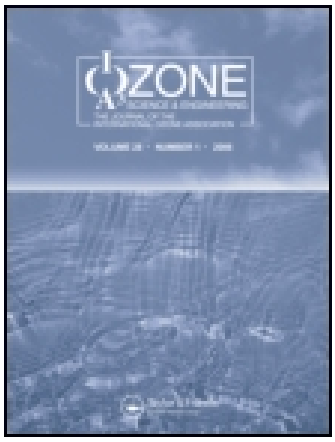

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The Practical Application of Ozone Gas as an Anti-fungal (Anti-mold) Agent

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The Practical Application of Ozone Gas as an Anti-fungal (Anti-mold) Agent

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We evaluated the ability of a portable ozone generating machine (Viroforce 1000) to inactivate 13 different species of environmental fungi. Samples, prepared as wet or dried films, were subjected to one or two cycles of treatment (35 ppm ozone for 20 minutes, with a short burst of >90% relative humidity), and measured for residual viability. Treatments could inactivate 3 log_{10} cfu (colony forming units) of most of the fungi, both in the laboratory and in simulated field conditions, on various surfaces. We conclude that the ozone generator would be a valuable decontamination tool for mold removal in buildings.

Keywords Ozone, Fungi, Mold, Anti-Fungal, Healthcare, Environmental Fungi, *Stachybotrys chartarium*, *Trichoderma viride*

INTRODUCTION

The presence of mold in buildings is associated with adverse health outcomes. Many different microbes, including a variety of fungal and bacterial species, thrive in the conditions offered by moist building materials, which contain both the nutrients and the moisture needed for microbial growth. This has given rise to the designation of "sick building syndrome" (SBS) to people afflicted directly or indirectly by the presence of fungal or bacterial products on surfaces and in the air. Similar situations have been documented in health care facilities, especially intensive care units, where there may be patients who are particularly vulnerable to microbial contaminants (Hope and Simon, 2007; Gniadek and Macura, 2007; Huttunen et al., 2008; Gottschalk et al., 2008). In addition to the risk of fungal infection in immune compromised individuals, microbial products can include mycotoxins, endotoxins, and various microbial volatile organic compounds (mVOC's). These products may be cytotoxic, allergenic, or inducers of pro-inflammatory mediators (Hope and Simon, 2007; Huttunen et al., 2008).

A variety of mold cleanup methods are available for remediating damage to building materials and furnishings caused by mold growth, with some success (Kleinheinz et al., 2006). These include mechanical methods, which have inherent drawbacks, including cost, labor, and inhalation of spores. For example wet vacuums may spread spores if sufficient liquid is not present. Porous materials that are wet and have mold growing on them may have to be discarded. Since molds can infiltrate porous substances and grow on or fill in empty spaces or crevices, the mold can be difficult or impossible to remove completely. Sodium hypochlorite aerosols have been found to be effective, but are not generally acceptable (Martyny et al., 2005).

Building remediation has been estimated to comprise a multibillion dollar industry in the USA alone (Kleinheinz et al., 2006); consequently there is a financial incentive, as well as health concerns, to come up with an acceptable, efficient, anti-mold treatment.

Ozone has been shown to be an effective anti-bacterial treatment, in aqueous solution and in gaseous form, in several types of application, such as laundries and food protection (Bialka and Demirci, 2007; Naitou and Takahara, 2008; Selma et al., 2008; Akbas et al., 2008). Ozone gas has several advantages over alternative liquid treatments; it is easy and cheap to produce from air or oxygen, it diffuses quickly into all parts of a room, including cracks and crevices, and, although toxic at high doses, dissociates naturally to oxygen. Dosages can be controlled remotely from outside a treated sealed room. It is also a strong oxidizing agent that has the potential of not only inactivating microbes on various surfaces (Sharma and Hudson, 2008), but is also an efficient

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de-odorizing agent, and might be capable of inactivating the microbial products referred to above.

Korzun et al. (2008) recently evaluated the efficacy of ozone gas against several environmentally important fungi, and obtained promising results, although their ozone dosage was below what we have described as effective anti-viral and anti-bacterial concentrations (Hudson et al., 2007; Sharma and Hudson, 2008). This report describes the use of a proprietary portable ozone generator as a practical anti-fungal device.

MATERIALS AND METHODS

Equipment

The laboratory test chamber was a molded polycarbonate box with a transparent plastic front window that could be lifted to allow access to samples. Within the test chamber was a small ozone generator (Treated Air Systems, Vancouver) fitted with a control dial that could be pre-set to known ozone doses in ppm, an ozone sampler tube connected to the exterior ozone measuring system (see below) and the probe of a hygrometer for measuring relative humidity and temperature. Humidity was applied manually as a mist of deionized sterile water.

The test room for field trials was an office, volume 34 m^3, containing normal office furniture, which was located adjacent to the laboratory. In this test system we used a model 1000 Viroforce ozone generator, which is a portable module containing multiple corona discharge units, a circulating fan, and an efficient catalytic converter (scrubber) to reconvert ozone to oxygen at the termination of the ozone exposure period (Figure 1). In addition a portable commercial humidifier (Humidifirst Inc, Florida)

FIGURE 1. Photograph of Viroforce 1000 programmable ozone generator (right) with accessory sonic humidifier and its control unit and water reservoir (left).

was used to provide a burst of water vapor when required. All the components were controlled remotely from outside the test room.

In both test situations ozone concentration was monitored continuously by means of an Advanced Pollution Instrumentation Inc. model 450 system (from Teledyne, San Diego, CA), which measured samples of ozonated air passed through a UV spectrometer. The input Teflon sampling tube could be taped in an appropriate location for the duration of the experiment. Relative humidity and temperature were recorded by a portable hygrometer (VWR Scientific, Ontario). The probe was taped in a convenient location inside the test room.

In preliminary trials we used 2–3 manual ozone sensors (Ecozone, USA) located at random sites within the room to confirm homogeneous distribution of the ozone gas. The variation in recorded levels between sensors and the monitor probe was less than ± 10%.

Materials

The lids of sterile polystyrene tissue culture trays were used as plastic surfaces. Samples of fabrics and cotton (typical of those used in hospital and hotel rooms) were cut into small pieces, cleaned in detergent, washed, dried, and sterilized by autoclaving. Cotton tips and other materials were heated for 2 min in a microwave oven. Fetal bovine serum and PBS (phosphate buffered saline) were obtained from Invitrogen (Ontario). Sterile plastic 24-well plates and other supplies were BD-Falcon brand obtained from VWR Scientific (Ontario). Sabouraud-dextrose plates were obtained from PML Microbiologicals, Willsonville, Oregon.

Fungal Strains

The following fungal strains were collected from various derelict buildings in B.C. and speciated by Dr. Eduardo Jovel, University of British Columbia, who kindly supplied them to us as colonies grown on agar plates: *Alternaria sp. Aspergillus species (flavus, niger, fumigatus), Aureobasidium sp, Botrytis sp. Cladosporium sp. Geotrichum sp. Mucor sp. Penicillium brevicompactum, Stachybotris chartarium, Trichoderma viride, Ulocladium sp.* We also used ATCC strains of *Candida albicans* and *A. flavus. C. albicans* was used both as yeast cells and hyphal form.

All organisms were propagated and maintained in our laboratory on standard Sabouraud-dextrose agar plates (PML Microbiologicals, Oregon). *C. albicans* colonies were obtained in the same way, and mixed populations of yeast cells and hyphae (approximately 1:1) were obtained by growing the cells in Dulbecco MEM cell culture medium supplemented by 10% fetal bovine serum. Cells and hyphae were harvested by centrifugation and resuspended in PBS for the tests.

Test Procedure

Inocula of filamentous fungal cultures were prepared by excision of 5 mm^3 blocks of agar containing actively growing mycelium and spores, and transferred to 5 mL of PBS (phosphate-buffered saline). The material was homogenized thoroughly on a vortex mixer. No attempt was made to separate mycelia from spores; thus each preparation comprised a mixture of both forms, and was assumed to be representative of growing cultures in the field. In some experiments, sterile fetal bovine serum was incorporated into the PBS to mimic an organic load (to demonstrate if organic load interferes with the fungicidal activity of ozone). The starting concentration of each preparation (cfu/mL) differed because of different growth rates and densities of the organisms within the agar blocks.

Quantitative tests were carried out as follows: Each sample, consisting of 100 μL drop of suspension, was applied, in duplicate, to the sterile surface of a plastic tray lid in a biosafety hood and allowed to dry (approximately 45 min). The dry samples were covered and placed at various locations within the test chamber or within the test room, and in the latter case the ozone generator and rapid humidifying device (RHD) were placed in a central location. These units were operated remotely from outside the room. All vents and windows in the test room were sealed. At the commencement of the test, the samples were uncovered, the door closed and sealed with tape, and the generator switched on by a programmable remote control.

The ozone level reached 35 ± 5 ppm within several minutes, and was maintained at this level for the balance of the 20 minutes. The RHD was then activated to produce a burst of water vapor for 5 min. Both generator and RHD were then switched off for another 5 min to allow "incubation" in the humid atmosphere (the relative humidity usually reached 99%). The catalytic converter was then turned on to remove all ozone gas. Ozone levels decreased to less than 1 ppm within 15 min. at which point the door was opened and the test samples were retrieved. All tests were conducted at ambient temperature, 19–21 °C.

In the laboratory tests, following the 20-min ozone exposure in the polycarbonate test chamber, the window was lifted briefly to allow the delivery of a mist of sterile pure water from a spray bottle. This resulted in a rapid increase in relative humidity to 90–99%.

All samples were reconstituted in PBS (phosphate buffered saline), and serial 10-fold dilutions were made in PBS. Recovery of samples from the non-plastic materials required additional vortexing and squeezing of the materials. However the controls for these samples were processed identically. Aliquots of 2.5 μL were spotted and spread out with plastic inoculating loops onto Sabouraud-dextrose plates. Control untreated samples were kept in the biosafety cabinet during the entire

operation. Agar plates were incubated at 35 °C for a minimum of 48 h, after which fungal colonies were counted.

In some experiments, as indicated, the wet fungal inocula were used without drying, and in some experiments the tests with wet and dry inocula were carried out in ambient relative humidity, 40–45% RH.

RESULTS

Laboratory Tests

All 13 fungi, including cells and hyphae of *Candida albicans*, were initially evaluated for susceptibility to ozone gas in the laboratory test system. Replicate 100 μL samples of the fungal suspensions in PBS were dried onto sterile plastic trays in a bio-safety cabinet, and exposed to the standard ozone dosage (35 ppm for 20 min). Preliminary tests had shown that lower concentrations of ozone were less effective.

The results are summarized in Table 1. Because of the losses in cfu on drying, and the dilution factors involved in the assay procedures, we were not always able to determine the exact end points of inactivation. Nevertheless in all fungal species, greater than 3 $\log_{10}$ decreases were consistently obtained provided the RH was greater than 90%. Not all species were tested at ambient RH. For *C. albicans* and *Trichoderma viride*, the corresponding values were $\geq$ 4.0 $\log_{10}$. These results also indicate that wet films of all the fungi were equally susceptible.

In some experiments, up to 50% by volume of fetal bovine serum was included in the samples prior to drying. This did not affect the outcome however (data not shown). *Candida albicans* and *Trichoderma viride* were also tested on several other surfaces, filter paper, cotton, cardboard, and fabric, and were found to be equally susceptible to the ozone treatment, as indicated in Table 2.

Field Tests

These were conducted in an office adjoining the laboratory. This room had a volume of 34 cu meters, and contained standard office furniture and a large window. Initially we used *C. albicans* to evaluate the efficacy of the prototype ozone generator and the rapid humidifier. During the standard program the ozone level rose to a maximum of 35 ± 5 ppm within a few minutes and was maintained at this level until the 20-min point, when the humidifier was automatically switched on.

The relative humidity increased rapidly from the ambient level (40–45% RH) to greater than 90% (usually 99%) within 5 min. After a further 5-min incubation at this level, the generator and humidifier were turned off

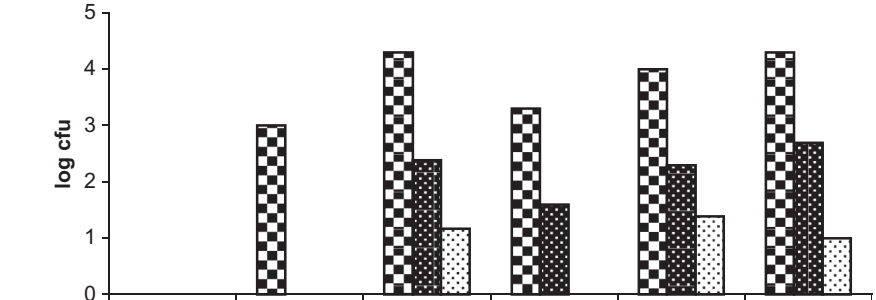
TABLE 1. Susceptibility of Fungi to Ozone Gas

Species	$\log_{10}$ Reduction in CFU (colony forming units)		
	Wet sample high RH (> 90%)	Dry sample high RH	Dry/wet sample low RH (45%)
Aspergillus species *(flavus, niger, fumigatus)*	➢ 3.0	➢ 3.0	~1.5
Aureobasidium species	➢ 3.0	➢ 3.0	nt
Botrytis species	➢ 3.0	➢ 3.0	nt
Candida albicans (cells and hyphae)	≥4.0	≥4.0	1.5–2.0
Cladosporium species	➢ 3.0	➢ 3.0	~1.0
Geotrichum species	➢ 3.0	➢ 3.0	nt
Mucor species	➢ 3.0	➢ 3.0	~1.0
Penicillium brevicompactum	➢ 3.0	➢ 3.0	nt
Stachybotris chartarium	➢ 3.0	➢ 3.0	~1.0
Trichoderma viride	≥4.0	≥4.0	~1.5
Ulocladium species	➢ 3.0	➢ 3.0	~1.0

nt = not tested.

TABLE 2. Susceptibility of *Trichoderma viride* and *Candida albicans* to Ozone on Different Surfaces: $\log_{10}$ Reduction in CFU (Colony Forming Units)

Fungi	Plastic	Cotton	Fabric	Filter Paper	Cardboard
Trichoderma viride	≥4	≥4	≥4	≥4	≥4
Candida albicans	≥4	≥4	≥4	≥4	≥4



	Candida	Asp.niger	Ulocladium	Trichoderm	Asp.fumig
C	3	4.3	3.3	4	4.3
1dose	0	2.39	1.6	2.3	2.69
2doses	0	1.17	0	1.39	1

FIGURE 2. Anti-fungal effects of one and two doses (cycles of treatment) of ozone with high humidity (> 90% RH). Values expressed as cfu (colony-forming units) per 2.5 μL reconstituted sample (details in Materials and Methods section). The 5 organisms shown (*Candida albicans, Aspergillus niger, Ulocladium sp. Trichoderma viride, Aspergillus fumigatus*) were simultaneously exposed to one cycle of standard treatment by the ozone generator in the test room. Half the samples (all in duplicate) were then retrieved from the room and reconstituted, while the other half were exposed to a second cycle of treatment, followed by reconstitution. Controls were kept in the biosafety cabinet for the duration of the entire experiment. The starting concentrations for the 5 organisms were different. No *C. albicans* cfu were detected after one cycle of treatment, and *Ulocladium* cfu were not detected after the second treatment cycle.

and the catalytic converter activated. The ozone concentration decreased to < 1 ppm after 15 min, at which point the samples were retrieved and taken to the laboratory for processing. A single exposure of *C. albicans* to this treatment program consistently inactivated more than 3 log $_{10}$ cfu (Figure 2), and in most tests eradication was achieved.

The other species of fungi were tested, in groups, with the same protocol. Most of them appeared to be more resistant than *C. albicans*, in that a single cycle of treatment was usually insufficient to kill all the test organisms. Nevertheless a second cycle of treatment, applied shortly after the first one, was much more effective, as can be seen from the results shown in Figure 2. In all cases there were fewer residual cfu following the second exposure. Consequently in subsequent tests two consecutive standard treatments were used, although the maximum ozone level attained during the second cycle was significantly lower (approximately 20 ppm) due to residual humidity from the first cycle (ozone generation efficiency was reduced somewhat in the presence of high RH).

Similar results were obtained for wet films of the species indicated in Figure 2. The remaining fungal species were then tested with the two cycle program, and all were found to be susceptible, although there did appear to be some variation in relative sensitivity among the species; but this possibility was not examined systematically. The variation in maximum $\log_{10}$ inactivation for some species was a reflection of their different starting concentrations. *Stachybotris* and *Botrytis* were especially difficult to obtain in high cfu/mL.

In additional tests, replicate samples of *Trichoderma viride* were dried onto plastic trays, as usual, and also on to samples of fabric, cotton, filter paper and cardboard. These were placed at various locations within the room to mimic possible contamination sites in buildings. All samples apparently showed similar sensitivity to ozone, regardless of their location or the surface on which they were dried. However, after several days of incubation of the assay plates, a few colonies appeared, in the samples reconstituted from non-plastic surfaces, and subsequently grew into the familiar mats, indicative of incomplete inactivation.

DISCUSSION

Many studies have shown that exposure to indoor air microbes from moisture-damaged buildings can cause adverse health problems including irritation, respiratory infections, an increased risk of asthma development and various other symptoms and diseases as a result of mycotoxins and VOCs (Lai, 2006; Gniadek and Macura, 2007; Hope and Simon, 2007; Gottschalk et al., 2008; Huttunen et al., 2008). In addition certain species have been associated with opportunistic life-threatening infections in immune-compromised patients (Marr et al., 2002).

Ozone decontamination has potential advantages. It can effectively penetrate every part of a room, including sites that might prove difficult to gain access to with conventional liquids and manual cleaning procedures. It can be switched on and off from the outside after the room has been made airtight. Ozone is cheap to generate

and although toxic, rapidly dissociates to oxygen with a half-life of about 20 min. The use of a catalytic converter speeds up the removal considerably. Ozone gas has been advocated as a safe disinfecting agent in the food and agriculture industries (Serra et al., 2003; Bialka and Demirci, 2007; Akbas and Ozdemir, 2008; Naitou and Takahara, 2008; Selma et al., 2008), although usually at lower concentrations than those used in the present study. Nevertheless, in view of the speed and efficiency of the catalytic conversion, resulting in a return to ambient levels within 15–20 minutes, and an overall treatment cycle of less than one hour, we believe that the Viroforce system is safe and practical.

This study has demonstrated that our prototype ozone generator produced a fungicidal concentration of ozone (of the order of 35 ppm). The potent biocidal activity of the ozone generator after 20 min exposure time and >90% RH was demonstrated across a range of fungi including spore producing mold. Inactivation of *Trichoderma* samples dried onto soft surfaces, such as fabric, cotton, and filter paper, was comparable to that observed for samples on plastic, thus confirming that ozone gas can be fungicidal to samples on curtains, linen, furniture and walls in the buildings or health care facilities. This is of particular concern since some authors have suggested that gaseous ozone might be less effective against surface bound organisms (Serra et al., 2003). However this was clearly not a limitation in our studies, possibly a reflection of the higher ozone levels used by us.

Korzun et al. (2008) raised several issues relevant to the use of ozone gas to remove mold contamination. In their study they achieved a significant reduction in fungal cfu, but not eradication, as a consequence of exposure to ozone concentrations up to 12 ppm. We found that it was necessary to use considerably higher levels, and expose to two cycles of treatment, in order to achieve a 3 $\log_{10}$ reduction in most of the fungi tested, with the exception of the more sensitive *C. albicans*, which was readily reduced by more than 4 $\log_{10}$ cfu by only one cycle. We overcame the toxic potential of high ozone concentration by operating the generator and catalytic converter remotely from outside the sealed room. In the case of larger rooms more than one machine could be used in parallel.

Korzun et al. (2008) tested fungal conidia on the surface of agar plates. We found that several different hard and soft surfaces could be used, and the addition of serum to the samples, to mimic an organic load, did not affect the results. They were also concerned about the size limitations of their ozone generation system. However our generators can be scaled up or down to suit the demand. Their other concern was the possible limitation of testing laboratory passaged strains which might not behave the same as wild strains. Most of our fungi were isolated from derelict buildings, and received minimal passaging in the laboratory.

While the use of liquid disinfectant for general decontamination requires a great deal of labor and is unsuitable for curtains, walls and ceilings, decontamination with gas is easier. Ozone treatment can also remove unpleasant odors, including those caused by mold. It is also feasible to give repeated exposures to achieve complete eradication of mold.

The mechanisms of action of ozone against fungi and other microbes are not understood; however its broad oxidizing activity against many macromolecules (Cataldo, 2006) suggest that microbial membranes, proteins and nucleic acids could all be vulnerable. Nevertheless the requirement of high humidity for optimal efficacy indicates that hydroxyl ions and possibly additional water-derived radicals could be involved, as suggested for the aqueous environments (Lin and Wu, 2006).

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