Effects Of Dynamic Fluid Activity From An Electric Toothbrush On *In Vitro* Oral Biofilms

Running Title: Biofilm removal by electric toothbrush

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ABSTRACT

Objectives. To determine the plaque removing ability of a Sonicare Plus® electric toothbrush in an *in vitro* model.

Materials and Methods. Multi-species oral biofilms derived from human saliva were grown on hydroxyapatite discs in a constant-depth film fermenter. The biofilms were placed in a typodont model so that they mimicked the interproximal plaque between teeth 46 and 47 and were then treated with an electric toothbrush, both activated and inactivated. The distance from the bristle tips to the edge of the disc was 2.65mm. Brushing action was controlled by a specially-constructed brushing machine. After brushing, the number of viable bacteria removed from, and remaining in, the biofilms were determined.

Results. 73.70% of viable bacteria in the biofilms were dislodged from the discs using the activated toothbrush An inactivated toothbrush removed only 3.66%. Scanning electron microscopy and confocal microscopy revealed differences between untreated and treated biofilms.

Conclusion. The fluid shear forces generated by the electric toothbrush penetrated at least 2.65 mm beyond the reach of the bristles and these forces contributed to the toothbrush's plaque-removal ability ($P<0.001$).
Keywords: biofilm, plaque, sonic toothbrush, constant-depth film fermenter

INTRODUCTION

Dental plaque is a biofilm that forms at the solid-liquid interface between the tooth and saliva (Wilson, M.2001). Dental caries is a plaque-associated disease characterised by a progressive disintegration of the tooth structure (Kidd, E. A. M & Joyston-Bechal, S.1997). Caries prevention is achieved by the removal of plaque by brushing and a number of electric devices are now available for this purpose (Fischman, S. L.1997). Electric toothbrushes commonly consist of a vibrating and/or oscillating brush head. There is evidence to suggest that plaque removal by electric toothbrushes is better than that achieved by manual brushes (van der Weijden, G. A. et al.1998) and that by regular use, electric toothbrushes can significantly improve periodontal health (Robinson, P. J. et al.1997;Ho, H. P. & Niederman, R.1997).

An electric toothbrush (Sonicare Plus®) has been developed by Philips Oral Healthcare (Snoqualmie, USA) which operates at a frequency of 260 Hz and removes plaque by generating localised hydrodynamic shear forces (Khambay, B. S. & Walmsley, A. D.1995). Microelectronics within the brush handle produce a rapidly-oscillating magnetic field which induces an oscillation in the brush head. The frequency and amplitude of oscillation produces a bristle tip velocity that, when inserted in a fluid/air environment, creates turbulent fluid and bubble activity and associated shear forces. *In*

vivo studies have shown the superior plaque removing ability of this brush compared to manual brushing (Tritten, C. B. & Armitage, G. C.1996;Stanford, C. M. et al.1997). However, confounding factors with *in vivo* plaque removal studies are the poor reproducibility of results, the inability to standardise treatment and variations in the human oral microflora. Patients involved in any form of dental regime tend to improve their oral hygiene, additionally, such studies are often conducted in academic research institutions where the 'dental IQ' of patients is relatively high (Overholser, C. D1988). A procedure, which has the advantage of the reproducibility of an *in vitro* model system married to the realism of *in vivo* plaque, was therefore developed for examining the plaque removal efficacy of an electric toothbrush. Both activated and inactivated electric toothbrushes can be studied to investigate the ability of fluid motion generated by an activated toothbrush's bristles to remove plaque in the absence of direct bristle contact.

The purpose of this study was to determine and quantify the effects of the fluid activity, beyond the bristles, delivered by an electric toothbrush on microcosm dental plaques grown on hydroxyapatite (HA) discs in a constant depth film fermenter (CDFF) under conditions similar to those which would exist *in vivo*.

MATERIALS AND METHODS

Preparation of Constant-depth film fermenter

Oral biofilms were grown in a CDFF (University of Wales, Cardiff, UK). The CDFF consists of a rotating turntable holding 15 polyterafluoroethylene (PTFE) pans, each pan containing five cylindrical holes filled by PTFE plugs. The CDFF was loaded with seventy five, 5 mm diameter HA discs (Clarkson Chromatography Products, South

Williamsport, USA) which were inserted on top of the PTFE plugs and recessed to a depth of 200 µm before autoclaving at 121ºC for 30 minutes. Following inoculation, nutrient fluid was dripped onto the turntable, spread over the discs and removed by the action of scraper blades giving biofilms of a fixed depth. The *modus operandi* for the CDFF (Peters, A. C & Wimpenny, J. W.1988) has undergone an evolution with experience at the Eastman Dental Institute, specifically for growing models of dental plaque (Wilson, M. et al.1995;Pratten, J. & Wilson, M.1999;Pratten, J. et al.1998b;Roberts, A. P. et al.2001),

Inoculation of the CDFF

Saliva was collected from 20 individuals (aged 20-40 years, in good oral health, a mixture of non-smokers and smokers) in sterile containers. 5 ml of phosphate buffered saline (PBS) was added to each of the samples and these were pooled. Glycerol (BDH Chemicals, Poole, UK) was added to 15% volume, the mixture divided as 2 ml aliquots in cryo-vials and stored at -80ºC. For each fermenter run, a thawed 2 ml aliquot was aseptically added to a flask containing 1 litre of an artificial saliva containing hog gastric mucin, without urea (Pratten, J. et al.1998a) at 37ºC. The inoculum was then pumped into the sterile CDFF at a rate of 0.72 l day^{-1}. Artificial saliva medium flow was then started the following day at 0.72 l day^{-1}, the mean resting saliva flow rate in humans: (Guyton, A. C & Hall, J. E.1997;Lamb, J. F et al.1991).

Biofilm Growth and Sampling

After at least 8 days growth, three sampling pans were removed from the CDFF and 10 of the 15 discs were used for treatment with a Sonicare Plus. The operation of the CDFF demands that the PTFE pans are removed sequentially, so sample randomisation was

incorporated by requesting impartial colleagues, blinded as to the subsequent treatment, to select biofilms.

Biofilm Challenging with Sonicare Plus

A pair of HA discs were carefully dipped into 1 ml of sterile PBS to wash off cells present in the liquid phase. The discs were then inserted into recesses located in plastic teeth (figure 1c) designed to simulate inter-proximal plaque between teeth 46 and 47. The teeth containing the discs were then placed in an exposure chamber (figure 1d) mounted on a specially-constructed brushing machine (figure 2). The exposure chamber was filled with 7ml PBS containing 0.8 g l^{-1} gastric mucin. Using an "activated" brush (i.e. with its electronically induced vibrating action operating), a reciprocating brushing action of 0.26 Hz was initiated with the brush at an angle of 40º with a horizontal and vertical load of 62 g (±5g; 88g vector total) between the brush and teeth, this was in accordance with the manufacturers data associated with typical use. The distance travelled by the brush during one brushing cycle was 9.5 mm and the duration of brushing (exposure time) was 15 seconds. The above procedure was repeated but with the brush in an "inactivated" condition (i.e. with its electronically induced vibrating action not operating).

In order to document the separation between the discs and the bristles of the brush when activated, the teeth were marked with lipstick which could easily be removed when contacted by the moving bristles (Figures 1a and 1b). The brushing machine was set up as described previously, but without PBS in the sample holder, because the aim was to determine whether there was any contact between the bristles and the discs rather than the

extent of any fluid shear activity. The experiment was conducted five times, returning ten bristle / disc separation values.

Post-Treatment Sampling

After the brushing, 5 ml of the suspension was transferred from the sample holder into a sterile container with glass beads. The samples were vortex-mixed for 30 seconds to break up bacterial clumps and serial dilutions prepared in PBS.

To determine the number of viable cells remaining on the discs after treatment, these were placed into 7 ml of sterile PBS containing glass beads, vortex-mixed for 30 seconds and serial dilutions prepared in sterile PBS. The discs were examined by SEM to determine whether all adherent bacteria were removed by vortex mixing. Aliquots of the dilutions were plated in quadruplicate onto blood agar (BA) (Becton Dickinson, Franklin Lakes, USA) for total anaerobic counts, Mitis salivarius agar (MSA) (Difco Laboratories, Detroit, USA.) for *Streptococcus* species, *Veillonella* agar (VA) (Difco) for the isolation of *Veillonella* species and cadmium fluoride-acriflavin-tellurite (CFAT) agar (Zylber, L. J. & Jordan, H. V.1982) for the isolation of *Actinomyces* species. All plates were incubated anaerobically at 37ºC for 48 hours and the resulting colonies counted.

Two other discs were brushed as mentioned previously whilst another two were only dipped in sterile PBS before microscopic examination by either scanning electron microscopy (SEM) or confocal laser scanning microscopy (CLSM) using LIVE/DEAD® *Bac*Light™ staining (Molecular Probes, Eugene, USA) to differentiate between viable and non-viable cells on the basis of membrane integrity.

Statistical Analysis

Where appropriate, confidence intervals were calculated at 95% to give a range of values, consistent with the data, that is believed to encompass the actual or "true" population value. P-values were calculated to measure the significance of the statistical evidence in favour of the null hypothesis (the activated and inactivated toothbrushes remove the same amount of bacteria).

RESULTS

Figures 1a and 1b show the results of a typical experiment to determine how close the brush bristles came to the hydroxyapatite discs. It can be seen in 1b that some of the lipstick was removed from the teeth and that this zone did not make contact with the recess holding the disc. The distance between the edge of the brushed zone and the closest edge of the disc recess was 2.65 mm (2.597 to 2.703 mm with 95% confidence intervals).

The proportions of the different bacterial species present in the biofilms (measured as viable counts on the selective agars) were 32.82% *Streptococcus* spp., 33.20% *Veillonella* spp. and 33.98% *Actinomyces* spp. The average density of bacteria in the biofilms was 1.03×10^7 cfu per mm^2 (6.10×10^6 to 1.45×10^7 cfu per mm^2 with 95% confidence intervals).

SEM of untreated biofilms showed structures typically found in *in vivo* plaques such as chains of streptococci (figures 4a and 4c) and 'corn-cobs'. CLSM of the biofilms showed a complex structure with chains of streptococci and water channels. Vital staining of the biofilms indicated that the majority of the live bacteria present were cocci.

The data obtained from the series of nine experiments (quadruplicate counts taken from each experiment, n=31 to 36) are summarised in figures 3a and 3b. These show that the total number of viable bacteria removed (in terms of viable counts on blood agar) by an activated toothbrush was 4.49×10^6 cfu mm^{-2} (3.59×10^6 to 5.39×10^6 with 95% confidence intervals) while an inactivated brush removed only 1.27×10^5 cfu mm^{-2} (7.27×10^4 to 1.81×10^5 with 95% confidence intervals). The activated toothbrush removed significantly ($p<0.001$) more bacteria than the inactivated brush for all bacteria counted on the different selective media.

The percentage of bacteria removed (based upon the sum of the number of bacteria removed and the number of bacteria remaining on the HA discs) by an activated toothbrush was 73.70% (57.70 to 89.7% with 95% confidence intervals), the percentage of bacteria removed by an inactivated toothbrush was 3.66% (0.48 to 6.84% with 95% confidence intervals). The activated toothbrush removed a significantly ($p<0.001$) higher percentage of bacteria than the inactivated brush for all bacteria counted on the different selective media.

CLSM images of untreated biofilms showed few non-viable cells (figures 4e and 4f). Treated biofilms contained only small amounts of residual biofilm and these fragments were viewed by CLSM. This showed that the viable cells (green channel) were mostly cocci (figure 4g) conversely, the dead cells were predominantly filamentous (red channel) (figure 4h).

DISCUSSION

Numerous studies have used the CDFF to grow biofilms with a composition and structure

similar to those found in supragingival dental plaque *in vivo*. The microcosm plaques grown in this series of experiments were similar in composition to those found in approximal dental plaques *in vivo* (Marsh, P. D. & Martin, M. V.1999). Prior to treatment, typical plaque structures such as chains of streptococci and 'corn-cobs' were visible by SEM. CLSM of the biofilms showed the presence of water channels and 'stacks' typical of many biofilms and previously found in oral biofilms grown *in vitro* and *in vivo* (Pratten, J. et al.2000;Wood, S. R. et al.2000). After treatment with an activated toothbrush, very few chains of streptococci could be seen in the remaining biofilm, however many individual cocci were still evident as were an increased number of filamentous morphotypes.

With regard to the removal of biofilms from the discs, the design of the mouth model and brushing machine used enabled the reproducible determination of the efficacy of a particular brushing regime in removing interproximal plaque. The presence of a mucin-containing fluid in the mouth model simulates the saliva present *in vivo*. This represents a better simulation of the *in vivo* situation than liquid-free models that have been used previously (Driesen, G. M. et al.1998)

A considerably greater proportion of the biofilm was removed from the hydroxyapatite discs when activated (73.70%) rather than the inactivated (3.66%) toothbrush was used. Biofilm removal from HA discs treated with an activated toothbrush tended to exhibit a definite 'cleared zone' proximal to the bristles. SEM images of the residual biofilms further away from these cleared zones showed few streptococcal chains with a visibly increased proportion of filamentous bacteria. Typical SEM images showed the biofilm to have an upper layer of streptococci with lower layers

of flilamentous bacteria (*Actinomyces* species and a number of spirochaete morphotypes). The upper layer of the biofilm was often cracked by SEM sample preparation, further revealing the details of the layered differentiation. It is suggested that the liquid shear forces generated by the sonic toothbrush affect, for the most part, the outer layers of dental plaque.

Statistical analysis of counts obtained from the selective agars elude to the preferential removal of *Streptococcus* species over *Actinomyces* species ($p = 0.183$) by Sonicare Plus and fewer streptococci remaining in the biofilm after brushing than *Veillonella* species ($p=0.110$) or *Actinomyces* species ($p=0.095$). While not *statistically* significant these data support the SEM observation of the preferential removal of streptococci.

Biofilms treated with an inactivated brush were relatively unaffected with very few viable bacteria removed from the discs. This is because the bristles of the brush did not make contact with the biofilms at any point during the experiment and there was insufficient kinetic energy in the brushing action alone to generate the fluid shear forces necessary to remove a significant number of bacteria beyond the reach of the bristles.

The disparity in cell viability between streptococcal chains and filamentous bacteria, as revealed by viable staining, after brushing suggests that spherical bacteria are more resilient than filamentous bacteria to the liquid shear forces generated by the brush. The vulnerability of filamentous organisms to liquid shear is likely to be due to a (relatively) long filament crossing a range of shear gradients. This would also explain the breaking up of streptococcal chains into their component cocci in treated samples.

In conclusion, the amount of biofilm removed (in terms of viable bacteria) by an activated Sonicare Plus was twenty times greater than that removed by an inactivated brush. This implies that the fluid shear effects induced by the oscillation of the brush head significantly contribute to the plaque removing ability beyond the bristles. The fluid shear forces induced by the activated toothbrush are of sufficient magnitude to remove oral biofilms from a distance of at least 2.65 mm from the bristles.

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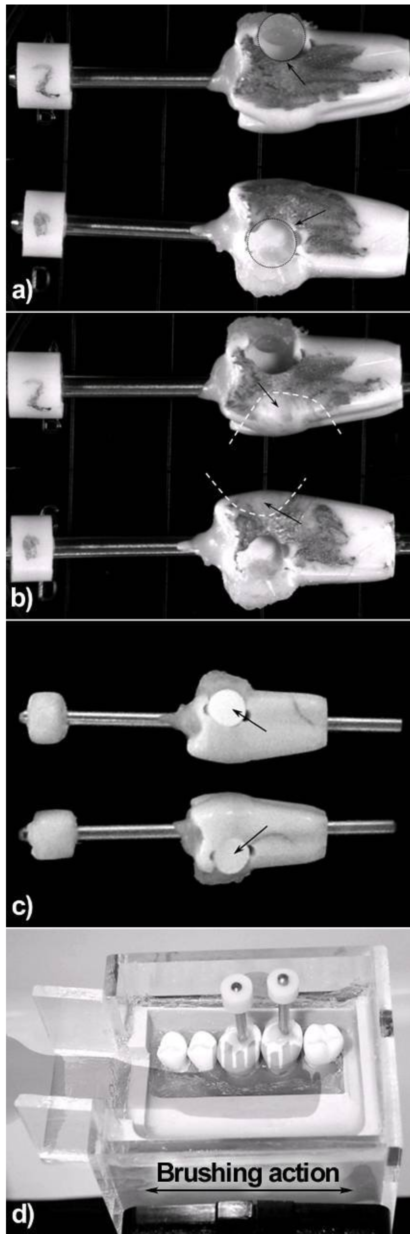


Figure 1. a) Model teeth covered in a standard cosmetic lipstick. The recesses for holding the 5 mm HA discs are shown by the arrows. b) Model teeth after brushing by an active Sonicare Plus without a liquid medium present to measure the separation between the disc recesses and the zone of contact by the bristles (bounded by the dashed line). The mean separation was measured at 2.65 mm. c) Model teeth with the 5 mm HA discs located in the recesses. The surface of the HA discs are flush with the contours of the teeth. d) Model teeth located in the exposure chamber. The two HA discs were adjacent to each other to represent the site of interproximal plaque. The approximate position of the brush is (shown as a shadow) is for illustrative purposes only. The direction of the 9.5 mm brushing action is perpendicular to the biofilm coated surfaces of the HA discs.

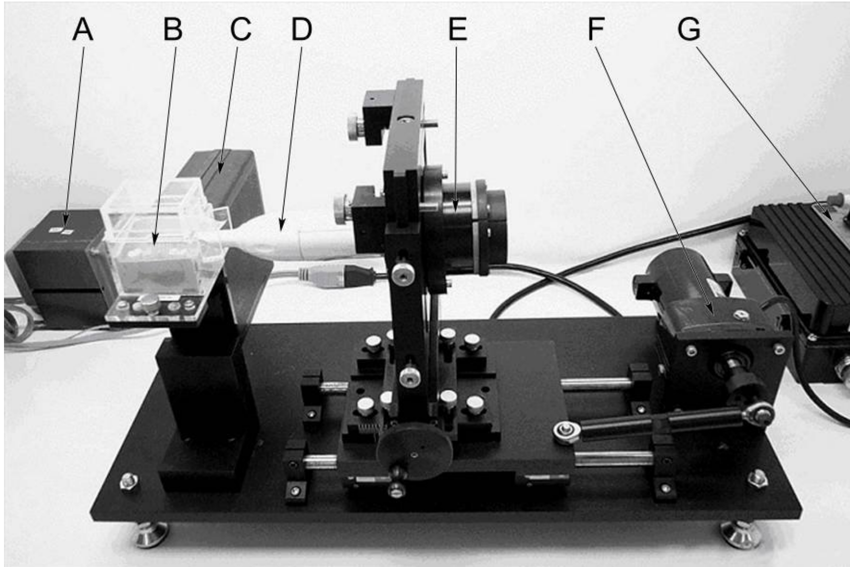
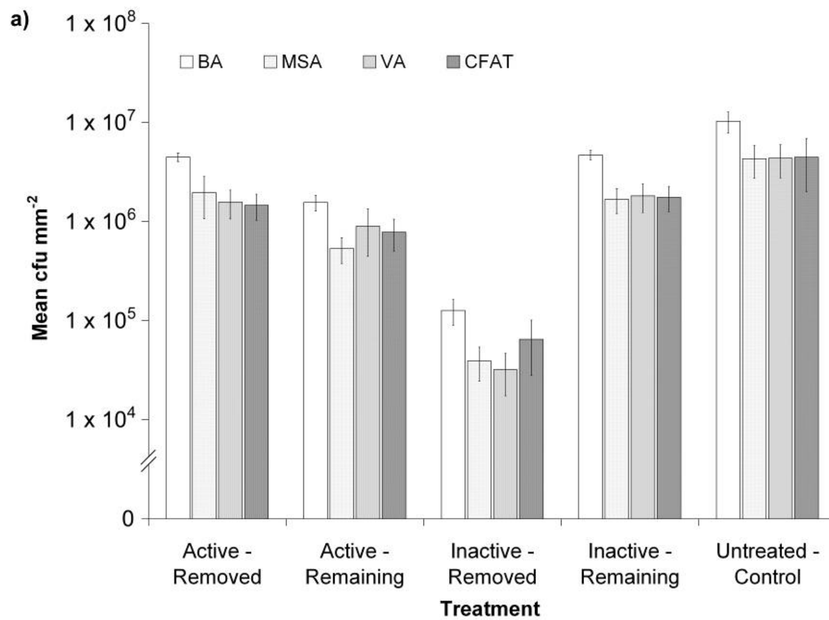


Figure 2. The brushing machine. A) Transformer. B) Exposure chamber. C) Load cell, for measuring the vertical and horizontal load between the brush and the exposure chamber (teeth and typodont). D) Sonicare Plus toothbrush. E) Toothbrush holder, containing a mechanism for the fine adjustment of the position of the toothbrush to achieve the correct loads and position of the brush head against the gingival margin. F) Electric motor with eccentric cam, for generating the 9.5 mm linear displacement of the toothbrush holder. G) Motor speed controller, normally set at 0.26 Hz (4 cycles during the 15 seconds exposure)



b)

Biofilm Removed (%)	Selective Media Type BA	MSA	VA	CFAT
Sonicare Active	73.70 ± 16.00	72.86 ± 20.25	71.29 ± 22.00	69.10 ± 22.26
Sonicare Inactive	3.66 ± 3.18	9.33 ± 11.47	7.88 ± 9.65	8.83 ± 11.69

Figure 3. a) The number of cfu mm^{-2} of biofilm for different samples obtained from the brushing experiments, bacteria removed and remaining on the HA disc with active or inactive brushes on four different media, blood agar (BA) non-selective growth, *Mitis salivarius* agar (MSA) for the selective growth of *Streptococcus* species, *Veillonella* agar (VA) for the selective growth of *Veillonella* species and cadmium fluoride-acriflavin-tellurite (CFAT) agar for the selective growth of *Actinomyces* species.. Error bars represent confidence intervals at 95%. The p-values associated with a comparison between active and inactive brushes (for both the number of bacteria removed and the number of bacteria remaining) were significant in every case (i.e. p=0.001 or less). b) Table showing the percentage of biofilm removed from HA discs by active and inactive brushes. These percentages were calculated based upon the sum of the numbers of bacteria removed and the numbers of bacteria remaining. The results of the four selective media types were significant in each case (i.e. p=0.001 or less).

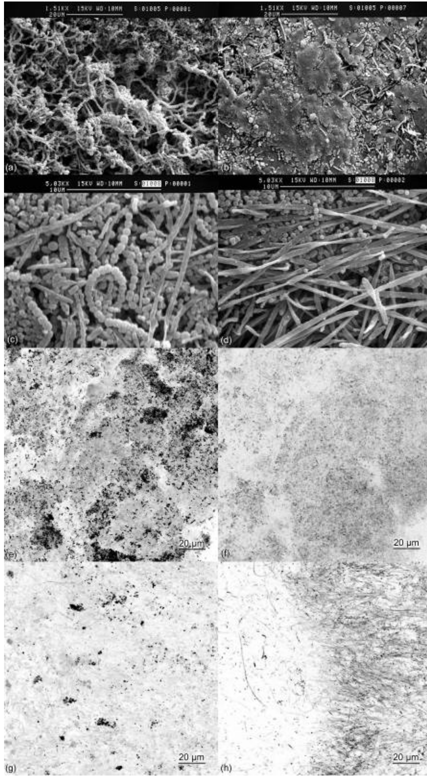


Figure 4. Microscopic analysis of treated and untreated biofilms, a-d scanning electron micrographs, e-f confocal laser scanning micrographs (shown as negatives for clarity). a) SEM (x 1 510) of plaque biofilm grown in a CDFF, on a hydroxyapatite substratum, showing some of the structural motifs found in *in vivo* plaque biofilm such as *Streptococcus* chains. b) SEM (x 1 510) of plaque biofilm grown in a CDFF, on a hydroxyapatite substratum, treated with an active Sonicare Plus showing the bare surface of the disc with a small number of bacteria retained in depressions. This area of disc was proximal to the site of brushing. c) SEM (x 5 030) of plaque biofilm grown in a CDFF, on an untreated HA disc, representative of the predominant morphotype including intact *Streptococcus* chains. d) SEM (x 5 030) of HA disc treated with active Sonicare Plus representative of the predominant morphotype including filamentous bacteria and individual cocci remaining after treatment with Sonicare Plus. This area of biofilm was located away from the site of brushing. e-f) CLSM projection image (159 x 159 µm: image depth = 76 µm), of untreated biofilm showing viable bacteria (e) and non-viable bacteria (f) from the exactly the same location. g-h) CLSM projection image (159 x 159 µm: image depth = 19.86 µm) of treated biofilm with an active Sonicare Plus showing viable bacteria (g) and non-viable bacteria (h) from exactly the same location. Non-viable filamentous bacteria can be seen on the right-hand side of figure h, suggesting that filamentous bacteria are susceptible to membrane damage by the shear forces produced by an active Sonicare Plus.