The role of chemical products at low doses in preventing the proliferation of bacteria in dental unit waterlines: the ICX® experience

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ABSTRACT

In this study we evaluated (1) the efficacy of a protocol that combines hydrogen peroxide (shock treatment) and ICX® tablets (continuous treatment) for the control of microbial contamination in dental unit water lines, and (2) the *in vitro* antimicrobial activity of ICX® tablets on collection and wild strains isolated from dental chair output waters. To assess the treatment effectiveness, the microbial load in the output water samples of three dental chairs were investigated: one control chair received only shock treatment. *In vitro* bactericidal activity was tested against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Data obtained from samples collected from chairs treated with ICX® and shock treatment and data from the control chair did not differ significantly on the basis of microbial load. In the *in vitro* study, the product was unable to kill Gram-negative bacteria. These results show that the continuous introduction of ICX® was not effective in maintaining low counts of the heterotrophic bacteria in the output water of dental devices, and shock treatment may be needed more frequently than monthly.

Key words | dental disinfectants, dental equipment, dental offices, infection control, water microbiology

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INTRODUCTION

Dental units are a reservoir for potential pathogens of human or environmental origin, and dental instruments are believed to be responsible for the transmission of microorganisms by direct contact or by spreading through aerosol sprays created by handpieces (high-speed drills, scalers, air and water syringes). A dental unit is furnished with a system of thin plastic tubes, called dental unit waterlines (DUWLs), which deliver water to the different handpieces.

Contamination occurs when oral organisms (Abel *et al.* 1971; Fitzgibbon *et al.* 1984) enter the unit's waterlines through back siphonage when the handpiece is momentarily turned off (Bagga *et al.* 1984; Crawford & Broderius 1990). Dental units can also become contaminated from the main water supply, which, although potable, still carries bacteria producing an adherent heterogeneous microbial accumulation called a biofilm. Once formed, the biofilm protects the organisms from desiccation, chemical insult and predation, and it serves as a reservoir that significantly changes the number of free-floating microorganisms in the water exiting the waterlines (Whitehouse *et al.* 1991; Barbeau *et al.* 1998). Most of the bacterial species found in DUWL output water are Gram-negative aerobic heterotrophic environmental bacterial species that exhibit very low pathogenicity, although they may be of concern in the treatment of vulnerable patients, such as immunocompromised and medically compromised individuals (Martin 1987) and dental staff (Fotos *et al.* 1985; Reinthaler & Mascher 1986). Because of these contaminants, it is important to establish

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control methods for cleaning and disinfecting dental water systems and for providing quality irrigant/dental treatment water. Depending on the nature of various germicidal agents and the various devices or systems provided with the dental unit, chemical treatment protocols could be used intermittently as a 'shock' treatment and/or continuously introduced into waterlines in small quantities.

Intermittently used cleaners are not intended for patient contact and require that the waterlines be flushed after each chemical treatment, which eliminates any potential adverse effects the chemical may have on the bond strength of dental adhesive materials. However, one possible drawback is that intermittent cleaning may allow for reformation of the biofilm between uses (Karpay *et al.* 1999; Tuttlebee *et al.* 2002; Montebugnoli *et al.* 2004; Schel *et al.* 2006; Puttaiah *et al.* 2012). In contrast, cleaners intended for continuous use are less concentrated and do not require flushing of waterlines. The main benefit of continuous chemical use is that it reduces the potential for recolonization of waterlines between treatments, although the potential still exists and water quality should be monitored to ensure that it is of acceptable quality (O'Donnell *et al.* 2005; Schel *et al.* 2006; Szymańska 2006; Bansal *et al.* 2011). In a study conducted in 2015 (Ditommaso *et al.* 2016), we found that although a significant proportion of the DUWLs tested were disinfected, samples taken from them were more heavily contaminated with bacteria and that their bacterial levels were much higher than the Italian (G.U. 52 2001) and European quality standards (European Union 1998) set for drinking water (≤100 colony forming units per millilitre (CFU ml^{-1}) of heterotrophic bacteria at 22 °C and ≤20 CFU ml^{-1} of mesophilic bacteria at 37 °C). Furthermore, the rate of recovery of *Legionella* from the DUWLs observed in our study greatly varied depending on the analytical method used. When we used the culture method to detect *Legionella* in the DUWL samples, we observed a low rate of contamination (6.6%); however, when we used propidium monoazide quantitative polymerase chain reaction (PMA-qPCR) for the same purpose, we found that 100% of the samples were positive. Cultivation has been the principal approach employed in the past, but the culture may result in false negative data or underestimated bacterial counts. In contrast, PCR detection methods also include viable but non-culturable (VBNC) legionellae, and legionella living within amoeba (Ng *et al.* 1997; Bates *et al.* 2000).

During the collection of samples (Ditommaso *et al.* 2016), information was also collected relating to waterline disinfection. We found that the most commonly used product for bacterial contamination control was hydrogen peroxide at various concentrations. Only one dental office had adopted a continuous disinfection system called ICX® (A-dec Inc., Newberg, OR, USA), but none of the dental units treated with ICX® tablets delivered potable water and were legionella free (negative by culture method and positive by PMA-qPCR).

From this finding, we decided to: (1) evaluate the chemical treatment of waterlines using a protocol that combines shock treatment with hydrogen peroxide and continuous treatment with ICX® tablets for effective control of microbial contamination; and (2) investigate the *in vitro* antimicrobial activity of ICX® tablets on collection strains and wild strains isolated from dental chair unit (DCU) output water.

METHODS

Dental chair units

In this study, we monitored two 3-year-old DCUs (DCU n°1 and n°2) and one 17-year-old chair (DCU n°3) as a control. The water source that fed all three DCUs was tap water (municipal water). All chairs had been in daily use; DCU n°1 and n°2 for conservative dentistry and dental surgery, and DCU n°3 for dental hygiene.

Both DCUs n°1 and n°2 were equipped with a self-contained dental unit water system that included a 2-litre water bottle that received ICX® (A-dec Inc., Newberg, OR, USA) tablets added to the water reservoir bottle at each refill. These DCUs had been sanitized with ICX® tablets from the date of installation in the year 2012. From 2012, these DCUs had never had biofilm removed or planktonic bacteria reduced using shock treatment. DCU n°3 had never been cleaned and used only municipal water as the irrigant.

ICX® effervescent tablets

ICX® are effervescent tablets (sodium percarbonate 6.96%, dimethyl benzyl ammonium chloride 0.85%, dimethyl ethylbenzyl ammonium chloride 0.85%, silver nitrate 0.14%, and

other ingredients 91.2%) specially formulated to maintain DUWLs and prevent accumulation of odours and foul-tasting bacteria and maintain DUWLs effluent $\leq$10 CFU ml^{-1}, as long as proper infection control is followed. The self-contained reservoir bottle was filled with 2 L of fresh tap water and one ICX® effervescent tablet (212.4 mg). A tablet placed in the self-contained water delivery system remains effective in the waterlines for up to 2 weeks. Additionally, the manufacturer reports that it 'eliminates the need to purge waterlines at night,' and the tablet remains 'continuously present in the water system and provides a preventive, proactive solution rather than a reactive one' (A-dec Inc. 2015).

This product is intended for use with potable water and in conjunction with regular dental unit water testing, following shock treatment with antimicrobial products recommended by the manufacturer.

Sampling of DUWLs

From March to September 2015, 18 water effluents were collected monthly in a private dental office. Samples were taken in the morning from the air-water syringes and turbines and were mixed together. Each sample was collected in a sterile, 1-litre plastic bottle containing thiosulfate sodium (10% w/v).

Sampling of tap water

Samples were also taken initially from the sink faucet to compare the CFU counts of the water supplied to the building with CFU counts of DUWLs. Only potable water provided for drinking, bathing or culinary purposes was supplied to plumbing fixtures of the building in which the dental office was located.

Before sampling, the taps were disinfected according to the following procedure: (1) removal of the water flow regulator, (2) internal disinfection of the tap with a solution of sodium hypochlorite at (10% w/v) for 2–3 min, (3) disinfection of the whole faucet with a Bunsen flame, and (4) water flow for 5 min. Finally, the water samples were collected in a sterile, 2-litre plastic bottle containing thiosulphate sodium (10% w/v).

Quantification of waterborne bacteria

To assess the effectiveness of shock treatment, the bacteria total viable counts (TVCs) in DUWL water samples were investigated according to ISO 6222 (International Standards Organization 1999). One millilitre of the water samples and 1 ml of a decimal dilution of the samples were tested using the pour plate method on yeast extract agar. The calculation of the number of CFU ml^{-1} of the sample was performed after 7 days of incubation at 22 °C and 5 days at 36 °C according to the US standard method (Eaton *et al.* 1995). If there were more than 300 colonies on the plates inoculated with the decimal dilution, the results were expressed as >3,000 CFU ml^{-1}.

Antimicrobial efficacy of ICX®: in field studies

Disinfection of waterlines using ICX® and hydrogen peroxide 3% (shock treatment)

The DCUs underwent intensive disinfection using 3% hydrogen peroxide, which was kept present in all waterline elements for 40 min due to the continual flow of water from the reservoir to the handpieces. Subsequently, fresh water is added to the bottle container, and from there, is used to flush the instrument waterlines and cup fill line. A total of 8 L of fresh main water is used to remove all hydrogen peroxide residue. After a rinsing cycle, the DCUs are ready for use. To assess the effectiveness of shock treatment, water samples were collected immediately following the first disinfection cycle and after 30 days of shock treatment; for each sample, the TVCs were measured. Later, throughout the study period (5 months of monthly shocks), samples were obtained 30 days after shock treatment. During the 6-month follow-up, chairs n°1 and n°2 received the tablet in addition to the treatments, while chair n°3 received only the shock treatment with hydrogen peroxide. The follow-up for DCUs n°2 and n°3 was extended for another 4 months.

Antimicrobial efficacy of ICX®: *in vitro* study

Evaluation of basic bactericidal activity against reference strains

Although this product is not classified as a disinfectant, to evaluate the statement from the producer, 'effective way to maintain clean DUWLs,' the efficacy of ICX® was tested according to the method described in EN 1040 (European Committee for Standardization 2005). The method specifies

the minimum requirements for bactericidal activity of chemical disinfectants and antiseptic products that form a homogeneous physically stable preparation in water. It is assumed that the product has antibacterial properties if it causes a minimum 5 log reduction in the number of viable *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 15442 cells within 60 min. To examine the intended specific use conditions (dental settings), additional specific bactericidal activity was determined by testing contact times at 24 hours; tests were performed in duplicate. Briefly, 1 ml of water and 1 ml of bacterial test suspension (N_0) were added to 8 ml of the examined product test solution (one tablet in 2 L of sterile distilled water) and then neutralized using Dey-Engley neutralizing broth (Sigma Aldrich, Milan, Italy), serially diluted tenfold and inoculated in duplicate using the pour plate technique. The numbers of surviving bacteria (N_a) in each sample was determined, and the reduction is calculated. Mean CFU ml^{-1} was converted into a decimal-log value for normalization, and decimal-log reduction (lgR) was calculated using the following equation:

$$lgR = lg\ N_0 - lg\ N_a \quad (1)$$

Evaluation of bactericidal activity against environmental strains

To evaluate the occurrence of microbial resistance to ICX® we compare bactericidal efficacy of ICX® on planktonic population living in DUWLs of DCU n°1, n°2 and n°3.

Output water samples from DCUs were collected from chairs n°1 and n°2 after the lack of ICX® in waterlines for 72 hours. We enumerated the background bacteria (N_0) according to ISO 6222 and also assessed the bactericidal efficacy of ICX® on planktonic population living in DUWLs (bacteria that are released from the biofilm and circulating in the waterline) according EN 1040.

Laboratory tests were conducted by dissolving ICX® tablets according to the manufacturer's instructions in output water sampled from each DCU. After 60 minutes and 24 hours of contact time, 1 ml of the mixture was neutralized using Dey-Engley, serially diluted tenfold and inoculated in duplicate using the pour plate technique. The numbers of surviving bacteria (N_a) were enumerated and lgR was calculated (Equation (1)).

Microbial profile of DUWLs

We decided to investigate the diversity and distribution of aerobic bacterial species in DUWLs' output water from dental chairs to determine whether the relative abundance of individual species is associated with the failure of waterline disinfection observed in the first part of the study described above.

At the same time, we identified the microorganisms present in the output water samples collected from DCUs before and after the *in vitro* contact with ICX®. Selected examples of the colony types present on each plate were purified by subculture and stored on nutrient agar slopes in the dark for subsequent identification; the colonies were Gram-stained, and then identified using Matrix Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) (Carbonnelle *et al.* 2011; Biswas & Rolain 2013) and API 20 NE (BioMérieux, Marcy l'Etoile, France).

Statistical analysis

Differences between the microbial loads detected in the three dental units were tested using standard one-way analysis of variance (ANOVA) with software package SPSS. A p value <0.05 was considered statistically significant.

RESULTS

Antimicrobial efficacy of ICX®: in field study

Disinfection of waterlines using hydrogen peroxide 3% (shock treatment) and ICX®

At baseline, samples from DCUs showed massive contamination with a high bacterial count at both 22 °C and 37 °C. Only samples from the taps were found to satisfy the Italian standards for drinking water.

Post-intervention monitoring samples (taken the same day as the first shock) achieved the lowest levels of TVCs at 37 °C and 22 °C, conforming to the standard. However, after 30 days of shock treatment, chairs began to show an increase in TVCs.

During the microbiological surveillance, the post-intervention monitoring (samples taken after 30 days from each treatment) produced the results reported in Table 1.

We performed one-way ANOVA on data obtained during the 6 months of monitoring; the samples collected from DCU n°1 and n°2 and samples collected from DCU n°3 did not differ significantly on the basis of TVCs at 37 °C and 22 °C (37 °C $p = 0.86$; f between treatment = 0.15; 22 °C $p = 0.52$; f between treatment = 0.67).

Additionally, in the following 4 months, there was no significant difference between the results obtained from DCU n°2 and DCU n°3 (37 °C $p = 0.63$; f between treatment = 0.24; 22 °C $p = 0.78$; f between treatment = 0.09).

Antimicrobial efficacy of ICX®: *in vitro* study

Basic bactericidal activity against reference strains

At the concentration recommended by the manufacturer, the product caused a 4.0 log reduction of *S. aureus* and a 1.3 log reduction of *P. aeruginosa* within 60 min of contact time. However, the reduction in number of bacterial cells of *S. aureus* strains was approximately 7.6 log after 24 hours of contact time, while in the *P. aeruginosa* strain, the reduction in the number of bacterial cells after 24 hours remained unchanged (Table 2).

Table 1 | Bacteriological quality of output water of dental chairs during the study period

	DCU n° 1 ICX® + shock treatment		DCU n° 2 ICX® + shock treatment		DCU n° 3 Only shock treatment	
	CFU ml^{-1} 22 °C	CFU ml^{-1} 37 °C	CFU ml^{-1} 22 °C	CFU ml^{-1} 37 °C	CFU ml^{-1} 22 °C	CFU ml^{-1} 37 °C
Baseline	3,000	3,000	3,000	3,000	2,100	220
Month 1	0	5	0	16	2	5
Month 2	1,170	870	370	160	300	300
Month 3	63	7	0	0	59	23
	All chairs shocked and dental office closed for the summer holidays					
Month 5	300	180	300	264	360	830
Month 6	44	68	10	14	9,440	910
Month 7	n.d.[a]	n.d.	3,000	3,000	2,860	2,200
Month 8	n.d.	n.d.	2	0	2,520	3,000
Month 9	n.d.	n.d.	68	0	28	2
Month 10	n.d.	n.d.	1,116	100	9	8

n.d., not determined.

[a]After 6 months of follow up DCU n°1 was no longer monitored because it received only ICX® tablet.

Bactericidal activity against environmental strains

Bacterial loads of output water from all DCUs were evaluated quantitatively and qualitatively before and after disinfection. Before *in vitro* treatment, all baseline TVCs measured in samples from the three chairs were above the Italian drinking water standard. After *in vitro* exposure to ICX®, only planktonic bacteria from chair n°3 were completely killed (lgR = 4.3), while in DCU n°1 and n°2, the bacteria survived (Table 3).

Microbial profile of DUWLs

The bacterial species recovered from the samples taken from the dental chairs were all Gram-negative and belonged to the families of aquatic and soil bacteria. Among our isolates, *Delftia acidovorans*, *Stenotrophomonas maltophilia*, *Mycobacterium flavescent*, *Listeria innocua*, *Comamonas testosteroni*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Vibrio metschnikovii*, *Sphingomonas adhaesiva*, *Vibrio alginolyticus*, and *Aeromonas salmonicida* were identified, and some of these are opportunistic human pathogens (Hsueh *et al.* 1998; Martins *et al.* 2010; Brooke 2012; Kim *et al.* 2012; Orsini *et al.* 2014). After *in vitro* exposure to ICX®, only planktonic bacteria from DCU n°3 were completely killed, while *Delftia acidovorans*,

Table 2 | Reduction of *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 15442 exposed to ICX® according to European Standard 1040

Time of exposure	Inoculum (N_0)	Outcome (N_a)	lgR
	Staphylococcus aureus ATCC 6538		
60 min	8.9×10^7 (8.9)	8.5×10^4 (4.9)	4.0
24 h	8.9×10^7 (8.9)	19 (1.3)	7.6
	Pseudomonas aeruginosa ATCC 15442		
60 min	1.2×10^8 (8.0)	5.7×10^6 (6.7)	1.3
24 h	1.2×108 (8.0)	5.1×10^6 (6.7)	1.3

lgR = lg N_0–lg Na; N_0: the number of cells per ml in the test mixture; N_a: the number of surviving cells per ml in the test mixture at the end of exposure; R: viable cell reduction factor.

Table 3 | Reduction of TVCs in output water of dental chair treated *in vitro*

	Temp. of incubation	N_0 (log)	N_a (log) 1 h	N_a (log) 24 h	lgR 1 h	lgR 24 h
DCU n°1	22 °C	3.9×103 (3.59)	1 (0)	4 (0.6)	3.59	2.99
	36 °C	3.7×10^3 (3.57)	14 (1.15)	8 (0.9)	2.42	2.67
DCU n°2	22 °C	1.86×10^4 (4.27)	874 (2.94)	338 (2.53)	1.33	1.74
	36 °C	49 (1.69)	0	0	>1.69	>1.69
DCU n°3	22 °C	9.59×10^4 (4.98)	0	0	4.98	4.98
	36 °C	1.94×10^4 (4.29)	0	0	4.29	4.29

N_0 = total viable counts in output water before contact with ICX®; N_a = surviving bacteria in output water after contact with ICX®; lgR = lg N_0 − lg N_a.

Mycobacterium flavescent, *Stenotrophomonas maltophilia*, and *Listeria innocua* survived in DCU n°1 and n°2 (Figure 1).

DISCUSSION

As previously described in the literature, the only method to lower bacterial counts in dental water to acceptable levels is to permanently eliminate the existing biofilm inside the tubes and prevent biofilm formation in new units. Most of the cleaners and disinfectants do not effectively remove biofilms: Walker & Marsh (2007) appraised a range of chemical DUWL treatment agents and reported that only a few disinfectants successfully remove biofilm and consistently reduce the microbial load of DUWL output water to <200 CFU ml^{-1}. However determining the bacterial load using culture does not predict the exact size of the problem because bacteria in a VBNC state cannot grow on standard growth media.

Different methods have been developed to avoid contamination of DUWLs and dental chairs by pathogens and environmental organisms, with one being chemical disinfection but frequent application may result in the development of disinfectant/biocides resistance contributing to resistance to antibiotics by co-selection of antibiotic resistance genes (Russell 2004).

Cleaners intended for continuous use are less concentrated, and these cleaners are introduced after an initial shock treatment that has acted upon the biofilm in the waterlines, or they are used with new waterlines where biofilms have not yet formed. The literature contains only two accounts (McDowell *et al.* 2004; Meiller *et al.* 2004) of the properties, germicidal effectiveness, and potential uses of the chemical waterline cleaner ICX® tablets. These studies, performed in a simulated use in DUWLs demonstrate that in the presence of a bacterial challenge of 100 to 1,000 CFU ml^{-1} in incoming water, ICX® effectively

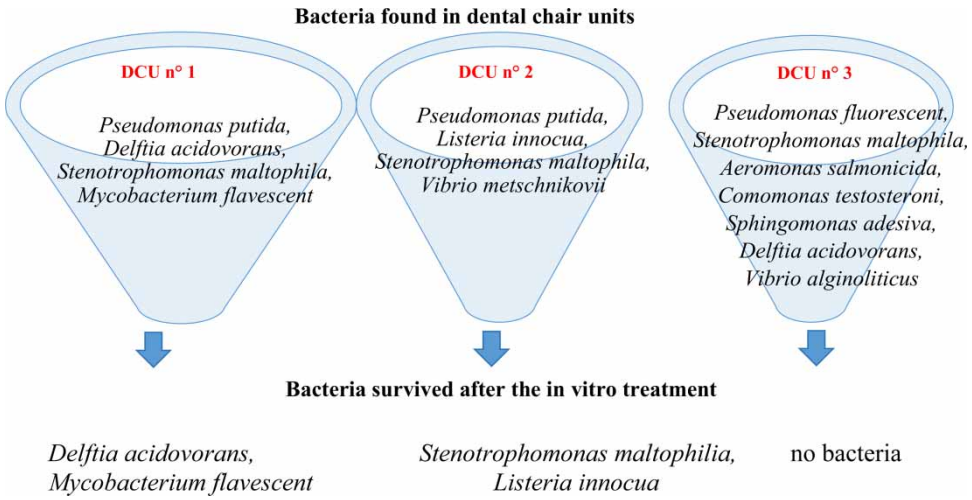

Figure 1 | Gram-negative bacteria in DUWLs before and after in vitro treatment. *Delftia acidovorans, Mycobacterium flavescent. Stenotrophomonas maltophilia, Listeria innocua,* no bacteria.

prevented the development of biofilm and maintained the water quality at a level consistently well below 200 CFU ml^{-1}. Moreover, the treatment prevented biofilm build-up during daily use and over weekends during a period of inactivity.

A-dec recommends in their Waterline Maintenance Guide a 'Maintain, Monitor, and Shock' approach to waterline care. This approach involves daily maintenance with ICX®, monitoring water quality regularly with devices such as the Millipore Heterotrophic Plate Counter (HPC) Sampler, and shocking the waterlines when 'test results are greater than the water quality action level.'

As a part of laboratory procedure, in the first stage of our study, the number of heterotrophic microorganisms in each water sample collected after the first shock treatment with hydrogen peroxide and in 6 months of monitoring was evaluated. However, the effects of a continuous disinfecting system proposed by the manufacturer for a daily disinfection of waterlines were not demonstrated in this study; no significant differences were found between the bacterial loads (TVCs at 37 °C and 22 °C) detected in the effluent water of the two dental chairs that received the ICX® tablet versus chair n°3. As a result, if dental offices are using disinfectant waterline tablets, shock treatment may be needed before 30 days. These results are similar to the finding of Bowen *et al.* (2015) who compared ICX® and Citrisil disinfectants in one clinical setting. They reported that bacterial loads increased to unacceptably high levels within 1–2 weeks of treatment, with a gradual increase after disinfection treatments ceased.

To explain this poor performance, we tested the product versus the collection strains and environmental strains *in vitro*. For *Staphylococcus aureus,* ICX® achieved a four decimal log reduction at 60 min and a seven decimal log reduction after overnight treatment (24 hours). This last contact time reflects a realistic period of inactivity in which the chemical product (although at low doses) could prevent the proliferation of bacteria. Instead, the product is ineffective in reducing the number of *Pseudomonas aeruginosa* cells after 60 min and 24 hours of contact time.

This inability to kill Gram-negative bacteria is a disadvantage because most of the bacterial species found in DUWL output water are Gram-negative. Furthermore, it has been recognized that *in vivo* bacteria are found predominantly attached to surfaces (biofilm), hence being more resistant to heat, dehydration, ultraviolet (UV) light, disinfectants, antibiotics, etc.

It is known that opportunistic pathogens may account for more than 30% of the total bacterial populations in water distribution systems (LeChevallier *et al.* 1980), and these bacteria have all been implicated in waterborne nosocomial infections (Barbeau *et al.* 1996).

A detailed analysis of the output water from these three DCUs showed that the most prevalent bacterial species recovered were Gram-negative, and when we treated DCU output samples *in vitro* with ICX®, we obtained a partial reduction of counts in dental chairs continuously treated with ICX® tablets, but the total killing of bacteria was observed from the output water of dental chair n°3.

CONCLUSION

Biofilms proved to be difficult to control despite all of the measures in this study. Our results show that the continuous introduction of ICX®, used at low levels to minimize potential toxic effects, was not effective in maintaining the heterotrophic bacterial counts within the recommended standards in the output water of dental devices, and shock treatment may be needed more frequently than monthly treatments. Moreover, the prolonged use of ICX® tablets was not always able to control microorganisms that are very resistant to disinfectants due to their intrinsic characteristics, because they are protected within the biofilm or because of the development of biofilm tolerance which we observed in the growth of *Delftia acidovorans* and *Stenotrophomonas maltophilia*.

The only way to lower the bacterial counts in the dental water to acceptable levels is to permanently eliminate the existing biofilms inside the tubes and to ensure that the frequency of the treatment is sufficient to successfully maintain a good quality of water when performing periodic water testing; therefore, routine monitoring is strongly recommended.

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CONFLICTS OF INTEREST

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