

Inactivation of *Legionella pneumophila* by Hypochlorite and an Organic Chloramine

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The susceptibility of a strain of *Legionella pneumophila* to disinfection by an organic halamine, free chlorine, and a mixture of the organic halamine and free chlorine was assessed. The organic halamine was found to have superior stability in solution and to exhibit adequate disinfectant potential over a period of 1 month of repeated reinoculations of fresh bacteria. The combined halamine exhibited great potential for use in maintaining closed-cycle cooling water systems free of *L. pneumophila*.

Reports that *Legionella pneumophila*, the causative agent of Legionnaires disease, could be isolated from a variety of potential human contact areas prompted us to examine the effectiveness of an organic *N*-chloramine as a control measure for this organism. The two main areas of concern are (i) populations of legionellae in heat rejection systems, cooling towers, and other nonpotable-water sources, such as hot tubs and spas, where there are risks of aerosol formation and (ii) subsequent transmission of the organisms to susceptible individuals (4, 6) and to the potable-water supply (3, 10, 13, 16, 18) through aerosols.

Various biocides have been examined for their efficacies in both laboratory simulations and field treatments of *Legionella* contamination of cooling towers and potable-water supply systems (1, 6-8, 10, 11, 14, 15). The most effective potable-water treatments have been found to be hyperchlorination, especially of hot water supplies (1, 10, 11), and heating to above 50°C (12, 18). Treatments for nonpotable water, such as water in cooling towers and in other heat rejection equipment, include chlorination (6, 10), the use of quaternary ammonium compounds with or without bis(tri-*n*-butyltin) (6, 8, 14, 15), and the use of an *N*-halamine, 1-bromo-3-chloro-5,5-dimethylhydantoin (7). The difficulty of maintaining adequate biocide residuals in cooling towers owing to high biocide demand has made microorganism control in these structures virtually impossible.

The effectiveness of organic halamines as water disinfectants has been demonstrated for a variety of microorganisms under several conditions of pH, temperature, and water quality (2, 20, 24). The response of *L. pneumophila* to one of these halamines, 3-chloro-4,4-dimethyl-2-oxazolidinone (henceforth referred to as compound 1), was assessed under a variety of treatment conditions. Disinfection experiments were carried out as previously described (20) with *L. pneumophila* ATCC 33152, and the contact time at which a 99.99% decline in viable CFU per milliliter occurred was determined. All disinfection experiments were conducted in chlorine-demand-free buffer or in controlled-high-chlorine-demand synthetic-demand water (20). Synthetic-demand water was buffered at pH 9.5 and held at 4°C because these conditions represented the worst-case scenario for disinfection of water in a field situation, as viewed by the sponsors of this research (21). Furthermore, it has been demonstrated recently (17) that cooling tower operation at high pH may

best exploit the sensitivity of *Legionella* species under these worst-case conditions. Aliquots removed from the reaction mixtures were quenched with sodium thiosulfate and plated on charcoal-yeast extract agar base with BCYE supplement SR110 (Oxoid Ltd). The initial bacterial densities in the reaction mixtures ranged from 7×10^6 to 1.8×10^7 CFU/ml. The most rapid disinfection by compound 1 was at pH 9.5 and 37°C (Table 1). Disinfection was most rapid under basic conditions, as has been previously described for this compound (20, 23, 24), and the rate of disinfection was related to the dose of the compound. Disinfection by free chlorine (calcium hypochlorite) was quite rapid in demand-free treatments, with a greater than 5-log decrease in bacterial density being observed within 1 min of contact time (data not shown) for all concentrations tested.

The ability of compound 1 to reduce extremely high densities of *L. pneumophila* to nondetectable levels is demonstrated in Fig. 1 and 2. The somewhat more-rapid inactivation by compound 1 in an alkaline-environment was found in contrast to the more-rapid inactivation by free chlorine under acidic conditions noted previously (10), when decreasing the pH from 7.6 to 7.0 resulted in a reduction of contact time from 60 to 10 min for a 3-log decrease in viable bacteria. The nonlinear segments of the inactivation curves (Fig. 1 and 2) were thought to have been due to clumping of cells during the disinfection experiment. From the Chick-Watson model (5, 9, 19), values for the constants *k* and *n* (the coefficient of dilution) were obtained for the data presented in Fig. 1 and 2; they were 2.15 liter^{*n*} per mg^{*n*} per min and 1.04, respectively, for pH 7.0 and 25°C and 1.95 liter^{*n*} per mg^{*n*} per min and 0.47, respectively, for pH 9.5 and 25°C.

Maintenance of a long-term residual, especially in cooling tower water treatment, would be a desirable disinfectant characteristic. Compound 1 and some other organic halamines studied in our laboratories have just such stabilities, even under conditions in which high halogen demand exists (23). The use of a mixture of compound 1 and Ca(OCl)₂ as a rapid initial disinfectant with slower long-term biocidal activity was evaluated in a series of rechallenge experiments in which solutions of compound 1, Ca(OCl)₂, or a 1:1 (wt/wt, of total Cl) mixture of the two were reinoculated with fresh cultures of *L. pneumophila* at weekly intervals over a period of 4 weeks. The solutions were prepared in demand-free buffer (pH 7.0) and were stored at room temperature (25 ± 2°C) for the duration of the experiment. Organism challenges consisted of the introduction of

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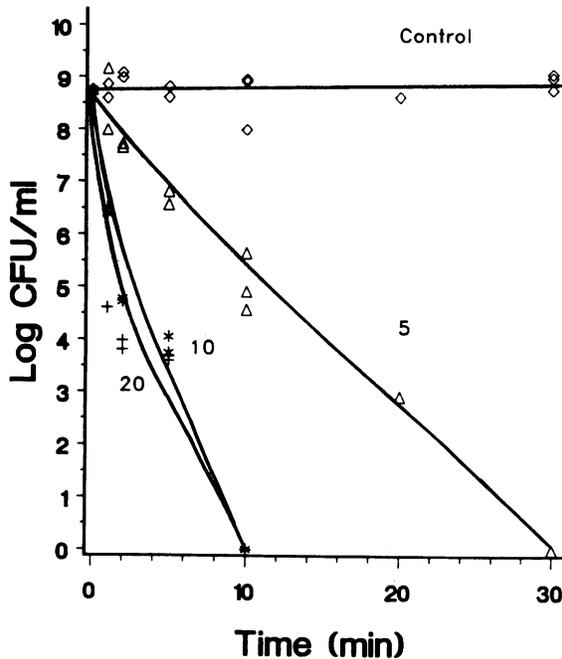


FIG. 1. Disinfection of *L. pneumophila* by compound 1 in demand-free phosphate buffer (pH 7.0; 25°C) at various total concentrations of chlorine (in milligrams per liter, indicated alongside curves).

ca. 10^7 CFU/ml, followed by the determination of survivor densities at 1, 2, 5, 10, 20, and 30 min after the addition of the cells. All samples removed were treated with sodium thiosulfate before dilution and plating. Predicted time for a 6-log kill was calculated as previously described (20). The ability of $\text{Ca}(\text{OCl})_2$ to produce a 6-log or greater kill within 30 min of contact declined after the first challenge (Table 2). An analysis of variance indicated that, for $\text{Ca}(\text{OCl})_2$, the storage time per rechallenge had a significant effect on disinfection ($F = 46.91$, $P > F = 0.0001$) when CFU per milliliter was taken as the response variable. Solutions of compound 1 did not show effects of storage time per rechallenge ($F = 0.33$, $P > F = 0.8491$) and maintained approximately the same rate of disinfection (14.53 to 16.9 min for a 6-log decline) during the 4-week course of evaluation. The mixture of compound 1 and $\text{Ca}(\text{OCl})_2$ functioned in a manner similar to that of calcium hypochlorite alone in the initial challenge, with a predicted 6-log kill within 0.64 min, but subsequent rechallenges indicated a disinfection rate more in keeping with that of compound 1. As was observed with $\text{Ca}(\text{OCl})_2$, storage time per rechallenge had a significant effect on the mean CFU per milliliter with the mixture, but this effect was limited to the comparison between the initial challenge and the first rechallenge ($F = 5.90$, $P > F = 0.025$). Control cultures (buffer plus cells) indicated no decline in CFU per milliliter during the 30-min sampling periods at any rechallenge point.

The effect of storage under demand-free conditions at three temperatures on the biocidal action of $\text{Ca}(\text{OCl})_2$, compound 1, and a 1:1 (wt/wt, of total Cl) mixture of the two was evaluated also. Solutions of the above-mentioned compounds and mixture were prepared in demand-free phosphate buffer (pH 7.0) at 4, 8, and 8 mg of total chlorine per liter, respectively, placed in demand-free screw-cap bottles, and kept in darkness at 4, 25, and 37°C. Samples of the

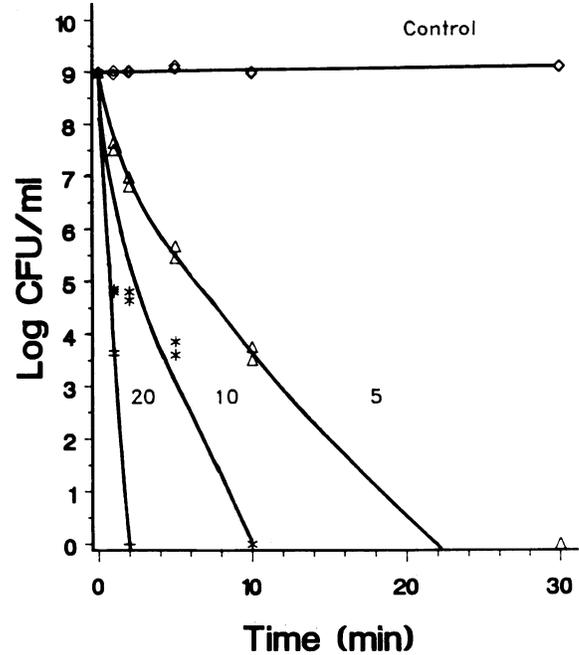


FIG. 2. Disinfection of *L. pneumophila* by compound 1 in demand-free borate buffer (pH 9.5; 25°C) at various total concentrations of chlorine (in milligrams per liter, indicated alongside curves).

solutions were removed for initial determinations of biocidal action and at weekly intervals for 4 weeks. An equal volume of the disinfectant solution was added to demand-free buffer (pH 7.0) at 25°C containing *L. pneumophila* at ca. 2×10^6 CFU/ml. Samples were removed, and excess chlorine was quenched before samples were diluted, and then samples were plated as described previously. The minimum contact time for a 6-log decline in CFU per milliliter was recorded. Chlorine residuals were determined at the end of each experiment by the *N, N*-diethyl-*p*-phenylenediamine method with a commercially available test kit (Hellige Inc.).

Elevated storage temperature had the greatest effect on the activity of calcium hypochlorite. After 2 weeks of storage at either 25 or 37°C, chlorine had completely disap-

TABLE 1. Observed inactivation times for disinfectant treatments of *L. pneumophila*

Assay condition	pH	Temp (°C)	Compound	Inactivation time (min) ^a at total Cl concn (mg/liter) of:		
				10	5	2
Demand free	7	4	1	5	30	30
	7	22	1	5	30	30
	7	37	1	5	10	30
	9.5	4	1	5	10	30
	9.5	22	1	2	10	10
	9.5	37	1	1	10	30
	9.5	4	$\text{Ca}(\text{OCl})_2$	1	1	1
High demand ^b	9.5	4	1	10	10	30
	9.5	4	$\text{Ca}(\text{OCl})_2$	2	2	5

^a Time at which a $>4 \log_{10}$ decrease in viable CFU per milliliter was observed. At 30 min of contact time the CFU per milliliter had decreased by more than 99.999%.

^b Synthetic-demand water containing inorganic salts, bentonite clay, horse serum, dead yeast cells, and humic acid was buffered to pH 9.5 and held at 4°C (20).

TABLE 2. Predicted 6-log inactivation times as a function of rechallenge by *L. pneumophila*^a

Compound	Concn ^b	Inactivation time (min) when rechallenged at wk ^c :				
		0	1	2	3	4
Ca(OCl) ₂	2.0	0.6 (2.0)	ND ^d (0.0)	ND (0.0)	ND (0.0)	ND (0.0)
1	4.0	14.53 (4.0)	16.9 (<4.0)	15.8 (3.9)	15.5 (3.7)	16.7 (3.35)
Mixture	4.0 ^e	0.6 (4.0)	— ^f (<2.0)	21.4 (1.75)	21.1 (1.5)	20.8 (1.25)

^a Inoculations at each rechallenge with fresh portions of cells at ca. 10⁷ CFU/ml. Assays and storage were at 25 ± 2°C in demand-free buffer (pH 7.0).
^b Milligrams of total chlorine per liter.
^c Values in parentheses are total chlorine residuals (in milligrams per liter) measured at the end of each rechallenge by the *N,N*-diethyl-*p*-phenylenediamine method.
^d ND, No decrease in CFU per milliliter during the 30-min contact period.
^e Contained 2.0 mg of total chlorine per liter from each compound.
^f —, Contact periods of up to 5 min only were considered for the mixture at week 1. Little reduction of viable CFU per milliliter was observed.

peared (Table 3). The rate of loss was more rapid at 37°C and was much less rapid at 4°C. Storage at 37°C had an extremely small effect on the stability of compound 1. After 4 weeks of storage, the total chlorine concentration in solutions of compound 1 was only slightly less than that at the beginning of the storage period. The mixture of compound 1 and Ca(OCl)₂ lost approximately one-half of the starting chlorine within 2 weeks at either 25 or 37°C, the same period during which the loss of chlorine residual from the Ca(OCl)₂ solution was observed, and then remained relatively stable during the remainder of the experiment. Disinfection rates were consistent with the levels of observed chlorine residuals measured during these experiments.

It has been demonstrated that organic halamines can function as suitable disinfecting agents against *L. pneumophila* under a variety of treatment conditions. The exceptional long-term stabilities of these compounds make them quite desirable for use in situations in which such stability is needed, such as in air-conditioning systems, cooling towers,

and hot tubs. Compound 1 is considerably more stable than the hydantoin or isocyanurates often used in such systems (22). Furthermore, compound 1 is noncorrosive (21), while hypochlorite is known to be quite corrosive; this property of compound 1 renders it more economical than free chlorine for the applications mentioned above. In addition, if extremely rapid initial-disinfection action is desired, mixtures of these halamines and free chlorine offer a method for rapid decontamination with long-term residual activity with only one treatment.

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TABLE 3. Effects of storage temperature and duration on the biocidal activities of compounds tested^a

Compound or mixture	Storage duration (wk)	Contact time (min) ^b and total residual chlorine concn ^c at:		
		4°C	25°C	37°C
Ca(OCl) ₂	0	— ^d (—)	1 (2.0)	— (—)
	1	— (—)	1 (1.25)	1.5 (0.8)
	2	1 (2.0)	>30 (0.0)	>30 (0.0)
	3	— (—)	>30 (0.0)	>30 (0.0)
	4	1 (<2)	>30 (0.0)	>30 (0.0)
Mixture of Ca(OCl) ₂ and compound 1 ^e	0	— (—)	1 (4.0)	— (—)
	1	— (—)	1.5 (3.2)	1.5 (2.75)
	2	1 (4.0)	30 (2.0)	30 (2.0)
	3	— (—)	30 (2.0)	30 (<2.0)
	4	1 (4.0)	30 (2.0)	30 (<2.0)
Compound 1	0	— (—)	20 (4.0)	— (—)
	1	— (—)	20 (4.0)	20 (4.0)
	2	20 (4.0)	20 (4.0)	20 (4.0)
	3	— (—)	20 (4.0)	20 (<4.0)
	4	20 (4.0)	20 (4.0)	20 (<4.0)

^a Compounds were stored at twice the final chlorine concentration and diluted with an equal volume of demand-free buffer (pH 7.0, 25°C) before assay.
^b For a >6-log decrease in CFU per milliliter.
^c Values in parentheses. In milligrams of total chlorine per liter, as measured by the *N,N*-diethyl-*p*-phenylenediamine method at the end of the assay.
^d —, Not done.
^e 1:1 (wt/wt of total chlorine) mixture.

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