ORIGINAL ARTICLE

Water and air ozone treatment as an alternative sanitizing technology

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Keywords

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Summary

Aims. *We investigated the effectiveness of ozone (aqueous and gaseous) treatment as an alternative sanitizing technology to common conventional disinfectants in reducing the microbial contamination of both water and air.*
Methods. *Ozone was added for 20 minutes to a well-defined volume of water and air by the system named "Ozonomatic®". The effectiveness of ozonation was determined by counting CFU/ m^3 or ml of bacteria present in samples of air or water collected before (T_0) and after (T_1) the addition of ozone and comparing the microbial load of different bacteria present in ozonized and non-ozonized samples.*
Results. *When the ozonisation equipment was located at 30 cm from the surface of the water in the bath tub in which the bacteria investigated were inoculated, the treatment was able to reduce the total microbial load present in the aerosol by 70.4% at a temperature of 36°C for 48 hours. Conversely, at 22°C for 5 days, only a modest decrease (9.1%) was observed. Escherichia coli and Pseudomonas aeruginosa were completely eliminated. A 93.9% reduction was observed for Staphylococcus aureus, followed by Streptococcus faecalis (25.9%). The addition of ozone to water was able to almost eliminate Staphylococcus aureus (98.9% reduction) and also to exert a strong impact on Legionella pneumophila (87.5% reduction). Streptococcus faecalis and Pseudomonas aeruginosa showed a decrease of 64.2% and 57.4%, respectively. Conversely, only a 26.4% reduction was observed for the bacterium Escherichia coli. This study showed that the addition of ozone in the air exerted a modest reduction on microbial load at 36°C, whereas no effect was observed at 22°C.*
Conclusions. *Aqueous and gaseous ozone treatments were effective against microbial contaminants, reducing the CFU of the microorganisms studied. These results confirm the efficacy of the ozone disinfection treatment of both water and air; particularly, it constitutes an extremely promising alternative, allowing the possibility to reuse contaminated water.*

Introduction

Alternative disinfection methods, such as gaseous disinfectant technologies, have recently been introduced into the market; these constitute an additional, efficient means to manual disinfection [1-3] or, in the case of water disinfection, a valid substitute for chlorine, enabling water to be reused (i.e. water reconditioning) [4, 5]. Chlorine is the most widely used commercial sanitizing agent, and is added to the water used for washing vegetables and fruit. However, its use in food applications is associated with various problems, such as the production of several carcinogenic disinfection by-products (DBP) [6], including trihalomethanes and haloacetic acids, derived from the reaction between chlorine and organic material [7, 8]. This concern has prompted some European countries to ban its use for washing organic produce [9, 10]. Furthermore, it has been demonstrated that a gaseous sanitizer (ozone) has a greater disinfectant ability than a liquid sanitizer, owing to its uniform distribution and penetration. Indeed, gaseous sanitizers display a four-fold higher diffusivity [11]. The choice of the appropriate sanitizer depends on the processing limitations, including the residual disinfectant needed to achieve sufficient disinfection. In the water used for washing freshly cut products, hydrogen peroxide (H_2O_2), organic acids, US, and ultraviolet (UV) irradiation are not recommended [12]. In the literature, the efficiency of a sanitizer is currently determined by evaluating the microbial reduction, investigating the process of decontamination and, to a lesser extent, assessing the prevention of cross-contamination [12].
Among the gaseous sanitizers investigated in recent years, such as ozone, chlorine dioxide (ClO_2), and cold plasma, ozone has proved the most effective [13], being a powerful oxidant for water treatment, after the hydroxyl radical. Conversely, chloramines are the least efficient. Moreover, they are not recommended for primary disinfection, but for secondary water disinfection, since they react more slowly than chlorine and persist for a longer time in distribution systems [13]. Compared with chlorine, ozone needs a lower concentration and shorter contact time in order to exert its disinfectant effect [14]. Ozone aerosolization could constitute an effective alternative antimicrobial delivery system, as it is able to penetrate into all surface irregularities and is applicable to a wide antimicrobial spectrum [11]. Furthermore, water treatment through the addition of ozone could maximize

water reusability [14]. Owing to its short half-life, its toxicity and reactivity, ozone must be produced on-site, where it reacts principally with carbon-carbon double bonds, activated aromatic structures, and non-protonated amines. Ozone reacts more slowly with fatty acids and carbohydrates, while it reacts faster with proteins, amines, amino acids, nucleic acids, and protein functional groups [12].
The aim of the present study was to evaluate the ability of the ozonised hydro massage system supplied by Ozonomatic® to reduce the bacterial load present in air and in water.

Materials and methods

Growth of bacteria in broth cultures

Staphylococcus aureus (*S. aureus*) (ATCC 13150), *Pseudomonas aeruginosa (P. aeruginosa)* (ATCC 27853), *Escherichia coli* (*E. coli*) (ATCC 25922) and *Streptococcus faecalis* (*S. faecalis)* (ATCC 10541) were obtained from LGC Standards (Sesto San Giovanni, Milan, Italy). A suspension of *Penicillium*-type mycetes had previously been isolated in the laboratory. The growth medium used were: Tryptone Soya Agar (P05012A), Brain Heart Infusion (BHI) Broth (CM225), Mannitol Salt Agar (CM85), Cetrimide Agar (Sigma-Aldrich Chemical C, St Louis, MO, USA), Sabouraud dextrose Agar (CM41), Endo Agar Base Oxoid (CM0479), and Slanetz & Bartley Medium (CM0377, ThermoScientific). With the exception of Cetrimide Agar and Slanetz & Bartley Medium, the growth media were obtained from Oxoid (Wesel, Germany) unless otherwise specified. Slabs with a diameter of 55 mm and sterile cellulose acetate membrane filters were provided by Sartorius (Italy).
The lyophilized growth media were re-hydrated and sterilised in accordance with the producers' instructions. They were then dispensed on Surfair-type slabs with a diameter of 55 mm. Different media were used according to the bacteria to be isolated. Specifically, Tryptone Soya Agar was used in the search for mesofila at 36°C; Mannitol Salt Agar for *S. aureus*; Centrimide agar for *P. aeruginosa* Endo Agar Base for *E. coli*; Slanetz & Bartley Medium for *S. faecalis*; and Sabouraud dextrose Agar for mycetes belonging to the *Penicillium* type. The BHI broth was dispensed in glass test tubes as growth medium. For every above-mentioned growth medium, tests of sterility and fertility were carried out.
Growth in BHI broth was investigated for all the bacteria (*E. coli*, *S. aureus*, *P. aeruginosa*, *S. faecalis*), whereas for suspensions of mycetes (*Penicillium*-type) NaCl solution was used.

Generation of experimental biofilms to evaluate microbiological control exerted in water by ozone

To generate an experimental biofilm of each bacterium, samples from each broth culture were added to the water in a bath tub. The tub contained 20 liters of distilled water at a temperature of 37°C and was located in a room measuring about 85 m^3.
After centrifugation for 20 minutes at 2,500 rpm, the supernatant was removed and 9 parts of HCl-KCl were added. After resuspension, 0.1 ml of the sample was seeded onto plates containing the specific medium for *Legionella* (Legionella CYE Agar Base). It was then dispensed onto slabs with a diameter of 90 mm after being supplemented with Legionella Growth Supplement and Legionella MWY Selective Supplement. The samples collected were incubated at 36°C in a humidified atmosphere for 10 days, with readings being taken daily.
To determine the total microbial load at 36°C, 1 ml of mixed cultivation ground of *E. coli*, *S. aureus*, *P. aeruginosa*, *S. faecalis* and *Legionella pneumophila* (*L. pneumophila*) was used to contaminate the water; to determine the total microbial load at 22°C, 1 ml of a suspension of *Penicillium*-type mycetes was used. At the time of inoculation into the bath tub, each inoculum had a minimum concentration not lower than 10^5 CFU/ml (colony-forming units per milliliter) for mycetes and 10^7 CFU/ml for all the bacteria. After shaking, one 10 ml and one 5 ml sample of water were collected and identified as T_0. These were filtered by cellulose acetate membrane filters with porosity of 0.45 μm, in order to not remove the microorganism of interest, and a diameter of 55 mm; these filters were located on the surface of the growth medium. The slabs for *E. coli*, *S. aureus*, *P. aeruginosa* and mesofila were incubated at 36°C for 48 hours, while mycetes were incubated for 5 days at 22°C. The microbial load was measured 5 times in both the 5 ml and 10 ml samples, and a mean value was calculated from the means of each set of samples. The microbial load was expressed as CFU/ml.

Evaluation of the microbiological control exerted in water by an ozonized hydromassage system produced by Ozonomatic®

After the experimental contamination of the water by inoculating it with the bacterial suspensions, the water was exposed to ozone for 20 minutes; 5 ml of water was then collected and identified as "T_1". The analysis was performed in the same way as for samples collected at T_0. In the case of *L. pneumophila*, the samples of water collected at T_0 and T_1 were centrifuged at 2,500 rpm for 20 minutes, in order to concentrate the bacteria. The upper and lower float layers present in the test tube containing 9 parts of HCI-KCI tampon were re-suspended and 0.1 ml of each sample was seeded onto slabs containing specific medium. The samples were incubated for 10 days at 36°C in a humidified atmosphere; during the period of incubation, readings were taken daily.

Microbiological control exerted in air by ozone

This part of the study was conducted in two phases with different aims. The first phase aimed to evaluate the possible "total microbial reduction" in the air in the room where the ozonization equipment was located; the sec-

ond phase aimed to evaluate the possible "reduction of each single microorganism" in the air.
Specifically, in the first phase, the "Microflow 60" equipment was set up in order to aspirate a volume of 180 litres of air. The apparatus was located at distance of 1m from the bath tub and at a height of 1.5 m from the floor. The first aspiration was carried out in order to measure the bacterial and mycotic loads present in the air before the action of the hydro-massage (T_0); the second aspiration was carried out 20 minutes after the production of ozone (T_1).
In the second set of experiments, the "Microflow 60" was positioned 30 cm from the surface of the water in the bath tub, which had previously been filled with 20 litres of water at a temperature of 37°C. A volume of 180 litres of air was aspirated after the addition of BHI broth culture containing *E. coli*, *S. aureus*, *P. aeruginosa*, and *S. faecalis*. A further 180 litre volume was aspirated after the addition of a suspension containing all the above-mentioned bacteria and a suspension of mycetes. Air samples were collected at T_0 and after 20 minutes of ozone treatment (T_1).
The samples collected were incubated at 36°C for 48 hours for the investigation of *E. coli*, *S. aureus*, *P. aeruginosa* and *S. faecalis* and of the total microbial load, and at 22°C for 5 days for mycetes.

Results

Evaluation of the antimicrobial effect of the ozonized bath produced by Ozonomatic® on water

Among the microorganisms investigated, *S. aureus* displayed the greatest reduction (98.9%) after ozonization treatment, being almost completely eliminated. Ozonization also exerted a strong impact on *L. pneumophila* (87.5% reduction). Regarding *S. faecalis* and *P. aeruginosa*, the addition of ozone to water was able to eliminate more than half of the microbial cells, obtaining a reduction of 64.2% and 57.4%, respectively. Conversely, only a 26.4% reduction in the bacterium *E. coli* was observed. A slight reduction (16.6%) was seen in mycetes, incubated at 22°C.

Tab. I. Total microbial load present in the water at 36°C and 22°C, and microbial load of each bacterium measured before (T_0) and after ozonization treatment (T_1).

	T_0 CFU/ml	T_1 CFU/ml	Microbial load reduction (%)
Microbial Load at 36°C	151	43	71.5
Microbial Load at 22°C	12	10	16.7
E. coli	87	64	26.4
P. aeruginosa	244	104	57.4
S. aureus	377	4	98.9
S. faecalis	162	58	64.2
L. pneumophila	495	62	87.5

The total microbial load measured at 36°C revealed a 71.5% diminution; this is in line with all the percentages found for the individual microorganisms, constituted by the totality of the above-mentioned bacteria. At 22°C, a smaller microbial reduction was observed (16.7%) (Tab. I).

Evaluation of the antimicrobial effect of ozone produced by Ozonomatic® on the air

The antimicrobial effect produced by ozone on the air in the environment where the bath tub and the ozonizing equipment were situated was evaluated by comparing the total microbial load measured at T_0 (no ozone treatment) and T_1 (ozone treatment). The total microbial load (*E. coli, P. aeruginosa, S. aureus* and *S. faecalis)* measured at 36°C and 22°C proved to be low at both temperatures (26 CFU/m^3 and 8 CFU/m^3, respectively) before ozone treatment. Consequently, ozone treatment did not significantly reduce the bacterial load, a modest reduction from 26 CFU/m^3 to 23 CFU/m^3 (11.5%) being observed at 36°C. No reduction was observed at 22°C (0%) (Tab. II).
When the ozonization equipment was placed 30 cm from the surface of the water in the bath tub, ozone treatment was able to reduce the total microbial load present in the aerosol by 70.4% at a temperature of 36°C. Conversely, at 22°C only a modest decrease (9.1%) was observed.
Ozonization was able to completely eliminate the microbial loads of both *E. coli* and *P. aeruginosa* (100% reduction). A reduction of 93.9% was observed for *S. aureus*. Regarding *S. faecalis*, a smaller reduction was seen (25.9%) (Tab. III).

Tab. II. Microbial load present in the air before (T_0) and after ozonization treatment (T_1) at 36°C and 22°C.

	T_0 CFU/m^3	T_1 CFU/m^3	Microbial load reduction (%)
Microbial Load at 36°C	26	23	11.5
Microbial Load at 22°C	8	8	0

Tab. III. Microbial load present in the air collected at 30 cm from the water surface contained in the bath tub where the ozone equipment was located, measured before (T_0) and after ozonization treatment (T_1) at 36°C and 22°C.

	T_0 CFU/m^3	T_1 CFU/m^3	Microbial load reduction (%)
Microbial Load at 36°C	655	194	70.4
Microbial Load at 22°C	33	30	9.1
E. coli	378	0	100
P. aeruginosa	233	0	100
S. aureus	5,955	361	93.9
S. faecalis	2,400	1,778	25.9

Discussion

This study reproduced a situation of water contamination due to microorganisms naturally present in the environment and in human organisms.
The search for *L. pneumophila* was conducted because this microorganism is frequently present in water and is extremely dangerous for human beings if it is found in aerosols [15-17]. The evaluation of the results highlights an effective diminution of the microbial load after 20 minutes of ozone treatment. We recorded some marked reductions, mainly regarding *S. aureus*, in agreement with a study by Cesar [18], and *S. faecalis*. *L. pneumophila* also showed a marked diminution. Ozone had a lower effect on *P. aeruginosa* and, particularly, on *E. coli*, although this latter microorganism is considered one of the bacteria most sensitive to ozone [18]. It is possible that, in this experimental condition, the concentration of the gas did not reach a sufficient level for the total elimination of *E. coli*, in accordance with a recent study conducted by the group of Heß, which reported that resistance to ozone inactivation probably depends on several factors [19].
In comparison with the other microorganisms, ozone exerted a small reduction on the mycetes load; we hypothesize that mycetes could be endowed with greater genetic resistance to this disinfectant.
The results on the microbial load present in the air in the room where the ozonization equipment was located showed limited significance, especially because the air which presented scant microbial contamination. Furthermore, another element that has to be considered is the large size of the room (85 m^3). As a consequence of the large volume of the room, ozone dispersion was elevated. The experiment should be repeated in a suitably smaller room in order to evaluate the positive impact of ozone on the microorganisms present in the air.
Furthermore, the surface of the mat which liberated the ozone was much smaller than that of a normal hydromassage bath tub. For these reasons, further studies should be performed, including the artificial contamination of the room; in the present study, this could not be done, since the room was used as a research laboratory.
Evaluation of the effect of the ozonized bath produced by Ozonomatic® on the microbial load in the aerosol yielded satisfactory results at a temperature of 36°C and regarding *E. coli, P. aeruginosa and S. aureus.* Concerning the *S. faecalis* load, a positive impact was also observed, though the reduction was less marked than in the other species analyzed.
The finding that at 22°C a moderate percentage reduction was found allows us to hypothesize that the microorganisms investigated could be endowed with greater resistance to this category of disinfectants.
The moderate effect exerted by ozone on the microbial load present in the air is strictly due to the mechanism of action of the ozone, which requires the presence of water.
The present study constitutes a preliminary investigation. Further research needs to be carried out in order to optimize sanitation parameters, including the evaluation of different times of ozone exposure, temperatures and volumes of the room where the ozonization equipment is located. Such factors can influence the effectiveness of antimicrobial ozone treatment.
In conclusion, ozone treatment is considered a safe and effective disinfectant tool for the decontamination of water and equipment [18] and even for food applications [20]; indeed, food safety is a top priority [21]. Ozone may therefore be regarded as a valid alternate means of disinfection.

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The authors declare no conflict of interest with Ozonomatic®.

Authors' contributions

MM analyzed the data and prepared the manuscript. GF and RS carried out data collection and analysis. EM designed the study and performed data analysis and manuscript preparation. CT carried out the technical revision of the manuscript. All authors have critically read and revised the manuscript and approved the final version.

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