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Colonization and Disinfection of Biofilms Hosting Coliform-Colonized Carbon Fines

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The documented release of carbon fines from granular activated carbon filters is a concern for drinking water utilities, since these particles may carry coliform and even pathogenic bacteria through the disinfection barrier. Such a breakthrough could have an impact on distribution system biofilms. Using total cell counts, specific monoclonal antibody staining, and computerized image analysis, we monitored the colonization of introduced *Klebsiella pneumoniae* associated with carbon fines in mixed-population biofilms. The particles transported the coliforms to the biofilms and allowed successful colonization. Chlorine (0.5 mg/liter) was then applied as a disinfectant. Most *K. pneumoniae* along with the carbon fines left the biofilm under these conditions. The impact of chlorine was greater on the coliform bacteria and carbon fines than on the general fixed bacterial population. However, 10% of the introduced coliforms and 20% of the fines remained in the biofilm. The possibility that this represents a mechanism for bacteria of public health concern to be involved in regrowth events is discussed.

To comply with the U.S. Environmental Protection Agency regulations on disinfection by-products in drinking water, utilities need to lower applied disinfectant concentrations (11, 13, 20, 29). However, adequate disinfectant residuals in distribution systems must be provided to control bacterial growth. These somewhat contradictory goals can be reached by decreasing the amount of natural organic matter in water (31). Natural organic matter is responsible for disinfectant demand in water and formation of disinfection by-products (27), and the fraction called biodegradable organic matter can be used by bacterial populations as their carbon and energy source. In biological treatment, the degradation of organics is ensured by microbiological processes, and the result is more biologically stable water (1, 10, 18, 19, 30, 31) which does not induce further bacterial growth in the distribution system (5, 24, 30). A common biological treatment process for drinking water consists of preozonation of the water followed by filtration through granular activated carbon (GAC) beds optimized for biological growth (11, 18, 21, 23, 28, 33). Such a process is called biologically activated filtration.

GAC is an ideal medium for the process, as it possesses large adsorptive capabilities and a large surface area (4). The adsorption phenomenon coupled with microbial degradation by the bacterial populations in the biologically activated filtration process is responsible for the biological stability of water (1, 10, 30). A major concern with the utilization of GAC filters optimized for microbiological growth is the potential introduction of microorganisms to the distribution system (breakthrough) in association with GAC fines (CF) released from the filter medium (6, 7, 35, 37). CF result from the mechanical abrasion of the GAC grains during backwash sequences of the filters. Organisms attached to particles are less susceptible to disinfection (7, 14, 35) and may therefore pass disinfection barriers and have a significant impact on the biofilm of distribution systems (7, 17). It has been shown that CF with an average length of 11.5 μm were able to reach and remain in biofilms for an extended period of time (22). If CF colonized by heterotrophic bacteria are released, there appears to be limited concern for public health (22). However, coliforms and opportunistic pathogens have been associated with GAC particles released from traditional GAC filters (7, 35). Thus, there is a concern over the release of fines colonized with opportunistic or frank pathogens and the potential induction of regrowth events in water distribution systems (3, 5, 7, 17). These chronic or periodic appearances of bacteria in finished water are associated with the proliferation of biofilm bacteria within distribution networks (5, 16); the inoculum for these biofilms probably originated with breakthrough events (5). Numerous studies have shown that, at least for environmental strains, coliforms such as *Klebsiella* spp. and *Escherichia coli* are capable of growth in the oligotrophic conditions prevalent in drinking water distribution system biofilms (8, 16, 24, 32, 38, 39). Multiplication of opportunistic pathogenic bacteria in potable water represents a potential human health problem, especially for immunodeficient people, such as the elderly, newborns, and AIDS and dialysis patients (12, 26). The work presented here focused on the colonization of biofilms by the environmental coliform and opportunistic pathogen *Klebsiella pneumoniae* carried by CF. Biofilms grown in laboratory reactors operated under conditions relevant to drinking water conditions were subjected to slug doses of colonized fines. The biofilms, along with the incorporated fines and coliforms, were then studied under disinfecting conditions with chlorine.

MATERIALS AND METHODS

Experimental system. The experimental system consisted of polycarbonate annular reactors, previously described (34), in which biofilms were grown. These reactors had 12 removable polycarbonate slides that allowed periodic biofilm sampling. The shear stress created by the rotation of the inner drum simulated a velocity of 0.3 m/s in a 4-in. pipe. Feed water was first treated with a GAC filter and then with a biologically activated carbon filter. These two filters removed chlorine and natural biodegradable organic matter from tap water and also

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provided a source of consistent microbial inoculum for the annular reactors. The treated water was supplemented with an assimilable organic carbon solution and a nitrate-buffer solution. The final assimilable organic carbon concentration in the reactor was 0.5 mg/liter, and the P and N concentrations were 0.1 mg/liter each, giving a C/N/P ratio of approximately 5/1/1. The assimilable organic carbon solution consisted of equimolar concentrations based on the carbon contents of acetic acid, benzoic acid, propionaldehyde, parahydroxybenzoic acid, and ethanol (Sigma Chemical Co.). This solution was tested to be chlorine demand free. The buffer-nitrate solution contained 0.1 mg of sodium nitrate per liter and 0.1 mg of potassium phosphate per liter that supplemented trace levels of N and P present in tap water. A 0.2-μm-pore-size filter (Gelman Sciences) was located between the dilution water reservoir (water with a low level of biodegradable organic matter) and the reactors to avoid the presence of external CF in the reactor. The retention time was 2 h in the reactors (total flow rate, 5 ml/min). This residence time was sufficiently short to minimize the contribution of planktonic growth of the heterotrophic plate count (HPC) bacteria; a mass balance across the reactor will reflect, therefore, net attachment or detachment of cells or CF from the surface. The reactors were partially immersed in a temperature-controlled water bath to maintain a constant temperature of 20 ± 1°C.

Microbial population. A mixed-population biofilm was grown in the reactors under conditions relevant to drinking water distribution systems. The source of the bacteria was water from the laboratory biologically activated carbon columns, which contained approximately 10^4 CFU/ml, as enumerated on R2A medium (Difco). To minimize the introduction of extraneous carbon particles from the columns but allow for sufficient inoculation, the reactors were initially run for 5 h with settled column effluent. After this time the systems were switched to filtered column effluent.

Carbon fine characteristics and colonization. A suspension of CF was obtained by washing and filtering powdered, activated carbon through a 50-μm-pore-size sieve and an 8-μm-pore-size nylon filter (Spectrum). The average size of the CF retained on the 8-μm-pore-size filter was 11.5 μm as determined by the observation of at least 50 CF with the aid of a computerized image analysis system (American Innovision). The CF were colonized with *K. pneumoniae*, a nonmotile gram-negative rod and opportunistic pathogen. This environmental strain was recovered from a drinking water network (8). The colonization of these fines was carried out by mixing them with a pure culture suspension in buffer containing approximately 6×10^5 bacteria per ml for 24 h. The suspension was then filtered through an 8-μm-pore-size polycarbonate membrane (Poretics Corp.) to separate the fines from the free bacteria. The CF recovered on the filter membrane were resuspended in 30 ml of sterile buffered solution (1.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and then introduced into the reactors.

Sampling and analytical methods. Reactors were run for at least 10 days before the first sampling and introduction of the colonized CF to allow for adequate colonization. Previous results obtained under identical conditions showed that steady-state numbers of biofilm HPC bacteria were achieved within 7 days of operation (data not shown). The biofilm was sampled 1 h before the addition of the CF suspension, and total bacteria, HPC bacteria, *K. pneumoniae*, and CF numbers were determined (see below). The reactors were then inoculated with suspensions of colonized CF to a final concentration of approximately 3×10^5 CF per ml. The second sample was taken 24 h after the addition of the CF suspension. One reactor was fed a chlorine solution (influent concentration, 0.5 mg/liter) immediately after the second sampling, while the other received the same amount of nanopure water. Chlorine solution was prepared with commercial bleach (5.25%) diluted with nanopure water. Solution concentrations were measured with an *N*,*N*-diethyl-*p*-phenylenediamine digital chlorine colorimetric kit (Lamotte). The effluent residual chlorine concentration was 0.1 mg/liter. Samples were then taken every 5 days for 20 days. Two slides were removed from each reactor at every sampling time. Biofilm was scraped from the slides and suspended in 10 ml of water. The suspensions were homogenized for 30 s at maximum speed with a tissue homogenizer (Tekmar). A control experiment (data not presented) showed that homogenization dispersed the biofilm without breaking up the large CF. The homogenized samples were used for bacterial and CF analyses. The sizes and the masses of the CF were determined by exposing the suspension for 5 min to an 8.5% phosphoric acid solution to remove biological material and then by filtration through a 1.2-μm-pore-size cellulose acetate membrane (Millipore Corp.). The filter was examined under a microscope at ×1,000 magnification. Counting and sizing of the CF were carried out with an image analysis system. Total bacteria were estimated by 4′,6-diamidino-2-phenylindole (DAPI) (0.1 g/liter; Sigma Chemical Co.) staining and direct microscopic count (16, 25). *K. pneumoniae* in the dispersed biofilms was monitored by a combined fluorochrome-antibody technique. An aliquot of the cell suspension was filtered on a 0.2-μm-pore-size nonfluorescent polycarbonate filter membrane (Poretics Corp.). A blocking solution (0.3% Tween 80 and 2% skim milk in phosphate-buffered saline [PBS]) was applied to the membrane for 1 h. After filtration and a rinse with PBS-Tween 80 solution, the monoclonal antibody anti-*K. pneumoniae* (antibody 1) was put in contact with the immobilized cells on the membrane for 1.5 h. Monoclonal antibody production and testing were as described elsewhere (36). After a new rinse, the second antibody, anti-antibody 1 conjugated with Texas red (antibody 2; Southern Biotechnology Associates), was then applied for the same period of time. Following a final rinse, the membrane filter was dried and mounted in nonfluorescent oil between a slide and coverslip. Because of the sizes of the targeted cells and the specificity of the monoclonal antibody, this combination allowed the count of total *K. pneumoniae* organisms on the membrane. The minimum sensitivity of the technique was 10^3 bacteria per cm^2. HPC bacteria were evaluated by the spread plate method in triplicate on R2A medium; CFU were counted after 7 days of incubation at 20°C.

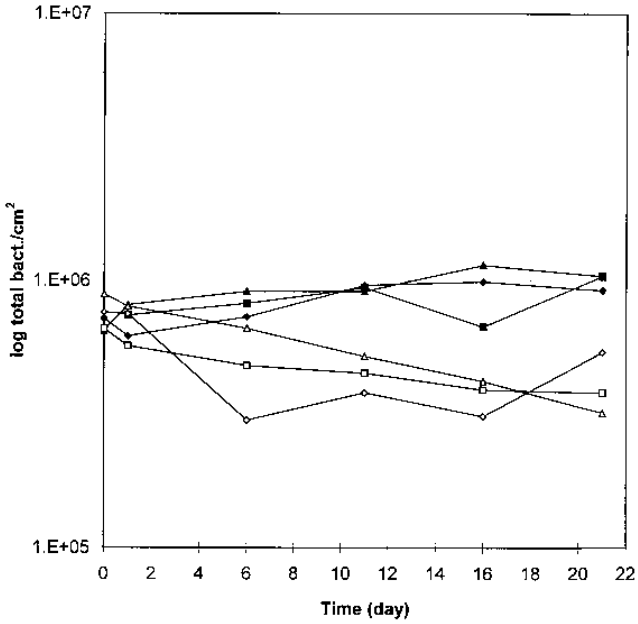

FIG. 1. Effect of the presence of carbon fines and chlorine disinfection on the total bacterial population in a biofilm. All reactors were inoculated with a suspension of *K. pneumoniae*-colonized carbon fines after the first sample at time zero. Closed symbols, control replicate experiments; open symbols, disinfection replicate experiments (0.5 mg of chlorine influent concentration per liter after day 1). bact., bacteria.

RESULTS

These experiments were designed to check the feasibility of the incorporation of CF colonized with *K. pneumoniae* in biofilms growing under conditions of drinking water distribution systems and to study the disinfection of the transformed biofilms.

Figures 1 and 2 present the overall population results versus time obtained, with total cell counts measured with DAPI (Fig. 1) and plate counts measured on R2A medium (Fig. 2). The concentrations of total bacteria and HPC bacteria in the nonchlorinated reactors were parallel and constant over the course of the experiments at 5.9 log and 5.27 log, respectively. No measurable effect of the introduction of the colonized CF on total bacteria or on HPC bacterial concentrations in the reactor biofilms was visible after 24 h (day 1). However, the results under disinfecting conditions showed different trends for total and viable cells. Chlorine appeared to result in a limited detachment of biofilm organisms as determined by total cell counts, with an average decrease of 0.23 log for the three replicates over the time period of the experiment (Fig. 1). The impact on the viable bacterial population (CFU) was greater, with an average decrease of 2.5 log after day 21 (Fig. 2).

Figures 3 and 4 present the results for *K. pneumoniae* counts assessed by the fluorescent antibody technique and for the CF larger than 5 μm, respectively. The total concentration of *K. pneumoniae* in the biofilms increased over a 24-h period after the inoculation of the CF suspension, exceeding 10^4 cells per cm^2 after 1 day. For the rest of the experiment in the control reactors, the total *K. pneumoniae* count remained essentially constant at this concentration. A marked decrease in the con-

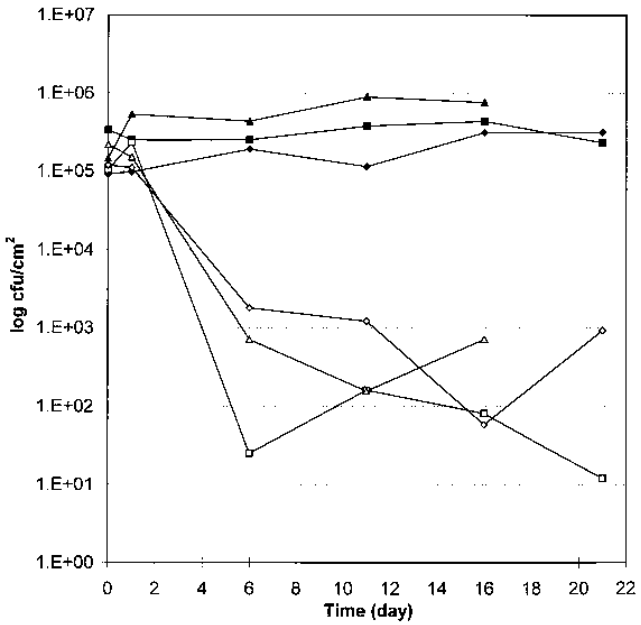

FIG. 2. Effect of carbon fine inoculation and chlorine disinfection on culturable HPC bacteria in a biofilm. All reactors were inoculated with a suspension of *K. pneumoniae*-colonized carbon fines after the first sample at time zero. Closed symbols, control replicate experiments; open symbols, disinfection replicate experiments (0.5 mg of chlorine influent concentration per liter after day 1).

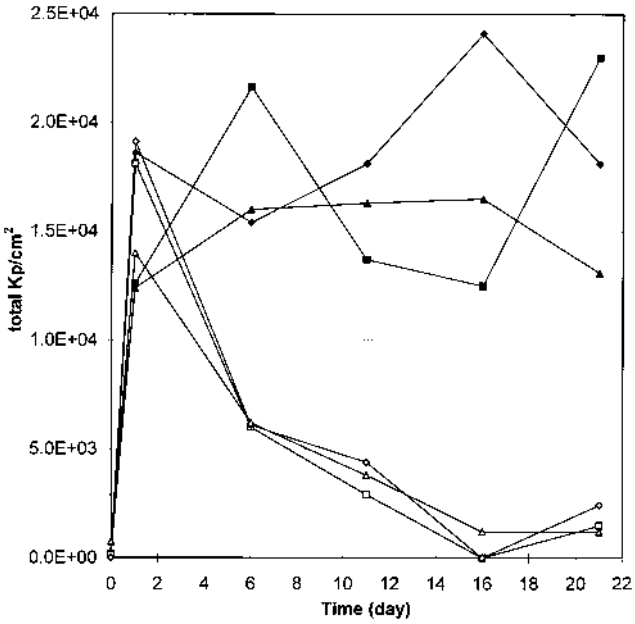

FIG. 3. Inoculation and disinfection of *K. pneumoniae* in the biofilms grown in the annular reactors. All reactors were inoculated with a suspension of *K. pneumoniae*-colonized carbon fines after the first sample at time zero. Closed symbols, control replicate experiments; open symbols, disinfection replicate experiments (0.5 mg of chlorine influent concentration per liter after day 1).

centration of the coliform bacteria in the biofilm in comparison with that of the control was observed following the application of chlorine. At day 21, *K. pneumoniae* was present in the biofilm of the chlorinated reactor at a concentration of approximately 1.7×10^3 bacteria per cm^2. Although this concentration was determined by microscopy (with its inherent inaccuracy at low cell concentrations), this result indicates that the coliform bacteria were able to persist in the biofilm and, to a limited extent, to resist the chlorination. Figure 4 presents the results obtained from the monitoring of the CF larger than 5 μm in the biofilm. A rapid increase in the number of fines in the biofilms followed the inoculation of the CF suspension in the reactors. The particles remained in the biofilm of the nonchlorinated reactors at a constant concentration of approximately 5.0×10^3 CF per cm^2. These results show that the CF introduced in the reactors at time zero were retained in the undisturbed biofilms over the time period of the experiments. The chlorinated biofilms, however, released approximately 80% of the particles larger than 5 μm by the end of the experiment. The similarities between the results presented in Fig. 3 and 4 for *K. pneumoniae* and the CF in the biofilm suggest that (i) the CF introduced in the reactors at time zero carried the coliforms to the biofilm and (ii) substantial numbers of coliform bacteria detached along with the particles when the disinfectant was applied.

Table 1 summarizes the disinfection results for total bacteria, *K. pneumoniae*, and CF. The removal rate coefficients were interpreted by calculating first-order detachment rate coefficients obtained by linear regression of the natural log of cell number or CF density versus time between 1 and 16 days. The detachment coefficient for total cells was more than four times smaller than the detachment coefficient for *K. pneumoniae* cells (0.038 and 0.162 day^{-1} on average for the three replicates, respectively). The detachment coefficient for CF larger than 5 μm was between those of the two bacterial populations (0.109 day^{-1} on average). Further, the fraction of the number of cells or CF remaining in the film after 21 days showed that CF and coliforms were eliminated to a greater extent than total cells.

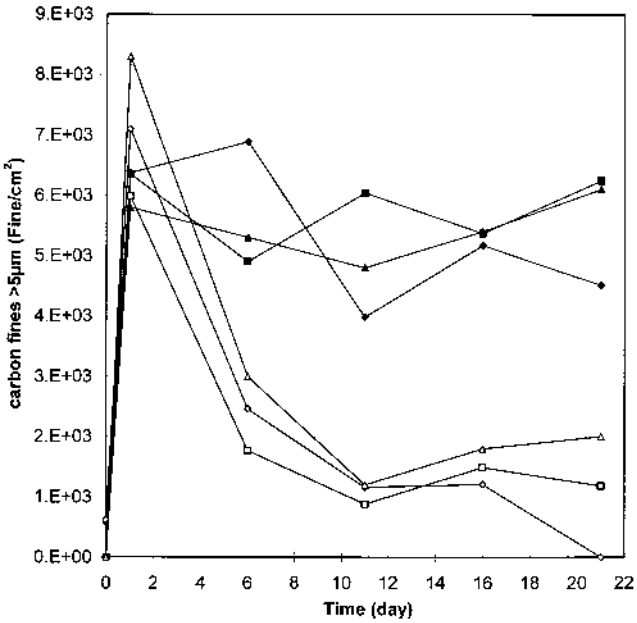

FIG. 4. Incorporation of and removal by disinfection of the carbon fines larger than 5 μm. All reactors were inoculated with a suspension of *K. pneumoniae*-colonized carbon fines after the first sample at time zero. Closed symbols, control replicate experiments; open symbols, disinfection replicate experiments (0.5 mg of chlorine influent concentration per liter after day 1).

TABLE 1. Removal rate coefficients and fractions remaining at day 21 for biofilms treated with 0.5 mg of chlorine per liter

Biofilm component	Replicate	Removal rate coefficient ± SE (day^{-1})	Fraction remaining after 21 days	n^a
Total cells	1	0.024 ± 0.002	0.66	4
	2	0.048 ± 0.031	0.72	4
	3	0.043 ± 0.001	0.40	4
K. pneumoniae	1	0.183 ± 0.021	0.08	3
	2	0.146 ± 0.047	0.12	3
	3	0.157 ± 0.017	0.08	4
Carbon fines	1	0.097 ± 0.056	0.19	4
	2	0.121 ± 0.035	0	4
	3	0.110 ± 0.048	0.24	4

[a] *n* is the number of datum points used in the regression analysis to determine coefficients and standard errors.

DISCUSSION

The coliform *K. pneumoniae* was used to study the ability of CF colonized by bacteria of public health relevance to attach to simulated drinking water distribution system biofilms. This opportunistic pathogen was isolated from a distribution system during a regrowth event (8) and is therefore representative of the bacteria present in real systems. This is of importance since many fecal strains of *K. pneumoniae* may not be able to colonize distribution system biofilms (15). The mean size of the CF in the suspension introduced into the reactors was 11.5 μm, and we monitored the CF greater than 5 μm in the biofilms. Long-term attachment of large carbon particles in mixed-population biofilms was shown in a similar study (22). CF with large surface areas would be able to carry bacteria (7, 12) and to optimize the colonization of the biofilms by the coliforms they transport. Although this is not a requirement for coliforms to colonize biofilms (6, 38, 39), a carrier may help bacteria reach and remain in the biofilm. In actual systems, attachment to particulate matter may result in advantages for the microorganisms. The protection conveyed to bacterial cells by their attached state (7, 14, 35) may allow them to pass through the disinfection barrier (2, 3, 14, 17) without being severely injured and therefore to reach the biofilm under conditions that may increase their capacity for colonization. Environmental strains of coliform bacteria grow under drinking water distribution system oligotrophic conditions (8, 16, 24, 32). However, the manner in which coliforms and pathogenic bacteria might actually colonize heterotrophic biofilms is not clear. As shown in Fig. 3 and 4, the appearance of *K. pneumoniae* in the biofilm, as monitored by the antibody technique, was paralleled by the appearance of large (>5 μm) CF. In the nonchlorinated biofilms (Fig. 3), the concentration of introduced coliforms remained constant over the time period of the experiment. These results show that the transport of CF carrying *K. pneumoniae* to heterotrophic biofilms can lead to a successful colonization of the coliform in the biofilm.

Because of the observation that particle-associated bacteria are less susceptible to disinfection (7, 14, 35), it was of interest to determine if the coliforms transported to the biofilms by CF would be selectively protected when chlorinated. As shown in Fig. 3 and 4, coliforms were released from the biofilm following continuous application of 0.5 mg of free chlorine per liter. Approximately 10% of coliforms and 20% of CF remained in the biomass at the end of the experiment (Table 1). When these results were compared with the weak effect of chlorination on total biomass (Fig. 1), it appeared that *K. pneumoniae* in the biofilm responded differently from the general population (90 versus 40% decrease, respectively) but not entirely in the same manner as carbon particles (80% decrease). The analysis presented in Table 1 helps explain the differences in overall removal between total cells, coliforms, and CF under disinfecting conditions. *K. pneumoniae* detached faster than the CF, which were released faster than the total bacterial population from the biofilm. This suggests that the coliform population was both detached and disinfected faster than the total population and that *K. pneumoniae* cells were not protected from chlorination by the carbon particles introduced into the reactors.

Despite the rapid removal of both *K. pneumoniae* and CF from the biofilm during chlorination, neither was removed completely. Preliminary results of related experiments in our laboratory (data not shown) are that an inoculum of freely suspended organisms is not retained in the biofilm as well as a comparable inoculum attached to carbon fines. Although the role of the CF in the long-term retention of coliforms is unclear, there is clear potential for incorporation of the coliforms into a biofilm when they are introduced with the fines, as previously suggested by Camper et al. (7). It is well established that bacterial growth in biologically activated filters (11, 21, 23, 28) can release colonized particles into the water (7, 35), and it has been suggested that coliform breakthrough in the final disinfection step of a drinking water treatment plant can provide the inoculum for later regrowth (3, 5, 17). Thus, the possibility remains that coliforms and other organisms that can survive biological treatment can act as inocula for the distribution system and that attachment to fines can play a role in the effectiveness of this process.

Regardless of the retention of coliforms in the biofilm, our assay technique was not capable of assessing either pathogenicity or viability. We identified and enumerated only those organisms that still possessed sufficient epitopes to react with the monoclonal antibody and give an epifluorescent signal. The mere presence of the organism in the biofilm was insufficient information to assess the actual public health significance of the colonization of distribution system biofilms by *K. pneumoniae*. On the basis of the discussion presented in the previous paragraph, however, several recommendations and observations can be made. First, appropriate ozonation as a treatment step prior to biological filtration should prevent significant colonization of the filter media by organisms of public health concern. It has previously been shown that biologically activated carbon does not support as high a population of coliforms as nonbiologically activated carbon (9). Second, the primary function of biological treatment—to remove biodegradable organic matter from finished water—should limit the ability of coliforms to multiply to any great extent, even if they do become incorporated into the distribution system biofilm. Third, it would be of interest to know the effect of a longer or shorter delay between inoculation and chlorine application (24 h in our experiments).

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