The Effect of Ozone on Common Environmental Fungi

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OBJECTIVE: To determine if gaseous ozone can effectively kill common environmental fungi.

DESIGN: This study was designed to test the null hypothesis that there is no significant difference in viability between fungal conidia treated with ozone and fungal conidia not treated with ozone. A single control group design was utilized.

SETTING: Academic research laboratory.

INTERVENTIONS: Freshly prepared suspensions of *Cladosporium* spp., *Stachybotrys* spp., and *Aspergillus niger* conidia were diluted and plated onto the surface of solid agar plates. The plates were exposed to room air or to different concentrations of ozone for up to four hours, as were uninoculated plates. All plates were then incubated at 25°C until quantitative colony counts could be performed.

MAIN OUTCOME MEASURE: The effect of ozone on fungal conidia viability was assessed by comparing quantitative colony counts from conidia exposed to ozone to quantitative colony counts from conidia exposed only to room air.

RESULTS: There was a significant ($p < 0.05$) decrease in viable conidia of all three fungi, at ozone concentrations of 5.0 – 12.8 parts per million, by four hours of exposure. However, in every case, some conidia remained viable even at the highest level of exposure.

CONCLUSIONS: These data suggest that ozone must be used in conjunction with other methods of remediation or for more prolonged exposure times in order to eliminate fungal contamination of buildings.

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ABBREVIATIONS: BRI = Building Related Illness; ppm = parts per million; SBS = Sick Building Syndrome.

INDEX TERMS: Building Related Illness; fungi; ozone; Sick Building Syndrome.

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Air and moisture-related problems in buildings have been on the rise since the early 1970s, contributing to a phenomenon termed Sick Building Syndrome (SBS).[1] As a result of humidity, reduced air exchange rates, and the composition of most building materials, a variety of saprophytic and potentially harmful fungal species have been isolated in cases of SBS.[2,3] These fungi are relatively harmless to healthy individuals at low levels; however they can pose risks for immuno-compromised individuals or for healthy hosts when present in elevated numbers. Although the cause-and-effect relationship is controversial, these fungi, as well as other

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microbial pathogens, have also been implicated in the more serious situation termed Building Related Illness (BRI). For example, hypersensitivity to *Aspergillus* may be manifested as allergic bronchopulmonary aspergillosis and/or allergic fungal sinusitis.[4] Sensitization to *Cladosporium* has been associated with severe cases of asthma.[5] Furthermore, cases of BRI have been alleged to involve mycotoxins released from *Stachybotrys* found in water-damaged buildings.[6]

A potential remediation for buildings contaminated with fungi is exposure to ozone, O_3. In the stratosphere, the formation of ozone from diatomic oxygen and the decomposition of ozone to diatomic oxygen both absorb potentially harmful ultraviolet and cosmic radiation. In the lower troposphere, ozone can be generated by gamma irradiation, ultraviolet lights, lightning, high voltage electrical equipment, and sunlight-induced reactions involving volatile organic compounds in smog and NO_2. The strong oxidation potential of ozone makes it fairly toxic to living cells by a variety of mechanisms including free radical formation, lipid peroxidation, oxidation of sulfhydryl and other functional groups in proteins, and alteration of membrane permeability.[7] According to the Clean Air Act, ozone exposure for humans should not exceed 0.12 parts per million (ppm) at a daily one-hour average.[8]

Several studies have shown ozone to be effective at killing a variety of bacteria and fungi. Castillo and others reported that an aqueous solution of ozone sprayed on beef carcasses significantly reduced the load of *Escherichia coli* and *Salmonella typhimurium* on the smooth surfaces of the carcasses.[9] Serra and others demonstrated a 10-fold decrease in viable airborne mold conidia in cheese ripening rooms after treatment with gaseous ozone.[10] Dyas and others reported 95% killing of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Aspergillus fumigatus*, and *Candida albicans* after exposure to gaseous ozone, with the fungi requiring higher concentrations of ozone for longer periods of time (1.0-1.5 ppm for six hours) than the bacteria to achieve the same level of killing.[11] Kowalski and others showed that *Escherichia coli* and *Staphylococcus aureus* could be killed with 99.99% efficiency with gaseous ozone. However, ozone concentrations as high as 1500 ppm were required to achieve that level of killing.[12]

There is a dearth of quantitative data on the efficacy of ozone to reduce the fungal burden in contaminated building environments. We investigated the effect of different ozone concentrations in the air and different exposure intervals on the viability of freshly prepared conidia of common environmental fungi.

MATERIALS AND METHODS

Preparation of fungal conidia

Samples of *Cladosporium* spp., *Stachybotrys* spp., and *Aspergillus niger* were cultured from buildings suspected of mold contamination. These fungi were utilized in this study because they were isolated in high numbers from the respective buildings, and because they are easily distinguishable from each other and from potential laboratory contaminants on the basis of colonial morphology and microscopic appearance. Cultures were grown on Sabouraud's dextrose agar in the dark at 25°C for seven days. Each culture was passed at least twice before use to ensure that the cultures were pure and that the isolates were viable. Conidia were harvested by agitating the agar with 1 mL of sterile water. The conidia suspensions were then diluted 1/5 with sterile water and allowed to settle for several minutes to remove larger fungal particles. Conidia suspensions were then adjusted by visual comparison to the turbidity of a 0.5 McFarland standard. Each suspension was examined microscopically to ensure that conidia were present. The suspensions of *Cladosporium* spp. and *Aspergillus niger* were then diluted 1/10 and the suspensions of *Stachybotrys* spp. were diluted 1/100 with sterile water. These dilutions were selected after preliminary experiments revealed that these dilutions would lead to colony counts between 50 and 500 colony-forming units per plate inoculated with 10 μL of conidia suspension. The difference in the dilution required for *Stachybotrys* spp. was undoubtedly due to differences in the size and refractive index of its conidia, compared to those from the other two fungi. Ten μL of supension was spread uniformly across the surface of a fresh Sabouraud's dextrose agar plate for exposure to ozone or control conditions.

For each day's experiment with each organism, three plates were inoculated for each ozone concentration and each time of exposure. Each experiment was performed in triplicate with each organism, on three different days with fresh conidia suspensions each day.

Ozonation conditions

Plates were exposed to ozone in a clean 2' x 4' x 2' chamber constructed of plywood and plexiglass. All joints were sealed with silicone caulking. An Air-Zone XT-800 ozonator was centrally located on the exterior of the top panel of the chamber, expressing ozone to the interior of the chamber. Inoculated test and control plates were evenly spaced throughout the interior floor of the chamber and exposed to ozone from one to four hours in one hour increments. Preliminary experiments revealed that ozone concentrations

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of <5 ppm had no effect on the viability of conidia from any of the three fungi. Ozone concentrations of 5.0-7.0 ppm and 11.0-12.8 ppm were achievable inside the chamber with the ozonator set to the two highest settings; and these two concentrations were tested over the selected times. Ozone concentration was monitored using a Model A-21ZX Ozone Sensor manufactured by Eco Sensors, Inc. The chamber was equilibrated to the appropriate ozone concentration for approximately 15 minutes prior to introducing the inoculated plates. Baseline atmospheric ozone readings using the Model A-21ZX Ozone Sensor of the ozonation chamber were taken prior to application of ozone to each test set. All baseline atmospheric ozone readings were 0.04 ppm or less.

Controls

For each experiment with each organism, five control plates were set up. One control plate was inoculated with 10 μL of the water used to prepare the conidia suspensions, and incubated without ozonation, to verify the sterility of the agar and the water. The second control was an uninoculated plate that was ozonated for the same time interval as the inoculated plates, to verify that the air in the chamber did not contribute to the number of viable conidia observed on a plate after ozonation. Three control plates were inoculated with the same conidia suspension as the rest of the plates in that experiment, but were not exposed to ozone prior to incubation.

Quantitation of viability

After ozonation for a prescribed time interval, inoculated plates and controls were removed from the chamber, incubated at 25°C in the dark, and examined daily for fungal growth. Colony counts were determined by manually counting colonies when they were first visible. In most instances, immature colonies could be observed with the naked eye within 48 hours to 72 hours; but plates were held until colonial characteristics could be distinguished to verify that there were no contaminants and that the appropriate organism was growing. The identity and purity of the organisms on the plates for colony counts was confirmed by microscopic examination with a lactophenol cotton blue stain.

Statistical analysis

The colony counts for each organism, exposed to each level of ozone, for each time interval, were compared to the colony counts obtained from the same conidia suspensions on the control plates that were not exposed to ozone, using the t-test for independent samples. A p <0.05 was considered a statistically significant difference in viability.

RESULTS

The graphs in Figures 1 through 3 illustrate the effect of ozone on the viability of conidia of *Aspergillus niger, Cladosporium* spp., and *Stachybotrys* spp., respectively. All three experienced statistically significant reductions in viability after exposure to 11.0-12.8 ppm of ozone, while *Aspergillus niger* and *Stachybotrys* spp. were also significantly affected at a level of 5.0-7.0 ppm. However, some conidia from all three fungi survived, even after four hours of exposure to the higher dose.

The data also reveals differences between the three fungi and between levels of exposure in the kinetics of the effect of ozone on the viability of their conidia. Although there are not enough time points to make quantitative comparisons, it appears that exposure to the higher level of ozone led to a more rapid decline in viability of the conidia of the three fungi than did exposure to the lower level of ozone. Furthermore, it appeared that with the higher level of ozone, there was a more dramatic decrease in viability of the conidia of *Aspergillus niger* and *Cladosporium* spp. after only one hour, whereas the most dramatic decrease in viability of the conidia of *Stachybotrys* spp. occurred after two hours of exposure.

There was also a qualitative difference in the effect that ozone had on *Cladosporium* spp. compared to the other two fungi. Colonies of *Aspergillus niger*, *Stachybotrys* spp., and *Cladosporium* spp. were typically visible within 48 hours of inoculation of a Sabouraud's dextrose agar plate with their respective conidia. Following exposure to ≥5 ppm ozone, however, colonies of *Cladosporium* spp. were not visible until 72 hours after inoculation, and the colonies were smaller and less pigmented than those from conidia exposed only to room air. This finding suggests that even though some conidia of *Cladosporium* spp. were able to survive ozonation, the gas causes damage to the metabolic and/or biosynthetic capabilities of the conidia that do survive.

DISCUSSION

The results of our study show that ozone is toxic to three environmental fungi commonly isolated from buildings contaminated with mold. We were able to demonstrate significant reductions in the viability of conidia from *Aspergillus niger, Cladosporium* spp., and *Stachybotrys* spp. following exposure to ozone at a concentration of 11.0-12.8 ppm for one to four hours. A potential limitation of our study design was that the conidia from these fungi were tested after the organisms had been cultured in a laboratory

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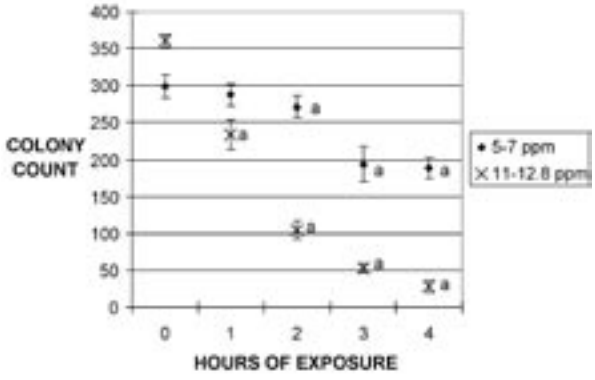
environment, with each culture passed at least twice. It cannot be determined from our study whether these fungi would be more or less susceptible to ozone-mediated damage in their natural environment.

Our results are consistent with earlier investigations into the efficacy of ozone to kill certain bacteria and fungi in two respects. First, while significant killing was achieved with ozone, the killing was not complete under the conditions of the experiments. Second, the concentrations of ozone required to achieve significant killing were well above the level that is considered safe for human exposure.[8-12] In our study, we used ozone concentrations that were approximately 40 times to 100 times higher than what is considered safe for human exposure. Even though the half-life of ozone in a confined space is reportedly less than ten minutes,[13] a building treated with ozone at that level would be uninhabitable for at least two hours after treatment.

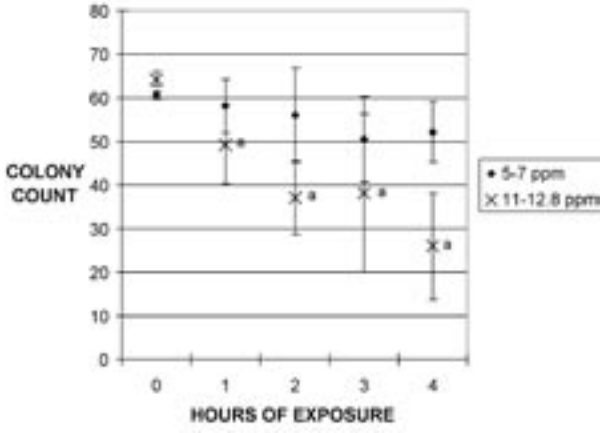
The results of our study reveal several limitations to the potential remediation of mold-contaminated buildings by ozonation. First, the level of killing we observed was significant but not complete, even after four hours of exposure. This suggests that ozonation would provide only a transient reduction in the total mold burden in a contaminated building. Second, the level of killing we observed was with the conidia exposed to gaseous ozone on the surface of agar plates. In contaminated buildings, molds frequently grow into or even behind materials such as sheetrock, plaster, or tiles that would present a diffusion barrier and protect the fungi from gaseous ozone generated in a room or other building spaces. Third, we were able to achieve an ozone concentration of 11.0-12.8 ppm with a commercial ozone generator operating on the "high" setting and discharging the ozone into a closed chamber with only 16 ft^3 of total volume. This,

Figure 1. Viability of *Aspergillus niger* following exposure to ozone



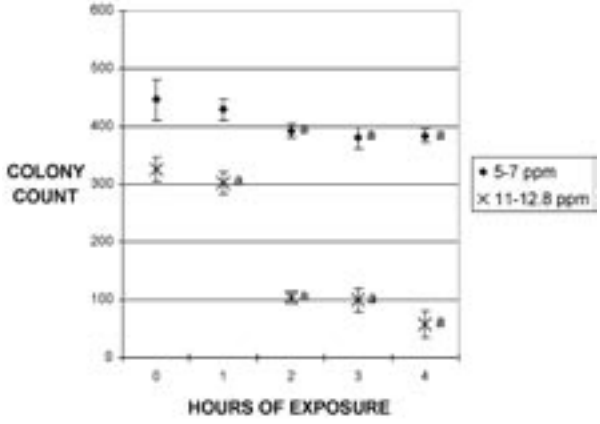
Plot symbols represent the means and standard deviations of nine replicates. 'a' denotes p <0.05 relative to zero hours of exposure.

Figure 2. Viability of *Cladosporium* spp. following exposure to ozone



Plot symbols represent the means and standard deviations of nine replicates. 'a' denotes p<0.05 relative to zero hours of exposure.

Figure 3. Viability of *Stachybotrys* spp. following exposure to ozone



Plot symbols represent the means and standard deviations of nine replicates. 'a' denotes p<0.05 relative to zero hours of exposure.

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along with the first two limitations, suggests that it would be difficult to achieve effective fungicidal concentrations of ozone inside large buildings.

Given the results of this study, further research is indicated to identify the specific macromolecules in fungal conidia that are oxidatively damaged by ozone, in order to elucidate the mechanisms whereby ozone is toxic to each of the species of mold that commonly contaminate buildings. An understanding of these mechanisms will facilitate future research into what other agents may be combined with ozone to potentiate its fungicidal activity and to overcome the physical barriers to its successful application to the process of building remediation.

In conclusion, gaseous ozone in high enough concentrations can significantly reduce the number of viable conidia from *Aspergillus niger, Cladosporium* spp., and *Stachybotrys* spp., but not completely eliminate them. Ozonation may be useful as one component of a multifaceted strategy for treating mold-contaminated buildings; however, it is unlikely to be effective by itself for building remediation.

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