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Low Current Electrical Enhancement of a Dental Unit Waterline Cleaner: Microbiological Effects

by

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THESIS

Submitted in partial satisfaction of the requirements for the degree of

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DEDICATION

This is dedicated to my parents, John and Jo, and my husband, Stephen. Thank you for all of your support and encouragement throughout my education.

ACKNOWLEDGEMENTS

This project began as the vision of Dr. Robert Wirthlin. It would not have been possible without his guidance and leadership. Dr. Chuck Hoover is deserving of recognition for his laboratory support and technical input. I would also like to acknowledge Dr. Art Miller and Dr. Gary Armitage for serving on my thesis committee.

Low Current Electrical Enhancement of a Dental Unit Waterline Cleaner: Microbiological Effects

Adrienne S. Gunstream, DDS

ABSTRACT

PURPOSE: The purpose of this study was to investigate the efficacy of applying a low voltage and current together with antimicrobials in the removal of bacterial biofilms from a simulated dental unit waterline (DUWL). The specific aim of this project was to report on the microbiological effects of this novel DUWL treatment method. **MATERIALS AND METHODS:** An experimental DUWL was designed which simulated the 15-feet of tubing, joined by three barb fittings, leading from the water reservoir to the air-water syringe in a standard dental unit. One length of tubing served as the test and a parallel length of tubing served as the control. A 0.2mm diameter platinum wire was passed through the lumen of the test tubing to allow current to be applied along the length of the test DUWL. *Pseudomonas aeruginosa* inoculated water was run through both lines at the start of each experiment to establish a biofilm. Effluent samples were collected on alternating days, plated on *Pseudomonas*-selective agar, and counted 2-3 days after plating. At the end of each experiment, the waterlines were disassembled and tubing sections were assessed for counts of viable bacteria. Trials with sterile tap water were compared to trials utilizing a dilute chlorine dioxide solution. **RESULTS:** Prior to applying current and chlorine dioxide to the test setup, effluent counts were >100,000 CFU/ml. After current and chlorine dioxide were applied, effluent counts initially dropped to 0 CFU/ml. Counts did increase periodically during the 4-week trial but never

rose above 3,020 CFU/ml. For the biofilm analysis, 0 CFU/cm^2 were observed for all experiments using a combination of current and chlorine dioxide. **CONCLUSION:** The combination of dilute chlorine dioxide and low current can effectively eliminate a *Pseudomonas aeruginosa* biofilm from a simulated DUWL. In addition, this combination can significantly reduce the effluent bacterial load when compared to the control.

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INTRODUCTION

Dental practitioners continually strive to provide a clean and safe environment for their patients. Although national and state regulations require adherence to strict standards for cleanliness in dental offices, patients may still be exposed to millions of potential pathogens during routine dental treatment. One source of these pathogens is the dental unit water line. For dental units connected to the municipal water supply, one mouthful of spray (20 ml) from the air-water syringe may contain as many as 2,000,000 bacteria or 100,000 colony forming units (CFU) per ml.[1] While municipal water is safe for consumption, it is not sterile. The Environmental Protection Agency (EPA) standard for bacterial contamination in drinking water is 500 CFU/ml.[2] The growth and persistence of microorganisms in dental unit water lines are to blame for the discrepancy between microbial contaminant levels of water flowing into and out of dental units.

Water system contamination and the use of chlorine dioxide

There are several sources of contamination for municipal drinking water. The United States Environment Protection Agency has identified contaminants in the following categories: microorganisms, disinfectants, disinfection byproducts, inorganic chemicals, organic chemicals, and radionucleotides.[2] The National Primary Drinking Water Regulations (NPDWR), set by the EPA, are standards meant to limit the Maximum Contaminant Levels (MCLs) of these contaminants in drinking water. Chlorine dioxide is regulated as a possible contaminant because of its wide use as a drinking water

disinfectant. In addition to its efficacy at reducing the microbial load of drinking water, chlorine dioxide has also been shown to improve the taste, odor, and color of raw water.[3] It does so by removing chlorinated phenols with unpleasant odors and tastes, and by oxidizing iron and manganese.[4] The NPDWR lists 0.8mg/L of chlorine dioxide as both the Maximum Residual Disinfectant Level (MRDL) and the Maximum Residual Disinfectant Level Goal (MRDLG). These designations indicate that there is a benefit to adding the disinfectant to the water and, in concentrations below the MRDLG, there are no known or expected risks to health.

Chlorine dioxide as a disinfectant

The disinfecting properties of chlorine dioxide are due its oxidizing activity. In the presence of *E. coli*, oxidative damage to the cell membrane inhibits respiration.[5] Chlorine dioxide has been shown to inactivate polioviruses by damaging the viral capsid.[6,7] Scatina *et al.*,[8] studied the activity of chlorine dioxide and found that it inhibited bacterial protein synthesis by removing methionine, which is the lead triplet for all bacterial protein synthesis. In the presence of chlorine dioxide, the tRNA for methionine was absent and the protein synthesis process was not initiated in the ribosomes.

Chlorine dioxide safety

In spite of its effects on bacteria and viruses, chlorine dioxide has been shown to be safe when used in appropriate concentrations in both human and animal studies. In a review by Calabrese *et al.*,[9] it was concluded that chlorine dioxide at concentrations less than 10mg/l is not toxic to animals. In rats, it has been shown that ingested chlorine dioxide (ClO_2) is converted to Cl^-, $ClO2^-$, and $ClO3^-$. The half-life for elimination was 44 hours with excretion occurring mostly in the urine.[10] Lubbers *et al.*,[11] examined the ingestion of increasing doses of chlorine dioxide, chlorite, and chlorate in 60 normal men for 12 weeks. They reported no undesirable physiological effects after extensive physical examination including: electrocardiogram, complete blood and urine analysis, thyroid function tests, Coombs tests, methemoglobin assay, glucose-6-phosphate dehydrogenase, glutathione, and sickle cell preparations. Michael *et al.*,[12] compared 197 persons in a community receiving chlorine dioxide through the municipal water to 112 controls over a 115-day period. No adverse effects were found.

Dental unit waterlines contamination: patient risks

It has been known for many years that dental unit waterlines (DUWLs) contain microbial contaminants. In 1963, Blake[13] identified bacteria in samples taken from dental units. Bacterial contamination of dental units was investigated by Abel *et al.*,[1] in 1971. This group reported average counts of 1000 colony-forming units (CFU)/ml from DUWLs with a range of 400 to $1x10^6$ CFU/ml. In comparison, samples taken from the domestic water supply had counts ranging from 0 to 90 CFU/ml. The significance of DUWL contamination may be substantial for individuals with a reduced immune

response. Martin[14] reported two cases of dental abscesses linked to the DUWL. Both cases involved immunocompromised patients who developed abscesses after restorative dental treatment. In each case, the same strain of *Pseudomonas aeruginosa* was recovered from the abscess and from the DUWL.

Dental unit waterlines contamination: provider risks

The potential health risks associated with DUWL contamination may exist with dental health care practitioners as well as with patients. The entire dental team is exposed to aerosols produced by high-speed handpieces, air-water syringes, and ultrasonic instruments. Higher *Legionella* antibody titers have been reported for dental healthcare workers when compared to the general population.[15,16] A lung tissue sample of a dentist who died from Legionnaire's Disease was examined along with water samples from his home water system and from his office DUWL. The water taken from the dentist's home had <100 *Legionella* per ml whereas the samples taken from the DUWL had >10,000 per ml. The predominant strain in the DUWL sample was consistent with the *Legionella pneumophila* strain found in the deceased dentist's lung tissue sample.[17]

Microbial introduction to the dental unit

Microorganisms may enter DUWLs from various sources. One source is the municipal drinking water flowing into the DUWL. However, ultrasonic scalers, high-speed handpieces, and air-water syringes may also introduce bacteria to the DUWL.

Retraction valves on older dental units are intended to create negative pressure in the DUWL and prevent dripping from the high-speed handpiece. This negative pressure is capable of retracting about 1ml of fluid through DUWL, depending on the magnitude of the pressure.[18]

Sudden drops in water pressure may also be capable of causing backsiphonage, or retraction of fluid into the DUWLs. The American Dental Association (ADA) Statement on Backflow Prevention states that the chance of a backsiphonage occurring is low, and the possibility of disease transmission after a backsiphonage is extremely unlikely.[19] Anti-retraction valves can be installed in dental units to prevent retraction of fluid through the water line. These devices may not always be effective. A recent study demonstrated that after 60 days of testing 9 anti-retraction valves, 8 of the valves demonstrated failure.[20] One clinic reported *Pseudomonas aeruginosa* contamination in over 86% of dental units fitted with anti-retraction valves. The authors postulated that the source of contamination was the oral flora of patients because *P. aeruginosa* was not found in the source water for half of the clinics.[21] Even with proper control of anti-retraction, a negative pressure capable of aspirating fluids can be developed within the turbine head when a highspeed dental handpiece coasts to a stop.[22]

Recommendations on control of dental unit waterlines

The problem of DUWL contamination has forced the ADA and the Centers for Disease Control (CDC) to issue statements regarding water quality in the dental office. In 1995, the ADA adopted a statement recommending that by the year 2000, dental

equipment should be designed to allow for no more than 200 CFU/ml of aerobic mesophilic heterotrophic bacteria in the output of DUWL for non-surgical procedures. The ADA statement lists the following devices as tools to improve the water quality in the dental office: independent water reservoirs, chemical treatment regimens, source-water treatment systems, daily draining and air purging regimens, and point-of-use filters.[23] The CDC issued a statement in 2003 recommending that water used in non-surgical dental procedures adhere to the EPA standards for drinking water at less than 500 CFU/ml for heterotrophic bacteria. The use of sterile water irrigation was recommended for surgical procedures.[24]

Microbial filters

Filtering may have some effect on the CFUs present in the effluent from DUWLs, but filters will not prevent bacterial contamination and biofilm formation downstream from the filter. Filters also present the problem of periodic clogging. In one study, filters placed several feet upstream from the air-water syringe did not reduce CFUs in the effluent while filters placed very close to the air-water syringe demonstrated a significant reduction in CFUs. However, the investigators were unable to reduce the CFUs to the CDC or ADA recommended levels for DUWLs.[25]

Disinfectants

Disinfectant treatment of DUWLs has been shown to reduce bacteria in the effluent to the ADA recommended <200 CFUs/ml for an experimental period of 6 weeks. The lines were treated overnight for 3 consecutive days with an alkaline peroxide solution. Thereafter, weekly follow-up treatments were performed.[26] Disinfectants may be effective at eliminating bacteria in a planktonic state, but may have less of an effect on biofilms. Wirthlin and colleagues[27] reported significant reductions in heterotrophic bacterial counts when DUWLs were treated with two types of chlorine dioxide solutions, but erratic effects of a peroxide–based agent. The same study showed reductions in some scanning electron microscopy (SEM) images of biofilm coverage of the DUWL after 10 days, but nothing statistically significant. Wu and Wirthlin[28] performed a 6-month trial of chlorine dioxide treatment of DUWL biofilms using a "shock" treatment of chlorine dioxide followed by continuous use of a 10:1 dilute chlorine dioxide solution in the dental unit reservoir, and compared results to a tap water control. The initial "shock" treatment reduced planktonic bacterial counts to zero overnight. Using SEM, significant reductions in biofilm coverage were demonstrated between the experimental and control groups, and within the experimental group after a period of 6 months. Although the biofilms were significantly reduced by chlorine dioxide, they were not completely eliminated.

Pseudomonas aeruginosa **pathogenesis**

Pseudomonads are gram-negative rods typically found in soil and water. They are aerobes that can survive in habitats with very few nutrients, such as tap water.[29] *P.*

aeruginosa is an opportunistic pathogen that typically causes disease in humans when host defenses are compromised. Some examples of weakened host defenses are neutropenia, corneal ulcerations, and skin burns. Additionally, hospitalized, intubated patients and those with urinary catheters are at risk for *P. aeruginosa* infection.[30] *P. aeruginosa* infections are commonly seen in the lungs, especially in patients with cystic fibrosis. The inability of cystic fibrosis patients to clear mucous from their lungs leads to chronic and ultimately fatal *P. aeruginosa* infections. This species adheres to mucous layers and damaged epithelium, but, when in contact with intact epithelium, will not cause infection.[31] If *P. aeruginosa* enters the blood and causes sepsis, the mortality rate is greater than 50%.[32]

One of the major virulence factors of *P. aeruginosa* is its ability to produce a thick, exopolysaccharide matrix, which is a type of alginate. Many *P. aeruginosa* biofilms are resistant to antimicrobials including antibiotics, detergents, and solvents. This resistance to treatment is due its ability to establish a complex biofilm. When *P. aeruginosa* contact a surface, a mature biofilm forms after about 6 days, and at 9 days, cells may disperse from the surface and enter the surrounding fluid.[33]

The exopolysaccharide alone is not completely responsible for *P. aeruginosa's* antimicrobial resistance. Both penetration of the biofilm and exopolymer neutralization of antimicrobials were addressed in one study. A mutant strain of *Pseudomonas aeruginosa* was identified which had a decreased resistance to the antibiotic tobramycin in the biofilm state when compared to the wild type. This same strain showed no difference in antibiotic resistance when in the planktonic state. Both biofilms demonstrated equal viability when antibiotics were not present, and the antibiotic was

able to diffuse through both biofilms with no difference. It was found that the mutant strain was unable to produce cyclic sugar polymers, which could sequester foreign molecules in the exopolymer matrix.[34]

Bacterial biofilm formation

When bacteria encounter a surface, the following series of events occur leading to the formation of a bacterial biofilm:

1. Reversible attachment influenced by the electrical charge of the bacteria
2. Irreversible attachment consisting of chemical and physical bonds facilitated by alterations in gene expression which allow for adherence
3. Growth and division of bacteria leading to the accumulation of autoinducers
4. Production of extracellular polymer substances (EPS)
5. Attachment of other organisms to the slime layer of EPS and bacteria.[35]

Within a few minutes of reversibly attaching to a surface, specific genes are expressed by the bacteria that lead to irreversible adhesion. In a bacterial species, these changes in gene expression may lead to a biofilm phenotype that is very different from the phenotype seen in the planktonic state. For instance, in *Pseudomonas aeruginosa,* the proteins in the cell envelope in the biofilm phenotype are 30-40% different from those seen in the planktonic phenotype.[36] Another study demonstrated that slight changes in overall gene expression for *P. aerugionsa* may be responsible for dramatic differences between planktonic and biofilm phenotypes. Differential gene expression for only 73 of 5500 genes was noted when planktonic and biofilm bacteria were compared.[37]

Biofilm organization

Biofilms serve as a survival strategy for bacteria. Key characteristics of biofilms aid in their persistence and survival, and make their eradicaton a challenge. These include the exopolysaccharide matrix, formation of microcolonies, water channels, steady-state shedding of planktonic bacteria from the surface, and coordinated activities.[38] The exopolysaccharide slime layer serves to cement the bacteria to the solid surface and provides resistance to physical removal.[39] It also holds the bacteria together and allows for the formation of microcolonies, which work together to maintain the biofilm. Water channels, voids within the biofilm, allow for communication and fluid flow between microcolonies.[38,40]

Quorum sensing

Quorum sensing is a method of coordinating activities among bacteria. Bacteria communicate with one another through the release signaling molecules or autoinducers. This cell-cell communication is called quorum sensing. When autoinducer concentration, which is influenced by cell density, reaches a threshold level, a signaling cascade occurs and individual bacteria display coordinated activities. *P. aeruginosa* release acyl homoserine lactones (AHLs) which cross cell membranes and bind Lux-R proteins. The Lux-R-AHL complex then binds DNA promoters and activates gene expression.[41]

These alterations in gene expression affect the way bacteria respond to changes in the environment. Quorum sensing allows bacteria to form a biofilm and resist antimicrobials and host responses. Bacteria may use quorum sensing to delay the release of virulence factors until cell density is great enough to overcome host defenses.[42]

Biofilm resistance to antimicrobial agents

Bacteria in a biofilm state may be 1000 to 1500 times more resistant to antimicrobials and antibiotics than bacteria in a planktonic state.[36] Armitage[43] described four possible mechanisms for bacterial biofilm antimicrobial resistance:

1. Failure to penetrate the biofilm
2. Neutralization or consumption of the drug
3. Presence of drug-resistant bacteria
4. Inability of the drug to affect "dormant" bacteria.

It seems probable that antimicrobials are able to penetrate the biofilm matrix. It may, however, be possible that population-induced stress responses of biofilm bacteria result in a phenotype that is resistant to antimicrobials.[44] When *Pseudomonas aeruginosa* in biofilms are exposed to high doses of tobramycin, alterations in gene expression for twenty genes have been demonstrated.[37] In the biofilm state, expression of a single gene in *P. aeruginosa* allows for the production of periplasmic glucans, which can protect the bacteria from foreign molecules.[34] Because of the close physical proximity of biofilm bacteria, genes for resistance can easily be passed between organisms through horizontal gene transfer.

Bacteria deep within the biofilm may show reduced growth rates due to low availability of nutrients and resistance to oxygen diffusion.[45] This "dormant" or slow-growing phenotype deep within the biofilm may have some role in the resistance of biofilms to antibiotics and antimicrobials.

While the mechanism for biofilm resistance to antibiotics and disinfectants is not completely understood, it is likely that a combination of the previously described defense systems is responsible. The ability of the exopolysaccharide slime layer to sequester foreign molecules may allow time for individual bacteria to express genes conferring resistance. The slowed growth of bacteria within the biofilm may offer an additional defense mechanism.

Biofilm formation on dental unit water lines

There are several reasons why biofilms form on small-diameter DUWLs. These include the hydrophobic polymeric tubing material, which may favor bacterial adhesion, long periods when the fluid in the lines is stagnant, a high surface area to volume ratio within the tubing, and the flow-profile through the tubing. Flow through DUWLs is laminar in nature. When flow is laminar, the fluid moving with the highest velocity is at the center of the tube. The flow profile tapers from the center of the tube to the wall of the tube, where the flow rate is zero. The low shear stresses at the wall of the tube allow for bacterial colonization.

The currently available systems intended to prevent microbes from flowing out of dental units have little to no affect on established biofilms. Disinfectants, cleaning

solutions, and filters are effective against bacteria in the planktonic state, but established biofilms have proven to be much more resilient.

Electrical enhancement of antimicrobials

The effects of electricity on bacterial growth have been studied for many decades. In 1974, Barranco, *et al.*,[46] demonstrated zones of inhibition for plated bacteria when weak currents were applied to electrodes incorporated into the plates. Additionally, the combination of low current and antimicrobials has shown promise in eliminating bacteria. Blenkinsopp, *et al.*,[47] studied 24-hour old *P. aeruginosa* biofilms growing on stainless steel surfaces in a modified Robbins device. These investigators reported decreases in CFUs when the biofilms were treated with combinations of low current and industrial biocides.

The problem of biofilm growth on tubing is not limited to DUWL. Urinary and intravenous catheters are major sources of nosocomial infections. Several *in vitro* studies have demonstrated some degree of inhibition of bacterial growth in catheters when low current was applied to wires within the catheters.[48,49,50] One of the studies compared wires used to apply current to the catheters and found that platinum wires were as effective as gold but lasted nearly twenty-times longer.[49] However, these reports were of tests on suspensions of bacteria, and results on sessile biofilms were not provided. An animal study demonstrated significantly less bacteria in the urinary tracts of sheep when currents were applied to platinum electrodes on urinary catheters in place for 21 days.[51] Again, only effects on planktonic forms were reported.

A recent study reported on the effect of a small electric current applied to dental unit waterlines.[52] In this study, a simulated dental waterline was developed, and fluid was cycled through the line with a pump. An electric current generator was incorporated into the loop, which created a current potential downstream from the pump. The results from this study showed initial decreases in CFUs from effluent samples during the first week. This decrease was followed by a gradual increase in CFU counts for the remaining five weeks of the study. Observations of SEM images of some tubing sections suggested an effect on matrix or surface of bacteria, but this was not quantified or tested through culture data.

Hypothesis

The intent of this study is to investigate the efficacy of applying a low voltage and current together with antimicrobials in the removal of bacterial biofilms in DUWL. The specific aim of this project is to report on the microbiological effects of low current and antimicrobials applied to a simulated DUWL. The hypothesis is that current applied along the length of a DUWL will greatly enhance the efficacy of a chlorine dioxide solution in the removal of bacterial biofilms from a simulated DUWL.

Significance of this research

This is the first description of a system designed to eradiate bacterial biofilms along the entire length of a DUWL through the use of electric current. The prevention and elimination of bacterial biofilms in DUWL may have a significant impact on the

health of both patients and dental health care providers, especially those with weakened immune systems.

MATERIALS AND METHODS

Model DUWL

A model DUWL was constructed to simulate an aDec dental unit water line between the reservoir and the 3-way, air-water syringe (AWS). A parallel system allowed for test and control experiments to be performed simultaneously. Each length of tubing was comprised of three 5-foot sections of 1/8" outside diameter polyurethane tubing (#036.005.04, aDec, Newberg, OR), connected with three "barb" fittings similar to those found in the dental unit. The tubing was in a new, clean condition.

The first barb fitting of each line of tubing was connected to a section of sterile IV tubing approximately 10-cm in length. Each section of IV tubing included an injection port. The IV tubing was connected by a brass Y-fitting to a neoprene tube. The neoprene tubing was connected to a peristaltic pump, which was connected by another neoprene tube to a 10-liter carboy reservoir (Figure 1).

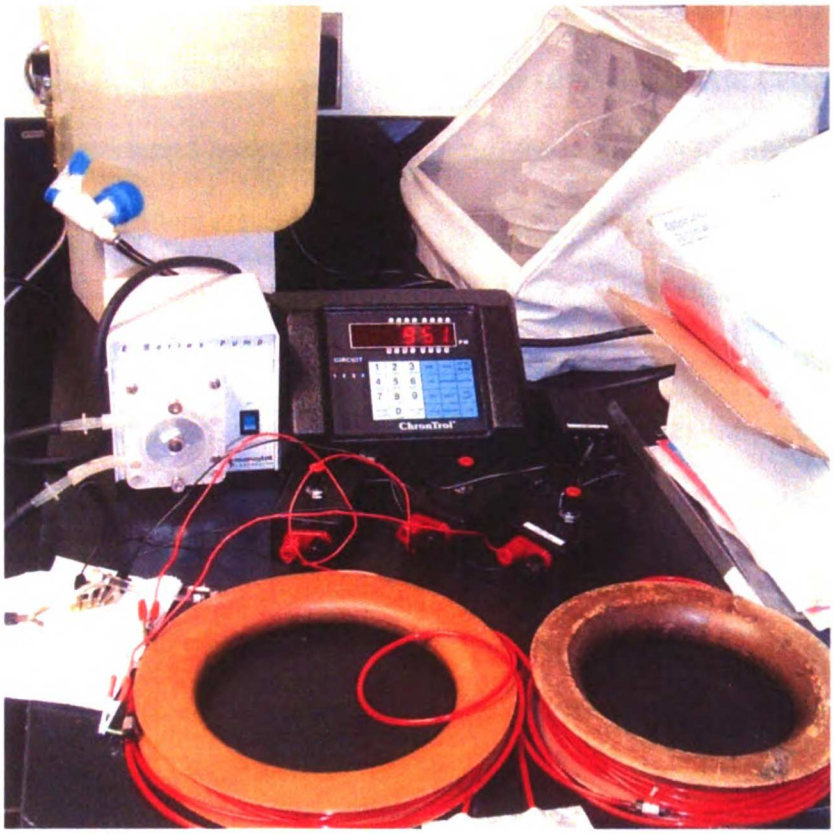
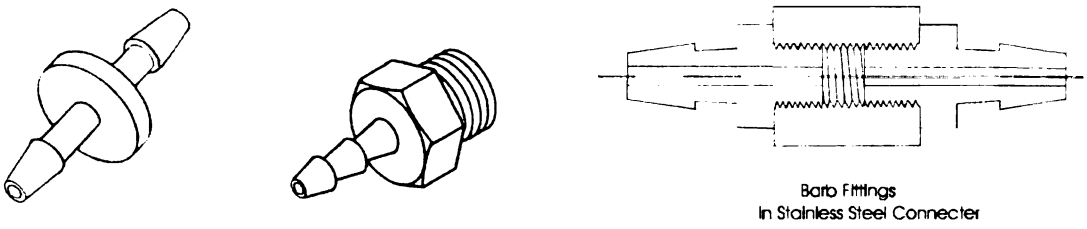


Figure 1: The experimental setup including the coil of test DUWL on the left side and the control DUWL on the right side. Behind the coils are the current controllers, the peristaltic pump and electronic timer, and the 10L carboy reservoir.

A platinum wire (15' long, 0.2mm dia.) with a rating of 0.03387ohms/cm was used as one electrode in the experimental model. The wire was autoclaved prior to set-up and handled with sterile gloves. After passing the wire through the IV injection port, it was continued along the length of the test water line. Approximately 5mm of the platinum wire was left extending from the injection port to allow attachment of a current source. As the wire was inserted into the injection port and waterline, care was taken to avoid bending or nicking its surface.

In this DUWL model, the barb fitting connectors were modified from those of the

standard dental unit, which uses straight 1/16" plated brass. For the model DUWL, two 10-32 x ¼" hex/male-1/16" barb Nylon plastic fittings (#101K, E-Z Air, C&OR, Rogue River, OR) were connected back-to-back to a metal sleeve with 10-32 female openings at each end. This arrangement created a 1/16" gap of metal inside the sleeve between the ends of the barb fittings to serve as opposite electrodes to the platinum wire electrode in the test system. The wire was able to pass through the barb fittings without creating an electrical short (Figure 2). In the first experiment, the metal sleeves were made of plated brass. But, after corrosion from the plated brass caused clogging of the test set-up (Figure 3), the sleeves were switched to type 316 stainless steel (#MF-1010-316, Beswick Engineering Co. Inc., Greenland, NH) for subsequent experiments. A 1" length of electrical wire was soldered to the outside of the metal sleeve for connection to the current source. The same barb fittings were used in the control water line, but no current was applied. The metal sleeves were autoclaved prior to each set-up, and the Nylon male barb fittings were always used in a new, clean condition.



A) B) C)

Figure 2: Schematic drawings of: A) standard dental unit straight barb, B) 10/32x1/4" hex male barb, and C) a cross-section of two hex male barbs connected by a stainless steel fitting with the platinum wire passing through the unit.

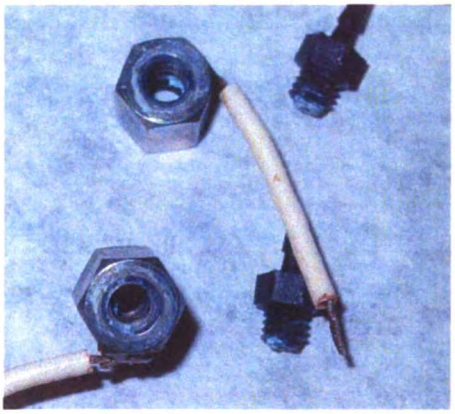


Figure 3: Corrosion present in plated brass barb fitting connectors in test setup after Experiment 1. Inside the connector and on the interior face of the barb fitting crusts of a greenish-blue color were seen and thought to be copper chlorides.

The current regulator

A current regulator was designed which could be easily attached to and removed from the experimental waterline. The current regulator device was similar to that of Mims[53] but with an added potentiometer. Alligator clips allowed the device to be connected to an anode (the barb fitting) and a cathode (the platinum wire) applying a voltage of 12-V DC and a current of 10 milliamperes (10-mA).

The input to the current regulator was 15-V DC from a wall transformer, protected by a 1-A fuse. Input was directed to three current regulators, one for each barbed fitting anode. The current regulators used a 7812T 12-V positive voltage regulator and a 30-ohm fixed resistor to limit current to no more than 400mA. The current to each anode was set to 10mA and verified with an ammeter. This was done by connecting the

ammeter between the current regulator and the barb fitting sleeve with test lead clips and manipulating the potentiometer until the current was set correctly. Then, the ammeter and test clips were removed, and the current regulator was connected to the barb fitting sleeve by its alligator clip.

Biofilm formation

To simulate the daily use of a dental operatory unit, sterile tapwater was pumped through the system at a rate of 50 ml/min. The pump (Manostat 72-410-014, Cole-Parmer, Vernon Hills, IL) was connected to a programmable timer (ChronTrol XT, ChronTrol Corp., San Diego, CA) and run continuously for a 5-minute interval every 30 minutes, 8 hours per day (8am to 5pm, no runs from 12 to 1), 5 days per week. The flow rate and effluent output were determined primarily by the internal diameter of the tubing used in the peristaltic pump, which cycled at 14 rpm. The action of the peristaltic pump introduced intermittent fluid shear as in a DUWL.

For each experiment, inoculated water was initially pumped through the system to create a biofilm on the internal wall of the DUWL tubing. First, a 10-L carboy was filled with tapwater and autoclaved. The sterile water in the carboy was then inoculated with the predominant DUWL microorganism, *Pseudomonas aeruginosa* ($1X10^3$ CFU/ml), strain 27853. After the first 10-L carboy of inoculated water was pumped through the system, another carboy containing either sterile tap water or a sterile 10:1 chlorine dioxide (MicroClear, Rowpar Pharmaceuticals, Inc, Scottsdale, AZ) solution was connected to the system.

Data collection, planktonic assessment

Samples of the effluent were collected at the terminations of the waterlines every 2 to 3 days. In Experiment 1, the samples were compared for turbidity in a spectrophotometer (Emax Precision Microplate Reader, Molecular Devices Corporation, Sunnyvale, CA). Throughout Experiment 1, no turbidity was detected, and this practice was discontinued for the subsequent experiments. The pH and oxidation-reduction potential (ORP) of the samples were measured with an electronic pH/ORP meter (Oyster 10, Extech, Waltham, MA).

For all experiments and at all times during the experimental periods, effluent samples were plated on *Pseudomonas*-selective agar (#17208, *Pseudomonas* Isolation Agar, Aldrich) plates, and CFUs were counted 2-3 days after incubating at room temperature. Samples were serially diluted and plated at concentrations of 10^{-1}, 10^{-2}, and 10^{-3}. The 10^{-1} sample was taken directly from the effluent. The 10^{-2} dilution was created by adding 1-ml of the effluent to 9-ml of phosphate-buffered saline (PBS). The 10^{-3} dilution was created by adding 0.1-ml of the effluent to 9.9-ml of PBS. For each dilution, 0.1-ml was spread on a *Pseudomonas*-selective agar plate. After 2-3 days of incubation, counts of CFUs were performed at 10X under a light microscope.

During the biofilm formation periods, CFU counts from consecutive days were compared. When the counts from two consecutive days did not increase and remained within one log of each other for both the test and control, steady state was assumed. A steady state biofilm was a requirement because bacterial biofilms in earlier phases of

development are more sensitive to antimicrobial agents. In a steady state they are more resistant, and more closely resemble conditions in a DUWL.

Assessment of biofilm

At the end of each experimental period, the water line setup was disassembled, and "biopsies" of the test and control lines were examined. The middle 5' section of polyurethane tubing was used for both the test and control line "biopsies." One 2.1-cm section was taken near the anode (the barb fitting), and another 2.1-cm section was taken from the middle of the tubing. This length was chosen because it would allow for a total inner surface area of $1cm^2$. Each "biopsy" was then split lengthwise with a sterile #15 blade. The inner surface was scraped with a sterile curette, which was shaken by hand into 10ml of phosphate-buffered saline. A small, sterile brush (Microbrush, Microbrush Corp., Grafton, WI) was then used to scrub the inner surface of the tubing. The end of the brush was removed with sterile scissors, and the brush head along with the tubing itself were added to the 10ml PBS solution. The PBS solution containing the tubing sample and the brush head was sonicated for 30 seconds, vortexed for 30 seconds, and homogenized for 30 seconds. These samples were serially diluted and plated in triplicate at concentrations of 10^{-1}, 10^{-2} and 10^{-3} as previously described.

Experiment 1: The initial 10-liter carboy of inoculated, previously sterilized tap water was run through the system followed by sterile tap water without inoculation. When CFU values from effluent plates indicated that steady state had been achieved (7 days),

current was applied. In this experiment, current was applied to the test setup only during working hours (8am-5pm) with the use of the previously described programmable timer. The effluent samples and biofilm samples were plated once at each dilution, and the experiment was conducted over a 10-day period. Plated brass metal sleeves were used in the barb fittings for this experiment only.

Experiment 2: The initial 10-liter carboy of inoculated sterile tap water was run through the system followed by sterile tap water without inoculation. After a steady state biofilm was achieved (26 days), current was applied to the test setup 24-h per day for 21 consecutive days. Effluent samples and biofilm samples were plated in triplicate at each dilution.

Experiment 3: The initial 10-liter carboy of inoculated sterile tap water was run through the system followed by sterile tap water without inoculation. For this experiment, current was applied to the test setup 24-h per day for 14 consecutive days from the initial moment that fluid was pumped through the system. In contrast to the previous experiments, biofilm steady state was not established prior to applying the current. Effluent samples and biofilm samples were plated in triplicate at each dilution.

Experiment 4: After the initial 10-liter carboy of inoculated sterile tap water was run through the system, it was replaced by sterile tap water until biofilm steady state was determined (12 days). At that point, chlorine dioxide in a 1:10 dilution with sterile tap water was pumped through the system, and current was applied to the test setup 24h per

day for 10d. Effluent samples and biofilm samples were plated in triplicate at each dilution.

Experiment 5: This experiment repeated experiment 4, however, the current and 10:1 chlorine dioxide dilution were applied to the test setup for 28 days after biofilm formation.

Analysis of data

Colony forming units (CFU) were compared between the control and experimental groups for differences at all time points. Apart from Experiment 1, all samples were plated in triplicate. Mean counts and standard deviations are reported for Experiments 2-5. Comparison of mean counts between control and experimental groups at each day were performed by unpaired Student's *t*-test with α=0.05. Comparisons of counts over time would require a generalized estimating equation analysis, as counts from the preceding day could have influenced those of the following day.

RESULTS

At the start of each experiment, the initial carboy of sterilized water was inoculated with *P. aeruginosa* at a concentration ranging from $3.6x10^4$ to $5.5x10^4$ CFU/ml.

Experiment 1: Experiment 1 tested the effect of current alone, applied during working hours on an established biofilm (Table 1, Figure 4). When steady-state biofilm was assumed, and before current was applied to the test setup, the counts from the effluent were $2.3x10^4$ CFU/ml (test) and $2.7x10^4$ CFU/ml (control). The effluent counts remained similar throughout the experiment for both systems. The counts of cultivable biofilm bacteria from the waterline specimen were lower in the test setup when compared to the control setup. This was true at both the anode ($6.0x10^1$ CFU/cm^2 vs. $2.4x10^2$ CFU/cm^2) and at the middle of the tubing ($2.0x10^1$ CFU/cm^2 vs. $4.0x10^3$ CFU/cm^2).

Table 1: Cultivable effluent bacterial CFUs (CFU/ml) when current alone was applied to the test system during working hours and after biofilm formation

	CFU Test		CFU Control	
Day	Current	Mean	Current	Mean
3	Off	1000	Off	0
5	Off	0	Off	2000
7	Off	23000	Off	27000
9	On	1700	Off	3200
11	On	33000	Off	46000
14	On	540000	Off	750000
16	On	380000	Off	190000
18	On	900	Off	9200

***P. aeruginosa* inoculated water**
sterile water

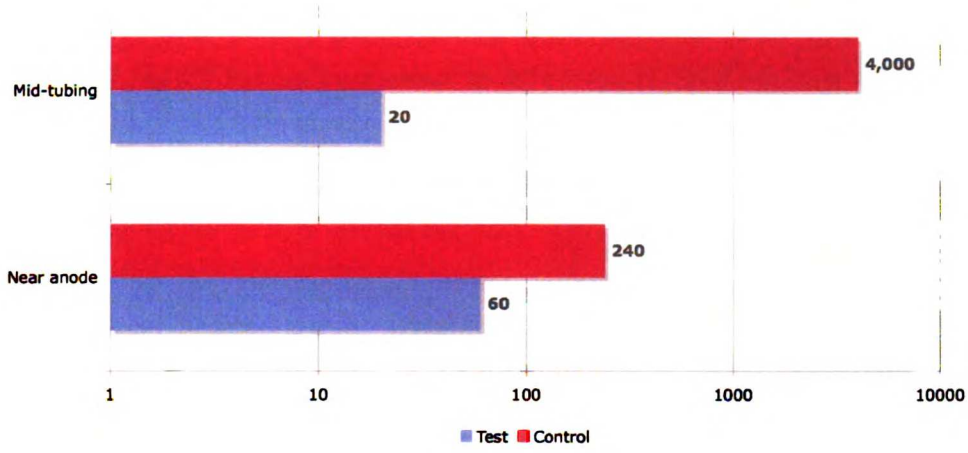


Figure 4: Cultivable biofilm bacterial counts (CFU/cm^2) from simulated DUWL when current was applied to the test system during working hours after biofilm formation. The lower counts in the test system may have been due to electrolysis and corrosion of plated brass connectors, with release of antimicrobial copper salts.

Experiment 2: Experiment 2 tested the effect of current alone, applied 24 hours per day, 7 days per week on an established biofilm (Table 2, Figure 5). When steady-state biofilm was assumed, and before current was applied to the test setup, the mean counts from the effluent were $2.2x10^4 \pm 2.7x10^3$ CFU/ml (test) and $2.6x10^4 \pm 2.3x10^3$ CFU/ml (control). There were no differences in the effluent CFUs between the test and control systems at that point in the experiment ($p=0.157$). Effluent CFUs were significantly different between the test and control systems at only one point in the experiment. This occurred in the first effluent sample taken after current was applied to the test setup. The test count, $4.5x10^4 \pm 8.5x10^3$ CFU/ml, was significantly higher than the control count, $1.1x10^4 \pm 3.2x10^3$ CFU/ml ($p=0.003$). The mean counts of cultivable biofilm bacteria from the waterline specimen were lower in the test setup than in the control setup near the anode, and this was statistically significant ($p=0.022$). For the mid-tubing specimen, the

cultivable biofilm CFUs were significantly higher (p=0.005) in the test system ($2.7x10^4 \pm 7.5x10^3$ CFU/cm^2) compared to the control ($1.7x10^3 \pm 4.2x10^2$ CFU/cm^2).

Table 2: Cultivable effluent bacterial CFUs (CFU/ml) when current alone was applied to the test system 24-hours per day after biofilm formation.

	CFU Test			CFU Control		
Day	Current	Mean	SD	Current	Mean	SD
2	Off	>80000	0	Off	>80000	0
4	Off	>100000	0	Off	>100000	0
**7	Off	21000	3300	Off	11000	3400
9	Off	520000	130000	Off	39000	28000
**11	Off	80000	0	Off	28000	3300
14	Off	3400	550	Off	2600	950
18	Off	380000	87000	Off	360000	49000
21	Off	250000	49000	Off	330000	13000
25	Off	22000	2700	Off	26000	2300
**28	On	45000	8500	Off	11000	3200
30	On	17000	2000	Off	16000	700
32	On	11000	5300	Off	4000	2600
**35	On	16000	4000	Off	8100	1000
37	On	19000	2300	Off	20000	2500
**39	On	24000	2600	Off	37000	2200
42	On	20000	4800	Off	15000	5400
44	On	38000	4500	Off	28000	9500
46	On	>60000	0	Off	>60000	0

***P. aeruginosa* inoculated water**
sterile water
****=significantly different at *P*<0.05 or less**

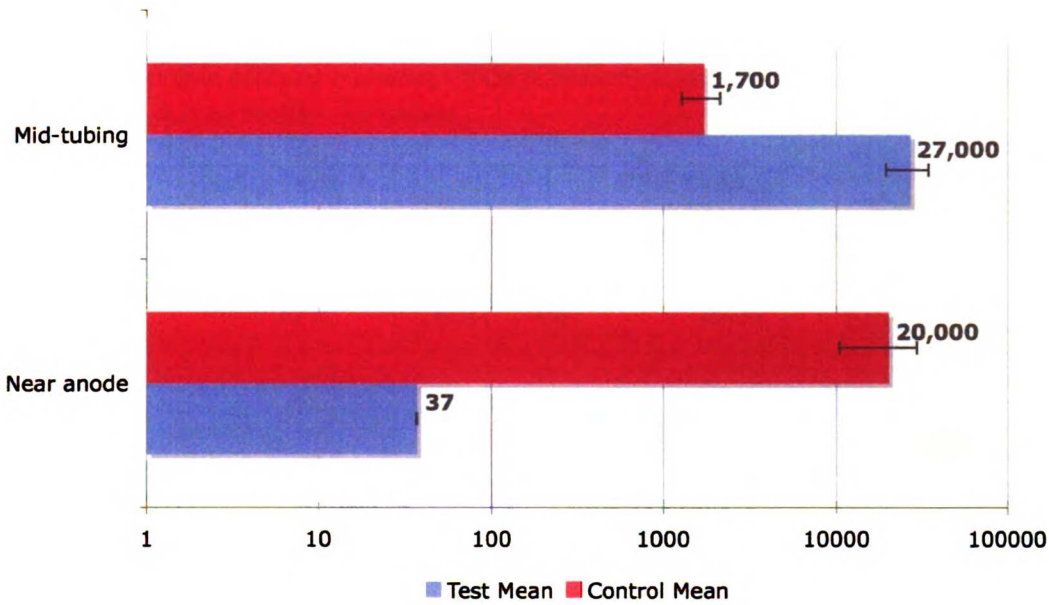


Figure 5: Cultivable biofilm bacterial counts (CFU/cm^2) from simulated DUWL when current was applied to the test system 24-hours per day after biofilm formation. The test was significantly less near anode (P=0.022), but control was less at mid-tubing (P=0.005). Error bars represent one standard deviation.

Experiment 3: Experiment 3 tested the effect of current alone, applied to the DUWL prior to the establishment of a steady state biofilm (Table 3). At days 3 and 9 the test effluent mean counts were significantly lower than the control mean counts. At the final day of the experiment, day 13, the control counts were significantly lower than the test counts. The mean counts of cultivable biofilm bacteria from the simulated DUWLs were higher in the test setup than in the control setup near the anode, but this was not statistically significant. For the mid-tubing specimen, the cultivable biofilm CFUs were significantly higher (p=0.001) in the test system ($1.49x10^3 \pm 2.6x10^2$ CFU/cm^2) compared to the control ($1.67x10^1 \pm 1.2x10^1$ CFU/cm^2).

Table 3: Cultivable effluent bacterial CFUs (CFU/ml) when current was applied 24-hours per day during biofilm formation

Day	CFU Test			CFU Control		
	Current	Mean	SD	Current	Mean	SD
1	On	33	32	Off	46.7	12
**3	On	83800	6500	Off	109000	7700
5	On	7230	2600	Off	6530	1700
7	On	16900	2100	Off	15800	1800
**9	On	3330	850	Off	19100	4500
11	On	2400	610	Off	1700	270
**13	On	8370	1900	Off	3070	600

***P. aeruginosa* inoculated water**
sterile water
****=significantly different at *P*<0.05 or less**

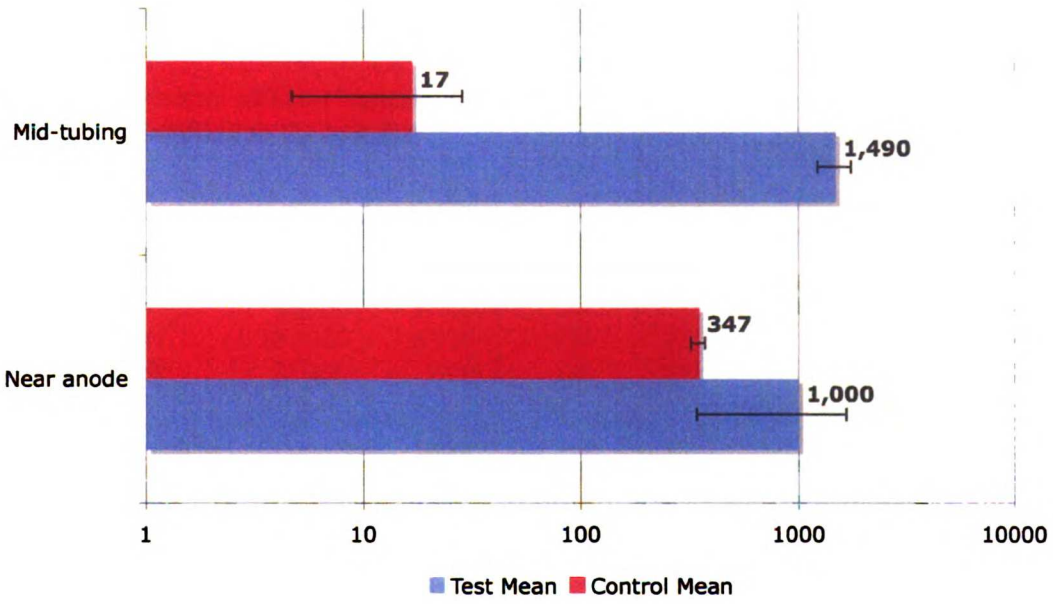


Figure 6: Cultivable biofilm bacterial counts (CFU/cm^2) from simulated DUWL when current was applied 24-hours per day during biofilm formation. The control was significantly less (P=0.001) at mid-tubing. The use of current from the outset did not prevent formation of biofilm in a water system. Error bars represent one standard deviation.

Experiment 4: Experiment 4 tested the effect of current combined with a 10:1 dilution of a chlorine dioxide solution on an established biofilm (Table 4). When the steady-state biofilm was assumed, the mean counts from the effluent were $2.33x10^3 \pm 5.7x10^2$ CFU/ml (test) and $3.39x10^2 \pm 5.7x10^2$ CFU/ml (control). This difference was statistically significant ($p=0.013$). After chlorine dioxide solution was added to the carboy and current was applied to the test setup, the test effluent counts dropped to zero and did not return for the remainder of the experiment. For the biofilm analysis, zero CFUs were detected in both the control and test systems at the mid-tubing waterline section. Near the anode, zero CFUs were detected in the experimental system while $4.50x10^2 \pm 1.7x10^1$ CFU/cm^2 were present in the control system. This difference was statistically significant ($p<0.001$).

Table 4: Cultivable effluent bacterial CFUs (CFU/ml) when current and chlorine dioxide solution were applied to the test system, after biofilm formation, for 10-days.

	CFU Test			CFU Control		
Day	**Current**	**Mean**	**SD**	**Current**	**Mean**	**SD**
1	Off	68300	19000	Off	46300	8000
3	Off	383000	59000	Off	300000	62000
****5**	Off	31400	3400	Off	21100	2700
8	Off	114000	12000	Off	97000	9200
10	Off	27100	1900	Off	24500	2400
****12**	Off	2330	570	Off	339	570
****15**	On	0	0	Off	3060	321
****19**	On	0	0	Off	10000	6100
****22**	On	0	0	Off	380	40
****25**	On	0	0	Off	140	52

***P. aeruginosa* inoculated water**
sterile water
chlorine dioxide
****=significantly different at *P*<0.05 or less**

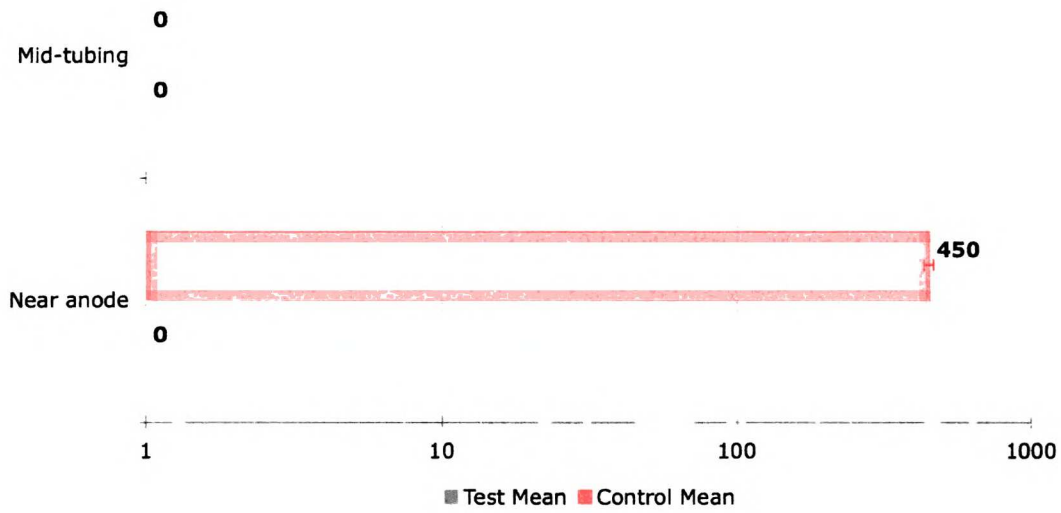


Figure 7: Cultivable biofilm bacterial counts (CFU/cm^2) from simulated DUWL when current and chlorine dioxide were applied to the test system, after biofilm formation, for 10-days. The test system was significantly less (P=0.000) than control near the anode. Error bars represent one standard deviation.

Experiment 5: Experiment 5 repeated experiment 4 but differed in that the current was applied for four weeks (Table 5, Figure 8). When a steady-state biofilm was assumed, CFU counts from the effluent of the test and control systems were both >1.00x10^5, and were not statistically different. After chlorine dioxide solution was added to the carboy and current was applied to the test setup, the test effluent counts dropped to zero. The test counts did rise at several time points throughout the experiment. Eight days after chlorine dioxide was added to the carboy, the control counts also dropped to zero. The control counts rebounded after this and never again reached zero throughout the experiment. At one time point during the experiment, the test counts rose to a level that was less than but not statistically significantly different from the control. This occurred

18 days after the current was applied. For all other days, while the current and chlorine dioxide were being used, the test and control effluent counts were significantly different, with the test counts being significantly lower.

Differences in maintenance of a viable, steady-state biofilm were observed as well. For the test system, zero CFUs were detected at both the mid-tubing and near anode sites. At the anode end, the mean count for the control system was $5.33x10^{1}\pm3.21x10^{1}$. This difference was statistically significant ($p=0.001$). At the mid-tubing section, the mean control count was $1.27x10^{2}\pm5.77$, and this difference was statistically significant from the mean test count ($p=0.000$).

Table 5: Cultivable effluent bacterial CFUs (CFU/ml) when current and chlorine dioxide were applied to the test system for 4-weeks after biofilm formation

	CFU Test			CFU Control		
Day	**Current**	**Mean**	**SD**	**Current**	**Mean**	**SD**
**1	Off	3000	3000	Off	18300	577
4	Off	4670	1530	Off	9000	3460
6	Off	19700	3220	Off	22000	11000
8	Off	6670	4510	Off	15000	3610
14	Off	>100000		Off	>100000	
**18	On	0	0	Off	4730	666
20	On	0	0	Off	0	0
**22	On	0	0	Off	110	45.80
**25	On	0	0	Off	1060	246
**27	On	153	32.10	Off	199000	27100
**29	On	0	0	Off	16000	777
32	On	3020	624	Off	17700	14300
**34	On	0	0	Off	311000	116000
**36	On	300	157	Off	21300	6030
**39	On	3.33	5.77	Off	1400	140
**41	On	0	0	Off	13300	2740
**43	On	0	0	Off	25300	577

***P. aeruginosa* inoculated water**
sterile water
chlorine dioxide
****=significantly different at *P*<0.05 or less**

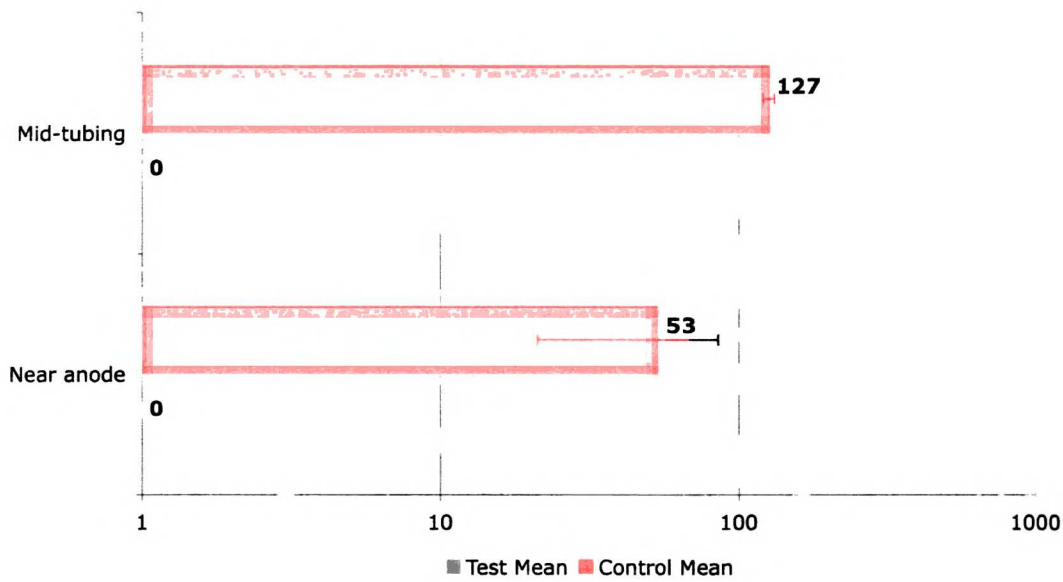


Figure 8: Cultivable biofilm bacterial counts (CFU/cm^2) when current and chlorine dioxide were applied to the test system for 4-weeks after biofilm formation. Undetectable biofilm counts in the test system were significantly less near the anode (P=0.045) and also at mid-tubing (P=0.000). Error bars represent one standard deviation.

DISCUSSION

The data from this study show that the combination of a weak electric current and a dilute solution of chlorine dioxide are capable of reducing CFUs in a simulated DUWL effluent to below the ADA recommended 200 CFU/ml. In experiment 4, CFUs in the effluent dropped to zero after current and chlorine dioxide were applied. *P. aeruginosa* CFUs in the effluent remained at zero and did not rise throughout the 10-day duration of the experiment.

In experiment 5, the CFUs in the effluent of the test system dropped to zero after chlorine dioxide and current were applied. In this experiment, the CFUs in the control system also dropped to zero shortly after chlorine dioxide was applied to the system.

This evidence indicates that chlorine dioxide solution does have some affect on planktonic bacteria in solution. However, the effect of chlorine dioxide solution alone on bacteria in the effluent was not capable of producing CFU counts at or below the ADA recommended 200 CFU/ml for the duration of this experiment.

During experiment 5, the test effluent CFU counts increased. This occurred 13, 18, 22, and 25 days after the current and chlorine dioxide were applied to the system. At each of these time points, the test CFU counts were lower than the control counts. One explanation for the rises demonstrated in this experiment is biofilm sloughing from the tubing upstream of the test waterline. In this experimental setup, current was only applied to the test system at the point where the test DUWL split from the common tubing leading from the pump. The upstream tubing was capable of facilitating a *P. aeruginosa* biofilm throughout the experiment. When a mature biofilm reaches steady state, bacteria will slough or "break-through" from its surface.[33] The shear force of liquid flowing across the biofilm surface may cause some surface bacteria to be sloughed off. But, other large groups will periodically dissociate from the biofilm, perhaps due to quorum sensing. These bacteria will demonstrate a reversion of their phenotype to that of a planktonic state, including the regrowth of flagellae. This process allows bacteria to migrate to a new location and begin forming a new biofilm. In this experiment, these "break-through" bacteria were washed through the experimental system and collected in the effluent samples.

More striking than the reduction in CFUs in the effluent was the ability of the test system to both eradicate and prevent new formation of *P. aeruginosa* biofilm in the lumen of the simulated DUWL. The results from experiments 1, 2, and 3 demonstrated

that *P. aeruginosa* was capable of maintaining a biofilm on the lumen of the test DUWL system, even after current was applied. During experiments 1, 2, and 3, it was noted that the flowrate appeared to slow in the test system throughout the experiment. When the systems were disassembled, a brown sludge was noted within the lumen of the tubing, along the platinum wire, and within the barb fittings. The current alone did not appear to have an effect on bacterial colonization within the tubing and may have allowed for more extensive colonization through a disruption in the flow profile within the tubing. The wire, itself, may have even provided additional surface area for the bacteria to colonize.

The chlorine dioxide solution alone did have some affect on the DUWL biofilm. In experiment 4, mid-tubing CFU counts dropped to zero for the control system. The chlorine dioxide solution may have removed some of the established biofilm and delayed biofilm re-establishment. This effect was not observed in experiment 5, and it is likely that, with time, *P. aeruginosa* were able to overcome the effects of chlorine dioxide and form a viable biofilm. It is also possible that when the setup was disassembled, the biofilm had partially but not completely covered the lumen of the DUWL, and the small biopsied section of tubing was devoid of bacteria. One study of SEM images of DUWL described biofilm coverage as incomplete or patchy.[28] A limitation of this study was the inability to quantify biofilm coverage along the entire 15-feet of simulated DUWL.

This series of experiments have demonstrated that current alone, and chlorine dioxide alone, are incapable of removing established biofilms from the lumen of DUWL tubing. But, the observations from experiments 4 and 5 provide strong evidence for the combined effects of low current and dilute chlorine dioxide solution in eliminating established biofilms. In both of these experiments, no viable bacteria were cultured from

the test waterline tubing. Because biofilms are extremely resilient, and their eradication is quite difficult, these results are substantial.

One possible explanation for the effectiveness of the chlorine dioxide and low current combination is the liberation of additional bactericidal chlorine dioxide radicals from the chlorine dioxide solution. The waterline cleaner used is a 0.1% sodium chlorite solution, stabilized by a 0.2% phosphate buffer. It is estimated to have 3-4ppm active chlorine dioxide when diluted 10:1. As stated earlier, these radicals affect methionine and prevent bacterial protein synthesis.[8] The oxidation-reduction potential of the effluent dropped (data not shown) in the test system once current and chlorine dioxide solution were run through the system. This may have been a result of changes in the strength or character of the chlorine dioxide in solution. It is possible that the immediate and rapid change in counts was due to electrochemical generation of nascent ClO_2 radicals, and electroporation of the *P. aeruginosa*.

The technology presented in this paper may have effects that extend beyond dental unit waterlines and impact other fields. For example, biofilms are a significant problem for hospitalized patients with urinary catheters. Biofilms have also been a problem in oil pipelines. Further studies involving scanning electron microscopy imaging could be used to examine whether biofilm fragments remain attached to the tubing surface after treatment with current and chlorine dioxide.

One practical limitation of this device is the expense of the platinum wire. Other studies may address whether different types of metal have the same effect without causing corrosion. For example, stainless steel wire may prove to be a sufficient and

inexpensive alternative. The magnitude of current and concentration of chlorine dioxide solution required to produce these results are also worthy of further investigation.

CONCLUSION

The combination of a low 10mA current, provided through a platinum wire extending the length of a dental unit waterline tubing and a 1:10 chlorine dioxide solution flowing through the tubing, is capable of eliminating an established *Pseudomonas aeruginosa* biofilm. This effect was maintained over a 4-week observation period. The effect was significantly greater than in tubing with chlorine dioxide and no current. Therefore the hypothesis is accepted, that current applied along the length of a DUWL will significantly enhance the efficacy of a chlorine dioxide solution in the removal of bacterial biofilms from a simulated DUWL.

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