Removal of oral biofilm by sonic phenomena

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ABSTRACT: ***Purpose:*** To investigate the role of sonic acoustic waves and related phenomena in removing a model plaque from a surface, and to determine if there was an optimal frequency for sonic-type toothbrushes. ***Methods:*** A mechanical system was built in which submerged biofilms of *Streptococcus mutans* were exposed to sonic energy in the range from 80 to 1,000 Hz. The system was calibrated by an accelerometer and pressure transducer, and biofilm removal was measured by optical techniques. ***Results:*** The results showed that the removal was a strong function of the acoustic intensity, but there was no significant dependence upon frequency. Biofilm was removed in very small amounts (up to 2% in 10 minutes) due to acoustic energy in the absence of convective fluid flow, even at high acoustic intensities. When the acoustic action was coupled with convective fluid flow, caused by the piston and well geometry, up to 80% of a biofilm was removed at a calculated acoustic intensity of 27 W/cm^2 in 2 minutes. When gas bubbles were entrained in the fluid, the removal approached 100% under intense sonic conditions. With respect to removal of plaque on teeth, the vigorous action of flowing fluid and bubbles is expected to remove plaque, and a maximum fluid velocity is recommended rather than a particular frequency. (*Am J Dent* 2005;18:345-352).

CLINICAL SIGNIFICANCE: The removal of plaque from teeth is effected more by convective fluid motion and bubbles than by any particular frequency of audio sound, and thus toothbrush designers should emphasize the production of fluid flow generated by the toothbrush.

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Introduction

A primary concern in oral hygiene is the removal of plaque, a community of various bacteria and their products residing on the enamel surface of teeth. This biofilm of plaque has been implicated as a causative agent in dental caries, gingivitis and periodontal disease.[1,2] Dental practitioners agree that proper and regular brushing is effective in removing the plaque. However, controversy exists over the best design of toothbrushes, particularly with the introduction of mechanical toothbrushes, some of which operate at sonic frequencies or ultrasonic frequencies.[3-5] Sonic toothbrushes vibrate at frequencies in the audible range (below 20 kHz), whereas ultrasonic toothbrushes emit sound waves above 20 kHz.

Given the popularity of sonic frequency toothbrushes to the public market, it is remarkable that there are so few reports of the interaction of oral bacteria with sonic acoustic energy. McInnes *et al*[6] reported that exposure of *Photobacterium phosphoreum* to pressures up to 67 kPa at frequencies from 100 to 800 Hz caused a decrease in luminescence but did not decrease viability. They also found[7] that the adhesion of *Actinomyces viscosus* to a salivary pellicle on hydroxyapatite was reduced by exposure to oscillations at 200 Hz and pressures up to 65 kPa. The reduction was attributed to damage to the bacterial fimbriae.[8]

Energy generated at ultrasonic frequencies is sometimes used in ultrasonic dental scalers to remove plaque and calculus from teeth and roots; however, this is more mechanical removal by direct contact with a vibrating tool that operates at a very high frequency rather than transmission of acoustic energy through liquid. High intensity low frequency ultrasound is commonly used to lyse bacteria,[9,10] but low intensity ultrasound causes no damage. For example, Qian *et al*[11] found that 500 kHz ultrasound at 10 mW/cm^2 could not disrupt the physical structure of a biofilm of *Pseudomonas aeruginosa*. These and other ultrasonic conditions could not reduce the viability of P. aeruginosa or *Escherichia coli* until the intensity was greater than about 2 W/cm^2 at 70 kHz. In fact, in the absence of other stressors to the cells (such as antibiotics), low intensity ultrasound increases the growth rate of some biofilms.[12] No reports were found on the use of audio frequency sound to lyse bacteria.

Any sound, whether audio or ultrasound, can interact with bacteria and biofilms by several mechanisms. The presence of any sound wave causes an oscillation in the position of water molecules; the displacement is very small at high ultrasonic frequencies, but becomes appreciable at low acoustic frequencies. For example, 1.6 MHz ultrasound in plane waves at 10 mW/cm^2 causes a 1.6-nm peak-to-peak (P-P) displacement, whereas 260 Hz audio plane waves at the same intensity cause a 7-μm displacement (P-P). However, this displacement alone has very little effect on bacteria or biofilm because the water and everything around also displaces in concert, so there is little net shear force on the bacteria.[13]

A more significant source of stresses on bacteria and biofilm is the fluid hydrodynamic forces generated in the region of the oscillating surface that produces the acoustic waves. As in this research for example, when a piston operates in a confined cylinder, there is significant shear stress in the liquid and on the surface of the annular space between the piston and the walls of the cylinder. Shear forces are known to break fimbriae from the surface of cells such as *Streptococcus mutans*.[14]

Perhaps the most disruptive factor during sonic or ultrasonic exposure is the presence of gas bubbles. Gas bubbles cause disruption by several avenues. First, the interface between the gas bubble and water creates a high surface energy, sufficiently strong that if this interface moves across

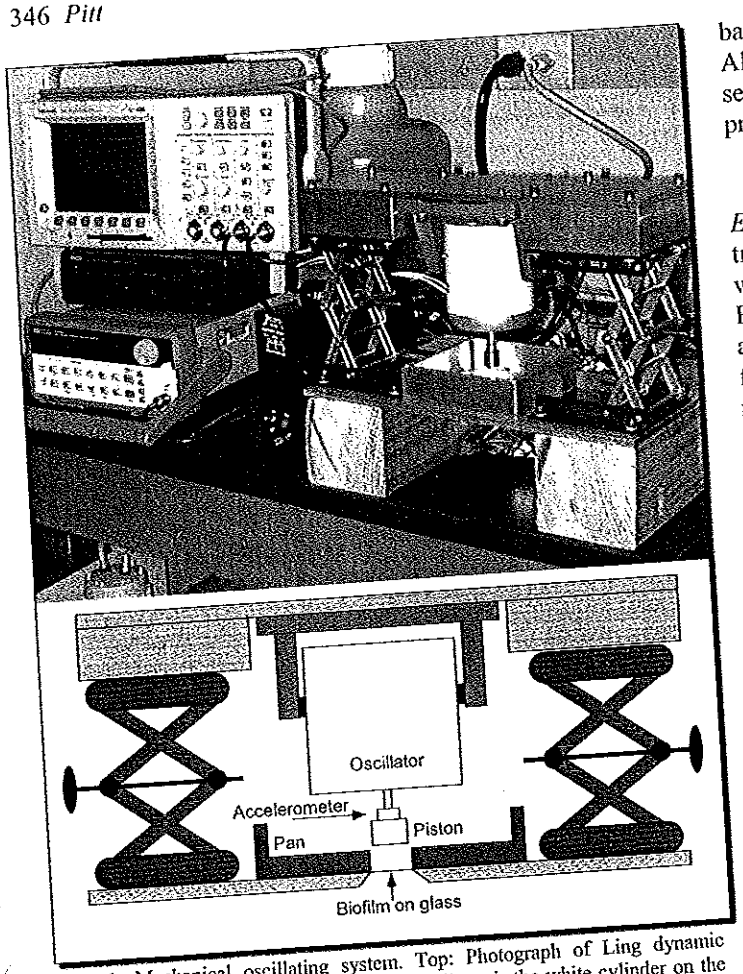

Fig. 1. Mechanical oscillating system. Top: Photograph of Ling dynamic oscillator and electronic equipment. The oscillator is the white cylinder on the right, held upside down in the trunnion. The jacks lower the piston into the pan. Attached to and beneath the oscillator are the accelerometer and the piston. To the left are the function generator (bottom) amplifier (middle) and oscilloscope (top). Bottom: Cross section schematic of the oscillator, accelerometer, piston and pan. The biofilm is placed at the bottom of the pan.

a surface containing adherent bacteria, the bacteria or other particles are stripped from the surface.[15-17] Thus a gas bubble entrained in flowing fluid that collides with a bacteria-covered surface could strip bacteria from the surface. Although these bubbles can remove bacteria, it is doubtful that they could kill bacteria; microbial bioreactors often employ a purge stream of air or oxygen bubbles to enhance bacterial growth.

A second interaction between bacteria and bubbles occurs in the presence of sound waves. The oscillating pressure causes the bubbles to oscillate in size, a phenomena called cavitation. At low intensities, and with a bubble that is not near the resonance size, the oscillations are at the same frequency as the sound waves. This oscillation causes microcirculation, called microstreaming, adjacent to the bubble. The microstreaming can generate shear forces sufficient to lyse red blood cells.[18] It is not reported if audio or ultrasonic microstreaming can lyse bacteria cells. However, ultrasonic microstreaming has been implicated in enhancing the permeability of bacterial cells.[19]

When the acoustic amplitude becomes large, and/or when the bubble approaches a resonance size, the bubble oscillations become non-linear. Under these conditions the bubble can collapse to a very small volume, compressing the gas to very high temperatures and pressures. This causes a shock wave and extremely high shear forces that can lyse bacteria,[20,21] and produce free radicals that can poison bacteria.[18] Although very common with ultrasonic devices in laboratory settings, it is very improbable that sonic toothbrushes could produce this type of violent "collapse" cavitation.

Materials and Methods

Equipment - A mechanical oscillator and accompanying trunnion (model V203[a]) were employed to create acoustic waves. This oscillator has a working range from 5 to 13,000 Hz, can generate up to 5 mm (peak to peak) of displacement at low frequencies (<100 Hz), and produce up to 17.8 N of force at higher frequencies (>100 Hz). The oscillator was mounted upside-down on an apparatus consisting of a base plate machined to accept a microscope objective from the bottom side and hold a glass cover slip on the top side, an aluminum pan that seals onto the cover slip and contains water, two lab jacks by which the oscillator is positioned vertically, and a top plate to which the trunnion holding the oscillator is bolted. A straight walled circular hole (16.0 mm diameter) in the bottom of the pan (4.6 mm thick) accepted a piston (15.0 mm) mounted to an accelerometer. Figure 1 shows the apparatus, the oscillator, and the associated electrical equipment, which consisted of a function generator (33120A[b]), a digital oscilloscope, and a 25 W stereo amplifier (RCA model SA-155[c]). A sine wave of specified frequency was amplified by the stereo amplifier, and both speaker outputs were connected to the input terminals of the oscillator. The amplitude of voltage entering the oscillator was controlled by both the function generator and the gain on the amplifier.

The amplitude of the displacement of the piston mounted on the oscillator was measured by an accelerometer (model 3220B[d]) that was factory calibrated to 5,000 Hz. The accelerometer, mounted between the piston and a shaft attached to the oscillator, was used to calculate the piston displacement amplitude and other acoustic parameters. The accelerometer was removed for experiments with biofilms.

The oscillator specifications indicated that the oscillator has a fixed impedance; thus at a fixed frequency, the stroke amplitude of the oscillator is proportional to the input voltage. This was confirmed in preliminary experiments. Therefore the oscillator was calibrated by measuring the acceleration of the piston as a function of applied voltage at numerous frequencies. The acceleration was integrated twice to obtain the piston displacement.[22]

Because the oscillator delivers constant average power independent of load, at a given input voltage and frequency, the stroke length amplitude is a function of the mass on the oscillator. Preliminary studies confirmed that the stroke amplitude was inversely proportional to the square root of the mass, as predicted by theory.[22] When the piston operated in the actual experiments without the accelerometer, the calibration was adjusted to account for the difference in mass.

Another adjustment to the calibration was made to account for the increase in "effective mass" of the piston as it pushes through water. When the piston is placed in water above the hole (or well) in the pan, the increase in effective mass was only about 4% at 80 Hz, and 7% at 200 Hz. When the piston was placed down into the well, there was additional pressure on the piston as it pushed water into the

well and out the narrow gap between the piston and sides of the well. This viscous force increased with the depth of the piston in the well, so the calibration was done at several positions of the piston into the well. Thus the final values of stroke displacement (as a function of voltage input into the oscillator) were corrected for the mass of the piston and the viscous forces acting on the piston. Once these corrections and correlations were made, the input voltage was used to calculate the piston stroke length, the piston velocity, and the calculated acoustic intensity of an acoustic wave generated by an ideally large piston at that same stroke length and frequency. This is only a theoretically calculated acoustic intensity because the piston diameter is much smaller than the wavelength of sound at these low acoustic frequencies; therefore the piston does not create plane waves, but instead acts as a point source and creates spherically radiating waves in which the intensity decreases with distance from the oscillator.

Because of the limits of the Ling oscillator, high acoustic intensities were generated at low frequencies, but these intensities decreased at higher frequencies. The maximum calculated acoustic intensities ranged from 44 W/cm^2 (at 80 Hz) to 0.9 mW/cm^2 (at 20,000) Hz. The actual intensities were probably much less because these are not plane waves.

The hydrodynamic pressure in the bottom of the well was also measured in separate experiments by placing a small pressure transducer (model EPX-N02-15P[e]) at the bottom of the well where the biofilm was normally placed. The dynamic response of this calibrated pressure transducer began to decline above 1,000 Hz; therefore, pressure measurements were made only up to 600 Hz. The hydrodynamic pressure oscillations were measured as a function of the acoustic input parameters. The pressures on the biofilm depended strongly on the depth of the piston in the well. For example, at a calculated acoustic intensity of 5.1 W/cm^2, when the piston was 1.25 mm into the well, the hydrodynamic pressures ranged from 3.1 psi at 600 Hz to 0.6 psi at 80 Hz, and when the piston was 1.25 mm above the well, the hydrodynamic pressures ranged from 2.3 psi at 600 Hz to 0 psi at 80 Hz.

Biofilm growth - Biofilms were grown on #1 thickness 1" x 1" microscope coverslips in a drip flow reactor developed at the Center for Biofilm Engineering. The general procedure for growing biofilms in these reactors has been published previously.[23] Unique to the present report is that three coverslips were attached to glass slides using a 10-µL drop of sucrose syrup (50% sucrose in water, boiled). The sucrose held the coverslips in place during assembly, autoclaving, and initial inoculation of the drip flow reactor. By the time the biofilms were grown, the sucrose had dissolved and the coverslips could be easily removed from the glass slide. A brief synopsis of the biofilm growth procedure is given below.

The drip flow reactor was loaded with 12 coverslips and autoclaved. Five mL of brain-heart infusion broth supplemented with 2 wt% sucrose (BHI-S) was inoculated from a frozen culture of S. mutans (strain UA 159, ATCC #700610) and incubated for 8 hours at 37°C in a CO_2-supplemented atmosphere. Then 50 mL of BHI-S were added to the culture, and 13 mL of this mixture was pipetted into each of the four chambers of the drip flow reactor. The reactor was incubated in batch culture at 37°C with CO_2 flow for 16 hours (overnight) in level position. The next morning the desired number of slides with biofilms was removed for the experiments of the day. These overnight biofilms were very uniform in appearance within one chamber, and from chamber to chamber in the reactor. In some experiments the drip flow reactor ran continuously for the next few days, at a 10° incline with a continuous supply of CO_2 and a 1/10 dilution of BHI-S dripping at a rate of about 0.2 mL/minute. If biofilms were needed at any time, the flow to a particular chamber was stopped, the cover removed, and the glass slide with three biofilms removed.

The thicknesses of some biofilms were measured on a Leica confocal scanning laser microscope. The viable colony forming units in the biofilm were determined by scraping the biofilm from a coverslip, using a Teflon "policeman", into a sterile 100 mL beaker containing 18 mL of dilution buffer. The biofilm was then sheared using a homogenizer (Ultra-Turrax T25[f]) for 3-5 minutes followed by insonation with an ultrasonic probe (Torbeo Ultrasonic Cell Disruptor model 36810[g]) for 1 minute at 6 watts. Preliminary experiments showed that the ultrasonication step did not reduce CFUs, but actually increased them, probably by dispersing the biofilm. The sample was then serially diluted (1:10 dilutions) and plated on BHI-S agar using the drop plating technique. Plates were incubated at 37°C for at least 48 hours in a CO_2-supplemented atmosphere before counting. The viability of the biofilms was assessed using the BacLight live/dead staining technique.[h]

Exposure to sound - Coverslips were removed by sterile forceps from the glass slide and placed in a clean Petri dish. The coverslips were rinsed gently twice by overflow with water. Puddled water was decanted from the Petri dish, and the biofilms were stored wet, but not underwater for a couple of hours before insonation.

The coverslip was placed in a recess on the oscillator stage, and the pan was bolted in place, sealing the coverslip with a rubber gasket. Then distilled water (50 to 125 mL) was poured into the pan, immersing the coverslip. The oscillator was lowered to the desired distance from the top of the well. The desired frequency and amplitude were applied for a predetermined time. Then water was poured from the pan by tilting the oscillator stage and pouring off the water into a collector, and the coverslips were carefully removed and placed biofilm-side down in a clean Petri dish and stored damp so they would not dry out. A black paper was placed over the biofilm (which is white) to provide contrast, and the whole Petri dish was scanned on a color scanner at 200 dpi resolution (Hewlett Packard Scanjet C7716[b]). This produced a digital image of the biofilm. NIH Image software[i] was used to quantitate the amount of biofilm removed by the exposure. Using this software, at least three measurements were made of the black background intensity, at least three measurements were made of the white non-insonated corners of the biofilm, and then an average intensity measurement was made of the area that was exposed. The amount of bacteria remaining was

calculated by the following formula,

$$\%\ \text{Removal} = \frac{I_{white} - I_{sample}}{I_{white} - I_{black}} \times 100\%$$

where I_{sample} is the grey scale intensity of the insonated sample, I_{white} is the grey scale intensity of the white untouched biofilm, and I_{black} is the insonated grey scale intensity of the black paper. This procedure gave consistent and fairly reproducible quantitation as long as the biofilm did not dry out before it was scanned.

Results

Biofilm growth - The drip flow reactor is a very convenient system for producing uniform biofilms of S. mutans. When 16-hour-old biofilms (overnight) are used, a single reactor can produce 12 biofilms per day, all of very uniform quality, completely covering the coverslip. Biofilms older than this are generally more varied in their uniformity on the coverslip, and in their repeatability from slide to slide and experiment to experiment. Confocal microscopy showed that the 16-hour, overnight biofilms were about 80 μm thick, 2-day-old biofilms (40 hours) were about 150 μm thick, 3-day-old biofilms (64 hrs) were about 250 μm thick, and 4-day-old biofilms were also about 250 μm thick. The thicknesses at 40 and 64 hours of growth are consistent with the thickness reported at 48 hours by other investigators.[24] The production of the overnight biofilms appears to be a good model of overnight plaque formation that is readily available and reproducible.

The overnight biofilms had a viable population of about 6×10^9 CFU/cm^2, based on plate counting of CFUs. The viability of overnight biofilms was >99% as assessed by the live/dead stain.

Kinetics of biofilm removal - As one would expect, longer exposure time removed more biofilm. Figure 2 shows the amount of removal of overnight biofilms at 120 Hz and 80 Hz. The biofilm was never completely removed, no matter how long and intense the insonation. At high intensities the biofilm was removed faster than at low intensities. Because different amounts were removed depending upon the type of experiment, different exposure times were employed in subsequent experiments.

Constant intensity experiments - Many experiments were conducted in which the acoustic intensity was kept constant while the frequency was varied in order to determine if there was an effect of frequency upon the amount of biofilm removal. Figure 3 shows several such experiments conducted on overnight biofilms. There are several important observations regarding this data. The first observation is that as the calculated acoustic intensity increased, the amount of removal increased, as expected. There was no apparent trend with removal as a function of frequency.

These data also show the magnitude of the scatter in reproducibility. Data with common symbols were collected on the same day, with biofilm from the same batch. Within data collected on the same day, there is less variation than there is from day to day. For example, the data at 11.1 W/cm^2 shows more removal on the first day than on the next day with a different set of biofilms that were slightly thicker than those of the first day. Therefore, comparisons in this report are made on data from biofilms grown on the same day.

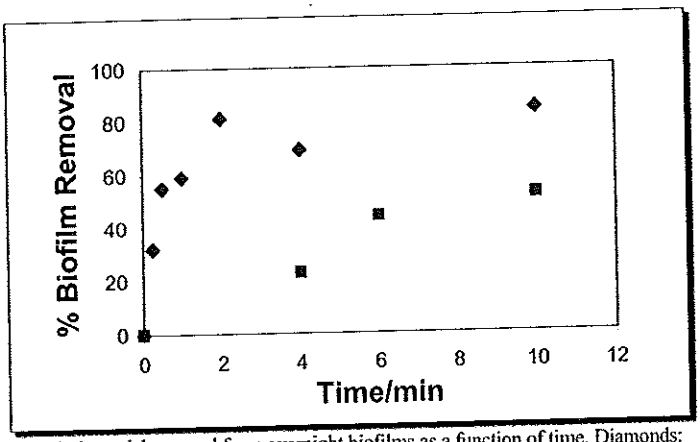

Fig. 2. Bacterial removal from overnight biofilms as a function of time. Diamonds: 120 Hz, the piston was 1.25 mm into the well, the piston displacement was 0.8 mm, the calculated acoustic intensity was 27 W/cm^2, and the pressure amplitude was 5.1 psi. Squares: 80 Hz, the piston was 1.25 mm into the well, the piston displacement was 0.8 mm, the calculated acoustic intensity was 12 W/cm^2, and the pressure amplitude was 1.7 psi. Each point represents a separate experiment.

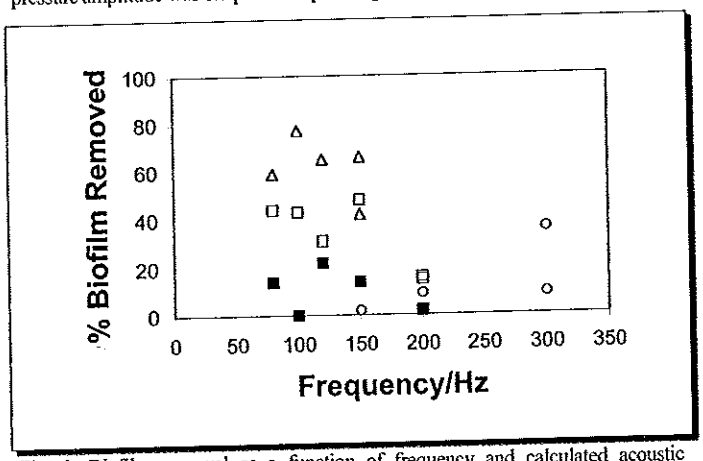

Fig. 3. Biofilm removal as a function of frequency and calculated acoustic intensity. All the data represented by open symbols were collected on the same day on the same batch of biofilm. The data represented by closed symbols were collected on the next day with a new batch of biofilm that was slightly thicker than the first day. In all experiments, the biofilm was exposed to the acoustic field for 10 minutes with the piston 1.25 mm into the well. The calculated acoustic intensity is: Circle, 5.1 W/cm^2; Square, 11.1 W/cm^2; Triangle, 18.0 W/cm^2.

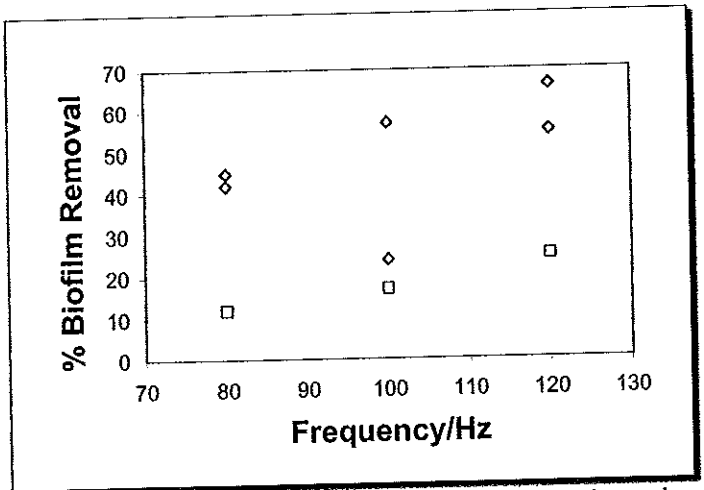

Fig. 4. Biofilm removal at two locations of the piston with respect to the top edge of the well. In these experiments, the biofilm was exposed to the acoustic field for 10 minutes with the piston 1.25 mm above (squares) or below (diamond) the top of the well. The calculated acoustic intensity was 27 W/cm^2.

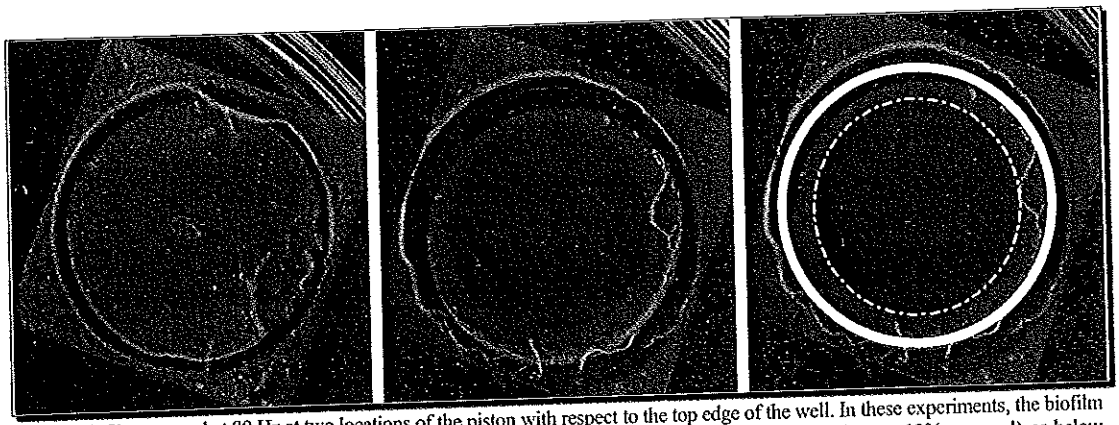

Fig. 5. Biofilm removal at 80 Hz at two locations of the piston with respect to the top edge of the well. In these experiments, the biofilm was exposed to the acoustic field (27 W/cm^2) for 10 minutes with the piston 1.25 mm above (left image, 12% removal) or below (middle image, 45% removal) the top of the well. On the right, the overlay shows the position of the gasket (solid circle) and the edge of the well (dotted circle). The gasket scraped some biofilm when the sample was removed.

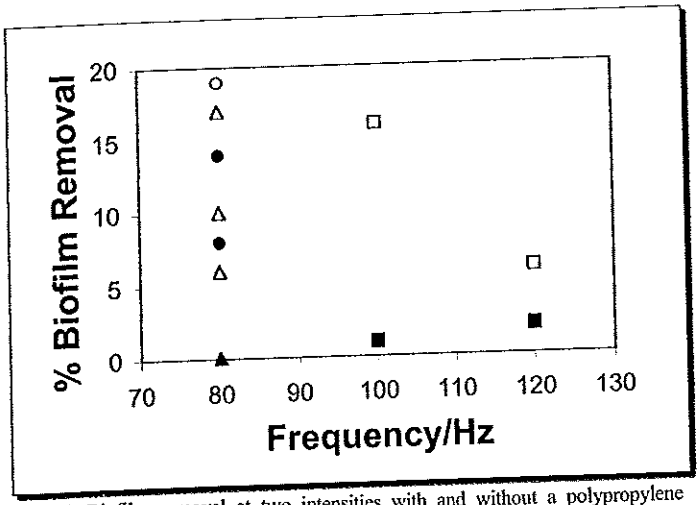

Fig. 6. Biofilm removal at two intensities with and without a polypropylene acoustic window. In these experiments, the biofilm was exposed to the acoustic field for 10 minutes with the piston 1.25 mm above the edge of the well. Open symbols depict no acoustic window present, whereas filled symbols indicate that the PP acoustic window was between the piston and the biofilm. Square: 30 W/cm^2. Triangle: 44 W/cm^2. Circle: 44 W/cm^2 with bubbles present.

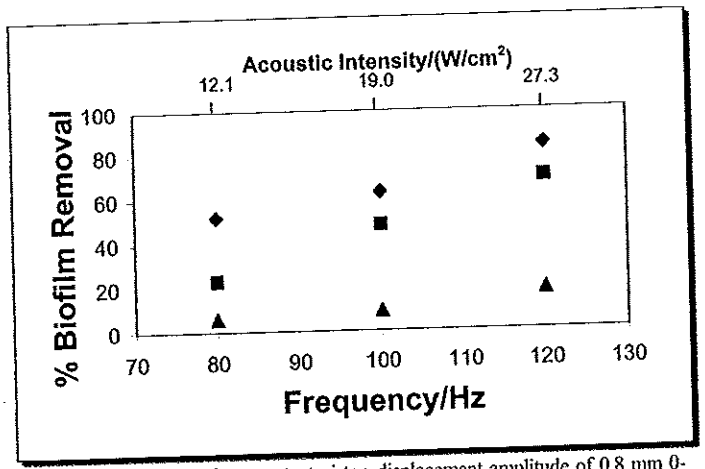

Fig. 7. Biofilm removal at constant piston displacement amplitude of 0.8 mm 0-peak. Piston was above or into the well. Diamond: 10-minute exposure, 1.25 mm into well. Square: 4-minute exposure, 1.25 mm into well. Triangle: 10-minute exposure, 1.25 mm above well. The corresponding calculated acoustic intensities are indicated on the upper x-axis.

The present observation that removal is a strong function of intensity but not frequency is similar to the observation of McInnes *et al*[6] that the decrease in bacterial luminescence upon exposure to low frequency pressure waves was greatly influenced by the pressure amplitude, but not by the frequency in the range studied (100 to 800 Hz).

Effect of the piston placement and fluid velocity - The piston could be placed at any height above or below the top of the well. The placement of the piston theoretically should not greatly change the near field acoustic intensity. However, it greatly changes the local fluid dynamics. When the piston is above the well, there was not much water forced directly into the well. However, when the piston was down in the well, the upstroke sucked in water at high velocities, and it was jetted down the sides of the well where it impinged upon the biofilm. Figures 4 and 5 illustrate the difference in biofilm removal produced by the fluid mechanics as a result of the piston position. Three to four times as much biofilm was removed by the additional fluid action caused by placing the piston in the well.

The effect of fluid dynamics and bubbles was decoupled from the effects of acoustic pressure waves by placing an acoustic window of polypropylene (PP) film over the well. This material is transparent to underwater sound, so the acoustic energy entered the well, but the convective flow and bubbles could not enter. Note that the near field acoustic pressure waves cause small-scale oscillatory motion in the well, so there is some fluid movement. The glass slide probably flexes, as well as the PP window, but there are no jets of fluid rushing down the sides of the well. These experiments were done with the piston 1.25 mm above the well. These data, shown in Fig. 6, indicate that less biofilm is removed when the acoustic window precludes convective flow into the well.

Effect of bubbles - The data in Figures 2-5 were carefully collected such that no gas bubbles were entrained into the well. This was done by placing 125 mL of water in the pan, and not applying full power at 80 and 100 Hz. When the volume of water in the pan was reduced to 50 mL, the piston naturally entrained gas bubbles, which always produced more biofilm removal. For example, for the open circle in Fig. 6 (44 W/cm^2, 80 Hz, 1.25 mm above well), the amount removed in the presence of bubbles was 19%, nearly double the average removal of 11% for conditions without bubbles.

In two separate experiments, a bubble was trapped under the PP film and sonified at 80 Hz and 44 W/cm^2. The average (n=2) removal was 11%; much more than when there was no bubble under the film (< 1% removal). These

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experiments indicated that there was some water movement under the film caused by the acoustic oscillations, and that the presence of gas bubbles within this acoustic field increased biofilm removal.

Constant amplitude experiments - The above experiments were conducted at constant acoustic intensity, which is also constant piston maximum velocity. Other experiments were done employing constant displacement amplitude of the piston to examine any correlations under these conditions. The piston stroke amplitude was fixed at 0.8 mm displacement (0 to peak) for frequencies of 80, 100, and 120 Hz with the piston 1.25 mm above and 1.25 mm below the top of the well. The results shown in Fig. 7 indicate that at constant amplitude, biofilm removal increases with frequency, but intensity also increases with frequency. Thus the increase noted in these experiments is attributed to the increase in intensity, since previous data showed that removal is independent of frequency (Fig. 3) but increases with intensity (Figs. 2, 3).

Discussion

The results and observations suggest that there are three components that contribute to removal of biofilm from the glass surface: acoustic energy, fluid shear forces, and the action of bubbles. Although each of these contributes, they do not seem to contribute equally to the removal of biofilm.

Effect of acoustic energy - One must remember that acoustic energy is the movement of fluid molecules in an oscillatory motion. Thus one cannot separate acoustic energy from oscillatory fluid motion and the shear forces produced by such fluid motion. In addition to fluid motion, there are pressure fluctuations associated with the acoustic field. However, there is a difference between the oscillatory motion of acoustic waves and the net convective and streaming motion of fluid that is generated by the moving surfaces of the apparatus that produces the sound. The experiments that employed an acoustic window produced a minimal amount of convective fluid motion in the well. In these experiments at high intensities and low frequencies (Fig. 6), there was a small amount of biofilm removed, but this removal was the lowest of any of the measurements made. Thus it appears that the acoustic field itself, at these high intensities, is capable of removing a small amount of biofilm during a 10-minute exposure. In these experiments, the vibrating surface was about 7.6 mm from the biofilm, and the acoustic intensity decreases with distance from the radiating source. Although the actual acoustic intensity values could not be computed, they are probably much lower than those values calculated herein for simple plane waves.

Effect of fluid motion - The fluid motion created by the displacement of the piston can be considered pure acoustic motion immediately adjacent to the piston surface. However, because the piston is not an infinitely wide flat plane, the fluid motion, even small distances from the plane, is no longer described by planar acoustic equations. When the piston in free field (away from a surface or well) moves downward, some fluid is pushed downward, but much of it also is pushed sideways and radially outward from the piston. Similarly, when the piston moves upward, the water rushes in from the sides, as well as upward. Pressure waves are also propagated in these same directions, but may not coincide with the convective fluid movement.

When the piston oscillates near to and perpendicular to a flat surface, then most of the fluid movement is in the radial direction. Thus when the piston operates above a well covered with an acoustic window, the fluid motion is radial, and not into the well, even though the pressure waves go through the window and interact (cause fluid movement) beyond the window.

Now consider the case in which the acoustic window is removed. Some fluid can move into the well, but this is a rather stagnant enclosure, and so most of the movement will still be radial along the flat plate at the top of the well. As the resting or average position of the piston is moved incrementally closer to the top of the well, there will be more radial shear of the water at the top of the well, and this will create some circulatory convection inside the well.

As the piston approaches the point where it enters the well, the fluid motion changes drastically because the fluid can no longer move sideways as the piston moves downward. When the piston is inside the well, and is moving upward, water is forced down through the annular gap between the side of the piston and the side of the well. This creates a sheet of jetting liquid that flows down the sides of the well and impinges with great force on the biofilm next to the side of the well. When the piston stroke is downward into the well, the jet of liquid shoots out of the well, and there is probably relatively little shear stress on the biofilm.

This description of the fluid mechanics is consistent with the observation of biofilm removal. As mentioned above, when the acoustic window was in place, very little biofilm was removed compared to the same situation without an acoustic window. Figure 6 shows the removal with and without an acoustic window when the piston was 1.25 mm above the top of the well. When the piston was placed in the well, a tremendous amount of biofilm was removed in a relatively short amount of time. For example, Figs. 4 and 6 show 10 to 20% removal when the piston is above the well, compared to 50 to 70% when the piston is 1.25 mm into the well. Figure 7 also illustrates this difference.

The images shown in Fig. 5 are typical of the biofilm removal that was observed when the piston was inside the well, and no bubbles were present. The removal was not from the center of the exposed circle, but from the edge adjacent to the side of the well. This pattern of removal is consistent with the predicted fluid motion jetting down the sides of the well and impinging onto the biofilm.

Because much more biofilm was removed when the fluid velocities were greater, this indicates that the shearing effect of fluid motion probably has a much greater effect than the simple acoustic oscillatory motion.

Effect of bubbles - The data obtained in this research show that when bubbles are present, in addition to fluid motion and acoustic pressure, there is a tremendous enhancement in the amount of biofilm removed. Most importantly, the bubbles tend to remove the biofilm all the way down to the glass surface. Without bubbles present, there was no removal down to the glass, but the biofilm became thinner, apparently through shear removal of the top layers of bac-

teria. Even gas bubbles in the absence of convective flow (placed under the acoustic window) increased the removal of bacteria from the surface (Fig. 6).

The three-phase interface between liquid, solid and gas creates very strong interfacial forces on a solid surface that can pull particles such as bacteria from a surface. There are four types of interactions of bubbles with bacteria on surfaces noted in the literature: 1) A slowly expanding bubble on a surface will push bacteria aside as it expands;[17] 2) A bubble or air/liquid interface moving across a surface will remove bacteria;[15] 3) A bubble undergoing stable (non-collapse) cavitation will pulsate rapidly in size, which produces a fluid convection near its surface called microstreaming; this in turn creates strong fluid shear forces[25] that may perturb bacteria or biofilm; and 4) A bubble undergoing collapse cavitation not only creates microstreaming shear forces, but creates a shock wave that can shear a biofilm[26] and generates free radicals that may poison bacteria.[27] In addition, if the collapse event occurs near a surface, a liquid jet of high velocity is projected at high speed toward the surface, and this may remove bacteria or biofilm from the surface.[28]

Thus the action of bubbles contacting a biofilm is very strong, and all of these events may play a role in the observed phenomena that much more biofilm is removed in the presence of bubbles. The first two effects listed in the paragraph above are independent of the presence of an acoustic field, and occur with or without application of acoustic energy. The latter two events occur only in an acoustic field.

Of the three methods of biofilm removal (acoustic, fluid shear, and bubble associated phenomena), the bubbles appear to have the greatest impact. Figure 6 shows that bubbles in the acoustic field, without convective fluid flow (blocked by the acoustic window), remove about the same amount of biofilm as convective flow without bubbles.

Relationship to plaque removal - The biofilms studied herein are not plaque, which are much more complex and more mature collections of many interacting species in a biofilm. Neither is the glass surface a good model of the enamel surface of a tooth. However, the biofilm model of an 80 μm thick, overnight layer of *S. mutans* is a first approximation to a plaque model. The important parameters established from this work on a monoculture biofilm probably apply to plaque also. These important findings include the following observations: 1) There appears to be no particular frequency that disrupts the biofilm by acoustic energy alone; 2) Acoustic oscillations alone appear to remove a small amount of biofilm, given enough time; and 3) The action of convective fluid motion and of bubbles are much more significant in removing biofilm than the acoustic oscillations.

Using this imperfect biofilm model, the removal of biofilm was a strong function of the acoustic intensity. Biofilm was removed in very small amounts (up to 2% in 10 minutes) due to acoustic energy in the absence of convective fluid flow, even at the higher intensities (30 W/cm^2). When the acoustic action was coupled with convective fluid flow caused by the piston and well geometry, up to 80% of a biofilm was removed at 27 W/cm^2 in 2 minutes. When gas bubbles were entrained in the fluid, the removal approached 100%. With respect to removal of plaque on teeth, the vigorous action of moving fluid and bubbles is expected to remove plaque, and a maximum permissible fluid velocity is recommended, rather than any particular frequency. Entrainment of bubbles in the flowing liquid, which naturally happens when dentifrice is used in the mouth, is anticipated to enhance the removal of biofilm from teeth.

Although these results do not point to a "magical" frequency in which biofilm can be removed, they do validate the role of acoustics and fluid movement in oral hygiene. The early work of McInnes showed that bacteria are negatively affected by sonic frequency fluid motion. While the present study did not address the impact on bacterial viability, it does corroborate the findings of McInnes with respect to stress on bacteria.[6-8] His experiments also created much fluid shearing motion (and perhaps bubbles), and this action probably created great stresses on the bacteria, sufficient to remove fimbriae, and apparently to perturb the cells in other manners. Impingement of bubble-filled fluid on a biofilm is also expected to create stress on bacteria from the fluid shear forces and from the interfacial stresses at the gas-liquid-bacterium boundary. Thus in development of powered toothbrushes, designers should consider maximizing fluid flow as a method of increasing plaque removal in interproximal spaces or other regions where direct contact with toothbrush bristles is difficult.

a. Ling Dynamic Systems, Royston, England.
b. Hewlett Packard Palo Alto, CA, USA.
c. Radio Shack, Bozeman, MT, USA.
d. Dytran Instruments, Chatsworth, CA, USA.
e. Entran, Fairfield, NJ, USA.
f. Janke & Kunkel, IKA-Labortechnik, Staufen, Germany.
g. Cole-Parmer, Vernon Hills, IL, USA.
h. Molecular Probe, Eugene, OR, USA.
i. National Institutes of Health, Bethesda, MD, USA.

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