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Inactivation of Biofilm Bacteria

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The current project was developed to examine inactivation of biofilm bacteria and to characterize the interaction of biocides with pipe surfaces. Unattached bacteria were quite susceptible to the variety of disinfectants tested. Viable bacterial counts were reduced 99% by exposure to 0.08 mg of hypochlorous acid (pH 7.0) per liter (1 to 2°C) for 1 min. For monochloramine, 94 mg/liter was required to kill 99% of the bacteria within 1 min. These results were consistent with those found by other investigators. Biofilm bacteria grown on the surfaces of granular activated carbon particles, metal coupons, or glass microscope slides were 150 to more than 3,000 times more resistant to hypochlorous acid (free chlorine, pH 7.0) than were unattached cells. In contrast, resistance of biofilm bacteria to monochloramine disinfection ranged from 2- to 100-fold more than that of unattached cells. The results suggested that, relative to inactivation of unattached bacteria, monochloramine was better able to penetrate and kill biofilm bacteria than free chlorine. For free chlorine, the data indicated that transport of the disinfectant into the biofilm was a major rate-limiting factor. Because of this phenomenon, increasing the level of free chlorine did not increase disinfection efficiency. Experiments where equal weights of disinfectants were used suggested that the greater penetrating power of monochloramine compensated for its limited disinfection activity. These studies showed that monochloramine was as effective as free chlorine for inactivation of biofilm bacteria. The research provides important insights into strategies for control of biofilm bacteria.

The most commonly used model for inactivation of microorganisms by disinfectants has been derived from the work of Chick (9) and Watson (55). The "Chick-Watson law" is $\ln (N/N_0) = -kC^n t$, where N/N_0 is the ratio of surviving organisms at time t, C is the disinfectant concentration, and k and n are empirical constants (n is also called the coefficient of dilution). The Chick-Watson law with its concentration and time ($C \times T$) factor is the basis for all other models which can be considered as deviations of this formula (for a review, see references 17, 18, 22, and 49). The model implies that disinfectant concentration and contact time are the two key variables determining disinfection efficiency. The equation is based on the observation that inactivation of microorganisms generally follows first-order kinetics. These concepts are so influential that current disinfection regulations are based on a $C \times T$ concept.

Important in the application of the kinetic models is recognition of the assumptions of derivation. These assumptions include complete and uniform mixing of microorganisms and disinfectant, whereby diffusion is not rate limiting, and constant disinfectant concentration with time (49). One criticism of the kinetic model has been that much of the data for the model's development has been based on laboratory studies of monodispersed microorganisms and do not reflect realistic circumstances.

In a distribution system, maintenance of a disinfectant residual is intended to control microbiological degradation of water quality (54). However, experience has shown that maintenance of a disinfectant residual cannot be relied on to totally prevent bacterial occurrences. Numerous investigators (13, 16, 23, 29, 43, 44, 57) have reported recovery of coliform bacteria from chlorinated drinking water. Reilly and Kippen (44) showed that coliform bacteria were isolated from 22 and 18%, respectively, of chlorinated water samples from the Salem and Beverly, Mass., distribution systems. Olivieri et al. (43) reported recovering coliform bacteria in 21% of the distribution system samples containing 1 to 3 mg of free residual chlorine per liter. Coliform bacteria were recovered in some samples containing 6 to 8 mg of free chlorine per liter. Earnhardt (13) reported recovering 51 coliform bacteria per 100 ml in samples containing 10 to 12 mg of free chlorine per liter. In that episode, free chlorine residuals as high as 15 mg/liter were necessary to control bacterial regrowth in the distribution system. Goshko et al. (16) indicated that maintenance of a free chlorine residual did not always correlate with reduced heterotrophic plate count (HPC) of bacteria in the water column.

Research has shown that increased resistance to disinfection results from attachment or association of microorganisms to various surfaces, including macroinvertebrates (*Crustacea*, *Nematoda*, *Platyhelminthes*, and *Insecta*) (31, 53), turbidity particles (19–22, 27, 46), algae (51), carbon fines (7, 28), and even glass microscope slides (43), provides increased resistance to disinfection. Ridgway and Olson (46) showed that the majority of viable bacteria in chlorinated drinking water were attached to particles. Presumably, microbes entrapped in particles or adsorbed to surfaces are shielded from disinfection and are not inactivated.

Biofilms in drinking water systems have also proved difficult to inactivate. Nagy et al. (39) reported bacterial levels in the Los Angeles, Calif., aqueduct biofilms as high as 10^4 CFU/cm^2 in the presence of a residual of 1 to 2 mg of chlorine per liter. Maintenance of a residual of 3 to 5 mg of chlorine per liter was necessary to reduce bacterial biofilms by more than 99.9%. In another study, these investigators found no correlation between free chlorine residuals (0.15 to 0.94 mg of chlorine per liter) and the densities of HPC bacteria in distribution pipeline biofilms (40). Seidler et al. (47) recovered *Klebsiella pneumoniae* in a potable water supply 1 week after scrubbing redwood tank biofilms with a solution of 200 mg of chlorine per liter. Ridgway et al. (45) found that a residual of 15 to 20 mg of chlorine per liter was

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necessary to control biofilm fouling of reverse osmosis membranes.

Recently, some water utilities have resorted to unconventional biocides to control bacterial problems in distribution systems. Application of low levels of heavy metals (copper and zinc) and alkaline pH adjustments have been reported to produce some benefits (10, 15, 34), although controlled studies have not been performed.

Previous research (29) at a water utility experiencing chronic bacteriological problems demonstrated that maintenance of a free chlorine residual was insufficient to eliminate coliform occurrences. Experiments collecting samples with and without sodium thiosulfate showed that coliform bacteria were not significantly affected by an additional 1-h contact time with a residual of 0.75 mg of free chlorine per liter. Free chlorine residuals as high as 4.3 mg/liter (monthly average) did not eliminate coliform occurrences. Statistical analyses of the sampling data showed that coliform densities were not influenced by maintenance of a free chlorine residual. Furthermore, the greatest occurrence of coliform bacteria was observed in distribution system trunk lines which always contained a residual of 1 to 2 mg of free chlorine per liter. In that study (29), it was shown that growth of coliform bacteria in distribution system biofilms was responsible for the occurrence of indicator organisms in the water column.

The current investigation was initiated to examine inactivation of biofilm bacteria and to characterize the interaction of biocides with microbial interfaces. The research examines four disinfectants (hypochlorous acid, hypochlorite, chlorine dioxide, and monochloramine), three types of surfaces (granular activated carbon, metal coupons, and glass microscope slides), two bacterial types (HPC bacteria and coliforms) as well as several alternate biocides (copper, zinc, sodium chlorite, and alkaline pH). The results reveal important properties of the compounds which can be exploited to improve inactivation of biofilm bacteria.

MATERIALS AND METHODS

Bacterial strains. (i) HPC bacteria. A population of HPC bacteria from the deionized water system of the Belleville Laboratory were grown without subculture in dechlorinated drinking water. This stable population (composed of 70% *Pseudomonas picketti*, 18% *Moraxella*, and 12% *Pseudomonas paucimobilis*) was used as an inoculum for subsequent experiments. Bacteria were identified using the Rapid NFT identification system (Analytab Products, Plainview, N.Y.). Subcultures inoculated with the HPC population were grown at room temperature for 5 days in 20 mM potassium dihydrogen phosphate buffer (pH 7.0) containing 25 μg of yeast extract (Difco Laboratories, Detroit, Mich.) per liter. Samples prepared in this manner typically contained 10^5 to 10^6 bacteria per ml.

Biofilms of HPC bacteria were grown on spent granular activated carbon (GAC) (Calgon Filtersorb 200; Calgon Corp.) and 3- by 0.5-in. (ca. 1.3- by 7.6-cm) metal corrosion coupons (Technical Products Corp., Portsmouth, Va.). Sterile spent GAC (500 g) was placed in a 1-liter wide-mouth jar and inoculated with the HPC suspension (described above). The jar was fitted with a stopper and connected to the deionized water supply (flow rate, approximately 50 ml/min). Deionized water entered at the bottom of the jar and flowed through the GAC and out at the top of the jar. This apparatus operated throughout the course of the experiments. The biofilm density was approximately 10^7 CFU/g of GAC.

Biofilms of the HPC bacteria were grown on metal coupons (copper, lead, and mild steel) at room temperature for 3 weeks in 20 mM phosphate buffer containing 1 mg of yeast extract per liter. Biofilms were grown evenly on both sides of the coupon by suspending the coupons in the medium with a nylon thread. This even growth allowed one side of the coupon to be a control while the other side was the test system. Biofilm density on the metal coupons were typically 10^6 bacteria per side.

(ii) *K. pneumoniae*. A strain of *K. pneumoniae* was obtained from Ian W. Sutherland, University of Edinburgh, Scotland, and was stored at −20°C in a 40% glycerol–2% peptone solution. For inactivation of suspended bacteria, cells were grown on EPS agar spread plates at 35°C for 24 h. EPS agar contained (per liter of deionized water) 7.0 g of K_2HPO_4, 3.0 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $(NH_4)_2SO_4$, 0.01 g of $CaCl_2$, 0.001 g of $FeSO_4$, 0.1 g of NaCl, 10.0 g of glucose, and 15.0 g of Bacto-Agar (Difco) (final pH 7.0). Bacteria were washed off the plates and suspended in 20 mM phosphate buffer (pH 7.0) at concentrations of 10^6 CFU/ml.

Biofilms of *K. pneumoniae* were grown on clean, sterile, glass microscope slides in EPS broth for 2 days at 35°C. Slides were held in a vertical position with cardboard inserts in the mouths of the 125-ml flasks.

Preparation of the disinfectants. Stock solutions of the disinfectants were prepared daily, and concentrations were checked by amperometric titration (1). A sodium hypochlorite solution (5%) was obtained from the J. T. Baker Chemical Co. (Phillipsburgh, N.J.). Data from Fair et al. (14) indicate that over 85% of the free chlorine in water at pH 7.0 and 1 to 2°C is in the form of hypochlorous acid. At pH 8.5 (1 to 2°C) over 90% of the free chlorine is in the form of the hypochlorite ion (14). Monochloramine solution was prepared by using a 3:1 molar ratio of ammonia (ammonium chloride; Baker) to chlorine (pH 9.0). Amperometric titration indicated that no detectable free chlorine or dichloramines were present in the monochloramine stock solution. Chlorine dioxide was produced by standard methods (1) with sodium chlorite (Aldrich Chemical Co., Milwaukee, Wis.) and concentrated sulfuric acid. The mixture was purged through both a saturated sodium chlorite solution and a packed sodium chlorite column to ensure that no free chlorine was present in the chlorine dioxide stock solution.

Depending on the experiment, doses of free chlorine ranged from 0.01 to 5.0 mg of chlorine per liter for 1 to 60 min. Free chlorine doses below 0.1 mg/liter were added by dilution of the stock solution and could not be reliably measured. In these cases, a consistant die-off curve indicated that the disinfectant residual was still present and effective. Monochloramine doses ranged from 1.0 to 100 mg of NH_3Cl per liter for 10 to 60 min. Chlorine dioxide doses ranged from 0.1 to 1.0 mg of ClO_2 per liter for 1 to 60 min.

Several alternate biocides were examined for inactivation of biofilm bacteria. The copper and zinc solutions were prepared in 20 mM phosphate buffer (pH 7.0) with cupric sulfate and zinc sulfate (Fisher Scientific Co., Fair Lawn, N.J.), respectively. Sodium chlorite was prepared using a weight-per-volume (20 mM phosphate buffer, pH 7.0) basis. A 20 mM phosphate buffer (pH 9.0) was used to determine the bactericidal effects of an alkaline pH.

Chlorine demand studies. Application of disinfection kinetics required that the disinfectant concentration remain stable during the contact time. Measurement of disinfectant residuals demonstrated that *K. pneumoniae* grown on glass microscope slides produced very little chlorine demand.

Less than 4% of the free chlorine dose was consumed during the 10-min time interval. Monochloramine tested under the same conditions showed no chlorine demand.

No significant chlorine demand was detected for HPC bacteria grown on metal coupons in experiments where monochloramine was used. For free chlorine, a slight chlorine demand (9.6% of the dose) was detected. The effect of this demand was compensated for by adding slightly higher free chlorine doses. A value of 0.10 mg · min per liter (instead of 0.08 mg · min per liter; Table 1) was used in calculating the free chlorine $C \times T$ doses. This calculation resulted in a 20% higher free chlorine dose to compensate for the chlorine demand of the system.

The chlorine demand of biofilms grown on GAC was more complicated. At low disinfectant doses, the demand of the system was small (20% of the dose). It was in this area of low demand (a dose of 1 mg of disinfectant residual per liter for 60 min or less) that comparisons of disinfection activity were made. To help compensate for the demand of the system, a higher dose (20%) of free chlorine was added to the GAC experiments.

The disinfectant demand of GAC increased as the disinfectant dose increased above 1 mg/liter. Apparently, breakdown of the GAC particles (from the reaction of GAC with the disinfectants and mechanical scraping from the stir bar) created new GAC surfaces, which increased the disinfectant demand. This process was most pronounced with free chlorine but was also observed with monochloramine. For these experiments, as little as 10 to 50% of the disinfectant remained at the end of the 60-min reaction time. In addition, breakdown of the GAC particles interfered with the disinfectant measurements.

Inactivation of suspended bacteria. HPC bacteria suspended in the low nutrient water were treated with various disinfectants at 1 to 2°C. Timed samples were removed and dechlorinated with sodium thiosulfate (0.01% final concentration) (1). Because HPC bacteria had used most of the available nutrients, no appreciable chlorine demand was detected in the low nutrient system. Surviving bacteria were enumerated in triplicate with R_2A agar (Difco Laboratories) and incubated at room temperature (20 to 24°C) for 7 days.

K. pneumoniae (10^6 CFU/ml) suspended in chlorine-demand-free 20 mM phosphate buffer (pH 7.0) were treated with various disinfectants at 1 to 2°C. Timed samples were removed, dechlorinated with sodium thiosulfate, plated in triplicate on R_2A agar, and incubated at 35°C for 24 h.

Inactivation of biofilm bacteria. Bacteria attached to 1 g of GAC, metal coupons, or glass microscope slides were washed with sterile phosphate buffer to remove unattached cells. Biofilms were disinfected in 100 ml of chlorine-demand-free phosphate buffer (pH 7.0) at 4°C in a 250-ml beaker agitated with a magnetic stir bar. GAC particles were suspended by the action of the stir bar. Metal coupons and glass microscope slides were placed in the beakers at a 45° angle so that the stir bar had room under the coupon to spin. At the end of the reaction time, sodium thiosulfate (0.01%) was added to stop the reaction. Viable bacteria attached to GAC were desorbed by a previously published homogenization procedure (6). This procedure has been shown to recover 80 to 90% of attached organisms. Biofilms on metal coupons and glass slides were scraped off with a sterile rubber policeman and homogenized by the same procedure used for the GAC particles (6). HPC and *K. pneumoniae* enumerations were made in triplicate with R_2A agar as described above.

Quality control and statistical comparisons. A quality assurance program, as outlined previously (1, 3), was used throughout the course of the study. Materials used during each experiment were checked for sterility. The temperatures of autoclaves and incubators were monitored on a per-use basis. Spectrophotometers, pH meters, and analytical balances were calibrated on a regular basis.

Statistical comparisons were made using the Stat-Pac statistics program (Northwest Analytical, Portland, Oreg.) on a Kaypro professional computer.

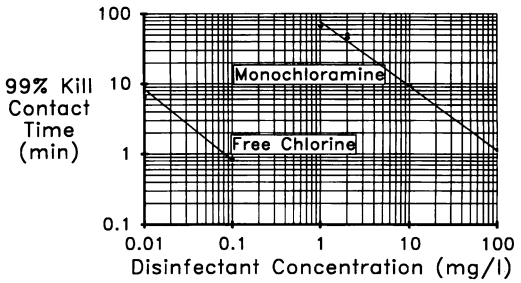

FIG. 1. Comparison of contact time versus disinfectant concentration. Each point represents the average of three replicate experiments. The slope (coefficient of dilution [n]) of the lines was 1.0 for free chlorine and 0.9 for monochloramine.

RESULTS

Inactivation of unattached bacteria. Inactivation of unattached bacteria in a chlorine-demand-free system generally followed first-order kinetics. However, variability was found even between identical replicates of the same experiment. In some experiments, variability approached 33%. From replicate die-off curves, the disinfectant concentration and the time for 99% reduction in viable counts were estimated. Log-log plots of the 99% kill contact time at various disinfectant concentrations showed that the slope of the lines (coefficient of dilution [n]) was 1.0 for free chlorine and 0.9 for monochloramine (Fig. 1).

A summary of the $C \times T$ coefficients for 99% inactivation of unattached HPC bacteria is shown in Table 1. For comparison, published data for *Escherichia coli* are also presented. For inactivation of unattached bacteria, the relative effectiveness of the disinfectants tested was found to be in the following order: $HOCl > ClO_2 \gg OCl^- \gg NH_2Cl$. HPC bacteria used in this study (grown under low-nutrient conditions) were two to three times more resistant to various disinfectants than *E. coli* (Table 1). The low-nutrient-grown

TABLE 1. Comparative efficacy of disinfectants for the production of 99% inactivation in demand-free systems

Disinfectant agent	*E. coli*[a]			HPC bacteria		
	pH	Temp (°C)	$C \times T$[b]	pH	Temp (°C)	$C \times T$
Hypochlorous acid	6.0	5	0.04	7.0	1–2	0.08 ± 0.02
Hypochlorite ion	10.0	5	0.92	8.5	1–2	3.3 ± 1.0
Chlorine dioxide	6.5	20	0.18	7.0	1–2	0.13 ± 0.02
	6.5	15	0.38	8.5	1–2	0.19 ± 0.06
	7.0	25	0.28			
Monochloramine	9.0	15	64	7.0	1–2	94.0 ± 7.0
				8.5	1–2	278.0 ± 46.0

[a] Values for *E. coli* were adapted from Olivieri (42).

[b] All $C \times T$ calculations (milligram minutes per liter) are based on a minimum of two trials. Each trial was performed in triplicate.

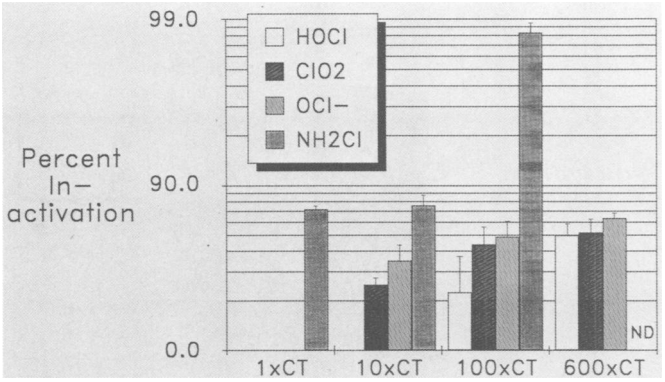

FIG. 2. Inactivation of biofilms grown on granular activated carbon particles. Compounds were compared on the basis of equal activity (equal $C \times T$ coefficients). The solution was stirred using a magnetic stir bar (pH 7.0, 4°C). ND, Not determined.

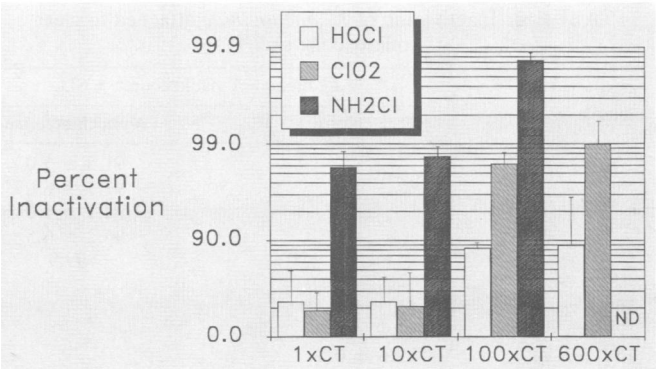

FIG. 3. Inactivation of biofilms grown on metal coupons. Compounds were compared on the basis of equal activity (equal $C \times T$ coefficients). The solution was stirred using a magnetic stir bar (pH 7.0, 4°C). ND, Not determined.

HPC bacteria, however, were slightly more sensitive to chlorine dioxide than *E. coli*.

Data from Table 1 demonstrate the known fact that hypochlorous acid is a much more effective disinfectant (for suspended bacteria) than monochloramine (over 1,000-fold more effective). The results showed a 40-fold decrease in disinfection efficiency for free chlorine with changes in pH from 7.0 to 8.5. Chlorine dioxide, however, showed no significant change in activity over the pH range of 7.0 to 8.5 (Table 1).

Inactivation of attached bacteria. The data in Table 1 allowed for the comparison of various disinfectants on the basis of activity. That is, a $C \times T$ value represents a unit of activity which will inactivate 99% of the test organisms. Comparison of equal activities (equal $C \times T$ units) of hypochlorous acid, hypochlorite, chlorine dioxide, and monochloramine for inactivation of HPC bacteria grown on GAC is shown in Fig. 2. The data indicate that, relative to the effectiveness for unattached bacteria, monochloramine inactivated biofilm bacteria more effectively than the other disinfectants tested. For example, hypochlorous acid at 10 times the concentration necessary to kill unattached bacteria showed little effect for bacteria grown on GAC. However, monochloramine under the same conditions (10 times the $C \times T$ for unattached bacteria) reduced viable counts by 65%. One $C \times T$ unit (required for 99% inactivation of unattached cells) of monochloramine was more effective for inactivating biofilm bacteria than 600 $C \times T$ units of hypochlorous acid (see Fig. 5).

Application of one $C \times T$ unit of hypochlorous acid or monochloramine to bacteria desorbed from the GAC surface inactivated 97.5 ± 3.7 and 97.5 ± 2.1%, respectively. These data indicate that HPC bacteria grown on GAC did not change disinfection resistance patterns.

Comparison of equal activities of disinfectants for biofilms of HPC bacteria grown on metal coupons is shown in Fig. 3. Similar to the results for GAC, data from Fig. 3 indicate that relative to inactivation of unattached bacteria, monochloramine was more effective than the other disinfectants tested for disinfection of biofilms on metal coupons. One $C \times T$ unit of monochloramine reduced viable counts by 95%, whereas equivalent activities of hypochlorous acid or chlorine dioxide had little effect. To match the disinfection activity of 1 $C \times T$ unit of monochloramine, it required 100 $C \times T$ units of chlorine dioxide and over 600 $C \times T$ units of hypochlorous acid.

Control experiments for HPC bacteria desorbed from the metal coupon surfaces showed no change in the susceptibility of the organisms to hypochlorous acid or monochloramine.

Data from the experiments presented above are summarized in Fig. 4. The results show that increased inactivation of biofilm bacteria was not solely dependent on $C \times T$. That is, doubling the disinfectant activity did not result in twice the bacterial inactivation. These results were especially important for hypochlorous acid, where disinfection kinetics showed a plateau effect. For biofilms grown on both GAC and metal coupon surfaces, hypochlorous acid activity plateaued at 200 $C \times T$ units. Increasing the disinfectant activity to 600 $C \times T$ did not increase disinfection efficiency. The results suggest that penetration of the disinfectant to the biofilm surface (mass transfer) was an important rate-limiting step.

Disinfection of *K. pneumoniae* biofilms. Similar experiments were conducted with *K. pneumoniae* attached to glass microscope slides. $C \times T$ values to achieve 99% inactivation for unattached *K. pneumoniae* were 0.065 ± 0.02 mg · min per liter for hypochlorous acid and 33 ± 18 mg · min per liter for monochloramine (pH 7.0, 1 to 2°C). Presented in Table 2 are multiples of the $C \times T$ units required to achieve 99% reduction of unattached *K. pneumoniae*. Because of the protection provided by attachment of the bacteria to glass surfaces, higher multiples were needed to achieve a 99% reduction. Attachment of *K. pneumoniae* to glass microscope slides increased resistance to hypochlorous acid over 125-fold, whereas resistance to monochloramine inactivation increased only 2-fold (Table 2).

Comparison on a weight basis. The previous experiments showed monochloramine (on activity basis) had a greater

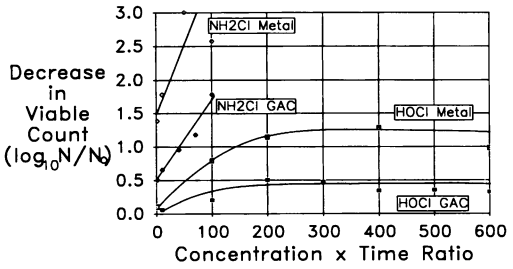

FIG. 4. Disinfection kinetics of hypochlorous acid and monochloramine for biofilms grown on GAC and metal surfaces.

TABLE 2. Inactivation of *K. pneumoniae* attached to glass microscope slides

$C \times T$ [a] multiple	% Reduction of viable count[b] ± SD	
	Free chlorine	Monochloramine
1.0		91.5 ± 5.0
1.5		91.1 ± 6.0
2.0		94.1 ± 5.0
2.5		96.2 ± 6.5
3.0		>99.9
75	34.0 ± 16.9	
100	51.3 ± 18.2	
125	91.1 ± 6.0	
150	99.6 ± 0.4	

[a] $C \times T$ values for unattached *K. pneumoniae* were 0.065 ± 0.02 mg · min per liter for free chlorine and 33 ± 18 mg · min per liter for monochloramine (pH 7.0, 1 to 2°C).

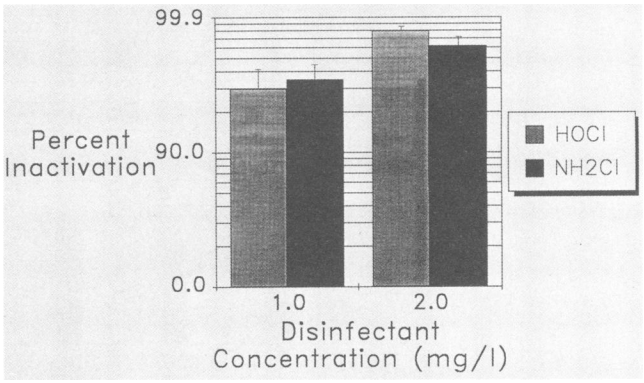

FIG. 6. Comparison of equal weights of disinfectants for inactivation of biofilms grown on metal coupons. The solution was stirred with a magnetic stir bar for 1 h (pH 7.0, 4°C).

ability to penetrate and inactivate biofilm bacteria. The results of inactivation of bacteria on GAC and metal coupon surfaces (Fig. 2 and 3) showed that, relative to the effectiveness for unattached bacteria, monochloramine was over 600 times better than hypochlorous acid for penetrating biofilms (i.e., 1 $C \times T$ unit of monochloramine produced greater inactivation than 600 $C \times T$ units hypochlorous acid). These data suggest, for treatment of biofilms, that the greater penetrating power of monochloramine may offset its limited disinfection activity.

Comparison of equal weights (expressed as chlorine equivalents) of hypochlorous acid and monochloramine for inactivation of HPC bacteria on GAC is shown in Fig. 5; 1 mg of monochloramine per liter had nearly the same disinfection effectiveness for biofilm bacteria as 1 mg of hypochlorous acid per liter (both reacted with GAC for 1 h). Monochloramine at concentrations between 2 and 5 mg/liter resulted in greater disinfection efficiency than did hypochlorous acid (Fig. 5). This difference, however, may be explained by the free chlorine demand of GAC at high disinfectant doses.

Hypochlorous acid at 5 mg/ml reacted with GAC for 1 h (3,000 times the $C \times T$ to inactivate 99% of unattached bacteria) reduced viable bacterial counts by 58% (Fig. 5). These results indicate that the plateau effect seen in Fig. 4 extends past 3,000 $C \times T$ units. At this high chlorine concentration and mixing speed, considerable deterioration of the GAC granules occurred. This deterioration resulted in an increased chlorine demand (50 to 90%) for both hypochlorous acid and monochloramine. Apparently, the more chlorine that was added, the greater the chlorine demand. Despite near destruction of the GAC particles, HPC bacteria were hardly affected.

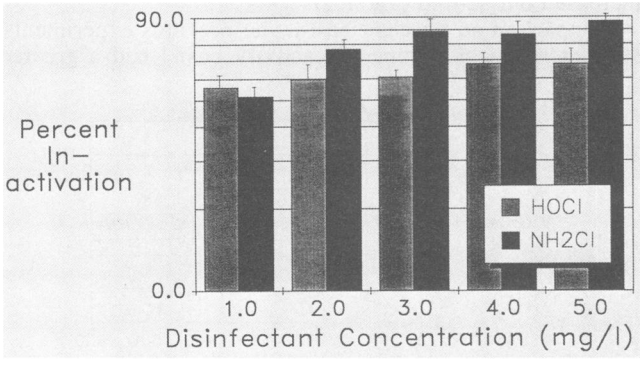

FIG. 5. Comparison of equal weights of disinfectants for inactivation of biofilms grown on GAC particles. The solution was stirred using a magnetic stir bar for 1 h (pH 7.0, 4°C).

Similar experiments were conducted with biofilms grown on metal coupons (Fig. 6). Equal weights (expressed as chlorine equivalents) of monochloramine and free chlorine reacted with biofilm bacteria for 1 h showed nearly the same disinfection efficiency. With these experiments, however, there was no appreciable chlorine demand. A hypochlorous acid concentration of 4 mg/liter for 1 h (2,400 $C \times T$ units) contact was necessary to inactivate greater than 99% of the attached bacteria (data not shown).

Alternate biocides. In addition to the common disinfectants used in water treatment, a number of alternate biocides were also examined (Table 3); 1 mg of copper per liter and 10 mg of sodium chlorite per liter exposed to cells for 24 h at 4°C had some bactericidal activity. Cupric sulfate (1 mg/liter as copper) inactivated over 99% of *K. pneumoniae* and HPC strain 2 but showed little activity against HPC strain 1, which was isolated from a copper service line. Sodium chlorite (5 mg/liter) at pH 6 reduced viable counts of HPC and *K. pneumoniae* by more than 99%. Zinc sulfate (3 mg/liter as zinc) and alkaline pH (9.0) showed little biocidal activity over the 24-h time period (Table 3).

Treatment of biofilm bacteria grown on GAC particles with 1 mg of copper per liter or 10 mg of sodium chlorite per liter for 24 h at 4°C, however, showed little activity. Copper and chlorite reduced viable counts of biofilm bacteria by 52 and 60%, respectively.

TABLE 3. Disinfection efficacy of alternate biocides for unattached bacteria

Test strain	PH	% decrease in viable count[a]						
		Copper		Zinc (3 mg/ml)	Chlorite			Alkaline (pH 9.0)
		1	0.5		10	5	1	
K. pneumoniae	7.0	99.4	68.0	90.0	99.9	99.5		68.0
HPC strain 1	7.0	75.0	22.0	63.0	99.9	61.0	21.0	0.0
HPC strain 1	6.0	63.0	21.0	61.0	99.9	99.8	62.0	
HPC strain 2	7.0	99.8	84.0	80.0				

[a] Cells were exposed for 24 h at 4°C in a phosphate buffer. Concentrations of biocides are given in milligrams per liter.

DISCUSSION

Inactivation of unattached bacteria. Inactivation of unattached bacteria in this study provided a perspective for evaluating the effectiveness of biocides on bacterial biofilms. By comparing disinfectants on the basis of activity, it was possible to evaluate more than just the oxidizing power of each compound. This type of perspective is similar to the approach used for evaluation of enzymes and other biochemical products (e.g., antibiotics). Comparisons based on activity reveal insights into the nature of the compound, whereas comparisons based on weight relate to practical application in the field. Both types of comparisons were used in this study.

The disinfection procedures used in this study gave results (Fig. 1, Table 1) similar to those published by other investigators (17, 22, 38, 42). Haas and Karra (17), using values from various sources, determined that 68 and 56% of the n values for free chlorine and combined chlorine, respectively, were within the range of 0.7 to 1.3. Research from this report found that the coefficient of dilution was 1.0 for free chlorine and 0.9 for monochloramine, which agreed with the majority of other investigators. The ranges of $C \times T$ values for a given disinfectant (represented as standard deviations) in Table 1 indicated intrinsic variability between replicate experiments. Values for replicate experiments varied up to 33%. It is difficult to compare the precision of these data with other reports because most other investigations have not presented $C \times T$ data with standard deviations. Nevertheless, our experience indicates that the shape and slope of the disinfection curve can show significant variation even between identical experiments. Without replicate data, interpretation of disinfection results must be guarded.

Inactivation of attached bacteria. The results of this study indicate that inactivation of attached bacteria was not solely related to the oxidizing power of the biocide. When comparisons were made on the basis of equal activity, monochloramine inactivated biofilm bacteria better than free chlorine or chlorine dioxide. These results were consistent even when the surfaces were GAC particles (Fig. 2), metal coupons (Fig. 3), or glass microscope slides (Table 2). The kinetics of the interaction of the disinfectants with the surfaces (the plateau effect) suggested that transport of the compounds from the bulk liquid phase to the bacterial surfaces was the major rate-limiting step (Fig. 4). W. G. Characklis (Institute for Process Analysis Bulletin, Montana State University, 1987), using known rate constants, calculated that the total chlorine consumption rate was determined by the diffusion rate of the disinfectant through a biofilm rather than by the reaction with pipeline wall material.

The mechanism of action of monochloramine may account for its effective penetration of bacterial biofilms. Jacangelo and Olivieri (24) reported that monochloramine reacted rather specifically with nucleic acids, tryptophane, and sulfur-containing amino acids. The researchers observed no reaction between chloramines and sugars such as ribose. Results presented in another study (26) showed that monochloramine did not react with extracellular polysaccharides. Free chlorine, however, is known to react with a wide variety of compounds (5, 38, 52). This difference in specificity may allow monochloramine to penetrate a biofilm and react with the microorganisms, whereas free chlorine is consumed before it can fully penetrate the biofilm surface. The importance of mass transfer from the bulk fluid and diffusion of compounds within biofilms has been modeled for several nutrients (8, 35). Additional research is necessary to describe the interaction of disinfectants with microorganisms growing on pipeline surfaces.

The results of this study also confirm the experience of many in the water industry who find effective control of bacterial levels in distribution water by using a combined chlorine residual (4, 11, 25, 33, 36, 37, 41, 50, 58). However, based on the $C \times T$ data alone (where chloramines are a 1,200-fold weaker disinfectant than free chlorine), one would expect chloramine use to be disastrous. As reviewed by Kreft et al. (25), over 70 utilities in the United States effectively use chloramines for disinfection of distribution water supplies. The Denver, Colo., Water Department has used chloramines for the past 70 years. Dice (11) reported that in 1946, George Turre, quality control engineer in the Denver Water Department at that time, indicated that "there was a complete lack of bacterial slime growth in the distribution system because of the persistence of the chloramine residual in the water." The Philadelphia Suburban Water Co. Pickering Creek Plant has used chloramines in its treatment process to reduce coliform levels by more than 99.999% (50). MacLeod and Zimmerman (33) reported that, before conversion to chloramines, 56.1% of the distribution system water samples were positive for coliform bacteria and that, after conversion, only 18.2% of the samples contained coliform organisms. Although there may be many reasons for the reduced coliform counts, the system has remained coliform free since February of 1984. MacLeod and Zimmerman (33), however, report a small but statistically significant increase in HPC bacteria after conversion to chloramines. Norman et al. (41), using limited data, reported a 52% reduction in HPC bacteria after conversion to chloramines.

Investigations by the Sanitation Districts of Los Angeles County (48) found that chloramines were almost as good bacteriocides and virucides as free chlorine for disinfection of wastewater. Data from Fig. 5 and 6 of this report, where the two disinfectants were compared on a weight basis, support this conclusion. The results suggest that the greater penetrating power of monochloramine compensated for its limited disinfection activity. For disinfection of complex biofilms monochloramine may be as effective as free chlorine.

One application of the results presented in this report may be the control of bacterial growth in GAC filters. The ability of monochloramine to interact with biofilms on GAC may make this disinfectant preferred for application on GAC filters. Recent research has shown that bacterial levels from GAC filters were lower when monochloramine was applied to the filters than when an equivalent dose of free chlorine was used (Ed Means, personal communication).

In the book *Handbook of Chlorination* (56), White reviewed efforts to rid distribution systems of biofilm problems. Although free chlorine has been successful for control of bacterial biofilms in many cases, new problems, including temporary production of chlorinous tastes and odors and complaints due to dirty water caused by sloughing of debris, were encountered in some cases when a free chlorine residual was used. It is significant to note that some successes were reported when a combined chlorine residual was used.

Other factors that need to be considered before changing disinfectants include microbial nutrients and trihalomethane formation potential. Addition of ammonia to nitrogen-limited water may cause further HPC bacterial problems. Recently, Wolf et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, Q59, p. 291) reported problems with nitrification in two

covered, finished water reservoirs after a changeover from chlorine to chloramine disinfection. A combination of slow turnover, excessive ammonia levels, and warm water temperatures were thought to be contributing factors. On the other hand, trihalomethane levels can be effectively reduced by chloramine disinfection (52).

Alternate biocides. Several reports have indicated that some water utilities have used novel approaches to combat biofilm problems. Copper sulfate has been applied in some systems to inactivate coliform organisms (10, 15). This practice was based on the observation that copper can injure and kill coliform bacteria (12). Martin et al. (34) found that elevated pH helped control coliform bacteria in drinking water, whereas Lowther and Moser (32) found that application of zinc orthophosphate seemed to alleviate problems in the Seymour and Muncie, Ind., episodes. The exact reason for the success of zinc orthophosphate is not known, but the toxicity of zinc for microorganisms was thought to be a possible factor.

Results of this study showed that these alternate biocides had some effectiveness on suspended bacteria but limited activity against biofilm microorganisms (Table 3). The combined effect of the biocides with other disinfectants, however, was not tested and could have some synergistic effect.

The development of resistance to heavy metals by microorganisms is well known. This trait is known to be plasmid mediated (30). Armstrong et al. (2) suggested that multiple antibiotic resistance in HPC bacteria was due in part to elevated heavy metal levels found on pipe surfaces. The addition of metals to drinking water supplies for disinfection purposes could select for microorganisms with increased resistance to antibiotics. We recommend that utilities using unconventional biocides (copper, zinc, alkaline pH) examine these practices under controlled experimental conditions.

Summary. Based on the data presented in this report, new criteria have been developed to evaluate the best disinfectant for control of biofilm bacteria. Although the solution will depend on unique circumstances of each system, considerations such as penetration of biofilms, taste and odor production, trihalomethane formation, corrosion control, and disinfectant stability may make monochloramine an effective choice. Further research is necessary to determine how to apply disinfectants for optimum biofilm control.

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