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Efficacy of Copper and Silver Ions and Reduced Levels of Free Chlorine in Inactivation of *Legionella pneumophila*

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Water disinfection systems utilizing electrolytically generated copper and silver ions (200 and 20, 400 and 40, or 800 and 80 μg/liter) and low levels of free chlorine (0.1 to 0.4 mg/liter) were evaluated at room (21 to 23°C) and elevated (39 to 40°C) temperatures in filtered well water (pH 7.3) for their efficacy in inactivating *Legionella pneumophila* (ATCC 33155). At room temperature, a contact time of at least 24 h was necessary for copper and silver (400 and 40 μg/liter) to achieve a 3-$\log_{10}$ reduction in bacterial numbers. As the copper and silver concentration increased to 800 and 80 μg/liter, the inactivation rate significantly ($P \leq 0.05$) increased from $K = 2.87 \times 10^{-3}$ to $K = 7.50 \times 10^{-3}$ ($\log_{10}$ reduction per minute). In water systems with and without copper and silver (400 and 40 μg/liter), the inactivation rates significantly increased as the free chlorine concentration increased from 0.1 mg/liter ($K = 0.397$ $\log_{10}$ reduction per min) to 0.4 mg/liter ($K = 1.047$ $\log_{10}$ reduction per min). Compared to room temperature, no significant differences were observed when 0.2 mg of free chlorine per liter with and without 400 and 40 μg of copper and silver per liter was tested at 39 to 40°C. All disinfection systems, regardless of temperature or free chlorine concentration, showed increase inactivation rates when 400 and 40 μg of copper and silver per liter was added; however, this trend was significant only at 0.4 mg of free chlorine per liter.

Legionella pneumophila is a ubiquitous aquatic organism which can survive under a wide range of environmental conditions (11). This organism is the causative agent of Legionnaires disease, a severe form of pneumonia, and may also cause Pontiac fever, a self-limiting nonpneumonial illness. Since its isolation following an outbreak of pneumonia at the 1976 American Legion Convention in Philadelphia (12), the annual number of reported cases of legionellosis in the United States has been increasing.

L. pneumophila has been isolated from cooling towers and evaporative condensers (7, 10, 16, 21), hot water tanks (13, 14, 29, 31), and whirlpools (32). These water-recirculating systems often possess favorable growth conditions for *L. pneumophila* such as elevated temperatures and mineral deposits (27) which can serve as a source of nutrients for biomasses, and in turn, support the growth of *Legionella* species (22). These devices are also known to produce aerosols and have been implicated in numerous outbreaks. Aerosolization of contaminated water sources is believed to be the route of transmission.

Hypochlorites have been used for many years in the disinfection of potable water because of their rapid inactivation of microorganisms. However, there are certain disadvantages with chlorination. The concentrations of hypochlorites required to be effective in inactivating *Legionella* species in cooling tower water are relatively high and would most likely be corrosive to plumbing systems in actual conditions (26). Furthermore, levels of chlorine may be reduced from initially high levels to background levels rapidly (10), especially in systems operating at elevated temperatures, and may not provide a long-lasting residual effect.

Although hypochlorites can reduce numbers of *L. pneumophila* in cooling tower water to environmental levels through continuous and shock chlorination (10), isolates of *Legionella* species from regions of high chlorination have been shown to develop increased chlorine resistance (18).

In seeking to address these concerns, alternative disinfection treatments have been investigated. Copper and silver have been used for numerous years in the disinfection of water. Copper and silver are known to affect a number of microorganisms including bacteria, viruses, and algae (4, 5, 25, 35, 36). They are believed to interfere with enzymes involved in cellular respiration (6) and to bind at specific sites to DNA (19, 20, 23).

Inactivation by combined copper and silver has been shown to be relatively slow when compared with that of free chlorine; however, when these metals were added to low levels of free chlorine, inactivation rates of bacterial indicator organisms (used to judge the sanitary quality of drinking and swimming pool water) were shown to be greater than those at comparable levels of free chlorine alone (36).

This research examined the efficacy of electrolytically generated copper and silver ions with and without concurrent low levels of free chlorine in inactivating pure cultures of agar-passaged *L. pneumophila*.

MATERIALS AND METHODS

Water source. The test water used in disinfection systems was obtained directly from the Martin Street well located at the University of Arizona, Tucson. After sufficient water was run from the faucet (to flush out the casing), an acid-washed 20-liter high-density polyethylene container was filled and the water sample was kept at 4°C. Within 24 h of each experiment, the water was filtered (0.2-μm pore size; Costar, Cambridge, Mass.) and adjusted to pH 7.3 ± 0.1 with 1 N HCl.

Chemical analyses were performed on well water by procedures adapted from *Standard Methods for the Examination of Water and Wastewater* (1). Analyses included total alkalinity, total and calcium hardness, total phosphate, turbidity, nitrogen (ammonia), nitrogen (nitrate), sulfate, free

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and total chlorine, chloride, total dissolved solids, conductivity, and pH. Copper and silver analyses were performed as described in *Official Methods of Analysis* (2).

Culture. A pure culture of *L. pneumophila* serogroup 3 (ATCC 33155) previously obtained from the American Type Culture Collection (Rockville, Md.) was lyophilized at the University of Arizona. At various times throughout the research, lyophilized samples were recovered in skim milk and grown on buffered charcoal-yeast extract (BCYE) agar plates (MicroBio, Tempe, Ariz.) for 4 to 5 days at 37°C. Serial transfers were made onto fresh BCYE plates from refrigerated (4°C) cultures.

On the day of each experiment, colonies of *L. pneumophila* (grown for 4 to 5 days at 37°C on BCYE plates) were removed and suspended in ca. 30 ml of filtered well water (pH 7.3). The cells were washed (centrifugation at 8,000 × *g* for 10 min) twice in filtered well water. The final pellet was resuspended in filtered well water and standardized by comparison with a McFarland no. 1 standard (a suspension of barium sulfate precipitate) to a cell density of ca. 3×10^8 organisms per ml (8).

Disinfectant preparation and determination. Free chlorine solutions were prepared from a stock solution of sodium hypochlorite (5.25%) and were diluted with filtered well water to the desired free chlorine concentration (0.1 to 0.4 mg/liter). Free chlorine concentrations were measured at the beginning and end of each experiment by the *N*,*N*-diethyl-*p*-phenylene diamine method adapted from *Standard Methods for the Examination of Water and Wastewater* (1). The accuracy of this method was confirmed by titration with As_2O_3 (2).

Copper and silver ions were generated electrolytically in filtered well water with copper- and silver-generating units (Electronic Pool Purity Units; Tarn-Pure USA, Las Vegas, Nev.) with expected copper/silver ratios of either 90:10 or 80:20, respectively. The unit (closed at one end) was rinsed with hot sterilized distilled water, filled with ca. 1.5 liters of filtered well water, and operated with continuous stirring for a period long enough to generate the desired concentrations (ca. 18 to 23 s for 400 and 40 μg of copper and silver per liter in the 90:10 unit at room temperature). Estimates of the level of copper were determined at the beginning of each experiment with a test kit (model EC-20; La Motte Chemical Products Co., Inc., Chestertown, Md.) provided with the unit. Actual copper and silver concentrations were determined at the beginning and end of each experiment with an atomic absorption spectrophotometer (Hitachi 180-70 with hollow cathode lamp) and standard solutions of $AgNO_3$ and $CuSO_4$ as described in *Official Methods of Analysis* (2). Linear regression equations were calculated from standard solutions for concentration versus absorbance, and a correlation coefficient value of 0.997 or greater was obtained for each experiment.

Glassware preparation. All glassware was soaked overnight in a 12.5% nitric acid bath to remove metal contaminants, rinsed with distilled water, and sterilized before use. Copper and silver in solution were transferred after generation to 1-liter polyethylene bottles. Glassware used for systems containing free chlorine was soaked overnight in 0.8 to 1.0 mg of free chlorine per liter to satisfy demands from the glassware which might reduce the amount of available free chlorine during the experiment. Test systems were conducted in 100-ml polyethylene beakers. Disposable pipettes were used for all chemical preparations and assay dilutions.

Experimental conditions. The disinfection systems tested included (i) well water containing 0.1, 0.2, 0.3, or 0.4 mg of free chlorine per liter; (ii) well water containing 0.1, 0.2, 0.3, or 0.4 mg of free chlorine per liter with copper and silver (ca. 200 and 20, 400 and 40, or 800 and 80 μg/liter); and (iii) well water containing copper and silver (ca. 200 and 20, 400 and 40, or 800 and 80 μg/liter. A control of well water with no added metals or free chlorine was also tested for each experiment. The majority of experiments were performed at room temperature (21 to 23°C), with some systems being tested at elevated temperatures (39 to 40°C). A minimum of duplicate separate experiments were performed for all disinfection systems.

Experimental design. The standardized culture suspension (1 ml; ca. 10^8 cells) was added to 99 ml of each test system to achieve a final concentration of ca. 10^6 cells per ml. The inoculated system was stirred continuously throughout the experiment with a magnetic stirring plate for even cell distribution.

At predetermined time intervals, a 1-ml sample was removed and neutralized with 10 μl of a neutralizer solution (3, 28) (14.6% sodium thiosulfate and 10% sodium thioglycolate in distilled water, filtered through a 0.2-μm-pore-size filter). After appropriate dilutions in 0.1% peptone, 0.1 ml from the dilution blank was spread plated onto duplicate BCYE plates and incubated at 37°C in a semiclosed system (to help prevent moisture loss) for 4 to 5 days. After incubation, colonies were enumerated and the bacterial inactivation rates and $\log_{10}$ reductions were calculated.

Data analyses. Inactivation rates (K values) for each experimental system were calculated by linear regression analysis. The inactivation rate can be expressed by the equation $K = -2.3[\log_{10}(C_0 - C_T)/T]$ (15), where C_0 and C_T are the initial and final bacterial concentrations, respectively, and T represents time (in minutes). The data were also calculated as $\log_{10} N_T/N_0$ CFU/ml, which expresses the $\log_{10}$ reduction in bacterial numbers at each time interval. Inactivation rates differing by more than one standard deviation from the mean value of the particular disinfection system were not used in the results. A statistical computer program (CoStat Statistical Software; CoHort Software, Berkeley, Calif.) was used for analysis of variance to determine significant differences between disinfection systems.

RESULTS AND DISCUSSION

Well water was chosen for disinfection systems over tap water and distilled water because it contained no detectable free chlorine, copper, or silver and contained adequate levels of alkalinity and conductivity necessary to generate copper and silver in a relatively short time (Table 1). Tap water had the disadvantage that detectable levels of copper (up to 80 μg/liter) occurred from leaching of the piping.

The initial pH of the well water ranged from 7.8 to 8.2 and was adjusted to pH 7.3 (with 1 N HCl) as this was the pH recommended by the manufacturer of the copper- and silver-generating units. The pH concentration may be important for the efficacy of free chlorine and the solubility of metals. At pH 7.3, ca. 60% of the chlorine should exist as HOCl (8).

Experiments were conducted in acid-washed polyethylene containers since experimental results indicated less adsorption of the metals to polyethylene than to polystyrene or Pyrex (Table 2). Over 48 h, the level of copper in solution decreased 2.5% when stored in polyethylene compared with a 10.0% decrease for Pyrex. Silver in solution was adsorbed even more than copper, with an 11.6% decrease when stored in polyethylene versus a 15.4% decrease when stored in

TABLE 1. Chemical analyses of Martin Street well water[a]

Parameter	Value
Free chlorine concn (mg/liter)	0
Total chlorine concn (mg/liter)	0
pH[b]	7.9
Total alkalinity (mg of $CaCO_3$ per liter)	110
Total hardness (mg of $CaCO_3$ per liter)	92
Calcium hardness (mg of $CaCO_3$ per liter)	88
Chloride concn (mg/liter)	30
Total phosphate concn (mg/liter)	2.2
Sulfate concn (mg/liter)	74
Nitrogen (nitrate) concn (mg/liter)	3.5
Nitrogen (ammonia) concn (mg/liter)	0.31
Copper concn (μg/liter)[c]	<10
Silver concn (μg/liter)[c]	<10
Turbidity (nephelometric turbidity units)	0.21
Total dissolved solids concn (mg/liter)	166
Conductivity (mS/cm)	0.414

[a] Unfiltered well water before pH adjustment.
[b] pH was adjusted to 7.3 with 1 N HCl for expeirments.
[c] Below limits of detection.

Pyrex. In general, the greatest amount of adsorption appeared to occur within the first 24 h. Therefore, to minimize this effect, all copper and silver solutions were used within 1 h after generation. Similar adsorbance behaviors have been observed by other investigators (3, 5, 35).

Acid washing of the glassware in HNO_3 proved useful in removing adsorbed copper from the surfaces of glassware. After autoclaving, glassware used for chlorine solutions was made demand-free by soaking in sterile distilled water which contained 0.8 mg of free chlorine per liter. Copper concentrations achieved during various contact times in the 90:10 (copper-silver) unit were determined (Fig. 1). The time required to generate ca. 400 μg of copper per liter in well water at room temperature was 18 to 25 s. The generation rate was found to be increased by elevated temperatures and decreased pH of the water (data not shown). Although the times required to generate silver concentrations were not determined separately, they usually remained within the expected copper/silver ratio.

Copper and silver were generated as ions; however, at the pH values tested and in the water type used, complexation of the metals would not be unexpected. A computer program (Mineql; Department of Civil Engineering, Massachusetts Institute of Technology, Cambridge) model based on the chemical parameters of the water predicted over 90% of the copper to exist in a complexed form. Of the copper that would be predicted to exist in an ionic form, ca. 90% would exist as Cu^{2+} (data not shown).

The experimental systems utilized no buffer. This was in part to simulate natural water systems as much as possible, and because phosphate buffers were found to interfere with the disinfection efficacy of copper in previous tests with

TABLE 2. Adsorbance of copper and silver to Pyrex and polyethylene container surfaces at room temperature

Time (h)	Copper concn (μg/liter)[a]		Silver concn (μg/liter)[a]	
	Pyrex	Polyethylene	Pyrex	Polyethylene
0	633	446	39	43
24	588	437	34	36
48	570	435	33	38

[a] Concentration in solution in 1-liter beaker held at room temperature for the times indicated.

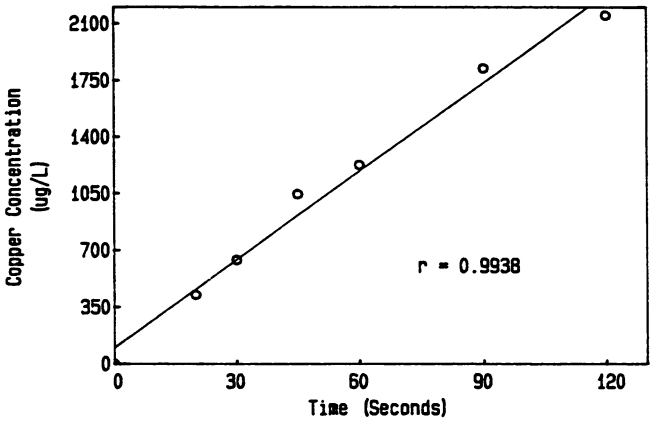

FIG. 1. Concentration of electrolytically generated copper in well water at room temperature with the 90:10 copper-silver unit.

Escherichia coli (ATCC 11229) (data not shown). When bacteria were exposed for 1 h to 302 μg of copper per liter in filtered distilled water, a 3.3-$\log_{10}$ reduction in bacterial numbers was observed. No detectable decrease in bacterial numbers occurred over the same period when bacteria were exposed to buffered distilled water containing over twice the amount of copper (669 μg/liter). Only a slight decrease was observed in the unbuffered distilled water system without copper (0.19-$\log_{10}$ reduction within 4 h).

Copper is known to bind to phosphate; therefore, the failure of copper in the buffered system to inactivate bacteria was most likely a result of complex formation between the copper in solution and phosphates from the buffer. Complexation of copper by phosphates has been shown (37) to reduce the microbicidal effects of copper. Silver has also been shown to complex with phosphate, resulting in increased bacterial inactivation times (35).

A neutralizer solution of sodium thiosulfate and sodium thioglycolate was found to be effective in neutralizing residual free chlorine and metals in previous tests with *E. coli* (ATCC 11229) (data not shown). Other investigators (28) using a neutralizer stock solution of thiosulfate and thioglycolate to neutralize the effects of silver found that samples receiving neutralizer had 30% higher bacterial counts than those samples not receiving neutralizer. Another study (3) demonstrated sodium thiosulfate and sodium thioglycolate to be effective in neutralizing high concentrations of silver.

Inactivation of *L. pneumophila* by copper and silver is relatively slow compared with that of free chlorine. The inactivation rates for exposure to 200 and 20, 400 and 40, or 800 and 80 μg of copper and silver per liter are listed in Table 3. The inactivation rate for copper and silver at 800 and 80

TABLE 3. Inactivation of *L. pneumophila* by exposure to various concentrations of electrolytically generated copper and silver

Copper and silver concn (μg/liter)	Mean K[a]	Significance[b]
200 and 20	9.83×10^{-4}	a
400 and 40	2.87×10^{-3}	a
800 and 80	7.50×10^{-3}	b

[a] Inactivation rate, $K = -2.3[\log_{10}(C_0 - C_T)/T]$.
[b] Treatment systems with different letters are significantly different ($P \leq 0.05$).

TABLE 4. Inactivation of *L. pneumophila* by exposure to electrolytically generated copper and silver (400 and 40 and 800 and 80 μg/liter) and/or free chlorine (0.2 and 0.3 mg/liter)

Free chlorine concn (mg/liter)	Copper and silver concn (μg/liter)	Mean K^a	Significance[b]
0.2	0	1.077	a
	400 and 40	1.322	a
	800 and 80	2.012	a
0.3	0	1.591	a
	400 and 40	2.282	a
	800 and 80	2.639	a

[a] Inactivation rate, $K = -2.3[\log_{10}(C_0 - C_T)/T]$.
[b] Treatment systems with different letters are significantly different ($P \leq 0.05$).

μg/liter ($K = 7.50 \times 10^{-3}$) was shown to be significantly ($P \leq 0.05$) faster than the rates for 400 and 40 ($K = 2.87 \times 10^{-3}$) or 200 and 20 ($K = 9.83^{-4}$) μg/liter. This may be a result of exceeding the capacity at which the cells can effectively handle toxic metals (33). In experiments involving *L. pneumophila* (24), up to 1.5 mg of copper per liter was found to be noninhibitory; however, copper was added to a metal-deficient agar medium, so the medium itself may have reduced the toxic effects of the copper.

Free chlorine (0.2 mg/liter) was tested separately and in combination with 400 and 40 and 800 and 80 μg of copper and silver per liter (Table 4). Although there were increased inactivation rates (K) between free chlorine alone and free chlorine with either 400 and 40 or 800 and 80 μg of copper and silver per liter, these were not shown to be statistically significant. The same effect was observed for 0.3 mg of free chlorine per liter when tested with and without copper and silver (Table 4); however, these differences were not significant either.

The inactivation of *L. pneumophila* after exposure to 400 and 40 μg of copper and silver per liter and low levels of free chlorine (0.1 to 0.4 mg/liter) showed increasing inactivation rates (Fig. 2) and greater $\log_{10}$ reductions (Fig. 3) as the concentration of free chlorine increased. Free chlorine (0.4 mg/liter) with or without the addition of 400 and 40 μg of copper and silver per liter was significantly ($P \leq 0.05$) greater than all other free chlorine concentrations separately or combined with copper and silver. Although inactivation rates at all the tested levels of free chlorine appeared to be enhanced when 400 and 40 μg of copper and silver per liter were added, this observation was significant only when the chlorine concentration was increased from 0.3 to 0.4 mg/liter.

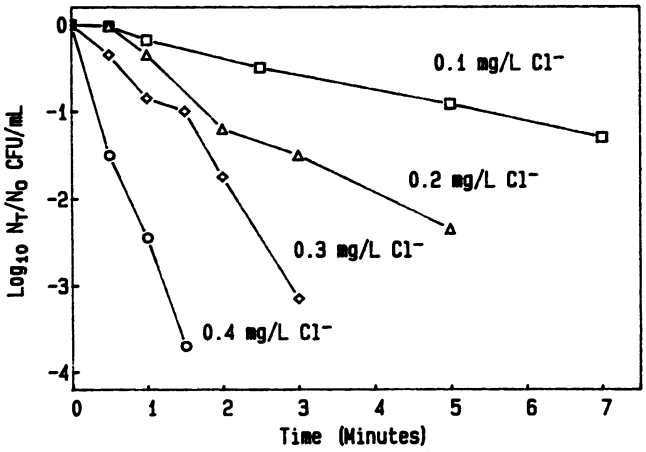

FIG. 3. Reduction of *L. pneumophila* by exposure to electrolytically generated copper and silver (400 and 40 μg/liter) and various concentrations of free chlorine.

The reduction of *L. pneumophila* numbers over time after exposure to free chlorine (0.4 mg/liter) with and without copper and silver (400 and 40 μg/liter) is shown in Fig. 4. After 1.5 min, a 3.7-$\log_{10}$ reduction was observed for the combined free chlorine and copper and silver system. In contrast, only a 2.6-$\log_{10}$ reduction was seen for free chlorine alone, and a contact time of at least 24 h was necessary for copper and silver alone to achieve a 3-$\log_{10}$ reduction in the bacterial numbers.

Statistical analysis of the inactivation rates of the water systems which contained only free chlorine compared with those with free chlorine and copper and silver (Table 5) showed that significantly greater ($P \leq 0.05$) inactivations occurred in systems in which copper and silver (400 and 40 μg/liter) was added to free chlorine (0.1 to 0.4 mg/liter) than

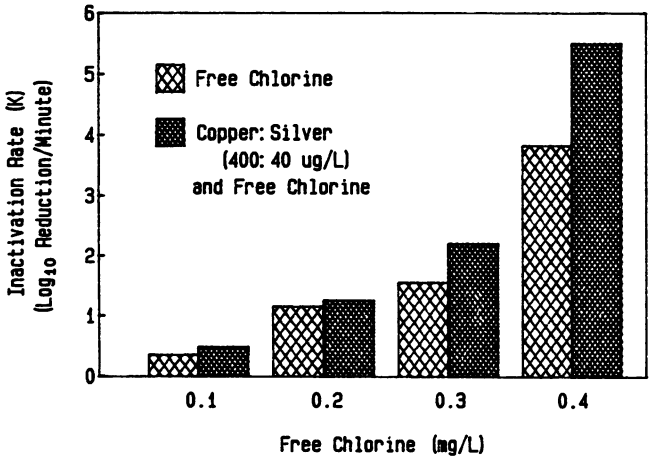

FIG. 2. Inactivation of *L. pneumophila* by exposure to electrolytically generated copper and silver (400 and 40 μg/liter) and/or various concentrations of free chlorine.

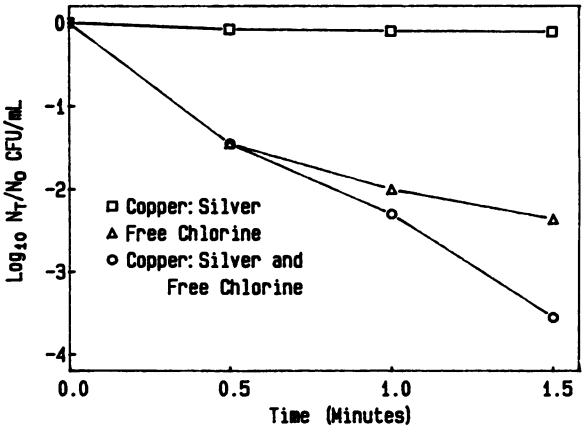

FIG. 4. Reduction of *L. pneumophila* by exposure to electrolytically generated copper and silver (400 and 40 μg/liter) and/or free chlorine (0.4 mg/liter).

TABLE 5. Determination of the effects of free chlorine concentration and addition of electrolytically generated copper and silver by two-way analysis of variance

Concn of:	Mean K^a	SD	n^b	Significance[c]
Free chlorine (mg/liter)				
0.1	0.397	0.208	4	a
0.2	1.151	0.907	10	ab
0.3	1.868	0.786	10	b
0.4	4.602	1.047	4	c
Free chlorine (0.1–0.4 mg/liter) and copper and silver (μg/liter)				
0	1.478	1.228	17	a
400 and 40	2.277	1.775	11	b

[a] Inactivation rate, $K = -2.3[\log_{10}(C_0 - C_T)/T]$.
[b] Number of experiments.
[c] Treatment systems with different letters are significantly different ($P \leq 0.05$).

for systems with free chlorine alone. The level of free chlorine was also significantly different for systems at 0.1, 0.2, 0.3, and 0.4 mg/liter when both free chlorine and free chlorine with copper and silver (400 and 40 μg/liter) were averaged together.

Inactivation rates of *L. pneumophila* at room (21 to 23°C) and elevated (39 to 40°C) temperatures are compared in Table 6. Although it appears that the rate for free chlorine (0.2 mg/liter) with copper and silver (400 and 40 μg/liter) at the higher temperature ($K = 2.559$) was greatly increased, this was not shown to be significantly different from the room temperature rates ($K = 1.077$ and $K = 1.322$) or the rate for free chlorine alone at the higher temperature ($K = 1.186$). Other investigators (18, 30), by using different disinfectants, have demonstrated an enhanced inactivation of *L. pneumophila* at elevated temperatures.

Differences in the inactivation rates of *L. pneumophila* by copper and silver and free chlorine are probably a consequence of the different mechanisms each disinfectant employs. Since organisms are known to have mechanisms to counter the microbicidal effects of these metals (34), it may be that concentrations must exceed levels at which the organism can successfully combat the metals. Significantly greater inactivation rates which occurred as the copper and silver concentration increased from 200 and 20 to 800 and 80 μg/liter could indicate that *L. pneumophila* has a tolerance to low levels of metals, but when concentrations exceed levels able to be controlled through defense mechanisms, inactivation of the organism occurs more rapidly.

Enhanced bacterial inactivation rates of agar-grown cultures of *L. pneumophila* in well water systems were shown when copper and silver were added to chlorinated water. Whether increased bacterial inactivations may occur with water-maintained isolates, which are known to be more resistant to disinfection (17), or in water systems of other chemical consistencies and environmental conditions (such as hardness, pH, turbidity, and presence of other microflora) remains to be investigated.

TABLE 6. Inactivation of *L. pneumophila* by exposure to electrolytically generated copper and silver (400 and 40 μg/liter) and free chlorine (0.2 mg/liter) at room and elevated temperatures

Temp (°C)	Copper and silver concn (μg/liter)	Mean K^a	Significance[b]
21–23	0	1.077	a
	400 and 40	1.322	a
39–40	0	1.186	a
	400 and 40	2.559	a

[a] Inactivation rate, $K = -2.3[\log_{10}(C_0 - C_T)/T]$.
[b] Treatment systems with different letters are significantly different ($P \leq 0.05$).

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