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In vitro efficacy of copper and silver ions in eradicating *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Acinetobacter baumannii*: Implications for on-site disinfection for hospital infection control

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ABSTRACT

Pseudomonas aeruginosa, *Stenotrophomonas maltophilia* and *Acinetobacter baumannii* are major opportunistic waterborne pathogens causing hospital-acquired infections. Copper–silver ionization has been shown to be effective in controlling *Legionella* colonization in hospital water systems. The objective was to determine the efficacy of copper and silver ions alone and in combination in eradicating *P. aeruginosa*, *S. maltophilia* and *A. baumannii* at the concentration applied to *Legionella* control. Kill curve experiments and mathematical modeling were conducted at copper and silver ion concentrations of 0.1, 0.2, 0.4, 0.8 and 0.01, 0.02, 0.04, 0.08 mg/L, respectively. The combinations of copper and silver ions were tested at concentrations of 0.2/0.02 and 0.4/0.04 mg/L, respectively. Initial organism concentration was ca. of 3×10^6 cfu/mL, and viability of the test organisms was assessed at predetermined time intervals. Samples (0.1 mL) withdrawn were mixed with 10 μL neutralizer solution immediately, serially diluted and plated in duplicate onto blood agar plates. The culture plates were incubated for 48 h at 37 °C and enumerated for the cfu (detection limit 10 cfu/mL). The results showed all copper ion concentrations tested (0.1–0.8 mg/L) achieved more than 99.999% reduction of *P. aeruginosa* which appears to be more susceptible to copper ions than *S. maltophilia* and *A. baumannii*. Silver ions concentration of 0.08 mg/L achieved more than 99.999% reduction of *P. aeruginosa*, *S. maltophilia* and *A. baumannii* in 6, 12 and 96 h, respectively. Combination of copper and silver ions exhibited a synergistic effect against *P. aeruginosa* and *A. baumannii* while the combination exhibited an antagonistic effect against *S. maltophilia*. Ionization may have a potential to eradicate *P. aeruginosa*, *S. maltophilia* and *A. baumannii* from hospital water systems.

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1. Introduction

Pseudomonas aeruginosa, *Stenotrophomonas maltophilia* and *Acinetobacter baumannii* ("waterborne pathogens") are Gram-negative bacteria commonly present in chlorinated potable water. These organisms are opportunistic pathogens which do not pose a threat to the general public. However, these organisms have been epidemiologically linked to hospital-acquired respiratory infections in the intensive care units (Squier et al., 2000; Lee et al., 1998; Rusin et al., 1997) which affect millions of hospitalized patients. The hospital potable water system can be the reservoir responsible for these hospital-acquired infections. It has been suggested that waterborne pathogen-related infections could be prevented by avoidance of non-sterile potable water among high-risk patients as well as disinfection of the water distribution systems (von Reyn et al., 1994).

Control of hospital-acquired Legionnaires' disease has been accomplished by disinfection of the hospital supply system (Alyssa et al., 1995; Blanc et al., 2005; Chen et al., 2005). Copper–silver ionization is a robust technology that has been used in more than 300 hospitals in the United States and the Europe to control *Legionella* in hot water systems. Copper and silver ions (Cu = 0.2–0.4 mg/L, Ag = 0.02–0.04 mg/L) are introduced into hospital water distribution systems via electrolysis. These positively charged metallic ions attach to the negatively charged bacterial cell wall and cause cell lysis and death (Bitton and Freihofer, 1977; Friedman and Dugan, 1968; Slawson et al., 1990). Copper–silver ionization has been successful in preventing outbreaks of hospital-acquired Legionnaires' disease (Stout and Yu, 2003). In vitro efficacies of copper and silver ions have also been demonstrated including *Legionella* (Landeen et al., 1989; Lin et al., 1996), *Naegleria fowleri* (Cassells et al., 1995), Coliphage MS-2 and Poliovirus (Yahya et al., 1992) and *Pseudomonas cepacia* (Pyle et al., 1992).

Given the efficacy of ionization against *Legionella*, it would be cost-effective if ionization is capable of eradicating other waterborne pathogens. However, no data are currently available. Thus, the objective of this study was to determine the in vitro efficacy of copper and silver ions in eradicating *P. aeruginosa*, *S. maltophilia* and *A. baumannii*. Furthermore, the efficacy of the combination of copper and silver ions was also determined as whether the combination demonstrated synergistic effect.

2. Materials and methods

2.1. Test organisms

The environmental isolates of *P. aeruginosa*, *S. maltophilia* and *A. baumannii* were selected as the test organisms. These isolates were transferred from −80 °C stock, inoculated on blood agar plate (BAP) media and incubated at 37 °C in a humidified incubator for 48 h. Inoculation was repeated overnight. The inocula were removed and suspended in 30 mL of sterile deionized water. The cells were washed twice by centrifugation at 1000*g* (2500 rpm) for 10 min. Ten milliliter of the suspension was removed and standardized by comparison with the turbidity of McFarland No. 1 standard (3×10^8 cfu/mL). One milliliter of the standardized suspension was transferred to 99 mL of the test solution to achieve the initial organism concentration of 3×10^6 cfu/mL for each experiment.

2.2. Copper and silver solutions

Copper and silver ion solutions were obtained by dissolving $CuCl_{2(s)}$ and $AgCl_{(s)}$ in deionized water (Sigma Chemical Co., St. Louis, MO, USA). Stock solutions of copper and silver ions containing 10 and 1 mg/L, respectively, were prepared in advance and transferred to test solution with a proper dilution scheme. Actual ion concentration was confirmed at the beginning of each experiment by Inductively Coupled Plasma Optical Emission Spectrometer. (PerkinElmer, Waltham, MA, USA)

2.3. Neutralizer

The purpose of using neutralizer, 0.1 N sodium thiosulfate solution ($Na_2S_2O_3 \cdot 5H_2O$), was to prevent any further disinfection effect of ions on the organism during incubation and enumeration. Ten microliter of neutralizer was mixed with 1 mL of sample withdrawn from the batch experiments.

2.4. Batch disinfection test

Batch disinfection tests were performed at different concentrations as described above. Approximately 3×10^6 cfu/mL of *P. aeruginosa*, *S. maltophilia* and *A. baumannii* were introduced into sterile teflon flasks. Actual bacterial concentration was determined using the plate count of the sample withdraw at time zero. Flasks were placed in a shaker with temperature control at 37 °C. All samples (1 mL) withdrawn from the flask were mixed with 10 μl neutralizer solution immediately, serially diluted and plated in duplicate with 0.1 mL sample solution onto BAP culture media. The culture plates were incubated for 48 h at 37 °C and enumerated for the cfu (Detection limit = 10 cfu/mL).

2.5. Study design

(A) Efficacy of individual ion
   1. The copper ions concentrations tested were 0, 0.1, 0.2, 0.4 and 0.8 mg/L and sampling time was 0, 0.5, 1, 1.5, 3, 6 and 24 h (seven sampling points).
   2. The silver ions concentrations tested were 0, 0.01, 0.02, 0.04 and 0.08 mg/L and sampling time was 0, 3, 6, 12, 24, 48, 72 and 96 h (eight sampling points).

(B) Efficacy of copper/silver combination
   Copper/silver ions concentrations tested were 0/0 (as control), 0.2/0.02 and 0.4/0.04 mg/L, and sampling time was 0, 1.5, 3, 6, 24 and 48 h.
   Each experiment was conducted twice at different days while each sample was analyzed in duplicate.

(C) Determination of individual and combined effect
   The efficacies of copper and silver ions against test

organisms were evaluated using the Ct value where C was the concentration of disinfectant (mg/L) and t was the contact time (h) between disinfectant and microorganism (J.M. Montgomery Engineers, Inc., 1985). Ct is used to calculate how much disinfectant is required to adequately disinfect the pathogens, and to determine the affectivity of a particular disinfectant against a certain microorganism under specified conditions. Ct value is commonly used to evaluate the efficacy of different disinfectants against the same microorganism at the same experimental conditions.

Inactivation rate for each experiment was modeled using a Gard model (Montgomery Eng, 1985). According to this model, the inactivation of organisms follows a declining rate as expressed by the following equation:

$$\frac{N}{N_0} = [1 + a(Ct)]^{-k/a},$$

where N_0 is the initial concentration of viable organisms at time t; N the concentration of viable organisms at time t; C the disinfectant concentration held constant over time; k the first-order rate of deactivation effected at time zero; a the rate coefficient and t the contact time. Coefficients k, a and Ct were determined for copper and silver ion using a non-linear regression analysis.

When two disinfectants were used in combination, the above equation can be modified as the Gard additive model. The differential equation below described the additive effect of the two disinfectants derived from the original Gard model:

$$\frac{N}{N_0} = \underbrace{[1 + a_1(C_1 t)]^{-K_1/a_1}}_{\text{effect of copper}} \underbrace{[1 + a_2(C_2 t)]^{-K_2/a_2}}_{\text{effect of silver}}.$$

Thus, the synergistic effect of two disinfectants is present if the inactivation rate observed from the experimental data is faster than the rate predicted by the Gard additive model using the parameters obtained from rate studies with individual disinfectants.

3. Results

3.1. Efficacy of ions on P. aeruginosa

Copper ion was effective in eradicating *P. aeruginosa*. All copper concentrations tested (0.1–0.8 mg/L) achieved more than 99.999% reduction of *P. aeruginosa* in 1.5 h (Fig. 1). This inactivation rate is similar to the rate of *Legionella* eradication (Lin et al., 1996). Silver concentrations of 0.04 and 0.08 mg/L also achieved more than 99.999% reduction of *P. aeruginosa* in 72 and 12 h, respectively (Fig. 2). The silver concentration of 0.02 mg/L achieved 99.999% reduction in 96 h. Silver concentration of 0.01 mg/L had an initial bactericidal effect on *P. aeruginosa* to nearly 99.99% reduction at 12 h, but subsequently growth returned to the baseline at 96 h.

3.2. Efficacy of ions on S. maltophilia

Copper concentration at 0.2–0.8 mg/L achieved more than 99.999% reduction of *S. maltophilia* in 6 h (Fig. 3). Copper concentration of 0.1 mg/L only achieved 99.99% reduction of *S. maltophilia* in 24 h. Silver concentration of 0.04 and 0.08 mg/L achieved more than 99.999% reduction of *S. maltophilia* in 6 h (Fig. 4). The silver concentration of 0.02 mg/L also achieved more than 99.999% reduction in 24 h. Silver concentration of 0.01 mg/L achieved 99.999% reduction of *S. maltophilia* initially at 6 h and finally at 72 h despite a 1.5 log regrowth was observed at 24 h.

3.3. Efficacy of ions on A. baumannii

Copper concentration at 0.4 and 0.8 mg/L achieved more than 99.999% reduction of *A. baumannii* in 24 h (Fig. 5). However, copper concentration of 0.1 and 0.2 mg/L only achieved 99.99% and 99.999% reduction for *A. baumannii* in 24 h. Silver concentration at 0.04 and 0.08 mg/L achieved more than 99.999% reduction of *A. baumannii* in 96 h (Fig. 6). The silver concentration of 0.02 mg/L achieved 99.999% reduction in

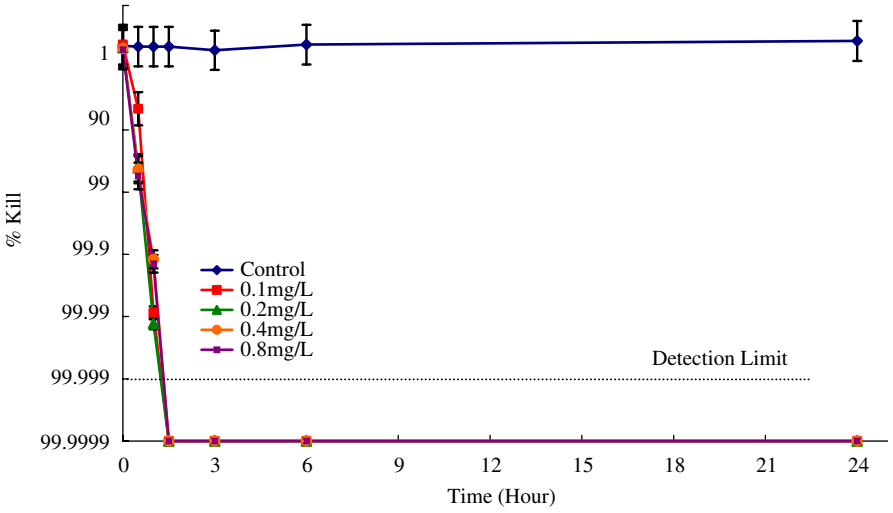

Fig. 1 – 0.1–0.8 mg/L of copper ions achieved more than 99.999% reduction of *P. aeruginosa* within 1.5 h.

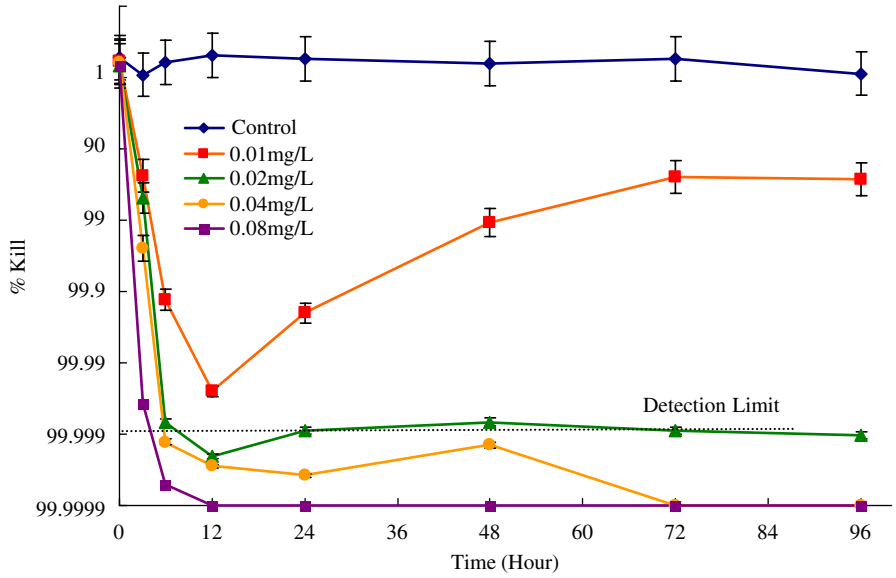

Fig. 2 – 0.04 and 0.08 mg/L of silver ions achieved more than 99.999% reduction of *P. aeruginosa* within 72 and 12 h, respectively.

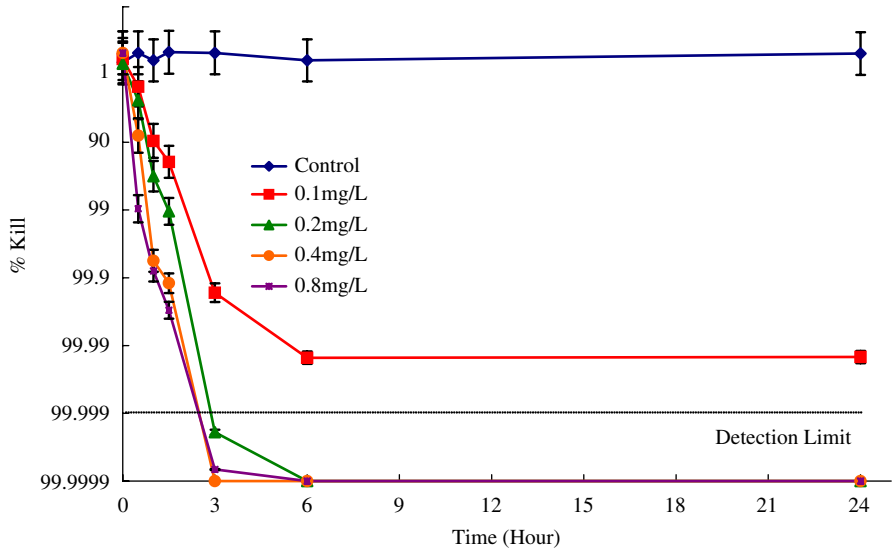

Fig. 3 – 0.2–0.8 mg/L of copper ions achieved more than 99.999% reduction of *S. maltophilia* within 6 h.

96 h, and 0.01 mg/L of silver only achieved 99.9% reduction of *A. baumannii* after 96 h.

3.4. *Susceptibility to copper and silver ions*

The results of Ct value (mg/L × h) and parameters *a* and *k* in Gard model were summarized in Table 1. The Ct value was calculated using the Gard model at 99.9% reduction. The susceptibility of the waterborne pathogens to the copper and silver ions was shown from sensitive to resistant as follow:

Copper—*L. pneumophila* → *S. maltophilia* → *P. aeruginosa* → *A. baumannii*,
Silver—*S. maltophilia* → *P. aeruginosa* → *L. pneumophila* → *A. baumannii*.

Among the four pathogens, *A. baumannii* appears to be the most resistant organism to copper and silver ions.

3.5. *Effect of copper and silver in combination*

Figs. 7–9 showed the results of batch disinfection studies at two copper–silver ions combinations (0.2/0.02 and 0.4/0.04 mg/L) on the eradication of *P. aeruginosa*, *S. maltophilia* and *A. baumannii*. Each figure also included the inactivation rate predicted by Gard Additive Model for each organism. Combination of copper and silver ions at concentrations of 0.2/0.02 and 0.4/0.04 mg/L exhibited synergistic effect against *P. aeruginosa* and *A. baumannii* (Figs. 7 and 9). However, the same combination of copper and silver ions exhibited antagonistic effect against *S. maltophilia* (Fig. 8).

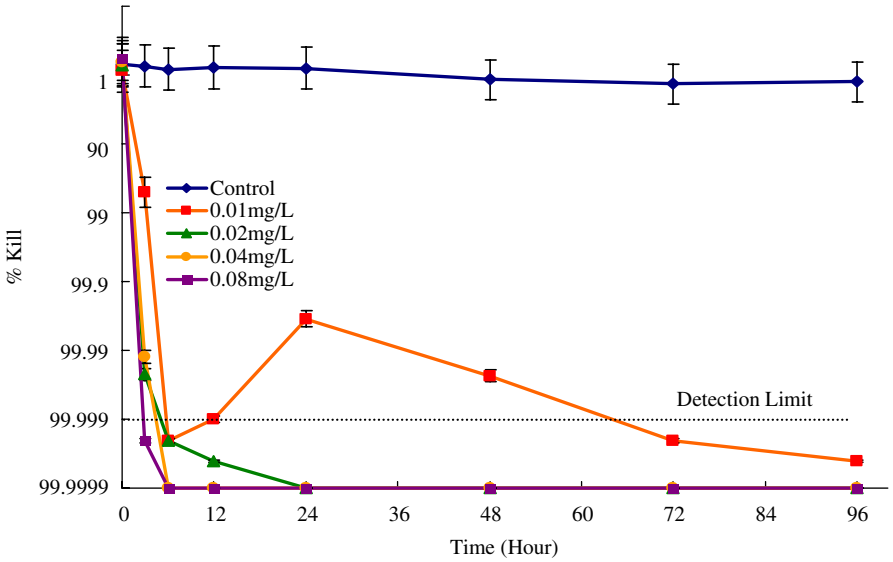

Fig. 4 – 0.02 and 0.04/0.08 mg/L of silver ions achieved more than 99.999% reduction of *S. maltophilia* within 24 and 6 h, respectively.

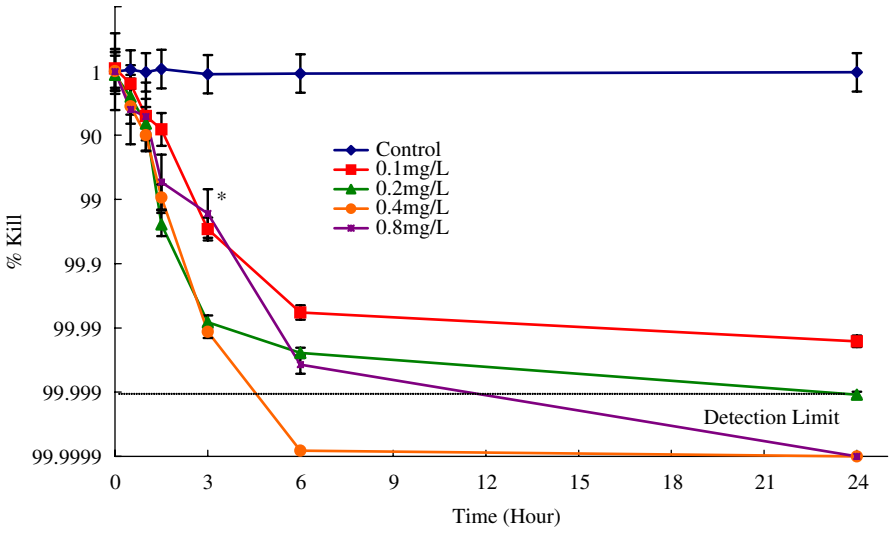

Fig. 5 – 0.4 and 0.8 mg/L of copper ions achieved more than 99.999% reduction of *A. baumannii* within 24 and 6 h, respectively. (*: an outlier was observed at Cu = 0.8 mg/L and t = 3 h. The average value of this data point may not reflect the real condition).

4. Discussion

P. aeruginosa, *S. maltophilia* and *A. baumannii* are waterborne pathogens which can be found easily in chlorinated finish water. These bacteria are opportunistic pathogens which do not affect healthy people. However, they can infect immunocompromised patients in the hospitals (especially patients in intensive care units) with infections such as pneumonia, bacteremia and urinary tract infections. Therefore, the presence of these bacteria in water may increase colonization with subsequent hospital-acquired infections. Although most of the published reports are outbreak-associated, endemic infections caused by the waterborne pathogens also occur.

Drinking water disinfection targeting these pathogens at the domestic water treatment plant may not be economical since these pathogens generally only affect patients in hospitals. Thus, on-site disinfection of hospital water systems might be cost-effective. Our finding showed that both copper and silver ions alone were effective in killing *P. aeruginosa*, *S. maltophilia* and *A. baumannii* at ion concentrations currently used in hospital water distribution systems for *Legionella*. *A. baumannii* appears to be the most resistant organism to copper and silver ions while *P. aeruginosa* and *S. maltophilia* exhibited similar susceptibility to copper and silver ions based on the *Ct* value, a value commonly used to evaluate the efficacy of different disinfectants against the pathogens under specified conditions. Furthermore, the

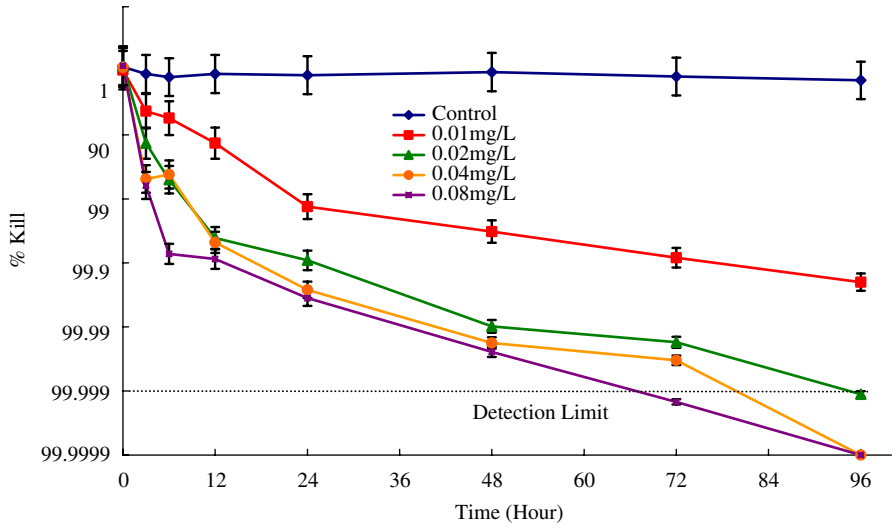

Fig. 6 – 0.04–0.08 mg/L of silver ions achieved more than 99.999% reduction of *A. baumannii* within 96 h.

Table 1 – *Ct* value and parameters *a* and *k* in *Gard* model of copper and silver ions in disinfecting *P. aeruginosa*, *S. maltophilia* and *A. baumannii*

Pathogens	Copper			Silver		
	Ct[a] (R^2)[b]	*a* (σ)[c]	*K* (σ)	Ct (R^2)	*a* (σ)	*K* (σ)
P. aeruginosa	0.39 (0.9827)	68.8 (43.94)	142.3 (62.52)	0.075 (0.8860)	1439.9 (4182.69)	2138.4 (5262.58)
S. maltophilia	0.35 (0.9609)	29.1 (28.11)	83.6 (57.51)	0.014 (0.9698)	6426.0 (16045.75)	9990.0 (21536.22)
A. baumannii	0.86 (0.9242)	14.9 (11.26)	39.1 (20.07)	0.59 (0.9863)	27.6 (7.53)	66.7 (13.10)
L. pneumophila (Lin et al., 1996)	0.08	176.9	502.8	0.35	30.9	91.0

[a] *Ct* value (*C* = the concentration of disinfectant in mg/L, *t* = the contact time in h) was used to evaluate disinfection efficacy of different disinfectants against the pathogens under specified conditions.
[b] R^2: adjusted *R* square.
[c] σ: standard deviation.

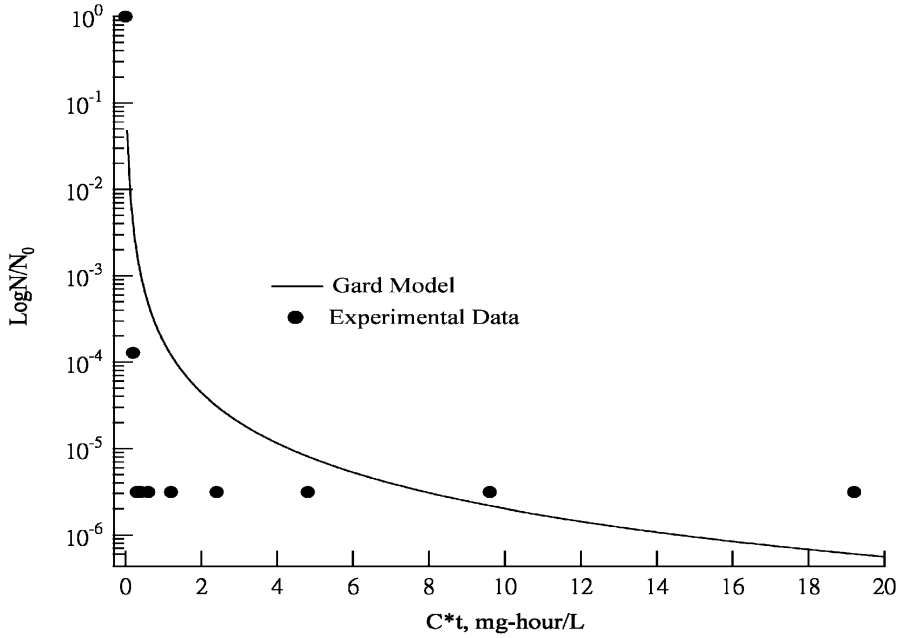

Fig. 7 – Combination of copper and silver ions exhibited synergistic effect on inactivation of *P. aeruginosa* using Gard model.

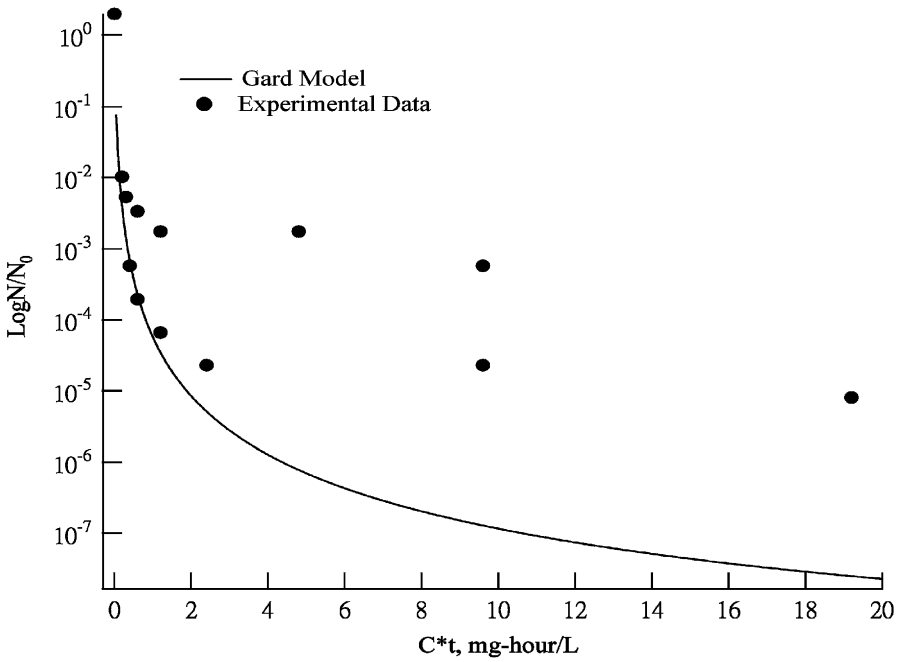

Fig. 8 – Combination of copper and silver ions exhibited antagonistic effect on inactivation of *S. maltophilia* using GARD model.

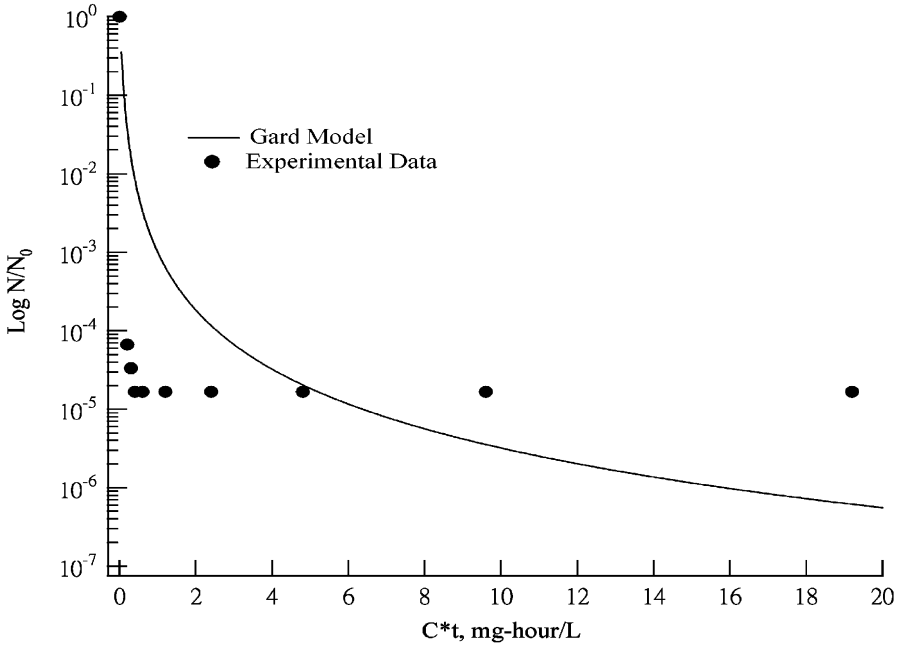

Fig. 9 – Combination of copper and silver ions exhibited synergistic effect on inactivation of *A. baumannii* using Gard model.

combination of copper and silver exhibited synergistic effect against *P. aeruginosa* and *A. baumannii*. The copper and silver ions in the concentrations allowable by EPA were effective in eradicating *P. aeruginosa*, *S. maltophilia* and *A. baumannii*.

Our finding may be the basis for implementing a proactive hospital infection control approach. The current infection control practices for waterborne pathogen-related infections in hospitals focus on interrupting the contact transmission. For example, healthcare workers are encouraged to wash hands rigorously, and use sterile water for all medical equipment rinsing and cleaning. Despite these efforts, waterborne-pathogens-related infections still occur. Thus, the new infection control strategy for waterborne pathogen infections has been shifted to hospital water supply disinfection which is derived from the knowledge of hospital-acquired Legionnaires' disease prevention. Our study showed that the copper and silver concentrations that are effective in eradicating *Legionella* are also effective in eradicating *P. aeruginosa*,

S. maltophilia and *A. baumannii*. Copper–silver ionization may be an attractive option for on-site disinfection of waterborne pathogens in hospitals.

The weakness of this study was that we only demonstrated the in vitro efficacy of copper and silver ions against these waterborne pathogens. However, as the bacteria residing in biofilms are more resistant to disinfectants, the study of ionization efficacy against waterborne pathogens in biofilms is necessary to determine the effective yet optimal copper and silver ion concentrations when applying ionization in hospital water systems. Furthermore, eradicating these waterborne pathogens from hospital water supply may only reduce part of the hospital-acquired infections. *P. aeruginosa*, *S. maltophilia* and *A. baumannii* are known to colonize medical equipment as a potential risk for patients (Exner et al., 2005). These pathogens can also colonize humans as part of the normal flora (Exner et al., 2005). Prevention of medical equipment contamination and patient-to-patient transmission of the pathogens should be focused in addition to the disinfection of hospital water supply.

5. Conclusion

The presence of waterborne pathogens in domestic finish water can cause opportunistic infections in hospitalized patients. On-site supplemental disinfection of hospital water systems might be one of the approaches to prevent these infections. Copper and silver ions are effective in eradicating *P. aeruginosa*, *S. maltophilia* and *A. baumannii* in vitro. Copper–silver ionization may have the potential to eradicate major waterborne pathogens in hospital distribution systems. The eradication efficacy of ionization under field conditions in institutional water systems and its significance in reducing hospital-acquired infections remain to be determined.

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