legionella pneumophila* has been demonstrated *in vitro* (Landeen *et al.*, 1989) and *in situ* (Liu *et al.*, 1994). Landeen *et al.*, reported enhanced inactivation of *Legionella* when 0.4 mg/l of copper and 0.04 mg/l of silver ions were combined with 0.4 mg/l of chlorine. Liu *et al.*, reported that at copper/silver concentrations of 0.4/0.04 mg/l or greater, *Legionella* was eliminated from a hospital water distribution system. These reports were limited to the study of the combined effect of these ions. Thus, the objective of this study was to determine the effect of each metal ion individually and to determine whether the effect of the combination of these metals was additive or synergistic. It would be difficult to make these determinations in a real water distribution system because of extraneous factors which would unpredictably affect the activity of copper and silver ions in eradicating *Legionella*. Therefore, this study was conducted *in vitro* using two different experimental systems, the microbiological checkerboard method and batch disinfection experiments.

The data observed in this study suggested that both copper and silver ions alone were effective in killing *L. p.* sg-1, and the killing rate for copper ions was faster than for silver ions. This study focused on only one serogroup of *Legionella*, namely *L. pneumophila* serogroup 1. While there are more than 30 serogroups

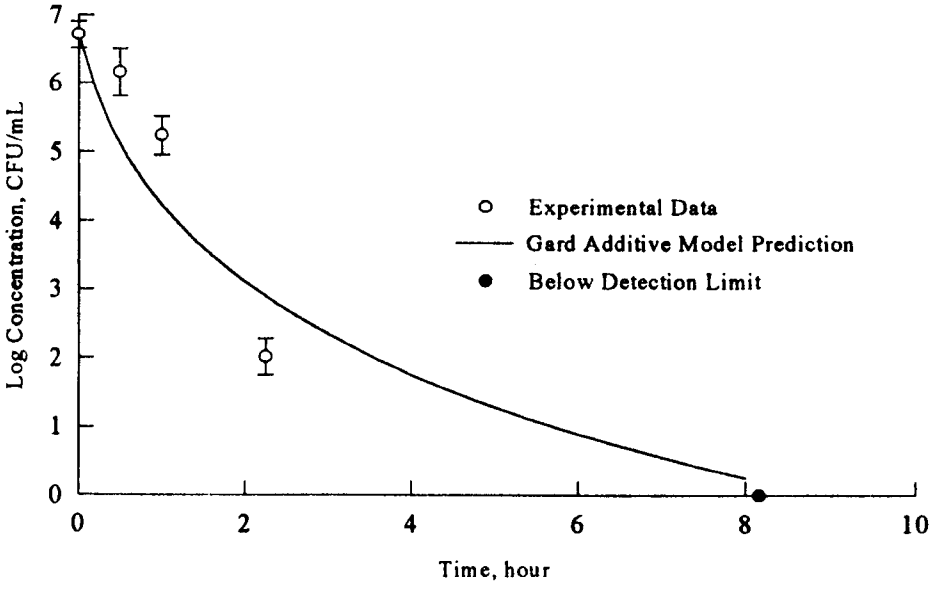

Fig. 6. Inactivation of *L. pneumophila* with 0.02 mg/l of copper and 0.02 mg/l of silver ions.

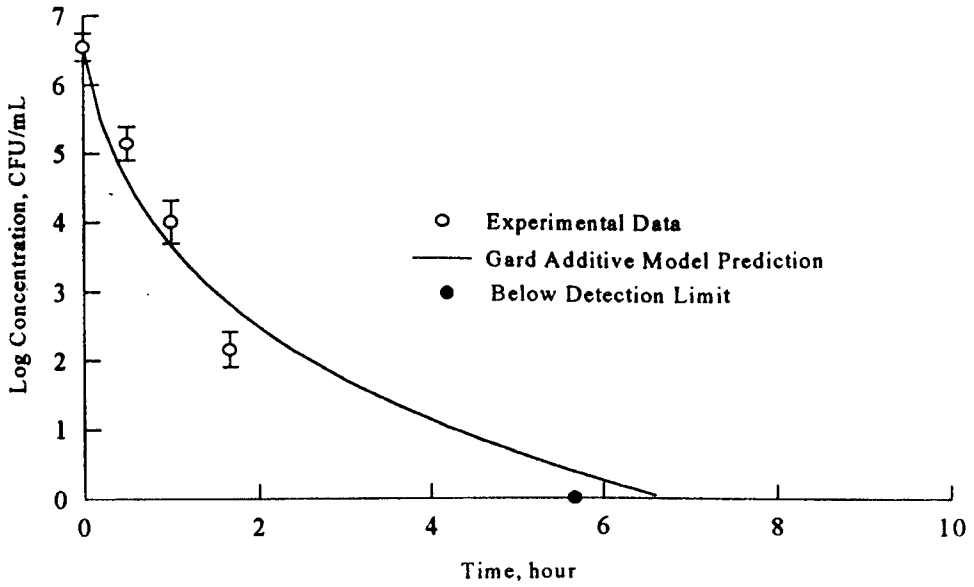

Fig. 7. Inactivation of *L. pneumophila* with 0.02 mg/l of copper and 0.04 mg/l of silver ions.

of *Legionella pneumophila*, *L. p.* sg-1 is the most prevalent and causes over 70% of reported cases of Legionnaires' diseases (Breiman, 1993). The effects of copper and silver ions on other serogroups of *L. pneumophila* or other species of *Legionella* were not investigated in this study and remain to be determined.

Figure 2 and Table 1 showed that the positively charged copper ions that were attached onto the cell wall remained bound to that cell and might become unavailable for inactivation of other cells (Zevenhuizen, 1979). The hypothesis in this study was that each organism requires a certain minimum copper loading to cause cell wall damage and any additional copper ions attached onto the cell wall will not accelerate the death rate of the injured organism. This may explain why higher copper concentrations, such as 0.4 and 0.8 mg/l, did not expedite the inactivation rate of *L. p.* sg-1 when compared to the rate obtained at 0.2 mg/l. In contrast, copper ion concentration in the solution could be the rate limiting factor for the values below 0.1 mg/l as evidenced by the slow rate of inactivation in the case of 0.05 mg/l (Fig. 1).

The concept of synergy is that the combined effect of two drugs is significantly greater than the sum of the effects of the two drugs independently. Both

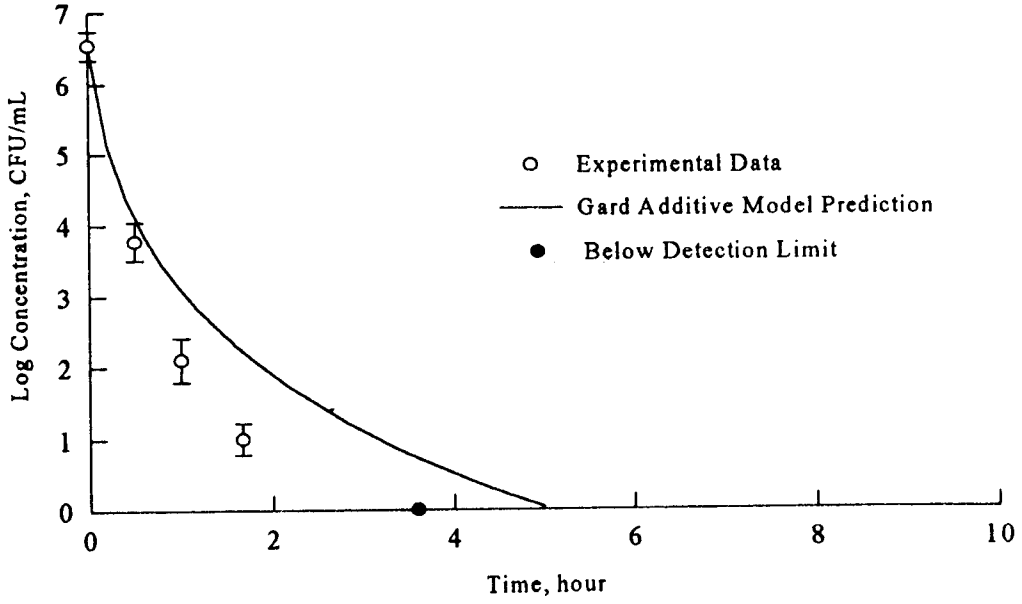

Fig. 8. Inactivation of *L. pneumophila* with 0.04 mg/l of copper and 0.02 mg/l of silver ions.

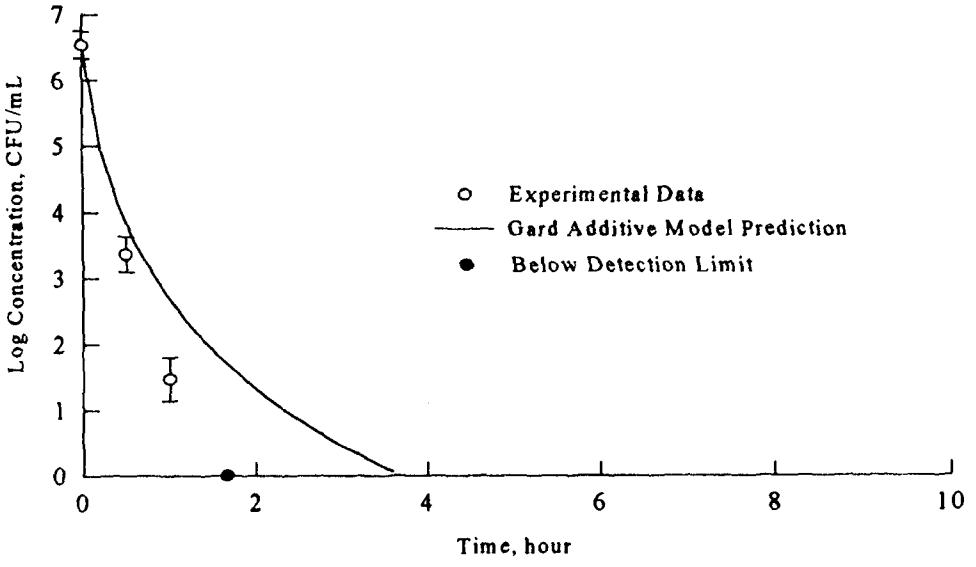

Fig. 9. Inactivation of *L. pneumophila* with 0.04 mg/l of copper and 0.04 mg/l of silver ions.

checkerboard and batch disinfection experiments demonstrated that copper/silver ions in combination resulted in both additive and synergistic interaction in eradicating *L. p.* sg-1. Copper ion is a cell-wall active agent which can destroy cell wall permeability while silver ions interfere with protein and enzyme synthesis (Chambers *et al.*, 1962; Domek *et al.*, 1984). In the batch disinfection studies, synergy was observed at higher copper ion concentrations (Figs 8 and 9). This behavior may be the result of greater intracellular penetration of silver caused by the cell wall disruption induced by the copper ions.

It is also important to note that the copper/silver concentrations that *inactivated L. p.* sg-1 in this study were lower than the 0.4/0.04 mg/l that was used in a controlled evaluation in a hospital water distribution system (Liu *et al.*, 1994). This may be explained by differences in culture condition and experimental systems. First, agar-grown bacteria are known to be more sensitive to disinfectants than water cultivated bacteria. Water-grown *Legionella* was found to be more resistant to iodine and chlorine than agar-grown strains (Kuchta *et al.*, 1985; Cargill *et al.*, 1991). In addition, *L. pneumophila* is predominantly present in water distribution systems in microbial biofilms which can absorb metal ions. Copper and silver ions may also be bound to the chemical compounds present in potable water and rendered biologically unavailable. Therefore, caution should be exercised in extrapolating these *in vitro* results to actual water distribution systems where numerous parameters may impact availability and activity of these metallic ions. Nevertheless, this study showed that both copper and silver ions are bactericidal for *Legionella pneumophila* and that synergism exists under certain conditions. Further, the demonstration of synergy in this study may have practical application for further evaluation of copper/silver ionization disinfection of hospital hot water systems. Specifically, a bactericidal effect may be achieved at lower copper/silver ion concentrations which is desirable given the potential for exposure to these ions via the drinking water.

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