

# Effects of antimicrobial agents on oral biofilms in a saliva-conditioned flowcell

J. S. Foster<sup>1</sup>, P. C. Pan<sup>2</sup> and P. E. Kolenbrander<sup>1\*</sup>

\* Corresponding author:  
Dr P. E. Kolenbrander  
NIDCR/National Institutes of Health  
30 Convent Drive MSC 4350  
Building 30  
Room 310  
Bethesda  
MD 20892-4350 USA  
T 1 301-496-1497  
F 1 301-402-0396  
E pkolenbrander@dir.nidcr.nih.gov

1 Oral Infection and Immunity Branch,  
National Institute of Dental and  
Craniofacial Research, National  
Institutes of Health, Bethesda,  
MD 20892-4350, USA

2 Pfizer Consumer Healthcare  
Research and Development,  
Morris Plains, NJ 07950, USA

## ABSTRACT

Oral bacteria form mixed-species biofilms known as dental plaque. Growth of these complex microbial communities is often controlled with the use of antimicrobial mouthrinses. Novel laboratory methods for testing the efficacy of antimicrobials *in situ* are necessary to complement current clinical testing protocols. In this study, we examined the effects of antimicrobial agents on a streptococcal biofilm grown in a saliva-conditioned flowcell. The flowcell coupled with confocal laser scanning microscopy enabled examination of growing oral biofilms *in situ* without disruption of the microbial community. Biofilms composed of *Streptococcus gordonii* DL1 were grown in an *in vitro* flowcell and treated with several commercially available antimicrobial mouthrinses containing essential oils, triclosan, cetylpyridinium chloride/domiphen or chlorhexidine. The results of this study revealed varying abilities of the antimicrobial agents to cause cellular damage on the growing biofilm *in situ*. This study therefore demonstrated the usefulness of the flowcell in the rapid assessment of antimicrobial efficacy.

## INTRODUCTION

Dental plaque is an oral microbial biofilm comprising a multi-species community that forms on the surfaces of the oral cavity. Understanding the underlying mechanisms of biofilm development has been facilitated by the use of *in vitro* model systems (Wilson & Pratten, 1999; Hope *et al.*, 2002; Pratten *et al.*, 2003). Similar model systems have also been applied to study the effects of antimicrobial agents on biofilm growth and viability. Examining the impact of antimicrobials on oral biofilms *in vivo* has been the focus of several recent investigations (Eley, 1999; Fine *et al.*, 2000; Pan *et al.*, 2000; Wecke *et al.*, 2000). However, such studies have relied on methods that do not look at the *in situ* effects of various antimicrobials. In many studies the biofilms are disassociated before analysis, therefore losing spatial information on the effects of antimicrobial efficacy. Furthermore, many of the studies disrupt the growing biofilm prior to antimicrobial treatment, potentially causing an alteration in the response of the biofilm to the agent (Fine *et al.*, 2001). Although some previously described studies do examine growing biofilms *in situ* (Guggenheim *et al.*, 2001; Zaura-Arite *et al.*, 2001), the biofilms are maintained statically and are not exposed to salivary flow, a factor contributing to biofilm formation (Palmer *et al.*, 2001). Adherence of bacteria to the substratum is a critical step to biofilm formation (Whittaker *et al.*, 1996) and salivary flow introduces an added selective pressure. Here we present a model that

examines the effects of antimicrobials on monospecies biofilms composed of *Streptococcus gordonii*, a known colonizer of the tooth surface (Nyvad & Kilian, 1987). The biofilms were grown within an *in vitro* flowcell that utilizes human saliva supplemented with sucrose as the nutrient source. The flowcell was initially designed to enable biofilms to grow in an environment that mimics the dynamic conditions of the oral cavity (Palmer & Caldwell, 1995). Oral microenvironments are difficult to model *in situ* because they are open systems subject to (1) fluctuations of the complex nutrients in the surrounding environment, (2) metabolic by-products of the colonizing microbes, and (3) variable growth rates of the microorganisms. The flowcell permits adherence of early colonizing bacteria to a saliva-conditioned solid substratum and direct examination by confocal laser scanning microscopy (CLSM) without disruption to the growing biofilm. To analyze the effects of antimicrobials on the *in vitro* biofilms, we utilized CLSM, thus enabling a three-dimensional reconstruction of biofilm structure and antimicrobial efficacy.

In this study, four classes of antimicrobial agents were used: essential oils, triclosan, cetylpyridinium chloride/domiphen bromide, and chlorhexidine. These categories of antimicrobial agents were selected to represent antimicrobial agents of diverse origin and widely differing modes of action. We chose to use these commercially available antimicrobial agents in their final formulation

in order to mirror many previous clinical studies (van der Hoeven *et al.*, 1993; Pratten *et al.*, 1998; Moran *et al.*, 2000; Pan *et al.*, 2000; Leyes Borrajo *et al.*, 2002). However, it is recognized that vehicle excipients can enhance or reduce activity of antimicrobials. By using CLSM, vitality staining and image analysis software, we have complemented previous clinical studies and can ascertain the efficacies of various antimicrobial agents on undisrupted oral biofilms.

## MATERIALS AND METHODS

### Saliva collection

Saliva was collected on ice from at least six healthy individuals who had refrained from eating for a minimum of 2 h prior to contributing. The saliva was pooled and treated with 2.5 mM dithiothreitol while being stirred for 10 min to reduce the potential aggregation of salivary proteins. The pooled saliva was then centrifuged at  $30\,000 \times g$  for 20 min at 4 °C as described (de Jong & van der Hoeven, 1987). The supernatant was diluted with three volumes of distilled water to produce a final saliva concentration of 25%, and then filtered through a 0.22 µm pore low-protein binding filter and frozen at -20 °C until use. Saliva was thawed immediately prior to use and centrifuged at  $1430 \times g$  for 5 min to remove any precipitate that might have formed during freezing and thawing. The saliva was then supplemented with 50 mM sucrose that facilitated biofilm growth.

### *In vitro* flowcell model

The *in vitro* flowcell model was modified from that described by Palmer & Caldwell (1995). Two flow chambers with a volume of 250 µl per track were milled into a high-density polyethylene block and a glass coverslip was attached with silicone adhesive, thus enabling the biofilms to be examined *in situ* without disruption. The flowcells were cleaned with 0.1 M HCl overnight and then rinsed with 5 ml of distilled water. The flowcell was disinfected by treatment with 100% bleach for 2 h and rinsed with 5 ml of distilled water. The flowcell chambers were treated with saliva for 10 min to coat the flowcell with host-derived molecules. A peristaltic pump maintained saliva flow at 200 µl/min to mimic human saliva flow rates (Dawes *et al.*, 1989; Palmer & Caldwell, 1995).

### Bacterial inoculum

*Streptococcus gordonii* Challis strain DL1 was inoculated into brain heart infusion (BHI) medium (Becton Dickinson Co., Sparks, MD) and incubated overnight in an anaerobic chamber (N<sub>2</sub>:CO<sub>2</sub>:H<sub>2</sub>, 90:5:5) at 37 °C. A 300 µl aliquot of the culture was transferred into 8 ml of anaerobically prereduced BHI medium and incubated for 4 h at 37 °C to achieve exponential growth. Cells were pelleted at  $1000 \times g$  for 15 min, washed three times in 25% saliva, and resuspended to an A<sub>600</sub> of 0.05. The

inoculum was then diluted to a final concentration of  $2.5 \times 10^5$  cells/ml of 25% saliva.

### Biofilm growth

Each flowcell was inoculated with 500 µl of the bacterial suspension containing  $2.5 \times 10^5$  cells/ml of 25% saliva and then inverted for 20 min without flow to facilitate colonization of the bacteria onto the underside of the glass coverslip. The biofilms were grown within flowcells for 12 h and were maintained in an ambient-atmosphere incubator at 37 °C.

### Antimicrobial treatments

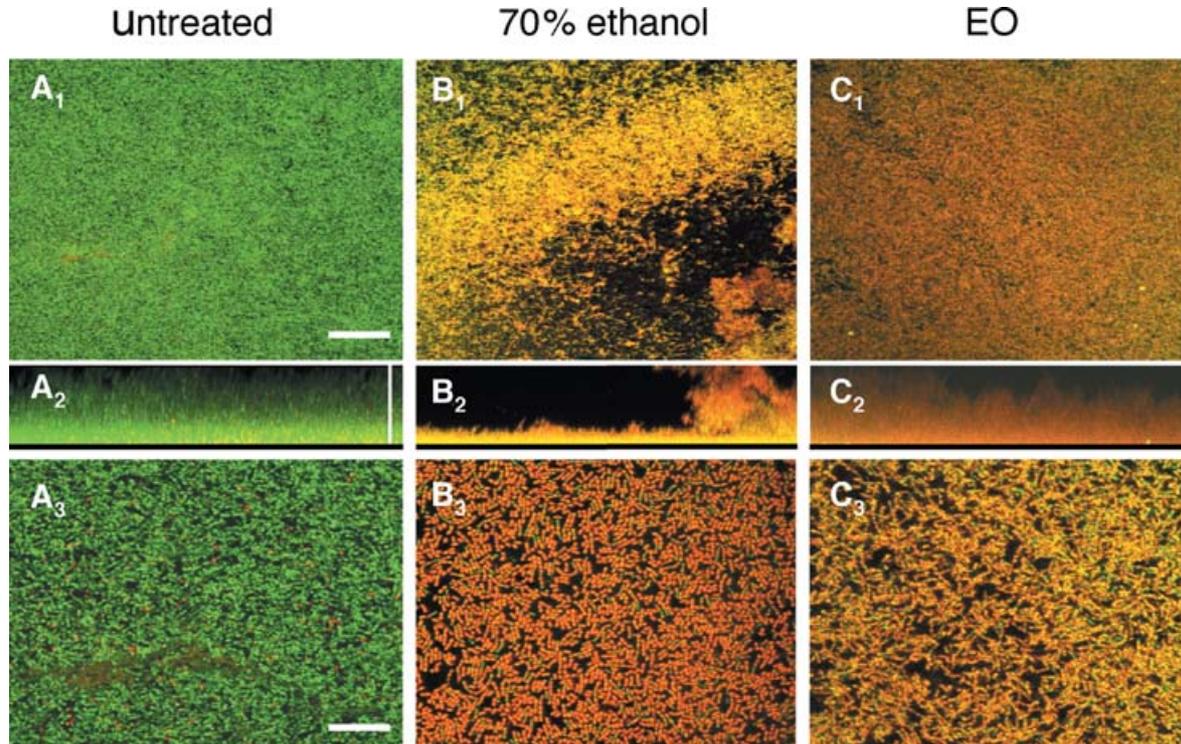
After the 12 h biofilm growth period, the peristaltic pump was stopped and 500 µl of antimicrobial agent was injected by syringe into the flowcell for either 30 or 60 s. The antimicrobial agents used in this study included: (1) Listerine® (Pfizer, Morris Plains, NJ), containing essential oils; (2) Plax® (Colgate, Sydney, Australia) with triclosan, (3) Scope® (Procter and Gamble, Cincinnati, OH) with cetylpyridinium chloride/domiphen bromide (CPC), and (4) Peridex® (Zila Pharmaceuticals, Phoenix, AZ) with chlorhexidine. Each antimicrobial treatment was examined in at least three independent flowcell experiments using different saliva pools. For control purposes additional flowcell tracks were injected with 70% ethanol for 30 s or left untreated. After treatment, the flowcells were rinsed with 25% saliva for 10 min.

### Bacterial vitality staining

To assess the effectiveness of the different antimicrobial treatments, the biofilms were stained with the LIVE/DEAD Bacterial Viability kit (Molecular Probes, Eugene, OR), which visualizes the integrity of bacterial plasma membranes. The viability kit, used in recent studies of oral biofilms (Guggenheim *et al.*, 2001; Hope *et al.*, 2002; Hope & Wilson, 2003) consists of two fluorescent markers: (1) SYTO 9, a green fluorescent stain which is indicative of intact plasma membranes; and (2) propidium iodide (PI), a red stain which targets those cells that have incurred membrane damage. A solution containing 1.67 µM of SYTO 9 and 10 µM PI was prepared in 25% saliva and then injected into each track of the flowcells. The stained biofilms were examined *in situ* through the glass coverslip of the flowcell with either a 40× or 63× oil immersion lens on a Leica TCS4D confocal laser scanning microscope.

### Image analyses

To quantify the effectiveness of the antimicrobial treatments, micrographs obtained by confocal microscopy were analyzed with Metamorph 4.6r9 image analysis software (Universal Imaging Corporation, Downingtown, PA). Fluorescence intensity thresholds were set for green,



**Fig. 1:** Effect of essential oils (EO) on 12 h biofilms. (A<sub>1</sub>) Confocal image of untreated negative control biofilm demonstrating extensive labeling with SYTO 9 (green) indicating viability. (A<sub>2</sub>) A three-dimensional reconstruction of A<sub>1</sub> rotated 90° in the *x-z* direction. (A<sub>3</sub>) High-magnification image of biofilm reveals that a few of the streptococcal cells within the biofilm are labeled with propidium iodide (red). (B<sub>1</sub>) Micrograph of positive control biofilm treated with ethanol. The majority of cells within the biofilm are stained yellow (colocalization of SYTO 9 with propidium iodide), suggesting that cells with damaged membranes are more numerous than in A<sub>1</sub>. (B<sub>2</sub>) Projection of ethanol-treated biofilm rotated 90° in the *x-z* direction. (B<sub>3</sub>) High magnification image of cells within the ethanol-treated biofilm. (C<sub>1</sub>) Representative image of biofilms treated with EO for 30 s. Propidium iodide staining indicates widespread membrane damage throughout the thickness of the biofilm. (C<sub>2</sub>) A three-dimensional reconstruction of C<sub>1</sub> rotated 90° in the *x-z* direction, demonstrating EO penetration to the base of the thick biofilm and efficacy of agent in disruption of vitality, as indicated by the red staining. (C<sub>3</sub>) High-magnification image of biofilm treated with EO. Few viable cells (green) remain within the biofilm. Most cells stain yellow or red indicative of membrane damage. Scale bars: 50 μm (A<sub>1</sub>, B<sub>1</sub>, C<sub>1</sub>), 55 μm (A<sub>2</sub>, B<sub>2</sub>, C<sub>2</sub>), and 10 μm (A<sub>3</sub>, B<sub>3</sub>, C<sub>3</sub>). Black line in A<sub>2</sub>, B<sub>2</sub>, and C<sub>2</sub> indicates substratum surface.

red and yellow pixels and normalized to account for empty spaces. The pixel areas and their intensities were measured using the image analysis software. The resulting values for green and red fluorescent intensities were then analyzed for statistical significance.

### Statistical analysis

Descriptive statistics, including the mean and standard error were calculated for each of the treatment groups. One-way analysis of variance (ANOVA) was used to determine whether significance differences existed between the various treatments. To determine whether the means were statistically different from each other a Tukey's pairwise comparison test was used at the chosen level of probability ( $P < 0.05$ ).

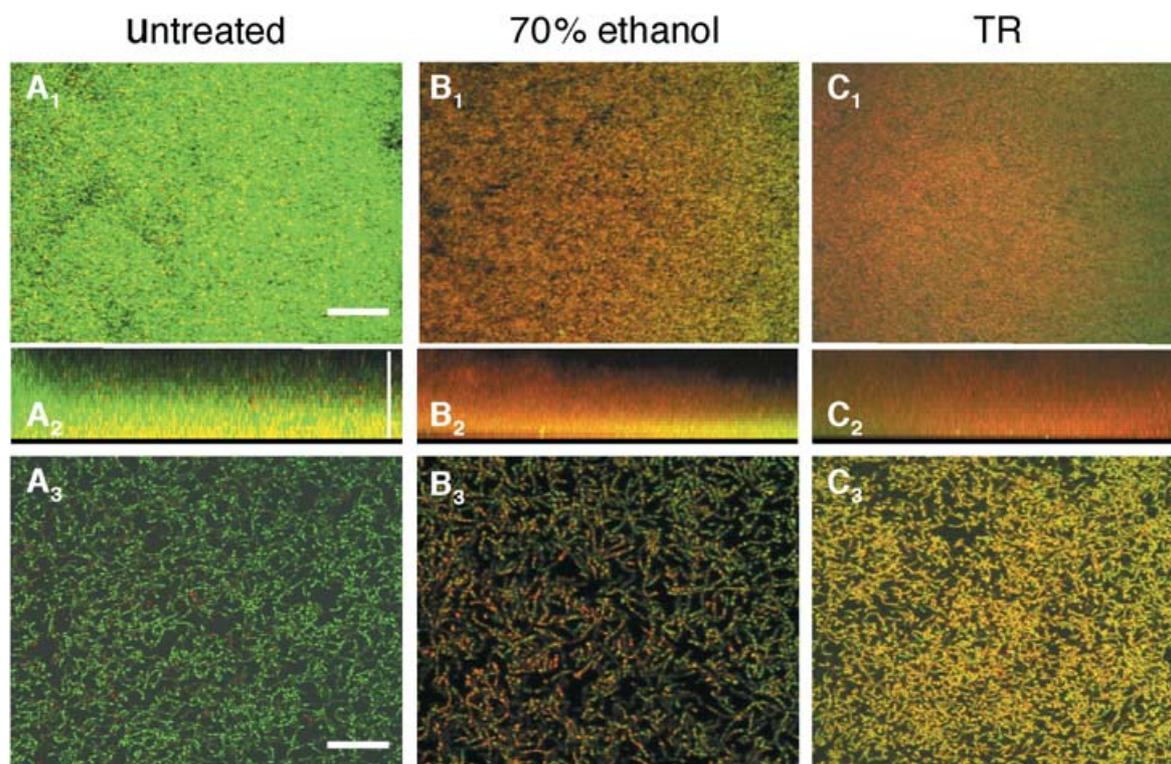
## RESULTS

Adherence to the saliva-conditioned glass surface by *S. gordonii* was observed in the flowcell chambers immediately after inoculation (data not shown). An untreated negative control and a 70% ethanol-positive

control were conducted in parallel with each antimicrobial treatment to demonstrate the minimal variability of staining present in this assay. Untreated biofilms were observed to be viable after the 12 h incubation. Examination of biofilms treated with essential oils for 30 s showed widespread levels of membrane damage, similar to those biofilms treated with 70% ethanol (Fig. 1). Confocal micrographs of the biofilm revealed effective penetration of both the antimicrobial agent and vitality stains throughout the growing biofilm (Fig. 1). High magnification images revealed that a few of the cells comprising the biofilms remained viable despite antimicrobial exposure (Fig. 1C<sub>3</sub>).

Treatment of biofilms with triclosan resulted in levels of membrane damage that mirrored those biofilms exposed to essential oils for similar periods (Fig. 2C<sub>1</sub>, C<sub>2</sub>, C<sub>3</sub>). Confocal micrographs taken at higher magnifications showed that only a few cells resisted the triclosan treatment, similar to treatment with essential oils.

In biofilms treated with CPC mixture, moderate levels of membrane damage were detected after 60 s exposures (Fig. 3). Profiles of the treated biofilms obtained by CLSM showed that CPC caused loss of membrane integrity only in the top two-thirds of the biofilm after 60 s of treatment



**Fig. 2:** Effect of triclosan (TR) on 12 h biofilms. Panels A<sub>1</sub>, A<sub>2</sub>, A<sub>3</sub> are untreated negative controls as described in Fig. 1. Panels B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub> are positive controls treated with 70% ethanol, as described in Fig. 1. (C<sub>1</sub>) Representative image of biofilm treated with TR for 30 s showing extensive labeling with propidium iodide (red) and SYTO 9 (green). Colocalization of stains appears yellow. (C<sub>2</sub>) A three-dimensional reconstruction of C<sub>1</sub> rotated 90° in the *x-z* direction, showing extensive propidium iodide labeling throughout biofilm thickness. (C<sub>3</sub>) High magnification confocal image of biofilm treated with TR shows the majority of cells stained with propidium iodide and SYTO 9 (yellow), indicating loss of membrane integrity. Scale bars: 50 μm (A<sub>1</sub>, B<sub>1</sub>, C<sub>1</sub>), 65 μm (A<sub>2</sub>, B<sub>2</sub>, C<sub>2</sub>), and 10 μm (A<sub>3</sub>, B<sub>3</sub>, C<sub>3</sub>). Black line in A<sub>2</sub>, B<sub>2</sub>, and C<sub>2</sub> indicates substratum surface.

(Fig. 3C<sub>2</sub>). Little or no change in biofilm viability was seen after 30 s (see Fig. 5). High magnification confocal micrographs of those cells in the lower one-third of the biofilm (i.e. close to the glass substratum) indicated that the majority of cells appear to be unaffected by CPC treatment (Fig. 3C<sub>3</sub>). Overall, the 30 and 60 s exposures of CPC appeared to be less effective than essential oils or triclosan in causing loss of plasma membrane integrity.

The antimicrobial chlorhexidine also required a 60 s exposure to cause widespread membrane damage in the 12 h growing biofilm (Fig. 4). Despite the longer time needed for effective induction of membrane damage, the damage appeared to be throughout the entire biofilm (Fig. 4C<sub>2</sub>). However, as seen in the treatments with essential oils and triclosan, high magnification images showed a subset of the cells that resisted 30 and 60 s chlorhexidine treatments (Fig. 4B<sub>3</sub> and C<sub>3</sub>, respectively).

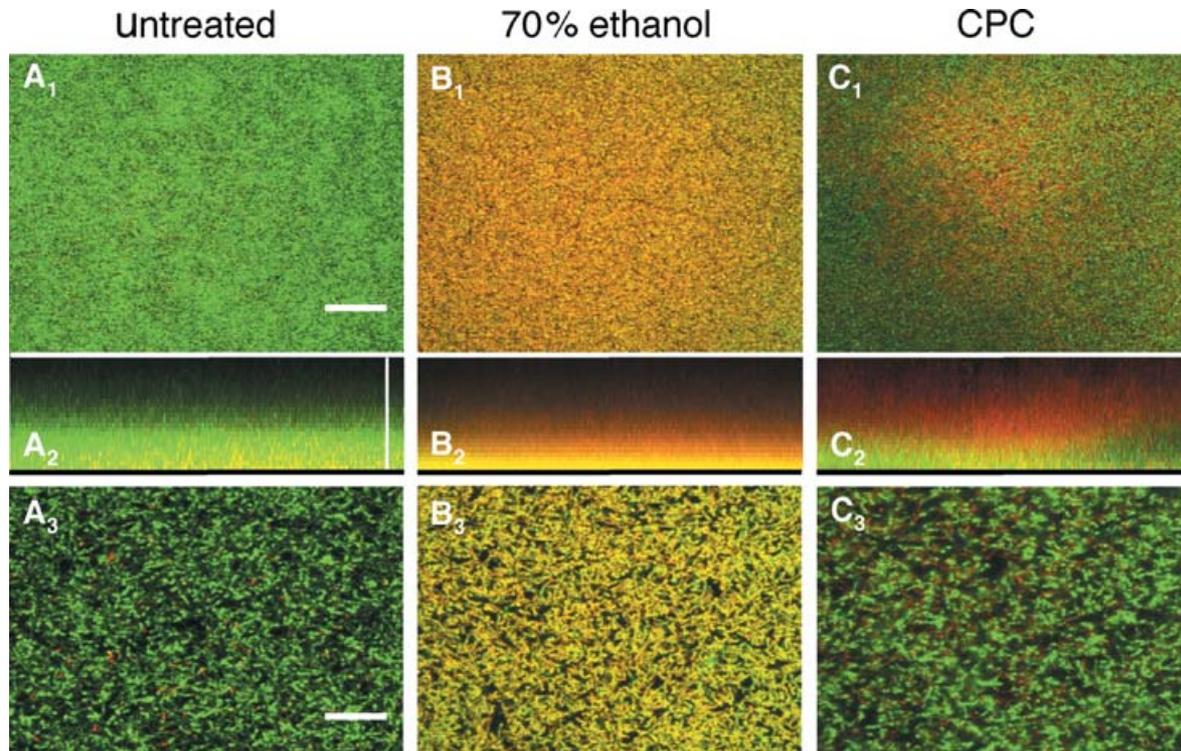
To quantify the percentage of viable cells in each treatment the fluorescence intensities of the confocal micrographs were separated on the basis of green and red pixels using image analysis software. A threshold was set for both the red and green pixels and the total area with green fluorescence was calculated for each treatment (Fig. 5). The resulting values were then compared to assess statistical significance. One-way ANOVA revealed a statistically significant difference between the mean

values ( $P < 0.001$ ) of the antimicrobial treatments. A Tukey's nonparametric pairwise comparison indicated that there was a statistically significant difference ( $P < 0.05$ ) between the untreated biofilms and 70% ethanol, essential oils (EO), triclosan (TR) and chlorhexidine (CHX) 60 s treated biofilms. Untreated biofilms were not significantly different from CPC 30 s, CPC 60 s and CHX 30 s treated biofilms (Fig. 5).

Some variation in the thickness of oral biofilm growth was observed throughout this study. To determine whether significant differences occurred in biofilm thickness between treatments, ten random samples were selected throughout each flowcell track and the biofilm thickness was measured using confocal microscopy z-sectioning. The average thickness of the biofilms was 53 μm and random measurements were statistically compared using a two-sample t-test. The analysis indicated that the thickness of the biofilms between treatments was not statistically different at the 95% confidence level ( $P < 0.05$ ).

## DISCUSSION

The results of this study demonstrate that (1) the saliva-conditioned flowcell provides a reproducible and quantitative tool for the testing of multiple antimicrobial



**Fig. 3:** Effect of cetylpyridinium chloride/domiphen bromide (CPC) on 12 h biofilms. Panels A<sub>1</sub>, A<sub>2</sub>, and A<sub>3</sub> are untreated negative controls as described in Fig. 1. Panels B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub> are positive controls treated with 70% ethanol, as described in Fig. 1. (C<sub>1</sub>) Biofilm treated with CPC shows very little membrane damage after 60 s exposure. (C<sub>2</sub>) A three-dimensional reconstruction of C<sub>1</sub> rotated 90° in the *x-z* direction, demonstrating the inability of this antimicrobial agent to cause membrane damage (red) throughout the thickness of the biofilm. (C<sub>3</sub>) High-magnification confocal image of CPC-treated biofilm showing that most of the cells are fluorescing green, indicative of viability. Scale bars: 50 μm (A<sub>1</sub>, A<sub>2</sub>, B<sub>1</sub>, B<sub>2</sub>, C<sub>1</sub>, C<sub>2</sub>), and 10 μm (A<sub>3</sub>, B<sub>3</sub>, C<sub>3</sub>). Black line in A<sub>2</sub>, B<sub>2</sub>, and C<sub>2</sub> indicates substratum surface.

agents *in situ*, and (2) the four tested antimicrobial agents had various levels of effectiveness on *S. gordonii* monoculture biofilms.

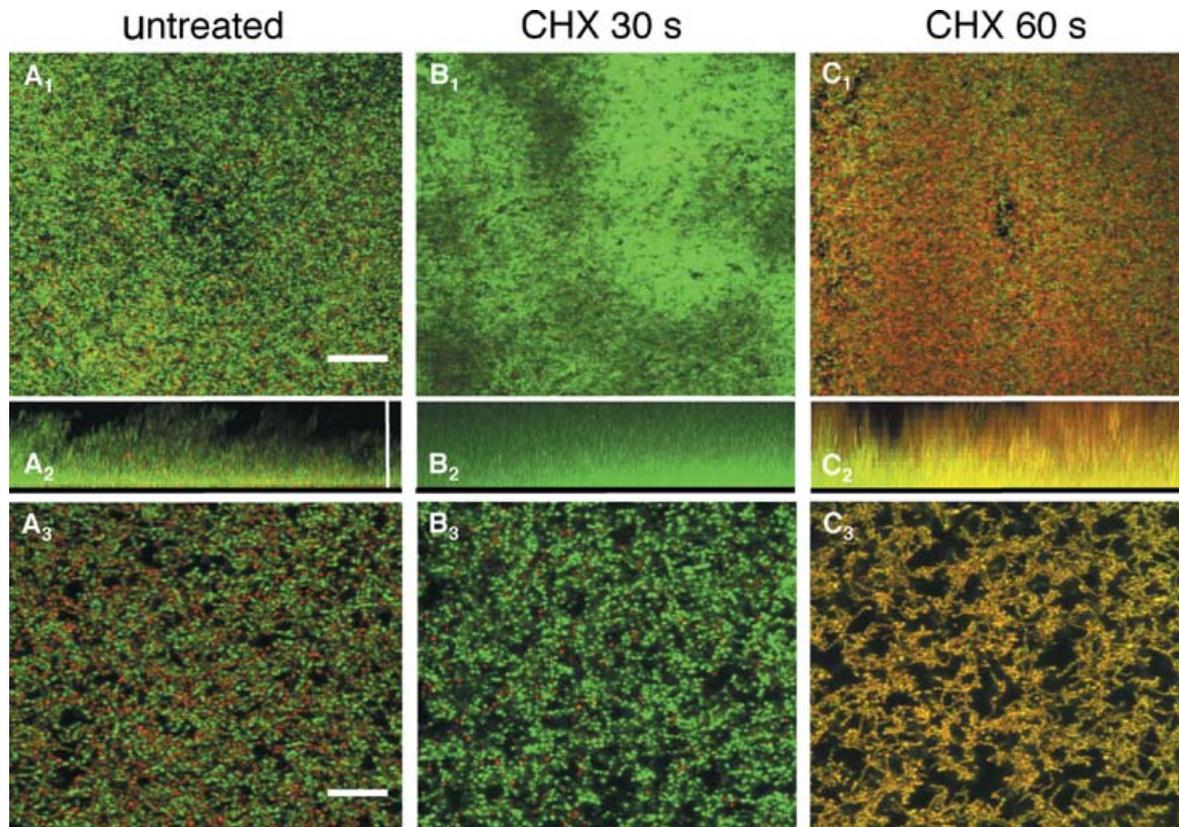
By using a saliva-conditioned flowcell, antimicrobial agents can be tested *in situ* on undisturbed biofilms thus providing spatial information on the efficacy of the different agents. Many of the previous methods measured the efficacy of antimicrobials on planktonic monocultures, not biofilms (Fine *et al.*, 2000; Pan *et al.*, 2000). Studies have shown that the susceptibility of oral bacteria to antimicrobials decreases when bacteria are in biofilms (Brown *et al.*, 1988). By using flowcell-grown biofilms the efficacy of antimicrobials can be tested and analyzed on naturally forming dental plaque *in situ*, thus overcoming many of the caveats associated with previously described methods.

An additional attribute, although not unique to this model system, is that biofilms grown in the flowcell can be manipulated to contain a specific mixture of organisms. However, in order to complement many previous studies examining the effects of antimicrobial agents on oral bacteria, we used a monoculture biofilm of *S. gordonii*, a known early colonizer that grows well in monoculture and in mixed-species biofilms (Palmer *et al.*, 2001). This simplified model enables the testing of various agents on a known oral bacterial species *in situ*, and differs from

those studies in which the antimicrobials were tested on bacterial cultures or disassociated biofilms. Information from monoculture studies with a variety of species can be used as a basis for additional studies with combinations of several known species or biofilms formed from a natural inoculum.

Of the antimicrobials tested, essential oils and TR were the most effective in causing damage to the plasma membranes of the *S. gordonii* biofilms in 30 s. Both CHX and CPC caused little if any visible membrane damage after a 30 s incubation. After 60 s, however, CHX caused a significant increase in membrane damage. These results are consistent with previous studies examining the effects of TR and CHX on oral biofilms (Pratten *et al.*, 1998; Zaura-Arite *et al.*, 2001), although in those studies the exposure time to CHX was 60 s or greater. In this study, we attempted to mimic exposure times often used in *in vivo* clinical studies (i.e. 30 and 60 s) (Moran *et al.*, 1997; Fine *et al.*, 2000; Pan *et al.*, 2000).

Although variation of biofilm thickness was not statistically significant between flowcells, one source of possible variation in thickness could be the result of differences in the acquired pellicle of host-derived proteins from saliva on the glass surface of the flowcell. To reduce inherent variations, saliva used in these experiments was collected and pooled from six or more individuals.



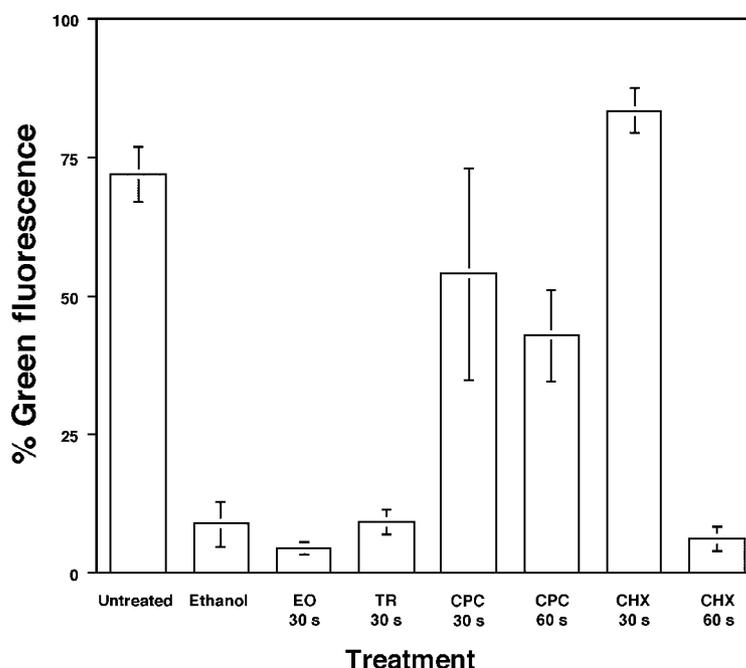
**Fig. 4:** Effect of chlorhexidine (CHX) on 12 h biofilms. Panels A<sub>1</sub>, A<sub>2</sub>, and A<sub>3</sub> are untreated negative controls as described in Fig. 1. (B<sub>1</sub>) Representative image of biofilm treated for 30 s with CHX. (B<sub>2</sub>) A three-dimensional reconstruction of B<sub>1</sub> rotated 90° in the *x*-*z* direction. (B<sub>3</sub>) High-magnification image of biofilm treated for 30 s with CHX. Biofilms are labeled extensively with SYTO 9, suggesting viability. (C<sub>1</sub>) Confocal image of biofilms treated with CHX for 60 s showing more membrane damage (red) than in 30 s treatment. (C<sub>2</sub>) A 90° rotation of a three-dimensional reconstruction of C<sub>1</sub>. (C<sub>3</sub>) High-magnification micrograph of a biofilm treated with CHX for 60 s showing extensive labeling of propidium iodide and SYTO 9 (yellow), thus indicating membrane damage. Scale bars: 50 μm (A<sub>1</sub>, B<sub>1</sub>, C<sub>1</sub>), 55 μm (A<sub>2</sub>, B<sub>2</sub>, C<sub>2</sub>), and 10 μm (A<sub>3</sub>, B<sub>3</sub>, C<sub>3</sub>). Black line in A<sub>2</sub>, B<sub>2</sub>, and C<sub>2</sub> indicates substratum surface.

Results from previous studies have shown that *S. gordonii* DL1 consistently grows planktonically to densities  $\geq 2 \times 10^7$  cells per ml in different samples of pooled saliva (Palmer *et al.*, 2001), thus making the pooled saliva an unlikely source of significant variation.

In three of the four antimicrobials, complete penetration of the agents throughout the biofilms was observed within 60 s as indicated by the fluorescent staining. Evidence of membrane damage extended from the glass substratum towards the lumen of the flowcell. In biofilms tested with the agent CPC, it remained unclear why the product did not cause widespread membrane damage. Several possibilities include: (1) that the molecule may not have been able to completely permeate the biofilms; (2) that the molecule could penetrate but was unable to cause membrane damage within the tested time period; or (3), as CPC diffused though the biofilm, its efficacy may have been reduced as it reacted with the cells of the biofilm. Despite this uncertainty with CPC, the vitality stains completely permeated the biofilm as visualized with CLSM. Another observation suggesting that the CPC molecule was able to completely penetrate was the presence of spaces (2 to 10 μm) between the cells

of the growing biofilm. Studies have shown that dextran beads with a relative molecular mass ( $M_r$ ) of 70 000 and immunoglobulin (Ig) G molecules with  $M_r$  up to 150 000 are able to successfully penetrate biofilms up to 60 μm (Thurnheer *et al.*, 2002). The relative molecular mass of CPC (cetylpyridinium chloride  $M_r$  400, and domiphen bromide  $M_r$  414) as well as the solubility of CPC in water suggest that CPC has the capacity to permeate the entire biofilm thickness, although its diffusion and reaction capabilities with the biofilm have yet to be tested in this *in vitro* flowcell model.

Regardless of the antimicrobial agent examined in this study, high magnification confocal micrograph images revealed that some of the bacterial cells were unaffected by the various treatments. These viable, intact cells were randomly located throughout the growing community. Previous studies have shown that, as compared with planktonic forms, bacteria associated in biofilm communities are 10 to 1000 times more likely to be resistant to antimicrobial agents (Fine *et al.*, 2001; Mah & O'Toole, 2001). Many biofilms utilize alternative resistance mechanisms such as reduced growth and stress responses to avert the cidal effects of the antimicrobial (Foley *et al.*,



**Fig. 5:** Efficacy of antimicrobial treatments on 12 h biofilms measured with image analysis software showing the percentage of confocal micrographs staining with SYTO 9, an indicator of viable cells. Each bar represents the average value obtained with at least four confocal micrographs (standard error of the mean is indicated), (EO, essential oils; TR, triclosan; CPC, cetylpyridinium chloride/domiphen bromide; and CHX, chlorhexidine).

1999). Another factor known to alter the response of biofilms to antimicrobial agents is age. Studies have shown that, as a biofilm matures, cells are less susceptible to the effects of antimicrobial agents (Carpentier & Cerf, 1993; Leriche *et al.*, 2003). In this study we examined 12 h biofilms to approximate the length of time between normal oral hygiene procedures. Additional time points would be required to examine whether there is an age-related reduction in the efficacy of those antimicrobial agents used in this study. These observations, however, reinforce the need to examine the effects of antimicrobials on undisturbed oral biofilms *in situ* rather than on planktonic bacterial cultures or disassociated biofilms.

This study represents an alternative, noninvasive method for the examination of antimicrobials on intact oral bacterial biofilms. The method complements current clinical testing methods; however, this technique is unique in that it mimics the natural salivary flow conditions of the human mouth and yet does not require human subjects for testing. Future work could utilize this method to rapidly test new antimicrobial agents on biofilms containing specific target species, mixed-species of a defined composition, or natural saliva inocula without disruption to the growing biofilm.

## ACKNOWLEDGEMENTS

The authors thank R. J. Palmer, P. Egland and D. Blehert for their useful comments on this manuscript and T. Bilbault

and M. Barnett for their continued interest in this research. The work was supported in part by a grant from Pfizer Inc.

## REFERENCES

- Brown, M. R., Allison, D. G. & Gilbert, P. (1988) Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? *Journal of Antimicrobial Chemotherapy* **22**, 777–780
- Carpentier, B. & Cerf, O. (1993) Biofilms and their consequences, with particular reference to hygiene in the food industry. *Journal of Applied Bacteriology* **75**, 499–511
- Dawes, C., Watanabe, S., Bigelow-Lecomte, P. & Dibdin, G. H. (1989) Estimation of the velocity of the salivary film at some different locations in the mouth. *Journal of Dental Research* **68**, 1479–1482
- de Jong, M. H. & van der Hoeven, J. S. (1987) The growth of oral bacteria on saliva. *Journal of Dental Research* **66**, 498–505
- Eley, B. M. (1999) Antibacterial agents in the control of supragingival plaque – a review. *British Dental Journal* **186**, 286–296
- Fine, D. H., Furgang, D., Barnett, M. L., Drew, C., Steinberg, L., Charles, C. H. & Vincent, J. W. (2000) Effect of an essential oil-containing antiseptic mouthrinse on plaque and salivary *Streptococcus mutans* levels. *Journal of Clinical Periodontology* **27**, 157–161
- Fine, D. H., Furgang, D. & Barnett, M. L. (2001) Comparative antimicrobial activities of antiseptic mouthrinses against isogenic planktonic and biofilm forms of *Actinobacillus actinomycetemcomitans*. *Journal of Clinical Periodontology* **28**, 697–700
- Foley, I., Marsh, P., Wellington, E. M., Smith, A. W. & Brown, M. R. (1999) General stress response master regulator *rpoS* is expressed in human infection: a possible role in chronicity. *Journal of Antimicrobial Chemotherapy* **43**, 164–165

- Guggenheim, B., Giertsen, W., Schupbach, P. & Shapiro, S. (2001) Validation of an *in vitro* biofilm model of supragingival plaque. *Journal of Dental Research* **80**, 363–370
- Hope, C. K., Clements, D. & Wilson, M. (2002) Determining the spatial distribution of viable and nonviable bacteria in hydrated microcosm dental plaques by viability profiling. *Journal of Applied Microbiology* **93**, 448–455
- Hope, C. K. & Wilson, M. (2003) Measuring the thickness of an outer layer of viable bacteria in an oral biofilm by viability mapping. *Journal of Microbiological Methods* **54**, 403–410
- Leriche, V., Briandet, R. & Carpentier, B. (2003) Ecology of mixed biofilms subjected daily to a chlorinated alkaline solution: spatial distribution of bacterial species suggests a protective effect of one species to another. *Environmental Microbiology* **5**, 64–71
- Leyes Borrajo, J. L., García Varela, L., López Castro, G., Rodríguez-Nuñez, I., García Figueroa, M. & Gallas Torreira, M. (2002) Efficacy of chlorhexidine mouthrinse with and without alcohol: a clinical study. *Journal of Periodontology* **73**, 317–321
- Mah, T. F. & O'Toole, G. A. (2001) Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology* **9**, 34–39
- Moran, J., Addy, M. & Newcombe, R. (1997) A 4-day plaque regrowth study comparing an essential oil mouthrinse with a triclosan mouthrinse. *Journal of Clinical Periodontology* **24**, 636–639
- Moran, J., Addy, M., Jackson, R. & Newcombe, R. G. (2000) Comparative effects of quaternary ammonium mouthrinses on 4-day plaque regrowth. *Journal of Clinical Periodontology* **27**, 37–40
- Nyvad, B. & Kilian, M. (1987) Microbiology of the early colonization of human enamel and root surfaces *in vivo*. *Scandinavian Journal of Dental Research* **95**, 369–380
- Palmer, R. J. & Caldwell, D. E. (1995) A flowcell for the study of plaque removal and regrowth. *Journal of Microbiological Methods* **24**, 171–182
- Palmer, R. J. Jr, Kazmerzak, K., Hansen, M. C. & Kolenbrander, P. E. (2001) Mutualism versus independence: strategies of mixed-species oral biofilms *in vitro* using saliva as the sole nutrient source. *Infection and Immunity* **69**, 5794–5804
- Pan, P., Barnett, M. L., Coelho, J., Brogdon, C. & Finnegan, M. B. (2000) Determination of the *in situ* bactericidal activity of an essential oil mouthrinse using a vital stain method. *Journal of Clinical Periodontology* **27**, 256–261
- Pratten, J., Wills, K., Barnett, P. & Wilson, M. (1998) *In vitro* studies of the effect of antiseptic-containing mouthwashes on the formation and viability of *Streptococcus sanguis* biofilms. *Journal of Applied Microbiology* **84**, 1149–1155
- Pratten, J., Wilson, M. & Spratt, D. A. (2003) Characterization of *in vitro* oral bacterial biofilms by traditional and molecular methods. *Oral Microbiology and Immunology* **18**, 45–49
- Thurnheer, T., Gmur, R., Shapiro, S. & Guggenheim, B. (2002) Time and size dependent diffusional transport of dextrans and other macromolecules in an *in vitro* oral biofilm model. *Journal of Dental Research* **81**, A-393
- van der Hoeven, J. S., Cummins, D., Schaeken, M. J. & van der Ouderaa, F. J. (1993) The effect of chlorhexidine and zinc/triclosan mouthrinses on the production of acids in dental plaque. *Caries Research* **27**, 298–302
- Wecke, J., Kersten, T., Madela, K., Moter, A., Gobel, U. B., Friedmann, A. & Bernimoulin, J. (2000) A novel technique for monitoring the development of bacterial biofilms in human periodontal pockets. *FEMS Microbiology Letters* **191**, 95–101
- Whittaker, C. J., Klier, C. M. & Kolenbrander, P. E. (1996) Mechanisms of adhesion by oral bacteria. *Annual Review of Microbiology* **50**, 513–552
- Wilson, M. & Pratten, J. (1999) Laboratory assessment of antimicrobials for plaque-related diseases. In *Dental Plaque Revisited, Oral Biofilms in Health and Disease, Proceedings of the Royal College of Physicians Conference*, pp. 503–521. Edited by H. Newman & M. Wilson. Antony Rowe Ltd, Wilts., UK
- Zaura-Arite, E., van Marle, J. and ten Cate, J. M. (2001) Confocal microscopy study of undisturbed and chlorhexidine-treated dental biofilm. *Journal of Dental Research* **80**, 1436–1440