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Effects of Three Oxidizing Biocides on *Legionella pneumophila* Serogroup 1

E. L. DOMINGUE,[1,2] R. L. TYNDALL,[2]* W. R. MAYBERRY,[3] AND O. C. PANCORBO[4]

Department of Environmental Health[1] and Department of Microbiology,[3] Quillen-Dishner College of Medicine, East Tennessee State University, Johnson City, Tennessee 37614; Department of Zoology, University of Tennessee, Knoxville, Tennessee 37996[2]; and Environmental Health Science Program, Department of Food Science and Technology, University of Georgia, Athens, Georgia 30602[4]

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A study was conducted to determine the bactericidal effects of ozone and hydrogen peroxide relative to that of free chlorine on *Legionella pneumophila* serogroup 1. In laboratory batch-type experiments, organisms seeded at various densities were exposed to different concentrations of these biocides in demand-free buffers. Bactericidal effects were measured by determining the ability of *L. pneumophila* to grow on buffered charcoal-yeast extract agar supplemented with α-ketoglutarate. Ozone was the most potent of the three biocides, with a greater than 99% kill of *L. pneumophila* occurring during a 5-min exposure to 0.10 to 0.30 μg of O_3 per ml. The bactericidal action of O_3 was not markedly affected by changes in pH or temperature. Concentrations of 0.30 and 0.40 μg of free chlorine per ml killed 99% of the *L. pneumophila* after 30- and 5-min exposures, respectively. A 30-min exposure to 1,000 μg of H_2O_2 per ml was required to effect a 99% reduction of the viable *L. pneumophila* population. However, no viable *L. pneumophila* could be detected after a 24-h exposure to 100 or 300 μg of H_2O_2 per ml. Attempts were made to correlate the biocidal effects of O_3 and H_2O_2 with the oxidation of *L. pneumophila* fatty acids. These tests indicated that certain biocidal concentrations of O_3 and H_2O_2 resulted in a loss or severe reduction of *L. pneumophila* unsaturated fatty acids.

The presence of *Legionella* spp. in a wide range of aquatic habitats, both natural and human-made, is well documented (10, 11, 20, 27, 28, 32, 34, 36). During the last decade, epidemiological and environmental investigations have shown that cooling system waters are potential environments for the amplification and dissemination of these bacteria. Cooling towers and evaporative condensers have been implicated as the common source of infectious *Legionella* spp. in some outbreaks of legionellosis (4, 5, 13, 18, 19). Conversely, these potential pathogens have also been found in cooling systems and natural waters in the absence of overt outbreaks of disease (20, 34). Questions about the virulence patterns, minimum infectious dose, resistance to drying, and ecological factors contributing to amplification and persistence of legionellae have yet to be fully answered. Although eradication of *Legionella* spp. from all cooling system aquatic habitats may not be practical or necessary, control of *Legionella pneumophila* serogroup 1 densities in selected systems might be desirable through effective biocidal treatment regimens. This is especially prudent in population areas with high-risk, immunocompromised individuals (17, 24, 33).

Inadequacies exist in current biocidal treatment practices for cooling system waters. Several organic biocides in use today have been shown to be ineffective in controlling *Legionella* densities (8, 12, 15, 20, 34). Fliermans and co-workers (9) have demonstrated the effectiveness of hyperchlorination for the initial reduction of *Legionella* populations in cooling system waters. This practice in certain systems, however, may be potentially deleterious to heat exchangers. Moreover, U.S. Environmental Protection Agency regulations governing periodic discharges of cooling system water into local rivers or streams have begun to restrict the amount of chlorine residual present in such discharges (35). The cooling system industry is therefore in need of environmentally safe biocides that are compatible with system operation and effective in reducing the burden of potentially infectious *Legionella* spp.

This study was undertaken to investigate the efficacy of alternative biocides such as ozone and hydrogen peroxide in killing *L. pneumophila* serogroup 1, the most common environmental and clinical isolate of the genus *Legionella*. Field studies, laboratory studies, or both by Pope et al. (30) and Edelstein et al. (6) have indicated the possible usefulness of ozone in controlling *Legionella* populations. In the present study, the biocidal effectiveness of ozone was studied at the different temperatures and pH values that reflect cooling tower operating conditions. In addition, the ozone sensitivity of *Pseudomonas aeruginosa*, another potentially pathogenic microorganism found in cooling system environments, was compared with that of *L. pneumophila*; and the efficacies of three oxidizing biocides, i.e., ozone, hydrogen peroxide, and free chlorine, in destroying *L. pneumophila* were compared under standard conditions of pH 7.2 and 25°C.

MATERIALS AND METHODS

Bacterial isolates. *L. pneumophila* serogroup 1 was kindly provided by Carl Fliermans (E. I. du Pont de Nemours & Co., Aiken, S.C.). This environmental isolate was cultured from thermally altered water. The *P. aeruginosa* isolate was cultured from cooling tower water in the Oak Ridge, Tenn., area. *P. aeruginosa* identification was confirmed by testing with the API-20E and Rapid-NFT biochemical test systems (Analytab Products, Plainview, N.Y.). Before the isolates were frozen in 1.0-ml portions at −50°C, the *P. aeruginosa* isolate was grown for 18 to 24 h on tryptic soy agar (TSA; Difco Laboratories, Detroit, Mich.), and *L. pneumophila* serogroup 1 was grown for 72 h on buffered charcoal-yeast

* Corresponding author.

extract supplemented with α-ketoglutarate (BCYE-α) agar (GIBCO Diagnostics, Madison, Wis.).

Generation and distribution of O_3. The generation of O_3 was accomplished by the corona electrical discharge method at 15,000 V by using pure oxygen (O_2) as the feed gas. The ozone generator (Dynamic Concepts Corp., Miami, Fla.) contained a series of three glass tubes (length, 10 in [25.4 cm]) which housed three aluminum electrodes. A small percentage (2 to 3%) of the O_2 flowing inside the glass tube series was converted to ozone during the electrical discharge. The equipment was modified to allow the flow of O_2 gas through the system without concomitant production of O_3. This latter feature provided the capability of oxygenating the various buffer solutions for control purposes. The gases, either O_2 or a mixture of O_2 and O_3, were distributed with Teflon (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) or tygon tubing and passed through several glass wool filters before contacting the liquid buffer systems via porous-fritted glass tubes.

Preparation of glassware. All glassware was washed with laboratory detergent (Bio-Mild; Green Mountain Research Corp., Waterbury, Vt.) and rinsed thoroughly with tap water, deionized water, and finally, deionized distilled water. Glassware was then subjected to several 1-h soakings in a strong O_3-water solution (2 to 4 μg/ml), followed by dry heat sterilization for 7 h at 180°C (356°F). This treatment allowed for the oxidation of any residual organic material which would otherwise have interfered with the determination of the oxidant concentrations necessary for inactivation of *L. pneumophila*.

Buffer solutions. Three buffer solutions were used in these experiments. A 0.01 M phosphate buffer was used to achieve pH 7.2 and 8.0. A 0.01 M sodium carbonate-bicarbonate buffer was used to achieve pH 8.9. High-purity deionized distilled water was treated with ozone in 6-liter volumes for 50 min to oxidize any substances that may have been present. This ozone-treated water was then boiled for 1 h to dissipate any remaining ozone residual. In this manner, water used to prepare the phosphate and carbonate buffers was rendered demand-free. All buffers were filter-sterilized through 0.22-μm-pore-size membrane filters (type L-S; Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.) and stored in sterile demand-free glass bottles.

Biocide preparations. (i) Ozone. Ozone was generated at 25°C and bubbled into approximately 200 ml of buffer solution contained in a beaker with a magnetic mixing bar rotating at 60 to 80 rpm. Dilutions were made directly from this O_3 solution to achieve the final concentrations in micrograms per milliliter. The standard iodometric method for ozone determination, as outlined previously (1), was modified for this study. Equal volumes of the ozone solution and a 5.5% KI solution were rapidly mixed, acidified with concentrated H_2SO_4, and then titrated with a standardized 0.01 N sodium thiosulfate solution by using an excess of thyodene starch (Fisher Scientific Co., Fairlawn, N.J.) as the indicator.

(ii) Chlorine. A solution of 4 to 6% sodium hypochlorite (Fisher Scientific) was diluted in demand-free buffer to prepare a stock sodium hypochlorite solution containing approximately 3.0 μg of free chlorine per ml. Triplicate 100-ml volumes of this stock were titrated by the DPD (*N*,*N*-diethyl-*p*-phenylenediamine)-ferrous ammonium sulfate method (1). After the mean concentration of free chlorine was determined, this stock solution was diluted further with demand-free buffer to achieve the final level in micrograms per milliliter for exposure to bacterial suspensions.

(iii) Hydrogen peroxide. Two solutions of H_2O_2, either 3% H_2O_2 (Medic, Smyrna, Tenn.) or 30% H_2O_2 (Mallinckrodt, Paris, Ky.), were diluted with demand-free buffer to achieve the final level in micrograms per milliliter for exposure to bacterial suspensions. The Kingzett iodide method was used to confirm the percentage of H_2O_2 in these solutions (14).

Experimental procedure. On the day of each experiment, frozen vials of organisms were thawed in a water bath at 37°C. Bacterial suspensions were then washed three times with the appropriate buffer. After each wash, organisms were pelleted by centrifugation at approximately 1,000 × *g* for 15 min at 25°C. For some experiments, the frozen stock of *L. pneumophila* was inoculated into yeast extract broth (1% yeast extract, 1% ACES (*N*-[2-acetamido]-2-aminoethanesulfonic acid) buffer, 0.025% ferric nitrate, 0.04% L-cysteine, 0.1% α-ketoglutarate; the pH was adjusted to 6.9 with 5 N KOH) or onto BCYE-α agar and incubated at 35°C until the culture was in the late-log or early-stationary phase. Suspensions of these organisms with a density equivalent to that of the frozen stock of *L. pneumophila* were washed as described above. The final density of all bacterial suspensions was adjusted with buffer to yield 10^5 to 10^6 CFU/ml when the suspensions were diluted in a 10-ml volume. In some experiments, the density of organisms was adjusted to 10^7 to 10^9 CFU/ml. Following the final wash, organisms were equilibrated in the appropriate buffer at the indicated temperature for approximately 2 h before they were exposed to biocides.

Biocides were divided into aliquots by using glass pipettes and placed into sterile glass culture tubes containing Teflon (du Pont) magnetic stirring bars. The total volume in each tube was held constant at 10.0 ml. Tube contents were mixed at 60 to 80 rpm with a magnetic stirrer for the duration of each experiment and incubated at 25, 35, or 45°C. Control tubes containing only the organisms suspended in buffer were included for every experiment.

Biocidal activity was quenched at various contact times by removing 1.0-ml volumes from each tube and quickly adding them to 9.0 ml of buffered dilution water (3.1×10^{-4} M KH_2PO_4 per liter, 1.0×10^{-3} M $MgSO_4 \cdot 7H_2O$ per liter [pH 7.2]) which contained 0.1 ml of a 10% sodium thiosulfate solution. This concentration of sodium thiosulfate quenched the oxidizing activity of ozone and free chlorine and reduced the activity of hydrogen peroxide. The rapid decay of ozone and chlorine, the low concentrations of ozone and chlorine, or both in the test systems precluded determination of the oxidant concentration at the various contact times. Hydrogen peroxide lost none of its oxidizing potential over the 24-h contact period after the addition of *L. pneumophila*. The sensitivities of *L. pneumophila* and *P. aeruginosa* to sodium thiosulfate were determined by exposing various dilutions of the organisms to sodium thiosulfate under the experimental conditions described above. No deleterious effect of the quenching agent was observed.

Following quenching of the biocides, the organisms were serially diluted in buffered dilution water. Duplicate 0.1-ml volumes of these dilutions were spread plated onto either TSA (for *P. aeruginosa*) or BCYE-α agar (for *L. pneumophila*). The TSA plates were incubated for 18 to 24 h at 35°C. Negative TSA plates were incubated for 7 days before they were discarded. The BCYE-α plates were incubated for 4 days at 35°C in a humid atmosphere. Negative BCYE-α plates were incubated for 10 days before they were discarded. Bacterial colonies were counted manually with the aid of a colony counter (New Brunswick Scientific Co., Inc., Edison, N.J.). All plates were used for enumeration, but in

most cases final determination of CFU per milliliter was made from plates that contained between 30 and 300 colonies. The limit of detectability for enumeration was 10 CFU/ml when 0.1 ml was plated directly. The absence of detectable colony formation was considered evidence of a biocidal effect, although the possibility exists that the *L. pneumophila* organisms were damaged to the extent that they could no longer replicate on BCYE-α but might have replicated in more complex environmental milieus.

For each experiment the lowest concentration of biocide that resulted in at least a 99% reduction in viable organisms was designated as the endpoint. Two or more experiments were performed, and determination of the mean biocide concentration for 99% inactivation in a group of related experiments was made. A 99% inactivation corresponded to a log survival ratio [log (N_t/N_0), where N_t and N_0 are bacterial concentrations at times t and zero, respectively] of −2.00.

Since aggregation of microorganisms can exert a protective effect during the disinfection process and can result in decreased bacterial sensitivity to biocides, an estimate of the proportion of aggregated organisms in *L. pneumophila* suspensions was made by using a direct fluorescent antibody technique (3). Less than 10% of the *L. pneumophila* population was aggregated in either the phosphate or the carbonate buffers.

Fatty acid analysis. Prior to fatty acid analysis, exposure of *L. pneumophila* to the various biocides and quenching of the biocidal activity was done as described above. After exposure for either 30 or 60 min, organisms were pelleted by centrifugation at 1,000 × *g* for 40 min. Organisms were suspended in approximately 2.0 ml of methanol and stored at room temperature until the fatty acid analysis could be performed as described by Mayberry (25).

RESULTS

Because of the reactive nature and natural decay tendency of ozone in solutions (16, 29, 38), ozone decay under the experimental conditions chosen for this study was determined. Ozone decay was defined as a loss of oxidizing capacity, as measured by the ability to liberate free iodine from a 5.5% KI solution. A greater decay rate occurred at pH 8.0 than at pH 7.2 during the first 5 min (Fig. 1).

Comparisons of the biocidal activities of ozone at different pHs and temperatures are shown in Table 1. Under standard conditions of pH 7.2 and 25°C, 0.10 to 0.30 μg of O_3 per ml effected at least a 99% inactivation of *L. pneumophila* within 5 min. Similar bactericidal effects were also obtained with *P. aeruginosa* within 5 min after exposure to 0.20 to 0.30 μg of O_3 per ml. No differences were noted in the O_3 sensitivities of stored frozen stock of *L. pneumophila* and freshly grown *L. pneumophila* harvested during the late-log or the early-stationary growth phase. By increasing the temperature to 35 and 45°C at pH 7.2, the amount of O_3 needed for at least 99% inactivation of *L. pneumophila* was 0.11 to 0.15 and 0.11 to 0.14 μg/ml, respectively. The control tubes of *L. pneumophila* exposed to these higher temperatures showed a less than 10% loss of viability at 35°C, while 25% loss of viability occurred at 45°C.

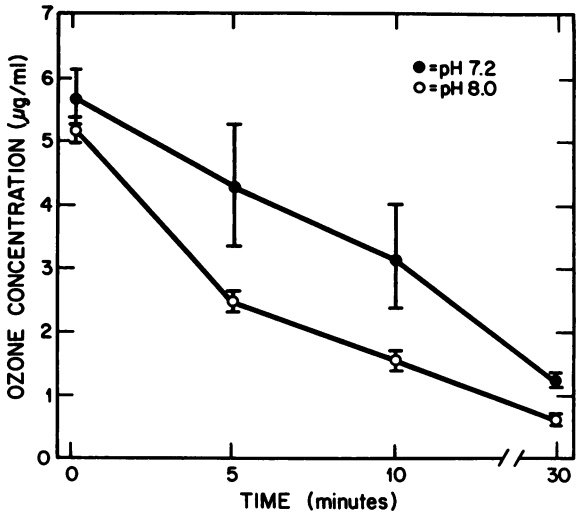

FIG. 1. O_3 decay in demand-free, 0.01 M phosphate buffer at 25°C. Points represent the mean of triplicate experiments. Bars represent ± 1 standard deviation.

The effect of increasing pH on O_3 inactivation of *L. pneumophila* is also shown in Table 1. At pH 8.0 and 25°C, a concentration of 0.20 μg of O_3 per ml effected at least a 99% inactivation within 5 min. When the pH was increased to 8.9 at 25°C, a level of 0.14 μg of O_3 per ml was required for inactivation. Inactivation for each group of experiments with O_3 consistently occurred within 5 min. Because of the temporal constraints when the various concentrations of

TABLE 1. Ozone inactivation of *L. pneumophila* serogroup 1 and *P. aeruginosa*

Organism	pH	Temp (°C)	No. of tests	O_3 concn (μg/ml) for approx. 99% inactivation[a]			Log(N_t/N_0)[b]		
				Mean	Range	SD	Mean	Range	SD
L. pneumophila	7.2	25	6[c]	0.21	0.10–0.30	0.10	−2.37	−2.03 to −2.60	0.22
L. pneumophila[d]	7.2	35	2	0.13	0.11–0.15	0.03	−2.21	−2.14 to −2.28	0.10
L. pneumophila[e]	7.2	45	2	0.13	0.11–0.14	0.02	−2.55	−2.51 to −2.59	0.06
L. pneumophila	8.0	25	2	0.20	0.19–0.20	0.01	−2.45	−2.10 to −2.79	0.49
L. pneumophila	8.9	25	2	0.14	0.13–0.14	0.01	−3.28	−2.64 to −3.92	0.91
P. aeruginosa	7.2	25	3	0.27	0.20–0.30	0.05	−2.19	−1.66 to −2.79	0.57

[a] Rate of inactivation for each group of experiments occurred within the minimum contact time of 5 min.

[b] Survival ratio is log(N_t/N_0), where N_t and N_0 are bacterial concentrations at time t and 0, respectively. Log(N_t/N_0) = −2.00 corresponds to a 99% inactivation. Mean $N_0 = 4.5 \times 10^5$ CFU/ml.

[c] Includes one test in which the bacteria were grown on BCYE-α agar and one in which bacteria were grown in yeast extract broth and tested immediately on harvest. The mean O_3 concentrations required for 99% inactivation were 0.15 and 0.25, respectively, which were indistinguishable from those for the frozen stock culture used in other replicate experiments.

[d] Less than 10% loss of viability occurred in the control tube at 35°C.

[e] A 25% loss of viability occurred in the control tube at 45°C.

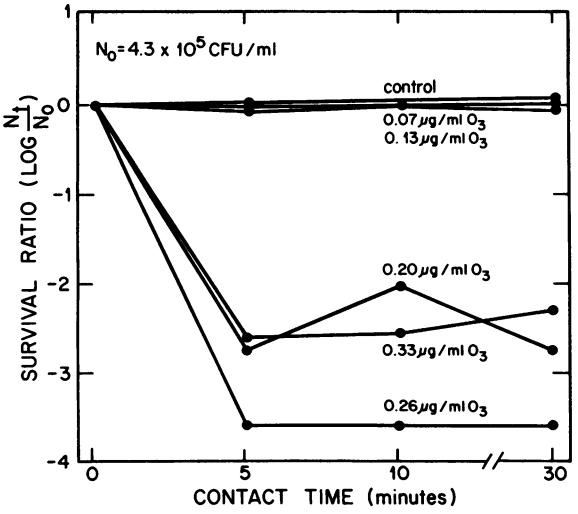

FIG. 2. Effect of O_3 concentration on the inactivation of *L. pneumophila* serogroup 1 in 0.01 M phosphate buffer (pH 8.0 and 25°C).

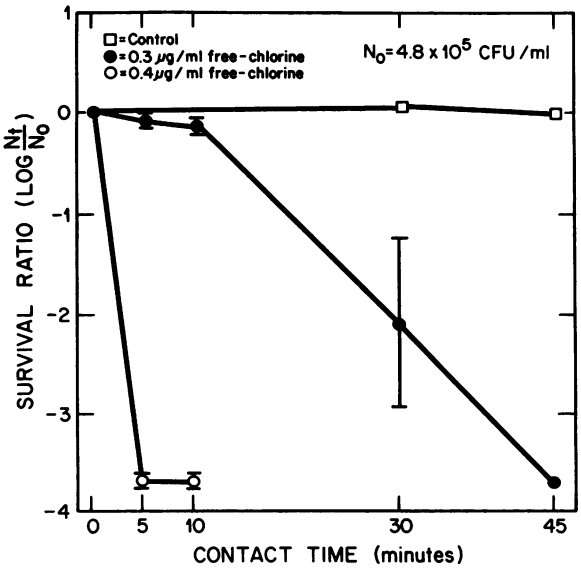

FIG. 3. Effect of free chlorine concentration on the inactivation of *L. pneumophila* serogroup 1 in 0.01 M phosphate buffer (pH 7.2 and 25°C). Points represent the mean of duplicate experiments. Bars represent ± 1 standard deviation.

ozonated buffer were divided into aliquots, 5 min was the minimum sampling time for each reaction tube.

The ozone inactivation kinetics for *L. pneumophila* at pH 8.0 is shown in Fig. 2. Inactivation of *L. pneumophila* occurred within 5 min once a critical concentration of ozone was reached. Ozone levels of 0.13 μg/ml or less had no effect, even at contact times of up to 30 min. Not until a concentration of at least 0.20 μg/ml was reached did inactivation occur.

Exposure of *L. pneumophila* to 0.3 μg of free chlorine per ml resulted in a 99% inactivation after a 30- to 45-min contact time (Fig. 3). At a concentration of 0.4 μg of free chlorine per ml, a >99% inactivation occurred within 5 min, indicating that there is a dose-response relationship between the concentration of free chlorine and the rate of inactivation. Evidence for the bacteriostatic effects of free chlorine on *L. pneumophila* were observed at the lower chlorine concentration of 0.3 μg/ml for prolonged contact times (30 min or greater). Late-occurring colonies were noted on days 6 and 8 after incubation. Control plates with comparable numbers of organisms did not show similar late-occurring growth.

In contrast to ozone and free chlorine, H_2O_2 inactivation of *L. pneumophila* required much higher concentrations under standard conditions. It is shown in Fig. 4 that >99% of the *L. pneumophila* population was inactivated at 1,000 μg of H_2O_2 per ml within 30 min. If the concentration was increased to 10,000 μg/ml, a >99.99% inactivation occurred at a faster rate. This was true for all initial concentrations of *L. pneumophila* from 10^5 to 10^9 CFU/ml. It is also evident from the results presented in Fig. 4 that lower concentrations of 100 and 300 μg of H_2O_2 per ml had no appreciable effect within 60 min, but required a longer contact time for inactivation. After a 24-h contact time, with an initial (N_0) *L. pneumophila* concentration of 7.1 × 10^5 CFU/ml, no viable organisms could be detected after exposure to 100 and 300 μg of H_2O_2 per ml.

The loss or severe reduction in *L. pneumophila* unsaturated fatty acids after exposure of approximately 2.0 × 10^9 CFU/ml to 7.2 μg of O_3 per ml can be seen in Table 2. Reduction in unsaturated fatty acids was also evident after exposure of *L. pneumophila* to 10,000 μg of H_2O_2 per ml, but not after exposure to 1,000 μg of H_2O_2 per ml. Organisms were also exposed to oxygenated buffer for the maximum 60-min contact time with neither a reduction in viable organisms nor an alteration of fatty acid composition.

DISCUSSION

The biocidal effect of ozone was demonstrated in this study. Ozone was effective against *P. aeruginosa* and *L.*

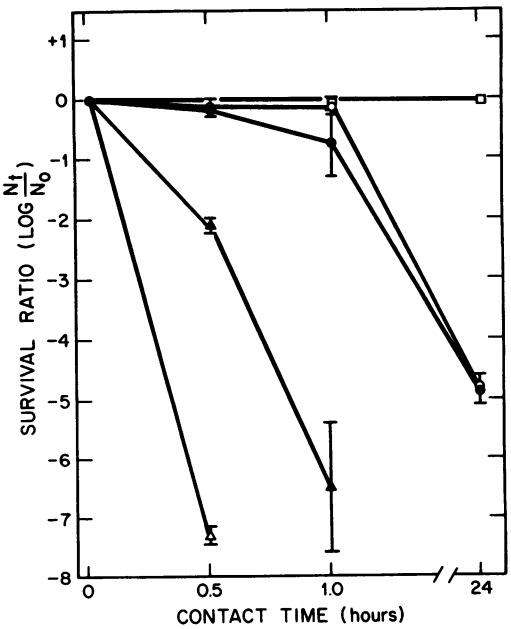

FIG. 4. Effect of H_2O_2 concentration on the inactivation of *L. pneumophila* serogroup 1 in 0.01 M phosphate buffer (pH 7.2 and 25°C). Points represent the mean of duplicate experiments. Bars represent ± 1 standard deviation. For 100 μg/ml (○) and 300 μg/ml (●), N_0 = 7.1 × 10^5 CFU/ml; for 1,000 μg/ml (▲) and 10,000 μg/ml (△), N_0 = 1.9 × 10^9 CFU/ml; control (□) is indicated.

TABLE 2. Effect of various biocide treatments on the nonhydroxy fatty acid composition of *L. pneumophila*, serogroup 1

Treatment[a]	Reaction time (min)	% Reduction in viability[b]	Fatty acid identification and relative abundance[c,d,e]					
			n15:1[d]	i16:1	i16:0	n16:1	i17:1	a17:1
Control + $Na_2S_2O_3$	60	0	4	4	100	26	tr	tr
Control of 7.2 μg of O_3 per ml + $Na_2S_2O_3$	60	0	4	5	100	22	tr	tr
Control O_2	60	0	4	5	100	23	tr	tr
1,000 μg of H_2O_2 per ml	30	99.0	3	4	100	19	1	tr
1,000 μg of H_2O_2 per ml	60	99.99	4	5	100	23	tr	tr
10,000 μg of H_2O_2 per ml	30	99.99	ND[f]	ND	100	1	ND	ND
10,000 μg of H_2O_2 per ml	60	99.99	ND	ND	100	ND	ND	ND
7.2 μg of O_3 per ml	30	99.98	ND	ND	100	1	ND	ND
7.2 μg of O_3 per ml	60	99.99	ND	ND	100	9	ND	ND

[a] Organisms were exposed to biocides in glass tubes containing approximately 50 ml of the biocides diluted in 0.01 M phosphate buffer (pH 7.2); tubes were seeded with approximately 2.0×10^9 CFU/ml from the frozen stock culture. Reactions occurred at 25°C with a mixing speed of 60 to 80 rpm. Biocidal activity was quenched on the addition of 0.1 ml of 10% $Na_2S_2O_3$ per 10-ml reaction volume.

[b] Viability was determined by serially diluting the organisms in buffered dilution water, followed by plating 0.1-ml volumes of these dilutions onto BCYE-α agar.

[c] Fatty acid profiles were analyzed as methyl esters and are expressed as relative abundance, i16:0 = 100 (see footnote *d* for explanation of i16:0).

[d] Designations indicate the number of carbons: the number of double bonds; abbreviations: i, iso-branched; a, anteiso-branched; cyc, cyclopropane; tr, trace; n, normal (straight chain).

[e] Nonhydroxy fatty acids not altered by these treatments included the following (mean relative abundance): i14:0(18), n14:0(tr), i15:0(tr), a15:0(49), n15:0(2), n16:0(12), i17:0(tr), a17:0(23), 17cyc(3), n17:0(1), i18:0(1), n17:0(5), a19:0(tr), n19:0(1), i20:0(tr), a21:0(tr), and n21:0(tr).

[f] ND, Not detected.

pneumophila serogroup 1 at the pHs and temperatures investigated. At low levels (0.10 to 0.30 μg of O_3 per ml), the rate of inactivation was very rapid (≤5 min). These data compare favorably with the minimal O_3 values found to be effective against other bacteria. Broadwater et al. (2) noted a lethal threshold level of 0.12 μg of O_3 per ml for *Bacillus cereus* and a level of 0.19 μg of O_3 per ml for *Escherichia coli* and *Bacillus megaterium* when these organisms were seeded at 10^6 CFU/ml; the rate of inactivation occurred within 5 min. Edelstein and co-workers (6) reported that a minimum concentration of 0.32 μg of O_3 per ml resulted in a 4-log-unit reduction of *L. pneumophila* serogroups 1 and 4, which were also seeded at 10^6 CFU/ml. In field studies, Pope et al. (30) showed that ozone effectively reduced the bacterial population in cooling tower water, including, to a degree, *L. pneumophila*. The pHs and rate of inactivation were not indicated in these studies.

At comparable microgram per milliliter levels in this study, ozone inactivated *L. pneumophila* more rapidly than did free chlorine. Such a rapid rate of inactivation may be a definite advantage in a large recirculating water system where retention time in a treatment loop is necessarily short. Ozone was effective against *L. pneumophila* over a pH range of 7.2 to 8.9, despite the natural decay and faster rate of decay of ozone at alkaline pH. In contrast, the reduced biocidal effectiveness of free chlorine at pH values of >7.0 has been well documented (9, 22). This comparison may make ozone a more efficient biocidal agent in recirculating water systems that tend to be alkaline. Also noteworthy was the observation of bacteriostatic effects at low levels of free chlorine for prolonged contact times. Evidence of chlorine resistance in *Legionella* populations has been cited by other investigators (21, 22, 37). The resistance of *Legionella* spp. to low levels of chlorine may help to explain, in part, the presence and persistence of legionellae in potable as well as recirculating water systems.

In contrast to both ozone and free chlorine, H_2O_2 inactivation of *L. pneumophila* required much higher microgram per milliliter levels. However, H_2O_2 inactivation did exhibit a dose-response relationship between the concentration of H_2O_2 and the rate of inactivation. This was similar to free chlorine inactivation but differed from ozone inactivation, which occurred very rapidly after a critical level of ozone was reached. The lowest concentration of H_2O_2 tested, i.e., 100 μg/ml, reduced the *L. pneumophila* population below the limit of detectability, but prolonged contact times of ≤24 h were required at pH 7.2 and 25°C. Unlike ozone, there was no decrease in the oxidizing potential of the various concentrations of H_2O_2 during the 24-h contact time. Yoshpe-Purer and Eylan (39) reported a similar prolonged rate of H_2O_2 inactivation against mixed populations of a variety of bacteria. Persistent killing effects of up to 13 days were noted in solutions containing 30 to 90 μg of H_2O_2 per ml. During an investigation of in vivo microbiocidal systems against *L. pneumophila*, Locksley et al. (23) were able to demonstrate a 99% inactivation of 10^6 CFU/ml at 66 μg of H_2O_2 per ml within 1 h at 37°C and with constant agitation. While perhaps not amenable to large cooling tower application, H_2O_2 may be useful in the control of *Legionella* populations in the treatment of evaporative condensers or other smaller volumes of water.

The loss or severe reduction of *L. pneumophila* unsaturated fatty acids following exposure to ozone was not unexpected, owing to the powerful oxidizing capability of ozone and previous studies of ozone oxidation of commercially prepared unsaturated fatty acids (31). The ability of O_3 to attack the unsaturated fatty acids of *L. pneumophila* and to kill the bacteria in this experimental system probably reflects the powerful oxidizing capability of this unstable biocide, its rapid rate of diffusion through bacterial cell walls, and its rapid rate of reaction with a wide range of organic compounds (26, 31, 38). Since fatty acids are an integral component of bacterial membranes, disruption of membrane structure and, hence, function may be a critical effect of O_3 exposure. In particular, membrane-associated proteins containing sulfhydryl groups can be readily oxidized by ozone (26).

The retention of unsaturated fatty acids after exposure of *L. pneumophila* to 1,000 μg of H_2O_2 per ml, concomitant with a 99% plus loss in viability, was unexpected. This was in contrast to the loss of unsaturated fatty acids and viability with exposure to higher levels of H_2O_2 and O_3. Such data suggest that the loss of *L. pneumophila* unsaturated fatty acids following exposure to H_2O_2 is probably not the lethal event per se. In addition, O_3 attacks the double bonds of unsaturated fatty acids more rapidly and possibly by a

different mechanism than does H_2O_2. Another factor which may effect the response of *L. pneumophila* to H_2O_2 compared with that of O_3 and chlorine is the presence of catalase in many species of *Legionella*. The increased levels of H_2O_2 necessary for the killing of *L. pneumophila* and oxidation of its fatty acids may reflect the destruction of H_2O_2 by the bacterial enzyme catalase.

One advantage to a study such as this one is that comparisons of amounts of biocides and their relative rates of inactivation can be made under standard controlled conditions. Such comparisons do not imply how effective these biocides would be in a complex environmental situation. Certainly, the interplay of many chemical, physical, and biological parameters within a cooling system environment determines the biocidal effectiveness of each particular water treatment, as does the dissociation species of a biocide. Competition of the biocides for reaction with organic matter is also a factor. Additionally, bacterial populations in their natural environment presumably grow more slowly than do those same populations cultivated on nutrient-rich media. *Legionella* populations, in their natural aquatic habitats, may exhibit different sensitivities to these biocides in comparison with their laboratory-grown counterparts.

Ozone and hydrogen peroxide are not currently in widespread use in the cooling system industry, but they are becoming more attractive as possible environmentally safe alternatives to the use of organic biocides and chlorine (7). The final decomposition products of both O_3 and H_2O_2 are O_2 and H_2O. In the case of O_3, no residual persists in water under normal conditions. Because of its persistent biocidal activity, H_2O_2 may be a useful adjunct biocide.

As with any biocide, the true test of effectiveness in environmental situations is through well-planned monitoring in the field. The results of this laboratory study give credence to and support the need for field studies into the effectiveness of both O_3 and H_2O_2 in controlling *Legionella* populations.

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