

***In Vitro* Efficacy of Biocides against Dental Unit Water Line (DUWL) Biofilm Bacteria**



I. Liaqat* and A. N. Sabri

Department of Microbiology and Molecular Genetics,
University of the Punjab, Quaid-e-Azam Campus,
Lahore-54590; Pakistan.

Abstract : Dental unit waterlines (DUWL) are an integral part of dental surgery equipment, supplying water as a coolant, primarily for air turbine and ultrasonic scalers. DUWL when not in use remain connected to main water supply providing conditions for biofilm development within 8 hours. Bacteria shed from the biofilm can maintain and support massive number of planktonic organisms. Characteristically biofilm bacteria exhibit 3000 fold more resistance to surfactants, biocides and antibiotics than organisms floating freely in fluids. Biofilms on tubings within DUWL provide a reservoir of microorganisms and must be controlled. This study compared different biocides for their ability to reduce and/ eliminate the biofilm bacteria. Sodium dodecyl sulphate (SDS), Hydrogen peroxide (H_2O_2), Sodium hypochlorite (NaOCl), Phenol (Phe), Tween 20 (Tw 20), Ethylene dihydro tetraoxide (EDTA), Chlorohexidine gluconate (CHX) and Povidine iodine (PI) were tested against DUWL biofilm bacteria. SDS, H_2O_2 , Tw 20 and EDTA completely eliminate viable bacteria when applied singly, however, combined forms of these were found to be more effective in eliminating the biofilm bacteria. Some combinations effectively reduced the biofilm bacterial population. The most effective combination was of CHX with rest of the six biocides, although CHX gave the most consistent and sustained antimicrobial effect over time. Applying all the biocides simultaneously resulted in elimination of most bacteria.

Key words : Biofilms, DUWL tubing samples, Biocides efficacy, DUWL isolates.

Introduction

Water delivered by dental units during routine dental practice is highly contaminated (Montebugnoli and Dolci, 2002; Liaqat and Sabri, 2008a, b). Dental unit water Lines (DUWL) are used to irrigate the oral cavity during dental treatment. Water delivered from these devices is not sterile and has been shown to contain large population of bacteria (Singh *et al.*, 2003). Biofilms developing on the inner surface of the tubing can be responsible for high levels of contamination of water delivered by DUWL (Liaqat and Sabri, 2008a, b).

Microorganisms in water supplied to DUWL, mainly aerobic heterotrophic gram-negative environmental bacteria, attach to the

internal surfaces of the waterlines and form microcolonies that eventually give rise to multispecies biofilm (Meiller *et al.*, 2000). DUWL biofilms are composed mainly of highly hydrated bacterial exopolysaccharide that hosts both microcolonies and single cells interspersed heterogeneously with channels or pores (Davey and O'Toole, 2000; Walker *et al.*, 2004). Biofilm develop result at the internal surface of the narrow-bore DUWL due to flow of slow water and low disturbance to the microbial group than at the centre. This further allows microorganisms to proliferate before eventually dispersing through the water supply as planktonic forms where they may be deposited at other sites within the waterline network or delivered directly into the mouths

* **Corresponding author :** Iram Liaqat, Department of Microbiology and Molecular Genetics, University of the Punjab, Quaid-e-Azam Campus, Lahore-54590; Pakistan; E-mail: iramliq@hotmail.com

of patients during dental procedures. Thus, DUWL biofilm acts as a reservoir for ongoing contamination of DUWL output water.

Many opportunistic or pathogens such as *Legionella pneumophila*, *Mycobacterium* sp., *Pseudomonas aeruginosa*, and *Candida* sp. have been recovered from DUWL (Watnick and Kolter, 2000; Tuttlebee *et al.*, 2002). Exposure of dental personnel to such pathogens can be inferred from the finding that dentists have significantly higher antibody titers to *L. pneumophila* than individuals in other, equivalent employment sectors. Asthma may be another condition associated with occupational exposure to endotoxin in aerosols from contaminated DUWS (Pankhurst, 2003). In addition, *P. aeruginosa* isolated from a DUWL was found to be responsible for the hospitalization of two patients following a visit to a dental surgery (Barbeau, 2000).

The presence of high concentrations of microorganisms in DU water (up to 10^6 cfu/mL has been recorded) is a potential risk of infection for dental patients and staff and is incompatible with good cross-infection control practices (Smith *et al.*, 2002). Previous studies have shown that waterborne bacteria are aerosolised during dental procedures, exposing dental personnel and patients to these microorganisms and biofilm fragments. DUWL contamination is of particular concern in the treatment of immunocompromised and medically compromised individuals.

Although studies have assessed the bacterial population in the bulk water delivered from dental units only few workers have identified the organisms (Smith *et al.*, 2002; Singh *et al.*, 2003). Even less attention has been given to the types of organisms present in the biofilm, the primary source of bacteria in the DUWL (Shapiro *et al.*, 2002). Uptil now, the microbial community in the bulk water and in the DUWL biofilm has only been assessed after flushing of biocides and cultivation. However,

the aim of this study was therefore to evaluate and compare the efficacy of biocides on DUWL isolates. This study provides the first *in vitro* examination of biocides efficacy against DUWL biofilm isolates by applying culture dependent techniques.

Materials and Methods

Sampling of DUWL biofilms

DUWL biofilm microorganisms were isolated from the tubing samples obtained from the Punjab Dental Unit (PDU), Lahore, Pakistan following the method of Liaqat and Sabri (2008b). SDS (1% w/v), H₂O₂ (35% v/v), Tw 20 (4% v/v), EDTA (1% w/v), NaOCl (5.25% v/v), Phe (35% w/v), CHX (0.2% w/v), PI. (1% v/v) were used as biocides to determine the resistance profile of the isolated strains. The tubings selected for investigation in this study had a significant degree of microbial contamination (Liaqat and Sabri, 2008b) and were obtained from principal dental unit, located within a dental teaching hospital to closely simulate usage patterns in a general dental practice.

Efficacy of biocides

The concentration of biocides where required was prepared using sterile distilled water. L- agar plates supplemented with biocides were prepared into the following three ways.

1. L- agar plates supplemented with biocides individually (100, 500, 1000 μgml^{-1}) were prepared. Growth of DUWL biofilm microorganisms was monitored.

2. Some biocides (NaOCl, EDTA, CHX, SDS) were introduced into the media as combined forms of two (100, 500, 1000 μgml^{-1}) and DUWL biofilm formed was observed.

3. Finally the L-agar plates supplemented with all the biocides (25, 50 and 100 μgml^{-1}) were prepared and resistant strains were isolated.

Results

Subculturing and purification of biofilm isolates from AWT, PT and MWP on different media resulted in the isolation of 66 morphologically different bacterial strains (Data not shown). Eight biocides (5.25% NaOCl, 35% H₂O₂, 4% tween 20, 1% PI, 0.2% CHX, 1% EDTA and 1% phe) were supplied singly and combination of two in L- agar medium to monitor the efficacy of biocides and resistance profile of the 66 isolates. Almost all strains were resistant to 100-500 µgml⁻¹ concentration of biocides in single and combined form of two. However at 1000 µgml⁻¹ resistance profile of

isolates was changed both singly and in combined form (Figure 1).

Supplementation of biocides in L-agar medium at 1000 µgml⁻¹ resulted in variation in resistance of AWT, PT and MWP isolates to eight biocides each. NaOCl, SDS, Tw 20, each exhibited 37% resistant AWT isolates, whereas NaOCl and EDTA were effective combinations in PT (10, 24% resistance) and MWP isolates with 6 and 28% resistance (Figure 2).

Four biocides SDS and CHX were tested by flushing method (Liaqat and Sabri, 2008b) while two (NaOCl and EDTA by culture

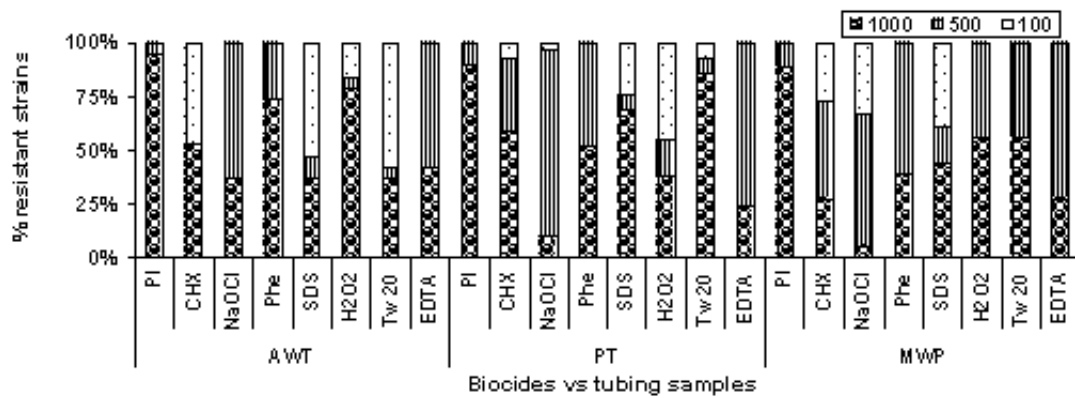


Fig. 1 : Introduction of all the biocides individually into the media and isolation of percentage resistant strains

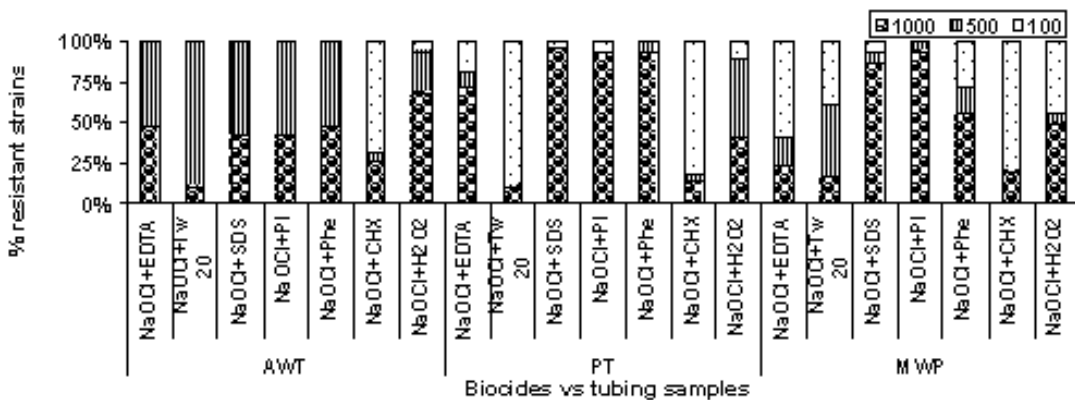


Fig. 2 : NaOCl in combined form with rest of the seven biocides and isolation of percentage resistant strains

dependent method), found to be effective in eliminating/ reducing DUWL bacterial contamination were combined with rest of the seven biocides, introduced into the media. Resistance profile of all 66 isolates from three tubing samples was checked at 100, 500 and 1000 μgml^{-1} of biocides in combined form of two.

Combination of NaOCl with other biocides

In AWT isolates, combination of NaOCl with other biocides resulted in variation of resistance level of isolates at different concentrations and combinations. The most effective combination was NaOCl and Tw 20 and NaOCl and CHX in all tubing isolates. At 1000 μgml^{-1} concentration, in AWT samples these two combinations resulted in 11, 26% resistant isolates compared to PT isolates where 10 and 14% resistance was observed. In MWP isolates, NaOCl combination with Tw 20 and CHX was effective. 17 and 6% resistant isolates were observed at 1000 μgml^{-1} concentration of these two combinations (Figure 2).

Combination of EDTA with other biocides

Combination of EDTA with seven other biocides resulted in variation in effectiveness

against bacterial isolates, isolated from different sources. In AWT isolates, the combined form of EDTA with CHX and in PT isolates, EDTA with Phe were observed to be most effective combinations resulting in 21, 24% resistant DUWL biofilm isolates (1000 μgml^{-1}). 17% resistance in MWP biofilm isolates was observed by applying EDTA in combination with Tw 20 and Phe (Figure 3).

Combination of CHX with other biocides

Combined form of CHX with each of seven biocides indicated CHX plus EDTA and CHX plus NaOCl, as effective combinations. At 1000 μgml^{-1} concentration, 21 and 14 % resistant isolates from AWT, PT samples were observed against these two combinations. CHX combination with PI and NaOCl, resulting in 0 and 6% resistance in MWP isolates, were the most effective combinations in reducing growth of MWP isolates compared to other combinations tested in this study (Figure 4).

Combination of SDS with other biocides

SDS plus Phe was observed to be the most effective combination in AWT and PT isolates, resulting in 37 and 48% resistant isolates (upto 1000 μgml^{-1} concentration) from DUWL biofilm respectively. In MWP isolates, this

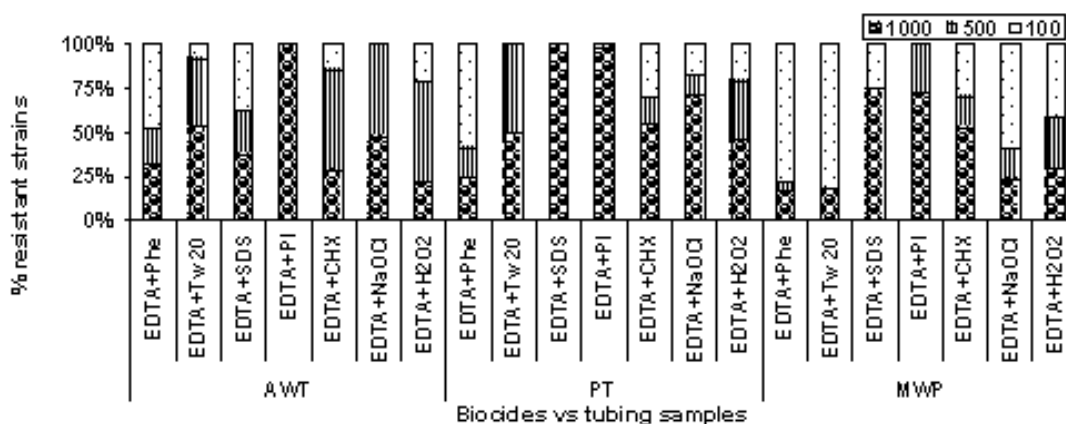


Fig. 3 : EDTA in combined form with rest of the seven biocides and isolation of percentage resistant strains

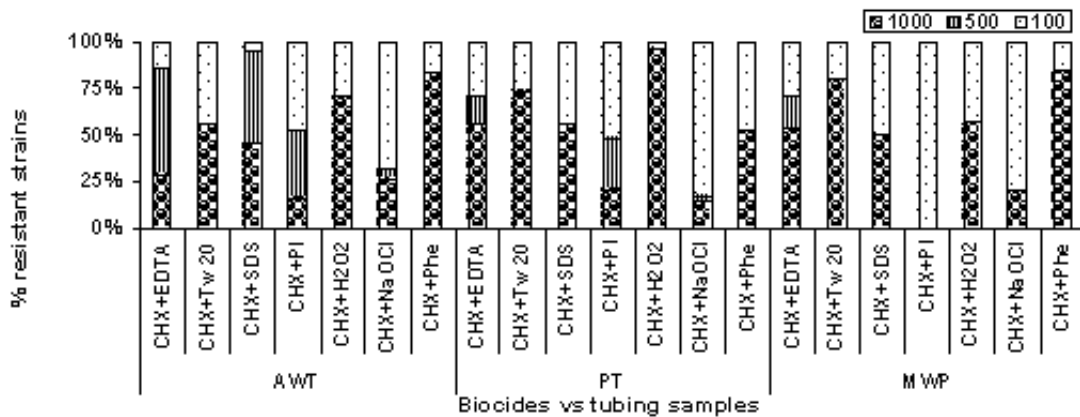


Fig. 4 : Introduction of all the biocides individually into the media and isolation of percentage resistant strains

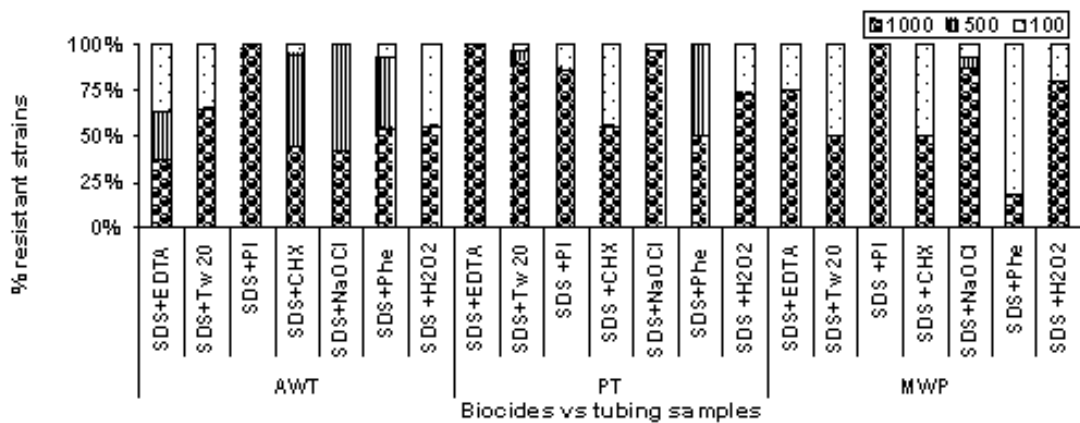


Fig. 5 : SDS in combined form with rest of the seven biocides and isolation of percentage resistant strains

combination resulted in 17% resistance at 1000 μgml^{-1} concentration (Figure 5).

In case, when all the biocides were introduced into the media simultaneously (100 μgml^{-1}), and microbial growth was checked. Twenty different strains (AWT 1, AWT 2, AWT 10, AWT 13, AWT 14, AWT 16a, AWT 21, AWT 25, AWT 28, AWT 33, PT 1, PT 2, PT 16, PT 19, PTNPF, PT PA, MWP 14, MWP 15, MWPNPC, MWPNPD) were observed to be resistant.

Statistical analysis

The effect of various biocides on DUWL isolates was determined by measuring % resistant level of isolates against each biocide alone as well as in combined form with rest of seven biocides at various concentrations (100, 500 and 1000 μgml^{-1}). All the data were presented in the form of table and figures.

Discussion

The source of bacterial contamination within the dental unit water supply is thought

to be caused biofilm composed of micro-colonies of proliferating bacteria, fungi and protozoa on the inner surface of the water lines. A wide range of micro-organisms can be isolated which include environmental organisms, opportunistic pathogens, such as *Pseudomonas* spp. and human pathogens, such as *Legionella pneumophila* (Walker *et al.*, 2000). Not only patients but also dentists and dental personnel are at risk of being infected with opportunistic pathogens such as *Pseudomonas* or *Legionella* species by means of cross-infection or following aerosol formation from water emanating from DUWL (Bennett *et al.*, 2000). The problem of microbial contamination of DUWL is compounded by the intricacy and complexity of dental units for which there appear to be no immediate solutions. The long-term solution to the problem lies in redesigning the water supply system within dental units to eliminate stagnant areas and biofilm build up. In the shorter term, disinfectants may have a role to play in controlling the levels of microbial contamination within dental unit water lines to more acceptable levels (Smith *et al.*, 2002).

This study describes the use of eight biocides (5.25% sodium hypochlorite; 35% H₂O₂, 4% tween 20, 1% povidine iodine, 0.2% chlohexidine gluconate, 1% ethylene di-amino tetra acetic acid and 1% phenol) alone and in

combined form for their ability to eliminate/ reduce the biofilm microorganisms contaminating dental unit waterlines. Biocides efficacy observed by culture independent (Liaqat and Sabri, 2008b) and by culture dependent method resulted in NaOCl, EDTA, CHX, SDS as effective biocides. All strains could tolerate to 100 µgml⁻¹ and 500 µgml⁻¹ concentration of biocides alone and in combined form so they were taken to higher level of biocides i.e., 1000 µgml⁻¹. By adding biocides into the media alone at 1000 µgml⁻¹ concentration, NaOCl and EDTA were found to be effective against DUWL biofilm isolates from all the three tubing samples. Resistant isolates observed against these two biocides (NaOCl, EDTA) were 8, 9% (AWT); 12, 6% (PT) and 2, 8% (MWP) (Table-1). H₂O₂ (9% PT resistant isolates) and CHX (8% resistant MWP isolates) were also observed to be effective. To further evaluate the efficacy of aforementioned two biocides (NaOCl and EDTA) and two other biocides (CHX and SDS, observed effective by culture independent method), these were applied in combination with rest of seven biocides. NaOCl in combination with Tw 20 resulted in 4% (AWT) , 3% (PT) and 5% (MWP) resistant isolates while 9 (AWT), 3 (PT) and 2% (MWP) isolates were found to be resistant against combined form of NaOCl plus CHX at 1000 µgml⁻¹ concentration (Table-1).

Table 1 : Effective combinations of biocides and isolation of % resistant strains

S. No.	Biocides combinations	Tubing samples (% resistant isolates)		
		AWT	PT	MWP
1	NaOCl + Tw 20	4	3	5
2	NaOCl + CHX	9	3	2
3	EDTA +Phe	11	6	7
4	EDTA +H2O2	6	9	11
5	CHX+PI	6	6	0
6	SDS+CHX	12	10	12
7	SDS+Phe	10	9	4

Combined form of EDTA with Phe, H₂O₂ was found to be effective in controlling the resistance of AWT, PT and MWP isolates. 11%, 6%, 7% (AWT, PT, MWP) and 6%, 9%, 11% (AWT, PT, MWP) resistant isolates were observed by supplying EDTA in combination with two other biocides (Phe, H₂O₂) at 1000 µgml⁻¹ concentration (Table-1). 6% (AWT), and again 6% (PT) resistant isolates were observed by supplying CHX plus PI combination in L-agar medium while all MWP isolate were sensitive at 1000 µgml⁻¹ concentration of this combination (Table-1). SDS with CHX resulted in 12 (AWT), 10 (PT) and 12% (MWP) resistant isolates. However, combination of SDS with Phe proved to be more effective in significant percentage reduction/ elimination (10% resistant) of AWT isolates, while 9% (PT) and 4% (MWP) isolates were noted as resistant at 1000 µgml⁻¹ of concentration (Table-1). Combination of NaOCl with Tw 20 or EDTA, might have the NaOCl as active agent. It has already been observed that NaOCl works effectively to control microbial contamination in dental settings (Montebugnoli and Dolci, 2002). Another possibility is that combined form of NaOCl with EDTA may result in a compound with properties identical to Alpron, a disinfectant which had NaOCl and EDTA as active agents. It works effectively against DUWL bacteria and biofilm (Smith *et al.*, 2002).

EDTA, a divalent cation chelator, has been reported among microbial control technique in DUWLs (Walker and Marsh, 2007). In combined form of EDTA with H₂O₂, possibly EDTA act as chelator thus enhancing the efficacy of H₂O₂ against DUWL isolates. Alternatively combined form of H₂O₂ with EDTA might be equivalent dentasept, a disinfectant which contains 1% H₂O₂ as active agent. Dentosept has been shown to be highly effective in reducing TVCs and maintaining the microbial load to levels below 200 cfu/ml

and gave the most consistent and substantial antimicrobial effect over time (Walker and Marsh, 2007).

Chlorhexidine gluconate (Pankhurst, 2003), povidone iodine (Pankhurst *et al.*, 2005), sodium hypochlorite (Meiller *et al.*, 2001), hydrogen peroxide (Decoret *et al.*, 2005) have been employed to variable effects to remove the biofilm and eliminate the planktonic bacterial count. It was found that over all combination of CHX with PI was very effective in eliminating/reducing the biofilm bacteria at 1000 µgml⁻¹ as compared to other combinations. The efficacy of CHX in reducing oral bacterial viability (has been demonstrated in many studies (Shapiro *et al.*, 2002; Clavero *et al.*, 2003). It's a commonly used antimicrobial in dentistry and hence to control bacteria in dental water lines (Epstein *et al.*, 2002). In combined form of CHX plus PI, the efficacy is possibly again due to CHX, since PI alone didn't prove to be affective agent both against DUWL biofilm and biofilm bacteria by applying culture dependent and independent techniques. This was the only biocide that was observed not very promising in this study.

Application of all biocides at 100 µgml⁻¹ versus 66 DUWL isolates resulted in elimination of all isolates. Combination of all biocides might result in the formulation of a compound having a broader antimicrobial spectrum. It has already been reported that new chemical disinfectants are often combinations of different compounds (Andersen and Hilsberg, 2007). Only 20 strains were found to be resistant. The mechanisms of resistance in these isolates may be intrinsic or acquired in nature.

In addition, microorganisms have adapted to biocide exposure by acquiring plasmids and transposons that confer biocide resistance, the same survival strategies to disseminate acquired mechanisms of resistance to biocides

as they have for resistance to antibiotics (Albert and Sheldon, 2005). Also modification of cell wall constituents has been reported to play a significant role in conferring microbial resistance against all above mentioned biocides (Liaqat and Sabri, 2008a).

Overall, the results of this study favour the use of biocides in combination rather than alone. In the long term, the redesign of dental units may be necessary to decrease biofilm and microbial contamination. However, in the short term, effective disinfectants are required that will control biofilm formation. Currently, not enough data are available that address how specific components attack the integrity of the biofilms. According there still remains a need for a protocol for cleaning biofilm coated surfaces, and it should effectively dislodge biofilm and optionally kill the microorganism flora in the dislodged biofilm. These protocols can be adapted to suit a variety of industrial uses and needs.

References

- Albert T. and Sheldon Jr. (2005): Antiseptic "Resistance": Real or Perceived Threat? *Clin. Infect. Dis.*, **40**, 1650-1656.
- Andersen L.P. and Hilsberg P. (2007): Disinfectants and disinfection methods. *Ugeskr. Laeger.*, **169**, 4243-4236.
- Barbeau J. 2000: Waterborne biofilms and dentistry: the changing face of infection control. *J. Can. Dent. Assoc.*, **66**, 539-541.
- Bennett A.M., Fulford M.R., Walker J.T., Bradshaw D.J., Martin M.V. and Marsh P.D. (2000): Microbial aerosols in general dental practice. *Br. Dent. J.*, **189**, 664-667.
- Blake G.C. (1963): The incidence and control of bacterial infection of dental units and ultrasonic scalers. *Br. Dent. J.*, **115**, 413-416.
- Clavero J., Baca P., Junco P. and Gonzalez M.P. (2003): Effects of a 0.2% chlorhexidine spray applied once or twice daily on plaque accumulation and gingival inflammation in a geriatric population. *J. Clin. Periodontol.*, **30**, 773-777.
- Davey M.E. and O'Toole G.A. (2000): Microbial biofilms: from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews.* **64**, 847-867.
- Decoret D., Amoussou Y., Lagneau C., Lissac M., Morrier J.J. and Barsotti O. (2005): Simulated-use evaluation of hydrogen peroxide disinfectant for preventing biofilm formation in dental unit waterlines. *Eur. Cell. Mater.*, **10**, 24-25.
- Epstein J.B., Dawson J.R., Buivids I.A., Wong B. and Le, N.D. (2002): The effect of a disinfectant/coolant irrigant on microbes isolated from dental unit water lines. *Spec. Care. Dentist.*, **22**, 137-141.
- Liaqat I. and Sabri A.N. (2008a): Analysis of cell wall constituents of biocide-resistant isolates from dental unit water line biofilms. *Curr. Microbiol.*, **57**, 340-347.
- Liaqat I. and Sabri A.N. (2008b): Effect of biocides on biofilm bacteria from dental unit water lines. *Curr. Microbiol.*, **56**, 619-624.
- Meiller T.F., Kelley J.I., Baqui A.A. and DePaola, L.G. (2001): Laboratory evaluation of anti-biofilm agents for use in dental unit waterlines. *J. Clin. Dent.*, **12**, 97-103.
- Meiller T.F., Kelley, J.I., Baqui A.A. and DePaola L. G. (2000): Disinfection of dental unit waterlines with an oral antiseptic. *J. Clin. Dent.*, **11**, 11-15.
- Montebugnoli L. and Dolci, G. (2002): A new chemical formulation for control of dental unit water line contamination: an in vitro and clinical study. *BMC. Oral. Health.*, **2**, 1.
- Pankhurst C.L. (2003): Risk assessment of dental unit waterline contamination. *Prim Dent. Care.* **10**, 5-10.
- Pankhurst C.L., Coulter W., Philpott-Howard J.N, Surman-Lee S., Warburton F. and Challacombe S. (2005): Evaluation of the potential risk of occupational asthma in dentists exposed to contaminated dental unit waterlines. *Prim. Dent. Car.*, **12**, 53-59.
- Shapiro S., Giertsen E. and Guggenheim B. (2002): An in vitro oral biofilm model for comparing the

- efficacy of antimicrobial mouthrinses. *Caries Res.*, **36**, 93-100.
- Singh R., Stine O.C., Smith D.L., Jr Spitznagel J.K., Labib M.E. and Williams H.N. (2003): Microbial Diversity of Biofilms in Dental Unit Water Systems. *Appl. Environ. Microbiol.*, **69**, 3412-3420.
- Smith A.J., McHugh S., Aitken I. and Hood J. (2002): Evaluation of the efficacy of Alpron disinfectant for dental unit water lines, *British. Dental. Journal.*, **193**, 593-596.
- Tuttlebee C.M., O'Donnell M.J., Keane C.T., Russell R.J., Sullivan D.J., Falkiner F. and Coleman D.C. (2002): Effective control of dental chair unit waterline biofilm and marked reduction of bacterial contamination of output water using two peroxide-based disinfectants. *J. Hosp. Infec.*, **52**, 192-205.
- Walker J.T. and Marsh P.D. (2007): Microbial biofilm formation in DUWS and their control using disinfectants. *J. Dent.*, **35**, 721-730.
- Walker J.T., Bradshaw D.J., Bennett A.M., Fulford M.R., Martin M.V. and Marsh P.D. (2000): Microbial biofilm formation and contamination of dental-unit water systems in general dental practice. *Appl. Environ. Microbiol.*, **66**, 3363-3367.
- Walker J.T., Bradshaw D.J., Finney M., Fulford M.R., Frandsen E., Ostergaard E., Ten Cate J.M., Moorer W.R., Schel A.J., Mavridou A., Kamma J.J., Mandilara G, Stosser L., Kneist S., Araujo R., Contreras N., Goroncy-Bermes P, O'Mullane D., Burke F., Forde A., O'Sullivan M. and Marsh P.D. (2004): Microbiological evaluation of dental unit water systems in general dental practice in Europe. *Eur. J. Oral. Sci.*, **112**, 412-418.
- Watnick P. and Kolter R. (2000): Biofilm, city of microbes. *Journal of Bacteriology.*, **182**, 2675-2679.