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ORIGINAL ARTICLE

Evaluation of disinfection efficacy of ozone and chlorinated disinfectant against the biofilm of *Klebsiella michiganensis* and *Pseudomonas aeruginosa*

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Abstract Household microorganisms mostly reside in the form of biofilms on wet surfaces in the bathroom and kitchen areas. Microorganisms constituting biofilm communities are less susceptible than their planktonic counterparts to antimicrobial agents and are thus difficult to control. Traditionally, chemical disinfectants have been used as cleaners in the household environment. Recently, incorporation of ozone as a microbial control agent in the consumer product industry has been initiated as an alternative method of disinfection. In this study, antimicrobial efficacy of ozone and a commercial chlorinated disinfectant was evaluated against the biofilm of a commonly occurring *Pseudomonas aeruginosa* and *Klebsiella michiganensis*, a newly described species recovered from toothbrush holder. Single species biofilm was grown on borosilicate glass and polycarbonate coupons using the Centers for Disease Control and Prevention (CDC) biofilm reactor. This is the first study demonstrating the biofilm forming capability of *K. michiganensis* under laboratory conditions using the CDC biofilm reactor. Planktonic cells and mature biofilms were exposed to ozonated water for 2 and 4 min and chlorinated cleaner for 2 min and 10 min. Based on the ozone stability study, the exposure experiments were carried out in two different ways. The type 1 treatment was carried out according to ASTM method E2871-12, with 5 mL of ozonated water as disinfectant in a 50 mL conical centrifuge tube; whereas in type 2 treatment, 14.0 mL of ozonated water was used to avoid any headspace in the capped culture tube. In biofilms, type 1 ozone treatment demonstrated an average log reduction of 0.88 (±0.13) and 0.12 (±0.01) for *K. michiganensis* and *P. aeruginosa*, respectively, in 2 min. Similarly, for type 2 treatment, the values were 1.56 (±0.58) and 0.27 (±0.08). In planktonic cells, log reduction was 2.61 (±0.11) in *P. aeruginosa* and>4.0 in *K. michiganensis*, within 2 min. Chlorinated cleaner demonstrated > 4.0 log reduction in all cases. The disinfection efficacy of ozone type 1 treatment varied between bacterial species ($p<0.001$) and exposure times ($p<0.04$), but not the coupon types ($p>0.95$). Within biofilms, log reduction varied significantly between ozone and chorine treatments ($p<0.001$). Significant differences in log reduction were also observed between planktonic cells and biofilms ($p<0.001$), target organisms ($p<0.05$) and the types of ozone treatment ($p<0.05$, for *K michiganensis* only). From the results, it can be concluded that *K. michiganensis* was more susceptible to the disinfectants compared to *P. aeruginosa.* Within a shorter contact time, commercial chlorinated cleaner was more effective against both biofilms and planktonic cells than ozonated water.

Keywords Biofilms · *Pseudomonas aeruginosa* · *Klebsiella michiganensis* · Ozone · Chlorine · Disinfection

Introduction

Household microorganisms mostly occur in the form of biofilms on wet surfaces in the bathroom and kitchen area (Flores et al. 2013; Yano et al. 2013). Biofilm is a complex community of microorganisms growing on a biotic or abiotic surface in an aqueous environment (Donlan 2001; Stoodley et al. 2002; Lynch and Robertson 2008). Microorganisms constituting biofilm communities are less susceptible to antimicrobial agents than their planktonic counterparts and are thus difficult to control (Xu et al. 2000; Stewart and Costerton 2001). The recent media exposure on outbreaks of microbial

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infections and household foodborne illnesses has led to an increase in use of wide varieties of antimicrobial agents, and has also triggered the development of alternative methods of disinfections in the consumer product industry (Levy 2001; Zekert 2009). For example, incorporation of ozone as a control agent in the consumer product industry has been initiated recently (FDA 2001). Ozone is primarily used in the treatment of water and waste water for disinfection and oxidation (Donofrio et al. 2013). The mode of action of ozone in aqueous solution is by direct oxidation of compounds by molecular ozone or oxidation of compounds by hydroxyl free radicals produced during the decomposition of ozone (EPA 1999). Ozone decomposes spontaneously in water, generating hydroxyl free radicals that have a half-life in the order of microseconds (EPA 1999; Yousef et al. 2011). The hydroxyl free radicals are the most reactive agents and are highly antimicrobial in nature (Greene et al. 2012). Due to the high oxidation potential, ozone oxidizes cell membrane materials, enters the organism and damages enzymes, DNA and RNA, thus causing cell destruction (Khadre et al. 2001). Some of the advantages of ozone over traditional antimicrobial agents such as chlorinated cleaner are that it has no harmful by-products, there is no re-growth of organisms, it has high penetrability and there is no additional disposal cost, as it naturally decomposes into water and air (Jin-Gab et al. 1999).

In this study, we have investigated the disinfection efficacy of ozone and a commercial chlorinated cleaner against the biofilms of *Klebsiella michiganensis* and *Pseudomonas aeruginosa. Klebsiella michiganensis* is a new described species belonging to the genus *Klebsiella* and was isolated from a toothbrush holder (Saha et al. 2013). Species belonging to the genus *Klebsiella*, such as *Klebsiella pneumoniae* and *Klebsiella oxytoca*, are clinically important bacteria and are well documented to cause nosocomial infections (Langley et al. 2001; Wollheim et al. 2011). They are also known as excellent biofilm former (Maldonado et al. 2007; Murphy and Clegg 2012). Since *K. michiganensis* belongs to the genus *Klebsiella* and was recovered from a bathroom environment, it was important to investigate its biofilm-forming capabilities on different surface materials, as well as its response to commonly used methods of household disinfection such as chlorinated cleaner, and also to an alternative method of disinfection such as ozonated water. *Pseudomonas aeruginosa* is a commonly occurring microorganism and is notoriously known for its biofilm formation in wide variety of environments (Ma et al. 2009). The specific objectives of this study was to: 1) investigate the biofilm forming capability of *K. michiganensis* on different surfaces under high sheer condition using the Centers for Disease Control and Prevention (CDC) biofilm reactor; and 2) investigate the disinfection efficacy of ozone and commercial chlorinated cleaner against the biofilms of *K. michiganensis* and *P. aeruginosa.*

Materials and methods

Bacterial cultures

Cultures of *P. aeruginosa* (ATCC 27313) and *K. michiganensis* (ATCC BAA 2403) were selected for the study. Both the bacteria were grown and maintained on Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) (BD, Franklin Lakes, NJ; Thermo Scientific, Lenexa, KS). The bacteria were grown for 24 h at 35±1 °C.

Disinfectants

Two types of disinfectants, ozone and chlorine, were evaluated in this study. For ozone, a faucet with a standard flow rate of 0.46 gal per minute (gpm) was used to deliver ozonated water at concentration ranging from 0.2 to 0.4 parts per million (ppm). The following characteristics of the ozonated water was monitored: pH 7.5±0.5; temperature 20.0±5 °C; turbidity 0.112±0.06 NTU; hardness 300 ppm; Total Organic Carbon (TOC) 0.1, ppm and the water was nonchlorinated. For chlorine, commercial chlorinated cleaner containing 1.94 % of sodium hypochlorite (NaOCl) was used in the study. A schematic of the ozone treatment is presented in Fig. 1.

Preparation of biofilm

Mature single species biofilms were prepared using a CDC biofilm reactor following the American Society for Testing and Materials(ASTM) method E2562-12. For the development of the *P. aeruginosa* biofilm no modification of the ASTM method was made. The following modification was performed for the development of the *K. michiganensis* biofilm: the batch mode was operated for 24 h at 35 °C with 120 rpm of sheer and the continuous mode was operated for additional 24 h at 25 °C with 120 rpm sheer and a flow rate of 11.7 mL/min. For both the bacteria, borosilicate glass (BGC) and polycarbonate (PC) coupons were used as the surface materials.

Disinfection efficacy study

The disinfection efficacy study was performed according to the ASTM method E2871-12. The only modification was in the amount of disinfectant used in the study, instead of adding 4.0 mL of disinfectant, 5.0 mL was added because to completely wet a 5″×5″ surface area, 5.0 mL was the required volume. The exposure times for the ozonated water were 2 min and 4 min, whereas for chlorinated cleaner it was 2 min and 10 min. The 4 min time point for ozone was selected based on the stability of ozone in the ozonated water. We performed an experiment to measure the stability of ozone

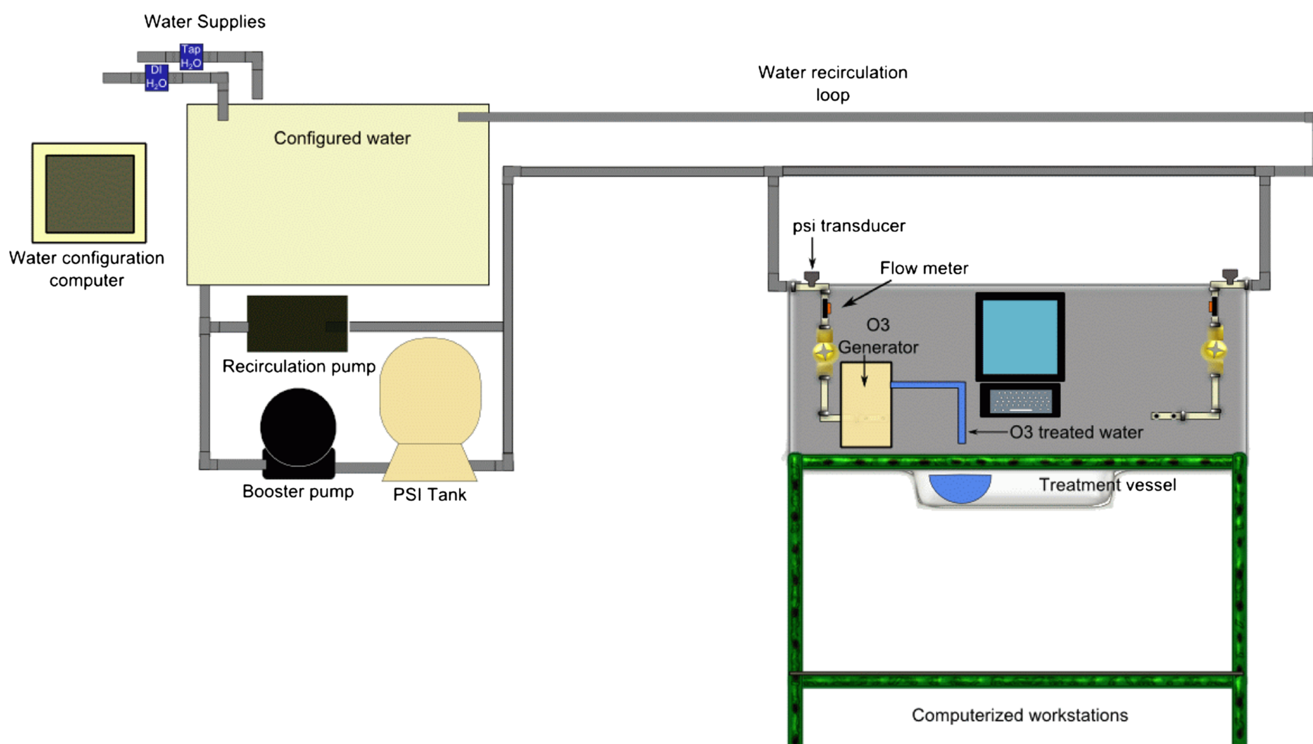

Fig. 1 Schematic of the ozone treatment unit

at different time points by taking 500 mL of ozonated water (0.35 ppm) in an open 1 L beaker and also in a capped 500 mL amber colored bottle. The level of ozone was measured at 2.0 min, 4.0 min and 6.0 min time points. At 4.0 min and 6.0 min, the level of ozone in the open beaker was found to be 0.08 and 0.03 ppm respectively, whereas in the closed container at 4.0 min and 6.0 min, the level of ozone was 0.15 and 0.14 ppm, respectively. For chlorinated cleaner, the 10 min time point was chosen because the manufacturer of the product recommended a contact time of 10 min for disinfection purposes. The 2 min time point was selected based on the feasibility of use of products in a household environment, from a consumer standpoint. Therefore, in this study, 2 min was the common time point used for the evaluation of both the disinfectants.

Based on the ozone stability study, the ozone exposure experiments were carried out in two different ways. One set of experiments (Type 1) was carried out according to ASTM method E2871-12 with 5 mL of ozonated water as disinfectant in a 50 mL conical centrifuge tube. In another set of experiments (Type 2), 14 mL of ozonated water was used to avoid any headspace in the capped culture tube, instead of the 50 mL conical centrifuge tube used for the exposure study. Following exposure, the 14 mL ozonated water, along with the coupon, was transferred to a 50 mL conical centrifuge tube containing 36 mL of Sterile Buffered Dilution Water (SBDW, neutralizer) to stop the reaction.

The neutralizer used for the chlorinated cleaner was SBDW containing a final concentration of 0.1 % sodium thiosulfate (1 mL of 10 % Sodium Thiosulfate was added to 1 L SBDW, pH of the solution was 7.4-7.5).

Staining and imaging of biofilms

Following incubation under continuous mode, the rods holding the coupons were carefully removed from the CDC biofilm reactor and gently rinsed twice in SBDW to remove unattached cells. The coupons were stained with Filmtracer™ Live/Dead Biofilm Viability Kit (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Image analysis was performed using the Zeiss Axioskop 2 Plus microscope with AxioCam fluorescence camera. Imaging was performed to confirm viability and biofilm formation. The viable bacteria with intact cell membranes stain fluorescent green and bacteria with damaged membranes stain fluorescent red.

Data and statistical analysis

The mean $\log_{10}$ density of treated and untreated/control coupons as well as the mean $\log_{10}$ reduction for each disinfectant were calculated according to the formula presented in ASTM method E2871-12. Univariate ANOVA was conducted to test if there was any significant difference in $\log_{10}$ reduction with: a)

coupon types, organisms and exposure times within biofilm treated with ozone; and b) treatment type (ozone and chlorinated cleaner) within biofilms. Another set of one-way ANOVA was performed to determine differences in killing effect with ozone between a) planktonic cells and biofilm, b) target organisms in planktonic forms, c) target organisms in biofilm forms, and d) between Type 1 and Type 2 ozone treatments in both *K. michiganensis* and *P. aeruginosa*. Prior to statistical analysis data was checked for deviations from normality by the using the Kolmogorov–Smirnov test. All statistical analyses were performed using SPSS 17.

Results

Biofilm formation under high sheer condition

Both *P. aeruginosa* and *K. michiganensis* were capable of forming mature biofilms within 48 h of incubation under high sheer (120 rpm) condition in the continuous flow mode (Fig. 2). The average $\log_{10}$ density of *P. aeruginosa* biofilm on BGC and PC coupons was 9.13 (±0.15) and 9.23 (±0.12) CFU/cm^2, respectively. Similarly, the average $\log_{10}$ density of *K. michiganensis* biofilm on BGC and PC coupons was 6.97 (±0.24) and 7.03 (±0.23) CFU/cm^2. There were no significant differences in average $\log_{10}$ density between the two coupon types in both *K. michiganensis* (F=0.23, df=11, p>0.63) and *P. aeruginosa* (F=1.6, df=11, p>0.23). However, significant differences in the biofilm formation were observed between *K. michiganensis* and *P. aeruginosa* for both the BGC (F= 346.3, df=11, p<0.05) and PC (F=418.8, df=11, p<0.05) coupons.

Disinfection efficacy study

The disinfection efficacy of ozonated water and commercial chlorinated cleaner was evaluated against the two target organisms, *K. michiganensis* and *P. aeruginosa*. Ozone demonstrated an average $\log_{10}$ reduction of 0.88 (±0.13) and 0.12 (±0.01) for *K. michiganensis* and *P. aeruginosa*, respectively, for 2 min, whereas for 4 min average $\log_{10}$ reductions of 1.15 (±0.16) (*K. michiganensis*) and 0.29 (±0.03) (*P. aeruginosa*) were observed within biofilms for Type 1 treatment (Fig. 3). In planktonic cells of *P. aeruginosa*, the $\log_{10}$ reduction was 2.61 (±0.11) and 3.31 (±0.23) for 2 and 4 min, respectively. Similarly, > 4.0 log reduction was observed for *K. michiganensis* (Fig. 4). In the Type 2 treatment ozone demonstrated an average $\log_{10}$ reduction of 1.56 (±0.58) and 0.27 (±0.08) for *K. michiganensis* and *P. aeruginosa*, respectively, for 2 min exposure. On the other hand, for 4 min of exposure, an average $\log_{10}$ reduction of 1.97 (±1.10) for *K. michiganensis* and 0.60 (±0.28) for *P. aeruginosa* was obtained (Fig. 5). Chlorinated cleaner demonstrated > 4.0 log reduction for both the bacteria in biofilm and planktonic form within 2 and 4 min (Fig. 4).

Results of the ANOVA indicated that the disinfection efficacy of ozone varied between the two species of bacteria and the exposure times, but there were no significant differences in log reduction between the types of coupons tested (Table 1). Within the biofilms, the log reduction in bacterial counts varied significantly between ozone and chorine treatments (F=48.26, p<0.001, df=6). Significant differences in log reduction were also observed between planktonic cells and biofilms (F=60.85, p<0.001, df=10), target organisms (in planktonic form: F=22.22, p<0.04, df=2 and in biofilms: F=54.54, p<0.001, df=6). Similarly, significant difference in log reduction was observed between the two types of ozone treatment (Type 1 and Type 2) only in *K. michiganensis* (F= 10.71, df=6, p<0.01), but not in *P. aeruginosa* (F=2.7, df=6, p>0.15).

Discussion

The newly described species *K. michiganensis* was capable of forming mature biofilm under high sheer conditions within 48 h in the CDC biofilm reactor. Capability to form biofilm is considered as a virulence factor (Maldonado et al. 2007). Due to its capability to form biofilm and its mucoid phenotype (Saha et al. 2013), *K. michiganensis* could be a potential pathogen. In this study, we found *P. aeruginosa* to be a better biofilm former compared to *K. michiganensis* under similar experimental conditions, based on the cell density on the coupons and image analysis (Fig. 2). Image analysis also revealed the architectural difference of the biofilms between the two organisms (Fig. 2). *Pseudomonas aeruginosa* exhibited a distinct clustering pattern, and the clusters were interconnected with layers of cells. The clustering pattern on BGC was different compared to the clustering pattern on the PC coupon. On BGC, the individual clusters appeared as mushroom-shaped growth, whereas the clusters on the PC coupon were flat. In contrast, *K. michiganensis* exhibited a unique pattern on both BGC and PC surfaces. It was observed that *K. michiganensis* formed separated microcolonies and cell clustering on the PC surfaces, and produced lot of exopolysaccharide materials leading to mature biofilm formation; whereas the architecture of the biofilm on BGC appeared as a monolayer of cells with less exopolysaccharide materials. From the average log density value and the image analysis, it is evident that PC provided a better surface for the biofilm formation of *K. michiganensis* than the BGC coupon. The fluorescent green stain indicated that all the cells were viable in the mature biofilm of both the surface types for the two bacteria. Biofilm architecture is reported to be heterogeneous in nature and constantly changing due to internal and external factors (Donlan 2002).

Fig. 2 Epifluorescent micrograph of: *Pseudomonas aeruginosa* ATCC 27313 biofilm on **a** borosilicate glass coupon, **b** polycarbonate coupon. *Klebsiella michiganensis* ATCC BAA 2403 biofilm on **c** polycarbonate coupon, **d** borosilicate glass. Biofilms were stained with Film Tracer™ Biofilm Stains. Bar, 10 μm

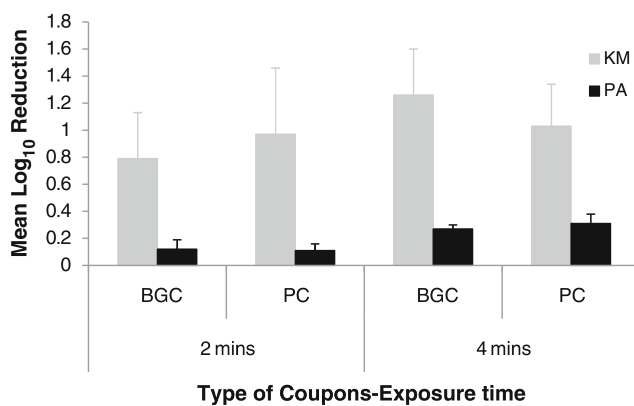

Fig. 3 Mean (±SD) Log_{10} Reduction in cell counts of *K. michiganensis* (*KM*) and *P. aeruginosa* (*PA*) treated with Ozone (Type 1 treatment) using Borosilicate (*BGC*) and Polycarbonate (*PC*) coupons for 2 and 4 min exposure time

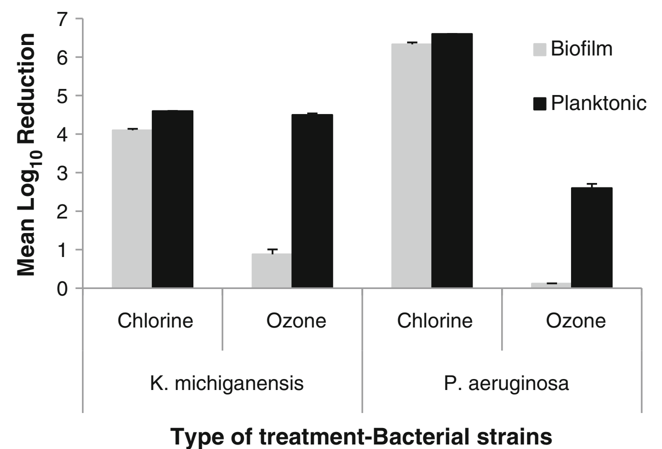

Fig. 4 Disinfection efficacy of ozone and chlorine on the biofilm and planktonic cells of *K. michiganensis* and *P aeruginosa.* The commercial chlorinated cleaner exhibited greater than 4.0 log reduction for both cell states in both the bacteria. Ozone exhibited greater efficacy against *K. michiganensis* compared to *P. aeruginosa*

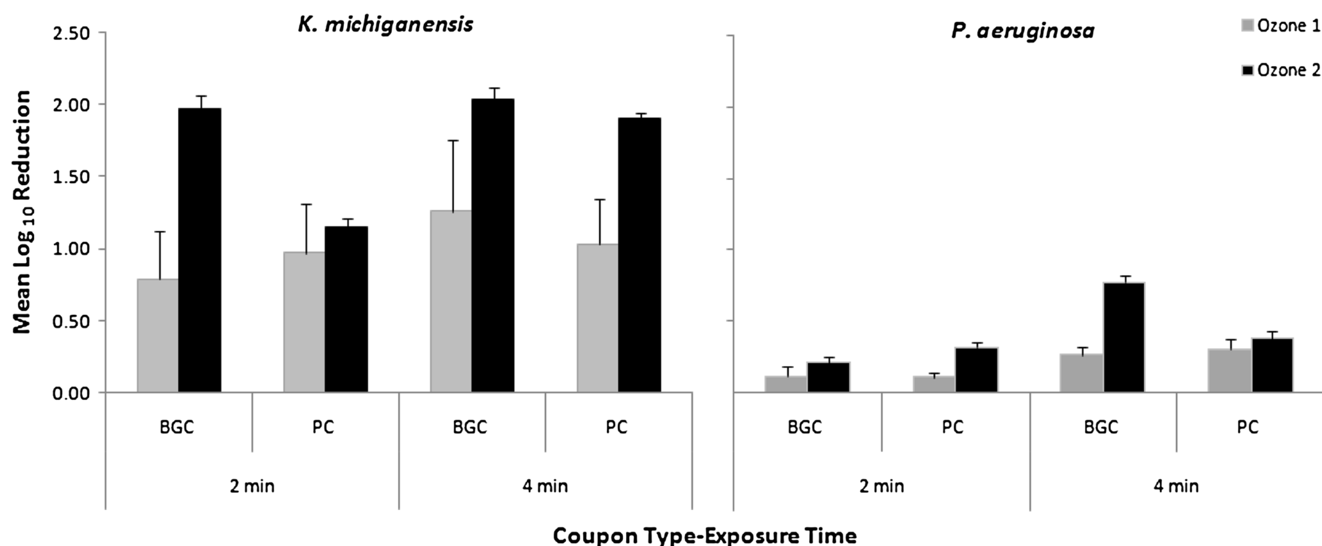

Fig. 5 Comparison of Type 1 and Type 2 ozone treatment on the biofilms of *K. michiganensis* and *P. aeruginosa* on Borosilicate Glass (*BGC*) and Polycarbonate (*PC*) coupons for 2 and 4 min exposure time. The values represent Mean (± SD) of log reduction

In this study, we observed that both Type 1 and Type 2 ozone treatments were more effective against the biofilm of *K. michiganensis* compared to *P. aeruginosa,* even though both are Gram-negative bacteria. Earlier studies have reported that the difference in architecture of biofilms might contribute to reduced susceptibilities to different methods of disinfections (Xu et al. 2000; Folkesson et al. 2008). The difference in the susceptibility of the two target organisms could be due to the architectural difference in the biofilm formation. Additionally, cell density and thickness of the biofilm might have also contributed to the difference in log reduction between the two bacteria. It was also interesting to note that Type 2 ozone treatment exhibited greater killing effect (Fig. 4) compared to Type 1 ozone treatment (Fig. 1). This could be due to a combination of factors, such as that ozone is partially soluble in water and is also capable of auto-decomposition (Summerfelt 2003). Therefore, the effectiveness of ozone depends on the concentration of ozone present at any given time in the ozonated water. For effective disinfection, the water is required to maintain a certain dissolved ozone concentration for a given contact time (Summerfelt 2003). In this study, we have demonstrated the stability of ozone in the test water when present in a container with a headspace (Type 1) and in a capped container without any headspace (Type 2). Rice et al. (1981) reported the half-life of ozone dissolved in pure water at 20 °C to be 165 min, whereas earlier studies on surface water disinfection by ozone reported that even in higher water quality with low concentrations of oxidizable organic material, iron and manganese, the ambient ozone demand reduced the half-life of ozone to less than a few minutes (Cryer 1992; Summerfelt 2003). In our study, we have similar observations. The capped container without any headspace (Type 2) was more effective against the biofilm of *K. michiganensis* and *P. aeruginosa.* The disinfection efficacy varied significantly ($p<0.01$) for *K. michiganensis* between the two treatment types. However, no significant difference ($p>0.15$) in log reduction was observed for *P. aeruginosa* biofilm. Interestingly, 4 min contact time exhibited higher log reduction compared to 2 min of exposure, which could be due to the fact that ozone decomposes spontaneously, and formed by-products that were still effective against the microbial cells. From a practical application standpoint, Type 1 treatment will need to be implemented, as the ozonated water will be interacting with wet surfaces harboring biofilms where there will always be ambient ozone demand with high decomposition of ozone. Due to toxicity associated with ozone gas, there is a limitation on the concentration of ozone that can be used in the production of ozonated water (Khadre et al. 2001; Palou et al. 2002). The commercial chlorinated cleaner was equally effective against the biofilm of *K. michiganensis* and *P. aeruginosa.* Greater than 4.0 log reduction was achieved

Table 1 Result of one-way ANOVA to test if coupon types, exposure time and the target organisms have significant effect on log reduction in biofilms treated with ozone (Type 1)

Source of variation	df	Mean square	*F*	*p*
Intercept	1	2.9	248.6	0.001
Coupon types (Borosilicate glass vs. Polycarbonate)	1	0.00005	0.004	0.9
Exposure time (2 min vs. 4 min)	1	0.1	8.2	0.046
Organism type (*K. michiganensis* vs. *P. aeruginosa*)	1	1.31	110.5	0.001
Error	4	0.01		
Total	8			

df degrees of freedom, *F* F ratio, *p* significance level of F

within 2 min of contact time. The 10 min contact time was used in the experiment as per the manufacturer's instructions, to be complaint with the disinfection claim of the product. The chlorinated cleaner exhibited the same level of disinfection efficacy against both planktonic and biofilm forms for the two target organisms compared to ozonated water, within a shorter exposure time (2 min). However, there are certain disadvantages of using chlorinated cleaner, such as it might form harmful by-products and also give rise to resistant organisms (Nozaic 2004). Therefore, future research is warranted to investigate the effect of ozone and chlorine (lower concentration) in combination against the biofilms of different organisms.

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