

# Biofilm penetration and disinfection efficacy of alkaline hypochlorite and chlorosulfamates

P.S. Stewart<sup>1,2</sup>, J. Rayner<sup>1</sup>, F. Roe<sup>1</sup> and W.M. Rees<sup>3</sup>

<sup>1</sup>Center for Biofilm Engineering and <sup>2</sup>Department of Chemical Engineering, Montana State University – Bozeman, Bozeman, MT and <sup>3</sup>S.C. Johnson & Son, Inc., Racine, WI, USA

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**Aims:** The purpose of this study was to compare the efficacy, in terms of bacterial biofilm penetration and killing, of alkaline hypochlorite (pH 11) and chlorosulfamate (pH 5.5) formulations.

**Methods and Results:** Two species biofilms of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were grown by flowing a dilute medium over inclined stainless steel slides for 6 d. Microelectrode technology was used to measure concentration profiles of active chlorine species within the biofilms in response to treatment at a concentration of 1000 mg total chlorine l<sup>-1</sup>. Chlorosulfamate formulations penetrated biofilms faster than did hypochlorite. The mean penetration time into ~1 mm-thick biofilms for chlorosulfamate (6 min) was only one-eighth as long as for the same concentration of hypochlorite (48 min). Chloride ion penetrated biofilms rapidly (5 min) with an effective diffusion coefficient in the biofilm that was close to the value for chloride in water. Biofilm bacteria were highly resistant to killing by both antimicrobial agents. Biofilms challenged with 1000 mg l<sup>-1</sup> alkaline hypochlorite or chlorosulfamate for 1 h experienced 0.85 and 1.3 log reductions in viable cell numbers, respectively. Similar treatment reduced viable numbers of planktonic bacteria to non-detectable levels (log reduction greater than 6) within 60 s. Aged planktonic and resuspended laboratory biofilm bacteria were just as susceptible to hypochlorite as fresh planktonic cells.

**Conclusions:** Chlorosulfamate transport into biofilm was not retarded whereas hypochlorite transport clearly was retarded. Superior penetration by chlorosulfamate was hypothesized to be due to its lower capacity for reaction with constituents of the biofilm. Poor biofilm killing despite direct measurement of effective physical penetration of the antimicrobial agent into the biofilm demonstrates that bacteria in the biofilm are protected by some mechanism other than simple physical shielding by the biofilm matrix.

**Significance and Impact of the Study:** This study lends support to the theory that the penetration of antimicrobial agents into microbial biofilms is controlled by the reactivity of the antimicrobial agent with biofilm components. The finding that chlorine-based biocides can penetrate, but fail to kill, bacteria in biofilms should motivate the search for other mechanisms of protection from killing by antimicrobial agents in biofilms.

## INTRODUCTION

Micro-organisms that attach to a surface and form a biofilm are usually found to be highly protected from killing by

Correspondence to: P.S. Stewart, Center for Biofilm Engineering, Montana State University – Bozeman, Bozeman, MT 59717-3980, USA (e-mail: phil\_s@erc.montana.edu).

antimicrobial agents (Stewart *et al.* 2000a). Even when challenged with a seemingly brute force agent such as free chlorine, micro-organisms in biofilms survive treatments that rapidly eradicate freely suspended cells (Ronner and Wong 1993; Oie *et al.* 1996; Ntsama-Essomba *et al.* 1997; Stewart *et al.* 1998). Chlorine is a benchmark biocide that is used to control biofilm contamination and fouling in diverse

application areas, including drinking water, food processing, hospital sanitization, industrial water systems and household cleaning. The basis for biofilm resistance to chlorine remains incompletely understood, but evidence that chlorine penetration into biofilms can be profoundly retarded has been presented recently (de Beer *et al.* 1994; Chen and Stewart 1996; Xu *et al.* 1996).

The poor penetration of hypochlorite/hypochlorous acid into biofilm is due to reactive neutralization of the active chlorine in the outermost regions of the biofilm matrix (Chen and Stewart 1996; Xu *et al.* 1996). In other words, the chlorine is consumed by reaction with organic matter in the surface layers of the biofilm faster than it can diffuse into the biofilm interior. The reaction–diffusion theory that explains this phenomenon predicts that a less reactive biocide should penetrate biofilm more effectively (Stewart and Raquepas 1995; Stewart 1997). This raises the possibility that stabilized forms of hypochlorite (unipositive chlorine), which may react at a slower rate with biofilm organic constituents than the highly reactive hypochlorite species, might penetrate more readily into a biofilm. Examples of halogen-stabilizing agents include ammonia, amines and hydantoin. A weakly reactive disinfectant that penetrates a biofilm may outperform, at least in terms of microbial killing, a stronger disinfectant that fails to penetrate fully. Exactly this scenario seems to be responsible for reports of superior biofilm antimicrobial efficacy by monochloramine compared with equivalent doses of free chlorine (LeChevallier *et al.* 1990; Neden *et al.* 1992; Griebel *et al.* 1993; Samrakandi *et al.* 1997; Stewart *et al.* 2000a). Monochloramine is known to be a weaker disinfectant than free chlorine when tested against suspended bacteria.

We have been interested in the potential utility of chlorosulfamates as alternative biofilm control agents. Mono- and dichlorosulfamate ( $\text{ClNHSO}_3^-$  and  $\text{Cl}_2\text{NSO}_3^-$ , respectively) form rapidly upon reaction of hypochlorite or hypochlorous acid with sulfamate over a wide range of pH (about pH 1–10). Although the chlorosulfamates are known to be weaker disinfectants than hypochlorous acid (Delaney and Morris 1972), they have several compensatory advantages: chlorosulfamates can be formulated at mild pH (typically pH 5–6) whereas hypochlorite is only stable for prolonged periods at highly alkaline pH ( $> 10.5$ ); these chlorosulfamate formulations are quite stable and they produce no objectionable fumes and, finally, lower reactivity of chlorosulfamates with soil and biofilm is anticipated compared with free chlorine. Lower reactivity with organics is predicted to facilitate penetration into a biofilm. These features of chlorosulfamates make them particularly attractive as potential biocidal systems in consumer products.

There were two main objectives to our work. The first was to compare biofilm penetration and disinfection efficacy of

chlorosulfamates with alkaline hypochlorite. The second was to determine how much of the biofilm resistance observed with either disinfectant could be attributed to poor penetration.

## MATERIALS AND METHODS

### Biofilm system

A continuous flow laboratory biofilm reactor system was used to grow mixed population biofilms of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. This system delivered a continuous flow of growth medium dropwise over four stainless steel coupons contained in separate parallel chambers, each of which measured 10.1 cm long by 1.9 cm wide by 1.9 cm deep. The chambered reactor was fabricated from polycarbonate plastic. Each of the chambers was fitted with an individual removable plastic lid that could be affixed with thumbscrews. During continuous flow operation, the reactor was placed on a stand that inclined the entire chamber at an angle of 10° from horizontal. Before each experiment the 316L stainless steel slides (7.8 × 1.2 × 0.1 cm) were cleaned by dipping in acetone, air drying, sonication for 5 min, rinsing in Nanopure water, soaking in 2 N HCl for 2 h, rinsing with deionized and then Nanopure water and allowing to air dry. The slides were placed in the reactor chamber and the reactor assembly wrapped in aluminium foil and autoclaved. In a biological hood, rubber tubing was attached to the effluent port of the sterilized reactor, clamped off and each chamber was then separately inoculated. To inoculate, each chamber was loaded with 15 ml 1/10-strength trypticase soy broth (TSB) and 1 ml each of overnight cultures of *Ps. aeruginosa* PAO1 and *Kl. pneumoniae* KP1. Overnight cultures were grown up in 1/10-strength TSB in a 35°C shaker. The inoculated reactor was allowed to stand (no flow) for 18–24 h. Each chamber was then drained and the flow of medium (50 ml h<sup>-1</sup>, 1/100-strength TSB) initiated by attaching the influent tubing and starting the peristaltic pump. Biofilms were grown at ambient temperature of 23°C for 6 d.

### Biofilm disinfection

Biofilms grown for 6 d were subjected to various chemical treatments *in situ* by simply switching solutions. The flow rate was maintained at 50 ml h<sup>-1</sup> during the treatment period of either 15 min or 1 h. The treatments applied were: (1) no treatment; (2) pH 5.5 citrate buffer; (3) 1000 mg total chlorine l<sup>-1</sup> chlorosulfamate in citrate buffer; (4) pH 11 carbonate buffer and (5) 1000 mg total chlorine l<sup>-1</sup> hypochlorite in pH 11 carbonate buffer. Citrate buffer was prepared by mixing 7.0 g citric acid, 3.2 g NaOH and 100 ml water. The pH was subsequently adjusted to 5.5 if

necessary. The chlorosulfamate formulations were produced by reacting sodium hypochlorite with sodium sulfamate in a citrate buffer. The resulting solutions contained a mixture of mono-*N*-chlorosulfamate, di-*N*-chlorosulfamate and non-chlorinated sulfamate. Carbonate buffer was prepared by dissolving 2.1 g sodium bicarbonate and 0.9 g sodium hydroxide in 727 ml water, adjusting the pH to 11 with NaOH and making up the volume to 1 l. The buffer solution was filter sterilized through a 0.4- $\mu\text{m}$  filter prior to use. Hypochlorite solutions were prepared by diluting titrated stock sodium hypochlorite (approximately 10–12% available chlorine) into carbonate buffer (pH 11) to attain 1000 mg chlorine  $\text{l}^{-1}$ . After a 1-h treatment, biofilm was scraped from individual slides into 50 ml phosphate buffer containing 0.2% sodium thiosulphate as a neutralizer, homogenized for 15 s with a tissue homogenizer and then analysed as described below.

### Planktonic disinfection

Suspended cultures of *Ps. aeruginosa* and *Kl. pneumoniae* were grown separately in TSB overnight at 35°C with shaking. Aliquots of each of the two cultures with similar optical densities were mixed in equal proportions. A volume (1 ml) of this mixed culture was added to 9 ml of either sterile water, pH 11 buffer, pH 5.5 buffer, 1000 mg  $\text{l}^{-1}$  total chlorine as hypochlorite in pH 11 buffer or 1000 mg  $\text{l}^{-1}$  total chlorine as chlorosulfamate in pH 5.5 buffer and the tube vortexed to mix. After 30 or 60 s exposure, a sample was serially diluted and plated on R2A agar. For chlorine treatments, the first dilution tube contained 2 g  $\text{l}^{-1}$  sodium thiosulphate as a neutralizer.

### Microelectrode measurements of active chlorine species and chloride ion

An amperometric microelectrode sensitive to both hypochlorite and chlorosulfamates was prepared. The electrode was constructed by tapering a 100- $\mu\text{m}$  platinum wire down to 10–15  $\mu\text{m}$ , fusing it in a glass pipette, grinding the end of the resulting electrode flat, recessing the tip 5  $\mu\text{m}$  and covering the tip with cellulose acetate dissolved in acetone. The resulting microelectrode tip was 15–20  $\mu\text{m}$  in diameter. The electrode was calibrated by placing it in a beaker of an appropriate buffer solution, adding aliquots of either sodium hypochlorite or chlorosulfamate solution and measuring the current produced by applying –0.2 V direct current between it and a calomel reference electrode.

Biofilm specimens were placed into a special reactor for microelectrode measurements. The reactor consisted of an open-top rectangular conduit. The channel was 1 cm wide and one side was made of optically clear glass through which

progress of the microelectrode penetration could be observed. Fluid was circulated through the reactor using a peristaltic pump. The biofilm-covered sample slide was laid on the platform in the reactor and a 1-mm mesh stainless steel screen was placed on top of the biofilm. The mesh was coarse enough to allow the microelectrode to penetrate the biofilm exposed through the mesh, yet rigid enough to prevent the biofilm from washing away. The microelectrode was mounted in a computer-controlled micropositioner above the sample in the reactor. Custom software was used to control the position of the microelectrode and collect and store data. The probe was positioned at the surface of biofilm exposed through the mesh. This was defined as 'zero position'. The probe was stepped down through the biofilm at 10- $\mu\text{m}$  intervals until there was a flex in the probe indicating that it had touched the substratum. A position 10  $\mu\text{m}$  higher than this was taken as the last point sampled in successive profile measurement experiments. The probe was then raised back to zero position in preparation for profile sampling. A volume (40 ml) of an antimicrobial solution was gently poured into the reactor to cover the biofilm to a depth of 1.5 cm. The time of this addition was recorded as time zero. Profile sampling was initiated immediately after all of the solution was added and successive profiles were sampled until little or no difference existed between top and bottom current readings.

A pair of chloride microelectrodes was employed to measure chloride ion penetration into biofilms. One of these was positioned in the bulk fluid and a second was positioned 1000  $\mu\text{m}$  below the biofilm–bulk fluid interface of 1200- $\mu\text{m}$  thick biofilm. The biofilm was conditioned by flowing 0.1 mol  $\text{l}^{-1}$  phosphate buffer (pH 6.8) over it for several minutes. The experiment was initiated by delivering 0.2 mol  $\text{l}^{-1}$  NaCl dissolved in the same buffer into the flow cell. The temperature was 24°C.

Potentiometric chloride electrodes were constructed by first tapering a 100- $\mu\text{m}$  silver wire down to 10–15  $\mu\text{m}$ , fusing it in a glass pipette and grinding the tip of the resulting electrode flat. The silver in the tip was recessed 5  $\mu\text{m}$ . Silver chloride was electrolytically generated at the exposed tip by applying a current of 2 mA  $\text{cm}^{-2}$  in a solution of 0.05 mol  $\text{l}^{-1}$  HCl. The resulting microelectrode tip was 15–20  $\mu\text{m}$  in diameter. The electrode was calibrated in serially diluted chloride solutions. The chloride potentiometric microelectrode gave a logarithmic response to a series of five chloride solutions between pCl 1 and 5. The potential between it and a calomel reference electrode was measured using an electrometer (model 6517; Kiethley, Cleveland, OH, USA). LAB-TECH Notebook (Laboratory Technologies Corporation, Wilmington, MA, USA) software was used to collect and store the data. The effective diffusion coefficient of chloride ion in the biofilm was estimated by fitting a mathematical model of diffusion in a slab (Bird *et al.* 1960) to the data.

## Analytical methods

Viable cell counts in cell suspensions were determined by serial dilution and drop-plating on R2A. Plates were incubated overnight at 35°C. The two microbial species were distinguished by differences in colony colour and morphology. Protein was assayed using a commercial kit (catalogue no. 5656; Sigma). Prior to protein analysis, samples were sonicated using a probe for 1–2 min. Total cell counts were determined by staining membrane-filtered samples with 4',6-diamino-2-phenylindole (DAPI) and performing direct microscopic counts. The total carbohydrate of sonicated aliquots was analysed by the modified Dubois method. Total chlorine concentrations were determined by amperometric titration. Biofilm thickness was determined either by the depth at which a microelectrode flexed, indicating direct contact with the substratum, or by image analysis of frozen cross-sections of biofilm. The combined effects of disinfection and removal were reported by calculating the  $\log_{10}$  reduction in viable cell numbers ( $-\log_{10}(X/X_0)$  where  $X$  = viable cell count after treatment and  $X_0$  = viable cell count before treatment). The effect of disinfection alone was reported by calculating the  $\log_{10}$  reduction in the fraction of the number of total cells that were viable ( $-\log_{10}(XT_0/X_0T)$  where  $X$  = viable cell count after treatment,  $X_0$  = viable cell count before treatment,  $T$  = total cell count after treatment and  $T_0$  = total cell count before treatment).

## RESULTS

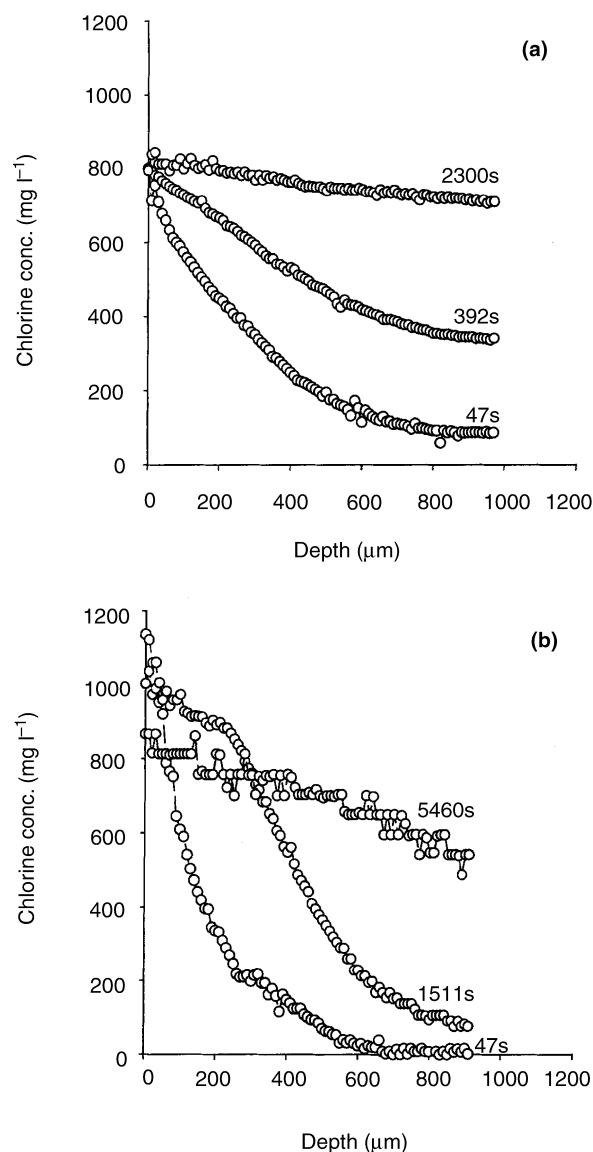
### Biofilm characteristics

After 6 d of growth in the laboratory reactor, bacterial biofilm accumulated to a thickness of almost 1 mm ( $900 \pm 160 \mu\text{m}$ ) and a mean areal cell density of  $8 \times 10^9 \text{ cfu cm}^{-2}$ . *Pseudomonas aeruginosa* outnumbered *Kl. pneumoniae* in the biofilm by a factor ranging from approximately 2 to 6. These biofilms contained an order of magnitude more protein ( $2.7 \pm 1.1 \text{ mg cm}^{-2}$ ) than carbohydrate ( $0.13 \pm 0.08 \text{ mg cm}^{-2}$ ).

### Solute penetration measurements

Amperometric microelectrode technology proved to be applicable to measurement of chlorosulfamate concentrations. Electrodes exhibited satisfactory signal stability in the  $1000 \text{ mg l}^{-1}$  total chlorine range. Excellent linear calibration curves were demonstrated for both chlorosulfamate formulations ( $r^2 \geq 0.989$ ).

Alkaline hypochlorite, chlorosulfamate and chloride ion penetrated biofilms, but chloride ion and chlorosulfamate penetrated more quickly than alkaline hypochlorite (Fig. 1). Biofilm thickness ranged from 890 to  $1200 \mu\text{m}$ . The mean and standard deviation of the 50% penetration time was

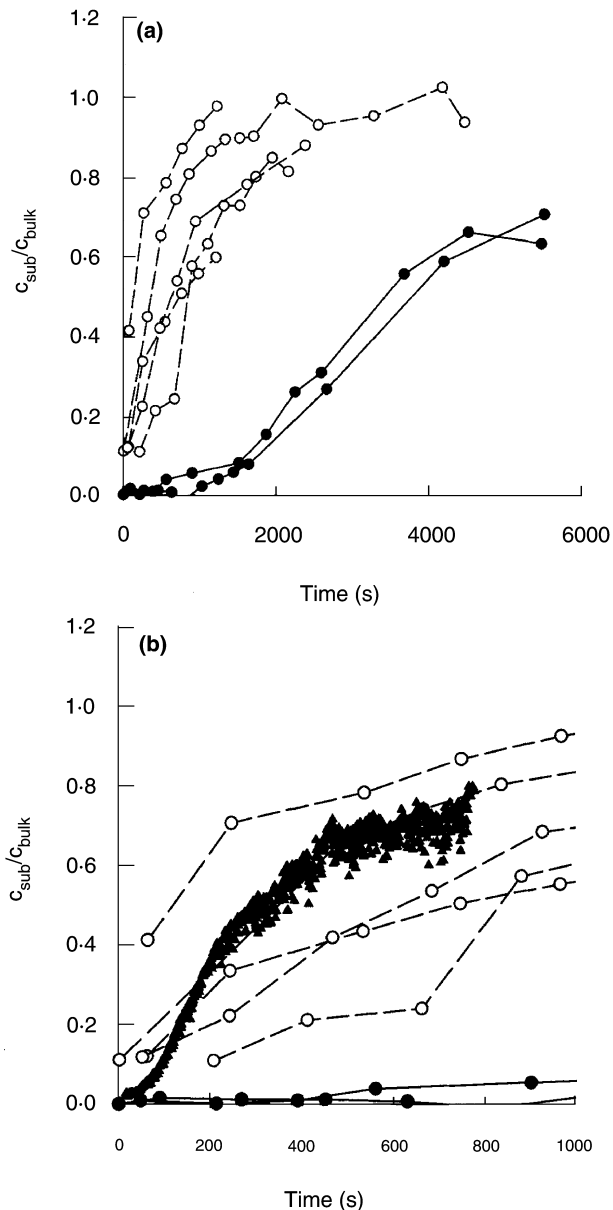


**Fig. 1** Representative total chlorine concentration profiles within biofilms treated with (a) chlorosulfamate or (b) hypochlorite. Zero on the x-axis corresponds to the biofilm–bulk fluid interface. In each case, the substratum was located between 900 and  $1000 \mu\text{m}$  deep. The time associated with each curve indicates the duration of hypochlorite treatment up to the time of measurement of the concentration profile

$6.2 \pm 3.0$  min for chlorosulfamate ( $n = 5$ ) and  $48 \pm 4$  min ( $n = 2$ ) for hypochlorite. The 50% penetration time measured for chloride in one experiment was 5.3 min. In these experiments, the initial total chlorine concentration ranged from 799 to  $1140 \text{ mg l}^{-1}$  and the final total chlorine concentration ranged from 586 to  $866 \text{ mg l}^{-1}$ .

One feature of these results that complicates their interpretation is the fact that the concentration of active chlorine species at the biofilm–bulk fluid interface changes

with time. To facilitate comparison of the extent of penetration, the ratio of the chlorine concentration at the substratum,  $C_{\text{sub}}$ , to the concentration at the biofilm–bulk fluid interface,  $C_{\text{bulk}}$  was plotted (Fig. 2). If this ratio was 0 there was no penetration and if the ratio was 1 penetration was complete. Both chlorosulfamate formulations penetrated into biofilm more rapidly than hypochlorite. The time



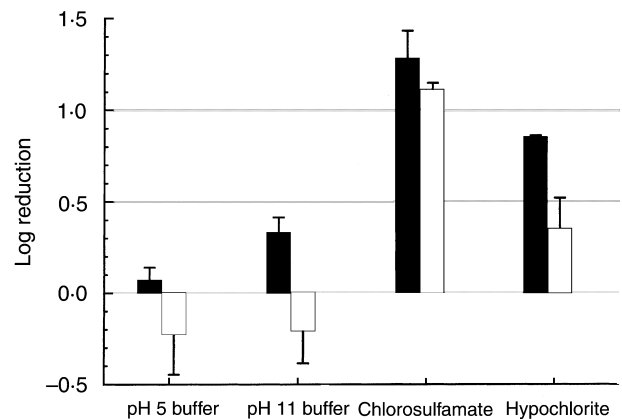
**Fig. 2** (a) Normalized chlorine concentration at the substratum as a function of duration of treatment for chlorosulfamate (○) and hypochlorite (●) treatments of approximately  $1000 \text{ mg l}^{-1}$ . (b) As (a) with data for penetration of chloride ion included for comparison (▲). Each trace represents a separate experiment. Note that the time scales of the two panels differ

required to attain, at the substratum, 50% of the biofilm–bulk fluid interface concentration of total chlorine averaged 6.2 min for chlorosulfamate and 48 min for regular hypochlorite. More rapid penetration of chlorosulfamate was statistically significant ( $P = 0.05$ ).

The penetration into biofilm of a non-reactive tracer ion, chloride, was also measured using microelectrodes (Fig. 2b) and indicates that chlorosulfamate and chloride ion penetrated at similar rates while regular hypochlorite was clearly retarded in comparison to chloride ion. The 50% penetration time for chloride was 5.3 min whereas it was, on average, 6.2 min for chlorosulfamate. From the transient chloride concentration profiles, the effective diffusion coefficient of chloride ion in the biofilm was estimated to be in the range of  $1.4 \times 10^{-5}$ – $1.9 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ .

### Biofilm disinfection and removal

Treatment of biofilms with hypochlorite or chlorosulfamate resulted in approximately a 1-log reduction in viable cell numbers (Fig. 3). Treatment with the buffer solutions in which the chlorine species were dissolved had little effect on viability (Fig. 3). To separate the effects of disinfection from those of removal, the log reduction in the surviving fraction of total cells was calculated. This parameter measures disinfection in particular whereas the log reduction in viable cells combines the effects of disinfection and detachment. The efficacy of alkaline hypochlorite as a disinfectant (log reduction in surviving fraction ranged from 0.2 to 0.5) was consistently less than the efficacy shown by chlorosulfamate formulations (log reduction in surviving fraction ranged from 1.0 to 1.2) for 60-min treatments. The difference in



**Fig. 3** Log reduction in viable cell numbers (■) and surviving fraction (□) in laboratory-grown biofilms treated for 60 min. The log reduction in viable cell numbers measures loss of viable bacteria by the combined effects of killing and removal. The log reduction in surviving fraction reflects the killing of those bacteria that remain in the biofilm

disinfection efficacy by this measure could not be demonstrated statistically ( $P = 0.14$ ), perhaps because there were too few replicates. Shorter duration treatments (15 min) resulted in about the same log reduction in viable cells as 60-min treatments (data not shown). Again, there was no statistical difference in disinfection efficacy between chlorosulfamates and hypochlorite ( $P = 0.37$ ).

Data for removal of total cells, protein and carbohydrate suggest a noisy but consistent picture of partial removal of the biofilm by all of the chlorine formulations as well as by the buffer solutions in which they were prepared. There was no apparent difference in the removal efficacy of either chlorine formulation and its corresponding chlorine-free buffer. The average removal effected by the pH 11 carbonate buffer or by hypochlorite dissolved in this buffer was 65% of total cells, 47% of protein and 19% of carbohydrate. The average removal effected by the pH 5.5 citrate buffer or chlorosulfamate dissolved in this buffer was 27% of total cells, 61% of protein and 16% of carbohydrate.

### Planktonic disinfection

Treatment of mixed planktonic cultures of the two test micro-organisms with 1000 mg l<sup>-1</sup> hypochlorite resulted in rapid disinfection (Fig. 4). After 30 s of exposure to alkaline hypochlorite, viable bacterial numbers experienced a mean log reduction of 5.5. After 30 s of treatment with chlorosulfamate, the mean log reduction was 2.9. These data show that hypochlorite was a more effective disinfectant than chlorosulfamate against laboratory micro-organisms grown in suspension culture and this difference could be demonstrated with statistical significance ( $P = 0.016$ ). Disinfection by chlorosulfamate continued so that after 60 s of treatment

bacterial survivors were near the detection limit with a mean log reduction of 6.9. There was no significant effect of either of the buffer solutions on planktonic bacterial viability (data not shown).

As some of the bacteria in a laboratory-grown biofilm can be as old as 6 d, it was hypothesized that mere ageing of planktonic cultures might confer resistance. To test this possibility, a planktonic culture was covered and allowed to stand unmixed for 6 d at room temperature. This aged bacterial suspension was then subjected to hypochlorite treatment. There was no difference between the disinfection susceptibility of fresh and aged planktonic cultures (Fig. 4). For example, mean log reductions of fresh (2.9) and 6-d-old (3.2) planktonic cells achieved by 30-s treatment with 1000 mg l<sup>-1</sup> chlorosulfamate were not statistically significantly different ( $P = 0.75$ ). When laboratory-grown biofilms were scraped and dispersed with a tissue homogenizer, the resuspended bacteria were also found to be just as susceptible to hypochlorite or chlorosulfamate as fresh planktonic cells (Fig. 4). For example, mean log reductions of fresh planktonic cells (2.9) and resuspended biofilm cells (2.8) achieved by 30-s treatment with 1000 mg l<sup>-1</sup> chlorosulfamate were not statistically significantly different ( $P = 0.92$ ).

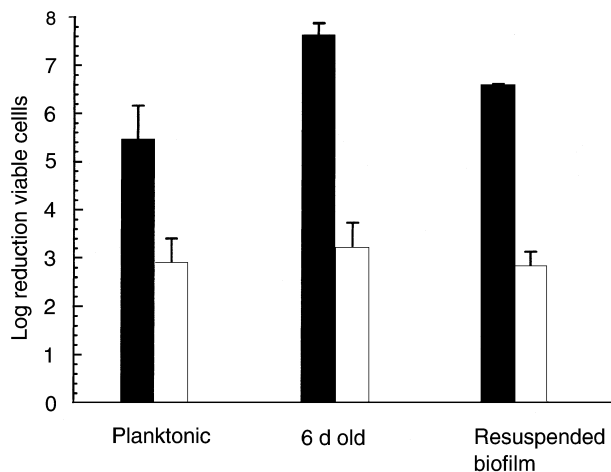
## DISCUSSION

### Biofilms resist disinfection

This study reproduced the nearly universally observed resistance of biofilm micro-organisms to disinfection when compared with their freely suspended counterparts. Planktonic bacteria challenged with alkaline hypochlorite or chlorosulfamate were rapidly killed whereas biofilm bacteria challenged with 1000 mg l<sup>-1</sup> of either agent could be only partially killed.

### Chlorosulfamate penetrates biofilms more rapidly than hypochlorite

Chlorosulfamate formulations penetrated biofilms approximately eight times faster than alkaline hypochlorite. In the case of a highly reactive antimicrobial, such as hypochlorite, the antimicrobial agent must chemically consume its way into the biofilm, depleting the neutralizing capacity in surface layers of the biofilm before it can penetrate into deeper layers. This mechanism of retarded penetration has been shown to be significant for hypochlorite and our results are consistent with those of previous investigations (de Beer *et al.* 1994; Chen and Stewart 1996; Xu *et al.* 1996). If an antimicrobial agent has a slower reaction rate with biofilm, then it will penetrate more rapidly. Chlorosulfamate probably penetrates biofilms more rapidly than hypochlorite due to a slower reaction rate with biofilm constituents.



**Fig. 4** Comparison of disinfection susceptibility of fresh planktonic, 6-d-old planktonic and resuspended laboratory biofilm cells to chlorosulfamate (□) and hypochlorite (■)

Mono- and dichlorosulfamates are both larger molecules (molecular weight 131 and 166, respectively) than hypochlorite ion (molecular weight 51.5). The aqueous diffusion coefficients of the mono- and dichlorosulfamates are estimated, by the Wilke–Chang correlation and at 25°C, to be  $1.1 \times 10^{-5}$  and  $1.0 \times 10^{-5}$  cm<sup>2</sup> s<sup>-1</sup>. The diffusion coefficient of the hypochlorite ion has been estimated to be  $1.9 \times 10^{-5}$  cm<sup>2</sup> s<sup>-1</sup> at 25°C (Stewart *et al.* 2000a). Better biofilm penetration by the chlorosulfamates than by hypochlorite is not due to inherently faster diffusion of these molecules; in fact, they are predicted to diffuse approximately 45% slower than hypochlorite ion.

Measurements of chloride ion penetration into laboratory-grown biofilms showed that the effective diffusion coefficient of chloride in the biofilm was close to its value in water. The effective diffusion coefficient of chloride ion in the biofilm relative to the diffusion coefficient of chloride in water was estimated to be  $0.84 \pm 0.14$  (the range indicates estimated upper and lower bounds on the relative effective diffusion coefficient). The 50% penetration time for chloride, a non-reactive tracer that should define the most rapid possible diffusive penetration, was 5.3 min. This is close to the 50% penetration time for chlorosulfamates of 6.2 min, indicating that chlorosulfamates experience little retardation.

These measurements are consistent with the following conceptual model of antimicrobial penetration into biofilm. Biofilms are highly hydrated structures in which small solutes the size of disinfectants and antibiotics move relatively freely (Stewart 1998). This expectation holds true, as we have shown here for chlorosulfamate, if the antimicrobial agent does not react appreciably with any component of the biofilm. If an antimicrobial agent is reactively neutralized in the biofilm, then its ability to penetrate the biofilm can be severely compromised. Hypochlorite reacts rapidly with organic constituents of the biofilm and its penetration is thereby retarded. Other examples of such a reaction–diffusion interaction leading to poor antimicrobial penetration have been described for hydrogen peroxide (Stewart *et al.* 2000b), which is degraded by catalases, and beta-lactam antibiotics (Anderl *et al.* 2000), which are cleaved by beta-lactamases.

One qualification that should be kept in mind in considering these results is that penetration times are predicted to depend on the antimicrobial dose concentration (Stewart and Raquepas 1995; Stewart 1997). In the case of a stoichiometric reaction, such as is thought to be the case for hypochlorite, the 50% penetration time is predicted to be inversely proportional to the dose concentration. As the treatment concentration is reduced, the penetration time will increase. For example, in the present characterization of hypochlorite we measured a 48-min 50% penetration time at a concentration of 1000 mg l<sup>-1</sup>. If the treatment concentra-

tion were only 10 mg l<sup>-1</sup>, the penetration time would be expected to be 100 times longer (4800 min (3 d)). Conversely, if the concentration were increased 10-fold to 1% chlorine, the penetration time should reduce to about 5 min. The penetration time will not reduce with further increases in hypochlorite concentration because at this point, in this particular system, the penetration time would be controlled by diffusion alone and would no longer depend on the reaction–diffusion interaction. At 1000 mg l<sup>-1</sup> the penetration of chlorosulfamate appears to be at the diffusion limit. At some lower concentration, chlorosulfamate penetration might be retarded by a reaction–diffusion interaction, but this threshold concentration cannot be predicted from the present data.

### Poor antimicrobial penetration is insufficient to explain biofilm resistance

After 60 min of treatment, both chlorosulfamate and hypochlorite had penetrated throughout the biofilms, yet neither antimicrobial was able to kill sessile micro-organisms very effectively. The mean log reduction in the viable fraction of total cells after 60 min exposure to 1000 mg l<sup>-1</sup> was just 1.1 for chlorosulfamate and 0.4 for hypochlorite (Fig. 3). If biofilm micro-organisms were just as susceptible as planktonic cells and the only protection afforded by the biofilm mode of existence were due to retarded delivery of an antimicrobial agent, then rapid killing of the biofilm should ensue once a cidal concentration of antimicrobial has fully penetrated. In our experimental system using chlorosulfamate, penetration requires about 6 min and complete kill (of a planktonic cell) requires an additional 1 min. The biofilm would be sterilized within 7 min of treatment according to this scenario. Similarly with hypochlorite the biofilm is penetrated in about 50 min and should be completely killed, if the cells have the same intrinsic susceptibility as planktonic cells, in an additional 1 min. The fact that we have measured poor biofilm killing despite direct measurement of effective physical penetration of the antimicrobial agent into the biofilm demonstrates that bacteria in the biofilm are protected by some mechanism other than simple physical shielding by the biofilm matrix.

### Chlorosulfamate as an alternative antimicrobial agent for biofilm control

Although chlorosulfamate is a weaker disinfectant than regular hypochlorite when tested against bacteria cultured in suspension (Fig. 4), it was as effective or more effective a disinfectant when tested against biofilm (Fig. 3). One explanation for this would be the more rapid penetration into the biofilm by chlorosulfamate. Experimental data

bearing on biofilm removal by stabilized and regular hypochlorite were noisy, but suggested that the two treatments were equally effective with regard to removal. These results underscore the inadequacy of planktonic testing methods for evaluating antimicrobial agents to be used against biofilms. As the use of biofilm testing methods expands, new antimicrobial formulations that are more effective against biofilm bacteria, because they penetrate the biofilm and target the distinct biofilm phenotype, will be discovered.

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