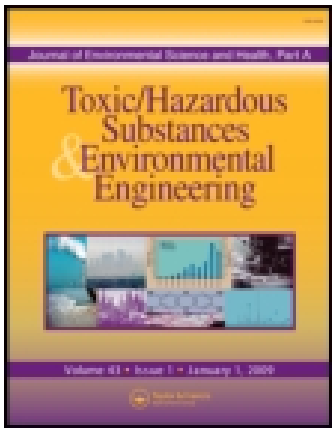

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The impact of dose, irradiance and growth conditions on Aspergillus niger (renamed A. brasiliensis) spores low-pressure (LP) UV inactivation

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The impact of dose, irradiance and growth conditions on *Aspergillus niger* (renamed *A. brasiliensis*) spores low-pressure (LP) UV inactivation

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The use of *Aspergillus niger* (*A. niger*) fungal spores as challenge organism for UV reactor validation studies is attractive due to their high UV-resistance and non-pathogenic nature. However *A. niger* spores UV dose-response was dependent upon sporulation conditions and did not follow the Bunsen–Roscoe Principle of time-dose reciprocity. Exposure to 8 h of natural sunlight for 10 consecutive days increased UV resistance when compared to spores grown solely in dark conditions. Application of 250 mJ cm^{-2} at high irradiance (0.11 mW cm^{-2}) resulted in a 2-log inactivation; however, at low irradiance (0.022 mW cm^{-2}) a 1-log inactivation was achieved. In addition, surface electron microscopy (SEM) images revealed morphological changes between the control and UV exposed spores in contrast to other well accepted UV calibrated test organisms, which show no morphological difference with UV exposure.

Keywords: *Aspergillus niger*, Bunsen–Roscoe law, reactor validation, scanning electron microscopy, ultraviolet disinfection.

Introduction

The use of ultraviolet light (UV) as a disinfectant for drinking water is growing among public water systems due to its ability to inactivate waterborne microorganisms without forming disinfection by-products. To implement UV technology as a treatment unit, it is required to validate each full-scale UV reactor to ensure the appropriate dose is delivered. The Long-Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR) prescribes a UV dose of 186 mJ cm^{-2} for a 4-log viral removal credit, based on low pressure (LP) data.[1] Biodosimetry is the recognized method of validation and utilizes a microorganism for which the UV response is well characterized. The reactor calibration test organism should have similar UV tolerability as the pathogenic microorganism that drives the risk and account for differences in UV-tolerability between strains. For example, certain adenoviruses have greater UV-tolerability than others; when using a low pressure (LP) UV lamp, adenovirus type 2 required a UV dose of 160 mJ cm^{-2} to achieve the desired 4-log inactivation, while adenoviruses 40 only required 124 mJ cm^{-2}.[2,3]

Ideally, the biodosimeter organism would be non-pathogenic, easily cultured to high titers, have reproducible dose-response curves, and follow the principles of the Bunsen-Roscoe Principle, also known as the law of time-dose reciprocity, which simply states that the intensity of the dose should not impact the UV-inactivation.[4] This is important because the intensity will inherently fluctuate within UV flow reactors. Currently in the United States, UV reactor validation is performed with viruses such as MS2 bacteriophage, while in Europe, *Bacillus subtilis* spores are the challenge organism of choice.[5] However, these surrogates are not resistant enough to accurately model UV-log reduction required for highly resistant organisms, such as adenovirus 2. *Bacillus subtilus* spores have a *k*-value 0.059 log (mJ cm^{-2}) $^{-1}$ that is 2–3 higher than that of adenoviruses (0.018–0.024 log (mJ cm^{-2}) $^{-1}$ under LP lamp.[6]

Fungal spores might also be considered for UV validation studies due to their high UV-resistance. Fungal UV tolerance is a function of their high melanin content, which can absorb damaging UV photons.[7] *Aspergillus niger* (*A. niger* renamed *Aspergillus brasiliensis*) spores, commonly referred to as black mold, are darkly pigmented due to their high melanin content. *A. niger* spores were evaluated

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at full-scale as a challenge organism and found to be a viable biodosimeter for high-dose UV reactor validation, such as those required to achieve multi-log virus credit.[8] However, no published work has investigated the suitability of *A. niger* spores for UV validation purposes by (a) measuring the impact of UV intensity (time-dose reciprocity) on *A. niger* spore inactivation, (b) determining the impact of sporulation conditions (growth in dark vs. light) on *A. niger* spore UV dose-response curves, (c) examining if any morphological changes following LP-UV exposure using SEM, which may help to determine if the spores are dormant.

Material and methods

Spore preparation and enumeration

Aspergillus brasiliensis (ATCC16404) deposited as *A. niger* was obtained from American Type Culture Collection (Manassas, VA, USA) and reconstituted according to manufacturer's instructions. *A. niger* cultures were inoculated on sterile OGYE agar plates (Neogen Corporation, Acumedia, Lansing, MI, USA) for 10 days at 25°C and sporulated under dark conditions. Spores were harvested aseptically with 0.9% NaCl (w/v) and 0.002% Tween 40 (v/v) (Fluka, Sigma-Aldrich, St. Louis, MO, USA) and shaken vigorously to break conidial chains. After 30 min incubation at room temperature, mycelial fragments were removed by cotton plug filtration. Filtrate was centrifuged (10 min, 3780 g) and pelleted spores were re-suspended in 30 mL of sterile saline (0.9% NaCl w/v) for storage at 4°C. Spore viability and concentration were measured by membrane filtration in sterile phosphate buffer saline (PBS) and vacuum filtered through 0.45-μm membrane (mixed cellulose ester; Millipore, Billerica, MA, USA). Membranes were incubated on sterile OGYE agar plates at 25°C for 5 days; colonies were enumerated as CFU/mL (colony forming unit). Unless otherwise stated, *A. niger* spores were prepared under dark conditions.

UV irradiation experiments

UV exposure was performed with an LP bench-scale UV collimated-beam apparatus (CBA), as outlined in the EPA method,[9] using two LP CBA units; a 15W LP (Philips Ltd., Einhoven, The Netherlands) located at Atlantium Technologies LTD and a 43W LP lamp (Trojan Technologies Inc, Canada) located at Tel-Aviv University (TAU). Both systems emit a monochromatic ultraviolet radiation at 253.7 nm. The UV dose (mJ cm^{-2}) was calculated according to USEPA methods,[9] where average irradiance (E_{avg}) was determined by the measured incident spectral irradiance using a spectroradiometer (USB4000, Ocean Optics, FL, USA). Water absorbance, sample surface reflection and measured petri-factor were taken into consideration. Absorbance was recorded by a spectrophotometer (Cary Bio100, Varian Inc., Palo Alto, CA, USA). UV dose was calculated by multiplying the actual E_{avg} by the exposure time. The height of the water sample was adjusted to provide similar incident irradiance for both LP lamps at Atlantium and TAU.

Scanning Electron Microscopy (SEM) of A. niger *spores*

A. niger spores were subjected to 100 mJ cm^{-2} dose of UV with an irradiance of 0.26 mW cm^{-2}, incubated in dark conditions for 9 days at 4°C, and compared to non-irradiated spores cultivated in dark conditions (control). All spores were filtered through an 3-μm polycarbonate isopore membrane (Millipore, Bedford, MA, USA). Following dehydration, samples were mounted on aluminum stub, coated by gold-palladium, and viewed with an Environmental Scanning Electron Microscope (ESEM, Quanta 200 field emission gun from FEI) operated in high vacuum mode.

Experimental procedures

A. niger spores were suspended in PBS at a final concentration of $\sim 10^6$ CFU mL^{-1} and used for UV exposure experiments.

Impact of sporulation conditions on A. niger *spore LP-UV dose-response curve*

A. niger cultures grown in dark conditions and natural sunlight conditions (8 h of natural sunlight per day for 10 days, under a quartz lid). Then 20-mL aliquots of 10^6 CFU mL^{-1} of each spore suspension grown under dark and light conditions were exposed to range of LP-UV CBA doses, where each dose was performed in triplicate. UV-exposed spores were serially diluted, filtered, plated, and enumerated after 5 days of incubation at 25°C.

Log-inactivation of A. niger *spores exposed to 8.5 h of sunlight*

The effect of 8.5 h of sunlight exposure (natural sunlight under a quartz lid) on the UV-resistance (LP-UV) in comparison to *A. niger* spores that were not exposed to light (dark conditions) was investigated. Each spore suspension was exposed to 200 mJ cm^{-2} using a LP-CBA and enumerated after 5 days' of incubation at 25°C.

Time-Dose reciprocity of A. niger *spores*

A. niger spores ($\sim 10^6$ CFU mL^{-1}) were suspended in 20 mL PBS and exposed to 100, 250 and 350 mJ cm^{-2} with LP-CBA at two UV lamp irradiances to evaluate the time-dose reciprocity behaviour. Different intensities were

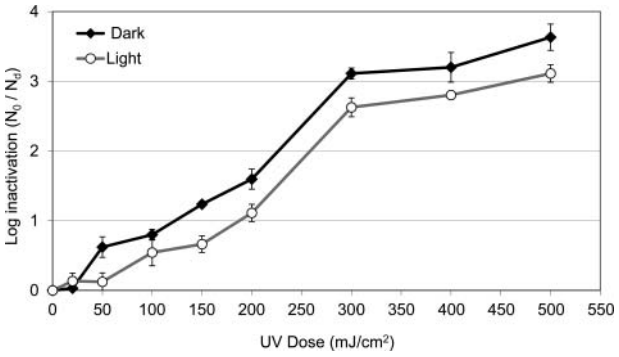

Fig. 1. UV log-inactivation curves for *A. niger* spores cultured under light (~8 h of natural sunlight for 10 consecutive days) or dark conditions, where the irradiance was 0.16 mW cm^{-2}. Error bars represent the standard deviation for replicate experiments ($n = 3$).

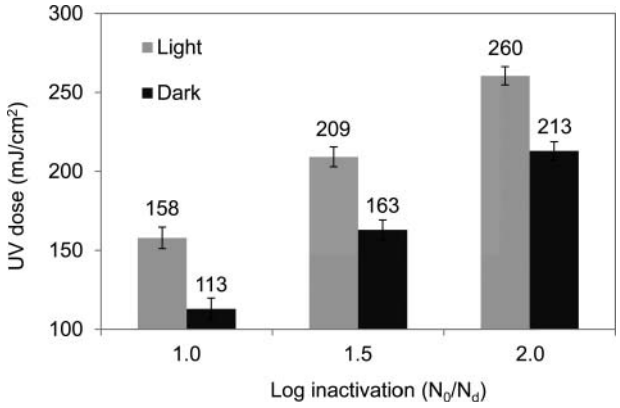

Fig. 2. LP-UV doses required to achieve 1, 1.5 and 2-log inactivation for *A. niger* spores propagated under dark and light (~8 h natural sunlight/day for 10 days) conditions.

achieved using Neutral Density (ND) filters (FDU1, OFR, POB 82, Caldwell, NJ, USA) with transmission percentage 15%; irradiances were measured spectrophotometrically. The two irradiance values examined were 0.11 and 0.022 mW cm^{-2} (without and with ND filter, respectively) termed high and low intensity.

Results and discussion

Impact of A. niger *sporulation conditions on LP-UV dose-response*

The UV inactivation of *A. niger* spores cultured under two growing conditions (8 h of natural sunlight for 10 consecutive days or dark) were evaluated. Sporulation condition had an impact on LP-UV dose response curves, where *A. niger* spores sporulated in sunlight were more resistant to UV exposure than spores grown in dark conditions (Fig. 1). The difference between the log inactivation curves is approx. 0.5-log. Of note, the standard deviations do not overlap and the difference in inactivation response is consistent for all UV doses applied, except for the 20 mJ cm^{-2} dose. The calculated k-value for *A. niger* spores assuming a first-order linear relationship between doses of 50–300 mJ cm^{-2} was approximately 0.010 log/(mJ cm^{-2}) for both the dark and light strains, with R^2 above 0.93. This value is below the *k*-value for adenoviruses under LP lamp (0.018–0.024 log/(mJ cm^{-2})).[6] Therefore *A. niger* spores have a *k*-value, which is more than a factor of ~1.8–2.4 below the *k*-value for adenoviruses.

Differences in UV tolerability are observed by comparing the UV doses required to achieve the same log inactivation for light and dark spores (Fig. 2). These values were determined by assuming a linear trend in the dose-response curve between 50–300 mJ cm^{-2}. To achieve a 1-log reduction, 113 ± 6.8 mJ cm^{-2} is required for spores grown in dark conditions whereas 158 ± 10.1 mJ cm^{-2} is needed for the light strain. In other words, the light strain is 40% more UV-resistant. In terms of log inactivation, a dose of 158 ± 10.1 mJ cm^{-2} resulted in a 1-log reduction for the light strain and a 1.4-log reduction for spores grown in the dark. These results indicate that *A. niger* spores adapt to their growth conditions and their UV response will differ depending on the amount of light present during sporulation.

This may have consequences for UV validation work when considering *A. niger* spores as biodosimeters since exposure to sunlight during the propagation can significantly impact UV tolerance. Each reactor is validated to ensure a dose of 186 mJ cm^{-2} is delivered in accordance with the LT2ESWTR regulations. Exposure of *A. niger* cultures to natural sunlight during sporulation may skew the UV-response curve and lead to inaccurate reactor dose estimations. In comparison to MS2, *A. niger* spores are highly UV-resistant, as previously discussed where 113 and 158 mJ cm^{-2} is required to achieve a 1-log inactivation of dark and light strain, respectively. A dose of 80 mJ cm^{-2} (LP-UV) is needed for 4-log inactivation of MS-2, or ~20 mJ cm^{-2} log^{-1}. However unlike *A. niger*, MS-2 dose response is linear.[10] With respect to UV resistance, *A. niger* spores are more suitable biodosimeters for 4-log virus credit as demonstrated herein and previously.[8] However UV dose-response of *A. niger* spores was dependent upon sporulation conditions. Additional indications of dormancy as sunlight exposure, intensity effect and morphological changes will be further evaluated in the next sections.

Impact of short sunlight exposure during A. niger *spore handling on UV tolerance*

The effect of short-term sunlight exposure on *A. niger* spore UV tolerance was examined. Spores propagated in dark conditions were exposed to 8.5 h of natural sunlight and tested for UV sensitivity. Figure 3 graphically compares the log inactivation between the control (dark) and

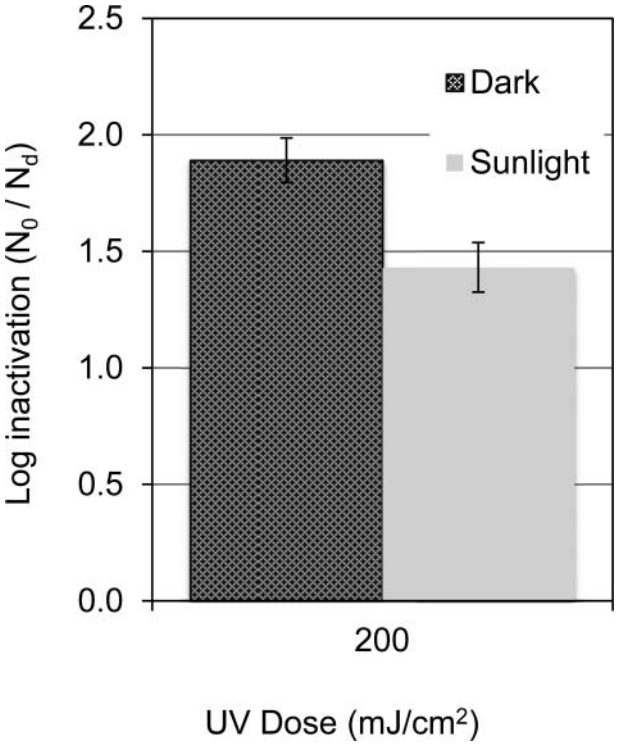

Fig. 3. Effect of short exposures to natural sunlight (8.5 h) on the log-inactivation of *A. niger* spores following an LP-UV dose of 200 mJ cm^{-2}. Error bars represent the standard deviation from $n = 3$.

sunlight spores, following exposure to 200 mJ cm^{-2} LP-UV dose. It is evident that sunlight exposure can alter the UV response, where 1.4 ± 0.11-log inactivation was observed for the sunlight exposed group but control spores had 1.9 ± 0.10-log removal (Fig. 3). This translates into a 25% increase in UV resistance with only 8.5 hours of sunlight exposure. For UV reactor validation experiments, these results highlight the importance of controlled handling practices, strict light control during validation tests, and the need for rigorous spore transportation and storage protocols.

Time-dose reciprocity

The log inactivation of *A. niger* spores as a function of LP-UV dose and irradiance (time-dose reciprocity, also known as the Bunsen–Roscoe principle) was investigated. Two light irradiances (0.11 and 0.022 mW cm^{-2}) were delivered at a dose of 100, 200 and 350 mJ cm^{-2} (Fig. 4). Immediately following UV exposure, spore plating was performed under dark conditions. The non-linear UV inactivation curve consisted of a shoulder at lower doses and tailing at higher doses (Fig. 4).

Initially, a shoulder is observed when 100 mJ cm^{-2} is applied at both high and low intensities. When 250 mJ cm^{-2} was applied, the survival of the low intensity group is significantly higher than the high intensity group. Once

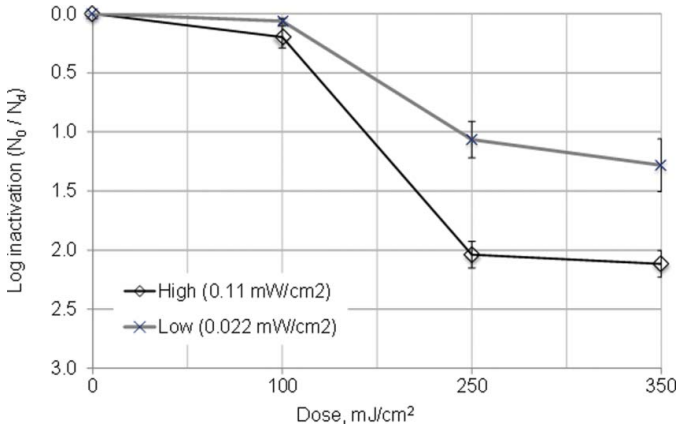

Fig. 4. Impact of intensity on *A. niger* UV dose-response when low irradiance (0.022 mW cm^{-2}) and high irradiance (0.11 mW cm^{-2}) are used to apply a 100, 250 and 350 mJ cm^{-2} LP-UV dose. Each point represents the average of four experiments; error bars represent the standard deviation.

the UV dose increased above 250 mJ cm^{-2}, the rate of inactivation of *A. niger* spores decreased. At this dose, 90% inactivation is achieved using low irradiance (0.022 mW cm^{-2}) versus a 99% inactivation when the irradiance was higher (0.11 mW cm^{-2}).

The *A. niger* spore concentration in the tailing or attenuated portion of the curve was above 10^2 CFU mL^{-1}, as recommended, therefore the change in the inactivation rate is not an artifact of low CFU counts.[11] Similar inactivation curves were observed for other organisms using a variety of disinfectants. For example, *B. subtilis* spores disinfected by chlorine, UV, monochloramine and ozone also displayed similar log-inactivation profiles consisting of a shoulder, linear first-order, and tailing dose-response patterns.[12,13] However unlike *A. niger*, *B. subtilis* UV inactivation followed the Bunsen–Roscoe principal, meaning the dose produced similar inactivation curves irrespective of the intensity or time required to deliver the dose.

Previously reported UV-inactivation of microorganisms with similar intensity-time ranges had displayed variances in log-inactivation with respect to intensity,[14] meaning they did not follow the Bunsen-Roscoe Principle. In the current study, the time-dose reciprocity was found to be invalid for *A. niger* spores grown under dark conditions. A removal of ~2-log was observed when 250 mJ cm^{-2} dose was applied using a high irradiance (0.11 mW cm^{-2}) for a short time, however, only a 1-log removal was observed when the same dose was applied using a low irradiance (0.022 mW cm^{-2}) over a longer period of time (Fig. 4). The time-dose reciprocity for several *E. coli* strains (ATCC 25922, ATCC 11229 and wild-type) also violated the Bunsen–Roscoe Principle when tested for three irradiation levels (0.2, 0.02, and 0.002 mW cm^{-2}).[14] High intensities with short exposure times resulted in higher log-inactivation when compared to low intensities conducted over longer exposure times. It was reasoned that this effect

may be the result of damage to repair enzymes within the cell in response to high UV intensities. This is in contrast to eukaryotic yeast cells of *Saccharomyces cerevisiae* (both haploid and diploid strains) log-inactivation, which were more susceptible to long exposure times at lower intensities.[4]

However, several organisms (*B. subtilis* spores, MS-2, Φ174 and B40-8 phage and *S. cerevisiae* yeast spores) had similar dose-response curves regardless of UV intensity and follow the time-dose reciprocity principle. It was suggested that the repair mechanisms are either inactive in these organisms or that the organisms are in a dormant state.[4,14] This is different from *A. niger* spores where long exposure times at low intensity may have elicited an adaptive mechanism to UV exposure, as evidenced by their prolonged survival. Previous studies suggest that *A. niger* spores can increase their UV-resistance by way of DNA repair mechanisms or increased melanin production.[15]

Another study examined the photocatalytic oxidation of *A. niger* spores using an LP-UV system and also found non-conformity to time-dose reciprocity rule.[16] To achieve a 0.5-log reduction of *A. niger* spores using high light intensity (0.1 mW cm^{-2}) required 6 min, while a 10-fold lower intensity (0.01 mW cm^{-2}) required 49 min, which is approximately 10 times slower. In the present study, 0.5-log reduction of *A. niger* spores at an irradiance of 0.1 mW cm^{-2} required 20-min exposure time, while a 0.5-log reduction of *A. niger* spores at an irradiance of 0.022 mW cm^{-2} required 140 min (Fig. 5).

Morphological changes following LP-UV exposure using SEM

The scanning electron microscope (SEM) micrographs of control *A. niger* spores reveal a characteristic dumpling shape with a diameter of 3.4–4.5 μm (Fig. 6). These images are similar in morphology and size to SEM images previously published.[17] Little is known about spore structure-function relationships; however, in general these spores are regarded as bacterial cells consisting of a cell membrane, cytoplasm, capsule, plasmid membrane, ribosomes, surrounded by pili.[17] Spores propagated under dark conditions were divided into two groups designated as control (Fig. 6A and 6C) and UV-treated with 100 mJ cm^{-2} at high intensity (0.26 mW cm^{-2}) (Fig. 6B and 6D). Following UV-exposure, spores were incubated for 9 days at 25°C to capture the morphological changes due to UV stress.

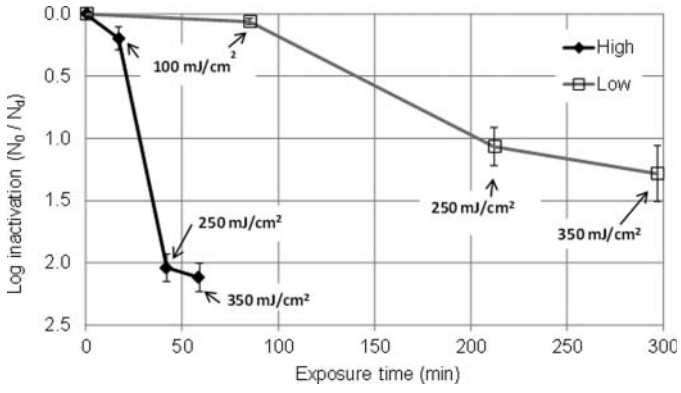

Fig. 5. Log-inactivation of *A. niger* spores as a function of exposure time and UV irradiance, under LP-UV lamp.

Distinct morphological changes between the control (6C) and UV exposed (6D) spores at the cell surface were observed. Control spores contained dark structures just below the pili surface; however, irradiated spores appeared smoother and devoid of any dark structures. While the identity and functional significance of the SEM structures remain unclear, the morphological changes are indicative of spore activity in response to UV-illumination. This is in contrast to *B. subtilis* spores, typically used in Europe for UV validation, which do not show any morphological changes when exposed to UV.[13]

Implications for reactor validation studies

The UV-inactivation of a validation test organism is significant as regulators and drinking water facilities must be confident the reactor is providing the correct dose for disinfection and regulatory inactivation credit. Dose delivery in a continuous flow UV reactor is considerably more complex when compared to a completely mixed batch reactor.[9] In effect, each microorganism passing through the continuous reactor may receive a different UV dose, resulting in a dose distribution,[18] as opposed to the average dose (or UV fluence) in a collimated beam. Current UV validation methods measure the inactivation of a test organism through the UV reactor from which one can back-calculate the delivered UV fluence, or biodosimetry method. However the determination of the reduction equivalent dose (RED) when performed with a dosimeter with non-linear characteristics, as in the case of *A. niger* spores, may skew the RED from the arithmetic mean of the dose distribution. Although there are marked differences in intensity and the residence times when comparing collimated beam tests to UV reactors, biodosimetry tests are still an effective means of estimating the average dose output by the reactor.

The residence time in UV reactors is in the order of milliseconds, delivering between 40–120 mJ cm^{-2} as stipulated for water disinfection and reuse purposes. For example, mean residence time of 0.2 sec can be achieved in a UV reactor with a ~5 m s^{-1} flow velocity and assuming the length of the reactor is 1 m. As the intensity in a UV reactor is not uniform, computer modeling using light maps have estimated, for example, the germicidal light intensities to be in the range of 1700 mW cm^{-2} in proximity to a certain examined UV reactor. An exposure time of 0.01 sec would result in a UV-dose of 17 mJ cm^{-2}, while a microbe exposed 0.1 sec will accumulate a dose of

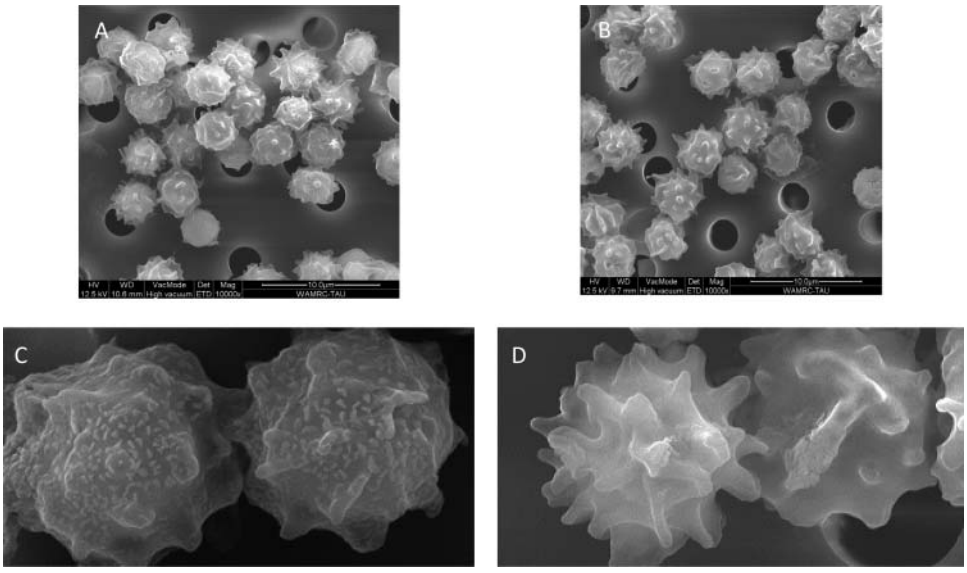

Fig. 6. Morphology of *A. niger* spores (6A and 6C) compared to *A. niger* spores exposed to a UV dose of 100 mJ cm^{-2} as captured by SEM after 9 days post-exposure (6B and 6D).

170 mJ cm^{-2} (personal communication, Atlantium Technologies Ltd., Israel).

Although *A. niger* spores are attractive challenge organisms for biodosimetry studies for UV reactor validation for 4-log virus credit due to their ease of cultivability, nonpathogenic nature and high resistance, dose-response of *A. niger* spores was dependent upon sporulation conditions and UV intensity, therefore is a questionable candidate. The handling and growth conditions of *A. niger* spores is critical to their performance therefore, if used as a challenge organisms their growth should be performed by an accredited lab and qualified personnel to ensure the dose-response curves accurately reflect the UV dose being delivered by the reactor.

Conclusion

In the present study, we conclude that *A. niger* spores are (1) not dormant and can undergo morphological changes when exposed to UV; (2) UV response is intensity dependent and do not follow the Bunsen–Roscoe principle; and (3) Exposure to natural sunlight will change the UV dose-response curve.

Acknowledgments

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