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# **Akinetes and ancient DNA reveal toxic cyanobacterial recurrences and their potential for resurrection in a 6700-year-old core from a eutrophic lake**

Running title: Cyanobacterial recurrence and survival over 6700yr

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## 1 **Abstract**

2 In order to evaluate the recurrence of toxic cyanobacterial blooms and to determine the  
3 survival capabilities of the resistance cells through time, a sedimentary core spanning 6700  
4 years was drilled in the eutrophic Lake Aydat. A multiproxy approach (density, magnetic  
5 susceptibility, XRF, pollen and non-pollen palynomorph analyses), was used initially to  
6 determine the sedimentation model and the land uses around the lake. Comparison with the  
7 akinete count revealed that Nostocales cyanobacteria have been present in Lake Aydat over a  
8 six thousand year period. This long-term cyanobacterial recurrence also highlights the past  
9 presence of both the *anaC* and *mcyB* genes, involved in anatoxin-a and microcystin  
10 biosynthesis, respectively, throughout the core. The first appearance of cyanobacteria seems to  
11 be linked to the natural damming of the river, while the large increase in akinete density  
12 around 1800 cal.yr BP can be correlated with the intensification of human activities  
13 (woodland clearance, crop planting, grazing, etc.) in the catchment area of the lake, and marks  
14 the beginning of a long period of eutrophication. This first investigation into the viability and  
15 germination potential of cyanobacteria over thousands of years reveals the ability of intact  
16 akinetes to undergo cell divisions even after 1800 years of sedimentation, which is 10 times  
17 longer than previously observed. This exceptional cellular resistance, coupled with the long-  
18 term eutrophic conditions of this lake, could partly explain the past and current recurrences of  
19 cyanobacterial proliferations.

20

## 21 **Keywords**

22 Sediment, past bloom, akinete, past toxic potential, ancient akinete germination

23

## 24 1. Introduction

25 Environmental problems caused by cyanobacteria proliferations have increased over  
26 the last few decades due to anthropization of watersheds and aquatic systems (Merel et al.,  
27 2013; Huisman et al., 2018). However, cyanobacteria have existed on Earth for at least 2.5  
28 billion years and past proliferations have also occurred naturally far from anthropic pressure  
29 (Canfield, 2005). Numerous environmental factors, both physicochemical and biological ones,  
30 can drive cyanobacterial proliferations, so the functioning of long-term cyanobacteria  
31 dynamics remains difficult to predict. Lacustrine sediments represent a means of  
32 reconstituting a lake's history, and benthic sediments are known to store evidence of past  
33 aquatic ecosystems, including microbial diversity (Domaizon et al., 2013; Martinez De la  
34 Escalera et al., 2014; Savichtcheva et al., 2015; Legrand et al., 2017a). Retro-observations  
35 obtained from paleolimnology make it possible to reconstruct the long-term dynamics of  
36 populations and to identify the background state and the tipping points for a lake. The two  
37 main ways used to trace the long-term dynamics of lacustrine organisms are by means of  
38 fossils, when they can be identified, and the analysis of DNA preserved in sediment. Both  
39 approaches have been applied to cyanobacteria, as certain species, including Nostocales and  
40 Stigonematales, can produce resistant cells, termed akinetes. These cells are able to resist  
41 unfavorable environmental conditions, such as anoxia, desiccation and lack of light (Kaplan-  
42 levy et al., 2010). Thus, akinetes present in sediments can be used as a record of past  
43 nostocalean recurrences (Capo et al., 2015). Some studies have already investigated past fossil  
44 akinete distributions in lacustrine sediments (Van Geel et al., 1994; Eilers et al., 2004; Miras  
45 et al., 2015) and have described nostocalean dynamics over a period of 1950 years calibrated  
46 Before Present (cal.yr BP). Investigating these resting cells in ancient sediments also makes it  
47 possible to look into their viability and of the idea of resurrection ecology. Wood et al. (2009)  
48 have shown the surprising viability of ancient akinetes whose cell germination goes back to

49 120 BP. Legrand et al. (2017a) have also investigated the long-term persistence of akinete  
50 integrity and viability in sediment using a 220 year-old core. However, while these findings  
51 are promising, very few other studies exist, especially for sediments dating back more than a  
52 century, that could help determine the driving factors involved in long-term persistence.

53 On the other hand, ancient cyanobacterial DNA can give relevant and complementary  
54 information about the diversity and toxicity of past cyanobacterial proliferations. Based on the  
55 detection of genes involved in cyanotoxin biosynthesis, several studies have revealed the  
56 recurrence of potentially toxic cyanobacterial taxa throughout the last century in Lake Zurich,  
57 involving the *mcyA* gene (microcystins biosynthesis) (Monchamp et al., 2016), and during the  
58 last 220 years in Lake Aydat (France), involving the *mcyA* and *anaC* genes (anatoxin-a  
59 biosynthesis) (Legrand et al., 2017a). The presence of genes involved in cyanotoxin  
60 biosynthesis is probably closely related to the recurrence of toxic cyanobacterial blooms (such  
61 as *Dolichospermum* (*i.e.* *Anabaena*) in Lake Aydat) over the same period, and the recent  
62 increase in anthropic pressure around lakes (Legrand et al., 2017a). While this relationship  
63 seems clear in the current context, it is more challenging to establish over a time period of  
64 thousands of years, given all the uncertainties with respect to past land use and human  
65 activities during that time. Nevertheless, tracing the long-term cyanobacterial dynamics would  
66 help to improve our understanding of their driving forces (climatic *versus* anthropic) and to  
67 create predictive models to better understand patterns in species diversity and toxicity.

68 In this context, it is essential to simultaneously integrate the quantitative  
69 characterization of past cyanobacterial proliferations, their past toxic potential and their  
70 capacity to survive long term in sediment. For this purpose, we used both microscopic and  
71 molecular approaches in order to reconstruct long term nostocalean recurrences in terms of  
72 abundance, viability and toxic potential in Lake Aydat (France). We identified the long term  
73 akinete recurrence in a sediment core covering 6700 years and went on to characterize the

74 physiological state of akinetes in the sediment, including the identification of a pool of intact  
75 akinetes. In these samples, we detected the presence of biosynthetic genes of two cyanotoxins:  
76 anatoxin-a and microcystin. The last challenging step was to link these cyanobacterial patterns  
77 with a number of anthropization indicators already described in Miras et al., (2015) (pollen,  
78 non-pollen palynomorphs) in the watershed area, to determine the potential factors driving the  
79 long-term recurrence of Nostocales in Lake Aydat. In parallel, in order to test the long-term  
80 viability of akinetes and the potential of cyanobacterial resurrection through time, we  
81 conducted germination experiments on selected samples.

## 82 2. Methods:

### 83 2.1 *Sampling and study site*

84 Lake Aydat (maximum depth: 15m; area: 6.105 m<sup>2</sup>; N45°39.809'/E 2°59.106'/837masl)  
85 is a eutrophic naturally dammed lake located in the French Massif Central where recurrent  
86 blooms of cyanobacteria have occurred in the late summer and autumn for the last few  
87 decades (Gerphagnon et al., 2013b). The lake was created by the damming of the Veyre river  
88 by a basaltic lava flow 8551 ± 400 cal.yr BP ago (Boivin et al., 2004). A detailed description  
89 of the lake's features (i.e geological and geomorphological setting) is given in Lavrieux et al.,  
90 (2013a). Coring was performed in the central, deepest part of the lake (Figure 1A). Based on  
91 sedimentological features, the record is divided into 2 main units separated by an erosive mass  
92 wasting deposit: i) the lower one (1076-1974cm depth which corresponds to 777-1600cm in  
93 the master core), and ii) the upper unit (0-829cm depth which corresponds to 0-775cm in the  
94 master core) (Lavrieux et al., 2013a). Akinete and molecular analysis were carried out on both  
95 the lower and the upper units (Figure 1B) to complement existing pollen, non-pollen  
96 palynomorph and diatom analyses (Miras et al., 2015).

### 97 *Chronology and accumulation rate*

98 Details of the chronology and accumulation rate can be found in Lavrieux et al., (2013a).  
99 Briefly, the age-depth model was firstly constructed from <sup>17</sup>AMS radiocarbon dates, based on  
100 analyses of leaf and wood fragments, distributed through the core. The radiocarbon dating  
101 was performed at the Laboratoire de Mesure du <sup>14</sup>Carbone (Gif-sur-Yvette, France) and  
102 calibrated (2σ) using CalPal Online. <sup>137</sup>Cs; <sup>241</sup>Am, <sup>210</sup>Pb and <sup>7</sup>Be measurements and  
103 correlation with major historical floods were also used for the chronology of the upper part of  
104 the core. The two sedimentary units were respectively dated at between 6700 ± 200 and 3180  
105 ± 90 cal.yr BP (lower unit) and 1770 ± 60 cal.yr BP to present (upper unit), and are separated  
106 by an erosive mass wasting deposit. In the lower unit and for the basal part of the upper unit,  
107 the mean sedimentation rate was 0.20cm.year<sup>-1</sup>. There was a sudden increase to 0.52cm.year<sup>-1</sup>  
108 between 660cm depth and the top, which is similar to the rate of 0.46cm.year<sup>-1</sup> estimated by  
109 Sarazin et al., (1992) for the 50 last years. Flood events caused the sedimentation rate to reach  
110 up to 0.6cm.year<sup>-1</sup> (Lavrieux et al., 2013a).

## 111 2.2 Akinete extraction and counting

112 Akinetes were extracted from 72 sediment samples (Figure 1C) along the core using  
113 protocols from Legrand et al., (2016a). To summarize, 0.5g of fresh sediment was diluted with  
114 4mL of ludox TM-50 (Sigma-Aldrich) and 9.5mL of distilled water. Solutions were sonicated  
115 and centrifuged. Then, the first 4mL of ludox supernatant were pipeted and homogenized and  
116 divided into 2 replicates. Two extractions were performed for each sample to obtain a total of  
117 4 replicates. 1μL of Sytox Green® (50mM) was then added to 1mL replicate solution  
118 (Gerphagnon et al., 2013a). Each replicate was filtered through 8μm (TEPT filters Merck,  
119 Milipore, Tullagreen, Ireland). Akinetes were counted in 40 fields per replicate using an  
120 epifluorescence microscope (Zeiss Axiovert 200M; HBO 103 mercury vapor short-arc lamp,  
121 200x magnification, Oberkochen, Germany). Two counts were performed for each field: i) at  
122 546nm to highlight chlorophyll autofluorescence in akinetes; and ii) at 488 nm to distinguish

123 damaged akinetes using Sytox Green® binding. The SYTOX green, which stained the nucleic  
124 acids of permeabilized cells after only 30 min of incubation, allowed the damaged akinetes to  
125 be clearly identified. In contrast, the intact akinetes (with intact cell walls) were not rendered  
126 visible by the SYTOX green staining, but they nevertheless contain photosynthetic pigments  
127 that are visible under green light (Legrand et al., 2016b). Akinetes were counted and  
128 discriminated based on morphological criteria (Cirés et al., 2013; Legrand et al 2017, a,b;  
129 Ramm et al., 2017) using taxonomic keys found in reference books (Geitler, 1932).  
130 Hormogonia and young filaments observed during germination experiments (see section 2.4)  
131 enable the specific identification of 2 akinete morphotypes: *Dolichospermum macrosporum*  
132 and *Dolichospermum flos-aquae* (Figure 1B). The observed filaments are the same those  
133 observed in studies which focused specifically on these 2 species (Legrand et al., 2016a,  
134 Legrand et al., 2017, a,b).

### 135 2.3 Reviviscence of preserved cells

136 Akinete germination experiments were performed on 7 samples which were selected for  
137 their high intact akinete concentrations and their diversity of akinete morphotypes. Samples  
138 were respectively dated to  $318 \pm 40$ ,  $566 \pm 60$ ,  $897 \pm 60$ ,  $1633 \pm 60$ ,  $1769 \pm 60$ ,  $4274 \pm 119$   
139 and  $5510 \pm 156$  cal.yr BP. For the akinete germination experiment, cells were extracted  
140 following the same ludox protocol described above, but using 4.5 g of sediment. Supernatants  
141 were filtered through a 10 $\mu$ m nylon filtration tissue. This tissue containing the akinetes was  
142 then washed with a minimal volume of distilled water. The solution was cleaned through a  
143 100 $\mu$ m and then a 50 $\mu$ m nylon filtration tissue to remove a maximum of residual sedimentary  
144 particles. Finally, the cells were put in 4mL of BG11 medium with cycloheximid (50 $\mu$ g.mL<sup>-1</sup>).  
145 Five replicates of 800 $\mu$ L of solution consisting of akinetes in BG11 medium were placed in a  
146 plate with 48 wells using the following procedure. At Days 0, 1 and then every 2 days until  
147 day 18, 50 $\mu$ L of homogenized solution were introduced through a 8 $\mu$ L TETP filter (Milipore,



148 Tullagreen, Ireland). Intact, germinating akinetes and hormogonia were counted using an  
149 inverse epifluorescence microscope (Zeiss Axiovert 200M; 200x magnification, Oberkochen,  
150 Germany). Counts were performed with an excitation light wavelength of 546nm. Samples  
151 were incubated at 20°C, 6μmol photons.m<sup>-2</sup>.s<sup>-1</sup> with white light and a photoperiod of 16 hours  
152 of light and 8 hours of dark.

#### 153 2.4 DNA extraction

154 The same 72 sediment samples described above were submitted to DNA analysis.  
155 Genomic DNA from these samples was extracted with the FastDNA<sup>®</sup> Spin kit for soil (MP  
156 biomedical<sup>®</sup>) following the manufacturer's instructions. Precautions laid out in Domaizon et  
157 al. (2017) concerning ancient DNA were applied. A control extraction was performed and  
158 checked with all samples in order to verify that there was no contamination from modern  
159 DNA.

160 The DNA concentration in each sample was estimated using a UV spectrophotometer  
161 (Nanodrop<sup>®</sup> ND 2000), and samples were kept at -20°C until further analysis.

#### 162 2.5 Target gene amplification

163 Three genes were targeted by classic or nested PCR during this study. The  
164 cyanobacterial 16S rRNA gene was amplified in sediment samples using the *cya359F* and  
165 *cya781R* set of primers (Nubel et al., 1997) (Table 1). Then one gene involved in the  
166 biosynthesis of anatoxin-a, *anaC*, and one of microcystin, *mcyB* gene, were also investigated.  
167 The first was detected with the sets of primers *anxgen-F2/R* and *anaC 80F/706R* for nested 1  
168 and 2 PCR respectively. For *mcyB* nested PCR, *MCY F1/R1* then *MCY F1/R2* were used.  
169 PCR mixtures were the same as in Legrand et al., (2016a) for the two *anaC* nested PCRs. For  
170 use with *mcyB*, the mix for both PCRs was composed of 5μL of colorless Go Taq<sup>®</sup> felxi 5X  
171 Buffer, 2.5mM of MgCl<sub>2</sub> solution, 0.2mM of dNTPs, 0.5μM of each primer (Table 1), 1

172 mg.mL<sup>-1</sup> of Bovine Serum Albumin (BSA), and 1.5 U of GoTaq<sup>®</sup> G3 Hot Start Polymerase in  
173 a final volume of 25µL. The PCR programs used were referenced in Legrand et al., (2016 a,b)  
174 for *anaC*. For *mcyB*, the program for PCR 1 was composed of an initial hybridization step of  
175 94°C for 5 min, 35 cycles of 94°C for 30 sec, 50°C for 1 min, 72°C for 30 sec, and a final  
176 extension of 72°C for 5 min, and for PCR 2 of an initial hybridization step of 94°C for 5 min,  
177 35 cycles of 94°C for 30 sec, 54°C for 1 min, 72°C for 20 sec, and a final extension of 72°C  
178 for 5 min. All PCR products were revealed with 0.5% agarose gel with 0.3 mg.L<sup>-1</sup> ethidium  
179 bromide and migrated in a TAE buffer 0.5x at 100V for 30 min. Gels were put under UV light  
180 and investigated using Gel Imager (ScienceTech) with the software VisionWorksLS vers.6.5  
181 (Upland, USA). Band localization and intensity in electrophoresis gels were defined with  
182 GelAnalyser vers. 2010 (<http://www.gelanalyzer.com/>) (Legrand et al., 2016a). For *anaC*,  
183 only those PCR products which had been digested with the restriction enzyme EcoRV  
184 (Biolabs, Ipswich, USA), following manufacturer's instructions, were considered as being  
185 positive. This gene possesses a restriction site for this enzyme which reinforces the detection  
186 of the expected gene (Figure S1). Furthermore, several samples have been sequenced to  
187 confirm the amplification of the expected genes (Legrand et al., in prep). Some of these  
188 sequences have been deposited in the GenBank-EMBL database under the accession numbers:  
189 MK646071-MK646074 and MK636679-MK636688.

### 190 3. Results

#### 191 3.1 *Total akinete abundance through the core*

192 The total akinete distribution was heterogeneous through the core and can be divided into  
193 two parts based on the two major units (Figure 2): i) the lower unit, between 6700 ± 200 and  
194 3180 ± 90 cal.yr BP) is characterized by low akinete abundance, with between 197 and  
195 97 900 akinetes per gram of dried sediment (gDW<sup>-1</sup>) with a mean value of 12 300

196 akinetes.gDW<sup>-1</sup> of sediment; and ii) the upper unit (from 1770 ± 60 cal.yr BP to present) is  
197 characterized by a significantly higher mean value of akinetes corresponding to 52 600  
198 akinetes.gDW<sup>-1</sup> of sediment. However, the upper part also shows a greater fluctuation, from  
199 22 000 akinetes.gDW<sup>-1</sup> (between 1391 ± 50 and 670 ± 50 cal.yr BP) to around 200 000  
200 akinetes.gDW<sup>-1</sup> of sediments (in the sections comprised between 1769 ± 60 and 1507 ± 40  
201 cal.yr BP, and 107 ± 40 and 10 ± 40 cal.yr BP). Four akinete morphotypes were counted along  
202 the core corresponding respectively to *Dolichospermum macrosporum*, *Dolichospermum flos-*  
203 *aquae*, *Dolichospermum sp.* and *Aphanizomenon sp.* (Figure 1B). The vertical distribution of  
204 these four morphotypes was different, for example *D. macrosporum* was particularly  
205 dominant in the upper unit where it composed up to 100% of the akinete morphotypes (Figure  
206 S2).

207 It can be noted that the total akinete abundance and the *D. macrosporum* akinete  
208 abundance are very similar (SC= 0.92,  $p < 0.001$ ). In the lower unit, *D. macrosporum* akinetes  
209 were often absent with values no higher than 15 500 akinetes.gDW<sup>-1</sup> of sediment. *D. flos-*  
210 *aquae* was almost always present through the core, but its akinete abundance was generally  
211 low, with between several hundred and a few thousand cells.gDW<sup>-1</sup> of sediment.  
212 Nevertheless, *D. flos-aquae* akinete abundance reached occasionally high values at 1339,  
213 1054, 840 and 767cm depth, levels respectively dated to 5510 ± 156, 4274 ± 119, 3421 ± 97  
214 and 1728 ± 60 cal.yr BP, with respective concentrations of 76 400, 42 300, 23 600 and 97 400  
215 cells.gDW<sup>-1</sup> of sediment. Finally, *Dolichospermum sp.* and *Aphanizomenon sp.* remained at  
216 low concentrations and presented a similar pattern: akinete presence of these 2 species was  
217 higher in the lower part, where they were found in 10 and 18 samples respectively. Akinete  
218 abundance of these 2 species was also generally low, between several hundred and several  
219 thousand, with a slight peak of 23 300 akinetes.gDW<sup>-1</sup> for *Aphanizomenon sp.* at 4648 ± 129  
220 cal.yr BP.

### 221 3.2 Akinete integrity along the core

222 Overall, the number of intact akinetes in the lower unit, where *D. macrosporum* was not  
223 dominant, followed the same pattern as that of total akinetes (SC= 0.70  $p < 0.0001$ ) (Figure 2).  
224 The number of intact akinetes was higher in the lower unit than in the upper unit, with a mean  
225 number of 8 620 and 3 300 cells.gDW<sup>-1</sup> of sediment respectively. However, there is no  
226 statistical difference due to large variations (from 0 to 86 200 akinetes.gDW<sup>-1</sup> of sediment)  
227 especially in the lower unit (Figure 2). These large variations are the result of numerous intact  
228 akinete peaks, especially for *D. flos-aquae*. For example, the number of intact cells for this  
229 species reached 41 500 and 76 400 akinetes.gDW<sup>-1</sup> at 4274 ± 119 and 5510 ± 156 cal.yr BP  
230 respectively. These variations seem to reflect a significant difference in integrity related to the  
231 akinete morphotype (Kruskal-Wallis test  $p < 0.0001$ ) (Figure 3). The mean percentages of  
232 intact akinetes through the core for *D. macrosporum*, *D. flos-aquae*, *Dolichospermum sp.* and  
233 *Aphanizomenon sp.* are 5.1, 54.1, 90.1, and 83.7% respectively. In addition, high temporal  
234 variations in the percentage of intact akinetes were observed, especially for *D. macrosporum*  
235 and *D. flos-aquae* (Figure 3). The variations for *D. flos-aquae* are marked between the two  
236 units (with a mean of 35 and 72% intact akinetes in the upper and lower unit respectively  
237 (Mann-Whitney test,  $p = 0.002$ ). The percentage of *D. macrosporum* is also lower in the upper  
238 unit, where the percentage of intact akinetes is low, between 0 and 13%. In the lower unit,  
239 there are alternations between an absence of this morphotype and a percentage of intact  
240 akinetes mostly comprised between 10 and 63%.

### 241 3.3 Potential of reviviscence of past cyanobacteria

242 At the beginning of the experiment, the number of akinetes.mL<sup>-1</sup> in BG11 was very  
243 different from one sample to another (Figure 4). *D. macrosporum* was largely dominant in  
244 318, 566 and 1663 cal.yr BP whereas *D. flos-aquae* was dominant in 897 and 1769 cal.yr BP

245 samples. Akinete distribution was heterogenous in 4274 and 5510 cal.yr BP samples. Four of  
246 the 7 studied samples showed no evidence of akinete germination after 18 days incubation  
247 (Figure 4B). Some germinating akinetes were observed in the samples dated to  $318 \pm 40$   
248 cal.yr BP and  $1769 \pm 60$  cal.yr BP after respectively 3-5 and 7-13 days after the beginning of  
249 the experiment. All the germinating akinetes corresponded to *Dolichospermum macrosporum*  
250 for  $318 \pm 40$  cal.yr BP and to *Dolichospermum flos-aquae* for  $1769 \pm 60$  cal.yr BP. These  
251 germinating akinetes were observed with 2 or 4 cells present inside the envelope (Figure 4)  
252 but no young filaments were observed. The ratio of germinating akinetes to total intact  
253 akinetes was very low, and varied between 6 and 8% for the  $318 \pm 40$  cal.yr BP sample, and  
254 between 0.31 and 1.23% for the  $1769 \pm 60$  cal.yr BP sample (Figure 4B). Some *D. flos-aquae*  
255 short filaments of a few cells in length (hormogonia) were observed in the sample dated to  
256  $897 \pm 50$  cal.yr BP after between 3 to 5 days of incubation. This percentage of young  
257 filaments remained relatively low, between 6 and 8%.

### 258 3.4 Target gene detection in sediment

259 The 16 rRNA gene was detected in all the studied samples through the core. The *mcyB*  
260 gene was detected with nested PCR in 19 samples through the entire core (Figure 5): it was  
261 detected in several samples of the lower unit (from  $6702 \pm 195$  to  $3269 \pm 93$  cal.yr BP) and  
262 also in the upper unit (at  $1316 \pm 40$  cal BP). This gene recurred at the top of the core with the  
263 12 more recent samples being positive (from  $422 \pm 40$  to  $10 \pm 40$  cal.yr BP). The *anaC* gene  
264 was found in 11 samples, mostly located in the lower unit but also in recent layers from  $227 \pm$   
265  $40$  cal.yr BP.

## 266 4. Discussion

### 267 4.1 Reconstruction of long-term nostocalean recurrences in Lake Aydat

268 The overall objective of this study was to characterize past recurrences of toxigenic  
269 nostocalean cyanobacteria in a 6700-year-old sediment core from Lake Aydat (France).  
270 Previously, the oldest evidence of akinete persistence had been found in Lake Gosciadz  
271 (Poland), with akinetes approximately 4000 years old (Van Geel, 1994) So, to our knowledge,  
272 our study is the first report of such a long-term recurrence of four morphotypes of akinetes  
273 belonging to two genus, *Dolichospermum* and *Aphanizomenon*. These 4 morphotypes have  
274 already been observed in the water column of Lake Aydat (Sabart et al., 2015; Lafforgue et  
275 al., 1995). Moreover, akinetes from *Dolichospermum* have already been observed in surface  
276 sediment (Legrand et al., 2016a), in a 220-year-old core (Legrand et al., 2017a) and in the  
277 same 6700-year-old core, but using a palynological approach (Miras et al., 2015). Total  
278 akinete profiles from Miras et al. (2015) are very similar to our results in the upper unit of the  
279 core and confirm the recent nostocalean bloom recurrences. However, Miras et al. (2015) did  
280 not observe akinetes in the lower unit, which suggests that it is possible to achieve a better  
281 resolution of akinete extraction and identification with ludox and Sytox-Green protocols than  
282 with the classical palynological method traditionally used in paleoecological research (Van  
283 Geel et al., 1994, 1996; Revelles et al., 2016). Standard palynological treatment of samples,  
284 which often includes acetolysis (Faagri and Iversen, 1989), seems thus to induce a loss of  
285 akinete material and must consequently be avoided for the characterization of the long-term  
286 evolution of akinetes. Our method using Sytox green also makes it possible to evaluate the  
287 number of intact akinetes present in the sediment, which provides fresh insight into the  
288 understanding of the long-term resistance of nostocalean blooms.

#### 289 4.2 *Establishment of baseline conditions at Lake Aydat*

290 One surprising result was to find sizeable concentrations of akinetes in the oldest part of  
291 the core, between ca 6500 and 6000 cal.yr BP. This suggests that only around 2 millennia after  
292 its formation, the baseline water conditions at Lake Aydat, defined as conditions prevailing

293 prior to human disturbances, were characterized by high nutrient levels (Miras et al., 2015),  
294 which is common for lakes formed by the damming of a river (Smol, 2014). This is also  
295 supported by other paleo-ecological bio-indicators such as different non-pollen palynomorphs  
296 (mainly *Turbellaria* (Haas, 1996), HdV-179 (Van Geel et al., 1983), and diverse rotifer resting  
297 eggs such as *Anuraeopsis fissa*-type, *Filinia longiseta*-type and *Keratella*-type (Barbiero et  
298 al., 2011; Haberman and Haldna, 2014) and diatom data (*Stephanodiscus medius*, *S. minutulus*  
299 and *S. parvus*) which are recorded as early as 6700 cal.yr BP (Miras et al., 2015).  
300 *Stephanodiscus* species are encountered in lakes with elevated nutrient concentrations and  
301 are typically found in eutrophic or hypertrophic conditions (Kirilova et al., 2009). This high  
302 trophic status is mainly driven by an influx of nutrients carried by the water flow into the  
303 drainage basin, which has been very vegetation-rich in Aydat since 6700 cal.yr BP. Pollen and  
304 non-pollen palynomorph data clearly indicate that woods (mainly constituted of beech and  
305 oak) were already present around the lake at this time (Miras et al., 2015).

#### 306 4.3 Chronological comparison between akinete concentrations and human impact in the 307 catchment basin

308 The oldest significant increase in akinete concentrations, dating from 5500 cal.yr BP,  
309 is coincident with the first palynological evidence of middle Neolithic human activity in the  
310 area (Miras et al., 2015). Grazed pastures and small-scale cultivated fields were created within  
311 the progressively more open wooded landscape of the Aydat catchment area (Figure S3). The  
312 combined effect of this human impact with a climatic trend toward cooler and more humid  
313 conditions, especially around 5600-5300 cal.yr BP (Magny et Hass, 2004), may have  
314 increased soil erosion on slopes within the catchment area. Higher detrital input may have  
315 consequently induced the increase in the trophic state of Lake Aydat, which is demonstrated  
316 by non-pollen palynomorphs (NPP) and the presence of eutrophic diatoms (Miras et al.,  
317 2015). This direct relationship between a high number of akinetes and phases of human

318 activity is also found in subsequent periods. High concentrations of akinetes observed in the  
319 lower unit at  $4648 \pm 129$ ,  $4274 \pm 119$  and  $3421 \pm 97$  cal.yr BP are close to, or concomitant  
320 with, different episodes of human activity (both temporary farming and agriculture  
321 accompanied by woodland clearances) in the Aydat catchment area, as revealed by pollen and  
322 non-pollen palynomorph data (Miras et al., 2015). The same trend is observed for the upper  
323 unit, with an increase in anthropogenic pressure and diversification of land use. High amounts  
324 of akinetes between  $1769 \pm 60$  and  $1507 \pm 40$  cal.yr BP and between  $670 \pm 50$  cal.yr BP and  
325 the sub-recent period ( $639 \pm 50$  cal.yr BP) are respectively encountered during phases of  
326 significant deforestation and diversification of human practices, such as the introduction of  
327 hemp retting (Lavrieux et al., 2013b). Hemp needs a large amount of nitrogen, phosphorus  
328 and potassium (Barron et al., 2012) and its cultivation in the catchment area, and in particular  
329 immediately surrounding Lake Aydat, could have caused an increase in nutrient loading into  
330 the lake through soil leaching. Moreover, hemp cultivation needs several periods of retting in  
331 the lake water, generally during May, August and October. These periods may induce nutrient  
332 enrichment in the water column that can be directly used by cyanobacterial proliferations.  
333 Thus, it is already well known that hemp retting provokes eutrophication of lakes and pools  
334 (Van Geel et al., 1994). This process can also be reinforced through the gradual development  
335 of an agricultural and grass-rich landscape. The recurrence of eutrophic conditions is also  
336 confirmed by the increasing number of diatoms through the core, related to higher phosphorus  
337 concentrations estimated in the upper unit relative to the lower unit (Miras et al., 2015).  
338 Eutrophic species such as *Aulacoseira subarctica* and *Fragilaria recta*, that require a high  
339 phosphorus content, become more dominant towards the top of the core (Kauppila, 2006).  
340 The dominance of *D. macrosporum* in this upper unit seems to attest to monospecific blooms  
341 and confirms stable eutrophic conditions. However, in this upper unit, there are strong  
342 temporal variations in akinete concentration, despite the consistently high number of diatoms,



343 which implies high total phosphorus concentrations. Other environmental parameters may  
344 thus have affected cyanobacterial growth, such as water column instability, light intensity and  
345 water temperature. For example, on the basis of sedimentological parameters, the period from  
346  $1391 \pm 50$  to  $670 \pm 50$  cal.yr BP is mainly characterized by an increase in the amount of  
347 detrital input and thus in the frequency of flooding events (Lavrieux et al., 2013a,b). It is  
348 noteworthy that a doubling of the sedimentation rate since 1200 cal.yr BP took place at the  
349 end of a wetter period, in a context of high anthropogenic pressure. Recurrent flooding may  
350 have had an effect on the water column stability, creating unfavorable conditions for the  
351 development of cyanobacterial biomass (Mitrovic et al., 2003; Lewis et al., 2011; Paerl et al.,  
352 2013). Nevertheless, the persistence of long-term high trophic conditions allowed  
353 cyanobacteria to regrow as soon as conditions become favorable again, as can be observed at  
354 the end of the Medieval period and during Modern Times.

#### 355 4.4 Long term akinete integrity: persistence and germination potential

356 Very few studies have considered the potential viability of ancient cyanobacterial cells.  
357 Rucker et al., (2009) and Ramm et al. (2012, 2017), focused on akinete integrity but were not  
358 able to discriminate intact akinetes from lysed ones. On the other hand, Legrand et al. (2017b)  
359 characterized akinete integrity in sediments, but only using the previous year's sediment  
360 surface. So, to our knowledge, this study is the first one to investigate the viability and  
361 germination potential of cyanobacteria over thousands of years. Interestingly, in Lake Aydat,  
362 the percentage of intact akinetes of *D. macrosporum* are in the same range at the sediment  
363 surface (around 7%) (Legrand et al., 2017b) and the upper unit of the core (a mean of 5%).  
364 This suggests that akinete degradation does not increase with time spent in sediment. Instead,  
365 the percentage of intact benthic akinetes seems to be linked to processes occurring in the  
366 water column and is thus representative of their state at the end of their sedimentation out of  
367 the water column. This hypothesis may also be confirmed by the higher percentage of

368 integrity of akinetes in the lower units, especially given the presence of *Dolichospermum flos-*  
369 *aquae*, *Dolichospermum sp.* and *Aphanizomenon sp.* These species composed between 60 and  
370 100 % of the intact akinetes, whereas *D. macrosporum* (dominant in the upper part) had a  
371 mean of 6%. This suggests that the different species had different resistance capacities as also  
372 suggested in Legrand et al., (2016b). The latter study showed that akinetes from *D.*  
373 *macrosporum* were less resistant than those of *D. flos-aquae*. In addition, the different species  
374 are known to have varying abilities to produce akinetes. Thus, some species produce a large  
375 number of akinetes, but which are less resistant (such as *D. macrosporum*), while others  
376 produce less akinetes, but ones that are more resistant to unfavorable environmental  
377 conditions (such as *D. flos-aquae*). These differences between species can be integrated into a  
378 broader ecological strategy allowing each species to adapt to its preferred environment.

379 Despite the variable percentages of integrity, the number of intact akinetes able to  
380 germinate remained extremely low in the oldest sediment (1% maximum) compared to  
381 surface sediments (up to 70%) (Tjsujimura et al., 2003; Legrand et al., 2017b). Even though  
382 old akinetes might have retained an intact envelope, they probably lost their potential for  
383 reviviscence with time. Current germination has been recorded after a storage time of 64 years  
384 (Livingstone and Jaworski, 1980) and 120 cal.yr BP (i.e. around 180 years) (Wood et al.,  
385 2009), but not longer. Nevertheless, in our study, some intact akinetes from *D. flos-aquae*  
386 showed the ability to undergo cell divisions after 1830 years of sedimentation (1769 cal.yr  
387 BP), which is 10 times more than that previously observed in the literature. Therefore, while  
388 the culture conditions and/or cellular capacity were able to produce hormogonia only in 1  
389 sample (897 cal.yr BP) (i.e. a young filament (Hence and Beckmann, 2006)), our results show  
390 that akinetes can remain alive 1800 years after their formation. Interestingly, these results may  
391 be due to the differential loss of viability depending on the species. For example, *D.*  
392 *macrosporum* only presents germinating akinetes in the more recent samples, whereas the

393 number of intact akinetes of this species was higher in older samples. On the contrary, *D. flos-*  
394 *aquae* filaments were observed in samples where intact akinete concentration was very low.  
395 The only presence of germinating akinetes without visible filament formation may be due to a  
396 loss of viability and/or to inappropriate culture conditions. Thus, samples which contain only  
397 germinating akinetes may represent the limit of viability for a species. This indicates that *D.*  
398 *flos-aquae* akinetes may be able to stay viable longer than *D. macrosporum* akinetes. This  
399 hypothesis may confirm observations in Legrand al. (2016a, 2017a,b) that akinete integrity  
400 could reflect different ecological strategies between these 2 species .

401 Finally, this work highlights the unexpected viability of akinetes through hundreds of  
402 years and may be the starting point for other research focusing on the isolation and  
403 characterization of ancient cyanobacterial strains. Knowing the host of conditions favorizing  
404 akinetes germination (Kaplan-levy et al., 2010; Sukenik et al., 2019), other investigations will  
405 be necessary to test the long-term viability of akinetes of other cyanobacterial species.

#### 406 4.5 Cyanotoxin genes detection as a marker of past toxic genotypes.

407 This study also highlighted the past recurrences of the *anaC* and *mcyB* genes which are  
408 involved in anatoxin-a and microcystin biosynthesis, respectively. For the first time, results  
409 revealed the presence of these genes back to 6700 cal yr. BP. Previously, the presence of the  
410 *mcyA* gene had only been demonstrated from the last century (Monchamp et al., 2016), and  
411 from 220 years ago for the *anaC* gene and *mcyA* (Legrand et al., 2017a). So, the presence of  
412 these genes in Lake Aydat provides the first long-term dynamics of both microcystin and  
413 anatoxin genotypes. Interestingly, *anaC* and *mcyB* genes are both detected in one of the oldest  
414 samples, 6702 ± 195 cal.yr BP, revealing an unexpected co-occurrence, or at least a close  
415 succession, of genotypes carrying these two genes. Numerous studies have already

416 highlighted the co-occurrence of these two cyanotoxins in the water column (Park et al., 1993,  
417 1997; Pawlik-Skowronska, 2004) including at Lake Aydat (Sabart et al., 2015)

418 Nevertheless, except for short periods of co-occurrence, *anaC* and the *mcy* genes are  
419 mainly found independent of each other. The *anaC* gene was mostly observed in deep  
420 sediment (from  $5674 \pm 161$  to  $4802 \pm 134$  cal.yr BP) rather than in surface sediments,  
421 whereas *mcyB* has been recurrent over at least the last 500 years. Monchamp et al. (2016)  
422 have linked the presence of *mcyA* gene from 1972 to the presence of *Planktothrix rubescens*.  
423 The same applies to *Microcystis* and the detection of *mcyA* in a sediment core spanning 30  
424 years from Lake Erie, USA (Rinto-Kanto et al., 2009). *Aphanizomenon* and *Dolichospermum*,  
425 genera found throughout the core from Lake Aydat, are both able to produce these  
426 cyanotoxins (Rapala et al., 1993, 1997; Ballot et al., 2010; Bernard, 2014). Furthermore,  
427 *mcyA* gene and *anaC* have already been associated with the recurrence of *D. macrosporum*  
428 and *D. flos-aquae* in Lake Aydat (Legrand et al., 2017a). So, the Nostoclean species may be  
429 at least partly responsible for these toxic patterns found through the studied core.  
430 Nevertheless, we were not looking for, and have no evidence of, the presence of non-  
431 Nostoclean groups in our sediment core. *Microcystis*, *Woronichinia* and *Pseudanabaena*  
432 have already been observed in the Lake Aydat water column (Sabart et al., 2015; Lafforgue et  
433 al., 1995) and are potential producers of the studied cyanotoxins (Bernard, 2014). So, it is also  
434 possible that these non-nostoclean cyanobacteria are also partly responsible for the toxic  
435 potential detected in the core. First results from sequencing of *anaC* seem to be in accordance  
436 with a combination of several species possessing this gene. This analysis from 4000-year-old  
437 sediment highlighted that the *Oscillatoria* and *Aphanizomenon* genera possessed this gene  
438 (Figure S4). More investigations are required to identify a formal link between past  
439 nostoclean biomass and the presence of genes which could create cyanotoxins.

## 440 5. Conclusion

441 Cyanobacteria are known to have been on Earth for more than 2.5 billion years, but  
442 much evidence suggests that harmful cyanobacterial blooms have been more recurrent and  
443 frequent on a global scale over the last few decades (Huisman et al., 2018). Our results  
444 confirm this trend and show a long recurrence of cyanobacteria through time, with the oldest  
445 noticeable concentration of akinetes at 6700 cal.yr BP. So the presence of cyanobacteria in  
446 Lake Aydat is not restricted to the modern period, but originates very early on in the history of  
447 the lake. The natural damming of the river probably provided the first favorable conditions for  
448 their development, which was subsequently accentuated by human activity (woodland  
449 clearance, crop planting, grazing around the lake, etc.) and diverse land use through time  
450 (hemp culture and retting). The next step is to characterize the ancient cyanobacterial strains  
451 through this long time period, using 16S phylogenetic analysis, to find out more about the  
452 total diversity of cyanobacteria, not just Nostocales. Demonstrating the akinetes' potential  
453 to germinate and the exceptional longevity of these cells in sediments (over 1800 years)  
454 illustrates the huge *in situ* stock of viable akinetes able to germinate again and to sustain  
455 future cyanobacterial blooms. The long-term recurrence of cyanobacteria, with the dominance  
456 of the same species over the last 1800 years, reveals that eutrophic conditions have been  
457 maintained over a long time period, illustrating the current difficulty to mitigate  
458 eutrophication. Thus, sedimentary archives constitute a good way to improve our  
459 understanding of the dynamic functioning of individual lakes and also to indicate means of  
460 management adapted to their life history and cyanobacterial sedimentary stock.

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468

469 **Conflict of Interest**

470 The authors declare no conflict of interest.

471           **References**

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674

## 675 **Figure legends**

676 Graphical abstract: Evolution of akinetes from a cyanobacterial bloom to several thousand years after  
677 landfill (DNA was preserved as free DNA in sediment or in intact akinete).

678 Table 1: Primers used to amplify targeted genes.

679 Figure 1: (A) Bathymetric map of Lake Aydat (the star represents the location of the core sample); (B)  
680 age model of the core from Lavrieux et al., (2013a) (blue lines represent analyzed samples, and blue  
681 circles samples used in the germination experiment); (C) different akinete morphotypes encountered:  
682 (a) *Dolichospermum macrosporum*, b) *D. flos-aquae* c) *Dolichospermum sp.* d) *Aphanizomenon sp.*

683 Figure 2: Distribution of *D. macrosporum* and *D. flos-aquae*, *Dolichospermum sp.* and  
684 *Aphanizomenon sp.* akinetes through the sediment core. Light color represents total akinete abundance  
685 for one condition whereas deep color represents intact akinete distribution. Intact akinetes are defined  
686 as akinetes which have an intact cellular wall (Legrand et al., 2016b).

687 Figure 3: Evolution of the percentage of intact akinetes for each studied species through the core.

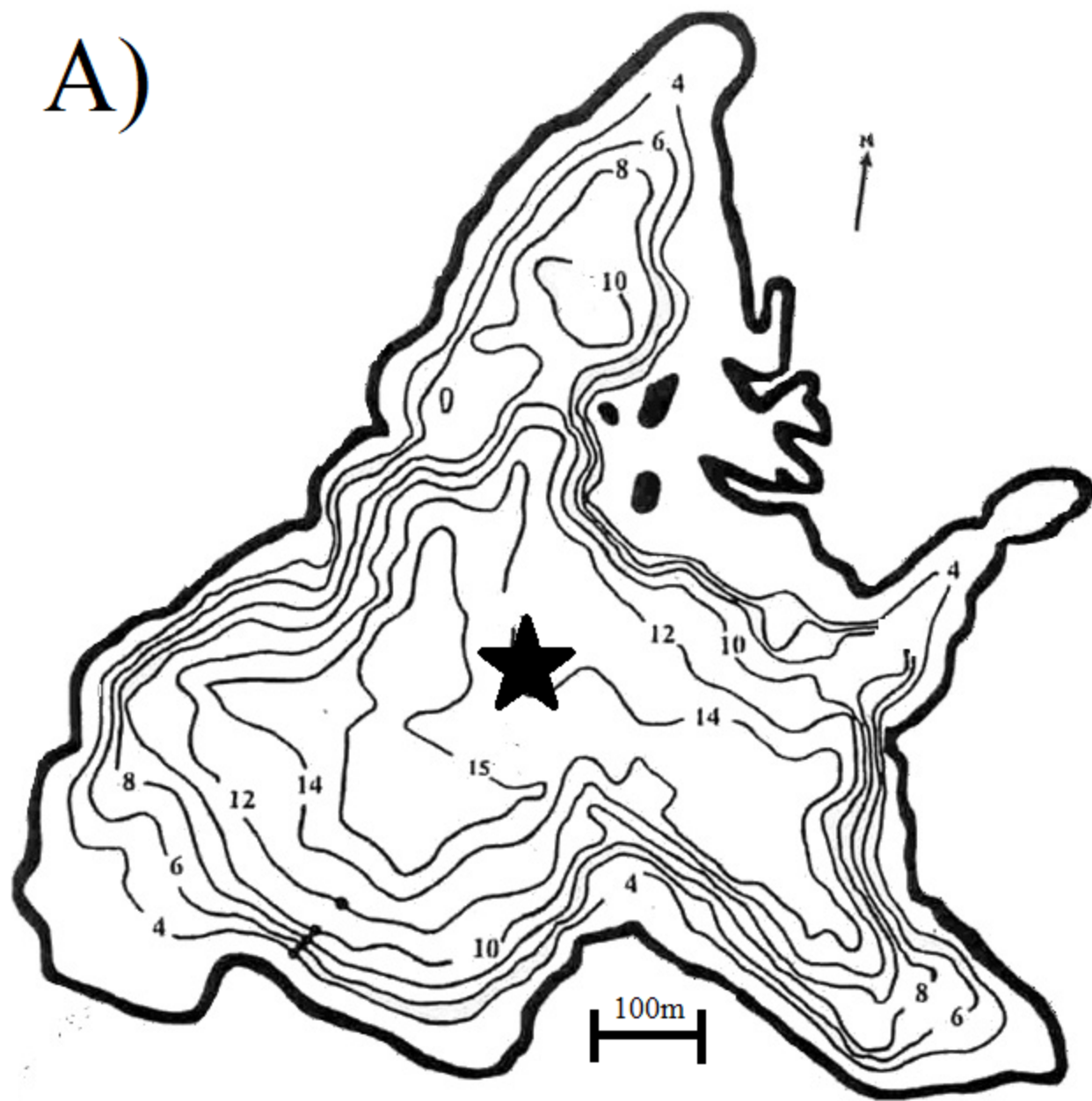
688 Figure 4: Proportion of akinete morphotypes observed in the sediment layers (A) and during the  
689 germination experiment (B). Red color represents samples for which intact akinetes have not been  
690 enumerated.

691 Figure 5: Detection of the targeted genes in total sediment through the core.

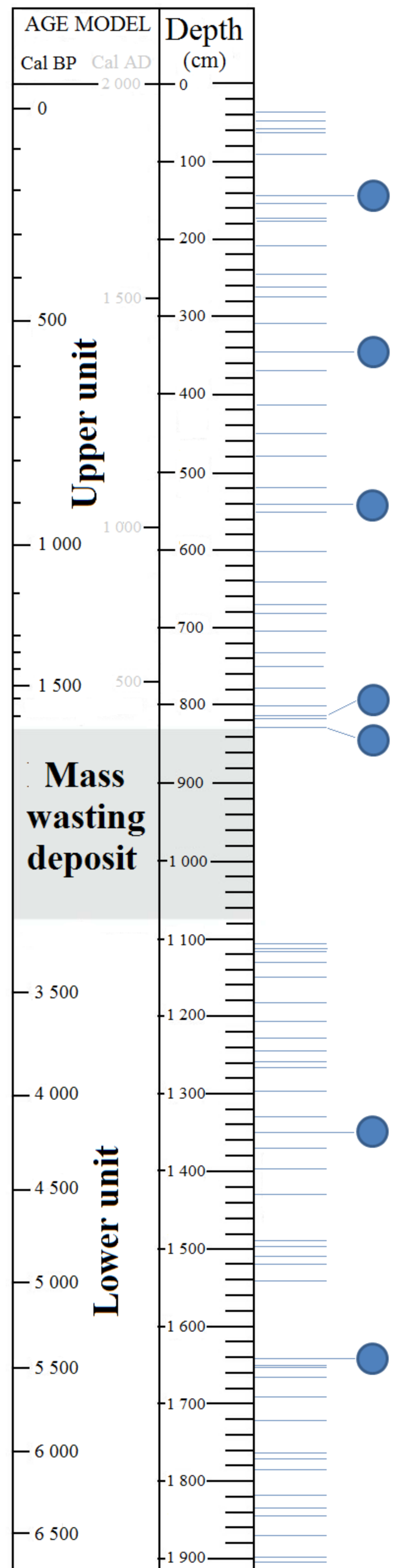
692



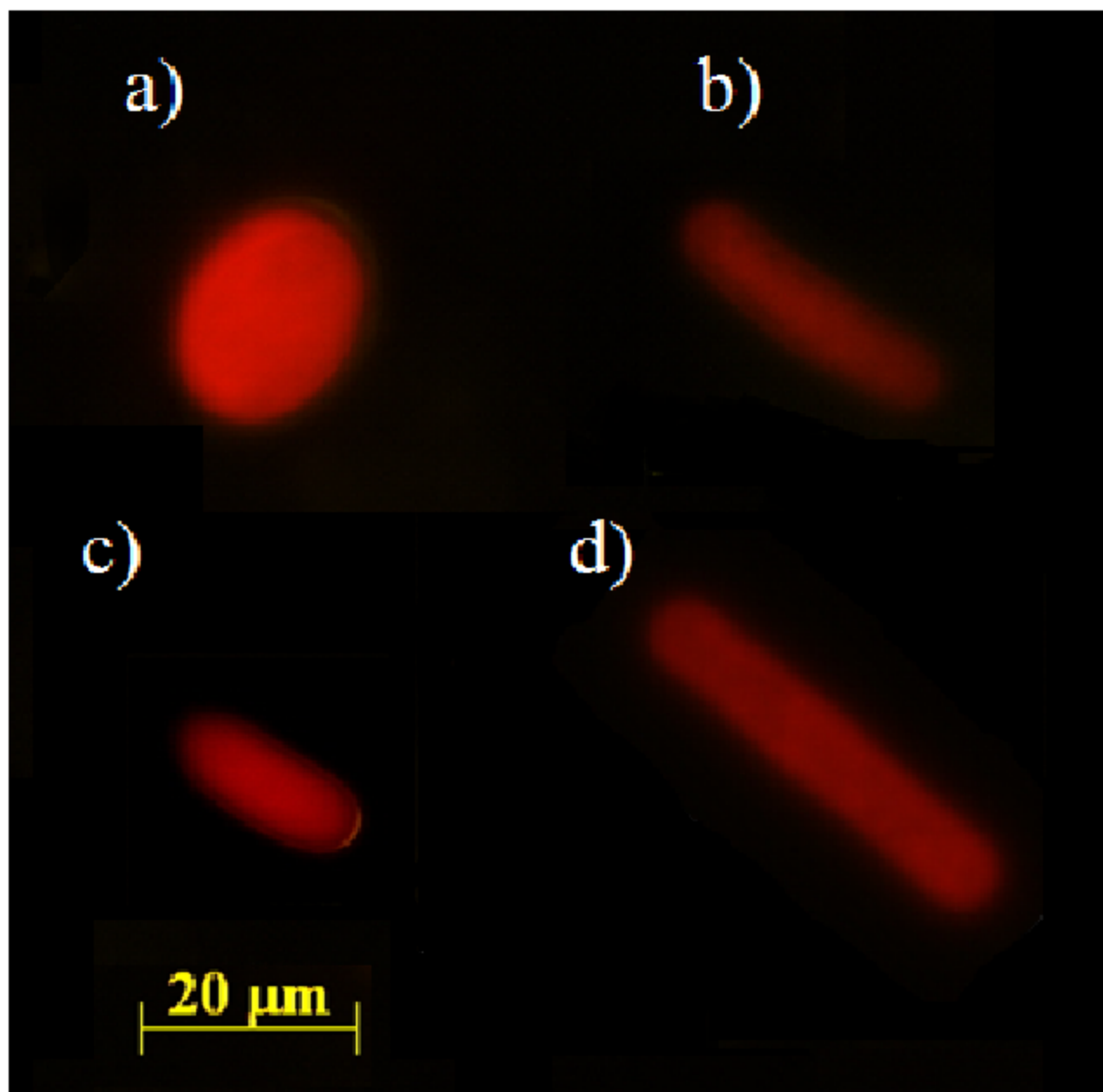
A)



B)



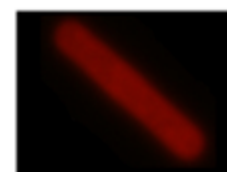
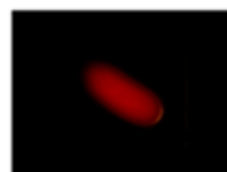
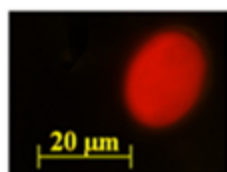
C)



**Legend:** — Analyzed samples

● Samples used for germination experiments

All akinetes morphotypes



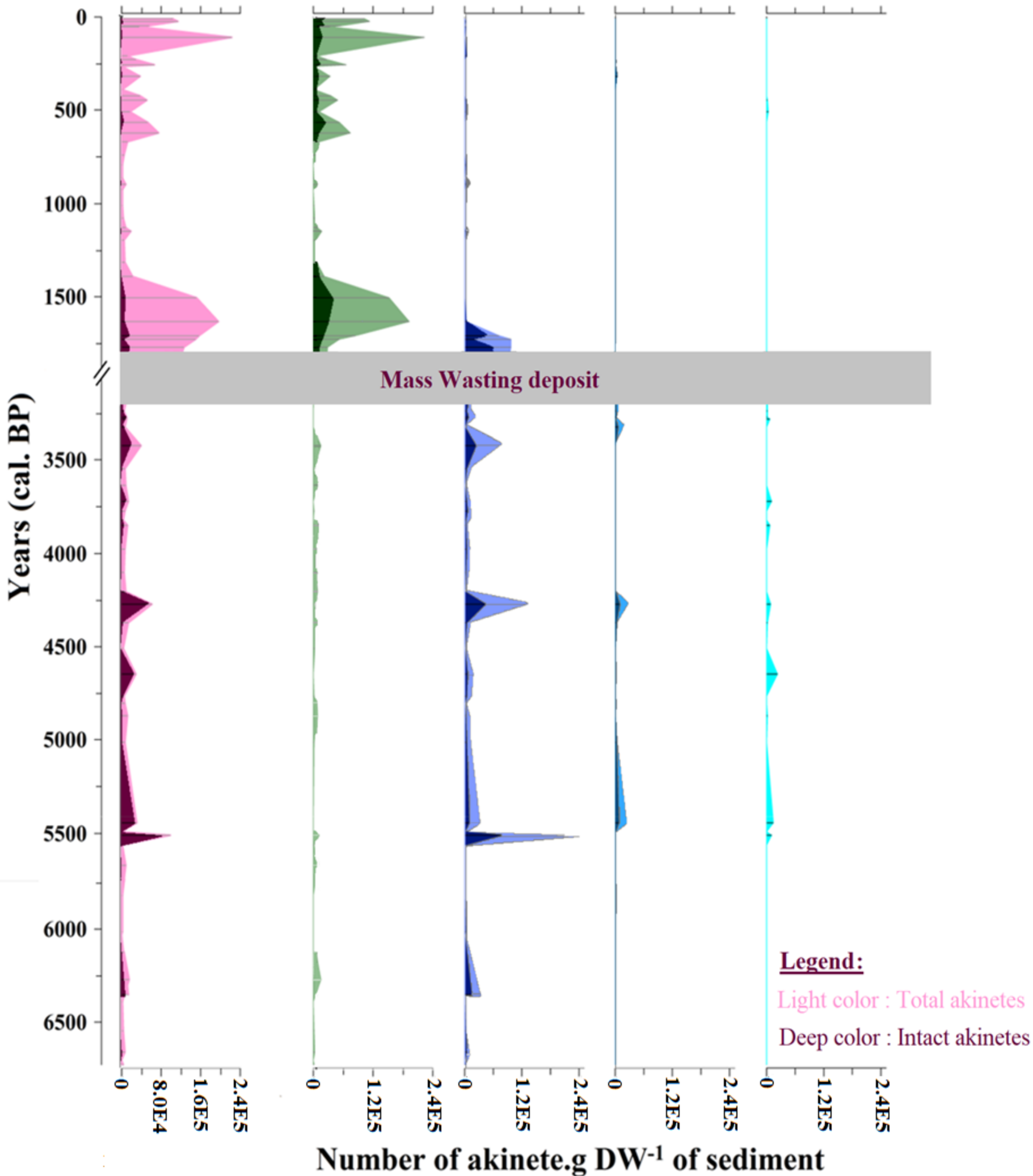
Total

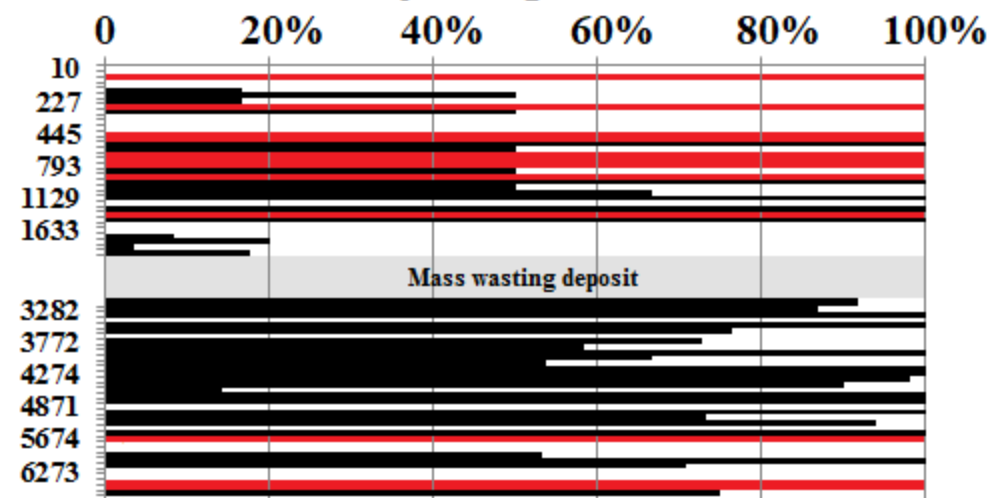
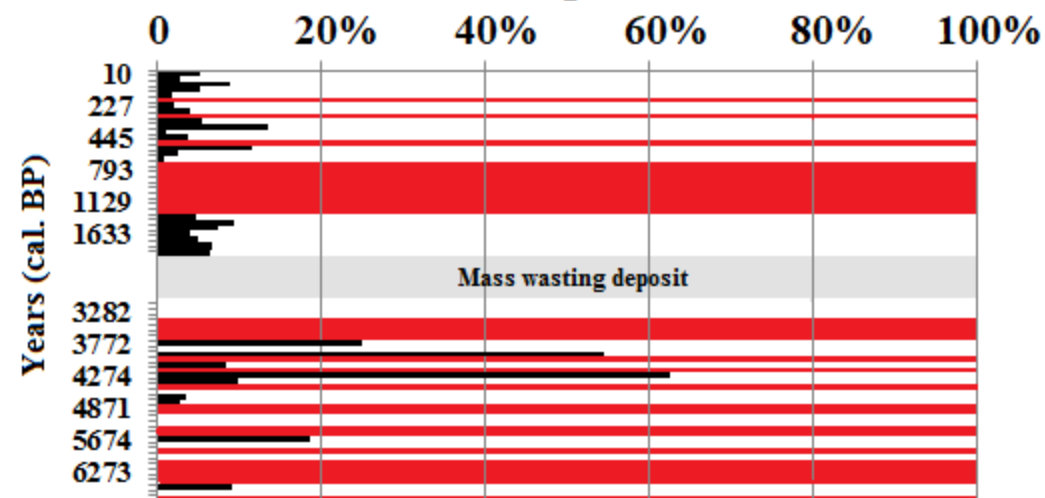
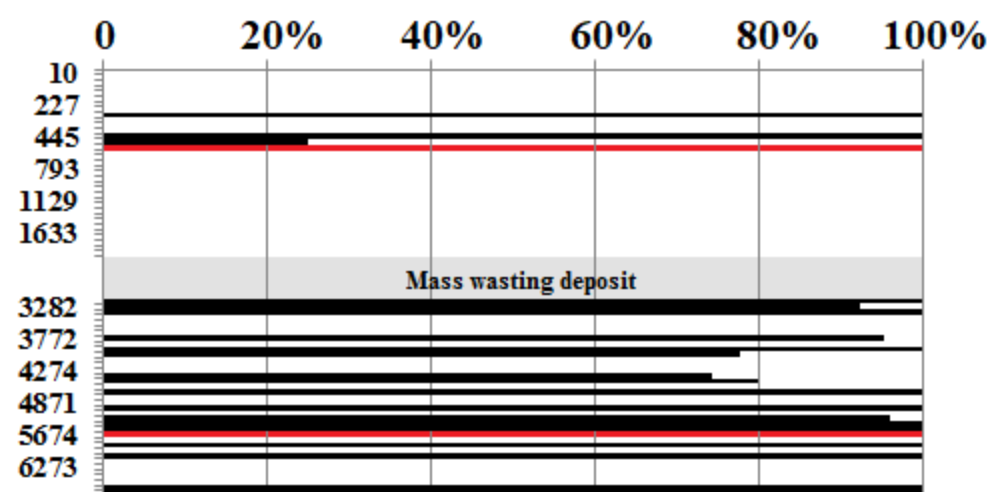
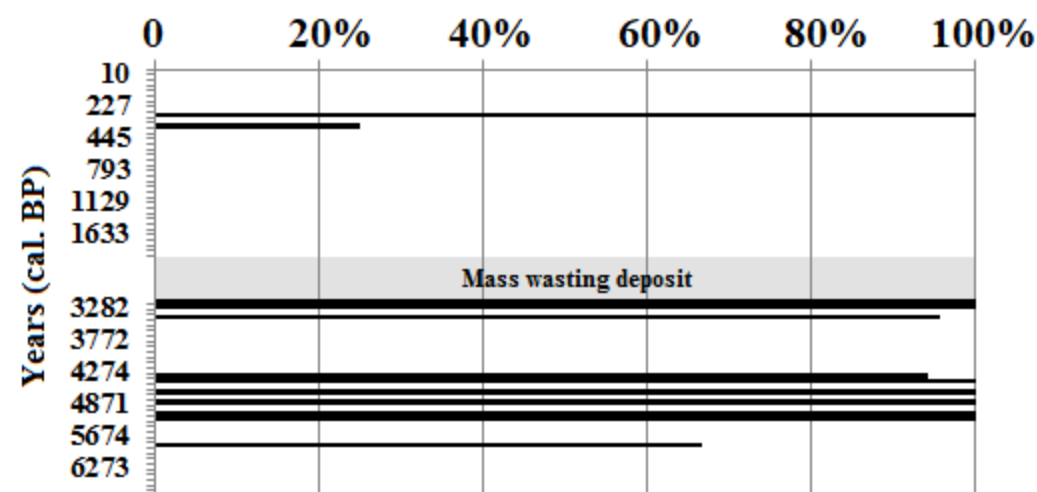
*D. macrosporum*

*D. flos-aquae*

*Dolichospermum sp.*

*Aphanizomenon sp.*



*D. macrosporum**D. flos-aquae**Dolichospermum sp.**Aphanizomenon sp.*

Percentage of intact akenetes



Percentage of intact akenetes

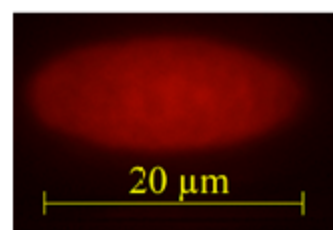
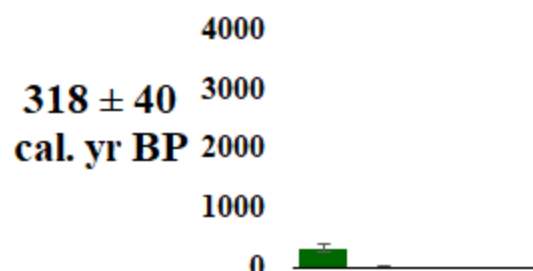


Samples characterized with 0% of intact akenetes

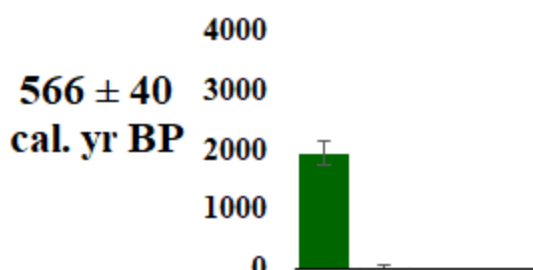
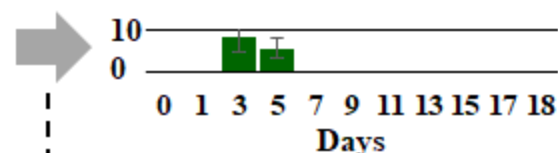
Number of akinetes.mL<sup>-1</sup> at the beginning of the experiment

Evidences of akinete germination

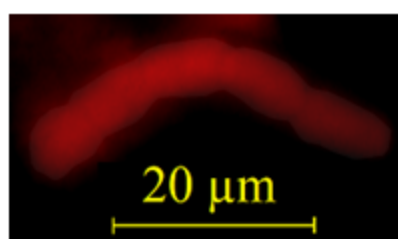
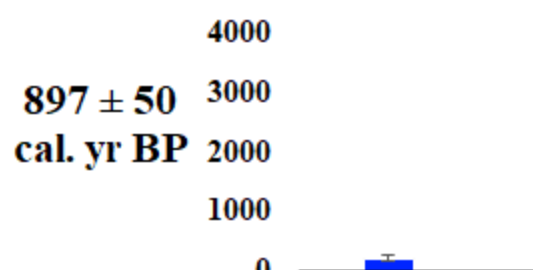
Kinetics of germination (% of germination)



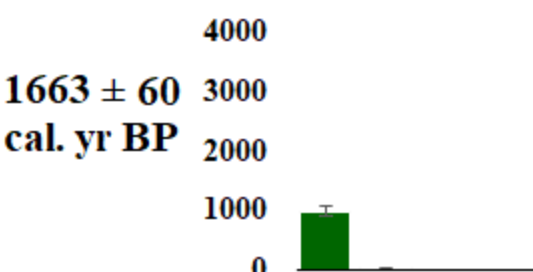
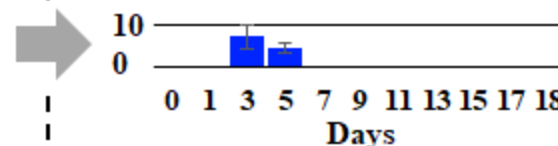
*D. macrosporum*  
germinating akinete



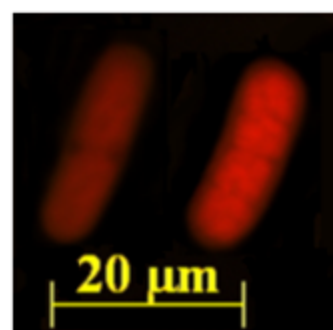
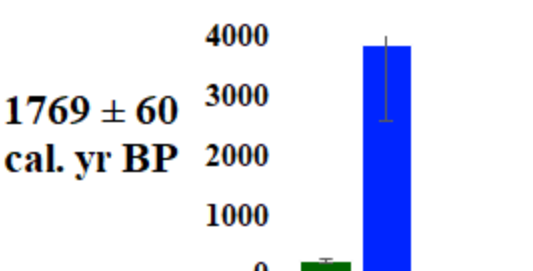
No akinete germination observed



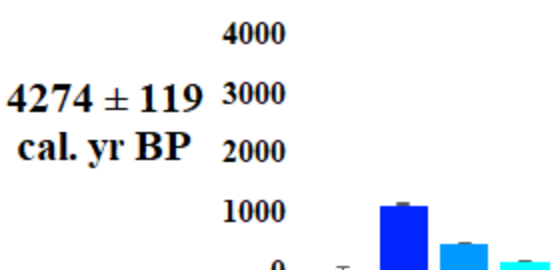
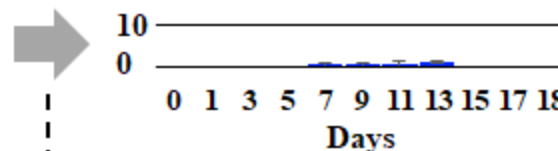
*D. flos-aquae*  
hormogonia



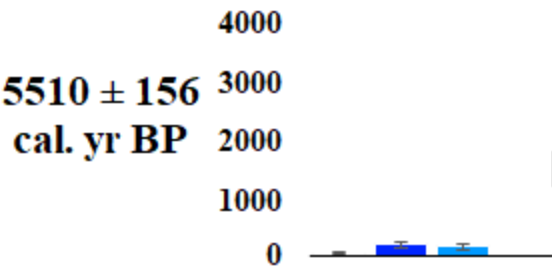
No akinete germination observed



*D. flos-aquae*  
germinating akinetes







No akinete germination observed



No akinete germination observed

**Legend:**

-  *D. macrosporum*
-  *D. flos-aquae*
-  *Aphanizomenon sp.*
-  *Dolichospermum sp.*

| Upper Unit           |             |             | Lower Unit          |             |             |
|----------------------|-------------|-------------|---------------------|-------------|-------------|
| Age (Years cal. BP)  | <i>mcyB</i> | <i>anaC</i> | Age (Years cal. BP) | <i>mcyB</i> | <i>anaC</i> |
| 10                   |             |             | 3238                |             |             |
| 25                   |             |             | 3269                |             |             |
| 48                   |             |             | 3282                |             |             |
| 52                   |             |             | 3322                |             |             |
| 107                  |             |             | 3421                |             |             |
| 208                  |             |             | 3544                |             |             |
| 227                  |             |             | 3638                |             |             |
| 258                  |             |             | 3721                |             |             |
| 268                  |             |             | 3772                |             |             |
| 318                  |             |             | 3820                |             |             |
| 387                  |             |             | 3848                |             |             |
| 422                  |             |             | 3976                |             |             |
| 445                  |             |             | 4102                |             |             |
| 566                  |             |             | 4208                |             |             |
| 620                  |             |             | 4274                |             |             |
| 670                  |             |             | 4374                |             |             |
| 675                  |             |             | 4512                |             |             |
| 739                  |             |             | 4648                |             |             |
| 793                  |             |             | 4768                |             |             |
| 864                  |             |             | 4802                |             |             |
| 897                  |             |             | 4871                |             |             |
| 924                  |             |             | 4871                |             |             |
| 1011                 |             |             | 5011                |             |             |
| 1080                 |             |             | 5446                |             |             |
| 1129                 |             |             | 5492                |             |             |
| 1151                 |             |             | 5510                |             |             |
| 1194                 |             |             | 5565                |             |             |
| 1316                 |             |             | 5674                |             |             |
| 1391                 |             |             | 5822                |             |             |
| 1507                 |             |             | 6013                |             |             |
| 1633                 |             |             | 6032                |             |             |
| 1708                 |             |             | 6126                |             |             |
| 1728                 |             |             | 6273                |             |             |
| 1769                 |             |             | 6354                |             |             |
| Mass wasting deposit |             |             | 6388                |             |             |
|                      |             |             | 6547                |             |             |
|                      |             |             | 6659                |             |             |
|                      |             |             | 6702                |             |             |
|                      |             |             | 6727                |             |             |

Positive with PCR detection

|              | Primer name                | Sequence (5'-3')   | Target gene                     | Gene Amplicon size (bp) | References   |
|--------------|----------------------------|--|---------------------------------|-------------------------|--|
| Classic PCR  | cya359F<br>cya781R         | GGGGAATYTTCCGCAATGGG<br>GACTACTGGGGTATCTAATCCCATT        | Cyanobacterial<br>16S rRNA gene | 403                     | Nubel et al., (1997)   |
| Classic PCR  | anaC-gen-F2<br>anaC-gen-R2 | TCTGGTATTCAGTMCCCTCYAT<br>CCCAATARCCTGTCATCAA            | <i>anaC</i>                     | 366                     | Sabart et al., (2015) modified<br>from Rantala-Ylinen et al., (2011) |
| Nested PCR 1 | anxgen-F2<br>anxgen-R      | ATGGTCAGAGGTTTTACAAG<br>CGACTCTTAATCATGCGATC             | <i>anaC</i>                     | 861                     | Rantala-Ylinen et al., (2011)  |
| Nested PCR 2 | anaC 80F<br>anaC 706R      | CCAATAGCCTGTCATCAACC<br>TATACATCTGGCTCAACAGG             | <i>anaC</i>                     | 626                     | This study   |
| Nested PCR 1 | MCY F1<br>MCY R1           | TGGGAAGATGTTCTTCAGGTATCCAA<br>AGAGTGGAAACAATATGATAAGCTAC | <i>mcyB</i>                     | 319                     | Nonneman et al., (2002)  |
| Nested PCR 2 | MCY F1<br>MCY R2           | TGGGAAGATGTTCTTCAGGTATCCAA<br>GAGATCCATCTGTTGCAAGACATAG  | <i>mcyB</i>                     | 287                     | Nonneman et al., (2002)  |

