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Efficacy of methylchloro/methylisothiazolone biocide against *Legionella pneumophila* in cooling tower water

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SUMMARY

Methylchloro/methylisothiazolone biocide was tested for efficacy in cooling tower water against *Legionella pneumophila* (strains Flint 1 and 2) and *L. gormanii.* These analyses are the first reports on the efficacy of isothiazolones against *Legionella* in actual cooling tower water; the results of data reported previously obtained from more arbitrary laboratory tests are discussed and compared. The biocide was effective in cooling tower water at the two pH levels tested (pH 8.0 and 6.7). The concentration of biocide required to eliminate the *L. pneumophila* depended on contact time and pH: 99% of the bacteria were killed after 6 and 3 h of contact by 1.07 and 3.13 ppm active ingredient, respectively, when the pH was 8.0: 99% of the bacteria were killed after 6 and 3 h of contact by 2.23 and 9.43 ppm active ingredient, respectively, when the pH was 6.7. 24 h of contact with 0.35 ppm active ingredient methylchloro/methylisothiazolone biocide reduced the concentration of viable bacterial cells by more than four orders of magnitude (> 99.99%), regardless of pH.

INTRODUCTION

The study of Legionnaire's disease has proceeded continuously in medicine, medical microbiology and environmental microbiology since the recognition of symptoms in 1976 [3,5,6]. Many previously undiagnosable pneumonias are now more effectively treated because of newly developed identification and isolation techniques for pathogenic *Legionella* [4]. The microbiological quality of the water used in cooling systems has been identified as one of several targets for the control of disease outbreak especially in public areas such as hotels, hospitals and apartment buildings [1,13,17]. The following study was performed in order to assess the efficacy of methylchloro/methylisothiazolone biocide against *Legionella* in cooling tower water. The need for this work was recognized after surveying the literature with regard to biocidal activity of isothiazolones against *Legionella pneumophila.* In particular, Soracco et al. [19] tested the efficacy of 11 biocides, including isothiazolones, against *L. pneumophila* in a nutrient broth that contained 0.2% yeast extract and 200 ppm L-cysteine hydrochloride. Skaliy et al. [18] tested the efficacy of 6 biocides in a sterile tap water laboratory assay. Thus, although isothiazolones performed well as antibacterial agents in both of these arbitrary lab-

oratory tests, the question as to efficacy in actual cooling tower water remained unanswered.

Cooling tower waters are generally enriched in mineral content (relative to the make-up water) with especially high levels of calcium, magnesium, silica, carbonate and phosphate (total hardness is usually 400 to 900 ppm calcium carbonate equivalents). Similarly, organic materials tend to concentrate in the cooling tower circuit and can become significant inactivators of certain oxidizing microbial biocides [10]. The pH of the water in cooling systems is an important factor to consider when describing efficacy of biocides, since the active ingredients in many commercial formulations are susceptible to alkaline hydrolysis. Sterilized tap water is perhaps an appropriate choice for simulating a cooling tower make-up supply but, since industrial cooling tower biocides are designed for use in the cooling tower circuit, an appropriate and nearly as convenient choice for laboratory efficacy tests is sterilized cooling tower water from typical industrial cooling systems. The results of laboratory efficacy tests that were designed to simulate industrial conditions in cooling tower water are reported in this paper. Methylchloro/methylisothiazolone efficacy against *Legionella* in industrial cooling systems is reported in terms of the actual concentrations of active ingredient and contact time required to control the organism.

MATERIALS AND METHODS

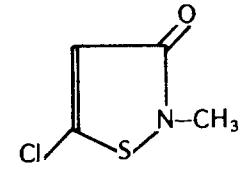
The biocide. Methylchloro/methylisothiazolone biocide is a broad-spectrum antimicrobial agent that is widely used in industrial cooling tower maintenance programs to control algae, bacteria and fungi. Commercial preparations of the compound for use as a cooling tower biocide (Kathon® WT) are water-based formulations of inorganic stabilizers and active ingredients. The active ingredients (Fig. 1), 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one, are present in an appproximate ratio of 3:1.

Isothiazolones are effective against microorganisms at extremely low concentrations (ppm range) and are highly resistant to the inhibitory effects of high pH and most organic and inorganic compounds [2,9,15,16]. The microbial activity of the biocide is probably due to its reactivity with cell membrane proteins; inhibition of macromolecular synthesis occurs as the result of this interaction (D.E. Greenley, Rohm and Haas Co., Spring House, PA, personal communication).

Bacteria. Cultures of *Legionella pneumophila* strain Flint 1 (serogroup 1), strain Flint 2 and *L. gormanii* [11,17] were maintained on (BCYE) buffered charcoal yeast extract agar plates [4] and transferred 2 days prior to use. Fresh suspensions were made in sterile distilled water immediately before testing.

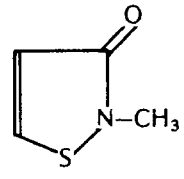
Cooling tower water. Water was obtained from taps in the Life Sciences Building at Wayne State University, MI and from the Saginaw Steering Gear Plant, Saginaw MI. The turnover time in the Wayne State cooling tower was about 2 days and there was no residual biocide in the water because all chemical treatment in the system had been suspended 1 week

5-Chloro-2-methyl-4-isothiazolin-3-one



CAS Reg. No. 26172-55-4

2-Methyl-4-isothiazolin-3-one



CAS Reg. No. 2682-20-4

Fig. 1. The active ingredients in the methylchloroisothiazolone biocide. Compounds are identified according to IUPAC nomenclature.

prior to sample collection. The concentration of bacterial cells in the Wayne State cooling tower water was approximately 10^5 cfu/ml; the sample was autoclaved immediately prior to use. The average temperature of Saginaw cooling tower water in the sump was 26°C and its pH 6.4; the total water hardness was between 400 and 500 ppm (EDTA colorimetric test strip method; Miles Environmental test Systems). The pH values of the test waters were adjusted to 6.7 or 8.0 with KOH and sterilized by filtration through 0.45 μm membrane filters. The Wayne State water was used for tests with *L. pneumophila* strain Flint 2 and *L. gormanii*; the Saginaw cooling tower water was used for tests with *L. pneumophila* strain Flint 1, serogroup 1.

Test systems. The tests were designed to determine the efficacy of methylchloro/methylisothiazolone against *Legionella* in cooling tower water. All tests were done at pH 6.7 or pH 8.0, 32°C in a volume of 100 ml. Sterile, filtered water (99 ml) was dispensed into sterile milk dilution bottles. Biocide was added volumetrically; the final concentration of active ingredient was calculated on a weight per volume basis. 1 ml of fresh viable cell suspension (see above) was added to the bottles. Inoculated cooling tower waters were sampled at 0, 3, 6 and 24 h by removing 1 ml aliquots and diluting into 9 ml phosphate-buffered saline. Samples were then assayed using a droplet plating method [12] on BCYE agar. The combined effects of dilution and plating

Table 1

The effect of methylchloro/methylisothiazolone biocide on *Legionella* in cooling tower water

Organism	pH	Active ingredients (ppm)	Logarithmic reductions		
			contact time (h): 24	6	3
L. pneumophila (strain Flint 1)	8.0	0.00	0.81	0.03	0.03
		0.35	> 4.20	0.66	0.13
		0.87	> 4.20	2.23	0.40
		1.74	> 4.20	3.60	1.03
		2.62	> 4.20	3.81	1.73
L. pneumophila (strain Flint 1)	6.7	0.00	0.22	0.00	0.02
		0.35	> 4.30	0.46	0.19
		0.87	> 4.30	0.82	0.05
		1.74	> 4.30	1.56	0.40
		2.62	> 4.30	1.56	0.43
L. pneumophila (strain Flint 2)	6.7	2.62	> 5.04[a]	n.t.	n.t.
		4.36	> 5.04	2.34	1.00
		8.72	> 5.04	2.74	1.09
		17.43	> 5.04	3.87	1.84
L. gormanii	6.7	0.00	1.00	1.00	0.00
		0.87	> 5.00[a]	n.t.	n.t.
		2.62	> 5.00[a]	n.t.	n.t.
		4.36	> 5.00[a]	4.30	2.70
		8.72	> 4.78	4.78	3.18
		17.43	> 4.78	> 4.78	3.00

[a] no cfu detected in 50 ml of inoculated cooling tower water.
n.t., not tested.

onto nutrient-rich media have been shown previously (data not included) as an effective means by which to inactivate these biocide treatments and thus, no neutralizer was required. The plates were incubated at 37°C for 7 days and colonies were enumerated in order to determine the concentration of colony forming units (cfu/ml). Plates with no growth were incubated an additional 7 days before final calculations were made. The detection of low levels of survivors was achieved by filtering 50 ml of the test suspension through sterile 0.45 μm membrane filters (Nuclepore) and incubating the filtered sample on BCYE plates as described above.

RESULTS

The results of the time course trial experiments are summarized in Tables 1 and 2 and Fig. 2. The concentrations of bacteria (cfu/ml) in the inoculated cooling tower waters at time zero were $2.0 \cdot 10^6$ and $1.6 \cdot 10^6$ (*L. pneumophila* strain Flint 1, at pH 8.0 and pH 6.7, respectively) and $1.1 \cdot 10^7$ and $1.0 \cdot 10^7$ (*L. pneumophila* strain Flint 2 and *L. gormanii*, respectively). Table 1 shows the reduction of viable bacteria as a function of biocide concentration: the data are expressed as the difference in the logarithms of cfu/ml at time 0 and after 24, 6 or 3 h of contact with the biocide. The reductions in viable bacteria were one log unit or less in all of the controls (no biocide). The 3 h and 6 h controls showed negligible log reductions of viable *Legionella* bacteria. Substantial reductions in the concentration of viable cells (4.2 to 5.0 orders of magnitude) were observed after 24 h of contact with the biocide regardless of the type of microorganism, type of cooling water, pH or concentration of active ingredient. Shorter contact times resulted in smaller reductions of viable cells in cooling tower waters; substantial reductions (greater than about two orders of magnitude) were observed after 6 h of contact if the concentration of biocide was 0.87 ppm active ingredient (at pH 8.0) or 2.62 ppm active ingredient (at pH 6.7). Similarly, 3 h of contact with the biocide resulted in smaller reductions of viable cells but substantial reductions were observed when the highest biocide concentrations were tested. Logarithmic reductions per ppm active ingredient were calculated by least-squares regression analysis; there was a statistically significant linear correlation between the logarithmic reduction of viable *L. pneumophila* (strain Flint 1) cells and the concentration of biocide; the data are shown in Table 2. Methylchloro/methylisothiazolone was more efficacious at higher pH: the biocide concentrations required for a 99% kill at pH 8.0 were about one

Table 2

Efficacy of methylchloroisothaizolone biocide against *Legionella pneumophila* (strain Flint 1, serogroup 1) in cooling tower water

Contact time (n)	pH	Log reduction per ppm A.I.	r^a	N^b	Conc. required to kill 99% (ppm A.I.)	Symbols (Fig. 2)
24	8.0	> 4.20	—	—	< 0.35	○
6	8.0	1.52	0.95	5	1.07	△
3	8.0	0.66	0.99	5	3.13	▽
24	6.7	> 4.30	—	—	< 0.35	■
6	6.7	0.87	0.99	4[c]	2.23	◇
3	6.7	0.21	0.96	6[d]	9.43	●

[a] r, product-moment correlation coefficient (Pearson).

[b] N, number of observations.

[c] log reduction of strain Flint 1 by 2.62 ppm active ingredient (A.I.) was omitted from the regression.

[d] log reduction of strain Flint 2 by 4.36 ppm active ingredient was included in the regression.

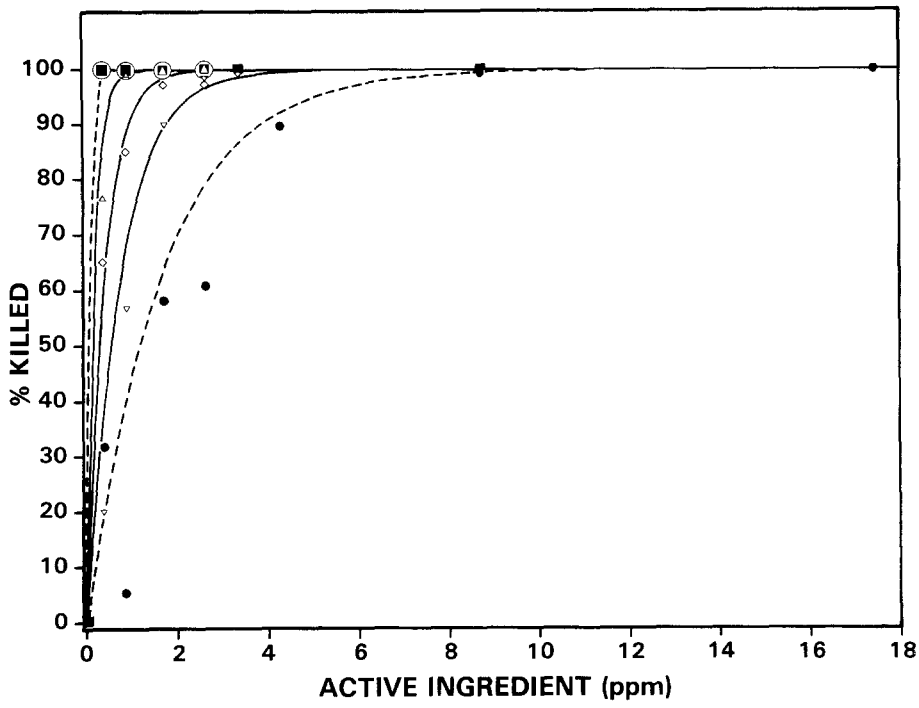


Fig. 2. The effect of methylchloroisothiazolone biocide on *Legionella pneumophila* in cooling tower water. The symbols are data recalculated from Table 1 and the curves were calculated from eqn. 3 (in Results). Symbols (also see Table 2): ○, △, ▽, pH 8.0 after 24, 6 and 3 h of contact, respectively; ■, ◇, ●, pH 6.7 after 24, 6 and 3 h of contact, respectively.

half (6 h contact time) or about one third (3 h contact time) of the concentrations required for the same kill when the pH was 6.7.

The number of survivors (N) as a function of biocide concentration was calculated with the following exponential expression (since the log reduction vs. concentration regressions were statistically significant):

$$N = N_i e^{-(k' \cdot \Delta t)c} \quad (1)$$

where N_i is the initial number of viable bacteria, $k' \cdot \Delta t$ is equal to the logarithmic reduction of viable bacteria per ppm active ingredient after contact time (Δt) per ppm active ingredient (from the regressions listed in Table 2) and c is the concentration of biocide (ppm active ingredient). Thus, the data could be significantly fitted with solutions to the following equation and are so depicted in Fig. 2 [10]:

$$\% \text{ killed} = 100\left(\frac{N_i^0 - N_i e^{-(k' \cdot \Delta t)c}}{N_i}\right)$$

$$100\,(1 - e^{-(k' \cdot \Delta t)c}) \quad (2)$$

DISCUSSION

Soracco et al. [19] tested the efficacy of methylchloro/methylisothiazolone against nine strains of *L. pneumophila* in a rich growth medium; efficacy was determined by observing growth/no growth in the presence or absence of the biocide. Methylchloro/methylisothiazolone was bacteriocidal towards all nine of the strains at the dosage recommended by the manufacturer. 10-fold and 100-fold dilutions of the biocide were also tested and shown to be inhibitory to some, but not all of the strains tested. Yeast extract and, especially, cysteine are known inactivators of methylchloro/methylsothiazolone at the high levels (0.2% and 200 ppm, respectively) present in these efficacy tests. Therefore, the efficacy of methylchloro/methylisothiazolone against *Legionella* in cooling tower water would be considerably underestimated by this laboratory test. Nevertheless, the data show that the biocide was effective against *L. pneumophila*; concentrations or contact times required for control of the

organism in industrial cooling systems cannot be inferred, however.

Skaliy et al. [18] showed that methylchloro/methylisothiazolone biocide was quite effective against *L. pneumophila* in tap water after 24 h of contact at the dosage recommended by the manufacturer and that higher concentrations of biocide gave significant reductions in viability after 6 h of contact. These data suggest that control of the microorganism in industrial cooling systems cannot be achieved immediately after contact with methylchloro/methylisothiazolone (as would be required of a disinfectant) but that the biocide may nevertheless maintain control if the treatment program provides contact times that are long enough. Maintenance of microbiological control in industrial cooling systems for example, is commonly achieved with methylchloro/methylisothiazolone because the contact time between biocide and microbial populations is long enough to be effective.

Our data essentially agree with those of Skaliy et al. [18] but they cannot be directly compared, unfortunately, because the pH of the tap water used in their efficacy test was not specified. Our data show a significant effect of pH on efficacy; the concentration of methylchloro/methylisothiazolone required to eliminate *Legionella* in cooling tower water was significantly lower at pH 8.0 than at pH 6.7. Tests run at alkaline pH (8.0) relate more closely to conditions in most cooling towers, especially those in the United States and South America, because the most popular corrosion-inhibition programs require alkaline conditions [10]. Cooling towers operated at a pH below 7.0 are rare because of the toxicity of corrosion inhibition programs that require acidic pH (e.g., chromate). Methylchloro/methylisothiazolone was shown to be more effective at pH 8.0 than at 6.7 in this study.

Additionally, our results are not directly comparable with those of Skaliy, et al. [18] because the concentration of dissolved minerals in the tap water (commonly expressed as hardness) was not described. The effect of water hardness (concentration of calcium and magnesium in solution expressed as $CaCO_3$) is important when considering the efficacy of methylchloro/methylisothiazolone because the compound is stabilized by the salts in solution. For example, the percent methylchloro/methylisothiazolone remaining in solution at pH 8.5 and 37°C after 3 days was 100% when the water hardness was equal to 896 ppm (as $CaCO_3$), 95% when the water hardness was equal to 192 ppm and 55% in distilled water (data available from manufacturer). Thus, the concentration of biocide that is in contact with the bacteria may not remain constant for the duration of the efficacy test if the water is very soft (low hardness); the present report shows data from tests run with hard water (400–500 ppm as $CaCO_3$). Water hardness is particularly important with respect to evaporative cooling systems in which there is usually more than 3- and as much as 10-times more dissolved solids in the recycle circuit relative to the make-up water. The stabilizing effects of dissolved solids (in particular, divalent cations) on methylchloro/methylisothiazolone in solution indicate that the biocide will be most efficacious in water with high calcium carbonate hardness.

Fig. 2 summarizes all of the data generated from the present study and shows fitted exponential curves based upon the regressions of log reduction onto the concentration of active ingredient. The data show that the lowest concentrations of methylchloro/methylisothiazolone tested (0.35 ppm) killed virtually all of the bacteria, regardless of pH when the contact time was 24 h. However, the 3 h contact time tests gave the least predictable results and required the highest concentrations of biocide in order to effectively control *Legionella*. In particular, the curve drawn through pH 6.7 test data is dotted to indicate that there is considerable scatter in the data. Nevertheless, the fit of the data in Fig. 2 shows that the rate of kill was probably dependent on biocide concentration according to first-order rate kinetics; this can be shown as follows. If the change in number of viable *Legionella* cells as a function of time is

$$dN/dt = k\,N$$

where k is the specific rate coefficient of kill for a given biocide concentration, t is time in hours [8] and the ratio of the rate coefficient and the biocide

concentration is constant (k') for each specific test [10],

$$k' = (k/c)$$

then these relationships can be used accurately to calculate the percent killed as a function of biocide concentration (substitute into eqn. 2):

$$\% \text{ killed} = 100\,(1 - e^{-(k/c)\,(\Delta t)c})$$

Thus, $k' \cdot \Delta t$ is shown to be equal to the logarithmic reduction of viable cells (as cfu's) per ppm active ingredient of biocide after contact time in hours. These interpretations of the data have allowed us to predict the contact time and concentration of methylchloro/methylisothiazolone biocide required for specific reductions in the number of viable planktonic *Legionella* in certain cooling tower waters (Table 2 and Fig. 2).

It is expressly noted that methylchloro/methylisothiazolone does not perform as a disinfectant against *Legionella* or any other microorganism in industrial cooling towers because the rate of kill is not sufficiently rapid. However, the goal of biocide treatment programs for industrial cooling circuits can never be to render the system completely disinfected, i.e., sterile. Of course, since this is obviously true, methylchloro/methylisothiazolone biocide can be effectively used as a part of the total water treatment program in industrial cooling systems in order to control the entire population of microorganisms, including *Legionella*. Control of the entire population of microorganisms with a broad-spectrum antimicrobial agent is especially important with respect to *Legionella* because it has been clearly shown that the metabolism of other microorganisms, including protozoa [21], green algae [14], cyanobacteria [20,22] and at least one species of *Flavobacterium* [7] can support the growth of the pathogen in aquatic environments. Treatment of the source of *Legionella* in industrial cooling systems should be considered paramount, however. It has been shown that the primary reservoir of *Legionella* in cooling systems is probably the sediment and sludge in cooling tower basins [22] and also, throughout the system in the adherent (sessile) populations on surfaces. Therefore, we are currently studying the efficacy of methylchloro/methylisothiazolone against *Legionella* in biofilms to determine the concentration required for the control of the organism on surfaces in industrial cooling systems.

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