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

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Running title: Cyanobacterial recurrence and survival over 6700yr

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Abstract

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

Keywords

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

1. Introduction

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

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

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

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

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

2. **Methods:**

2.1 *Sampling and study site*

Lake Aydat (maximum depth: 15m; area: 6.105 m²; N45°39.809'/E 2°59.106'/837masl) is a eutrophic naturally dammed lake located in the French Massif Central where recurrent blooms of cyanobacteria have occurred in the late summer and autumn for the last few decades (Gerphagnon et al., 2013b). The lake was created by the damming of the Veyre river by a basaltic lava flow 8551 ± 400 cal.yr BP ago (Boivin et al., 2004). A detailed description of the lake's features (i.e geological and geomorphological setting) is given in Lavrieux et al., (2013a). Coring was performed in the central, deepest part of the lake (Figure 1A). Based on sedimentological features, the record is divided into 2 main units separated by an erosive mass wasting deposit: i) the lower one (1076-1974cm depth which corresponds to 777-1600cm in the master core), and ii) the upper unit (0-829cm depth which corresponds to 0-775cm in the master core) (Lavrieux et al., 2013a). Akinete and molecular analysis were carried out on both the lower and the upper units (Figure 1B) to complement existing pollen, non-pollen palynomorph and diatom analyses (Miras et al., 2015).

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 ^{17}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 ^{14}Carbone (Gif-sur-Yvette, France) and
102 calibrated (2σ) using CalPal Online. ^{137}Cs; ^{241}Am, ^{210}Pb and ^{7}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^{-1}. There was a sudden increase to 0.52cm.year^{-1}
108 between 660cm depth and the top, which is similar to the rate of 0.46cm.year^{-1} 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^{-1} (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 morprhotypes: *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 ± 40, 566 ± 60, 897 ± 60, 1633 ± 60, 1769 ± 60, 4274 ± 119
139 and 5510 ± 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 10µm 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 100µm and then a 50µm nylon filtration tissue to remove a maximum of residual sedimentary
144 particles. Finally, the cells were put in 4mL of BG11 medium with cycloheximid (50µg.mL^{-1}).
145 Five replicates of 800µL 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, 50µL of homogenized solution were introduced through a 8µL TETP filter (Milipore,

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

2.4 *DNA extraction*

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

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

2.5 *Target gene amplification*

Three genes were targeted by classic or nested PCR during this study. The cyanobacterial 16S rRNA gene was amplified in sediment samples using the cya359F and cya781R set of primers (Nubel et al., 1997) (Table 1). Then one gene involved in the biosynthesis of anatoxin-a, *anaC,* and one of microcystin, *mcyB* gene, were also investigated. The first was detected with the sets of primers anxgen-F2/R and *anaC* 80F/706R for nested 1 and 2 PCR respectively. For *mcyB* nested PCR, MCY F1/R1 then MCY F1/R2 were used. PCR mixtures were the same as in Legrand et al., (2016a) for the two *anaC* nested PCRs. For use with *mcyB*, the mix for both PCRs was composed of 5µL of colorless Go Taq® felxi 5X Buffer, 2.5mM of $MgCl_2$ solution, 0.2mM of dNTPs, 0.5µM of each primer (Table 1), 1

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

3. **Results**

3.1 *Total akinete abundance through the core*

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

196 akinetes.gDW^{-1} 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^{-1} of sediment. However, the upper part also shows a greater fluctuation, from
199 22 000 akinetes.gDW^{-1} (between 1391 ± 50 and 670 ± 50 cal.yr BP) to around 200 000
200 akinetes.gDW^{-1} 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^{-1} 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^{-1} 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^{-1} 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^{-1} 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^{-1} of sediment respectively. However, there is no
226 statistical difference due to large variations (from 0 to 86 200 akinetes.gDW^{-1} 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^{-1} 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^{-1} 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

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

3.4 *Target gene detection in sediment*

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

4. **Discussion**

4.1 *Reconstruction of long-term nostocalean recurrences in Lake Aydat*

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

4.2 *Establishment of baseline conditions at Lake Aydat*

One surprising result was to find sizeable concentrations of akinetes in the oldest part of the core, between ca 6500 and 6000 cal.yr BP. This suggests that only around 2 millennia after 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 nutriment 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 ± 129, 4274 ± 119 and 3421 ± 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 ± 60 and 1507 ± 40 cal.yr BP and between 670 ± 50 cal.yr BP and
325 the sub-recent period (639 ± 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 ± 50 to 670 ± 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 Rücker 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

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

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

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

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

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

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

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

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

5. **Conclusion**

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

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Conflict of Interest

The authors declare no conflict of interest.

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Figure legends

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

Table 1: Primers used to amplify targeted genes.

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

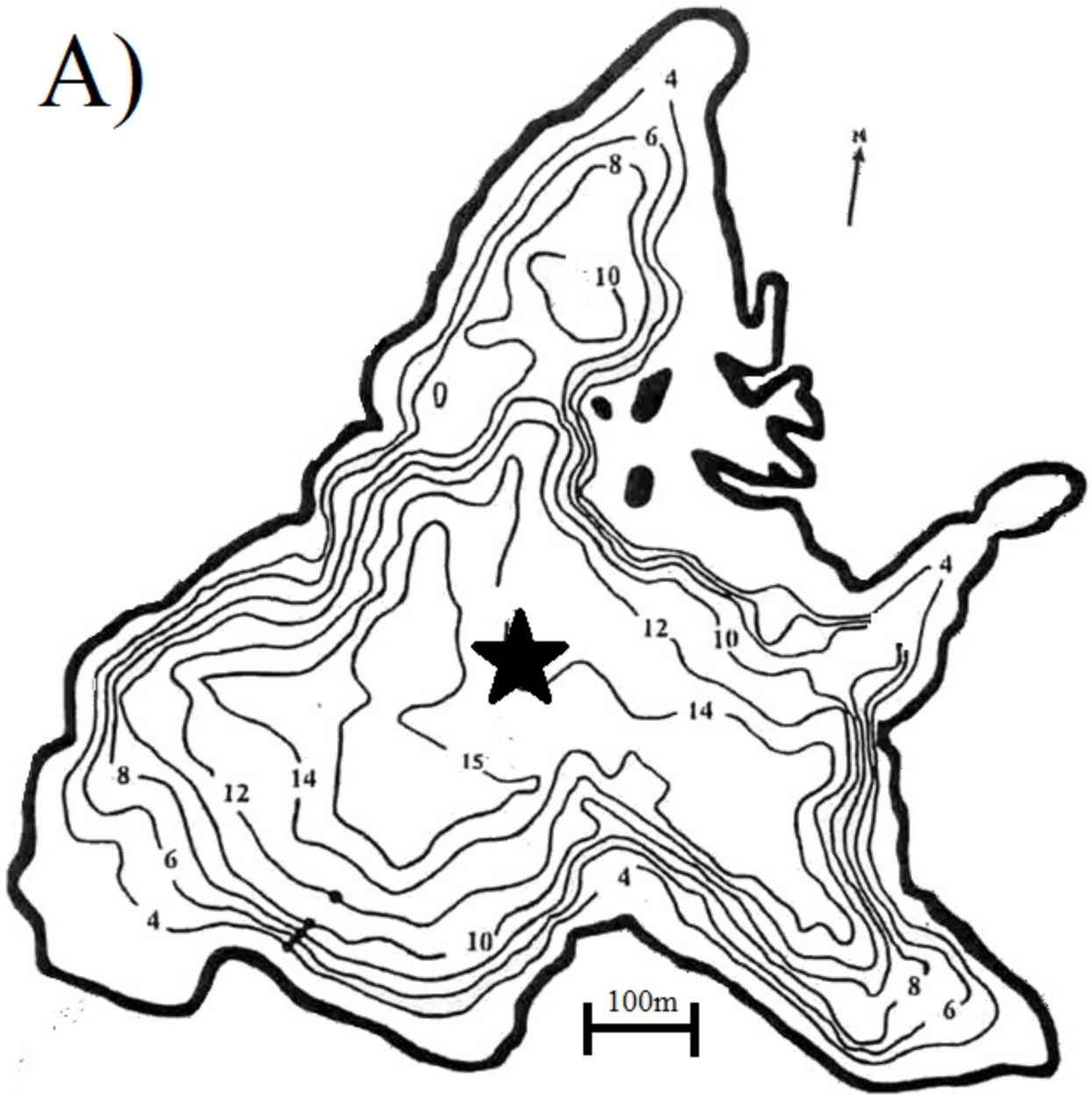
Figure 2: Distribution of *D. macrosporum* and *D. flos-aquae, Dolichospermum sp.* and *Aphanizomenon sp*. akinetes through the sediment core. Light color represents total akinete abundance for one condition whereas deep color represents intact akinete distribution. Intact akinetes are defined as akinetes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

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

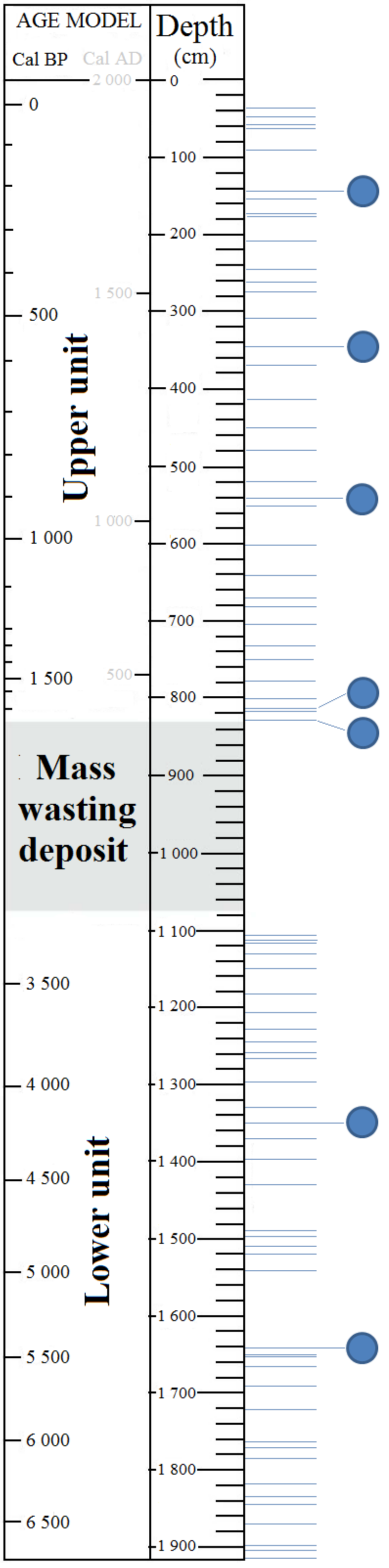
Figure 4: Proportion of akinete morphotypes observed in the sediment layers (A) and during the germination experiment (B). Red color represents samples for which intact akinetes have not been enumerated.

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

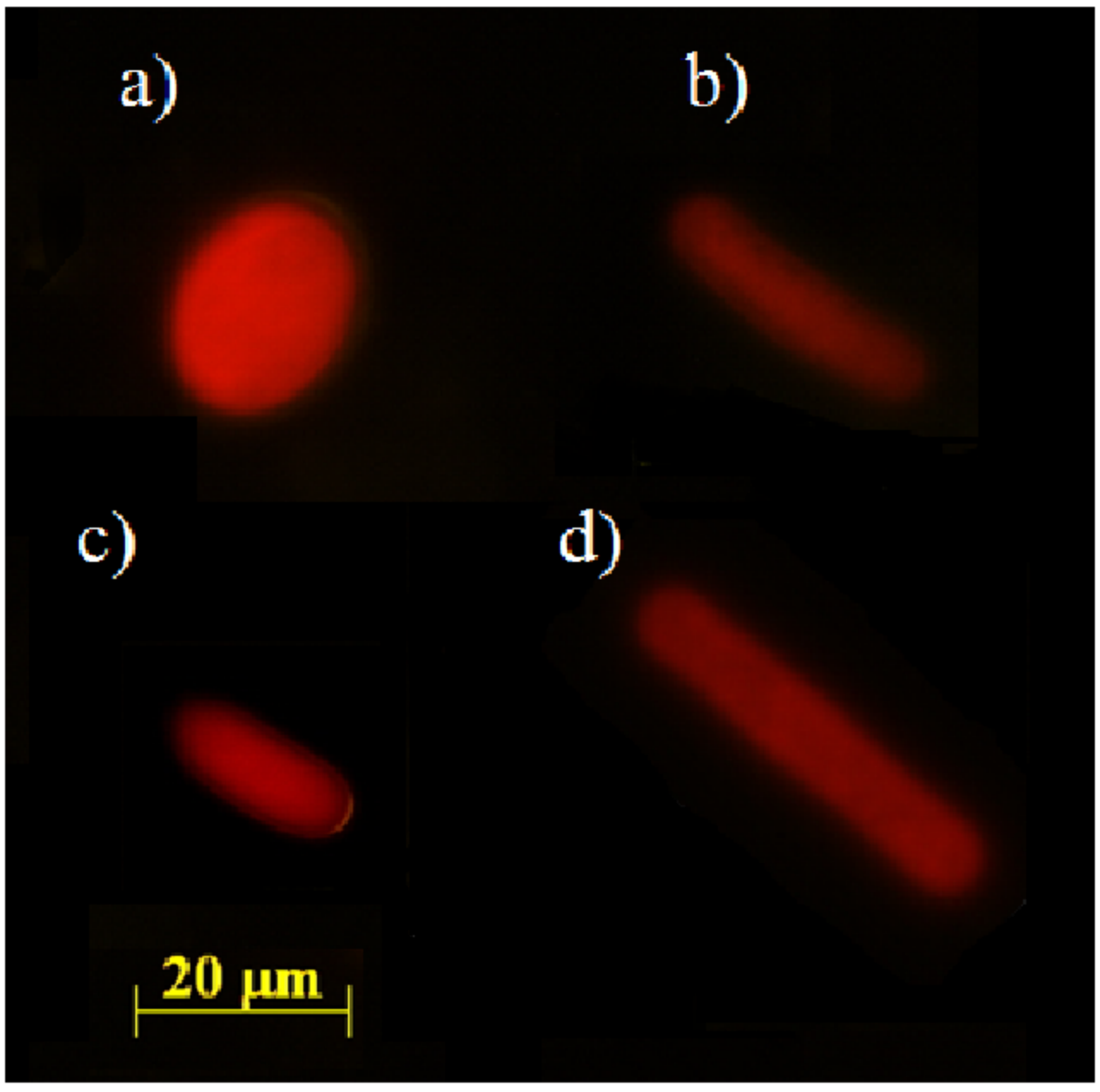
A)



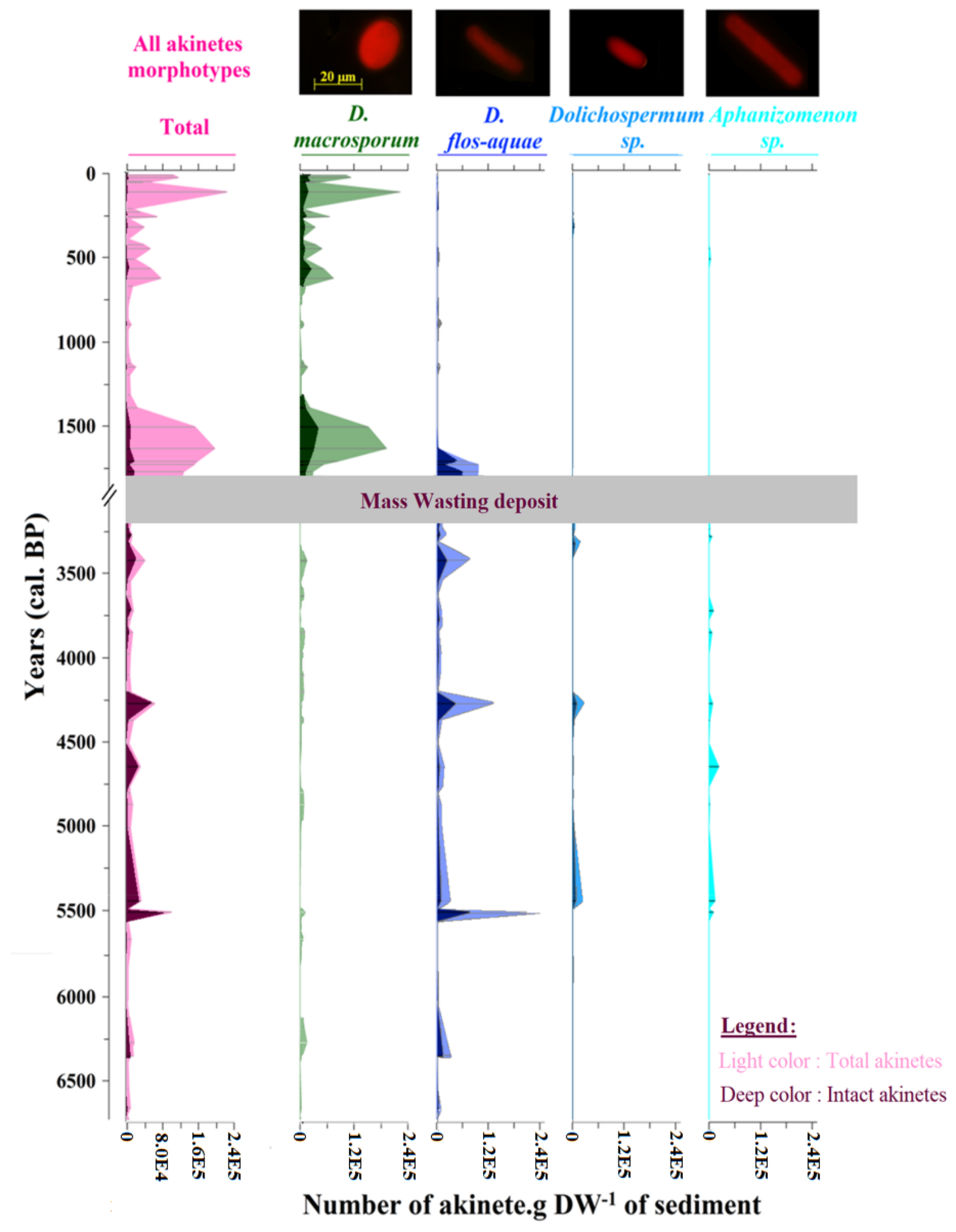
B)

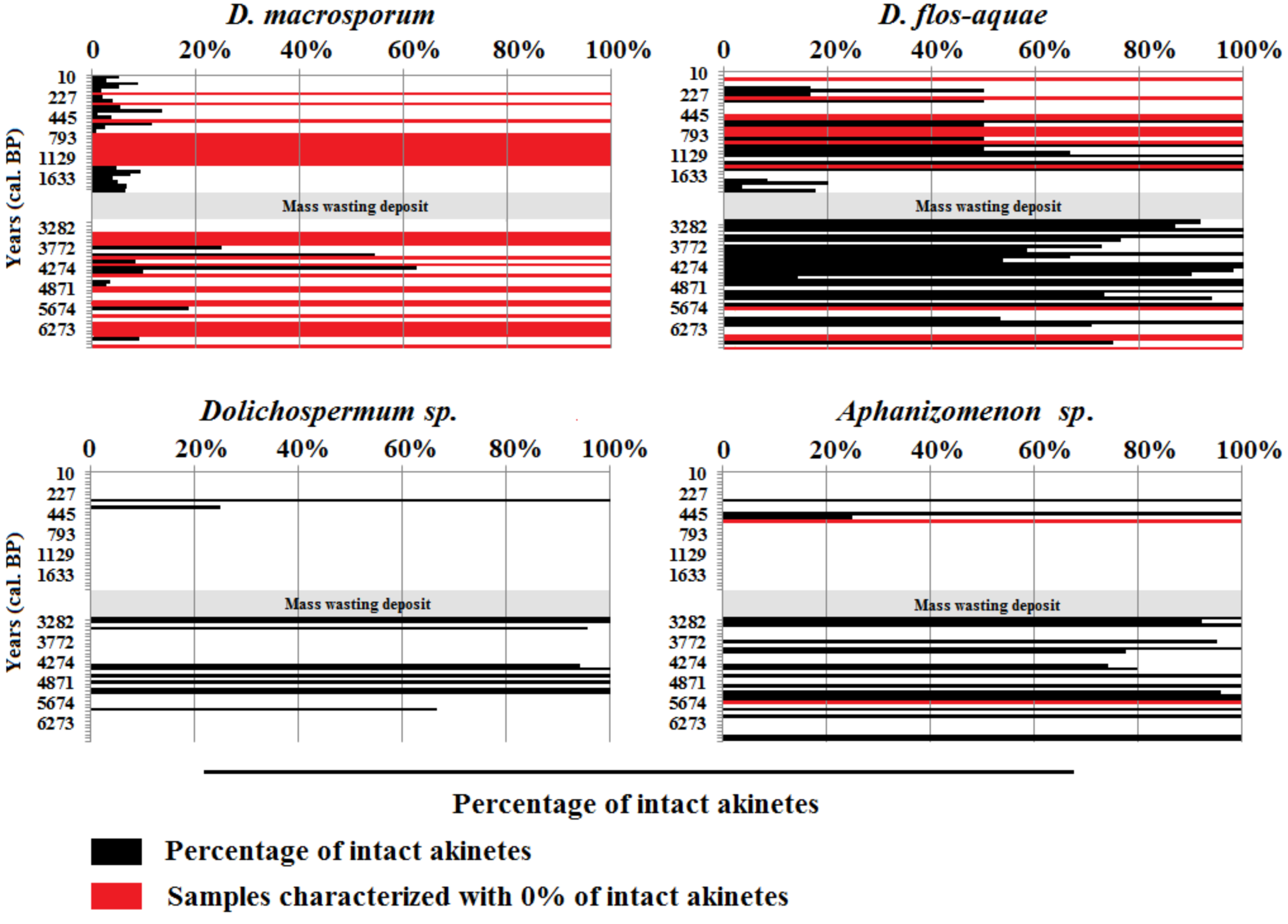


C)



Legend: Analyzed samples — Samples used for germination experiments


All akinetes morphotypes
20 µm
Total
D. macrosporum
D. flos-aquae
Dolichospermum sp.
Aphanizomenon sp.
0
500
1000
1500
Mass Wasting deposit
3500
4000
4500
5000
5500
6000
6500
Years (cal. BP)
0
8.0E4
1.6E5
2.4E5
0
1.2E5
2.4E5
0
1.2E5
2.4E5
0
1.2E5
2.4E5
0
1.2E5
2.4E5
Number of akinete.g DW-1 of sediment
Legend:
Light color : Total akinetes
Deep color : Intact akinetes

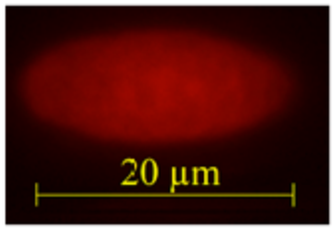

D. macrosporum
D. flos-aquae
Dolichospermum sp.
Aphanizomenon sp.
0
20%
40%
60%
80%
100%
Years (cal. BP)
10
227
445
793
1129
1633
3282
3772
4274
4871
5674
6273
Mass wasting deposit
Percentage of intact akinetes
Percentage of intact akinetes
Samples characterized with 0% of intact akinetes

Number of akinetes.mL^{-1} at the beginning of the experiment

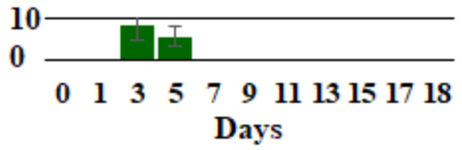
Evidences of akinete germination

Kinetics of germination (% of germination)

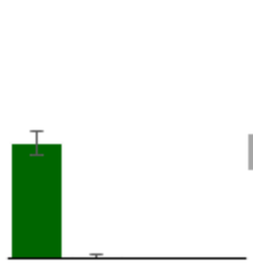
318 ± 40 cal. yr BP



D. macrosporum germinating akinete

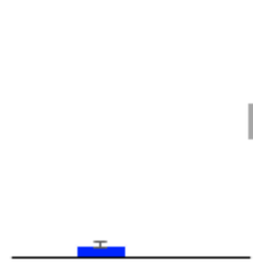


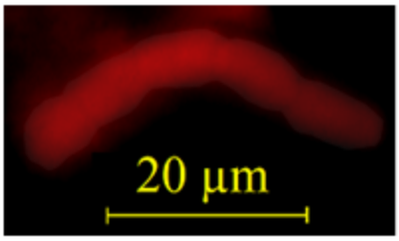
566 ± 40 cal. yr BP



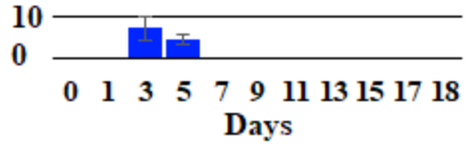
No akinete germination observed

897 ± 50 cal. yr BP

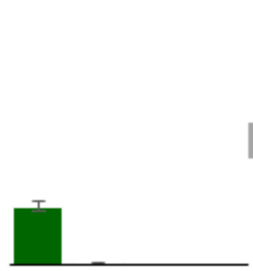




D. flos-aquae hormogonia

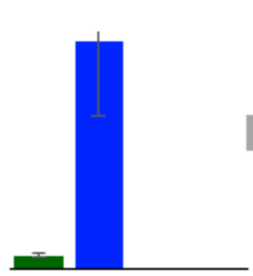


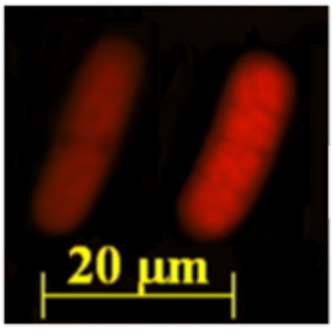
1663 ± 60 cal. yr BP



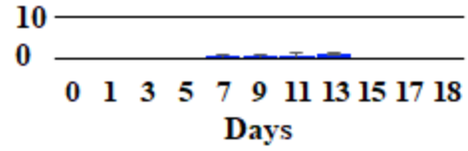
No akinete germination observed

1769 ± 60 cal. yr BP

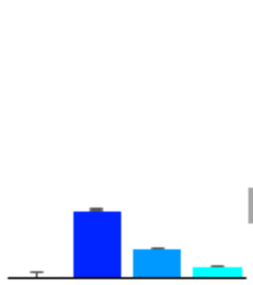




D. flos-aquae germinating akinetes

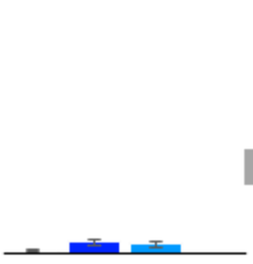


4274 ± 119 cal. yr BP



No akinete germination observed

5510 ± 156 cal. yr BP



No akinete germination observed

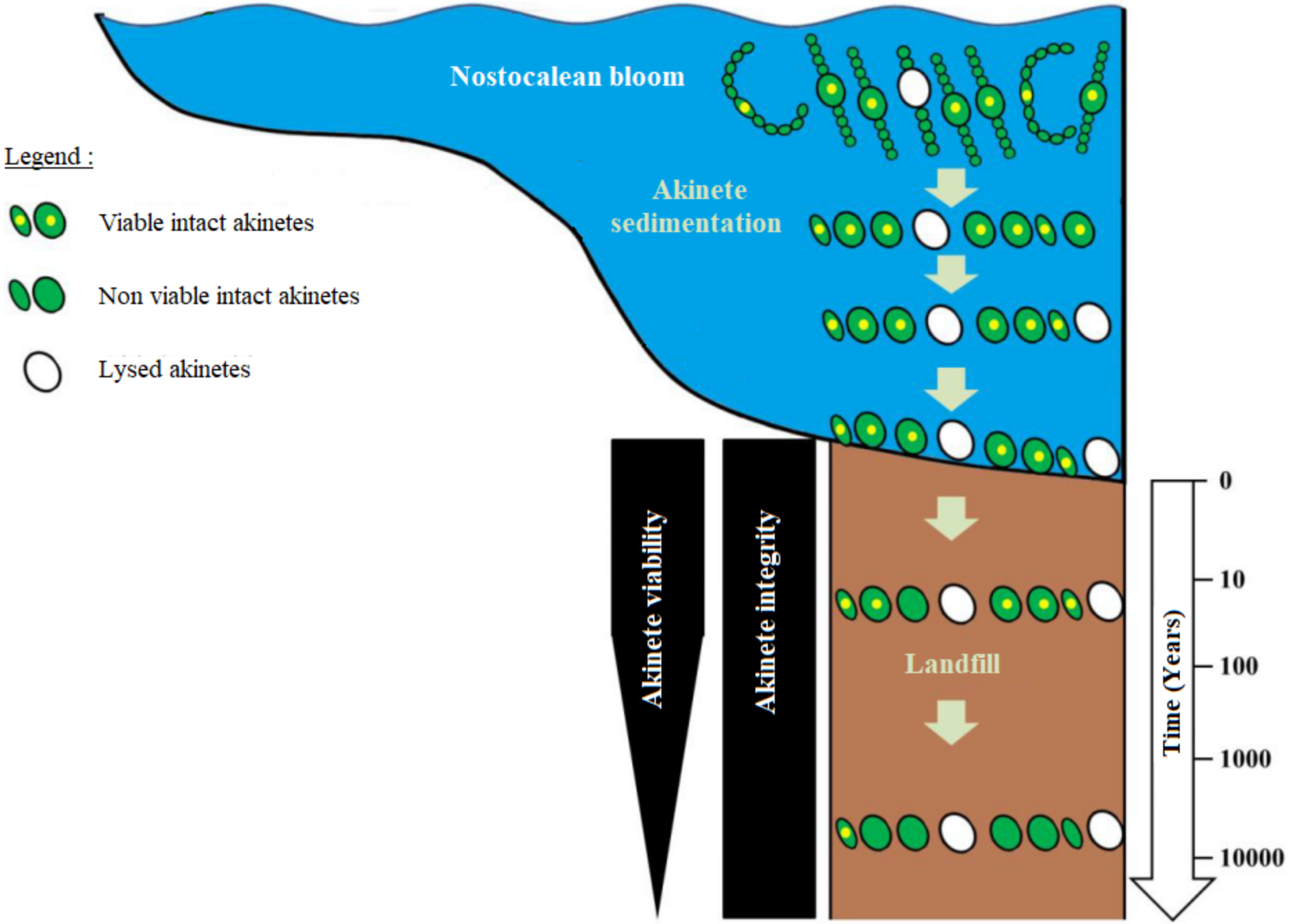
Legend:

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

Upper Unit			Lower Unit		
Age (Years cal. BP)	*mcyB*	*anaC*	Age (Years cal. BP)	*mcyB*	*anaC*
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			5011		
1011			5446		
1080			5492		
1129			5510		
1151			5565		
1194			5674		
1316			5822		
1391			6013		
1507			6032		
1633			6126		
1708			6273		
1728			6354		
1769			6388		
Mass wasting deposit			6547		
			6659		
			6702		
			6727		

Positive with PCR detection

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


Nostocalean bloom
Legend :
Viable intact akinetes
Non viable intact akinetes
Lysed akinetes
Akinete sedimentation
Akinete viability
Akinete integrity
Landfill
Time (Years)
0
10
100
1000
10000