MONOCHLORAMINE DISINFECTION OF BIOFILM-ASSOCIATED *LEGIONELLA PNEUMOPHILA* IN A POTABLE WATER MODEL SYSTEM

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Microbial biofilms are ubiquitous in potable water systems and may provide a habitat tor the survival of pathogenic organisms. *Legionella pneumophila* has been shown to survive in water system biofilms. Biofilm-associated microorganisms are significantiy more resistant to antimicrobial agents either due to the mass transport limitations provided by the biofilm matrix or because these organisms are physiologically different from planktonic organisnıs (2). A recently published study provided evidence that hospitals supplied with drinking water containing free chlorine as the residual disinfectant were more likely to have a reported outbreak of Legionnaires' disease than those that used drinking water containing monochloramine as the residual disinfectant (4). Other published studies showed that monochloramine inactivated biofilm bacteria more effectively than free chlorine when compared on the basis of equal activity (5).

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Legionella, Edited by Reinhard Marre et al.

We undertook a study to compare free chlorine and monochloramine as disinfectants against L. *pneumophila* cells in a mixed culture bacterial biofilm. Our approach was to develop a heterotrophic-bacterial biofilm containing the amoeba *Hartmannella vermiformis* in a laboratory potable water system, then colonize these biofilms with L. *pneumophila.* The goal of this study was to determine the susceptibility of biofilm-associated L. *pneumophila* to free chlorine and monochloramine.

MATERIALS AND METHODS

A biofilm reactor, developed in the Centers for Disease Control and Prevention (CDC) Biofilm Laboratory and containing 316L stainless-steel coupons (1.3-cm diameter), was used for all experiments (Fiğ. l). Temperature was maintained at 30°C by placing the reactor in a water bath. The biofilm reactor had a lid with a vent, a sampling port for the bulk liquid, and an inlet through which fresh nutrients were added. A side arm at the 400-ml level provided overflow drainage. Mixing was provided by a digitally controlled mixing plate (Mirak Thermolyne, Fisher Scientific, Pittsburgh, Pa.) placed beneath the water bath. The medium initially used in the biofilm reactor contained 0.05 g of yeast extract, proteose peptone no. 3, Casamino Acids, and

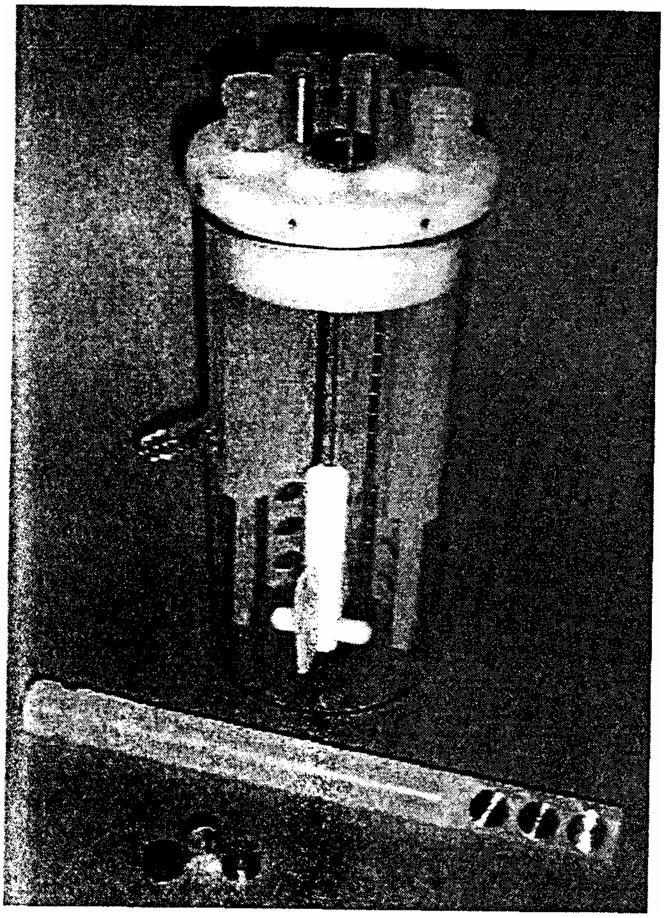

FIGURE l Potable water biofilm reactor.

dextrose; 0.03 g of sodium pyruvate and dibasic potassium phosphate; and 0.005 g of magnesium phosphate per liter of filter-sterilized reverse-osmosis water. it was operated in batch mode tor the first 72 h to establish the biofilms on the steel substrata. The system was then operated as an open system by continuously pumping a 1/10 dilution of the mediun formulation given above at a flow rate of l mi/nün tor 24 h to dilute the medium, after which the feed to the reactors was changed to filter sterilized, dechlorinated tap water (Atlanta, Ga., municipal tap water dechlorinated using 0.1 N sodium thiosulfate) tor the remaining 21 days.

Each of two reactors was inoculated with *Pseudomonas aerugmosa* (ATCC 7700), *Klebsiella pncumoniae* (CDC Strain Designation DMDS Lab No. 92-08-28a), and *Flavobacterium* spp. (CDC-65). Cultures were stored at -70°C, transferred to R2A ağar plates, and resuspended in phosphate buffered water (pH 7.2) to a concentration equivalent to a 0.5 McFarland Standard. A total of l ml of each celi suspension ($l0^8$ cells per ml) was added to each reactor. *H. vermiformis* (CDC-19) stocks were kept in axenic growth medium at 35°C without CO^2 and were split twice a week into T75 cell culture flasks. Flasks were tapped to dislodge *H. vermiformis* trophozoites from the growth surface, and 50 mi of the culture was transferred to individual conical tubes that were centrifuged to pellet the amoebae and then was resuspended in phosphate buffered saline. Reactors were inoculated with *H. vermiformis* to obtain a final concentration of $l0^4$ cells/ml. A virulent strain of L. *pneumoplıila* (RI-243-GFP) was stored as a suspension in defibrinated rabbit blood in liquid nitrogen (-120°C). Four days before the isolate was needed, the mutant was cultured on buffered charcoal-yeast extract medium containing kanamycin and incubated at 36°C with 2.5% CO^2. After 4 days, the isolate was resuspended in sterile reverse-osmosis-treated water and diluted to the desired concentration. A total of l ml of this suspension was added to each reactor.

Coupons were removed from the reactor, rised in sterile phosphate buffered water, and placed into 250-ml beakers containing 200 mi of either 0.2-, 0.5-, or 1.5-mg/liter concentrations of either free chiorine or monochloramine tor contact times of either 15, 60, or 180 min at ambient temperature. All glassware used was demand-free. Free chlorine and monochloramine residual concentrations were measured with a Hach Amperometric Digital Titrator (model 19300, Hach Co., Loveland, Colo.). Monochloramine stock solution was prepared by mixing 0.11 g of ammonium chloride with 100 ml of phosphate buffered water and then slowly adding l ml of 5% sodium hypochlorite and stirring tor 20 min. Final concentration of this stock solution was 300 to 400 mg of total chlorine per liter. Reverse-osmosis (RO) water was used to dilute the stock solution to obtain the desired working concentration. Free chlorine was prepared by adding 20 to 25 μl of 5.25% sodium hypochlorite (commercial bleach) to 1 liter of RO water to obtain a stock solution final concentration of 1.5 mg/liter. Additional RO water was added as required to obtain the desired concentradon. The pH of the monochloramine working solution was 7.5; the pH of the free chlorine solution was 7.8.

Coupons were then placed into 10 ml of a 0.12 M solution of sodium thiosulfate in phosphate buffered saline, processed to remove the attached organisms by three 30-s cycles of sonication and vortexing, homogenized for 1 min, then spread plated onto R2A medium tor quantification of the base biofilm. For recovery of H. *vermiformis,* 100/xl from several dilutions were plated onto nonnutritive agar, which had been spread with viable *Escherichia coli.* Plates were read tor the presence or absence of *H. venniformis* at the dilution plated. For recovery of L. *pneumophila,* the remaining suspension was treated *with* a KC1-HC1 solution, filtered through a 0.2-/xm-pore-size filter (part no. GTTP, Millipore Corp., Bedford, Mass.), resuspended, and plated onto glycine-polymyxin B-anisomycin-vancomycin plates.

RESULTS AND DISCUSSION

A biofilm reactor was developed to grow biofilms containing L. *pncumophila* on 24 replicate stainless-steel surfaces in potable water. The ability of this reactor to produce reproducible biofilms was validated by the fact that the standard deviations of the base biofilm densities on stainless-steel coupons (n = 3) ranged from 0.06 to 0.18 log CFU per coupon. Tik and Hamilton (7) reviewed the literature to determine the range of repeatability (within laboratory precision of log reduction data during germicide testing) and found that standard deviation values ranged between 0.25 and 1.21, and that a test can be considered very good if it achieves a value of less than 0.3. Our results were well within this range.

Legionella levels in the biofilms exceeded 10^3 per coupon surface on the pretreated coupons. When *Legionella-containing* biofilms were exposed to three different concentrations of free chlorine (0.2, 1.0, 1.5 mg/liter) for three different contact periods (15, 60, 180 min), this disinfectant was relatively ineffective, providing a 1.1 log reduction only at the highest dosage/contact time tested (1.5 mg/liter for 3 h) (Fig. 2). When *Legionella*-containing biofilms were exposed to the same dosages of monochloramine for identical contact periods, the treatments were significantly more effective (Fig. 3). Even though the 0.2-mg/liter treatment was ineffective for all contact times examined, the 0.5-mg/liter dosage

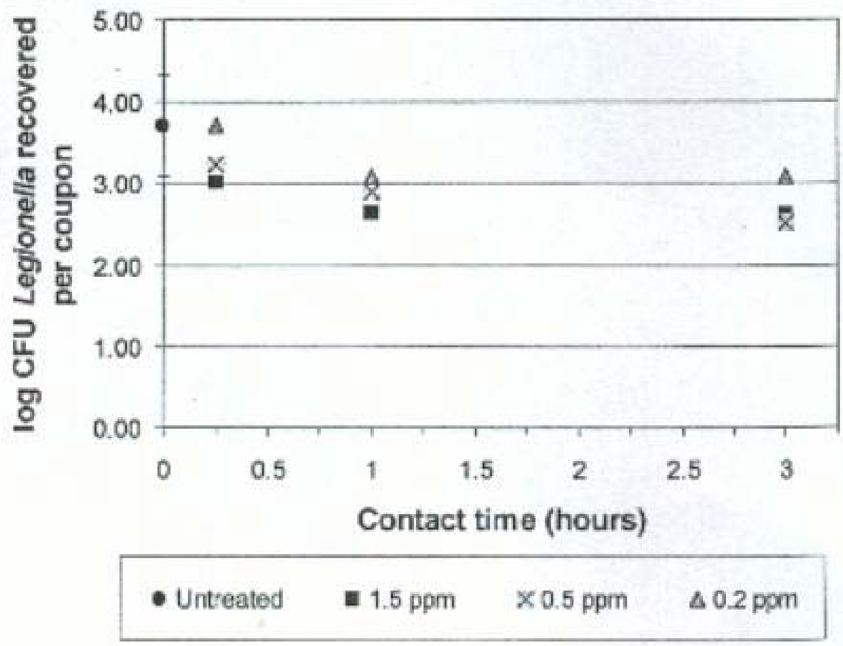

FIGURE 2 Biofilm-associated *Legionella* disinfection with free chlorine.

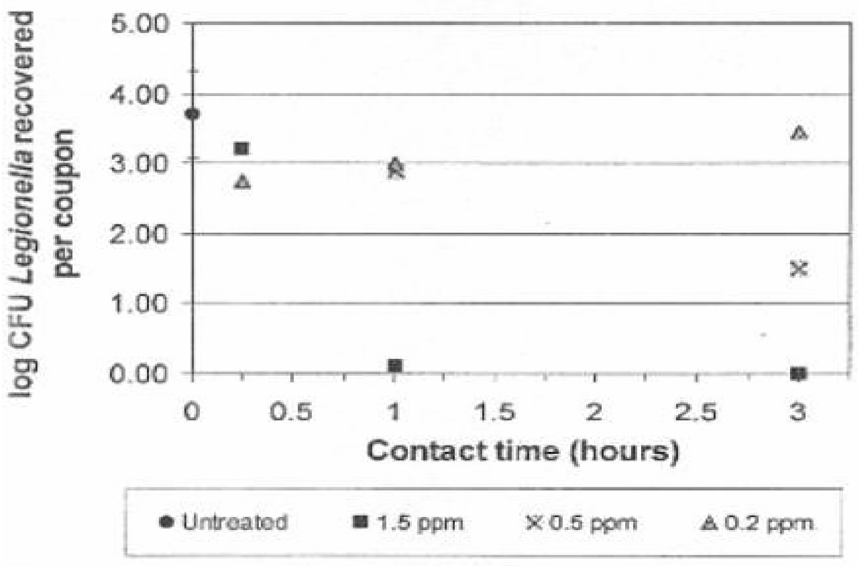

FIGURE 3 Biofilm-associated *Legionella* disinfection with monochloramine.

reduced *Legonella* levels by 99% for the 180-min contact period. The 1.5-mg / liter concentration reduced levels by more than 99.9% for both the 60- and 1-80-min contact periods. A second study cornparing 1.5 mg of free chlorine per liter with the same concentration at monochloramine showed that monochlormine was significantly more effective than free chlorine for a 3-h contact period. These results are shown in Fig. 4.

LeChevallier et al. (5) found that the concentration-times-time coefficient (CT) values (milligram minutes per liter to achieve a 99% reduction in viability) for hypochlorous acid (pH 7.0, 1 to 2°C) against heterotrophic plate count bacteria was 0.08. For monochloramine under the same conditions, the value was 94. They showed that three times the CT was required to inactivate biofilm-associated *Klebsiella* cell on glass slides using mono-

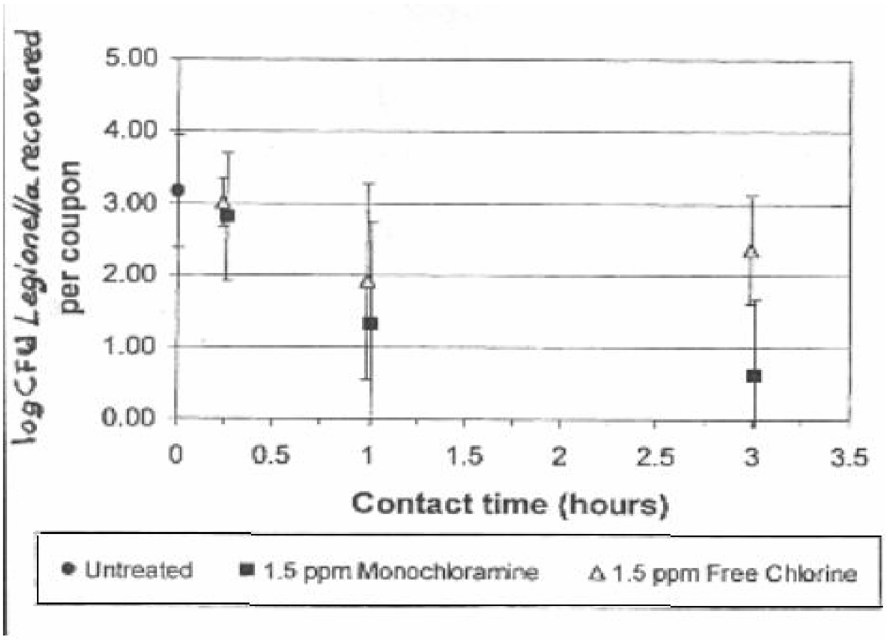

FIGURE 4 Comparison of free chlorine and monochloramine disinfection efficacy of biofilm-associated *Legionella*. Error bars represent standard deviations ($n = 3$).

chloramine; one CT was required to inactivate 99% of heterotrophic bacteria on metal surfaces with monochloramine. Our results show that the CT value for inactivation of biofilm-associated *Legionella* was 90, which is similar to the results obtained by LeChevallier et al. for biofilm-associated heterotrophic bacteria.

In summary, we have shown that biofilm-associated L. *pneumophila* are significantly less susceptible to chlorine than are planktonic L. *pneumophila,* while susceptibility of planktonic and biofllm-associated L. *pneumophila* to monochloramine are similar. When monochloramine and free chlorine were compared under identical conditions, monochloramine was significantly more effective, indicating that monochloramine may be an effective disinfectant for the inactivation of L. *pneumophila* within potable water distribution systems. since it is known that L. *pneumophila* is indigenous to potable water system biofilms (8), and that biofilms readily form in these systems (3), an effective control strategy could incorporate monochloramine as a disinfectant for these organisms. This has been suggested by LeChevallier et al. (6) for general biofilm control and by Cunliffe (l) specifically for *Legionella* control. Further research using open system biofilm reactors and model distribution systems is needed to determine the utility of monochloramine as a disinfectant against biofilm-associated L. *pneumophila.*

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