CONTROL OF BIOFILM BACTERIA

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REFERENCE: Proceedings of the 1995 Georgia Water Resources Conference, held April 11 and 12, 1995, at The University of Georgia, Kathryn J. Hatcher, Editor, Carl Vinson Institute of Government, The University of Georgia, Athens, Georgia.

Abstract. Based on problems associated with the use of chlorine as a water disinfectant and with the control of bacterial populations in biofilms, 1,3-dichloro-2,2,5,5-tetramethyl-4-imidazolidinone (compound DC) and 1-bromo-3-chloro-2,2,5,5-tetramethyl-4-imidazolidinone (compound DBC) were used to control planktonic and sessile populations of *Klebsiella*. Following the suspension of stainless steel disks in tubes, the tubes were capped with gauze/cotton plugs and autoclaved. Sterile chlorine demand-free buffers and inocula of mucoid and nonmucoid variants of *Klebsiella* were introduced to the tubes to initiate biofilm formation. After 24 to 48 hours equilibration time, both sessile and planktonic cells were exposed to varying concentrations of the disinfectants. Fluid samples and disk surface scrapings were used to determine the length of time required to achieve a 99.9999% decrease in viable cells. While disinfection rates were slower with the sessile organisms than with the planktonic organisms, both organic N-halamine disinfectants achieved 6 log decreases in viable *Klebsiella* in less than 12 hours.

INTRODUCTION

Relevance

Biofilms develop where bacteria adhere to a surface, form colonies, and adapt to a sedentary existence. Such biofilms are actually combinations of bacterial cells and extracellular polymers such as polysaccharides. Microbial ecology studies have estimated 99% of the microorganisms found on earth are associated with biofilms (Costeron, *et al.*, 1987). Biofilms are advantageous to the bacteria through incorporation of nutrients into the matrix (Flemming, 1991), resistance to toxic substances (Nichols, 1989; v. Loosdrecht, *et al.*, 1990; Fletcher, 1991), and protection from biocides (Flemming, 1991). With these advantages, biofilms have the potential to create problems in drinking water systems (LeChevallier, *et al.*, 1988) including physical blockage of the system plus increased cost and environmental problems through reliance on increased biocide concentrations. Nutrient availability in recycled water systems may increase biofilm formation (Flemming, 1993).

Disinfectant Development

With industrial development and population expansion resulting in increased water recycling, effective water treatment is becoming a higher priority for many Georgia communities. Although chlorination procedures are commonly used for disinfecting potable water, the free chlorine, hydantoins, and isocyanurates employed have brief useful lifetimes in water. These products can react with organic impurities to produce trihalomethanes which have been linked to cancer in laboratory animals. Chlorine dioxide and ozone do not provide long-term residuals and, being strong oxidizing agents, could react with organic matter in water to produce byproducts of unknown health risks. Many of the organic and inorganic chloramines lack stability in solid form or in aqueous solutions (Tsao, *et al.*, 1991). Ideally, a disinfectant will be stable in solid and aqueous forms, nontoxic, noncorrosive, tasteless, odorless, and effective against a broad spectrum of organisms (Barnela, *et al.*, 1986). These qualities are present in the organic N-halamines described below.

Initial research focused on 3-chloro-4,4-dimethyl-2-oxazolidinone (compound I) and 3-bromo-4,4-dimethyl-2-oxazolidinone (compound IB). A recent review of N-halamine water disinfectants provides stability and biocidal efficacy data for these compounds as a function of pH, temperature, and water quality (Worley and Williams, 1988). While compound I tends to be slow in the eradication of many water-borne organisms, it is more efficacious than free chlorine in the eradication of *Giardia lamblia*. Compound IB is probably not sufficiently stable for use as a general purpose biocide. No N-bromamine will be as stable as the N-chloramine analogue due to the longer, weaker N-Br bond compared with the N-Cl bond. The key to the stability of compound I is the presence of the methyl substituents at the 4 position of the oxazolidinone ring.

Understanding the stability of compound I led to the development of 1,3-dichloro-4,4,5,5-tetramethyl-2-imidazolidinone (compound A), 1,3-dibromo-4,4,5,5-tetramethyl-2-imidazolidinone (compound AB), and 1-bromo-3-chloro-4,4,5,5-tetramethyl-2-imidazoidinone (compound ABC). Compound A is the most stable N-halamine in water or dry storage, compound AB is the most stable N-bromamine ever reported, and compound ABC is an ideal disinfectant with the bromine moiety providing rapid disinfection and the chlorine moiety providing long-term disinfection. Some limitations in the use of the compound A series come as a result of the cost and difficulty in synthesis of the 4,4,5,5-tetramethyl-2-imidazolidinone precursor.

Alterations in the synthetic process lead to the development of compound MC (1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone), compound DC (1,3-dichloro-2,2,5,5-tetramethyl-4-imidazolidinone), compound DB (1,3-dibromo-2,2,5,5-tetramethyl-4-imidazolidinone), and compound DBC (1-bromo-3-chloro-2,2,5,5-

tetramethyl-4-imidazolidinone). These isomers are inexpensive to synthesize, reasonably stable in aqueous and dry storage, and more actively biocidal than the A series analogues (Tsao, *et al.*, 1991).

Early Applications

The organic N-halamine research undertaken in the past ten years has proven these compounds to be effective against a broad spectrum of potentially pathogenic organisms including *Enterobacter, Escherichia, Klebsiella, Legionella, Pseudomonas, Salmonella, Serratia, Sphaerotilus,* and *Staphylococcus* as well as *Giardia* (Elder, *et al.*, 1986; Williams, *et al.*, 1985; Worley, *et al.*, 1985; Worley, *et al.*, 1983; Worley, *et al.*, 1981). All studies undertaken have utilized the compounds in concentrations of 10 mg/L Cl^+ or less. Combining the broad spectrum with other characteristics such as being stable, noncorrosive, nontoxic, tasteless, and odorless make these compounds particularly promising biocides.

Biofilm Applications

The early research, which has shown the organic N-halamines to be effective water disinfectants, primarily dealt with planktonic bacteria. The potential for various bacteria to establish biofilms in water distribution systems and the efficacy of the N-halamines against organisms such as *Klebsiella* has led to this study which utilized compounds DC and DBC against *Klebsiella* biofilms.

METHODS

The major thrust of the work was to determine whether two organic N-halamine disinfectants, compound DC and compound DBC, can control sessile and planktonic *Klebsiella* populations. Variables included in the study were the *Klebsiella* variant (mucoid or nonmucoid), the location of the organisms (sessile or planktonic), the disinfectant, and the disinfectant concentration. All experiments were undertaken in sterile chlorine demand-free conditions using pH 7.0 phosphate buffer at 25° C. The ability of the disinfectants to control the bacteria was based on the amount of time required to achieve 6 log decreases in viable cells.

The growth surfaces were circular coupons of stainless steel (304 S stainless steel, 1/32" thick, 3/4" diameter, with 1/16" hole set 1/8" into circle). The coupons were tied to 12" pieces of thread, suspended in tubes, capped with gauze/cotton plugs, autoclaved, and dried. Each experimental and control tube was partially filled with 10 mL of sterile pH 7.0 phosphate buffer. Each inoculum was prepared by swabbing the surface of a nutrient agar plate of *Klebsiella* (K63M) or *Klebsiella* (K63N) which had been incubated at 37° C for 24 hours. The bacteria were suspended in a spectrophotometer tube containing sterile saline. The saline/bacterial solution was calibrated to 50% transmission using a broad spectrum spectrophotometer set at 420 nm. Based on previous calibration procedures, each tube was inoculated with 0.1 mL of the suspension to result in $1\text{-}2x10^6$ cfu/tube.

To determine the initial bacterial concentration, one tube was immediately used to determine the numbers of viable suspended and attached organisms. For the viable suspended organisms, a 1.0 mL sample of the buffer/bacteria solution was collected. To maintain consistency, the sample was mixed with 1.0 mL of sterile sodium thiosulfate which served as a quench for the disinfectant. Serial saline dilutions were spot plated (10 µL/spot; 3 spots/dilution) on nutrient agar and incubated at 37° C. Counts were made at 24 and 48 hours to allow for the growth of injured cells.

For the viable cells attached to the stainless steel disk, the disk was shaken in the tube to remove excess fluid then transformed to 2.0 mL of sterile sodium thiosulfate in a petri dish. Using one sterile swab to anchor the disk, a second sterile swab was used to wipe both sides of the disk in two directions to remove all attached bacteria. The bacteria/thiosulfate suspension was used in preparing sterile saline dilutions which were handled as described above.

To continue this experiment, the assembled tubes were placed in a temperature controlled, orbital water bath set at 50 rpm to maintain the bacterial suspension. The tubes were maintained at 25° C throughout the study. The bacteria were allowed to form biofilms for 24 and 48 hour periods. At the end of the respective equilibration periods, the above methods were applied to determine the planktonic and sessile organisms present.

Once the initial samples were collected, compounds DC and DBC were added to the experimental tubes resulting in 10 and 20 mg/L Cl^+ concentrations or the molar equivalents of compound DBC. The disinfectants were left in contact with the coupons for 1, 2, 4, 8, and 12 hour time periods. At the end of each time period a tube from each concentration of each disinfectant was treated by the procedures described above.

RESULTS

Results are presented in Tables 1 and 2. Several factors should be considered while assessing the results of this study. Data manipulations such as linear regressions to predict precise disinfection times have not been applied. The times recorded are those required to observe 6 log decreases in viable cells. Smaller decreases, such as 3 or 4 logs, could be obtained in shorter periods. Six log decreases were selected to challenge the disinfectant capabilities of the compounds. The combination of pH 7.0 and 25° C was also selected to challenge the disinfectant capabilities. In earlier studies of suspended cells these conditions were found to require longer disinfection periods than pH 4.5 or pH 9.5 and 37°C. The concentrations used were low considering the resistance of biofilm organisms to disinfectants. Changes in any of these factors could alter the results obtained.

As found in the earlier experiments using suspended organisms, both the 10 mg/L and 20 mg/L concentration of compounds DC and DBC achieved 6 log decreases in viable planktonic cells within 1 hour exposure of the organisms to the disinfectants. As expected by the protection biofilms afford bacteria, longer periods were necessary to achieve 6 log decreases in the attached cells. The lower concentration of compound DC achieved 6 log decreases in less than 12 hours exposure while the higher concentration required less than 8 hours. As expected with the

Table 1. Time Required (hrs) for Compound DC to Achieve 6 Log Decreases in Viable *Klebsiella* (pH 7.0, 25°C)

	Sessile 10 mg/L	Planktonic 10 mg/L	Sessile 20 mg/L	Planktonic 20 mg/L
Nonmucoid Variety				
24 Hour, Mucoid *Klebsiella*				
Initial	0	$1.97x10^6$	0	$1.83x10^6$
0	$2.37x10^4$	$2.04x10^4$	$2.16x10^4$	$2.04x10^4$
1	$1.45x10^4$	0	$4.26x10^3$	0
2	$7.45x10^3$	0	$6.05x10^2$	0
4	$1.92x10^2$	0	$5.11x10^1$	0
8	$1.18x10^2$	0	0	0
12	0	0	0	0
48 Hour, Mucoid *Klebsiella*				
Initial	0	$1.72x10^6$	0	$1.92x10^6$
0	$2.42x10^4$	$2.04x10^4$	$2.69x10^4$	$2.40x10^4$
1	$1.01x10^4$	0	$9.74x10^3$	0
2	$6.43x10^3$	0	$1.05x10^2$	0
4	$9.05x10^2$	0	$3.04x10^2$	0
8	$1.25x10^2$	0	0	0
12	0	0	0	0
Mucoid Variety				
24 Hour, Mucoid *Klebsiella*				
Initial	0	$1.74x10^6$	0	$1.96x10^6$
0	$2.42x10^4$	$2.13x10^4$	$2.75x10^4$	$2.12x10^4$
1	$1.04x10^4$	0	$9.64x10^3$	0
2	$6.95x10^3$	0	$1.82x10^2$	0
4	$1.75x10^2$	0	$6.04x10^1$	0
8	$1.09x10^2$	0	0	0
12	0	0	0	0
48 Hour, Mucoid, *Klebsiella*				
Initial	0	$1.69x10^6$	0	$1.86x10^6$
0	$2.12x10^4$	$2.44x10^4$	$1.97x10^4$	$2.32x10^4$
1	$1.45x10^4$	0	$8.92x10^3$	0
2	$7.42x10^3$	0	$4.81x10^2$	0
4	$9.12x10^2$	0	$2.62x10^2$	0
8	$1.18x10^2$	0	0	0
12	0	0	0	0

presence of free bromine, compound DBC achieved the desired decreases more rapidly than compound DC. The lower concentration of compound DBC required less than 4 hours while the higher concentration required less than 2 hours to obtain 6 log decreases. The control tubes counts were stable during the study period; the counts remained in the $1\text{-}2.0x10^4$ cfu/mL or cfu/disk range that was present on the disks and in the fluid of the experimental tubes prior to disinfectant exposure. Since the counts for the control tubes were stable, the data were not presented in the tables.

Table 2. Time Required (hrs) for Compound DBC to Achieve 6 Log Decreases in Viable *Klebsiella* (pH 7.0, 25°C)

	Sessile 10 mg/L	Planktonic 10 mg/L	Sessile 20 mg/L	Planktonic 20 mg/L
Nonmucoid Variety				
24 Hour, Mucoid *Klebsiella*				
Initial	0	$1.73x10^6$	0	$1.76x10^6$
0	$2.42x10^4$	$2.11x10^4$	$2.33x10^4$	$2.14x10^4$
1	$7.85x10^2$	0	$1.35x10^2$	0
2	$3.82x10^1$	0	0	0
4	0	0	0	0
48 Hour, Mucoid *Klebsiella*				
Initial	0	$1.93x10^6$	0	$1.76x10^6$
0	$2.54x10^4$	$2.24x10^4$	$2.19x10^4$	$1.82x10^4$
1	$4.56x10^3$	0	$3.74x10^2$	0
2	$7.82x10^1$	0	0	0
4	0	0	0	0
Mucoid Variety				
24 Hour, Mucoid *Klebsiella*				
Initial	0	$1.74x10^6$	0	$1.96x10^6$
0	$2.42x10^4$	$2.13x10^4$	$2.75x10^4$	$2.12x10^4$
1	$1.04x10^3$	0	$9.64x10^2$	0
2	$6.95x10^1$	0	0	0
4	0	0	0	0
48 Hour, Mucoid *Klebsiella*				
Initial	0	$1.42x10^6$	0	$1.75x10^6$
0	$2.55x10^4$	$2.32x10^4$	$2.30x10^4$	$2.56x10^4$
1	$1.45x10^4$	0	$8.92x10^3$	0
2	$7.42x10^3$	0	0	0
4	0	0	0	0

CONCLUSIONS

This study indicated compounds DC and DBC were capable to controlling sessile and planktonic populations of mucoid and nonmucoid *Klebsiella* variants. Future studies will utilize other organic N-halamines such as compound MC, a very stable monochlorinated compound. The stability of compound MC may be especially desirable in applications such as air conditioning cooling towers. Variations in disinfectant concentration will also be utilized to determine optimums for controlling cell viability. Different buffers, including buffers with added organic materials, and temperatures will be utilized since these factors can impact the disinfectant efficacy. Other organisms, plus combinations of organisms, may be utilized for biofilm formation since the responses of other organisms to disinfectants may vary.

By decreasing the disinfectant concentration required for drinking water, wastewater, and municipal swimming pools and by decreasing the distribution system corrosion, the organic N-halamine disinfectants may decrease treatment costs. As with chlorination, the disinfectants are effective against a broad spectrum of suspended microorganisms. Unlike chlorination, the proposed disinfectants are effective against attached microorganisms. Also unlike chlorination, toxic byproducts do not result from the application of these disinfectants. The organic N-halamine disinfectants can provide safe, palatable water for current and future Georgia municipalities and industries.

ACKNOWLEDGMENT

This research was supported by the National Aeronautics and Space Administration through the JOVE Project.

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