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1 Spectroscopic and microscopic characterization of dipicolinic acid and its salt photoproducts  
2 - A UVc effect study on DPA in solution and in bacterial spores.

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14

15 **Abstract:**

16 Bacterial spores can cause significant problems such as food poisoning (like neurotoxin or emetic  
17 toxin) or serious illnesses (like anthrax or botulism). This dormant form of bacteria, made of several  
18 layers of barriers which provide extreme resistance to many abiotic stresses (radiation, temperature,  
19 pressure, etc.), are difficult to investigate *in situ*. To better understand the biological and chemical  
20 mechanisms involved and specific to spores resistance, the acquisition of environmental parameters  
21 is necessary. For that purpose, our research has been focused on the detection and analysis of a  
22 unique spore component, dipicolinic acid (DPA), used as the main *in situ* metabolite for sporulating  
23 bacteria detection. In its native form, DPA is only weakly fluorescent but after Ultraviolet irradiation  
24 at the wavelength of 254 nm (UVc), DPA photoproducts (DPAp) exhibit a remarkable fluorescence  
25 signal. These photoproducts are rarely identified and part of this study gives new insights offered by  
26 mass spectrometry (MS) in the determination of DPA photoproducts. Thanks to DPA assay  
27 techniques and fluorescence spectrometry, we highlighted the instability of photoproducts and  
28 introduced new assumptions on the effects of UVc on DPA. Studies in spectroscopy and microscopy  
29 allowed us to better understand these native probes in bacterial spores and will allow the  
30 implementation of a new method for studying the physico-chemical parameters of spore resistance.

31 **Keywords:**

32 Bacterial spore – DPA – Photoproducts – Native probe – Fluorescence lifetime – Phasor plot – Two-  
33 photon microscopy

34

## 35 **1. Introduction**

36 Bacterial spores are a dormant form of bacteria structured in several layers: exosporium (absent  
37 from *B. subtilis*), coats, outer membrane, cortex, inner membrane and a core; which result in high  
38 resistance to extreme conditions [1,2]. These structures are mainly composed of lipids,  
39 polysaccharides or proteins. Because of its multilayer structure, the molecules and their structure  
40 involved in the resistance properties of this bacterial form are difficult to study separately. Moreover,  
41 analysis often requires invasive or destructive methods. There is a need to determine the physico-  
42 chemical parameters of bacterial spores leading to their resistance. These parameters should be  
43 measured inside the spore core. The investigation difficulties explain the lack of knowledge  
44 concerning the physical state and mobility of spore core. The form of CaDPA inside the spore core is  
45 not clear, some authors suggest, for example, that CaDPA is probably present in a powder form  
46 inside the spore [3]. Moreover, pH inside the spore core and water activity are still not been easily  
47 measured *in vivo* and require destructive or complex methods to be investigated. These parameters  
48 are important for spore resistance against physical stresses used in sterilization processes like wet  
49 heat resistance or even UV resistance. Having new insights of what are the precise internal physico-  
50 chemical parameters could lead to the development of more precise and destructive methods to  
51 sterilize spores in food matrix and avoid spoilage or diseases caused by pathogenic sporulated  
52 bacteria. To achieve this objective, it is interesting to look at native probes (intrinsic probes in the  
53 medium) present in bacterial spores. There are two native biomolecules sufficiently concentrated in  
54 the core that could govern spore resistance, particularly against temperature and UV exposure.  
55 Water is the first of these molecules as H<sub>2</sub>O makes up 27-55% of the spore core wet weight  
56 depending on the species [4–6]. Dipicolinic acid, also named 2,6-pyridinedicarboxylic acid (DPA) is the  
57 second core molecule involved in spore resistance [7–9]. DPA is involved in low water activity of the  
58 spore core. This gives the bacterial spore its high thermal resistance properties. DPA is a specific  
59 spore molecule present in large quantities in bacterial spores (5-15% of spore dry weight) [10]. DPA is  
60 located only in the core, and Raman spectroscopy showed that it should be present in equimolar  
61 amounts with calcium, as CaDPA [3]. The use of DPA and CaDPA as a probe for the study of bacterial  
62 spores has been largely documented [11–16]. Those studies used destructive methods of the spore  
63 structures to extract DPA outside the core. Another approach consists in analyzing the photoproduct  
64 forms of DPA and CaDPA (DPAp and CaDPAp), because of their fluorescent emission properties

65 [17,18]. DPA and CaDPA are only very weakly fluorescent thus irradiation at 254 nm is required to  
66 create DPAP and CaDPAP that will emit fluorescence signals. This method has already been used for  
67 the analysis of bacterial spores [19,20] but very few publications succeeded in determining the  
68 characteristics of these photoproducts [21,22]. Even if DPA seems present mainly in chelated forms,  
69 particularly with calcium, studies showed the interest in studying both compounds (DPA and CaDPA)  
70 because of the sensitivity of fluorescence to diagnose environmental conditions of bacterial spores  
71 [18].

72 This paper aims to discuss the photochemical effect of UVC on DPA and CaDPA and to characterize  
73 the resulting molecules DPAP and CaDPAP to detect them in bacterial spores. For that purpose, the  
74 fluorescence of these photoproducts was investigated with excitation emission matrices of  
75 fluorescence (EEMF) and emission spectra at fixed excitation wavelengths. The characterization of  
76 photoproducts and their stability has been studied using various techniques, such as the dosage of  
77 Terbium-DPA complex and mass spectrometry. The visualization of its photoproducts was carried out  
78 by multi-photon microscopy techniques and by analysis of the fluorescence lifetimes associated with  
79 these molecules.

80

## 81 **2. Materials and Methods**

### 82 **2.1. Chemicals**

83 DPA (Sigma-Aldrich, Germany, 167.12 g/mol, purity > 98 %) solution (10 mM) was prepared in water  
84 and stored at room temperature. The pH was adjusted to 8 with a Tris base buffer (Sigma-Aldrich,  
85 Germany, 121 g/mol, purity > 99.9 %). CaDPA solution (50 mM) was prepared with a 1:1 mix of DPA  
86 solution and CaCl<sub>2</sub> (Sigma-Aldrich, Germany, 110.98 g/mol, purity > 96 %).

### 87 **2.2. Bacterial strains and culture conditions**

88 *B. subtilis* strains used in this study were PS533 (wild-type), PS4150 and FB122. PS533 was obtained  
89 from the courtesy of P. Setlow from the Department of Molecular, Microbial, and Structural Biology,  
90 University of Connecticut Health Center, USA. This strain was carrying a plasmid encoding resistance  
91 to kanamycin. PS4150 was a strain that was unable to form the majority of proteins from the outer  
92 coat layer because of the deletion of *cotE* and *gerE* coding sequences, making this strain non  
93 autofluorescent [23]. FB122 was a strain deleted for the *spoVF* operon and *SleB* gene, resulting in a  
94 strong depletion of DPA content [7].

95 For spores production, the different strains were first isolated on LB agar medium containing  
96 antibiotics (10µg/mL of kanamycin for PS533, spectinomycin 100 µg/ml + 10µg/mL tetracycline for  
97 PS4150 and FB122) at 37°C for 12h. An isolated colony was then placed on double strength  
98 Schaeffer's-glucose liquid medium (2xSG) at 37°C under agitation until reaching an OD of 0.6. Then  
99 the culture was diluted 10 times on a fresh 2xSG medium and incubated under agitation 5 days at  
100 37°C. All purified spores were washed at least 3 times with sterilized distilled water at 4°C until 98%  
101 purity was reached and stored at 4°C.

### 102 **2.3. Photolysis conditions**

103 Ultraviolet (UV) corresponds to wavelengths of light between about 100 and 400 nm, that are  
104 divided into several categories according to their ranges in this spectrum (UVA, UVb and UVC). The  
105 wavelength used for the photolysis is 254 nm, which correspond to UVC. UVC irradiation of DPA and  
106 CaDPA solutions was performed in distilled water, by means of a UV irradiation system  
107 CROSSLINKER® CL-508 (UVItec Ltd, UK) with 5 UVC tubes at 0.120 J/cm<sup>2</sup>. For the mass spectrometry  
108 (MS) experiment, DPA with greater purity (Sigma-Aldrich, Germany, 167.12 g/mol, purity > 99.5 %)  
109 (3 g/L) was diluted in ultrapure water. Water was removed after irradiation using a rotary  
110 evaporator. The residues of DPAP for each irradiation time were dissolved in methanol solution  
111 (Sigma-Aldrich, Germany, Chromasolv™ for HPLC min. 99.9 %). Irradiations of bacterial spores were  
112 performed in a liquid sample by means of 2 minutes UVC irradiation at 0.120 J/cm<sup>2</sup>, at a  
113 concentration of 1.10<sup>7</sup> cell/ml to avoid any shielding effect [24].

### 114 **2.4. Steady state fluorescence**

115 Excitation-emission matrices of fluorescence (EEMF) was acquired on a Horiba Aqualog (Horiba  
116 Scientific, Kyoto, Japan) unit by setting the excitation wavelengths from 225 nm to 600 nm (3 nm  
117 interval) and the emission wavelengths from 200 to 600 nm (3.22 nm). Each EEMF was corrected  
118 daily for Rayleigh scattering and inner filtering. It was normalized to a 1ppm quinine sulphate  
119 solution using the manufacturer's software (Aqualog software).

120 Emission spectra were acquired on a Jobin Yvon Fluorolog 3 (Horiba Scientific, Kyoto, Japan) by  
121 setting the excitation wavelengths at 300 nm and 340 nm and the emission wavelengths from 380 to  
122 500 nm with a 5 nm bandpass and a 1 nm step.

### 123 **2.5. DPA assay**

124 DPA assay was performed according to the protocol proposed by Hindle and Hall (1999). This stock  
125 solution of 0.1 mM DPA was prepared in a 16 g/L NaOH solution (Sigma-Aldrich, Germany, 40.00  
126 g/mol, purity > 98 %). Different volumes of this DPA were added to 3 mL of 1 M acetate buffer

127 (C<sub>2</sub>H<sub>3</sub>NaO<sub>2</sub>·3H<sub>2</sub>O, Sigma-Aldrich, Germany, 136.08 g/mol, purity > 99 %) (pH 5.6) and 3 μL of 10 mM  
128 TbCl<sub>3</sub> solution (Sigma-Aldrich, Germany, 373.38 g/mol, purity = 99.9 %) in quartz cuvettes to make  
129 the standard curve. Emission spectra were acquired on a Jobin Yvon FluoroMax-4 (Horiba Scientific,  
130 Kyoto, Japan). The excitation and emission wavelengths were 280 ± 10 nm and 545 ± 5 nm  
131 respectively. A glass slide was placed in front of the detector to filter the Rayleigh diffusion of water.  
132 The standard curve can be found in Supplementary Information (S.I.) Fig. S1 & S2.

### 133 **2.6. Q-ToF MS analysis**

134 Direct injection of DPA solution (3g/L in methanol) and DPAP identification was performed with a  
135 MaXis plus MQ ESI-Q-TOF mass spectrometer (Bruker, Bremen, Germany). MS experiments aim of  
136 identifying new photoproducts, and was not a quantitative method. Acquisitions were done in the  
137 *m/z* 100 to 1500 mass range in positive ionization mode with an electrospray ionization source. The  
138 injection flow was 200 μL/h, the nebulization pressure was 2 bar and the dry nitrogen flow was 10  
139 L/min. The mass spectrometer parameters were 500 V for end plate offset and 4500 V for capillary  
140 voltage. Before analysis, the mass spectrometer was calibrated by injecting Na Formate clusters. This  
141 calibration was realized in “enhanced quadratic” mode with an error of less than 0.5 ppm. Data were  
142 treated using Compass DataAnalysis v4.3 software (Bruker, Bremen, Germany). Elementary formula  
143 were determined using an isotopic profile.

### 144 **2.7. Multi-photon & fluorescence lifetime microscopy**

145 Fluorescence lifetime images were collected using a time-correlated single-photon counting (TCSPC)  
146 module (Picoquant, Germany) on a Nikon A1-MP scanning microscope equipped with a Plan Apo IR x  
147 60 objective (NA: 1.27, Water Immersion, Nikon, Japan) at a scanning speed of 1 frame per second. A  
148 700 nm excitation was provided by an IR laser (Chameleon, Coherent, California) delivering  
149 femtosecond pulses at a repetition rate of 80 MHz. Fluorescence emission of spores was collected on  
150 three detection channels for multiphoton imaging: FF01-492/SP-25 (400-492 nm), FF03-525/50-25  
151 (500-550 nm) and FF01-575/25-25 (563-588 nm) (Semrock, USA). For fluorescence lifetime imaging,  
152 fluorescence was collected through a band-pass emission filter 680/SP (Semrock, USA). TCSPC  
153 recording was performed over 200 temporal channels (final resolution 0.64 ps). We performed  
154 fluorescence lifetime image acquisition on spores using Symphotime software (Picoquant, Germany).  
155 Then fluorescence lifetimes were calculated by using the MAPI software (See next paragraph).

### 156 **2.8. Time resolved fluorescence analysis**

157 Fluorescence lifetime is a photo-physical parameter that varies with the conformation of the  
158 fluorophore molecule and with the external physico-chemical conditions of the local environment.

159 Fluorescence lifetime analysis was performed using the phasor plot representation provided by the  
160 MAPI software (IRI, USR 3078 CNRS, BCF, available on request at: [http://biophotonique.univ-](http://biophotonique.univ-lille1.fr/spip.php?rubrique60)  
161 [lille1.fr/spip.php?rubrique60](http://biophotonique.univ-lille1.fr/spip.php?rubrique60)) [26].

### 162 **2.8.1. Phasor plot representation**

163 The phasor plot is a graphical representation of the raw TCSPC data in a vector space obtained by the  
164 mathematical Fourier transform decomposition. Each fluorescence decay curve is converted into  
165  $[g ; s]$  coordinates, calculated by the cosine and sine transforms of the measured fluorescence  
166 intensity decay  $I(t)$ , defined by:

$$167 \quad g(\omega) = \frac{\int_0^T I(t) \cdot \cos(n\omega t) dt}{\int_0^T I(t) dt} \quad (3)$$

$$168 \quad s(\omega) = \frac{\int_0^T I(t) \cdot \sin(n\omega t) dt}{\int_0^T I(t) dt} \quad (4)$$

169 Where  $\omega$  is the laser repetition angular frequency,  $n$  is the harmonic frequency and  $T$  is the finite  
170 width of the temporal measurement window. For a mono-exponential decay, the  $[g ; s]$  coordinates  
171 are located on a semicircle centered on point  $[0.5;0]$  with a radius of  $s = 0.5$ . Whereas for a multi-  
172 exponential decay, the  $[g ; s]$  coordinates will no longer be located on the semicircle but rather  
173 inside this semicircle. In the semicircle, the short fluorescence lifetimes are close to the coordinates  
174  $[1;0]$ , and the long fluorescence lifetimes approach the origin  $[0;0]$ .

### 175 **2.9. Statistical analysis**

176 The DPA concentration and fluorescence intensity of bacterial spores were statistically analyzed with  
177 a variance analysis (ANOVA performed with Origin® (OriginLab®, USA),  $p$ -value  $<0.05$ ) to evaluate the  
178 differentiation significance between before and after UVC irradiation.

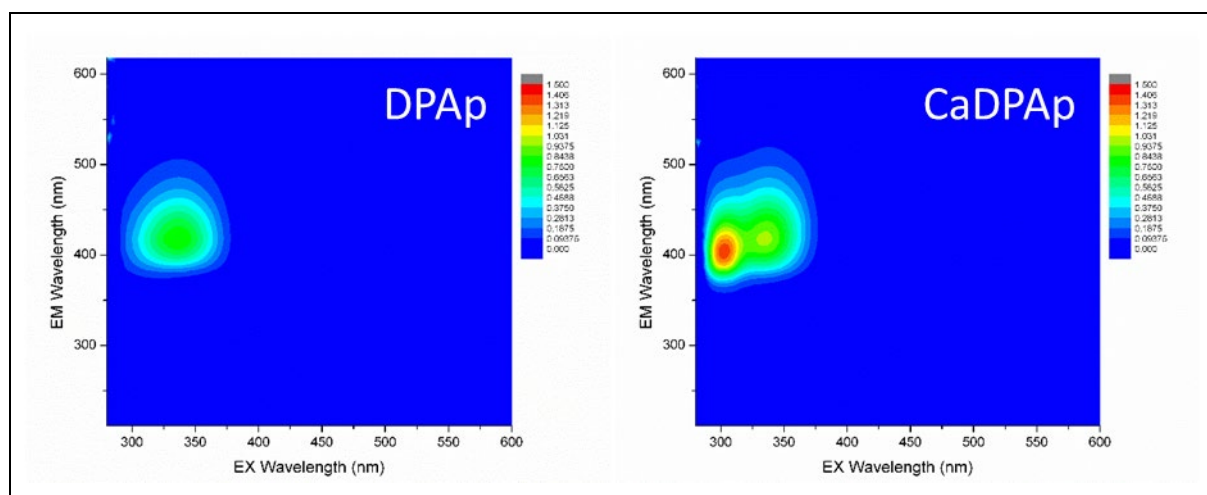
179

## 180 **3. Results & Discussion**

### 181 **3.1. Stationary spectroscopy on DPAP and CaDPAP**

182 Fig. 1 shows the effect of UVC irradiation on DPA (10 mM) and CaDPA (50 mM). Untreated DPA and  
183 CaDPA were very weakly fluorescent, which was already known [17]. After the UVC treatment, there  
184 were no changes in wavelengths of maximum absorbance (to collect the absorbance spectra, the  
185 solutions were diluted which did not change the couples excitation/emission wavelengths (EX/EM)  
186 (S.I. Fig. S3 & S4)). The EEMFs of DPAP and CaDPAP solution highlight the presence of two groups of  
187 fluorophores at EX/EM (nm): 340/430 nm and another one at 300/400 nm. The first group of

188 fluorophores with the EX/EM couple of 340/430 nm were mainly present in the DPAP solution but  
189 also observed in the CaDPAP solution. However, in CaDPAP solution, it was the group of fluorophores  
190 at the couple EX/EM of 300/400 nm that had the most influence on the fluorescence intensity. These  
191 results were in accordance with the literature [17–19]. Those EEMF showed at the same time the  
192 influence of the calcium salt on the fluorescence of the photoproducts and the importance of  
193 working at two different EX/EM couples to target and distinguish DPAP/CaDPAP fluorophores.



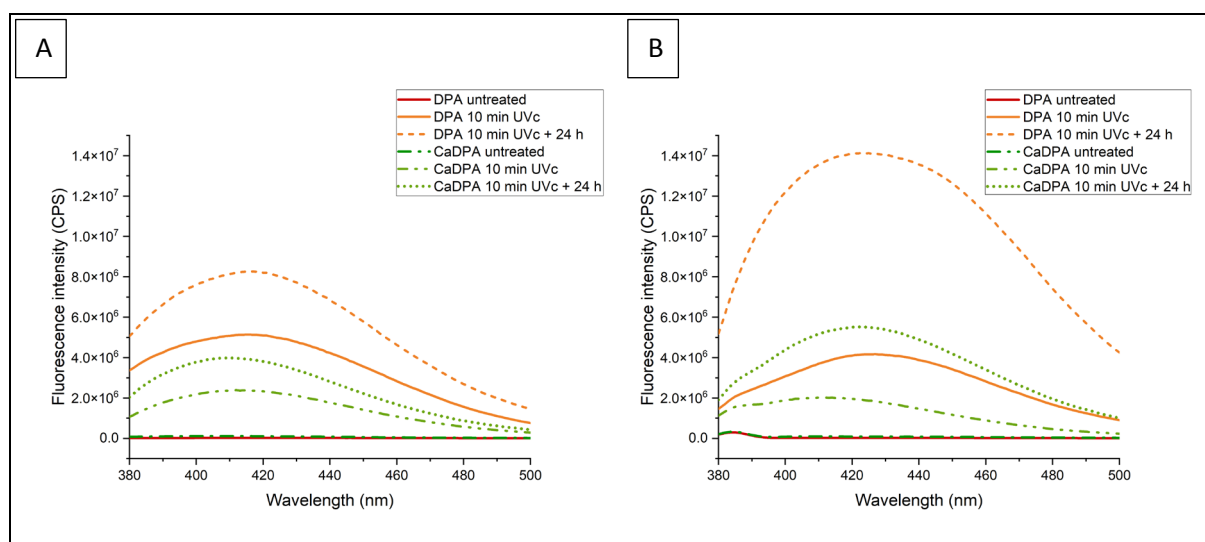
194 *Figure 1: UVC irradiation ( $\lambda_{ex}$ : 254 nm, 10 min at 0.120 J/cm<sup>2</sup>) on DPA (10mM) and CaDPA (50mM). EEMF of resulting*  
195 *photoproducts: DPAP and CaDPAP. DPA and CaDPA EEMF were not shown because the compounds did not emit enough*  
196 *fluorescence to be detected. The same intensity (a.u.) scale was used for all spectra.*

197

198 To confirm the previous results and observe the stability of the photoproducts, emission spectra  
199 were obtained for DPA and CaDPA before and after UVC treatment. As shown in Fig. 2, the  
200 fluorescence intensity significantly increased with irradiation (10 min at 0.120J/cm<sup>2</sup>). For DPA, the  
201 maximum of fluorescence intensity increased respectively by 156 and 216 times for EX/EM couples  
202 of 340/430 nm and 300/400 nm. For CaDPA, the maximum of fluorescence intensity increased  
203 respectively by 21 and 22 times. However, these experiments also highlighted that the  
204 photoproducts were partly unstable. For DPAP and CaDPAP, and for each EX/EM couple, the  
205 fluorescence intensity blatantly increased, by 1.6 to 3.4 times after 24 hours. A possible hypothesis of  
206 such instability of DPAP/CaDPAP obtained after UVC treatment could be attributed to the aromatic  
207 ring stretch and the bonds of the acidic functional groups becoming shortened [27]. These changes in  
208 the conformation of the molecule, and possibly others, may be responsible for the instability of  
209 DPAP. Subsequently the rearrangement of part of excited DPA or CaDPA (DPA\* or CaDPA\*) is  
210 probably oriented towards the production of photoproducts, which may explain the slight increase in  
211 fluorescence intensity.

212





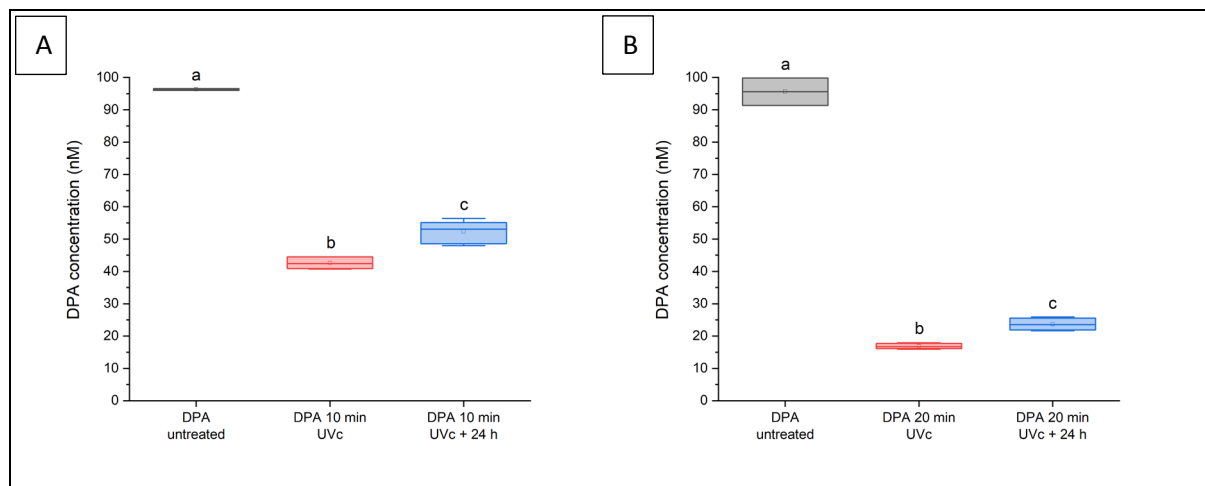
213 *Figure 2: Fluorescence emission spectra of DPA (10 mM) and CaDPA (50 mM) for two excitation wavelengths:  $\lambda_{ex} = 300$  nm*  
 214 *(A) and  $\lambda_{ex} = 340$  nm (B). The red (DPA) and the dark green (CaDPA) lines were obtained before UVc treatment (irradiation of*  
 215 *10 min. at  $0.120$  J/cm<sup>2</sup>). The orange and light green solid line correspond to DPAp and CaDPAp emission spectra after*  
 216 *irradiation. The corresponding dotted lines come from the same samples after 24h.*

217

### 218 3.2. UVc effect on DPA

219 Complexation between DPA and certain lanthanides makes it possible to drastically increase DPA  
 220 fluorescence, with emission bands characteristic of these complexes, it allows a quantitative and  
 221 specific detection of DPA. Terbium was the best lanthanide for the detection of DPA, as it provides a  
 222 greater fluorescence compared to other lanthanides [25]. For the DPA assay, we used TbCl<sub>3</sub> as  
 223 lanthanide able to form highly fluorescent complex with DPA. As shown in Fig. 3(A), after UVc  
 224 irradiation of 10 minutes at  $0.120$  J/cm<sup>2</sup>, the concentration of a DPA solution initially close to 100 nM  
 225 decreased to a mean value of 43.2 nM. For the same group of samples, after 24 hours of waiting, the  
 226 DPA dosage leads to a significant increase of the DPA concentration up to a mean value of 59.8 nM.  
 227 The same behavior was observed with another set of samples submitted to 20 minutes irradiation, as  
 228 shown in Fig. 3(B). In this case, the DPA concentration decreased up to a mean value of 16.8 nM after  
 229 the 20 minutes irradiation and increased up to a mean value of 23.6 nM after 24 hours. This behavior  
 230 was observed on the same set of samples irradiated twice 10 minutes (S.I. Fig. S5). This experiment  
 231 highlights the fact that excited DPA\* reverts to the DPA form some time after irradiation, which to  
 232 our knowledge, has never been demonstrated until now. Our hypothesis was clarified as follows:  
 233 after a UVc irradiation, the DPA\* will partly be transformed in photoproducts but a part of this DPA\*  
 234 stays in an unstable form. This unstable form of DPA\* will then break down into two parts, a first that  
 235 will return to its initial form of DPA and another part that will rearrange itself to form DPAp. Only a  
 236 small part of this DPA\* was supposed to rearrange into the photoproducts form, would have

237 explained the slight increase in fluorescence intensity after 24 hours, as observed in part 3.1 (Fig.  
238 2(A) and 2(B)).

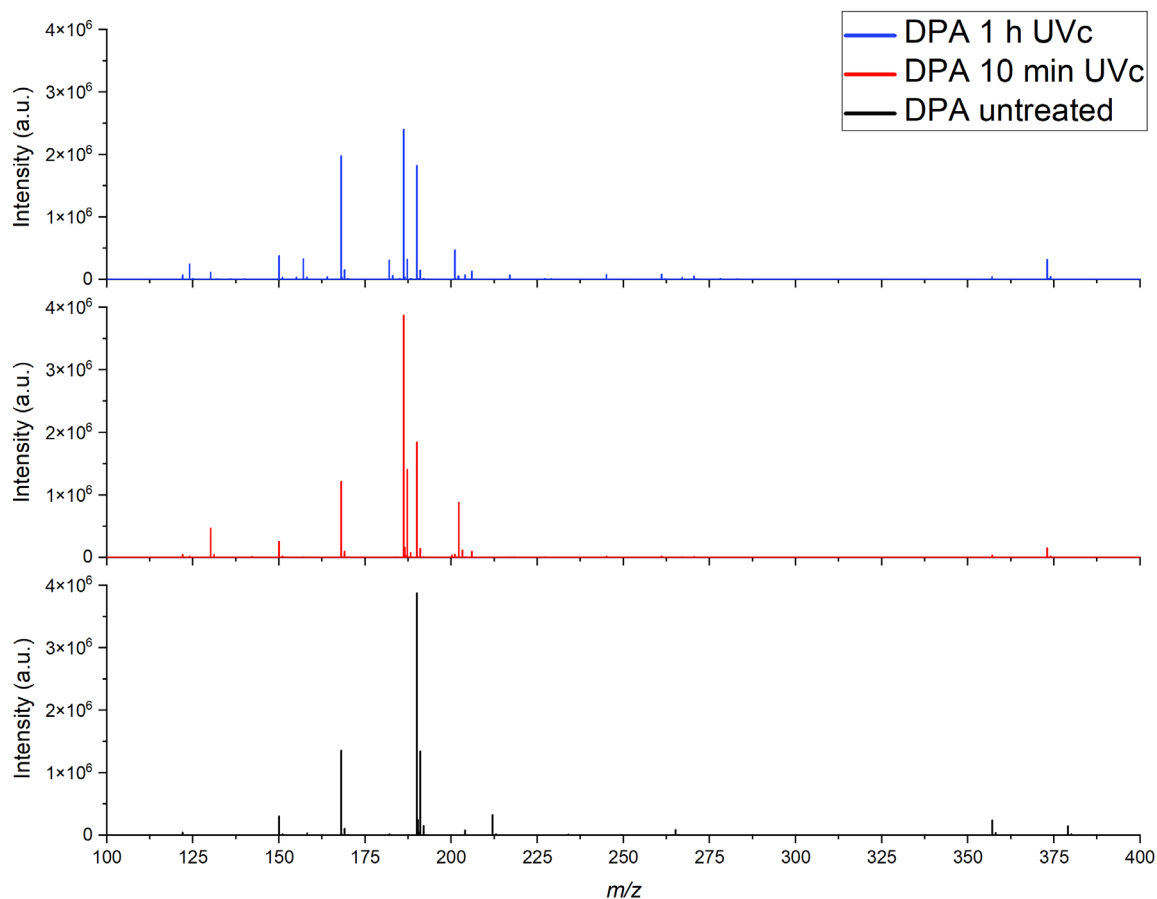


239 *Figure 3: Analysis of variance of DPA concentration (nM) depending if DPA was dosed immediately after or 24 hours after*  
240 *the UVc irradiation. Two irradiation times were used: 10 min at 0.120 J/cm<sup>2</sup> (A) and 20 min at 0.120 J/cm<sup>2</sup> (B) with an initial*  
241 *DPA concentration of 100nM. Each irradiated group (N(A)=6; N(B)=4) was dosed directly after irradiation and 24 hours after*  
242 *irradiation. Different letters above box plot (a,b) represent significant differences (p=0.05) between the time conditions.*

243

### 244 3.3. Identification of DPAP

245 The influence of the UVc treatment on the DPA was directly visible on our mass spectrometry  
246 spectra, shown in Fig. 4. There were five MS peaks significantly present before irradiation  
247 corresponding to  $m/z$  of 122.0236, 150.0184, 168.0290, 182.0447 and 190.0110. After irradiation of  
248 10 min, many MS peaks appear at  $m/z$  of 12.0392, 130.1589, 157.0758, 186.2217, 201.0658,  
249 202.2165, 217.0607, 245.0556, 261.0505, 270.5129 and 372.9975. We also observe the  
250 disappearance of a peak at 182.0447 that reappears for prolonged irradiation. By using longer  
251 irradiation time, the amount of certain photoproducts was amplified such as the molecules  
252 corresponding to the  $m/z$  at 124.0392, 157.0758, 201.0658 and 217.0607. This prolonged irradiation  
253 highlighted the consumption of one of the photoproduct present at the 202.2165  $m/z$  peak.



254

255 *Figure 4: MS spectra obtained for DPA before and after UVC treatment (0.120 J/cm<sup>2</sup>) at different times (10 min and 1 h).*  
 256 *Only m/z values from 100 to 400 were representing on the X-axis, as all changes occur in this range.*

257

258 Based on the previous recent literature [21,22] and knowing the configuration of the DPA molecule,  
 259 we were able to provide information concerning the identification of certain photoproducts and to  
 260 propose structural formula, as shown in Table 1. These results were accompanied by a precision, Err  
 261 in ppm, which represented the deviation from the theoretical monoisotopic mass, as well as an  
 262 mSigma value, which represented the correspondence between theoretical and experimental  
 263 isotopic profiles. The lower Err and mSigma values were, the stronger the correspondence was. The  
 264 UVC treatment may allow the decarboxylation of one of the acidic functions of dipicolinic acid that  
 265 would lead to the production of picolinic acid. This acid would serve as a precursor for the creation of  
 266 bipyridine complexes such as Dimethyl 2,2'-Bipyridine-6,6'-dicarboxylate and Dimethyl 2,3'-  
 267 Bipyridine-6,6'-dicarboxylate. The molar masses obtained in mass spectrometry were consistent with  
 268 these compounds and support the proposed models.

269 The following putative compounds: C<sub>6</sub>H<sub>4</sub>NO<sub>2</sub>, C<sub>7</sub>H<sub>4</sub>NO<sub>3</sub>, C<sub>7</sub>H<sub>6</sub>NO<sub>4</sub>, C<sub>8</sub>H<sub>8</sub>NO<sub>4</sub> and C<sub>4</sub>H<sub>4</sub>N<sub>3</sub>O<sub>6</sub> were present  
 270 in the DPA solution before UVC, which means that all these compounds were already present in

271 either DPA or the results of chemical reactions due to the solvent which was methanol. Among this  
272 list of compounds, some of them might participate to the weak fluorescence of DPA. Knowing the  
273 compounds present before UVC treatment, we were able to determine which molecules were  
274 produced.

275 The first effect was the decarboxylation of the acid functions of dipicolinic acid and maybe a double  
276 decarboxylation of this compound. As the mass of DPA is around 168  $m/z$ , a double decarboxylation  
277 would have resulted in a molecular weight around 78  $m/z$ , which would have been outside of the  
278 mass scan range of 100-1500  $m/z$ . Therefore, the double decarboxylation hypothesis could not be  
279 verified. These molecules,  $C_6H_6NO_2$  and perhaps  $C_5H_6N$  (not seen) could have been precursors to  
280 other molecules.

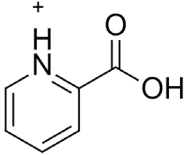
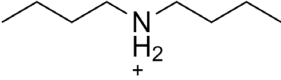
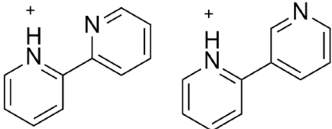
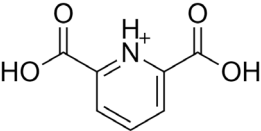
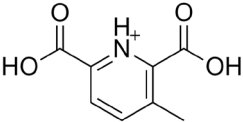
281 The first hypothesis was that the UVC treatment would break the pyridine and favour a polymeric  
282 form with an azote at the center of this structure, as shown in the following compounds:  $C_8H_{20}N$ ,  
283  $C_{12}H_{28}N$  and  $C_{12}H_{28}NO$ . The second hypothesis was the production of bipyridine complexes with two  
284 isomers in 2,2' or 2,3' for the following compounds:  $C_{10}H_9N_2$ ,  $C_{11}H_9N_2O_2$ ,  $C_{11}H_9N_2O_3$ ,  $C_{12}H_9N_2O_4$  and  
285  $C_{12}H_9N_2O_5$ .

286 Some molecules, which showed a negligible intensity on the mass spectra, could not be determined  
287 based solely on their molecular weights:  $C_6H_4NO_2$ ,  $C_7H_4NO_3$  and  $C_4H_4N_3O_6$ . A literature review of DPA  
288 and its photoproducts did not provide any additional information. Other molecules on the MS  
289 spectra, whose molecular mass ( $m/z$ ) were 270.5129 and 372.9975, could not be determined.  
290 Further experiments, like molecular fragmentation or separation and purification followed by  
291 infrared or NMR studies, will be needed to determine these molecules and characterize still  
292 undetermined photoproducts.

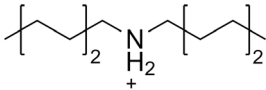
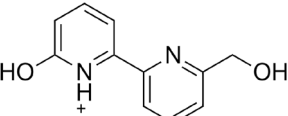
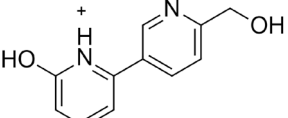
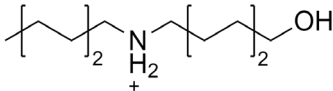
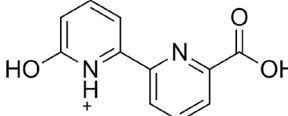
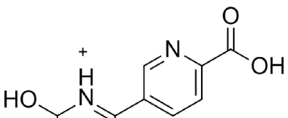
293 It should be noted that the irradiation time had an important influence on the production of  
294 photoproducts. Even if mass spectrometry direct injection can not be used to accurately quantify the  
295 detected compounds, a strong change in intensity gives an indication of the shift in relative  
296 proportions. We noticed that the compounds that present the main shift after prolonged irradiation  
297 were the picolinic acid and the majority of the bipyridine complexes. There was also an increase in  
298 the proportion of the polymeric structure  $C_{12}H_{28}NO$ . The shift of other compound intensities was not  
299 large enough to draw any conclusions. It seems that a longer irradiation time would promote the  
300 production of precursors such as picolinic acid and thus increase the proportion of complexes formed  
301 from this compound.

302  
303

Table 1: List of the major compounds found by mass spectrometry by direct injection of DPA solution, before and after UVC treatment at different irradiation times (10 min. and 1h.). ND: Not Determined. NP: Not Proposed, a.u.: arbitrary unit.

$m/z_{\text{experimental}}$ [M+H] <sup>+</sup>	Err [ppm]	mSigma	Ion putative formula	Proposed structural formula	Intensity (a.u.)		
					DPA untreated 1 x 10 <sup>-2</sup> M	DPA 10 min UVC	DPA 1 h UVC
122.0236	0.7	5.8	C <sub>6</sub> H <sub>4</sub> NO <sub>2</sub>	NP	5.26 x 10 <sup>4</sup>	4.58 x 10 <sup>4</sup>	7.26 x 10 <sup>4</sup>
124.0392	0.8	3.7	C <sub>6</sub> H <sub>6</sub> NO <sub>2</sub>		ND	2.51 x 10 <sup>4</sup>	2.47 x 10 <sup>5</sup>
130.1589	0.7	2.5	C <sub>8</sub> H <sub>20</sub> N		ND	4.67 x 10 <sup>5</sup>	1.14 x 10 <sup>5</sup>
150.0184	1.2	1.1	C <sub>7</sub> H <sub>4</sub> NO <sub>3</sub>	NP	3.10 x 10 <sup>5</sup>	2.58 x 10 <sup>5</sup>	3.78 x 10 <sup>5</sup>
157.0758	1.1	3.6	C <sub>10</sub> H <sub>9</sub> N <sub>2</sub>		ND	1.25 x 10 <sup>4</sup>	3.33 x 10 <sup>5</sup>
168.0290	0.6	1.5	C <sub>7</sub> H <sub>6</sub> NO <sub>4</sub>		1.36 x 10 <sup>6</sup>	1.23 x 10 <sup>6</sup>	1.97 x 10 <sup>6</sup>
182.0447	0.2	n.a.	C <sub>8</sub> H <sub>8</sub> NO <sub>4</sub>		2.28 x 10 <sup>4</sup>	ND	3.08 x 10 <sup>5</sup>

304

$m/z_{\text{experimental}}$ [M+H] <sup>+</sup>	Err [ppm]	mSigma	Ion putative formula	Proposed structural formula	Intensity (a.u.)		
					DPA untreated 1 x 10 <sup>-2</sup> M	DPA 10 min UVc	DPA 1 h UVc
186.2217	-0.3	0.6	C <sub>12</sub> H <sub>28</sub> N		ND	3.88 x 10 <sup>6</sup>	2.39 x 10 <sup>6</sup>
190.0110	-8.2	12.9	C <sub>4</sub> H <sub>4</sub> N <sub>3</sub> O <sub>6</sub>	NP	3.87 x 10 <sup>6</sup>	1.86 x 10 <sup>6</sup>	1.81 x 10 <sup>6</sup>
201.0658	0.5	1.7	C <sub>11</sub> H <sub>9</sub> N <sub>2</sub> O <sub>2</sub>	 	ND	5.28 x 10 <sup>4</sup>	4.71 x 10 <sup>5</sup>
202.2165	0.3	12.9	C <sub>12</sub> H <sub>28</sub> NO		ND	8.83 x 10 <sup>5</sup>	4.44 x 10 <sup>4</sup>
217.0607	0.4	13.5	C <sub>11</sub> H <sub>9</sub> N <sub>2</sub> O <sub>3</sub>	 	ND	7.71 x 10 <sup>3</sup>	7.04 x 10 <sup>4</sup>

$m/z_{\text{experimental}}$ [M+H] <sup>+</sup>	Err [ppm]	mSigma	Ion putative formula	Proposed structural formula	Intensity (a.u.)		
					DPA untreated 1 x 10 <sup>-2</sup> M	DPA 10 min UVC	DPA 1 h UVC
245.0556	0.2	13.3	C <sub>12</sub> H <sub>9</sub> N <sub>2</sub> O <sub>4</sub>		ND	2.37 x 10 <sup>4</sup>	8.16 x 10 <sup>4</sup>
261.0505	0.5	15.2	C <sub>12</sub> H <sub>9</sub> N <sub>2</sub> O <sub>5</sub>		ND	2.30 x 10 <sup>4</sup>	8.60 x 10 <sup>4</sup>
270.5129	ND	ND	NP	NP	ND	1.60 x 10 <sup>4</sup>	5.33 x 10 <sup>4</sup>
372.9975	ND	ND	NP	NP	ND	1.56 x 10 <sup>5</sup>	3.19 x 10 <sup>5</sup>

309 **3.4. Application of DPAP and CaDPAP analysis in bacterial spores**

310 UVc treatment made it possible to observe photoproducts formation using fluorescence  
311 measurement, as shown in Fig. 5. Nevertheless, the treatment applied *in vitro* (0,120 J/cm<sup>2</sup> during 10  
312 min) killed all bacterial spores (inactivation of more than 6 log) in our sample as given by CFU  
313 experiment (not shown). A DPA assay has been carried out to check if CaDPA and its fluorescent  
314 photoproducts were not released outside the spore after this UVc treatment. No release of CaDPA  
315 was detected, except for strain PS4150 that shown partial permeability by releasing a very small part  
316 of its CaDPA content (data not shown). This CaDPA release after UVc exposure can be explained by a  
317 greater impact of UVc on PS4150 spore structure, particularly on the inner membrane, due to the  
318 lack of the spore coat. A treatment just as lethal as the previous one was carried out by reducing the  
319 irradiation time to 2 minutes, which allows the strain PS4150 to retain its DPA and CaDPA. However,  
320 shorter treatments in terms of irradiation time, and especially at non-lethal dose, did not make it  
321 possible to clearly distinguish the increase in fluorescence intensity. Thus, although our treatment  
322 was lethal, it allowed our bacterial spores to retain the intracellular DPA and CaDPA.

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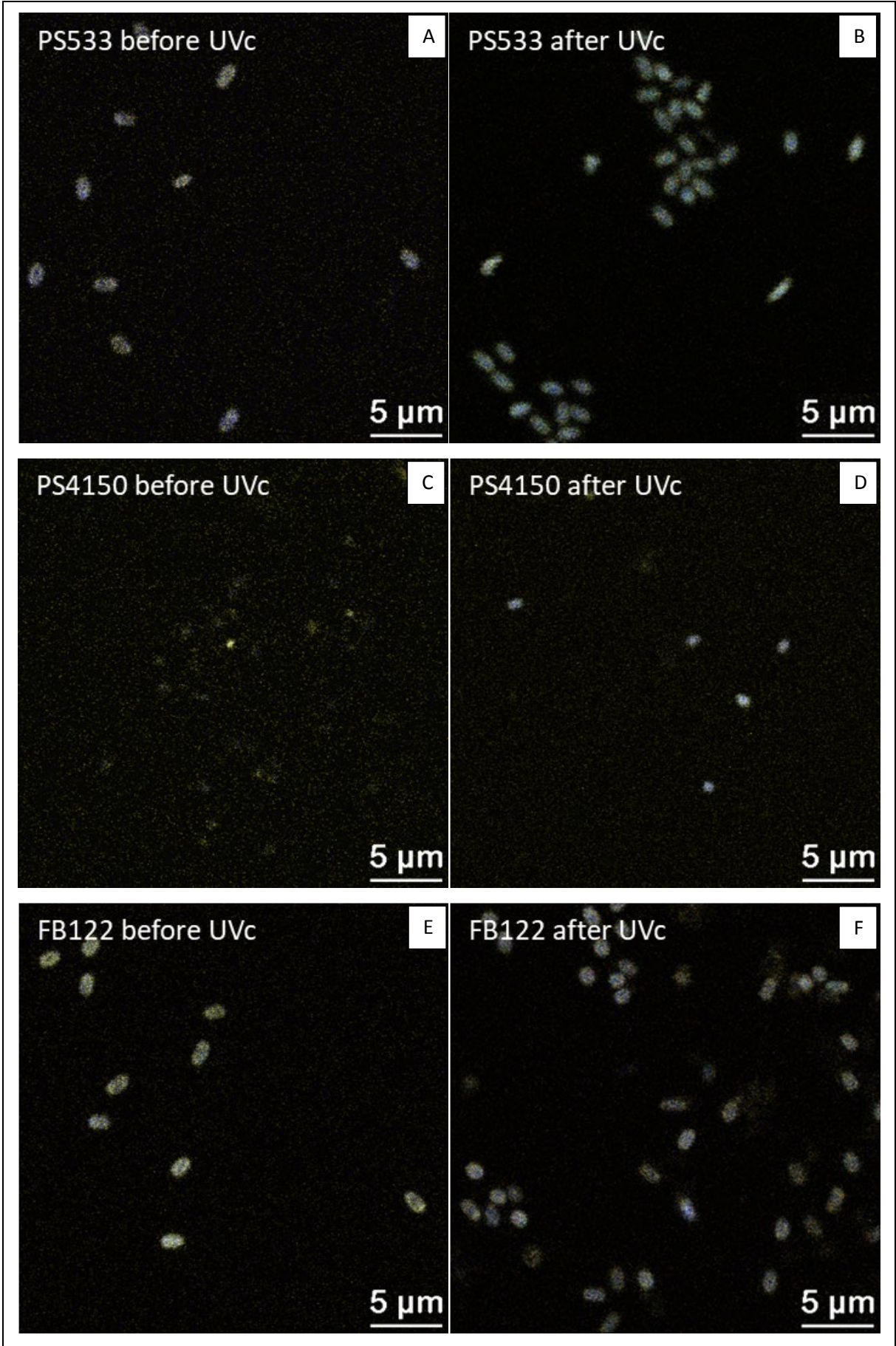
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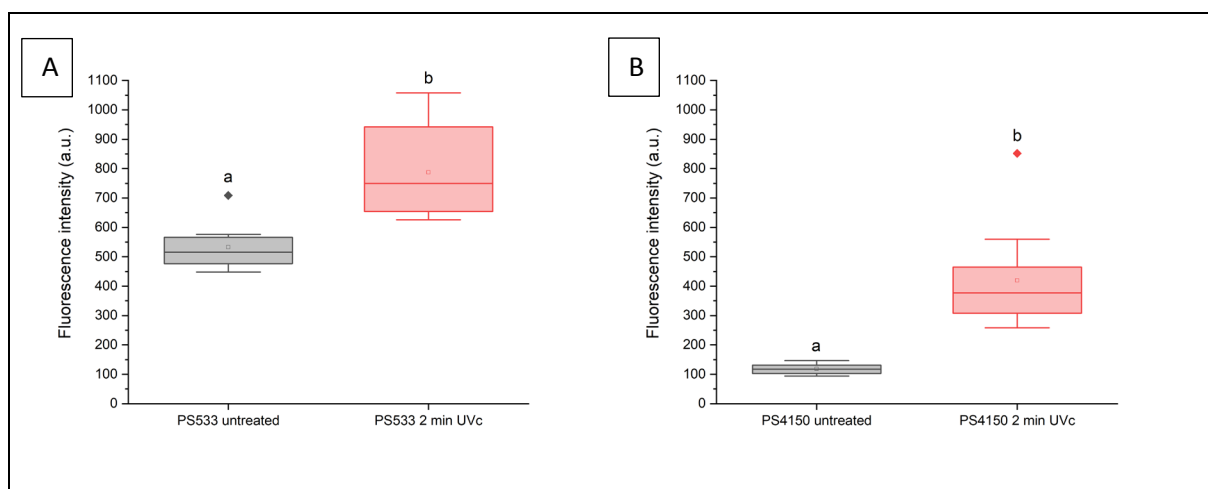


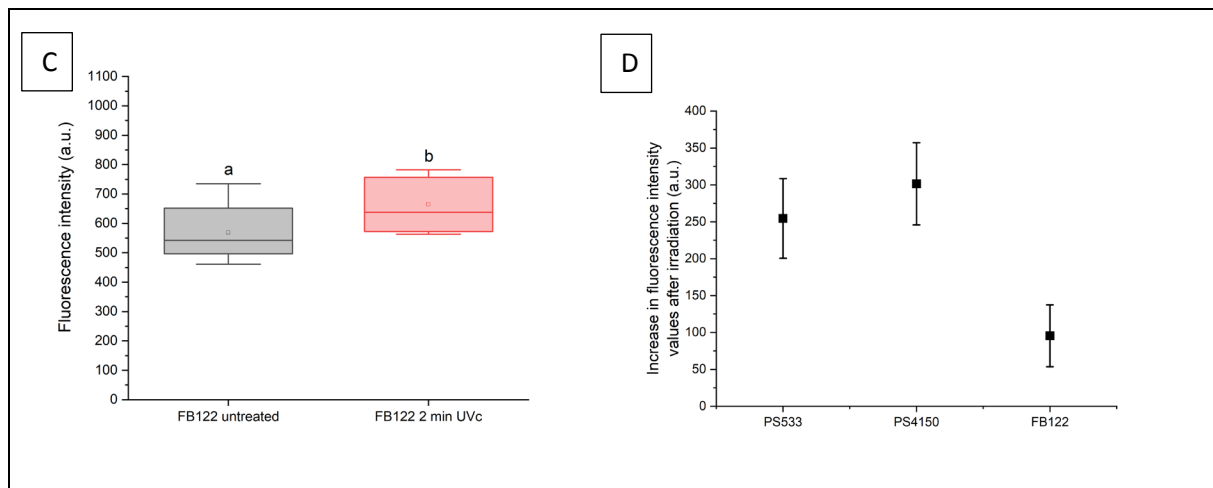


330 *Figure 5: Two photon microscopy images for each strain before and after UVC treatment (2 minutes at 0.120 J/cm<sup>2</sup>) with the*  
 331 *same contrast. PS533 (wild-type) before irradiation (A) and after irradiation (B). PS4150 (without coat) before irradiation (C)*  
 332 *and after irradiation (D). FB122 (lack of DPA) before irradiation (E) and after irradiation (F).*

333

334 Fig. 6 showed the fluorescence intensity variations after UVC irradiation of 2 minutes at 0.120 J/cm<sup>2</sup>  
 335 for all the strains. It increased in all cases. The reference was the strain PS533 which was a wild type.  
 336 For this strain, the mean value of fluorescence intensity (a.u.) changes from 532 to 787, as shown in  
 337 Fig. 6(A). PS4150, the strain whose coat was highly degraded, serves as a control for the spores  
 338 autofluorescence. In Fig. 6(B), its mean value of fluorescence intensity increased from 117 to 419  
 339 (a.u.). Fig. 6(C) clearly showed that our strain control without DPA and CaDPA, FB122, had the  
 340 weakest increase. Its mean value of fluorescence intensity changed from 568 to 664 (a.u.). Fig. 6(D)  
 341 showed the increase in fluorescence intensity for each strain before and after UVC treatment. This  
 342 figure allows us to compare the direct effect of UVC treatment between our different strains and to  
 343 see the influence of the coat in the resistance to UVC. The PS4150 strain had a much higher increase  
 344 in fluorescence intensity than the other strains which was approximately  $301 \pm 55$  (a.u.). We have  
 345 hypothesized that the coat may have absorbed a part of the UVC, which could explain the significant  
 346 reduction in the production of DPAP and CaDPAP for the strains possessing the coat. The FB122  
 347 strain, lacking DPA and CaDPA, had the lowest increase in fluorescence intensity after irradiation,  
 348 which was around  $95 \pm 41$  (a.u.). This weak UVC effect might be generated by photochemical  
 349 reactions with the coat or with compounds present in the bacterial spore, or could have been due to  
 350 the low remaining presence of DPA and CaDPA in this strain [7]. The increase in fluorescence  
 351 intensity for the PS533 spores which was approximately around  $254 \pm 53$  (a.u.), had suggested that  
 352 the UVC effect might be mainly governed by the production of DPAP and CaDPAP.





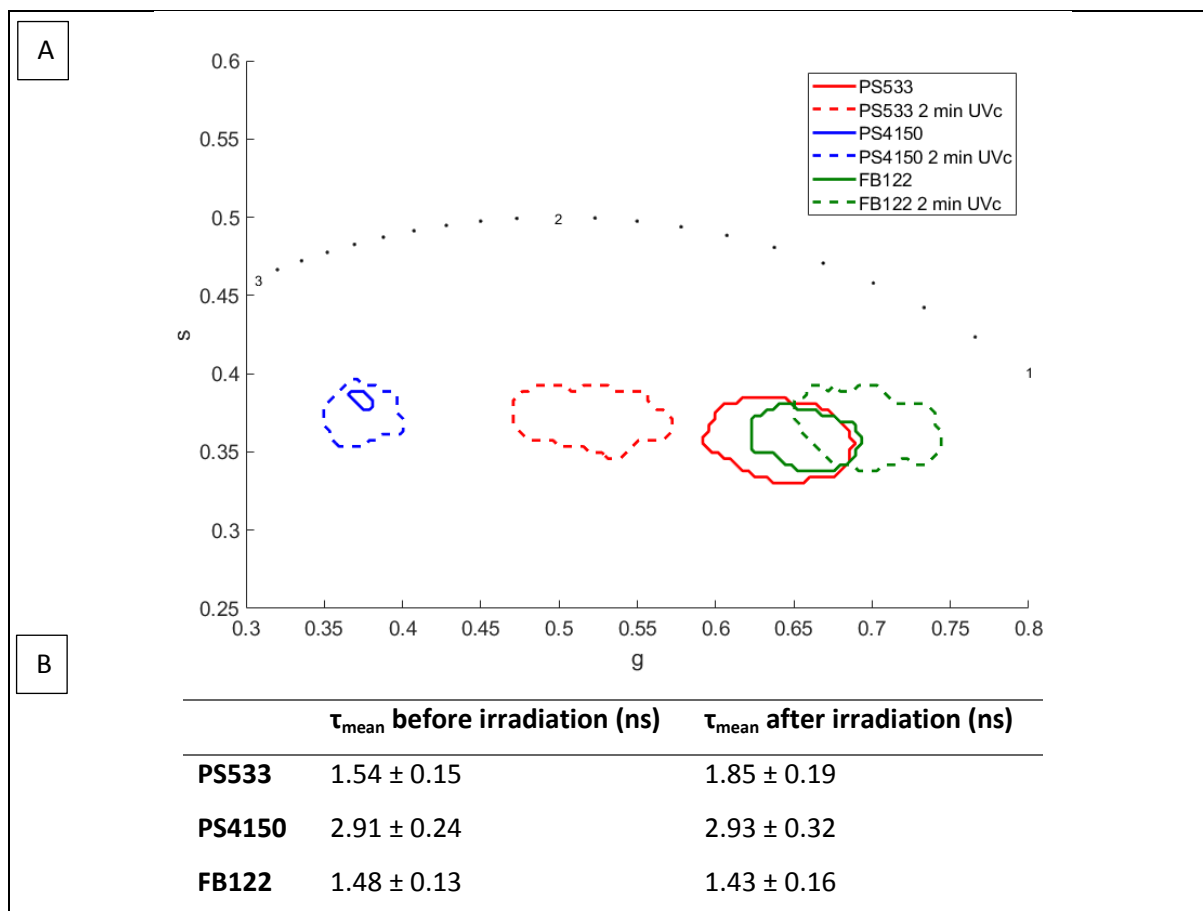
353 *Figure 6: Fluorescence intensity of bacterial spores (N=10) before and after irradiation for strains PS533 (wild-type) (A),*  
 354 *PS4150 (without coat) (B), FB122 (lack of DPA) (C). Increase in fluorescence intensity values after irradiation for each strain*  
 355 *(D). Different letters above box plot (a,b) represent significant differences ( $p=0.05$ ) between the time conditions.*

356

357 Moreover, we have performed an analysis of the contours of each of our phasor plot representations  
 358 to be able to observe the shifts in our fluorescence lifetimes as a function of irradiation, Fig. 7(A).  
 359 This allows us to highlight the elongation of lifetimes for strain PS533 that moved mainly on a  
 360 horizontal axis, suggesting an increase in the proportion of long fluorescence lifetimes. Strain PS4150  
 361 seemed to move weakly towards the inside of the semicircle, which means the appearance of  
 362 multiple fluorescence lifetimes. For strain FB122, the irradiation did not seem to shift the phasor plot  
 363 representation to higher values, but on the contrary moved it towards a greater proportion of short  
 364 fluorescent lifetimes. With the lack of DPA, the effect of irradiation could be due to many  
 365 compounds, such as coat proteins. The associated table, Fig. 7(B), shows the mean fluorescence  
 366 lifetimes obtained for all the studied strains. For PS533 strain, the mean fluorescence lifetime  
 367 increased significantly from  $1.54 \pm 0.15$  ns to  $1.85 \pm 0.19$  ns. The two other strains had mean  
 368 fluorescence lifetime that did not change significantly. The constant PS4150 fluorescence lifetimes  
 369 before and after irradiation could be explained by a lower production of photoproducts, but this is  
 370 not consistent with the intensity changes reported (cf. Figure 6). Otherwise, we may assume that  
 371 natural DPA photoproducts are already present in the bacterial spore, explaining the initial presence  
 372 of a long lifetime. For PS4150, no short fluorescence lifetime from coat proteins will reduce the spore  
 373 mean lifetime, as they do for PS533, so irradiation of PS4150 will increase the rate of DPA  
 374 photoproducts without changing the lifetime. For strain FB122, there was a very small decrease in  
 375 fluorescence lifetime from  $1.48 \pm 0.13$  ns to  $1.43 \pm 0.16$  ns with a small increase in fluorescence  
 376 intensity. Thus, we propose that the slight observed changes should be related to proteins rather to  
 377 residual DPA. These results were in line with our previous observations, since we expected no change  
 378 for this strain without DPA/CaDPA. The irradiation seemed to increase significantly the fluorescence

379 intensity and lifetime only for strain PS533, counterbalancing the short lifetime of coat proteins. The  
 380 PS533 lifetime shifts closer to the PS4150 one, which stay stable after irradiation because of the  
 381 absence of coat proteins. Conversely, strain FB122 do not change significantly in lifetime nor in  
 382 intensity due to its deficiency in DPA.

383 We hypothesize that fluorescence lifetime changes should be driven primarily by the creation of  
 384 CaDPA photoproducts. To explain this increase, we know that the pH inside the bacterial spore,  
 385 regardless of its state (dormant or during sporulation), was greater than pH 6 [28,29]. This  
 386 intracellular pH corresponds to a pH value greater than its  $pK_{a3}$  ( $pK_{a1}$ : 0.49;  $pK_{a2}$ : 2.2 and  $pK_{a3}$ : 4.6)  
 387 [30–32]. In such an environment, the DPA is in a twice deprotonated form ( $DPA^{2-}$ ) with a mean  
 388 fluorescence lifetime of 4.3 ns and a mean fluorescence lifetime of 3.2 ns for CaDPA in their  
 389 irradiated form (data not shown). These lifetimes were greater than those of the spore before  
 390 irradiation for the strain PS533 and FB122. This allows us to explain the increase in lifetime with to  
 391 the appearance of photoproducts that will lengthen the lifetimes for strain PS533, and could explain  
 392 the slight change for the strain PS4150, that had fluorescence lifetimes close to these values.



393 Figure 7: (A) Phasor plot image outlines before (line) and after irradiation (dotted line) for each strain: PS533 (red), PS4150  
 394 (blue) and FB122 (green). (B) Table: Mean fluorescence lifetime ( $\tau_{mean}$ ) obtained by phasor plot representation for all strains  
 395 used before and after irradiation ( $0.120 J/cm^2$  for 2 min). Where  $g$  and  $s$  represent the values at the two coordinates [ $g$ ;  $s$ ] of  
 396 the phasor plot.

#### 398 4. Conclusions

399 In conclusion, this study demonstrates the instability of the photoproducts of DPA and CaDPA, which  
400 continue to reorganize as DPAP and CaDPAP, 24 hours after irradiation. To complete this  
401 observation, we measured the consumption of DPA for the production of these compounds, in  
402 amounts linked to the irradiation time. The loss of DPA was about 60% of the initial concentration  
403 after 10 minutes of irradiation. This study also demonstrates the rearrangement of unstable  
404 photoproducts into the form of DPA 24 hours after irradiation. It allows us to hypothesize that the  
405 DPA excited via UVc produces photoproducts, but that part of this DPA\* will take longer to either  
406 rearrange in the form of DPAP or in the form of DPA. Mass spectrometry experiments confirm the  
407 presence of photoproducts already identified and suggest the presence of still unidentified  
408 molecules. All of these data enable us to better understand the effects of UVc on DPA and its CaDPA  
409 salt, which may be useful for further studies on bacterial spores. This work also highlights the  
410 benefits of using fluorescence microscopy and fluorescence lifetime imaging to study the inner  
411 compartment of bacterial spores and more specifically to use the DPA photoproducts as  
412 environmental probes.

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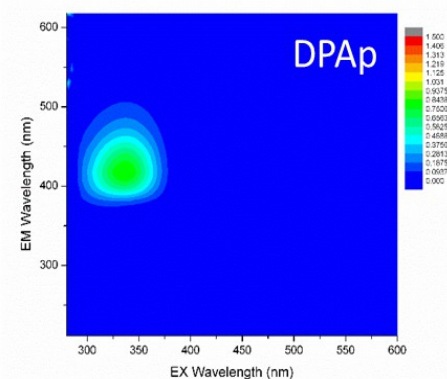
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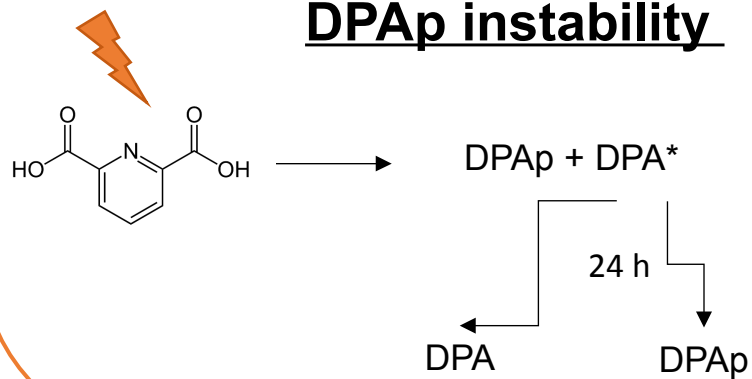
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## UVC effects

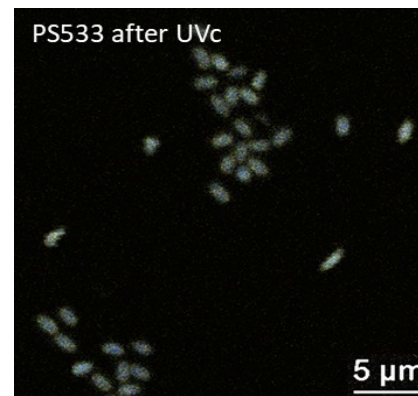
DPA  
photoproducts  
(DPAp)



## DPAp instability

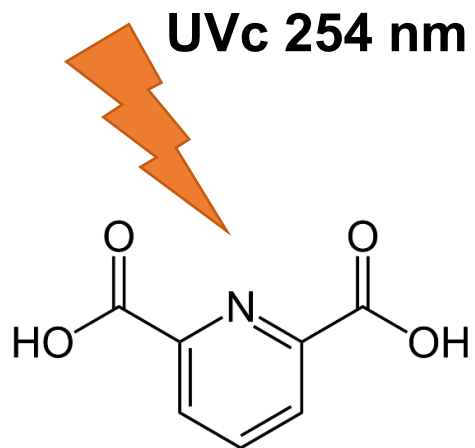
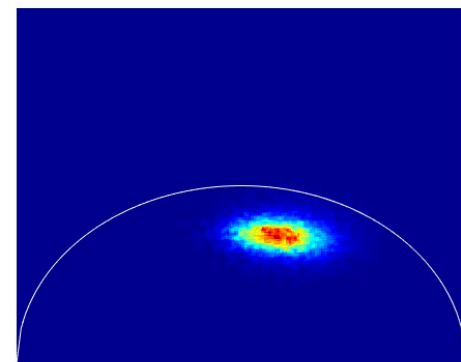


## DPAp visualization



Multi-photon  
microscopy  
on bacterial  
spores

FLIM &  
Phasor  
plot on  
bacterial  
spores



2,6-pyridinedicarboxylic acid (DPA)