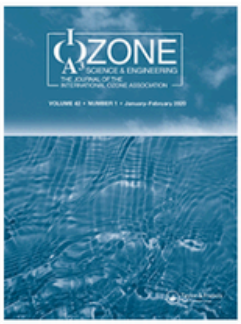

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Optimization of Ozonation Process for Disinfection of Dental Unit Waterlines Using Response Surface Methodology

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ABSTRACT

The reduction of microbial contamination in dental unit waterlines (DUWLs) appears to be necessary because of a potential risk of infections in immunocompromised patients and medical staff, which are regularly exposed to water and aerosols generated from DUWLs. In the present study, the qualitative and quantitative microbial contamination of water in DUWLs were determined and the conventional biomedical diagnostic tests were applied to identify microorganisms. A 3-level, 2-factor central composite design was utilized to investigate the effects of chief operating parameters and optimize ozone disinfection conditions. Also, the activity of three disinfectant (ozone, NaOCl, and peracetic acid) in microbial decontamination of DUWLs were compared. The results indicated that *Microbacterium laevaniformans* were the most prevalent genera (21%) among both Gram-negative and positive species in all samples. Regression analysis illustrated the good fit of the experimental data to the predicted model with R^2 and R^2_{adj} correlation coefficients of 0.988 and 0.980, respectively. Moreover, under the optimal circumstances (Ozone concentration = 1.2 ppm and reaction time = 13.5 min) the disinfection efficiency was 97.52%. The results also revealed that ozone was effective disinfectant to reduce prevalent genera (with the removal of 93.75%, 92.57% and 96.15% of *Pseudomonas aeruginosa, Microbacterium laevaniformans*, and *Alcaligenes faecalis*, respectively) and already formed biofilms under optimum conditions. Based on achieved results, ozone was highly effective on microbial decontamination compared to peracetic acid and NaOCl disinfectant and can be used for disinfection of DUWLs.

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Dental Unit Waterlines; Microbial Biofilm; Ozonation; Disinfection; Optimization; Central Composite Design

Introduction

Dental unit waterlines (DUWLs) provide an ideal site for bacterial growth and biofilm formation composed of various types of microorganisms (Costa et al. 2015). The microbial contamination of DUWLs output water can originate from various sources of contamination including water stagnation, the water supply of dental unit and patients by suck-back (Costa et al. 2016; Dallolio et al. 2014). Albeit, bacterial biofilms are fixed in the inner surface of the tubing, microbes, and their toxic by-products from biofilms are frequently shed as the water flows through the tubing and led to continuous contamination of patient water treatment (Ji et al. 2016; Porteous et al. 2013; Tuttlebee et al. 2002). Hence, output water from DUWLs can be a potential source of infectious diseases for dental health-care personnel and especially immunocompromised and diabetics patients (Dallolio et al. 2014; Walker et al. 2004). The American Dental Association (ADA) and the Center for Disease Control and Prevention (CDC) have recommended <200 and ≤500 CFU/mL for microbial load delivered by dental units, respectively (Szymańska, Sitkowska, and Dutkiewicz 2008). Nevertheless, many studies have reported that the bacterial count can be different from 10^2 to 10^8 colony-forming units per milliliter (CFU/mL) (Arvand and Hack 2013; Barbot et al. 2012). Therefore, the reduction of microbial contamination in DUWLs is a significant subject in the field of water pollution and diseases control. During recent decades, several strategies have been applied to control DUWLs bacterial colonization, such as waterline flushing, ultraviolet light, inline microfiltration and continuous disinfection by chemical compounds (Linger et al. 2001). However, such methods are unable to reduce the microbial contamination in the standard range and generate

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disinfection by-products which is a critical issue for economic and public health (Pan et al. 2017). Accordingly, an alternative convenient technology with high ability to fight biofilms, safety for the patient and compatible with the material of DUWLs must be presented (Özcan, Kulak, and Kazazoglu 2003). Ozone is an appealing disinfectant which has a potent and reliable antibacterial ability to eliminate the pathogenic microorganisms including viruses, bacteria, and fungi (Azarpazhooh and Limeback 2008; Demir and Atguden 2016). Ozonation can greatly inactivate microorganisms (up to 99%) by the demolition of cell walls and protoplasm of bacteria and fungi in a short time with low dosage when compared to other chemical disinfectants (Alwi and Ali 2014; Rojas-Valencia 2011; Verma, Gupta, and Gupta 2016). Also, Ozonation prevents the production of detrimental by-products after the disinfection process and finally provides effluent with better physicochemical and microbiological quality in DUWLs (Zucker et al. 2014). It should be noted that the disinfection process is affected by various operating parameters such as reaction time, disinfectant concentration, etc. Thus, using experimental design methodology as a statistical technique can be evaluated the interactions of main influencing parameters with a limited number of experiments (Bilici Baskan and Atalay 2015; Khorsandi et al. 2016). Therefore, in the present study Response Surface Methodology (RSM) based on Central Composite Design (CCD) was utilized to optimize the disinfection of DUWLs using ozonation process. Although some studies have been conducted on the disinfection of DUWLs, and RSM has been widely used to optimize various process (Fujita, Mashima, and Nakazawa 2017; Kaur, Jindal, and Kaur Bhatia 2018; Khorram and Fallah 2018; Patel, Desai, and Owen 2016), there is no accessible and comprehensive information concerning the optimization of the disinfection process in DUWLs by ozone, from the literature. The aim of the current study is (a) to determine the qualitative and quantitative contamination of DUWLs (b) detection of microbial species (c) to evaluate of ozonation process and flushing for removal of a variety of microbial contamination (d) optimization of disinfection process for maximum microbial decontamination using CCD (e) and finally, comparison of ozone disinfectant with other chemicals.

Method and material

Water sampling at baseline

The samples were collected from 20 dental units (supplied with tap water) located in the public dental clinic in the city of Tehran, Iran during two working days from the different therapeutic wards. Water samples were taken from various water outlets of dental units (150 mL) in sterile glass bottles containing 0.1 ml sodium thiosulfate (15 g/L) in order to remove residual chlorine. All samples were kept refrigerated at 4–8 °C and were transported immediately to the laboratory in an insulated cool box, microbiological analyses were done within a period of 2 h.

Microbiological analysis of water samples

The conventional microbiological methods recommended in the Standard Examination Methods for drinking water were used to isolate and identify microorganisms (Ji et al. 2016). Bacteria were cultured on nutrient agar with 5% sheep blood and plates incubated at 37 °C for 2 days under aerobic conditions. After incubation, the number of colonies forming units (CFU/mL) was counted in each plate. Subsequently, the samples were tested for the detection of microbial species using biochemical micro tests. In order to define bacterial genus or species, a catalase test, a Gram stain, an oxidase test, and nitrate reduction test were performed. API 20NE and API 20E tests (bioMerieux, France) were applied to identify Gram-negative bacteria, while for Gram-positive bacteria were identified with API staph and API 20 strep tests (Güngör, Kadaifçiler, and Peker 2014; Kadaifciler and Cotuk 2014).

Disinfectants preparation and delivery to DUWLs

Ozone was generated by connecting pure oxygen cylinder to the ozone generator (OZ-DC800MG), Ozonefac, China. After the ozone generation, it was connected to the unit's water bottle and ozone was bubbled through the distilled water for several minutes, followed by flushing the ozonated water using an automatic flush device (Castel-lini Autosterile) and remained in the waterlines. The concentration of ozonated water was measured before and after disinfection by UV absorption ozone analyzer (Model T400), Teledyne, USA. On the other hand, the disinfectants solutions (peracetic acid and NaOCl) freshly prepared each night and flushed through DUWLs by Autosterile.

Process variable and central composite design (CCD)

In the present study, the concentration of ozone and reaction time were identified as the critical variables to investigate the disinfection efficiency of DUWLs

Table 1. Independent process variables, range, and levels used for CCD.

Independent variables	Factors	−1	0	+1
Ozone concentration (ppm)	A	0.5	1	1.5
Time (min)	B	5	10	15

by ozonation process. A three-level two-factor CCD (Design-Expert 7) was utilized to evaluate disinfection process parameters affecting the microbial decontamination using ozone (Table 1). A total of 13 experiments were conducted in this study to assess the effects of the two chief independent parameters on the disinfection process. Finally, the obtained results were analyzed using analysis of variance (ANOVA) and statistical response plot.

Scanning electron microscopy and confocal laser scanning microscopy analysis

To investigate the morphology of biofilm formed inside the DUWLs, scanning electron microscopy (SEM-HITACHI S-4160, Japan) was used. Dental unit tubes washed twice with 0.1 M phosphate-buffered saline (PBS) for 10 min, fixed in 2.5% glutaraldehyde for 48 h at 4°C, and dehydrated in a graded series of ethanol from 30 to 100%. Then, the tube was divided longitudinally, dried naturally, mounted onto studs, and sputter-coated with gold-palladium before detection.

The viability of the biofilms formed on the inner surface of DUWLs and their thickness was analyzed before and after ozonation using a confocal laser scanning microscope (CLSM, ZEISS LSM 800, Switzerland). All samples were stained with a LIVE/DEAD® BackLight™ Viability Kit (Invitrogen, Carlsbad, CA), and propidium iodide (3 µl) and SYTO® 9 (3 µl) were diluted in 1 ml of distilled water and were used as a reagent. The reagent mixture (100 µl) was added to the tubing section, and the tubing was stained at 25 °C for 20 min. afterward, the reagent was removed and the tubing was rinsed with distilled water and was investigated through CLSM. When observed with CLSM, live bacteria were stained fluorescent green, whereas dead bacteria were stained fluorescent red.

Results

Qualitative and quantitative evaluation of DUWLs

According to the results (Tables 2–4), most of the samples (66%) had lower contamination than the guideline value recommended by the American Dental Association (ADA). However, the percentage of samples with high bacterial load (>1000 CFU/mL) is notable and were similar (13.8%) on both the first and last days of work. Periodontal surgery ward had the highest bacterial load among other wards that mainly showed to have microbial contamination lower than the standard limit. We also sampled different parts of dental unit waterline (DUWL). The highest and lowest microbial loads observed in the cup water and after flushing, respectively.

Genera of bacteria

The samples were tested for the identification of bacteria's genera. The results are presented in Table 5. According to this table, mesophilic bacteria are present in all of the samples. About 61% of the bacteria are Gram-negative, and among them, *Pseudomonas aeruginosa* had the highest prevalence (18.2%). On the other hand, *Microbacterium laevaniformans* was the most prevalent genera (21%) among both Gram-negative and positive species. Two Gram-positive genera, i.e. *Corynebacterium auris* and *Pediococcus pentosaceus* had the lowest prevalence.

Effect of flushing and working day

The results of the effect of flushing are illustrated in Figure 1. This Figure indicates that the significant effect

Table 2. Results of microbial quality of DUWLs based on a working day.

	The first working day (Saturday)		The Last working day (Wednesday)	
CFU/mL	n	%	n	%
<500*	27	33.8	26	32.5
500–1000#	2	2.5	3	3.8
>1000‡	11	13.8	11	13.8

*Acceptable density for potable water according to regulations in the USA
Middle bacterial load
‡ high bacterial load

Table 3. Results of microbial quality of DUWLs in different wards.

	Treatment wards											
	Periodontics		Root canal therapy		Scaling		Children		Restorative		Dental Prosthesis	
CFU/mL	n	%	n	%	n	%	n	%	n	%	n	%
<500	6	7.5	12	15	4	5	5	6.3	12	15	14	17.5
500–1000	1	1.3	1	1.3	0	0	0	0	3	3.8	0	0
>1000	9	11.3	3	3.8	4	5	3	3.8	1	1.3	2	2.5

Table 4. Results of microbial quality of DUWLs in various parts of the dental unit.

	Sampling location							
	Cup water		Air/water syringe		Handpiece		Flushing	
CFU/mL	n	%	n	%	n	%	n	%
<500	14	17.5	11	13.8	12	15	16	20
500–1000	1	1.3	1	1.3	2	2.5	1	1.3
>1000	5	6.3	8	10	6	7.5	3	3.8

Table 5. The presence of bacteria in water sampled from DUWLs.

Genius/species	Gram	Percentage share
Pseudomonas aeruginosa	-	18.2
Pseudomonas putida	-	2.1
Ralstonia pickettii	-	7.9
Stenotrophomonas maltophilia	-	1.1
Alcaligenes faecalis	-	15.8
Sphingomonas paucimobilis	-	11.6
Moraxella lacunata	-	4.7
Brevibacterium epidermidis	+	1.65
Corynebacterium auris	+	0.74
Pediococcus pentosaceus	+	0.85
Staphylococcus Aureus	+	11.8
Microbacterium laevaniformans	+	21
Micrococcus lylae	+	2.56

of flushing (handpiece water line without ozone) on the microbial load in the first and last days of work. In overall, the microbial load was decreased with increasing the time of flushing, so that the 6.4 logs microbial load in the first day and before flushing was reduced to 5.5-logs microbial load after 120 s of flushing. In the last working day, the microbial load was decreased from 4.3 to 2.8-log after 120 s flushing.

Central composite analysis and fitting the process models

CCD matrix is proper for building a quadratic response surface and constructingthe second-order polynomial models (Singh et al. 2012). In order to investigate the incorporated effect of process variables (concentration of ozone and reaction time) on disinfection of DUWLs, various experiments were performed using CCD technique and observed results along with predicted values for the microbial decontamination using ozonation process are presented in Table 6. All the experiments were carried out in order to determine the optimal conditions and evaluate the effect of operating parameters on the disinfection process. The predicted values were obtained by fitting the model with Design-Expert software, and the results illustrated these values have a good correlation with experimental values. Several models such as linear, quadratic models and cubes were managed to determine the coefficient of the response equation (Haghighi et al. 2019). Based on the CCD and input variables, a second-order polynomial equation of coded units for disinfection of DUWLs was expressed as the following equation:

$$R = 88.29 + 15.93A + 9.29B - 4.4AB - 8.35A^2 - 4.55B^2 \quad (1)$$

where R represents predicted disinfection efficiency, A and B are coded variables corresponding to the initial concentration of ozone and reaction time, respectively. Also, validation of the selected model and testing hypotheses on the parameters of the model were assayed by means of an analysis of variance (ANOVA). Analysis of variance is a collection of statistical models and their associated estimation procedures used to analyze the differences among variables of the model (Huiping et al. 2007). The ANOVA for the proposed model is presented in Table 7. The results of ANOVA indicated that probability>F less than 0.0001 for the model, which it confirms the model is significant, while values greater

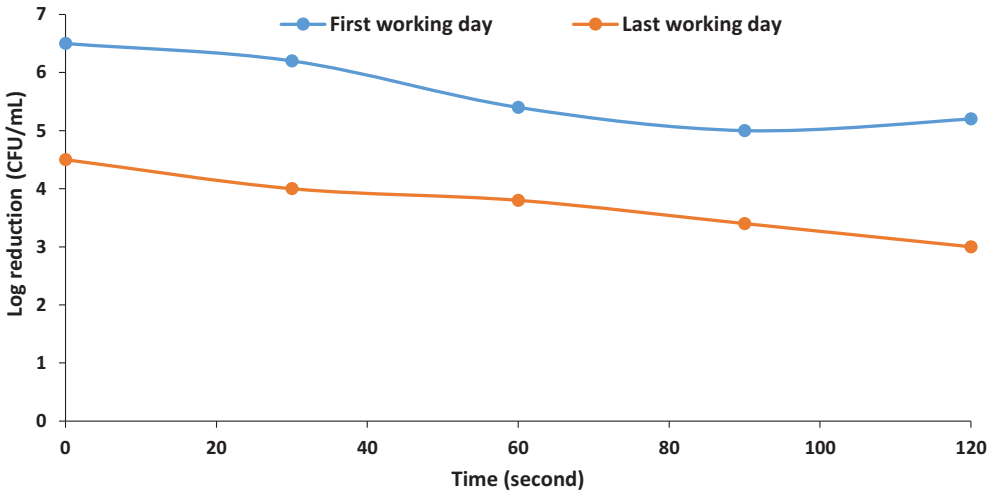

Figure 1. The effect of flushing (without ozone) on reducing the microbial load at different times.

Table 6. Experimental and predicted values of disinfection efficiency by ozonation.

Run	Ozone (ppm)	Time (min)	Experimental (%)	Predicted (%)
1	1	10	88.43	88.29
2	1	5	76.50	74.45
3	0.5	15	73.60	73.14
4	1	10	88.43	88.29
5	1	10	88.43	88.29
6	0.5	5	43.82	45.77
7	1.5	15	98.52	96.21
8	1	10	90.26	93.02
9	0.5	10	65.50	64.01
10	1.5	5	86.34	86.44
11	1	10	88.43	88.29
12	1	15	90.26	93.02
13	1.5	10	93.67	95.88

than 0.0500 shows that the model is not significant under selected circumstances (Kousha et al. 2012). Moreover, the calculated *F* value (123.50) is higher than the tabulated *F*-ratio value ($F_{0.05,\ 9,\ 5} = 4.77$) at 0.05 significant level, which can conclude that the quadratic model fitted well for disinfection of DUWLs by ozonation process (Montgomery 2001). Additionally, the quality of the fit of the model was represented by the correlation coefficient (R^2). According to the results (Table 8), the R^2 (0.9888) and adjusted R^2 (0.9808) indicate that 98.88% of the response variability is described by the model (Torrades and García-Montaño 2014). On the other hand, these values are close to 1, we can state that there is a high correlation between the experimental and predicted values (Garg et al. 2008).

The main operational parameters (reaction time and initial concentration of ozone) and the interaction of them simultaneously are very significant ($P < .0001$). The significance level (*P*-value) plays the most important role in determining whether the interaction of the variables is significant or not. Also, *p*-value less than 0.001 means much higher than 95% significance level (Tripathi, Srivastava, and Kumar 2009).

Table 7. ANOVA results of response surface quadratic model for disinfection of DUWLs.

Source	Sum of Squares	df	Mean Square	F_{Value}	P-value	Status
Model	2503.81	5	500.76	123.50	<0.0001	Significant
A-Ozone	1523.55	1	1523.55	375.75	<0.0001	Significant
B-Time	517.45	1	517.45	127.62	<0.0001	Significant
AB	77.44	1	77.44	19.10	0.0033	Significant
A^2	192.35	1	192.35	47.44	0.0002	Significant
B^2	57.19	1	57.19	14.10	0.0071	Significant
Residual	28.38	7	4.05	-	-	-
Lack of fit	28.38	3	9.46	-	-	-
Pure Error	0.000	4	0.000	-	-	-
Core total	2532.20	12	-	-	-	-

Table 8. The correlation coefficients for the second-order polynomial quadratic model.

Parameter	Value	Parameter	Value
Standard deviation	2.01	R-Squared	0.9888
Mean	82.34	Adj R-Squared	0.9808
C.V.%	2.45	Pred R-Squared	0.0.8869
PRESS	286.49	Adeq Precision	34.874

Adequacy of the model

In order to validate the proposed model, various analyses were carried out. The experimental data versus the predicted data indicated in Figure 2. It is evident that the experimental points are uniformly and consistently aligned along a straight line and are highly correlated. In the statistical analysis of empirical data, it is necessary to check that data distribution is normal or not. The normal plot of residuals illustrates how well the model satisfies the assumptions of the analysis of variance. Hence, a normal probability plot (Figure 3) was applied to verify the normality of residuals. In the normal distribution, data points are very close to each other and following the right line are descending (Ponnusami et al. 2007; Ruiz Espejo 2006). According to the corresponding diagram, it is clear that no abnormalities of the model since the errors are distributed normally for all the responses (Zahrim, Nasimahand, and Hilal 2015).

Effects of different parameters on disinfection efficiency

The influence of the initial concentration of ozone (0.5, 1, 1.5 ppm) and reaction time (5, 10, 15 min) on disinfection efficiency is indicated in Figure 4. The results exhibit that the increasing reaction time and initial concentration of ozone cause an increase of disinfection efficiency. From the plot, it was observed that the maximum decontamination (98.52%) was found to be at 15 min and 1.5 ppm of ozone concentration. Based on plot and ANOVA results, the disinfection efficiency using ozone forcefully depends on the initial concentration of ozone (*F*-value = 375.75) and reaction time (*F*-value = 127.62), respectively. Also, the interaction of ozone concentration with reaction time (AB) is statistically significant (*P*-value = 0.0033).

Optimization of independent variable and validation experiment

Optimization of disinfection parameters (initial concentration of ozone and reaction time) was performed using numerical technique according to the predicted model and factors in their critical range as the constraints (Nandiwale and Bokade 2016).

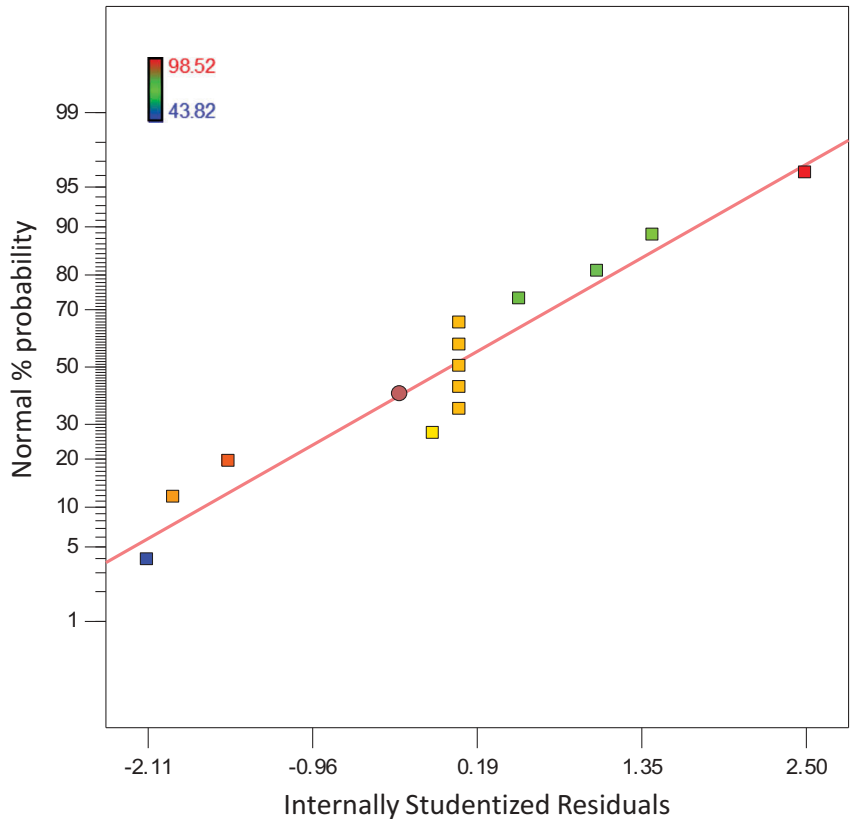

Figure 3. Normal probability plots of studentized residuals.

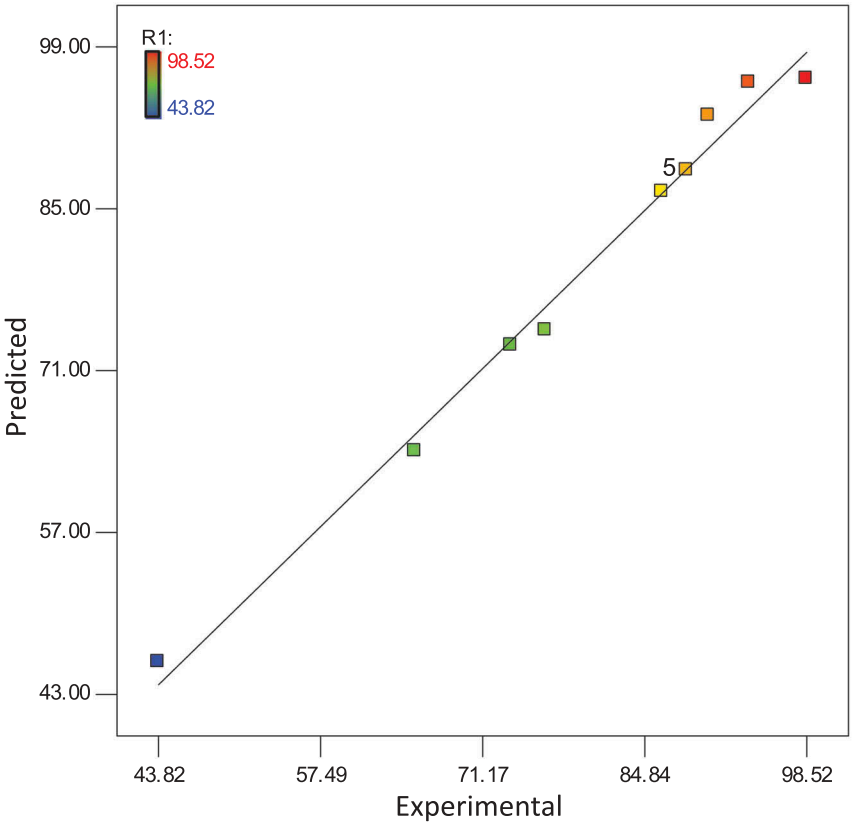

Figure 2. Comparison between predicted and experimental values of DUWLs disinfection.

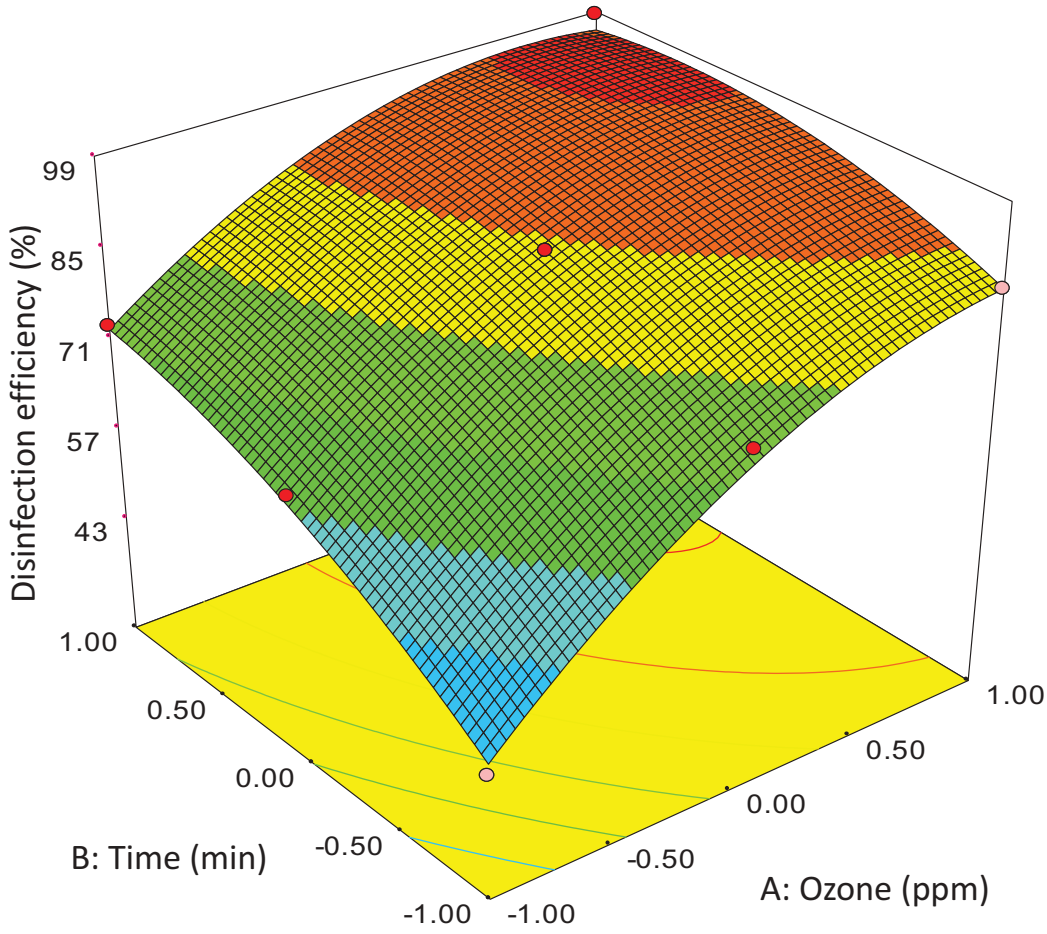

Figure 4. Contour plot showing the interactive effect of the initial concentration of ozone and reaction time on DUWLs disinfection.

Independent parameters applied in numerical optimization includes the concentration of ozone and reaction time was set within the range between low (−1) and high (+1) while the disinfection efficiency was set to the maximum value. The results (Figure 5) exhibited that in optimum circumstances (Ozone concentration = 1.2 ppm and reaction time = 13.5 min) the disinfection efficiency of DUWLs was maximum (97.522%) with an overall desirability value of 0.982. To assess the accuracy of the optimization predicted by the model, the verification test was done under optimum conditions. The disinfection efficiency was found out from the verification experiment to be 95.24%, which closely agree with the predicted results. Moreover, the Efficiency of ozone to remove the three most prevalent microbial species in DUWLs (*Pseudomonas aeruginosa, Microbacterium laevaniformans, Alcaligenes faecalis*) samples under optimum conditions were evaluated, and the results are provided in Table 9. The lowest and highest removal efficiencies were observed for Micro bacterium laevaniformans and Alcaligenes faecalis, respectively.

Viability evaluation of bacterial biofilm using CLSM and SEM

The viability of biofilm was evaluated using CLSM and SEM analysis. Figure 6 shows the bacterial biofilm viability before and after disinfection, respectively. Before ozonation, biofilm has a high density, and the bacteria are active (green fluorescence). However, after 15 min of ozonation, the bacteria in the biofilm are damaged or completely dead. The orange areas show the dying and inactive bacteria (Red fluorescence).

SEM images also endorsed our finding from CLSM and showed a decrease in biofilm density after disinfection by ozone.

Comparison of the efficiency of different disinfectants in microbial decontamination of DUWLs

The performance of three disinfectants (ozone, Peracetic acid, and NaOCl) was compared in disinfection of the DUWLs. The log-transformed CFU/mL data that were achieved after ozonation process in various

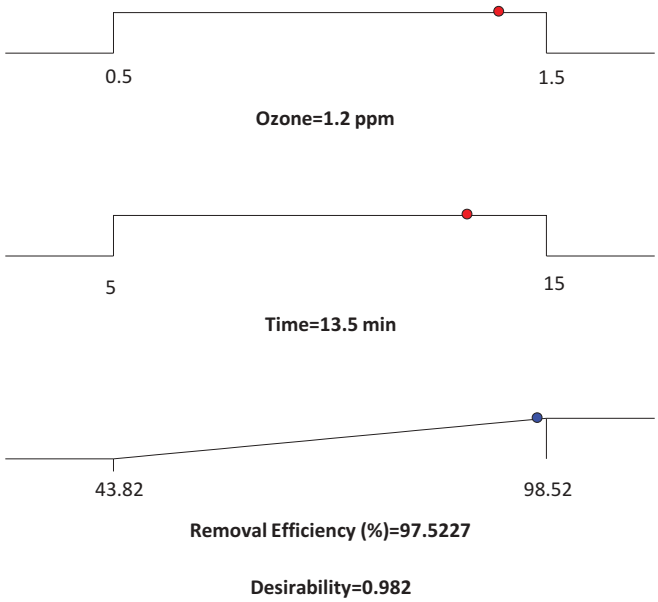

Figure 5. Desirability ramp for optimization of DUWLs disinfection process.

Table 9. Effect of ozonation against dominant bacteria under optimum conditions.

Genius/species	Colony (CFU/mL) before ozonation	Colony (CFU/mL) after ozonation	Reduction Efficiency (%)
Pseudomonas aeruginosa	800	50	93.75
Microbacterium laevaniformans	970	72	92.57
Alcaligenes faecalis	650	25	96.15

reaction time (5, 10, 15 min), are shown in Figure 7. As can be seen, with increasing the reaction time, the efficiency of disinfection increases, which is very significant about ozone (the reduction from 6 logs to 0.5 logs after 15 min disinfection). The chemical agent group, Peracetic acid, and NaOCl at their highest performance (after 15 min) indicated disinfection effects similar to that of the 5-min ozonation. The Practice acid exhibited higher effectiveness than NaOCl; however, it was not very impressive.

Discussion

This study investigated the bacterial load of DUWLs in different wards of a dental clinic during the first and last working days in a week. Then, the effects of the most important factors such as reaction time and initial

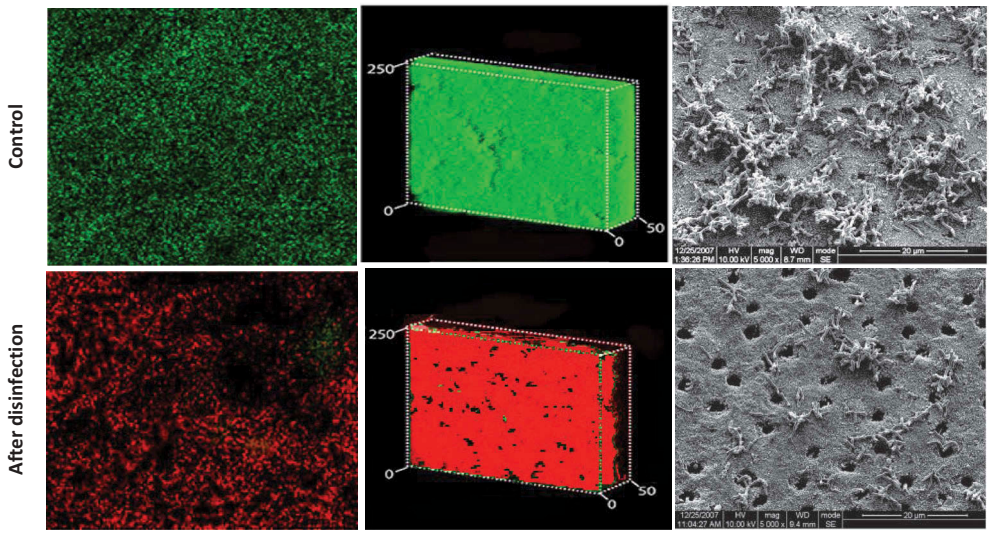

Figure 6. CLSM and SEM images of bacterial biofilm before and after disinfection by ozone.

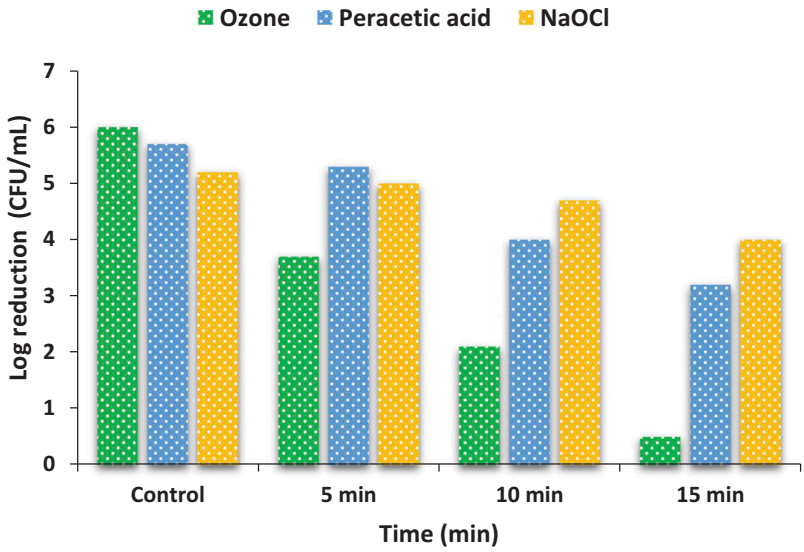

Figure 7. Activity of disinfectants in microbial decontamination of DUWLs.

concentration of ozone and interaction between them on disinfection efficiency using ozonation process were investigated by response surface methodology based on CCD.

The results showed that most of the samples meet the standard limit of the American Dental Association (less than 200 CFU/mL) (Tuttlebee et al. 2002). However, some samples had a bacterial load higher than 1000 CFU/mL. Similar results have been observed in previous studies (Blake 1963; Hami, Lim, and Salleh 2018; Lal, Ravindra, and Biswal 2018; Pederson et al. 2002). Although there has not been reported any significant relationship between DUWL contamination and any certain diseases, the high load of bacteria in DUWL cannot be ignored (Cleveland et al. 1999; Dallolio et al. 2014).

Due to the fact that the stagnation of water inside DUWLs creates a proper environment for increasing bacterial growth and biofilm formation. Increasing the microbial load in some samples can be due to some other factors, such as aging, and the improper sterilization and disinfection of DUWLs. In the case of aged DUWLs, biofilm width grows with the increase of operation years (Arvand and Hack 2013). On the other hand, the bacterial load was different at different parts of DUWLs. This can be due to the difference in water speed (Göksay, Çotuk, and Zeybek 2008). Due to the long length and narrow width of DUWLs, a large surface-to-volume ratio is created that is suitable for bacterial growth and biofilm formation (Dodds, Grobe, and Stewart 2000).

Our results indicated the presence of heterotrophic bacteria that can be originated from the waterline or oral microorganisms (Montebugnoli et al. 2004). In most samples, opportunistic species such as Pseudomonas and Mycobacterium were observed. This is consistent with the results of other studies. Pseudomonas is an environmental bacterium and normal flora of human that can be present in aqueous solutions (Cleveland et al. 1999). This bacterium participates in biofilm formation and has a high antibiotic resistance, so it is important in clinical wards, especially for people with immune deficiency. In addition, Staphylococcus spp. were identified in some samples that can be due to the contamination with patients' saliva (Memarian et al. 2008). As well as other studies, our results showed that most of the bacterial species are Gram-negative (Göksay, Çotuk, and Zeybek 2008; Nikaeen et al. 2009). In addition to infection, Gram-negative bacteria create endotoxin that can be aggregated in DUWLs and released in dangerous concentration for patients' health (Walker et al. 2003).

Effect of flushing on microbial load was evaluated in two different working days (first and last days). As well as other studies, our results showed that increasing the time of flushing decreases the microbial load. However, this reduction did not lead to an acceptable level of bacterial load (ADA standard). Flushing only affects the suspended bacteria in the system, not the attached biofilm; therefore, it cannot be used as an infection-control method in DUWLs (Memarian et al. 2008). This fact is approved in previous studies (Montebugnoli et al. 2004). Our study also revealed that the microbial load on the first day of work is higher than the last day. This is because of the stagnation of water in DUWL and improper disinfection. Dentists should perform flushing and disinfection of DUWL's water at the beginning and end of each working day.

Effect of ozone on the most prevalent bacterial species under optimum conditions was evaluated, and it was revealed that there is a difference between its effects on various species. The difference in resistance can be due to the structure of the cell wall (Gram-positive or negative) and other bacterial resistance factors (Azarpazhooh and Limeback 2008). Ozone is a natural biocide that can remove a wide range of microorganisms in a short period of time. Ozone is extensively used for the treatment of water because its efficiency is not affected by pH, reduces total dissolved solids, and does not produce disinfection byproducts (Am Water Works Res et al. 1991; Powell and Scolding 2018). Ozone oxidizes the organic compounds in the cell membranes of microorganisms causing dissociation and damage to these membranes, thus affecting the viability of the cells. In addition, free radicals produced by ozone react with the nucleic acid present in the structure of microorganisms and damage their DNA or RNA (Gray 2014). As the results show, with increasing ozone concentrations and reaction time, there is an increasing trend in bacterial removal and disinfection. As the ozone oxidizing property is due to the production of nascent oxygen during decomposition of ozone in water, when the concentration of ozone and reaction time is increased, the amount of nascent oxygen production increases, which also significantly increases the disinfection rate (Altmann et al. 2014; Jyoti and Pandit 2004).

Electron Microscope Imaging was used to evaluate the effect of ozonation. The results showed significantly reduction of bacteria in the biofilm (Figure 2). This phenomenon can be attributed to the oxidation of organic materials of cell lysis or convert them into small molecules. Hence, the major accumulation of cellular materials were not observed after the ozonation process. That was consistent with other studies (Srinivasan and Chitra 2015). Disinfection with chemical agents (Peracetic acid and NaOCl) do not show satisfactory results compared to ozone. Since ozone is highly soluble in water, it easily penetrates bacterial biofilms and in less time it can reduce the number of microorganisms (Baysan and Lynch 2005; Filippi 2000). So it can be concluded, ozone is the best alternative for disinfection of water units at low concentrations and at the fastest possible time.

Conclusion

The comprehensive evaluation of microbial contamination of DUWLs was carried out, the ozonation system was utilized for disinfection of DUWLs containing various bacteria and polymicrobial biofilms. Moreover, to our knowledge, for the first time, a CCD was applied to investigate and optimize disinfection variables such as ozone concentration and reaction time. According to the results following the conclusion reached:

- Some of the samples showed a significant bacterial load (>1000 CFU/mL) and periodontal surgery ward had the highest microbial contamination.
- Gram-negative bacteria were dominant species (approximately 61%) and Pseudomonas were more prevalent among them.
- Flushing after 120 s could reduce microbial contamination rates, but this was not of an acceptable in standard terms (≤200 CFU/mL).
- The quadratic model was highly precise and predictive (F= 123.5) in disinfection of DUWLs by ozone.
- From the results of statistical design, disinfection efficiency dramatically increased with increasing of ozone concentration and reaction time.
- The optimum conditions were found to be reaction time of 13.5 min and the ozone concentration of 1.2 ppm.
- Ozonation had a high efficiency in disinfection of DUWLs, which the efficiency was higher than 90% in the removal of dominant species.
- SEM and CLSM analysis confirm that ozonation can effectively eliminate bacterial biofilms

The obtained results demonstrated that the disinfection of DUWLs using ozone is an effective way to the removal of microbial contamination, which is recognized as the most important risk factor in DUWLs.

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