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Inactivation of bacterial and fungal spores by UV irradiation and gaseous iodine treatment applied to air handling filters

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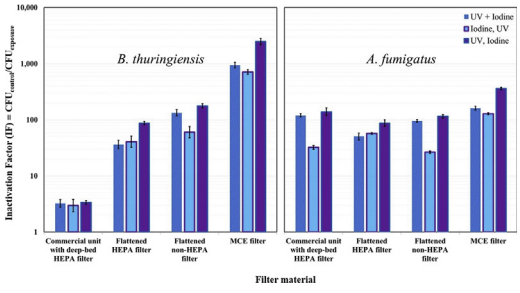
HIGHLIGHTS

- UV and gaseous iodine treatment can inactivate Btk and *A. fumigatus* on filters.
- UV combined with iodine produced a synergistic inactivation effect for Btk spores.
- Applying UV first and iodine second produced the highest inactivation level.
- Filter type influenced the inactivation for Btk, but not as much for *A. fumigatus*.

GRAPHICAL ABSTRACT



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ABSTRACT

Exposure to viable bacterial and fungal spores re-aerosolized from air handling filters may create a major health risk. Assessing and controlling this exposure have been of interest to the bio-defense and indoor air quality communities. Methods are being developed for inactivating stress-resistant viable microorganisms collected on ventilation filters. Here we investigated the inactivation of spores of *Bacillus thuringiensis* var. *kurstaki* (Btk), a recognized simulant for *B. antracis*, and *Aspergillus fumigatus*, a common opportunistic pathogen used as an indicator for indoor air quality. The viability change was measured on filters treated with ultraviolet (UV) irradiation and gaseous iodine. The spores were collected on high-efficiency particulate air (HEPA) and non-HEPA filters, both flattened for testing purposes to represent "surface" filters. A mixed cellulose ester (MCE) membrane filter was also tested as a reference. Additionally, a commercial HEPA unit with a deep-bed (non-flattened) filter was tested. Combined treatments of Btk spores with UV and iodine on MCE filter produced a synergistic inactivation effect. No similar synergy was observed for *A. fumigatus*. For spores collected on an MCE filter, the inactivation effect was about an order of magnitude greater for Btk compared to *A. fumigatus*. The filter type was found to be an important factor affecting the inactivation of Btk spores while it was not as influential for *A. fumigatus*. Overall, the combined effect of UV irradiation and gaseous iodine on viable bacterial and fungal spores collected on flat filters was found to be potent. The benefit of either simultaneous or sequential treatment was much lower for Btk spores embedded inside the deep-bed (non-flattened) HEPA filter, but for *A. fumigatus* the inactivation on flattened and non-flattened HEPA filters was comparable. For both species, applying UV first and gaseous iodine second produced significantly higher inactivation than when applying them simultaneously or in an opposite sequence.

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1. Introduction

Ultraviolet (UV) radiation is conventionally used as a germicide for "killing" (inactivating) of viable microorganisms, including bacteria, fungi, and viruses (Al-Gabr et al., 2013; Koivunen and Heinonen-Tanski, 2005; Kujundzic et al., 2006, 2007; Xu et al., 2003). UV irradiation in the "C" band (wavelength of 200–280 nm) can generate the highest germicidal effect compared to other bands such as UV-A (320–400 nm) and UV-B (290–310 nm) (Braga et al., 2015; Guerrero-Beltran and Barbosa-Canovas, 2004; Ozcelik, 2007). UV-C can be used for disinfecting surfaces and water, treating food, and purifying air in health-care facilities because, in contrast to some other microbial inactivation methods, it does not generate hazardous by-product in the process (Begum et al., 2009; Kujundzic et al., 2006). The primary action of UV irradiation affecting microorganisms is DNA damage (Lin and Li, 2002; Setlow, 2006).

Iodine is a well-known disinfectant (Gottardi, 2014; Russell, 1990). It has been shown to effectively inactivate bacterial vegetative cells and viruses in water media (Aviv et al., 2013; Mazumdar et al., 2010; Pennell et al., 2008). Additionally, combustion products of iodine-containing reactive materials have been demonstrated to produce a very high inactivation effect on aerosolized bacterial spores with inactivation factors (*IF*) reaching and exceeding ~ 10^5 (Aly et al., 2014; Grinshpun et al., 2017; Nakpan et al., 2018; Wang et al., 2018). *IF* is defined as the inverse of the survived microbial fraction, which is determined as the ratio of concentrations of the colony forming units (CFU) in the control sample and the one exposed to the treatment. Some studies have shown that the iodine interaction with the coat and cortex of spores causes the loss of viability (Bloomfield and Arthur, 1994; Li et al., 2017). Yet, no sufficient knowledge has been acquired to understand the biocidal effect of gaseous iodine, particularly on microorganisms collected on filters, and very little is known about the combined inactivation effect of gaseous iodine and UV irradiation on bacterial and fungal spores collected on filters.

An air filtration unit is usually a part of any heating, ventilation and air conditioning (HVAC) system. While the HVAC filters are capable of removing aerosol particles including dust and microorganisms from indoor air environments, they can become reservoirs for potentially hazardous particles, including bacterial and fungal spores. Several studies have shown that stress-resistant spores can remain viable over a prolonged period of time and even grow in the filter media (Forthomme et al., 2014; Kemp et al., 1995; Maus et al., 2001; Simmons and Crow, 1995). The spores collected on filters can be re-aerosolized during replacement or maintenance (Morisseau et al., 2017), which poses a health risk.

The main objective of this study was to investigate source-specific and combined effects of UV irradiation and gaseous iodine on the viability of bacterial spores of *Bacillus thuringiensis serovar kurstaki* (Btk) and fungal spores of *Aspergillus fumigatus* collected on filters. Inactivation factors were determined for "flat" filters including flattened HEPA and non-HEPA ones as well as mixed cellulose ester (MCE) membrane filters.

2. Materials and methods

2.1. Microorganisms

Both species chosen for testing are very stress-resistant (Alshareef and Robson, 2014; Nicholson et al., 2002; Russell and Furr, 1996; Setlow, 2006, 2014). Btk has been widely utilized as a nonpathogenic surrogate for *Bacillus anthracis* (Ba) in defense research (Greenberg et al., 2010; Rice et al., 2005; Tufts et al., 2014). *A. fumigatus* is a ubiquitous fungus found in the outdoor environment and has been used as an indicator for mold problems in indoor environments (Cabral, 2010). *A. fumigatus* is non-pathogenic for healthy individuals, but it can be pathogenic for immunocompromised ones, causing infectious diseases, *e.g.*, aspergillosis (Alshareef and Robson, 2014; Fuller et al., 2013; O'Gorman, 2011; Youngchim et al., 2004).

Spores of Btk, strain SA-11 (product # SA-11 SDTC; technical grade concentrate developed for US Army and Air Force) were prepared in suspension as described in our previous publications (Grinshpun et al., 2017; Nakpan et al., 2018). Briefly, the dry-frozen Btk spores purchased from Certic USA Inc. (Columbia, MD, USA) were washed in sterile deionized water by vortexing and centrifugation, repeatedly. Then the spores were re-suspended in sterile filtered deionized water. Prior to an experiment, the suspension was vortexed for 2 min using a touch vortex mixing (Fisher Scientific Inc., Pittsburgh, PA, USA), then placed in an ultrasonic bath (42 KHz Fisher Ultrasonic Cleaner, Fisher Scientific Inc., Pittsburgh, PA, USA) for 15 min to reduce the spore agglomeration. The ready-to-use suspension had a culturable spore concentration of ~10^7–10^9 CFU mL^{-1}.

A strain of *A. fumigatus* (ATCC 34506™) was obtained from the American Type Culture Collection (Manassas, VA, USA) and propagated on malt extract agar (MEA) by incubating in the dark for 7 days at 25 °C. Before the experiments, *A. fumigatus* spores were harvested from plate surfaces by aseptic shaking with 3–mm glass beads. Next, the spores were washed off from the glass beads by vortexing and suspended in de-ionized water. The initial concentration of culturable *A. fumigatus* spores in the suspension was ~10^8–10^{10} CFU mL^{-1}.

2.2. Filter materials

The tests were performed with HEPA (HEPA type R, Kaz USA, Inc., Marlborough, MA, USA) and non-HEPA air filters (True Blue model, Protect Plus Industries, LLC, Hickory, NC, USA), which were flattened to eliminate the structure configuration factor (depth). The materials were cut to fit a 47–mm open face filter holder. Additionally, a perfectly flat filter ideally representing the "surface" aerosol filtration, a mixed cellulose ester (MCE) membrane filter 47 mm diameter, 0.45 μm pore size (Millipore Corporation, Billerica, MA, USA), was tested as a reference. Among other characteristics, the fibrous density of the tested filter materials was different. Finally, a full commercial HEPA unit with a 4.5 cm deep-bed (non-flattened) filter was tested.

2.3. Experimental protocol

The tests were performed in a chamber of approximately 90 L volume made of transparent Plexiglas. The chamber was placed in a Biosafety Level II cabinet (SterilchemGARD, Baker Co., Sanford, ME, USA).

The challenge microorganisms were aerosolized from the suspension using a 6-jet Collison nebulizer (BGI Inc., Waltham, MA, USA) operating at 6 L min^{-1}. The tested "flat" 47-mm pre-cut filter piece was placed into a stainless steel, open-face filter holder tightened with a rubber O-ring (Model KS47, Thomas Scientific, NJ, USA). The filter assembly was placed inside the chamber and connected to an air pump (Air Diagnostics and Engineering, Inc., Harrison, ME, USA). The sampling flow rate of 7.5 L min^{-1} was set to match a linear air face velocity of 0.07 m s^{-1} calculated for a commercial HEPA air purifying unit (HPA106, Honeywell, Palatine, IL, USA) considering its full filter area and the nominal flow rate of 2550 L min^{-1} ("Germ mode").

Following a 10-min collection of the aerosolized spores, a loaded filter was assigned as non-treated (control). The deposit was incubated and analyzed using the culture-based method (see Section 2.3.4 below) to determined $CFU_{control}$. To generate a treated sample, the same procedure was repeated, but the filter loaded with spores was subsequently exposed to a specific treatment (UV or iodine or their combinations). Similarly, the deposit was incubated and analyzed, and the culturable spore count $CFU_{treated}$ was determined. The inactivation was quantified according to Eq. (1).

$$IF = CFU_{control}/CFU_{treated} \quad (1)$$

As a reference material, an MCE filter loaded with spores was tested with a single source-specific treatment (either UV or iodine). Other filters were tested only with combined treatments involving both the UV irradiation and gaseous iodine.

2.3.1. UV irradiation

An 11–W low-pressure mercury lamp radiating primarily (>90%) at 254 nm with an intensity of 6 mW cm^{-2} (measured at the test-relevant source-to-filter distance) was used as a UV source in this study. The irradiance measurements were made using a radiometer (ILT-1700, International Light Technologies, Peabody, MA, USA) connected to a UV-C sensor (SED033–25, International Light Technologies, Peabody, MA, USA). The UV lamp was placed in front of the open-face filter holder (when testing "flat" filters) or in front of the air purifier (when testing with the full HEPA deep-bed filtration unit). The duration of the UV treatment was 10 min.

2.3.2. Iodine treatment

Crystal iodine acquired from Carolina Biological Supply Company (Burlington, NC, USA) was utilized to generate gaseous iodine in the chamber. The crystal iodine was placed in a ceramic plate at the chamber entrance and heated by an electrical heater at a temperature of 110 °C (±10 °C). The concentration of vaporized iodine in the chamber was in a range of 3 to 5 ppm as measured by a photoionization detector (PGM 7340, ppbRAE3000, RAE Systems, Inc., San Jose, CA, USA). The duration of the iodine treatment was 10 min.

2.3.3. Combination of treatments

The combined treatment involving UV irradiation and gaseous iodine was done in three ways: simultaneous application (labeled as "UV + Iodine") and sequential applications including UV followed by iodine (labeled as "UV, Iodine") and iodine followed by UV (labeled as "Iodine, UV"). In the sequential applications, each treatment was applied for 10 min – one immediately following the other.

2.3.4. Sample extraction and viability quantification

The collected microbial samples were analyzed to determine the concentration of viable spores using the conventional culture-based method. Immediately after sample collection, the filters were placed in a 50–mL sterile centrifuge tube and immersed in 10 mL of 0.05% Tween–80 (Sigma Aldrich Inc., St. Louis, MO, USA) solution. After the tube was vortexed for 2 min, volumes of aliquots ranging from 100 μL to 1 mL were taken for cultivation in triplicate. The proper volume was determined based on the anticipated CFU counts on the culture plates. Btk samples were incubated on tryptic-soy agar (TSA) plates at 37 °C for 24 h. *A. fumigatus* samples were incubated on MEA at 25 °C for 5–7 days.

2.4. Data analysis

The data set was log-normally distributed. Therefore, the geometric mean (GM) and the geometric standard deviation (GSD) were used to calculate *IF* values for each set of replicates. The spore inactivation levels produced by different treatments were compared using an analysis of variance (ANOVA), with *post hoc* comparisons of the means by the Tukey test, as required. Significant differences were considered where p-value ≤0.05.

3. Results and discussion

3.1. Inactivation effect of single and combined treatments on MCE filter

Since the MCE was selected a reference filter as a perfectly "flat" one (to eliminate the depth factor), the results obtained with this filter are presented first. Fig. 1 shows the *IF* values for Btk and *A. fumigatus* spores collected on MCE filters as a result of a source-specific and combined treatments with UV irradiation and gaseous iodine. The iodine treatment alone showed a small inactivation effect on Btk spores (*IF* ~2), while its effect on *A. fumigatus* spores was found to be much greater (*IF* ~60) ($p < 0.01$). It is not surprising to observe a loss of microbial viability in spores originated by the iodine treatment. Indeed, it has been reported that oxidizing agents, including iodine compounds as well as chlorine compounds and hydrogen peroxide, can cause damage to the spore coat, rupture cells, disable proteins inside the cortex, and break DNA bonds (Melly et al., 2002; Russell, 2003; Tennen et al., 2000; Young and Setlow, 2003). Vastly different responses produced by Btk and *A. fumigatus* spores can be attributed to the substantial differences in their structure and composition. For instance, Btk spores possess multi-layer coats including exosporium, outer membrane, cortex, inner membrane, and center core (Nicholson et al., 2000; Plomp et al., 2005; Russell, 1990, 2003; Setlow, 2006). This presents a challenge for gaseous iodine to penetrate into the cortex and inactivate the bacterial spores (Bloomfield and Arthur, 1994). Meanwhile, *A. fumigatus* spores are likely to be more susceptible to iodine treatment because it has lower and thinner outer cell layers compared to Btk (Bernard and Latgé, 2001; Latgé, 2001; Russell and Furr, 1996; Setlow, 2014).

As seen in Fig. 1, UV treatment alone generated about the same inactivation on Btk and *A. fumigatus* spores (*IF* values ~30, $p > 0.05$). This is consistent with the fact that UV irradiation (especially UV-C) can directly damage DNA inside the core of the microorganism (Nicholson et al., 2000, 2002) and inhibit the cell germination (Setlow et al., 2014; Setlow, 2001). Similar to the results of this study, Lin and Li (2002) reported that low UV doses can produce comparable inactivation levels in aerosolized bacterial and fungal spores. The inactivation effect of UV irradiation is expected to increase at a longer treatment time or higher UV irradiance intensity for both species (Chang et al., 1985; Kujundzic et al., 2006; Levetin et al., 2001; Lin and Li, 2002; Luna et al., 2008; Ozcelik, 2007).

Further, the effect produced by a combination of the two treatments was investigated for the following scenarios: simultaneous application ("UV + Iodine") and two sequential applications ("UV, Iodine" and "Iodine, UV"). As presented in Fig. 1, UV irradiation combined with gaseous iodine exhibited a synergistic inactivation effect for Btk spores collected on MCE filters. The principal target of UV irradiation is DNA bonds, while chemical disinfectants, such as iodine or others in halogen group usually affect the spore coats, membranes, and enzyme systems (Braga et al., 2015; Nicholson et al., 2000; Setlow et al., 2014; Setlow, 2006). It has been found that UV irradiation not only can energize and break down the DNA bonds, but also can degrade the cell membranes of *Bacillus* spores (Raguse et al., 2016). Therefore, the possible mechanism of synergy discovered in this study can be associated with the UV irradiation that damages cell walls, thus enhancing the penetration of gaseous iodine through these walls. Other studies on inactivation of *Bacillus* spores using UV irradiation followed by the application of disinfectants such as hydrogen peroxide showed a similar synergy (Reidmiller et al., 2003; Zhang et al., 2014). The data analysis showed that there was no statistical difference in *IF* values ($p > 0.05$) generated by the sequential application "Iodine, UV" and the simultaneous application "UV + Iodine", but both combined treatments produced lower inactivation levels than the sequence of "UV, Iodine" ($p < 0.05$). The latter may be associated with differences in iodine penetration with the highest one achieved when UV is applied first "prepping" the bacterial spore coat for the subsequent damage by iodine. However, even if the iodine is deployed simultaneously with UV or prior to UV, the synergy of both treatments is still generated. It is because iodine compounds are deposited on the cell membrane, and when the spore is exposed to the UV irradiation, these compounds can be carried through the cracks and produce additional bacterial inactivation. Overall, we concluded that under the experimental conditions tested in this study, UV irradiation was a predominant factor inactivating Btk spores on an MCE filter.

On the other hand, none of the tested combination modes of UV and iodine showed a synergistic inactivation of *A. fumigatus* spores ($p >$

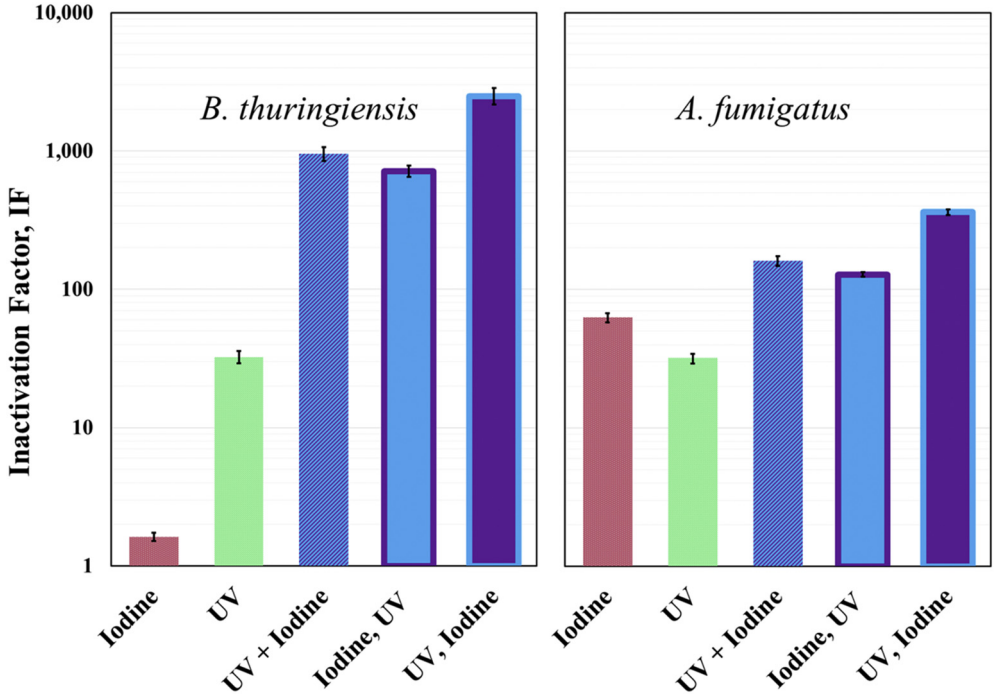

Fig. 1. Inactivation of *B. thuringiensis* (Btk) and *A. fumigatus* spores collected on MCE filter by the source-specific (UV irradiation or gaseous iodine) and their combined treatments.

0.05). The *IF* values from the combined treatments were higher than those produced by each of the single ones but lower than their product representing the two working independently. Since *A. fumigatus* can normally be found in the outdoor environment (Alshareef and Robson, 2014), the spores usually contain stress-resistant pigments called melanin (Bernard and Latgé, 2001). The melanin is produced as a protective component for fungal spores from environmental stresses such as UV irradiation as well as oxidizing agents, acids, and gamma radiation (Eisenman and Casadevall, 2012; Fuller et al., 2013; Gessler et al., 2014; Youngchim et al., 2004). While some spores are inactivated by these stressors, others survive as they may have developed an additional resistance due to melanin. Thus, a population of *A. fumigatus* spores may show a mixed response to the UV irradiation and iodine treatment due to the defense mechanism associated with melanin. Furthermore, a relatively short-term exposure to UV irradiation may be insufficient to substantially destroy the cell structure of *Aspergillus* spores. Consequently, gaseous iodine would not be able to penetrate the cell wall as effectively as for Btk spores (Taylor-Edmonds et al., 2015). However, the sequential treatment "UV, Iodine" was still found the most efficient among tested for inactivating *A. fumigatus* spores (as well as for Btk).

Overall, Btk spores were approximately an order of magnitude more susceptible to all three combination modes of UV irradiation and gaseous iodine on MCE filters compared to *A. fumigatus* spores. The findings

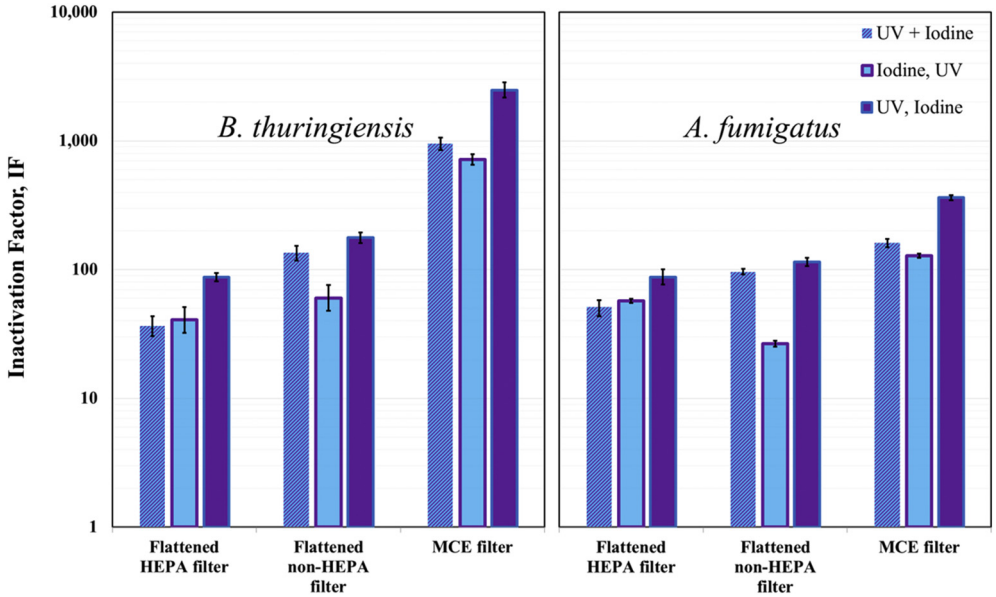

Fig. 2. Inactivation of *B. thuringiensis* (Btk) and *A. fumigatus* spores collected on "flat" filters of three different types by combined treatments of UV irradiation and gaseous iodine.

are consistent with other studies, which report that the fungal spores can be more resistant than bacterial spores (American Air and Water, 2018; Kowalski, 2009).

3.2. Inactivation on different filter materials

The data on inactivation of both species collected on three tested "flat" filter materials due to different combined treatments of UV and gaseous iodine are presented in Fig. 2. Some results shown for the MCE filter in Fig. 1 are plotted here again for comparison. For Btk, the highest *IF* values are observed on MCE filter followed by the flattened non-HEPA and flattened HEPA filters. The differences may be attributed to the thickness and density of filter materials as an increase in both is expected to reduce bacterial inactivation. It should be noted that even after flattening, both HEPA and non-HEPA filters allowed for some depth filtration so that some spores were deposited inside the filter material (not only on its surface). Therefore, the accessibility of UV irradiation to these spores is likely impeded. With a lower amount of the UV exposure, which was shown to be the predominant factor for the Btk inactivation investigated in this study, it is explainable with the viability loss was significantly lower ($p < 0.05$) for flattened HEPA and flattened non-HEPA filters compared to the perfectly "flat" MCE filter. The data analysis revealed that the type of "flat" filter and the type of combined treatment are factors significantly influencing ($p < 0.05$) the inactivation of Btk spores. Similar to the MCE filter, the sequence "UV, Iodine" caused significantly greater ($p < 0.05$) inactivation on two other "flat" filter materials compared to other combinations. The *IF* values obtained for the simultaneous application of UV and iodine ("UV + Iodine") were either statistically the same ($p > 0.05$) or slightly higher ($p < 0.05$) than that of the sequential application "Iodine, UV" (Fig. 2).

The trends found for *A. fumigatus* showed some difference compared to those obtained with Btk (Fig. 2). The properties of filter materials are likely to have low influence on the inactivation of *A. fumigatus* by a specific treatment. While the reason behind this difference is not entirely clear at this point, it may be attributed to the fact that *A. fumigatus* is more susceptible to gaseous iodine treatment than to UV irradiation as compared to Btk. Therefore, differences in filter materials producing different UV access to the deposited spores were not as important for fungal spores compared to bacterial spores. The sequence of "UV, Iodine" was still the most effective combination compared to others ($p < 0.05$). Similar to Btk, the inactivation effect generated by the simultaneous application of the two treatments ("UV + Iodine") was observed to be either statistically the same ($p > 0.05$) or somewhat higher ($p < 0.05$) than "Iodine, UV".

3.3. Comparisons between two configurations of HEPA filters

An additional experiment was conducted to assess the filter configuration effect on inactivation of spores exposed to the three above-listed combinations of UV and gaseous iodine treatments. In order to compare "flat" and deep-bed filters made of the same material, we deployed a full commercial HEPA air purifying unit in addition to the flattened HEPA filter. Both species, Btk and *A. fumigatus*, were utilized for this experiment. The results are presented in Fig. 3 (the flattened HEPA data are adopted from Fig. 2 for visual comparison). For all combinations of UV and iodine, the inactivation of Btk spores collected on flattened HEPA filters was significantly greater ($p < 0.05$) than that measured on the deep-bed filter of the commercial HEPA unit. The difference is attributed to the depth of the HEPA filter. Most Btk spores collected by the deep-bed filter of the commercial HEPA unit are embedded inside the pleated structure of the dense fibrous material. As discussed above, the efficiency of UV irradiation, which was identified as the predominant inactivation source in this investigation, especially for Btk spores, may be drastically impeded due to the lack of access of these "hidden" bacterial spores. Surprisingly, the filter depth was not as influential for *A. fumigatus* spores. When tested on the deep-bed filter of the commercial HEPA unit, *A. fumigatus* spores were inactivated by any of the three combinations of the UV and iodine treatments more efficiently ($p < 0.05$) than Btk spores (Fig. 3). This is consistent with the finding that iodine, capable of penetrating the deep-bed filter unlike UV, caused a greater inactivation in *A. fumigatus* spores than in Btk spores. The *IF* values for *A. fumigatus* presented in Fig. 3 are generally comparable; furthermore, in two out of three cases a slightly higher inactivation was found in the deep-bed filter as compared to the "flat" one (Fig. 3).

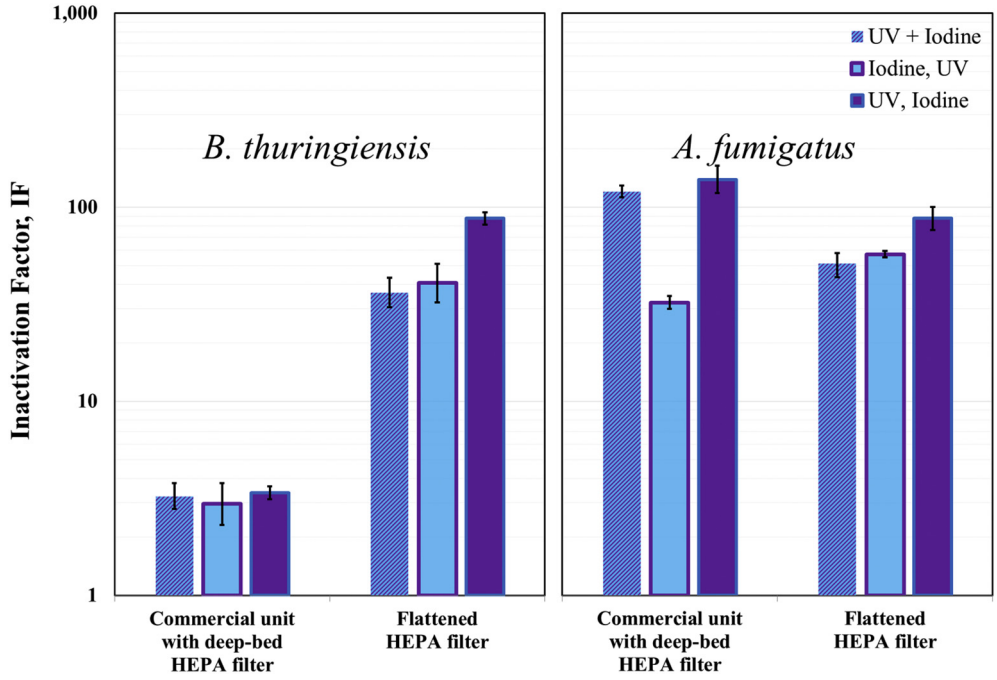

Fig. 3. Inactivation of *B. thuringiensis* (Btk) and *A. fumigatus* spores collected on HEPA filters of two different configurations ("flat" and deep-bed) by combined treatments of UV irradiation and gaseous iodine.

This study has several limitations. Among them is the method used for quantifying microbial viability. The culture-based method may underestimate the viable spore count due to the spores commonly referred to as viable but not culturable (VBNC). At the same time, the numerical alteration caused by the VBNC spores is not expected to be substantial under the conditions of this investigation. The culture-based method is widely used for assessing microbial inactivation.

4. Conclusions

Inactivation of Btk and *A. fumigatus* spores collected on different filters by UV irradiation and gaseous iodine was investigated. The source-specific treatments (involving either UV irradiation or gaseous iodine), as well as their different combinations, were tested. The combinations included the simultaneous application as well as two sequential applications (UV first and iodine first). Several differences were observed in the results obtained for the two species. All combined applications on Btk spores revealed a pronounced synergy of inactivation by UV and iodine. However, no similar synergistic effect was found for *A. fumigatus*. The type of the "flat" filter (MCE, flattened non-HEPA, and flattened HEPA) was found to significantly affect inactivation of Btk spores; however, the influence of this factor was not prominent for *A. fumigatus* spores. For viable bacterial spores, the use of the deep-bed filter resulted in a significantly lower inactivation effect generated by any of the three tested combinations of UV and iodine compared to the "flat" filters. However, this trend was not observed for fungal spores of *A. fumigatus*. For both species, the results obtained with all tested filters demonstrated that utilizing UV irradiation before the iodine treatment produced significantly greater inactivation than utilizing gaseous iodine before UV or applying the two treatment simultaneously.

Most of the findings were explained with the help of the existing knowledge pertaining to complex interactions of bacterial and fungal spores with UV irradiation and gaseous iodine. Further research should be conducted to enhance our understanding of these interactions and further assess the feasibility of using UV, gaseous iodine and other treatments for inactivating viable stress-resistant microorganisms collected on different media, including HVAC filters.

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