Formation and Decontamination of Biofilms in Dental Unit Waterlines

M. Robert Wirthlin,* Grayson W. Marshall Jr.,† and Randal W. Rowland*

Background: Biofilms are a natural occurrence in aquatic environments, including community drinking water systems. The interior of small-diameter tubings in dental unit waterlines (DUWL) are also sites of biofilm formation. In the lumen of the tubings, the flow is minimal, and the water becomes stagnant when the units are not in use. Molecules precipitate from the water onto the interior wall and promote the adherence of planktonic microorganisms from the water. Once they become sessile, the microorganisms change their phenotype. After adherence, there is a so-called surface-associated lag time, and the organisms then enter a growth phase and produce exopolysaccharides that coat the organisms in a slime layer. Within the biofilm, the microorganisms can signal one another, transfer nutrients, and exchange genetic material. The insoluble exopolysaccharides shield the microorganisms from displacement and from penetration by predator organisms, antibiotics, and disinfectants. The external surface layer of microorganisms is faster growing and may detach as "swarmer" cells. Detachment of microorganisms from dental unit biofilm flushed into the oral cavity could theoretically infect the patient. Splatter and aerosols from dental procedures may possibly infect health care personnel.

Methods: This study compared three DUWL cleaners (an alkaline peroxide product, a freshly mixed chlorine dioxide product, and a buffer-stabilized chlorine dioxide product) in 16 dental units with self-contained water systems, 6 months after installation in a periodontal teaching clinic. One unit treated by flushing and drying served as a control. Units were sampled daily for 10 days with heterotrophic plate count (HPC) sampler plates. The plates were incubated for 7 days at room temperature, and colonies were counted at 10.5× magnification. Samples of internal water tubing before and after the use of waterline cleaners were processed and examined by scanning electron microscopy.

Results: The estimated mean HPC was derived from original and replicate independent counts of two investigators of undiluted and diluted samples, reported as colony forming units (CFU)/ml. Shock treatments with the alkaline peroxide product (n = 5) reduced the HPC from baseline, but in the ratio of daily counts to control, there was a large variance and a trend to return of high counts as days passed. The mean daily HPC was significantly better than the control for only 3 of the 9 days of treatment and exceeded the goal of 200 on 3 days. Freshly mixed chlorine dioxide (n = 4) and the buffer-stabilized chlorine dioxide (n = 5) both reduced HPC to near 0 on all days. Their ratios of daily estimated means to that of the control were significantly (P <0.001) better at all times. In comparing treatments, the freshly mixed chlorine dioxide was better (P <0.001) than the alkaline peroxide on 8 of 9 days. The buffered chlorine dioxide treatment was better than the alkaline peroxide at all times. The two chlorine dioxide treatments each had so many HPC counts of 0 that a meaningful statistical difference between them was not calculated. Scanning electron microscopy of plastic waterline tubing samples taken before and after treatments showed reductions in biofilm coverage, but the differences were not statistically significant.

Conclusions: Chlorine dioxide waterline cleaners are effective in decontaminating DUWL biofilm. Chlorine dioxide has advantages over other chlorine products. Controlling DUWL biofilm may have beneficial effects on nosocomial infections. *J Periodontol 2003;74:1595-1609.*

KEY WORDS

Biofilms; cleansing agents; comparison studies; dental equipment; infection control; risk factors; water pollution/prevention and control.

* Department of Stomatology, Division of Periodontology, University of California-San Francisco, San Francisco, CA.
† Department of Preventive and Restorative Sciences, Division of Biomaterials and Bioengineering, University of California-San Francisco.

Dental unit waterlines (DUWL) are sites for the development of biofilms of aerobic, mesophilic, heterotrophic microorganisms commonly found in fresh drinking water systems.[1] Dental units contain many fine-diameter tubings (Fig. 1). The inside diameter of these tubings is about 1 to 2 mm, so the intraluminal surface-to-volume ratio is greater than in the water mains and pipes that bring the water to the units.[1] The flow of water in the mains, pipes, and tubings is laminar; therefore, the flow at the lumen surface is almost at a standstill.[2] In this zone, the bacteria may move by fluid flow, Brownian motion, sedimentation, and flagellae. Molecules in the water may adhere to the lumen surface by physical adsorption and chemisorption, providing a conditioned substratum that can attract other molecules projecting from the surface of the microorganisms by means of van der Waal's forces, electrostatic forces, hydrophobic forces, or chemisorption of bacterial fimbriae, pili, or adhesins.[3-5] Divalent cations may contribute to microbial adherence as they "bridge" anions precipitated on the lumen to those on the bacterial "fuzzy coat" or glycocalyx. These initial attractions may be considered weak and reversible.

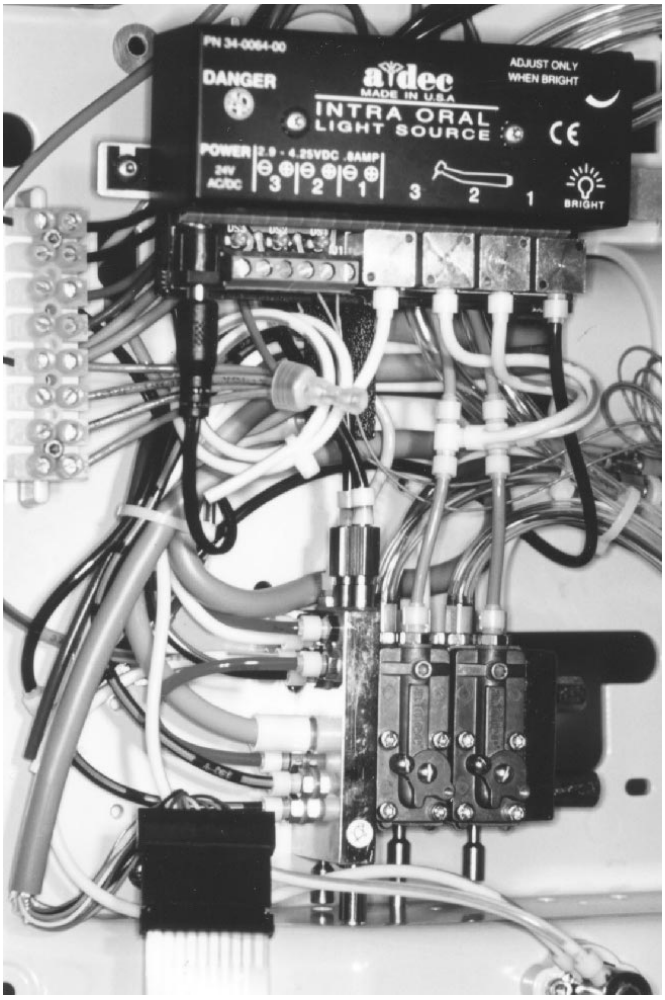

Figure 1.
Part of the interior of a modern dental unit, showing a portion of the many small tubings used to distribute compressed air and water.

After their initial adherence on a conditioned surface, the microorganisms enter a quiet phase, termed the surface-associated lag time, during which they may be changing the expression of their genes.[6,7] Once they make the phenotypic shift and divide, the microorganisms enter a rapid growth phase and begin to secrete complex exopolysaccharides, a mucilaginous slime that cements the organisms to the surface and resists detachment by fluid shear forces.[8] The exopolysaccharides form a coating on the bacteria and a fibrous matrix. Silt and other microorganisms may be trapped in the tangled matrix or adhere by the molecular interactions of ligand-substrate nature to the "forest" of protruding molecules.[9]

The growth of microcolonies within the matrix and the coaggregation of other bacteria increase the depth of the biofilm; however, it might not exceed 1,000 µm in thickness in a turbulent flow setting.[10] The adherence of bacteria increases their density compared to their former free-floating planktonic state, and the signals they express may become concentrated enough to serve as autoinducer signal molecules.[11] Thereby the concentration may exceed a threshold, and the bacteria sense they have a "quorum."[12,13] Gram-negative waterline bacteria *Pseudomonas aeruginosa* use N-acyl homoserine lactones and quinolone type signals. Gram-positive *Bacillus subtilis* and *Enterococcus faecalis* are also quorum-sensing. The signals are thought to allow cross-talk between species, causing them to increase their production of exopolysaccharide and the factors that increase their virulence.[14] The exopolysaccharides are a heterogeneous group of substances consisting in general of neutral sugars, amino sugars, and some uronic acids. Streptococci may make glucans, dextrans, and levans.[15] Gram-negative bacteria make acetylated polymers of uronic acids termed alginates. The reaction of the alginates with calcium ion in the water causes precipitation[16] (as in dental impression materials). The exopolysaccharides are mostly insoluble in water.

Deep inside the accumulated biofilm, nutrients are transferred from one species to another, but the inward diffusion of oxygen and absorbed nutrients decreases. Studies with microelectrodes showed that oxygen penetrated no deeper than 25 or 30 µm.[17] As a result, bacterial growth becomes very slow or almost static. The close quarters of the interior of biofilms may expedite gene sharing through conjugation, plasmid transfer, absorption of DNA from lysed cells, or cellular fusion of organisms like mycoplasma without walls.[18] The matrix resists the physical displacement of biofilm bacteria, and it limits the inward diffusion of adverse agents

by consuming them through chemical reactions. Because the matrix is polyanionic, it resists the diffusion of cations; it also prevents the intrusion of antibodies, protects against invasion of amoebae and paramecia (or macrophages and neutrophils, in a living host), and increases the concentration of antibiotic-neutralizing enzymes such as beta-lactamase. The overall result is that microorganisms in a biofilm are many times more resistant to disinfection than in their planktonic phase.[2]

Although sheltered within the biofilm, the microorganisms may be iron-starved and compensate by corroding the surface of a metal substratum.[7] The biofilm may start as a surface patch, but as it spreads and develops, it may take on the form of pillars or mushroom-like shapes, forming channels that improve the circulation of nutrients and the discharge of wastes and toxins.[19,20] In a severely starved biofilm, stacks of colonies may extend up into the fluid bulk like dendrites; in a richly fed biofilm such as dental plaque, the colonies form a thick, dense film.[21]

Microorganisms on the surface are not as strongly embedded as those deep within the biofilm and are faster growing. Surface bacteria are susceptible to detachment by predator protozoans, abrasion or shear of fluids or particles in the stream, and periodically slough individuals or clumps.[22] The detachment may serve the community of microorganisms by seeding downstream surfaces with the start of a new colony,[23] much like the swarming of bees.

CONTROL OF WATERLINE CONTAMINATION

Detachment of surface microorganisms from the biofilms in DUWL allows them to exit in the coolant of high-speed dental handpieces, in the flow of air-water syringes (AWS), and from ancillary equipment such as ultrasonic scalers attached to the dental units. These bacteria can then be flushed into the mouths of dental patients and become airborne as aerosols and droplets of splatter. When the dental units are not in use–between patients, at night, over weekends–the planktonic bacteria entering from the city water distribution system and those shed from the biofilm surfaces accumulate in large numbers. Counts as high as 1,000,000 colony forming units (CFU)/ml have been recorded.[24]

It is a general principle that since dentists are entrusted with the care of their patients, they should eliminate the risk of high counts of bacteria from the effluent of their dental units. The American Dental Association (ADA) has called for dental researchers and dental manufacturers to design equipment and measures to reduce waterline contamination. The announced goal was to match the standards for kidney dialysate, or 200 CFU/ml.[25]

Flushing

Flushing for 2 minutes in the morning and for 20 to 30 seconds between patients should be considered the norm for dental office procedures, and longer flushing is suggested after weekends.[26] Flushing the waterlines removes the bulk of amassed bacteria. A 2-minute flush in the morning can reduce bacterial counts by 99%,[24] but it might take 8 minutes of constant flushing to get a count of 0,[27,28] and the counts quickly return to high levels.[29] The microorganisms that are flushed from DUWL are varied. Even though a clinic may have multiple dental units all connected to the same community water system, counts and identifiable species will differ from unit to unit. Table 1 lists some of the species recovered by flushing from DUWL.[24,29-42] However, not all of those recovered are viable, and not all of those viable are cultivable.[31] In addition, fungi, protozoa, and aquatic nematodes have been identified.[1]

Table 1.

Microbial Species Isolated from DUWL[24,29-32]

Achromobacter spp.	*Pasturella haemolyticus*
Acinetobacter calcoaceticus	*Pasturella multocida*
Actinomyces spp.	*Pasturella pneumotrophica*
Aeromonas spp.	*Pseudomonas acidovorans*
Alcalignes faecalis	*Pseudomonas aeruginosa*
Bacillus spp.	*Pseudomonas cepacia*
Brevundimonas vesicularis	*Pseudomonas fluorescens*
Burkholderia cepacia	*Pseudomonas maltophila*
Burkholderia picketii	*Pseudomonas paucimoblis*
Cephalosporum spp.	*Pseudomonas picketii*
Cladosporium spp.	*Pseudomonas putida*
Flavobacterium spp.	*Pseudomonas putrefaciens*
Legionella bozemanii	*Pseudomonas stutzeri*
Legionella dumoffi	*Pseudomonas vesicularis*
Legionella longbeachae	*Psychrobacter phenylpyruvica*
Legionella pneumophila	*Sphingomonas paucimoblis*
Methylobacterium mesophilicum	*Staphylococcus aureus*
Moraxella phenylpyruvica	*Staphylococcus cohnii*
Moraxella urethalis	*Staphylococcus warneri*
Mycobacteria chelone	*Streptococcus mitis*
Mycobacteria flavescens	*Streptococcus salivarius*
Mycobacteria fortuitum	*Xanthomonas maltophila*
Mycobacteria kansasii	*Mycobacteria terrae*

Filters

To stop the effluent of waterline bacteria, filters near the end of a waterline have been recommended,[43] but they often foul rapidly and may have to be changed daily or more often. Downstream from the filter, there is almost certain to be more biofilm.[40] Filters trap the planktonic bacteria only; they do nothing to remove the biofilm.

Drying

Since biofilms are usually thin (200 or 300 µm) and are mostly water (>95%), drying the DUWL at night and on weekends by purging the waterlines with compressed air would seem to be rational. Furuhashi and Miyamae reported that air drying resulted in no CFU/ml when used in combination with flushing and 70% ethanol.[44] In other tests, however, drying alone seemed to offer no benefit.[45,46] The exopolysaccharide matrix and the static growth conditions probably protect the bacteria from dessication.

Biocides

Treatment of DUWL biofilms with biocides may be done as periodic shock treatments or by continuous treatment systems.[1] Sodium hypochlorite solutions (NaOCl), or diluted bleach, have effectively reduced planktonic counts, but biofilms are 150 to 3,000 times more resistant to hypochlorite.[47] Using a combination of periodic shock and continuous treatment in dental units with independent reservoirs, Karpay et al.[48] reduced the heterotroph plate counts (HPC) in 10 dental units to less than 10 CFU/ml 5 days after the shock treatment. Examination by scanning electron microscopy (SEM) showed no morphological features of biofilm in six of 10 units. Fiehn and Henriksen[49] reported that the use of 1 to 2 ppm NaOCl in intermittent doses reduced the HPC in ultrasonic scalers to 100 to 6,500 CFU/ml, while continuous treatment resulted in 270 to 610 CFU/ml. Removal of tubings from dental units and treatment in the laboratory with 5.25% bleach for 15 hours eliminated counts in the tubing effluent, but they tended to recur by 15 days.[37] In a clinic water distribution system, use of a chlorinator to provide a shock treatment with 50 ppm overnight and then to dispense 1 ppm for 4 weeks failed to eliminate counts or resident *Legionella*.[40] The use of 0.5% to 1% bleach once a week for 10 minutes over a 4-year period kept microbial contamination in check but caused a slow corrosion of metal fittings in the dental units. An energy dispersive x-ray study found traces of the corroded metals in residual biofilms.[50] Treating DUWL for 10 minutes with 1:6 bleach solution and then flushing it out eliminated bacteria in the effluent, but there were compliance problems in private practice settings.[51] Another study in a school clinic found that bleach reduced counts to 0 to 30 CFU/ml but did not remove the biofilm within the tubings.[52]

Another problem with chlorine or its hypochlorite solution is that it reacts with the organic matrix to create chlorinated by-products. The chemical reactions in the surface layers of the biofilm would prevent its penetration. In vitro tests on biofilms of waterline bacteria showed that the matrix reduced the penetration of chlorine over time.[47,53] Chlorinated by-products such as trihalomethanes are considered carcinogenic[54] and were detected in effluent water from DUWL treated with bleach at an average of 40 ppb, which is less than the 100 ppb safe standard.[48] Chloramine and chlorine dioxide do not form chlorinated by-products, and therefore penetrate deeper into biofilms in water systems and kill more effectively.[47]

Our periodontal postgraduate teaching clinic had 16 new chairs and chair-mounted dental units‡ installed in March 2001. The manufacturer recommended that the waterlines of the self-contained water system be purged nightly with air and disinfected weekly with 1:10 solution of household bleach for at least 10 but never more than 30 minutes. The manufacturer also issued supplemental advice on an alkaline peroxide product. Clinical reports indicated that after 4 or 5 weeks of treatment with alkaline peroxide, all units had counts under 200 CFU/ml,[55-58] and the biofilm on tubing samples examined by SEM was absent [56] or spotty.[58]

Chlorine dioxide is a biocide that effectively controls biofilm in many applications. It prevents the fouling of reverse osmosis membranes and is kinder to the membranes than chlorine.[59] Dose for dose, chlorine dioxide was more effective than chlorine on biofilms containing *Legionella pneumophila* and *Escherichia coli*.[60,61] In a papermill, stabilized chlorine dioxide controlled slime, and was less toxic and irritating than chlorine.[62] In in-dwelling silicone tubing vascular catheters, chlorine dioxide controlled biofilms more effectively than an antibiotic.[63] On stainless steel and polyvinyl surfaces, chlorine dioxide was effective against biofilms containing *E. coli*.[64] In a hospital water supply, stabilized chlorine dioxide was more effective than chlorine in controlling *Legionella* and did not corrode pipes or cause odors.[65]

Chlorine dioxide was also more effective than chlorine in controlling biofilms on stainless-steel heat exchangers in a water treatment plant[66] and in drip irrigation waterlines.[67] In trials on biofilms containing *L. pneumophila* in waterline systems, chlorine dioxide (0.5 ppm) reduced counts by 2 to 3 logs and significantly reduced biofilm as seen by SEM.[68] Chlorine dioxide was more effective than chlorine in preventing slime, odors, and foul tastes in a food-processing plant.[69]

‡ Adec, Newberg, OR.

In the dental setting, 0.1% stabilized chlorine dioxide mouthrinse reduced bacterial counts in the effluent of four simulated DUWL to less than 200 CFU/ml and did not adversely affect metal fittings.[70] In a private practice setting, stabilized chlorine dioxide used in independent reservoirs reduced HPC significantly ($P < 0.05$).[71] Stabilized chlorine dioxide mouthrinse used as a lavage with ultrasonic scalers also reduced HPC counts significantly ($P < 0.05$) more than a water lavage control after 4 minutes of flushing, and significantly more than water controls after being held in the waterlines overnight; the mouthrinse product also left significantly less biofilm as seen by SEM of waterline tubing samples.[72]

In the fall of 2001, we decided to conduct parallel tests of DUWL cleaning products in the 16 new dental units. The null hypothesis was that there would be no differences in the ability of products to control HPC counts and biofilm in the DUWL. Evaluations were designed to answer logistical questions of time, cost, and safety.

MATERIALS AND METHODS

The 16 chair-mounted dental units with self-contained water systems in the University of California-San Francisco postgraduate periodontology clinic had been in use for 6 months. Their reservoirs were filled with sterile saline during surgical procedures and with tap water at other times. The units were randomly divided into three DUWL treatment groups: five were treated with an alkaline peroxide product,§ five with a freshly mixed chlorine dioxide solution,‖ and five with a stabilized chlorine dioxide solution.¶ One unit served as an untreated control (Fig. 2). All units were provided with a quick-connect fitting and about 8 feet of tubing to be attached to the base of the chair where ultrasonic scaler waterlines are connected.

Control and Treatment Regimens

Control. The control regimen was that suggested by the dental unit manufacturer, except that no disinfectant was used. Each night, the water bottle was emptied and reinstalled, and the quick-connect was attached. The two air turbine handpiece hoses (handpieces removed), the two air-water spray (AWS) handpieces (tips removed), and the quick-connect hose were held over the sink while the foot control, the water buttons on the AWS, and the unit flush valve were depressed to purge the DUWL of water. Each morning, the bottle was filled with tap water and reinstalled, the quick-connect was attached, the lines and hoses were held over the sink, and all lines were flushed with water for 2 minutes. The quick-connect was removed, and the empty bottle was filled with tap water or sterile saline during the day as required.

Treatment 2. Alkaline peroxide treatment. According to the material safety data sheet (MSDS), the alkaline peroxide waterline cleaner contains sodium carbonate, sodium carbonate peroxy-hydrate, N-alkyl-dimethylbenzylammonium chloride dihydrate, and tetra-sodium salt of ethylenediamine-tetracetic acid tetrahydrate. The concentrations were not given. It is readily soluble in water to form a pink liquid. The alkaline peroxide regimen was that stated by the manufacturer. One single-unit dose packet was emptied into 8 oz of hot water, stirred until dissolved, and put into the water bottle. The bottle was reinstalled, the quick-connect was attached, and the handpiece hoses, AWS, and quick-connect hose were held over the sink while the foot control, water buttons, and unit flush valve were operated until the pink solution appeared at the ends of the lines. The unit was then shut off, and the alkaline peroxide allowed to stand in the DUWL overnight. The next morning the bottle was emptied, filled with hot water, and reinstalled; the quick-connect was attached, and the handpiece hoses, AWS, and quick-connect hose were held over the sink while all lines were flushed with water until the bottle was empty. The quick-connect was removed, and the bottle was filled with tap water or sterile saline during the day as required. This shock treatment was repeated on the first 3 days of week 1 and on the first day of week 2. On the remaining days, the units were treated with the control regimen.

Treatment 3. Freshly mixed chlorine dioxide treatment. According to the MSDS, part A of the freshly mixed chlorine dioxide product is sodium chlorite (the salt of chlorous acid), and part B is a combination of phosphoric acid, lactic acid, and a non-toxic catalyst. The concentrations were not given. The concentration of the active chlorine dioxide created by acidifying the chlorite was not given. Four of the five units allotted to this product were treated with the waterline cleaner, and one was treated with a related product designed for use as a mouthrinse.# The treatment was that stated by the manufacturer. On the first night, the bottle was removed and half filled with cool tap water; 60 ml of part A and 60 ml of part B were added, and the bottle was filled with tap water and reinstalled. The quick-connect was attached, the handpieces were removed, and the quick-connect hose, handpiece hoses, and AWS were held over the sink while the foot control, AWS buttons, and unit flush valve operated until the bottle was empty. The bottle was then refilled with tap water, all lines were flushed until the bottle was empty, and all fluids were purged from the unit with compressed air. The next morning, the water bottle was

§ Ultra (formerly Ultra-Kleen), cat. no. 513P, Sterilex, Owings Mills, MD.
‖ DioxiClear (formerly Ciderm Disinfectant), Frontier Pharmaceutical, Melville, NY.
¶ MicroClear Dental Unit Waterline Cleaner, Rowpar Pharmaceuticals, Scottsdale, AZ.
# DioxiRinse, Frontier Pharmaceutical Inc., Melville, NY.

half filled with tap water, and 1 ml of part A and 1 ml of part B were added. The bottle was filled with water and reinstalled, the quick-connect was attached, and the handpiece hoses, AWS, and quick-connect hose were held over the sink while the foot control, AWS water buttons, and flush valve were operated for 2 minutes. As required during the day, the bottle was refilled with the two 1 ml doses and tap water or with sterile saline for surgical procedures.

Treatment 4. Stabilized chlorine dioxide treatment. According to the MSDS, chlorine dioxide is the active ingredient, and the ingredients listed on the label are chlorine dioxide, trisodium phosphate, and purified water. The active chlorine dioxide concentration was not given. The bulk of the product is sodium chlorite, and nascent chlorine dioxide is formed when the product meets a reactive compound or a low pH. The regimen was that of the manufacturer, except that the amount used in the initial shock treatment was increased from 100 ml to 150 ml, to compensate for the volume of the quick-connect hose. At the end of the first day, the unit was turned off, the handpieces were removed, the quick-connect was attached, and 150 ml of the concentrate was put into the water bottle. The handpiece hoses, AWS, and quick-connect hose were held over a sink, and the foot control, AWS water buttons, and flush valve were operated for 30 seconds. The unit was turned off, the quick-connect was removed, and the concentrate was allowed to sit in the DUWL overnight. The next morning, the water bottle was removed, 75 ml of concentrate was added, and the bottle was filled with tap water, making a 1:10 dilution. The quick-connect was attached, and the handpiece hoses, AWS, and quick-connect hose were held over a sink while the foot control, AWS water buttons, and unit flush valve were operated for 30 seconds. The unit was turned off, and the quick-connect was removed. During the day, the bottle was refilled with the 1:10 dilution as it was emptied, or sterile saline used for surgical procedures. On subsequent days, the water bottle was filled with fresh 1:10 dilution and flushed for 30 seconds each morning.

The units were operated and cleaned by the periodontal residents. They also kept a log of the time to complete the waterline cleaning procedures and noted any unusual incidents of odor, taste, or irritation reported by patients, or effects on clothing.

Microbiological Evaluation

All samples were taken in the morning from the AWS on the dental assistant's side of the dental units. Clean examination gloves were put on; a clean, disposable tip was inserted into the AWS; and the AWS waterline was flushed for 2 minutes. This flush was done after any flush required by the waterline cleaning regimen. Two samples were taken from the AWS into HPC dental samplers,** the first a 1:10 dilution with sterile water, and the second undiluted. The HPC sampler paddle was then reinserted for 30 seconds or until bubbles of air no longer exited from the vent. The paddle was removed and the case emptied, the paddle and case were shaken to remove drops of water, and the paddle was reinserted into the case. The samplers were incubated for 7 days at room temperature.

After the incubation was complete, the paddle was removed and held at a slight angle under a spotlight so that any rounded colonies would be easier to see by a highlight on the far side and a shadow on the near side. The colonies were counted under a dissecting microscope at 10.5× magnification. The membrane filter on the surface of the culture pad has inked squares to facilitate counting. Only full squares were counted; partial squares on the edges or corners were not counted. There were usually six or seven complete squares from top to bottom and 11 or 12 across. Colonies were counted as individuals if they did not touch or if they touched, but differed in form or color. When the pad had areas of colony spread, squares with distinct colonies were counted, and the counts were adjusted for the total countable squares. The counts were repeated for accuracy and were made without reference to the dental unit number, treatment regimen, or previous count. Since the media pad of the sampler absorbs 1 ml of water, counts were reported as CFU/ml. A second investigator, also blind to the unit number and waterline treatment, did an independent count of samplers.

On most days, each unit had eight counts performed: two investigators made replicate counts of the diluted and undiluted samplers. However, on the second day, counts were made by only one investigator. Two of the treatments had many counts of 0. Because of skewed distributions with the many 0 counts, parametric assumptions for simple linear regression were not met. We therefore used negative binomial regression.†† This is a generalization of Poisson distribution, which is often used for count data. Because of the different timing of the treatment regimens and differing impacts of gaps over the weekend, we did not fit one overall model including all days but instead modeled each day separately. To account for likely dependence among the multiple counts from the same unit, we used the generalized estimating equations method with robust variance estimation.[73] All models included as covariates 1) whether the sample was diluted, 2) investigator, and 3) the logarithm of the unit's pretreatment estimated mean count of the first day baseline sample (to control for the possibility that some units were more bacteria-laden than others). Predicted counts

** HPC Dental Sampler, cat. no. MHPC 100 25, Millipore, Bedford, MA.
†† Stata 7.0, Stata Corporation, College Station, TX.

shown are from the negative binomial models with covariates equal to the mean of the two investigators, the mean of the logarithms of the pretreatment counts, and no dilution. For counts on treated days with all 0s, we list the upper confidence bound as 1, because the chance of all five units showing 0 counts is <0.05 if the true mean were 1.

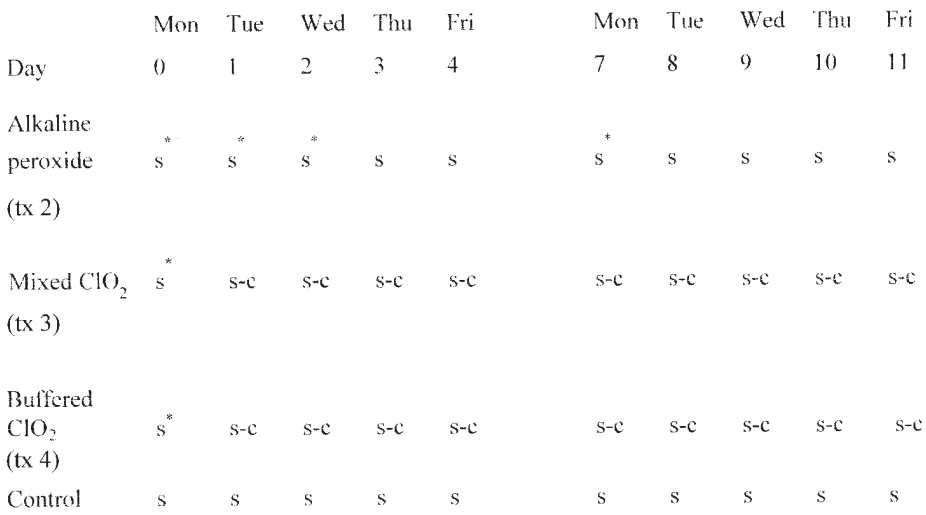

Figure 2.
*Timeline for waterline treatment regimen. Day 0, baseline; s, morning HPC sample; *, evening shock treatment; c, daily use of diluted agent.*

Scanning Electron Microscopy

The week before and the week after the clinical testing, biopsies were taken from the AWS waterline on the assistant's side of the dental unit. The terminal 5 mm of the waterline adjacent to the barb fitting and its sleeve were cut off with a fresh sterile scalpel blade and processed for SEM. The tubing samples were fixed in 3% glutaraldehyde in 0.2 M sodium cacodylate buffer and stored in the fixative for several weeks. The samples were then removed, washed in purified water, cut into sections, and split lengthwise to expose the interior surface. After fixation, the specimens were dehydrated in a series of ethyl alcohol, with a minimum of 1 hour at each step (50%, 70%, 80%, 90%, 95%, and 100%, and 100% repeated), and dried with 100% hexamethyldisilazane to minimize shrinkage due to drying, as described by Perdigao et al.[74]

The tubing samples were mounted on double-sided copper tape, and a 10 nm layer of gold was sputtered onto the samples. The samples were examined by SEM‡‡ at an operation voltage of 5 KeV, a beam diameter of about 8.5 nm, and a beam current of 1.5 nA. The images were digitally captured (1,000×) at the midpoint of the tubing.

The amount of biofilm coverage in each of the resulting 32 images was scored from 1 (lowest) to 32 (highest). After scoring, the key was opened that described the specimen code (before treatment or after treatment) and the treatment group. The means of the scores of each group were statistically analyzed for change in biofilm coverage with the Wilcoxon signed-rank test.

RESULTS

Microbiological

The intraexaminer correlations of original and replicate counts were very high (r = 0.998, P <0.0001; and r = 0.987, P <0.0001). The independent microbial counts of the two investigators were in excellent agreement (Spearman rank correlation coefficient of 0.894, P <0.0001).

The results are presented in Table 2. The estimated mean counts and 95% confidence interval (CI) of the control (treatment 1) seldom made the ADA goal of 200 CFU/ml. Treatment 2 met the goal on days just after shock treatments, but with time began to revert toward baseline. Figure 3 shows the counts of this group in relation to those of the control and waterline standards.

Treatments 3 and 4 had dramatic drops from baseline to 0 or near to 0. We did not see any counts of 200 or more for treatments 3 or 4 on any day, even after multiple counts on each of the units. Figure 4 shows the counts of treatment 3 in relation to those of the control and waterline standards.

Treatment 4, after the initial shock treatment, had all five treated units with HPC values of 0. Figure 5 shows the counts of treatment 4 in relation to those of the control and waterline standards.

In the unit treated with the mouthrinse variation of freshly mixed chlorine dioxide, the HPC decreased by about 95% from its baseline level of 2,105 CFU/ml to 112 CFU/ml the following day. However, there were several high counts, which were interpreted as possible sloughing of patches of biofilm, and these data were not included in the analyses.

Table 3 presents the results of treatment comparisons. The estimated ratios of counts for treatment 2 versus the control were significant (P <0.05) on only 3 days. Treatments 3 and 4 always performed better than the control (P <0.001).

Treatment 3 was better than treatment 2 in eight of nine comparisons. Treatment 4 was better than treatment 2 in all comparisons. Counts were too low in both

‡‡ XL-30sFEG, FEI Co., Hillsboro, OR.

Table 2.

Estimated Mean Counts and 95% Confidence Interval of DUWL in CFU/ml from Baseline Through 2 Work Weeks (treatments were not done on the weekend [days 5 and 6])

Day	Treatment 1 (control)	Treatment 2†	Treatment 3‡	Treatment 4§
0*	992 (976-1009)	1,343 (1061-1699)	1,123 (876-1440)	1,260 (909-1746)
1	444 (289-684)	47 (3-697)	0 (0-1)	0 (0-1)
2	171 (63-468)	21 (2-190)	2 (0-33)	0 (0-1)
3	408 (162-1027)	56 (16-203)	0 (0-1)	0 (0-1)
4	290 (240-350)	575 (257-1287)	0 (0-1)	0 (0-1)
7	162 (91-291)	45 (30-68)	3 (0-17)	0 (0-1)
8	378 (228-627)	67 (11-421)	0 (0-1)	0 (0-6)
9	199 (116-342)	257 (110-600)	0 (0-1)	0 (0-1)
10	265 (167-419)	69 (24-198)	0 (0-1)	0 (0-1)
11	130 (103-163)	438 (251-765)	0 (0-1)	0 (0-1)

* Day 0 is baseline, prior to treatments.
† Treatment 2 = alkaline peroxide.
‡ Treatment 3 = mixed ClO_2.
§ Treatment 4 = buffered ClO_2.

treatment 3 and treatment 4 to allow meaningful statistical calculations. They would appear to be equivalent in terms of microbiological results.

Scanning Electron Microscopy

SEM was performed to determine whether the test agents merely reduced the amassed planktonic forms of the heterotrophs or had a significant effect on removal of the biofilm itself. Biofilm coverage decreased in four of the five samples in each of the experimental groups, but increased in the control sample. Before treatments, the mean scores were comparable: treatment 3 (freshly mixed ClO_2), 22.8; treatment 4 (stabilized ClO_2), 19.8; and treatment 2 (alkaline peroxide), 18.8. After treatments, the mean scores were lower: treatment 2 was 2.6 less; treatment 3 was 10.2 less; and treatment 4 was 9.0 less. However, statistical analysis with the Wilcoxon signed-rank test showed no significant difference in coverage for any group. This was most likely due to the small number of samples in each group (n = 5) and control (n = 1).

In general, images obtained before treatment showed a nearly complete covering of biofilm, which had rod-shaped or filamentous microorganisms either scattered on the surface or clumped in masses and bits of unrecognizable debris (Figs. 6 and 7). Images after treatment showed fewer surface microorganisms on the biofilm, and occasional patches of bare tubing (Figs. 8 and 9).

Logistical Matters

Treatment of the control unit by drying and flushing required 4.6 minutes in the evening and 4.3 minutes in the morning. In the alkaline peroxide group, each of the five units had four shock treatments. The average time for the evening administration of the product was 4.4 minutes, and the morning flush averaged 4.6 minutes. Daily care on other days averaged 4.1 minutes in the evening and 4.6 minutes in the morning. Based on the retail price of the product, the daily cost per unit was $0.86 for the 10-day trial. There were no reports of odor, taste, or irritation by patients. However, there was some mess when the pink product foamed out of the AWS or handpiece lines after the shock treatment. Repeated tests of the pink solution showed it had a pH of 11.2.

The units treated with freshly mixed chlorine dioxide each had an initial shock treatment, followed by daily care with diluted product. The average time of the initial treatment, including the administration of the 60 ml doses of parts A and B and the succeeding flush, was 17 minutes in the evening and 9 minutes the next morning. Daily care with diluted product required an average of 5.9 minutes in the evening and 5.9 minutes in the morning. Based on the retail price, the daily cost per unit was $0.11. One of the residents commented that the odor of the concentrate was irritating to nasal and air passages. The solution used in the shock treatment had a pH = 3.6 (part A, pH = 13.2; part B, pH = 1.3). The solution used for daily care had a pH of 6.8.

In the buffer-stabilized chlorine dioxide group, the units also had an initial shock treatment and daily care with diluted product. The average time for the shock treatment was 7.7 minutes in the evening and 6.0 minutes in the morning. Thereafter, daily care took 3.6 minutes in the evening and 4.1 minutes in the morning. Based on the retail price, the daily cost per unit was $0.35. Only one resident remarked on a slight chlorine scent when dispensing the concentrate. The concentrate used for the shock treatment had a pH of 6.8. The 1:10 dilution used for daily care had a pH of 7.0.

DISCUSSION

Reviews of DUWL contamination and its significance as a factor in nosocomial infection of patients and health

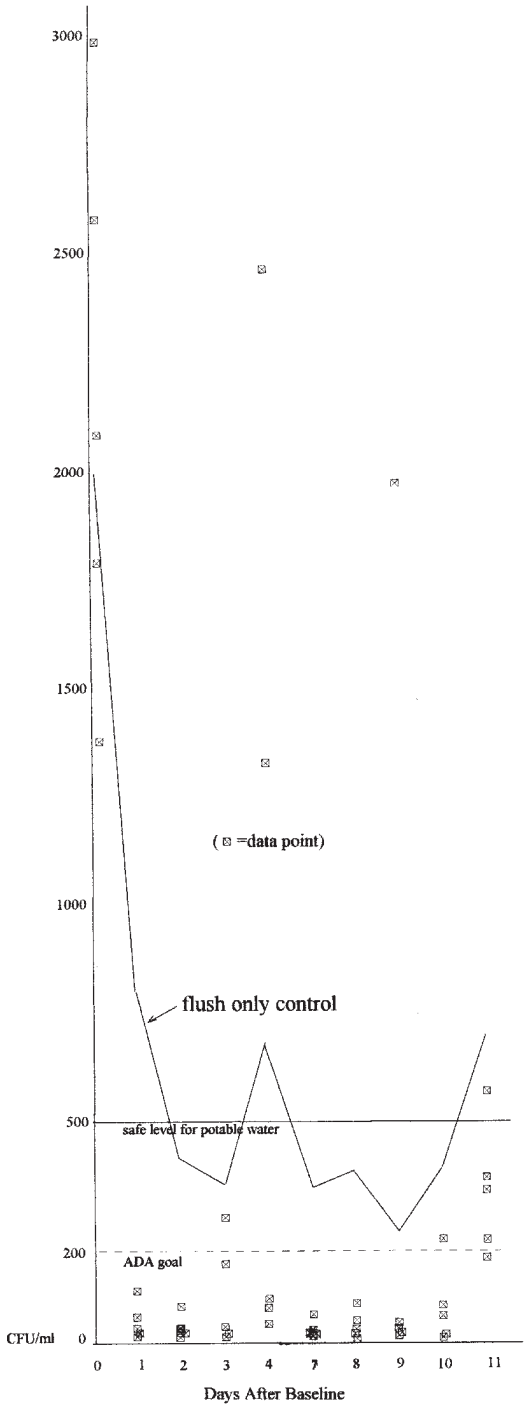

Figure 3.
A graph plotting the morning HPC (CFU/ml) of five dental units assessed at baseline (day 0), and daily for 9 days. Alkaline peroxide shock treatments were done in the evening on days 0, 1, 2, and 7. The estimated mean daily HPC ranged from 21 to 575 CFU/ml on treated days.

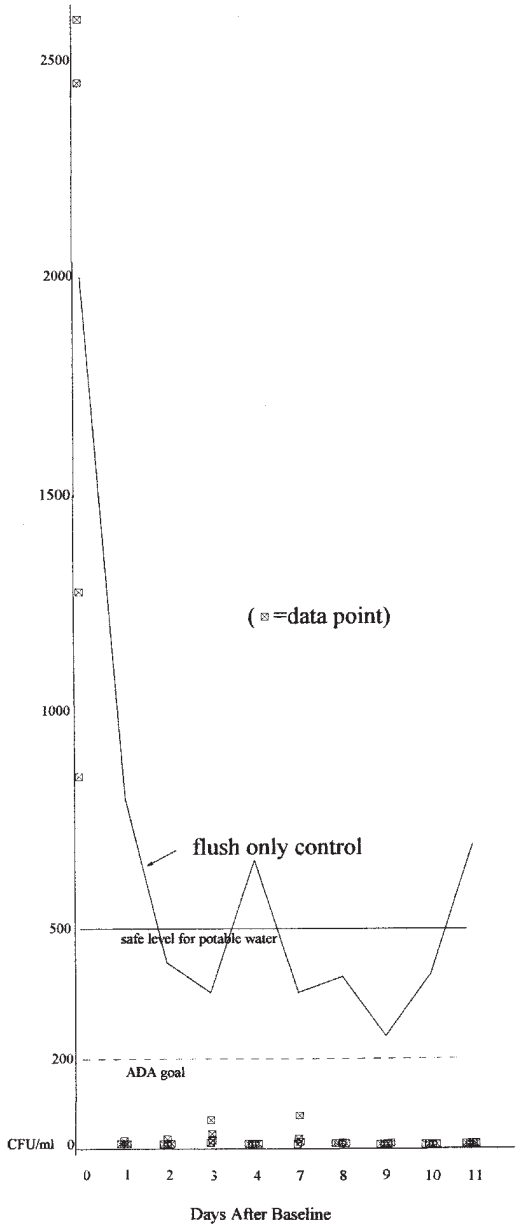

Figure 4.
A graph plotting the morning HPC (CFU/ml) of four dental units assessed at baseline (day 0), and daily for 9 days. A shock treatment with freshly mixed chlorine dioxide was done the evening of day 0, and continuous diluted treatments were done daily. The estimated mean daily HPC ranged from 0 to 3 CFU/ml on treated days.

care workers have stressed the threat to immunocompromised persons. The microorganisms found in the DUWL effluent have been considered to be of low pathogenicity, and there is little evidence that any have directly caused a human infection.[75,76] Opportunistic infections by the planktonic forms of these bacteria (Table 1) are conceivable. *Pseudomonas cepacia* may be associated with cystic fibrosis, *Pseudomonas aeruginosa* with burns, and *Legionella pneumophila* with Legionnaire's pneumonia or Pontiac fever; nontuberculous mycobacteria are considered emerging pathogens. There are many reports of infections in hospitalized patients from these organisms growing as

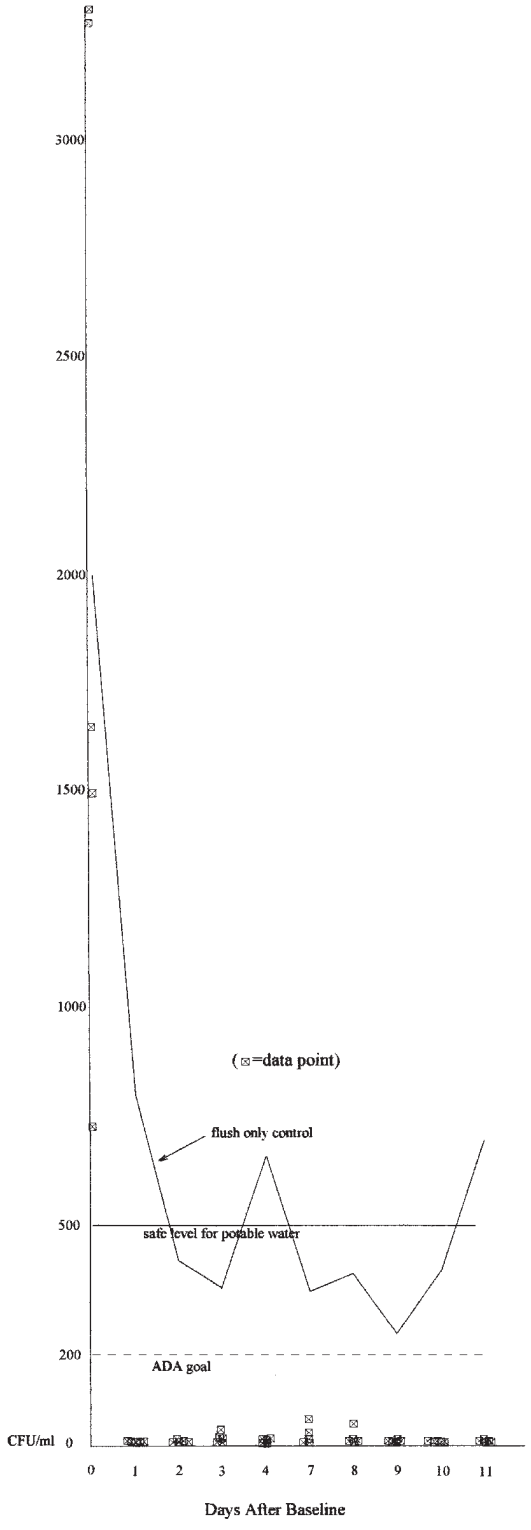

Figure 5.
A graph plotting the morning HPC (CFU/ml) of five dental units assessed at baseline (day 0), and daily for 9 days. Shock treatment with a buffer-stabilized chlorine dioxide was done on the evening of day 0, and continuous diluted treatments were done daily. The estimated mean daily HPC ranged from 0 to 1 CFU/ml.

Table 3.

Comparison of DUWL Treatments

	Treatment 2/Control		Treatment 3/Control*	
Day	Effect (95% CI)	*P*	Effect (95% CI)	*P*
1	0.11 (0.01-1.16)	0.066	0 (0-0.001)	<0.001
2	0.12 (0.01-2.8)	0.19	0.01 (0.001-0.09)	<0.001
3	0.14 (0.02-0.99)	0.049	0 (0-0)	<0.001
4	1.99 (0.86-4.6)	0.11	0†	<0.001
7‡	0.28 (0.11-0.70)	0.007	0.017 (0.003-0.081)	<0.001
8	0.18 (0.02-1.7)	0.13	0†	<0.001
9	1.29 (0.54-3.1)	0.57	0 (0-0)	<0.001
10	0.26 (0.07-1.04)	0.058	0 (0-0)	<0.001
11	3.4 (1.7-6.6)	<0.001	0 (0-0)	<0.001

Estimated effects are the ratios of counts from the first treatment listed to those from the second listed; counts had too many 0s in treatments 3 and 4 to allow comparison of those treatments.
* Entries of "0" indicate a value <0.001.
† Confidence intervals not available due to infinite parameter estimates. *P* values are from likelihood ratio tests.
‡ Samples not taken over weekend days 5 and 6.

biofilms in hospital water systems, medical devices, and even in antiseptic solutions.[75,76]

Another concern may be more relevant to the daily practice of periodontics. "Refractory periodontitis" has been an ill-defined diagnosis, generally meaning a type of periodontitis that does not respond to conventional oral hygiene instruction, deep scaling and root planing, gingival surgery, and routine maintenance therapy.[77] Retrospective studies of practices have described 4% to 28% of patients as "downhill" or "extreme downhill;"[78-80] these categories were especially associated with deeper pockets in molars.

Case reports and clinical studies of refractory periodontitis have had problems with the vague criteria for that diagnosis, and investigations of microorganisms associated with refractory disease have sought relationships to putative periodontal pathogens. Screening the subgingival microflora for 40 recognized taxa found that the only statistically significant difference between refractory and successfully treated cases was in the levels of *Prevotella nigrescens*.[81] Enteric rods have been reported in cases of human periodontitis; in a study of 500 patients with recurrent loss of attachment refractory to therapy including antibiotics, yeasts were isolated in 84 patients and enteric rods or pseudomonads in 51 patients.[82] The reported isolates included *Achromobacter* spp., *Acinetobacter calcoaceticus*, *Alcaligenes* spp., *Citrobacter freundii*, *Cladosporium*, *Enterobacter aerogenes*, *E. agglomerans*,

Table 3. (continued)

Comparison of DUWL Treatments

Treatment 4/Control*		Treatment 3/Tx 2*		Treatment 4/Tx 2*	
Effect (95% CI)	*P*	Effect (95% CI)	*P*	Effect (95% CI)	*P*
0 (0-0)	<0.001	0.003 (0-0.043)	<0.001	0 (0-0)	<0.001
0†	<0.001	0.08 (0.001-10.0)	0.31	0†	<0.001
0 (0-0.002)	<0.001	0 (0-0)	<0.001	0.002 (0-0.013)	<0.001
0†	<0.001	0†	<0.001	0†	<0.001
0 (0-0.001)	<0.001	0.06 (0.007-0.48)	0.008	0 (0-0.06)	<0.001
0.002 (0.001-0.01)	<0.001	0†	<0.001	0.01 (0-0.42)	0.014
0.001 (0.0-0.007)	<0.001	0 (0-0)	<0.001	0.001 (0-0.005)	<0.001
0.001 (0.0-0.004)	<0.001	0 (0-0)	<0.001	0.004 (0.00-0.018)	<0.001
0.003 (0.001-0.012)	<0.001	0 (0-0)	<0.001	0.001 (0-0.004)	<0.001

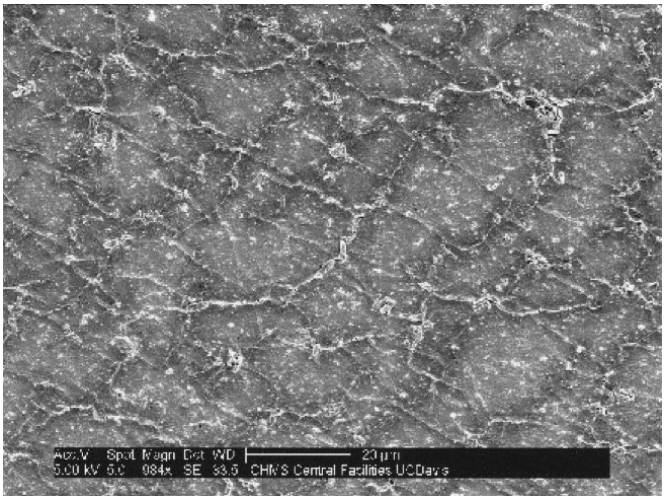

Figure 6.
SEM of DUWL inner surface of plastic tubing from the dental assistant's side of the dental unit before treatment. There is a continuous sheet of adherent biofilm. Rods and filamentous microorganisms of various sizes are scattered on the surface. (Original magnification ×1,000.)

E. cloacae, *Escherichia coli*, *Flavobacterium* spp., *Klebsiella oxytoca*, *K. pneumoniae*, *Proteus mirabilis*, *Pseudomonas acidovorans*, *P. aeruginosa*, *P. cepacia*, *P. maltophila*, and *Serratia marcescens*.[82-86] Many of these organisms have been found in DUWL (Table 1).

The majority of these Gram-negative, facultative rod strains isolated from cases of refractory periodontitis were resistant to thorough mechanical treatment and to tetracycline, penicillin, and erythromycin.[87] Ciprofloxacin systemic therapy required a 10-day regimen.[83,84] The antibiotic resistance of these microorganisms suggests that they may have been inoculated into periodontal pockets from contaminated DUWL and taken up residence as a biofilm in their new site.

In addition to the possible inoculation of microorganisms into periodontal pockets in patients undergoing treatment, one needs to consider the harm that might occur from the instillation of endotoxins released by these Gram-negative heterotrophs. The mean endotoxin unit (EU) level in samples of DUWL effluent has been measured at 80.7 EU/ml, which is considered enough to cause a fever in a normal, healthy patient.[88] In samples from 11 dental units with extensive biofilm contamination and high viable counts, the endotoxin level was 2,560 EU/ml before flushing. The correlation of HPC to endotoxin levels was significant ($r = 0.69$, $P < 0.05$). Even after 10 minutes of flushing, the endotoxin level was 220 EU/ml.[89] Filters would not stop the flow of endotoxin. Using sterile saline in a self-contained dental unit water system would not prevent the flow of heterotrophs and endotoxin from biofilms located downstream from the reservoir bottle.

Biomaterials pose a risk for the creation of biofilms and subsequent chronic infections. Sutures and orthopedic implants may acquire the seeds of a biofilm at the time of surgery, or they could arrive after placement by swarms of heterotrophs released from the biofilms on a catheter or pacemaker.[90] Direct examination of necrotic bone removed from two patients with osteomyelitis after treatment for leg fractures revealed biofilms consisting of Gram-positive and Gram-negative bacteria within a ruthenium red-positive glycocalyx on the bony surface; *Bacteroides melaninogenicus*, *Clostridium clostridiforme*, *Corynebacterium*, *Enterococcus*, *Fusobacterium*, *Proteus mirabilis*, and *Streptotoccus morbillorium* were isolated from cultures of the bone samples.[91]

The bottom line for clinicians is that they cannot risk letting DUWL contamination cause "refractory peri-

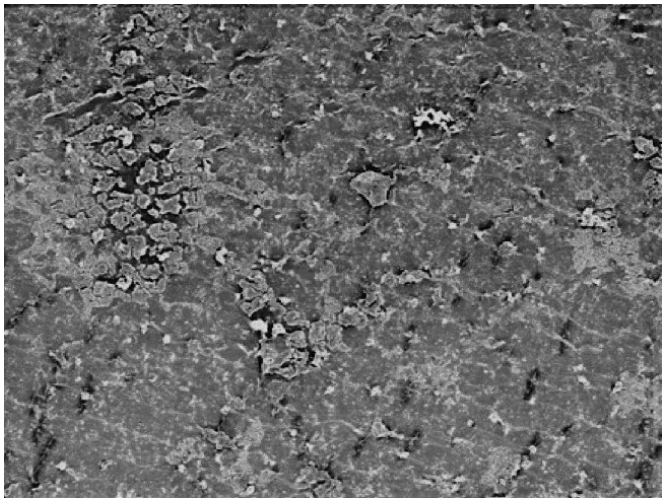

Figure 7.
SEM of DUWL inner surface of plastic tubing from the AWS on dental assistant's side of the dental unit before treatment. On its surface are scattered bits of debris and a cluster of rods and filaments. (Original magnification ×1,000.)

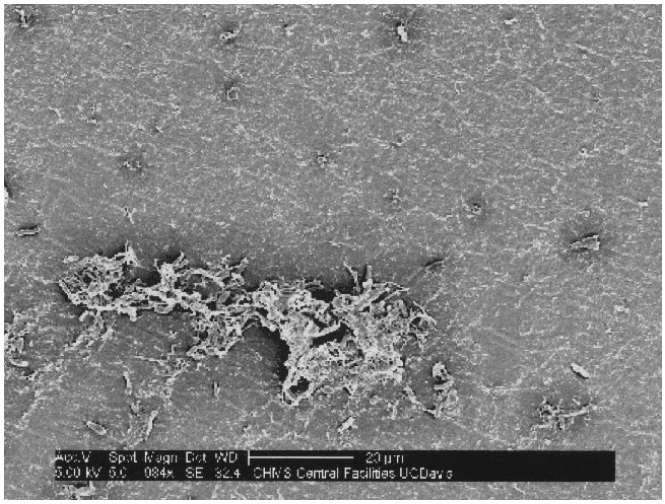

Figure 8.
SEM of DUWL inner surface of plastic tubing from the AWS on dental assistant's side of the dental unit after alkaline peroxide treatment. Fewer rods and filaments are visible, and some patches of tubing surface are exposed. (Original magnification ×1,000.)

odontitis" in their patients, or possibly the removal of a failing dental implant that has been carefully and skillfully placed. Clinicians need a sure remedy for control of biofilms in dental unit waterlines. An agent that is only partly effective may cause even higher HPC than existed before the DUWL treatment.[92]

Flushing the waterlines for 2 minutes before patients were treated and dehydrating the units when they were not in use, as done in our control unit, were ineffective and inconsistent in reducing HPC to

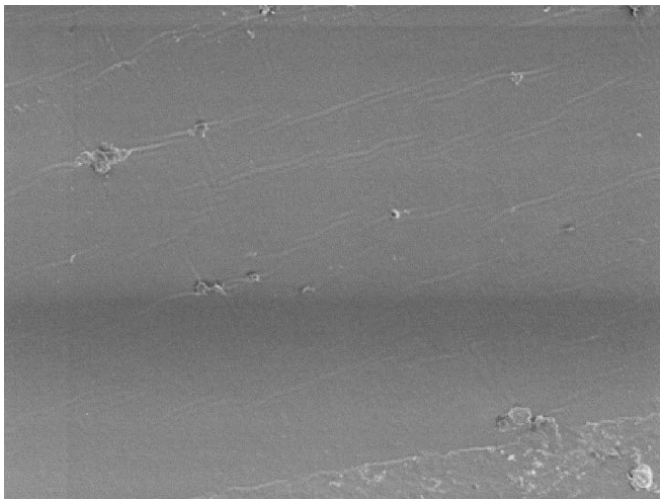

Figure 9.
SEM of DUWL inner surface of plastic tubing from the AWS of dental assistant's side of the dental unit after stabilized chlorine dioxide treatment. There is a nearly complete sloughing of biofilm. Only a few microorganisms and bits of debris remain. This specimen was the only one with such an effect; most of the images were similar to Figures 6, 7, and 8. (Original magnification ×1,000.)

acceptable levels. Alkaline peroxide treatments might be beneficial, as hydrogen peroxide has been recommended,[3,34,93] and chelation of cations by EDTA is a possible treatment ploy.[3] The HPC levels in this study after use of the alkaline peroxide cleaner were too inconsistent to be acceptable; the cost was higher than other agents tested; and although the time for carrying out the required procedures did not differ from the control, there is a safety concern about the high pH of the product and its handling by auxiliary personnel.

The chlorine dioxide products were very effective in lowering the HPC. From the standpoint of efficacy, there was no significant difference in the CFU/ml they achieved or in the mean scores of SEM images. The freshly mixed chlorine dioxide product had a higher initial cost per gallon than the buffer-stabilized chlorine dioxide, but less is used each day, so there is eventually some savings. However, both the shock treatment and daily care for the freshly mixed chlorine dioxide took longer than treatment with the buffer-stabilized chlorine dioxide. The high pH of part A, the low pH of part B, and the low pH and irritating odors of the freshly mixed chlorine dioxide are negative factors in its acceptance.

Overall, the evaluations of this study favor the use of the buffer-stabilized chlorine dioxide to control biofilms in DUWL. It was not expensive, took the least time to implement, had a neutral pH, and was very effective in reducing dental unit waterline CFU/ml.

ACKNOWLEDGMENTS

The authors are indebted to Grace Nonomura for SEM specimen preparation, to Michael Dunlap of the University of California-Davis for assistance with scanning electron microscopy, and to Dr. Peter Bacchetti, PhD, of UCSF for statistical assistance. The chlorine dioxide products used were gifts from their respective manufacturers; the alkaline peroxide product was purchased by the authors. The authors have no financial relationship with the manufacturer of any product used or mentioned in this article.

REFERENCES

1. ADA Council on Scientific Affairs. Dental unit waterlines: Approaching the year 2000. *J Am Dent Assoc* 1999;130:1653-1664.
2. Fletcher M. The physiological activity of bacteria attached to solid surfaces. *Adv Microb Physiol* 1991;32: 53-85.
3. Carpentier B, Cerf O. Biofilms and their consequences, with particular reference to hygiene and the food industry. *J Appl Bacteriol* 1993;75:499-511.
4. O'Toole GA, Kolter R. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* 1998;30:295-304.
5. Pratt LA, Kolter R. Genetic analysis of *Escherichia coli* biofilm formation: Roles of flagella, motility, chemotaxis and type I pili. *Mol Microbiol* 1998;30:285-293.
6. Sauer K, Camper AK. Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. *J Bacteriol* 2001;183:6579-6589.
7. Rice AR, Hamilton MA, Camper AK. Apparent surface associated lag time in growth of primary biofilm cells. *Microb Ecol* 2000;41:8-15.
8. Zobell CE. The effect of solid surfaces upon bacterial activity. *J Bacteriol* 1943;46:39-56.
9. Costerton JW, Cheng K-J, Geesey GG, et al. Bacterial biofilms in nature and disease. *Annu Rev Microbiol* 1987; 41:435-464.
10. Characklis WG. Fouling biofilm development: A process analysis. *Biotechnol Bioeng* 1981;23:1923-1960.
11. DeKievil TR, Iglewski BH. Bacterial quorum sensing in pathogenic relationships. *Infect Immun* 2000;68: 4839-4849.
12. Rashid MH, Rumbaugh K, Passador L, et al. Polyphosphate kinase is essential for biofilm development, quorum sensing, and virulence of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci* (USA) 2000;97:9636-9641.
13. del Pulcini E. Biofilms: Sensing and signaling. *CDA J* 2001; 29:351-353.
14. Eberl L. N-acyl homoserinelactone-mediated gene regulation in Gram-negative bacteria. *Syst Appl Microbiol* 1999;22:493-506.
15. Sutherland IW. Biosynthesis and composition of Gram-negative bacterial extracellular and wall polysaccharides. *Annu Rev Microbiol* 1985;39:243-270.
16. Hoyle BD, Alcantara J, Costerton JW. *Pseudomonas aeruginosa* biofilm as a diffusion barrier to penicillin. *Antimicrob Agents Chemother* 1992;36:2054-2056.
17. Wimpenny JW, Coombs JP. Penetration of oxygen into bacterial colonies. *J Gen Microbiol* 1983;129:1239-1242.
18. Roberts AP, Pratten J, Wilson M, Mullany P. Transfer of a conjugative transposon, Tn 5397 in a model oral biofilm. *FEMS Microbiol Lett* 1999;177:63-66.
19. Costerton JW, Lewandowski Z, DeBeer D, Caldwell D, Korber D, James G. Biofilms, the customized microniche. *J Bacteriol* 1994;176:2137-2142.
20. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. *Annu Rev Microbiol* 1995;49:711-745.
21. Wimpenny JWT. A unifying hypothesis for the structure of microbial films based on cellular automation models. *FEMS Microbiol Ecol* 1997;22:1-16.
22. Rittman BE. Detachment from biofilms. In: Characklis WG, Wilderer PA, eds. *Structure and Function of Biofilms*. Chichester, UK: John Wiley & Sons; 1989:49-58.
23. Costerton JW. The etiology and persistence of cryptic bacterial infections: A hypothesis. *Rev Infect Dis* 1984; 6(Suppl. 3):S608-S616.
24. Abel LC, Miller RL, Micik RE, Ryge G. Studies on dental aerobiogy: IV. Bacterial contamination of water delivered by dental units. *J Dent Res* 1971;50:1567-1569.
25. American Dental Association. ADA Statement on Dental Unit Waterlines. *J Am Dent Assoc* 1996;127:185-186.
26. ADA Council on Dental Materials and Devices, and Council on Dental Therapeutics. Infection control in the dental offices. *J Am Dent Assoc* 1978;97:673-677.
27. Beierle JW. Dental operatory water lines. *CDA J* 1993; 21:13-15.
28. Scheid RC, Rosen S, Beck FM. Reduction of CFUs in high-speed handpiece water lines over time. *Clin Prev Dent* 1990;12:9-12.
29. Santiago JI, Huntington MK, Johnston AM, Quinn RS, Williams JF. Microbial contamination of dental unit waterlines: Short- and long-term effects of flushing. *Gen Dent* 1994;42:528-535.
30. Atlas RM, Williams JF, Huntington MK. *Legionella* contamination of dental-unit waters. *Appl Environ Microbiol* 1995;61:1208-1213.
31. Barbeau J, Tanguay R, Faucher E, Trudel L, Cote L, Prevost AP. Multiparametric analysis of waterline contamination in dental units. *Appl Environ Microbiol* 1996; 62:3954-3959.
32. Challacombe SJ, Fernandes LL. Detecting *Legionella pneumophila* in water systems: A comparison of various dental units. *J Am Dent Assoc* 1995;126:603-608.
33. Furuhashi M, Miyamae T. Prevention of bacterial contamination of water in dental units. *J Hosp Infect* 1985;6: 81-88.
34. Kelstrup J, Funder-Nielsen TD, Theilade J. Microbial aggregate contamination of water lines in dental equipment and its control. *Acta Pathol Microbiol Scand* 1977;85(Sect. B):177-183.
35. Lück PC, Bender L, Ott M, Helbig JH, Witzleb W, Hacker J. Analysis of *Legionella pneumophila* serogroup 6 strains isolated from dental units. In: Barbaree JM, Breiman RF, Dufour AP, eds. *Legionella Current Status and Emerging Perspectives*. Washington, DC: American Society for Microbiology; 1993:240-242.
36. Martin MV. The significance of the bacterial contamination of dental unit water systems. *Br Dent J* 1987;163: 152-154.
37. Meiler TF, Depaola LG, Kelley JI, Baqui AA, Turng B-F, Falkler WA. Dental unit waterlines: Bio films, disinfection and recurrence. *J Am Dent Assoc* 1999;130:65-72.
38. Mills SE, Lauderdale PW, Mayhew RB. Reduction of microbial contamination in dental units with povidone-iodine. *J Am Dent Assoc* 1986;113:280-284.
39. Oppenheim BA, Sefton AM, Gill ON, et al. Widespread *Legionella pneumophila* contamination of dental stations

in a dental school without apparent human infection. *Epidemiol Infect* 1987;99:159-166.
40. Pankhurst CL, Philpott-Howard JN, Hewitt JH, Casewell MW. The efficacy of chlorination and filtration in the control and eradication of *Legionella* from dental chair water systems. *J Hosp Infect* 1990;16:9-18.
41. Schulze-Robbecke R, Feldman C, Fischeder R, Janning B, Exner M, Wahl G. Dental units: An environmental study of sources of potentially pathogenic mycobacteria. *Tuber Lung Dis* 1995;76:318-323.
42. Tall BD, Williams HN, George KS, Gray RT, Walch M. Bacterial succession within a biofilm in water supply lines of dental air-water syringes. *Can J Microbiol* 1995; 41:647-654.
43. Murdoch-Kinch C, Andrews NL, Atwan S, Jude R, Gleason MJ, Molinari JA. Comparison of dental water quality management procedures. *J Am Dent Assoc* 1997; 128:1235-1243.
44. Furuhashi M, Miyamae T. Prevention of bacterial contaminatrion of water in dental units. *J Hosp Infect* 1985;6: 81-88.
45. Fiehn NE, Larsen T. Effect of drying dental unit water lines on bacterial contamination. *J Dent Res* 2000;79 (Spec. Issue):567(Abstr. 3392).
46. Wang MY, Wirthlin MR, Hoover CI. Evaluation of air-purging and ClO_2 treatment on dental unit waterlines. *J Dent Res* 2001;80(Spec. Issue):622(Abstr. 762).
47. LeChevalier MW, Cawthon CD, Lee RG. Inactivation of biofilm bacteria. *Appl Environ Microbiol* 1988;54: 2492-2499.
48. Karpay RI, Plamondon TJ, Mills SE, Dove SB. Combining periodic and continuous sodium hypochlorite treatment to control biofilms in dental unit water systems. *J Am Dent Assoc* 1999;130:957-965.
49. Fiehn NE, Henriksen K. Methods of disinfection of the water system of dental units by water chlorination. *J Dent Res* 1988;67:1499-1504.
50. Sherman LR, Mills SE, Plamondon TJ. Identification of mineralization in clean and biofilm contaminated dental unit water lines. *J Penn Acad Sci* 1995;69:31-34.
51. Williams HN, Kelley J, Folineo D, Willams GC, Hawley CL, Sibiski J. Assessing microbial contamination in clean water dental units and compliance with disinfection protocol. *J Am Dent Assoc* 1994;125:1205-1211.
52. Williams HN, Johnson A, Kelley JI, et al. Bacterial contamination of the water supply in newly installed dental units. *Quintessence Int* 1995;26:331-337.
53. deBeer D, Srinivasan R, Stewart PS. Direct measurement of chlorine penetration into biofilms during disinfection. *Appl Environ Microbiol* 1994;60:4339-4344.
54. Bull RJ, Birnbaum LS, Cantor KP, et al. Water chlorination: Essential process or cancer hazard? *Fundam Appl Toxicol* 1995;28:155-166.
55. Shojaei M, Staat RH. Disinfection of dental unit water lines using hydroperoxide: Preliminary data. *J Dent Res* 1997;76(Spec. Issue):435(Abstr. 3372).
56. Leonard RH, Tart ND, Simmons E, Ambrose WW, Caplan DJ. Controlling DUWL bacterial contamination and biofilm by chemical biocides. *J Dent Res* 2000;79(Spec. Issue):567(Abstr. 3388).
57. Sims CA, Young JM, Dodge W, et al. Antimicrobial and corrosion effects of three dental unit waterline treatments. *J Dent Res* 2000;79(Spec. Issue):568(Abstr. 3394).
58. Linger JB, Molinari JA, Forbes WC, Farthing CF, Winget WJ. Evaluation of a hydrogen peroxide disinfectant for dental unit waterlines. *J Am Dent Assoc* 2001;132:1287-1291.
59. Adams WR. The effects of chlorine dioxide on reverse osmosis membranes. *Desalination* 1990;78:439-453.
60. Berg JD, Hoff JC, Roberts PV, Matin A. Disinfection resistance of *Legionella pneumophila* and *Escherichia coli* grown in continuous and batch culture. In: Jolley RL, Bull RJ, Davis WP, Katz S, Roberts MH Jr., Jacobs VA, eds. *Water Chlorination Chemistry, Environmental Impact and Health Effects*, vol 5. Chelsea, MI: Lewis Publ.; 1985:603-613.
61. Berg JD, Hoff JC, Roberts PV, Matin A. Resistance of bacterial populations to disinfection by chlorine dioxide. *J Am Water Works Assoc* 1988;80:115-119.
62. Cravens BB. Stabilized chlorine dioxide for microorganism control. *TAPPI* 1966;49:53A-55A.
63. Dennis MB, Jones DR, Tenover FC. Chlorine dioxide sterilization of implanted right atrial catheters in rabbits. *Lab Animal Sci* 1989;39:51-55.
64. Foshino R, Nervegna I, Motta A, Galli A. Bactericidal activity of chlorine dioxide against *Escherichia coli* in water and on hard surfaces. *J Food Prot* 1998;61:668-672.
65. Hamilton E, Seal DV, Hay J. Comparison of chlorine and chlorine dioxide disinfection for control of *Legionella* in a hospital potable water supply. *J Hosp Infect* 1996;32:56-60.
66. Mayack LA, Soracco RJ, Wilde EW, Pope DH. Comparative effectiveness of chlorine and chlorine dioxide biocide regimens for biofouling control. *Water Res* 1984; 18:593-599.
67. Rav-Acha C, Kummel M, Salaman I, Adin A. The effect of chemical oxidants on effluent constituents for drip irrigation. *Water Res* 1995;29:119-129.
68. Walker JT, Roberts AD, Lucas VJ, Roper MA, Brown RG. Quantitative assessment of biocide control of biofilms including *Legionella pneumophila* using total viable counts, fluorescence microscopy, and image analysis. *Methods Enzymol* 1999;310:629-637.
69. Welch JL, Folinazzo JF. Use of chlorine dioxide for cannery sanitation and water conservation. *Food Technol* 1959;13:179-182.
70. Puttaiah R, Sargent CM, Anderson K, Spears R, Cederberg R, Matheson K. Effects of a ClO_2 based mouthwash on dental unit waterline contamination. *J Dent Res* 2000;79 (Spec. Issue):227(Abstr. 666).
71. Matheson K, Brannon MD. The effects of stabilized chlorine dioxide on bacterial concentrations in dental unit water. *J Dent Res* 2000;79(Spec. Issue):568(Abstr. 3398).
72. Wirthlin MR, Marshall GW. Evaluation of ultrasonic scaling unit waterline contamination after use of chlorine dioxide mouthrinse lavage. *J Periodontol* 2001;72:401-410.
73. Liang K-Y, Zeger SL. Longitudinal data analysis using generalized linear models. *Biometrika* 1986;73:13-22.
74. Perdigao J, Lambrechts P, van Meerbeek B, Tome AR, Vanherle G, Lopes AB. Morphological field emission-SEM study of the effect of six phosphoric acid etching agents on human dentin. *Dent Mater* 1996;12:262-271.
75. Miller CH. Microbes in dental unit water. *CDA J* 1996;24: 47-52.
76. Barbeau J, Gauthier C, Payment P. Biofilms, infectious agents, and dental unit waterlines: A review. *Can J Microbiol* 1998;44:1019-1028.
77. Flemmig TF. Periodontitis. *Ann Periodontol* 1999;4: 32-37.
78. Hirschfeld L, Wasserman B. A long-term survey of tooth loss in 600 treated periodontal patients. *J Periodontol* 1978;49:225-237.
79. Goldman MJ, Ross IF, Goteiner D. Effect of periodontal therapy on patients maintained for 15 years or longer.

A retrospective study. *J Periodontol* 1986;57:347-353.
80. McFall WT. Tooth loss in 100 treated patients with periodontal disease. *J Periodontol* 1982;53:539-549.
81. Columbo AP, Haffajee AD, Dewhirst FE, et al. Clinical and microbiological features of refractory periodontitis subjects. *J Clin Periodontol* 1998;25:169-180.
82. Slots J, Rams TE, Listgarten MA. Yeasts, enteric rods and pseudomonads in the subgingival flora of severe adult periodontitis. *Oral Microbiol Immunol* 1988;3:47-52.
83. Slots J, Feik D, Rams TE. Prevalence and antimicrobial susceptibility of *Enterobacteriaceae, Pseudomonadaceae* and *Acinetobacter* in human periodontitis. *Oral Microbiol Immunol* 1990;5:149-154.
84. Rams TE, Feik D, Young V, Hammond BF, Slots J. Enterococci in human periodontitis. *Oral Microbiol Immunol* 1992;7:249-252.
85. Rams TE, Babalola OO, Slots J. Subgingival occurrence of enteric rods, yeasts, and staphylococci after systemic doxycycline therapy. *Oral Microbiol Immunol* 1990;5:166-168.
86. Helovuo H, Hakkarainen K, Paunio K. Changes in the prevalence of subgingival enteric rods, staphylococci and yeasts after treatment with penicillin and erythromycin. *Oral Microbiol Immunol* 1993;8:75-79.
87. Listgarten MA, Lai C-H, Young V. Microbial composition and pattern of antibiotic resistance in subgingival microbial samples from patients with refractory periodontitis. *J Periodontol* 1993;64:155-161.
88. Puttaiah R, Cederberg RA. Assessment of endotoxin levels in dental effluent water. *J Dent Res* 1998;77(Spec. Issue):263(Abstr. 1257).
89. Putins EE, DiGiovanni D, Bhular AS. Dental unit waterline contamination and its possible implications during periodontal surgery. *J Periodontol* 2001;72:393-400.
90. Gristina AG, Costerton JW. Bacterial adherence and the glycocalyx and their role in musculoskeletal infection. *Orthop Clin North Am* 1984;15(3):517-535.
91. Marrie TJ, Costerton JW. Mode of growth of bacterial pathogens in chronic polymicrobial human osteomyelitis. *J Clin Microbiol* 1985;22:924-933.
92. Walker JT, Bradshaw DJ, Bennett AM, Fulford MR, Martin MV, Marsh PD. Microbial biofilm formation and contamination of dental-unit water systems in general dental practice. *Appl Environ Microbiol* 2000;66(8):3363-3367.
93. Rosenzweig AL. Hydrogen peroxide in prevention of water contamination (letter to editor). *Lancet* 1978;1:944.

Correspondence: Dr. M. Robert Wirthlin, Department of Stomatology, University of California, Box 0762, San Francisco, CA 94143-0762.

Accepted for publication April 18, 2003.