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High variability of microbial diversity from lampenflora of two bas-reliefs in the Pommery Champagne cellar

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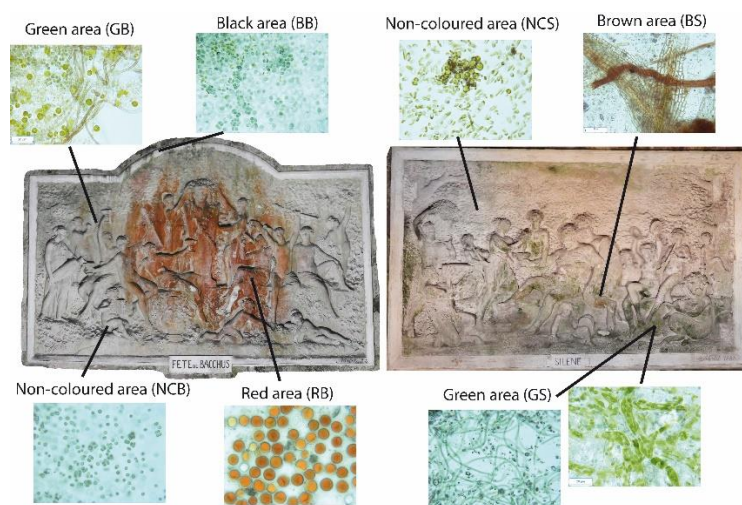
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Abstract

Pommery Champagne cellar has the originality to be in the underground of Reims city (NE - France) and has got monumental bas-reliefs carved in chalk walls. Bas-reliefs were illuminated for tourist visits and led to a lampenflora growth with different coloured areas which mar the beauty of the artworks. This study was focused on the characterization of the lampenflora of two bas-reliefs (named Fete de Bacchus and Silene) with the hypothesis that the different visual aspects of biofilms are associated to different phototrophic components. The goal was to control the proliferation of the lampenflora. Molecular analysis revealed distinct exogenous and opportunistic microorganisms in the two bas-reliefs though the bacteria:fungi ratio was remarkably constant. Pseudomonadota, Acidobacteriota, Bacteroidota and Actinomycetota were surprisingly dominant on Cyanobacteria. Bacchus had a higher microbial diversity than Silene. The different areas within Bacchus were composed of distinct communities while this was not the case for Silene. Phototrophs reflected specificity of each pit, Bacchus was mainly colonised by *Chromochloris* algae whereas Embryophyta and *Stichococcus* rather grew in Silene. The perspectives of this study are to propose a sustainable management of the cellar, notably the lighting strategy and visitor circuit, as well as preventive and curative treatments.

Key words

Lampenflora, microbial communities, DNA sequencing, subterranean site, Pommery Champagne cellar



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39 1. Introduction

40

41 The beauty and cultural significance of underground sites gave rise to a notable interest for tourist
42 attraction. In contrast with brief scientific visits, the massive people influx required installation of
43 walking paths and artificial lights. The consequence was to irreversibly alter the natural environmental
44 conditions with an increasing of temperature and CO₂ level by visitors and air currents (Martin-Sanchez
45 et al., 2014) which gave access to complex communities called lampenflora. Such biofilms replaced
46 progressively the original microbiota as biovermiculations combining clayey sediments and microbial
47 populations of oligotrophic cave environments composed of chemolithoautotrophic microorganisms
48 (Tomczyk-Żak and Zielenkiewicz, 2016). The lampenflora is characterized by phototrophic organisms
49 developed near lamps and at distances over 10 m (Mulec et al., 2012; Baquedano Estévez et al., 2019).
50 Such biofilms are composed of colonizers from outdoor habitats selected according to their
51 adaptability to new microclimatic conditions: no natural light, weaker aeration but increased humidity
52 (Ariño et al., 2010) and an oligotrophic substrate but with organic matter provided by native
53 communities (Addesso et al., 2021). Over time, the lampenflora usually gathers different microbes,
54 aerophytic algae, cyanobacteria, bryophytes, mosses and ferns (Mulec and Kosi, 2009; D'Agostino et
55 al., 2015; Duan et al., 2021). Despite the invasive and unsightly aspect, the lampenflora is sometimes
56 considered as a part of the underground natural environment but it is also considered as
57 biodeteriogens and can lead to irreversible damage when biofilms alter paintings and high-value
58 geological formations (Bastian and Alabouvette, 2009; Saiz-Jimenez et al., 2011; Cennamo et al., 2016;
59 Perez, 2018).

60 In the city of Reims located in the North-East of France, the basement made of chalk rock was quarried
61 for building houses and monuments. Quarries were reused centuries later by winemakers to age
62 Champagne wine from the 19th century, thanks to perfect environmental conditions: darkness, low
63 variation of temperature and high humidity all over the year. They were listed as a UNESCO World
64 Heritage site in 2015. The lampenflora clearly occurred on stone walls where the illumination was
65 intensified especially on monumental bas-reliefs carved in the 19th century. These artworks were
66 previously highlighted by incandescent lamps and in recent years by LED for tourist visits (180,000
67 people/year). Once lampenflora installed, recurring cleanings do not prevent its quick recolonisation
68 causing spread discolorations which visually mar and physically degrade the artworks as some visible
69 "scars" and stone replacement testify to the damage. Moreover, the lampenflora grows in spite of a
70 current light control by a direct limited lighting time on the bas-reliefs but contemporary artworks
71 exhibited in the cellar pits imply an indirect thus low irradiances on bas-reliefs but enough for the
72 lampenflora growth (Albertano et al., 2003; Mulec et al., 2008).

73 The purpose of this study is (1) to characterize contrasted microbial communities as lampenflora which
74 thrive on two monumental bas-reliefs of the cellar, named "Fete de Bacchus" and "Silene", (2) to test
75 the hypothesis that the difference in the macroscopic aspect of the biofilms mainly corresponds to
76 differences in the phototrophic component of the stone-colonizing communities (and thus, pigmented
77 organisms) and (3) to get a better understanding of the parameters influencing biofilm development
78 in cellars and caves that are open to the public and, in turn, helping to elaborate preventive and
79 curative strategies, and suggesting a management reconciling heritage preservation with tourism
80 exploitation in the cellar. This investigation and analyses were conducted to compare the different
81 microbial communities developed on each bas-relief and communities between both bas-reliefs which
82 are located in two different pits. We finally define the environmental origin of the microorganisms and
83 their potential involvement in the stone biodeterioration.

84 This work started *in-situ* by sampling different areas detected by their colour and analysing by
85 colourimetry, chlorophyll fluorescence and pH measurement. A laboratory investigation was carried
86 out to quantify chlorophyll pigments, carotenoids and phycobiliproteins. Finally, the biodiversity of
87 biofilm areas was analysed by means of optical microscopic observation of biofilm samples, cultivation
88 and rRNA sequencing-based identification of isolates as well as Illumina-based high-throughput
89 sequencing of 16S and 18S rRNA genes from biofilm-extracted DNA. Statistical and analyses were
90 performed in order to correlate the different biological results with all the metadata associated.

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2. Materials and methods

2.1. Description of the study sites and cleaning history of the bas-reliefs

The specificity of Pommery cellar is the monumental bas-reliefs carved in chalk rock between 1882 and 1885 by a French sculptor, Gustave Navlet. The lampenflora invaded bas-reliefs whose walls are always saturated of water, available resource for the microbial growth. Pits and galleries are at 30 m depth and were quarried in a fractured chalk, limestone with a high porosity (40.4 %) and a high water saturation (94.4 %) (Franco-Castillo et al., 2022). The temperature is from 14°C in winter up to 17°C in summer and a relative humidity from 95 to 99 %.

The cleaning frequency of the lampenflora varied according to the artwork and went from nil or once to three times between 2014-2023 and no other details before this period was listed by the stakeholders of the cellar. It was carried out by a commercial biocide or/and by carbonate air-scrub but a visual recolonization was observed after six months causing spread discolorations which visually mar the artwork. In addition, stone replacements are also observed by a clear difference in biocolonisation which suggests physical biodeterioration implications from the lampenflora over time and a restoration by repair compound varying from the original stone. Fete de Bacchus bas-relief was never cleaned so biofilms are growing for a long time, and Silene bas-relief has already been cleaned in 2014 by a commercial biocide and bicarbonate airbrushing.

2.1.1. Fete de Bacchus bas-relief

The bas-relief Fete de Bacchus (Bacchus) basement is at 4 m from the ground and dimensioned 5 m wide and 8 m high (Fig.S1a). The sculpture thickness is around 15-20 cm (Fig. 1) (Franco-Castillo et al. 2022). It is located on the south wall of a pyramid-shaped pit with a ground dimension around 10 x 12 m and 30 m high up to the outdoor ground from which a dim light comes from a glazed opening 2 m in diameter. In the pit, a spotlight is currently fixed on the wall on the opposite of the artwork at 6 m high to illuminate it (Fig. 2a). This light is turned on by a switch for a few minutes for visits which limits the light supply. Moreover, the Vranken-Pommery Domain creates an art exhibit every year with contemporary artworks in the different pits and galleries of the cellar (Experience Pommery #16). That induces lighting to illuminate them, and some artworks are luminous themselves. That is the case in Bacchus pit, where artworks are luminous, nevertheless the light intensity measured at different points on the bas-relief through the photosynthetically active radiation (PAR) is currently very low (0.1 $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$).

In-situ lampenflora observations clearly show regular non-colonised zones always located to the left of the statues where the red lampenflora is largely expanded (Fig. S2). Those zones suggest shadow zones where a lack of light prevented phototrophic microorganism development, since the light source came from the west wall of the pit. This orientation of the red lampenflora can be explained by the settlement of a large red and green luminous artwork in 2009 (Experience Pommery #6) which was hanged up on the west wall and probably fostered the growth of biofilms.

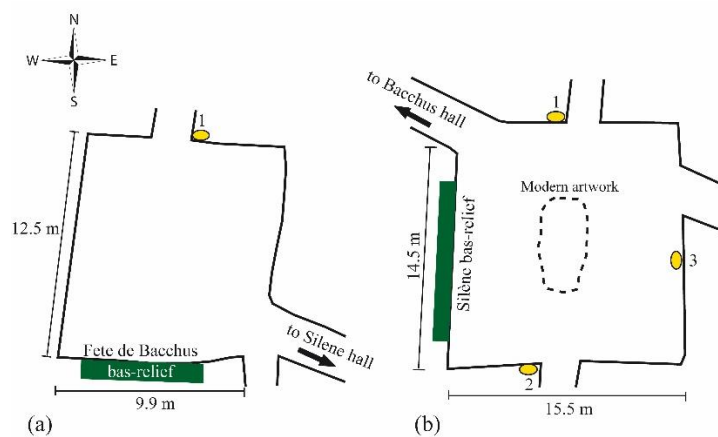


132 (a) Fete de Bacchus bas-relief (8 x 5 m) and (b) Silene bas-relief (15 x 6 m) carved in the chalk rock of two different pits
 133 in the Pommery Champagne cellar. Bacchus image was built by photogrammetry from 94 photos and Silene image from
 134 respectively 153 photos (Coolpix B700) by Agisoft Metashape v.1.7.2 software.
 135
 136

137 2.1.2. Silene bas-relief

138 Silene bas-relief is located at 3 m from the ground, and it is 15 m wide, 6 m high and inclined of 10°
 139 (Fig.S1b) and the sculpture thickness is around 25 cm (Fig. 1) (Franco-Castillo et al. 2022). The artwork
 140 was carved in the west wall of a pyramid shape pit. This pit is around 10 x 12 m to the ground and 30 m
 141 high up to the outdoor well which illuminate naturally the pit.

142 Artificial spotlights also illuminated the bas-relief, located on the lateral walls at 5 m high (1)-(2) and a
 143 third one is on the opposite wall of the bas-relief at 10 m high (3) (Fig. 2b). Nevertheless, the light
 144 intensity measured from the bas-relief is currently low ($0.1 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and the lighting time
 145 is limited to a few minutes like in Bacchus pit. Nevertheless, a ramp of 10 halogen spotlights settled at
 146 2 m below the bas-relief, ran for several years but it is no longer operating now. However, the in-situ
 147 observations displayed a net lampenflora growing in the lower part of the artwork, closer to the spot
 148 ramp and in the highest part because of the inclination of the bas-relief, well exposed to the ramp (Fig.
 149 S3). Finally, a modern artwork is currently exposed in the middle of the pit and always lit by LED lights
 150 on the ground.
 151



152
 153
 154 Fig. 2. Schematic map of the pits with bas-reliefs and current locations of spotlights.
 155

156 2.2. Sampling and characterization of the lampenflora

157 2.2.1. Sampling method

158 The sampling was carried out from a visual selection of 7 coloured areas and non-coloured areas (Fig.
 159 S4-Table S1). Bacchus showed three coloured areas: the major and central red area (Red Bacchus-
 160 further referred to as RB), in the secondary and peripheral green area (Green Bacchus, GB), in the black
 161

162 upper border of the bas-relief (Black Bacchus, BB) and finally in the peripheral non-coloured area (Non-
163 Coloured Bacchus, NCB). Silene had two coloured areas: the major green area (Green Silene-GS) and a
164 minor brown area (Brown Silene, BS), and a non-coloured area (Non-Coloured Silene, NCS). Every area
165 was sampled by means of a scalpel cleaned with ethanol, samples were transferred into sterile tubes,
166 and stored at 4 °C until analysis. For culture-based and molecular characterization of the biological
167 diversity, in order to account for the small-scale variability, 8 to 10 replicate samples (150 ± 32 mg
168 fresh weight) from each area were collected. The corresponding sampling surface was in the order of
169 1 cm^2 but not determined precisely. For pigment quantification, the biofilms were totally scraped and
170 collected from two $5 \times 5 \text{ cm}$ zones from each area (1 for liposoluble pigments and 1 for
171 phycobiliproteins).

172

173 *2.2.2. Characterization of colonized and non-colonized areas on the bas-reliefs*

174

175 *2.2.2.1. Colourimetry*

176 Colour change was performed with 10 measurements in a significative spot for each area with a
177 Chroma Meter CR-400 from Konica-Minolta with a light projection tube CR-A33c of 11 mm diameter
178 (corresponding to the measurement zone). Calibrations were previously performed with a white
179 ceramic plate CR-A43. Values are given in the CIE- $L^*a^*b^*$ colour space (European committee for
180 Standardization, 2011), with three dimensionless parameters determining the colour: L^* indicates
181 lightness; a^* and b^* are the chromaticity coordinates. Then the average and the standard deviation
182 were calculated.

183

184 *2.2.2.2. Photosynthetic activity*

185 Fifteen in-situ measurements were carried out on each area by means of a photosynthesis yield
186 analyser, Junior PAM Chlorophyll Fluorometer (Walz, Effeltrich, Germany) comprising a pulse-
187 amplitude modulation device (PAM) in combination with saturating pulse analysis of fluorescence
188 quenching. It is a non-destructive technique for the characterization of phototrophic biofilms on
189 substrates commonly employed now in cultural heritage (Miller et al., 2011; Bruno et al., 2019; Del
190 Rosal et al., 2021). This handheld device was connected on one side to a laptop with WinControl-3
191 software (Walz) and on the other side to the studied area via a 50 cm long flexible fibre optic with
192 1.5 mm active diameter. Junior PAM released a blue power LED (460 nm) for pulse modulated
193 fluorescence excitation and saturating pulses (1 s, 10 kHz) and collected chlorophyll *a* fluorescence
194 emitted by phototrophic microorganisms especially algae.

195 Site conditions (artwork at several meters high) prevented from performing a dark-adaptation before
196 measurements, thus the two recorded parameters were the minimal fluorescence of light-adapted
197 biofilm (F_0') and the maximal fluorescence during the saturating light (F_M'). From those parameters,
198 ϕ_{PSII} was calculated from $F_V'/F_M' = (F_M' - F_0')/F_M'$, it corresponds to the relative effective quantum yield
199 of photochemical energy conversion at steady-state photosynthesis (Pfendler et al., 2018a; Eyssautier-
200 Chuine et al., 2021). For each area defined previously, 15 measurements were carried out before
201 sample collection, the average and standard deviation of ϕ_{PSII} were then calculated.

202

203 *2.2.2.3. pH measurements*

204 pH of biofilms was measured with the Pocket pH-meter, LAQUAtwin pH-11 from Horiba with pH 7.00
205 and 4.01 pH buffers. This devise requires a low sample volume (0.1 ml) by means of a flat electrode
206 enabling accurate (± 0.1 pH) direct measurements of solid micro samples. Three measurements were
207 carried out on the non-coloured areas (NCB-NCS) but only one measurement was done on biofilm
208 areas because of the coloration of the electrode by liposoluble pigments.

209

210 *2.2.3. Characterization of areas in laboratory*

211

212 *2.2.3.1. Liposoluble pigment concentrations*

213 Lampenflora colour is partly related to photosynthetic microorganisms as algae and cyanobacteria that
 214 produce photosynthetic pigments. A square biofilm sample (5 x 5 cm) was scraped in each area (non-
 215 coloured and coloured), they were weighed and transferred into sterile tubes, then 10 ml of 80%
 216 acetone was added. Tubes were kept in the dark for 2 h at 4°C. After centrifugation at 4°C for 10 min
 217 at 2075 rpm (Beckman Coulter allegraX-15R centrifuge), the supernatant was taken and the
 218 absorbance measured with Genesys 10 uv Scanning spectrophotometer (Thermoscientific) at 646, 663,
 219 and 470 nm. The concentrations of chlorophyll a, b and carotenoids were calculated with the following
 220 equations (1) (Wellburn, 1994). Pigment concentrations per surface unit (mg.m⁻²) were then calculated
 221 by multiplying the volume of the extracted solution (10 ml) and finally by dividing the surface of the
 222 sample (0.0025 m²).

$$\begin{aligned}
 & \text{- [Chlorophyll } a] \text{ mg.l}^{-1} = 12,21 \times A_{663} - 2,81 \times A_{646} = C_a \\
 & \text{- [Chlorophyll } b] \text{ mg.l}^{-1} = 20,13 \times A_{646} - 5,03 \times A_{663} = C_b \\
 & \text{- [Carotenoids] mg.l}^{-1} = [1000 \times A_{470} - 3,27 \times C_a - 104 \times C_b] / 198
 \end{aligned}
 \tag{1}$$

2.2.3.2. Concentration of phycobiliproteins

229 For phycobiliproteins (PBP) quantification, 5 x 5 cm biofilm samples were scraped with the same
 230 protocol as samples for liposoluble pigments. Then they were suspended in 5 ml of 0.01 M Na₂HPO₄
 231 buffer, pH 7, 0.15 M NaCl. Disruption of cells were carried out on first by cycles of freezing (-80°C) -
 232 thawing (90°C) method. After light microscope examinations, red round cells in RB were still not lysed,
 233 the disruption of cells was then carried on by sonication in an ice bath at with Bioblock Scientific
 234 vibracell sonifier at 20 kHz ± 50 Hz, 40% amplitude for 5 min. Ten sonication pulses, alternated with a
 235 cooling period, were needed for complete cell disruption. Sonicates were centrifuged at 8000 g for
 236 30 min and the optical density (OD) of the supernatant was measured at 562, 615 and 652 nm (Bruno
 237 and Valle, 2017). Finally, concentrations of the most well-known phycobiliproteins (Allophycocyanin :
 238 APC, Phycocyanin: PC, and Phycoerythrin: PE) were calculated from equations (2) (Bennett and
 239 Bogorad, 1973). PBP concentrations per surface unit (mg.m⁻²) were calculated by multiplying the
 240 volume of the extracted solution (5 ml) then by dividing the surface of the sampled zone (0.0025 m²).

$$\begin{aligned}
 & \text{PC} = [A_{615} - 0.474 (A_{652})]/5.34 \\
 & \text{APC} = [A_{652} - 0.208 (A_{615})]/5.09 \\
 & \text{PE} = [A_{562} - 2.41 (PC) - 0.849 (APC)]/9.62
 \end{aligned}
 \tag{2}$$

2.3. Microbial diversity

2.3.1. Isolation of strains by culture-based techniques

249 Isolation of bacterial, fungal, and phototrophic strains was performed to characterize the diversity of
 250 cultivable microorganisms present in the biofilms. One sample per area was suspended in 10 ml of
 251 distilled water and diluted from 10⁰ to 10⁻⁵. Then 100 µl of each dilution was inoculated on duplicate
 252 Petri dishes containing agar medium with Luria Bertani medium at 1/10 concentration (LB10) for
 253 bacterial isolation (Ezraty et al., 2014), Malt medium for fungal and yeast isolation (Sarkar et al., 2009)
 254 and Hoagland medium for phototroph isolation (Salamah et al., 2019) (Table S2). Cultures were
 255 maintained at 20 °C, 80% relative humidity and included an 12/12 light/dark period with by means of
 256 two neon lights (Sylvania Gro-Lux) with a photosynthetically active radiation (PAR) of 25 µmol
 257 photons.m⁻².s⁻¹. Microbial growth was monitored every week by binocular magnifier observations to
 258 isolate every strain time after time.

2.3.2. Identification of microbial strains

261 Strains were isolated from a morphological description. Bacterial DNA extraction was performed using
 262 Bacterial Genomic DNA kit from GenElute, fungi and yeast DNA was extracted with Plant/fungi DNA
 263 isolation kit from Norgen Biotek corp. and eukaryotic phototroph DNA with DNeasy PowerBiofilm from

264 Qiagen. DNA concentration and quality were determined by NanoDrop One UV-visible
265 spectrophotometer (ThermoFisher).
266 Prokaryotic 16S and eucaryotic 18S rRNA genes were amplified by polymerase chain reaction (PCR),
267 using specific primers (Eurogentec - Belgium): 8F (5'- AGAGTTTGATCCTGGCTCAG-3') and 1391R (5'-
268 GACGGGCGGTGTGTRCA-3') for bacteria, NS1F (5'-GTAGTCATATGCTTGTCTC-3') and NS8R (5'-
269 TCCGCAGGTTACCTACGGA-3') for fungi and yeast, *rbcL* primers for algae and plants with S1F (5'-
270 ATGTCACCACAAACAGAGACTAAAGC-3') and S1R (5'-GAAACGGTCTCTCCAACGCAT-3') . PCR reactions
271 were carried out in 200 µl PCR tubes with a reaction volume of 50 µl: 41.25 µl of sterile ultra-pure H₂O
272 (Millipore Milli-Q Water System), 5 µl of PCR buffer (Promega, France), 1 µl of dNTP (Promega, France),
273 1 µl forward primer, 1 µl reverse primer, 0.25 µl Go taq Polymerase (Promega, France) and 0.5 µl
274 template DNA.

275
276 For the qualitative analysis, the amplified PCR products were checked by migration of a 5 µl of the PCR
277 product on 1 % agarose gel electrophoresis (0.5 M TAE Buffer) before commercial Sanger sequencing
278 (Genoscreen, France). The nucleotide sequences were aligned with the closest relative sequences of
279 representatives in the GenBank database by BLASTN (Basic Local Alignment Search Tool), an algorithm
280 using the CLUSTALW program (Thompson et al., 2002), available at NCBI (National Center for
281 Biotechnology Information). Sequences were then deposited in the NCBI Genbank database under
282 accession numbers listed in tables S5-S6.

283
284 *2.3.3. Molecular analysis of the microbial community diversity in biofilm samples*
285 The total microbial DNA was extracted with DNeasy PowerBiofilm (Qiagen) from 4 samples for each
286 non-coloured and coloured area on the bas-reliefs. The DNA quality was determined by NanoDrop One
287 UV-visible spectrophotometer (ThermoFisher) and 3 samples per area were sent to Integrated
288 Microbiome Resource (IMR) at Dalhousie University in Halifax (Canada) for ILLUMINA MiSeq-based
289 high-throughput sequencing, with primer pairs B969F (5' ACGCGHNRAACCTTACC 3') and BA1406R (5'
290 ACG GGC RGT GWG TRC AA 3') for bacteria (Comeau 2011), and E572F (5' CYG CGG TAA TTC CAG CTC
291 3') and E1009R (5'AYG GTA TCT RAT CRT CTT YG 3') for eukaryota (Comeau et al., 2011). Sequencing
292 data were processed using the Mothur software (Schloss et al., 2009). Operational taxonomic units
293 (OTUs) were defined at 3% dissimilarity threshold using the optclust algorithm (Westcott and Schloss,
294 2017), abundance tables and Alpha diversity indices were produced by Mothur at the phylum level and
295 at the genus level. Corresponding data were plotted with the software R (see below) and Excel. Non-
296 metric multidimensional scaling (NMDS) and analysis of molecular variance (AMOVA) on the Bray-
297 Curtis inter-sample distance matrix were performed using Mothur.

298 The unprocessed sequencing read dataset has been deposited in the NCBI Sequence Read Archive
299 (SRA) and is freely available under accession number PRJNA1045856
300 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA1045856>).

301
302 *2.3.4. Quantitative PCR*
303 Fungal 18S and bacterial 16S rRNA gene copy numbers were determined by quantitative PCR (qPCR)
304 amplification of biofilm extracted DNA from three or four samples per area, using the primers FR1 (5'
305 AIC CAT TCA ATC GGT AIT 3') and FF390 (5' CGA TAA CGA ACG AGA CCT 3'), and 341F (5' CCT ACG GGA
306 GGC AGC AG 3') and 515R (5' ATT ACC GCG GCT GCT GGC A 3') respectively (Lienhard et al., 2013).
307 Genomic DNA of a cultivated fungal strain and a cultivated bacterial strain were amplified by PCR using
308 the previous primers and diluted to serve as a standard range from 10³ to 10⁸ copies/µl. qPCR runs
309 were performed on a Bio-rad CFX thermal cycler using ABsolute Blue QPCR Mix (ThermoFisher) and
310 bacteriophage T4 gene 32 protein (New England Biolabs) as anti-inhibitor. After 15 min at 95 °C for
311 enzyme activation, 40 cycles comprised 10 s at 95°C for denaturation, 45 s at 60°C for annealing and
312 extension. The melt curve was performed from 65°C to 95°C, for 1 s at each 0.5°C. Raw results were
313 processed using the software CFX Manager 3.1 (Bio-rad) before statistical analysis.

314
315 **2.4. Statistical analysis**

316 Multivariate statistical analyses were carried out with R.4.2.1 software (R Core Team, 2023) for
317 graphics were produced with ggplot2 package (Wickham, 2009). For qPCR, XLSTAT was used for
318 ANOVA test (checking the normality of the data distribution) then Fisher test (LSD).

319

320 **3. Results**

321

322 **3.1. Colour measurements**

323 Several different areas have been defined and characterized according to their colour (Table S3). On
324 Bacchus, NCB was a clear yellowish stone area with high L^* of 83.4 and b^* of 12.2. Data were compared
325 to dry and water saturated chalk colour and displayed that NCB L^* was between L^* of dry and saturated
326 chalk and a^* and b^* were similar to the same parameters of the saturated chalk. Consequently, NCB
327 was close to a saturated chalk. (RB was characterized by a red-orange colour of RB (a^* of 25.3 and b^*
328 of 32.4). The green and black areas (GB and BB) had the same b^* with respectively 19.9 and 19.1 that
329 displayed a yellow part in both biofilm areas. The parameter a^* was negative for GB (-4.2) and for BB
330 (-5.8) which revealed a greening for both and more intense for BB. Moreover, L^* was darker for BB
331 with 44.3 than GB with 57.9. Therefore, colour data showed that BB was rather dark green.

332 Silene bas-relief had a non-coloured area whom the colour was characterized by L^* of 70.8 which was
333 darker than the dry and water saturated chalk. Moreover, b^* was at 16.0 which is more yellow than
334 NCB and the saturated chalk. The darkening and yellowing of NCS were probably not associated to the
335 water content in the stone but to a colonization which had not been detected to the naked eye. The
336 green area (GS) had a L^* of 31.2, a^* of -7.4 and b^* of 11.4; all parameters are lower than GB and BB,
337 especially much darker and greener. The brown area (BS) was characterized by L^* of 44.9 which was
338 similar to RB and BB, a^* of 7.7 and b^* of 22.9 that corroborated the brown colour with much less red
339 colour than RB.

340 Accordingly, the colour analysis could specify that GB was characterized by a low L^* and a^* thus green
341 and dark colour which intensified in BB. RB had a low L^* , a high a^* and b^* so it was a dark red-orange
342 colour. NCB was characterized by a high L^* thus a clear white colour probably associated to the water
343 content in the stone whereas NCS on Silene had a weaker L^* showing a darker stone than a water
344 saturated stone. BS was characterized by a weak L^* and a high b^* so a dark yellow colour and GS had
345 the weakest L^* and a^* thus it was a dark green area, darker than GB and BB.

346

347 **3.2. pH measurements**

348 On Bacchus bas-relief, pH of NCB was 8.6 thus basic (Table S3). BB and GB had respectively a pH of 8.3
349 and 7.5, therefore the pH remained basic but was lower than for NCB. On Silene bas-relief, NCS pH was
350 neutral (7.1) and lower than NCB and GS had a pH at 7.4 and BS at 6.8. Consequently, areas on Silene
351 had neutral to slightly acid pH whereas on Bacchus, areas were basic. Nonetheless, green area pH on
352 both bas-reliefs were similar.

353

354 **3.3. Liposoluble pigment concentrations**

355 The characterization of Bacchus areas showed that chlorophyll *a* concentrations (chl.a) increased
356 progressively between NCB, GB and BB with 0.77, 1.79 and 4.16 mg.m⁻² while RB chl.a was much lower
357 at 0.41 mg.m⁻² (Table S4). NCB chl.b was 1.05 mg.m⁻² surprisingly higher than GB which chl.b was 0.70
358 mg.m⁻² whereas GB was greener than NCB. BB chl.b was the highest with 3.52 mg.m⁻². RB chl.b was
359 also low at 0.36 mg.m⁻². Carotenoid concentrations displayed the same trend as chl.a with an increase
360 between NCB, GB and BB (0.12, 0.99 and 5.49 mg.m⁻²). RB carotenoids were high with 4.55 mg.m⁻².

361 Accordingly, RB was characterized by a high concentration of carotenoids while chl. a and b were low.
362 NCB area which is visually non-coloured, had low concentrations of the three pigments. Contrarily, all
363 pigments in BB had high concentrations and GB had intermediate values between NCB and BB.

364

365 On Silene bas-relief, NCS displayed weak contents of pigments with respectively 0.83, 0.64 and 0.15
366 mg.m⁻² for chl.a, chl.b and carotenoids. GS was characterized by the highest chl.a concentration with
367 16.55 mg.m⁻², a high chl.b which was at 6.77 mg.m⁻² and carotenoids at 2.01 mg.m⁻². Finally, BS had

368 4.37 mg.m⁻² lower than chl.a GS but similar to BB, chl.b was at 2.30 mg.m⁻² and carotenoids were
369 surprisingly low with 1.02 mg.m⁻² that suggested the brown colour of this area did not result from
370 those pigments.

371

372 **3.4. Photosynthetic activity**

373 On Bacchus, the relative quantum yield of the photosystem II varied from 0.30 for NCB and 0.31 for
374 GB to 0.44 on RB (Table S4). On Silene, NCS GB ϕ_{PSII} was 0.39 so higher than NCB which had the same
375 visual aspect whereas GS ϕ_{PSII} was higher with 0.43. On BS with brown colour, the chl. fluorescence
376 was 0.35.

377

378 **3.5. Phycobiliprotein concentrations**

379 On Bacchus, phycobiliprotein (PBP) concentrations varied in function of the areas, RB had the highest
380 values for the three measured PBP with a maximum of 7.32 mg.m⁻² for APC while NCB had low contents
381 for all PBP (Table S4). On Silene, the green area (GS) had the highest concentrations (5.52 mg.m⁻²)
382 though lower than RB on Bacchus. In addition, in both sites and all areas, allophycocyanin (APC) were
383 higher than PC or PE concentrations.

384

385 **3.6. Microbial diversity identified by high-throughput sequencing**

386

387 *3.6.1. Bacterial diversity*

388 The number of 16S rRNA raw sequencing reads for the 22 samples was 1,457,140 but two samples had
389 to be deleted due to poor quality (one replicate of NCS and one replicate of RB). After all quality control
390 steps, the number of high-quality 16S rRNA reads was 1,233,252 in total and was between 12,197 (RB4)
391 and 127,453 (BS4) per sample. At the threshold of 3% of dissimilarity, these reads were clustered into
392 10,621 OTUs. Seventeen OTUs comprised more than 1% of the total reads each, the largest OTU
393 comprising 46,489 sequences, i.e. 3.8 % of the total reads. Contrarily, 58 % of the OTUs comprised only
394 one read. After sub-sampling randomly each sample down to 12,197 reads, 4,360 OTUs were present.
395 Fourteen OTUs comprised more than 1 % of the total reads each, the largest OTU comprising 9,844
396 sequences, i.e. 4.0 % of the total reads. Contrarily, 48 % of the OTUs comprised only one read.

397

398 The rarefaction curves (Fig. S5) did not reach a plateau suggesting that 16S sequencing depth did not
399 enable to saturate the microbial diversity in the samples.

400 The observed community richness (number of OTUs per sample, S_{obs}) ranged from 239 to 1,093
401 (Table 1). S_{obs} was lower for GS samples and higher for BB samples. As a whole, Silene samples had
402 lower S_{obs} than Bacchus samples, which was also the case for the Shannon diversity index.
403 Furthermore, Good's coverage index values were lower for Bacchus samples than for Silene samples,
404 suggesting that the Bacchus samples have a total diversity even higher than observed. The Chao1
405 richness estimator values suggested that for every sample, the total richness was 1.4-2.6 times as high
406 as the observed richness (Chao1 divided by S_{obs}).

407 In Bacchus, BB had a higher richness and diversity than the other areas; on the opposite, RB had a
408 poorer richness and diversity. In Silene, BS had a higher richness and diversity compared to NCS and
409 GS.

410 The non-metric multidimensional scaling (NMDS) plot based on the OTU distribution table (Fig. S6a)
411 indicated a clear difference between Bacchus and Silene biofilms, and within each bas-relief a clear
412 grouping of the replicates of all areas defined for the Bacchus bas-relief though only for the NCS for
413 the Silene bas-relief. AMOVA performed on the OTU distribution table confirmed the significant
414 difference between the overall Silene and Bacchus bacterial diversities (Bonferroni p-value < 0.001),
415 that was observed on the NMDS plot. AMOVA revealed significant differences between the 4 types of
416 biofilm defined on the Bacchus bas-relief (p < 0.001) as well as between the 3 types of biofilm defined
417 on the Silene bas-relief (p = 0.028). More specifically, among all possible pairs, AMOVA revealed
418 significant differences between Non-Coloured Bacchus and either Black Bacchus (p = 0.031), Green
419 Bacchus (p = 0.024), Brown Silene (p = 0.024) or Green Silene (p = 0.029).

Bas-relief	Samples	S _{obs}	Chao 1	Shannon	Coverage
	NCB1	591	892	4.56	0.981
	NCB2	611	1145	4.46	0.978
	NCB3	552	905	4.63	0.983
	NCB4	494	749	4.41	0.985
Bacchus	BB1	803	1388	4.92	0.972
	BB2	743	1267	4.51	0.974
	BB4	1093	2460	4.87	0.952
	RB3	426	631	4.00	0.988
	RB4	412	580	3.80	0.988
	GB1	545	854	3.94	0.981
	GB3	591	1085	4.04	0.977
	GB4	706	1855	4.28	0.968
Silene	NCS1	338	486	3.83	0.991
	NCS2	396	798	3.95	0.986
	BS1	369	683	4.07	0.987
	BS2	451	1069	4.18	0.980
	BS4	311	550	3.78	0.990
	GS1	372	620	4.32	0.989
	GS3	239	440	3.70	0.993
	GS4	321	483	3.88	0.991

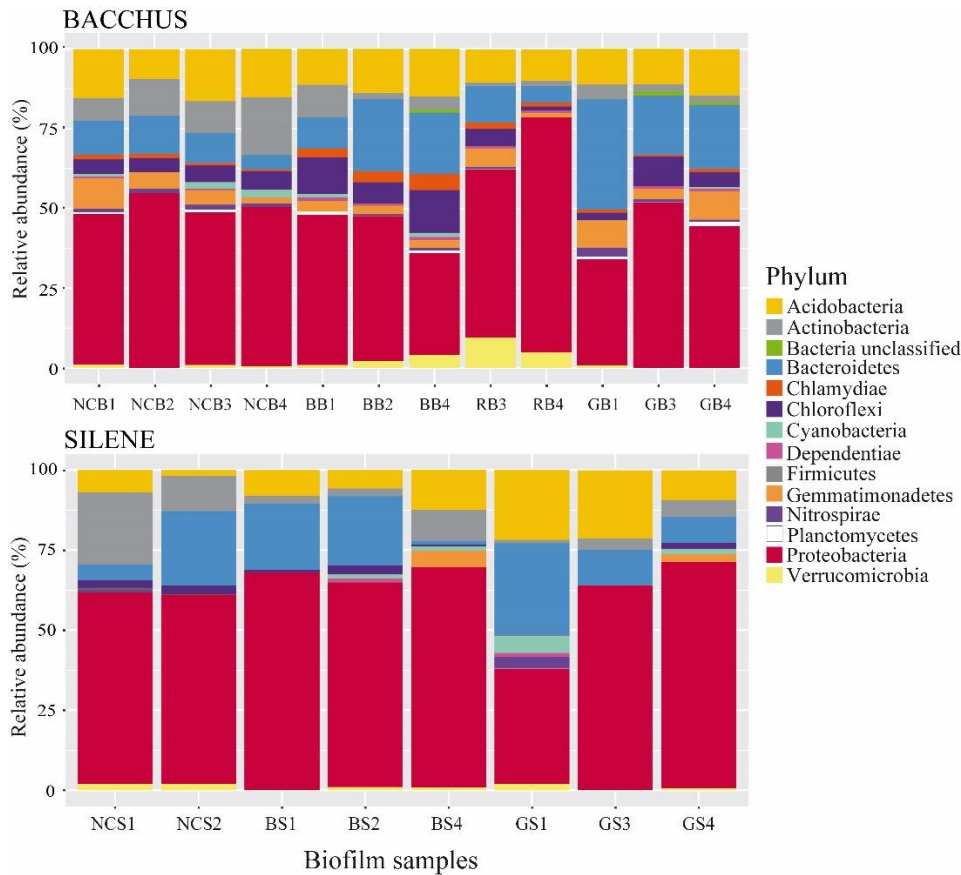
420

421 **Table 1.** Alpha diversity indices of samples from bas-relief areas, calculated from the bacterial OTU abundance table. S_{obs} is
422 the observed richness, Chao1 is the Chao1 richness estimator, Shannon is the Shannon diversity index, Coverage is Good's
423 coverage index.

424

425 After taxonomic affiliation of the OTUs, only phyla representing more than 0.5 % of the total sequence
426 reads for each area were further considered (Fig. 3). As a result, 14 main phyla of *Bacteria* dominated
427 the microbial communities (8 phyla were under 0.5 %). The major phylum was *Pseudomonadota*, it
428 was represented by a relative abundance between 31 % for BB4 and 73 % for RB4 in Bacchus and
429 between 36 % for GS1 and 70 % for GS4 in Silene. In both bas-reliefs, the other main phyla were
430 Bacteroidota with a maximum abundance (35 % for GB1), Acidobacteriota (22 % for GS1),
431 Actinomycetota (23 % for NCS1), Chloroflexota (14 % for BB4), Gemmatimonadetes, Chlamydiota,
432 Planctomycetota, Cyanobacteria, Dependenteae, Bacillota, Nitrospirota, Verrucomicrobiota, and
433 Latescibacteria (Oren and Garrity, 2021).

434 Not all OTUs could be classified at the genus level. On Bacchus, NCB samples had a homogeneous
435 diversity in genera (Fig. S7) with a majority of OTUs classified as *Hyphomicrobium*, *Dongia*,
436 *Pseudonocardia* but also unclassified *Gemmatimonadaceae* and subgroup 6 (*Acidobacteriota*). BB
437 samples had a majority of OTUs classified as unclassified *Microscillaceae*, *SWB02* (*Hyphomonadaceae*),
438 *Bryobacter* or *Dongia* but also *Gemmatimonadaceae*, *Ardenticatenaceae*. RB samples had a majority
439 of OTUs classified as *Hirschia* but also unclassified *Blastocatellaceae*, *Gemmatimonadaceae* and, for
440 RB4, Xanthomonadales. Although three GB samples were clustered on the NMDS plot, the
441 predominant groups were not the same for them, with a majority of OTUs classified as
442 *Chitinophagaceae* for GB1, *Dongia* and *Microscillaceae* for GB3 and *Hyphomonadaceae* for GB4.



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Fig. 3. Bacterial diversity at phylum level in Bacchus and Silene areas determined by Illumina-based high-throughput sequencing. Only the phyla representing more than 0.5 % of total sequences were considered.

448 On Silene areas, NCS samples displayed the same major OTUs with unclassified *Rhizobiaceae*,
449 *Hyphomicrobium*, *Pseudonocardia*. Then BS and GS displayed a heterogeneity in the genera diversity,
450 BS samples had not clear predominant groups, BS1 had *Pseudomonobacter*, *Bryobacter*, unclassified
451 *Rhizobiaceae*, BS2 rather had unclassified *Rhizobiaceae* and *Microscillaceae*, BS4 had *Hyphomicrobium*,
452 unclassified *Hyphomonadaceae* and *Blastocatellia*. Finally, GS showed 3 samples without main OTUs,
453 GS1 had *Bryobacter* and *Stenotrophobacter*, GS3 had unclassified *Blastocatellaceae* and
454 *Shingomonadaceae*, GS4 had unclassified *Microscillaceae* and *Gemmatimonadaceae*.

455 Cyanobacteria displayed 7 genera. On Bacchus, *Chalicogloea* was the genus in all GB samples,
456 representing 0.1 to 1.3 % of bacteria. They were comprised between 0.2 and 1.0 % in BB samples and
457 0.7 % in NCB1. Oxyphotobacteria_ofg were the vast majority of Cyanobacteria in NCB3 (1.8 %) and
458 NCB4 (2.3 %), they were also present though less abundant lower in RB samples 0.02 % in RB4, RB3,
459 NCB1 and GB4. RB3 sample was also characterized by *Obscuribacterales_fg* (0.02 %) and RB4 by
460 *Chloroplast* (0.03 %). *Leptolyngbya* were only present in all BB samples, representing 0.05 to 0.2 % of
461 bacteria. On Silene, *Chloroplast* were the only Cyanobacteria genus in NCS2 (0.02 %) and were in
462 majority in NCS1. BS samples had Oxyphotobacteria_ofg as the main Cyanobacteria genus (with 1.0
463 and 1.2 %) except in BS1 which was different with *Chloroplast* (0.06 %) and *Leptolyngbya* (0.1 %). Finally
464 in GS samples, *Leptolyngbya* was the main genus in GS1 (5.3 %) whereas *Chalicogloea* were in majority
465 in GS3 0.3 %) and GS4 (0.4 %).

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3.6.2. Eukaryotic diversity

468 The 18S rRNA gene sequence dataset was of poor quality. The number of raw sequencing reads for the
469 22 samples was 34,690 but 8 samples had to be deleted due to an excessively low number of high-
470 quality sequences (1 replicate of each of BB-NCB-NCS, two replicates of GB and all three replicates of
471 RB). After all quality control steps, the number of high-quality 18S rRNA reads was 26,842 in total and

472 was between 154 and 6,361 per sample. Sequences affiliated to animal phyla (Annelida, Arthropoda,
473 Rotifera), representing 16 % of the total sequence set, were removed for further analysis, resulting in
474 a total number of sequence reads for eukaryotic phototrophs and fungi of 22,259, and between 112
475 and 6,305 per sample. At the threshold of 3 % of dissimilarity, these reads were clustered into 693
476 OTUs. Twelve OTUs comprised more than 1 % of the total reads each, the largest OTU comprising 8,893
477 sequences, i.e. 40 % of the total reads. Contrarily, 78 % of the OTUs comprised only one read. Contrary
478 to the 16S, the 18S data set was subsampled only for the calculation of the diversity indices and not
479 for the NMDS and AMOVA in order not to obtain an excessively low number of reads for all samples.
480

481 The NMDS plot based on the OTU distribution table (Fig. S6b) indicated a difference between Bacchus
482 and Silene biofilms, but within each bas-relief, no clear grouping of samples from the defined zones.
483 AMOVA performed on the OTU distribution table confirmed the significant difference between the
484 overall Silene and Bacchus fungal and phototrophic diversities (Bonferroni p-value = 0.007), that was
485 observed on the NMDS plot. AMOVA revealed significant differences between the 3 types of biofilm
486 defined on the Bacchus bas-relief ($p < 0.047$) but no significant differences between the 3 types of
487 biofilm defined on the Silene bas-relief.
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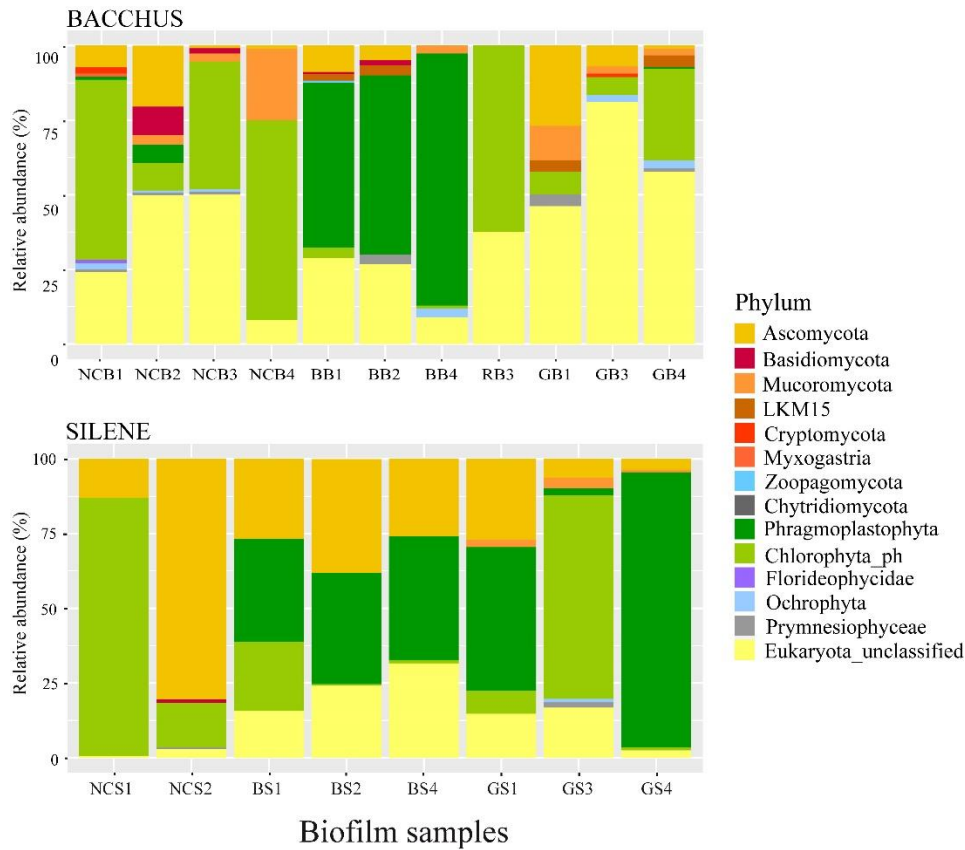
489 On Bacchus, Chlorophyta were the major phylum for the NCB1 and NCB4 samples with respectively
490 60 % and 67 % (Fig. 4) whereas they accounted only for 9 % and 43 % of reads for NCB2 and NCB3,
491 respectively (50 % of reads being unclassified at the phylum level).

492 Muromycota represented 24 % of reads in NCB4, Ascomycota and Basidiomycota represented 20 %
493 and 10 % of reads in NCB2, respectively. BB seemed the most homogeneous area, whose samples were
494 characterised by Phragmoplastophyta as the major phylum (57 % in BB1, 64 % in BB2 and 87 % in BB4),
495 then by unclassified Eukaryota (9 to 29 %), Ascomycota (9 % in BB1), Mucoromycota (3 % in BB4) and
496 Basidiomycota. GB samples displayed a majority of reads unclassified at the phylum level (46, 81 and
497 58 % in GB1, GB3 and GB4, respectively). Then Chlorophyta ph. represented 31 % of reads in GB4 and
498 Ascomycota 27 % in GB1. Finally, RB3 had a majority of Chlorophyta (62 %) and reads unclassified at
499 the phylum level (37 %).

500 On Silene, NCS1 and NCS2 were different, NCS1 had 86 % of Chlorophyta and 13 % of Ascomycota
501 whereas NCS2 had 81 % of Ascomycota and only 15 % of Chlorophyta. BS samples seemed
502 homogeneous, dominated by Phragmoplastophyta (between 34 and 42 % of reads), Ascomycota
503 (between 26 and 38 % of reads), 15 to 32 % of reads being unclassified at the phylum level. Chlorophyta
504 represented 23 % of reads in BS1, compared to 1 % in BS2 and BS4. GS samples showed a high
505 abundance of Phragmoplastophyta (92 % in GS4 and 48 % in GS1) whereas Chlorophyta dominated in
506 GS3 (68 %). Ascomycota were also present at 27 % in GS1.

507 More details were disclosed at a genus level (Fig. S8). *Chromochloris* (Chlorophyta) were dominant in
508 all NCB samples (between 36 and 49 %) except in NCB2 (2%), *Stichococcus* representing between 1 and
509 16 %. In NCB4, *Mortierella* represented 24 % of reads. *Chromochloris* were also in majority in RB3
510 (85 %). In BB samples, reads of *Embryophyta* unclassified at the genus level were dominant (between
511 49 and 85 %). In GB samples, *Chromochloris* represented 25% of reads in GB4, though a majority of
512 reads were unclassified at the genus level. In GB1, *Calcarisporiella* and *Mortierella* represented
513 respectively 17 and 13 %.

514 On Silene, NCS samples differed from one another, with a majority of *Stichococcus* (83 %) in NCS1 and
515 *Plectosphaerella* (52 %) in NCS2. BS samples were dominated by *Embryophyta* reads unclassified at the
516 genus level (29 to 37 %) but *Eukaryota* reads unclassified at the phylum level were abundant in BS4.
517 Reads of *Hypocreales* unclassified at the genus level represented at 21 and 27 % for BS1 and BS2.
518 *Stichococcus* represented 23 % of reads in BS1. Finally, GS1 and GS4 were dominated by unclassified
519 Embryophyta (48 and 92 %) whereas GS3 was dominated by *Stichococcus* (63 %).
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Fig. 4. Eukaryotic diversity at phylum level in Bacchus and Silene areas determined by Illumina based high-throughput sequencing. Only the phyla representing more than 0.5% of total sequences were considered.

3.7. Quantitative PCR

qPCR was carried out on 4 samples for each area except BB where only DNA could be extracted from only 2 samples and finally one sample gave results. In consequence, BB was excluded from the statistical analysis though its sole sample had the highest 16S and 18S rRNA gene copy numbers. Other samples had 16S and 18S gene copy numbers of $2.8\text{-}5.4 \times 10^5$ cp/ng and $1.3\text{-}2.1 \times 10^5$ cp/ng, respectively (Table 2). The 16S/18S ratio was remarkably similar for all areas with 2.6 (2.5 for GS).

Area	16S rRNA	18S rRNA	Ratio 16S/18S
NCB	$4.5 \cdot 10^5 \pm 2.2 \cdot 10^4$ ^{ab}	$1.7 \cdot 10^5 \pm 8.2 \cdot 10^3$ ^{ab}	$2.6 \pm 6.5 \cdot 10^{-3}$ ^a
GB	$3.6 \cdot 10^5 \pm 8.1 \cdot 10^4$ ^{ab}	$1.4 \cdot 10^5 \pm 3.0 \cdot 10^4$ ^{ab}	$2.6 \pm 3.0 \cdot 10^{-2}$ ^{ab}
BB	$6.0 \cdot 10^5$	$2.3 \cdot 10^5$	2.6
RB	$4.5 \cdot 10^5 \pm 1.4 \cdot 10^4$ ^{ab}	$1.7 \cdot 10^5 \pm 5.3 \cdot 10^4$ ^{ab}	$2.6 \pm 5.2 \cdot 10^{-2}$ ^a
NCS	$5.4 \cdot 10^5 \pm 1.5 \cdot 10^4$ ^a	$2.1 \cdot 10^5 \pm 5.5 \cdot 10^4$ ^a	$2.6 \pm 3.7 \cdot 10^{-3}$ ^a
GS	$2.8 \cdot 10^5 \pm 1.6 \cdot 10^4$ ^b	$1.3 \cdot 10^5 \pm 7.3 \cdot 10^4$ ^b	$2.5 \pm 8.2 \cdot 10^{-2}$ ^b
BS	$5.0 \cdot 10^5 \pm 9.6 \cdot 10^4$ ^a	$1.9 \cdot 10^5 \pm 3.5 \cdot 10^4$ ^a	$2.6 \pm 2.4 \cdot 10^{-2}$ ^a

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Table 2. qPCR data with 16S rRNA and 18S rRNA (cp/ng DNAG) and the ratio 16S/18S. ^a, ^b correspond to statistically no-significant differences (Fisher's test, $p < 0.05$).

3.8. Identification of isolated microbial strains

The culture-based approach led to the isolation of 24 distinct bacterial strains whose partial 16S rRNA gene sequences were deposited into the NCBI Genbank database (Tables S5-S6). Thirteen and eleven strains were isolated and identified respectively from Bacchus and Silene. On both bas-reliefs, most strains belonged to the phylum Pseudomonadota with 9 bacterial strains in Bacchus and 7 in Silene. In

542 Bacchus, the area with the highest number of isolated bacterial strains was NCB with 5 strains
543 comprising 3 Pseudomonadota affiliated to Alpha-Pseudomonadota (*Sphingopyxis macrogoltabida*,
544 *Afipia massiliensis*, *Rhodospirillum sp.*) and 2 Actinomycetota (*Streptomyces cirratus* and *Saccharothrix*
545 *sp.*). In RB, 3 Pseudomonadota were identified, one belonging to Alpha-Pseudomonadota and the
546 genus *Mesorhizobium sp.* and two belonging to Gamma-Pseudomonadota and the species
547 *Pseudomonas baetica* and *Acinetobacter radioresistens*. Four strains were identified in GB with 2
548 Alpha-Pseudomonadota (*Mesorhizobium sp.* and *Sphingopyxis sp.*), 1 Bacteroidota (*Pedobacter sp.*)
549 and 1 Cyanobacteria (*Timaviella Dunensis*). Finally, only 1 Gamma-Pseudomonadota affiliated to
550 *Lysobacter sp.* was isolated from BB. In Silene, BS was the area with the highest number of isolated
551 bacterial strains (7), comprising 5 Pseudomonadota including 4 Alpha-Pseudomonadota
552 (*Mesorhizobium sp.*, *Sphingopyxis alaskensis*, *Phyllobacterium sp.*, *Phyllobacterium trifolii* and 1
553 Gamma-Pseudomonadota (*Pseudomonas sp.*), 1 Actinomycetota (*Arthrobacter sp.*) and 1 Bacillota
554 (*Paenibacillus sp.*). Then only 2 bacterial strains were sequenced in NCS with 1 Alpha-Pseudomonadota
555 (*Aminobacter sp.*) and 1 Bacteroidota (*Dyadobacter sp.*) and in GS, 1 Alpha-Pseudomonadota
556 (*Sphingopyxis sp.*) and 1 Bacteroidota (*Dyadobacter sp.*).
557 Twenty-five eukaryotic strains were isolated, including 15 fungal strains identified as Ascomycota with
558 *Penicillium sp.* as main species present in every area and 10 phototrophs with Chlorophyta microalgae
559 in most areas. In Bacchus, NCB had the highest number of fungi with 5 species all belonging to
560 Ascomycota (*Aspergillus terreus*, *Cladosporium sp.*, *Verticillium leptobactrum*, *Penicillium sp.*). The
561 other areas had only 2 isolated strains like in GB with 1 Ascomycota (*Penicillium sp.*), 1 Basidiomycota
562 (*Rhodotorula mucilaginosa*), in BB with 1 Ascomycota (*Penicillium limosum*), 1 Mucoromyceta
563 (*Mortierella sp.*). Finally, in RB, only 1 strain classified as Ascomycota (*Penicillium brevicompactum*)
564 was present. Eukaryotic phototrophs isolated in GB were 3 algae with *Diplosphaera sp.*, *Bracteacoccus*
565 *sp.* and *Chromochloris zofingiensis*. In NCB, only *Diplosphaera sp.* was identified whereas in RB, only
566 *Chromochloris zofingiensis* was sequenced. In Silene, from BS 3 fungi were identified with 2
567 Ascomycota (*Penicillium sp.*, *Penicillium roqueforti*) and 1 Basidiomycota (*Sporidiobolus pararoseus*).
568 In NCS, 1 Ascomycota as *Penicillium sp.* was also sequenced and 1 Basidiomycota (*Sporobolomyces*
569 *ellipsoideus*). In GS, only 1 Ascomycota (*Penicillium sp.*) was also isolated. Finally, Chlorophyta
570 microalgae with *Diplosphaera sp.* were identified in every area, then *Pseudostichococcus*
571 *monallantoides* were sequenced in GS. The Bryophyta with *Eucladium verticillatum* was identified in
572 BS.

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574 3.9. Microscopic observations of phototrophs in biofilms

575 On Bacchus, in the NCB, a rod-like green morphotype similar to *Diplosphaera sp.* (Fig. S9), a green-blue
576 filamentous morphotype similar to *Timaviella dunensis* and blue-green coccoid morphotype with 4 or
577 2 cells surrounded by a colourless mucilage which could associated to Cyanobacteria (Barberousse et
578 al., 2006; Nugari et al., 2009) were observed. In GB, two green coccoid morphotypes similar to
579 *Chromochloris zofingiensis* and bigger cells similar to *Bracteacoccus sp.* were observed, in addition to
580 a green-blue coccoid morphotype with 4 or 2 cells in a colourless mucilage previously observed in NCB.
581 In BB, the green-blue coccoid morphotype in NCB and GB also dominated but the cells were
582 surrounded by an orange mucilage and the filamentous morphotype similar to *Timaviella dunensis* was
583 secondarily observed. A coccoid green and yellow orange morphotype similar to *Chromochloris*
584 *zofingiensis* algae and thick green filamentous morphotype were also described in this biofilm. RB was
585 mainly composed of coccoid big red morphotype algae similar to *Chromochloris zofingiensis* and
586 secondarily a green-blue tetra-shape morphotype as Cyanobacteria.

587 On Silene, a rod-like green morphotype similar to *Diplosphaera sp.* and a coccoid green morphotype
588 were mostly observed in NCS. In GS, both previous morphotypes were observed together with a
589 filamentous blue-green morphotype similar to *Timaviella dunensis*. Moreover, a new thick filamentous
590 green morphotype, was noticed but not similar to any isolated strain. Finally, in BS, cells similar to
591 *Diplosphaera sp.* and *Timaviella dunensis* were also observed, the green-blue coccoid morphotype with
592 4 or 2 cells surrounded by brownish mucilage and thick brown plant tissue-like filaments were very
593 frequent and only observed in this area.

594

595 **4. Discussion**

596 This work investigated the lampenflora grown in two monumental bas-reliefs carved in the Pommery
597 Champagne cellar, unique underground site as original chalk quarries transformed into a giant wine
598 cellar in the 19th century. This subterranean environment with low variation of temperature, high
599 relative humidity throughout the year and a water saturated stone, promotes the development of a
600 lampenflora when the illumination of the bas-reliefs was settled for tourist visits. A strategy to control
601 and prevent lampenflora needs to be found which includes a combination of several targets like the
602 change of the environment management and the test of treatments to avoid the lampenflora growth
603 for a longer time (Franco-Castillo et al., 2022). That firstly requires to characterise the different biofilm
604 areas composing each artwork in the aim to define a relationship between them, and between the two
605 artworks each located in the different pit. Measurements were carried out on first on the bas-reliefs
606 by colourimetry, chlorophyll *a* fluorescence, pH measurements and sampling. That was completed by
607 laboratory analyses of pigments, culture of microorganisms for genetic identification and high-
608 throughput sequencing of 16S and 18S rRNA to determine the microbial diversity of the biofilms.

609 The molecular analyses carried out on 16S RNA firstly revealed that Bacchus had a higher bacterial
610 richness than Silene. On Bacchus, GB and BB areas had the highest richness of OTUs while RB had the
611 lowest. On Silene, GS had a lower diversity than NCS and BS. Bray-Curtis NMDS showed that the
612 bacterial composition of the lampenflora of Bacchus was distinct from that of Silene. Moreover, it
613 clearly clustered the samples from the different defined areas, more specifically in Bacchus where each
614 area was characterized by a distinct bacterial diversity. Nonetheless, communities were mainly
615 composed of Pseudomonadota in both bas-reliefs, mainly Alpha-Pseudomonadota, then Bacteroidota,
616 Acidobacteriota and Actinomycetota. The dominance of those main phyla and especially
617 Pseudomonadota was reported in show caves with illumination and, also karst caves in the darkness.
618 Here proportions of Pseudomonadota were 45.5 % in Bacchus and 60 % in Silene, which was
619 comparable to the lampenflora in French and Swiss show caves in which this phylum also dominated
620 the bacterial community with 57.3 % (Pfundler et al., 2018b), and in Lehman caves in the USA with 35-
621 70 % (Burgoyne et al., 2021). In karst caves, it also dominated in vermiculations with 41-54 % in
622 Pertosa-Auletta Cave, southern Italy (Addesso et al., 2021).

623 Actinomycetota are often cited as a dominant phylum on par or with next to Pseudomonadota in show
624 caves (20.2 %, 10-30% and 7-17 % in the last cited studies) but in the Pommery Cellar, they represented
625 only 7 % for both bas-reliefs. Here, the ratio of Pseudomonadota to Actinomycetota was similar to
626 vermiculations observed in alpine cave with between 36 to 46 % of Pseudomonadota and 4 to 11 % of
627 Actinomycetota (Jurado et al., 2020). The dominance of Pseudomonadota in caves was already
628 reported in communities exposed to high levels of human contact whereas natural cave bacterial
629 community with low human contact was dominated by Firmicutes (Ikner et al., 2007). Here,
630 Pseudomonadota were mostly represented by Alpha-Pseudomonadota and Gamma-Pseudomonadota
631 among isolates, nonetheless the most abundant genera determined in the amplicon-sequencing were
632 different than isolated microorganisms.

633 Those phyla are commonly identified outdoor in soils and on stone surface of monuments (Mihajlovski
634 et al., 2017; Li et al., 2018; Romani et al., 2019), also in vermiculations thanks to the migration of
635 bacteria from soils (Lavoie et al., 2017). The Pommery cellar was dug in highly fractured chalk
636 promoting rainwater circulation and helping the migration of microorganisms from the upper surface.
637 Pseudomonadota, Actinomycetota, Acidobacteriota are commonly described in karst caves (Burgoyne
638 et al., 2021; Ai et al., 2022) and in catacombs often with a dominance of Pseudomonadota or
639 Actinomycetota (Saarela et al., 2004; Urzi et al., 2010).

640 The comparison of the taxonomic diversity showed that Bacchus had a higher abundance of secondary
641 phyla than Silene with Chlamydiota, Chloroflexota, Gemmatimonadota. In Bacchus, samples from each
642 area were composed of a similar community at the genus level except for GB in which the three
643 samples had distinct communities. NCB could be characterized by a high abundance of *Dongia* and
644 *Rhizobiales* (*Hyphomicrobium*, *Rhizobiales Incertae sedis*), BB by *Microscillaceae* unclassified and RB by
645 *Hirschia* which are bacteria from soil and water (Hahnke et al., 2016; Kim et al., 2016). In Silene, NCS

646 samples had a relative similar community with a dominance of *Rhizobiaceae* and *Hyphomicrobium*
647 (both from *Rhizobiales* order) as bacteria from soil origin. *Rhizobiaceae*, which were the most abundant
648 Pseudomonadota in the amplicon sequencing and were also sequenced in culture with *Mesorhizobium*
649 *sp.* in RB, GB and BS, are nitrogen-fixing species (Addesso et al., 2021) and reported as responsible to
650 the deterioration of tomb (Diaz-Herraiz et al., 2014), and *Hyphomicrobium g.* can correspond to iron
651 and manganese-oxidizing bacteria involved in Mn precipitation (Northup et al., 2003; Miller et al.,
652 2020).

653 Cyanobacteria, phototrophic bacteria whose metabolic activity produces carbonic acid and dissolves
654 limestone, were weakly abundant in every area of both bas-reliefs (up to 5.5 % in GS) which suggested
655 that the green colour of GB, BB and GS was weakly associated with them. This result was surprising
656 since Cyanobacteria are considered the pioneering inhabitants in the colonisation of caves (Czerwik-
657 Marcinkowska and Mrozińska, 2011) and the main taxa with 72 % of the phototrophic microbiota in
658 hypogean habitats (Popkova et al., 2019) and up to 100 % when no Chlorophyta were detected (S.
659 Pfendler et al., 2018b). Chroococcales and Nostocales represented here by *Chalicogloea cavernicola*
660 (up to 1.3 % in GB), could correspond to the observed coccoid blue green morphotype in NCB, GB and
661 BB. Those phyla were often recorded as main cyanobacteria with respectively for the both 40.7 % and
662 51.2 % (Pfendler et al., 2018b). *Leptolyngbya* was the genus mainly sequenced in GS but also present
663 in BB on Bacchus and in NCS and BS on Silene. This phylum was dominated cyanobacteria in hypogea
664 (Bruno et al., 2009). Here, it could be associated to *Timaviella Dunensis* isolated from BS. Those species
665 were recently described from sand dunes (Mikhailyuk et al., 2022), other species, *Timaviella* Sciuto &
666 Moro, as recent cryptic genus created from the separation of the morphologically close *Leptolyngbya*,
667 were from underground environment similar to Pommery cellar environment but species determined
668 by Sciuto & Moro corresponded to red-brownish morphotype (Sciuto et al., 2017).

669
670 The eukaryotic 18S rRNA sequencing displayed a weak content of OTUs which did not reflect the real
671 microbial richness of the areas specially for phototrophic microorganisms abundantly observed in
672 microscopy. Nonetheless, the relative abundance showed the net higher proportion of phototrophs
673 than organoheterotrophs. Moreover, it revealed the same results than in 16S rRNA analysis, that
674 meant distinct microbial communities in Bacchus and Silene. Bacchus displayed a weaker content of
675 fungi than Silene. Ascomycota were dominant especially in Silene with *Hypocreales* unclassified,
676 Chaetothyriales unclassified, *Plectosphaerella* and *Fusarium*; whereas in Bacchus, Ascomycota were
677 most represented by *Calcarisporiella* and Mucoromycota by *Mortierella*. They were the most
678 frequently sequenced phylum from cultured species with *Penicillium sp.* and were found in show caves
679 with 71.3 % (Pfendler et al., 2018b) and a cave church 73.8 % (Ljaljević Grbić et al., 2022) and in above-
680 ground heritage sites (Mascaro et al., 2021). Furthermore, despite a high proportion of eukaryote
681 unclassified in the sequencing of samples, each area of Bacchus (except GB) had a distinct eukaryotic
682 community with a dominance of phototrophs: NCB was characterized by *Chromochloris* (except NCB2),
683 BB by Embryophyta unclassified, RB by *Chromochloris*. Only GB samples displayed a heterogeneous
684 community in samples with the dominance of fungi (*Chitinophagaceae* unclassified - GB1) or
685 *Chromochloris* (GB4). In Silene, more dissimilarities in the community were showed within areas, NCS
686 was dominated by the algae *Stichococcus* or the fungus *Plectosphaerella*. Nonetheless, GS and BS were
687 dominated by Embryophyta unclassified (except GS3) like *Eucladium verticillatum* which were cultured
688 from BS.

689 Notably, *Chromochloris* algae were specially developed in Bacchus whereas *Stichococcus* specially grew
690 in Silene. The environmental conditions of both bas-reliefs were similar, comprising temperature,
691 relative humidity and the high content of water in chalk on which lampenflora developed, only the
692 lighting conditions varied over time which could explain the variation of the composition of the
693 microbial communities located in the two different pits of the same cellar. *Diplosphaera sp.* and
694 *Pseudostichococcus sp.* sequenced from isolated strains from both Bacchus and Silene, belong to
695 *Stichococcus* genus and are widely in many environments (freshwaters, marine and terrestrial
696 ecosystems and in harsh polar environments (Proeschold and Darienko, 2020; Chiva et al., 2022).

697 The genus *Chromochloris* was the main phototrophs in RB and belongs to the Chlorophyta or green
698 algae, although the taxonomy has been revisited several times (Fucikova and Lewis, 2012). Cells
699 showed, however, marked red pigmentation in this area. Strains have been cultivated from RB and
700 identified as *Chromochloris zofingiensis*, also showing red morphotype, whereas isolates from GB were
701 green. Moreover, *C. zofingiensis* cultivated in laboratory in Hoagland liquid medium always showed
702 green morphotype whereas the showed red-orange colour when grown on the surface of chalk stone
703 at the natural daylight for many months. This genus was often studied and selected for the abilities to
704 produce a high content of carotenoids for potential applications in pharmaceutical industries, and
705 more specifically secondary carotenoids as astaxanthin accumulated in lipid bodies outside the
706 chloroplast (Liu et al., 2014). The accumulation of astaxanthin was associated with stress conditions
707 (high light, nitrogen starvation, high salinity) to protect algal cells against oxidative damage (Liu et al.,
708 2014; Maltsev et al., 2021) and in a mixotrophic medium with glucose content (Azaman et al., 2017;
709 Sun et al., 2019), which were on the opposite of the natural and drastic cellar conditions. Nonetheless,
710 an accumulation of astaxanthin was already performed in laboratory under limited light condition and
711 nitrogen depletion (Mulders et al., 2015) and it was also associated to mature cells from cultures more
712 than 4 months old (Fucikova and Lewis, 2012).

713
714 The results of qPCR revealed that bacterial 16S rRNA gene copy numbers 2.6 times as high as 18S, with
715 remarkably constant ratio values, suggesting equal proportions of bacteria and fungi or other
716 eucaryotes in all samples. Fisher test noticed that there was no statistical difference of 16S rRNA and
717 18S rRNA of every area on Bacchus and only GS was significantly different from the others. However,
718 the number of genes encoding for 16S/18S rRNA can vary among the microorganisms (Větrovský and
719 Baldrian, 2013; Gong and Marchetti, 2019) and this variability may influence the diversity and
720 abundance of the species determined in the different samples. One limit of the qPCR approach we
721 followed is that it is, as such, unable to investigate differences in the extent of biological colonization.
722 To this end, biofilm sampling from precisely measured areas and recording of the collected mass,
723 followed by DNA extraction and dosing as well as qPCR analysis, would be necessary to estimate the
724 biomass per surface unit and will be included in future studies. Furthermore, all extracted DNA does
725 not necessarily correspond to living cells, extracellular DNA being present in the biofilms after cell
726 death.

727 The alkaline pH observed for all Bacchus areas was in agreement with the dominance of the limestone
728 in this area. The non-coloured NCB area was the most alkaline with pH = 8.6 whereas pH in the
729 coloured areas BB and RB was slightly lower and the lowest for GB, which suggested its microbial
730 community could decrease the alkalinity with the production of extracellular compounds (enzymes,
731 acids) by bacteria and fungi (Warscheid and Braams, 2000; Saarela et al., 2004; Stanaszek-Tomal,
732 2020). All Silene areas had a neutral pH. This result was probably due to distinct communities on each
733 artwork and to the dominance of bacteria most of which prefer an inert or slightly alkaline pH
734 (Stanaszek-Tomal, 2020).

735
736 Analysis of pigments in the Bacchus areas showed a low chl. *a* content for NCB, it was higher in GB and
737 much higher in BB which coincided with the progressive greening showing by a * of those areas. BB had
738 the highest values of chl. *a* and *b* pigments and carotenoids which could explain the dark green colour
739 measured in colourimetry. The high content of carotenoids in RB confirmed the high potential of
740 *Chromochloris* to provide this pigment probably when a light intensity was much higher in the Bacchus
741 pit and presently because of a low nutriment substrate and a growth over a very long period.
742 Moreover, those algae were probably not the only source of carotenoids since the 16S rRNA
743 sequencing indicated RB had 18.5 % of the carotenoid-producing Xanthomonadales reads (Chen et al.,
744 2009; Porca et al., 2012; Adesso et al., 2021) and 13 to 27 % of *Hirschia* reads (Bontemps et al., 2023).
745 Concentrations of carotenoids were low in NCB and GB, suggesting that the cells of *Chromochloris*, the
746 most abundant algal genus present in 18S rRNA dataset, were green and that the biofilm was at an
747 earlier development stage than in RB, given the expansion of this biofilm from the middle to the
748 periphery of the artwork and maybe because of a competition with other microorganisms. In addition,

749 the presence of Rhizobiales (21 and 31 % respectively in NCB and GB) provide nitrogen to algae leading
750 to limit stress conditions. RB area displayed a high content of the three phycobiliproteins which was
751 not in accordance with Cyanobacteria 16S rRNA analysis since the abundance was very weak. PBPs,
752 common pigments in Cyanobacteria, can also be produced by algae like Rhodophyta and Cryptophyta
753 (Maltsev et al., 2021) but such algae were not detected here. Consequently, 16S rRNA analysis could
754 under-estimated the abundance of Cyanobacteria in the lampenflora areas of Bacchus. Moreover, the
755 photosynthetic activity of RB measured through the effective quantum yield of the photosystem II
756 (Φ_{PSII}) from the chl. *a* pigment in Chlorophyta, was higher than in green areas (GB and BB) despite the
757 lowest chl. *a* concentration which could limit the photosynthetic activity but the high content of PBPs
758 in RB promoted the absorption of excitation energy by phycobilisomes and transferred to the
759 chlorophyll molecules in the PSII and ascertained the presence of Cyanobacteria (Grossman et al.,
760 1994).

761 In Silene, GS had a high PBP content, the Cyanobacteria abundance was the highest compared with
762 the other areas, but it remained low (5.5 %). Besides, this area displayed the highest concentrations of
763 chlorophyll pigments probably associated to Embryophyta and *Stichococcus*. The high PBP content in
764 RB and GS highlighted the ability of Cyanobacteria to adapt to various wavelengths of the
765 electromagnetic spectrum that chlorophyll pigments and carotenoids cannot absorb (Albertano et al.,
766 2003; Korbee et al., 2005; Muñoz-Fernández et al., 2021) and to an intensity which is currently weak
767 ($0.1 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) that suggested those species can survive at a photon flux lower than their
768 photosynthetic compensation point (Mulec and Kosi, 2009; D'Agostino et al., 2015).

769 The lack of information concerning the history of cellar outfitting (including lighting), bas-relief cleaning
770 and repairing does not allow to establish a precise chronology of biofilm development. The tourist
771 attractiveness of the cellar required to improve the lighting to secure the premises and to highlight the
772 monumental artworks. The frequent air flow and people from outside provide an easy seeding and
773 spreading of opportunistic microorganisms which coat mineral surfaces and deteriorate them over
774 time. Controlling the air flow seems complicated due to the activities of the wine cellar but the artificial
775 illumination specifically on monumental bas-reliefs could be more adapted to avoid the lampenflora
776 growth. Efforts have already been carried out to illuminate bas-reliefs with for a reduced time.
777 Cyanobacteria and algae are, however, able to survive and develop in low lighting conditions
778 (Baquedano Estévez et al., 2019) and the constant lighting of modern artworks laid on the ground,
779 though low, is sufficient for their growth. Moving the temporary artworks to other pits would deprive
780 the lampenflora of this growth energy. Finally, the design of a lighting including lamps with a spectral
781 light emission of less photosynthetic effectiveness, such as green light less absorbed by chlorophylls
782 and carotenoids, could also be beneficial (Sanmartín et al., 2017). On the opposite, red-orange and
783 blue lights are the most effective for the algal proliferation (Cigna and Forti, 2013), but cyanobacteria
784 are well-known to develop in humid and dimly lit environments and are able to adjust the content of
785 phycobiliproteins in response to a short shift on the light quality (Maltsev et al., 2021). Nonetheless,
786 the best results to limit the photosynthetic growth were achieved with a 'testing light' corresponding
787 to an emission between 420-470 nm and between 500-670 nm (Bruno and Valle, 2017).

788

789 5. Conclusion

790 Lampenflora growing on bas-reliefs is mainly exogenous and opportunistic. Contrary to our hypothesis,
791 both phototrophic and non-phototrophic microorganisms differ in diversity and abundance between
792 bas-reliefs and between areas defined within the bas-reliefs, in spite of a remarkably constant bacteria-
793 to-fungi ratio. Bacchus, with *Chromochloris* algae as the main phototrophs, had a higher microbial
794 diversity than Silene, on which Embryophyta and *Stichococcus* were dominant. Areas defined visually
795 from the biofilm colour matched a specific community in Bacchus, which was not the case for Silene.
796 The strategy to control the lampenflora growth on the bas-reliefs should raise a sustainable
797 preservation program including the preservation of bas-reliefs and the tourist visits by refine the
798 heritage management on two sides: an adaptation of the environment with a new tourist path in
799 moving the contemporary artworks in pits without bas-reliefs and by changing of the lighting system
800 by the selection of wavelengths less favourable for the photosynthetic growth. The second side

801 intervenes on the bas-reliefs by the application of a preventive chemical treatment to delay the
802 recolonization or the installation of UV-C lamps that would work at night in absence of visitors.

803

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806

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