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Activity of ozonated water and ozone against *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: The known bactericidal properties of ozone have not been checked in relation to its action on bacterial biofilms. This is especially true of ozonated fluids. The aim of this study was to investigate the bactericidal activity of ozonated water and that of a mixture of ozone and oxygen against biofilms.

Material/Methods: Eighteen clinical strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* exhibiting various levels of antibiotic sensitivity were investigated. Bacteria were cultured in biofilm form on polystyrene titration plates for periods of 2 to 72 hours. The biofilms formed in this way were exposed to *in statu nascendi* ozonated water produced in a prototype device that had been tested in clinical conditions, or to a mixture of oxygen and ozone generated in the same device. Live cells in the biofilm were stained with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide solution. The degree of reduction of viable bacteria following ozone exposure was determined.

Results: Ozonated water was found to be an effective bactericidal agent against biofilms after as little as 30 seconds of exposure, while the bactericidal activity of the ozone-oxygen solution was much lower. Prolongation of the duration of biofilm exposure to the gaseous disinfectant to 40 minutes led to a reduction in the viable cell count, which nevertheless remained high.

Conclusions: Unlike the ozone-oxygen mixture, ozonated water effectively destroys bacterial biofilms *in vitro*.

key words: ***Staphylococcus aureus*** • ***Pseudomonas aeruginosa*** • **biofilm** • **ozone** • **ozonated water**

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Background

Ozone is well known for its antimicrobial effects and has been used as a disinfectant for applications such as water disinfection and sterilization of rooms [1]. Ozone therapy has also been increasingly used in medicine as an adjunct to primary treatment. Ozone is an allotrope of oxygen, and has interesting physical properties. It is an unstable gas that decomposes after approximately 20 minutes, generating a diatomic oxygen molecule and very active atomic oxygen. The antimicrobial activity of ozone stems from its oxidative properties. As any residual ozone spontaneously decomposes into non-toxic oxygen, ozone can be used in the food industry and also in medicine [2]. Ozone has an effect on metabolism in inflamed tissues, activates the body's immune response and destroys bacteria, fungi and viruses [3]. The chemical properties of ozone are utilized in ozone therapy to treat infected wounds, decubitus ulcers, burns, ulcerations, inflammation of skin and bone tissue, or radiation therapy-related changes in cancer patients. Ozone therapy is also used to treat inflammation and infections of certain internal organs, especially when antibiotic therapy has failed to control multidrug-resistant bacteria [4,5].

Previous studies have revolved around the use of ozone in dentistry, mainly in endodontics and for the elimination of biofilms in the oral cavity [3,6–8]. Ozone is used in dentistry for disinfecting cavities and root canals and in the treatment of inflammation of periodontal pockets and early caries. Very short ozone exposure times have been shown to reduce viable bacterial cell counts in both Gram-positive and Gram-negative bacteria, as well as yeast (*Candida albicans*) cell counts [9].

In Poland, pioneering research into the bactericidal efficacy of ozone therapy in the prevention and treatment of septic complications in orthopaedics and musculoskeletal traumatology has been carried out since 2001, utilizing a prototype device for the intraoperative application of ozone [10–12].

Our previous study [9] demonstrated an *in vitro* antimicrobial effect of ozonated water against bacterial and fungal strains from international collections and against 60 clinical isolates of planktonic pathogenic bacteria. It now appears interesting to determine whether ozonated water has similarly high biocidal activity against pathogenic microorganisms in biofilm form, as can be found, for example, on endoprostheses or in tissues (e.g., in the lungs), and to obtain comparative data on the bactericidal activity of gaseous ozone against biofilms.

The action of ozone on bacterial biofilms has been poorly studied. Most studies have focused on biofilms forming in

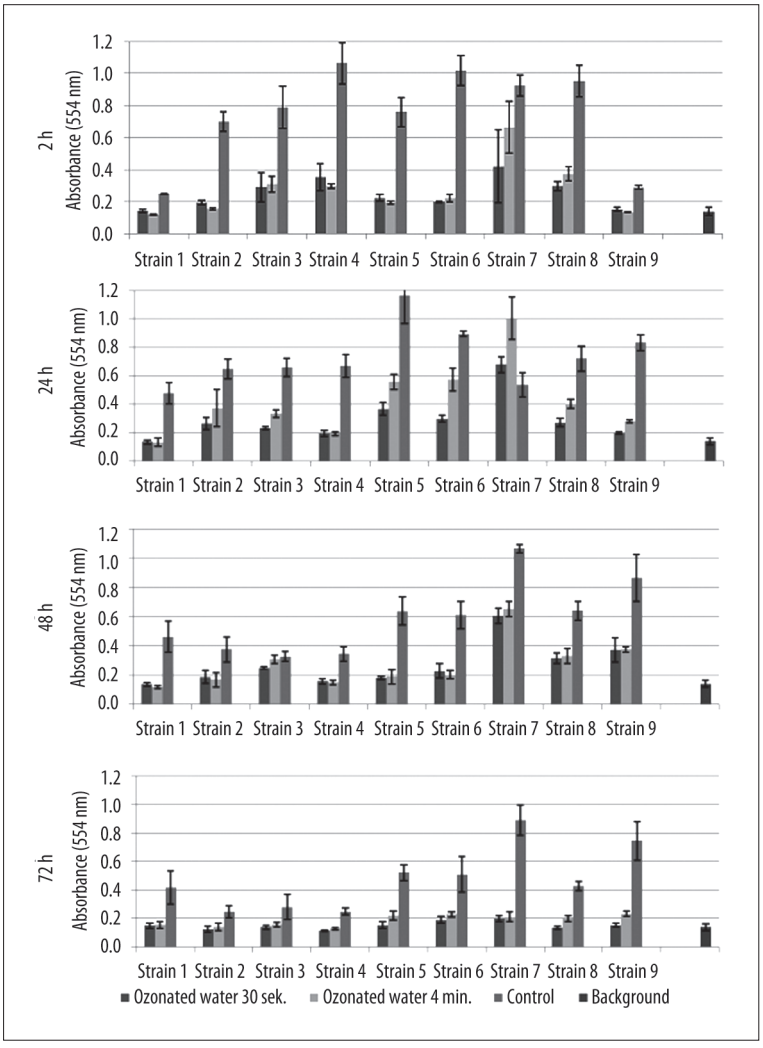

Figure 1. Effect of ozonated water on biofilms of 9 strains of *P. aeruginosa* after 2, 24, 48 and 72 hours of incubation.

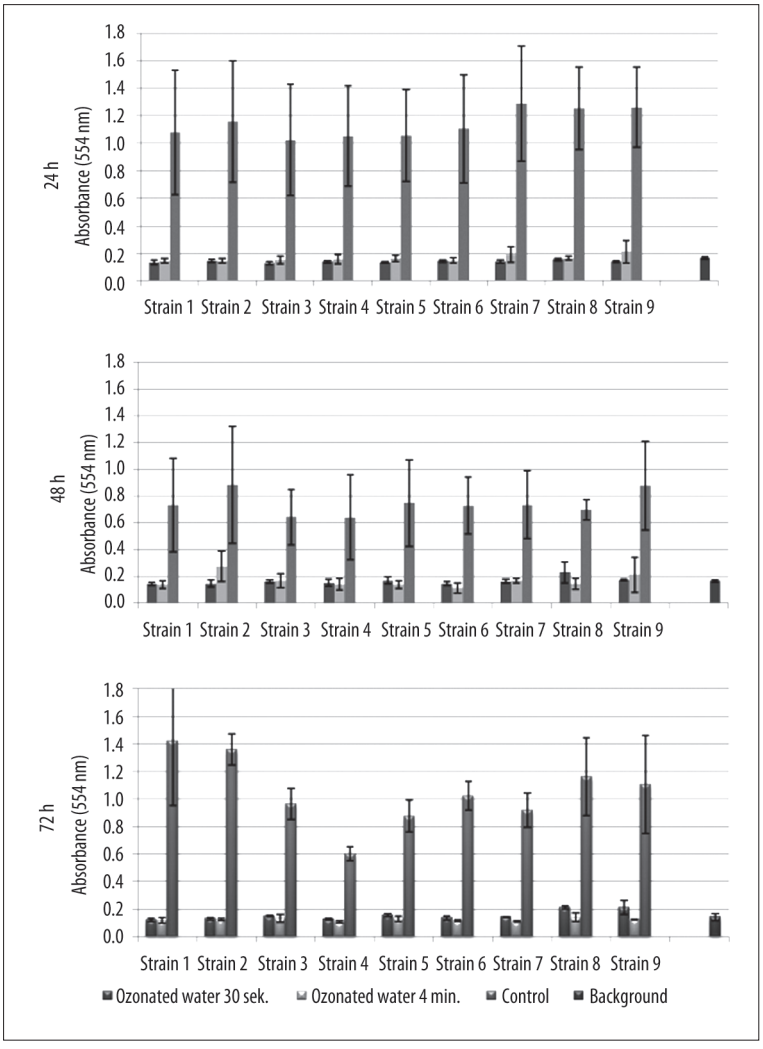

Figure 2. Effect of ozonated water on biofilms of 9 strains of *S. aureus* after 24, 48 and 72 hours of incubation

the oral cavity and the use of ozone as a disinfectant in endodontics and prevention of oral cavity disease [6,7], with isolated reports of research on biofilms covering bony implants and endoprostheses in septic complications of hip replacement surgery, none of which, however, has discussed the use of ozone therapy in such patients [13,14].

The aim of the present study was to investigate the bactericidal activity of ozonated water and that of a mixture of ozone and oxygen against biofilms formed by clinical isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, particularly isolates obtained from cystic fibrosis patients. The course of this severe genetic condition is often complicated by infections caused by these bacterial species.

Material and Methods

The study investigated 6 clinical strains each of *Staphylococcus aureus* and *Pseudomonas aeruginosa* obtained from the sputum or BAL samples of patients with known cystic fibrosis treated at the Children's Memorial Health Centre Institute in Warsaw and 3 strains of either species obtained from other types of biological material (3 isolated from blood, 2 from post-operative wounds, and 1 from urine), from the collection of the Department of Pharmaceutical Microbiology, Medical University of Warsaw. All strains had been stored frozen at –70°C in a BHI medium with 10% glycerol before the study. Frozen strains were subcultured onto an agar medium and incubated at 37°C for 24 hours. Pure cultures of the study strains were transferred from the agar medium to 5 mL of the Luria-Bertani liquid medium (LB; Pepton Tryptone – BTL, Yeast extract – Difco, NaCl – Chempur, Glucose - POCH) and allowed to multiply at 37°C for 24 hours, following which they were transferred to Petri dishes with the LB medium solidified with 1% agar and incubated for another 24 hours at 37°C. The resultant homogeneous bacterial colonies were suspended in NaCl until bacterial inocula were obtained with densities of approximately 3.2 McFarland units for *S. aureus* and 2.9 units for *P. aeruginosa*, corresponding to approximately 10^9 CFU/mL. The inocula were diluted 10-fold with fresh LB medium. Volumes of 200 µL were placed in cavities on microtitration plates (Kartell S.p.A., Medlab) and incubated at 37°C for 2 hours (only *P. aeruginosa*), or for 24, 48, and 72 hours (*P. aeruginosa* and *S. aureus*). At the end of the incubation, the suspension of planktonic cells was removed and the bacterial biofilm that had formed and settled on the polystyrene surface was exposed either to freshly ozonated water for periods of 30 seconds to 4 minutes or to an oxygen-ozone mixture for 20 and 40 minutes. Following

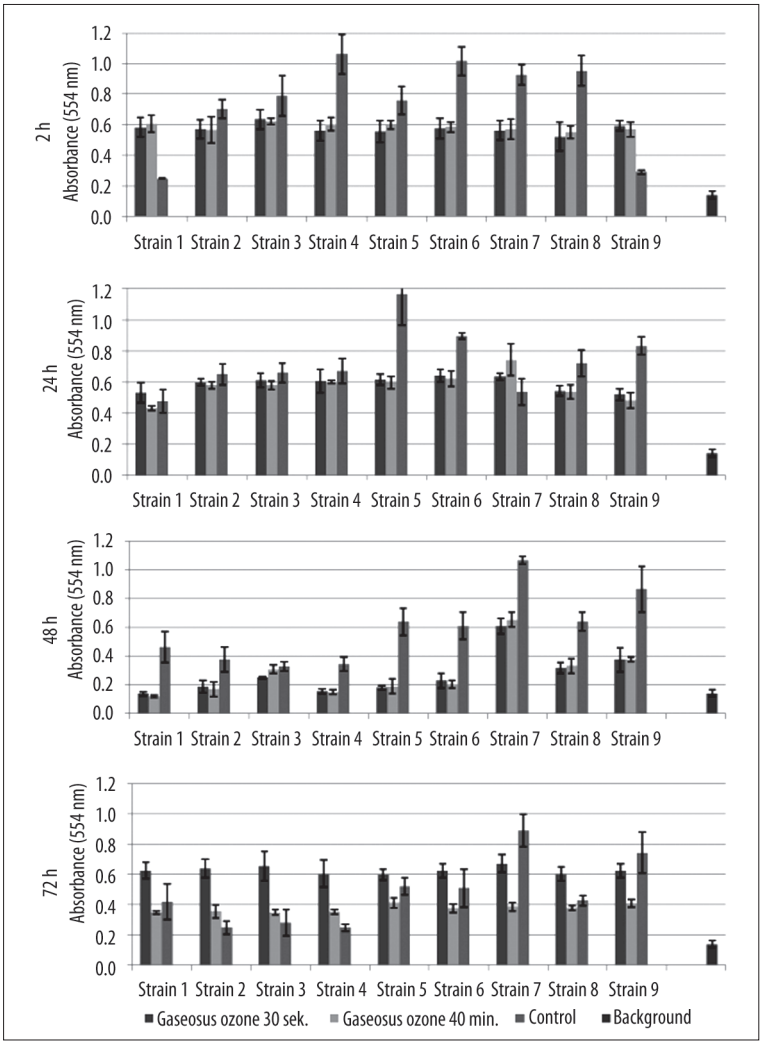

Figure 3. Effect of oxygen-ozone mixture on biofilms of 9 strains of *P. aeruginosa* after 2, 24, 48 and 72 hours of incubation.

specified exposure times, the cavities on microplates were cleansed with a sterile PBS solution in order to remove the ozone. Viable, metabolically active bacterial cells in the biofilm were stained with a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (in PBS, MTT, AppliChem) at 37°C for 2 hours. Simultaneously cultured biofilms that were not exposed to ozone served as controls. After 2 hours of staining, the MTT solution was removed and 150 µL DMSO (Merck) was added together with 25 µL glycine buffer (0.1 M, pH 10.2) in order to dissolve the produced formazan crystals. Color intensity in microtitration plate cavities was determined by spectrophotometry at 554 nm (PowerWave XS; BioTek).

Each experiment was performed 3 times in 5 replications, and means and standard deviations were calculated.

The oxygen-ozone mixture and ozonated water were generated using the prototype apparatus that was also used in our previous study [9]. Ozone concentration in the ozone-saturated water was determined by iodometric titration with a 0.02 M sodium thiosulphate solution. One g potassium iodide (KJ) and 3 mL starch solution (1 g/100 mL) was added to 100 mL ozonated water and then titrated with a 0.02 M sodium thiosulphate solution as described above [9].

Results

P. aeruginosa strains formed biofilms on microtitration plates much earlier and much more vigorously than the *S. aureus* strains investigated in this study (Figure 1). After 2 hours of culturing at a temperature of 37°C, *P. aeruginosa* strains formed biofilms with different A_{554} absorbance values for different isolates, ranging from 0.3 to 1.1. At 24 hours of incubation, absorbance values rose for most strains, ranging from 0.5 to 1.2. At 48 and 72 hours, *P. aeruginosa* biofilms in nearly all strains demonstrated a gradual reduction in viable cell counts to absorbance levels of 0.3–0.9.

The biofilms of 9 strains of *S. aureus* (Figure 2) after 24 hours incubation was being formed at a relatively high uniform level, with absorbance values of 1.0 to 1.3. After 24 and 72 hours, the number of viable cells either remained unchanged or decreased, but never to absorbance values below 0.6.

Biofilms whose absorbance levels were determined spectrophotometrically were exposed to ozonated water with ozone concentrations in the range of 1.2–3.6 µg/mL. Ozonated water caused a very abrupt fall in viable bacterial cell counts in biofilms, generally to background levels, in all *S. aureus*

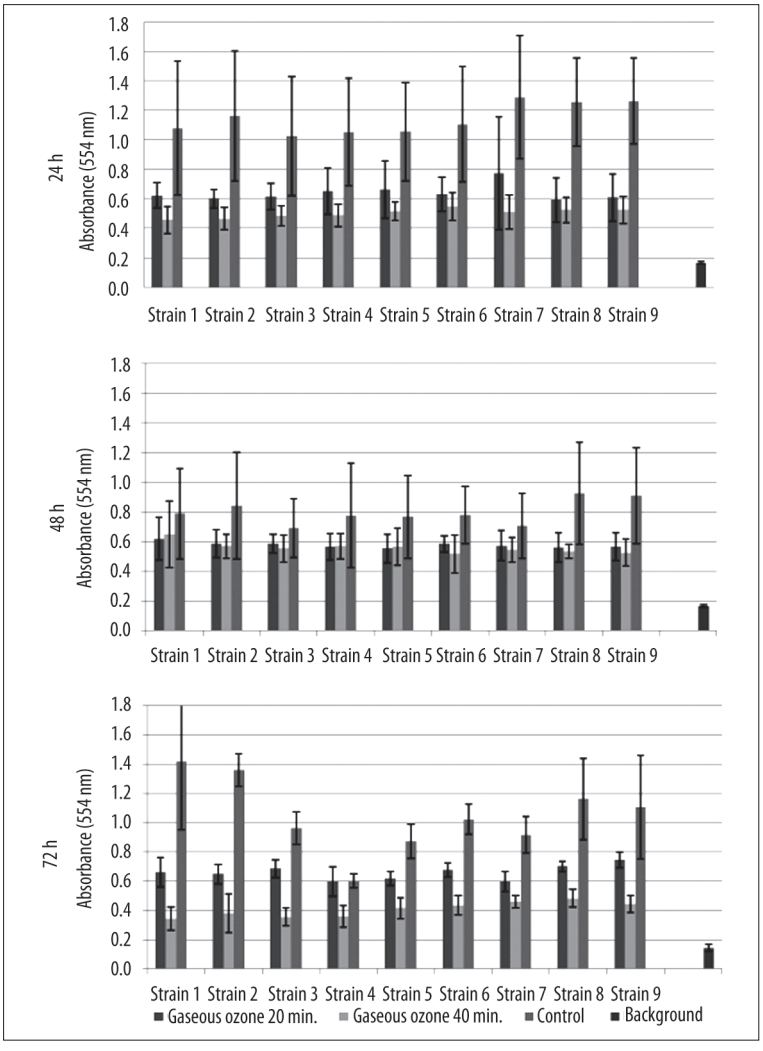

Figure 4. Effect of oxygen-ozone mixture on biofilms of 9 strains of *S. aureus* after 24, 48 and 72 hours of incubation.

strains, regardless of incubation time, after as little as 30 seconds of exposure (Figure 2).

The bactericidal effect of ozonated water on *P. aeruginosa* was somewhat less pronounced (Figure 1). Particularly, biofilms of strain no. 7, incubated for 2–48 hours, tolerated ozone exposures more than the other strains. However, reductions in viable cell counts of *P. aeruginosa* were observed in nearly all cases, with prolongation of exposure time to 4 minutes not resulting in total eradication of cells of all strains to background absorbance levels. However, older biofilms (incubated for 48 hours, and particularly 72 hours) were noted to be more sensitive to the bactericidal activity of ozone in ozonated water, with post-exposure absorbance levels close to background absorbance in all 9 strains.

The mixture of gaseous oxygen and ozone had a weaker effect on biofilms of both *P. aeruginosa* (Figure 3) and *S. aureus* (Figure 4) strains. No distinct differences were noted between the effects of exposure to gaseous ozone for 20 *vs.* 40 minutes. The staphylococcal biofilms were slightly more sensitive to gaseous ozone compared to *P. aeruginosa* strains. However, even after the 40 minute exposure, the levels of viable cells remained high in all strains. For *S. aureus*, the best effects were obtained for biofilms after 24 hours of incubation, and the least pronounced bactericidal effects of the oxygen-ozone mixture were observed in the case of 48-hour biofilms. *P. aeruginosa* biofilms were more tolerant to the action of gaseous O_3. A relatively stronger bactericidal effect was seen in the case of 2-hour biofilms, but this was observed only for 5 out of the 9 strains investigated. More mature biofilms of most *Pseudomonas* strains did not demonstrate reduced viability even after 40 minutes exposure, with some instances of increased viable counts registered after 20 minutes of exposure to the gaseous mixture.

Discussion

The bactericidal effect of gaseous ozone and aqueous ozone solutions on planktonic bacterial cells is well recognized and documented. At the same time, there is a dearth of studies targeting the activity of ozone against bacterial biofilms. Biofilms ensure bacterial colonization and growth on biotic surfaces (tissues) such as oral mucosa, ureters or lungs, and abiotic surfaces such as catheters or implants. Our study demonstrated bactericidal efficacy of freshly ozonated water against biofilms formed by clinical strains of *S. aureus*, as well as a considerable reduction in the number of viable cells in biofilms of *P. aeruginosa*. *P. aeruginosa* biofilms were more tolerant to ozonated water. The *P. aeruginosa* strains

investigated in the present study had been obtained from cystic fibrosis patients, in whom the peculiar pathological conditions in the bronchial tree may influence the expression of virulence factors, including an increase in alginate production, owing to which the biofilms acquire a more mucus-like form and are thus more difficult to eradicate [15]. The observed weaker effect of ozone against these strains may be secondary to poorer penetration of both water and gaseous ozone across the extracellular matrix. Bronchoscopic procedures are often carried out in cystic fibrosis patients whose airways had been colonized by bacterial strains, including strains of *S. aureus* and *P. aeruginosa*. As inappropriate sterilization of the equipment may lead to the formation of biofilms inside bronchoscopes, it is important to look for effective low-toxicity disinfectants. In the case of *S. aureus*, ozonated water proved to be effective against biofilms; however, some strains of *P. aeruginosa* investigated in this study were resistant to the action of ozonated water.

Microbial adaptations to disinfectant compounds and bacterial tolerance to biocidal concentrations of these compounds have recently been discussed by Meyer and Cookson [16].

Huth et al. [6] studied the effect of gaseous ozone and ozonated water on biofilms formed by bacteria that can colonize root canals. Exposure to a 5 μg/mL ozone solution for 1 minute led to complete elimination of planktonic cells of *Enterococcus faecalis* and *Candida albicans*, with lower concentrations (2.5 and 1.25 μg/mL) considerably reducing microbial counts, but not eliminating microbes completely. Gaseous ozone at a concentration of 32 g/L eliminated microorganisms completely after just 1 minute of exposure. With regard to biofilms, an aqueous ozone solution at 20 μg/mL nearly completely eradicated biofilms of *E. faecalis*, *C. albicans* and *P. aeruginosa* following only 1-minute exposure. In the present study, nearly complete eradication of *S. aureus* biofilms was observed after 30 seconds of exposure to ozonated water at lower ozone concentrations of 1.2–3.6 μg/mL.

Nagayoshi et al. [17] studied *Enterococcus faecalis* and *Streptococcus mutans* strains to find evidence of bactericidal effects of ozonated water used for irrigation of root canals. The water also exhibited low-grade cytotoxicity to murine fibroblasts. Simultaneous experiments with planktonic cells and biofilms of *Enterococcus faecalis*, however, showed a bactericidal effect of aqueous solutions of ozone on the former, with only a slight effect on biofilms [18].

In the light of both our previous [9] and present study, ozonated water is shown to be an effective antimicrobial with regard to both planktonic cells and biofilms. Gaseous ozone has much weaker activity; it appears unlikely that it will be widely used as an effective bactericide, but perhaps longer exposure times and higher concentrations would solve this problem.

Conclusions

Freshly ozonated water can be an effective solution for destroying bacterial biofilms. In the case of biofilms formed by strains of *Staphylococcus aureus*, ozonated water reduced viable cell counts to background levels following very brief exposures (30 seconds). Some cell populations of the strains of *P. aeruginosa* investigated in the present study exhibited tolerance to the action of ozonated water.

The effect of gaseous ozone was much less pronounced and it does not appear to be likely that gaseous ozone will be widely used for disinfection purposes. Prolongation of biofilm exposure times to gaseous ozone to 40 minutes did result in lower viable cell counts, but the figures still remained high.

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