THE TAKE-AWAYS

- It is important to decrease and prevent microbial contamination in DUWLs to reduce the risk of potential health problems.
- The study showed Citrisil was effective in controlling microbial contamination in dental tubing and preventing biofilm formation, but more research needs to be done.

Reducing contamination in your DUWLs

An overview of a study designed to evaluate the efficacy of Citrisil tablets in controlling opportunistic bacterial pathogen contamination in DUWLs.

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ABSTRACT

The purpose of this study was to evaluate the efficacy of Citrisil tablets in controlling microbial contamination, preventing biofilm formation and enhancing biofilm removal from dental unit waterlines. Test materials included Citrisil™, Citrisil Shock™, Aseptisil™ liquid and Aseptisil™ powder. Sterilex-powder served as control during the shock treatment. *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were used to inoculate regular polyurethane dental tubing. Experiments were conducted using a dental unit waterline biocide test apparatus and methodology in the ANSI/ADA's draft standard. Overnight shock treatments were performed using biofilm accumulated tubing. Citrisil™ eradicated 1x10^5 bacteria from the inocula ($p<0.001$). Biofilm TVC data showed significant differences between the control and Citrisil group ($p=0.029$). Citrisil Shock, Aseptisil liquid and Aseptisil powder effectively eradicated planktonic cells from tubing effluent and biofilm TVC during the shock treatments. The results were comparable to Sterilex treatments. A single treatment of 10 minutes with Aseptisil liquid or Aseptisil powder killed all planktonic bacteria. Daily use of Citrisil effectively controls opportunistic pathogenic contamination and prevents biofilm formation. Citrisil Shock, Aseptisil liquid and Aseptisil powder are capable of inactivating biofilm and keeping tubing uncontaminated.

Over recent years, biofouling has been recognized as the prime source of microbial contamination in dental unit waterlines (DUWLs). Although most of the microorganisms recovered from the DUWLs are heterotrophic mesophilic bacteria, oral flora and opportunistic pathogens such as Pseudomonas species, nontuberculous *mycobacteria* and *Legionella pneumophila* have been isolated from DUWLs.[1] Pathogens such as these may be harmful to medically compromised patients, especially those with cancer and immunodeficiency. Consequently, it is important to find ways to decrease and eventually prevent microbial contamination to reduce the risk of potential health problems to both patients and dental health care professionals.

The standards

The ADA recommends that water delivered to patients from DUWLs meet the same standard deemed necessary for drinking water.[2] The Centers for Disease Control and Prevention (CDC), the Environmental Protection Agency (EPA), the American Public Health Association (APHA) and the American Water Works Association (AWWA) have established guidelines recommending the amount of heterotrophic bacteria in drinking water be no more than 500 colony forming units per mL (CFU/mL). However, if the DUWL system is not treated with chemicals to control bacterial proliferation, just using source water containing ≤500 CFU/mL of bacteria will not eliminate existing bacterial contamination in DUWLs.[3]

Dental Unit Waterlines Biocide Test Apparatus.

Photos courtesy of Wu Zhang.

Contamination contributors

Multiple factors may contribute to high levels of bacterial contamination in DUWLs. Dental tubing is extremely narrow, typically having a diameter of 1.5 mm. The high surface area-to-volume ratio combined with the fact that dental tubing is usually constructed of polyurethane, which offers a substantial carbon source, promotes and sustains bacterial growth and biofilm formation. Additionally, intermittent operation in the clinic setting results in long periods in which DUWLs are stagnant, typically for 16 hours overnight and 64 hours over weekends—a condition that favors microbial colonization of lumenal surfaces of the tubing.[4]

Research has demonstrated that microbial counts can reach up to 200,000 CFU/mL five days after new dental unit waterlines are installed.[5] DUWL cleaners and disinfectants are available to combat microbial contamination, but a new challenge has emerged because of the increasing numbers of biocide-resistant microorganisms that have been isolated from the DUWLs. The number of resistant microorganisms is increasing, while our knowledge of antimicrobial resistance remains limited.[6] Overall, controlling microbial contamination of DUWLs is not a simple task as it involves coordination from multiple fields.

The study

The present study evaluated the effectiveness of four chemical cleaners, all of which contain high-purity multicomplexed stabilized silver ions as the active ingredient. Silver ion is an effective antimicrobial agent, with silver ions exhibiting antimicrobial properties in preventing biofilms on catheters, in other medical equipment and in water filters and cooling towers.

One mechanism through which silver functions as a bactericidal agent is its interaction with disulfide or thiol (sulfhydryl) groups within the amino acids of bacterial cell wall proteins. Silver also can bind to cellular DNA, which in turn interferes with normal metabolic functioning of microorganisms, eventually leading to cell death.[7,8]

The protocol for laboratory evaluation of chemical disinfectants followed draft standard of ANSI/ADA Specification 107, "Chemical Agents for the Control of Biofilm in Dental Unit Water Systems".[9] The protocol provides clinically relevant information pertaining to the effectiveness of tested cleaners as it relates to reducing microbial number in the unfiltered output of dental treatment water or maintaining the bacterial counts at or below 500 CFU/mL. Therefore, the objective of this study was to examine the potential of four Citrisil high-purity multicomplexed, stabilized silver ion-based DUWL cleaners in controlling microbial contamination, preventing biofilm formation and inactivating biofilm from contaminated dental lines using the dental unit waterline biocide testing model.

Material and methods

Test model

The test model followed draft standard of ANSI/ADA Specification 107, "Chemical Agents for the Control of Biofilm in Dental Unit Water System".[9] Modifications were made to meet the manufacturer's requirements of testing Citrisil's efficacy in preventing biofilm formation from new dental tubing by using sterile deionized (DI) water and new tubing.

Test materials

Citrisil, Citrisil Shock, Aseptisil liquid and Aseptisil powder were provided by the study sponsor, Sterisil (**sterisil.com**). Sterile DI water was used as source water; Sterilex Powder (**sterilex.com**) served as the control during the biofilm inactivation procedure. Polyurethane regular dental tubing (size 1/16 inch internal diameter, 1/8 inch outer diameter) from Freeman Manufacturing (**freeman-mfg.com**) was used in the study.

Microbial culture

The two-species microbial consortium was prepared using *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*.

Outcome parameters

Effects of the test agents on controlling contamination in DUWLs were evaluated using the CFU/mL of planktonic cell counts of effluent; biofilm total viable counts (TVC, CFU/cm^2) in

Part one (4-week)	**Daily treatments**		
	Groups	Sterile DI water	Citrisil™
	Group 1	Yes	No
	Group 2	Yes	Yes
Part two (3-day)	**Biofilm inactivation procedures**		
	Treatments		
		Overnight	Day
	Group 3	Aseptisil™ liquid	Citrisil™
	Group 4	Aseptisil™ powder	
	Group 5	Sterilex Ultra powder	Sterile DI water
Part three (1-day)	**Part three**		
		Treatments	Contact times
	Group 6	Aseptisil™ liquid	10-minute
	Group 7	Aseptisil™powder	
	Group 8	Citrisil Shock™	Overnight

Table 1 Study design.

dental tubing; and scanning electron microscopy (SEM) photomicrographs of tubing biofilms.

- **CFU/mL of planktonic cells:** The effluent from the control and test tubing was collected aseptically daily at a set time. Samples were processed within 1 hour of sampling. Samples placed on ice were homogenized at 18,000 rpm using Tissue Tearor, a rotor/stator type tissue homogenizer from Biospec Products (**biospec.com**) for 30 seconds and then serially diluted to 1:1, 10^{-1}, 10^{-2} and 10^{-3} with phosphate buffered saline (PBS) containing the neutralizer, sodium thiosulfate. Samples were then plated on R2A agar plates (Becton, Dickinson and Co., Sparks, Md., **bd.com**), in duplicate, to determine the CFU/mL. The plates were incubated at room temperature for seven days and counted for colonies using a ProtoCol automatic colony counter from Microbiology International (**800ezmicro.com**) to obtain the CFU/mL values.

TVC of biofilm (CFU/cm²): Samples of biofilms were collected in sterile PBS containing sodium thiosulfate using a sterile metal scraper. The samples were homogenized, serially diluted to 1:1, 10^{-1}, 10^{-2} and 10^{-3} concentrations, plated in duplicate onto R2A agar plates, and then incubated at room temperature for seven days. Plates were counted using ProtoCol to obtain the CFU values. The counts of CFU/cm² were calculated based on the tubing's length and size as well as the dilution factor.

- **SEM evaluation of biofilms morphology:** Dental tubing, in duplicate, was collected aseptically at the baseline, 2-weeks and 4-weeks for SEM evaluation. The samples were fixed with glutaraldehyde-cacodylate buffer and osmium tetroxide, dehydrated with an ascending series of alcohol solutions and sputter coated with gold-palladium. Samples were coded to ensure all evaluations were performed without knowledge of treatment. Specimens were examined using an SEM at an accelerating voltage of 5 kV. Each sample was examined at magnifications of 200X, 2,000X and 4,000X for biofilm morphology. Representative photomicrographs were taken from each group for documentary and illustrative purposes.

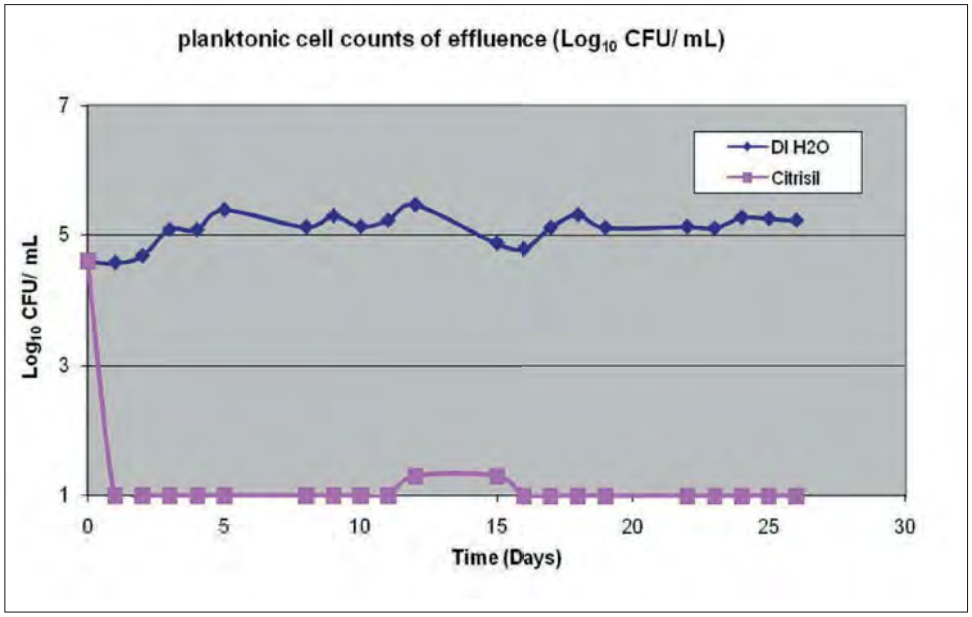
Experimental procedures: Treatment regimens

Part one: Citrisil daily treatment. Fresh cultures of *P. aeruginosa* and *K. pneumoniae* were grown in separate chemostats and pumped intermittently into mixing chambers. Per manufacturer's recommendations, sterile DI water was used to dilute the two-species consortium in the control mixing chamber, which was connected to control dental tubing. Citrisil Shock was used for the initial cleaning of dental tubing overnight, then Citrisil solution (each Citrisil tablet dissolved in 750 mL DI water) was used to dilute the two-species consortium in the test mixing chamber, which was connected to the test dental tubing. The solutions were retained in mixing chambers for less than one hour and then were pumped at the same flow rate into dental tubing, Monday through Friday, from 9 a.m. to 5 p.m. at 5-minute ON and 25-minute OFF intervals for four weeks, which simulated dental operation.

Part two: Shock treatment. Biofilm inactivation with Aseptisil liquid or Aseptisil powder. After 4-week treatment, dental tubing from the control group with accumulated biofilm was sampled to evaluate the biofilm inac-

tivation procedure. Groups 3 and 4 were tested using Aseptisil liquid and Aseptisil powder, respectively. Group 5 was treated with Sterilex powder and served as the positive control. Per manufacturer's instructions, sterile DI water was used to freshly prepare the treatment solutions. The treatment solutions were then flushed through the tubing and remained in the dental tubing overnight. During the day, dental tubing of Groups 3 and 4 was treated with Citrisil daily solution, while Group 5 tubing was treated with sterile DI water (**Table 1**). The procedure was conducted for three consecutive days.

Part three: Quick disinfection. Citrisil Shock overnight treatment was compared with one 10-minute quick treatment of Aseptisil liquid or Aseptisil powder. DUWLs with existing biofilm were assigned to be Groups 6, 7 and 8, respectively (**Table 1**). The DUWLs of Groups 6 and 7 were flushed with Aseptisil liquid or Aseptisil powder with 10-minute contact time, while Group 8 was treated with Citrisil Shock overnight.



[a] There is a statistically significant difference between the control and Citrisil™ group as determined using the Mann-Whitney Rank Sum Test ($p<0.001$).

Fig. 1 Efficacy of Citrisil daily treatment in controlling planktonic cell counts of effluence.

Analyses and interpretation of data

Means and standard deviations of CFU/mL and CFU/cm² were calculated. The biofilm TVC data were analyzed using the Mann-Whitney Rank Sum Test (**Table 2**). The Kruskal-Wallis One Way Analysis of Variance on Ranks Test was used to determine the overnight efficacy of three different shock treatments and quick cleaners (**Tables 3 and 4**). Wilcoxon Signed Rank Test was used to compare the pre-treatment and post-treatment CFU/mL data (**Table 4**).

Results

Part one: Test results obtained from the initial 4-week period are presented in **Fig. 1**. The control group, consisting of sterile DI water with two-species inocula, was kept at $1x10^5$ CFU/mL in the effluent. The Citrisil daily solution produced a 5-log reduction of two-species planktonic opportunistic pathogen levels throughout the 4-week period, maintaining the effluent at <20 CFU/mL. The average CFU data of the 20 time points were significantly different ($p<0.001$) between the two groups as determined using the Mann-Whitney Rank Sum Test. Analysis of the biofilm TVC data (**Table 2**) also indicated significant differences between the control and Citrisil groups ($p=0.029$) using the Mann-Whitney Rank Sum Test. The SEM images showed the biofilms accumulated only in control tubing (**Fig. 2**). Daily treatment with Citrisil prevented biofilm formation for up to 4 weeks (**Fig. 3**). The overall findings from SEM photomicrographs support the data obtained from the effluent CFU and biofilm TVC experiments.

Part two: The dental tubing had accumulated $3.6x10^5$ CFU/cm² in biofilm TVC and $7.6x10^4$ CFU/mL in effluent. Both Aseptisil liquid and Aseptisil powder eradicated microbes in the effluent in all three overnight treatments. Sterilex-powder served as the positive control, in which an average count of 40 CFU/mL was detected on day one but no detection after the first overnight treatment. Wilcoxon Signed Rank Test found the significance of the post-treatment compared with the pre-treatment was marginal ($p=0.058$) although the post-treatment data consistently showed zero CFU counts. There were no statistically significant differences among Aseptisil liquid, Aseptisil powder and Sterilex-powder (**Table 3**).

Part three: The dental tubing used for this portion of the study had accumulated $6.8x10^4$ CFU/mL in effluent. The results showed one overnight Citrisil

Groups	Treatment	Biofilm total Viable counts (CFU/cm²)			*p-value*
		Mean	±	SD[a]	
Group I	Control	362,824	±	148,335	0.029
Group 2	Citrisil™	0	±	0	

[a] The data was analyzed using the Mann-Whitney Rank Sum Test. N=4.

Table 2 Efficacy of Citrisil™ in controlling biofilm TVC: 4-week Data

Treatments		Planktonic cell counts of effluent (CFU/mL)a			
Days	Nights	Day-0	Day-1	Day-2	Day-3
Citrisil™	Aseptisil liquid	76,000 ± 9,899	0.0 ± 0.0[a]	0.0 ± 0.0	0.0 ± 0.0
Citrisil™	Aseptisil powder	76,000 ± 9,899	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
DI water	Sterilex powder	76,000 ± 9,899	40.0 ± 56.6	0.0 ± 0.0	0.0 ± 0.0

a The values within brackets are not significantly different as determined using the Kruskal-Wallis One Way ANOVA on Ranks. N=2.

Table 3 Comparison of Aseptisil liquid and Aseptisil powder with Sterilex on planktonic CFU/mL during three consecutive overnight treatments.

Shock treatment kept the dental tubing free of planktonic microbes. A single 10-minute treatment with either Aseptisil liquid or Aseptisil powder also eliminated all planktonic microbes from the contaminated dental tubing. Statistically significant differences (p=0.024) were detected for all three products tested by using the Wilcoxon Signed Rank Test when comparing the pre- and post- treatment CFU/mL values of each product (**Table 4**). Statistical differences were not found when comparing the efficacy among the Citrisil Shock, Aseptisil liquid and Aseptisil powder using the Kruskal-Wallis One Way Analysis of Variance on Ranks Test indicating that all three products are effective in eradicating microbes in DUWLs.

Why silver works

The key element in maintaining clean water in DUWLs is controlling biofilm accumulation. Biofilm serves as a reservoir of microorganisms, and if not controlled, the biofilm will lead to continual contamination of DUWLs and consequently the water they deliver. Any product that can remove existing biofilm or prevent biofilm formation should aim to comply with the CDC recommendations for DUWL microbial quality. The four Sterisil products tested in this study are capable of achieving the CDC goal.

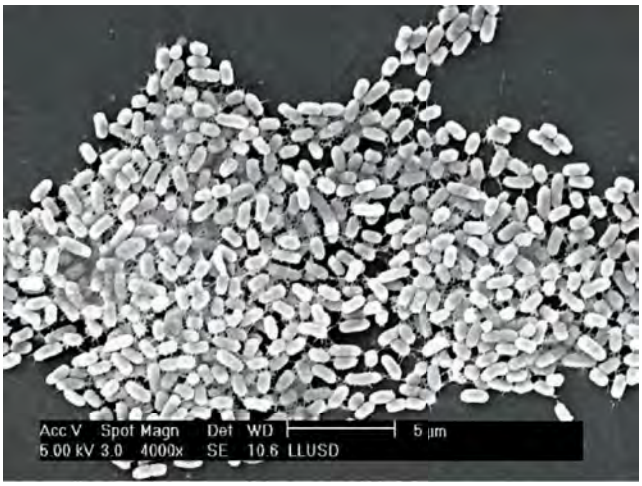


Fig. 2 Biofilm accumulated in control tubing: DI water and two-species inocula for four weeks.

Silver is an effective antimicrobial agent, though different forms of silver exhibit varying efficacies.[7,8,10] The antimicrobial properties exhibited by Citrisil, Citrisil Shock, Aseptisil liquid and Aseptisil powder are primarily associated with their chemical composition, which is a stabilized silver complex known as high-purity multicomplexed, stabilized silver. Silver complexes have demonstrated significant bactericidal properties. Djokić reported that silver complexes, such as silver citrate/citric acid solutions, exhibit stronger bactericidal properties than free silver ions such as silver nitrate in solution.[10]

Previous studies of various silver type as DUWL disinfectants have shown these cleaners and disinfectants can reduce effluent microbial contamination. In 2006, Schel and colleagues examined the efficacy of various disinfectants.[11] It was reported that Dentosept, Oxygenal and Sanosil, all of which contain hydrogen peroxide or silver ion as active agents, could effectively reduce effluent TVCs below CDC guidelines. These results were consistent with our findings.

Overnight treatment vs. quick disinfection

Because of the nature of most dental offices, it can be difficult to consistently use overnight treatments to prevent biofilm contamination. In certain cases, it is more effective and even becomes necessary to use quick disinfection measures. The present study shows an overnight Citrisil Shock treatment controls the contaminated DUWLs, as does the quick disinfection with Aseptisil liquid or Aseptisil powder, indicating their ability to quickly and effectively eradicate planktonic microbes in contaminated DUWLs.

We still need to learn more

Along with manufacturer's recommended modifications, this study followed procedures of the proposed ADA specification when testing Citrisil's efficacy in preventing biofilm. In an actual clinical setting, there are a wide variety of DUWL apparatuses, as well as variations in the type of water used within the system and different dental tubing along with various types of microbes that can be harbored inside. Additionally, long-term monitoring of microbial contamination and the potential for a biocide resistance situation in the clinical settings need to be evaluated and studied. Therefore, further investigations are necessary to reveal the potential of Citrisil's ability in a wide variety of situations.

What to think about

There are a number of factors that need to be considered when choosing a DUWL disinfectant. Roberts and colleagues indicated that problems with corrosion, disinfectant byproducts and decreased enamel and dentin bond strength have been caused by certain DUWL disinfectants.[12] However, in a comparison of eight disinfectant-treated water samples—including a silver-based DUWL disinfectant, PureTube™ from Sterisil®—demonstrated there was no change in microtensile bond strength of composite resin to dentin when using disinfectant-treated water.[13]

It is important to examine the effects treatment chemicals and cleaners may have on a patient's health, especially if these agents are used regularly. The United States Environmental Protection Agency and the World Health Organization have indicated that silver levels of <100 µg/L are safe for drinking water.[14,15] In a dental clinic, water is used solely for irrigation purposes; therefore there is little potential for ingestion. Pathways into the patient's bloodstream are often made through dental tools, such as high-speed handpieces or air/water coolants when preparing the subgingival tooth structure. Therefore, it is important to consider the mode of clinical application and the level of exposure to correctly evaluate the potential risks to patients.

Solving the problem

Over the last two decades, increasing efforts have been made by researchers and manufacturers in an attempt to solve the microbial contamination problem. As a variety of new products and approaches become available, new questions have been raised: What is the best way to evaluate the efficacy of any new product? How should the test results from different laboratories be compared? The solution is a bit complex as different dental units not only carry different microbes, but also use different source water and dental tubing. Consequently, standardizing the evaluation apparatus and methods is necessary to solve this universal problem.

The gold standard method for the examination of microorganism effluent is the "Heterotrophic Plate Counts by Membrane Filtration" published by the American Public Health Association, Method 9215D.[16] However, variations occur during biofilm evaluation. Currently, the SEM, laser confocal microscopy (LCM), TVC and protein profile contents in biofilm samples are among the most popular methods for biofilm evaluation. Each method has its advantages as well as limitations.

The photomicrographs obtained from SEM directly show if the biofilm exists and whether the surface morphology of biofilms is intact or disrupted. SEM is a useful tool in surface morphology analysis, though it does not provide a means to differentiate between viable and non-viable cells within the biofilm matrix.

Other techniques, such as LCM, have been used to compensate for SEM's inability to differentiate cell viability. Puttaiah and colleagues demonstrated that in addition to examining the viability, LCM is more sensitive than SEM in detecting the amount of bacterial contamination within the DUWLs.[17] Because the LCM is useful in determining cell viability, inorganic contaminants are not as clearly shown as those that were analyzed with the SEM.

While the LCM can recognize the amount and viability of the cells, the SEM is useful in examining the presence of contaminants.[17,18] Consequently, using both the SEM and LCM will provide a more complete examination of DUWLs fouling. However, availability and costs for the SEM and LCM could be a limitation. The most economic way is to evaluate TVC and protein contents within a biofilm.

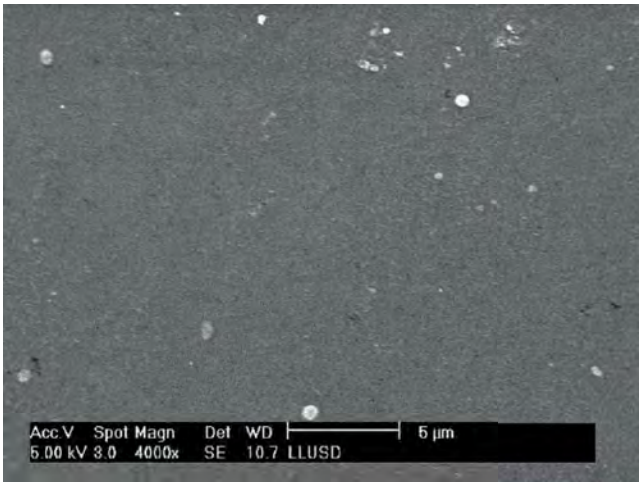


Fig. 3 No biofilm formation in test tubing: Citrisil daily solution and two-species inocula for four weeks.

It is important to note that more variations in test results may be expected because of the sample size and sampling technique and evaluation criteria. Increasing the sample size, improving sampling technique and using standard evaluation criteria can compensate the limitation of these methods.

What's next

Under conditions of the present study, Citrisil was effective in controlling microbial contamination in dental tubing and preventing biofilm formation during the 4-week study period. Three consecutive overnight treatments with Citrisil Shock, Aseptisil liquid or Aseptisil powder inactivates biofilm formation and keeps the dental tubing free of opportunistic pathogenic microbes. A single 10-minute quick treatment with Aseptisil liquid or Aseptisil powder eliminates the planktonic *P. aeruginosa* and *K. pneumoniae* in the DUWLs.

Further studies should be conducted to investigate the full potential of Citrisil's efficacy on various microbes and confirm its ability in removing existing biofilm from DUWLs. Additionally, it is necessary to monitor biofilm formation and evaluating potential biocide resistance problems with Citrisil in actual clinical settings. ●

Groups	Treatment	Contact Time	CFU/mLa		*p-Value*[b]
			Pre-treatment	Post-treatment	
Group 6	Aseptisil™ liquid	10 min.	67,500 ± 3,536	0.0 ± 0.0	0.024
Group 7	Aseptisil™ powder	10 min.	67,500 ± 3,536	0.0 ± 0.0	0.024
Group 8	Citrisil Shock™	Overnight	67,500 ± 3,536	0.0 ± 0.0	0.024

[a] The values within brackets are not significantly different as determined using the Kruskal-Wallis One Way ANOVA on Ranks.
[b] Pre- and Post-treatment values were analyzed using the Wilcoxon Signed Rank Test.

Table 4 Comparison of the quick disinfection with either Aseptisil liquid or Aseptisil powder and Citrisil Shock on planktonic CFU.

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References are available online at **dentalproductsreport.com.**

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