Journal of Microbiological Methods, 17 (1993) 167–180

MIMET 00557

A direct viable count method for the enumeration of attached bacteria and assessment of biofilm disinfection

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(Received 17 July 1992; revision received 26 October 1992; accepted 27 October 1992)

Summary

This report describes the adaptation of an in situ direct viable count (in situ DVC) method in biofilm disinfection studies. The results obtained with this technique were compared to two other enumeration methods, the plate count (PC) and conventional direct viable count (c-DVC). An environmental isolate (*Klebsiella pneumoniae* Kp1) was used to form biofilms on stainless steel coupons in a stirred batch reactor. The in situ DVC method was applied to directly assess the viability of bacteria in biofilms without disturbing the integrity of the interfacial community. As additional advantages, the results were observed after 4 h instead of the 24 h incubation time required for colony formation and total cell numbers that remained on the substratum were enumerated. Chlorine and monochloramine were used to determine the susceptibilities of attached and planktonic bacteria to disinfection treatment using this novel analytical approach. The planktonic cells in the reactor showed no significant change in susceptibility to disinfectants during the period of biofilm formation. In addition, the attached cells did not reveal any more resistance to disinfection than planktonic cells. The disinfection studies of young biofilms indicated that 0.25 mg/l free chlorine (at pH 7.2) and 1 mg/l monochloramine (at pH 9.0) have comparable disinfection efficiencies at 25°C. Although being a weaker disinfectant, monochloramine was more effective in removing attached bacteria from the substratum than free chlorine. The in situ DVC method always showed at least one log higher viable cell densities than the PC method, suggesting that the in situ DVC method is more efficient in the enumeration of biofilm bacteria. The results also indicated that the in situ DVC method can provide more accurate information regarding the cell numbers and viability of bacteria within biofilms following disinfection.

Key words: In situ; Direct viable count; Disinfection; Chlorine; Monochloramine; Viability

Introduction

The activities of surface-associated microorganisms, frequently called biofilms, were first studied in soil [1], and then later by microbiologists who submerged slides in

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aquatic environments [2–4]. Since then, progress has been made in understanding the ecology and physiology of adherent microorganisms [5]. In addition, attachment of bacteria to surfaces has important practical implications since biofilms may result in a significant increase in disinfection [6–10] and heavy metal resistance [11]. Attachment to surfaces also changes the physiology of microorganisms in a variety of ways as reviewed by van Loosdrecht et al. [12], including substrate uptake [10,13,14], and one report [15] indicated that sand-associated bacteria can adapt more quickly to, and have a greater degradative activity for, nitrilotriacetate (NTA).

Difficulties associated with studying surface-associated cells have hindered work characterizing the activities of adherent bacteria, compared to the progress made with free-living bacteria. For instance, it is necessary to remove the cells from the substratum prior to the enumeration of viable bacteria attached to surfaces. Differences in physiological activity between attached and free-living bacteria [16] may explain the diverse susceptibilities and growth requirements after cells being removed from the substratum. In addition, enumeration of viable bacteria by plate count (PC) methods may not detect all viable cells, particularly those injured by environmental stress [17–19]. Also, detached bacteria that are aggregated may be problematic in the plate counting technique.

Chlorine is the most widely used disinfectant for the chemical treatment of water. Monochloramine (NH_2Cl) penetrates biofilms and inactivates bacteria more effectively than hypochlorous acid (HOCl), hypochlorite (OCl^-) and chlorine dioxide (ClO_2) [20]. Therefore, we chose to use free chlorine (hypochlorous acid) and monochloramine as disinfectants in this study. Hypochlorous acid tends to undergo partial dissociation to produce a hypochlorite and a hydrogen ion, and the equilibrium is maintained mainly by the hydrogen ion concentration. Thus, pH is believed to have a strong influence on the antimicrobial activity of chlorine in solution [21]. In water between pH 6.5 and 8.5, the reaction is incomplete and both species (HOCl and OCl^-) are present in varying ratios. Because the bactericidal activity of HOCl is believed to be far stronger than OCl^- [22], an increase in pH (lower $[H^+]$) substantially decreases the biocidal activity of chlorine with the formation of higher OCl^- concentrations, while lower pH values increase the biocidal activity. Thus, we selected two pH conditions (6.0 and 7.2) in this study to determine if higher activity for biofilms disinfection would be found at a lower pH.

This study was initiated to seek an in situ method to enumerate quickly viable attached bacteria and assess the effect of bactericides on surface-associated bacterial cells without disrupting the integrity of biofilms. This was based on our previous studies [23], which applied image analysis and the conventional direct viable count (c-DVC) method to obtain a rapid enumeration of viable planktonic bacteria, as well as a report by Lytle et al. [24] who used a DVC method to examine bacteria in a gravel filter. We adapted the c-DVC method [25] to examine the susceptibilities of attached bacteria after disinfection. This novel approach offers a non-destructive means to study the biofilm community in situ. An environmental isolate (*Klebsiella pneumoniae* Kp1) was used to form biofilms on stainless steel coupons, which were limited to monolayers in order to be compatible with enumeration using epifluorescence microscopy. The disinfectants were used as tools to evaluate the efficiencies of the three enumeration methods. The in situ DVC method showed higher viable cell

counts than the PC and c-DVC methods without the bias caused by aggregated bacteria. As an additional advantage, it took 4 h to complete the enumeration process in contrast to the 24 h period required for plate counting.

Materials and Methods

Biofilm apparatus

The vessel used to grow bacterial biofilms was a wide-mouth pint Mason jar (Kerr, 450 ml), which was redesigned for this application. This device is described elsewhere [26]. The jar, containing stainless steel coupons, inoculated culture and a magnetic stir bar, was incubated on a thermally insulated magnetic stirrer with a constant speed (285 rpm).

Bacterial strains and growth conditions

Klebsiella pneumonia Kp1, isolated from drinking water, was obtained from Dr. D. Smith, South Central Connecticut Water Authority, New Haven, CT. The culture was stored in a solution that consisted of 2% peptone (Difco) and 20% glycerol (Sigma Chemical Company) in reagent grade water at −70°C.

Frozen cultures were inoculated into 1/10 tryticase soy broth (TSB) (Difco) and incubated at 35°C for 24 h. Cultures were then inoculated (1:100) into sterile medium and incubated for another 24 h, followed by dilution in the biofilm apparatus with sterile 1/10 TSB at a final cell density of ca. 10^2 CFU/ml. The diluted cultures were stirred at room temperature (25°C) for various time intervals to allow accumulation of cells on coupon surfaces. Biofilms were formed on coupons (12 × 76 mm) of 316 stainless steel.

Procedures for coupon cleaning

A cleaning method was used that yielded coupons with the same surface characteristics after being reused. Coupons were dipped into acetone to remove grease and allowed to air dry, then transferred to fresh RBS 35 (Pierce) working solution and heated to 50°C for 5 min and sonicated (Sonogen Automatic Cleaner, Branson Instruments Inc.) for 5 min. The coupons were then rinsed with sterile reagent grade water (Milli-Q, Millipore Corp.) until foaming ceased (approximately 5 times), sonicated again for 5 min then rinsed three more times. Coupons were transferred to a vial containing 30 ml of reagent grade water, sealed and autoclaved for 20 min then stored at room temperature in the same sealed vial until use.

Preparation of disinfectants

The disinfectants used were chlorine (sodium hypochlorite) and monochloramine. The chlorine solution was prepared as described earlier [27] using sodium hypochlorite, and the monochloramine solution was made according to LeChevallier et al. [20]. The bacterial cells were exposed to disinfectants in chlorine-demand-free sterile phosphate buffered water (PBW) without $MgCl_2 \cdot 6H_2O$ [28] at different pH values for 10 min at 25°C.

Disinfection

The planktonic cells within the reactor were diluted and transferred to filtered PBW (final concentration 10^5–10^6 CFU/ml) in an acid-washed bottle, then treated with disinfectants (0.25 mg/l hypochlorous acid and 1 mg/l monochloramine) at 25°C. The dilution process reduced the chlorine demand caused by medium carryover. Samples were removed after 5 and 10 min and the disinfectant was neutralized with sodium thiosulfate (final concentration 0.01 %). Surviving bacteria were enumerated by the PC and DVC methods [29].

Bacteria attached on the metal coupons were rinsed by transferring coupons to another biofilm apparatus filled with sterile distilled water to removed loosely attached cells. Biofilms were disinfected in chlorine-demand-free sterile PBW at 25°C in an acid-washed bottle mixed with a magnetic stir bar. The procedures for disinfection were similar to those described above. Biofilm bacteria on coupons were either removed from the coupons by scraping with a sterile rubber policeman or processed directly with in situ DVC incubation (described below) for enumeration.

The disinfection exposure was performed at different pH values in PBW (6.0 and 7.2 for chlorine and 9.0 for monochloramine). Tests were also done to determine the effect of pH on cell viability in PBW before disinfectants were added. The Hach DPD free chlorine and total chlorine test kit (Hach Co. Loveland, CO.) was used to measure the concentrations of residual chlorine before and after disinfection.

Optimization of the direct viable count method

The conventional direct viable count (c-DVC) [25] method was optimized as described by Singh et al. [23]. The optimal concentrations of nalidixic acid (Sigma) used for *Klebsiella pneumoniae* strain Kp1 were 15 μg/ml for untreated cells and 12 μg/ml for disinfectant-treated cells. The planktonic cells in suspension and the attached cells on the coupons did not show any difference in the concentrations of nalidixic acid required for optimal elongation without cell division.

The removal of attached cells from coupons with a sterile rubber policeman had a 99.9% efficiency based on microscopic examination of the unscraped and scraped coupons.

Determination of viable bacteria

The PC count method was performed by a modified drop plate method [30] using five 10 μl drops per dilution with tryptone lactose yeast extract agar medium (TLY) as described by McFeters et al. [18]. Colonies were counted after 24 h incubation at 35°C.

After disinfection treatment, coupons were placed flat in a Petri dish containing the medium used for c-DVC incubation, which prevented further sloughing of attached cells during the 4 h incubation. The c-DVC method was performed using phosphate buffered saline (PBS) containing 0.3% casamino acids (Difco), 0.03% yeast extract (Difco) and different concentrations of nalidixic acid to incubate attached bacteria scraped from the coupons and planktonic cells at 35°C for 4 h. Samples were then withdrawn, fixed with formalin (5%, final concentration) and stained with acridine orange according to the method described by Hobbie et al. [31]. Before staining, the bacteria in suspension were filtered onto 25-mm diameter (0.2 μm pore size), black

polycarbonate membranes (Nuclepore).

The in situ DVC method was similar to the c-DVC method. Coupons with attached cells were transferred into medium without scraping. After incubation, coupons were withdrawn and fixed with formalin followed by immersing in 0.02% acridine orange solution for 2 min. The coupons were then allowed to air-dry, placed on glass slides and examined using epifluorescence microscopy (Leitz Ortholux II). Both total and elongated cells in 10 microscopic fields were counted. If the total cell number was less than 400, more than 10 fields were examined.

Statistical analysis

Statistical analyses were performed on all data from three replicates using mathematical functions within SigmaPlot™ (Version 4.1 by Jandel Scientific) and one-way analysis of variances (ANOVA) with InStat™ (Version 1.1 by GraphPAD) computer software. The ANOVA were done on log data, and zero values were not encountered.

Results

Enumeration of surface-associated bacteria with disinfection

To enumerate viable biofilm bacteria, the in situ DVC assessment was used and compared with approaches where surface-associated cells were removed from the substratum followed by PC and c-DVC enumeration. Different concentrations of disinfectants (0.25 mg/l for free chlorine and 1 mg/l for monochloramine) were used to disinfect Kp1 cells grown on stainless steel coupons. Decreases in viable cell numbers were measured after 5 and 10 min exposure to disinfectants in order to describe the patterns of disinfection. Both PC and c-DVC methods were used to enumerate surviving cells after scraping attached bacteria from coupon surfaces. Results obtained with these two approaches were used to evaluate the efficiency of the non-destructive in situ DVC method. The viable cell densities are expressed as log cfu per mm^2 (on coupons) $\pm$ S.E. for the PC method, and log cells per mm $\pm$ S.E. for the c-DVC and in situ DVC methods. Comparisons of disinfection efficiency were determined by log n/n_0 values (the log ratio of surviving bacterial numbers to initial bacterial numbers) after biocide exposure for 5 and 10 min.

Fig. 1 shows that the viable cell numbers determined by three different methods at time 0 were similar. The counts were 4.22 $\pm$ 0.08 (in situ DVC), 4.03 $\pm$ 0.02 (c-DVC) and 3.98 $\pm$ 0.02 (PC) logs respectively. After 10 min of chlorine disinfection, the viability of attached cells on coupons decreased by 2.4 logs when determined by the in situ DVC assessment. A slightly larger reduction (2.6 logs) was observed when the attached cells were scraped off then enumerated with the c-DVC method. However, the conventional procedure of scraping followed by PC enumeration showed a much larger (3.3 logs) decline in bacterial viability within the disinfected biofilms. These data reveal a large portion of the biofilm bacteria that were not enumerated by the PC method since there was a 1.2 log discrepancy (0.65 $\pm$ 0.56 vs. 1.88 $\pm$ 0.11) in bacterial enumeration between the PC and in situ DVC methods after 10 min of chlorine disinfection, whereas the difference at time 0 (3.98 vs. 4.22) was not significant ($p > 0.05$). Furthermore, comparing the viable counts determined with the PC and

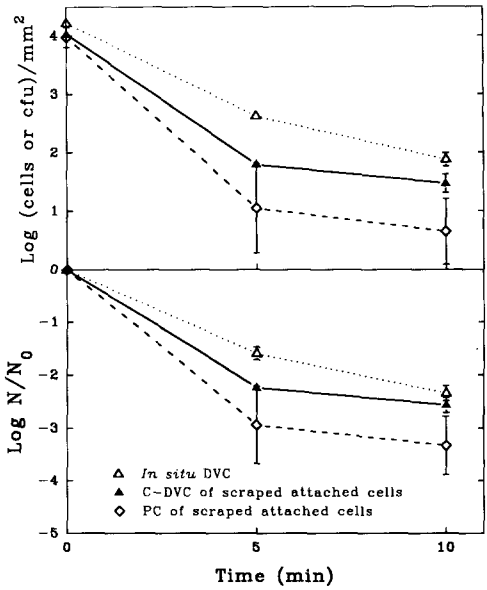

Fig. 1. Effect of chlorine treatment (0.25 mg/l) on *Klebsiella pneumoniae* Kp1 biofilms at pH 7.2. Surviving bacteria were enumerated by three different methods (n = 3, bars = SE).

c-DVC methods after removing attached bacteria by scraping, there was still a 0.82 log difference (0.65 ± 0.56 vs. 1.47 ± 0.16) which implied scraping was not the sole factor influencing the enumeration efficiencies. However, the differences between these three enumeration methods are statistically significant ($p < 0.05$).

The results of a similar experiment where bacterial biofilms were treated with monochloramine are shown in Fig. 2. The initial viable cell densities on the coupons were 4.16 ± 0.10 (in situ DVC), 3.80 ± 0.01 (c-DVC) and 3.51 ± 0.15 (PC) logs respectively. Following 10 min of disinfection, a 1.8-log decrease was observed by the in situ DVC assessment. With c-DVC and PC methods, lower counts that were similar (1.14 ± 0.08 and 1.13 ± 0.48) were observed following enumeration of attached cells after scraping. Nevertheless, statistical analyses showed that these three methods used for enumeration following monochloramine treatment gave significantly different results after 10 min of disinfectant exposure ($p < 0.05$).

Utilizing the in situ DVC method, we were able to enumerate the remaining surface-associated cells following disinfection. The data (Fig. 3) indicate that monochloramine removed approximately three times more attached bacteria from the substratum than free chlorine.

Enumeration of planktonic cells with disinfection

The PC and c-DVC methods were also used to assess the viability of planktonic bacteria following disinfection. Prior to incubation, the log counts/ml of planktonic cells enumerated with c-DVC and PC methods were 5.54 ± 0.06 and 5.08 ± 0.12 respectively, and they were inactivated 2.29 ± 0.17 and 3.09 ± 0.24 logs after 10 min exposure to free chlorine (Fig. 4). During the period of biofilm formation in the

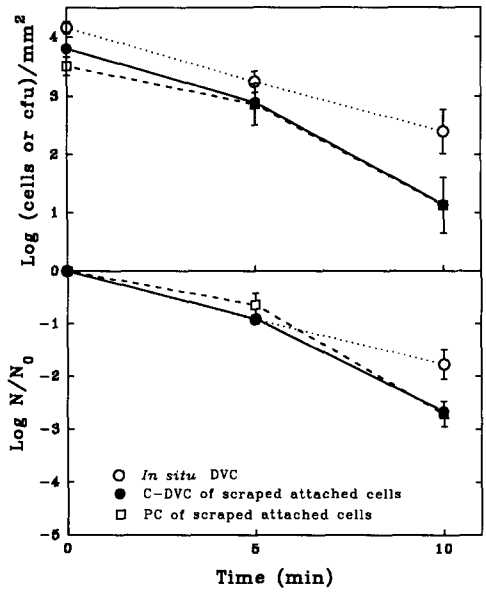

Fig. 2. Effect of monochloramine treatment (1 mg/l) on *Klebsiella pneumoniae* Kp1 biofilms at pH 9.0. Surviving bacteria were enumerated by three different methods (n = 3, bars = SE).

reactor, the culture medium contained planktonic plus aggregated cells detached from the coupons, which were observed microscopically. Following 43 h incubation

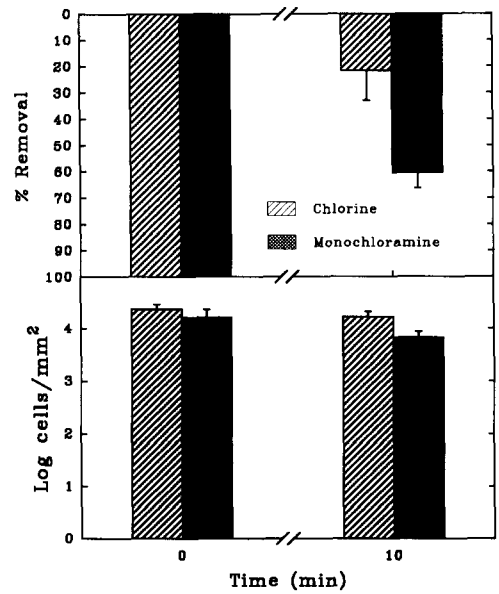

Fig. 3. Percent removal of attached *Klebsiella pneumoniae* Kp1 cells from substratum after 10 min treatment with disinfectants. The total cell numbers are displayed on the lower panel (n = 3, bars = SE).

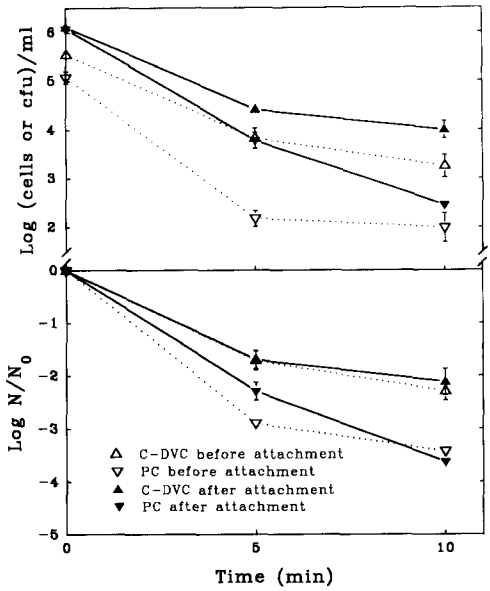

Fig. 4. Enumeration of planktonic *Klebsiella pneumoniae* Kp1 before and after attachment when treated with chlorine (0.25 mg/l) at pH 7.2 (n = 3, bars = SE).

in the biofilm apparatus, the log counts/ml of planktonic cells were 6.11 ± 0.11 and 6.08 ± 0.04, with inactivation of 2.11 ± 0.24 and 3.63 ± 0.05 logs when enumerated with c-DVC and PC methods under the same conditions. As expected, the viable cell numbers determined by the c-DVC method were significantly higher than those by the PC method ($p<0.05$), but not by the three-orders of magnitude as previously reported [25]. This population did not show any statistical difference in susceptibility to chlorine treatment ($p>0.05$) when enumerated either by the c-DVC or PC methods.

For the monochloramine treatments, the log counts/ml of planktonic cells were 5.47 ± 0.04 (c-DVC) and 5.12 ± 0.05 (PC) before attachment (Fig. 5). Following 10 min of exposure to 1 mg/l monochloramine at pH 9.0, planktonic bacteria were inactivated 1.74 ± 0.16 (c-DVC) and 2.84 ± 0.41 logs (PC). After biofilm attachment, the total numbers (log counts/ml) were 5.97 ± 0.01 (c-DVC) and 6.05 logs (PC) respectively. The disinfectant reduced the viable cell numbers by 2.40 ± 0.11 (c-DVC) and 3.21 ± 0.22 logs (PC). Statistical analyses indicated that planktonic cells became slightly more susceptible to monochloramine disinfection during the process of biofilm attachment when enumerated by the c-DVC method ($p<0.05$). However, that difference was not apparent when the cells were enumerated by the PC method ($p>0.05$).

Effect of pH on chlorine disinfection

The pH of PBW (see Materials and Methods) was varied to study activity of chlorine disinfection under different conditions. The attached and planktonic cells were examined using different procedures (Table 1). Most of the results showed that

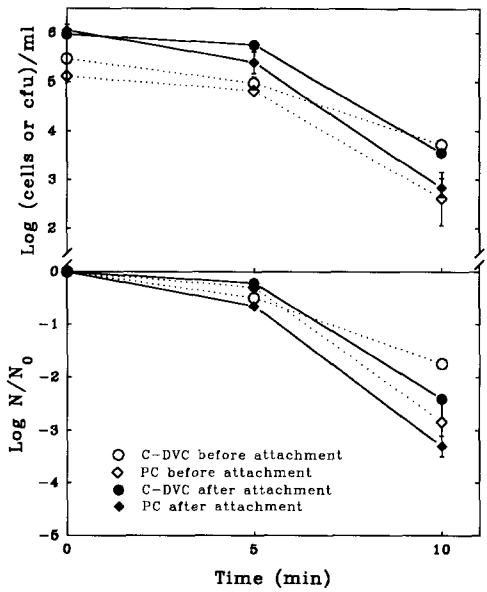

Fig. 5. Enumeration of planktonic *Klebsiella pneumoniae* Kp1 before and after attachment when treated with monochloramine (1 mg/l) at pH 9.0 (n = 3, bars = SE).

at 25°C, the bactericidal activity of chlorine under pH 7.2 and pH 6.0 was comparable ($p > 0.05$). The exception was from procedure #2 which indicated higher bactericidal activity at pH 7.2 when attached cells were enumerated with the PC method.

Tests were also done to determine the effect of pH on detachment and bacterial viability in the absence of disinfectant (data not shown). No significant changes were observed ($p > 0.05$) in total cell numbers and viable cell numbers which were determined by the in situ DVC method.

TABLE 1

One-way analysis of variance (ANOVA) of the effect of pH on chlorine disinfection (0.25 mg/l for 10 min) of *Klebsiella pneumoniae* Kpl. ($n = 3$, ± SE)

Procedure[a]	Log n/n_0		ANOVA
	pH 6.0	pH 7.2	
1	−2.10 ± 0.33	−2.56 ± 0.14	P = 0.2687
2	−2.04 ± 0.36	−3.80 ± 0.52	P = 0.0497
3	−2.30 ± 0.44	−2.29 ± 0.17	$P > 0.8$[b]
4	−3.79 ± 0.51	−3.09 ± 0.24	P = 0.2821

[a]Procedure 1: DVC method with scraped attached cells. Procedure 2: PC method with scraped attached cells. Procedure 3: DVC method with planktonic cells before attachment. Procedure 4: PC method with planktonic cells before attachment.

[b]The exact p-value was not given by the InStat program.

TABLE 2

Comparison of log n/n_0 values between planktonic and attached cells after 10 min of disinfection obtained with two DVC methods ($n=3$, $\pm$ SE)

		Log n/n_0	
		Chlorine (0.25 mg/l)	Monochloramine (1 mg/l)
Planktonic cells	c-DVC[a] (before attachment)	-2.29 ± 0.17	-1.74 ± 0.08
	(after attachment)	-2.11 ± 0.24	-2.41 ± 0.10
Attached cells	in situ DVC	-2.40 ± 0.12	-1.66 ± 0.13
	c-DVC	-2.56 ± 0.14	-2.34 ± 0.15

[a]Conventional direct viable count.

Discussion

Without environmental stresses such as disinfectants, the enumeration results of bacterial biofilms obtained using the three methods that were studied (in situ DVC, c-DVC and PC) were comparable. However, differences between the methods became apparent after the introduction of disinfectant, as illustrated in Figs. 1 and 2. The in situ DVC enumeration up to the 10 min exposure showed higher bacterial densities than those determined with either the c-DVC or the PC methods after removal of attached bacteria from coupon surfaces. Furthermore, the large error bars (Fig. 1) obtained with the PC method after chlorine treatment implied aggregation of scraped bacteria may have caused greater fluctuations in colony forming units. That conclusion was supported by microscopic observations. The significant difference in viable cell numbers determined by the c-DVC and the PC methods after the removal of attached bacteria from the coupons was only observed following chlorine and not monochloramine treatment. This may be explained by the suggestion that monochloramine is more effective in dispersing aggregated cells [32].

Our findings with the DVC analytical approach might be used to assess the physiological status of the bacteria under conditions of biofilm formation. An examination of Table 2 as well as a comparison of the lower panels in Figs. 1 and 4 indicate that the attachment of *K. pneumoniae* to stainless steel coupons does not markedly alter their susceptibility to free chlorine when either of the DVC enumeration methods were used. This suggests that bacterial attachment in early biofilm formation did not result in a significant change in cellular physiology relating to chlorine susceptibility although a slight increase in susceptibility to monochloramine was seen (Table 2, lower panels of Figs. 2 and 5). These results are in contrast to the findings of others showing that attached bacteria are more resistant to disinfection [6–9], but those workers either did not compare phenotypic responses before and after attachment as we have done, or used different experimental circumstances (high level of nutrients, longer incubation time, unencapsulated bacterium and glass microscopic slides as substratum) as reported by LeChevallier et al. [9]. It should be noted, however, that biofilm formation was limited to bacterial monolayers in this study and it is likely that more mature biofilm communities respond differently. Such

altered properties of surface-associated bacteria have been attributed to both changes in cellular physiology with biofilm formation [16,33] and protection by extracellular polymers [9].

Results of similar experiments-using planktonic bacteria provided a perspective for describing the kinetics of disinfection in biofilms. During the biofilm colonization process in the reactor, surface-associated bacteria detached from the substratum and became a part of the planktonic cell population which were observed microscopically as cell aggregates. Therefore, these formerly surface-associated bacteria, which are more resistant to disinfection [6,8,9], could have influenced the susceptibility of the planktonic populations in the reactor to disinfection.

There were no obvious changes in the susceptibility of planktonic bacteria to free chlorine following biofilm formation (Table 2 and lower panel of Fig. 4). However, there was a slight increase in the susceptibility of the planktonic cells to monochloramine with biofilm formation (Table 2 and lower panel of Fig. 5). These observations might also be indicative of a lack of a significant physiological change with biofilm initiation by *K. pneumoniae*, although it is important to note that the experimental design used here with cellular monolayers may have underestimated any physiological responses in the bacteria as they attached to the substratum.

From the shapes of the inactivation curves of chlorine disinfection over time, we repeatedly found that disinfection was more rapid for the first 5 min (Figs. 1 and 4). With monochloramine, it appeared that there was less rapid disinfection for the first 5 min followed by a period of more rapid disinfection (Figs. 2 and 5). These different reaction kinetics might result from distinct reaction mechanisms with the two disinfectants used, as suggested by other investigators [34–36].

The disinfecting efficiency of chlorine is considered to be directly related to the concentration of undissociated hypochlorous acid, which is greater at pH 6.0 (97.18%) than at pH 7.2 (68.52%) at 25°C [21]. Aqueous solutions of chlorine exhibit rapid bactericidal action by the formation of hypochlorous acid [21]. Hypochlorous acid tends to undergo a partial dissociation to produce hydrogen and hypochlorite (OCl^-) ions in water at pH values between 6.5 and 8.5. HOCl is far stronger in bactericidal action than OCl^-, thus, the disinfecting efficiency of chlorine theoretically decreases with increasing pH values [32]. However, results in Table 1 indicated that pH was of negligible effect on the bactericidal activity of chlorine disinfection with the possible exception of one procedure (no. 2). In addition to pH, numerous other environmental factors, alone or in combination, determine the antimicrobial action of chlorine compounds [32].

The relationship between disinfectant concentration (C) and contact time (t) might appear simple in bacterial disinfection. Nevertheless, the empirical $C^n \times t$ equation [37] does not adequately predict the exponential rate of disinfection. Higher concentrations are not always more effective. Differences in antimicrobial resistance between species, levels of aggregation, physiological status, and prior growth conditions are all factors that affect the outcome. In addition, the properties of biofilms vary with environmental factors (e.g. population distribution in film, nutrient loading rate, shear stress, etc.) and physiological properties of the bacteria. Therefore, the conclusions of this study apply only to the model system used.

The DVC method [25] distinguished viable cells by their ability to elongate while

cell replication is inhibited by nalidixic acid. Although the in situ DVC method has limits in assessing the viability of bacterial biofilms due to certain constrains imposed by optical microscopy, this approach provided rapid and accurate enumeration of viable cells without disturbing the biofilm integrity. Accordingly, this technique is valuable in studying the effect of disinfectants on attached bacteria in situ. In addition, this technique can reveal not only viable cell numbers within thin biofilms but also the total number of bacteria remaining on the substratum throughout the disinfection period. The resulting data may be applied to studies comparing the physiological status and disinfection kinetics of attached and planktonic cells. However, bacteria in thicker mature biofilms may have dramatically different responses when compared with free-living cells.

Our previous studies [23,29] indicated that image analysis can be used for rapid enumeration and is able to determine cell viability as well as injury in planktonic suspensions of bacteria. With the aid of image analysis, the in situ DVC method would provide further quantitative information that may be used in determining the viability of bacteria within intact biofilms. Recently, progress has been reported on the use of scanning laser confocal microscopy, especially with the software developed for 2- and 3-dimension image processing [38]. It, therefore, might be possible to utilize the in situ DVC method in mature biofilms with the assistance of scanning laser confocal microscopy.

Acknowledgements

This work was supported by grants from Industry/University Cooperative Research Center for Biosurfaces (IUCB) and the National Aeronautics and Space Administration (contact NAG 9-241).

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