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Inactivation of bacterial and fungal species during ozonation

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ABSTRACT

The present study was undertaken to evaluate the antibacterial and antifungal effect of ozone in aqueous condition by using an apparatus made by standard appliances Varanasi. The bacterial samples were collected from the hospital patients of BHU, Varanasi (U.P.) India. The fungal strains were collected from different fields of BHU. The samples were examined for isolation and identification of bacteria and fungi.The isolates of fungi and bacteria were subjected to ozone treatment at different time duration.The system was subjected to ozonation for 5, 10, 15, 20 and 25min for bacteria and 20, 40, 60, 80, 100 and 120min. for fungal spore. *Escherichia coli, Pseudomonas aeruginosa, Vibrio cholerae* and *Salmonella typhi* were killed after 15min of ozonation. Still, *Klebsiella pneumoniae* grew after 20min of ozonation and survived up to 25min. Among fungi *Fusarium oxysporum* f. sp. *lini* showed much susceptibility followed by *Trichoderma harzianum, Aspergillus luchuchensis, A. niger, A. flavus* and *A. terreus*. There was reduction in colony count from 40min to 120min of ozonation but all the fungi survived even after 2h of ozonation.The exposure of bacterial strainto ozone doses of 20ppm to 25ppm for 5 to 25min were sufficient for inhibition of bacterial growth. While the growth inhibition of fungi required exposure to ozone doses of 15ppm to 25ppm for 40 to 120min.The increase in the exposure time at any ozone concentration resulted in a significant reduction in fungal and bacterial growth.

Figures : 04 References : 23 Tables : 05
KEY WORDS : Antimicrobial, Inhibition, Ozone, Water.

Introduction

Ozone is a powerful oxidant, its oxidation potential of 2.07 V in an alkaline solution being second only to that of fluorine among the readily available water treatment chemicals. Ozone (O_3) in gaseous or aqueous phase is robust and reliable antimicrobial agent against bacteria, fungi, protozoa, and viruses[1].

Its oxidative capacity at 100ppm, 200ppm and 400ppm can also induce severe toxicity due to lipid peroxidation and ultimately cause DNA damage. A low concentration of ozonated water is sufficient to inactivate bacterial cells (0.12–0.19 mg/L) and their spores (2.29 mg/L).In 1893 the first drinking water treatment plant to employ ozone was established at Oudshoorn, Netherlands.Today there are more than 1000 drinking water treatment plants worldwide using ozonation for disinfection and many other purposes. Furthermore, when ozone is employed as a disinfectant, no residual effect of ozone is observed in the ozonated effluent. Thus, there does not appear to be any toxicity problem in the receiving streams. It was demonstrated that the lethal threshold concentration for the cells of *B. cereus* was 0.12 mg/L while that for *E. coli* and *B. megaterium* was 0.19 mg/L[4]. The threshold concentration required to kill the spores of both species was 2.29 mg/L. These microbial cells and spores exhibited the “all-or-none” die-away phenomenon generally associated with ozone treatment. Workers[19] studied the antimicrobial effects of ozonated water in a recirculating concurrent reactor which were evaluated against four Gram-positive and four Gram-negative bacteria, two yeasts, and spores of *Aspergillus niger*. In ozonated water, death rates among the gram-negative bacteria *S. typhimurium, E. coli, Pseudomonas aeruginosa* and *Yersinia enterocolitica* were not

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TABLE-1: Estimation of the amount of ozone dissolved in one litre of water ozonated for twenty minutes.

S. No.	A (ml)	O_3 (mg)
1	6.9	0.9
2	6.8	0.89
3	6.8	0.78
4	6.6	0.78
5	6.7	0.89

A= volume of sodium thiosulphate used to titrate 500ml of KI in trap

significantly different ($p>0.05$). *Listeria monocytogenes* had significantly ($p<0.05$) more sensitivity among Gram-positive bacteria than either *Staphylococcus* sp. or *Enterococcus faecalis*.Ozone appears to inactivate bacteria by the same mechanism as chlorine-based disinfection: by disruption of membrane permeability[21]; impairment of enzyme function and/or protein integrity by oxidation of sulfhydryl groups and nucleic acid denaturation[3]. Workers[6] reported ozone-killing action against bacterial and fungal species. Compared with bacteria, the fungi were less sensitive. *Aspergillus* sp. was found to be of intermediate sensitivity and required 1-1.5 ppm for six h to inhibit germination.Bacteria, including *Escherichia coli, Staphylococcus aureus, Bacillus cereus, B. megaterium, Salmonella Typhimurium, Shigella flexneri* and *Vibrio cholerae* are sensitive to ozonated water under various conditions[4,5,9,13].

Limited information is available on ozone's effectiveness against bacterial endospores[4,9]and viruses[5] and against eukaryotic pathogens including *Cryptosporidium parvum*[9,14,15,18]and *Giardia lamblia*[9,22,23] which also exists.Important design consideration for the ozone contact basis is transfer efficiency. Transfer efficiency is important whether ozone is being utilized for disinfection or for oxidizing organic and inorganic in the water. Either of the functions requires contact of ozone with the reactant. Transfer efficiency is a function of several factors including diffusion depth, ozone bubble size and liquid temperature. Diffusion depth and bubble size are physical factors that can be controlled in the ozone contact basin's design in ozone transfer which increases as the depth of the diffusion increases[14]. When disinfection is the primary purpose, it is vital that short, circulating be eliminated to ensure that all organisms contact the ozone.

Materials and Methods

Standard Appliances Varanasi manufactured the ozone generator. It consists of the air pump, two photochemical reactors connected in series, outlet and mechanical timer.

The iodometric method was used to calculate the amount of ozone in this water. The ozone from this water was displayed by air into potassium iodide trap. The decay of ozone dissolved in 1 litre of water was studied. After bubbling ozone through 1 litre of water, it was allowed to stand for 20, 40, 60, 80, 100 and 120 min. At the end of each time interval sample was washed with atmospheric air, and this washing was passed through 500 ml of standard potassium iodide solution.Therefore,the efficacy

TABLE-2 : Ozonated water sample was allowed to stand for 20,40,60,80,100 and 120 min and amount of ozone remaining after each time interval was estimated [Value of ozone (mg)]

Time (min)	0	20	40	60	80	100	120
1	0.702	0.396	0.202	0.099	0.048	0.028	0.013
2	0.692	0.396	0.201	0.098	0.048	0.026	0.012
3	0.792	0.396	0.202	0.101	0.049	0.024	0.013
4	0.782	0.402	0.201	0.088	0.050	0.026	0.013
5	0.692	0.386	0.202	0.099	0.050	0.024	0.013
Mean	0.732	0.394	0.202	0.097	0.049	0.049	0.013

TABLE- 3: Saturation kinetics of reactor system [Value of ozone (mg)]

Time (min)	5	10	15	20	25	30
1	0.768	1.5	2.292	3.048	3.9	407.6
2	0.768	1.5	2.292	3.048	3.9	4.716
3	0.768	1.5	2.292	3.036	3.88	4.728
4	0.768	1.548	2.282	3.024	3.88	4.716
Mean	0.768	1.512	2.289	3.039	3.89	4.719

TABLE- 4: Percent survival of bacteria at different time duration of ozonation

	Time (min)					
	0	5	10	15	20	25
Species	Percent Survival					
K. pneumoniae	100	90.0	65.9	40.5	0.1	0.01
E. coli	100	80.8	47.2	0.01	0.0	0.0
P. aeruginosa	100	83.1	52.3	0.01	0.0	0.0
V. cholerae	100	67.4	0.5	0.01	0.0	0.0
S. Typhi	100	87.2	49.7	0.01	0.0	0.0

TABLE- 5: Percent survival of fungal species at different time duration of ozonation

	Time (min)					
	20	40	60	80	100	120
Species	Percent Survival					
A. luchuensis	100	80.0	65.0	40.0	32.0	25.0
A. flavus	100	87.0	73.6	52.6	39.5	28.5
A. terreus	100	93.8	83.6	64.4	42.8	32.3
A. niger	100	85.6	68.5	45.0	34.1	27.0
Fusarium oxysporum f.sp. *lini*	100	81.5	63.0	32.3	26.0	22.0
Trichoderma harzianum	100	76.9	60.8	37.7	28.7	24.0

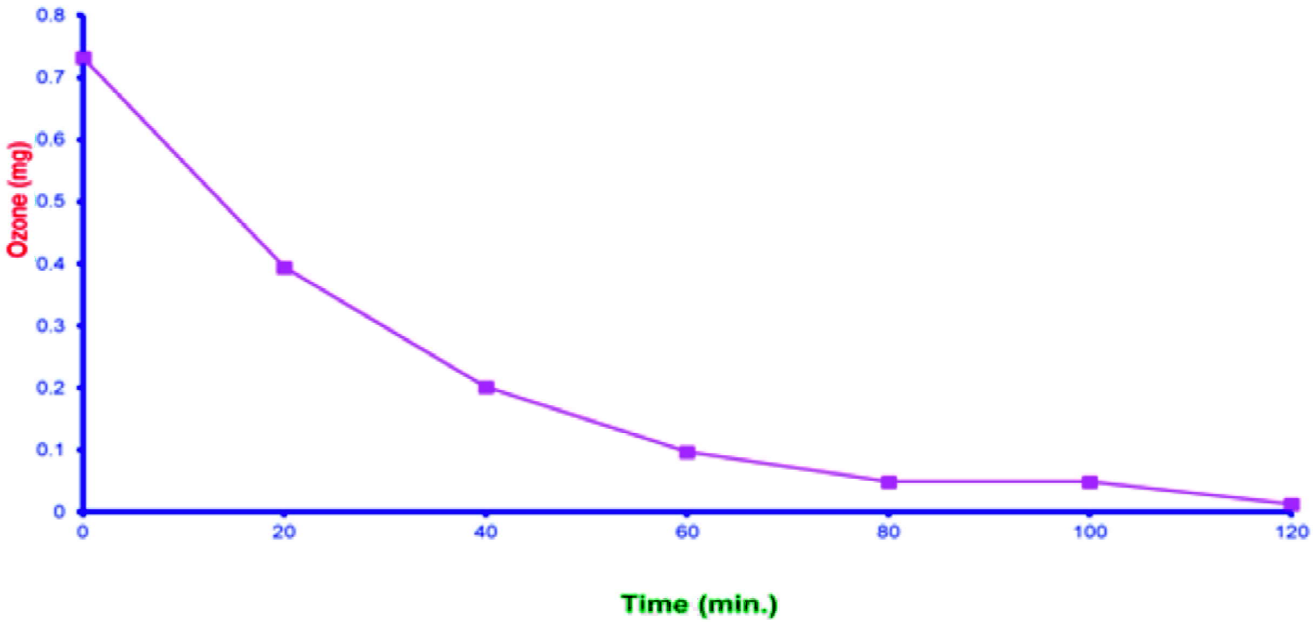

Fig. 1: Decay of ozone in solution with time.

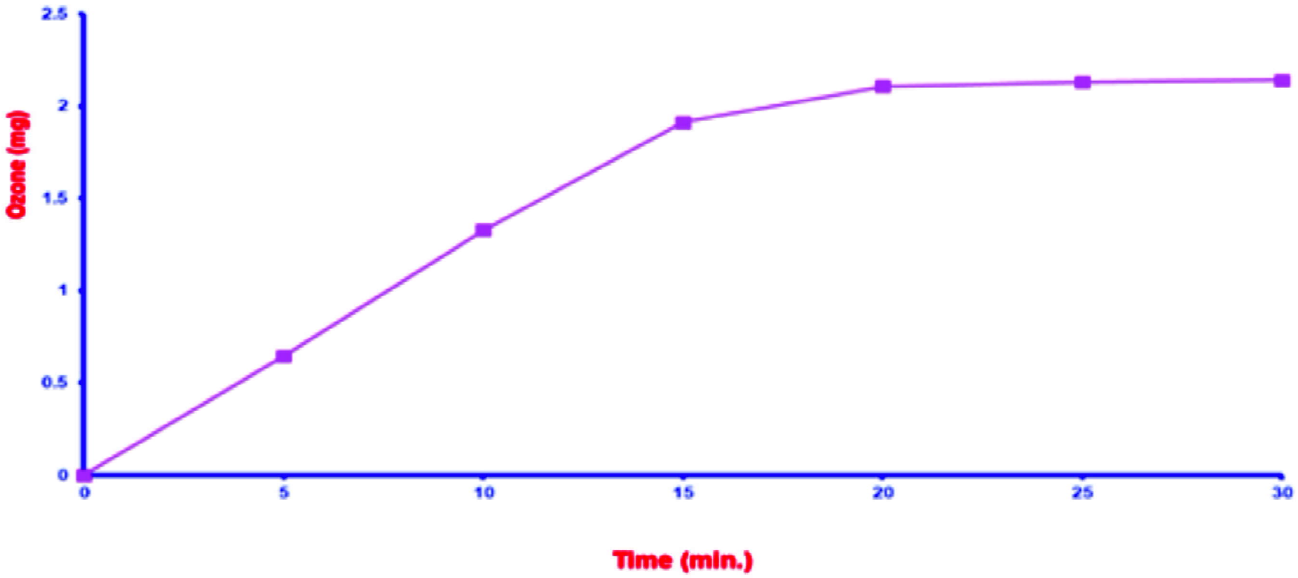

Fig. 2: Saturation kinetic for the reaction system

of ozone bubbles for killing the microbes was assessed. The reactor systems, were used for this study.A fixed number of bacteria (5×10^9) and fungi were suspended in each reactor system. Reactor system was exposed to ozone bubbles for 15 min, and cultures were done at each 5 min interval. All culture plates were incubated for 24 h at 37^0C for bacteria. For fungi the incubation period was 7 days at 25^0C. The concentration of 5×10^9 bacteria and fungi/ml were taken. Before ozonation, bacterial suspension was diluted 10^6 times and then 10 ml of the diluted suspension was spread over a nutrient agar plate and Potato Dextrose Agar (PDA) plate for fungi. After incubation, the colony counting was done.During the ozonation process after every three minutes interval, serial dilutions were made from the bacterial suspension to achieve a dilution of 10^4. Ten microlitres of this suspension

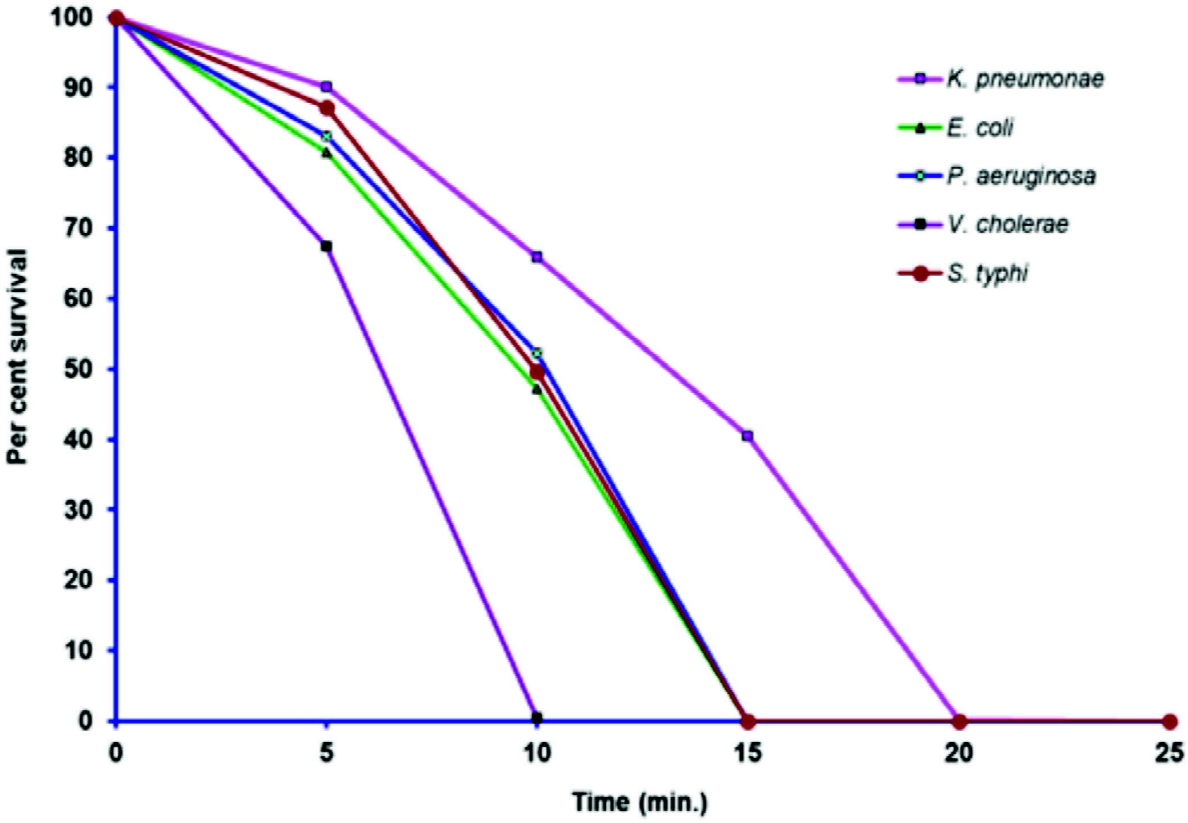

Fig. 3: Percent survival of bacteria at different time duration of ozonation.

were spread over nutrient agar plates. These plates were incubated for 24 h at 37^0C and colony counting was done for *E. coli* only.

Result and Discussion

The decay of ozone concentration in ozonated water followed first order kinetics with $t_{1/2}$ at 20 min. (Fig.1 & Table-1). The ozone saturation curves of the reactor system were plotted up to 30 minutes, shown in Tables 2, 3 and Fig.2. The reactor system has a slope of 0.16 mg/min. There is no plateau phase.Approximately $5´10^9$bacterial cell and fungal spore suspension were taken in the reactor system. The system was subjected to ozonation for 5, 10, 15, 20 and 25 minutes for bacteria,while 20, 40, 60, 80, 100 and 120 min for fungal spore. *E. coli, Pseudomonas aeruginosa, Vibrio cholerae,* and *Salmonella Typhi* were killed after 15 minutes of ozonation. Still, *Klebsiella pneumoniae* grew after 20 minutes of ozonation and survived upto (0.01%) 25 min. It was observed that *V. cholerae* was more sensitive than other bacteria. It was killed upto 99.5% after 10 minutes of ozonation. (Table- 4 & Fig.3).Among fungi *Fusarium oxysporum* f. sp. *lini* showed much susceptibility followed by *Trichoderma harzianum, Aspergillus lichuchensis, A. niger, A.flavus* and *A. terreus*. There was a reduction in colony count from 40 minutes to 120 minutes of ozonation, but all the fungi survived even after 2 h of ozonation (Table-5, Fig. 4).A significant variation was recorded p = 0.01/ 0.001 between the percent survival of microorganisms and ozone treatment duration. Ozone has been used for disinfection of water since1906. Earlier workers[4] studied the bactericidal activity of ozone on three bacterial species. They observed that the vegetative cells and their spores did not die. This means that above threshold concentration, all bacteria and spores died and below it, no one was killed.Other workers[10] reported 0.4 to 0.5 mg/l of ozone as the threshold concentration for *E. coli* after an exposure time of 1 minute at 1^oC and suggested that ozone killed microbes via an “all or none” phenomenon. Thus, either high ozone concentration for a short period (1 minute) or lower ozone concentration for a single extended period (5 minutes) provided sufficient activity to reach the minimal threshold for complete killing.

It was further observed that the reaction of ozone with bacteria was similar to the ozone response with potassium iodide in solution. Thus, only the amount of ozone reacting with the microorganisms was important. No effort was made to quantify the amount of ozone in the reactor system at any moment. In the reactor system, ozone bubbles by five-nozzle impinger from the bottom of the cylinder moved vertically upwards to the solution’s surface where ozone was dissipated to the atmosphere.

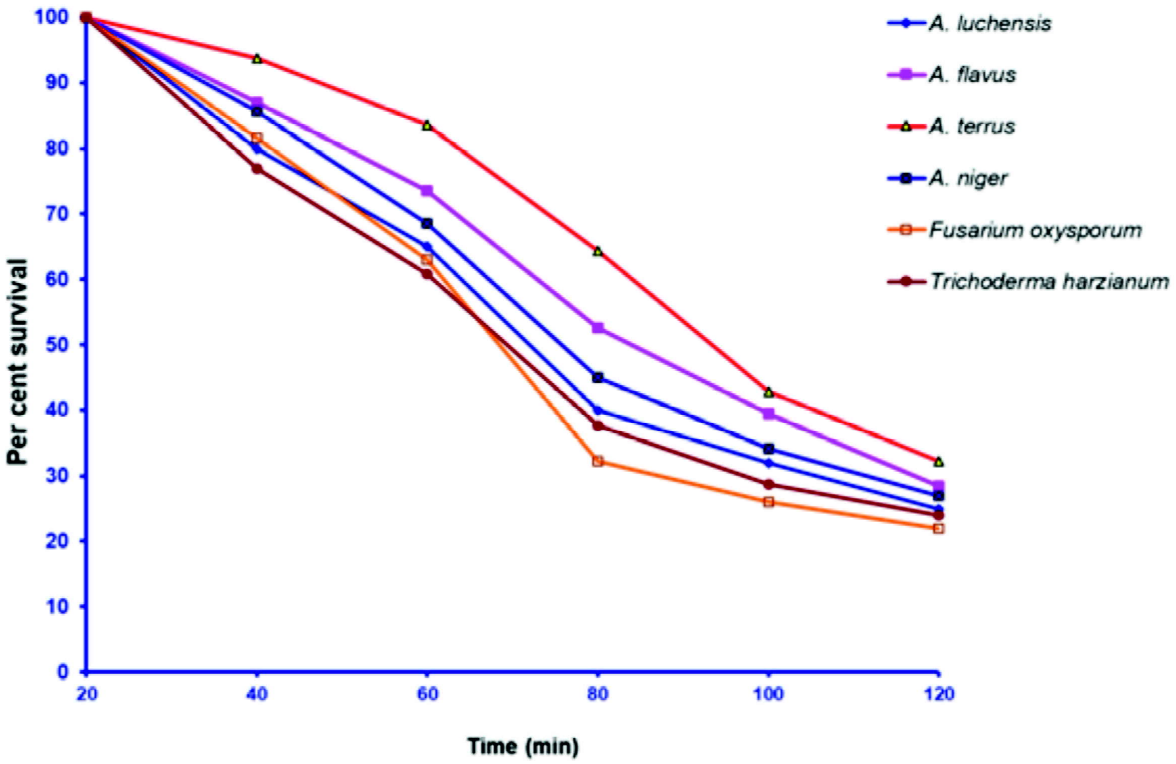

Fig. 4:Percent survival of fungal species at different time duration of ozonation.

Most of the bacteria except for *Klebsiella pneumoniae* were killed after 20 minutes of exposure to ozone. Among fungi *Fusarium oxysporum* f. sp. *lini* showed much susceptibility. There was a reduction in colony count from 40 minutes to 120 minutes of ozonation, but all the fungi survived even 2 hours of ozonation.In a similar type of experiment[4], It was found that the threshold of toxicity for *Bacillus cereus* was approximately 0.12 mg/L, whereas for *B. megaterium* and *E. coli* was about 0.19 mg/L. These values correlate well with those cited in the literature, especially for *E. coli*. There were cited[2,20] values ranging from 0.1 to 0.2 mg/L of residual ozone as effective for killing *E. coli*. It was[6] demonstrated that, when operating in a small space, the ozone generator produced a bactericidal concentration of ozone[6] (in the order of 1ppm). However, its use in a single room of a hospital produced undetectable ozone concentrations and no bactericidal effect could be demonstrated. Previous workers have shown that the minimum bactericidal concentration of ozone is 1ppm[11], while others have mentioned values between 20 ppm and 40ppm[7,17]. Other workers[12] examined the effect of ozone on fungal spore germination. They found that fungi are less sensitive than bacteria. *Aspergillus* species were found to be of intermediate sensitivity and required 1-1.5 ppm of ozone concentration for six hours to get their spore germination inhibited. Similar results were recorded for fungi.

Conclusions

The antimicrobial potential of ozone is due to the damage of the microbial cells' cell wall leading to leakage of the cell contents and finallycelldeath.During the present investigation, an effort was made to examine the possible use of this small ozone generator to decontaminate the drinking water. It could be concluded that the ozone generator was more effective against bacterial contaminant than the fungal spores. The exposure of bacterial strain to ozone doses of 20 ppm to 25 ppm for 5 to 25 min was sufficient for inhibition of bacterial growth. In contrast,fungi' growth inhibition required exposure to ozone doses of 15 ppm to 25ppm for 40 to 120 minutes. The increase in the exposure time at any gaseous ozone concentration resulted in a significant gradual retardation in the growth of fungi and bacteria.

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