



## Inactivation efficiency of *Bacillus* endospores via modified flow-through PUV treatment with comparison to conventional LPUV treatment

Title	Inactivation efficiency of <i>Bacillus</i> endospores via modified flow-through PUV treatment with comparison to conventional LPUV treatment
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22 exercised when manipulating media formulation to propagate endospores as this may lead to inconsistencies in  
23 reporting efficacy of intervention treatments with implications for validation and environmental evaluation.

24

25 **Keywords:** low-pressure UV, pulsed UV, bacillus endospores, disinfection, media formulation.

26 **Nomenclature:**

27	CE	Coefficient of efficiency
28	DVGW	German Association for Gas & Water
29	DPA	Pyridine-2,6-dicarboxylic acid
30	HRT	Hydraulic residence time
31	ÖNORM	Austrian Standards Institute
32	LPUV	Low Pressure UV
33	MnSO <sub>4</sub> .H <sub>2</sub> O	Manganese sulphate monohydrate
34	PPS	Pulses per second
35	PUV	Pulse UV
36	RED	Reduction equivalent dose
37	UV	Ultraviolet
38	UVDGM	UV Disinfection Guidance Manual

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42 **1.0 Introduction**

43 Adoption of ultraviolet (UV) treatment as a method of tertiary disinfection for drinking water treatment and for  
44 wastewater treatment has increased significantly worldwide in recent years (1,2). A key driver of this uptake

45 may be attributed to its' success of inactivating chlorine-resistant pathogens, in particular *Cryptosporidium*  
46 *parvum* (3,4). UV treatment is also a physical disinfection method that is not associated with the formation of  
47 harmful disinfection by-products e.g., trihalomethanes and haloacetic acids which are typically associated with  
48 free chlorines/chloramines disinfection applications. In addition, advanced oxidation processes that utilise UV  
49 treatment in combination with other chemical treatments (e.g. hydrogen peroxide) are effective in the  
50 degradation of a number of organic pollutants (5,6). UV irradiation is effective at inactivating a variety of  
51 pathogens, where UV dose or fluence ( $\text{mJ}/\text{cm}^2$ ) is defined as the UV intensity ( $\text{mW}/\text{cm}^2$ ) multiplied by the  
52 exposure time (s). In a laboratory setting, a collimated beam apparatus can be used to deliver a controlled amount  
53 of UV energy to receiving samples, thus the UV dose may be accurately determined. In contrast, UV reactors  
54 installed at drinking water treatment plants may be subject to varying water flow dynamics, varying influent  
55 water quality and varying UV intensities within the reactor (due to lamp aging and fouling etc) (7–10). These  
56 variations can in turn lead to inaccuracies when determining the exact UV dose delivered. For this reason, each  
57 UV reactor should undergo validation testing whereby biosimetry is used to determine a 'reduction equivalent  
58 dose' (RED) from the reactor (7,11–13). The process involves measuring the inactivation of a robust or  
59 'challenge' microorganism after it has been exposed to UV light within the reactor and then comparing the  
60 inactivation level to a UV dose response curve of the same microorganism ascertained under controlled  
61 laboratory conditions using a collimated beam apparatus - from this a RED may be determined (14). There are  
62 three primary UV reactor validation guidelines/protocols that are observed internationally; the US  
63 Environmental Protection Agency (US EPA) UV Disinfection Guidance Manual (UVDGM), the German  
64 Association for Gas & Water (Deutsche Vereinigung des Gas und Wasserfaches (DVGW) W294)) and the  
65 Austrian Standards Institute (Österreichisches Normungsinstitut (ÖNORM) M 58373-1)) (7,12,13). The  
66 UVDGM is typically applied for UV reactor validation testing in North America however as a guideline  
67 document it does not set out one standard protocol but rather recommends validation protocols based on the type  
68 of water source to be treated and the target pathogen in question. The RED is determined using the biosimetry

69 of a surrogate challenge microorganism with a similar UV sensitivity to the specific target pathogen requiring  
70 inactivation e.g. MS2 bacteriophage may be used a surrogate organism for the inactivation of *Cryptosporidium*  
71 (7). In Europe, the primary accepted protocols for UV reactor validation tend to be the ÖNORM and the DVGW  
72 protocols. Both protocols stipulate that all UV reactors are required to supply a constant minimum UV dose of  
73 40 mJ/cm<sup>2</sup> with the DVGW specifying *Bacillus subtilis* endospores to be used as the challenge microorganism  
74 for biosimetry analysis (12,13).

75 *Bacillus* endospores are commonly linked to UV disinfection studies. *B.subtilis* endospores have historically  
76 been used as pathogen surrogates in UV reactor validation trials as they are non-pathogenic and exhibit an  
77 increased resistance to UV irradiation in comparison to most waterborne pathogens (7,15). *B.pumilus* endospores  
78 have recently been suggested as suitable surrogates for the highly UV resistant waterborne pathogen human  
79 adenovirus (hAdV) due to exhibiting similar levels of UV resistance (16,17). Moreover, studies have shown  
80 *B.pumilus* endospores and hAdV to be almost ten times more sensitive to UV irradiation at 220 nm in comparison  
81 to the standard 254 nm wavelength (18,19). Thus, its potential as a surrogate challenge organism for hAdV in  
82 PUV reactor validation trials is significant.

83 The cultivation of *Bacillus spp.* endospores in a laboratory typically involves the addition of supplement  
84 manganese sulphate monohydrate (MnSO<sub>4</sub>.H<sub>2</sub>O) for rapid endospore propagation (20). A concentration of  
85 approximately 0.05 mM MnSO<sub>4</sub>.H<sub>2</sub>O is recommended by the German National Resource Center (DSMZ) for  
86 the sporulation of *Bacillus spp.* strains while a concentration of up to 0.1 mM MnSO<sub>4</sub>.H<sub>2</sub>O has been applied in  
87 a number of research studies (16,18,21–27). Recent studies have shown a relationship between *Bacillus spp.*  
88 endospores cultivated on agar supplemented with MnSO<sub>4</sub>.H<sub>2</sub>O and increased UV resistance to LPUV  
89 disinfection (16,17,19). However, little information is available regarding *Bacillus spp.* endospores cultivated  
90 on agar supplemented with MnSO<sub>4</sub>.H<sub>2</sub>O and enhanced UV resistance to pulsed UV (PUV) disinfection. The  
91 modification of *Bacillus spp.* endospore UV resistance may be an important consideration in laboratory scale

92 analysis particularly as *Bacillus subtilis* endospores are currently the challenge organism of choice for UV  
93 system validation under DVGW regulations.

94 PUV systems have been widely researched for application within the food industry and are characterised by high  
95 energy outputs and shorter contact times in comparison to standard LPUV disinfection systems (28–30).  
96 Typically, a polychromatic xenon lamp is used which emits light within the broadspectrum of the UV, visible  
97 and infrared range (200-1100nm) with approximately 25% of the energy output in the UV spectrum (31).  
98 Electricity is stored in a capacitor and released in extremely short pulses that lasts microseconds, the frequency  
99 of pulses typically range between 1 and 10 pulses per second (PPS). Low pressure mercury lamps are reported  
100 to have a coefficient of efficiency (CE) of approximately 35-50% for the conversion of electrical energy to UV  
101 irradiation (at 254 nm) whereas high pressure pulsed lamps i.e. xenon lamps are reported to have a lower CE of  
102 approximately 10-15% (32,33). In addition, the power emitted from pulsed UV lamps is generally at least an  
103 order of magnitude higher than the power emitted from LPUV lamps. Table 1 compares the UV dose  
104 requirements of PUV and LPUV systems for the log inactivation of various microorganisms. The UV dose  
105 calculated for the PUV systems typically includes the full polychromatic broadspectrum energy output in  
106 comparison to monochromatic energy output for LPUV systems at 254 nm. Some studies investigating PUV  
107 disinfection efficiency include only the energy emitted from wavelengths in the UV range (approximately 200-  
108 400 nm) and calculate the UV output of the system accordingly while others include both the ‘total broadband  
109 fluence/dose’ and the ‘UV fluence/dose’ (34–36).

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**Table 1. Comparison of UV output/dose (mJ/cm<sup>2</sup>) requirements of PUV and LPUV systems for the log**

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**inactivation of microorganisms.**

Microorganism	Medium	UV System	UV output/dose (mJ/cm <sup>2</sup> ) <sup>e</sup>	Log inactivation	Reference
<i>E.coli</i> DSM 498	Polysaccharide gel	PUV	450 <sup>a</sup>	3-4	(37)
<i>E.coli</i> DSM 787	Saline suspension	LPUV	8 <sup>b</sup>	4.2	(38)
<i>E.coli</i> DSM 498	Synthetic wastewater	PUV	7,200 <sup>c</sup>	5.6	(39)
<i>B.subtilis</i> DSM 10	Spore suspension	PUV	1,000 <sup>a</sup>	2.7	(21)
<i>B.subtilis</i> ATCC 6633	Synthetic wastewater	PUV	8,800 <sup>c</sup>	5.3	(39)
<i>B.subtilis</i> ATCC 6633	Spore suspension	LPUV	40.4 <sup>b</sup>	5.0	(40)
<i>L.monocytogenes</i> 10493S	Culture liquid medium	PUV	3,200 <sup>a</sup>	3.10	(41)
<i>L.monocytogenes</i> 10493S	Culture liquid medium	LPUV	33 <sup>b</sup>	3.14	(41)
Murine Norovirus	PBS solution	Static PUV	1,450 <sup>c</sup>	3.77	(42)
Murine Norovirus	Groundwater	Flow-through PUV	4,300 <sup>c</sup>	3.35	(42)
Murine Norovirus (CW3)	Liquid suspension	LPUV	29 <sup>b</sup>	4.0	(43)

117 <sup>a</sup>broad spectrum dose118 <sup>b</sup>UV dose119 <sup>c</sup>output120 <sup>d</sup>Not specified

121 °Where energies presented in Table 1 were calculated by the authors based on system characteristics the term “output” has been used.  
122 If the energies were measured at the sample surface the term “dose” has been used. This is because measured energies dissipate quickly  
123 the further a sample is from the PUV light source.  
124

125 Flow-through PUV systems are relatively unexplored in the water/wastewater disinfection sector but are noted  
126 as a promising alternative to LPUV as studies suggest high energy outputs may decrease contact times and  
127 inhibit photoreactivation (44). The use of xenon gas is considered more environmentally friendly than the  
128 standard mercury vapour LPUV lamps (30). However, questions remain as to whether the intensity and/or peak  
129 power of the applied PUV fluence for water treatment applications has a considerable impact on microbial  
130 inactivation in comparison to LPUV applications. A lack of clarity regarding how the UV dose for PUV systems  
131 is determined can hinder replication studies and does not allow for direct comparisons (45). Studies analysing  
132 LPUV systems which are set up as a continually mixing flow-through system also appear to be limited; such  
133 experimental set-ups may aid comparisons to LPUV operations on-site.

134 Thus, this study compared continuous LPUV and PUV flow-through irradiation systems for reliable and  
135 repeatable disinfection of *Bacillus* endospores that were artificially grown and harvested from agar  
136 supplemented with manganese sulphate.

## 137 **2.0 Materials and Methods**

### 138 **2.1 PUV System Analysis**

139 A bench-scale pulsed power source (PUV-01, Samtech Ltd., Glasgow) was used to power a low pressure (60  
140 kPa) xenon-filled flash lamp (Heraeus Noblelight XAP type NL4006 series constructed from a clear UV  
141 transparent quartz tube) which produced a high intensity beam of polychromatic pulsed light (200-1100 nm). A  
142 schematic figure of the system is available in the Supplementary material (Figure S1). The lamp was placed  
143 10.75 cm above a sterilised aluminium flow-through vessel (with a plan surface area of 290 cm<sup>2</sup>) through which

144 water was pumped at various flow rates corresponding to hydraulic residence times (HRT) being investigated.  
145 HRTs ranged between 65 s and 100 s. The PUV system allowed for the input voltage and the pulse rate to be  
146 varied between 400 and 1000 V and for a pulse frequency of between 0.1 and 10 pulses per second. The UV  
147 output was determined by calculating the output voltage energy in combination with the sample distance from  
148 the xenon lamp, the area of the vessel, the PPS and the HRT. It is important to note that the adjustable factors  
149 listed as well as the type of sample medium influences the penetration of the PUV light thus the energy delivered  
150 by the lamp is different to the energy received by the sample. For example, at 900V, 2PPS and 100s HRT, the  
151 UV output was calculated to be 2052 mJ/cm<sup>2</sup> (20.5 mW/cm<sup>2</sup>; 112 mW/cm<sup>2</sup> broadband output). All PUV outputs  
152 are presented to include only the wavelengths below 300 nm.

153 The total broadband dose received by the sample was analysed using a thermopile power detector (Model:  
154 XLP12-3S-H2-IN, Gentec-EO, Quebec, Canada) in conjunction with Integra software. The detector (12 mm  
155 aperture) was placed on the aluminium vessel 10.75 cm below the xenon lamp. Pulse energies were measured  
156 three times at each voltage setting after which an average value was obtained. In addition, two longpass colour  
157 glass filters; FGL 400 and FGL 280 (Thorlabs GmbH, Dachau, Germany) were also used to filter out the  
158 wavelengths emitted from the lamp below the UV range and below the UV-C range respectively. The filters  
159 were placed separately on top of the detector head and the power measurements were taken as previously  
160 described. The differences in the power values obtained with and without the filters was used to calculate the  
161 power emitted from the lamp within the UV and the UV-C range. Table 2 outlines the calculated total broadband  
162 PUV power output per voltage setting and the measured PUV dose as measured by the thermopile detector in  
163 addition to the measured UV and UV-C power. The UV filters were applied to 800 and 900V settings only as  
164 these were the two voltage settings used throughout the experimental analysis.

165 **Table 2. Comparison of PUV lamp energy output per pulse vs. energy received by sample at 10.75 cm.**

PUV Voltage (V)	Calculated lamp power output (1PPS) (mW/cm <sup>2</sup> )	Measured lamp power (1PPS) (mW/cm <sup>2</sup> )	% power received by sample	Measured UV lamp power <400 nm (1PPS) (mW/cm <sup>2</sup> )	% measured power < 400 nm	Measured UV-C lamp power <280 nm (1PPS) (mW/cm <sup>2</sup> )	% measured power < 280 nm
400	11	3.0	28%				
500	17	5.0	29%				
600	25	7.5	30%				
700	34	8.6	25%				
800	44	11.4	26%	0.7	6%	0.3	2%
900	56	14.4	26%	1.1	8%	0.2	1%
1000	69	17.8	26%				

166

## 167 2.2 LPUV System Analysis

168 The continuous-flow monochromatic LPUV system (LCD 412 Plus, S.I.T.A., Halpin & Hayward Ltd.) had a  
169 fixed power output of 40 W at a UV-C wavelength of 254 nm. The UV output could be altered by varying the  
170 influent flow rate thereby altering the HRT which equated to exposure time. The flow rates ranged between 11  
171 L/min and 27 L/min which corresponded to HRTs of between 1.66 s and 0.42 s to give a UV output range  
172 between 12.6 mJ/cm<sup>2</sup> and 31.9 mJ/cm<sup>2</sup> respectively. The UV output was calculated using the rated energy of the  
173 system. In addition, a transmissivity sensor was also used to monitor % UV transmittance which remained above  
174 80% at all times. A schematic figure of the system is available in the Supplementary material (Figure S2).

## 175 2.3 Endospore analysis

176 Bacterial test strains DSM 492 *Bacillus pumilus* and DSM 347 *Bacillus subtilis* were sourced from the German  
177 National Resource Center (DSMZ, Braunschweig, Germany). The strains were equivalent to ATCC 27142  
178 *Bacillus pumilus* and ATCC 6633 *Bacillus subtilis*. Vegetative cells of both strains were cultivated on nutrient  
179 agar (Biolab, Lennox, Ireland) at 35°C for 24 hours to produce pure colonies. One pure colony was inoculated  
180 into 100 mL tryptic soy broth (Sigma-aldrich, Wicklow, Ireland) and placed on a rotary shaker at 90 rpm for 24  
181 hours at 30-35°C. After incubation 0.5 mL of the 24-hour culture was spread onto nutrient agar plates and

182 incubated for at least four days at 30-35°C to encourage endospore production. Endospores cultivated in the  
183 presence of MnSO<sub>4</sub>.H<sub>2</sub>O were spread onto nutrient agar plates which were supplemented with various  
184 MnSO<sub>4</sub>.H<sub>2</sub>O (Honeywell, Sigma-aldrich, Wicklow, Ireland) concentrations ranging from 0.005 mM to 0.5 mM.  
185 After incubation, a sporulation rate of approximately 90% was achieved that was confirmed by endospore  
186 staining (Schaeffer and Fulton spore stain kit, Sigma-aldrich, Wicklow, Ireland) using light microscopy.  
187 Endospores were collected by flooding the plate with 8 mL distilled water after which a sterile spreader was  
188 used to gather endospores from the seeded plates. The water/endospore emulsion was then aseptically transferred  
189 to a sterile 30 mL glass vial before being placed into a water bath at 80°C for 20 minutes to kill any remaining  
190 vegetative cells. Once heat-treated, endospores suspended in water were used in UV trials within 4 hours.  
191 *B.pumilus* and *B.subtilis* endospores were exposed in separate batches to a range of PUV outputs from 0 to 2052  
192 mJ/cm<sup>2</sup> and LPUV outputs from 12 to 32 mJ/cm<sup>2</sup>. For PUV experiments, batches of distilled water (2.5 L) were  
193 spiked with 1 mL of the heat-treated endospores while for LPUV experiments 25 L of tap water was spiked with  
194 5 mL of the heat-treated endospores to give a starting influent concentration of  $6 \log_{10} \pm 0.5$ . Influent and effluent  
195 samples were analysed by triplicate in each run pre and post UV treatment via pour plate technique (1 mL) using  
196 non-selective nutrient agar (Biolab, Lennox, Dublin, Ireland) as per standard methods (46). Each UV output run  
197 was performed at least three times. Log inactivation was determined as the difference between log influent  
198 concentration ( $N_0$ ) – log effluent concentration (N).

## 199 **2.4 Statistical Analysis**

200 As some of the data sets were not normally distributed and small in sample size it was decided to apply non-  
201 parametric analysis. The Kruskal-Wallis test (one-way non-parametric ANOVA) was used to determine  
202 significant differences between sample populations. If the null hypothesis for the Kruskal-Wallis test was  
203 rejected, this was then followed by a post-hoc Dunn Test for pairwise multiple comparisons to determine  
204 significant differences between sample populations. Analysis was carried out using IBM SPSS Statistics 23.

## 206 **3.0 Results and Discussion**

### 207 **3.1 Disinfection of *Bacillus spp.* endospores using LPUV and PUV continuous flow-through water** 208 **systems with particular focus on influence of varying $\text{MnSO}_4\cdot\text{H}_2\text{O}$ concentration in sporulation media**

209 Studies were conducted to compare the disinfection efficacy of using LPUV and PUV flow-through water  
210 systems using pre-determined numbers of *B. pumilus* and *B. subtilis* endospores. *Bacillus* endospores are more  
211 UV resistant than vegetative bacterial cells and have been used as surrogates for assessing UV reactor  
212 performance for the water industry (23). *Bacillus* endospores are also used for monitoring important cross-  
213 cutting disinfection and sterilisation processes including determining sterility assurance levels for the medical  
214 device sector, however there is a commensurate dearth of knowledge on the potential influence of media  
215 formulation on this inactivation process. In addition, the broadspectrum output of the PUV system was compared  
216 to a monochromatic LPUV system for potential enhanced germicidal properties of *B.pumilus* endospores  
217 inactivation. *B.subtilis* endospores are currently the challenge microorganism of choice for UV reactor validation  
218 biosimetry as per the DVGW drinking water standards protocol (12). *B.pumilus* endospores are reported to  
219 exhibit a similar UV wavelength sensitivity to the highly resistant pathogen hAdV and have been suggested as  
220 potential surrogate challenge organisms for hAdV in UV reactor validation trials (16,17,19). However, the  
221 artificial modification of endospore resistance via inorganic compound supplementation to emerging UV  
222 technologies has yet to be established.

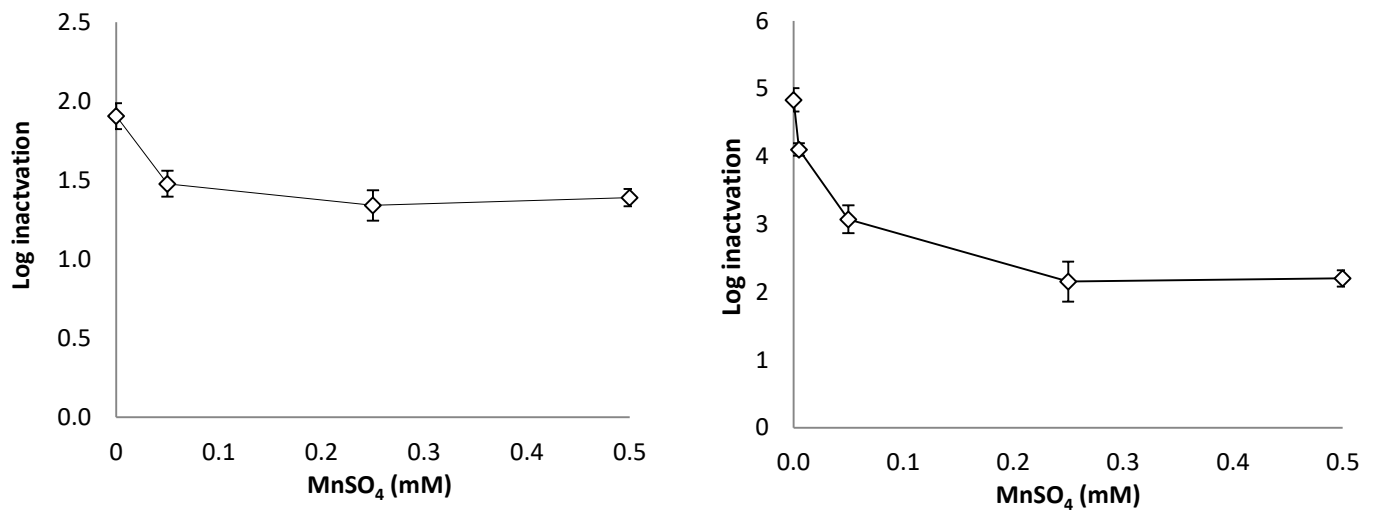
223 Findings on the impact of increasing  $\text{MnSO}_4\cdot\text{H}_2\text{O}$  concentrations (0 mM, 0.05 mM, 0.25 mM and 0.5 mM) in  
224 culture agar media on the log inactivation of *B.pumilus* endospores using a single PUV output of 2,052  $\text{mJ}/\text{cm}^2$   
225 are exhibited in Figure 1(a). Results showed that 1.5 log inactivation of *B.pumilus* endospores was observed  
226 using a concentration of 0.05 mM  $\text{MnSO}_4\cdot\text{H}_2\text{O}$ . In comparison, endospores cultivated in the absence of

227 MnSO<sub>4</sub>.H<sub>2</sub>O (or 0 mM) were inactivated by almost 2 log orders when treated at the same UV output. The log  
228 inactivation at 0 mM MnSO<sub>4</sub>.H<sub>2</sub>O was found to be significantly different ( $P<0.05$ ) to the two highest  
229 MnSO<sub>4</sub>.H<sub>2</sub>O concentrations analysed; 0.25 mM and 0.5 mM. There appeared to be a saturation point for  
230 MnSO<sub>4</sub>.H<sub>2</sub>O supplementation at 0.25mM as no significant difference was found between log inactivations at  
231 0.25mM and 0.5mM MnSO<sub>4</sub>.H<sub>2</sub>O ( $P>0.05$ ). The impact of the MnSO<sub>4</sub>.H<sub>2</sub>O agar supplementation for *B.pumilus*  
232 endospores inactivation using LPUV at a single output of 20 mJ/cm<sup>2</sup> is shown in Figure 1(b). Five concentrations  
233 of MnSO<sub>4</sub>.H<sub>2</sub>O were analysed, namely 0 mM, 0.005 mM, 0.05 mM, 0.25 mM and 0.5 mM. Endospores  
234 cultivated in the absence of supplement MnSO<sub>4</sub>.H<sub>2</sub>O were inactivated by 4.8 log. In comparison, the same  
235 endospores cultivated with the maximum concentration of 0.5 mM were inactivated by 2.2 log (UV output of  
236 20 mJ/cm<sup>2</sup>). Statistical analysis yielded similar results to those found with the PUV system; a significant  
237 difference was observed between endospore log inactivations at 0 mM and the two highest concentrations of  
238 0.25 mM and 0.5 mM MnSO<sub>4</sub>.H<sub>2</sub>O( $P<0.05$ ) while no significant difference was found between the log  
239 inactivations at the two highest MnSO<sub>4</sub>.H<sub>2</sub>O concentrations of 0.25 mM and 0.5 mM. Thus, for both the PUV  
240 and LPUV systems, *B.pumilus* endospores UV resistance appeared to be enhanced with the addition of minimal  
241 MnSO<sub>4</sub>.H<sub>2</sub>O supplementation. It was also clear that for both systems the 0.25mM concentration appeared to be  
242 saturation point for MnSO<sub>4</sub>.H<sub>2</sub>O supplementation and UV resistance therefore this concentration was selected  
243 for use in the following experiments.

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(a)

(b)

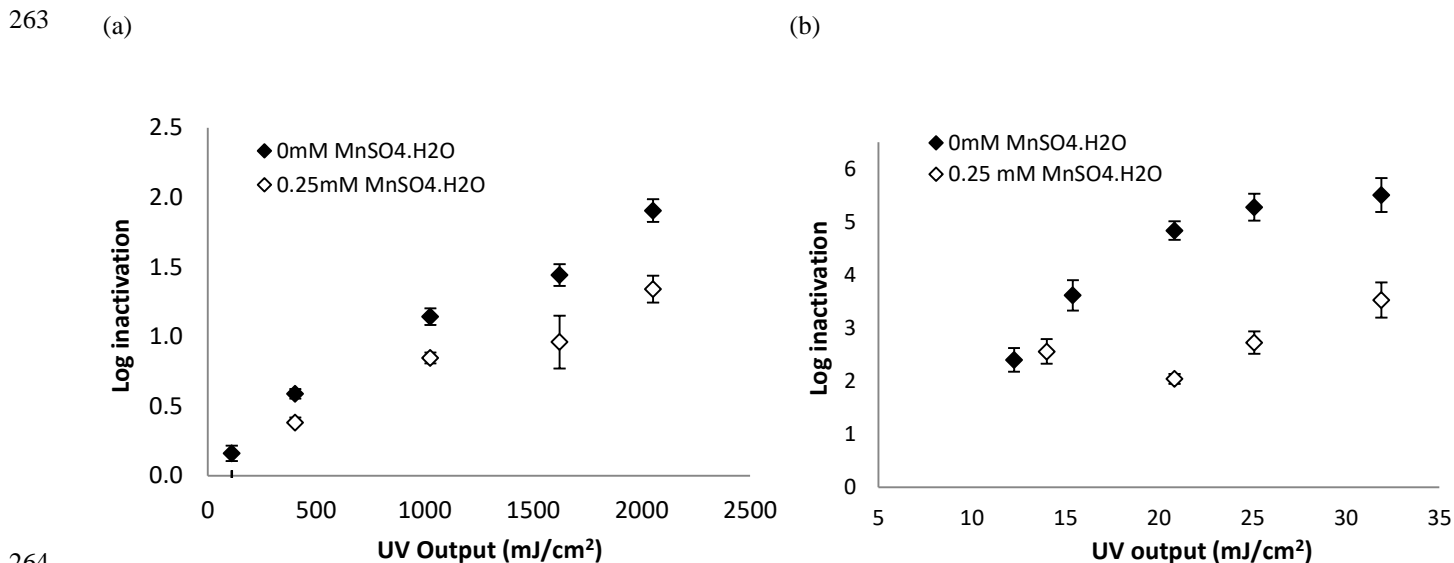


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246 **Figure 1. Impact of agar supplement  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  concentration on *B. pumilus* inactivation via (a) PUV**  
 247 **output at 2,052  $\text{mJ}/\text{cm}^2$  and (b) LPUV output at 20  $\text{mJ}/\text{cm}^2$ . Points are averages of at least three runs**  
 248 **and error bars are standard deviations.**

249 Figures 2(a) and 2(b) illustrate the UV output response of *B. pumilus* endospores post PUV and LPUV treatment  
 250 respectively, in the absence of  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  and those cultivated with agar supplemented with  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  (0.25  
 251 mM). The inactivation profiles of *B. pumilus* endospores (cultivated in the absence of  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  supplement)  
 252 via PUV and LPUV disinfection was carried out and results showed a PUV output of approximately 2,052  
 253  $\text{mJ}/\text{cm}^2$  was required to inactivate *B. pumilus* endospores by almost 2 log in a flow-through system while a LPUV  
 254 output of 12  $\text{mJ}/\text{cm}^2$  was required for the same inactivation level of 2 log. Complete inactivation of the  
 255 endospores via LPUV disinfection was achieved at a UV output of approximately 30  $\text{mJ}/\text{cm}^2$ . Further detailed  
 256 information is available in Supplementary Material (Figure S3 (a) and (b)). For PUV inactivation, the impact of  
 257  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  supplement on endospore UV resistance was observed to increase with the UV output applied.  
 258 LPUV results do not appear to follow the same trend; a difference of 2.7 log inactivation was found at a UV  
 259 output of 20  $\text{mJ}/\text{cm}^2$  while at the highest UV output (approximately 30  $\text{mJ}/\text{cm}^2$ ) a difference of 2 log inactivation  
 260 was found. Findings suggest cultivation agar supplemented with 0.25 mM  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  has an impact on the

261 UV resistance of *B.pumilus* endospores in particular when inactivated using LPUV disinfection (albeit higher  
262 disinfection rates were still observed than with the PUV system).

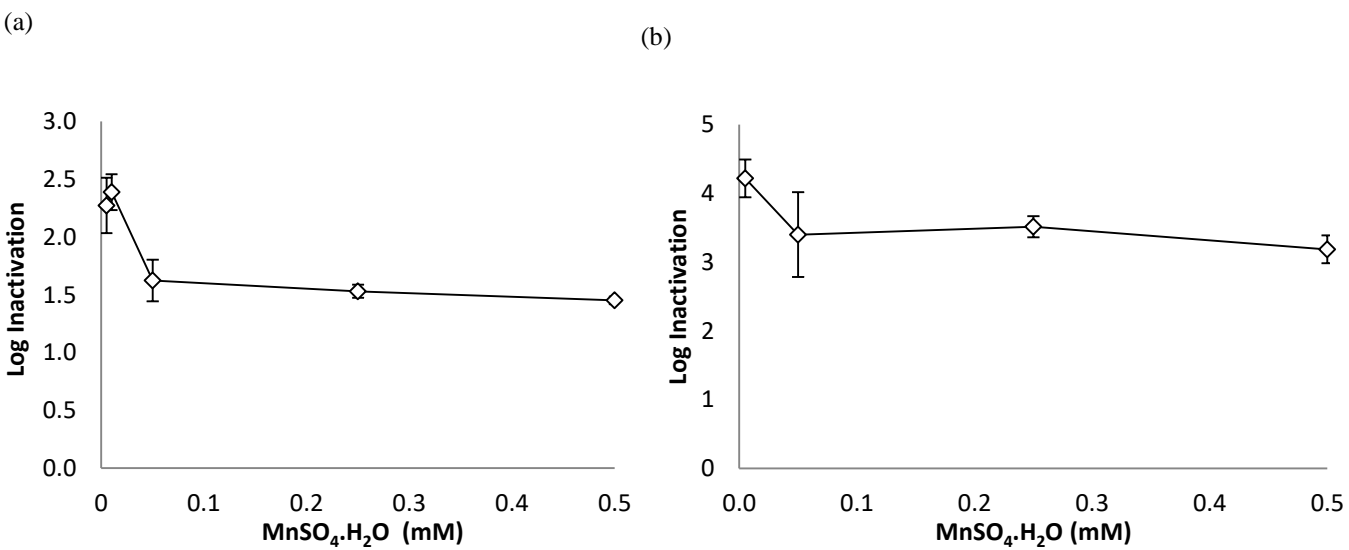


264  
265 **Figure 2. UV response curve of *B.pumilus* inactivation via (a) PUV and (b) LPUV with (0.25mM) and**  
266 **without (0mM) MnSO<sub>4</sub>.H<sub>2</sub>O supplement.**

267 The propagation of *B.subtilis* endospores in the absence of MnSO<sub>4</sub>.H<sub>2</sub>O was not possible in this study thus the  
268 minimum concentration of MnSO<sub>4</sub>.H<sub>2</sub>O that could be applied while still cultivating *B.subtilis* endospores was  
269 0.005 mM. This is likely to be attributed to the short propagation of endospores within ca. 4 days on media  
270 supplemented with MnSO<sub>4</sub>.H<sub>2</sub>O at above ambient temperatures in contrast to natural aging process in agar that  
271 occurs after typically 7 to 10 days due to the conversion of vegetative bacteria to endospores as a consequence  
272 of nutrient deprivation, build up of microbial waste products and reduction in water activity values. There is  
273 very limited knowledge on the specific role of media formulation on triggering the conversion from vegetative  
274 to endospore state. However, recent studies have reported that maintaining levels of the chemical compound  
275 Ca<sup>2+</sup> and pyridine-2,6-dicarboxylic acid (dipicolinic acid (DPA)) at 10% in the spore cortex appears critical as  
276 it has been shown to influence endospore resistance to environmental stresses and spore stability (47,48). Thus,  
277 it can be postulated that manganese sulphate monohydrate, in part, may influence the formation of this Ca-DPA

278 and concentrations thereof in spores, but this needs to be substantiated. One function of Ca-DPA in spore  
279 resistance is to lower the core water content which may also function to protect the endospore DNA by a variety  
280 of damaging agents, including UV radiation (49). Findings from this present study showed a similar log  
281 inactivation of *B. subtilis* endospores at the two lowest MnSO<sub>4</sub>.H<sub>2</sub>O concentrations (no significant difference; P  
282 > 0.05) (Figure 3(a)), but a decrease in log inactivation efficiency of approximately 0.6 log when supplemented  
283 with 0.05 mM – a similar result was found for *B.pumilus* inactivation via PUV (Figure 1(a)). A significant  
284 difference was found between the log inactivations at the two lowest concentrations in comparison to the other  
285 three higher concentrations (P<0.05). For the LPUV system, *B. subtilis* did not appear to be as readily affected  
286 by MnSO<sub>4</sub>.H<sub>2</sub>O supplement in comparison to *B.pumilus* (Figure 3(b)). Four concentrations of MnSO<sub>4</sub>.H<sub>2</sub>O were  
287 investigated; 0.005 mM, 0.05 mM, 0.25 mM and 0.5 mM. A log inactivation difference of 0.7 was observed  
288 between 0.005 mM MnSO<sub>4</sub>.H<sub>2</sub>O concentration and the 0.25 mM MnSO<sub>4</sub>.H<sub>2</sub>O concentration whereas a difference  
289 of 2 log was found between the same ranges for the *B.pumilus* experiments. Statistical analysis confirmed a  
290 significant difference (P<0.05) in endospore inactivation at the lowest MnSO<sub>4</sub>.H<sub>2</sub>O concentration (0.005 mM)  
291 and the highest concentration MnSO<sub>4</sub>.H<sub>2</sub>O concentration (0.5 mM) but not at 0.25 mM indicating a decreased  
292 sensitivity of *B. subtilis* endospores to MnSO<sub>4</sub>.H<sub>2</sub>O supplementation in comparison to *B.pumilus* for LPUV  
293 inactivation.

294



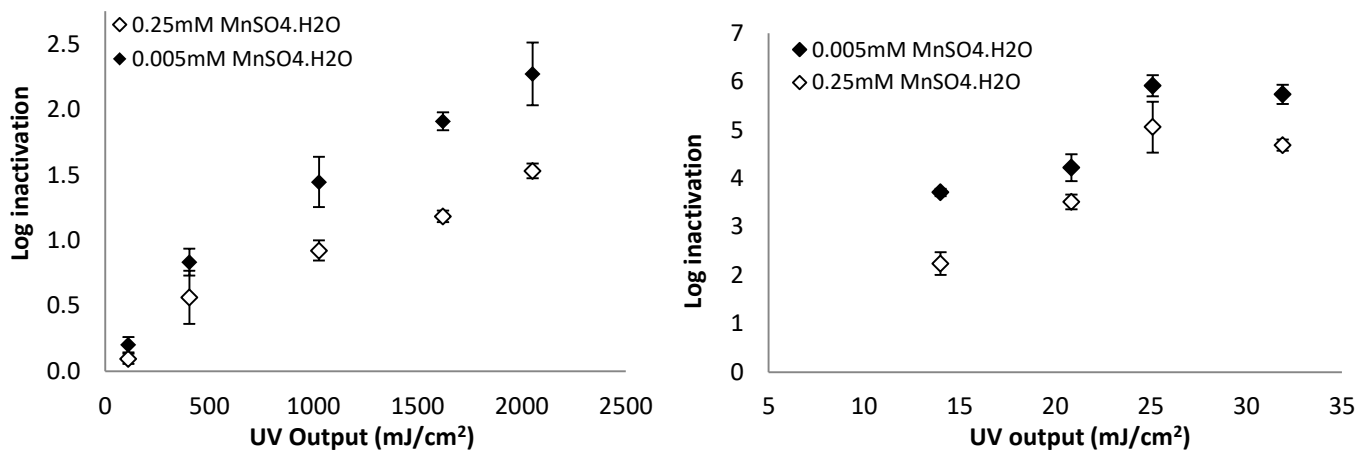
295

296 **Figure 3. Impact of agar supplement MnSO<sub>4</sub>.H<sub>2</sub>O concentration on *B.subtilis* inactivation via (a) PUV**  
297 **output (< 300 nm) at 2052mJ/cm<sup>2</sup> and (b) LPUV output at 20mJ/cm<sup>2</sup>.**

298 A UV response curve was generated for *B.subtilis* endospore inactivation via both flow-through PUV and LPUV  
299 (Figures 4(a) and 4(b)). The results of the PUV experiments followed a similar trend to the *B.pumilus* curve  
300 (Figure 2(a)) whereby as the UV output increased, so too did the impact of the MnSO<sub>4</sub>.H<sub>2</sub>O concentration on  
301 PUV inactivation efficiency. However, overall, *B.subtilis* endospores appeared to be more readily inactivated  
302 via PUV in comparison to *B.pumilus* endospores. At the maximum UV output of 2,052 mJ/cm<sup>2</sup> *B.subtilis*  
303 endospores were inactivated by 2.27 log and 1.53 log at 0.005 mM and 0.25 mM MnSO<sub>4</sub>.H<sub>2</sub>O concentrations  
304 respectively. In comparison, *B.pumilus* endospores were inactivated by 1.91 log and 1.34 log at 0.005 mM and  
305 0.25 mM MnSO<sub>4</sub>.H<sub>2</sub>O concentrations respectively. The UV response curve of *B.subtilis* endospores to LPUV  
306 disinfection at both low (0.005 mM) and high (0.25 mM) MnSO<sub>4</sub>.H<sub>2</sub>O concentrations (Figure 4(b)) is notably  
307 different to the same analysis with *B.pumilus* endospores (Figure 2(b)). *B.subtilis* endospores were inactivated  
308 by 5.91 log at 0.005 mM MnSO<sub>4</sub>.H<sub>2</sub>O at 25 mJ/cm<sup>2</sup>, at the same UV output the endospores were inactivated by  
309 5.06 log when supplemented with 0.25 mM MnSO<sub>4</sub>.H<sub>2</sub>O. In contrast, a difference of 2.56 log inactivation was  
310 found for *B.pumilus* endospores when analysed at the same MnSO<sub>4</sub>.H<sub>2</sub>O concentration and UV output. These  
311 results are in agreement with those found in Figure 3(b) and further indicate *B.subtilis* endospores are less  
312 affected by MnSO<sub>4</sub>.H<sub>2</sub>O supplementation in terms of UV resistance when compared to *B.pumilus* endospores.

313 (a)

(b)



314  
 315 **Figure 4. UV response curve of *B. subtilis* inactivation via (a) PUV and (b) LPUV when cultivated with**  
 316 **a high MnSO<sub>4</sub>.H<sub>2</sub>O concentration (0.25mM) versus a low (0.005mM) MnSO<sub>4</sub>.H<sub>2</sub>O concentration.**

317 **3.2 Flow-through UV system comparison for *Bacillus* endospores inactivation**

318 **3.2.1 *Bacillus pumilus* Inactivation**

319 Table 3 outlines the relevant literature to date investigating the inactivation of *B. pumilus* endospores via both  
 320 PUV and LPUV disinfection in comparison to results found in this study. To the best of our knowledge the  
 321 inactivation of *B. pumilus* endospores via pulsed UV disinfection in a flow-through system has not previously  
 322 been published in the literature. Moreover, the results demonstrate that when 0.25 mM of MnSO<sub>4</sub>.H<sub>2</sub>O was  
 323 added to cultivation agar, an increased UV output was required for the PUV system to achieve a similar log  
 324 inactivation to those endospores cultivated in the absence of MnSO<sub>4</sub>.H<sub>2</sub>O supplement. This finding is in  
 325 agreement with results for LPUV system analysis.

326 Previous research involving static PUV disinfection reported a UV dose of 500 mJ/cm<sup>2</sup> to achieve a 3 log  
 327 inactivation of the endospores on agar using the same strain of *B. pumilus* endospores utilised in this study (50).  
 328 The applied lower UV dose of 500 mJ/cm<sup>2</sup> which achieved a higher endospore log inactivation in comparison  
 329 to this research may be attributed to PUV set-up (static) and/or the disinfection medium (e.g. agar or hard  
 330 surfaces). Findings reported by Levy *et al.*, (2012) showed higher endospore inactivation rates on polystyrene,

331 glass and aluminium surfaces in comparison to agar surfaces (50). In addition, previous research has shown an  
 332 increased PUV output requirement for the inactivation of viruses in water via flow-through PUV in comparison  
 333 to static PUV while other studies have illustrated a difference in the inactivation mechanism of vegetative  
 334 *B.subtilis* cells when exposed to PUV in either a dry or liquid state (42,51). The method of UV dose/output  
 335 measurement may also be an important consideration when comparing the literature. Garvey *et al.*, (2013) used  
 336 chemical actinometry to determine the UV dose which measures the UV light absorbed by the sample and not  
 337 UV light emitted by the system, thus direct comparisons of UV dose/output and endospore inactivation levels  
 338 are often not possible (23).

339 For LPUV disinfection using *B.pumilus* endospores, previous research appears to have been carried out via static  
 340 system set-up only whereby a collimated beam apparatus was used to disinfect continuously stirring water in a  
 341 petri dish (Table 3). The LPUV doses required for endospore inactivation are higher in comparison to this study  
 342 however the continually mixing flow-through system may have aided endospore inactivation. Nevertheless,  
 343 despite the experimental set-up, the results for all of the LPUV studies in Table 3 comparing the impact of  
 344 MnSO<sub>4</sub>.H<sub>2</sub>O supplement on the enhanced UV resistance of *B.pumilus* are in agreement with the results found in  
 345 this study whereby the addition of MnSO<sub>4</sub>.H<sub>2</sub>O supplement aids endospore UV resistance (16,17,19). It is also  
 346 clear from data presented in Table 3 that the LPUV dose/output response of *B.pumilus* endospores tends to vary  
 347 by strain. It can also be concluded that *B.pumilus* endospores exhibit a similar response to PUV disinfection in  
 348 comparison to LPUV disinfection whereby cultivation agar supplemented with MnSO<sub>4</sub>.H<sub>2</sub>O enhances the UV  
 349 resistance of the endospore in flow-through systems.

350

351 **Table 3. Comparison of research studies for *B.pumilus* inactivation via PUV and LPUV disinfection.**

UV system &	Strain	MnSO <sub>4</sub> .H <sub>2</sub> O	System output/dose	Log	Reference
-------------	--------	-------------------------------------	--------------------	-----	-----------

set-up		(mM)	(mJ/cm <sup>2</sup> )	inactivation	
PUV static	ATCC 27142	FNA <sup>a</sup>	500 <sup>b</sup>	3.0	(50)
PUV static	ATCC 14884	0.02	0.00432 <sup>c</sup>	4.0	(23)
PUV flow-through	ATCC 27142	0	1,026 <sup>d</sup> (1,440 <sup>b</sup> )	1.4	This study
		0.25	2,052 <sup>d</sup> (2,880 <sup>b</sup> )	1.3	
LPUV static	ATCC 27142	0.1	138 <sup>e</sup>	2.0	(16)
LPUV static	ASFUVRC	0.1	348 <sup>e</sup>	2.0	(16)
LPUV static	ASFUVRC	0.1	174.7 <sup>e</sup>	2.0	(18)
LPUV static	ASFUVRC	0	77 <sup>e</sup>	3.0	(19)
		1	>150 <sup>e</sup>	2.0	
LPUV static	ASFUVRA	0	59 <sup>e</sup>	3.0	(19)
		1	200 <sup>e</sup>	2.0	
LPUV static	SAFR-032	0	76 <sup>e</sup>	3.0	(19)
		1	250 <sup>e</sup>	2.0	
LPUV flow-through	ATCC 27142	0	12 <sup>f</sup>	2.4	This study
		0.25	25 <sup>f</sup>	2.2	

<sup>a</sup>FNA – fortified nutrient agar used – typically pre-supplemented with 30mg/L MnSO<sub>4</sub>.H<sub>2</sub>O

<sup>b</sup>Broad spectrum dose measured with a joulemeter or radiometer

<sup>c</sup>UV dose measured as per actinometry method i.e. the amount of UV energy absorbed

<sup>d</sup>Calculated UV output (< 300nm) based on system specifications at 0 cm from lamp

<sup>e</sup>UV dose measured with a radiometer

<sup>f</sup>UV output based on system specifications

### 3.2.2 *Bacillus subtilis* Inactivation

360 *B.subtilis* endospores were less resistant to UV irradiation in comparison to *B.pumilus* endospores when  
361 cultivated with both low and high MnSO<sub>4</sub>.H<sub>2</sub>O concentrations when exposed to the PUV system. This may be  
362 due to differences in coat structure and/or proteins; it has been shown *B.subtilis* spores are more resistant to  
363 pulsed irradiation when expressing proteins linked to coat formation in comparison to mutated strains where  
364 said proteins were absent (52). The spectral sensitivity of both strains differ with *B.subtilis* endospores being  
365 most sensitive to wavelengths around 265 nm while *B.pumilus* endospores are most sensitive to wavelengths at  
366 approximately 220 nm (18,53). Spectral peaks of the PUV system used in the study were observed at 247 nm  
367 and 260 nm therefore the UV-C portion of the lamp output may favour inactivation of *B.subtilis* endospores over  
368 *B.pumilus* endospores. The disinfection of *B.subtilis* endospores in wastewater effluent via a flow-through PUV  
369 system has previously been reported, however the applied UV dose/output was not calculated. Rather, the log  
370 reduction per flow rate was given in synthetic municipal wastewater effluent (e.g. a flow rate of 8 L/min resulted  
371 in a 3.89 log reduction) (44). The authors completed a similar study investigating *B.subtilis* endospore  
372 inactivation via static PUV and in that study a broadband PUV output of 2,900 mJ/cm<sup>2</sup> was reported to give a  
373 1.72 log inactivation (39). Information on whether MnSO<sub>4</sub>.H<sub>2</sub>O supplement was added to cultivation agar for  
374 both studies was not provided. A similar study investigating the static PUV disinfection of *B.subtilis* endospores  
375 in suspension did report agar supplementation of 0.006 mM MnSO<sub>4</sub>.H<sub>2</sub>O. Samples were subjected to PUV doses  
376 of 500 mJ/cm<sup>2</sup> and 1000 mJ/cm<sup>2</sup> which reduced endospore counts by 1.1 log and 2.7 log respectively (21). The  
377 evaluation of PUV disinfection efficiency of *B.subtilis* endospores on a number of static surfaces; agar,  
378 polystyrene and glass was also investigated and reported a UV dose requirement of 500 mJ/cm<sup>2</sup> to achieve a 3  
379 log inactivation of the endospore on all three surfaces (50). Moreover, in-depth analysis of the germicidal  
380 effectiveness of the PUV broadspectrum applied confirmed the PUV system to be almost completely ineffective  
381 against *B.subtilis* endospores when UV wavelengths of <300 nm were removed from the treatment. This result  
382 has been corroborated in other studies for the PUV disinfection of *B.pumilus* endospores, *Listeria*  
383 *monocytogenes* and *Listeria innocua* (41,52,54,55). Thus, differences between inactivation results and PUV

384 dose/output measurements within the literature underline the necessity to consider the method by which the UV  
385 dose/output is determined for each system.

386 A LPUV output of 25 mJ/cm<sup>2</sup> yielded a log inactivation of 5.91 when *B.subtilis* endospores were  
387 cultivated with 0.005 mM MnSO<sub>4</sub>.H<sub>2</sub>O supplement. In contrast, endospores cultivated in 0.25mM MnSO<sub>4</sub>.H<sub>2</sub>O  
388 illustrated a greater UV resistance with a log inactivation of 5.06 at the same UV output. The inactivation of  
389 *B.subtilis* endospores via LPUV disinfection has been widely reported, perhaps due to its routine use as a  
390 challenge microorganism in UV reactor validation trials. The strain ATCC 6633 was chosen for this study as it  
391 has been widely analysed in the literature and hence may allow for greater intercomparisons. A peer review of  
392 the literature by Malayeri *et al.*, (2016) cited a total of 16 studies which met specific selection criteria for the  
393 investigation of *B.subtilis* endospore inactivation via LPUV disinfection (56). To achieve a 2 log inactivation, a  
394 UV dose range of between 15 mJ/cm<sup>2</sup> and 48 mJ/cm<sup>2</sup> was reported for all studies. The majority of the studies  
395 did mention the use of sporulation mediums which would typically contain the supplement MnSO<sub>4</sub>.H<sub>2</sub>O however  
396 the addition of MnSO<sub>4</sub>.H<sub>2</sub>O to cultivation agar or broth was not specified. In comparison, this study observed  
397 that at a UV dose of 14 mJ/cm<sup>2</sup>, log inactivations of 3.71 and 2.24 were achieved when cultivation agar was  
398 supplemented with 0.005 mM and 0.25 mM MnSO<sub>4</sub>.H<sub>2</sub>O respectively. Thus, results are agreeable with those  
399 cited in the literature though in this study MnSO<sub>4</sub>.H<sub>2</sub>O doses are indicated. Differences in endospore log  
400 inactivation depending on whether the endospores were cultivated in liquid or from the surface of agar have  
401 been reported previously. Bohrerova *et. al.*, (2006) demonstrated that *B.subtilis* endospores (ATCC 6633)  
402 cultivated in liquid broth required a LPUV dose 30 mJ/cm<sup>2</sup> to yield a 1 log inactivation while those cultivated  
403 on agar required a lower dose of approximately 10 mJ/cm<sup>2</sup> for the same log inactivation (57). As appears to be  
404 the case with *B.pumilus* spores, the LPUV sensitivity of *B.subtilis* may also vary depending on the cultivation  
405 method applied.

406 While a typical xenon gas PUV system is comprised of light emissions within the broadspectrum of UV, visible

407 and infrared light, it may be an important consideration to prioritise pulsed UV dose/output calculations in terms  
408 of ‘biocidal PUV dose/output’ i.e. the energy applied from wavelengths below 300 nm ahead of the ‘total PUV  
409 dose/output’ which infers the total energy applied across the whole broadspectrum output. This has been  
410 previously demonstrated in a number of studies with the aid of spectrometers/pyroelectric detectors and in some  
411 cases the UV dose/output from PUV systems is within the same order of magnitude as LPUV dose/output outputs  
412 e.g. 1-100 mJ/cm<sup>2</sup> (35,36,55). In this study measurements indicated that only 26% of the lamp energy reached  
413 the sample (at 900V and a distance of 10.75 cm) and of that, only 8% was within the UV wavelength range  
414 (Table 2). The system type and the amount of UV energy produced by individual systems may also influence  
415 the UV dose/output. For example, Vimont *et al.*, (2015) illustrated that just 2% of the broadband energy was  
416 within the UV range which is in agreement with findings in this study which also found the UV power to account  
417 for 2% of the total broadspectrum output of the lamp. Recent research by Gómez-López & Bolton (2016)  
418 attempted to address the challenges associated with determining the exact pulsed UV dose delivered by  
419 suggesting a standard method for the UV fluence or dose measurement from PUV disinfection systems in water  
420 by way of chemical actinometry methods (45). The protocol calls for energy calculations to be reported in terms  
421 of photon irradiance using the units einstein/(m<sup>2</sup>/s) which may be better suited to the photobiological processes  
422 associated with polychromatic lamps. However, specific instrumentation and strict experimental set-up is  
423 required. As highlighted in Table 3, there are various ways UV dose/output can be measured and reported which  
424 can lead to disparities when comparing studies in the literature, particularly in the case of PUV system analysis.  
425 Furthermore, some studies omit UV dose/output calculations entirely and report only system specifications and  
426 experimental set-up which may not always allow for direct comparisons to similar work. These are important  
427 factors which should be noted when reporting analysis using UV systems.

428 Rowan *et al.*, (1998) have previously studied the influence of variations in media formulation on bacterial  
429 physiology and subsequent tolerance to environmental stresses (58–62). It has been reported that the  
430 supplementation of growth media with food additive maltodextrin augmented the diarrhoeal enterotoxin

431 production in *Bacillus cereus* (61). Moreover, variations in media formulation and storage affected the virulence  
432 and pathogenesis of *B. cereus* in hospitalised HIV patients. Enhancing the osmotic environment of culture media  
433 with increasing concentrations of glucose has also been proven to affect the tolerance of *Listeria monocytogenes*  
434 to PUV treatments (59). The specific mechanistic involvement of  $MnSO_4 \cdot H_2O$  on enhanced endospore resistance  
435 to UV disinfection has yet to be elucidated but would be potentially unravelled by mapping gene transcripts  
436 using microarray and quantitative PCR given the informative role of metagenomics in modern day research.

437 The findings of this study highlight the need for international consensus on agreed standard methods in  
438 addition to a broader evaluation of the research to inform the validation of emerging technologies for water and  
439 adjacent food and pollination industries (63, 64, 65). PUV has been adopted commercially for food packaging  
440 but has yet to be applied for other industrial applications on a large scale, which may be attributed in part to lack  
441 of global harmonisation of metadata (58, 66). This particular study highlights the need to consider a holistic  
442 inter-laboratory study to assess the effectiveness of pulsed light where multi-parameters and trials are conducted  
443 to validate its application for water treatment (63). Frits *et al.*, (2018) recently highlighted this gap in the  
444 harmonisation of knowledge when it came to comparing the effectiveness of existing and emerging innovation  
445 for inactivating complex food-borne parasites and their transmission stages (67). Findings from this present  
446 study also supports the need for the development of appropriate risk assessment and predictive models that will  
447 help the uptake of new technologies for the water industry along with satisfying adjacent environmental policy  
448 and decision-making needs (68, 69). The aforementioned may explain why LPUV is still a preferred method of  
449 choice for the water industry over the past 50 years as it is a globally accepted technology supported by decades  
450 of validated disinfection data.

451

#### 452 **4.0 Conclusions**

453 This study showed that supplementing cultivation agar with  $MnSO_4 \cdot H_2O$  to aid the sporulation of *B.pumilus* and  
454 *B.subtilis* enhanced their resistance to both PUV and LPUV irradiation in flow-through systems that have

455 implications for comparative evaluation of water-based disinfection research and validation of innovation.  
456 Moreover, the level of UV resistance increased significantly when relatively low concentrations (0.05 mM) of  
457  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  were added to cultivation agar. *B.pumilus* endospores appeared to be more readily affected by the  
458 addition of the supplement in terms of increased UV resistance, particularly in the case of LPUV disinfection.  
459 In general, *B.subtilis* endospores were inactivated to a higher degree by the PUV and LPUV systems in  
460 comparison *B.pumilus* endospores. The energy required by the PUV system to inactivate both *Bacillus spp.*  
461 endospores was two orders of magnitude higher than the energy required by the LPUV. This is in keeping with  
462 previous work whereby significantly higher energy outputs were necessary to achieve similar log inactivations  
463 of various microorganisms. The flow-through PUV system appeared to offer no clear advantage over the LPUV  
464 in terms of bacterial endospore inactivation, however further research is warranted in terms of whether the high  
465 energy pulses of the PUV system might inhibit the photoreactivation of bacteria often associated with LPUV  
466 disinfection processes. In addition, studies are merited by way of optimising (i) the delivery of the pulsed UV  
467 light source in flow-through applications and (ii) system configuration in terms of energy delivery. It is  
468 recommended that future studies involving *Bacillus spp.* endospores and UV inactivation clarify whether  
469 manganese sulphate supplementation was applied during the cultivation stage and at what concentration to allow  
470 for stricter inter-comparison in terms of UV requirements. In the case of the PUV system, it is suggested that  
471 studies report full UV dose/output details e.g. whether the reported UV dose/output accounts for the full  
472 broadspectrum or the UV portion alone, whether it is calculated or measured, the number of pulses applied, the  
473 pulse rate and energy per pulse. In this way, clear comparisons can be drawn between PUV inactivation studies.  
474 This study highlights the need to consider future harmonisation of data for PUV and other emerging treatment  
475 technologies through pursuit of holistic, multi-parameter, inter-laboratory studies so as to validate these new  
476 innovations for uptake by the water industry.

477

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481

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