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Effect of Gaseous Chlorine Dioxide on Indoor Microbial Contaminants

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ABSTRACT
Traditional and modern techniques for bioaerosol enumeration were used to evaluate the relative efficiency of gaseous chlorine dioxide (ClO_2) in reducing the indoor microbial contamination under field and laboratory conditions. The field study was performed in a highly microbially contaminated house, which had had an undetected roof leak for an extended period of time and exhibited large areas of visible microbial growth. Air concentrations of culturable fungi and bacteria, total fungi determined by microscopic count and polymerase chain reaction (PCR) assays, endotoxin, and (1→3)-β-D-glucan were determined before and after the house was tented and treated with ClO_2. The laboratory study was designed to evaluate the efficiency of ClO_2 treatment against known concentrations of spores of *Aspergillus versicolor* and *Stachybotrys chartarum* on filter paper (surrogate for surface treatment). These species are commonly found in damp indoor environments and were detected in the field study. Upon analysis of the environmental data from the treated house, it was found that the culturable bacteria and fungi as well as total count of fungi (as determined by microscopic count and PCR) were decreased at least 85% after the ClO_2 application. However, microscopic analyses of tape samples collected from surfaces after treatment showed that the fungal structures were still present on surfaces. There was no statistically significant change in airborne endotoxin and (1→3)-β-D-glucan concentration in the field study. The laboratory study supported these results and showed a nonsignificant increase in the concentration of (1→3)-β-D-glucan after ClO_2 treatment.

IMPLICATIONS
ClO_2 gas was effective in reducing culturable and total fungi and bacteria in indoor air. The reduction of total count on surfaces was less efficient. Furthermore, the treatment process appeared to have no effect or increase the concentrations of endotoxin and (1→3)-β-D-glucan, which have been associated with respiratory symptoms in some individuals. Thorough cleaning of interior air and surfaces to remove the particulate matter are recommended to achieve acceptable conditions before reoccupancy.

INTRODUCTION
There have been numerous health concerns over exposures to indoor air contaminants, especially bioaerosols (a mixture of microbial, animal, and plant particles). The health effects are highly dependent upon individual responses to the various bioaerosols and fall into three main categories: allergic, infectious, and toxic. A wide range of symptoms have been associated with indoor biological contaminants ranging from irritation of the eyes, headache, fatigue, and respiratory tract symptoms to aggravation of asthma; however, the causal agents for these symptoms remain an area of ongoing scientific research.[1,2] Suspected biological causal agents include fungi (e.g., molds and yeasts) and bacteria, and their associated components (i.e., endotoxin and (1→3)-β-D-glucan). Endotoxin, a lipopolysaccharide complex in the cell wall of Gram-negative bacteria, has been associated with respiratory symptoms. (1→3)-β-D-glucan are the most abundant glucans from the cell walls of fungi as well as some bacteria and plants. They have been suspected to cause respiratory symptoms; however, the epidemiological data for this association are not conclusive.[3] There is also evidence that exposure to fungi may occur through fungal fragments that can contain allergens, toxins, and (1→3)-β-D-glucan.[4–6]

There has been increased interest in the use of new technologies for the remediation of mold and bacteria, especially since the prolonged flooding after Hurricanes Katrina and Rita in the New Orleans area resulted in highly contaminated buildings.[7–9] Among several techniques, the generation of gaseous chlorine dioxide (ClO_2) is currently being explored for the remediation of structures that have been impacted by microbial growth. The advantage of using a gas is that it can penetrate into building cavities.

ClO_2 has been approved by the U.S. Environmental Protection Agency (EPA) as a disinfectant, sanitizer, and sterilant.[10] Gaseous ClO_2 is used as a disinfectant and sterilant in the paper, fruit, vegetable, dairy, poultry, and beef processing industries, as well as in industrial wastewater processing.[11–13] Aqueous ClO_2 has been frequently used to treat drinking water and for wood-pulp bleaching in the paper industry. It has also been used to control mold in libraries.[14,15] Under a crisis exemption from EPA, ClO_2 gas was used to treat *Bacillus anthracis* spores in 2001 and 2002 in contaminated buildings and the exterior of mail packages.[16,17] Additional studies have been completed on the efficiency of ClO_2 for inactivation of *Bacillus* endospores, as surrogates for *B. anthracis* spores.[18–21]

Wilson and associates[22] conducted a laboratory study investigating the effect of ClO_2 gas on the colonies of four fungal species (*Chaetomium globosum*, *Cladosporium cladosporioides*, *Penicillium chrysogenum*, and *Stachybotrys chartarum*), ascospores (*C. globosum*), and mycotoxins produced by *S. chartarum*. The investigators exposed fungal colonies grown on filter paper and purified ascospores to ClO_2 at concentrations of either 500 or 1000 ppm in a sealed chamber for 24 hr. Both concentration levels were found effective in rendering *C. cladosporioides*, *P. chrysogenum*, and *S. chartarum* colonies nonculturable after exposure to both ClO_2 concentrations. *C. globosum* colonies showed a reduction of 91% at the 500-ppm concentration and 87% at the 1000-ppm concentration of ClO_2. The *C. globosum* ascospores were almost totally inactivated and spore count decreased, indicating that some ascospores were destroyed by the treatment. The ClO_2 did not detoxify the *S. chartarum* mycotoxins as determined by a yeast toxicity assay. The referred study did not aim at examining the effect of ClO_2 on total fungal count (except for *C. globosum* ascospores), (1→3)-β-D-glucan concentrations, or bacterial count for species commonly observed in indoor environments. It should also be noted that the ClO_2 generation method and concentrations presented by Wilson and his colleagues were different than those used in the anthrax remediation projects.

ClO_2 is a strong oxidizing agent which has been shown in laboratory studies to interact with amino acids, proteins, and viral ribonucleic acid (RNA).[23] The cellular mechanisms that are affected by exposure to ClO_2 are not totally understood. Young and Setlow[21] found that liquid ClO_2 did not damage the DNA of *Bacillus subtilis* endospores. It has been proposed that the oxidation process damages the inner membrane of bacterial endospores. Furthermore, ClO_2 has been demonstrated to preferentially inactivate the outer protein layers rather than the nucleic acids for viruses.[24]

The purpose of this paper is to describe the investigation of the effect of gaseous ClO_2 exposure on bioaerosol contaminants in a contained indoor environment in a field setting, using traditional and modern bioaerosol enumeration techniques: culture-based assay, microscopic counting, quantitative polymerase chain reaction (PCR), and Limulus amebocyte lysate assay (LAL). Additional data were obtained in the laboratory to determine the effect of ClO_2 on known concentrations of fungal spores commonly associated with damp indoor environments (*Aspergillus versicolor* and *Stachybotrys chartarum*).

METHODS

Field Evaluation Decontamination Procedures

The field study was performed in a 1890s Victorian house located in a small city in Upstate New York. The house had three stories and a dirt/stone basement and had been purchased by a nonprofit organization to be renovated and used as a shelter for women and children. Several agencies volunteered their services to help with the project, including a commercial remediation firm. The house had been occupied until approximately 6 months before the ClO_2 treatment. A major roof leak was identified and repaired following the completion of an asbestos abatement project. Visible mold was present in most areas of the first, second, and third floors. Some rooms had bird and cat droppings present on the floor.

After a walk-through survey of the house, eight environmental sampling stations were chosen (two on each floor and the basement as shown in Figure 1). Floor fans used to circulate ClO_2 during the treatment phase were turned off or redirected to prevent interference with the collection of air samples. An outdoor sampling station was set up in the backyard of the house. The entire structure was tented (using the standard procedure for whole-house pesticide treatment) and the interior was heated, ventilated, and humidified before the application of ClO_2 gas to maintain the tent under positive pressure and provide optimal conditions for the ClO_2 treatment by the commercial remediation company.[25] The tent positive pressure was monitored on a regular basis using pressure gauges and maintained under positive pressure for the duration of the environmental monitoring. National Institute for Occupational Safety and Health (NIOSH) investigators were responsible for the microbial contamination assessment.

The ClO_2 solution was created on-site by the commercial remediation company using household bleach (5–6% sodium hypochlorite), 6-N hydrochloric acid, 25%

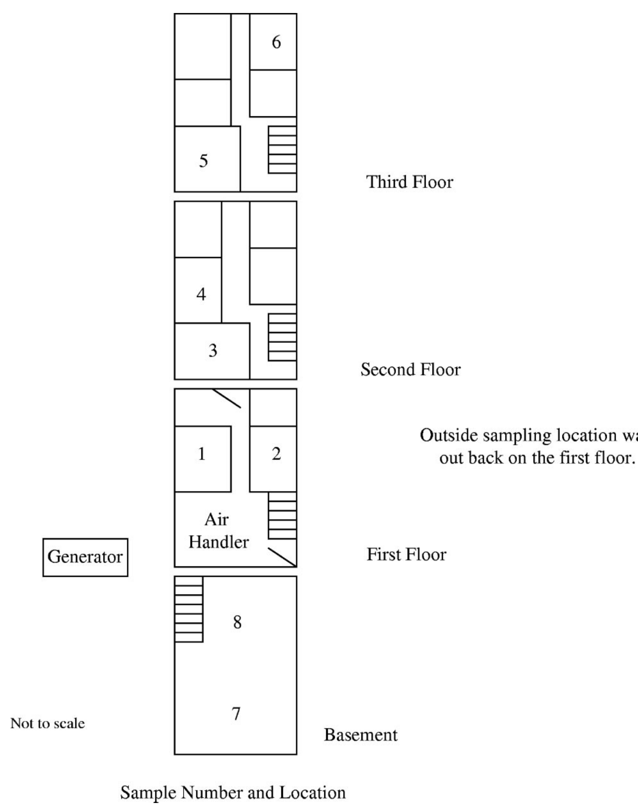

Figure 1. Simplified drawing of Victorian house plan showing sample, generator, and air handler locations. Outside sampling location was in back on the first floor.

sodium chlorite, and distilled water. This solution can generate a ClO_2 concentration of approximately 3,000 ppm.[16] The ClO_2 gas generator used a sparging column into which the ClO_2 solution was pumped. Air from the house was pumped into the sparging column (countercurrent to the ClO_2 solution). The air picked up the ClO_2 from the solution and was then returned to the house. When the ClO_2 concentration reached the desired level (650 ppm), the pumping of the liquid solution into the gas generator was stopped. The ClO_2 concentration was monitored during the treatment and additional ClO_2 was added to the house using this method to keep the ClO_2 at the above-specified level. The air inside the house was neutralized using a negative air scrubbing system with activated charcoal after the target contact time of 12.5 hr had been obtained. The spent liquid and remaining ClO_2 generator solution was treated with 10% sodium hydroxide.[16]

During the treatment process, ClO_2 concentrations were monitored inside the house on each floor remotely (using polyvinyl chloride tubing) and outside the house every 15 min by the commercial remediation company. Samples were collected into a midget impinger containing 5% potassium iodide phosphate buffer solution in conjunction with a Gillen sampling pump at 1 L/min. Pre- and postcalibration of the impinger/pump were performed using a mini-Buck calibrator (A.P. Buck, Inc.). The samples were analyzed by the commercial remediation company using a sodium thiolsulfate titration method.[23,26] An average total exposure level of 10,351 ppm · hr (calculated as ppm × hrs) was achieved for treatment. The average concentrations over the 12.5-hr treatment period for the basement and first, second, and third floors were 739, 902, 845, and 821 ppm, respectively. The treatment process did not start until all floors had measured ClO_2 concentrations above 500 ppm to assure appropriate air mixing. Relative humidity (RH) and temperature inside the house were measured in real-time with HOBO units (Onset Computer Corp.). The house was maintained at approximately 75 °F temperature and 70% RH.

To ensure that safe entry could be made, Dräger colorimetric detector tubes were used to determine the remaining concentration of ClO_2 48 hr after treatment. This time frame was similar to the decontamination procedure applied to anthrax-contaminated buildings to allow any remaining ClO_2 gas inside the house to off-gas and react with any remaining organic materials.[27] The tent was kept under positive pressure to prevent fungi and bacteria from entering the house from the outside environment.

Field Study Microbial Sampling

Environmental microbial sampling was conducted by using the same protocol before and after ClO_2 treatment. As presented in Table 1, microbial contamination was assessed using a series of standardized microbial monitoring techniques at the established environmental sampling locations. Airborne culturable count was determined by collecting samples with an Andersen N-6 single stage impactor on malt extract agar (MEA) and tryptic soy agar (TSA) in triplicate. Air samples for total microbial counting were collected using an Air-O-Cell spore trap sampler (Zefon International, Inc.). Three parallel filter samples of the air were collected: one for PCR, one for endotoxin, and one for (1→3)-β-D-glucan assay. PCR samples were collected on a 0.3-μm pore-size, 37-mm polytetrafluoroethylene (PTFE) filter. The PCR analysis was conducted to determine the spore equivalent count of 23 selected fungal species using standard protocols and prepared primer sequences for biological agents as patented by EPA.[28] Endotoxin and (1→3)-β-D-glucan samples were collected on 5-μm pore size, 37-mm polycarbonate (PC) and 0.3 μm pore size 37-mm PTFE filters, respectively. The samples were analyzed by the LAL assay. Microscopic analysis of tape samples collected from surfaces was performed to determine the level and form of fungal growth. The samples were collected on commercially prepared slides by placing the slide directly on the surface of interest and examined under a direct optical microscope using a lactophenol cotton blue stain. The laboratory analyses for the field samples were conducted by Aerotech/P&K Laboratories, Inc., with the exception of the endotoxin analysis, which was conducted by DataChem Laboratories, Inc.

The relative efficiency of the treatment for each of the measured microbial sample type for each sample location was calculated as:

Table 1. Sampling and analytical methods utilized in the field study.

Analyte	Sample Locations	Sampler	Media	Analytical Method	Average Sampling Time (minutes)	Flow Rate (L/min)
Air samples						
Culturable fungi	1–9	Andersen N-6	MEA	Cultivation (identification based on colony morphology)	3	28.3
Total fungi	1–9	Air-O-Cell	Slide	Microscopic counting (identification based on spore morphology)	3	15
PCR fungi	1–9	3-piece cassette	Filter (0.3-μm PTFE)	Real-time PCR (DNA)	320	2
(1→3)-β-D-glucan	3,4	3-piece cassette	Filter (0.3-μm PTFE)	LAL	320	2
Culturable bacteria	3,4	Andersen N-6	Trypticase soy agar	Culturability (identification by MIDI, Inc., gas chromatography)	3	28.3
Endotoxin	1–9	Cassette	Filter (5-μm PVC)	LAL	320	2
Surface samples						
Total fungi	Various	Sticky tape	Slide	Microscopic counting	N/A	N/A

Notes: N/A = not applicable.

$$\textit{Relative Efficiency} = \frac{\textit{Concentration}_{\text{before}} - \textit{Concentration}_{\text{after}}}{\textit{Concentration}_{\text{before}}} \times 100\% \quad (1)$$

Two fungal species were used for the laboratory study (1): *A. versicolor* (Research Triangle Institute [RTI] 367, RTI International), and (2) *S. chartarum* (No. 29-51-05, NIOSH). The two test organisms were chosen because they were found in culture-based and PCR samples collected before and after ClO_2 treatment in the field study. Pure cultures of *A. versicolor* were grown on MEA for 7 days at room temperature. The resultant *A. versicolor* spores were harvested from the plates using glass beads with sterilized, deionized water, as described by Schmechel et al.[29] Pure cultures of *S. chartarum* were grown on MEA for 4 weeks at room temperature. The *S. chartarum* spores were collected using sterilized, deionized water. Glass beads were not used because of the sticky nature of the mature spores. The spore count of the original fungal spore suspension was determined using a hemacytometer and was adjusted to 10^6 spores/mL. To mimic spores on surfaces, 1 mL of the fungal spore suspension was vacuum filtered through a 0.2-μm pore size 25-mm PC filter. The loaded PC filters were placed in 37-mm cassettes for transport to the laboratory location.

The laboratory exposure was conducted in an exposure chamber that was filled with gaseous ClO_2. The loaded filter samples were taken out of the cassettes and loaded into a tray, which was slid into the exposure chamber. The gaseous ClO_2 was manufactured on-site using a Sabre patented generator using the same ClO_2 generation and monitoring procedures that were used for the field project. Concentrations were monitored in the exposure chamber every 15 min for the 12 hr of the exposure experiment using the sodium thiolsulfate titration method, similar to the field study.[23,26] The spores were exposed to ClO_2 gas using three time periods (4, 8, and 12 hr) at 750 ppm to achieve total contact times of ClO_2 of 3000, 6000, and 9000 ppm · hr. The chamber was purged and opened at 4, 8, and 12 hr to remove the sequentially exposed samples. Three replicate filters were used for each contact time and for the controls. The control samples were handled similarly as the other samples, except that they were not exposed to ClO_2. The average temperature and RH in the chamber were 79 °F and 84%, which are optimal conditions for gaseous ClO_2 exposure.

The exposed and control filters were placed in sterile, 50-mL centrifuge tubes containing a 10-mL extraction fluid of 0.1% (w/v) sterile peptone water with 0.01% Tween 80. The filters were allowed to soak for 10 min. An extraction method of vortexing for 2 min followed by 15-min shaker agitation under ambient temperatures was used.[30] The extraction suspensions were analyzed for total spore count, spore-equivalent count (PCR), and (1→3)-β-D-glucan.

The total spore count was determined using an epifluorescence microscope after 1 mL of the extraction fluid was stained by acridine orange and filtered through a 25-mm black PC filter.[31] Quantitative PCR assays for the two organisms was performed from 5 mL of the extraction fluid by Aerotech/P&K Laboratories, Inc. using the same protocol as for field samples. One milliliter of extraction fluid was used by the University of Cincinnati Department of Environmental Health for (1→3)-β-D-glucan analysis using a LAL kinetic Glucatell test kit (Associates of Cape Cod, Inc.). The (1→3)-β-D-glucan samples were corrected for (1→3)-β-D-glucan contamination in the sterile reagent water used as part of the extraction fluid.

DATA ANALYSES

Data analyses were performed using the SAS statistical package version 9.1 (SAS Institute, Inc.). Paired *t* tests were conducted to compare the average sample concentrations before and after the ClO_2 treatment in the field study. A one-way ANOVA was used to compare the microbial concentrations and relative efficiency values obtained for the three ClO_2 exposure levels for the two fungal species in the laboratory study. Scheffe's test was used to locate the difference that ANOVA indicated. For samples with nondetectable concentrations, one-half of the limit of detection was used in the analyses. A significance level of 0.05 was used for all statistical tests.

RESULTS

Field Study

Table 2 shows the results for the microbial sampling that was conducted at the house before and after ClO_2 treatment. Initial concentrations of culturable fungi in the house were extremely high. All plates were overgrown with a laboratory estimate of over 400 colonies per plate, which after adjusting for multiple particle impaction yields an estimate of approximately 10^6 colony forming units per cubic meter (CFU/m^3) or greater. After treatment, the geometric mean for culturable fungi was 252

Table 2. Geometric mean and range for indoor bioaerosol concentrations before and after ClO_2 treatment.

Analyte	Number of Samples before and after Treatment	Geometric Mean (range)		Relative Efficiency (% ± standard deviation)
		Before ClO_2 Treatment	After ClO_2 Treatment	
Culturable fungi (CFU/m^3)	24 + 24	≥1,000,000	252 (129–435)	97.40 ± 0.45
Total fungi (S/m^3)	8 + 8	73,454 (16,311–195,289)	1552 (978–2,267)	97.55 ± 2.45
PCR fungi (SE/m^3)	8 + 8	5,535 (943–23,598)	332 (118–706)	90.45 ± 11.2
(1→3)-β-D-glucan (pg/m^3)	3 + 3	<125 (LOD)	736 (580–1100)	
Culturable bacteria (CFU/m^3)	6 + 6	1,077 (718–1,319)	158 (82–353)	84.93 ± 7.74
Endotoxin (EU/m^3)	8 + 8	10.32 (1.31–34.19)	18.59 (4.45–41.4)	

Notes: Values in parentheses are from results from the field study.

CFU/m^3. Paired t tests comparing the culturable fungal concentrations before and after treatment showed a statistically significant difference ($p = 0.0001$). The average relative efficiency against culturable fungi was 97.4% using the 10^6-CFU/m^3 estimate. The predominant fungal types in the house before ClO_2 treatment were *Aspergillus niger*, *A. versicolor*, *Cladosporium* sp., *Mucor* sp., and *Penicillium* sp.; whereas after ClO_2 treatment, *A. versicolor*, *Penicillium* sp., and *Sporobolomyces* sp. dominated. The data for the outside samples are presented in Table 3. The geometric means for the outside samples were 548 CFU/m^3 before treatment and 144 CFU/m^3 after treatment. For both before and after ClO_2 treatment, the geometric mean for the outside samples was significantly different when compared with the inside samples (before $p < 0.0001$; after $p = 0.0015$) The predominant general class for the outside samples before treatment were *Basidiomycetes*, *Cladosporium* sp., *Penicillium* sp., and *Epicoccum nigrum* and, after treatment, were *Aspergillus fumigatus*, *Aureobasidium pullulans*, *Basidiomycetes*, *Cladosporium* sp., *E. nigrum*, *Penicillium* sp., and *Pithomyces chartarum*.

The geometric mean for indoor total spore counts determined from Air-O-Cell samples was 73,454 spores per cubic meter (S/m^3) before the ClO_2 treatment, and 1552 sec/m^3 after treatment, and this difference was statistically significant ($p = 0.0052$). The average relative efficiency against total fungi was 97.55%. *Aspergillus/Penicillium*, *Stachybotrys*, *Basidiospores*, *Cladosporium*, and *Chaetomium* were the most commonly detected fungal spores before treatment in the spore trap samples. After treatment, *Ascospores*, *Aspergillus/Penicillium*, *Basidiospores*, and *Cladosporium* were found in the air samples. Outside concentrations of total fungi were 3556 sec/m^3 before treatment and 444 sec/m^3 after treatment. The predominant general classes for the outside samples both before and after treatment were *Ascospores*, *Basidiomycetes*, *Cladosporium*, and *Aspergillus/Penicillium*. *Curvularia* and *Myxomycetes* were also detected before treatment and *Torula* after treatment.

The geometric means for the PCR samples before and after ClO_2 treatment were 5535 and 332 spore equivalents per cubic meter (SE/m^3), respectively. These concentrations were significantly different ($p = 0.0249$), and the average relative efficiency was 90.45%. The five most commonly detected fungal species in the house using PCR analyses were *A. versicolor*, *Eurotium (Aspergillus) amstelodami*, *C. cladosporioides*, *Penicillium brevicompactum*, and *S. chartarum*. Figure 2 shows the relative efficiency of the treatment processes for these five species. *A. versicolor* and *S. chartarum* showed the highest relative efficiency (~100%), followed by *E. (Aspergillus) amstelodami* (95%), *P. brevicompactum* (90%), and *C. cladosporioides* (85%). Outside fungal spore concentrations were 375 SE/m^3 (before) and 76 SE/m^3 (after).

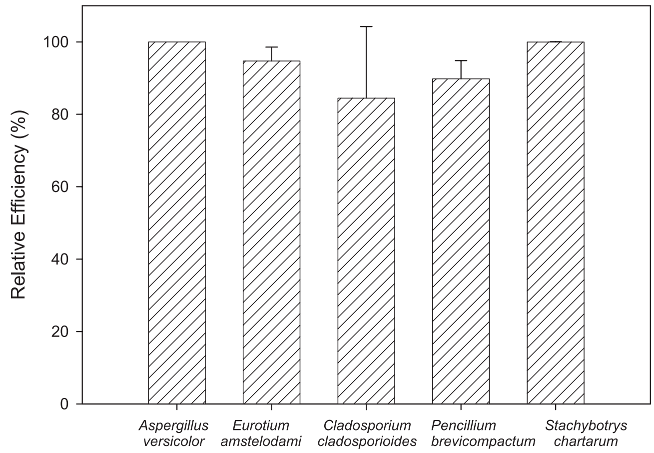

Figure 2. Relative efficiency of treatment (average and standard deviation) for the five most common fungal species detected in the PCR analyses.

The geometric means for (1→3)-β-D-glucan samples before and after ClO_2 treatment were below the limit of detection (LOD) and 736 pg/m^3, respectively. Paired t tests comparing the (1→3)-β-D-glucan concentrations before and after treatment showed no significant difference ($p = 0.0512$).

The bacterial species detected were highly variable between the sampling locations in the house and included both Gram-negative and Gram-positive organisms. Most commonly found species both before and after treatment were *Aeromonas caviae*, *Bacillus mycoides*, *Bacillus sphaericus*, *Brevibacillus brevis*, *Brevibacterium casei*, *Brevundimonas vesicularis*, *Chryseobacterium indologenes*, *Comamonas testosteroni*, *Enterococcus durans*, *Flavimonas orzyhabicans*, *Micrococcus luteus*, *Pseudomonas fluorescens*, *Psychrobacter phenylpyruvicus*, *Rhizobium radiobacter*, *Sphingomonas paucimobilis*, *Staphyococcus xylosus*, and *Streptomyces*. The majority of these bacteria are environmental species. The geometric means for indoor culturable bacteria samples before and after ClO_2 treatment were 1077 and 158 CFU/m^3, respectively. These concentrations were significantly different ($p = 0.0067$), resulting in an average relative efficiency of 85%.

Table 3. Geometric mean and range for outdoor bioaerosol concentrations before and after ClO_2 treatment.

Analyte	Number of Samples before and after Treatment	Geometric Mean (Range)	
		Before ClO_2 Treatment	After ClO_2 Treatment
Culturable fungi (CFU/m^3)	3 + 3	548 (380–710)	144 (94–188)
Total fungi (S/m^3)	1 + 1	3556	444
PCR fungi (SE/m^3)	1 + 1	375	76
Culturable bacteria (CFU/m^3)	3 + 3	1197 (1119–1319)	67 (35–94)
Endotoxin (EU/m^3)	1 + 1	0.74	21.92

Notes: Values in parentheses are from results from the field study.

Endotoxin concentrations before and after ClO_2 treatment were 10.32 and 18.59 endotoxin units per cubic meter (EU/m^3), respectively. Paired *t* tests comparing endotoxin concentrations before and after treatment showed no significant difference ($p = 0.2289$). Outside endotoxin levels were 0.74 EU/m^3 before and 21.92 EU/m^3 after the gas application.

As shown in Table 4, tape sampling results showed the presence of spores, hyphae, and conidiophores of *Aspergillus*, *Cladosporium*, *Penicillium*, *Scopulariopsis*, and *S. chartarum* on the surfaces before treatment. After treatment, it was still possible to identify spores, hyphae, and conidiophores of *Aspergillus*, *Cladosporium*, and *Penicillium* using microscopic techniques at the same levels of contamination as found before ClO_2 treatment.

Laboratory Study

Table 5 presents the total count, PCR, and (1→3)-β-D-glucan results for the laboratory study. Spores were easily visible using the acridine orange stain for total counting. A one-way ANOVA analysis of the total count concentrations for *A. versicolor* found no significant differences between the control samples and samples treated with the three ClO_2 contact times ($p = 0.6676$). The average relative efficiencies for the contact times of 3000, 6000, and 9000 ppm · hr were 51.7, 17.7, and 23.6%, respectively, and these values were not significantly different ($p = 0.6454$). The *S. chartarum* spores were not evenly distributed on the filters and therefore the direct count totals from the filters were considered unreliable and are not reported.

The PCR samples for both species showed lower counts as the ClO_2 exposure increased. The PCR total count was especially low for the *S. chartarum* samples. One-way ANOVA analyses of the PCR concentrations for *A. versicolor* showed only a borderline significant difference between the controls and the three ClO_2 exposure levels ($p = 0.0543$), whereas for *S. chartarum,* this difference was more pronounced ($p = 0.0410$). Post-hoc analysis of *S. chartarum* results showed that the means for the three ClO_2 contact times were similar to each other but significantly lower than the control. For *S. chartarum*, the relative efficiency value was 99.99% for the exposure level of 3000, and 100% for the two higher levels.

The (1→3)-β-D-glucan concentrations increased with increasing exposure to ClO_2. (1→3)-β-D-glucan levels for *A. versicolor* ranged from 8.48 to 13.75 ng/mL and for *S. chartarum* from 43.11 to 399.5 ng/mL. A one-way ANOVA analysis of the (1→3)-β-D-glucan concentrations for *A. versicolor* revealed significant differences between the controls and the three ClO_2 contact

Table 4. Fungal identification from tape samples using optical microscopy.

Sample Location	Sample Site	Fungal Identification	Category[a]
Before ClO_2 treatment			
Basement	Galvanized duct	*Penicillium*	A few
Basement	Wood board over utility sink	*Cladosporium*	A few
First floor	Door frame between dining room and porch	*Penicillium*	A few
First floor	Painted fireplace mantel	*Aspergillus*	A few
		Cladosporium	A few
		Penicillium	Many
First floor	Wallpaper between two front windows	None	None
Second floor	Shelving between windows in pink room	*Aspergillus*	A few
		Stachybotrys chartarum	A few
Second floor	Plaster wall under wallpaper near front window	*Aspergillus*	A few
Second floor	Yellow room wall by safe	*Aspergillus*	Many
		Cladosporium	Many
		Penicillium	Massive
		Scopulariopsis	Massive
Third floor	Sink countertop in back room	*Aspergillus*	Many
		Chaetomium	A trace
		Cladosporium	A few
		Penicillium	A few
After ClO_2 treatment			
Basement	Galvanized duct	*Aspergillus*	Many
		Zygomycetes	A few
Basement	Wood board over utility sink	*Aspergillus*	Many
		Cladosporium	Many
First floor	Painted fire place mantel	*Aspergillus*	A few
		Penicillium	Numerous
First floor	Door frame between dining room and porch	*Penicillium*	Many
First floor	Wallpaper between two front windows	None	None
Second floor	Pink room on mold growth	*Gliocladium*-like	Massive
Second floor	Plaster wall under wallpaper near front window	None	None
Second floor	Plaster wall near front window	None	None
Third floor	Sink countertop in back room	*Aspergillus*	Many

Notes: [a]massive > numerous > many > a few > a trace.

Table 5. Average total counts and relative efficiency with standard deviations obtained in the laboratory study.

Analyte	Concentration (average ± standard deviation)				Relative Efficiency (average ± standard deviation)		
	Control	3000 ppm · hr	6000 ppm · hr	9000 ppm · hr	3000 ppm · hr	6000 ppm · hr	9000 ppm · hr
A. versicolor							
Total fungi	$3.39 \times 10^6 \pm 2.12 \times 10^6$	$1.64 \times 10^6 \pm 7.65 \times 10^5$	$2.79 \times 10^6 \pm 1.05 \times 10^6$	$2.59 \times 10^6 \pm 2.35 \times 10^6$			
PCR fungi (no./mL)[a]	$3.20 \times 10^5 \pm 2.79 \times 10^5$	$3.34 \times 10^3 \pm 5.76 \times 10^3$	$7.10 \times 10^2 \pm 1.20 \times 10^3$	2.33 ± 2.31			
(1→3)-β-D-glucan (ng/mL)	6.52 ± 0.81	8.48 ± 1.4	13.70 ± 3.9	13.75 ± 1.9	−30.1 ± 21.5	−110.1 ± 59.6	−110.9 ± 29.4
S. chartarum							
PCR fungi	$2.89 \times 10^4 \pm 2.38 \times 10^4$	3.33 ± 4.04	1 ± 0	1 ± 0	99.99 ± 0.01	100 ± 0	100 ± 0
(1→3)-β-D-glucan	64.61 ± 82.33	43.11 ± 38.5	139.95 ± 80.4	399.50 ± 266.8			

Notes: Three filters were used for each set of samples. [a]#/mL = number of spores per milliliter of solution.

times (p = 0.0100), whereas no significant differences were found for *S. chartarum* (p = 0.0599). The post-hoc analysis of *A. versicolor* results showed that the control values were significantly lower than the 6000 and 9000 ppm · hr contact times.

DISCUSSION

In the field study, the relative efficiencies obtained using ClO_2 for culturable fungi and bacteria, total fungal spore counts from spore traps, and total fungal spore counts from PCR analyses ranged from 84.9 to 97.6%. In contrast, the relative efficiency for the (1→3)-β-D-glucan and endotoxin showed negative values (−515 and −96%). These results indicate that the ClO_2 treatment decreased both the culturable and total counts of airborne microorganisms but had no measured effect on the concentrations of their components, i.e., (1→3)-β-D-glucan and endotoxin.

The outside fungal concentrations after the treatment process may have been lower than anticipated because of a snow event prior to and during the sampling period. However, the initial indoor culturable fungal concentrations indoors were much higher than outside concentrations and the species profile in indoor air differed from that in outdoor air. Therefore, the decrease in the indoor air concentrations cannot be explained by the decrease in the outdoor air concentration. ClO_2 levels had been measured around this site using the EPA trace atmospheric gas analyzer (TAGA) van which had maximum concentration spikes of 2.5 ppb; the steady state for the ClO_2 was below the LOD (parts per trillion).[32]

The field samples were collected from the air to gain information on inhalation exposures. The laboratory study simulated fungal spores on surfaces, which is the "worst-case" scenario for ClO_2 treatment because it is harder to deactivate spores on surfaces than in the air. Compared with the field study, the laboratory study showed similar, although less significant trends for relative efficiencies in terms of decrease in PCR total counts of fungi and increase in (1→3)-β-D-glucan concentrations after exposure to ClO_2 gas. Some of the variability for the total spore counts obtained in the laboratory study may be explained by differences in the extraction process or in the initial spore suspension. The temperature and RH of the laboratory study was higher than the field study. This was because of the difficulty of maintaining the optimal conditions for treatment in the winter. This may explain some of the variability between the two studies. Three contact times were used in the chamber experiment to investigate the effect of increasing dose on the fungal spores in terms of total count, PCR count, and (1→3)-β-D-glucan. The 6000-ppm · hr contact time was reflective of the original contact time approved by EPA's waiver for the treatment of *B. anthracis*. This was later revised to 9000 ppm · hr. The current laboratory study showed a trend of higher relative efficiency with increasing contact time for total fungi for both fungal species and the opposite trend for (1→3)-β-D-glucan for the *A. versicolor* spores.

Our results are consistent with those obtained in a previous laboratory-based study by Wilson and associates who used initial ClO_2 concentrations of 1000 ppm in an enclosed chamber against fungal colonies and spores.[22] They found a reduction of more than 87% in the culturable spore count. There was no difference reported in the hemacytometer-measured total spore counts between control and exposed samples when treating fungal colonies with ClO_2, but a significant decrease was observed when treating purified spores. Furthermore, the gaseous ClO_2 exposure did not decrease the activity of two trichothecene mycotoxins (roridin A and verrucarin A) or trichothecene mycotoxins from *S. chartarum* spores.[22,33] These results are consistent with the data from our field study because no change was observed in the semiquantitative microscopic evaluation of tape samples to assess surface contamination, but a clear decrease was seen in the culturable and total count of airborne spores. Furthermore, an increasing trend was observed for endotoxin and (1→3)-β-D-glucan both in the laboratory and field study supporting the results of Wilson et al. on the inefficiency of ClO_2 treatment in reducing the concentrations of fungal components.[22,33]

Rendering microorganism nonculturable will prevent microbial growth and further production of harmful agents, such as endotoxin, (1→3)-β-D-glucan, mycotoxins, and allergens. However, the destruction of bacterial cells, fungal spores, and hyphae during the treatment will not remove these components. Exposure to endotoxin has been associated with respiratory complaints in indoor environments and a wide-range of symptoms such as fever, cough, and shortness of breath. There are some studies that have shown (1→3)-β-D-glucan to have proinflammatory capacities and are associated with adverse nonallergic respiratory health effects.[3,34,35] Therefore, attention has to be paid to removal of particulate matter from the air and surfaces after treatment with ClO_2 gas so that acceptable conditions are achieved before reoccupancy. Assessment of microbial components, such as endotoxin or (1→3)-β-D-glucan, should be part of clearance sampling after ClO_2 treatment.

Before the treatment, the levels of total fungi, culturable fungi, and endotoxin found in the house were similar or lower when compared with those reported in two recent studies performed in flooded homes in New Orleans. Rao and associates reported a geometric mean of 2.8×10^5 S/m^3 for total fungi, 0.7×10^5 CFU/m^3 for culturable fungi, and 22.3 EU/m^3 for endotoxin in moderately (n = 5) to heavily flooded homes (n = 15) in New Orleans.[7] Chew et al.[8] found the following ranges for total fungi, culturable fungi, and endotoxin in three homes before renovation: $(0.8–6.3) \times 10^5$ S/m^3, $(0.22–5.2) \times 10^5$ CFU/m^3, and 17–139 EU/m^3, respectively. In this study, the respective concentrations were approximately 0.7×10^5 S/m^3, 10×10^5 CFU/m^3, and 18.6 EU/m^3. Park et al.[36] reported lower endotoxin levels, with a geometric mean 0.64 EU/m^3 (range: 0.02–19.8 EU/m^3) from the bedrooms of 15 homes located in the greater Boston, MA, area. Outdoor levels of endotoxin before treatment were comparable to the ones in California outdoor air (0.44 EU/m^3),[37] and Denmark outdoor air (0.33 EU/m^3).[38] It should be noted, however, that short-term exposure to endotoxin levels above 45 EU/m^3 has been associated with decreases in lung function and respiratory inflammation, although these levels are higher than the levels found in this study.[39,40]

The (1→3)-β-D-glucan concentrations obtained in the field and laboratory studies were similar to those reported in other studies, which used LAL for (1→3)-β-D-glucan analysis.[3] The reported concentrations ranged from nondetected to 19 ng/m^3 in indoor environments. In this study, the average (1→3)-β-D-glucan concentration was 0.74 ng/m^3 in the house after treatment. Bacteria concentrations in the house were much higher (1077 CFU/m^3) than those found in non-problem buildings in the United States (average 102 CFU/m^3).[41] Levels of bacteria in microbially contaminated homes are not readily available for the United States.

Traditionally, monitoring for bioaerosols has consisted of culturing and microscopic counting of fungi and bacteria using short-term samples.[42] There are many advantages to using newer PCR technologies for indoor air environments, including quick turnaround of sample results, accurate identification and reproducibility, and the detection of nonviable fungi and fungal spores. The technology also allows for a long sampling time to get a better understanding of environmental exposures.[43] However, the PCR method is also very sensitive to environmental interferences in field settings that are difficult to identify.[18] The PCR method was used in this study along with traditional cultivation and microscopic counting techniques to assess the efficiency of ClO_2 treatment against fungi. The predominant species for fungal contamination in the house were similar for these three methods. *Aspergillus, Penicillium*, and *Cladosporium* species were among the five most commonly detected by the three methods before the treatment. *Stachybotrys* was detected by both the microscopic counting and PCR, but not by cultivation. For the relative efficiency of ClO_2 treatment, these methods exhibited similar trends, but the highest efficiency was found with the culture-based technique. In the field study, both the total microscopic count and the PCR count obtained for air samples decreased significantly. This could be caused by direct reduction of spores in the air or reduction of spores on surfaces that would serve as the source for the airborne spores. However, no decrease was observed in the semiquantitative analysis of amount of spores and hyphae in the sticky tape samples collected from surfaces. Furthermore, the laboratory study, which evaluated the efficiency of ClO_2 treatment on spores on surfaces, did not show any decrease in the total microscopic count of spores. In contrast, a decrease was observed for PCR count on surfaces.

The discrepancy between the microscopic, culturable, and PCR counts could be caused by injury to the fungal spores, deactivation of DNA, or inhibition of the PCR assay by the ClO_2 gas. Previous studies have shown that environmental contaminants in the indoor environment can inhibit PCR analyses, which may also give a false-negative result.[18,44,45] Buttner and fellow investigators[46] also identified the issue of inhibition of PCR for environmental samples in their surface disinfection study using gaseous ClO_2 and foam decontaminant. They also found that DNA and other compounds capable of producing immune responses were still present after treatment.

CONCLUSIONS

This study showed that gaseous ClO_2 treatment can be used to kill fungi and bacteria in a field setting after the source of moisture incursion has been addressed. The treatment also reduced the total fungi in the air of the treated house. The laboratory study supported the results obtained in the field study in terms of reduction of PCR-determined total counts but it remains unclear if this was due to inhibition of the PCR assay caused by ClO_2 gas. The fungal spores were visible using microscopic techniques both in the field and laboratory settings. To document the effectiveness of the ClO_2 treatment of microbially contaminated houses, environmental sampling techniques should include the collection of samples for culturable microorganisms as well as endotoxin and (1→3)-β-D-glucan before and after the treatment process. These results call for additional clean-up techniques such as use of air cleaners and cleaning surfaces with vacuums using high-efficiency particle air filters to reduce exposures to remaining spores and microbial components after gaseous ClO_2 treatment in microbially contaminated indoor environments.

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