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Authors: Thomas Griebe, Ching-I Chen, Rohini Srinivasan, and Philip S. Stewart

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I. INTRODUCTION

A biofilm consists of microbial cells embedded in an extracellular polymeric substance matrix attached to a substratum.[1] Prevention and control of biofilm accumulation is a challenging task to industry. The biofouling problem has usually been alleviated by application of oxidizing biocides, such as free chlorine (hypochlorous acid/hypochlorite), which have been used for decades. Although most of the microorganisms in industrial systems are associated with surfaces, biofilms have historically received less attention than have suspended (planktonic) microorganisms. Most disinfection studies have been carried out with suspended bacteria, thereby neglecting the influence of the substratum and cell aggregation.

It has been shown that various biocides are less effective against biofilm cells than dispersed cells of the same organism.[2–4] Chen et al.[5] demonstrated that *Pseudomonas aeruginosa* cells grown in a biofilm were less susceptible to biocide treatment, in part because the intact biofilm matrix constituted a diffusion barrier for the biocide. However, the mechanism of disinfection of biofilm organisms is not yet fully understood due to the lack of accurate methodology and to the misleading assumption that biocide reaction kinetics of planktonic and biofilm bacteria are similar.

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Previous comparisons between free chlorine and monochloramine for the inactivation of biofilm bacteria in pipe systems[6-8] were inconclusive with respect to the superior disinfectant. The objective of this study was to compare the disinfection efficacy of the oxidative biocides, free chlorine and monochloramine, on intact biofilms and mechanically dispersed biofilms of *P. aeruginosa*. A further goal of this study was to develop an experimental methodology for the testing of biocide efficacy against biofilms using a bioreactor system.

II. MATERIALS AND METHODS

A. Experimental Biofilm System

A RotoTorque annular reactor was used for the biofilm experiments. As shown in Figure 1, the reactor consists of a stationary outer casing and a rotating inner cylinder, both made of polycarbonate. Twelve removable polished stainless steel slides (SS304, 1.7 × 19 cm) form an integral part of the inside wall of the outer cylinder and permit sampling of biofilms growing on them. The bulk liquid is completely mixed by virtue of the cylinder rotation and angled draft tubes bored through the solid inner cylinder. Prior to inoculation, the system was autoclaved at 121°C (17 psi) for 25 min. All influent solutions except dilution water were autoclaved at 121°C (17 psi) for 4 h. Dilution water was sterilized via filtration using two capsule filters (0.2 μm, Gelman Sciences) in series connected to the RotoTorque (Figure 1). Concentrated glucose solution was sterilized via filtration (0.2 μm) before it was added to the sterilized, concentrated mineral salt solution. Three reservoirs containing dilution water (distilled), substrate/mineral solution, and phosphate buffer solution (pH = 7.0), respectively, were connected to the RotoTorque through silicone tubing (Masterflex 6411–14 and -16) and peristaltic pumps (Masterflex 7553–30, Cole-Parmer). The flow rates of the dilution water, concentrated substrate-mineral solution, and buffer solution were 30, 1, and 1 ml/min, respectively. The composition of the resulting influent was the same as used by van der Wende[9] with glucose (20 mg/l) and potassium nitrate (13.6 mg/l) as the carbon and nitrogen sources, respectively. Temperature was maintained at 25 ± 0.5°C with a thermostated water bath. The rotation of the inner drum in the RotoTorque was set at 150 rpm (92 cm/s) throughout all experiments, corresponding to a shear stress of approximately 1.4 N/m^2.

B. RotoTorque Experiments

A frozen stock culture (1 ml) of *P. aeruginosa* (10^8/ml) was inoculated into a 250-ml Erlenmeyer flask containing 50 ml of medium with the same

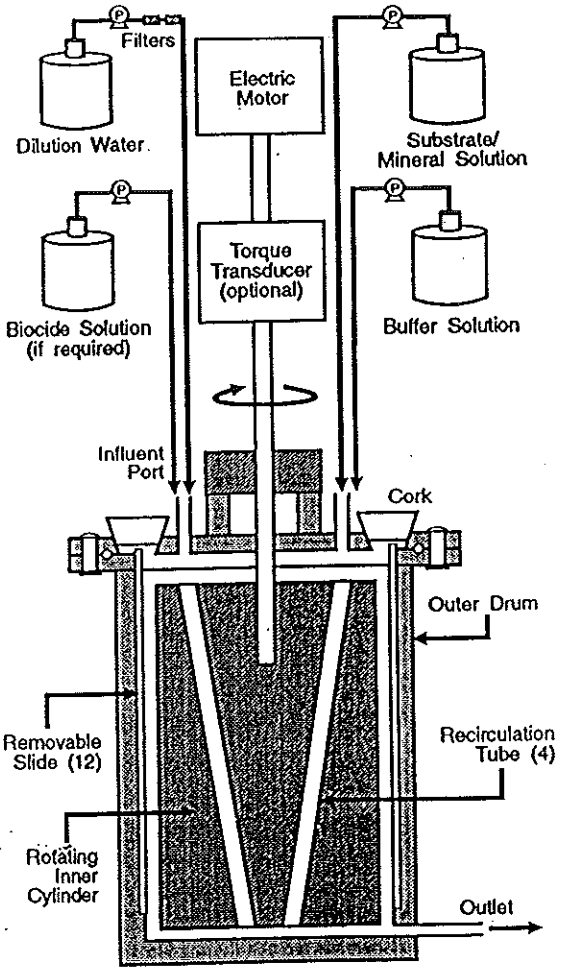

FIGURE 1. The RotoTorque system.

composition as the RotoTorque feed. The flask was incubated with shaking at 25 ± 0.5°C and 150 rpm (G24 Incubator Shaker, New Brunswick Scientific) for 24 h. The log phase culture (10 ml) was inoculated into the RotoTorque filled with medium (600 ml). After 24 h of batch cultivation at 25 ± 0.5°C and 150 rpm, all continuous influent flows were started. Since the dilution rate was very high ($3.2h^{-1}$), no planktonic cells were retained. Essentially all planktonic cells were detached biofilm cells.

Two RotoTorques were treated for 1 h with 4 mg/l monochloramine by a pulse injection of concentrated monochloramine, resulting in 4 mg/l monochloramine in the bulk fluid, followed immediately by a step feeding of 4 mg/l monochloramine (contained in the influent) after biofilms were accumulated to steady state (7 to 8 d after inoculation). One RotoTorque was used for *in situ* biofilm disinfection study and the other was used for biocide residual measurements.

C. Batch Experiments

Batch experiments were conducted to investigate disinfection of biofilm cells vs. suspended cells. Intact biofilms (slides) were removed from the RotoTorque, scraped into 150 ml of phosphate buffer (pH = 7.0, 5 m*M*), homogenized for 1 min with a Tekmar Tissumiser™, and gently stirred after addition of 1, 2, 3, or 4 mg/l monochloramine or 1 or 2 mg/l free chlorine (final concentration). At various times, samples were withdrawn, and the biocide was inactivated with 10 mg/l sodium thiosulfate and analyzed for viable cells. Aliquots were also removed to determine the residual monochloramine concentration.

D. Analytical Methods

Samples were taken daily from the effluent (representing the bulk fluid in the RotoTorque) and biofilm slides. The biofilm (7 to 8 d old) was scraped from the stainless steel slide into 150 ml of phosphate buffer (pH = 7.0, 5 m*M*) using a sterile cell scraper (Fisher Scientific) and homogenized via a Tekmar Tissumiser™ for 2 min with 100% power input. Effluent samples were also homogenized to disperse cell aggregates. Viable cells were counted by plating dilutions (in 5 m*M* phosphate buffer, pH = 7.0) of homogenized samples in triplicate (effluent and biofilm) on R2A™ agar (Difco). The results were expressed as number of colony forming units (cfu) per ml or m^2. Monochloramine-treated samples were diluted by phosphate buffer containing 10 mg/l sodium thiosulfate (biocide neutralizer) prior to plating. Total cells of homogenized biofilms and effluent samples were counted according to Griebe[10] by epifluorescent microscopy using a double staining procedure. Samples were filtered onto 25-mm-diameter (0.2-μm pore size), black polycarbonate membrane filters (Nuclepore) and stained successively with 4′,6-diamidino-2-phenylindole (DAPI, 10 μg/ml) for 10 min and acridine orange (0.1 μg/ml) for 3 min. The bacteria were counted randomly in at least 20 microscope fields using an Olympus BH2 epifluorescence microscope with DPlan Apo UV objectives and the U (DAPI) dichroic mirror cube.

E. Disinfectant Preparation and Detection

Concentrated monochloramine stock solution was prepared using a 3:1 molar ratio of ammonia (ammonium chloride, Fisher) to free chlorine (pH 9.0). Monochloramine concentration was measured by the DPD colorimetric method using a Hach test kit (Hach Co., Model CN-66) for total chlorine (detectability limit 0.05 mg/l). Since monochloramine was the only chlorine compound and there was no other strong oxidant in the system, monochloramine would not be expected to be transformed

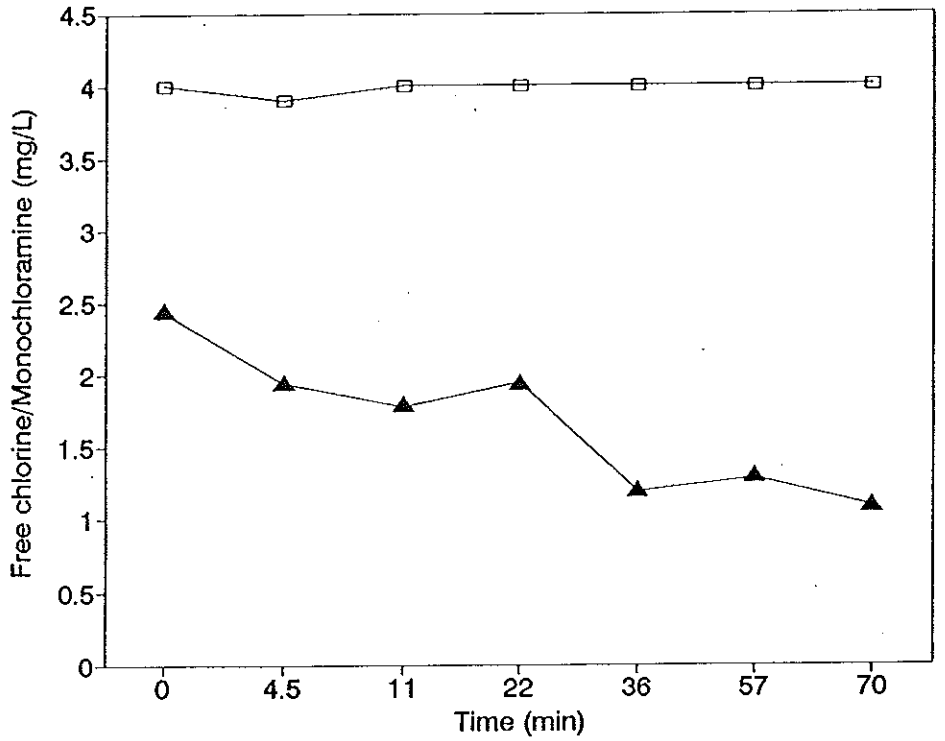

FIGURE 2. Biocide concentration in RotoTorque influent (stirred batch) after the addition of 4 mg/l monochloramine and 2.4 mg/l free chlorine (van der Wende[9]). (□) Monochloramine, (▲) free chlorine.

to dichloramine and trichloramine. Therefore, the total chlorine measurement was assumed to represent the concentration of monochloramine.

III. RESULTS AND DISCUSSION

A. Monochloramine and Free Chlorine Demands of the RotoTorque System

To determine the monochloramine demand of a sterile, clean RotoTorque system, experiments were conducted using exactly the same RotoTorque system, but with no microorganisms. The sterilized system was fed an influent containing 4 mg/l of monochloramine. The monochloramine concentration in the effluent remained at 4 mg/l, indicating that the RotoTorque system had no monochloramine demand under the stated operating conditions. The results were compared with those from van der Wende[9] treating the same system with free chlorine. Figure 2 shows the free chlorine demand of the RotoTorque system. A concentration of 2.5 mg/l free chlorine dropped about 50% within 60 min because free chlorine reacted with oxidative matter of the influent.

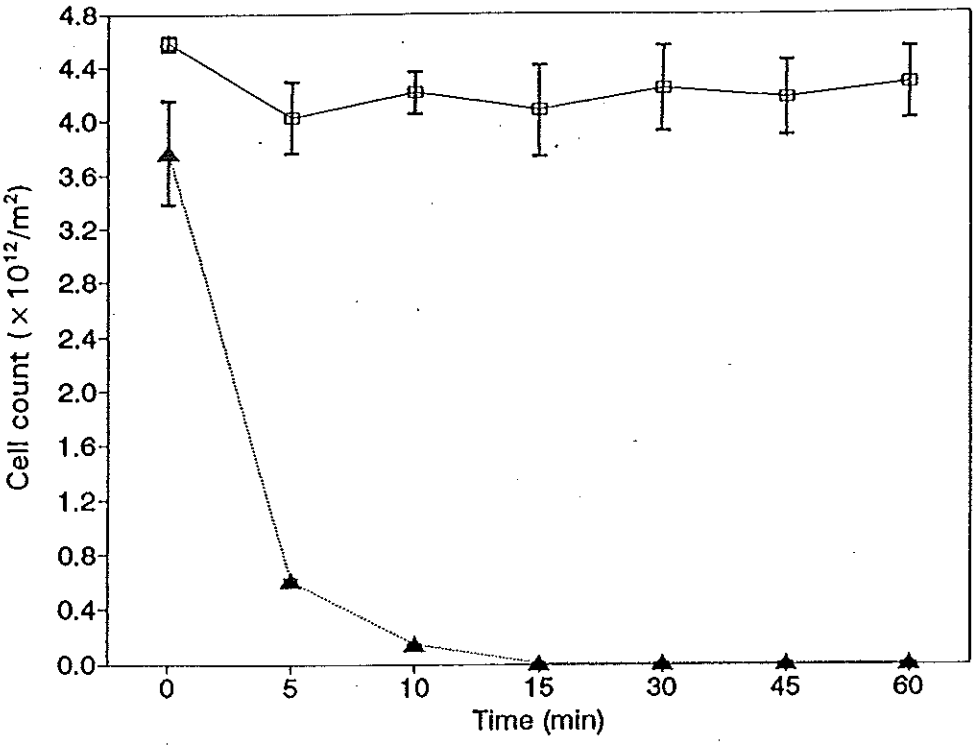

FIGURE 3. *In situ* biofilm dynamics during treatment with 4 mg/l monochloramine. (▲) Viable cell count, (□) total cell count. Time $t = 0$ corresponds to the beginning of biocide addition.

B. Disinfection of Biofilm Cells by Monochloramine and Free Chlorine

The results of *in situ* biofilm disinfection with 4 mg/l monochloramine are shown in Figure 3. The number of viable cells in the biofilm on the stainless steel surface decreased from 3.76 ($\pm$0.38) $\times$ 10^{12} cfu/m^2 to 1.68 ($\pm$0.13) $\times$ 10^{10} cfu/m^2 at 15 min, and to 2.52 ($\pm$0.24) $\times$ 10^{8} cfu/m^2 a decrease of about four orders of magnitude, at 60 min (Figure 3). Duplicate biofilm samples were taken at the first (t = 0 min) and last (t = 60 min) samplings to confirm the efficacy. There was no evidence of detachment or sloughing because the areal density of total cells (4.22 [$\pm$0.16] $\times$ 10^{12} cells/m^2) did not change during the biocide treatment. The residual monochloramine concentration in the bulk fluid was constant at 0.45 mg/l after the first sampling at 2.5 min through the end of the treatment.

The disinfection of biofilms by free chlorine is shown in Figure 4. A dose of 5.8 mg/l free chlorine reduced the areal density of viable cells in *P. aeruginosa* biofilms by one order of magnitude during the 1-h treatment. The residual free chlorine was 0.15 mg/l after the first sampling at 2.5 min. This indicates that the biofilm has higher free chlorine demand than monochloramine demand. Disinfection using a higher dose

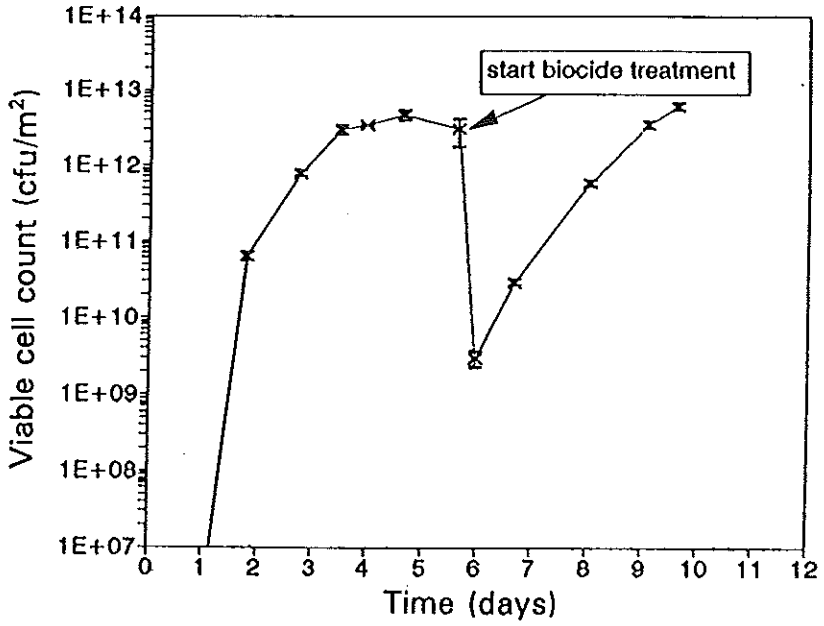

FIGURE 4. Viable cell numbers of *in situ* biofilm during treatment with a dose of 10.8 mg/l free chlorine (van der Wende).[9]

of free chlorine was also studied. A dose of 10.8 mg/l free chlorine inactivated viable cells by three orders of magnitude after the same interval. The residual free chlorine was at 0.5 mg/l after 2.5 min and slowly increased to 1.3 mg/l after 60 min. Therefore, a dose of 4 mg/l monochloramine was even more effective for biofilm inactivation than a dose of 10.8 mg/l free chlorine. van der Wende[9] also reported significant detachment and sloughing during the treatment with free chlorine, whereas no cell detachment was observed when applying monochloramine. The results of these studies indicate that monochloramine was more effective than free chlorine for inactivating biofilms of *P. aeruginosa*.

LeChevallier et al.[6] have demonstrated that biofilms in a model pipe macrosystem were successfully controlled using monochloramine levels ranging from 2 to 4 mg/l, but that free chlorine residuals from 3 to 4 mg/l were ineffective for biofilm disinfection. van der Wende et al.[11] hypothesized that the biofilm environment protects cells against the activity of chlorine by diffusional resistance and neutralization of chlorine through the reaction with biofilm and pipe wall materials. Planktonic cells find no such protection. LeChevallier et al.[3] also indicated that monochloramine and free chlorine might act differently at surfaces of biofilms of various bacteria.

C. Disinfection of Homogenized Biofilm Cells by Monochloramine and Free Chlorine

Intact biofilms on stainless steel from the RotoTorques were homogenized prior to disinfection. The disinfection of mechanically dispersed

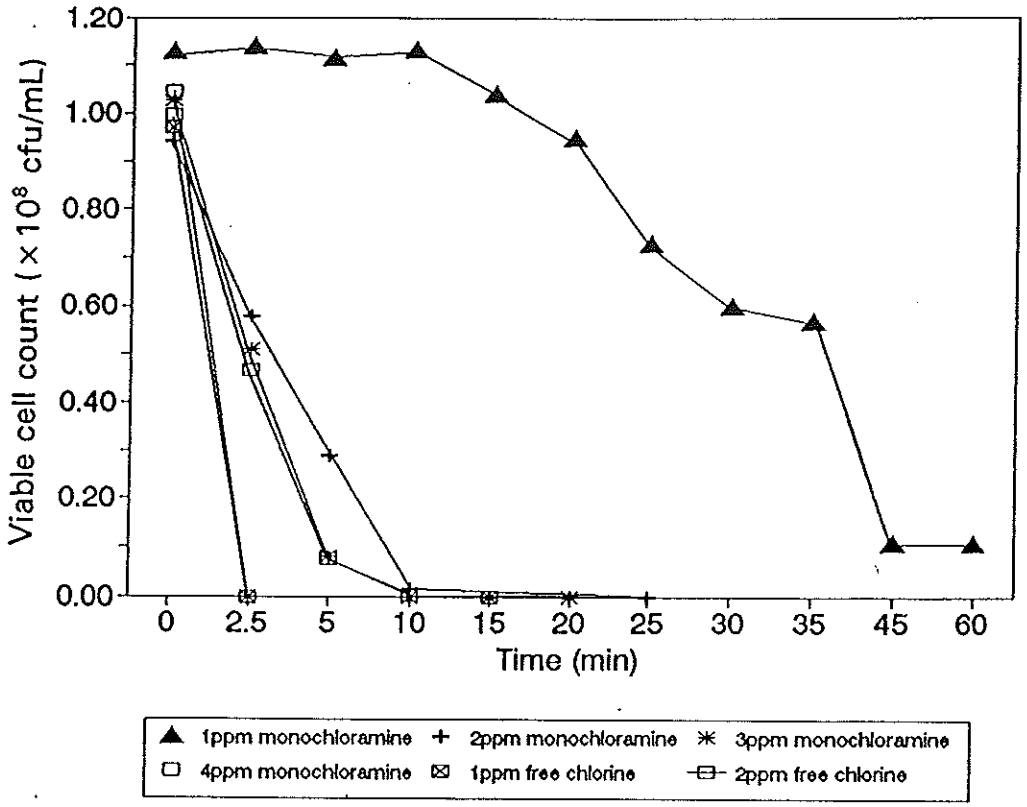

FIGURE 5. Disinfection of homogenized biofilms with monochloramine and free chlorine.

biofilm cells with either 3 or 4 mg/l monochloramine resulted in 99% killing of viable cells within the first 5 min of exposure (Figure 5). For all treatments (1, 2, 3, and 4 mg/l monochloramine), the biocide demand remained steady after 2.5 min at 0.45 mg/l. For the 2 mg/l monochloramine treatment, 99% of viable cells were killed at 15 min. Dispersed biofilm cells exposed to 1 mg/l monochloramine were not affected by the biocide within the first 15 min. However, the viability dropped moderately to 9.73% at 45 min and remained steady thereafter. These results indicate that there was a threshold level (between 1 and 2 mg/l) at which monochloramine was effective for a rapid inactivation of suspended biofilm cells.

Disinfection of suspended biofilm cells with 1 and 2 mg/l free chlorine (residuals 0.8 and 1.85 mg/l, respectively) resulted in 100% killing of viable cells within the first 2 min. Thus, free chlorine inactivated suspended biofilm cells more rapidly than monochloramine.

The commonly used CT coefficient (defined as biocide residual concentration [mg/l] multiplied by treatment time [min] to achieve 99% inactivation) enables comparison of various biocide activities. The disinfection data presented above were used to calculate CT values for monochloramine and free chlorine (Table 1). The mean monochloramine

TABLE 1. Comparative Efficiency of Monochloramine and Free Chlorine for 99% Inactivation of Intact Biofilms and Homogenized Biofilms

	CT value for monochloramine[a]	CT value for free chlorine
Intact biofilms	6.7 mg·min/l	69 mg·min/l[b]
Homogenized biofilms	28 mg·min/l	<1.6 mg·min/l

[a] CT is defined as biocide residual (mg/l) multiplied by treatment time (min) for achieving 99% inactivation of viable bacteria.
[b] Value was adopted from van der Wende.[9]

CT value for homogenized biofilm cells was 28 mg·min/l, whereas intact biofilms had a CT value of 6.75 mg·min/l. Nearly 100% inactivation of homogenized biofilm cells was achieved with a free chlorine residual of 0.8 or 1.85 mg/l after 2 min. The CT value for free chlorine was below 1.6 mg·min/l. Therefore, free chlorine is more effective than monochloramine for disinfecting homogenized (mechanically dispersed) biofilm cells. LeChevallier et al.[6] observed the same phenomena: monochloramine CT values for unattached bacteria were higher than for biofilms, and free chlorine was less effective for biofilm control.

D. Regrowth after Treatments with Monochloramine and Free Chlorine

The growth of biofilms reached steady state at 144 h, as revealed by steady viable cell and total cell counts in the bulk fluid (Figure 6). Viable and total cell counts were very consistent and indicated that about 95% of total cells were viable. After 1 h of treatment with 4 mg/l monochloramine, resulting in inactivation of 99.99% of viable cells, the regrowth of survived biofilm cells to pretreatment level required only 48 h (Figure 6, curve after 216 h). Based on the steady state biofilm-specific cell growth rate of 0.071 h^{-1}, calculated by Chen et al.,[5] and ignoring cell detachment during regrowth, predicted biofilm recovery would take about 141 h. If detachment occurs, this period would be longer. Thus, cell growth rates were considerably higher during biofilm recovery than at steady state.

The regrowth of biofilms after free chlorine treatment was similar. It took 48 h to reach the pretreatment level (Figure 4). Both regrowth studies showed that biofilm recovery to steady state was about three times faster than initial biofilm development. This was probably caused by structural change of biofilms after treatment, which facilitated nutrient transport through the film, resulting in a higher nutrient level within the biofilm and a higher specific growth rate for the survived cells. These results support the view that biofilms represent a protected

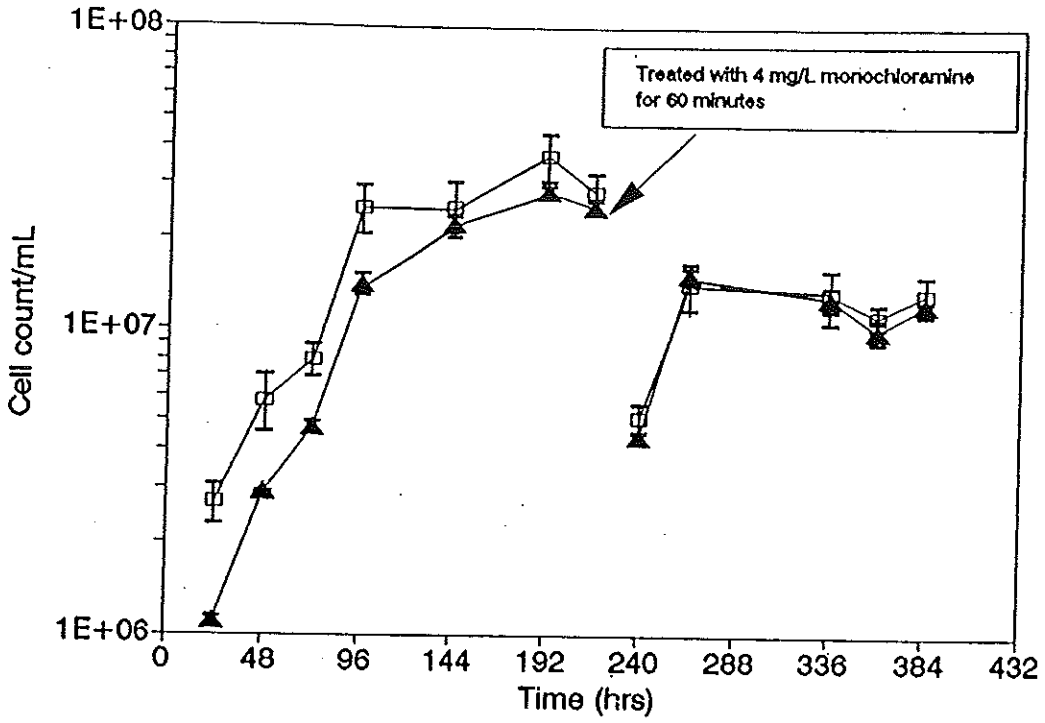

FIGURE 6. Changes of cell counts in the bulk fluid during growth of biofilms and regrowth after biocide treatment with 4 mg/l monochloramine, (▲) viable cell count, (□) total cell count.

niche where bacteria can survive stressful environmental changes. These results are consistent with field experiences which suggest that uncontrolled biofilm organisms can cause severe biofouling problems in industrial systems.

IV. CONCLUSION

1. Monochloramine was not reactive toward system components, whereas free chlorine showed a high reactivity with the medium.
2. Monochloramine was more effective than free chlorine for inactivation of *P. aeruginosa* biofilms. There was no significant biofilm detachment during monochloramine treatment, whereas free chlorine caused sloughing.
3. Mechanically dispersed biofilms were disinfected more rapidly by free chlorine than by monochloramine.

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